RECENT ADVANCES ON GRAPEVINE-MICROBE INTERACTIONS: FROM SIGNAL PERCEPTION TO RESISTANCE RESPONSE

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Editorial: Recent Advances on Grapevine-Microbe Interactions: From Signal Perception to Resistance Response

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Editorial on the Research Topic

Recent Advances on Grapevine-Microbe Interactions: From Signal Perception to Resistance Response

Grapevine (*Vitis vinifera* L.) is one of the most economically important cultivated perennial plants worldwide and is susceptible to an array of diseases caused by pathogenic fungi, bacteria, viruses, and oomycetes. In recent decades, extensive efforts have been made to reduce the use of chemical pesticides in viticulture by developing and deploying more sustainable alternative solutions. This Research Topic covers the most recent advances on both basic and applied research on grapevine-microbe interactions. It provides an overview on (i) the different resistance loci/genes evolved in grapevine species and their application for breeding new resistant cultivars; (ii) the molecular basis of grapevine susceptibility and resistance to pathogens; (iii) the biocontrol and integrated grapevine disease management including induced plant innate immunity by beneficial microbes and natural elicitors; and (iv) the functional properties of endophytic or rhizospheric microbial communities that promote plant health.

Breeding for resistant genotypes represents a promising alternative to control powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*) in grapevine. The dissection of the different resistance loci/genes evolved in grapevine species might provide information on the mechanisms involved in resistance. In this regard, using quantitative trait locus (QTL) mapping Vezzulli et al. have investigated metabolic homeostasis related to some loci, as a means to better improve grapevine resistance. They reported the identification of 46 novel metabolic QTLs linked to 30 phenolics-related compounds. They further analyzed the expression of 11 genes in genotypes resistant to *P. viticola* (*Rpv3-3*+/−) and revealed that the *Rpv3-3* haplotype is associated with stilbenoid induction. The evaluation of genetic resistance of vegetative organs is traditionally based on measuring pathogen growth and/or disease symptoms on leaf discs. Bove et al. described another method based on the measurement of components of resistance (RCs) taking into account the infection frequency, duration of latent period, lesion size, production of sporangia, infectious period,

and infectivity of sporangia produced. The RC scores were compared with the visual score proposed in the 2nd Edition of the International Organization of Vine and Wine (OIV). According to the authors, the measurement of the RCs and their incorporation into a model that simulates their effect on downy mildew development, would be a more accurate method for phenotyping resistance level. A review article by Pirrello et al. presented an overview on studies that focused on the genetic variability of grapevine pathogens, especially of the causal agents of powdery mildew, black rot and anthracnose. They explored various aspects including disease symptom assessment, nonsynthetic chemicals, and organic control measures. They also highlighted the usage and development of molecular markers and barcoding, coupled with genome sequencing, to better understand genetic variability and resistance mechanisms of these pathogens to fungicides, and to identify candidate effectors involved in the pathogenicity and host resistance mechanisms.

An important aspect dealing with the energy costs of pathogen infection and inducible defenses in terms of carbon production and utilization by the host was also addressed in this Research Topic. Four original research papers provided new insights into aspects of biological function of sugar partitioning and photosynthesis by examining different pathosystems. Based on different cultivars with contrasting phenotypes, Cardot et al. highlighted that the availability of sugar resources for either the host or the fungus is crucial for the outcome of the interaction between grapevine and *Eutypa lata*, the causal agent of eutypa dieback. Membrane transporters, like SWEET (Sugar Will be Eventually Exported Transporters) transporters, are the targets of extracellular pathogens and seem to be decisive in the control of the competition for sugar. Meteier et al. showed that the overexpression of *SWEET4* in grapevine hairy roots leads to improved resistance to *Pythium irregulare*, a soilborne necrotrophic pathogen. This work highlights the key role of sugar transport mediated by SWEET transporters for secondary metabolism regulation and pathogen resistance in grapevine. Breia et al. reported on the importance of SWEET transporters, especially *SWEET7* in sugar mobilization during grape berry development and in response to *Botrytis cinerea* infection. Nogueira Junior et al. evaluated the photosynthetic cost of the activation of leaf defense responses in a downy mildew resistant cultivar. The authors showed that the defense response against *P. viticola* causes a photosynthetic cost to grapevines, which is not reversible even 12 days after the pathogen infection.

Grapevine is also colonized by a wide variety of beneficial rhizospheric and endophytic microbes, which can promote plant growth and health. Some of these microbes can act as biological control agents, thanks to their ability to prime plant immunity, known as induced systemic resistance (ISR), and to their antagonism against pathogens. Like in pathogens, microbe-associated molecular patterns (MAMPs) are associated with beneficial microorganisms. The perception of MAMPs triggers the activation of the plant's immune response. The recognition of these natural elicitors and their function in grapevine immunity were reviewed by Héloir et al. The authors described in detail the chemical nature, the structural diversity, and how MAMPs, as well as host-derived damage-associated molecular patterns (DAMPs), are perceived by grapevine cells and subsequently activate grapevine immunity, resulting in some cases in disease resistance. Using cultivars with different levels of susceptibility to downy mildew, Lakkis et al. showed that the endophytic bacterium *Pseudomonas fluorescens* PTA-CT2 affects hormonal status and enhances photosynthetic efficiency in both susceptible and partially resistant cultivars. The beneficial bacterium also induces ISR against *P. viticola* and *B. cinerea* by priming common and distinct defensive pathways, depending on the basal resistance of the cultivar and the pathogen lifestyle. The biocontrol activity of *Bacillus subtilis* PTA-271 was demonstrated by Trotel-Aziz et al. against Botryosphaeria dieback, caused by the fungus *Neofusicoccum parvum*. The authors reported that the main toxins (-)-terremutin and (R)-mellein produced by *N. parvum* may suppress grapevine immunity to promote Botryosphaeria dieback symptoms. Interestingly, the beneficial bacterium *B. subtilis* PTA-271 not only antagonizes the pathogen, but also primes host immune response and detoxifies both fungal phytotoxins. Cobos et al. characterized the virulence activity of necrosis and ethylene inducing peptide 1 (NEP1)-like proteins of another Botryosphaeria dieback pathogen, namely *Diplodia seriata*. By analyzing the expression of the NEP encoding genes in conditions mimicking plant infection and expressing two of the four identified NEPs as recombinant proteins, the authors highlighted differential necrotic and cytolytic activity of NEP1-like proteins under *in vitro* and greenhouse conditions.

Other contributions to this Research Topic focused on the endophytic and rhizospheric microbiome with respect to their functional and beneficial effect on plant fitness and health. Niem et al. characterized the beneficial effect of endophytic bacteria isolated from grapevine wood to control trunk diseases. The authors identified *Pseudomonas* spp. as dominant taxon among the bacterial community in canker-free grapevine tissues, indicating their ability to colonize and play a role in grapevine health. Further tests revealed antagonistic activity of distinct fluorescent Pseudomonas isolates against Esca, Botryosphaeria and Eutypa dieback, making them potential candidates to control trunk diseases. In their review, Pacifico et al. summarized the current understanding of the role of endophytes in grapevine fitness and tolerance to biotic and abiotic stresses. The authors explored different mechanisms related to colonization and the interaction of endophytes with plant metabolism, as well as their efficiency to confer plant fitness and disease resistance. The authors also highlighted the role of the environment and discussed strategies of endophyte application for a more sustainable viticulture. Other works used amplicon metagenomic approaches to explore the plant microbiome, in order to understand the diverse structure and functions in plant fitness and health. Berlanas et al. evaluated the effects of rootstock genotype on bacterial and fungal communities in the rhizosphere, and found that microbial composition and diversity were significantly driven by the rootstock type in mature vineyards. Similarly, Del Frari et al. characterized the wood fungal composition in different areas of the stem and canes, and assessed the link between mycobiome and leaf symptoms of Esca disease. Although the spatial analysis revealed differences in diversity, taxa abundances and in tissue specificity, foliar symptoms were not directly linked with the fungal

community in the wood. In another report, Deyett and Rolshausen performed a comprehensive culture-independent microbiome analysis from the sap of grapevine in the context of Pierce's disease caused by *Xylella fastidiosa*. The authors identified a core microbiome of the sap composed of bacterial and fungal taxa with antimicrobial and plant growth promoting capabilities that were present throughout the growing season, while microbial community profiles were influenced by disease condition and plant phenology. In a second contribution, Del Frari et al. dissected how fungicides commonly applied against downy and powdery mildew agents affect the wood mycobiome, including wood pathogens such as *Phaeomoniella chlamydospora* under greenhouse conditions. The authors suggested that the resident fungi that colonize the endosphere of grapevines are affected by different fungicides, some of which would be effective against *P. chlamydospora*, as well as the early colonization success of a consortium of fungal endophytes. Lastly, Gan et al. provided a valuable contribution to the field of microbiota associated with the grapevine crown gall disease caused by *Agrobacterium* spp. or *Allorhizobium* spp. in seven infected vineyards with separate geographic distributions. Using 16S amplicon sequencing the authors showed that the crown gall microbial community varies significantly across sampling sites and/or climate conditions, and harbors a core microbiome containing *A. vitis*.

Overall, this Research Topic reports on recent advances in grapevine-microbe interactions in both fundamental and applied terms. The papers compiled revealed a growing interest for sustainable solutions for grapevine disease control. This includes the breeding/phenotyping programs towards aerial and trunk diseases, biocontrol strategies and priming plant immunity, the physiological cost linked to plant defense, as well as the exploration of the functional properties of the plant microbiome for a sustainable modern viticulture.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Bacillus subtilis PTA-271 Counteracts Botryosphaeria Dieback in Grapevine, Triggering Immune Responses and Detoxification of Fungal Phytotoxins

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Plant pathogens have evolved various strategies to enter hosts and cause diseases. Particularly *Neofusicoccum parvum*, a member of Botryosphaeria dieback consortium, can secrete the phytotoxins (-)-terremutin and (*R*)-mellein during grapevine colonization. The contribution of phytotoxins to Botryosphaeria dieback symptoms still remains unknown. Moreover, there are currently no efficient control strategies of this disease, and agro-environmental concerns have raised increasing interest in biocontrol strategies to limit disease spread in vineyards, especially by using some promising beneficial bacteria. Here, we first examined *in planta* the biocontrol capacity of *Bacillus subtilis* PTA-271 against *N. parvum Np*-Bt67 strain producing both (-)-terremutin and (*R*)-mellein. We then focused on the direct effects of PTA-271 on pathogen growth and the fate of pure phytotoxins, and explored the capacity of PTA-271 to induce or prime grapevine immunity upon pathogen infection or phytotoxin exposure. Results provided evidence that PTA-271 significantly protects grapevine cuttings against *N. parvum* and significantly primes the expression of *PR2* (encoding a β-1,3-glucanase) and *NCED2* (9-*cis*-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis) genes upon pathogen challenge. Using *in vitro* plantlets, we also showed that PTA-271 triggers the expression of salicylic acid- and jasmonic acid-responsive genes, including *GST1* (encoding a glutathione-*S*-transferase) involved in detoxification process. However, in PTA-271-pretreated plantlets, exogenous (-)-terremutin strongly lowered the expression of most of upregulated genes, except *GST1*. Data also indicated that PTA-271 can detoxify both (-)-terremutin and (*R*)-mellein and antagonize *N. parvum* under *in vitro* conditions. Our findings highlight (-)-terremutin and (*R*)-mellein as key aggressive molecules produced by *N. parvum* that may weaken grapevine immunity to promote Botryosphaeria dieback symptoms. However, PTA-271 can efficiently attenuate Botryosphaeria dieback by enhancing some host immune responses and detoxifying both phytotoxins produced by *N. parvum*.

Keywords: grapevine, biocontrol, Botryosphaeria dieback, *Bacillus subtilis*, *Neofusicoccum parvum*, phytotoxins

INTRODUCTION

Causal agents of grapevine trunk diseases (GTDs) are very damaging for viticulture since their effect leads to plant death, and to date no grape variety is known to be resistant (Surico et al., 2006; Bertsch et al., 2013; Spagnolo et al., 2014; Fontaine et al., 2015; Magnin-Robert et al., 2016). Botryosphaeria dieback, one of the most threatening GTDs (Bertsch et al., 2013), is caused by several Botryosphaeriaceae fungi, including *Diplodia seriata*, *Diplodia mutila,* and *Neofusicoccum parvum* (Úrbez-Torres, 2011; Larignon et al., 2015). Because of the diversity of these hemibiotrophic fungal pathogens and their virulence characters, understanding the interactions that lead to the disease symptomatology is a major challenge in viticulture. Moreover, the virulence of Botryosphaeriaceae is highly variable within the same species, depending on plant tissue, grapevine cultivar, and environmental conditions (Úrbez-Torres, 2011). A common feature is that Botryosphaeriaceae fungi are mainly found in woody tissues but not in leaves, drawing the hypothesis that secreted fungal toxins delocalized via the xylem sap to the leaves could be involved in the emergence of foliar symptoms (Mugnai et al., 1999). Indeed, several secondary metabolites have been characterized in the Botryosphaeriaceae species (Djoukeng et al., 2009; Evidente et al., 2010; Andolfi et al., 2011; Abou-Mansour et al., 2015), and particular attention has been paid to *Neofusicoccum* spp. regarding its aggressiveness (Úrbez-Torres, 2011). Compounds belonging to two chemical families, the dihydroisocoumarin (*R*)-mellein and the epoxytoluquinol (-)-terremutin as well as their derivatives are considered as the most phytotoxic (Abou-Mansour et al., 2015). Both (*R*)-mellein and (-)-terremutin were detected in wood from vines with Botryosphaeria dieback symptoms (Abou-Mansour et al., 2015), and the produced amounts of (*R*)-mellein were proportional to pathogen aggressiveness (Ramírez-Suero et al., 2014).

(*R*)-Mellein and its derivatives have been isolated not only from pathogens of grapevine, but also from those of apple, pine, citrus and tomato, and are known for their toxicity in different tissues during plant development (Venkatasubbaiah et al., 1991; Parisi et al., 1993; Cabras et al., 2006; Djoukeng et al., 2009; Evidente et al., 2010). It has been shown that (*R*)-mellein induced partial necrosis on grapevine leaves and calli (Djoukeng et al., 2009; Ramírez-Suero et al., 2014; Abou-Mansour et al., 2015), and inhibited the growth of wheat embryo culture (Keller et al., 1994). The (*R*)-mellein derivative methylmellein also exerted a strong antigerminative effect on garden cress (Chooi et al., 2015), while 6-hydroxymellein as a key precursor of (+)-terrein exerted a phytotoxic effect leading to necrotic lesions on fruits (Zaehle et al., 2014; Gressler et al., 2015). (-)-Terremutin and its precursor 6-methylsalicylic acid (6-MSA) as non-host-specific phytotoxins induced necrosis in leaf tissues of grapevine and *Arabidopsis thaliana*, and showed a mild-antibacterial activity (Venkatasubbaiah et al., 1992; Ding et al., 2010). Similarly, the (-)-terremutin derivative terreic acid also showed an antibacterial activity (Yamamoto et al., 1980; Han et al., 2010) and was suspected to be an important antibiotic compound in soil (Chen et al., 2016). In mammals, terreic acid can affect cell's immunity (Kawakami et al., 1999).

Attention was further paid to the role of fungal toxin systems in the modulation of the plant immune response leading to plant tolerance or susceptibility to pathogens (Pusztahelyi et al., 2015). In this context, (*R*)-mellein and (-)-terremutin were shown to induce a late expression of defense-related genes in grapevine calli, including *Pathogenesis Related* (*PR*) genes and those involved in the detoxification of reactive oxygen species (Ramírez-Suero et al., 2014; Abou-Mansour et al., 2015), but the extent of these responses remained lower compared to those induced by total extracellular pathogen compounds (Ramírez-Suero et al., 2014). More recently, it has been shown that various defense-related genes are not upregulated in grapevine artificially infected with *N. parvum* (Reis et al., 2016; Spagnolo et al., 2017). However, in naturally Botryosphaeria-infected grapevine in vineyards, abundant PR proteins and antioxidant enzymes, as well as stilbene accumulation were reported in the brown striped wood (Spagnolo et al., 2014). Similar trends of gene expression and protein upregulation were observed in grapevine leaves infected with another GTDs, namely Esca-complex (Magnin-Robert et al., 2011; Spagnolo et al., 2012). Interestingly, Magnin-Robert et al. (2016) showed the accumulation of (*R*)-mellein and derivatives in Esca-symptomatic grapevine tissues. However, unlike other pathogens that use specific polyketides as virulence mediators (Uppalapati et al., 2007; Dalmais et al., 2011), to date no relationship was clearly established between (*R*)-mellein or (-)-terremutin accumulation and modulation of the host immune response.

Grapevine like herbaceous or perennial plants can be colonized by an immense number of microbial organisms in the rhizosphere and aboveground parts (Trotel-Aziz et al., 2008; Pinto et al., 2014; Zarraonaindia et al., 2015). Some of these microorganisms can exert either beneficial or detrimental effects (Möbius and Hertweck, 2009; Schroeckh et al., 2009; Pusztahelyi et al., 2015; Zeilinger et al., 2015, 2016). In asymptomatic and symptomatic GTDs-affected grapevines, the bacterial communities also differed in necrotic and non-necrotic tissues. This microbial shift can impact the tolerance or susceptibility of the vine wood to fungal attacks (Bruez et al., 2015). Indeed, some bacteria belonging to *Bacillus* spp. (i.e., *B. subtilis* PTA-271), *Pseudomonas* spp. and *Pantoea* spp. isolated from healthy vineyards, are known to induce systemic resistance against the necrotroph *Botrytis cinerea* (Magnin-Robert et al., 2007; Trotel-Aziz et al., 2008; Verhagen et al., 2011). Beneficial bacteria can directly inhibit pathogen growth and prime plants for enhancing their basal immunity (Verhagen et al., 2004, 2011; Trotel-Aziz et al., 2008; Bakker et al., 2013; Gruau et al., 2015; Aziz et al., 2016). The complex patterns of microbial interactions occurring inside/outside the plant might thus ensure the beneficial outcome of plant association with beneficial/mutualist bacteria in the dieback context. Since 2000, several biocontrol agents have been tested against the numerous pathogens responsible for GTDs, the most efficient to date being antagonistic bacteria and fungi (Haidar et al., 2016; Mondello et al., 2018). For instance, *Trichoderma* spp. generally showed high efficiency in wound protection against all GTDs pathogens (Di Marco et al., 2002, 2004;

John et al., 2008; Halleen et al., 2010) as well as *Bacillus* spp. (Schmidt et al., 2001; Halleen et al., 2010; Kotze et al., 2011; Rezgui et al., 2016). The benomyl-resistant mutant *Fusarium lateritium* strain was especially effective as a wound protectant against *Eutypa lata* (McMahan et al., 2001; John et al., 2005). This strain can degrade *in vitro* some phytotoxins involved in the expression of foliar symptoms, namely eutypine, 4-hydroxybenzaldehyde, and 3-phenyllactic acid produced by *E. lata* and pathogens from Esca consortium (Christen et al., 2005). In contrast, the rhizospheric *Pythium oligandrum* was shown to reduce *Phaeomoniella chlamydospora* wood necrosis (Esca complex) by stimulating host plant defenses (Benhamou et al., 2012; Yacoub et al., 2016).

Although several biocontrol agents were successfully tested against GTDs pathogens (Mondello et al., 2018), few studies tried to decipher mechanisms involved in plant protection against Botryosphaeria species and their aggressive molecules. Especially, the molecular mechanisms underlying induced protection, and the extent by which beneficial bacteria modulate grapevine immunity and detoxification of the virulent-phytotoxins (*R*)-mellein and (-)-terremutin, remain largely unknown. In this study, we first examined the capacity of the beneficial bacterium *B. subtilis* PTA-271 (hereafter PTA-271) to counteract grapevine infection by a *N. parvum* strain producing both (-)-terremutin and (*R*)-mellein (namely *N. parvum*-Bt67). We then focused on the effects of PTA-271 on pathogen's growth and removal of pure phytotoxins from growth medium. We finally explored the capacity of PTA-271, which was initially isolated from grapevine rhizosphere, to induce or prime grapevine immunity upon pathogen inoculation or after plant exposure to exogenous phytotoxins.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Three-node-long cuttings of grapevine (*Vitis vinifera* L., cv. Chardonnay) were collected from 10-year-old plants in Pommery's vineyards in Reims (France) and kept in a cold chamber at 4°C for 1 month. Cuttings were surface-sterilized with 0.05% cryptonol (8-hydroxyquinoline sulfate) and rooted as described by Lebon et al. (2005). They were placed in 350 mL pots containing the soil Gramoflor Special (Gramoflor GmbH & Co. KG, Vechta, Germany) in a culture chamber (25°C day/night, 60% relative humidity, and 16 h photoperiod at 400 μmoles/m^2/s) and watered twice a week. Only cuttings that have developed roots were conserved for further experiments.

Grapevine plantlets (*V. vinifera* L. cv. Chardonnay, clone 7535) were produced from nodal explants transferred on 15 mL of agar-modified Murashige-Skoog (MS) medium (Trotel-Aziz et al., 2008) in 25-mm test tubes. Plantlets were grown at 25°C day/night, with a 16/8 h photoperiod.

Bacterial Growth and Treatment

Bacillus subtilis PTA-271 (GenBank Nucleotide Accession No. AM293677) was isolated from the rhizosphere of healthy field-grown Chardonnay grapevines in Champagne area, France (Trotel-Aziz et al., 2008). Bacterial growth starts by adding 100 μl of the glycerol stock suspension to sterile Luria Bertani (LB) medium, before incubating at 28°C under continuous shaking (75 rpm). Experiments were performed with the bacteria at the exponential growth phase. After centrifugation (5000 *g*, 10 min), the pellet was washed once and resuspended in sterile 10 mM $MgSO_4$ medium. Bacterial density was measured by spectrophotometry at 450 and 650 nm, and the mean concentration was adjusted with sterile $MgSO_4$ medium before treatment.

Bacterial suspension was applied twice at the root level of cuttings at a final concentration of 10^8 cfu/g soil. The first inoculation was performed when cuttings were 8 weeks old and the second inoculation when cuttings were 10 weeks old. Control cuttings were thus similarly drenched twice with $MgSO_4$ solution.

For *in vitro*-plantlets, bacterial suspension was adjusted to 10^8 cfu/mL with sterile liquid MS medium then added in new sterile 25-mm culture-tubes (15 mL per tube). Six-week-old plantlets were then transferred in these new tubes for 2 weeks of bacterial treatment in a growth chamber at 22°C with a photoperiod 16/8 h. Control plantlets were transferred in liquid MS medium without bacteria under the same conditions.

Fungal Strain and Growth

The *N. parvum* strain *Np*-Bt67 (Reis et al., 2016) isolated from Portuguese vineyards (Estremadura area) is inscribed in HIA collection (Lisbon University, Portugal). Fungi was maintained on potato dextrose agar (PDA, Sigma, Saint-Quentin-Fallavier, France) plates and stored at 4°C. Resulting mycelium was plated on PDA medium and incubated in the dark at 22°C for 7 days before used to inoculate cuttings.

Production and Quantification of Phytotoxins

(*R*)-mellein (log Kow ∼ 2.5) and (-)-terremutin (log Kow ∼ 0) were extracted and purified from a 10-day-old culture of the *Np* strain, according to Abou-Mansour et al. (2015). Both toxins were prepared as concentrated stock solutions in sterile MS or 10 mM $MgSO_4$ medium and stored in the dark at 4°C. Before each experiment, daughter solutions were prepared for the biological experiments, and the phytotoxin concentrations were determined before and after treatment using HPLC coupled to a diode array detector (Ultimate 3000 Dual-Gradient, Dionex, Voisins-le-Bretonneux, France). Analyses were done on a C18 reversed phase column (100 mm× 3 mm, 5 μm, Kromasil 100, Dionex) using isocratic elution with acetonitrile (ACN, LC–MS quality, Merck, France) and water (H_20) containing 0.1% phosphoric acid (H_3PO_4). Detection was recorded at 210 and 273 nm for (*R*)-mellein and (-)-terremutin, respectively. Phytotoxin identification was confirmed by UV spectrum and retention time; (*R*)-mellein was eluted with 1 mL/min of ACN:H_2O 60:40 v/v at 3.8 min, while (-)-terremutin was eluted with 0.7 mL/min of ACN:H_2O 10:90 v/v at 5.8 min. Concentration was determined using standard curves.

Fungal Inoculation and Disease Expression

Cuttings pretreated 1 month with bacteria were then wounded (5 mm diameter, 1 mm deep) at 12 weeks old at the second node of the green stem and inoculated with a 3 mm diameter mycelial plug from the 7-day-old culture of *Np*-Bt67 strain. Inoculation site was then covered with moisten hydrophilic cotton before sealing with parafilm. Without bacteria, cuttings were pretreated 1 month with $MgSO_4$, then pathogen-inoculated also at 12 weeks old using the same method. To confirm that lesions were really due to pathogen infection and not to the injury, controls were inoculated with sterile 3-mm PDA plugs. After inoculation, cuttings were kept in the same culture chamber conditions to quantify Botryosphaeria dieback symptoms at 4 months post-inoculation. As potentially indicative, phytotoxins were also extracted from the same leaf powder (1 g FW in 5 mL of methanol – LC–MS quality, Merck, France – for 1 h at 37°C before analysis in supernatant as described below) at least twice in triplicates, and phytotoxins were not detectable in leaves of infected cuttings. At 4 months post-inoculation, symptoms of Botryosphaeria dieback were evaluated by measuring both the canker and necrotic surface area on green shoots as described by Espinosa et al. (2009) and Laveau et al. (2009), and by quantifying the percentage of dead branch for inoculated cuttings.

Evaluation of Direct Effect of *B. subtilis* PTA-271 on *N. parvum* Growth

PTA-271 grown in LB medium was inoculated (5 μL drop at 10^9 cfu/mL) on the one side of a Petri plate (9 cm diameter) containing PDA medium, then incubated at 28°C in the dark. After 24 h, a mycelium plug of 4-day-old pathogenic fungus was co-inoculated on the other side of PDA plates, and the plates were incubated in the same conditions. Controls are PDA plates with a mycelium plug and a LB-drop incubated until mycelial growth reached the edge of the control plate. The same experiment was also performed at 22°C as an optimal temperature for pathogen growth (Trotel-Aziz et al., 2008), while 28°C was optimal for PTA-271 growth. Antagonistic effect was characterized by an inhibition zone around bacterial colony.

Detoxification Assays With *B. subtilis* PTA-271

PTA-271 was collected at exponential phase in LB medium, diluted to reach a final density of 10^4 to 2×10^8 cfu/mL, and centrifuged at 5000 *g* (4°C, 15 min). Pellet was then resuspended either in a sterile MS medium (nutrient rich) or in a 10 mM $MgSO_4$ medium (nutrient poor) containing or not (*R*)-mellein 350 μg/L (= 100%) or (-)-terremutin 750 μg/L (= 100%). Detoxification tests were performed after assessing the toxicity of (*R*)-mellein and (-)-terremutin on both bacteria and plantlets (see data in **Supplementary Figures S1**, **S2**). For both molecules, no toxic effect was observed from 0 to 1500 μg/L neither on the plant nor on the bacterium. Detoxification assays were done in triplicate at 28°C under continuous shaking for 72 h. Percentage of each phytotoxin was determined daily in both bacterial pellet and supernatant (culture medium) obtained after centrifugation. Phytotoxins were extracted from bacterial pellet with acetone (HPLC quality, VWR, France) by shaking for 48 h in darkness at 4°C. Mixture was then centrifuged (5000 *g*, 15 min, 4°C) and clean supernatant was collected for direct phytotoxin analysis with HPLC as described before. (-)-Terremutin as a highly hydrophilic molecule was directly analyzed in the culture medium by direct injection into HPLC system. However, (*R*)-mellein was extracted from the culture medium with hexane (10:2 v/v, extraction yield > 90%). After a vigorous shake of 1 min, the upper organic phase was directly used for (*R*)-mellein analysis with HPLC. Two controls were carried out: living bacteria in a toxin-free medium as a biological control, and medium containing only toxin without living bacteria as a physicochemical control.

Treatment of Grapevine Plantlets With (*R*)-Mellein and (-)-Terremutin

To investigate phytotoxin's capacity to modulate plant immunity, 6 weeks old plantlets were treated with bacterial suspension in liquid MS medium at the root level. After 2 weeks, roots of were washed three times in sterile liquid MS, then plantlets were transferred in a new sterile liquid MS medium supplemented or not with (*R*)-mellein 350 μg/L or (-)-terremutin 750 μg/L for 72 h under growth chamber conditions. Controls consisted of 8 weeks old plantlets on MS medium, further transferred for 3 days on liquid MS medium with or without phytotoxins.

In the meantime, phytotoxins were quantified from plantlet's incubating medium as described before, and extracted from shoot and roots with methanol (weight/volume: 1/5) in darkness under continuous shaking for 48 h at 4°C. The homogenate was then centrifuged at 5000 *g* for 15 min at 4°C and the clean supernatant was directly used for phytotoxin analysis by HPLC. All experiments were repeated four times at least in triplicate. Two different controls were carried out: living plants in a toxin-free medium and medium containing only toxin without living plants.

RNA Extraction and qRT-PCR Analysis

Leaf samples from cuttings and shoots from plantlets were collected respectively at 4 days post-inoculation with pathogen and at 3 days post-treatment with phytotoxins, ground in liquid nitrogen then stored at −80°C. Total RNA were extracted from 50 mg of leaf powder for cuttings or from 100 mg of powdered plantlet shoots with PlantRNA Purification Reagent according to manufacturer instructions (Invitrogen, Pontoise, France), and DNase treated as described by Gruau et al. (2015). RNA quality was checked by agarose gel electrophoresis, and total RNA concentration was measured at 260 nm for each sample and adjusted to 100 ng μL^{-1}. First-strand cDNA was synthesized from 150 ng of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, United States). PCR conditions were those described by Gruau et al. (2015). Quantitative RT-PCR

was performed with Absolute Blue qPCR SYBR Green ROX Mix according to manufacturer instructions (Thermo Fisher Scientific, Inc., Waltham, MA, United States), in a BioRad C1000 thermocycler using the BioRad manager software CFX96 Real Time PCR (BioRad, Hercules, CA, United States). A set of 13 defense-related genes, selected for their responsiveness to pathogen or priming state induced by beneficial bacteria (Spagnolo et al., 2012, 2014; Gruau et al., 2015; Magnin-Robert et al., 2016), was tracked by quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) using specific primers (**Supplementary Table S1**). qRT-PCR reactions were carried out in duplicates in 96-well plates in a 20-µl final volume containing Absolute Blue SYBR Green ROX mix including Taq polymerase ThermoPrime, dNTPs, buffer and $MgCl_2$ (Thermo Fisher Scientific, Inc., Waltham, MA, United States), 280 nM forward and reverse primers, and 10-fold diluted cDNA according to the manufacture's protocol. Cycling parameters were 15 min of Taq polymerase activation at 95°C, followed by 40 two-step cycles composed of 10 s of denaturation at 95°C and 45 s of annealing and elongation at 60°C. Melting curve assays were performed from 65 to 95°C at $0.5°C{\cdot}s^{-1}$, and melting peaks were visualized to check amplification specificity. *EF1* and *60SRP* genes were used as references and experiments were repeated five times. Relative gene expression was determined with the formula fold induction: $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta$Ct = [Ct TG (US) – Ct RG (US)] – [Ct TG (RS) – Ct RG (RS)], where Ct is cycle threshold, Ct value is based on the threshold crossing point of individual fluorescence traces of each sample, TG is target gene, RG is reference gene, US is unknown sample, and RS is reference sample. Integration of the formula was performed by the CFX Manager 3.0 software (BioRad). The genes analyzed were considered significantly up- or down-regulated when changes in their expression were > 2-fold or < 0.5-fold, respectively. Control samples for the cuttings model are cDNA from leaves of cuttings untreated with bacteria and inoculated with sterile PDA plugs (1x expression level), while for the *in vitro* model it corresponds to shoots from plantlets grown on MS medium without PTA-271 and phytotoxins (1x expression level).

Statistical Analysis

To quantify phytotoxins, standard curves were first established with pure phytotoxins through titrations repeated at least three times from two independent experiments. Biocontrol assays with cuttings model were repeated at least three times with at least 10 cuttings per treatment. The confrontation tests between PTA-271 and fungal pathogen were triplicated in experiments conducted twice. Detoxification assays with *in vitro* PTA-271 or *in vitro* plantlet model were repeated four times with each sample at least triplicated. Data are means ± standard deviations. Analyses of gene expression by qRT-PCR were repeated five-times from independent experiments. RNAs were extracted from powdered 20 leaves of 10 grapevine cuttings, and from powdered shoots of four plantlets. Results correspond to means ± standard deviation from one representative out of at least three showing the same trends. Statistical analyses were carried out using the SigmaStat 3.5 software. For treatment effect, mean values were compared by Tukey's test ($P < 0.05$).

RESULTS

B. subtilis PTA-271 Attenuates Botryosphaeria Dieback Symptoms in Grapevine Cuttings

PTA-271 was used to evaluate its capacity to control the occurrence of Botryosphaeria dieback symptoms. Bioassays with Chardonnay cuttings from control or bacteria-pretreated plants at root level further inoculated with *Np*-Bt67 showed that PTA-271 significantly reduced the dead branch development (**Figure 1A**) by approximately 50% compared to non-bacteria pretreated plants (**Figure 1E**). Similarly, the size of canker (**Figure 1B**) and those of external and internal stem lesions (**Figures 1C,D**) were reduced in PTA-271-pretreated cuttings after challenge with *Np*-Bt67. Both canker and stem lesions were reduced by about 63 to 75% compared to non-bacteria pretreated plants (**Figures 1F–H**). These results indicate that PTA-271 could efficiently protect Chardonnay cuttings from the *N. parvum* strain *Np*-Bt67.

B. subtilis PTA-271 Antagonizes *N. parvum* and Detoxifies Both (*R*)-Mellein and (-)-Terremutin

In regard to *in vitro* test with pathogen mycelium, results showed that PTA-271 clearly antagonizes *Np*-Bt67 by a fungistatic effect compared to control treatment at 28°C (**Figure 2A**) in both time points. Antifungal effect was detected approximately 4 days after pathogen inoculation at 22°C (**Figure 2B**). Thereafter, mycelial growth increased progressively and became comparable to the control.

We also investigated whether PTA-271 can affect fungal toxins, (*R*)-mellein and (-)-terremutin exogenously applied to their culture medium. Results showed that the percentage of both (*R*)-mellein and (-)-terremutin was significantly decreased in the presence of PTA-271 (**Figure 3**). The (*R*)-mellein decrease was effective after a 48 h latency period in the presence of PTA-271 at 10^8 cfu/ml, and reached 40% after 72 h of exposure (**Figure 3A**). Similar effect was observed after 72 h of incubation with PTA-271 at low (10^4 cfu/ml) or high (2×10^8 cfu/ml) bacterial density (**Figure 3B**). In addition, the bacterium seems to be effective to remove (*R*)-mellein, whether suspended in MS medium or in the less nutrient rich $MgSO_4$ medium. The amount of (*R*)-mellein decreased significantly with the high bacterial density whether in MS medium or in the less nutrient rich $MgSO_4$ medium (**Figure 3C**). Interestingly, the (-)-terremutin decrease was effective after a 24 h latency period in MS medium in the presence of PTA-271 at 10^8 cfu/ml to reach 50% after 72 h (**Figure 3D**). Such a (-)-terremutin decrease was not observed in the presence of the bacterium at lower density (**Figure 3E**), or in the less nutrient rich $MgSO_4$ medium compared to MS one (**Figure 3F**).

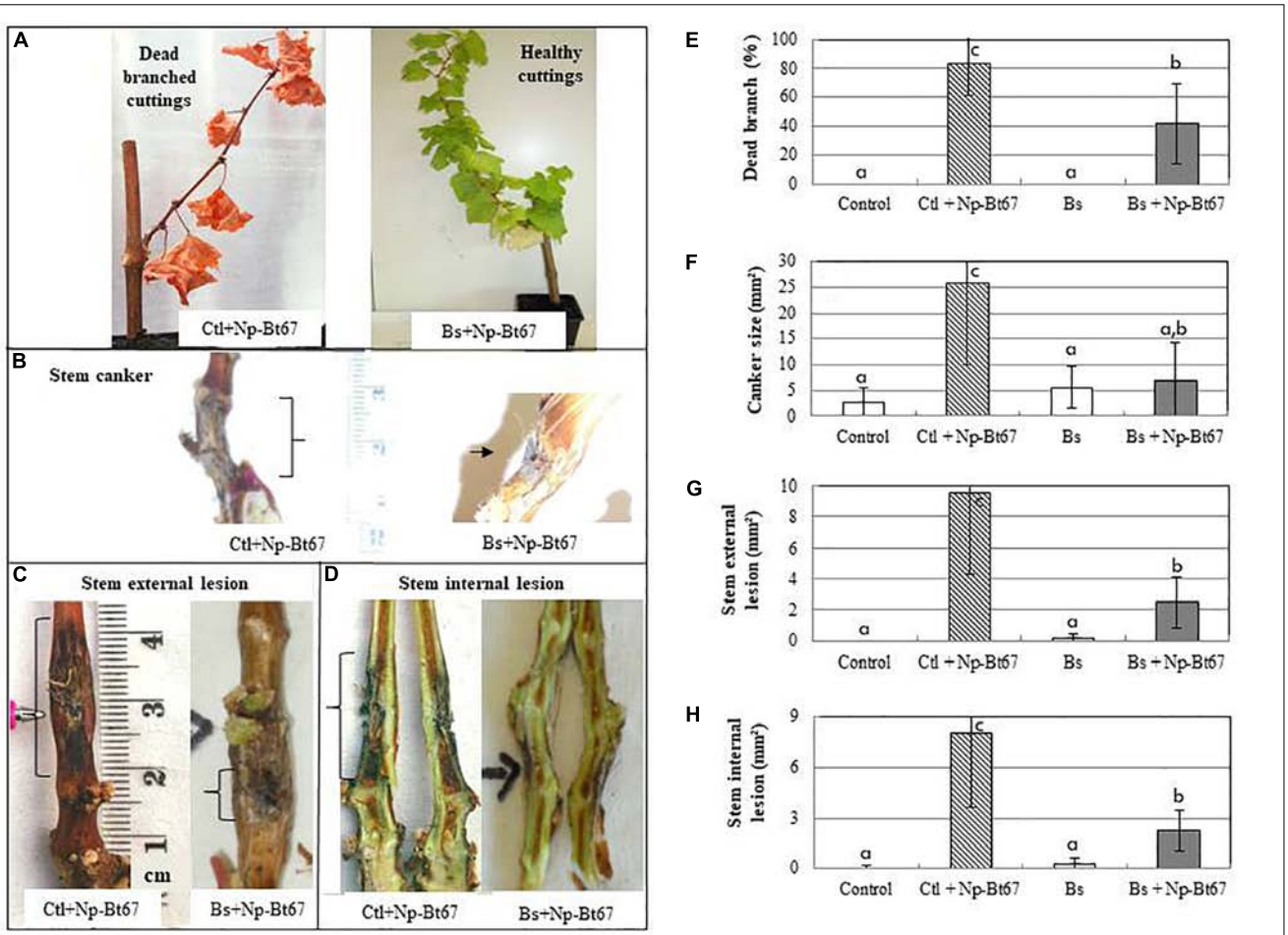

FIGURE 1 | The beneficial bacterium *Bacillus subtilis* PTA-271 attenuates the characteristic Botryosphaeria dieback symptoms induced in Chardonnay cuttings by the *Neofusicoccum parvum* strain *Np*-Bt67. One month pretreated grapevine cuttings with PTA-271 (Bs, 2×10^8 cfu/g soil) and non-bacteria pretreated ones (Ctl) were inoculated with pathogen mycelium (+Np-Bt67). Non-infected plants were inoculated with sterile medium without pathogen (Control). Compared to PTA-271 treated healthy asymptomatic cuttings **(A)**, the infected symptomatic cuttings showed the typical Botryosphaeria dieback symptoms: dead branch **(A,E)**, stem canker **(B,F)**, stem internal necrosis **(C,G)**, and stem external necrosis **(D,H)** that were photographed **(A–D)** and quantified **(E–H)** at 4 months post-inoculation. Data are means ± standard deviation (SD) for at least three independent experiments with 10 biological replicates per treatment. Vertical bars with different letters are significantly different (Multiple Comparison procedures with Tukey's test, $P < 0.05$).

B. subtilis PTA-271 Strongly Primes the Expression of *a* β-*1,3-Glucanase* After *N. parvum* Inoculation in Grapevine Cuttings

In leaves of control cuttings inoculated with *Np*-Bt67, data from qRT-PCR (**Figure 4** and **Supplementary Figure S3**) showed that, except for *PR1* (1.4-fold expression), the expression of defense genes responsive to salicylic acid (SA) including *PR2*, *PR5*, and *PR10* was significantly up-regulated from 6.6- to 7.3-fold. Expression of *PAL* (phenylalanine ammonia-lyase) and *STS* (stilbene synthase) involved in the synthesis of phytoalexins was also increased by 1.6- and 3.5-fold, respectively. In the meantime, expression of *GST1* encoding a glutathione-*S*-transferase putatively involved in the detoxification process, and that of *PR3* and *PR4* as responsive to jasmonic acid/ethylene (JA/ET), was upregulated by 2.7-, 3.3- and 1.3-fold, respectively. Data also showed a low upregulation of the *NCED2* gene involved in abscisic acid biosynthesis (1.6-fold), while that of *LOX9* was not upregulated by *N. parvum*.

The ability of PTA-271 to enhance grapevine immunity was addressed. Gene expression levels after pretreatment with PTA-271 was similar to control plants, before pathogen challenge (**Figure 4** and **Supplementary Figure S3**). However, after *Np*-Bt67 inoculation, bacteria-pretreated plants showed a significant priming of *PR2* (encoding a β-1,3-glucanase), *NCED2 and PAL* expressions compared to non-bacteria pretreated plants. *PR2* mRNA level was more markedly primed in the leaves (23.7-fold). However, only slight differences were observed regarding transcript levels of *LOX9*, *GST1*, and *STS*, while the expression levels of *PR1*, *PR3*, *PR4,* and *PR10* did not change in bacteria-treated plants compared to control after pathogen infection.

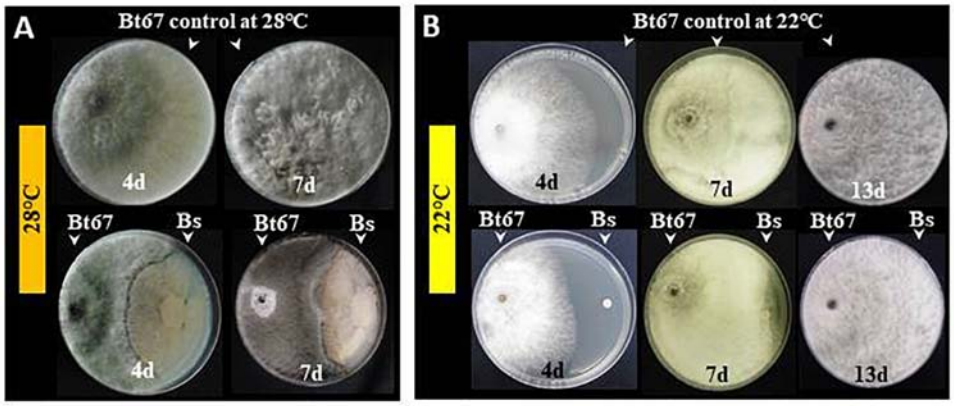

FIGURE 2 | Antagonistic activity of *B. subtilis* PTA-271 toward the *N. parvum* strain *Np*-Bt67. The beneficial bacterium PTA-271 (Bs) and the *N. parvum* strain *Np*-Bt67 (Bt67), co-inoculated on the opposite sides of PDA plates, were incubated at 28°C **(A)** or 22°C **(B)**. Pictures of representative plates among nine were taken from 4 to 13 days depending on mycelial growth. Top photographs are the plates without bacteria (pathogen control) and bottom ones are the plates co-inoculated with pathogen and Bs. Antagonism effect is characterized by an inhibition zone between the bacterial colony (right side) and the fungus (left side).

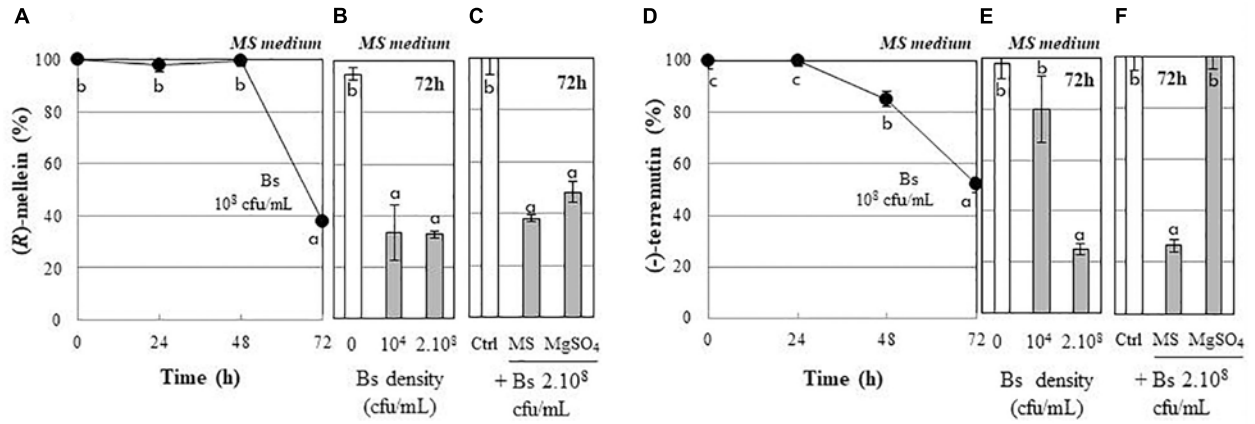

FIGURE 3 | Detoxifying capacity of *B. subtilis* PTA-271 (Bs) toward the purified (*R*)-mellein **(A–C)** and (-)-terremutin **(D–F)** from *N. parvum*. Phytotoxin concentrations were determined as remaining percentages in the bacterial incubating media either: **(A,D)** daily form MS medium containing PTA-271 (Bs) at 10^8 cfu/mL, or **(B,E)** 72 h post-exposure to two distinct bacterial densities 10^4 and 2×10^8 cfu/mL in MS, or **(C,F)** 72 h post-exposure to the two distinct incubating media Murashige-Skoog medium (MS) and $MgSO_4$ with *Bs* at 2.10^8 cfu/mL. Data are means ± SD of three independent experiments, each with triplicates. The toxin controls (Ctrl) indicated none physicochemical disappearance. Phytotoxins were not detectable inside bacterial pellet. Vertical bars with different letters are significantly different (Multiple Comparison procedures with Tukey's test, $P < 0.05$).

N. parvum Phytotoxins Repress PTA-271-Mediated SA- and JA-Responsive Gene Expression in Grapevine Plantlets

To focus on the repression of gene expression induced by PTA-271 after toxin application, we first examined how the bacterium affects gene expression in plantlets leaves. Data (**Figure 5** and **Supplementary Figure S4**) showed that PTA-271 alone induced significant changes in the expression of genes responsive to JA/ET (*PR3*, *PR4*, *LOX9*), SA (*PR1*, *PR2*, *PR5*, *GST1*, *PR10*) or abscisic acid ABA (*NCED2,* involved in ABA synthesis) compared to control plantlets. Transcript level was increased from 2.4- to 6.9-fold for JA/ET-responsive genes, from 2.0- to 5.8-fold for SA-responsive ones, and by 4.3 for *NCED2*. Expression of *PAL* and *STS* was also increased by 2.2 and 2.3-fold, respectively, and to a lesser extent for *CHI* (chalcone isomerase) and *NPR1.1* (non-expresser of *PR1*) reaching 1.3- and 1.9-fold expression, respectively.

After a subsequent exposure to toxins, most of the defense genes induced by PTA-271 were repressed. (-)-Terremutin and (*R*)-mellein significantly repressed the expression of genes responsive to JA/ET (*PR3*, *PR4*, *LOX9*) and SA (*PR2*, *PR5*, *PR10*), and that of PAL and STS (involved in phenylpropanoid pathway). (*R*)-mellein additionally repressed the expression *GST1*, another gene responsive to SA. Expression of the SA-dependent *PR1* gene was the sole gene still over induced in PTA-271 treated plantlets after toxin application, as in control plantlets treated

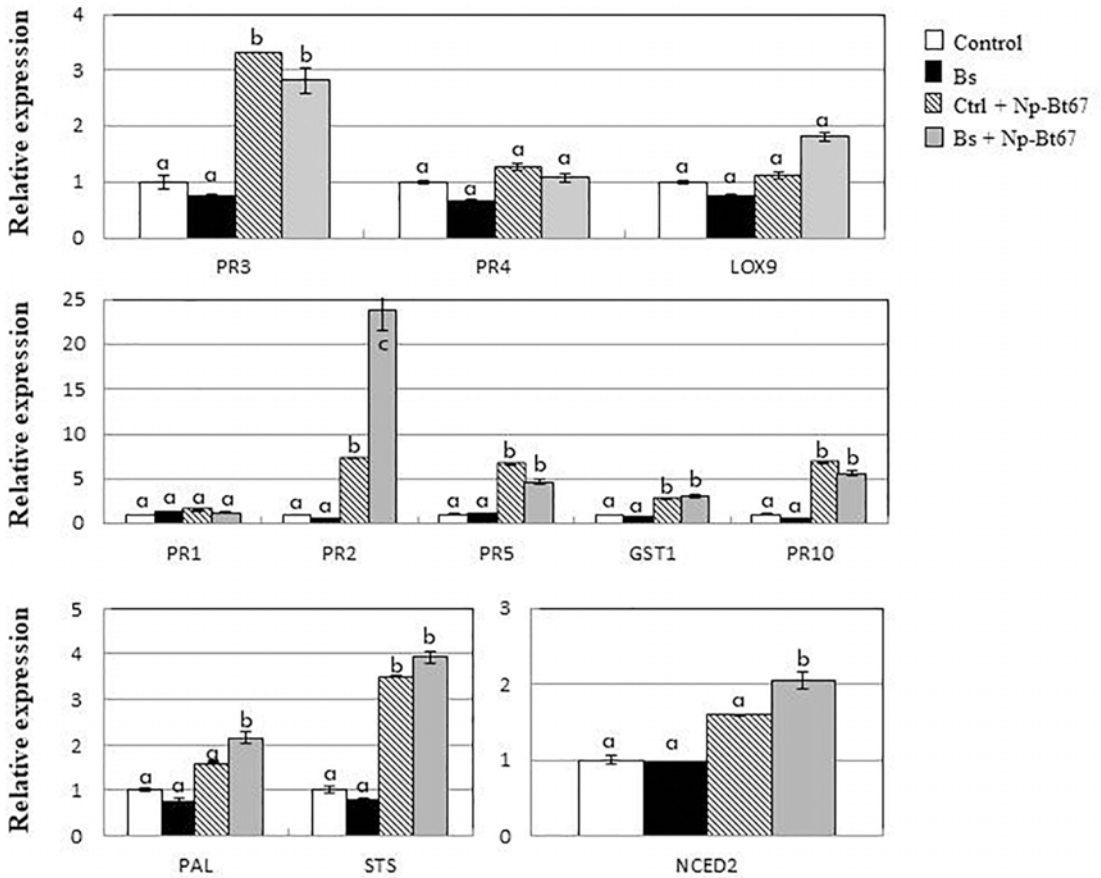

FIGURE 4 | *B. subtilis* PTA-271 strongly primes *PR2* gene in leaves of grapevine cuttings after infection with the *N. parvum* strain *Np*-Bt67. Twelve weeks old plantlets untreated or pretreated with PTA-271 were both infected with sterile PDA plugs (Control and Bs, respectively) or with mycelium plugs of *Np*-Bt67 (Ctl + Np-Bt67 and Bs + Np-Bt67, respectively). Transcript levels of defense-related genes were monitored by qRT-PCR in leaves at 4 days post infection. Results are from one representative replicate among three independent experiments showing the same trends. Different letters indicate significant differences. *PR3* = class IV chitinase (*chit4c*); *PR4* = PR-4 type protein; *LOX9* = lipoxygenase 9; *PR1* = pathogenesis-related protein 1; *PR2* = class I β-1,3-glucanase; *PR5* = thaumatin-like protein; *GST1* = glutathione-S-transferase 1; *PR10* = pathogenesis-related protein 10; *PAL* = phenylalanine ammonia lyase; *STS* = stilbene synthase; *NCED2* = 9-cis-epoxycarotenoid dioxygenase 2.

with both toxins. Expression of another SA-dependent *GST1* gene was the sole gene still over induced in PTA-271 treated plantlets after (-)-terremutin application, as in control plantlets treated with (-)-terremutin. In contrast, expression of the ABA-dependent *NCED2* gene was the sole gene still over induced in PTA-271 treated plantlets after each toxin application, while not significantly in control plantlets treated with toxins.

(*R*)-Mellein and (-)-Terremutin Are Mobilized or Accumulated Differently by PTA-271-Pretreated Plantlets

To investigate the fate of phytotoxins in the incubating medium of plantlets, control and PTA-271-pretreated plants were exposed to (*R*)-mellein or (-)-terremutin at their root level. As shown in **Figure 6A**, (*R*)-mellein quickly decreased in the MS growth medium of control plantlets. The amount of (*R*)-mellein decreased by about 83.5% within 24 h and by 97.5% after 48 h. *In planta* (**Figure 6B**), roots accumulated about 50% of (*R*)-mellein within 72 h. Experiments with PTA-271-pretreated plantlets showed a partial (*R*)-mellein removal even after 72 h exposure (**Figure 6A**), thus confining 20% of (*R*)-mellein in the incubating medium (**Figure 6A**), while only 20% of (*R*)-mellein was accumulated inside the plantlet roots (**Figure 6B**).

Supplied (-)-terremutin also decreased significantly from plantlets incubating medium (**Figure 6C**), especially after a 24 h period of exposure, to reach about 35% from 48 h. At 72 h, no apparent accumulation of (-)-terremutin was noticed in plantlet tissues (**Figure 6D**). With PTA-271-pretreated plantlets, a similar trend appeared for (-)-terremutin removal from the MS medium (**Figure 6C**), without any apparent accumulation inside the plant tissues (**Figure 6D**).

DISCUSSION

The contribution of (*R*)-mellein and (-)-terremutin to *N. parvum* aggressiveness was strongly suspected in grapevine, considering

	Plantlets		Plantlets challenged with phytotoxins			
	Control	Bs	Ctl + T	Bs + T	Ctl + M	Bs + M
PR3	1,00	3,46	0,57	0,47	1,21	0,56
PR4	1,00	2,44	1,02	1,42	1,90	1,27
LOX9	1,00	6,92	1,36	1,31	1,46	1,47
PR1	1,00	1,99	2,74	4,74	6,72	5,16
PR2	1,00	3,35	0,89	0,69	0,76	1,46
PR5	1,00	4,06	1,28	1,09	2,52	2,80
GST1	1,00	5,81	7,94	4,90	1,90	1,43
PR10	1,00	3,25	1,03	1,40	1,74	0,83
NPR1.1	1,00	1,86	0,77	1,12	0,85	1,03
PAL	1,00	2,21	0,55	0,77	1,19	0,68
STS	1,00	2,33	0,70	0,75	1,09	0,65
CHI	1,00	1,31	0,22	0,80	0,44	1,10
NCED2	1,00	4,33	1,26	3,31	1,86	4,56

0,22 1 7,94

FIGURE 5 | (*R*)-mellein and (-)-terremutin repress the *B. subtilis*-PTA-271-induced immune responses in grapevine plantlets. Eight weeks old plantlets untreated or pretreated with PTA-271 were further challenged with MS medium (Ctl and Bs, respectively) supplemented with (-)-terremutin (Ctl+T and Bs+T, respectively) or (*R*)-mellein (Ctl+M and Bs+M, respectively). Transcript levels of defense-related genes were monitored by qRT-PCR in plantlets shoots after 3 days of exposure. Results are from one representative replicate among five independent experiments showing the same trends. A three-color scale was used to show the expression level of each gene. Red shades indicate overexpression and deep red corresponds to an induction factor of 7.94 or more; white represents the basal expression level and signifies that the expression level is not different from the Control; blue shades symbolize repression and dark blue corresponds to a 0.22-fold induction or less. Legends for genes are as in **Figure 4**. *CHI* = chalcone isomerase; *NPR1.1* = non-expresser of *PR* genes 1.

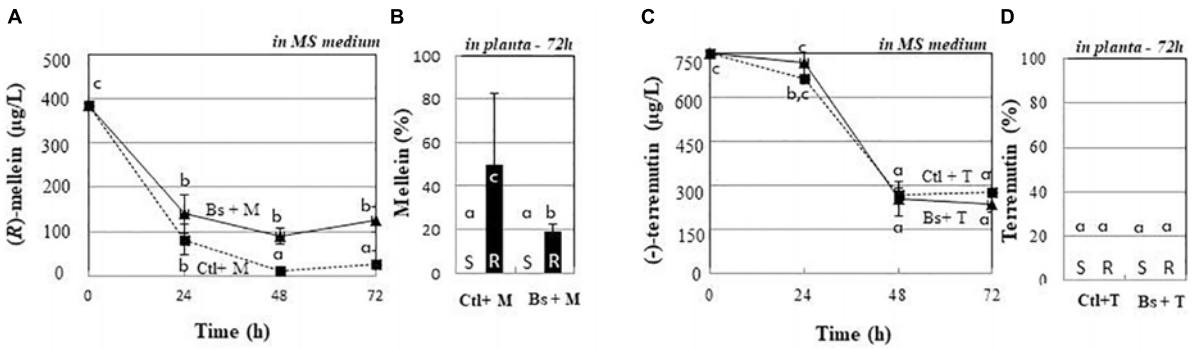

FIGURE 6 | Fate of (*R*)-mellein and (-)-terremutin from plantlets incubating medium or from medium of *B. subtilis*-PTA-271-pretreated plantlets. Eight weeks old plantlets treated with PTA-271 were transferred in a new MS medium containing (*R*)-mellein (Bs + M) 350 µg/L **(A,B)** or (-)-terremutin (Bs + T) 750 µg/L **(C,D)**. The same experiment was performed with non-bacteria pretreated plantlets, then transferred on (-)-terremutin 750 µg/L (Ctl + T) or (*R*)-mellein 350 µg/L (Ctl + M). Phytotoxin concentrations were determined either: daily in the plant culture media from 0 to 72 h **(A,C)**, or 72 h post-exposure as percentage accumulated in shoots (S) and roots (R) **(B,D)**. Data are means ± SD of three independent experiments, each with triplicates (at least four plantlets by replicate). The toxin controls indicated none physicochemical disappearance. Phytotoxins were not detectable inside bacterial pellet. Vertical bars with different letters are significantly different (Multiple Comparison procedures with Tukey's test, $P < 0.05$).

their detection in the wood and leaves of Botryosphaeria dieback affected plants (Abou-Mansour et al., 2015) while their secreting pathogens were exclusively wood-confined (Mugnai et al., 1999). However, the role of such phytotoxins in Botryosphaeria infectious process and their potential control by beneficial microbes remain unknown. In this study, we used a *N. parvum* strain that produces both (*R*)-mellein and (-)-terremutin, as well as these purified toxins, to understand their role in the *N. parvum* aggressiveness. We also investigated the capacity of the beneficial bacterium *B. subtilis* PTA-271 to counteract Botryosphaeria dieback symptoms, and explore whether the bacterium can affect pathogen growth, detoxify pure toxins and prime grapevine immunity after pathogen infection.

Our data provide evidence that *N. parvum Np*-Bt67 which produces high amount of (-)-terremutin provoked Botryosphaeria dieback symptoms within 10 days on grapevine

cuttings, including dead branch, canker and both external and internal stem necrosis (**Figure 1**). Interestingly, after treatment of cutting at the root level with PTA-271, the Botryosphaeria dieback symptoms were significantly reduced. The PTA-271-pretreated plants showed a reduced dead branch of 50% after *Np*-Bt67 challenge, accompanied with a strong reduction of canker and stem lesions. This study reports for the first time: (i) the expression of a severe form of Botryosphaeria dieback on Chardonnay plants in controlled conditions, and (ii) that PTA-271 seems to be a very effective bacterium to protect Chardonnay plants against a Botryosphaeria pathogen. This protective effect appears to be related to the ability of the bacterium to antagonize *N. parvum* by delaying its mycelial growth, to detoxify both (*R*)-mellein and (-)-terremutin, and to prime few defense genes including *PR2* (a β-1,3-glucanase), *NCED2* (involved in ABA synthesis) and *PAL* at systemic level after pathogen inoculation. Indeed PTA-271 was initially isolated from grapevine rhizosphere, while inducing leaf defense responses (Trotel-Aziz et al., 2008). But Santoyo et al. (2016) indicates that all of the genera described as common inhabitants of the rhizosphere, are also bacterial endophytes. Especially *Bacillus* sp. is the most commonly isolated species from all kinds of grapevine tissues including the wood of both Esca-foliar symptomatic or asymptomatic plants (Bruez et al., 2015). In this study, PTA-271 succeeds to protect grapevine. Whatever the inhabiting zone of PTA-271 or its active molecules, PTA-271 impacts were sought both on mycelium and toxins of fungal pathogen and on plant immunity.

The antagonistic activity of *B. subtilis* PTA-271 against *Np*-Bt67 (**Figure 2**) showed some dependency on temperature, since it is only effective at 28°C. PTA-271 could thus impact the life cycle of *N. parvum*, especially at 28°C since it clearly appears that PTA-271 grows less at 22°C while using identical bacterial densities at day 0. Thus the less fungal inhibition at 22°C might result from the fact that PTA-271 grows less at 22°C. This fungistatic effect might be explained by the release of various antifungal compounds by PTA-271, including surfactins or other lipopeptides which production was shown to depend on temperature (Ongena and Jacques, 2008; Pinto et al., 2018). Interestingly, PTA-271 can also detoxify the two main phytotoxins of *N. parvum* to different extents (**Figure 3**). The detoxifying activity of the bacterium seems to be more active in a nutrient rich medium for (-)-terremutin, but not for (*R*)-mellein. This suggests that (*R*)-mellein would be directly metabolized by PTA-271, while (-)-terremutin would require a co-substrate to be co-metabolized by this bacterium. This is consistent with the short latency period needed for (-)-terremutin mobilization from the medium, as already reported for some organic pesticides (Cycon and Piotrowska-Seget, 2016). It has been reported that bacteria can use root exudates such as catechin and coumarin as co-substrates to detoxify recalcitrant organic molecules *in situ* (Makova et al., 2006). It is thus speculated that grapevine and beneficial bacteria might interact together to improve detoxification process and then ensure an active protection against Botryosphaeria dieback. However, in the case of (*R*)-mellein, its detoxification rate by bacteria is characterized by a long latency phase followed by a rapid disappearance, even at a low bacterial density (10^4 cfu/mL). This latency period would be necessary for bacteria to express its detoxifying pathways.

Our results also suggested that PTA-271 might prime the expression of some plant defense genes responsive to different phytohormone pathways (**Figure 4**). In leaves of control cuttings challenged with *Np*-Bt67, some genes were slightly up-regulated, especially *PR2*, *PR5*, *PR10,* as SA-responsive genes (Dufour et al., 2013; Naznin et al., 2014; Caarls et al., 2015), while the expression of *PR4*, *LOX9*, as JA/ET responsive genes (Hamiduzzaman et al., 2005; Naznin et al., 2014) remained low. This suggests that the early activation of SA-signaling during pathogen's biotrophic phase could antagonize the expression of JA-dependent-defenses useful for grapevine once pathogen entered its necrotrophic phase as indicated by Yang et al. (2015). This could result from the pathogen strategy to overcome host defenses and thus promote disease. In the same sense, a late and weak defense's expression has been already observed in grapevines developing Botryosphaeria dieback symptoms in vineyards (Spagnolo et al., 2014). In PTA-271-pretreated plants, *PR2* was highly primed after pathogen inoculation, and to a weakest extent for *LOX9* as JA-dependent, *GST1*, *PAL,* and *STS* associated to secondary metabolism, or *NCED2* involved in ABA biosynthesis. Interestingly, the expression of *PR2* gene is described to be regulated by various phytohormones such as SA, JA, and ET (Liu et al., 2010). Up to date, it is still unclear how the SA-induced cellular changes can influence JA-inducible responses (Caarls et al., 2015). Pretreatment with PTA-271 might thus up-regulate *PR2* expression in a JA-dependent way. *PR2* encodes a β-1,3-glucanase, which could play an important role in grapevine defense, either directly, through the degradation of pathogen cell wall, or indirectly, by releasing oligosaccharide elicitors that could induce additional plant defenses (Renault et al., 2000). Although this priming effect essentially but indisputably concerns more *PR2* following Np-Bt67 challenge, it does not counter balance the PTA-271 priming capacity toward grapevine pathogens. Indeed, primed plants usually show no enhanced expression of phenotypic defense traits, but they respond faster or more strongly following the pathogen challenge inoculation (Conrath et al., 2006; Goellner and Conrath, 2008), as observed for *PR2* with PTA-271 treated cuttings at this time point of analysis (4 dpi). Verhagen et al. (2011) also showed a PTA-271 capacity to induce slight plant leaf defense responses, but further potentiated upon *B. cinerea* challenge (from 3 to 7 dpi, using a plantlet model). We also showed (Trotel-Aziz et al., 2008) that PTA-271 can stimulate JA/ET-dependent defenses in grapevine against the necrotrophic fungus *B. cinerea*. Regarding the expression of *NCED2* primed by PTA-271 upon pathogen challenge, our data cannot exclude a possible contribution of ABA biosynthesis to an enhanced JA biosynthesis (Adie et al., 2007) that remains to be further elucidated.

Deciphering now grapevine immune response using plantlets directly exposed to (*R*)-mellein and (-)-terremutin (**Figure 5**), our data showed that application of (*R*)-mellein and (-)-terremutin resulted in up-regulation of the SA-responsive genes *PR1* and *GST1*, respectively (Devadas et al., 2002). *GST1* is also part of the array of defense-related genes induced in response to oxidative burst produced after pathogen infection (Bhattacharjee,

2012). Contrary to SA-responsive genes, the expression of JA/ET-responsive genes remained weak as shown previously during grapevine-*Np*-Bt67 interaction, or even down-regulated by (-)-terremutin (i.e., *PR3*). This can be supported by the fact that (-)-terremutin is a derivative of 6-methyl-SA (Guo et al., 2014) as a mobile signal easily hydrolysable to active SA (Park et al., 2007; Kumar and Klessig, 2008). It is thus tempting to correlate (-)-terremutin to the necrotrophic stage of *Np*-Bt67 lifestyle, and to speculate the mimicking of SA effect to antagonize JA-dependent defenses. In contrast, (*R*)-mellein induced both the SA-responsive *PR1* and *PR5* and to a weaker extent the JA/ET-dependent *PR4* gene. Thus, (*R*)-mellein produced by *N. parvum* might be mainly in link with the biotrophic and early necrotrophic stages of pathogen with hemibiotrophic lifestyle (Duan et al., 2014; Ross et al., 2014; Yang et al., 2015). In PTA-271 pretreated plantlets (**Figure 5**), both JA/ET- and SA-responsive genes were up-regulated, as well as an ABA biosynthetic gene (*NCED2*) and phenylpropanoid pathway genes (*PAL*, *STS*) in *in vitro*-plantlets. These data are in agreement with those of Trotel-Aziz et al. (2008) using the same plantlet model. However, exogenous application of (-)-terremutin and (*R*)-mellein repressed the expression of almost all of the PTA-271 up-regulated host-defense-genes. The enhanced expression of *GST1* by PTA-271 was weakly repressed by (-)-terremutin, suggesting that GST could take part to the detoxification process of (-)-terremutin or maybe in the redox regulation in SA/JA crosstalk. Some authors have indicated that overexpression of *GST1* might mediate redox changes to prevent some pathogen aggressive molecules to mimic SA-signaling to overcoming host immunity (Tada et al., 2008; Vidhyasekaran, 2015). Interestingly, up-regulation of *NCED2* by PTA-271 was not altered by fungal toxins, emphasizing the role of ABA as a central component to overcome toxin effects by a possible enhancement of JA synthesis (Adie et al., 2007; Mohr and Cahill, 2007; Spoel and Dong, 2008). Many studies reported that endogenously accumulated SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent resistance over JA-dependent defense (Pieterse et al., 2012; Van der Does et al., 2013). Indeed *PR1* was the sole gene still over-induced in PTA-271 pretreated plantlets exposed to each pure toxin. This is consistent with our hypothesis of a SA mimicking effect to antagonize the host JA-dependent defenses. Deciphering the extend of cross-communication in the hormone signaling pathways, through fine tuning of transcriptional programs, would thus enable to better understand the mechanisms contributing to grapevine basal and induced resistances to GTD pathogens. The potential roles of *GST1* overexpressed in the presence of (-)-terremutin, and of *NCED2* upregulated in the presence of PTA-271, would now merit a greater attention.

PTA-271 beneficial effect might also target grapevine detoxifying capacity on GTD-secreted phytotoxins. Control plantlets can mobilize both (*R*)-mellein and (-)-terremutin when exogenously applied at the root level (**Figure 6**). (*R*)-Mellein is entirely mobilized and may be accumulated *in planta* in its native chemical form, while (-)-terremutin was partly mobilized and was not accumulated *in planta*. In contrast, in PTA-271-pretreated plantlets, only (*R*)-mellein mobilization was slightly reduced. Treatment with PTA-271 might thus slow down the (*R*)-mellein uptake by grapevine plantlets. The distinct chemical structures of each toxin still remain to be investigated (i.e., toxin conjugates), as well as the mechanisms slowing down (*R*)-mellein entry in plantlets, to better understand how PTA-271 might exert its beneficial effects on grapevine's detoxifying capacity.

CONCLUSION

Altogether, our results provide evidences that (-)-terremutin and (*R*)-mellein are usefull molecules for *N. parvum* that can secrete them inside the host to fully express its virulent character. Once inside the plant (-)-terremutin and (*R*)-mellein may reprogram grapevine immunity enabling the pathogen to overcome host defenses and thus promote disease. However, the beneficial bacterium PTA-271 significantly attenuated the Botryosphaeria dieback symptoms, by antagonizing *N. parvum* growth, inducing plant systemic resistance as shown by the strong *PR2* priming among the few host defense responses in the tested time point, and detoxifying both (*R*)-mellein and (-)-terremutin produced by *Np*-Bt67.

AUTHOR CONTRIBUTIONS

PTA planned and designed the research, performed most of the experiments, analyzed the data, and wrote the manuscript with the contributions and discussion from AA, FF, EAM, and CC. FF, AA, EAM, and CC validated the planned research and gave their expertise for all steps of this work. EAM prepared all purified toxins for the experiments. BC performed most of the qRT-PCR experiments and prepared grapevine plantlets. FR ensured the quality of qRT-PCR analysis and data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00025/full#supplementary-material

FIGURE S1 | Toxicity assessment of (*R*)-mellein and (-)-terremutin toward *B. subtilis* PTA-271 (Bs) in 24 h. PTA-271 was sprayed on PDA plates containing one central phytotoxin drop (5 μL) from 0 to 60 mg/L for (*R*)-mellein (M0 to M60) or (-)-terremutin (T0 to T60).

FIGURE S2 | Toxicity assessment of (*R*)-mellein and (-)-terremutin toward *in vitro*-plantlets. Eight weeks old plantlets were exposed to MS medium containing (*R*)-mellein or (-)-terremutin at concentrations ranging from 0 to 1500 μg/L. Three days post-exposure (dpe) to phytotoxin, toxicity was assessed through maximum quantum yield of photosynthesis (Fv/Fm), fresh weight and pigment concentrations. The maximum photosynthetic capacity of the plants was obtained by measuring the Fv/Fm parameter given by a PAM-Pulse Amplitude Modulated fluorimeter equipped with the Modfluor v2.00 software (Hansath, London, United Kingdom) according to the recommendations of Genty et al. (1990). The content of chlorophylls a, b, and carotenoids was obtained by colorimetric assay (spectrophotometry at 470, 652.4, and 665.2 nm) after pigments extraction in pure methanol (20 min at 65°C) and quantified according to Wellburn (1994) formulas. Data are means ± SD of three independent experiments, each with four replicates. None bars were headed with asterisks, indicating none significant differences (Multiple Comparison procedures with Tukey's test, $P < 0.05$).

FIGURE S3 | *B. subtilis* PTA-271 primes some defense-related genes in leaves of grapevine cuttings after infection with the *N. parvum* strain *Np*-Bt67. Legend as in **Figure 4**. Three-color scale as in **Figure 5**, with deep red corresponding to an induction factor of 23.71 or more, and dark blue corresponding to a 0.55-fold induction or less.

FIGURE S4 | (*R*)-Mellein and (-)-terremutin repress the *B. subtilis*-PTA-271-induced immune responses in grapevine plantlets. Different letters indicate significant differences. Legend as in **Figure 5**.

TABLE S1 | Primer sequences used for qRT-PCR analysis of defense-related genes.

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The *Rpv3-3* Haplotype and Stilbenoid Induction Mediate Downy Mildew Resistance in a Grapevine Interspecific Population

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The development of new resistant varieties to the oomycete *Plasmopara viticola* (Berk.& Curt) is a promising way to combat downy mildew (DM), one of the major diseases threatening the cultivated grapevine (*Vitis vinifera* L.). Taking advantage of a segregating population derived from "Merzling" (a mid-resistant hybrid) and "Teroldego" (a susceptible landrace), 136 F1 individuals were characterized by combining genetic, phenotypic, and gene expression data to elucidate the genetic basis of DM resistance and polyphenol biosynthesis upon *P. viticola* infection. An improved consensus linkage map was obtained by scoring 192 microsatellite markers. The progeny were screened for DM resistance and production of 42 polyphenols. QTL mapping showed that DM resistance is associated with the herein named *Rpv3-3* specific haplotype and it identified 46 novel metabolic QTLs linked to 30 phenolics-related parameters. A list of the 95 most relevant candidate genes was generated by specifically exploring the stilbenoid-associated QTLs. Expression analysis of 11 genes in *Rpv3-3*$^{+/-}$ genotypes displaying disparity in DM resistance level and stilbenoid accumulation revealed significant new candidates for the genetic control of stilbenoid biosynthesis and oligomerization. These overall findings emphasized that DM resistance is likely mediated by the major *Rpv3-3* haplotype and stilbenoid induction.

Keywords: disease symptom phenotyping, "Merzling" *Plasmopara viticola*, peroxidase, polyphenols, QTL analysis, stilbenes, *Vitis* spp.

INTRODUCTION

Around 68,000 tons of fungicides per year are used in Europe to manage grape diseases, i.e., 65% of all fungicides used in agriculture although viticulture encompasses only 4% of the EU arable land (Eurostat, 2007). A useful strategy to reduce the impact of pesticides on humans, animals and environment is based on genetic improvement, in particular on the introgression of resistance traits from ancestral species into domesticated varieties. The cultivated grapevine (*Vitis vinifera* L.) is

highly susceptible to downy mildew (DM), caused by the biotrophic oomycete *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni, the major disease of temperate-humid climate among various pathogen threats.

A total of 27 Quantitative Trait Loci (QTLs) associated with DM resistance in different genetic backgrounds are known and described (VIVC, 2018). In particular, the major *Rpv* loci originated from *Muscadinia rotundifolia* (Merdinoglu et al., 2003), *Vitis riparia* (Marguerit et al., 2009; Moreira et al., 2011), *V. amurensis* (Blasi et al., 2011; Schwander et al., 2012; Venuti et al., 2013), *V. cinerea* (Ochssner et al., 2016), and *V. rupestris* (Divilov et al., 2018). To close the list, the *Rpv3* locus is a major determinant of grapevine DM resistance. Seven conserved *Rpv3* haplotypes were identified in five descent groups of resistant varieties and traced back to their founders, which belong to *V. rupestris, V. lincecumii, V. riparia,* and *V. labrusca* (Di Gaspero et al., 2012). Until now only two haplotypes at this locus were validated in segregating populations derived from different DM resistance donors (Welter et al., 2007; Zyprian et al., 2016).

The related resistance mechanisms are partially known and are due to gene-for-gene recognition, thus signal cascade and finally defense response. A widespread hot spot of NBS-LRR genes was identified within the genomic region where the *Rpv3* locus resides, providing a distinctive advantage for the adaptation of native North American grapevines to resist to *P. viticola* (Moroldo et al., 2008). The defense response also involves the synthesis of secondary metabolites including the stilbenoids. In fact, besides necrosis (e.g., Boso Alonso and Kassemeyer, 2008; Peressotti et al., 2010) and callose deposition (e.g., Gindro et al., 2003), DM resistance can be accompanied by stilbene accumulation (e.g., Alonso-Villaverde et al., 2011; Malacarne et al., 2011; Mattivi et al., 2011) as activated defense mechanisms. To date no study has investigated the genetic basis of polyphenol, in particular stilbenoid, synthesis or its variation upon *P. viticola* infection. QTLs associated with synthesis of most polyphenols are not known; only a few research studies on proanthocyanidins, anthocyanins, and flavonol berry composition have recently been attempted in grapevine (Fournier-Level et al., 2009, 2011; Huang et al., 2012, 2013, 2014; Viana et al., 2013; Ban et al., 2014; Azuma et al., 2015; Costantini et al., 2015; Guo et al., 2015; Malacarne et al., 2015).

In this work, we aim to characterize the locus conferring resistance against *P. viticola* and to identify new polyphenol-related loci in order to shed light on the DM resistance mechanisms in an interspecific segregating population derived from the source "Merzling.'

MATERIALS AND METHODS

Segregating Population

An interspecific segregating population of 136 putative full-sib individuals derived from the cross between the complex *Vitis* hybrid "Merzling," descending from *V. vinifera, V. rupestris,* and *V. lincecumii*, and the *V. vinifera* cv "Teroldego" was studied. This cross between "Merzling," mid-resistant to DM and high-stilbenoid producer, and "Teroldego," susceptible to DM and low-stilbenoid producer, was performed at FEM (Edmund Mach Foundation, San Michele all'Adige, Italy) in 1989. During the 2012 growing season the progeny were propagated as grafted plants in 1L-pots filled with soil:sand:peat:vermiculite (3:1:3:3, v/v) in a greenhouse at 25/20°C day/night temperature, with a 16 h photoperiod and relative humidity (RH) of 70 ± 10%. The propagation of the two parental lines was carried out during 2013.

Phenotyping

DM Resistance Assessment

To collect sufficient fresh inoculum, *P. viticola* propagation was performed on leaves of the susceptible *V. vinifera* cv "Pinot gris" following the protocol by Vezzulli et al. (2018). Six potted plants (PP) per each progeny individual were maintained in two different growth chambers as three biological replicates for *P. viticola*- and mock-inoculation, respectively. Firstly, in June 2012 fully expanded leaves of the 10 week-old PP were *P. viticola*-inoculated (PI) by spraying a suspension of 1×10^5 sporangia/ml onto the abaxial leaf surface and were kept overnight in the dark in a growth chamber at 24°C with 80% RH; mock-inoculated (MI) samples were obtained by analogously spraying distilled water on PP kept under equal conditions. Secondly, in August 2012 the 4–5th leaves from the MI-PP apex of each progeny individual were collected to generate leaf disks (LD) that were inoculated according to Vezzulli et al. (2018). In June and August 2013 the same experiments were respectively repeated on PP and LD of 11 representative progeny individuals along with "Merzling" and "Teroldego" parental lines. Finally, the DM response was evaluated at 8 days post-inoculation (dpi) on PP and at 4, 5, and 6 dpi on LD by means of three parameters evaluated by visual inspection: disease severity (percentage of the disc area showing symptoms of sporulation) and disease incidence (number of discs with sporulation/total number of discs), according to OEPP/EPPO (2001), along with the descriptors OIV 452 for PP or OIV452-1 for LD (overall degree of resistance), recommended by the Organization Internationale de la Vigne et du Vin (OIV, 2009) and adapted according to Bellin et al. (2009) (**Table S1A**). The latter descriptors, ranking from 1 to 9, are positively correlated with the magnitude of plant response and inversely correlated with the severity of DM symptoms. All percentage data were Arcsin transformed in view of following statistical analysis.

Polyphenol Content Measurement

The 2nd−3rd leaves from the PI- and MI-PP apex were collected at 6 dpi and analyzed for the content of 42 phenolics (18 different stilbenoids) by targeted metabolomics (Vrhovsek et al., 2012), according to Chitarrini et al. (2017) with some modifications. For each metabolite, missing values (indicating concentrations below the quantification limit) were imputed by random sampling from a uniform distribution taking values in the range from 0 to the metabolite specific detection limit. Genotype specific levels were estimated as the median value of each metabolite across three biological replicates. To compensate for the expected non-normal distribution of metabolite concentration, genotype-specific data were subjected to logarithmic transformation (ln). Metabolomic data were used to calculate 22 sum/ratio parameters

(**Table S1B**). In all analyses, the difference (delta) between the PI and MI genotype specific values were considered.

Genotyping and Map Construction

Genomic DNA from the overall 138 studied genotypes was isolated from young leaves using DNeasy Plant Mini Kit (Qiagen, The Netherlands). The 136 putative full-sib individuals and the two parental lines were characterized at the genotypic level by means of 192 microsatellite (Simple Sequence Repeat, SSR) markers, corresponding to an average of ca. 10 SSRs well-scattered along each of the 19 grapevine chromosomes. The microsatellites were chosen based on their polymorphism information in "Merzling" and/or "Teroldego" reported by Salmaso et al. (2008), as well as their unique position and physical distance along the reference genome (http://www.genoscope.cns.fr) (**Table S2**). The applied mid-throughput genotyping strategy was as reported in Peressotti et al. (2015). Prior to building the "Merzling" × "Teroldego" (M×T) genetic map, SSR markers were tested against the expected segregation ratio using a $\chi 2$ goodness-of-fit implemented in JoinMap v.4.1 (JM, Van Ooijen, 2006). Highly distorted ($p > 0.05$) markers were discarded, while the others ($p \leq 0.05$) were used for linkage analysis unless they affected the order of neighboring loci. Molecular markers were grouped and ordered along linkage groups (LGs) using the Kosambi mapping function implemented in JM. Mapping parameters were set at a logarithm of odds (LOD) value of 8 and at a recombination frequency of 0.45. LG number was assigned according to Adam-Blondon et al. (2004) and the linkage map was visually represented with MapChart v.2.2 (Voorrips, 2002).

QTL Analysis and Candidate Gene Selection

Genetic map data were integrated with phenotypic data and QTL mapping was performed separately per each experiment by using the simple Interval Mapping algorithm in MapQTL v.6.0 (Van Ooijen, 2009). QTLs were declared significant if the maximum LOD exceeded the LG-specific LOD threshold (calculated using 1,000 permutations) and mean error rate was <0.05. Linkage groups and QTLs were visualized with MapChart2.2 (Voorrips, 2002) and a R *ad-hoc* script (R Core Team, 2018). Through the associated markers, each reliable QTL interval was further anchored and aligned on the assembled version of the grapevine reference genome (Jaillon et al., 2007). For the ease of calculation, base pairs (bp) were converted into centiMorgans (cM) by dividing by 433,989, which is the mean physical distance corresponding to 1 cM derived in this mapping work.

In order to characterize these genomic regions, the 12X PN40024 reference genome (http://genomes.cribi.unipd.it) was exploited to extract version 2 (V2) of the gene predictions (GPs) underlying QTLs. The gene annotation adopted was the one reported by Vitulo et al. (2014) and candidate genes (CGs) were selected adopting the following criteria:

i. Proximity to LOD peak offset (in case of large genomic intervals);
ii. Involvement in trait regulation based on literature (reference genes);
iii. Assignment to significantly over-represented functional categories. In particular, a Fisher's exact test was applied to evaluate the over-representation of specific functional categories within QTLs controlling a given parameter. Reference to our analysis was the distribution in the same categories of 31,922 12Xv2 GPs assigned to chromosomes (Vitulo et al., 2014). We considered GPs annotated at the third level, with the exception of GPs annotated at the first and second level if not characterized at a deeper level and significantly represented in the genome. Adjusted *p*-values by the Benjamini and Hochberg (1995) method for multiple-testing correction were considered significant when ≤ 0.05. For the functional categories significantly enriched a fold enrichment (Fold-change), as the ratio between the frequency of a category in the QTLs controlling a given parameter vs. the one in the genome, was calculated. The functional categories that passed the Fisher's test and having a Fold-change > 0 were considered as over-represented;
iv. Involvement in functional categories of interest.

Quantitative RT-PCR Expression Analysis

Total RNA was isolated from 60 to 80 mg of ground leaves, collected at 6 dpi from each biological replicate (the same powder used for biochemical analysis stored at −80°C), using Spectrum Plant Total RNA Kit (*Sigma-Aldrich*) according to manufacturer's instructions. cDNAs were synthetized using the SuperscriptVILO™ cDNA Synthesis Kit from 1.5 μg of DNAseI-treated RNA (*Thermo Fisher Scientific*). Primer sequences were derived from literature or designed on the reference CG sequence using Primer3 v.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) according to MIQE guidelines suggestions (Bustin et al., 2009) (**Table S3**). Due to the high sequence homology among different family members, it was not always possible to design gene-specific primers. Each primer pair was tested by semi-quantitative RT-PCR on cDNAs from one F1 individual, "Merzling" and "Teroldego" parental lines. The corresponding amplicons were checked by electrophoresis and, prior purification by ExoSAP (*Euroclone*), were Sanger sequenced to verify the specificity of the primer pair. Forward and reverse reads were aligned to the reference CG sequences using Staden Package software (http://staden.sourceforge.net/) in order to confirm their uniqueness.

Finally, the primer pairs that passed this step were employed in qRT-PCR analyses, carried out using the Platinum SYBR Green qPCR SuperMix-UDG in a ViiA™7 thermocycler (*Thermo Fisher Scientific*). The 384-well plates were set up according to the sample maximization strategy proposed in Hellemans et al. (2007). Each sample was examined in three technical replicates, and dissociation curves were analyzed to verify the specificity of each amplification reaction. Reaction conditions and the analysis protocol were the same as adopted in Malacarne et al. (2015). Six housekeeping genes (*VvACT, VvATP16, VvEF1α*, *VvGAPDH*, *VvSAND*, and *VvUBIQ*; **Table S3**) were tested for their stability using GeNorm software (Vandesompele et al., 2002). Normalized relative quantities (NRQs) were then calculated by dividing

the RQ by a normalization factor, based on the expression of the two most stable reference genes (*VvATP16* and *VvGAPDH*) (Reid et al., 2006).

Statistical Analysis

Statistical analyses applied to phenotypic data were performed in R (R Core Team, 2018) equipped with tidyverse (Wickham, 2017) https://CRAN.R-project.org/package=tidyverse and ggplot2 (Wickham, 2016) packages. The reproducibility between years was assessed by checking (*t*-test, $p \leq 0.05$) for the presence of a significant difference in the values of the three phenotypic parameters in a selected group of genotypes. In the case of the 64 polyphenol-related parameters, the reproducibility between years was assessed by testing the difference between PI and MI with a non-parametric Wilcoxon test ($p \leq 0.05$, corrected for multiplicity applying a Bonferroni correction). The same test was applied to evaluate the presence of a significant induction of the 64 parameters in the PI *vs.* MI samples.

Phylogenetic Analysis

A total of 90 GPs, including isoforms, encoding peroxidases (IPR000823) were identified in the 12Xv2 PN40024 reference genome (http://genomes.cribi.unipd.it/DATA/V2; **Table S6**), while the 76 peroxidases from Arabidopsis (Tognolli et al., 2002) were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Codon-based alignments of all the coding gene sequences were made using Macse program (Ranwez et al., 2011). Automatically produced alignments of individual genes were loaded into the Seaview alignment editor and manually edited in amino acid mode to discard obviously misaligned regions. The final selection of alignment columns was saved to produce a 206 pos. long curated alignment containing 167 aminoacid peroxidase sequences. Phylogenetic analyses were performed with the help of Iq-Tree multicore version 1.6. beta4 (Nguyen et al., 2015). All sequences in alignment passed $\chi 2$ test of compositional heterogeneity ($p < 0.05$). The optimal substitution model for the observed alignment was automatically selected under the Bayesian Information Criterion (BIC, Schwarz, 1978) among a set of 468 substitution models. The model assumed LG (Le and Gascuel, 2008) amino-acid replacement matrix with across sites rate heterogeneity modeled via FreeRate model (Soubrier et al., 2012) assuming six rate classes. Branch support values were inferred based on 1,000 bootstrap replicates employing an ultrafast bootstrap approximation (Hoang et al., 2018) as implemented in the IQ-TREE program. Branches were named as suggested by the Super-Nomenclature Committee for Grape Gene Annotation (sNCGGa) (Grimplet et al., 2014). A bootstrap value of 70 (recommended by the Committee) distinguished the genes within the majority of the classes. Whenever a branch containing both *Vitis* and *Arabidopsis* gene/genes was found in a subtree, a subclass was defined. The remaining *Vitis* genes were named independently. Moreover, different members of a subclass were distinguished by a letter and different splicing variants had the same name but followed by a number. In the few cases in which the direct hortolog from Arabidopsis was found, both names were retained.

RESULTS

Downy Mildew Resistance and Polyphenol Content

P. viticola inoculation was performed on the entire progeny (2012) and on the two parental lines along with 11 repeated F1 individuals (2013). The reproducibility of both LD and PP experiments between the 2 years was verified by *t*-test on the three phenotypic parameters (**Figures S1A,B**) and it confirmed an overall coherence between 2012 and 2013 infection both for LD and PP. Altogether the phenotypic results indicated an approximately normal distribution of severity, incidence and OIV452(−1) in the progeny and confirmed a mid-resistant and susceptible phenotype for "Merzling" and "Teroldego," respectively. It is relevant to underline that several F1 individuals were transgressive with respect to the resistance donor "Merzling" exhibiting a higher level of resistance, while none resulted more susceptible than the parental "Teroldego" (**Figure 1**).

Analogously to the DM resistance, the polyphenol-related data—the measurement of 64 parameters on PP of 11 repeated F1 individuals (**Figure S2**)—performed between 2012 and 2013 were found to be reproducible ($p \leq 0.05$, corrected for multiplicity applying a Bonferroni correction). The differences turned out to be significant ($p \leq 0.05$) in 13 out of 64 cases, exhibiting good reproducibility overall. Moreover, in many cases (e.g., 2,6-DHBA, fertaric acid, *cis*-piceid, *cis*-resveratrol, pterorostilbene, *cis*-ε-viniferin) the range of metabolic content identified in the progeny was greater than in the parents, suggesting a transgressive segregation (**Figure S3**). A significant delta value between PI and MI plants ($p \leq 0.05$, corrected for multiplicity applying a Bonferroni correction) was detected for 32 different value distributions, each representing variation in the content of polyphenols: 22 of stilbenoid class, out of which seven monomeric (*trans*-resveratrol, piceatannol, pterostilbene, *cis*-piceid, astringin, isorhamnetin, and the sum of monomeric stilbenoids), five dimeric (*trans*-ε-viniferin, *cis*/*trans* ω-viniferin, pallidol, ampelopsin D+quadrangularin A, and the sum of dimers), four trimeric (α-viniferin, E-*cis*-miyabenol C, Z-miyabenol C, and the sum of trimers), three tetrameric (isohopeaphenol, ampelopsin H + vaticanol C-like isomer, and the sum of tetramers), the ratio between tetramers and monomers, the sum of polymers and all stilbenoids; two of benzoic acid class (2,6-DHBA and the sum of benzoic acids); the coumarin fraxin; the t-coutaric hydroxycinnamic acid; the flavanone naringenin; four flavonols (quercetin-3-glucoside, rutin, quercetin-3-glucuronide, and the sum of flavonols); and finally the sum of all polyphenols (**Figure 2**).

QTL Mapping

The M×T Linkage Map

Out of 136 initial putative full-sib individuals, 129 resulted to be true-to-type F1 individuals upon the genotyping analysis. Of these, three were discarded because they presented >20% missing data (progeny information in **Table S4**). Out of the 192 scored, 181 markers were ordered into 19 LGs allowing the

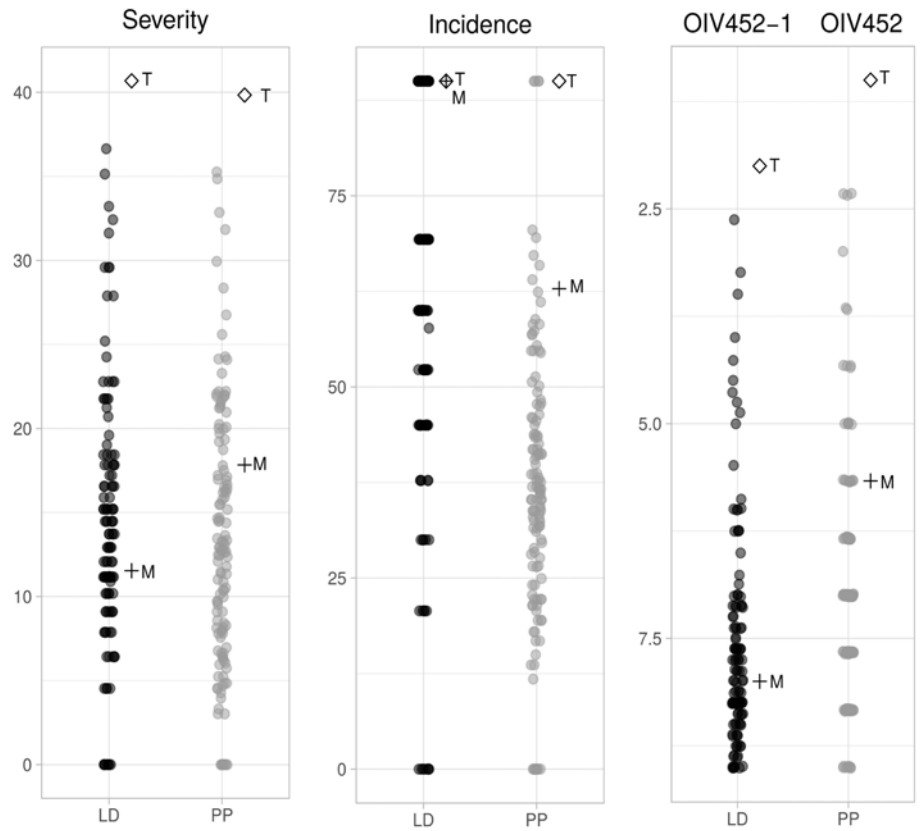

FIGURE 1 | Phenotypic distribution of the three different parameters associated with resistance to *P. viticola* in the M×T progeny. Severity and incidence are expressed in percentage while the OIV descriptor follows a discrete scale from 1 to 9. These parameters were scored at 6 days post-inoculation (dpi) for leaf disks (LD) and at 8 dpi for potted plants (PP). The data were collected in June and August 2012 for the segregating population and in June and August 2013 for the two parental lines. The reproducibility of the two experiments was tested ($p \leq 0.05$) on data collected in August 2012 and 2013 for the same genotypes as shown in **Figure S1**. M, "Merzling"; T, "Teroldego".

construction of the M×T consensus map. Marker order was generally consistent between parental and consensus homolog LGs, with local inversion of tightly linked markers, and reflected the backbone of previous published maps (Salmaso et al., 2008; Vezzulli et al., 2008). The remaining 11 markers consisted of one unlinked and 10 showing distorted segregation ratios with a probability $p \leq 0.05$. The distribution of the 181 mapped markers into different segregation types showed that 91.1% allowed discrimination between paternal and maternal inherited allele. The total length of the consensus map was 1,162.7 cM with a mean distance between adjacent markers of 6.4 cM (**Figure S4**). The overall linkage map statistics are reported in **Table S4**. This map was finally employed in a QTL mapping survey to identify putative genomic regions involved in the genetic control of DM resistance and polyphenol variation upon *P. viticola* inoculation; a total of 49 significant QTLs associated with 33 different parameters were detected on 12 LGs (**Figure 3**, **Table 1**).

The DM Resistance QTLs

Regarding the phenotypic data associated with DM resistance recorded on PP at 6 dpi, the first QTL identified was related to the severity parameter, showing a maximum LOD value of 7.12 (22.9% of explained variance), and located along the distal arm of chromosome 18 (88.3 cM). A co-localized QTL resulted associated with the OIV 452 descriptor with an LOD peak of 5.99 (19.7% of explained variance). Concerning the incidence parameter, the detected QTL was slightly shifted and showed a maximum LOD value of 3.18 (11% of explained variance). These results based on PP phenotypic data were confirmed by the results obtained on LD where a lower LOD peak was calculated per each parameter, except for disease incidence, which is more sensitive to the intra-plant leaf variability (**Table 1**, **Figure 3**). This overall genomic interval, ranging from 22.6 to 27.8 Mb of LG 18, co-localizes with the known *Rpv3* locus which is characterized by the flanking UDV305 and UDV737 SSR markers (Di Gaspero et al., 2012). Given their genetic profile in this work, the haplotype *Rpv3* $^{null-271}$ was identified as associated with DM resistance in "Merzling" and named *Rpv3-3*, according to the rules established by the international grapevine research community (www.vitaceae.org; http://www.vivc.de/). Not reliable, confirmed twice between PP and LD experiments, minor QTLs were also identified (data not show).

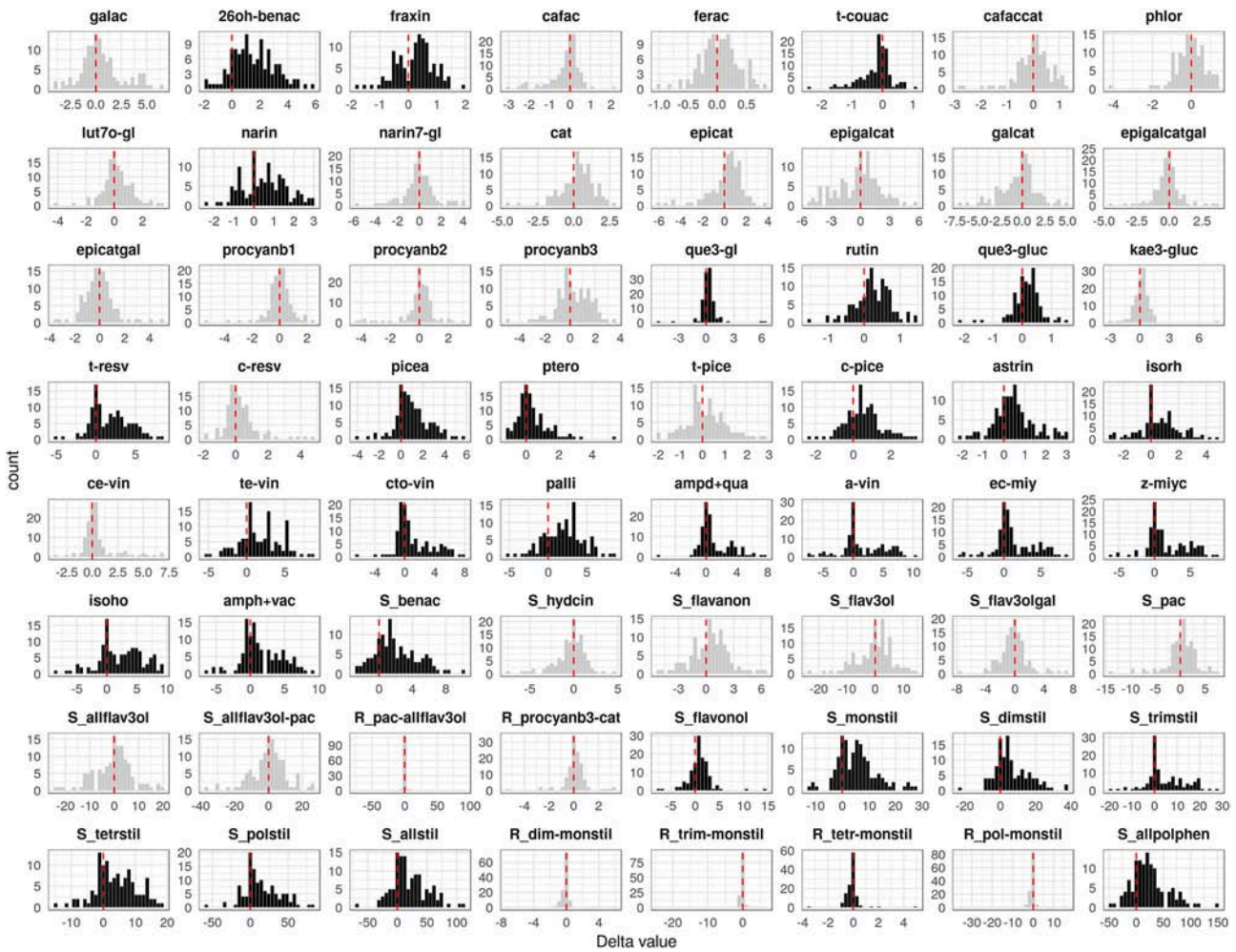

FIGURE 2 | Distribution of the difference between *P. viticola*-inoculated (PI) and mock-inoculated (MI) values (delta) associated with 64 polyphenol-related parameters recorded in the M×T progeny. Black distributions highlight significantly modulated compounds. The meaning of the parameter abbreviations is reported in **Table S1B**.

The Polyphenol-Related QTLs

As concerning polyphenol variation, significant QTLs were newly detected for a total of 30 parameters, including both the original and the derived ones. LOD peak values spanned from 2.52 to 4.28 (**Table 1**, **Figure 3**).

Overall, considering the QTL physical distribution, four clusters were located on LGs 18, 17, 16, and 15 by abundance order. In particular, a comprehensive cluster of QTLs positioned on LG 18 was associated with caffeic acid+catechin condensation, five parameters related to monomeric stilbenoids, *cis*-ε-viniferin, epicatechin gallate and procyanidin B1, and did not result overlapping with the distal region associated with DM resistance (**Figure 3**).

Regarding hydroxycinnamic acids, one QTL associated with fertaric acid (LOD peak value of 3.68) and one to caffeic acid+catechin condensation (LOD peak value of 2.64) were located on LGs 2 and 18, explaining, respectively 13.2 and 9.6% of the total phenotypic variance. In terms of benzoic acids, gallic acid, and 2,6-DHBA were correspondingly mapped on LGs 12 and 16, with 9.8 and 10.1% of explained variance.

Within the group of monomeric stilbenoids, three QTLs were found to be associated with *cis*-resveratrol on LGs 5, 9, and 3, explaining 10.8, 9.8, and 9.4% of total variance, respectively. A QTL for pterostilbene was located on LG 16 with a LOD peak value of 3.89, which corresponded to 13.9% of explained variance. Two QTLs, positioned on LGs 16 and 18, were related to the *cis*-piceid content (11.8 and 12.5% of explained variance), while *trans*-piceid was mapped only on the LG 18 region which explained a similar variance. Astringin was under control of three genomic regions (LGs 16, 4, and 18) which corresponded to a range from 10.1 to 12.7% of the total phenotypic variance. Two QTLs associated with isorhapontin were positioned on LGs 18 and 17, with 13.2 and 11.8% of explained variance. Finally, a QTL corresponding to the region controlling the content of all the monomeric stilbenoids mentioned above was found on LG 18 for the sum of monomeric stilbenoids. Concerning

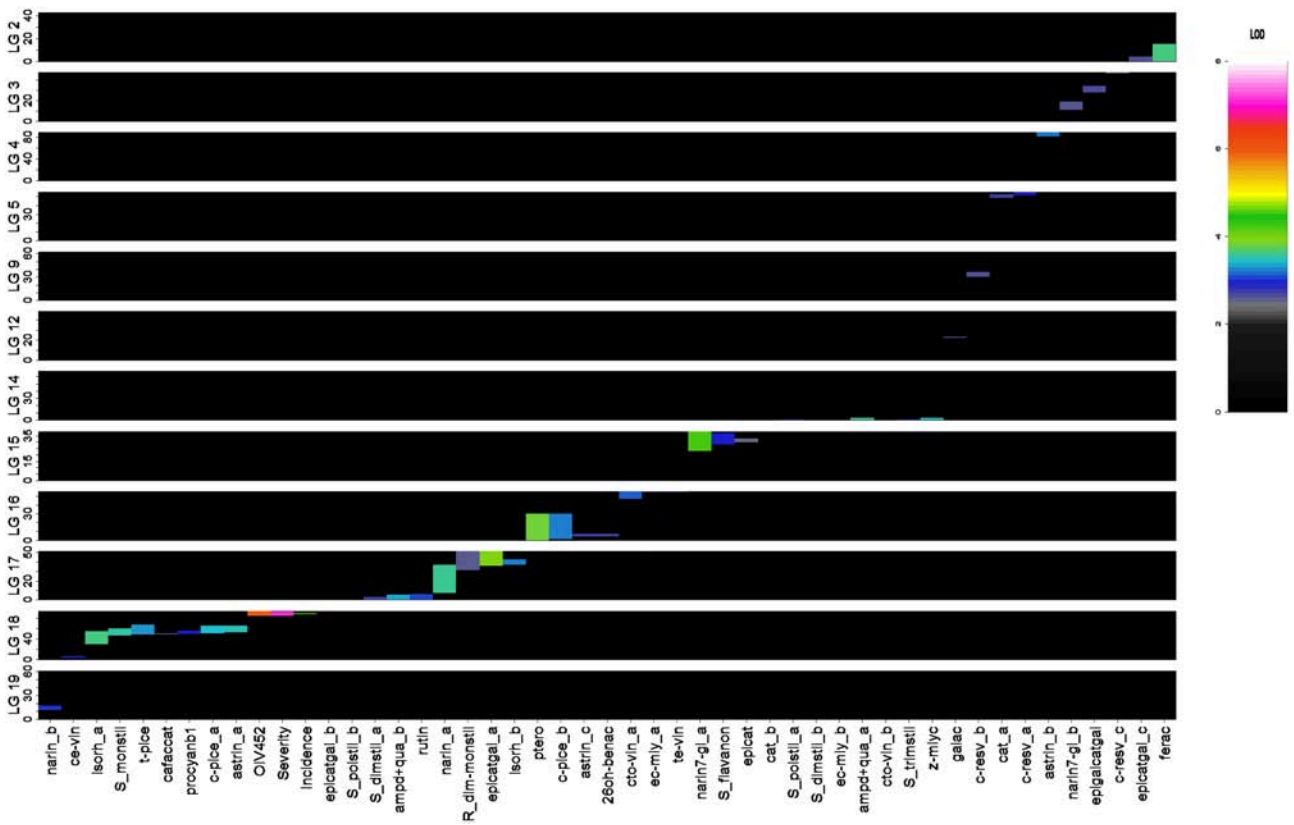

FIGURE 3 | QTL distribution across the 19 M×T LGs. The meaning of the parameter abbreviations is reported in **Table S1**.

polymeric stilbenoids, *cis*-ε-viniferin was mapped on LG 18, in a region upstream to the one associated with monomeric stilbenoids and encompassing 10.9% of the total phenotypic variance; indeed, a QTL related to the *trans*-ε-viniferin content, explaining a highly similar variance, was detected on LG 16 in a region far from the one associated with the monomeric stilbenoids and coincident with the one explaining the 9.7% of the variance of E-*cis*-miyabenol content described below. *Cis*+*trans*-ω-viniferin was under control of two genomic regions (LGs 16 and 14) which corresponded to the 11.5 and 10.7% of the total phenotypic variance. In particular, 85% of the LG 16 region was specifically associated with this specific dimer. Two genomic regions associated with ampelopsin D+quadrangularin A were identified on LGs 14 and 17; these QTLs showed a LOD peak value of 3.66 and 3.42, with 13.1 and 12.3% of explained variance, respectively. Two QTLs, positioned on LGs 16 and 14, were related to the E-*cis*-miyabenol content (9.7 and 9.6% of explained variance), while Z-miyabenol C was mapped only on the LG 14 region which explained 12.6% of the total phenotypic variance. Considering derived parameters, two QTLs were identified on LGs 17 and 14 for the sum of dimeric stilbenoids (a mean of 10% of explained variance), whereas the sum of trimeric stilbenoids was associated only with the QTL on LG 14, explaining 11.2% of the total phenotypic variance. For total polymeric stilbenoids, including tetrameric stilbenoids, the two QTLs identified on LGs 14 and 17 were confirmed, with 10.7 and 9.9% of explained variance. Finally, a QTL was detected as associated with the ratio of dimeric to monomeric stilbenoids on a different region of LG 17, explaining 9.6% of the total variance. This region was also controlling the isorhapontin content as previously highlighted. Contrary to LG17, on LG14 the region associated with all the parameters previously described was coincident.

Within the flavanon group, two QTLs related to the naringenin content were found on LGs 17 and 19 (LOD peak value of 3.64 for the major QTL), with 13 and 11.1% of explained variance, respectively. The 56% of the first and the 100% of the second region were specifically associated with naringenin. Indeed, naringenin-7-glucoside was mapped on LGs 15 and 3 (LOD peak value of 4.28 for the major QTL), which respectively explained 15.2 and 9.7 of the total phenotypic variance. In particular, the 72% of the region on LG 13 was specific for this flavanon. For total flavanones, only the QTL located on LG 15 was shared, with 10.8% of explained variance. Regarding flavan-3-ol monomers and dimers, for catechin two QTLs were detected on LGs 5 and 15—of which the first one was private—explaining 10.1 and 9.1% of the total phenotypic variance, respectively. Two QTLs, the first one positioned on LGs 15 coincidently with the region associated with catechin and the second one on LG 3, were respectively related to the epicatechin and epigallocatechin gallate content, explaining a similar percentage of the total

TABLE 1 | List of the 49 identified significant QTLs associated to three DM resistance and 30 polyphenol-related parameters.

Trait	Class	Parameter	Experiment	LG	LOD threshold ($\alpha = 5$)	LOD peak	LOD peak position (cM)	LOD peak offset (bp)	% Expl Var	Close-to-QTL-start SSR	Close-to-QTL-end SSR
Downy mildew resistance	–	Severity	Leaf Disks	18	2.7	5.73	89.312	26483989	19.9	UDV737	UDV737
			Potted Plants	18	2.8	7.12	88.312	24699030	22.9	VVIN16	UDV737
		Incidence	Leaf Disks	18	2.8	4.33	89.312	26483989	15.4	UDV737	UDV737
			Potted Plants	18	2.8	3.18	88.312	24699030	11.0	VVIN16	UDV737
		OIV 452-1	Leaf Disks	18	2.7	4.17	89.312	26483989	14.9	UDV737	UDV737
		OIV 452	Potted Plants	18	2.7	5.99	88.312	24699030	19.7	VVIN16	UDV305
Polyphenol content	Hydroxycinnamic acids	ferac	Potted Plants	2	2.4	3.68	0.000	2349171	13.2	VVIB01	VMC6F1
		cafaccat	Potted Plants	18	2.6	2.64	50.020	10721189	9.6	VVCS1H	VVCS1H
	Benzoic acids	galac	Potted Plants	12	2.5	2.69	22.593	6174660	9.8	VCHR12A	VCHR12A
		26oh-benac	Potted Plants	16	2.5	2.78	6.388	9994586	10.1	UDV104	UDV009
	Flavanones	narin	Potted Plants	17	2.6	3.64	23.182	3562640	13	VVIQ22-2	VMC9G4
				19	2.6	3.07	14.411	2124093	11.1	UDV023	UDV023
		narin7-gl	Potted Plants	15	2.5	4.28	30.709	16280816	15.2	VMC5G8	VMC8G3-2
				3	2.5	2.65	14.000	1817755	9.7	VMC8F10	VMC8F10
		S_flavanon	Potted Plants	15	2.4	2.99	30.709	16292317	10.8	VVIV24	VMC4D9-2
	Flavan-3-ol monomers and dimers	cat	Potted Plants	5	2.7	2.79	50.351	20620536	10.1	VMC9B5	VMC9B5
				15	2.4	2.52	30.709	15943274	9.2	VVIM42-2	VMC4D9-2
		epicat	Potted Plants	15	2.4	2.57	30.709	16292317	9.4	VVIV24	VMC4D9-2
		epigalcatgal	Potted Plants	3	2.5	2.70	30.471	4136318	9.9	UDV021	VMC1A5
		epicatgal	Potted Plants	17	2.5	4.02	44.395	8038301	14.3	VVIB09	VVIP16
				18	2.7	2.80	93.424	24868000	10.2	UDV305	UDV305
				2	2.5	2.68	0.000	2349171	9.8	VVIB01	VVIB01
		procyanb1	Potted Plants	18	2.9	2.99	53.134	12072631	10.9	VVCS1H	VVIM10
	Flavonol	rutin	Potted Plants	17	2.5	3.10	2.000	3033023	11.2	VMC3C11-1	VMC2H3
	Monomeric stilbenoids	c-resv	Potted Plants	5	2.6	2.99	55.224	24864769	10.8	VMC4C6	VMC4C6
[-10pt]				9	2.5	2.69	34.482	15302997	9.8	VCHR9A	VCHR9A
				3	2.5	2.57	47.034	11617504	9.4	VVMD28	VVMD28
		ptero	Potted Plants	16	2.5	3.89	16.522	9583955	13.9	UDV013	SC80189026
		t-pice	Potted Plants	18	2.8	3.37	56.134	13374598	12.1	VVCS1H	VVIP08
		c-pice	Potted Plants	18	2.9	3.48	55.134	12175152	12.5	VVIM10	VVIP08
				16	2.5	3.28	6.388	4092596	11.8	UDV104	SC80189026
		astrin	Potted Plants	18	2.8	3.53	57.134	12138633	12.7	VVIP08	VVIP08
				4	2.6	3.26	88.528	23105311	11.8	VMC6G10	VMC6G10
				16	2.4	2.78	6.000	9826198	10.1	UDV104	UDV009
		isorh	Potted Plants	18	2.8	3.69	49.020	9521743	13.2	VCHR18A	VVIM10
				17	2.5	3.29	39.977	9113940	11.8	VVIB09	VVIB09
		S_monstil	Potted Plants	18	2.8	3.58	54.134	12506620	12.8	VVCS1H	VVIM10
	Polymeric stilbenoids	ce-vin	Potted Plants	18	2.8	3.02	5.715	3362208	10.9	VMC8B5	VMC8B5
		te-vin	Potted Plants	16	2.6	2.74	55.429	21844881	10	SCU14	SCU14
		cto-vin	Potted Plants	16	2.6	3.18	51.154	21027861	11.5	VMC5A1	SCU14
				14	2.8	2.94	0.000	1413666	10.7	VMCNG1E1	VMCNG1E1
		ampd+qua	Potted Plants	14	2.7	3.66	0.000	1413666	13.1	VMCNG1E1	VMCNG1E1
				17	2.6	3.42	0.000	2165045	12.3	VMC3C11-1	SCU06

(Continued)

TABLE 1 | Continued

Trait	Class	Parameter	Experiment	LG	LOD threshold ($\alpha = 5$)	LOD peak	LOD peak position (cM)	LOD peak offset (bp)	% Expl Var	Close-to-QTL-start SSR	Close-to-QTL-end SSR
		ec-miy	Potted Plants	16	2.5	2.65	55.429	21844881	9.7	SCU14	SCU14
				14	2.6	2.64	0.000	1413666	9.6	VMCNG1E1	VMCNG1E1
		z-miyc	Potted Plants	14	2.8	3.52	0.000	1413666	12.6	VMCNG1E1	VMCNG1E1
		S_dimstil	Potted Plants	17	2.4	2.79	0.000	2165045	10.2	VMC3C11-1	VMC3C11-1
				14	2.7	2.69	0.000	1413666	9.8	VMCNG1E1	VMCNG1E1
		S_trimstil	Potted Plants	14	2.7	3.09	0.000	1413666	11.2	VMCNG1E1	VMCNG1E1
		S_polstil	Potted Plants	14	2.6	2.95	0.000	1413666	10.7	VMCNG1E1	VMCNG1E1
				17	2.7	2.73	0.000	2165045	9.9	VMC3C11-1	VMC3C11-1
		R_dim-monstil	Potted Plants	17	2.1	2.64	45.395	8472290	9.6	VMC9G4	VVIP16

phenotypic variance. Three genomic regions associated with epicatechin gallate were identified on LGs 17, 18, and 2; these QTLs spanned from a LOD peak value of 4.02 to 2.68, with a corresponding range of explained phenotypic variance from 14.3 to 9.8%. Procyanidin B1 was mapped on LG 18 with 10.9% of explained variance. Finally, a QTL was associated with the flavonol rutin on LG 17, which explain 11.2% of the total phenotypic variance.

Relationship Among Genetic Background, Resistance Level, and Polyphenol Induction

Of the 30 polyphenolic compounds with an associated QTL, infection significantly affected production of 23. Out of these, synthesis of four compounds was induced in *Rpv3-3*$^{+}$ genotypes only, synthesis of another four significantly decreased in *Rpv3-3*$^{-}$ genotypes only, and one (ampelopsin D+quadrangularin A) was significantly induced and repressed in both *Rpv3-3*$^{+}$ and *Rpv3-3*$^{-}$ genotypes, respectively (**Figure 4A**).

Considering the *Rpv3-3* haplotype, the OIV 452 parameter and the polyphenol delta values, a relationship was highlighted exclusively for the stilbenoids. For instance, *cis*/*trans*-ω-viniferin and polymeric stilbenoids, on average induced in the progeny, resulted significantly induced in *Rpv3-3*$^{+}$ genotypes showing a high OIV 452 value. By contrast, ampelopsin D+quadrangularin A, on average induced in the progeny as well, showed an opposite profile between *Rpv3-3*$^{+}$ and *Rpv3-3*$^{-}$ genotypes with high OIV 452 values. In addition, the induction of 2,6-DHBA, observed on average in the progeny, was very low in *Rpv3-3*$^{-}$ genotypes with a high OIV 452 value (**Figure 4B**). In the *Rpv3-3*$^{-}$ resistant genotypes no compound synthesis was significantly induced by the fungus (data not shown).

Candidate Gene Identification and Characterization

Candidate Genes Underlying DM Resistance and Polyphenol-Related QTLs

The number of genes identified within each QTL region was extremely variable from a minimum of 5 (LG 18) to a maximum of 984 (LG 16). Due to the high number of GPs underlying the QTLs associated with the 33 assessed parameters, four different main criteria were adopted to select CGs for trait regulation (e.g., **Figure S5** for enrichment analysis results). Upon this selection (**Table S5**), a refined list of the 95 most relevant unique CGs was generated, of which a few were previously characterized as involved in DM response and in the regulation of polyphenol synthesis (the so called "reference genes"), while the majority were newly identified. CGs mainly referred to the functional categories of signaling, secondary metabolism, regulation of transcription, and response to abiotic and biotic stimulus (**Table 2**).

Besides disease-related (NBS-LRR) genes alone representing a significant part of the grapevine genome (Malacarne et al., 2012) and found also in high numbers within the major QTL on LG 18 associated with DM resistance parameters, four laccases (*VIT_218s0117g00590, VIT_218s0117g00600, VIT_218s0117g00610, VIT_218s0117g00625*), which can be part of the defense response due to pathogen recognition mediated by the LRR domain, drew our attention (**Table S5**).

Regarding the polyphenol content trait, we focused on CGs (i) underlying QTLs associated with polyphenols induced by infection and (ii) showing disparity between *Rpv3-3*$^{+}$ and *Rpv3-3*$^{-}$ genotypes characterized by a different level of DM resistance. Within the category of signaling, many selected genes belong to the kinase protein family, as well as to ethylene, ABA and JA signaling pathways. Two genes appear to be of special interest, one coding for the LRK10 Receptor kinase homolog (*VIT_216s0013g01390*) and the other encoding CLL1B clavata1-like receptor S/T protein kinase (*VIT_216s0013g01990*) located within the QTL region on LG16 linked to 2,6-DHBA and to pterostilbene. In addition, a gene coding for a Receptor serine/threonine kinase (*VIT_216s0148g00300*) resulted associated with *cis*-ω viniferin, as well as two genes encoding the Ethylene-responsive transcription factor ERF105 (*VIT_16s0013g00900* and *VIT_16s0013g01070*) were linked to 2,6-DHBA, pterostilene, and *cis*-piceid content. In addition, five genes (*VIT_203s0132g00040, VIT_203s0132g00050, VIT_203s0132g00080, VIT_203s0132g00090, VIT_203s0132g00100*), coding for a cluster of HVA22-like abscisic acid-induced proteins, were linked

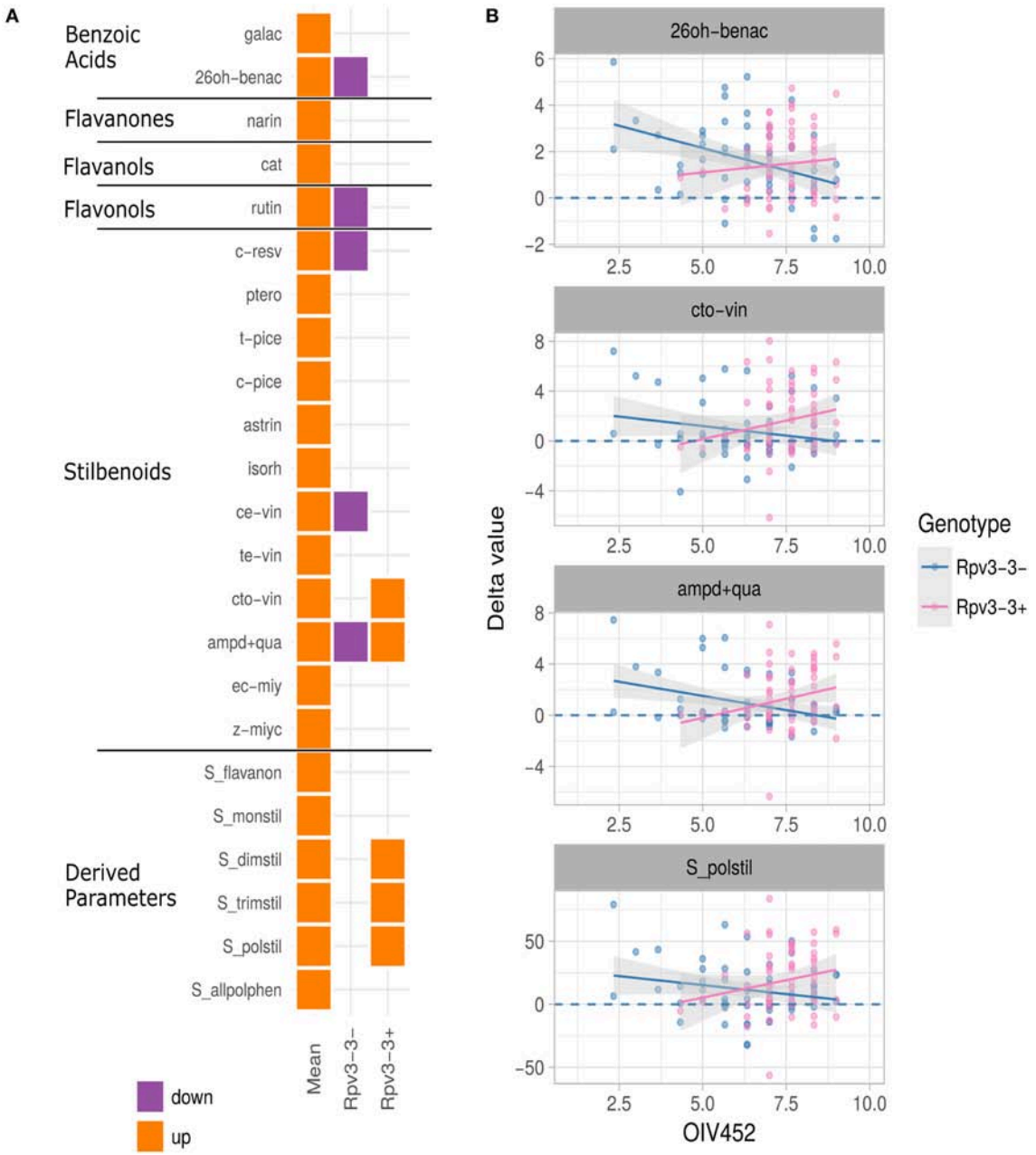

FIGURE 4 | (A) Summary of univariate analysis performed on the 30 polyphenol-related parameters—with a QTL region associated—measured in the M×T progeny. The plot summarizes the outcomes of a series of regression analyses performed to assess the presence of a significant induction ($p \leq 0.05$) and its dependence upon the resistance level (measured with the OIV 452 descriptor) for *Rpv3-3*$^{+}$ and *Rpv3-3*$^{-}$ genotypes. Orange and violet colors highlight up and down regulation, respectively. The first column (Mean) indicates the average value of the parameter distribution in the M×T progeny. **(B)** Examples of the univariate regression analyses. The meaning of the parameter abbreviations is reported in **Table S1B**.

to *cis*-resveratrol content and the gene encoding the bZIP factor VvAREB/ABF2 (*VIT_218s0001g10450*) was exclusively associated to isorhapontin. Three JA O-methyltransferases (*VIT_204s0023g03790*, *VIT_204s0023g03800*, *VIT_204s0023g03810*) were associated with astringin, an additional JA O-methyltransferase (*VIT_218s0001g12900*) was related to astringin and isorhapontin, five genes (*VIT_217s0000g07370, VIT_217s0000g07375, VIT_217s0000g07400, VIT_217s0000g07420, VIT_217s0000g07560*) coding for EDS1 were linked to isorhapontin and the ratio between dimeric and monomeric stilbenoids, and finally one gene (*VIT_216s0098g00330*) encoding ORG1 was associated with the regulation of *cis*+*trans*-ω-viniferin content (**Table 2**).

TABLE 2 | List of the 95 most relevant candidate genes associated with DM resistance and polyphenol content.

	Gene ID	Description	Functional category	Criteria of selection	Reference	Gene name
Severity (LG 18)						
1	VIT_218s0117g00590	Laccase	Single reactions	Enriched	This work	
2	VIT_218s0117g00600	Laccase	Single reactions	Enriched	This work	
3	VIT_218s0117g00610	low quality protein: laccase-14-like	Single reactions	Enriched	This work	
4	VIT_218s0117g00625	low quality protein: laccase-14-like	Single reactions	Enriched	This work	
Incidence (LG 18)						
5	VIT_218s0041g01620	R protein L6 (TMV resistance protein N-like)	Biotic stress response	LOD max peak	This work	
2,6-diOH-benzoic acid (LG 16)						
6	VIT_216s0013g01920	Ser/Thr protein kinase	Protein kinase	Enriched, closed to LOD max offset	This work	
7	VIT_216s0013g01940	Kinase-like protein TMKL1	Protein kinase	Enriched, closed to LOD max offset	This work	
8	VIT_216s0013g01990	CLL1B clavata1-like receptor like K (RLK)	Protein kinase	Enriched, closed to LOD max offset, reference gene	This work	
9	VIT_216s0013g02130	Protein kinase	Protein kinase	Enriched, closed to LOD max offset	This work	
10	VIT_216s0013g02170	Protein kinase	Protein kinase	Enriched, closed to LOD max offset	This work	
11	VIT_216s0013g01390	Receptor kinase homolog LRK10	Protein kinase	Enriched, closed to LOD max offset	This work	
12	VIT_216s0013g00900	Ethylene-responsive TF ERF105	Ethylene Signaling	Enriched, reference gene	This work	
13	VIT_216s0013g01070	Ethylene-responsive TF ERF105	Ethylene Signaling	Enriched, reference gene	This work	
14	VIT_216s0013g01570	Myb domain protein 92	Regulation of transcription	Category of interest	This work	VvMYB194
Rutin						
15	VIT_217s0000g02660	MYBC2-L2	Regulation of transcription	Category of interest, reference gene	Cavallini et al., 2015	VvMYBC2-L2
16	VIT_217s0000g02710	Myb domain protein 4R1	Regulation of transcription	Category of interest	This work	
17	VIT_217s0000g02730	Myb domain protein 4R1	Regulation of transcription	Category of interest	This work	
***Cis*-resveratrol**						
18	VIT_205s0094g00480	Ethylene-responsive protein	Ethylene Signaling	Category of interest		
19	VIT_209s0018g00240	WRKY40 like	Regulation of transcription	Category of interest	Corso et al., 2015	VvWRKY28
20	VIT_209s0018g00300	N-acetyltransferase hookless1 HLS1	Ethylene Signaling	Category of interest		
21	VIT_203s0132g00040	ATHVA22A	ABA Signaling	Enriched		
22	VIT_203s0132g00050	ATHVA22A	ABA Signaling	Enriched		
23	VIT_203s0132g00080	ATHVA22A	ABA Signaling	Enriched		
24	VIT_203s0132g00090	ATHVA22A	ABA Signaling	Enriched		
25	VIT_203s0132g00100	ATHVA22A	ABA Signaling	Enriched		
26	VIT_203s0097g00700	Pathogenesis-related protein 1 (PRP 1)	Jasmonate salicylate signaling	Category of interest		
***Cis*-piceid (LG=16)**						
12	VIT_216s0013g00900	Ethylene-responsive TF ERF105	Ethylene Signaling	Enriched, reference gene	This work	
13	VIT_216s0013g01070	Ethylene-responsive TF ERF105	Ethylene Signaling	Enriched, reference gene	This work	
27	VIT_216s0100g00750	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS7
28	VIT_216s0100g00760	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS8
29	VIT_216s0100g00770	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS9
30	VIT_216s0100g00780	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS10

(Continued)

TABLE 2 | Continued

	Gene ID	Description	Functional category	Criteria of selection	Reference	Gene name
31	VIT_216s0100g00800	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS12
32	VIT_216s0100g00810	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS13
33	VIT_216s0100g00830	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS15
34	VIT_216s0100g00840	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS16
35	VIT_216s0100g00850	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS17
36	VIT_216s0100g00860	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS18
37	VIT_216s0100g00880	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS19
38	VIT_216s0100g00900	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS20
39	VIT_216s0100g00910	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS21
40	VIT_216s0100g00920	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS22
41	VIT_216s0100g00930	Stilbene synthase	Phenylpropanoid metabolism	Enriched	this work	VvSTS23
42	VIT_216s0100g00940	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS24
43	VIT_216s0100g00950	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS25
44	VIT_216s0100g00960	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS26
45	VIT_216s0100g00990	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS27
46	VIT_216s0100g01000	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS28
47	VIT_216s0100g01010	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS29
48	VIT_216s0100g01020	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS30
48	VIT_216s0100g01020	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS31
49	VIT_216s0100g01040	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS32
50	VIT_216s0100g01060	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS33
51	VIT_216s0100g01070	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS35
52	VIT_216s0100g01100	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS36
53	VIT_216s0100g01110	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS37
54	VIT_216s0100g01120	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS39
55	VIT_216s0100g01130	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS41
56	VIT_216s0100g01140	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS42
57	VIT_216s0100g01150	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS43

(Continued)

TABLE 2 | Continued

	Gene ID	Description	Functional category	Criteria of selection	Reference	Gene name
58	VIT_216s0100g01160	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS45
59	VIT_216s0100g01170	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Vannozzi et al., 2012	VvSTS46-VvSTS47-VvSTS48
***Trans*-piceid (LG=18)**						
60	VIT_218s0001g12900	JA O-methyltransferase	Amino acid metabolism	JA signaling	This work	
61	VIT_218s0001g13110	Peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII34a
62	VIT_218s0001g15390	Gaiacol peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII21a
Pterostilbene (LG=16)						
6	VIT_216s0013g01920	Ser/Thr protein kinase (PK)	Protein kinase	Enriched, closed to LOD max offset	This work	
7	VIT_216s0013g01940	Kinase-like protein TMKL1	Protein kinase	Enriched, closed to LOD max offset	This work	
8	VIT_216s0013g01990	CLL1B clavata1-like receptor like K (RLK)	Protein kinase	Enriched, closed to LOD max offset, reference gene	This work	
9	VIT_216s0013g02130	Protein kinase (PK)	Protein kinase	Enriched, closed to LOD max offset	This work	
10	VIT_216s0013g02170	Protein kinase (PK)	Protein kinase	Enriched, closed to LOD max offset	This work	
11	VIT_216s0013g01390	Receptor kinase homolog LRK10	Protein kinase	Enriched, closed to LOD max offset	This work	
12	VIT_216s0013g00900	Ethylene-responsive TF ERF105	Ethylene Signaling	Enriched, reference gene	This work	
13	VIT_216s0013g01070	Ethylene-responsive TF ERF105	Ethylene Signaling	Enriched, reference gene	This work	
27	VIT_216s0100g00750	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS7
28	VIT_216s0100g00760	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS8
29	VIT_216s0100g00770	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS9
30	VIT_216s0100g00780	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS10
31	VIT_216s0100g00800	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS12
32	VIT_216s0100g00810	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS13
33	VIT_216s0100g00830	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS15
34	VIT_216s0100g00840	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS16
35	VIT_216s0100g00850	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS17
36	VIT_216s0100g00860	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS18
37	VIT_216s0100g00880	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS19
38	VIT_216s0100g00900	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS20
39	VIT_216s0100g00910	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS21
40	VIT_216s0100g00920	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS22
41	VIT_216s0100g00930	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS23
42	VIT_216s0100g00940	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS24

(Continued)

TABLE 2 | Continued

	Gene ID	Description	Functional category	Criteria of selection	Reference	Gene name
43	VIT_216s0100g00950	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS25
44	VIT_216s0100g00960	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS26
45	VIT_216s0100g00990	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS27
46	VIT_216s0100g01000	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS28
47	VIT_216s0100g01010	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS29
48	VIT_216s0100g01020	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS30
48	VIT_216s0100g01020	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS31
49	VIT_216s0100g01040	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS32
50	VIT_216s0100g01060	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS33
51	VIT_216s0100g01070	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS35
52	VIT_216s0100g01100	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS36
53	VIT_216s0100g01110	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS37
54	VIT_216s0100g01120	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS39
55	VIT_216s0100g01130	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS41
56	VIT_216s0100g01140	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS42
57	VIT_216s0100g01150	Stilbene synthase	Phenylpropanoid metabolism	Enriched	This work	VvSTS43
58	VIT_216s0100g01160	Stilbene synthase	Phenylpropanoid metabolism	Enriched, reference gene	Höll et al., 2013	VvSTS45
59	VIT_216s0100g01170	Stilbene synthase	Phenylpropanoid metabolism	Enriched	Vannozzi et al., 2012	VvSTS46-VvSTS47-VvSTS48
63	VIT_216s0022g02470	Cationic peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII08a
64	VIT_216s0100g00090	Cationic peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII08b
65	VIT_216s0022g01690	Band 7 family (Hrp_c)	Auxiliary transport proteins		Casagrande et al., 2011	
66	VIT_216s0022g02040	PBS2 (PPHB susceptible 2)	Biotic stress response		Casagrande et al., 2011	
Astringin (LGs=18, 16, 4)						
60	VIT_218s0001g12900	JA O-methyltransferase	Amino acid metabolism	JA signaling	This work	
61	VIT_218s0001g13110	Peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII34a
67	VIT_216s0013g01560	Myb domain protein 92	Regulation of transcription	Regulator	This work	VvMYB193
14	VIT_216s0013g01570	Myb domain protein 92	Regulation of transcription	Regulator	This work	VvMYB194
68	VIT_204s0023g03790	Jasmonate methyltransferase	Lipid metabolism	JA signaling	This work	
69	VIT_204s0023g03800	Jasmonate methyltransferase	Lipid metabolism	JA signaling	This work	
70	VIT_204s0023g03810	Jasmonate O-methyltransferase	Lipid metabolism	JA signaling	This work	
71	VIT_204s0044g01510	Histone deacetylase HDA14	Cell growth and death	LOD max peak	This work	
72	VIT_204s0044g01205	chitinase 1	Biotic stress response	Defense response	This work	
73	VIT_204s0023g03710	Myb domain protein 4B	Regulation of transcription	Regulator, reference gene	Cavallini et al., 2015	VvMYB4B
74	VIT_204s0044g01380	Myb domain protein 52	Regulation of transcription	Regulator	This work	

(Continued)

TABLE 2 | Continued

	Gene ID	Description	Functional category	Criteria of selection	Reference	Gene name
Isorhapontin (LGs=18, 17)						
75	VIT_218s0001g10450	VvAREB/ABF2	ABA Signaling	Category of interest	Nicolas et al., 2014	VvbZIP045
76	VIT_218s0001g11630	Allene oxide synthase	Lipid metabolism	reference gene	Casagrande et al., 2011	
60	VIT_218s0001g12900	JA O-methyltransferase (JMT)	Amino acid metabolism	Reference gene	Casagrande et al., 2011	
61	VIT_218s0001g13110	Peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII34a
77	VIT_217s0000g07750	Peroxidase 65	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII15a
78	VIT_217s0000g07370	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
79	VIT_217s0000g07375	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
80	VIT_217s0000g07400	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
81	VIT_217s0000g07420	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
82	VIT_217s0000g07560	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
***Cis*-ε-viniferin (LG=18)**						
83	VIT_218s0001g02400	Laccase 14	Single reactions	Involved in oligomerization	This work	
84	VIT_218s0001g02410	Laccase/Diphenol oxidase family protein	Single reactions	Involved in oligomerization	This work	
***Cis*+*trans*-ω-viniferin (LG=16)**						
85	VIT_216s0148g00300	Receptor serine/threonine kinase	Protein kinase	Enriched, reference gene	This work	
86	VIT_216s0098g00820	Peroxidase 3	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII23a
87	VIT_216s0098g00330	ORG1 (OBP3-responsive gene 1)	Jasmonate salicylate signaling	JA signaling	This work	
Ampelopsin D+quadrangularin A (LG=17)						
88	VIT_217s0000g02650	MYBC2-L4	Regulation of transcription	Regulator	Cavallini et al., 2015	VvMYBC2-L4
15	VIT_217s0000g02660	MYBC2-L2	Regulation of transcription	Regulator, reference gene	Cavallini et al., 2015	VvMYBC2-L2
16	VIT_217s0000g02710	Myb domain protein 4R1	Regulation of transcription	Regulator	This work	
17	VIT_217s0000g02730	Myb domain protein 4R1	Regulation of transcription	Regulator	This work	
S_monomeric stilbenoids (LG=18)						
61	VIT_218s0001g13110	Peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII34a
62	VIT_218s0001g15390	Gaiacol peroxidase	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII21a
S_dimeric stilbenoids						
8	VIT_217s0000g02650	MYBC2-L4	Regulation of transcription	Regulator	Cavallini et al., 2015	VvMYBC2-L4
15	VIT_217s0000g02660	MYBC2-L2	Regulation of transcription	Regulator, reference gene	Cavallini et al., 2015	VvMYBC2-L2
16	VIT_217s0000g02710	Myb domain protein 4R1	Regulation of transcription	Regulator	This work	
17	VIT_217s0000g02730	Myb domain protein 4R1	Regulation of transcription	Regulator	This work	
R_dimeric-monomeric stilbenoids (LG=17)						
77	VIT_217s0000g07370	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
78	VIT_217s0000g07375	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
79	VIT_217s0000g07400	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
80	VIT_217s0000g07420	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	

(Continued)

TABLE 2 | Continued

	Gene ID	Description	Functional category	Criteria of selection	Reference	Gene name
81	VIT_217s0000g07560	EDS1	Jasmonate salicylate signaling	Enriched	Casagrande et al., 2011	
89	VIT_217s0000g09070	Histone deacetylase HDA6	Jasmonate salicylate signaling	Enriched	This work	
77	VIT_217s0000g07750	Peroxidase 65	Amino acid metabolism	Involved in oligomerization	This work	VviPrxIII15a
S_trimeric stilbenoids (LG=14)						
90	VIT_214s0060g02280	C3HC4-type ring finger	Regulation of transcription	Regulator	This work	
Z-miyabenol C (LG=14)						
91	VIT_214s0060g01710	Ribosomal protein L18	Protein metabolism and modification	LOD max peak	This work	
S_polymeric stilbenoids (LG=14)						
92	VIT_214s0060g02420	JmjC domain-containing protein	Regulation of transcription	Regulator	This work	
93	VIT_214s0060g02440	Indeterminate(ID)-domain 2	Regulation of transcription	Regulator	This work	
94	VIT_214s0060g02640	Myb family	Regulation of transcription	Regulator	This work	
95	VIT_214s0060g02660	Nuclear transcription factor Y subunit B-5	Regulation of transcription	Regulator	This work	

(i) The gray background indicates the genes for which expression was evaluated in 12 F1 individuals of the M×T progeny by qRT-PCR; (ii) genes associated with different parameters are repeated in the Table; (iii) Gene ID, code of 12Xv2 gene predictions as retrieved by CRIBI database (http://genomes.cribi.unipd.it/DATA/V2/); functional category: functional category at the second or third level of description.

A cluster of stilbene synthase genes, mostly not specifically related to DM response previously, was found in the QTL intervals associated with *cis*-piceid and pterostilbene. In addition, six peroxidase genes (herein named *VviPrxIII08a*, *VviPrxIII08b*, *VviPrxIII15a*, *VviPrxIII21a*, *VviPrxIII23a*, *VviPrxIII34a,* **Figure 6**) underlied monomeric and oligomeric stilbenoid-related QTL regions and two laccase genes (*VIT_218s0001g02400 and VIT_218s0001g02410*) were associated with ε-viniferin.

Two of the genes recently identified as encoding a set of R2R3-MYB C2 repressors of phenylpropanoid levels (Cavallini et al., 2015) were linked to the regulation of ampelopsin D+quadrangularin A, the sum of dimeric stilbenoids and rutin (*VvMYBC2-L2*) and to the regulation of *trans*-piceid and astringin content (*VvMYB4B*). Another seven *MYB* genes were identified: *VIT_216s0013g01560* (*VvMYB193* in Wong et al., 2016) and *VIT_216s0013g01570* (*VvMYB194* in Wong et al., 2016) in the region controlling both 2,6-DHBA and astringin content, *VIT_204s0044g01380* in the region controlling astringin content, *VIT_217s0000g02710* and *VIT_217s0000g02730* as associated with ampelopsin D+quadrangularin A, the sum of dimeric stilbenoids and rutin content, and finally *VIT_214s0060g02640* related to the sum of polymeric stilbenoids. Finally, a WRKY factor (precisely VvWRKY28 in Wang et al., 2014) was associated to *cis*-resveratrol content.

Newly Identified Genes Candidate to Stilbenoid Oligomerization

Among all CGs, further investigation was dedicated to a set of 11 genes by assessing their transcript level in a set of 12 F1 individuals. The objective was to get further evidence of the association between the identified genomic regions and the traits under investigation. These genotypes exhibited disparity in pathogen resistance level, stilbenoid-related parameters with an mQTL associated and haplotype status at the *Rpv3* locus (**Figure S6**). These genes encoded: (i) five out of six peroxidases identified within monomeric and oligomeric stilbenoid-related QTL regions (*VviPrxIII08a*, *VviPrxIII08b*, *VviPrxIII15a*, *VviPrxIII21a*, *VviPrxIII23a*); (ii) two of the six laccases described above (*VIT_218s0117g00590* and *VIT_218s0001g02400*), the first associated with disease severity and the other with ε-viniferin and therefore putatively involved in its oligomerization; (iii) three stilbene synthases (*VvSTS27-8-9*, *VvSTS41*, and *VvSTS48*) already associated with DM response (Vannozzi et al., 2012; Höll et al., 2013) and here related to the regulation of pterostilbene and *cis*-piceid content; (iv) one Histone deacetylase HDA14 (*VIT_204s0044g01510*) found in correspondence of the LOD peak offset of the QTL on LG 4 associated with astringin.

The gene expression study revealed a significant correlation between (i) the content of *cis*-piceid and pterostilbene and the expression level both of *VviPrxIII08a* and *VviPrxIII08b,* (ii) the content of ω-viniferin and the expression level of *VviPrxIII23a,* and finally (iii) the content of *cis*-piceid and pterostilbene and the expression level both of *VvSTS41* and *VvSTS48* at 6 dpi (**Figure 5**).

In order to gather additional information about the peroxidases found in the QTL and correlated with stilbenoid induction upon *P. viticola* infection we performed a phylogenetic analysis of the whole grapevine peroxidase gene family, which allowed us to attribute the 90 GPs to 45 different classes. The six peroxidases herein associated to stilbenoid metabolism are part of different classes: *VviPrxIII08a* and *VviPrxIII08b* belong to class 08 and are the putative orthologs of *AtPrx66*, *VviPrxIII15a* and *VviPrxIII21a* are the unique members of class 15 and 23, respectively, *VviPrxIII23a* has three different isoforms which belong to class 23 together with another four members, and finally *VviPrxIII34a*

belongs to class 34 and is the putative ortholog of *AtPrx09* (**Figure 6**; **Table S6**).

DISCUSSION

Characterization of the *Rpv3-3* haplotype

The M×T genetic map built in this study is an improved version in terms of progeny individuals and LG number as well as marker number/order compared to the one by Salmaso et al. (2008). The high percentage of markers (68%) that was mapped in both parents is close to the 62% of markers positioned into the reference grapevine genetic map, while the 5% of distorted markers is about half of the value previously observed (Adam-Blondon et al., 2004). This upgrading should result in visible improvement in the parental and consensus genetic maps to be suitable for further applications as the association to new phenotypes.

Herein, at the hypervariable microsatellite markers UDV305 and UDV737, we identified the haplotype null-271 as conferring resistance against *P. viticola*; this genetic variant is located on chromosome 18 and it derives on the "Merzling" side from the grand-parent "Seyval." This haplotype was named *Rpv3-3* according to the VIVC nomenclature, given its co-localization with the *Rpv3* locus, which was subjected to selective sweep during grapevine breeding activities. In fact, a seminal study identified seven conserved haplotypes, which are overrepresented in grapevine breeding lines historically selected for DM resistance compared to their wild relatives, and are absent from the susceptible *V. vinifera* varieties (Di Gaspero et al., 2012). These genetic variants may carry *Rpv3* alleles or adjacent *Rpv3* paralogs that constantly remained linked with the diagnostic markers. Upon this reported association analysis, nowadays only two wild relative *Rpv3* haplotypes have been characterized in segregating populations. The *Rpv3-1* haplotype—preserved in the "Seibel 4614" lineage—was firstly identified in the German hybrid "Regent" (Welter et al., 2007; van Heerden et al., 2014) and in the Hungarian hybrid "Bianca" (Bellin et al., 2009) through QTL analyses. The second of these resistance haplotypes—named *Rpv3-2,* conserved in the "Munson" lineage—has recently been confirmed by QTL mapping (Zyprian et al., 2016). In this work, we have validated the *Rpv3-3* haplotype—derived from the descent group founder "Noah"—by means of a QTL analysis on the M×T progeny.

Given this outcome, we attempted the characterization of several genotypes related to Merzling (data not shown). Unlike the comprehensive *Rpv3* survey reported by Di Gaspero et al. (2012), we detected the *Rpv3-3* haplotype also in the "Merzling" offspring "Solaris," so far known to carry only the *Rpv10* locus derived from *V. amurensis.* This was due to the masking effect of a different *Rpv3* haplotype (*Rpv3-1*) derived from the second resistance donor Gf.Ga-52-42 in the QTL study (Schwander et al., 2012). Analogously, besides the *Rpv 10* locus, we found that "Bronner" and "Cabernet Cortis" unexpectedly inherited the *Rpv3-3* haplotype from their parent "Merzling" and "Solaris," respectively; all these genotypes used as parental lines demonstrated to transmit this acquired haplotype to their

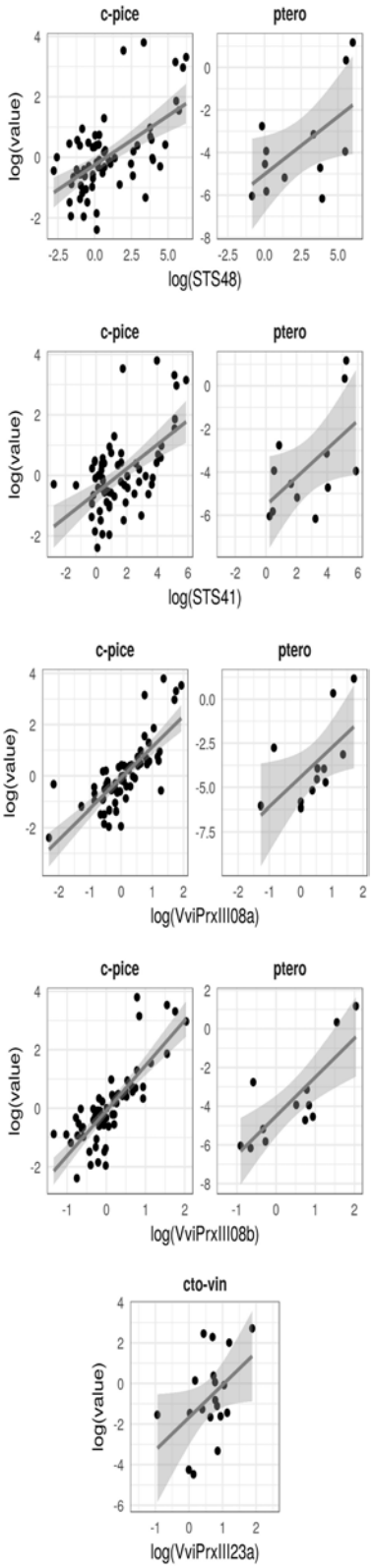

FIGURE 5 | Regression analysis which highlights the association between metabolic induction and normalized relative expression of selected candidate genes in *Rpv3-3*$^{+}$ and *Rpv3-3*$^{-}$ selected genotypes. c-pice, *cis*-piceide; ptero, pterostilbene; cto-vin, *cis* + *trans*-ω-viniferin.

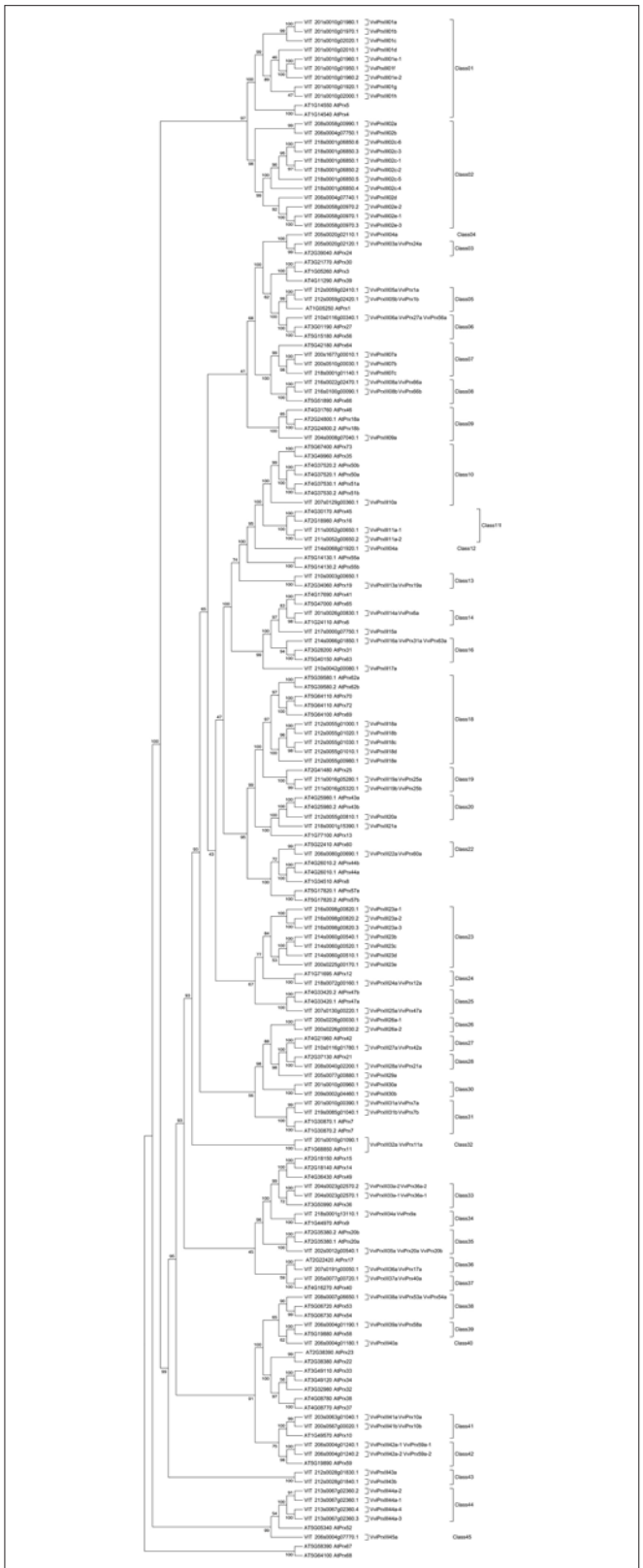

FIGURE 6 | Phylogenetic analysis of the grapevine peroxidase gene family.

respective progenies (Vezzulli S., personal communication). Finally, herein this specific genetic variant was detected also in the related Baron, Prior, and Cabernet Cantor (data not shown). These findings confirmed that grapevine breeders traditionally selected genotypes with a certain degree of pyramiding, namely accumulation of more than one *R*-locus (Töpfer et al., 2011), suggesting a reinforcement role of the *Rpv3-3* haplotype on other *R*-locus effects. For this reason our investigation can be considered a "genetic" upgrade of the pioneer *Rpv3* study by Di Gaspero et al. (2012).

Genes belonging to the NBS-LRR superfamily were detected in the regions underlying QTLs associated with all disease resistance parameters, in agreement with the first studied *Rpv3* resistance haplotype encoding NB-LRR and LRR-kinase receptors (Di Gaspero and Foria, 2015). According to the Effector-Triggered Immunity model, *R* gene products sense the pathogen effectors and activate signal transduction pathways (Cui et al., 2015). In grapevine, *Rpv3*-dependent resistance follows this model of gene-for-gene interaction (Casagrande et al., 2011). In previous studies, the resistance haplotype was revealed to be necessary and sufficient to trigger a hypersensitive response (HR) leading to cell death in the proximity of sites infected by *P. viticola* (Bellin et al., 2009; Zyprian et al., 2016). In the current study, weakened or delayed HR was observed on LD of *Rpv3-3*$^{+}$ genotypes (data not shown) with lower OIV452-1 values; this overall phenomenon can be due to the combination of different factors—high inoculum concentration and RH—leading to shorter incubation time, more infection sites and faster hyphal growth under highly conducive conditions (Gessler et al., 2011). In addition, it is relevant to highlight that the phenotypic distribution (ranges: OIV 452 from 1.6 to 9, Severity from 0 to 33.3%, Incidence from 0 to 100%) on PP revealed an average mid-resistance level of *Rpv3-3*$^{+}$ genotypes, in agreement with the recent study by Foria et al. (2018) which shed light on various *Rpv3* haplotypes responsible for different disease resistance degrees. Finally, unlike the expectation in the case of an *R*-locus, the non-binomial distribution of each DM resistance parameter suggests that this trait is controlled by multiple factors in this genetic background. The M×T QTL study refers to the concept of Advanced Backcross-QTL (AB-QTL)—recently recovered in grapevine—which combines QTL analysis and variety development by designing a mapping/breeding scheme for the simultaneous identification and introgression of wild haplotypes. AB-QTL relies on segregating populations in which most of the wild-parent genome that donates the trait of interest has been purged in early segregating generations by phenotypic selection (Tanksley and Nelson, 1996). This is relevant to guarantee QTL stability once the associated markers are screened in derived breeding materials. In fact, favorable QTL alleles identified in early generations often disappear in later back-cross generations, once the modifier genes that have epistatic interactions with the beneficial QTL alleles are removed from highly *V. vinifera* genetic backgrounds (Di Gaspero and Foria, 2015). Herein, we did

not actually face cases of susceptible individuals carrying the *Rpv3-3*$^{+}$ haplotype, whereas we found a few *Rpv3-3*$^{-}$ individuals displaying DM resistance. Since unreliable minor QTLs were identified in both the M×T consensus and the maternal genetic map (data not shown), and no intra-locus recombination was detected, this phenomenon has still to be elucidated.

Survey of Stilbenoid-Associated Regions Among the Discovered Polyphenol-Related QTLs

In the present study 46 novel metabolic (m)QTLs associated with 30 phenolics-related parameters were discovered. Among the new mQTLs, more than half were associated with stilbenoid-related parameters (monomers, dimers, and trimers), two with hydroxycinnamic acids, two with benzoic acids, five with flavanones, and eight with flavan-3-ol monomers and dimers. Pleiotropic effects, i.e., single gene producing multiple effects on various traits, were recorded. Except for few cases encompassing different classes (e.g., ratio between dimeric and monomeric stilbenoids and naringenin), pleiotropy was detected along genomic intervals associated with parameters falling into the same class, such as monomeric stilbenoids and flavanones. Moreover, epistatic effects have recently been considered in many studies as relevant for complex traits, such as polyphenol biosynthesis. Epistasis, i.e., an additive-by-additive interaction between QTLs, assayed in populations segregating for an entire genome, has been found at a frequency close to that expected by chance alone (Bocianowski, 2013). Therefore, we cannot exclude epistatic effects in the case of characters controlled by more than one region, such as *cis*-piceid, astringin, and naringenin.

Although few research studies on proanthocyanidin and flavonol berry composition have recently been attempted in grapevine (Huang et al., 2012; Malacarne et al., 2015), most polyphenols do not have a known QTL associated. The mQTLs here identified fill this gap and reveal polyphenols with central role in *P. viticola*-grapevine interaction. In particular, this analysis allowed the identification of mQTLs associated with 17 different stilbenoid-related parameters, therefore representing a thorough characterization of stilbenoid regulation upon *P. viticola* infection on leaves.

A cluster of stilbene synthase genes, mostly not specifically related to DM response previously, was identified in the QTL intervals associated with *cis*-piceid and pterostilbene. Previous works revealed different patterns of transcript accumulation between the different *VvSTS* family members (Dai et al., 2012; Vannozzi et al., 2012; Höll et al., 2013; Shi et al., 2014) depending on the high variability in their regulatory regions (Chialva et al., 2018). Moreover, six interesting peroxidase and two laccase genes were identified as associated with stilbenoids therefore representing good candidates for ROS-mediated stilbene oligomerization (Calderón et al., 1994; Barceló et al., 2003; Pezet et al., 2004b). Indeed, there is some evidence supporting the enzymatic biotransformation of stilbenes by plant peroxidases (Takaya et al., 2005; Wan et al., 2011) and/or fungal laccases (e.g., Pezet et al., 1991; Breuil et al., 1998, 1999). It is still a debate if the oligomerization is driven by plant or fungal laccases, which catalyze the degradation of stilbene monomers allowing the fungus to escape from the action of grapevine phytoalexins. By phylogenetic reconstruction of the entire grapevine peroxidase gene family, we determined that *VviPrxIII08a* and *VviPrxIII08b* are the putative orthologs of *AtPrx66* involved in lignification (Tokunaga et al., 2009), *VviPrxIII34a* is the putative ortholog of *AtPrx09* which is closed to Arabidopsis members involved in lignin biosynthesis (Tognolli et al., 2002; Herrero et al., 2013), while *VviPrxIII15a*, *VviPrxIII21a,* and *VviPrxIII23a* have no orthologs in Arabidopsis. A validation of the results obtained at the metabolic level came from a transcriptional investigation in a set of 12 F1 individuals of the progeny, which highlighted a significant association between some monomeric and dimeric stilbenoids and the transcript level of three newly identified peroxidases besides known stilbene synthases.

The time dependent regulation of different inducible stilbenes upon abiotic and biotic stresses was reported to be at least transcriptional (e.g., Vannozzi et al., 2012; Höll et al., 2013; Wong et al., 2016) and coordinated by the action of both MYB and WRKY transcription factors (Malacarne et al., 2018; Vannozzi et al., 2018; Jeandet et al., 2019; Jiang et al., 2019). In the present work additional *MYB* genes were associated with stilbenoid formation; in particular, the known R2R3-MYB C2 repressors of phenylpropanoid levels (Cavallini et al., 2015) were located along the regions on LG4 and LG17 controlling some monomeric and dimeric stilbenoids, respectively. In addition, it is worth noting that the WRKY factor VvWRKY28 already found co-expressed with *VvSTS* transcripts in root and leaves (Corso et al., 2015) was here associated with *cis*-resveratrol content extending the list of WRKY factors identified as involved in the regulation of stilbenoid metabolism. It has recently been shown that the expression level of several members of the stilbene synthase gene family and genes responsible of the oxidative polymerization of phenolic compounds in the phenylpropanoid pathway is highly influenced by the "location" variable in a G×E interaction study (Dal Santo et al., 2018).

Our results showed that ABA and SA/JA signaling play an important role in the regulation of 2,6-DHBA, reported to be the de-activated form of SA (Bellés et al., 2006; Campos et al., 2014; Nawrocka et al., 2018), and of stilbenoid synthesis. A cluster of five genes encoding HVA22-like abscisic acid-induced proteins, belonging to a class of ABA- and stress-inducible proteins (Chen et al., 2002), was identified on LG3 associated with *cis*-resveratrol. Moreover, the transcriptional regulator VvbZIP045, recently characterized by Nicolas et al. (2014) as a key factor activating down-stream genes of the ABA signaling cascade during the grape berry ripening process, was here associated with the regulation of isorhapontin content. It should be noted that (Wang et al., 2018) have recently showed that *Muscadinia rotundifolia* "Noble" defense response to *P. viticola* infection is mediated by stilbene accumulation induced by ABA and SA phytohormones. Moreover, two genes (*VIT_216s0013g00900* and *VIT_216s0013g01070*) encoding

Ethylene-responsive transcription factor ERF105, known to regulate the plant response to abiotic and biotic stress (Mizoi et al., 2012; Mishra et al., 2015), were selected within the 2,6 DHBA, *cis*-piceid, and pterostilbene associated regions. Remarkable was also the significant enrichment of protein kinases in the region on LG16 associated both with 2,6 DHBA and pterostilbene: two genes encoding respectively for CLV1 and LRK10, which are receptor-like kinases (RLK) previously related to plant-microbe interaction and stress responses (Shiu and Bleecker, 2001), and four calcium-dependent protein kinases involved in the translation of pathogen signal-induced changes in the Ca^{2+} concentration during plant defense reactions (Schulz et al., 2013).

Combination of *Rpv3-3* Haplotype, Stilbenoid Induction, and DM Resistance

In our previous study we found evidence of a putative involvement of stilbenoids in conferring DM resistance in the M×T segregating population (Malacarne et al., 2011), without taking into account the genetic background. To shed light into the found *Rpv3*-mediated DM resistance mechanism we have investigated the genetic bases of this mechanism. Although no overalapping QTLs controlling both DM resistance and stilbenoid-related traits were detected, we could highlight a significant induction of most stilbenoids, especially the polymeric ones, in a high fraction of the individuals. To date several studies have reported on (i) stilbenoid induction upon *P. viticola* infection (e.g., Langcake and Pryce, 1976; Alonso-Villaverde et al., 2011), (ii) a more rapid and extensive accumulation of stilbenoids in DM-resistant vs. DM-susceptible genotypes (*V. vinifera* cultivars) (Chitarrini et al., 2017), (iii) an antifungal activity carried out by stilbenoids on *P. viticola* and other fungi (e.g., Pezet et al., 2004a; Adrian and Jeandet, 2012; Gabaston et al., 2017). What still remains to be elucidated is the defense mechanism underlying DM resistance conferred by different *Rpv* loci. This is crucial for pyramiding various DM resistance mechanisms, derived from different sources, in the process of genetic improvement.

A deeper look into the *Rpv3-3* haplotype within the M×T population highlighted a clear link among genetic background, DM resistance, and stilbenoid induction upon *P. viticola* infection. Unlike the *Rpv3-3*$^{-}$ genotypes, the *Rpv3-3*$^{+}$ showed on average a significant induction of two dimeric and one trimeric stilbenoids and a positive correlation between the extent of induction and DM resistance. A clear explanation of the resistance in the *Rpv3-3*$^{-}$ individuals should be further elucidated, since in the present work we could not find any minor reliable DM resistance QTLs, as well as any metabolite exclusively induced in this group of genotypes.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.

AUTHOR CONTRIBUTIONS

SV conceptualized the project, carried out genetic mapping and QTL analysis, and drafted the manuscript. GM conceptualized the project, performed the candidate gene selection with related expression analysis, and drafted the manuscript. DM and ZHM carried out the metabolite analysis. UV supported the metabolite analysis. AV and LZ performed the phenotyping assays. MS supported the phenotyping assays. CD and EB carried out the genotyping analysis. RV supported the genotyping analysis. VG performed the phylogenetic study and contributed to the manuscript revision. PF performed the statistical analysis. CM conceptualized as well as coordinated the project, and contributed to the discussion of the results. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00234/full#supplementary-material

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frontiers in Microbiology

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The Fungal and Bacterial Rhizosphere Microbiome Associated With Grapevine Rootstock Genotypes in Mature and Young Vineyards

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The microbiota colonizing the rhizosphere and the endorhizosphere contribute to plant growth, productivity, carbon sequestration, and phytoremediation. Several studies suggested that different plants types and even genotypes of the same plant species harbor partially different microbiomes. Here, we characterize the rhizosphere bacterial and fungal microbiota across five grapevine rootstock genotypes cultivated in the same soil at two vineyards and sampling dates over 2 years by 16S rRNA gene and ITS high-throughput amplicon sequencing. In addition, we use quantitative PCR (qPCR) approach to measure the relative abundance and dynamic changes of fungal pathogens associated with black-foot disease. The objectives were to (1) unravel the effects of rootstock genotype on microbial communities in the rhizosphere of grapevine and (2) to compare the relative abundances of sequence reads and DNA amount of black-foot disease pathogens. Host genetic control of the microbiome was evident in the rhizosphere of the mature vineyard. Microbiome composition also shifted as year of sampling, and fungal diversity varied with sampling moments. Linear discriminant analysis identified specific bacterial (i.e., *Bacillus*) and fungal (i.e., *Glomus*) taxa associated with grapevine rootstocks. Host genotype did not predict any summary metrics of rhizosphere α- and β-diversity in the young vineyard. Regarding black-foot associated pathogens, a significant correlation between sequencing reads and qPCR was observed. In conclusion, grapevine rootstock genotypes in the mature vineyard were associated with different rhizosphere microbiomes. The latter could also have been affected by age of the vineyard, soil properties or field management practices. A more comprehensive study is needed to decipher the cause of the rootstock microbiome selection and the mechanisms by which grapevines are able to shape their associated microbial community. Understanding the vast diversity of bacteria and fungi in the rhizosphere and the interactions between microbiota and grapevine will facilitate the development of future strategies for grapevine protection.

Keywords: bacterial and fungal recruitment, black-foot disease, microbial ecology, microbiome, rhizosphere, rootstock selection

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INTRODUCTION

Plants have evolved to cope with biotic and abiotic stresses in association with soil microorganisms (Lemanceau et al., 2017). These microorganisms are known as plant microbiota and, together with the plant, they form an holobiont (Liu et al., 2018). Plant-soil microbiome interactions are complex and, until recent times, the study of these relationships has been mainly focused in the pathogenicity of some microbial agents and how they use and compete for the resources (Philippot et al., 2013; Zancarini et al., 2013; Gilbert et al., 2014; Sapkota et al., 2015). Recent investigations have shown that soil microbiota can directly and indirectly interact with the plants improving their fitness and health (Sapkota et al., 2015). For example, these interactions help plants to deal with abiotic stress and diseases, improving the exchange of substances such as nitrogen or phosphate, or by acting as biocontrol agents through competition with pathogens (Reinhold-Hurek et al., 2015; Vega-Avila et al., 2015; Gallart et al., 2018).

Roots are surrounded by a narrow zone of soil known as rhizosphere. This area, which is influenced by the roots, has a high microbial diversity and its community structure is expected to be different than the one found in the bulk soil (Reinhold-Hurek et al., 2015). The rhizosphere microbiome community composition is affected by different factors, such as ambient conditions, soil properties, and background microbial composition (Qiao et al., 2017). In addition, plants are able to shape their rhizosphere microbiome, as evidenced by the fact that different plant species host specific microbial communities when grown on the same soil (Aira et al., 2010; Berendsen et al., 2012; Bazghaleh et al., 2015).

As reviewed by Philippot et al. (2013), plant roots release a huge variety of carbon-containing compounds known as rhizodeposits (nutrients, exudates, border cells, and mucilage) which make the rhizosphere more nutritive than the bulk soil, which is mostly mesotrophic/oligotrophic, inducing therefore changes on soil microbial communities. It has been reported that the biodiversity in the rhizosphere is lower than in the corresponding bulk soil (Reinhold-Hurek et al., 2015; Lemanceau et al., 2017) since carbon availability often limits microbial growth (Dennis et al., 2010). Rhizodeposits released by the plants considerably vary according to the age and development of plants, among species and even among different genotypes of the same species (Inceoğlu et al., 2010; Philippot et al., 2013; Gilbert et al., 2014; Bazghaleh et al., 2015; Hacquard, 2016; Wagner et al., 2016; Lemanceau et al., 2017; Qiao et al., 2017).

The rhizosphere is also the infection court where soil-borne pathogens establish a parasitic relationship with the plant. To infect root tissue, pathogens have to compete with members of the rhizosphere microbiome for available nutrients and microsites (Chapelle et al., 2016). Exploiting genetic variation in host plant species and understanding interactions between microbiota and their hosts plants will allow the rhizosphere microbiota to be incorporated into plant breeding programs to promote beneficial associations between plants and microorganisms.

Common grapevine (*Vitis vinifera* L.) is one of the most extensively grown and economically important woody perennial fruit crop worldwide with an annual production in 2014 exceeding 74 million tons of grapes and 30 million tons of wine (FAO, 2018). Since the late 19th century, *V. vinifera* cultivars have been grafted onto resistant rootstock of other *Vitis* species and hybrids to combat the devastating root phylloxera pest. Several major criteria have been outlined for choosing rootstocks: resistance to phylloxera and nematodes, and adaptability to drought, salinity, limestone content, and poor mineral nutrition (Reynolds and Wardle, 2001). In addition, the rootstock influence may affect scion vigor, yields, and fruit and wine qualities (Warschefsky et al., 2016).

Plant genetic control over microbial communities in the rhizosphere has been reported for different genotypes of the same species (Aira et al., 2010; Bouffaud et al., 2012; Peiffer et al., 2013; Marques et al., 2014; Jiang et al., 2017; Gallart et al., 2018). However, within grapevine species, the impact of genetic variation on the composition of the bacterial and fungal microbiota is poorly understood. In a recent study, Marasco et al. (2018) observed that five grapevine genotypes influenced the bacterial microbiome from both the root tissues and the rhizosphere fractions at a single vineyard, sampling date and year.

To better understand the players and processes that operate in the rhizosphere, a variety of molecular techniques, such as metagenomics have been applied over the past decade. Here, we characterize the rhizosphere bacterial and fungal microbiota across five grapevine rootstock genotypes cultivated in the same soil at two vineyards and sampling dates over 2 years by 16S rRNA gene and ITS high-throughput amplicon sequencing (HTAS). This design allowed us to evaluate the effect of the growing region, year, sampling date, grapevine genotype, and their interactions on the bacterial and fungal community diversity. In addition, we used quantitative Polymerase Chain Reaction (qPCR) approach to measure the relative abundance and dynamic changes of fungal pathogens associated with black-foot disease, one of the main soil-borne fungal diseases affecting grapevine production worldwide.

MATERIALS AND METHODS

Sample Collection

Grapevine rhizosphere samples of five rootstocks (110 R, 140 Ru, 1103 P, 41 B, and 161-49 C) were collected at two vineyards located in Aldeanueva de Ebro (abbreviated as "Aldea") (La Rioja, Spain) and Olite (Navarre, Spain). Features of the selected rootstocks are reported in **Supplementary Table 1** (Martínez-Cutillas et al., 1990; Hidalgo, 2002; Keller, 2010). All the selected rootstocks were cultivated in the same vineyard and had been grafted onto Tempranillo cultivar. Soil physicochemical properties showed significant differences between soil types. Climate and soil management practices

Abbreviations: ITS, Internal transcribed spacer; OTU, Operational taxonomic unit; PCoA, Principal coordinate analysis; PCR, Polymerase chain reaction; PERMANOVA, Permutational multivariate analysis of variance; QIIME, Quantitative insights into microbial ecology; qPCR, Quantitative polymerase chain reaction; rRNA, Ribosomal RNA; SIMPER, Similarity percentages.

for fertilization, irrigation, and disease control also varied between vineyards (**Supplementary Table 2**). Aldea vineyard was 25-year-old vines at the moment of sampling and contained four randomized blocks of 48 vines per rootstock and block. Olite vineyard was 7-year-old vines at the moment of sampling and contained three randomized blocks of 15 vines per rootstock and block. In each vineyard, three rhizosphere samples were randomly collected per rootstock at two sampling dates (June and November) over 2 years (2016 and 2017). Sampled vines did not show any symptom of disease or nutrient deficiency. A total of 60 samples were collected per vineyard.

Rhizosphere soil samples were collected with a sterile spade close to the stem at depths of 40 to 50 cm, where the root system was denser. All samples were stored in sterile bags on dry ice at the time of sampling, and brought to the laboratory for further processing within 24 h from the time of sampling. The sampled roots with rhizosphere soil particles attached were placed in sterile tubes containing 9 mL of physiological solution (9 g/L NaCl). The tubes were vortexed for 5 min to detach the soil particles and then centrifuged at 4000 rpm for 5 min. The supernatant was discarded and the remaining soil fraction was used for DNA extraction.

DNA Extraction and Sequencing

The rhizosphere DNA was extracted from 0.5 g sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) and DNA samples were randomized across plates. The bacterial V4 region of the 16S rRNA gene was amplified using the protocol described by Lundberg et al. (2013). The universal primer pair 515F and 806R was used to generate bacterial-derived 16S rRNA amplicons. PNA PCR clamps were used to reduce host organelle contamination. The fungal ITS2 region was amplified using the universal primers ITS3/KYO2 and ITS4 (Toju et al., 2012). All primers were modified to include Illumina adapters[1]. Each 25 μl reaction contained 12.5 μl of HiFi HotStart Ready Mix (KAPA Biosystems, Woburn, MA, United States), 1.0 μl of each primer (10 μM), 2.5 μl of DNA template (5 ng/μl), and 8.0 μl PCR-grade water. PCR amplifications (performed in triplicate for each sample) consisted of a 3 min denaturation at 95°C; 25 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; and 5 min at 72°C. Samples were cleaned using the AMPure beads XP purification system (Beckman Coulter, United Kingdom) and sequenced on the Illumina MiSeq platform at the Fundación FISABIO (Valencia, Spain) facility using a 2 × 300 nucleotide paired reads protocol.

Data Analysis

Raw forward and reverse reads for each sample were assembled into paired-end reads considering the minimum overlapping of 50 nucleotides and a maximum of one mismatch within the region using the fastq-join tool from the ea-tools suite (Aronesty, 2011). The paired reads were then quality trimmed with a minimum of Q20. Sequences without either primer were discarded. Chimeric sequences were identified and filtered using the Usearch tool (Edgar, 2010, 2018). The UClust algorithm (Edgar, 2013) in QIIME (Caporaso et al., 2010) was used to cluster sequences at a 97% sequence similarity against UNITE dynamic database (Abarenkov et al., 2010) for ITS reads and Greengenes database (DeSantis et al., 2006) using the QIIME implementation of the RDP classifier for 16S rRNA reads (Caporaso et al., 2010). A tree was constructed from a gap-filtered alignment using FastTree (Price et al., 2009). A final OTU table was created excluding unaligned sequences and singletons. OTUs with no kingdom-level classification or matching chloroplast, mitochondrial, or Viridiplantae sequences were then removed from the data set. Good's coverage values were calculated using the Mothur computer software (Schloss et al., 2009). The rarefied OTU table and the phylogenetic tree were used as inputs for the subsequent analyses of α- and β- diversity. The OTU table was log transformed for statistical analysis (McMurdie and Holmes, 2014). As a final filter, taxa whose total abundances were less than 1% of the mean abundance were excluded, and only the OTUs present in at least two-thirds of the replicates of each sample were selected.

Bacterial and Fungal Diversity, Taxonomy Distribution and Statistical Analysis

Biodiversity indexes and principle statistics analyses on taxonomic profiles were analyzed in R version 3.5 using the vegan (Oksanen et al., 2018) and Phyloseq packages (McMurdie and Holmes, 2014). Data in each vineyard was analyzed separately due to the differences in soil chemistry and climate (**Supplementary Table 2**). Technical noise (variation attributable to sequencing depth or batch effects) was controlled by including MiSeq run as a random effect.

Within sample type, α-diversity estimates were calculated by analyzing the Chao1 richness and Shannon diversity in Phyloseq package, as implemented in the tool MicrobiomeAnalyst (Dhariwal et al., 2017). The normalized OTU table was analyzed using Bray Curtis metrics (Bray and Curtis, 1957) and utilized to evaluate the β- diversity and to construct PCoA plots (Vázquez-Baeza et al., 2013) using MicrobiomeAnalyst. In order to compare bacterial and fungal communities composition and to partition of variance in different categories, Bray–Curtis distance matrices were subjected to PERMANOVA (Anderson, 2001) using the adonis function with a permutation number of 999 available in the vegan package of R. PERMANOVA was performed to investigate which OTUs significantly differed in abundance among experimental factors.

The variance-partitioning model tests for effects of year, sampling date and genotype on microbiome communities, while year-by-genotype and date-by-genotype interaction terms describe how the distinct fungal and bacterial communities at different common rootstocks respond differently to each of these factors. The linear mixed models were fit using the lme4 package (Bates et al., 2015). Statistical significance of fixed predictors (Year + Sampling Date + Genotype + Year × Genotype + Date × Genotype) was assessed using Type III ANOVA with Satterthwaite's approximation of denominator degrees of freedom in the package InnerTest (Kuznetsova et al.,

[1]www.illumina.com

2016), and of random effects (MiSeq run) using likelihood ratio tests. This model was used to predict community descriptors that were continuous and approximately normally distributed in α-diversity metrics (Shannon entropy and Chao1 estimated richness) as described above.

The Linear Discriminant Analysis Effect Size (LEfSe) algorithm was used to identify taxa (genus level or higher) that differed in relative abundance between the rootstocks (Segata et al., 2011). The online Galaxy Version 1.0 interface (The Huttenhower Lab, 2018) was used, the threshold for the logarithmic LDA score was set at 1.0 and the Wilcoxon *p*-value at 0.05. The results are displayed in a cladogram and a bar graph. A Similarity Percentages (SIMPER) analysis was performed with PRIMER 6 software to explore the dissimilarities between the rootstock factor. Summarized taxa tables at the phylum and genera levels were used to investigate the phylogenetic groups that contribute to the dissimilarity. Unclassified OTUs amounting to less than 3% of the relative abundance in the rhizosphere were discarded from the analysis, according to Marasco et al. (2018). The bacterial and fungal OTUs shared among vineyards and rootstocks were defined by a Venn-diagram analysis using the software available at (Van de Peer et al., 2018).

Quantitative PCR Amplification and Quantification of Black-Foot Disease Pathogens

Quantitative PCR analyses were performed with the DNA extracted from the soil samples, as Agustí-Brisach et al. (2014) developed in previous research, using the primers YT2F and Cyl-R (Dubrovsky and Fabritius, 2007; Tewoldemedhin et al., 2011). These primers amplify the main *Cylindrocarpon*-like asexual morphs associated with black-foot disease, in particular those belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria*. Rotor-Gene 6000 real-time rotary analyzer (Qiagen, Hilden, Germany) was used to perform the qPCR amplifications. Each reaction contained 2 μl of DNA, 1× of SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara Bio Inc., Shiga, Japan) and 0.4 μM of each primer. The reaction mix was adjusted to a final volume of 20 μl with sterile distilled water. The thermocycling profile consisted of 30 s at 95°C and 50 cycles of 10 s at 95°C, 10 s at 60°C, and 30 s at 72°C. To evaluate amplification specificity, melting curve analysis was performed at the end of the qPCR runs according to the manufacturer's recommendations. Each analysis included three replicates of each sample, a non-template control reaction (water) and a positive control containing DNA extracted from a pure culture of the *Dactylonectria torresensis* isolate GTMF DT097, obtained from the collection of the Instituto Agroforestal Mediterráneo, Universitat Politècnica de Valencia, Spain. *D. torresensis* is the most common fungal species associated with black-foot diseased vines in Italy (Carlucci et al., 2017), Portugal (Reis et al., 2013), and Spain (Berlanas et al., 2017). For DNA extraction, fungal mycelium of this isolate grown on potato dextrose agar (PDA, Biokar-Diagnostics, Zac de Ther, France) for 2 weeks at 25°C in darkness, was scraped from the surface of the plate with a sterile scalpel. Total DNA was extracted using the E.Z.N.A. Plant Miniprep kit (Omega Bio-Tek, Doraville, United States) following the manufacturer's instructions and mycelia was previously homogenized with 4 steel beads of 2.38 mm and 2 of 3 mm diameter (Qiagen, Hilden, Germany) using a FastPrep-24TM5G (MP Biomedicals, California, United States) at 5 m/s for 20 s twice. DNA extracted was quantified with Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, United States).

DNA of the *Cylindrocarpon*-like asexual morphs species was quantified using a standard curve constructed with the isolate GTMF DT097, consisting of a dilution series from 275 μg/μL to 0.275 fg/μL. Quantitative PCR analysis were perform as previously explained and the standard curve was generated following the MIQE guidelines (Bustin et al., 2009), by plotting quantification cycle (C_q) values obtained for each specific DNA concentration, versus the logarithm of the initial concentration of isolate DNA. The mean DNA concentration and the standard deviation were determined from three replicates per dilution. Sensitivity of the qPCR assay was assessed using the standard curve to determine the minimum DNA concentration that can be detected. The amplification efficiency (E) and the coefficient of determination (R^2) of the standard curve were obtained using the Rotor-Gene 6000 Series software v. 1.7 (Qiagen, Hilden, Germany). Signal threshold levels were set automatically by the instrument software and the limit of detection (LOD) was identified by the last dilution when successful qPCR amplification of DNA occurred, accompanied by a melting curve peak temperature specific to *D. torresensis*.

Values from the *Cylindrocarpon*-like asexual morphs number of OTUs and DNA concentration were transformed by log ($n/N * 1000 + 1$). Where n was the number of OTUs or the DNA concentration detected on each sample and N was the total number of OTUs and the total DNA concentration detected. An analysis of correlation between both transformed datasets was performed in R version 3.5 using the corrr package.

RESULTS

High-Throughput Amplicon Sequencing

After paired-end alignments, quality filtering and deletion of chimeric, singletons, and mitochondrial and chloroplast sequences, a total of 4,337,395 bacterial 16S rRNA sequences and 6,216,366 fungal internal transcribed spacer (ITS) sequences were generated from 117 (three samples were removed from the analysis due to the low number of sequence reads) and 120 samples, respectively, and assigned to 975 bacterial and 567 fungal operational taxonomic units (OTUs) (**Supplementary Table 3**). Good's coverage values indicated that on average 94.5 and 90.1% of the total species richness were accounted for in bacteria and fungal communities, respectively (**Supplementary Table 4**). Chao1 diversity estimator ranged from 143.6 to 549.5 in the bacterial microbiome, and from 90.5 to 254.9 in the fungal microbiome. Shannon diversity estimator ranged from 1.80 to 4.68 in the bacterial microbiome, and from 1.80 to 3.84 in the fungal microbiome (**Supplementary Table 4**).

Core Grapevine Phylogeny Between Vineyards

The two habitats used as vineyard sites (Aldeanueva del Ebro, abbreviated "Aldea" in the figures and tables; and Olite) were separated by 45 km, and varied in most of soil physicochemical properties (**Supplementary Table 2**). Bacterial communities of rhizosphere soil samples did not differ significantly between vineyards (**Supplementary Table 5**). However, α-diversity differed among sites when studying the fungal microbiota, and principal coordinates analysis (PCoA) of Bray Curtis data demonstrated that vineyard was the primary source of β-diversity (**Supplementary Figure 1**). Comparing the fungal and bacterial microbiota of the two vineyards, 82.9 and 58.7% of bacterial and fungal OTUs, respectively, were shared between vineyards, demonstrating the existence of a "core" grape phylogeny that is independent of the growing region (**Figure 1**).

The relative abundance of bacterial and fungal phyla detected across all samples is shown in **Figure 1**. In both vineyards, the bacterial phyla Proteobacteria (26.1 and 28.1% in Aldea and Olite, respectively) and Actinobacteria (24.1 and 18.5%) represented almost 50% of the total bacteria detected. These phyla were followed by Acidobacteria (13.7 and 16.4%), unidentified bacteria (11.4 and 11.7%), and Bacteroidetes (5.2 and 6.1%) (**Figure 1**). The most abundant families within the Proteobacteria phylum were unidentified families from the order Rhizobiales (13.0 and 10.4% in Aldea and Olite, respectively), unidentified families from the class Betaproteobacteria (9.8 and 13.0%) and Sphingomonadaceae (7.6 and 10.7%). The most abundant families within the Actinobacteria phylum were unidentified Actinobacteria (29.1 and 22.5% in Aldea and Olite, respectively), Gaiellaceae (16.0 and 15.2%) and Streptomycetaceae (6.2 and 6.7%) (**Supplementary Figure 2**). Regarding the fungal taxa, the most abundant fungal phylum was Ascomycota (66.6 and 69.9% in Aldea and Olite, respectively), followed by Basidiomycota (20.1 and 11.5%) and Zygomycota (8.9 and 15.2%) (**Figure 1**). The most abundant families within the Ascomycota phylum were Nectriaceae (15.4%), unidentified Ascomycota (8.8%), and Bionectriaceae (9.1%) in Aldea vineyard, and Nectriaceae (17.7%), unidentified Ascomycota (11.1%), Pyronemataceae (9.6%), and Trichocomaceae (8.4%) in Olite vineyard (**Supplementary Figure 2**).

Host Genetic Influence on the Rhizosphere Microbiota

Bacterial and fungal diversity in rhizosphere soil samples differed significantly among rootstocks in Aldea vineyard. However, plant genotype did not predict Chao1 diversity (**Table 1**). Host genotype was the most important factor in structuring bacterial (R^2 = 0.65, $P < 0.001$) and fungal (R^2 = 0.86, $P < 0.001$) communities in the entire dataset, and also when the data were split by year and date (**Table 2**). A PCoA further demonstrated the variation in the total dataset could be attributed to host genotype in Aldea vineyard (**Figure 2**). In Olite vineyard, plant genotype had a much weaker influence on rhizosphere-associated bacterial and fungal communities. Host genotype did not predict any summary metrics of rhizosphere α and β-diversities (**Tables 1**, **2**).

The linear discriminant analysis effect size (LEfSe) detected 27 bacterial and 36 fungal clades in the rhizospheres, which discriminated the microbial communities between the different rootstock genotypes in Aldea vineyard (**Figures 3**, **4**). Both rootstocks 1103 P and 41 B showed higher number of differentially abundant bacterial clades (8 each) than the other rootstocks (5, 4, and 2 in 161-49 C, 110 R, and 140 Ru, respectively). The dominant bacterial phyla were Firmicutes

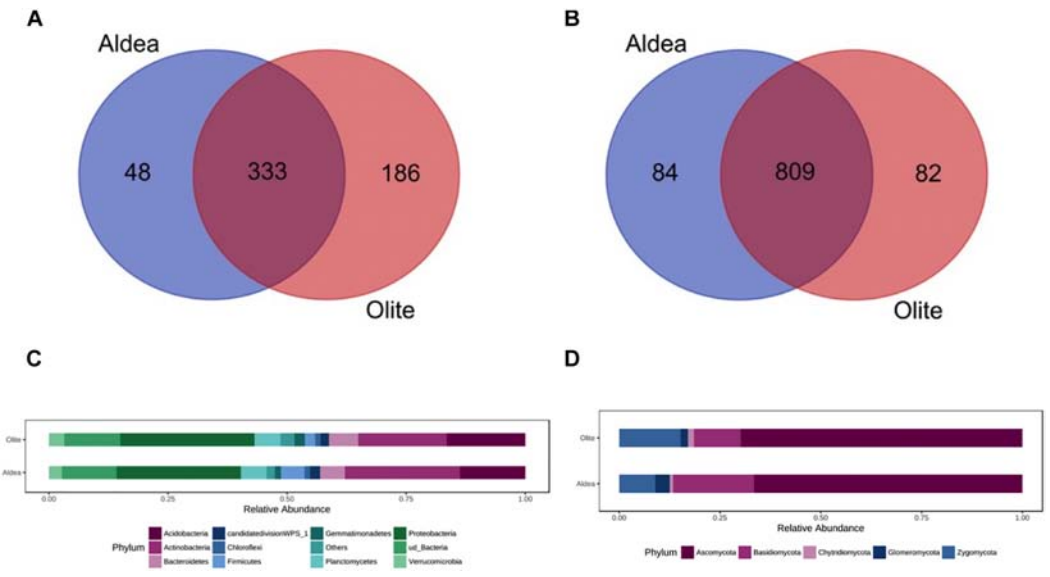

FIGURE 1 | Venn diagram illustrating the overlap of the OTUs identified in the bacterial **(A)** and fungal **(B)** microbiota between vineyards. Relative abundance of different bacterial **(C)** and fungal **(D)** phyla in the rootstock rhizospheres in both vineyards representing OTUs showing more than 1% relative abundance of all reads and present in at least 2/3 of replicates. Phyla representing less than 1% of the total reads are grouped in "Others".

TABLE 1 | Experimental factors predicting α-diversity of rhizosphere associated fungal and bacterial communities in Aldea and Olite vineyards.

Bacteria	**Aldea**		**Olite**	
	Shannon	**Chao1**	**Shannon**	**Chao1**
Genotype	$F_{4,54}$ = 3.47 **P = 0.0134**	$F_{4,54}$ = 0.34 P = 0.8480	$F_{4,54}$ = 0.90 P = 0.4693	$F_{4,54}$ = 0.32 P = 0.8648
Year	$F_{1,57}$ = 6.83 **P = 7.3e-09**	$F_{1,57}$ = 17.39 **P = 1.5e-20**	$F_{1,57}$ = 4.66 **P = 1.6e-04**	$F_{1,57}$ = 7.55 **P = 4.7e-10**
Year × Genotype	$F_{4,49}$ = 0.73 **P = 0.0122**	$F_{4,49}$ = 1.48 P = 0.3661	$F_{4,49}$ = 2.33 P = 0.0623	$F_{4,49}$ = 6.08 P = 0.2143
Date	$F_{1,57}$ = 0.05 P = 0.9555	$F_{1,57}$ = 0.18 P = 0.8502	$F_{1,57}$ = 0.68 P = 0.4989	$F_{1,57}$ = 0.13 P = 0.8941
Date × Genotype	$F_{4,49}$ = 1.55 P = 0.1812	$F_{4,49}$ = 0.74 P = 0.7702	$F_{4,49}$ = 0.19 P = 0.1802	$F_{4,49}$ = 1.67 P = 0.2561
MiSeq run	χ^2_1 = 0.55 P = 0.3623	χ^2_1 = 0.74 P = 0.4565	χ^2_1 = 0.28 P = 0.7712	χ^2_1 = 1.59 P = 0.3421
Fungi				
Genotype	$F_{4,55}$ = 2.80 **P = 0.0232**	$F_{4,55}$ = 1.12 P = 0.3529	$F_{4,55}$ = 0.82 P = 0.5130	$F_{4,55}$ = 2.27 P = 0.0929
Year	$F_{1,58}$ = 0.95 P = 0.3415	$F_{1,58}$ = 10.62 **P = 3.2e-15**	$F_{1,58}$ = 0.37 P = 0.7112	$F_{1,58}$ = 5.25 **P = 3.5e-06**
Year × Genotype	$F_{4,50}$ = 2.85 P = 0.1126	$F_{4,50}$ = 1.15 P = 0.3601	$F_{4,50}$ = 0.35 P = 0.1831	$F_{4,50}$ = 3.85 P = 0.3126
Date	$F_{1,58}$ = 8.52 **P = 1.08e-11**	$F_{1,58}$ = 2.17 P = 0.0640	$F_{1,58}$ = 0.44 P = 0.6597	$F_{1,58}$ = 1.31 P = 0.1937
Date × Genotype	$F_{4,50}$ = 0.71 **P = 0.0112**	$F_{4,50}$ = 0.91 P = 0.2903	$F_{4,50}$ = 1.91 P = 0.6351	$F_{4,50}$ = 6.81 P = 0.7443
MiSeq run	χ^2_1 = 0.74 P = 0.4912	χ^2_1 = 2.92 P = 0.2551	χ^2_1 = 1.77 P = 0.8135	χ^2_1 = 0.12 P = 0.7331

ANOVA, analysis of variance. Statistics describe linear random-intercept models of Shannon diversity and Chao1 richness in the rhizosphere. All P-values were corrected for multiple comparisons using the sequential Bonferroni correction. Significance was assessed using Type III ANOVA with F-tests for fixed effects and likelihood ratio tests for the random effect. Bold values indicate statistically significant results after correction for multiple comparisons. $P < 0.05$.

TABLE 2 | Adonis test of category effect on bacterial and fungal Bray–Curtis distance matrix.

Bacteria	**Aldea**			**Olite**		
Dataset	**Factor**	R^2	***P*-value**	**Factor**	R^2	***P*-value**
Total	Genotype	0.658	0.001	Genotype	0.058	0.015
	Year	0.163	0.001	Year	0.494	0.001
	Date	0.109	0.002	Date	0.059	0.004
110 R	Year	0.564	0.002	Year	0.438	0.005
	Date	0.028	0.116	Date	0.204	0.066
140 Ru	Year	0.235	0.006	Year	0.458	0.005
	Date	0.355	0.002	Date	0.092	0.333
1103 P	Year	0.220	0.011	Year	0.379	0.005
	Date	0.461	0.002	Date	0.174	0.036
41 B	Year	0.087	0.071	Year	0.453	0.005
	Date	0.670	0.002	Date	0.129	0.092
161 49 C	Year	0.228	0.003	Year	0.471	0.005
	Date	0.228	0.005	Date	0.221	0.040
2016	Genotype	0.868	0.001	Genotype	0.206	0.031
	Date	0.067	0.035	Date	0.165	0.001
2017	Genotype	0.768	0.001	Genotype	0.240	0.001
	Date	0.135	0.004	Date	0.138	0.002
June	Genotype	0.634	0.001	Genotype	0.145	0.365
	Year	0.110	0.005	Year	0.331	0.001
November	Genotype	0.831	0.001	Genotype	0.240	0.020
	Year	0.123	0.004	Year	0.354	0.001
Fungi						
Total	Genotype	0.864	0.001	Genotype	0.096	0.027
	Year	0.052	0.004	Year	0.564	0.001
	Date	0.084	0.001	Date	0.042	0.005
110 R	Year	0.183	0.122	Year	0.438	0.005
	Date	0.501	0.002	Date	0.204	0.066
140 Ru	Year	0.142	0.137	Year	0.458	0.005
	Date	0.615	0.002	Date	0.092	0.333
1103 P	Year	0.266	0.031	Year	0.379	0.005
	Date	0.496	0.002	Date	0.174	0.036
41 B	Year	0.241	0.033	Year	0.453	0.005
	Date	0.425	0.002	Date	0.129	0.092
161 49 C	Year	0.191	0.066	Year	0.471	0.005
	Date	0.472	0.002	Date	0.221	0.040
2016	Genotype	0.841	0.001	Genotype	0.144	0.305
	Date	0.110	0.002	Date	0.070	0.002
2017	Genotype	0.928	0.001	Genotype	0.274	0.001
	Date	0.130	0.002	Date	0.127	0.002
June	Genotype	0.808	0.001	Genotype	0.220	0.012
	Year	0.066	0.080	Year	0.289	0.001
November	Genotype	0.753	0.001	Genotype	0.200	0.003
	Year	0.105	0.004	Year	0.208	0.001

(37%) in rootstock 41B, Actinobacteria and Planctomycetes (50% each) in rootstock 140 Ru, and Actinobacteria in rootstocks 161-49 C, 110 R, and 1103 P (60, 75, and 75%, respectively) (**Figure 3**). The dominant fungal phyla were Basidiomycota (73%) in rootstock 140 Ru, and Ascomycota in rootstocks 41 B, 161-49 C, 110 R, and 1103 P (75, 100, 36, and 71%, respectively) (**Figure 4**).

The rootstock-pairs dissimilarity, due to phyla and genera contribution in the rhizosphere was calculated by SIMPER (similarity percentages) analysis (**Supplementary Table 6**). Higher microbiome dissimilarity among rootstocks was revealed in Aldea vineyard compared to Olite vineyard, considering bacterial (**Supplementary Table 6A**) and fungal phyla (**Supplementary Table 6C**), and bacterial (**Supplementary Table 6B**) and fungal genera (**Supplementary Table 6D**) distribution. Firmicutes and Acidobacteria were the major phyla that contribute to differentiate the bacterial communities associated with the different rootstock types in Aldea and Olite vineyards, respectively (**Supplementary Table 6A**). Several genera were predominant and determined the dissimilarities among rootstocks such as *Bacillus* in Aldea vineyard or *Aridibacter* in Olite vineyard. The genus *Bacillus* appeared to be rhizosphere genotype biomarker of 140 Ru and 161-49 C rootstocks (**Supplementary Table 6B**). The fungal phyla Ascomycota and Basidiomycota contributed to the dissimilarity among rootstocks in Aldea vineyard, while only the phylum Basidiomycota contributed to differentiate fungal communities among rootstocks (**Supplementary Table 6C**).

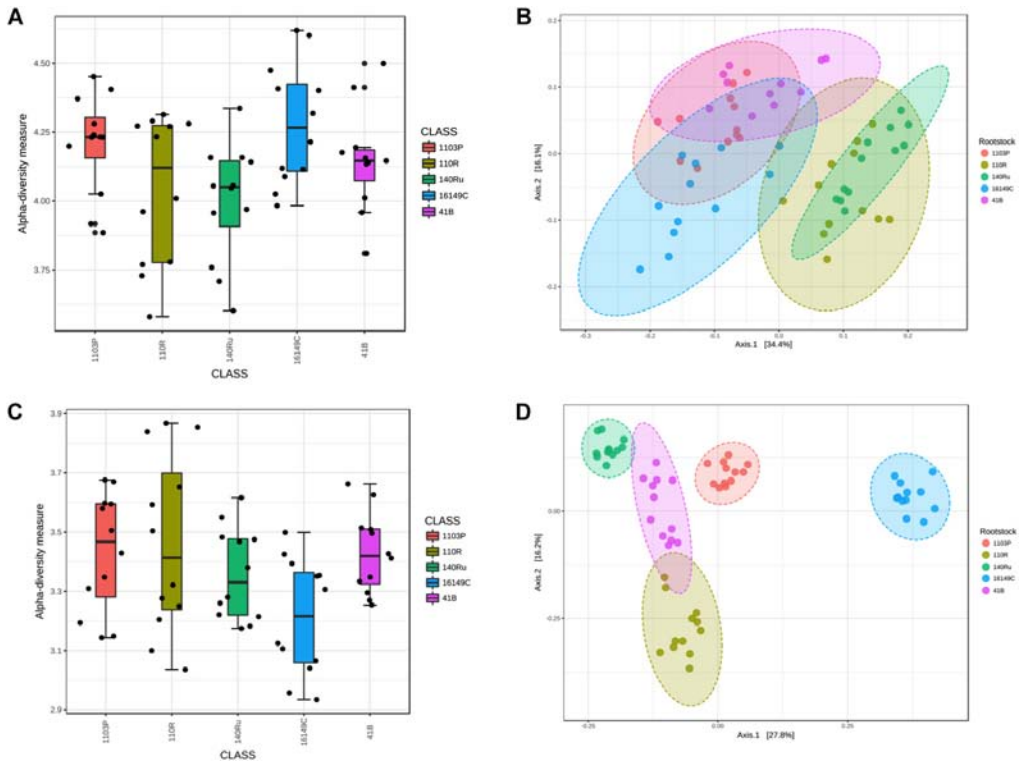

FIGURE 2 | Box plot illustrating the differences in Shannon diversity measures of the bacterial **(A)** and fungal **(C)** communities in the grapevine rootstocks in Aldea vineyard. Principal Coordinate Analysis (PCoA) based on Bray Curtis dissimilarity metrics, showing the distance in the bacterial **(B)** and fungal **(D)** communities among grapevine rootstocks.

The fungal genera *Geopyxis*, *Clonostachys*, and *Lecanicillium* determined the dissimilarities among rootstocks in Aldea vineyard, being *Geopyxis* a rhizosphere genotype biomarker of 110 R rootstock and *Clonostachys* of 1103 P and 140 Ru rootstocks (**Supplementary Table 6D**). In Aldea vineyard, 161-49 C rootstock showed the highest dissimilarity with the other rootstocks in bacterial and fungal microbiome distribution.

Year Strongly Influenced Microbiomes

Our results demonstrate that bacterial microbiome varied profoundly between years. This pattern was consistent to community-level measure of α- diversity in both Aldea and Olite vineyards (**Table 1**) Richness increased between 2016 and 2017 in both vineyards (**Supplementary Figure 3**). However, year of sampling affected the Bray Curtis metric of β-diversity in only Olite vineyard (R^2 = 0.494) (**Supplementary Figure 3**). Regarding the fungal microbiome, richness also varied between vineyards and increased between 2016 and 2017 in both vineyards (**Table 1** and **Supplementary Figure 4**). However, year of sampling did not predict Shannon diversity and affected the Bray Curtis metric of β-diversity in only Olite vineyard (**Table 2** and **Supplementary Figure 4**). Sampling date also contributed to α-diversity variation indicating temporal changes in relative abundance of fungal OTUs in Aldea vineyard. Fungal composition decreased between June and November (**Table 1** and **Supplementary Figure 5**). Fungal community structure varied individually in each rootstock with date (R^2 ranging from 0.42 to 0.61), but not in the total dataset ($R^2 < 0.1$) (**Table 2**).

Rootstock-Specific and Shared Bacterial and Fungal Assemblages

The rhizosphere compartments of grapevine rootstocks showed specific fungal and bacterial OTUs for each rootstocks and a cluster of shared OTUs. In Aldea, specific OTUs associated with most of the rootstocks ranged from 4.3 to 5.8% of their bacterial communities (**Figure 5**). Specific OTUs associated with the rootstocks 140 Ru, 1103 P, 41 B and 110 R represented less than 9% of their fungal communities, where the 161-49C-specific OTUs enriched only 4.5% of the relative abundance (**Figure 5**). In Olite, specific OTUs associated with most of the rootstocks represented less than 9% of their bacterial and fungal communities, with the exception of bacterial communities associated with 140 Ru rootstock that represented 21.3% of its total (**Figure 6**). The OTUs that were unique in each of the grapevine rootstock are shown in **Supplementary Tables 7, 8**.

Quantification of Black-Foot Disease Pathogens Using Quantitative PCR

The standard curve, constructed with serial dilutions of the DNA of *D. torresensis* isolate GTMF DT097, revealed high correlations between C_q and DNA, with R^2-value of 0.99 and reaction

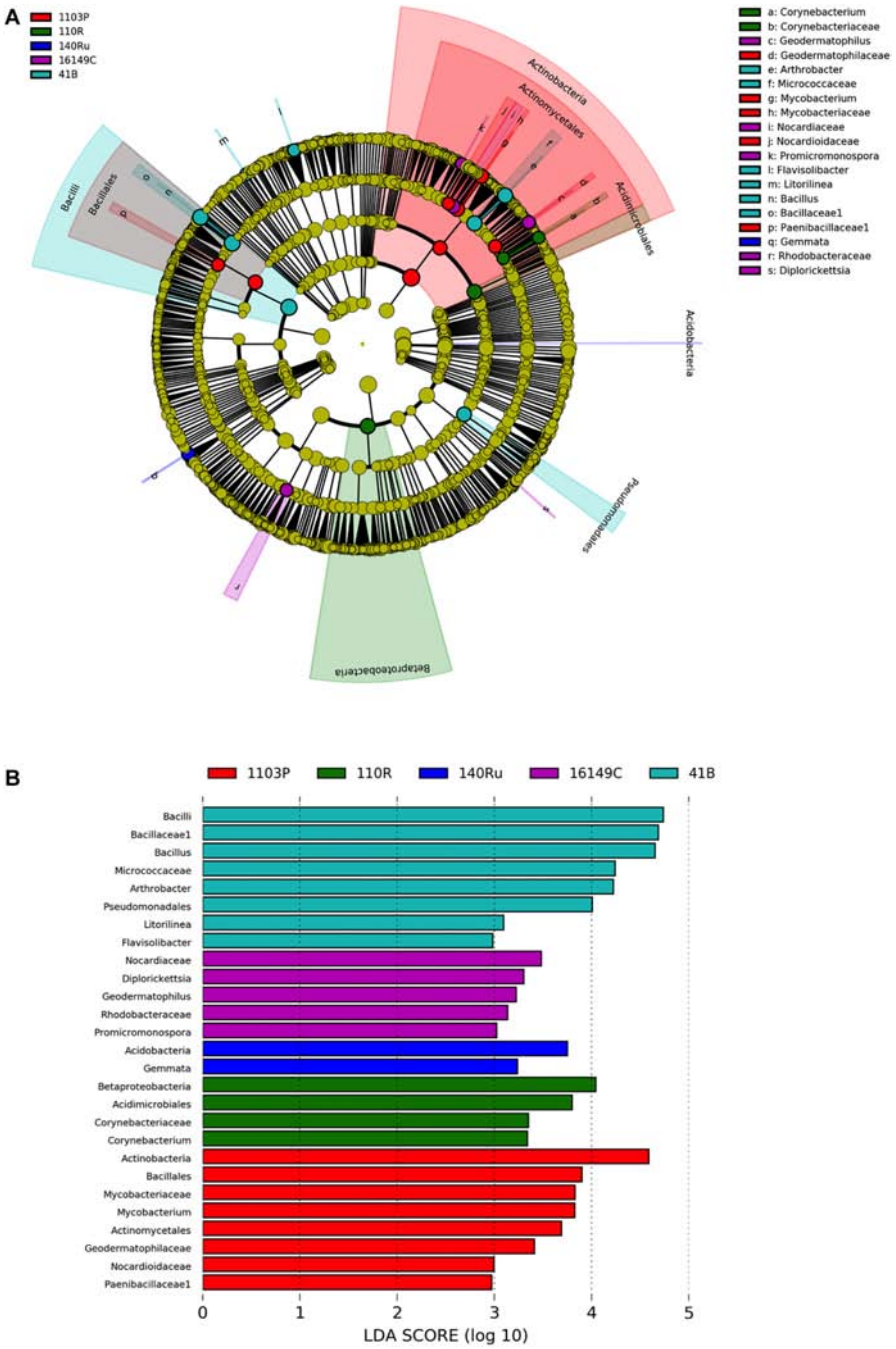

FIGURE 3 | LEfSe was used to identify the most differentially abundant taxa among grapevine rootstocks in Aldea vineyard. Cladogram generated by LEfSe indicating differences of bacteria **(A)** at phylum, class, family, and genus levels between the five groups (relative abundance ≤0.5%). Each successive circle represents a phylogenetic level. Color regions indicate taxa enriched in the different rootstocks. Differing taxa are listed on the right side of the cladogram. Bar graph showing LDA scores for bacteria **(B)**. Only taxa meeting an LDA significant threshold >2 are shown.

efficiency of 0.90. The minimum DNA concentration detectable of *D. torresensis* was at C_q value of the dilution D7 thus, the limit of detection (LOD) was established at 2.75 fg/μL.

DNA of *Cylindrocarpon*-like asexual morphs was detected in all rootstock rhizosphere samples, in both vineyards and years, with concentrations ranging from 0.39 pg/μL to 4.06 pg/μL in Aldea 2016, from 3.52 pg/μL to 14.14 pg/μL in Aldea 2017, from 0.88 pg/μL to 8.45 pg/μL in Olite 2016 and from 2.65 pg/μL to 59 pg/μL in Olite 2017. The year and vineyard factors had a significant effect on *Cylindrocarpon*-like asexual morphs DNA concentration detected ($P < 0.01$). The concentration of DNA detected was significantly higher in Olite vineyard compared with

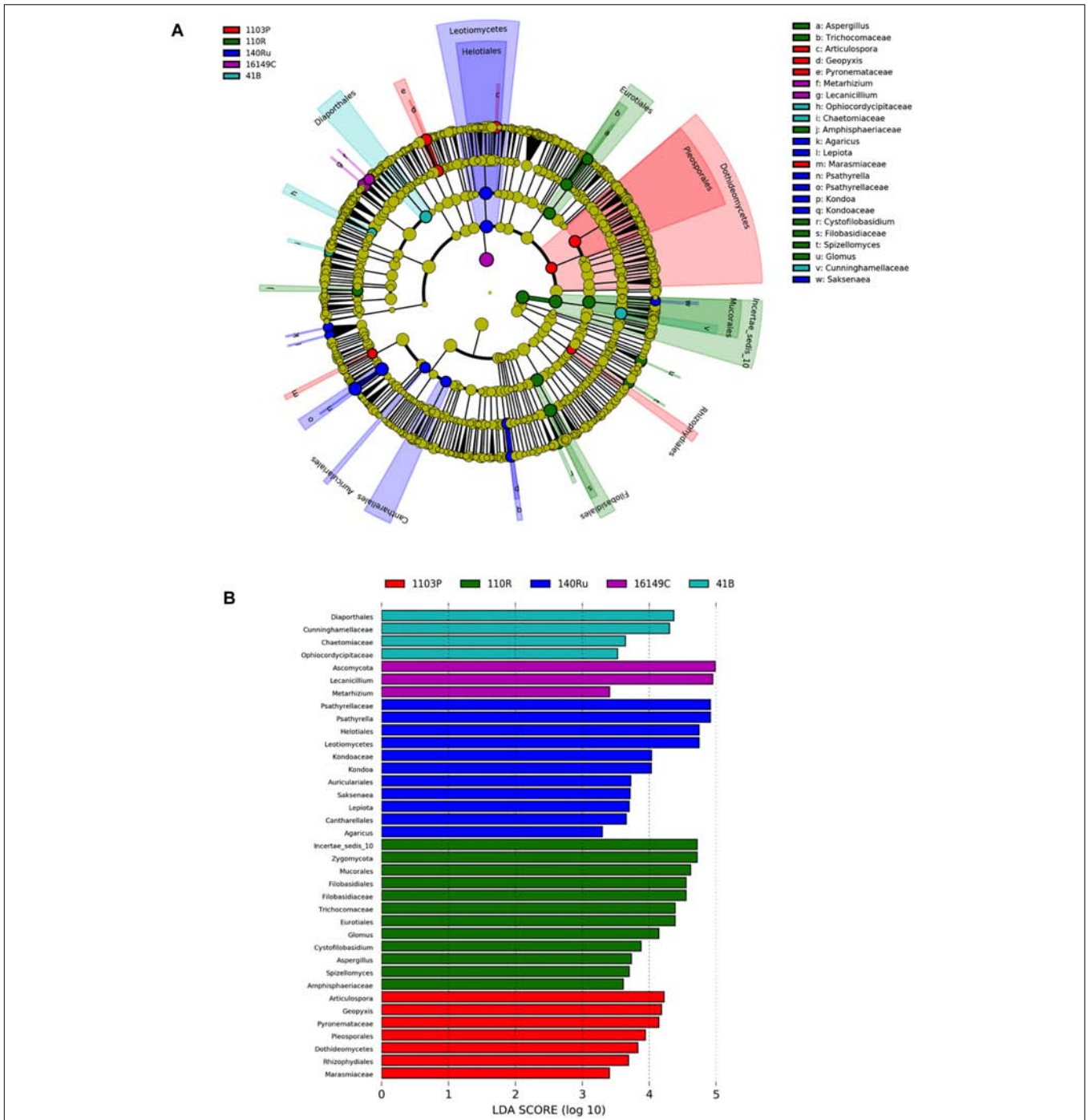

FIGURE 4 | LEfSe was used to identify the most differentially abundant taxa among grapevine rootstocks in Aldea vineyard. Cladogram generated by LEfSe indicating differences of fungi **(A)** at phylum, class, family, and genus levels between the five groups (relative abundance ≤0.5%). Each successive circle represents a phylogenetic level. Color regions indicate taxa enriched in the different rootstocks. Differing taxa are listed on the right side of the cladogram. Bar graph showing LDA scores for fungi **(B)**. Only taxa meeting an LDA significant threshold >2 are shown.

Aldea vineyard, especially in year 2017. The rootstock factor had a significant effect on the DNA concentration detected in Aldea vineyard for 2017 samples (P = 0.0156). Rootstocks 161-49 C, 140 Ru, 1103 P, and 110R showed similar DNA concentrations values that were significantly lower when compared with 41 B rootstocks (**Supplementary Figure 6**). The analysis showed a positive significant correlation between the number of OTUs and the *Cylindrocarpon*-like asexual morphs DNA quantified using the real-time approach ($P < 0.01$, Spearman correlation coefficient = 0.72) (**Supplementary Figure 7**).

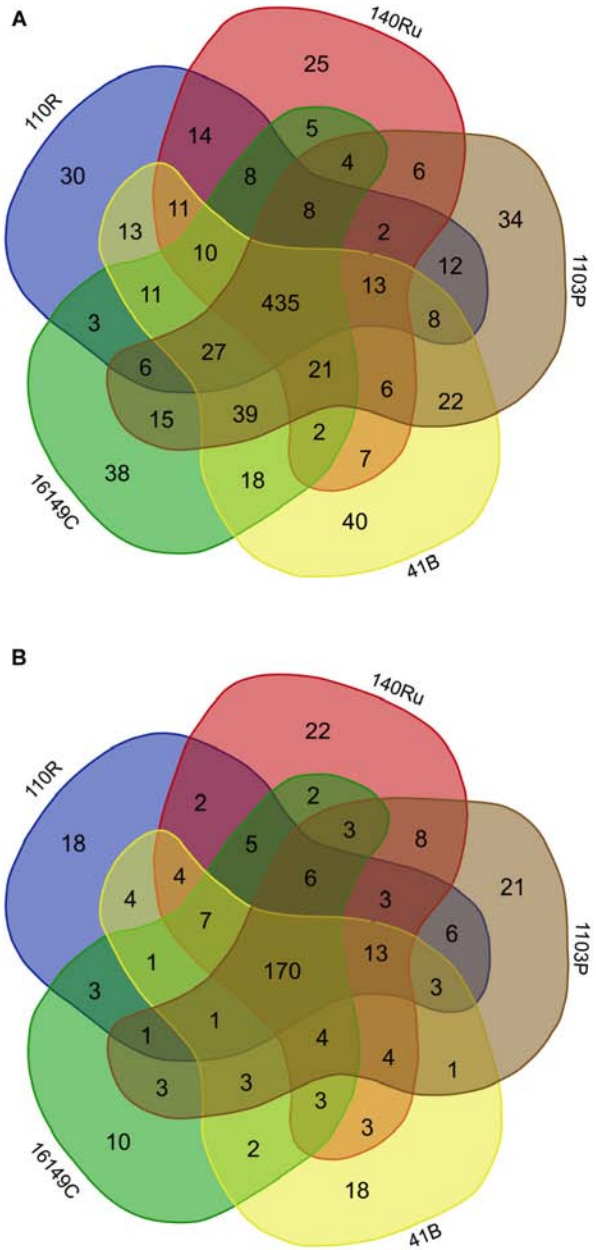

FIGURE 5 | Venn diagrams showing the common and exclusive bacterial **(A)** and fungal **(B)** OTUs of the rhizosphere of the grapevine rootstocks in Aldea vineyard.

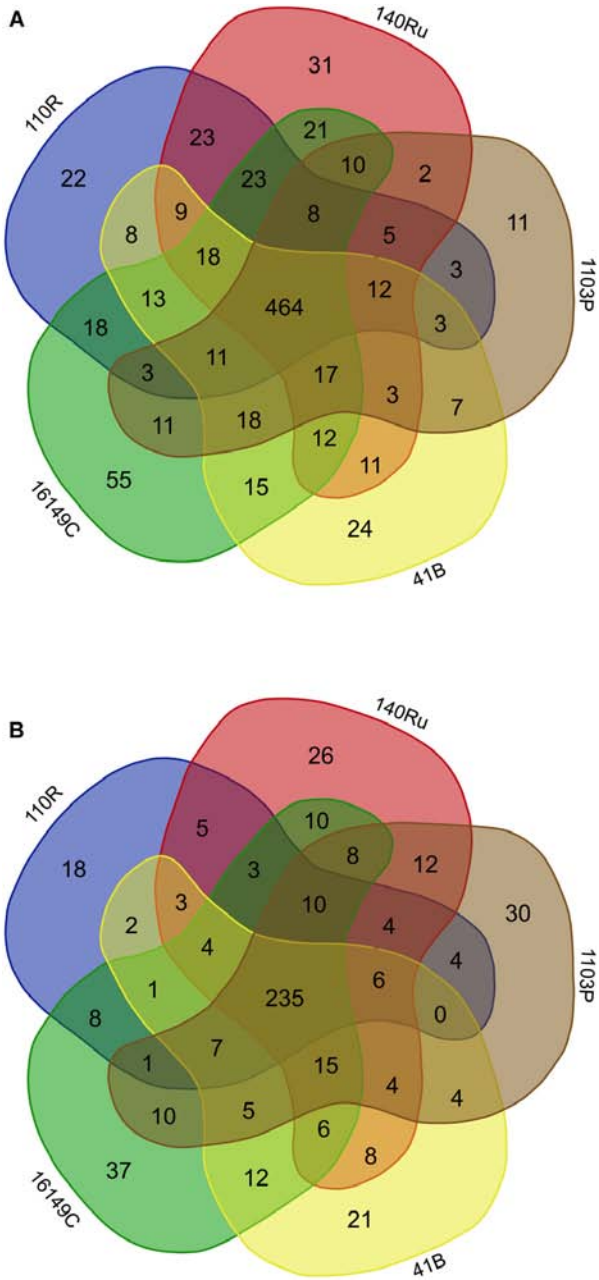

FIGURE 6 | Venn diagrams showing the common and exclusive bacterial **(A)** and fungal **(B)** OTUs of the rhizosphere of the grapevine rootstocks in Olite vineyard.

DISCUSSION

In this study, we characterized the rhizosphere microbial community composition across five commercial grapevine rootstock genotypes cultivated in the same soil at two vineyards and sampling dates over 2 years. The analysis of bacterial and fungal populations in the grapevine rhizosphere targeting 16S rRNA and ITS region, respectively, have been proved effective in previous studies (Corneo et al., 2014; Holland et al., 2016; Longa et al., 2017; Manici et al., 2017; Stefanini and Cavalieri, 2018). Especially for bacterial barcoding, the choice of partial sequence regions is pivotal and can significantly affect the results because the 16S rRNA gene regions have different divergence (Youssef et al., 2009). In our study, we used the V4 region because according to recent *in silico* studies (Youssef et al., 2009), V4 along with V5-V6, and V6-V7 regions were considered as the most suitable regions for metagenomic purposes because

they provided estimates comparable to those obtained with the complete 16S rRNA gene sequence (Youssef et al., 2009).

Our study represents the first approach to investigate the rhizosphere fungal microbiome of grapevine by HTAS. In grapevine, the ecology of fungal communities is so far largely derived from the studies using pyrosequencing approach in bulk soil (Holland et al., 2016; Castañeda and Barbosa, 2017; Longa et al., 2017) or ARISA fingerprinting (Likar et al., 2017) and PCR-DGGE (Manici et al., 2017) approaches in rhizosphere soil. Even though the ITS region was ratified by The Fungal Barcoding Consortium (Schoch et al., 2012) as the universal DNA barcode for the fungal kingdom using the same gene section proposed by White et al. (1990), some recent reports point out its limitations for specific taxa. This region does not work well with taxa having narrow or no barcode gaps in their ITS regions, such as *Fusarium* or *Trichoderma* (Schoch et al., 2012). In addition, the correct identification of morphologically similar cryptic species using the ITS regions is still problematic due to the lack of consensus in the lineage-specific cut-off value for species determination (Nilsson et al., 2008).

The bacterial microbiomes of the different rootstocks were largely composed of Proteobacteria and Actinobacteria that accounted for almost 50% of the relative abundance in both vineyards. The predominant bacterial phyla found in this work is consistent with the results obtained in other studies in vineyard soil (Opsi et al., 2014; Vega-Avila et al., 2015; Castañeda and Barbosa, 2017; Longa et al., 2017; Marasco et al., 2018). Proteobacteria and Actinobacteria are known for their role in the carbon biochemical cycle and their production of second metabolites (Jenkins et al., 2009). The major fungal phyla detected in our study were largely composed of Ascomycota and Basiodiomycota that accounted for almost 75% of the relative abundance in both vineyards. Previous studies also agree on the most common fungal phyla detected in grapevines fields (Castañeda and Barbosa, 2017; Longa et al., 2017; Manici et al., 2017). These results suggest that vineyard microbiome in Navarre and La Rioja regions is partially conserved.

The results obtained in the Aldea vineyard showed a significant fraction of variation in fungal and bacterial diversity (both the α- and β-diversity) that could be attributed to host genetics. Recent research indicated that rootstock genotypes could have a notable influence in shaping the bacteria taxa distribution in the root and rhizosphere systems of grapevine (Marasco et al., 2018). This effect of the host genotype in the rhizosphere microbiome has been reported in other woody crops, such as apple (Liu et al., 2018) and pines (Gallart et al., 2018), as well as in several annual crops, such as maize (Peiffer et al., 2013), potato (Inceoğlu et al., 2010), and chickpea (Bazghaleh et al., 2015). This could be due to the influence of the genotype in the root metabolism, including immune response and exudate composition, which impact in the rhizosphere microbiome (Wagner et al., 2016). Rootstocks show different level of tolerance to distinct diseases; and this could be decisive in their effect in the microbiome (Sapkota et al., 2015). Moreover, as reviewed by Liu et al. (2018), several studies hint to a possible co-evolution of the holobiont. However, further research is needed to validate this hypothesis. On the other hand, the Olite vineyard showed a lower microbiome dissimilarity among rootstocks, suggesting that the effect of genotype in shaping the microbiome might be influenced by other factors.

The differences between Olite and Aldea vineyards could lie in the soil physicochemical properties, in the soil and cultivar management practices, or in the age of the plants, being vines cultivated in Olite vineyard younger than in Aldea vineyard. Environmental heterogeneity, such as the soil physicochemical properties and moisture content have been identified as major factors shaping the spatial scaling of the rhizosphere microbiome in many previous studies (Costa et al., 2006; Tan et al., 2013; Schreiter et al., 2014), including grapevine (Fernández-Calviño et al., 2010; Corneo et al., 2014; Burns et al., 2015; Zarraonaindia et al., 2015; Holland et al., 2016). Soil physicochemical properties can also influence the population structure of specific soil-borne pathogens. For instance, Berlanas et al. (2017) observed that excessive calcium carbonate in soil may increase black-foot disease inoculum density.

Field management practices have been also reported as an important driver of the microbiome diversity (Santhanam et al., 2015; Sapkota et al., 2015; Hacquard, 2016; Gallart et al., 2018), including the grapevine soil microbiome (Vega-Avila et al., 2015; Likar et al., 2017; Longa et al., 2017). Nevertheless, other studies showed a long-term effect of cultivation rather than field management on soil microbial diversity (Buckley and Schmidt, 2001; Peiffer et al., 2013). Microbiome studies should consider the high degree of temporal variability in the sample design, because sampling the same point in different times can give different results due the variability of the own microbial community through time (Redford and Fierer, 2009). The year to year variation found in our study could be explained by the different root response to distinct environmental factors, such as temperature or precipitation (Wagner et al., 2016). Further research is needed to determine if environment plays a much greater role than host genetics in determining the composition of the rhizosphere microbiome of grapevine.

Several studies have remarked the effect of the growth stage of the plant in its associated rhizosphere microbiome (Baudoin et al., 2002; Inceoğlu et al., 2010; Li et al., 2014; Okubo et al., 2014; Yuan et al., 2015; Wagner et al., 2016; Qiao et al., 2017). Changes in the quantity and quality of root exudates as plants develop have been proposed as the main source of variation of the rhizosphere microbiome composition present during different developmental stages of maize cultivars (Baudoin et al., 2002). However, most of the published studies are focused in annual plant systems. In grapevine, Manici et al. (2017) recently investigated shifts in bacterial and fungal communities between mature and young replaced vines in Italy. At a single sampling moment, these researchers concluded that long-term growth legacy overcame plant age in shaping rhizosphere microbiome (Manici et al., 2017). Further research is therefore needed to determine the long-term effect of the grapevine age on the associated microbiome as plants develop. This could be accomplished by comparing the rhizosphere microbiome (i) in a single vineyard over time, or (ii) in two vineyards in close proximity with identical environmental conditions and soils, but with vines on different aging process.

Our results showed that the root system type is able to select specific bacterial and fungal OTUs as biomarkers for the different genotypes. Members of the bacterial genus *Bacillus*, which was only found in 140 Ru and 161-49 C rootstocks in Aldea vineyard, has wide diversity of physiological ability with respect to heat, pH, and salinity. Therefore, *Bacillus* species can be found in a wide range of habitats, being a few of them pathogenic to vertebrates or invertebrates (Holt et al., 1994). *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been described as potential biocontrol agents against *Aspergillus parasiticus* and stem rot disease (Le et al., 2018; Siahmoshteh et al., 2018). *In vitro* assays of the heat stable metabolites of *B. subtilis* showed promising results in reducing the growth of the fungal trunk pathogens *Lasiodiplodia theobromae*, *Phaeomoniella chlamydospora*, and *Phaeoacremonium minimum* (Alfonzo et al., 2009). Rezgui et al. (2016) recently identified several *B. subtilis* strains inhabiting the wood tissues of mature grapevines in Tunisia with antagonistic traits against fungal trunk pathogens. On the other hand, some species of the arbuscular mycorrhizal (AM) fungal genus *Glomus*, one of the most differentially abundant taxa for 110 R rootstock in Aldea vineyard, are cataloged as biocontrol agents (Tahat et al., 2010). For instance, inoculation of grapevine roots with *Rhizophagus irregularis* (syn. *Glomus intraradices*) reduced both the disease severity and the number of root lesions caused by black-foot disease pathogens (Petit and Gubler, 2006). AM fungi form one of the most interesting beneficial plant–microorganism associations (Smith and Read, 2008) and are known to colonize the roots of the majority of land plants, including grapevines (Schreiner and Mihara, 2009; Trouvelot et al., 2015). Several genera within the Glomeromycota phylum have been identified from the rhizosphere samples obtainted in this study, namely *Claroideoglomus*, *Diversispora*, *Entrophosphora*, and *Rhizophagus*. Trouvelot et al. (2015) reported that soil management can greatly impact the diversity of AM fungi. In fact, AM fungal communities are highly influenced by the soil characteristics but also to a smaller extent by the host plant development stage (Schreiner and Mihara, 2009; Balestrini et al., 2010).

High-throughput amplicon sequencing is a powerful method for the analysis of microbial populations. It is accomplished by sequencing specific marker genes amplified directly from environmental DNA without prior enrichment or cultivation of the target population (Franzosa et al., 2015). The advantages of this approach is the detection of rare taxa at the genus level given the availability of large and comprehensive reference databases as well as several pipelines for bioinformatics analysis (Stefanini and Cavalieri, 2018). Drawbacks of HTAS include the biased relative quantification of bacterial communities since bacterial species bear various number of copies of 16S rRNA genes, the sequencing of matrix (e.g., grape ITS, chloroplast 16S) and the low confidence for taxonomic assignment at the species level (Stefanini and Cavalieri, 2018). A step forward consists of the understanding of how changes in the composition of microbial communities impact the population's biological functions (Ravin et al., 2015). Unfortunately, HTAS only allows inference of functional annotation while in whole-genome sequencing, functional annotation can be carried out by gene enrichment (Stefanini and Cavalieri, 2018). A further drawback of using DNA-based metagenomic data to infer the biological functions potentially exploited by microbial populations is that the detected DNA may belong to dead organisms. However, an approach based on RNA sequencing would give a direct report of the functions achievable by the viable microbial populations. In grapevine, the study of the active fungal communities of internal grapevine wood by HTAS in extracted total RNA has been recently accomplished by Eichmeier et al. (2018).

The quantitative significance of next-generation sequencing data for microorganisms is often debated (Amend et al., 2010). Fortunately, we were able to compare the relative abundance of reads with the relative abundance of DNA of black-foot disease pathogens, and we observed significant positive correlation. From the fungal soilborne pathogens affecting grapevine, *Cylindrocarpon*-like asexual morphs associated with black-foot disease are among the most important limiting factor of the production worldwide (Halleen et al., 2006; Agustí-Brisach and Armengol, 2013). Therefore, *Cylindrocarpon*-like asexual morphs can be considered model pathogens to monitor the healthy status of the grapevine planting material when analyzing the fungal microbial composition of soil/rhizosphere samples.

Grapevine rootstocks have different susceptibilities toward pathogens, including trunk disease pathogens (Eskalen et al., 2001; Alaniz et al., 2010; Gramaje et al., 2010; Brown et al., 2013; Billones-Baaijens et al., 2014), which may be an important factor in shaping not only pathogens abundance but also entire communities. Nevertheless, we did not observe a clear correlation between known disease resistances in individual genotypes and the fungal communities, although *Cylindrocarpon*-like asexual morphs were found in lower abundance in 161-49 C rootstock by both high-throughput amplicon sequencing and qPCR approaches. The use of 161-49 C rootstock was previously recommended within an integrated management program for other grapevine trunk diseases, such as Petri disease and esca (Gramaje et al., 2010).

CONCLUSION

We have studied the effects of genotype, year, sampling date, and location on bacterial and fungal communities in the grapevine rhizosphere. We found that grapevine genotype was the most important factor in shaping the microbiome in the mature vineyard. Many bacterial and fungal species were found in all rootstocks and in both locations in our study, demonstrating the existence of a "core" grape phylogeny that is independent of the growing region. Interestingly, the rhizosphere compartments of 140 Ru and 161-49 C rootstocks, the latter showing high tolerance to esca and Petri disease pathogens in previous research (Gramaje et al., 2010), harbored lower number of black-foot pathogens than the other grapevine rootstocks. Also of interest was the presence of high relative abundance of the genus *Bacillus* in both grapevine rootstocks, a bacterial genus recognized as biocontrol agents. A more comprehensive study is needed to decipher the cause of the rootstock microbiome selection and the mechanisms by which grapevines are able to shape their associated microbial community. Understanding the vast diversity of bacteria and fungi in the rhizosphere and the interactions between microbiota

and grapevine will facilitate the development of future strategies for grapevine protection.

AUTHOR CONTRIBUTIONS

CB, MB, and DG conceived the study. All authors contributed to the data collection. CB, MB, GE, and DG performed the data interpretation and manuscript preparation. CB, MB, DG, GE, and ML performed the experiments. CB, MB, GE, and DG contributed to the bioinformatics data analysis. All authors critically reviewed and edited the manuscript, and approved its publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01142/full#supplementary-material

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Overexpression of the *VvSWEET4* Transporter in Grapevine Hairy Roots Increases Sugar Transport and Contents and Enhances Resistance to *Pythium irregulare*, a Soilborne Pathogen

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Sugar transport and partitioning play key roles in the regulation of plant development and responses to biotic and abiotic factors. During plant/pathogen interactions, there is a competition for sugar that is controlled by membrane transporters and their regulation is decisive for the outcome of the interaction. SWEET sugar transporters are the targets of extracellular pathogens, which modify their expression to acquire the sugars necessary to their growth (Chen et al., 2010). The regulation of carbon allocation and sugar partitioning in the interaction between grapevine (*Vitis vinifera*) and its pathogens is poorly understood. We previously characterized the SWEET family in *V. vinifera* and showed that SWEET4 could be involved in resistance to the necrotrophic fungus *Botrytis cinerea* in Arabidopsis (Chong et al., 2014). To study the role of VvSWEET4 in grapevine, we produced *V. vinifera* cv. Syrah hairy roots overexpressing *VvSWEET4* under the control of the CaMV 35S promoter ($VvSWEET4_{OX}$). High levels of *VvSWEET4* expression in hairy roots resulted in enhanced growth on media containing glucose or sucrose and increased contents in glucose and fructose. Sugar uptake assays further showed an improved glucose absorption in *VvSWEET4* overexpressors. In parallel, we observed that *VvSWEET4* expression was significantly induced after infection of wild type grapevine hairy roots with *Pythium irregulare*, a soilborne necrotrophic pathogen. Importantly, grapevine hairy roots overexpressing *VvSWEET4* exhibited an improved resistance level to *P. irregulare* infection. This resistance phenotype was associated with higher glucose pools in roots after infection, higher constitutive expression of several genes involved in flavonoid biosynthesis, and higher flavanol contents. We propose that high sugar levels in $VvSWEET4_{OX}$ hairy roots

provides a better support to the increased energy demand during pathogen infection. In addition, high sugar levels promote biosynthesis of flavonoids with antifungal properties. Overall, this work highlights the key role of sugar transport mediated by SWEET transporters for secondary metabolism regulation and pathogen resistance in grapevine.

Keywords: grapevine, SWEET transporter, sugar, pathogen interaction, flavonoid

INTRODUCTION

Grapevine (*Vitis vinifera*) is an economically important crop that is susceptible to diverse pathogens. Current strategies to control diseases in grapevine rely on the massive use of pesticides, making viticulture a major consumer of chemicals in agriculture, with important economic and environmental consequences. Source to sink transport and allocation of sugars are major determinants in the control of crop productivity and play key roles in the regulation of plant responses to biotic and abiotic factors (Lemoine et al., 2013). Since biotrophic and necrotrophic pathogens act as additional sinks within the host tissue, sugar transport and partitioning are modified following infection (Lemoine et al., 2013). Increasing evidence shows that during plant-pathogen coevolution, microorganisms have evolved sophisticated mechanisms to highjack sugar fluxes from their hosts (Baker et al., 2012). In grapevine, regulation of sugar allocation during the interaction with pathogens is poorly understood.

In plants, apoplastic sugar levels are controlled by glycoside hydrolases and membrane transporters regulating the levels of nutrients provided to the pathogen (Bezrutczyk et al., 2018). Enhanced sugar efflux from host cells and invertase activity can lead to sucrose and hexose accumulation into the apoplast, which are taken up by fungal sugar transporters (Lemoine et al., 2013; Veillet et al., 2016). Increased invertase activity and plant monosaccharide transporter expression have indeed been reported in several plant-pathogen interactions (Fotopoulos et al., 2003; Doidy et al., 2012). In *Arabidopsis thaliana*, apoplastic hexose retrieval mediated by the activity of Sugar Transport Protein (STP) is an important component of disease resistance. Whereas AtSTP13-deficient plants exhibit a reduced rate of glucose uptake and an enhanced susceptibility to *B. cinerea*, plants with a high constitutive level of AtSTP13 protein have improved capacity to absorb glucose and a better resistance to the fungus (Lemonnier et al., 2014).

On another side, it has also been proposed that sugars could represent signals for the induction of defense genes (Herbers et al., 1996; Herbers and Sonnewald, 1998; Gebauer et al., 2017). Several studies reported that high sugar levels in plant tissues are associated with a high level of resistance to fungal infection (Morkunas and Ratajczak, 2014). It has been hypothesized that sugars could provide the energy source to fuel defense responses and could also interact with hormonal signaling pathways regulating immune responses. Indeed, addition of sugars to plant culture media induced oxidative burst, lignification of cell walls, synthesis of flavonoids and PR protein expression (Morkunas and Ratajczak, 2014).

A class of sugar transporters playing a crucial role in plant-pathogen interactions is the SWEET family of sugar transporters. This family of sugar facilitators (PFAM code PF03083) comprises 17 members in Arabidopsis and 21 in rice (Chen et al., 2010). SWEETs are integral membrane proteins, with seven transmembrane domains, involved in sugar diffusion across membranes and playing important roles in nectar production as well as in seed and pollen development. Remarkably, SWEET transporters are targeted by extracellular pathogens, which modify their expression to obtain the sugar necessary for their growth (Chen et al., 2010). The role of SWEET proteins in plant resistance to pathogens has been well studied in rice. In this crop plant, the pathogenic bacteria *Xanthomonas oryzae* pv. *oryzae* uses different TAL (Transcription Activator-Like) effectors to up-regulate the expression of *SWEET* genes, resulting in sugar efflux into the apoplast, which supports the pathogen growth. The rice protein Xa13 is a SWEET transporter essential for pollen development and is also required for *X. oryzae* infection, Xa13-deficient plants being resistant to this bacterial pathogen (Chu et al., 2006). Several *xa13* alleles conferring recessive resistance to *X. oryzae* have been identified. In all cases resistance is associated with a mutation in the *Xa13* promoter (Chu et al., 2006; Römer et al., 2010). In rice, three out of 21 *SWEET* genes are targeted by pathogenic *X. oryzae* pv. *oryzae* (Yuan et al., 2014). This supports the general hypothesis that *X. oryzae* pv. *oryzae* induces developmentally regulated host genes to cause disease susceptibility. A similar mechanism has been described for the citrus bacterial canker disease caused by *Xanthomonas citri* subspecies *citri* (Hu et al., 2014) and the cotton blight caused by *X. citri* subsp. *malvacearum* (Cox et al., 2017).

Whereas the role of plant SWEET transporters is well characterized in the interaction with pathogenic bacteria, it is less documented in other pathosystems. In Arabidopsis, *SWEETs* are differentially regulated following infection with different types of pathogens (Chen et al., 2010), suggesting that pathogens with different lifestyles deploy specific strategies to divert host carbohydrates for their growth (Slewinski, 2011). AtSWEET2, which localizes to the root tonoplast, is involved in glucose sequestration in root vacuoles and limits carbon efflux from roots. Loss of function in *AtSWEET2* leads to enhanced susceptibility to the common root pathogen *Pythium irregulare*. This transporter has thus an important role in sugar retention and carbon availability modulation, with important consequences for root pathogen resistance (Chen et al., 2015).

Sugar partitioning is crucial in the major fruit crop grapevine, since berries accumulate high levels of sugars, which constitute a major component of fruit quality. Despite the importance of sugar allocation in grape berries, regulation of carbon allocation and sugar partitioning in the interaction with different pathogens

is poorly understood. Hayes et al. (2010) reported an enhanced expression of the hexose transporter *VvHT5* and of a cell wall invertase, both regulated by abscisic acid, during the infection with biotrophic pathogens. Similarly, Gamm et al. (2011) showed an increase in both invertase expression and soluble sugar contents in leaves infected with the biotrophic oomycete *Plasmopara viticola*.

Previous work from our laboratory identified 17 *SWEET* genes in the *V. vinifera* 40024 genome and showed that they are differentially expressed in different vegetative and reproductive organs, as well as after grapevine infection with biotrophic (*Erysiphe necator*, *P. viticola*) and necrotrophic (*B. cinerea*) pathogens (Chong et al., 2014). Our results showed a strong up-regulation of *VvSWEET4* upon infection with *B. cinerea*. Moreover, *A. thaliana* knockout mutants in the orthologous *AtSWEET4* were found more resistant to *B. cinerea*, suggesting a role of VvSWEET4 in resistance to necrotrophs in grapevine (Chong et al., 2014). In this study, we investigated the role of VvSWEET4 in a homologous system by stable overexpression in hairy roots of *V. vinifera* cv. Syrah. We show that high levels of *VvSWEET4* expression resulted in enhanced root growth, higher glucose and fructose contents and higher radiolabeled glucose passive uptake. We further demonstrate that hairy roots overexpressing *VvSWEET4* are more resistant to *P. irregulare*, a common root pathogen. Enhanced resistance could result from high sugar levels and/or constitutive enhanced flavonoid biosynthesis.

MATERIALS AND METHODS

Biological Material

Agrobacterium rhizogenes A4 strain (Tepfer, 1984) was maintained on MG/L medium as described in Torregrosa et al. (2015).

Vitis vinifera cv. Syrah *in vitro* plantlets were cultured on Mc Cown woody plant medium including vitamins (Duchefa, pH 6.2) supplemented with 15 g. L^{-1} sucrose and 0.7% bacto-agar in a growth chamber at 25°C, under a 16/8 h photoperiod and were subcultured every 2 months.

Vitis vinifera cv. Syrah hairy roots were cultured in Petri dishes on LG0 medium (Torregrosa and Bouquet, 1997; Torregrosa et al., 2015) in a growth chamber at 25°C, under a 16/8 h photoperiod. Every 3 weeks, root tip fragments of 1–2 cm length were harvested and subcultured on fresh LG0 medium.

Pythium irregulare strain isolated from carrot was provided by the Phytodiagnostique research and development centre of Vegepolys (Angers, France) and subcultured every week on PDA medium.

Transformation of *V. vinifera* cv. Syrah With *Agrobacterium rhizogenes* and Hairy Roots Regeneration

Agrobacterium rhizogenes A4 strain (Tepfer, 1984) was transformed by electroporation with empty pBin61 plasmid or pBin61 containing *VvSWEET4* cDNA under the control of the 35S cauliflower mosaic virus promoter obtained as described in Chong et al. (2014). Four to six-week-old plantlets of *V. vinifera* cv. Syrah were used for *A. rhizogenes* transformation. Hairy root lines were obtained after transformation of stems of *in vitro* plantlets with *A. rhizogenes* as described in Torregrosa et al. (2015). Different hairy root lines were regenerated: lines transformed with pBin61 containing *35S::VvSWEET4* ($VvSWEET4_{OX}$), lines transformed with empty pBin61 (pBin) and lines only transformed with *A. rhizogenes* A4 strain (A4).

Measurement of Hairy Root Growth

Four to six root tip fragments of approximately 1 cm length were harvested from 3-week-old hairy roots and placed at the center of 9 cm diameter Petri dishes. The initial position of the root tip was marked on the plate. Hairy roots were cultured at 25°C under a 16/8 h photoperiod. Pictures of the roots were taken 4, 7, 11, and 14 days after subculture. Primary root length was measured from the marked start position with the Image J software as described in Corrales et al. (2014).

Soluble Sugar Analysis

Frozen ground *V. vinifera* cv. Syrah hairy root tips (approximately 150 mg fresh weight) were extracted with 1.5 mL of methanol/chloroform/water (60/25/15, v/v/v). After vortex, the mixture was centrifuged at 5000 *g* for 10 min at 20°C. Supernatant was collected and pellet was reextracted 2 times with 900 μL of methanol/chloroform/water (60/25/15, v/v/v). Supernatants were pooled and mixed with 1.8 mL of water and centrifuged at 1200 *g* for 15 min at 20°C. The supernatant was collected and evaporated in a centrifugal vacuum evaporator (Eppendorf, 5301 concentrator) at 50°C for 3 h. The soluble glucose, fructose and sucrose contents of sample extracts were measured using the Sucrose/D-Fructose/D-Glucose Assay Kit (Megazyme) according to the manufacturer instructions.

Measure of Invertase Activities

Cytoplasmic (CIN), vacuolar (VIN), and cell wall (CWIN) invertase activities were extracted and determined as described in Veillet et al. (2016).

Sugar Uptake Assays With Radiolabeled Glucose

The sugar uptake assay was adapted from Veillet et al. (2016). Five primary root fragments of the same age (30-day-old) and size were placed for 60 min (2 times) in 6-well plate containing the equilibration buffer (20 mM MES-KOH pH 5.8, 1 mM $CaCl_2$) under agitation. After equilibration, samples were transferred into the incubation buffer (20 mM MES pH 5.8, 1 mM $CaCl_2$, 10 mM glucose) containing radiolabeled glucose (0.5 μCi. ml^{-1} of D-[U-^{14}C]-glucose) for 15 min under agitation. To measure CCCP- independent glucose uptake (diffusion), CCCP (20 μM) was added into the equilibration buffer 10 min before addition of incubation buffer. After incubation, samples were washed three times for 2 min in equilibration buffer. Samples were left overnight in the digestion buffer (36.4% perchloric acid w/v, 0.017% triton X-100 w/v and 8.1% hydrogen peroxide w/v)

at 60°C. Incorporated radioactivity was determined by liquid scintillation counting (Tri-Carb 2910 PR, PerkinElmer). Active glucose uptake results from the difference between total uptake and diffusion. Four independent repetitions have been realized with three technical replicates each comprising five roots for each repetition.

Inoculation of Hairy Roots With *Pythium irregulare* and *in planta* Quantification of Pathogen Growth

Eight hairy root tip fragments of 2 cm length and approximately the same diameter were selected, cut and placed 2 cm apart in square Petri dishes containing water agar (8 g/L) medium to favor infection. Water agar plates contain low levels of free sugars (approximately 2 $mg.L^{-1}$ sucrose, 0.16 $mg.L^{-1}$ fructose, and 0.2 $mg.L^{-1}$ glucose). One mycelium plug (0.5 cm^2 square) from the edge of a 1-week-old *P. irregulare* culture was placed at equal distance between two root fragments. Square Petri dishes were partly covered with aluminum foil and incubated in upright position inside a growth chamber at 22°C with a 12 h photoperiod. The experiment was repeated 3 times.

Real-time quantitative PCR has been reported as an accurate method to monitor fungal development in plant tissues (Gachon and Saindrenan, 2004) and specific primers from the ITS region have been developed for *P. irregulare* detection (Spies et al., 2011b). *P. irregulare* growth in roots was determined by relative quantification of fungal and plant DNA by means of qPCR analysis. Total fungal and plant DNA were extracted from 8 root tips 3 days after pathogen inoculation as described (Gachon and Saindrenan, 2004). The relative quantity of *P. irregulare* was calculated according to the abundance of the oomycete *ITS* sequence (Spies et al., 2011b) relative to the grapevine-specific *ACTIN* and *EF1α* genes measured by qPCR as described in Berr et al. (2010). qPCR reactions were performed as described in the Section "Gene Expression Analysis by Real-Time Quantitative RT-PCR." Primers used for real-time quantitative PCR are listed in **Supplementary Table S1**.

Gene Expression Analysis by Real-Time Quantitative RT-PCR

RNA extraction and DNase I treatment were performed as described in Chong et al. (2008). Reverse transcription was performed on 1 μg RNA using the SuperScript II Reverse Transcriptase (Invitrogen) and oligodT priming as recommended by the supplier.

Real-time PCR reactions were carried out on the CFX96 system (Biorad, France). PCR reactions were carried out in duplicates in a reaction buffer containing 1X iQ SYBR® Green Supermix, 0.2 mM of forward and reverse primers, and 10 ng of reverse transcribed RNA in a final volume of 25 μL. Thermal cycling conditions were: 30 s at 95°C followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The calibration curve for each gene was obtained by performing real-time PCR with serial dilutions of the purified PCR product (from 10^2 to 10^8 cDNA copy number). The specificity of the individual PCR amplification was checked using a heat dissociation curve from 55 to 95°C following the final cycle of the PCR and by sequencing the final PCR products. The results obtained for each gene of interest were normalized to the expression of two reference genes (see legend of Figures) as described in Vandesompele et al. (2002) and relative expression (fold induction) compared to appropriate controls (see legend of Figures) was calculated as described by Pfaffl (2001). Mean values and standard deviations were obtained from two technical and three biological replicates. Primers used for real-time quantitative PCR are listed in **Supplementary Table S1**.

Determination of Tannins With the Vanillin Method

Vanillin assay is quite specific to a narrow range of flavanols (monomers and polymers) and dihydrochalcones that have a single bond at the 2,3-position and free meta-oriented hydroxy groups on the B ring (Sun et al., 1998). Frozen ground *V. vinifera* cv. Syrah hairy root tips (approximately 150 mg fresh weight) were extracted twice with 80% methanol. After each extraction, the mixture was centrifuged at 10 000 *g* for 10 min at 20°C. Supernatants of both extractions were pooled. The content of tannins in root methanolic extracts was determined as described by Nakamura et al. (2003). For sample reactions, 100 μL root methanolic extract were mixed with 250 μL vanillin solution (1% in methanol) and 250 μL HCl 9 N. Control reactions were realized with 100 μL methanol, 250 μL vanillin solution (1% in methanol) and 250 μL HCl 9 N. Reactions were incubated 30 min at 30°C and the absorbance at 500 nm was determined. Absorbance of control reactions were subtracted to absorbance of sample reactions as described by Nakamura et al. (2003). Results were expressed as Absorbance units at 500 nm per g of fresh weight.

Identification and Quantification of Flavonoids by LC-MS

Metabolites were extracted from hairy roots as described above (see section "Determination of Tannins With the Vanillin Method"). Identification and quantification of flavonoids was performed using an Agilent 1100 series High Performance Liquid Chromatography system coupled to Agilent 6510 accurate-mass Quadrupole-Time of Flight (Q-TOF) Mass spectrometer with ESI interface in negative ionization mode (Agilent Technologies, California, United States). A Zorbax SB-C18 column (3.1 × 150 mm, ϕ3.5 μm), equipped with a 2.1 × 12.5 mm ϕ 5 μm Zorbax Eclipse plus C18 precolumn (Agilent Technologies), was used at 35°C. The injected volume was 3 μL, the elution gradient was performed with binary solvent system composed of 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in MeOH (solvent B) at a constant flow-rate of 0.35 $mL.min^{-1}$. The gradient elution program was as follows: 0–3.0 min, 5% B; 3.0–23.0 min, up to 100% B; held for 10.0 min, followed by 7 min of stabilization at 5% B. LC-MS grade water, methanol and formic acid were purchased from Thermo Fisher Scientific (Illkirch, France).

The mass spectrometer operated by detection in scan mode with the following settings: drying gas 13.0 $L.min^{-1}$ at 325°C;

nebulizer pressure 35 psi; capillary voltage −3500 V, fragmentor 150 V. Negative mass calibration was performed with standard mix G1969-85000 (Agilent Technologies).

Data were acquired with Agilent MassHunter version B.02.00 software and processed with Agilent MassHunter Qualitative and Quantitative software version B.07.00. Absolute flavonoid contents were calculated from external calibration curves prepared with pure standards: catechin, epicatechin, and procyanidins B1 and B2 were purchased from Extrasynthese (Genay, France).

Statistical Analysis

Data were analyzed by using a multifactorial ANOVA and a multiple comparison of means using the Tukey test ($p \leq 0.05$) performed with R 3.3.2 software (R Development Core Team, 2016).

RESULTS

Overexpression of *VvSWEET4* in *V. vinifera* cv. Syrah Hairy Roots Results in Enhanced Growth on Culture Media Supplemented With Sucrose or Glucose

In order to study the sugar transport function of VvSWEET4 in grapevine, the coding sequence of this transporter was placed under the control of the 35S CaMV promoter and used to create transformed hairy roots from stem tissue of *V. vinifera* cv. Syrah plantlets. The resulting hairy root lines (*VvSWEET4*$_{OX}$) contained the Ri plasmid of *A. rhizogenes* strain A4 along with the binary vector containing the *35S::VvSWEET4* construct. Lines transformed with the Ri plasmid of *A. rhizogenes* strain A4 and the empty binary vector (pBin) as well as lines transformed only with the Ri plasmid (A4) were also generated as controls.

After obtention of transformed calli, several independent lines of hairy roots expressing different levels of the *VvSWEET4* transcript (*VvSWEET4*$_{OX}$) were regenerated (**Figure 1**). During subcultures of the different hairy root lines, we noticed a faster growth of *VvSWEET4*$_{OX}$ compared to controls (**Figure 2A**). Growth of the hairy root lines was studied in more detail by measuring the length increase of primary roots at different times after subculture on media supplemented with glucose (**Figure 2B**) or sucrose (**Figure 2C**). Roots from *VvSWEET4*$_{OX}$ lines showed a greater length increase compared to roots from pBin and A4 lines, both on glucose and sucrose containing media, especially 11 and 14 days after subculture (**Figures 2B,C**). However, *VvSWEET4*$_{OX}$6A, which had low levels of *VvSWEET4* transcripts did not show enhanced growth compared to control lines.

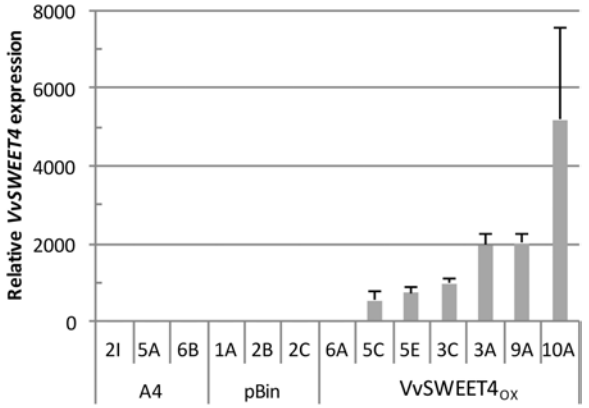

FIGURE 1 | *VvSWEET4* expression levels in hairy root lines. Relative expression was measured 2 weeks after subculture in control lines transformed with the *A. rhizogenes* A4 strain alone (A4), the A4 strain with empty vector (pBin) and lines transformed with *35S::VvSWEET4* (*VvSWEET4*$_{OX}$). Transcript levels of *VvSWEET4* were normalized to grapevine *GAPDH* and *EF1*α transcript levels. Relative expression indicates normalized expression levels in *VvSWEET4*$_{OX}$ lines compared to normalized expression levels observed in control lines. Results are mean ± SD of three biological replicates.

VvSWEET4$_{OX}$ Hairy Root Lines Have Higher Contents in Glucose and Fructose

Increased growth rate observed in *VvSWEET4*$_{OX}$ lines prompted us to determine their sugar contents 2 weeks after subculture. Levels of glucose, fructose and to a smaller extent sucrose were significantly higher in *VvSWEET4*$_{OX}$ lines compared to pBin and A4 control lines when hairy roots were grown on a culture medium containing sucrose as carbon source (**Figure 3A**). Levels of glucose were especially high in *VvSWEET4*$_{OX}$ lines, reaching up to 8 mg g^{-1} FW, except for *VvSWEET4*$_{OX}$ 6A which had low levels of transgene expression and sugar levels comparable to those found in control lines. Sugar contents were also determined after 2 weeks of culture on a medium containing glucose as carbon source (**Figure 3B**). *VvSWEET4*$_{OX}$ lines had significant higher contents in glucose except for *VvSWEET4*$_{OX}$ 6A. Fructose levels also tended to increase but to a smaller extent, with the exception of lines *VvSWEET4*$_{OX}$ 9A and 10A, where the increase was clear. When roots were grown on glucose as carbon source, sucrose contents were not significantly different in *VvSWEET4*$_{OX}$ lines, except for *VvSWEET4*$_{OX}$ 9A, and 10A (**Figure 3B**).

High levels of glucose and fructose found in *VvSWEET4*$_{OX}$ hairy roots grown on sucrose containing medium are likely to result from extracellular invertase activity releasing glucose and fructose from sucrose, followed by hexose import through VvSWEET4 transport activity. Cytoplasmic, vacuolar and cell wall invertase activities were thus measured in hairy root lines. Although differences in activities could be observed between lines, no strong difference was found between *VvSWEET4*$_{OX}$ and control lines (**Figure 3C** and **Supplementary Figure S1**).

VvSWEET4$_{OX}$ Hairy Root Lines Show Improved Glucose Absorption

Increased sugar levels in *VvSWEET4* overexpressors is likely to result from the sugar transport activity of VvSWEET4. We previously showed that VvSWEET4 is able to complement the growth on glucose medium of the yeast strain EBYVW4000, deficient in hexose transporters (Chong et al., 2014). Radiolabeled

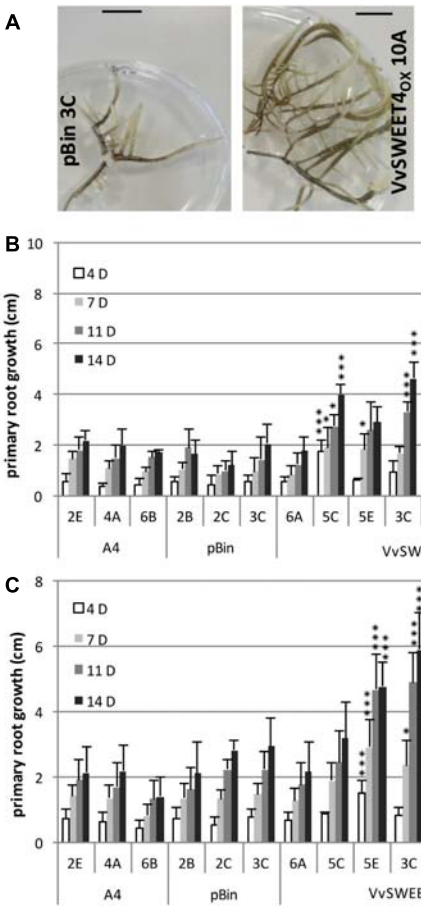

FIGURE 2 | Growth of hairy root lines. **(A)** Representative pictures of pBin 3C and *VvSWEET4*$_{OX}$ 10A lines after 3 weeks of culture on sucrose containing medium. Bar = 1 cm. **(B,C)** Growth of the hairy roots lines cultured on glucose **(B)** or sucrose **(C)** containing medium. Growth of the primary root (in cm) was measured at different times after subculture in control A4 and pBin lines and several *VvSWEET4*$_{OX}$ lines. Mean ± SD was obtained from 6 biological replicates. Asterisks indicate a significant different mean root growth in *VvSWEET4*$_{OX}$ lines compared to control A4 and pBin lines (ANOVA and multiple comparison of means, ***$p < 0.001$, **$p < 0.01$, *$p < 0.1$).

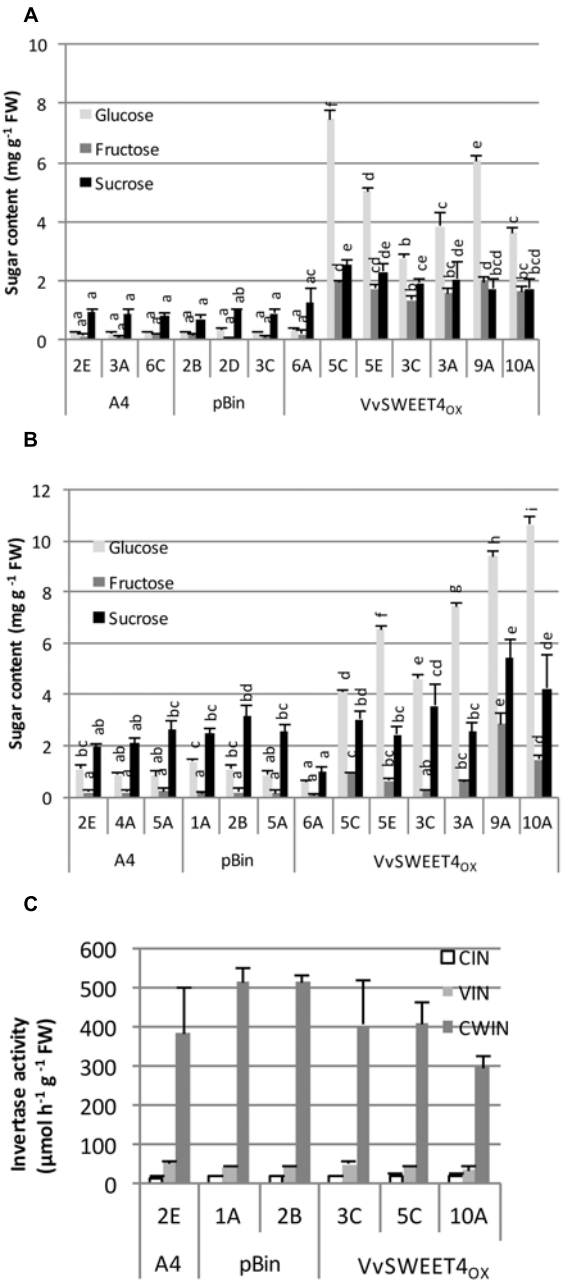

FIGURE 3 | Sugar contents and invertase activities of hairy root lines. Levels of glucose, fructose and sucrose were measured in hairy root tips 2 weeks after subculture on sucrose **(A)** or glucose **(B)** containing medium. Data are the mean ± SD of 4 independent experiments. Means with different letters are significantly different at $p \leq 0.05$ (Tukey Contrasts). **(C)** Cytoplasmic (CIN) vacuolar (VIN), and cell wall (CWIN) invertase activities. Data are the mean ± SD of three technical replicates. A second biological replicate with another set of lines produced similar results (**Supplementary Figure S1**). The different letters correspond to the different groups after the statistic Tukey Test (precized in the figure legend).

glucose absorption experiments were conducted on 2 control lines (A4 2B and pBin 2C) and 3 *VvSWEET4*$_{OX}$ lines (5C, 9A, and 10A). Fragments of primary roots were incubated in medium containing high concentration of [^{14}C]-glucose (10 mM) in order to create a steep gradient that favors glucose influx via facilitator transporters, such as SWEETs. After incubation, the amount of radiolabeled glucose was determined inside the roots.

Total glucose uptake rates were approximately two times higher in *VvSWEET4*$_{OX}$ roots than in control roots (**Figure 4**). To discriminate between passive diffusion and proton-coupled transport, uptake assays were conducted in the presence of the protonophore carbonylcyanide m-chlorophenylhydrazine (CCCP), which causes an uncoupling of the proton gradient. Accordingly, proton-coupled transport (active uptake) is calculated as the difference between total glucose uptake and CCCP-independent uptake (diffusion). As shown in **Figure 4**, glucose retrieval into the control roots occurred by diffusion and active mechanisms with similar rates. In contrast, radiolabeled glucose retrieval by *VvSWEET4*$_{OX}$ roots mainly occurred by

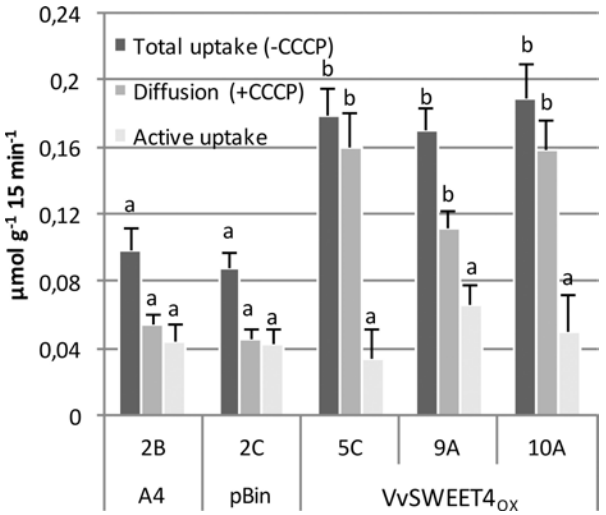

FIGURE 4 | Radiolabeled glucose uptake in hairy roots. Five root fragments (30-day-old) from A4, pBin and *VvSWEET4*$_{OX}$ lines were collected, and placed for 60 min (2 times) in equilibration buffer. Total glucose uptake of roots was measured after 15 min of incubation with 10 mM D-[^{14}C]glucose. To measure glucose diffusion, CCCP (20 µM) was added into the equilibration buffer 10 min before addition of incubation buffer. Active glucose uptake results from the difference between total uptake and diffusion. Data are the mean ± SE of four independent replicates. Means with different letters are significantly different at $p \leq 0.05$ (Tukey Contrasts).

a passive diffusion mechanism, since it was weakly inhibited by the protonophore CCCP. As expected, glucose uptake measured in the presence of CCCP was significantly increased in *VvSWEET4*$_{OX}$ lines compared to controls. Moreover, active glucose uptake rates, resulting from the activity of hexose/H^+ transporters was not significantly different between *VvSWEET4*$_{OX}$ and control lines (**Figure 4**). Altogether, these results indicate that the expression of functional VvSWEET4 resulted in an increased glucose absorption into the roots through a facilitated diffusion mechanism.

Overexpression of *VvSWEET4* in Hairy Roots Leads to Reduced Infection With *Pythium irregulare*, a Common Root Pathogen

We further investigated the consequences of improved glucose absorption and sugar levels in *VvSWEET4* overexpressors on the resistance to a common root pathogen, *P. irregulare*, which has been reported as a common pathogen in grapevine nurseries and is also one of the most prevalent *Pythium* species in vineyards in South Africa (Spies et al., 2011b). To favor infection of hairy roots by the pathogen, primary root fragments were placed on a water agar medium and inoculated with a *P. irregulare* mycelium plug placed between two root fragments (**Figure 5A**).

To monitor *P. irregulare* development in hairy roots, relative quantification of fungal and plant DNA in infected roots was realized by real-time quantitative PCR 3 days after inoculation as described in Berr et al. (2010). Relative quantities of *P. irregulare* DNA were markedly reduced in *VvSWEET4*$_{OX}$ lines compared to A4 and pBin control lines (**Figure 5B**). These results show a less intense colonization of hairy roots with high *VvSWEET4* levels compared to A4 and pBin lines.

In order to better study the role of VvSWEET4 in the interaction with *P. irregulare*, we monitored the expression of *VvSWEET4* in control A4 and pBin lines 3 days after inoculation. As shown in **Figure 6A**, *VvSWEET4* was induced by *P. irregulare* infection suggesting that it could play a role in the interaction of grapevine roots with this pathogen. Levels of sugars were also measured in hairy roots 3 days after infection (**Figure 6B**). As a consequence of culture on water agar medium and pathogen infection, sugar levels were much lower compared to levels measured in hairy root lines on sugar containing medium (**Figure 3**). Levels of sucrose and fructose were especially low after infection and could not be accurately determined (data not shown). However, levels of glucose were significantly higher after infection in *VvSWEET4*$_{OX}$ lines compared to controls (**Figure 6B**).

Overexpression of *VvSWEET4* Is Associated With Constitutive Induction of Genes Involved in Flavonoid Biosynthesis and Higher Flavanol Contents

To know if improved resistance of *VvSWEET4*$_{OX}$ lines could result from differences in defense gene activation, the expression of several defense genes was studied in healthy and infected roots. Expression of genes involved in the phenylpropanoid pathway (*VvPAL*, *VvSTS*), and genes encoding PR10 protein, callose synthase (*VvCalS*) or EDS1 signaling protein was similar in *VvSWEET4*$_{OX}$ and control lines, both in healthy and diseased roots (**Supplementary Figure S2**). However, a significant higher constitutive expression of several genes involved in flavonoid biosynthesis (*VvCHS1*, *VvCHS2*, *VvLDOX*, and *VvFMT*) and of a MYB transcription factor (*VvMYBF1*) that regulates several genes involved in flavonoid synthesis in grapevine (Czemmel et al., 2009), was detected in most healthy hairy roots with high *VvSWEET4* levels (**Figure 7**).

Higher expression of genes involved in flavonoid biosynthesis and regulation prompted us to investigate the flavonoid contents of *VvSWEET4* overexpressors. It has been previously reported that the main flavonoids in grapevine and especially hairy roots are proanthocyanidins (Huang et al., 2014). Proanthocyanidin contents were thus first determined using the colorimetric vanillin assay. As show in **Figure 8A**, total flavanol contents were significantly higher in *VvSWEET4*$_{OX}$ hairy root lines compared to controls. Flavanols (catechin, epicatechin, procyanidins B1 and B2) were further quantified by LC-MS in hairy root extracts (**Figures 8B–E**). As shown on **Figures 8B,D**, contents in catechin and procyanidin B1 were significantly increased in *VvSWEET4*$_{OX}$ hairy root lines compared to controls. Levels in epicatechin and procyanidin B2 were only significantly enhanced in *VvSWEET4*$_{OX}$ 3A and were not significantly different in the other overexpressor lines compared to controls (**Figures 8C,E**).

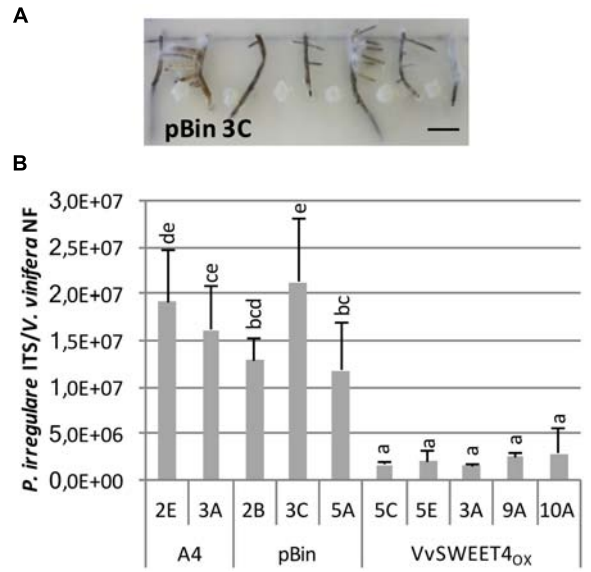

FIGURE 5 | Overexpression of *VvSWEET4* in hairy roots reduces *Pythium irregulare* infection. Eight two-week-old root tips (2 cm length) from A4, pBin, and $VvSWEET4_{OX}$ lines were placed 2 cm apart in square Petri dishes on water agar medium. Roots were inoculated by placing a 0.5 cm^2 agar plug from the edge of a 1-week-old *P. irregulare* culture at equal distance between each root. Roots were harvested 3 days after inoculation. **(A)** Picture of hairy roots 3 days after inoculation. Bar = 1 cm. **(B)** qPCR was used to analyze the relative quantity of *P. irregulare* according to the abundance of fungal *ITS* sequence relative to grapevine *ACTIN* and *EF1*α genes. NF: normalization factor calculated from the geometric mean of grapevine *ACTIN* and *EF1*α relative DNA levels. Data represent means ± SD of four biological replicates each containing eight roots. Means with different letters are significantly different at $p \leq 0.05$ (Tukey Contrasts).

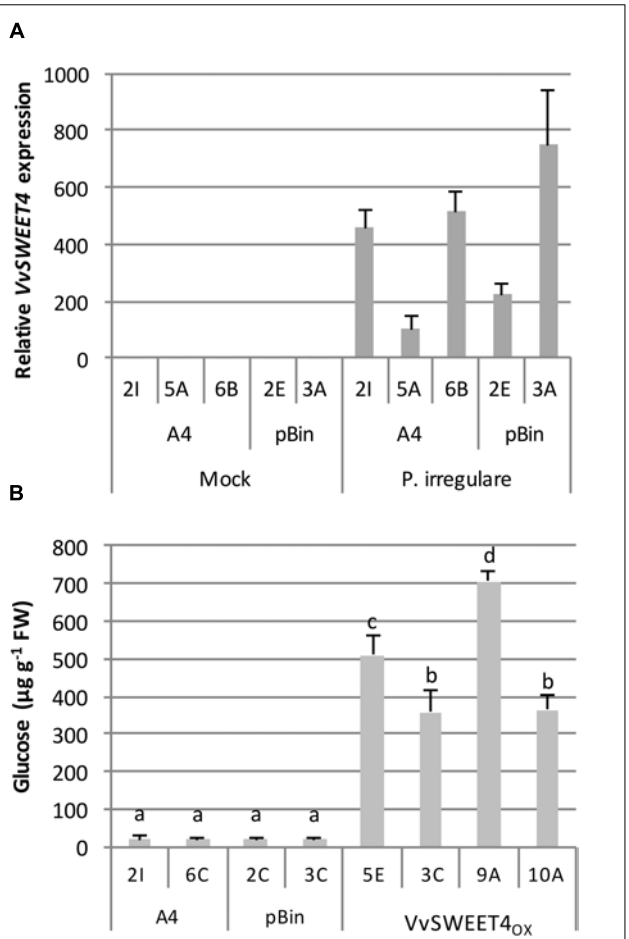

FIGURE 6 | Endogenous *VvSWEET4* expression and glucose contents in hairy root lines after infection with *P. irregulare*. **(A)** *VvSWEET4* expression was measured in control hairy root lines (A4 and pBin) 3 days after infection with *P. irregulare*. Transcript levels of *VvSWEET4* were normalized to grapevine *GAPDH* and *EF1*α transcript levels. Relative expression indicates normalized expression levels in inoculated roots compared to normalized expression levels observed in mock-inoculated roots. Data represent means ± SD of three biological replicates. **(B)** Levels of soluble glucose were determined in hairy root lines 3 days after *P. irregulare* infection. Data represent means ± SD of 4 to 6 biological replicates. Means with different letters are significantly different at $p \leq 0.05$ (Tukey Contrasts).

DISCUSSION

VvSWEET4 Is an Hexose Passive Transporter

In this study, we demonstrated that *VvSWEET4* overexpression led to enhanced root elongation and higher contents in hexoses such as glucose and fructose. Similarly, overexpression of *AtSWEET4* in Arabidopsis also triggered an increase in plant size and in glucose and fructose contents (Liu et al., 2016). Enhanced root elongation likely results from higher sugar contents in roots. Indeed, several studies reported that root elongation depends directly on the carbon supply (Hennion et al., 2019).

SWEET transporters have been described as sugar uniporters, transporting sugars along the concentration gradient (Chen et al., 2010; Chandran, 2015). In our hairy root system, sugar uptake assays with radiolabeled glucose showed that VvSWEET4 functions in passive transport of hexoses (especially glucose) independent of the proton-motive force. In the hairy root system, high extracellular sugar levels favors hexose import mediated by VvSWEET4 in the roots resulting in enhanced contents in glucose and fructose. These results are consistent with the restoration of growth on glucose medium of the yeast EBYVW4000 mutant deficient in hexose transporters (Wieczorke et al., 1999) by *VvSWEET4* expression (Chong et al., 2014). Hairy roots overexpressing *VvSWEET4* cultured on sucrose-containing medium are characterized by significant higher glucose and fructose levels than controls. VvSWEET4 belongs to the clade II of SWEET transporter family, which has been described as monosaccharide (glucose, galactose, and fructose) but not sucrose transporters (Chen et al., 2010; Chandran, 2015). The presence of hexoses in hairy roots grown on sucrose medium is likely a consequence from extracellular cell wall invertase activity and a higher invertase activity in $VvSWEET4_{OX}$ lines could explain their higher hexose contents. However, activities of cell wall, cytoplasmic and vacuolar invertases were not different between control and $VvSWEET4_{OX}$ lines. It is probable that basal cell wall invertase activities found

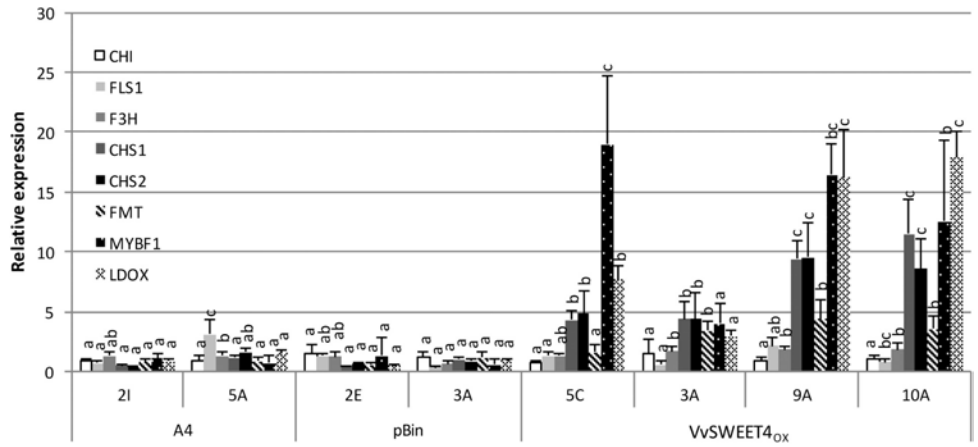

FIGURE 7 | Expression analysis of grapevine flavonoid biosynthesis genes in hairy root lines. Expression of chalcone synthases (*VvCHS1*, *VvCHS2*), chalcone isomerase (*VvCHI*), flavanone-3-hydroxylase (*VvF3H*), flavonol synthase (*VvFLS1*), leucoanthocyanidin dioxygenase (*VvLDOX*), flavonoid-*O*-methyltransferase (*VvFMT*), and a R2R3-MYB transcription factor (*VvMYBF1*) was studied by qPCR in 2-week-old root tips from A4, pBin and *VvSWEET4*$_{OX}$ lines. Transcript levels of the different genes were normalized to *Vitis vinifera GAPDH* and *EF1*α transcript levels. Relative expression indicates normalized expression levels in the different hairy root lines compared to the mean of the normalized expression levels measured in control A4 and pBin lines. Data are mean ± SD of three biological replicates. Means with different letters are significantly different at $p \leq 0.05$ (Tukey Contrasts).

in control and *VvSWEET4*$_{OX}$ hairy roots is sufficient to release glucose and fructose from sucrose. Enhanced hexose levels in *VvSWEET4*$_{OX}$ lines thus results from enhanced import activity and not from enhanced sucrose cleavage activity.

Enhanced Resistance of *VvSWEET4*-Overexpressing Hairy Roots to *Pythium* Infection

One of the disadvantages of the hairy root system is that it does not allow the regeneration of whole transformed plants, which represents a drawback for the test of pathogen resistance because it is limited to root pathogens. In our study, we thus chose to test the resistance of *VvSWEET4*$_{OX}$ lines to a soil-borne root pathogen, *P. irregulare*, which exhibits a high virulence and represents one of the most important *Pythium* species in agriculture (Spies et al., 2011a). This necrotrophic pathogen is an important and widespread pathogen of grapevines in South Africa and could be a major cause of vine decline in nurseries and vineyards (Spies et al., 2011a,b). Interestingly, hairy roots overexpressing *VvSWEET4* were clearly less colonized after infection with *P. irregulare* compared to control lines. One hypothesis to explain this enhanced resistance is the high sugar content of these roots. It is known that plants with high sugar contents in their tissues are more resistant to several fungal diseases, a phenomenon called "high sugar resistance" (Morkunas and Ratajczak, 2014). Indeed, high sugar contents provide more energy for plant cells to fuel plant defenses, which are cost intensive. In accordance with high sugar levels in healthy *VvSWEET4*$_{OX}$ hairy roots, glucose levels were also significantly higher compared to controls 3 days after infection with *P. irregulare*.

A recent study also reported the involvement of a SWEET transporter in the interaction between Arabidopsis and *P. irregulare* (Chen et al., 2015). The sugar facilitator AtSWEET2 is localized to the tonoplasts of cortex and epidermis root cells, and is involved in vacuolar glucose sequestration, thereby limiting the efflux of carbon from roots. The expression of *AtSWEET2* in roots is induced by infection with the soil-borne pathogen *P. irregulare* and the loss of function *sweet2* mutant displays an enhanced susceptibility to this pathogen. This study revealed an important function for AtSWEET2 in modulating sugar secretion in the rhizosphere, that could support the growth of pathogenic root microorganisms (Chen et al., 2015). In the case of interaction with pathogenic bacteria, SWEET transporters have been mainly described as sugar effluxers favoring pathogen development (Chen et al., 2010; Chandran, 2015). In this study, we found that *VvSWEET4* expression is low in non-inoculated control hairy roots and is induced after *P. irregulare* infection. In Arabidopsis, promoter-GUS fusion analysis also showed that *AtSWEET4*, the *VvSWEET4* ortholog, is expressed in the stele of roots (Liu et al., 2016). It is thus possible that *VvSWEET4* plays a role in the interaction of grapevine roots with *P. irregulare*. VvSWEET4 is a plasma membrane localized transporter that would allow sugar leakage from plant cells into the apoplasmic space, which is favorable to pathogen development. Indeed, knockout mutants in *AtSWEET4* (the Arabidopsis *VvSWEET4* ortholog) were shown to be more resistant to *B. cinerea* infection (Chong et al., 2014). In the context of natural interaction of grapevine roots with *P. irregulare*, the induction of *VvSWEET4* expression following infection may facilitate pathogen development. Unexpectedly, hairy roots overexpressing *VvSWEET4* were found more resistant to *P. irregulare* but this resistance most likely results from high preexisting sugar levels rather than from impaired pathogen nutrition. Measurement of apoplastic sugar contents would be an interesting way to explain the various phenotypes after infection (Bezrutczyk et al., 2018).

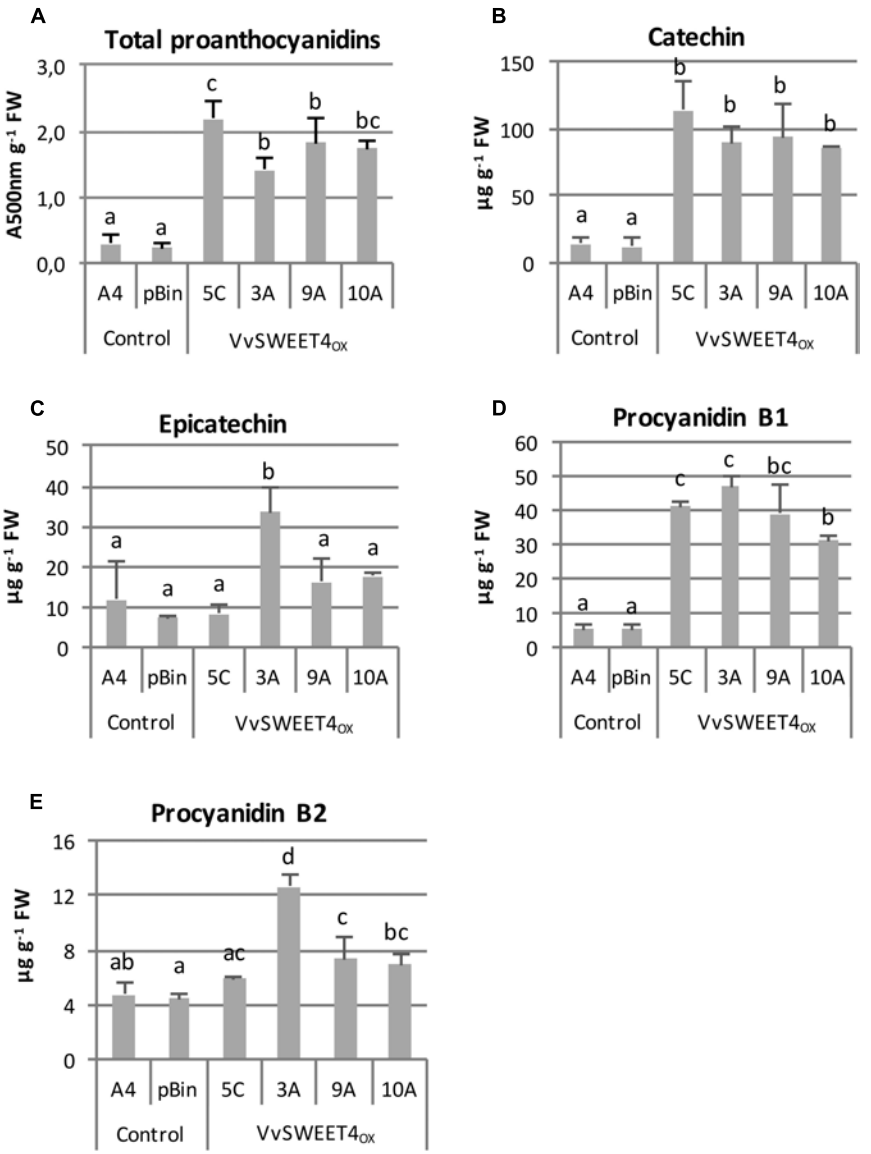

FIGURE 8 | Flavanol contents in hairy root lines. **(A)** Total proanthocyanidin contents were determined in 3-week-old hairy roots by the vanillin method. Results are expressed as absorbance units at 500 nm per g of fresh weight. Data are mean ± SD of n biological replicates (n = 6 for controls, n = 4 for *VvSWEET4*$_{OX}$). Means with different letters are significantly different at $p \leq 0.05$ (Tukey Contrasts). **(B–E)**: quantification by LC/MS of catechin **(B)**, epicatechin **(C)**, procyanidin B1 **(D)** and procyanidin B2 **(E)** in 3-week-old hairy root lines. Data are mean ± SD of n biological replicates (n = 3 for controls, n = 4 for *VvSWEET4*$_{OX}$). Means with different letters are significantly different at $p \leq 0.05$ (Tukey Contrasts).

Overexpression of *VvSWEET4* and Consequences on Flavonoid Biosynthesis

In addition to their role in energy supply, several studies revealed that sugars can act as signaling molecules to trigger the expression of defense genes (Lemoine et al., 2013; Trouvelot et al., 2014). For example, it has been reported that sucrose induced the expression of *PR* genes (Thibaud et al., 2004; Gómez-Ariza et al., 2007). Morkunas and Bednarski (2008) also studied defense responses induced in *in vitro* cultured embryo axes of yellow lupine after infection by the hemibiotrophic fungus *Fusarium oxysporum*. They showed that several defense responses such as ROS production, peroxidase activities and lignin contents are more intense when embryos are nourished with exogenously supplied sucrose (Morkunas and Gmerek, 2007; Morkunas and Bednarski, 2008). In a recent study, Gebauer et al. (2017) showed a positive influence of sugar metabolism on the salicylic acid (SA) signaling pathway. They studied the *atsweet11/atsweet12* double knockout mutant, which presents constitutively elevated levels of soluble sugars, especially hexoses. Elevated sugar levels were associated with enhanced SA levels, priming of defense and signaling genes of the SA pathway and

a better resistance to the hemibiotrophic fungus *Colletotrichum higginsianum* (Gebauer et al., 2017). However, in our study, no higher activation of defense genes regulated by SA in grapevine [*VvEDS1*, *VvPR1*, *VvPAL* (Chong et al., 2008)] was observed in *VvSWEET4*$_{OX}$ lines compared to controls both in healthy and *P. irregulare* infected roots.

An important result from this study is that the flavonoid biosynthesis is enhanced in *VvSWEET4* overexpressors. Expression of several genes involved in the flavonoid pathway is constitutively enhanced in most *VvSWEET4*$_{OX}$ hairy roots, whereas expression of other defense genes is not affected. We found that expression of chalcone synthases (*VvCHS1, VvCHS2*), putative flavonoid *O*-methyltransferase (*VvFMT*), leucoanthocyanidin dioxygenase (*VvLDOX*) and *VvMYBF1* transcription factor are constitutively up-regulated in most *VvSWEET4*$_{OX}$ hairy root lines before infection, whereas expression of other flavonoid biosynthesis genes (Chalcone isomerase *VvCHI,* Flavanone-3-hydroxylase *VvF3H*, Flavonol synthase *VvFLS1*) is not affected. The flavonoid biosynthetic pathway genes are predominantly regulated at the level of transcription. The R2R3-MYB transcription factor VvMYBF1 is a specific transcriptional regulator of several genes involved in flavonoid synthesis in grapevine. VvMYBF1 was shown to activate promoters of genes involved in the general flavonoid pathway such as chalcone synthase and chalcone isomerase and also the *LDOX* promoter (Czemmel et al., 2009). Activation of *VvCHS*, *VvFMT* and *VvLDOX* in *VvSWEET4*$_{OX}$ hairy root lines is thus consistent with the activation of VvMYBF1, which probably results from high sugar contents. However, the expression of *VvCHI* and *VvFLS1*, which are known targets of VvMYBF1 (Czemmel et al., 2009) was not significantly enhanced in *VvSWEET4*$_{OX}$ hairy root lines. It is possible that in the hairy roots, activation of several MYB transcription factors by sugars does not lead to the same results as transient expression of a single MYB transcription factor in suspension cells of Chardonnay. In future studies, it will be also interesting to test the expression of other grapevine MYB transcription factors such as *VvMYBPA1* and *VvMYBPA2* reported to more specifically regulate the proanthocyanidin pathway (Bogs et al., 2007; Terrier et al., 2009). In several plant species, it has been demonstrated that sugars regulate the accumulation of flavonoids. Anthocyanins are a widespread class of plant flavonoids and their accumulation is modulated by sucrose, a well-characterized endogenous developmental signal (Solfanelli et al., 2006; Meng et al., 2018). Whole-genome transcript profiling reveals that sucrose treatment up-regulated the flavonoid and anthocyanin biosynthetic pathway and affects both flavonoid and anthocyanin contents (Solfanelli et al., 2006). In yellow lupine embryo axes infected with *F. oxysporum,* enhanced expression of flavonoid biosynthesis genes and higher flavonoid content were also reported when explants were supplied with sucrose (Morkunas et al., 2011). In Arabidopsis, the expression of a MYB transcription factor, AtMYB56, a potent regulator of anthocyanin accumulation, is induced by sucrose (Jeong et al., 2018). In addition, the expression of several structural genes and transcription factors involved in the anthocyanin biosynthesis pathway is regulated by sugars in petunia (Neta-Sharir et al., 2000).

In grapevine, it is known that genes involved in the phenylpropanoid pathway and anthocyanin biosynthesis are regulated by sugars (Lecourieux et al., 2014). First, there is a correlation between anthocyanin content of the grape berry and sugar accumulation at the post-veraison stages (Lecourieux et al., 2014). Second, the addition of sucrose, glucose or fructose to suspension cultures of *V. vinifera* cv. Gamay Fréaux increases the anthocyanin production up to 12-fold (Larronde et al., 1998). Similarly, increasing sucrose concentrations promote cell growth and phenylpropanoid biosynthesis in grape cell cultures obtained from *V. vinifera* cv. Barbera immature berries, leading to anthocyanin, catechin and stilbene accumulation or secretion in the culture medium (Ferri et al., 2011). Stimulation of the transcription of phenylpropanoid biosynthetic enzymes, such as phenylalanine ammonia lyase, chalcone synthase, chalcone-flavanone isomerase and stilbene synthase paralleled enhanced polyphenol production after sucrose treatment (Ferri et al., 2011). Other studies reported induction of flavonoid biosynthesis gene expression by exogenous sugar supply in grapevine. The expression of both *LDOX* (leucoanthocyanidin dioxygenase) and *DFR* (dihydroflavonol-4-reductase) was up-regulated by treatment of cv. Gamay Red cell cultures with sucrose (Gollop et al., 2001). The transcripts and protein amounts of F3H (flavanone 3-hydroxylase) protein also increased in grape berries incubated with different concentrations of glucose, fructose or sucrose (Zheng et al., 2009). In grapevine, the main flavonoids are proanthocyanidins which are major determinants for fruit and wine quality (Huang et al., 2014). We further showed that up-regulation of flavonoid biosynthesis genes results in higher total proanthocyanidin levels and especially enhanced contents in catechin and procyanidin B1 in *VvSWEET4*$_{OX}$ lines compared to controls. Catechin and procyanidin B1 levels were comparable between *VvSWEET4*$_{OX}$ 3A, 5C, 9A, and 10A. However, expression of flavonoid biosynthesis genes were lower in *VvSWEET4*$_{OX}$ 3A and to a lesser extent 5C compared to expression in *VvSWEET4*$_{OX}$ 9A and 10A. It is possible that expression of flavonoid biosynthesis genes observed in *VvSWEET4*$_{OX}$ 3A is sufficient to result in higher flavanol contents. On another side, absence of correlation between flavonoid biosynthesis gene expression and flavanol contents could also be explained by the fact that gene expression was measured 2 weeks after hairy root subculture and flavanol contents was determined 3 weeks after subculture.

Overall, our results show that decreased susceptibility of *VvSWEET4*$_{OX}$ lines to *P. irregulare* may be not caused by impaired sugar provision to the pathogen but could be a consequence of sugar induction of the flavonoid pathway. Flavonoids are secondary metabolites with antimicrobial and especially antifungal properties (Treutter, 2006). Study of barley mutants impaired in proanthocyanidin accumulation in the seed testa layer revealed that these compounds are necessary to resistance to *Fusarium* infection (Skadhauge et al., 1997). In Arabidopsis, plants with depleted flavonoid contents by silencing MYB transcription factors involved in flavonoid biosynthesis

activation were more susceptible to infection with necrotrophic or hemibiotrophic pathogens. Conversely, stronger accumulation of flavonoids such as naringenin and kaempferol resulted in resistance to fungal pathogens (Camargo-Ramirez et al., 2018). In grapevine, study of calli with differential susceptibility to *P. viticola* revealed that resistant callus contained greater contents of gallocatechin derivatives (Dai et al., 1995). In addition, treatment of grapevine (cv Merlot) with benzothiadiazole (BTH) induced resistance to the gray mold *B. cinerea* and resistance was correlated with enhanced polyphenol contents in berry skins, especially the procyanidin fraction (Iriti et al., 2005).

CONCLUSION

In conclusion, our study characterized the sugar transport activity of a grapevine SWEET transporter in grapevine hairy roots, confirming the usefulness of this system for the functional characterization of grapevine sugar transporters. Grapevine hairy roots circumvent the time-consuming process of generating stable transgenic lines in grapevine. *A. rhizogenes* transformation is indeed an interesting efficient alternative to embryogenic calli transformation with *Agrobacterium tumefaciens* followed by the regeneration of whole transformed plants, which has a particularly low efficiency in grapevine. Hairy roots have been already used as an efficient system for characterization of regulators of anthocyanin accumulation and transport in grapevine (Gomez et al., 2011; Matus et al., 2017). Using this homologous system, we showed that the VvSWEET4 transporter is an important component for sugar accumulation in grapevine cells and that sugar fluxes are crucial for pathogen resistance. Moreover, our study points out a key role of sugars as signaling molecules for the regulation of flavonoid biosynthesis. In plant-bioagressor interactions, modulation of sugar pools acting either as a source of energy or as regulators of defense responses such as plant secondary metabolite synthesis has critical consequences for pathogen resistance. Future work will try to elucidate the role of VvSWEET4 in the interaction with *P. irregulare* in natural conditions.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

JC, PM, EM, and SL conceived and designed the experiments. EM, HL, SL, JC, and M-LG carried out the experiments. EM, JC, PM, SL, and M-LG analyzed the experiments. M-LG contributed to the materials and analysis tools. JC, PM, and SL wrote the manuscript. JC acquired the funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00884/full#supplementary-material

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Characterization of the Wood Mycobiome of *Vitis vinifera* in a Vineyard Affected by Esca. Spatial Distribution of Fungal Communities and Their Putative Relation With Leaf Symptoms

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Esca is a disease complex belonging to the grapevine trunk diseases cluster. It comprises five syndromes, three main fungal pathogenic agents and several symptoms, both internal (i.e., affecting woody tissue) and external (e.g., affecting leaves and bunches). The etiology and epidemiology of this disease complex remain, in part, unclear. Some of the points that are still under discussion concern the sudden rise in disease incidence, the simultaneous presence of multiple wood pathogens in affected grapevines, the causal agents and the discontinuity in time of leaf symptoms manifestation. The standard approach to the study of esca has been mostly through culture-dependent studies, yet, leaving many questions unanswered. In this study, we used Illumina® next-generation amplicon sequencing to investigate the mycobiome of grapevines wood in a vineyard with history of esca. We characterized the wood mycobiome composition, investigated the spatial dynamics of the fungal communities in different areas of the stem and in canes, and assessed the putative link between mycobiome and leaf symptoms. An unprecedented diversity of fungi is presented (289 taxa), including five genera reported for the first time in association with grapevines wood (*Debaryomyces*, *Trematosphaeria*, *Biatriospora*, *Lopadostoma,* and *Malassezia*) and numerous hitherto unreported species. Esca-associated fungi *Phaeomoniella chlamydospora* and *Fomitiporia* sp. dominate the fungal community, and numerous other fungi associated with wood syndromes are also encountered (e.g., *Eutypa* spp., *Inonotus hispidus*). The spatial analysis revealed differences in diversity, evenness and taxa abundances, the unique presence of certain fungi in specific areas of the plants, and tissue specificity. Lastly, the mycobiome composition of the woody tissue in proximity to leaves manifesting 'tiger stripes' symptoms of esca, as well as in leaf-symptomatic canes, was highly similar to that of plants not exhibiting any

leaf symptomatology. This observation supports the current understanding that leaf symptoms are not directly linked with the fungal communities in the wood. This work builds to the understanding of the microbial ecology of the grapevines wood, offering insights and a critical view on the current knowledge of the etiology of esca.

Keywords: microbial ecology, *Vitis*, metabarcoding, mycobiome, esca disease, grapevine trunk diseases

INTRODUCTION

The phyllosphere, rhizosphere, and endosphere of grapevine (*Vitis vinifera* L.) are characterized by the presence of complex communities of microorganisms that constantly interact with one another and with the plant, affecting it positively, neutrally or negatively (Bruez et al., 2014; Pinto et al., 2014; Zarraonaindia et al., 2015). Until a decade ago, the approach to characterize the mycobiome – namely the fungal community present in/on an organism – of grapevines, focused on culture-dependent studies in which fungi were isolated *in vitro* and identified morphologically and/or molecularly (Morgan et al., 2017). This approach remains valid to this day, however, it presents several limitations, such as the impossibility of detecting uncultivable fungi, the bias of the cultivation conditions (e.g., growth medium, incubation parameters) and the difficulty of isolating species present in low abundances (Morgan et al., 2017). In recent years, technologies like next-generation sequencing (NGS) have improved in quality and reduced in cost, which, in combination with ever more efficient bioinformatics tools, have allowed the exploitation of this method in the study of the molecular ecology of environmental DNA (eDNA) samples. In particular, DNA metabarcoding approaches have taken the investigations of microbiomes to a new level, surpassing some of the limitations which characterize culture-dependent studies. In fact, NGS studies have revealed a higher diversity of taxa and accurate relative abundances in samples coming from different environments, including the vineyard (Peay et al., 2016; Morgan et al., 2017; Jayawardena et al., 2018). Despite these recent advances, culture-independent studies describing the microbial endosphere of grapevines are still scarce.

DNA metabarcoding is a promising tool to investigate the microbial communities present in the wood of grapevines, as it may lead to a new understanding of the complexity that characterizes grapevine trunk diseases (GTD). This cluster of fungal diseases affects primarily the perennial organs of the plants, such as the trunk and roots, however, secondary symptoms may be observed in leaves, bunches and shoots. Overall, GTD cause a loss in vigor, productivity, quality of the yield and lifespan of the plants, with conspicuous economic consequences (Hofstetter et al., 2012; Bertsch et al., 2013; Fontaine et al., 2016; Calzarano and Di Marco, 2018). GTD pathogens are phylogenetically unrelated, belonging to different families, orders and even phyla, although plants infected may reveal similar symptomatology. For example, wood discoloration and necrosis are symptomatology shared by all GTD, whereas the 'tiger stripes' pattern in the leaves are attributed to grapevine leaf stripe disease (GLSD) and, by some authors, to Botryosphaeriaceous fungi responsible for the syndrome 'black dead arm' (Mugnai et al., 1999; Larignon et al., 2009; Bertsch et al., 2013). Moreover, the simultaneous presence of several possible causal agents in infected grapevines complicates the outline of a clear etiological pattern (Edwards and Pascoe, 2004; Bruez et al., 2016; Mondello et al., 2017). This is especially true in the case of esca, a disease complex consisting of five separate syndromes (brown wood streaking of rooted cuttings, Petri disease, GLSD, white rot and esca proper) in which, according to current literature, several pathogenic fungi play a role (Surico, 2009). These fungi may infect vines in the field, where conidia or other propagules reach fresh pruning wounds and start colonizing the xylem, or during the propagation process in nurseries (Gramaje et al., 2018). The pathogens most frequently associated with the first three syndromes are *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*, two tracheomycotic ascomycetes; while the latter two syndromes are associated with the presence of the wood rotting basidiomycete *Fomitiporia mediterranea* (white rot), especially common in Europe, or with the simultaneous presence of both tracheomycotic and wood rotting pathogens (esca proper) (Surico, 2009). Along with these three players, other wood pathogens including, but not limited to, members of the Diatrypaceae and Botryosphaeriaceae are often found in symptomatic plants (Edwards and Pascoe, 2004; Hofstetter et al., 2012; Bruez et al., 2014; Travadon et al., 2016). Studies that used the NGS approach to learn more about the grapevine endosphere are scarce (Dissanayake et al., 2018; Jayawardena et al., 2018) and none of them investigated the mycobiome of GTD-affected plants.

This work aims to investigate the fungal communities present in the wood of grapevines, in a vineyard with history of esca proper. Three main objectives were set, namely (1) to characterize the mycobiome of the wood of *V. vinifera* cv Cabernet Sauvignon, in a vineyard located in the Lisbon area (Portugal), using Illumina® NGS; (2) to understand the spatial distribution of the communities present in different areas of perennial wood and in canes (annual wood); (3) to understand whether there is a link between the microbial communities of the wood and the expression of leaf symptoms of esca.

MATERIALS AND METHODS

The Vineyard

This study focuses on a vineyard in Portugal, which ranks 11th in the world for wine production, with a total vineyard area of 195 kha (Aurand, 2017). *V. vinifera* cultivar Cabernet Sauvignon is the most cultivated worldwide, with a total vineyard

area of 340 kha. It is considered susceptible to trunk diseases (Darrieutort and Pascal, 2007; Eskalen et al., 2007) and has already been cited in studies concerning the microbial ecology of wood, phyllosphere and grapes (González and Tello, 2011; Bruez et al., 2014; Morgan et al., 2017; Singh et al., 2018).

Field sampling took place in the experimental vineyard (Almotivo) of the Instituto Superior de Agronomia, in Lisbon (38°42′32.7″N, 9°11′11.5″W). The vineyard has a density of 3,333 plants/ha, the soil is classified as vertisoil, it is managed under conventional agricultural practices and there is no irrigation system. The selected cultivar was Cabernet Sauvignon grafted on 140 RU rootstock (*Vitis berlandieri* × *Vitis rupestris*), 19 years-old at the moment of sampling (planted in 1998), trained as Cordon Royat Bilateral and spur pruned. The field has a history of esca, with leaf-symptomatic grapevines accounting for less than 1% of the total plants in all recorded years (2015, 2016, and 2017). The selection of the plants used in this experiment was restricted to a block of 450 m^2.

The immediate surroundings of the vineyard, within a 25 m radius from the perimeter, are characterized by the presence of diverse vegetation. Most of the trees are subject to winter pruning and present exposed heartwood from which wood decay have developed. The majority of the species are listed in **Supplementary Table S1**.

Sampling and Experimental Setup

Samples of perennial wood (PW) were taken in a non-destructive way, in April 2017, by means of hand-drilling the plants with a gimlet (**Figure 1B**). The sampling procedure occurred as follows. The bark, on each sampling point, was removed with the use of a knife and the wounds were disinfected with ethanol (70% v/v); the gimlet was placed perpendicularly on the open wounds and manually forced in the wood until it went through the whole width of the plant. This allowed us to extract cores of 19 years-old wood (5 mm of diameter and approximately 60 mm long) which were immediately placed in sterile 15 mL falcon tubes and temporarily stored in ice (**Figure 1C**). Samples were then transferred to a freezer, freeze-dried and stored at −80°C. After extracting each core of wood, the gimlet was sterilized by dipping it in a sodium hypochlorite solution (0.35 w/w of active chlorine) for 1 min, followed by a rinse with ethanol (70% v/v) and then double-rinsed with sterile distilled water (SDW), in order to minimize cross-contamination.

Canes grown in the 2017 growing season were sampled in September, as annual wood, detaching them with pruning scissors, approximately 3 cm above the spur from which they departed (length of wood sampled: 50 mm; age of wood: 5 months-old; **Figure 1F**). Each sample was deprived of its bark, frozen, freeze-dried and stored at −80°C until processing.

Concerning the PW, five grapevines with healthy leaves were sampled in 7 precise areas each (n = 35, **Figure 1D**) and five grapevines with symptomatic leaves were sampled in 9 precise areas each (n = 45, **Figure 1A**). The former five plants did not show leaf symptoms of esca during the previous two growing seasons (years 2015 and 2016), while the latter five manifested leaf 'tiger stripes' symptoms, only in one of the two cordons (cordon 2; **Figure 1A**), during the previous growing season (year 2016). The terms 'asymptomatic' and 'symptomatic,' which will often be encountered in the rest of the text, refer exclusively to leaf symptomatology and not to wood symptomatology (unless specifically stated).

The tissue types corresponding to the nine sampling areas are: 'Graft Union' (GU), located approximately (3 ± 1 cm) above the soil, on the graft union; 'Trunk' (T), (22 ± 1 cm) above GU; 'Upper Trunk' (UT), (22 ± 1 cm) above T; 'Arm 1' (A1), was located on the cordon, (36 ± 2 cm) away from UT; the sample point 'Spur 1' (S1) is located on the cordon, right below the spur, (10 ± 1 cm) from A1; 'Arm 2' (A2), located (22 ± 1 cm) to the right of A1; 'Spur 2' (S2), (10 ± 1 cm) from A2 (**Figure 1**). All canes departing from cordon 1 (S1, S2) did not exhibit leaf symptoms, for all 10 sampled plants. Sampling points (SA, symptomatic arm) and (SS, symptomatic spur) are the equivalent of points (A1) and (S1), but located in cordon 2. In this case, 5 out of the 10 sampled grapevines presented symptoms in the leaves of canes departing from (SS), while the other 5 plants had non-symptomatic leaves.

Only one tissue type was examined in annual wood, namely the 'Canes,' where 15 canes were sampled from 10 plants. Five of them came from asymptomatic plants, while the other 10 came from symptomatic plants. Within these 10, 5 canes were leaf-symptomatic (cordon 2, **Figure 1**), while the other 5 were asymptomatic and sampled in the cordon 1 (**Figure 1F**).

To address the three objectives of this study, the sample points corresponding to different tissue types were combined as follows. (1) To characterize the mycobiome of the wood of the vineyard, all sample points were taken in consideration (n = 80 from PW, n = 15 from canes; **Figure 1A**). (2) To learn about the spatial distribution of the mycobiome in the different areas of the plants, we used tissue types (GU – S2) (n = 10 per tissue type, total n = 70; **Figure 1D**), along with asymptomatic canes (n = 10). (3) To understand the link between leaf symptoms expression and mycobiome of PW or canes, we created three groups per category. In the category 'PW,' group (i) consisted in asymptomatic plants (cordon 1, points 'A1 and S1'; n = 10), group (ii) consisted in symptomatic plants, sampled in the asymptomatic cordon (cordon 1, points 'A1 and S1'; n = 10), group (iii) consisted in symptomatic plants, sampled in the symptomatic cordon (cordon 2, points 'SA and SS'; n = 10; **Figure 1E**). The same applies to the category 'canes,' where group (i) consisted in asymptomatic canes, sampled from asymptomatic plants, group (ii) consisted in asymptomatic canes, sampled from symptomatic plants, group (iii) consisted in symptomatic canes (n = 5 per group).

DNA Extraction, Amplification, Library Preparation and Sequencing

Wood samples were ground to dust using sterile mortars and pestles, aiding the process with liquid nitrogen. An aliquot of ground wood (0.25 ± 0.01 g) of each sample was added to DNA extraction columns (FastDNA™ SPIN Kit for Soil, MP Biomedicals® LLC) and total DNA was extracted as described by the kit manufacturer. Three negative controls of the DNA extraction procedure were added.

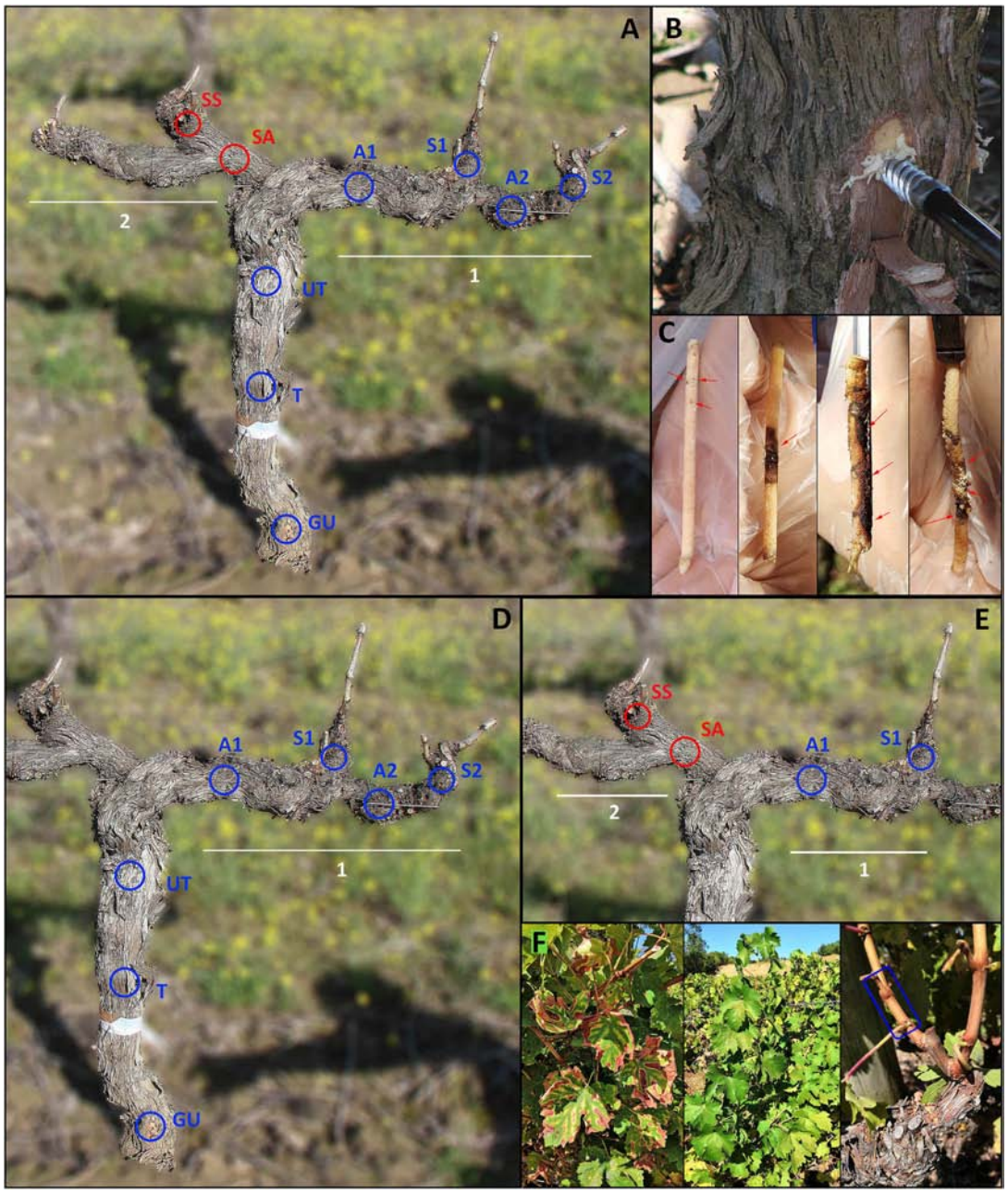

FIGURE 1 | Sampling points in perennial wood or canes of grapevine cv Cabernet sauvignon. (GU) Graft union, (T) Trunk, (UT) Upper trunk, (A1) Arm 1, (S1) Spur 1, (A2) Arm 2, (S2) Spur 2, (SA) Symptomatic arm, (SS) Symptomatic spur. Cordon (1) presented healthy leaves in all ten sampled plants, while cordon (2) presented leaf symptoms, in canes departing from SS, in five of the sampled plants. Red circles indicate wood sampled in proximity of symptomatic leaves, blue circles indicate wood not associated with leaf symptoms. **(A)** Sampling points used to characterize the mycobiome of perennial wood – objective 1 –. **(B)** Sampling procedure involved using a gimlet to drill the wood and extract wood cores. **(C)** Cores of wood extracted with a gimlet (red arrows indicate wood symptomatology). From left to right: brown wood streaking, wood necrosis, extensive wood necrosis, wood decay-white rot-wood necrosis. **(D)** Sampling points used to test the spatial distribution of fungal communities – objective 2 –. **(E)** Sampling points used to examine the mycobiome present in the wood in proximity of symptomatic (AS, SS) and healthy (A1, S1) leaves – objective 3 –. **(F)** From left to right: symptomatic canes sampled from plants with leaf symptoms, asymptomatic canes sampled from plants with no leaf symptoms in either of the cordons or with leaf symptoms in only one of the two cordons; the sampling area for each cane is indicated by the blue rectangle.

Among the several possible informative genes (e.g., β-tubulin, elongation factor), the amplicon chosen in this study targeted the Internal Transcribed Spacer ITS1 region, and the primer set selected was ITS1F2 – ITS2 (Gaylarde et al., 2017) with overhang recommended by Illumina. For building libraries, we used a double-step PCR approach as

reported by Feld et al. (2015). The full sequence of the primers, including Illumina overhangs, is the following: ITS1F2 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GAACC WGCGGARGGATCA-3′) and ITS2 (5′-GTCTCGTGGGC TCGGAGATGTGTATAAGAGACAG-GCTGCGTTCTTCATC GATGC-3′) (Gaylarde et al., 2017).

Each first-step PCR reaction contained 12.5 μL of Supreme NZYTaq II 2x Green Master Mix^{TM} (NZYtech^{TM}), 0.5 μL of forward and reverse primers from a 10 μM stock, 1.5 μL of sterile water, and 5 μL of template. Each reaction was pre-incubated at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 75°C for 10 s, 55°C for 15 s, 72°C for 40 s; a further extension was performed at 72°C for 10 min.

Second PCR-step for barcoding, fragment-purification by using MagBio beads and Qubit quantification was performed as reported in Gobbi et al. (2019). Final pooling was performed at 10 ng/sample. DNA Sequencing was performed using an in-house Illumina® MiSeq instrument and 2 × 250 paired-end reads with V2 Chemistry.

Bioinformatics

After sequencing, demultiplex was performed using our Illumina MiSeq platform and the raw data were analyzed using QIIME 2 v. 2018.2 (Caporaso et al., 2010) using the same pipeline described in Gobbi et al. (2019); denoised reads were trimmed 15 bp on the left to remove the adapters and then they were analyzed using DADA2 with the exact sequence variants (EVS) methods (Callahan et al., 2017). Each ESV appears at least twice in the dataset. Singletons were discarded. Taxonomic assignments were performed at 99% identity using qiime feature-classifier classify-sklearn with a Naïve-Bayes classifier trained with UNITE (Nilsson et al., 2013) v7.2 for ITS. To test the three hypotheses underlying this work we separate the frequency table in three sub-tables, which were tested under different conditions. The raw data of this study is available in the European Nucleotide Archive (ENA accession number PRJEB31028).

Statistics

The frequency table and its taxonomy were combined, converted to biom format in QIIME (Caporaso et al., 2010), then merged with a table of metadata into an S4 object and analyzed in R (v. 3.4.3) using the following packages: phyloseq, v. 1.22.3 (McMurdie and Holmes, 2013); biomformat, v. 1.10.0 (McMurdie and Paulson, 2018), vegan, v. 2.5.2 (Oksanen et al., 2007); ggplot2, v. 3.0.0 (Wickham, 2016); igraph, v. 1.2.2 (Csardi and Nepusz, 2006), MetacodeR, v. 0.2.1.9005 (Foster et al., 2017); adespatial, v. 0.1.1 (Dray et al., 2018); data.table, v 1.10.4.3 (Dowle and Srinivasan, 2017); and microbiome, v. 1.4.0. R code is publicly available at https://github.com/Marieag/EMG/blob/master/vine_microbiome_init.R.

To assess the alpha diversity, Shannon diversity index and Pielou's evenness were calculated and tested using a one-way ANOVA with *post hoc* Tukey's HSD (Honestly Significant Difference), determining differences in these indexes between tissue types or tissue groups.

We analyzed the β-dispersion to measure between-sample variances in abundance, computing average distances of the individual samples. The resulting ordination was plotted using the non-metric multidimensional scaling (NMDS) combined with a Jaccard index matrix. These ordinations were also performed with a Bray–Curtis dissimilarity matrix. To assess overall inter-group variance, we performed a PERMANOVA, using a Jaccard distance matrix with 999 permutations. The *post hoc* test for the PERMANOVA (performed on the EVS counts) was done using the adonis function from the vegan package in R.

In order to illustrate this effect size compared to the relative abundance of the taxa, we created differential heat trees using MetacodeR, illustrating the log2 fold change in species abundance. A Wilcoxon Rank Sum test was applied to test differences between the same species in different tissue types or tissue groups, and the resulting *p*-values were corrected for multiple comparisons using FDR, as implemented in MetacodeR. *P*-value threshold was set to 0.05.

RESULTS

Sequencing Dataset Description

This dataset, obtained by sequencing, consists of a total of 95 samples. It includes 80 samples collected from different parts of the PW and 15 from canes. All of them represent in total 20 plants, and each one of them counts as an independent biological replicate. This dataset contains 2,805 exact sequence variants which appear a total of 8.184.885 times among all the different samples. The raw tables of EVSs (read counts), for each of the three objectives of this study, are available in **Supplementary Data Sheet 1**.

Visual Examination of the Sampled Wood

Before processing the samples for the molecular analysis, wood cores of PW and samples of canes were visually examined to assess the presence of wood symptomatology. Approximately 10% of the wood cores were fully asymptomatic, 75% presented symptoms of tracheomycosis (e.g., brown wood streaking and/or wood necrosis), and 15% showed the presence of white rot, which was always associated with other tracheomycosis symptoms. The examination of the canes wood revealed that 100% of the samples were fully asymptomatic.

The Wood Mycobiome

The identification of sequences in our dataset revealed an unprecedented diversity. Taxa that were assigned to genus or species level are 289, 50 of them are found in relative abundance (RA) greater than 0.1%, while the remaining 239 are considered rare taxa (RA < 0.1%). Within these 239 taxa, 146 are found in a RA included between 0.1 and 0.01%, while the remaining 93 have a RA lower than 0.01%. The full list of taxa is available in **Supplementary Table S2**.

The qualitative overview of the wood mycobiome will focus mainly on the 30 most abundant taxa in PW and the 12 most abundant in canes, which account for 79.1 and 80.8% of the total RAs, respectively (**Table 1**), while the remaining percentages represent unidentified taxa or fungi found in lower abundances.

TABLE 1 | List of most abundant taxa, identified to genus or species level, found in grapevine wood.

Phylum	Family	Species	Relative abundance (%)		Ecology in wood *	Presence in different tissue type			
			PW	C		GU/T/UT	A1/A2	S1/S2	C
Ascomycetes (66.7–76.3)	Biatriosporaceae (0.6– 0)	*Biatriospora mackinnonii*‡	0.6	–	E[a]	–/–/–	–/–	–/–	–
	Bionectriaceae (0.4–0)	*Clonostachys rosea*	0.4	–	E, S, P[b]	–/–/–	–/–	–/+	–
	Davidiellaceae (2.0–8.2)	*Cladosporiu m* sp.	1.9	8.2	E, S[b]	+/+/+	+/+	+/–	+
	Diaporthaceae (<0.1–0.8)	*Diaporthe* sp.	<0.1	0.8	E, S, P[b]	–/–/–	–/–	–/–	+
	Diatrypaceae (2.6–0)	*Anthostoma gastrinum*†	0.9	–	S, P[c,d]	+/+/+	+/+	+/–	–
		Eutypa lata	0.7	–	P[b]	–/–/–	–/–	–/+	–
		Eutypa leptoplaca	0.9	–	P[b]	–/–/+	–/–	+/+	–
	Dothioraceae (0.4–4.0)	*Aureobasidium pullulans*	0.4	4.0	E, S[b]	–/+/+	+/+	–/–	+
	Glomerellaceae (<0.1–0.7)	*Colletotrichum* sp.	<0.1	0.4	P[b]	–/–/–	–/–	–/–	–
	Herpotrichiellaceae (27.1–3.9)	*Exophiala xenobiotica*†	0.5	–	na	–/–/–	–/+	+/+	–
		Phaeomoniella chlamydospora	25.8	3.9	P[d]	+/+/+	+/+	+/+	+
	Hypocreales (0.3–0.2)	*Acremonium* sp.	0.2	–	E[e]	–/–/–	–/–	–/+	–
	Lophiostomataceae (3.8–0)	*Angustimassarina acerina*†	0.5	–	S[f]	–/–/+	+/+	+/+	–
		Lophiostoma sp.	2.7	–	E, S[b]	+/+/–	+/–	+/–	–
		Lophiostoma cynaroidis†	0.3	–	E[g]	–/+/–	–/–	–/–	–
		Lophiotrema rubi	0.3	–	na	+/–/–	–/–	–/–	–
	Massarinaceae (0.5–0)	*Massarina* sp.	0.5	–	E[h]	+/+/+	–/–	+/–	–
	Mycosphaerellaceae (3.3–9.4)	*Ramularia* sp.	3.1	9.4	na	+/+/+	+/+	+/+	+
	Pleomassariaceae (2.9–0)	*Trematosphaeria pertusa*‡	2.9	–	S[i]	+/+/–	–/+	+/+	–
	Pleosporaceae (3.9–14.8)	*Alternaria* sp.	3.2	14.6	E[b,j]	+/+/+	+/+	+/+	+
			PW	C					
	Saccharomycetaceae (10.9–31.8)	*Debaryomyces prosopidis*‡	10.4	31.5	na	+/+/+	+/+	+/+	+
	Xylariaceae (0.7–0)	*Lopadostoma meridionale*‡	0.3	–	S[k]	–/–/–	–/–	–/–	–
		Lopadostoma quercicola‡	0.4	–	S[k]	+/–/–	–/–	–/–	–
Basidiomycetes (26.7–18.8)	Filobasidiaceae (0.2–0.2)	*Filobasidium magnum*†	0.1	0.2	na	+/–/–	–/+	–/–	–
	Hymenochaetaceae (15.2–0)	*Fomitiporia* sp.	14.6	–	P[d]	+/+/+	+/+	+/+	–
		Fomitiporia mediterranea	0.2	–	P[d]	–/+/–	+/–	–/–	–
		Inonotus hispidus	0.3	–	S, P[l]	+/–/–	–/+	–/–	–
	Malasseziaceae (0.3–0.3)	*Malassezia restricta*‡	0.2	0.2	na	+/–/–	–/–	–/–	+
	Psathyrellaceae (0.5–0)	*Psathyrella* sp.	0.5	–	S[m]	+/–/–	–/–	–/–	–
	Sporidiobolaceae (0.6–0)	*Rhodotorula mucilaginosa*†	0.4	<0.1	S[n]	–/+/+	+/+	–/–	–
	Tremellaceae (6.4–8.3)	*Cryptococcus* sp.	2.7	7.6	E, S[b]	+/+/+	+/+	+/+	+
		Cryptococcus heimaeyensis†	0.3	–	–	–/–/+	–/–	–/–	–
		Cryptococcus victoriae†	2.9	0.7	–	+/+/+	+/+	+/+	+
		Total	**79.1**	**80.8**					

*The list includes the 30 most abundant taxa found in perennial wood and the 12 most abundant in canes, for a total of 32 taxa. The numbers between brackets represent the relative abundance of that Phylum or Family in perennial wood or canes (PW% – C%) based on the table created to address objective (1). The ecology of the identified taxa in wood of grapevines or of other plants is shown based on available literature (E, endophyte; S, saprophyte; P, pathogen; na, unknown ecology). The presence of taxa in different tissue types is based on the table created to address objective (2), (+) indicates presence (RA ≥ 0.1%), (–) indicates absence or presence in RA < 0.1%. (GU) Graft union, (T) Trunk, (UT) Upper trunk, (A1) Arm 1, (S1) Spur 1, (A2) Arm 2, (S2) Spur 2 and (C) Canes. *References. [a](Kolařík et al., 2017), [b](Jayawardena et al., 2018), [c](Haynes, 2016), [d](Gramaje et al., 2018), [e](González and Tello, 2011), [f](Thambugala et al., 2015), [g](Xing et al., 2011), [h](Casieri et al., 2009), [i](Suetrong et al., 2011), [j](Pancher et al., 2012), [k](Jaklitsch et al., 2014), [l](González et al., 2009), [i](Bruez et al., 2016), [n](Haňáčková et al., 2017). ‡First report of genus and species in grapevine wood. †First report of species in grapevine wood.*

Within this group of taxa, five genera and nine species of fungi are described for the first time as part of the grapevine wood mycobiome, while the other 18 taxa have already been reported in literature (**Table 1**).

The community encountered in PW is characterized by the presence of both ascomycetes and basidiomycetes (66.7% and 26.7% RA), with high abundances of tracheomycotic pathogen *P. chlamydospora* (25.8%) and white rot agent *Fomitiporia* sp. (14.6%), two organisms directly associated with esca proper and other esca-related syndromes. Among all sampled wood cores of PW (n = 80), *P. chlamydospora* was present in 68 of them (85%; RA > 0.1%), while *Fomitiporia* sp. in 58 (64%; RA > 0.1%) or 14 (17.5%; RA > 35%). Other GTD pathogens among the 30 most abundant taxa are *Eutypa lata* (0.7%) and *Eutypa leptoplaca* (0.9%), within the Diatrypaceae. Other members of this family are *Anthostoma gastrinum* (0.9%), a potential wood pathogen, as well as *E. flavovirens*, *Eutypella citricola,* and *Cryptovalsa ampelina*, identified as rare taxa. Members of the Botryosphaeriaceae (e.g., *Diplodia pseudoseriata*, *Neofusicoccum parvum*, *Neofusicoccum australe*), *Ilyonectria* sp. and *Neonectria* sp. are also found, although represented only as rare taxa (**Supplementary Table S2**). Decay agents, such as *Fomitiporia* sp., *F. mediterranea* (0.2%) and *I. hispidus* (0.3%), were also identified in this study, along with several others represented in minor abundances (e.g., *Fomitiporella* sp.). Among the endophytes and saprophytes, *Alternaria* sp. (3.2%), *Cladosporium* sp. (1.9%), *Aureobasidium pullulans* (0.4%), and *Psathyrella* sp. (0.5%) are the most abundant. Several other genera or species, identified for the first time in association with grapevine wood, amount to 14 taxa out of the 33 most abundant in PW or canes (**Table 1**).

Canes were also colonized by both ascomycetes (76.3%) and basidiomycetes (18.8%). The most abundant taxa are endophytic and saprophytic fungi, with *Alternaria* sp. (14.6%), *Ramularia* sp. (9.4%) and *Cladosporium* sp. (8.2%) being among most abundant, as well as other species reported for the first time (e.g., *Debaryomyces prosopidis*; **Table 1**). Only two wood pathogens are present in moderate abundances in canes, namely *P. chlamydospora* (3.9%) and *Diaporthe* sp. (0.8%), while other pathogenic agents are found in minor abundances (RA < 0.2%; e.g., *N. australe*).

The core mycobiome, namely the taxa shared between PW and canes, is constituted by 44 taxa. Only 10 taxa are unique to canes and the remaining 235 are unique to PW. All the 10 unique taxa found in canes are considered rare taxa, as their RAs are lower than 0.1% of the total, while among the many taxa unique to PW we find organisms belonging to the Hymenochaetaceae, Lophiostomataceae, Pleomassariaceae, Xylariaceae and numerous others (**Supplementary Table S2**).

Diversity and Spatial Distribution of the Mycobiome

Alpha Diversity

The Shannon (H') and Pielou's (J') indexes vary significantly among tissue types, according to Tukey's HSD (**Figure 2** and **Table 2**). The Shannon diversity analysis reveals that one sample point, namely the Upper Trunk, differs from both the spur points S1 and S2 ($P < 0.05$). The spur tissue is also significantly different from canes ($P < 0.05$). No differences are observed when comparing GU, T, UT, the two sample points in the arm (A1 and A2) and canes. Exact p-values of the significant differences are available in **Supplementary Table S3**.

Different tissue types also vary in mycobiome evenness, with fungal communities of canes, GU, T and UT being more evenly distributed than those of the spurs ($P < 0.05$; **Figure 2**). All other tissue types examined do not differ in evenness (**Figure 2**).

Beta Diversity

The Jaccard's index, when visualized in a non-metric multidimensional scaling (NMDS) plot, shows a considerable overlap for different tissue types (**Figure 3A**). The PERMANOVA indicates significant difference between groups ($P < 0.001$), but looking at the ordination, the difference is arguably in the clustering of the observations, rather than a distinct difference in sample composition. A pattern emerges when examining each tissue type separately (**Figure 3B**). We observe a reduction in between-sample variability starting from the GU to T and until UT. The UT variability is very similar to that of both the Arm points (A1 and A2), while the Spurs (S1 and S2) have higher between-sample variability, when compared to the Arms, but similar to one another. Concerning the canes, the variability of this tissue type is very low (**Figure 3B**). Bray–Curtis test revealed a similar profile and significant differences ($P < 0.001$; data not shown). Jaccard and Bray–Curtis matrixes are available in the **Supplementary Materials**.

Mycobiome Composition and Differentially Represented Taxa

The bar plot in **Figure 4** shows the relative abundance of the 20 most abundant taxa in different tissue types, giving an overview of the presence/absence of taxa and their differential representation. For example, *E. lata* and *Acremonium* sp. are present exclusively in the wood below the spurs, while *Trematosphaeria pertusa* was detected in the same tissue type and in the graft union. *Fomitiporia* sp., abundant in most of PW, is nearly absent in the graft union. This tissue type contains three unique taxa, *Psathyrella* sp., *Lophiotrema rubi* and *Lopadostoma quercicola*, as well as other taxa which are present in higher abundances when compared to the rest of the plant, such as *Lophiostoma* sp. and *Massarina* sp. The heat trees shown in **Figures 5**, **6** give a thorough view of the differently abundant taxa, when comparing each tissue type, for taxa with RAs > 0.1% (n = 50). The majority of the statistical differences observed, for both ascomycetes and basidiomycetes, concern the comparison between canes and all other tissue types of PW. Among the ascomycetes, canes present lower abundance of *P. chlamydospora* and Diatrypaceae, and higher abundance of *A. pullulans*, *D. prosopidis*, *Diaporthe* sp., Capnodiales, depending on the woody tissues compared.

Mycobiome and Leaf Symptoms

Alpha and Beta Diversity

The examination of the mycobiome of PW in proximity of leaves that exhibited 'tiger stripes' symptoms revealed no significant differences in term of Shannon diversity (H') and evenness

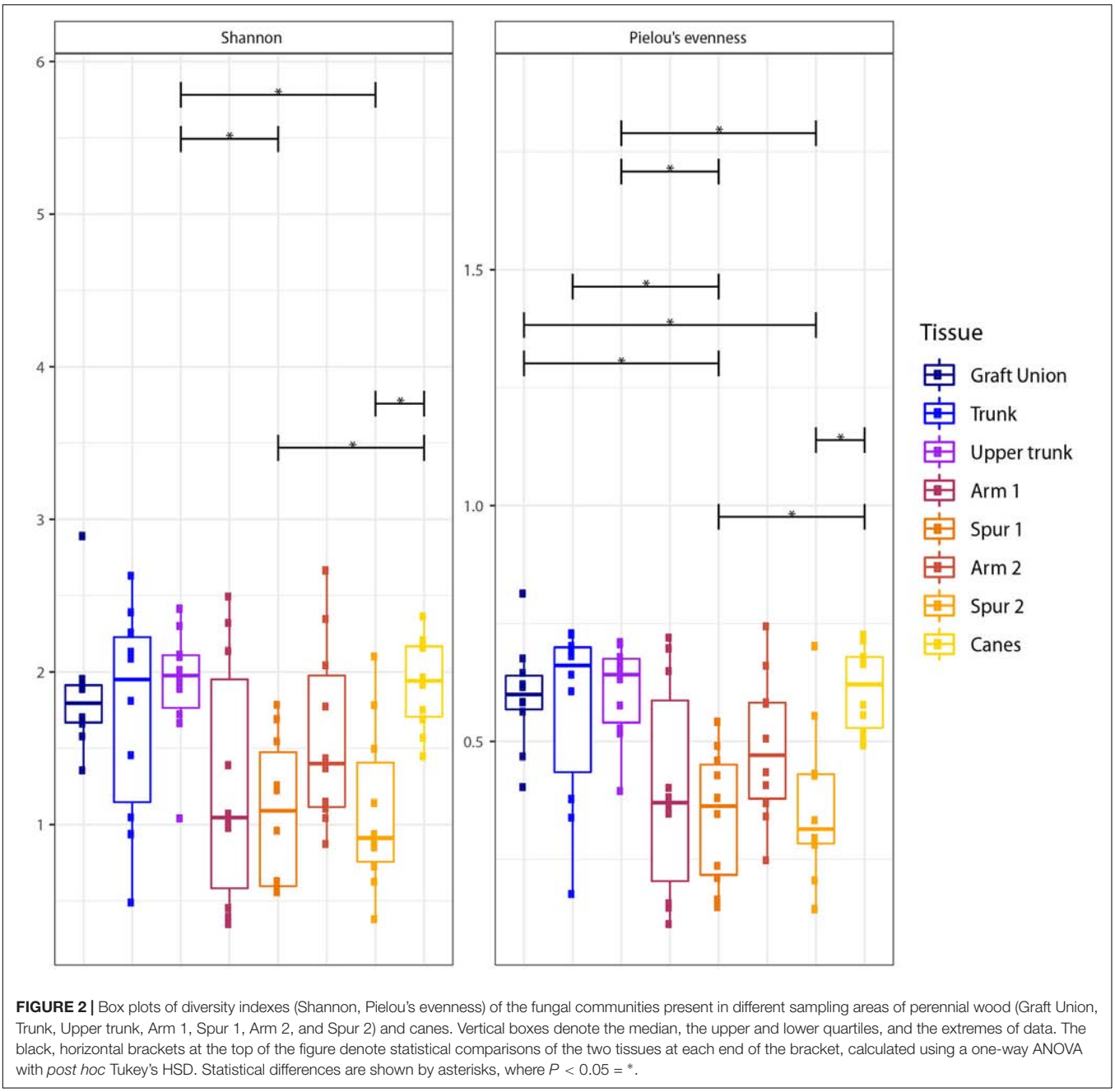

FIGURE 2 | Box plots of diversity indexes (Shannon, Pielou's evenness) of the fungal communities present in different sampling areas of perennial wood (Graft Union, Trunk, Upper trunk, Arm 1, Spur 1, Arm 2, and Spur 2) and canes. Vertical boxes denote the median, the upper and lower quartiles, and the extremes of data. The black, horizontal brackets at the top of the figure denote statistical comparisons of the two tissues at each end of the bracket, calculated using a one-way ANOVA with *post hoc* Tukey's HSD. Statistical differences are shown by asterisks, where $P < 0.05$ = *.

(J'), when compared with the fungal communities in the wood in proximity of non-symptomatic leaves, both in symptomatic and asymptomatic vines, according to Tukey's HSD ($P > 0.05$; **Table 2** and **Supplementary Figure S1**). The same results were obtained when comparing the mycobiome of canes presenting leaf symptoms or non-symptomatic leaves, in both symptomatic and asymptomatic plants. The Jaccard indexes, when plotted in a NMDS matrix, reveal overlapping communities with a very similar between-sample variability, suggesting an overall similarity in beta diversity (**Figure 7**). In fact, the PERMANOVA analysis revealed no statistical differences among the three tissue groups in both PW ($P = 0.067$) and canes ($P = 0.429$).

Mycobiome Composition and Differentially Represented Taxa

The mycobiome of PW in proximity of symptomatic or non-symptomatic leaves is characterized by high abundances of *P. chlamydospora*, *Fomitiporia* sp., and *D. prosopidis* (**Figure 8A**). The most frequent taxa of known GTD-associated pathogens are presented in **Table 3**.

The MetacodeR analysis revealed no statistical differences among taxa for their differential abundance in the three tissue groups compared in PW and in canes. Nevertheless, trends of change are present for some taxa, and they are shown in the heat trees in **Figure 9**.

TABLE 2 | Shannon diversity (*H'*) and Pielou's evenness (*J'*) indexes of the spatial distribution analysis (objective 2) and the mycobiome analysis in the wood associated with symptomatic leaves (objective 3).

	Shannon (*H'*)		Pielou's evenness (*J'*)	
	Value	***P* < 0.05**	**Value**	***P* < 0.05**
Spatial distribution (objective 2)				
Graft Union (GU)	1.85	–	0.60	S1, S2
Trunk (T)	1.73	–	0.57	S1
Upper trunk (UT)	1.92	S1, S2	0.61	S1, S2
Arm 1 (A1)	1.26	–	0.40	–
Spur 1 (S1)	1.08	UT, C	0.34	GU, T, UT, C
Arm 2 (A2)	1.58	–	0.49	–
Spur 2 (S2)	1.09	UT, C	0.37	GU, UT, C
Canes (C)	1.93	S1, S2	0.61	S1, S2
Leaf symptoms (objective 3)				
Perennial wood				
Asymptomatic arm in asymptomatic plant	1.29	–	0.41	–
Asymptomatic arm in symptomatic plant	1.05	–	0.33	–
Symptomatic arm	1.25	–	0.39	–
Canes				
Asymptomatic cane in asymptomatic plant	2.04	–	0.63	–
Asymptomatic cane in symptomatic plant	1.81	–	0.59	–
Symptomatic cane	2.09	–	0.68	–

Objective 2 aims to asses differences between tissue types sampled in different areas of grapevines; objective 3 aims to asses differences between non-leaf-symptomatic and leaf-symptomatic woody tissue, in perennial wood or canes. Tissue types significantly different, according to Tukey's HSD (P < 0.05), are listed under the column 'P < 0.05.'

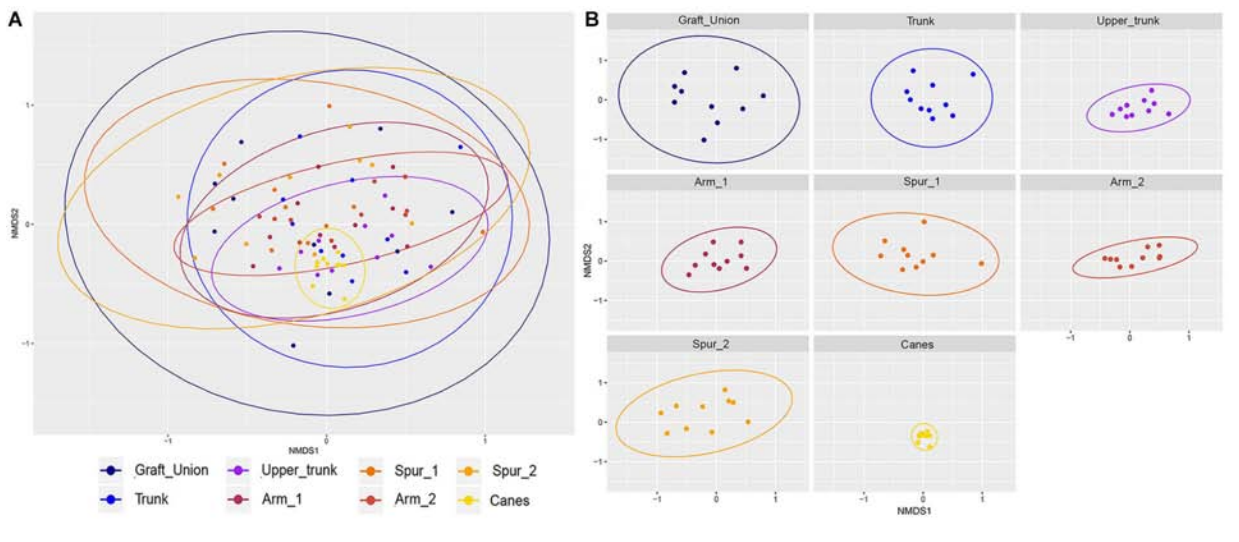

FIGURE 3 | Non-metric multidimensional scaling (NMDS) plots based on Jaccard's index. Fungal communities present in different tissue types in grapevine. **(A)** Shows all samples ordinated together, while **(B)** is the same data, split up per tissue type. Ellipses illustrate the multivariate normal distribution of samples within the same tissue type.

The PW in proximity of non-symptomatic leaves in non-symptomatic plants presents higher abundances of *Ramularia* sp., *Cladosporium* spp., *Alternaria* sp., *Debaryomyces* sp., *Cryptococcus* sp. and, to a lower extent, *P. chlamydospora*; while *Fomitiporia* spp. is under-represented, when compared with the wood near symptomatic leaves. When comparing the PW in proximity of non-symptomatic and symptomatic leaves, both collected from a symptomatic vine, similar trends are observed. In fact, *Ramularia* sp., members of the Pleosporales and *Aureobasidium pullulans* are over-represented in the former, along with a minor over-representation of the other taxa previously mentioned. Also, in this case a trend of underrepresentation is observed for *Fomitiporia* spp. Some differences are also

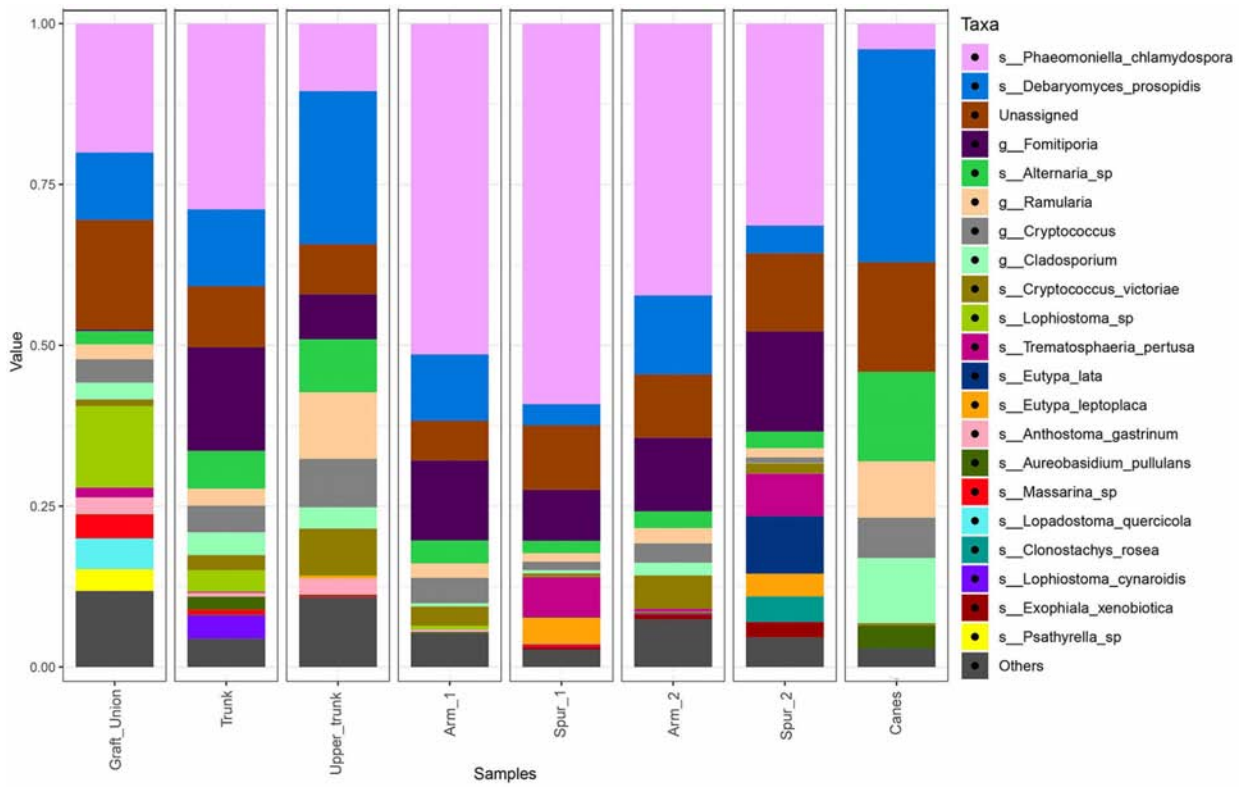

FIGURE 4 | Barplots of the relative abundance of the 20 most abundant taxa identified to species (s_) or genus (g_) level, found in different sampling areas of the stem and in the canes of grapevine. 'Unassigned' are taxa identified to a lower taxonomic level than genus, 'Others' are taxa not included in the 20 most abundant.

observed when comparing the PW near asymptomatic leaves coming from either asymptomatic or symptomatic plants. In the former we observe an over-representation of *Alternaria* sp., *Debaryomyces* sp. and *Cryptococcus* spp., and an under-representation of *A. pullulans* and *Anthostoma* sp. (**Figure 9A**).

Fungal communities found in canes are characterized by similar relative abundances, among the most abundant taxa, for all three groups analyzed (**Figure 8B**). Several trends of variation are shown in **Figure 9B**, involving primarily genera *Malassezia*, *Cryptococcus*, *Acremonium,* and *Diaporthe*.

DISCUSSION

Characterization of the Mycobiome in Perennial Wood and Canes

Research performed over the past decades on the grapevine mycobiome, using both culture-dependent and independent approaches, revealed over 900 fungal taxa (Jayawardena et al., 2018). The richness of the wood mycobiome in esca infected vineyards was estimated to be 88 taxa in Montpellier, France (Travadon et al., 2016); 85 species in Bordeaux, France, from 108 single strand conformation polymorphism (SSCP) profiles (Bruez et al., 2014); and 150 operational taxonomic units (OTUs) in Switzerland (Hofstetter et al., 2012). This study, the first using a NGS approach, reveals an even greater richness (289 taxa), adding numerous fungi to the known list of 900+ known taxa. Several factors may play a role in shaping the fungal community composition, such as location, cultivar and age of the plants (Travadon et al., 2016; Dissanayake et al., 2018). This is the primary reason why we expect that using NGS to assess the diversity of the wood mycobiome in other vineyards will considerably increase the number of fungal species found in association with grapevine wood.

Interestingly, 239 out of the 289 taxa (80%) are rare taxa, namely taxa that are detected at relative abundances lower than 0.1%. The ecology of several of them is known (e.g., *Trichoderma* spp., *Neofusicoccum* spp., *Ilyonectria liriodendri*; Jayawardena et al., 2018), while that of many others needs to be assessed (e.g., *Candida* spp., *Cryptococcus* spp., *Ganoderma* spp., and *Pyrenochaeta* spp.), moreover, the extent to which they contribute to the grapevine-mycobiome and fungus–fungus interactions remains to be elucidated. Hypothetically, grapevines perennial wood may function as a reservoir of fungal diversity, where species that are found in minor abundances may thrive under specific environmental conditions (e.g., extreme weather conditions, mechanical injuries), leading to positive or negative effects for the plants wellbeing.

Unsurprisingly, within this massive richness, numerous fungi associated with GTD were identified, both as frequent (e.g., *P. chlamydospora*, *Fomitiporia* sp., and *Eutypa lata*) and rare

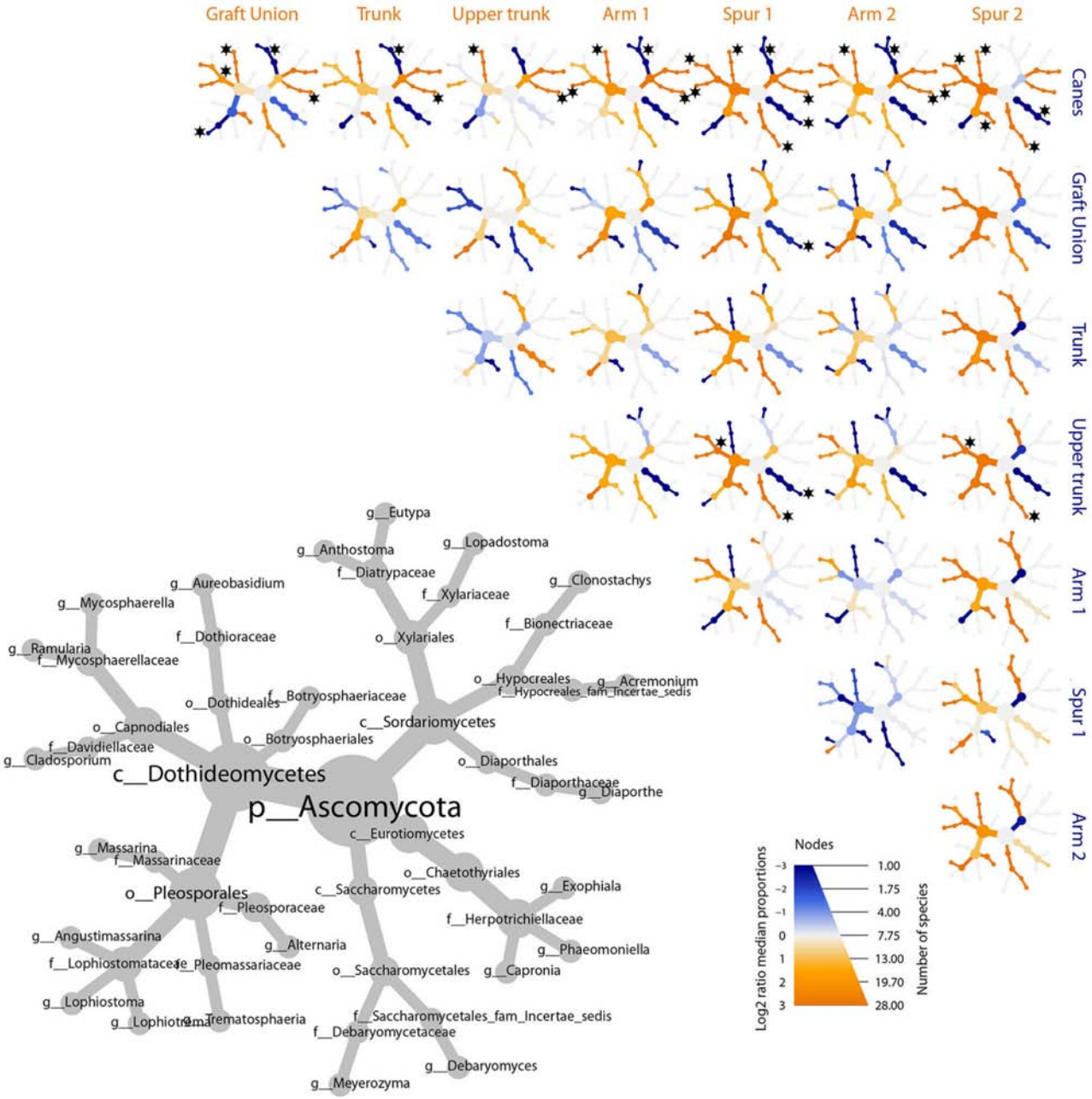

FIGURE 5 | Differential heat tree matrix depicting the change in taxa abundance between different tissue types, for ascomycetes, represented in the dataset (RA > 0.01%). The size of the individual nodes in the gray cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each tissue type: an orange node indicates a higher abundance of the taxon in the tissue type stated on the abscissa, than in the tissue type stated on the ordinate. A blue node indicates the opposite. Taxa identified as statistically differently represented, according to the Wilcoxon test, are tagged with a black star.

taxa (*Neofusicoccum* spp., *E. citricola*, *Fomitiporella* sp., *Diplodia* sp.). This agrees with previous reports showing that esca-affected plants may host numerous other wood pathogens (Rumbos and Rumbou, 2001; Edwards and Pascoe, 2004). However, *Phaeoacremonium* spp., a genus of tracheomycotic fungi often involved in esca-associated syndromes (Mostert et al., 2006), was not detected. The vineyard under study, as well as other esca-symptomatic fields examined in previous works (Hofstetter et al., 2012; Bruez et al., 2014; Travadon et al., 2016), are dominated by wood pathogens (e.g., *P. chlamydospora* or Botryosphaeriaceae), and the presence of decay agents was confirmed in all studies. On the other hand, the wood mycobiome of healthy vineyards, characterized by other authors, is dominated by endophytes and occasionally

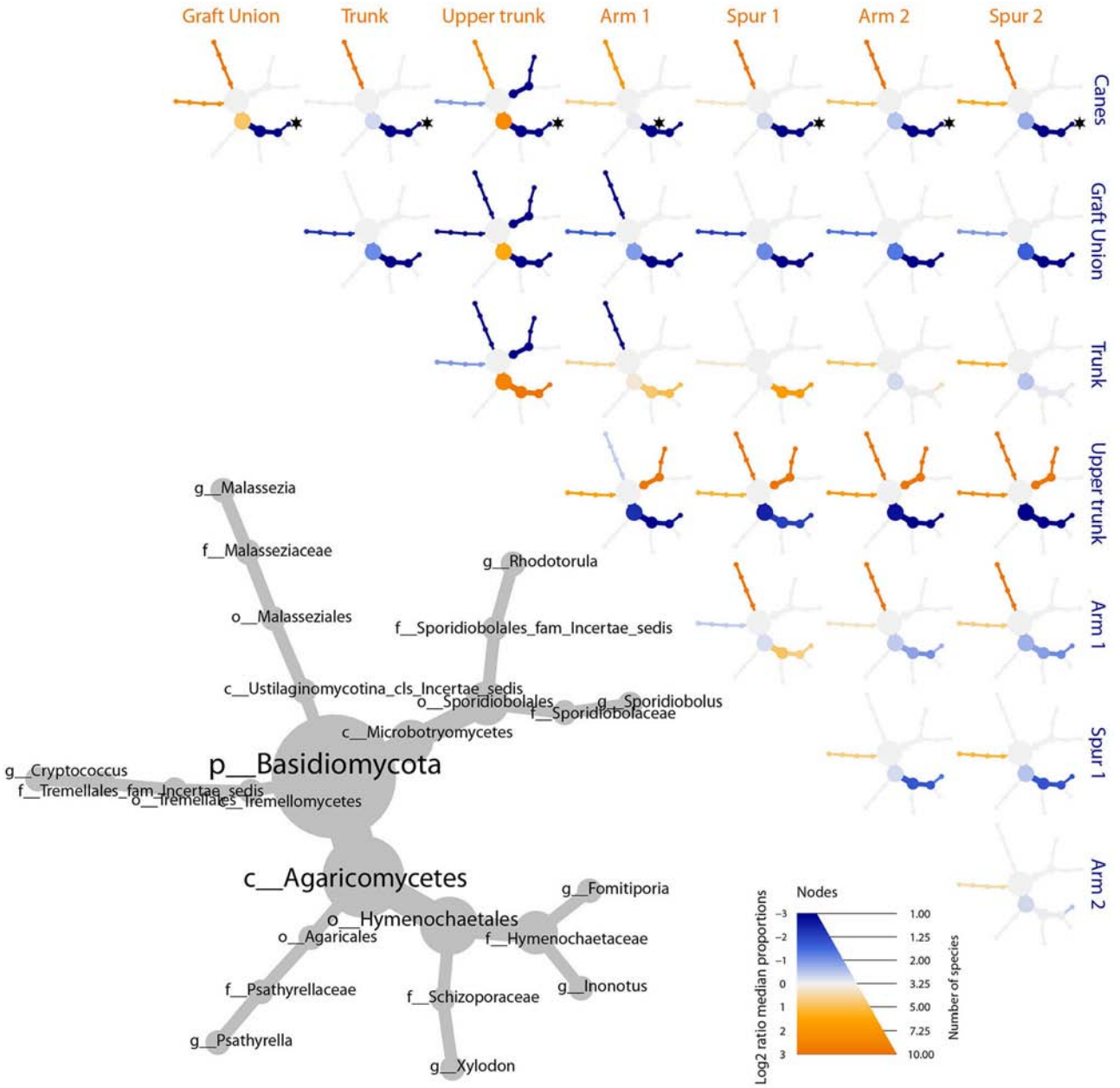

FIGURE 6 | Differential heat tree matrix depicting the change in taxa abundance between different tissue types, for basidiomycetes, represented in the dataset (RA > 0.01%). The size of the individual nodes in the gray cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each tissue group: an orange node indicates a higher abundance of the taxon in the tissue group stated on the abscissa, than in the tissue group stated on the ordinate. A blue node indicates the opposite. Taxa identified as statistically differently represented, according to the Wilcoxon test, are tagged with a black star.

saprobes, some of which hold potential for biological control of wood pathogens, with genera *Trichoderma*, *Aureobasidium*, *Acremonium*, *Cladosporium*, *Alternaria,* and *Epicoccum* being predominant (González and Tello, 2011; Pancher et al., 2012; Dissanayake et al., 2018). Despite the fact that wood pathogens were encountered, in minor abundance, also in healthy vineyards (González and Tello, 2011; Pancher et al., 2012), decay agents were not reported. Unfortunately, previous studies did not report data on rare taxa, therefore it is not possible to establish if other wood pathogens – including decay agents – were present but unable to thrive (e.g., antagonistic interactions with other fungi, strong plant defenses) or if they were completely absent.

The differences observed between PW and canes (e.g., alpha diversity, beta dispersion, taxa composition and abundance), some of which have also been observed in a previous study

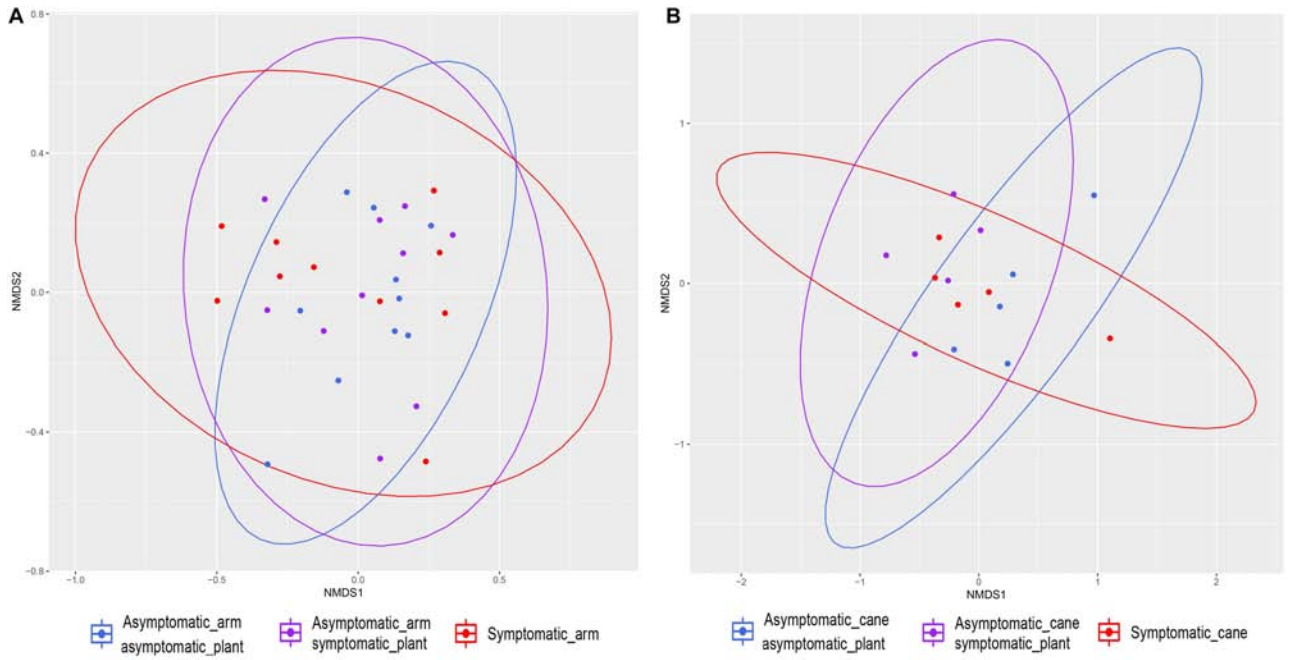

FIGURE 7 | Non-metric multidimensional scaling (NMDS) plots based on Jaccard's index. Fungal communities present in different tissue types in grapevine. **(A)** Communities found in the perennial wood in proximity of symptomatic leaves ('Symptomatic_arm') or of asymptomatic leaves, either in symptomatic plants ('Asymptomatic_arm symptomatic_plant') or in asymptomatic plants ('Asymptomatic_arm asymptomatic_plant'). **(B)** Communities found in canes with manifested leaf symptoms ('Symptomatic_cane') or asymptomatic, but coming from symptomatic plants ('Asymptomatic_cane symptomatic_plant') or asymptomatic plants ('Asymptomatic_cane asymptomatic_plant'). Ellipses illustrate the multivariate normal distribution of samples within the same tissue group.

in grapevines (Hofstetter et al., 2012) and other plants (Qi et al., 2012), can be due to two main factors. First factor is the time that fungi had to colonize PW (up to 19 years in the present study), which is also subjected to yearly pruning, leaving wounds that are the optimal entry point for colonizers. The canes had approximately 5 months of age from bud-burst to sampling and no wounding occurred in the shoots (e.g., summer pruning). A second factor that explains the these differences is the tissue-specificity, as considerable anatomical, biochemical, and physiological differences characterize perennial wood and annual wood, which may prevent some fungi, such as decay agents, from colonizing the latter. In fact, the absence of decay agents in canes (e.g., *Fomitiporia* sp., *I. hispidus*, and *Fomitiporella* sp.) observed in this study, and also reported in canes and nursery propagation material in other studies (Rumbos and Rumbou, 2001; Halleen et al., 2003; Casieri et al., 2009; Hofstetter et al., 2012), suggests that the infection by these pathogens occurs exclusively in older wood and under field conditions.

A brief mention of the five genera identified for the first time in grapevines wood follows.

Debaryomyces

Debaryomyces hansenii has been isolated from shoots of *Sequoia sempervirens* and from the soil under the tree (Middelhoven, 2003), as well as from white and brown rot of several woody species (González et al., 1989). *Debaryomyces* sp. was also detected in slime fluxes of *Prosopis juliflora*, a deciduous woody plant, as well as in insects associated with it (*Drosophila carbonaria* and *Aulacigaster leucopeza*) (Ganter et al., 1986). Molecular analysis identified some of these strains as a new species, *D. prosopidis* (Phaff et al., 1998), which is the only record of this organism in literature. Concerning the vineyard, *Debaryomyces* sp. has been identified on grape berries (Jara et al., 2016) and during wine fermentation (Varela and Borneman, 2017).

Trematosphaeria

Trematosphaeria pertusa, from this study, is known to grow on the surface of decaying terrestrial wood (Suetrong et al., 2011), however, it has also been retrieved from wood of *Fagus sylvatica* and *Pinus sylvestris* submerged in a river (Kane et al., 2002). Terrestrial woody hosts of *T. pertusa* include also *Fraxinus excelsior*, *Fagus* sp. and *Platanus* sp.

Lopadostoma

Members of the Xylariaceae are typically saprobes, despite some of them are endophytes and others are plant pathogens (Mehrabi and Hemmati, 2015). To date, due to the lack of studies on this genus, the ecology of *Lopadostoma* remains to be fully understood. There are 12 species described in this genus, most of which have been isolated from hosts *Quercus* spp. or *F. sylvatica* (Jaklitsch et al., 2014).

Biatriospora

This genus contains species that have been isolated as endophytes of both terrestrial and marine-associated plants (Kolařík et al.,

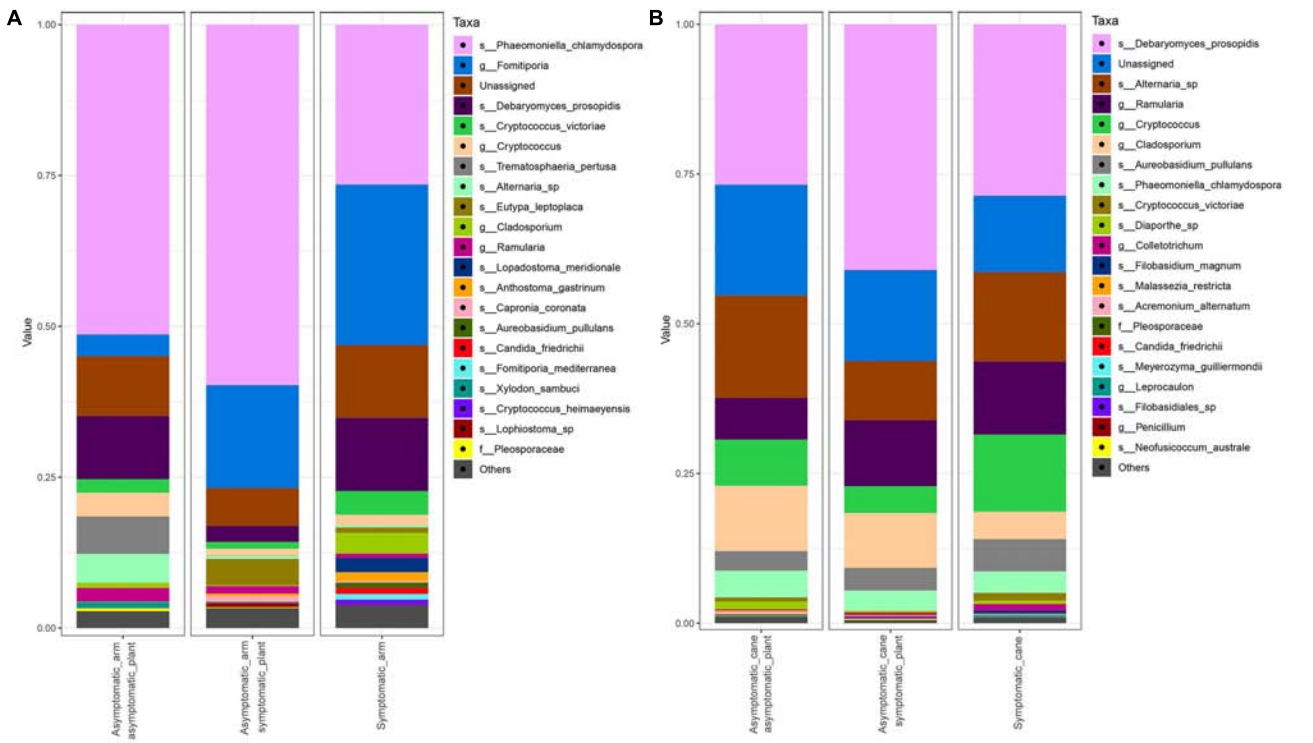

FIGURE 8 | Barplots of the relative abundance of the 20 most abundant taxa identified to species (s_), genus (g_) or family (f_) level. 'Unassigned' are taxa identified to a lower taxonomic level than family. 'Others' are taxa not included in the 20 most abundant. **(A)** Communities found in the PW in proximity of symptomatic leaves ('Symptomatic_arm') or of asymptomatic leaves, either in symptomatic plants ('Asymptomatic_arm symptomatic_plant') or in asymptomatic plants ('Asymptomatic_arm asymptomatic_plant'). **(B)** Communities found in canes with manifested leaf symptoms ('Symptomatic_cane') or asymptomatic, but coming from symptomatic ('Asymptomatic_cane symptomatic_plant') or asymptomatic plants ('Asymptomatic_cane asymptomatic_plant').

2017), as well as from lichens, as an endolichenic fungus (Zhou et al., 2016). Known hosts of this genus, in temperate climate, are *Ulmus* spp. and *Acer pseudoplatanus*, along with several other hosts from tropical climates (Haňáčková et al., 2017; Kolařík et al., 2017). *Biatriospora* sp. is a source of bioactive compounds (heptaketides) with antifungal properties (Zhou et al., 2016) and preliminary studies suggest some potential in biological control (Haňáčková et al., 2017). *Biatriospora mackinnonii*, from this study, has been isolated from terrestrial plant material and it has also been linked with human mycetoma, a skin disease (Ahmed et al., 2014; Kolařík et al., 2017).

Malassezia

Malassezia restricta, from this study, has been identified as endophyte of both woody and herbaceous plants (e.g., *Eucalyptus* sp., *Populus deltoides*, *Spiranthes spiralis*, *Solanum tuberosum*) as well as found on orchid roots, and in soil (Connell and Staudigel, 2013; Abdelfattah et al., 2016; Miguel et al., 2017).

It is important to mention the genera *Cryptococcus*, *Angustimassarina,* and *Exophiala*, found in this study, which have been previously associated to grapevines wood just once, either as saprobes or endophytes, in a culture-independent study (Jayawardena et al., 2018). Lastly, species of *Ramularia* have been isolated from leaves (both as epiphytes and endophytes) of several hosts, including *Platanus* sp., *Prunus cerasus,* and *V. vinifera* (Bakhshi and Arzanlou, 2017), although never in association with wood.

Among the genera and species that were found associated with grapevines for the first time in this study, some have previously been detected in the endosphere or phyllosphere of other woody plants that were found in the proximities of the vineyard used in the present study (**Supplementary Table S1**). For example, *Lopadostoma* spp. and *A. gastrinum* were reported in *Quercus* (Jaklitsch et al., 2014; Haynes, 2016), *Rhodotorula mucilaginosa* and *T. pertusa* in *Fraxinus* (Suetrong et al., 2011; Haňáčková et al., 2017), and *M. restricta* in *Eucalyptus* (Miguel et al., 2017). This suggests that the fungi present in the endosphere and phyllosphere of the flora in proximity of a vineyard might influence the composition of the mycobiome of grapevines wood, acting as a reservoir of multi-host fungi, with wind, rain and insects being possible vectors for mycobiome exchange.

Spatial Distribution of the Fungal Communities

The concept of spatial distribution and tissue specificity in woody and herbaceous plants is not new in microbial ecology (Wearn et al., 2012; Miguel et al., 2017; Singh et al., 2017; Kovalchuk et al., 2018). However, studies that investigated the spatial distribution of fungal communities that colonize different areas or tissues

TABLE 3 | Relative abundances (RA) of genera or species of fungi known to be involved in GTD, encountered in the perennial wood (PW) or canes, relative to objective (3).

Genus/Species	Pathogens RA in PW			Pathogens RA in canes		
	SYM	ASYM-SYM	ASYM	SYM	ASYM-SYM	ASYM
Ascomycetes						
*Anthostoma gastrinum**	1.3	0.7	–	–	–	–
Diaporthe sp.	–	–	<0.1	0.6	<0.1	1.2
Eutypa lata	0.2	0.1	<0.1	–	–	–
Eutypa leptoplaca	0.7	3.7	<0.1	–	–	–
Neofusicoccum parvum	0.1	–	–	–	–	–
Neofusicoccum australe				–	0.2	–
Phaeomoniella chlamydospora	21.8	50.1	43.1	3.5	3.4	4.9
Basidiomycetes						
Fomitiporia sp.	34.9	24.1	7.7	–	–	–
Fomitiporia mediterranea	0.9	<0.1	0.1	–	–	–
Total Ascomycetes	22.7	53.9	43.1	4.1	3.6	6.1
Total Basidiomycetes	35.8	24.1	7.8	–	–	–
TOTAL	**58.5**	**78.0**	**50.9**	**4.1**	**3.6**	**6.1**

*Three groups of wood were examined, namely the PW in proximity of symptomatic leaves (SYM); asymptomatic leaves, but in vines that presented leaf symptoms (ASYM-SYM); asymptomatic leaves, in vines that did not present leaf symptoms (ASYM). The same groups were examined in canes, namely canes with manifested leaf symptoms (SYM); asymptomatic canes, but in vines that presented symptomatic leaves (ASYM-SYM); asymptomatic canes, in vines that did not present symptomatic leaves (ASYM). Taxa listed are found in RA > 0.01% of the total dataset, other known wood pathogens (RA < 0.01%) are not included. *Genus Anthostoma has been associated to pathogenicity in grapevine wood, but not A. gastrinum, being this the first report of this species in grapevine wood. The RAs of this taxon were not calculated in the Total(s).*

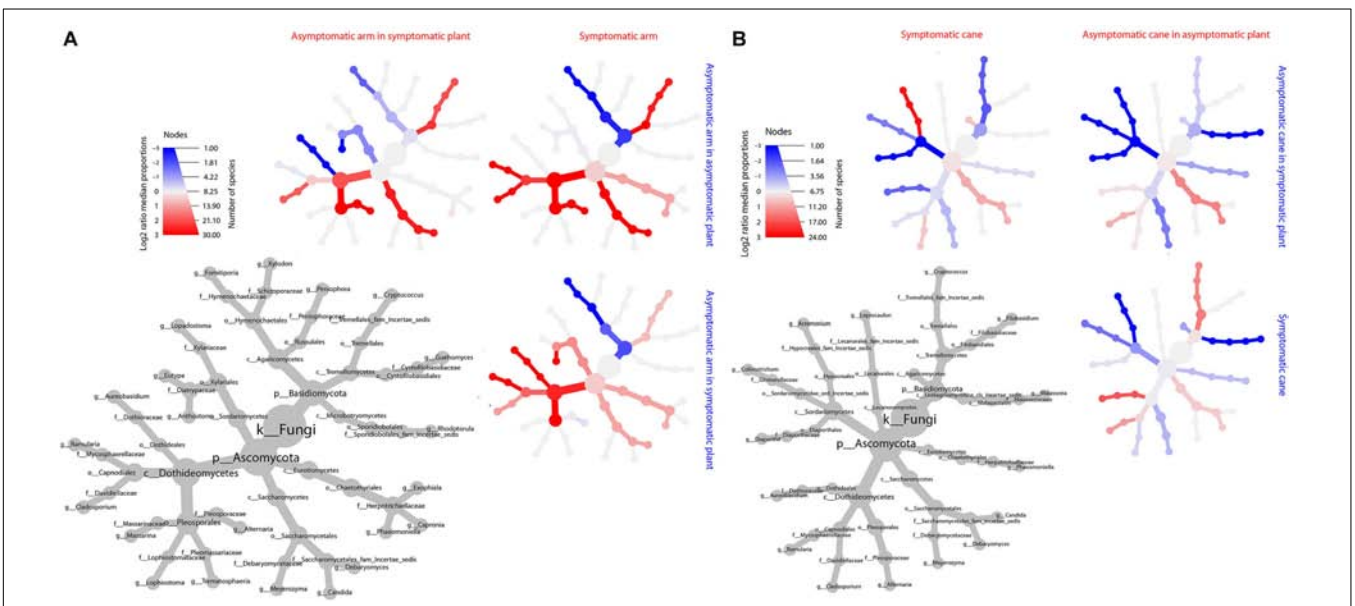

FIGURE 9 | Differential heat tree matrix depicting the change in taxa abundance between different tissue groups, perennial wood **(A)** and canes **(B)**, represented in the dataset (RA > 0.01%). The size of the individual nodes in the gray cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each tissue group, with the color illustrating the log2 fold change: a red node indicates a higher abundance of the taxon in the tissue group stated on the abscissa, than in the tissue group stated on the ordinate. A blue node indicates the opposite. No taxa were identified as statistically differently represented, according to the Wilcoxon test.

in the wood of adult grapevines are scarce (Bruez et al., 2014; Travadon et al., 2016), and none of them used a NGS approach. When examining the relative abundances of identified taxa with NGS, it is important to remember that the ITS marker, our target amplicon, can be found in multiple copies in the genome of fungi and the number of copies may vary considerably from species to

species (Schoch and Seifert, 2012), which inherently leave fungal relative distributions with great uncertainties.

From a qualitative point of view, the communities of various areas in PW shared numerous taxa, overall in agreement with the findings of Bruez et al. (2014), although some tissue types were more variable than others (**Figure 3**). Also, considerable differences are evident from a quantitative point of view. High variability was observed among individual plants, as already noted in grapevines (Travadon et al., 2013), suggesting the need to increase the amount of biological replicates in future studies.

The graft union and the wood below the spurs host unique taxa or taxa that are found only in low percentages (RA < 0.1%; **Table 1**), or completely absent, in other parts of the plant. Supposedly, the root system (in this study *V. berlandieri* × *V. rupestris*), whose endosphere is influenced by the soil microbiome (Zarraonaindia et al., 2015) and by the rootstock used in the propagation process, harbor unique fungi that have a limited capacity of colonizing the stem of *V. vinifera*. Interestingly, *Fomitiporia* sp., a species that is well represented in all PW tissues (8.9% < RA < 22.9%), is nearly absent in the graft union (0.6%; **Figure 4**). This suggests that other species of *Vitis* may be more resistant than *V. vinifera* to *Fomitiporia* sp., in fact, artificial inoculations of *Fomitiporia punctata* in rootstock Kober 5BB (*V. berlandieri* × *Vitis riparia*) led to low re-isolation percentages of this pathogen (8% of inoculated plants; Sparapano et al., 2000a). We are not aware of any other paper in the literature that describes the presence of *Fomitiporia* sp. in the root system of naturally infected vines.

The spur tissue is located in proximity of pruning wounds, which are considered the main entry point of GTD-associated fungal pathogens (Bertsch et al., 2013). Through this woody tissue some colonizers can successfully spread throughout the cordon and trunk (e.g., *P. chlamydospora* or *Fomitiporia* sp.), while others may be restrained by the antagonistic interactions with the resident mycobiome and/or plant defenses (e.g., *Eutypa* spp., *Clonostachys rosea*, *Acremonium* sp.; **Table 1**).

The remaining tissues, namely the trunk, upper trunk and arm points, do not differ by any of the parameters measured in this study (e.g., alpha and beta diversity, MetacodeR; **Figures 2**, **3**, **5**, **6**), where unique taxa among the most represented fungi are nearly absent (**Table 1**), and relative abundances differ primarily in the representation of *P. chlamydospora* (**Figure 4**).

The results of this study show that the wood mycobiome of grapevines may vary and, in order to have a representative understanding of the diversity and abundance of the fungal communities, multiple sampling areas are recommended. We propose four samples per plant. The first two are the graft union and the wood below one spur, where some taxa are uniquely represented and abundances vary considerably; the third is any point of the trunk or arm; and the forth is canes.

Fungal Communities and Leaf Symptoms

The outcome of the statistical analyses of the mycobiome of both PW and canes highlights that fungal communities were not affected by the manifestation of leaf symptoms, or *vice versa* (**Figures 7**, **8** and **Supplementary Figure S1**). The manifestation of 'tiger stripes' leaf symptoms, always associated with an advanced stage of infection by tracheomycotic pathogens, is in large part cryptic due to its discontinuity and unidentified causal agents (Mugnai et al., 1999). Greenhouse and field trials often failed to reproduce such symptoms with artificial inoculations of *P. chlamydospora* and/or *P. minimum* (Zanzotto et al., 2007; Gramaje et al., 2010), except for one study in which esca-like leaf symptoms were replicated in a very small percentage of inoculated plants (Sparapano et al., 2001). Other microbial ecology studies of the wood from leaf-symptomatic and asymptomatic vines showed that there are no differences in the fungal community composition, finding similar abundances of wood pathogens (Hofstetter et al., 2012; Bruez et al., 2014). Nevertheless, tracheomycotic fungi are currently believed to be directly or indirectly responsible for leaf symptoms as they are frequently isolated from symptomatic wood (Surico et al., 2008). The two arguments supporting this hypothesis are that the translocation of (1) fungal toxins, (2) byproducts of the wood degradation or (3) a combination of both, via xylem sap, from PW to leaves, are responsible for the appearance of leaf symptoms (Mugnai et al., 1999). While other studies showed that some toxins or culture filtrates of tracheomycotic fungi could lead to leaf discoloration and necrosis, solid evidence of the replication of the 'tiger stripes' pattern, as well as symptoms fluctuation, is lacking (Evidente et al., 2000; Sparapano et al., 2000b); Abou-Mansour et al., 2004; Andolfi et al., 2011. No evidence is currently available in support of the second and third hypotheses, namely those involving the byproducts of wood degradation.

The results of the present study are, to some extent, in agreement with the findings of Hofstetter et al. (2012) and Bruez et al. (2014). In fact, the fungal communities present in the PW in proximity of leaves that exhibited symptoms were overall similar to those found in plants with healthy leaves (in symptomatic or asymptomatic plants). When considering trends, *P. chlamydospora* is more abundant in the wood near non-symptomatic leaves, while *Fomitiporia* sp. is the most represented taxa in the wood near symptomatic leaves. This observation suggests a higher wood decay activity, which likely leads to a greater presence of byproducts of wood degradation, therefore supporting the second hypothesis for leaf symptoms manifestation. Regardless, it does not explain the manifestation of leaf symptomatology in grapevines not infected by *Fomitiporia* sp. (Edwards et al., 2001).

The similarity in the community structure of symptomatic and non-symptomatic canes is an additional evidence in support of the current understanding that fungi present in canes are not directly linked with leaf symptomatology (**Figures 7**, **8** and **Supplementary Figure S1**).

Leaf symptoms manifestation remains cryptic, however, it is important to note that this study, as well as the one by Bruez et al. (2014), analyzed the microbial communities of PW several months after the plant had exhibited leaf symptoms. It is not known whether the pathogens abundance in the moment pre-/during/post-symptomatology varies. In fact, the study by Bruez et al. (2014) revealed that alterations in the wood mycobiome may occur in different seasons, therefore further research is needed in this direction. However, this may not apply to the canes, as

leaf symptoms were still present, and manifested only 2 months before wood tissue sampling.

Further Considerations

In this study, the mycobiome of the canes is composed principally by endophytes and saprobes, with the exception of pathogens *P. chlamydospora* and *Diaporthe* sp., whose presence did not induce the appearance of wood symptoms. Concerning the former, this observation suggests that this fungus may fall along a continuum 'from mutualist to saprophyte or latent pathogen' (Wearn et al., 2012), as also proposed by other authors (Rumbos and Rumbou, 2001; Hofstetter et al., 2012), and whose pathogenicity is triggered by external factors. Interestingly, also *Fomitiporia* sp. was often identified in woody tissue that did not present the typical white rot symptom. This fungus may live asymptomatically in wood when found in low abundances, and produce white rot when its presence increases considerably. Hypothetically, the wood cores showing this symptom (15% of the total) may be the same in which *Fomitiporia* sp. was present in a RA $>$ 35% (17.5%).

The presence of *P. chlamydospora*, and that of other wood pathogens, in vineyards around the world might be currently blatantly underestimated due to the elusiveness of internal symptoms. The Almotivo vineyard (this study) presented an incidence of leaf symptoms manifestation of ca. 1%, for three consecutive years, nevertheless, 100% of the sampled plants were colonized by both *P. chlamydospora* (in PW and canes) and *Fomitiporia* sp. (in PW). It is already known that leaf symptoms are not a reliable parameter to assess the health status of a vineyard (Pollastro et al., 2000), although it is the one most frequently employed, along with the count of dead vines (Lecomte et al., 2018). It is of utmost importance to develop tools that allow vine growers to assess the real extent of infections in the wood, and apply appropriate control measures.

CONCLUSION

The characterization of the grapevine wood mycobiome, using NGS, in a vineyard affected by esca proper is an important step that lays the foundations for future studies to compare microbial community structures of vineyards affected by esca or other GTD. Some parameters that may influence the mycobiome composition and which may be of interest to investigate upon are: the flora surrounding the vineyard, the climatic conditions and seasonality, geographical location and year. Moreover, useful comparisons can be made between cultivars and vineyard management strategies (e.g., conventional, organic, and biodynamic). Eventually, a meta-analysis of the mycobiome that takes into account several of these parameters may reveal a pattern that could elucidate some of the obscure points that still prevent a full understanding of the etiology of this disease complex.

AUTHOR CONTRIBUTIONS

GDF conceived the study, performed field sampling and wet-lab work, interpreted and discussed the results, and wrote the manuscript. AG performed the wet-lab work and bioinformatics analyses, interpreted the results, and wrote the manuscript. MA performed statistical analyses and data visualization. HO, LH, and RF supervised the study and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00910/full#supplementary-material

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Comparison of the Molecular Responses of Tolerant, Susceptible and Highly Susceptible Grapevine Cultivars During Interaction With the Pathogenic Fungus *Eutypa lata*

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Eutypa lata is the causal agent of eutypa dieback, one of the most destructive grapevine trunk disease that causes severe economic losses in vineyards worldwide. This fungus causes brown sectorial necrosis in wood which affect the vegetative growth. Despite intense research efforts made in the past years, no cure currently exists for this disease. Host responses to eutypa dieback are difficult to address because *E. lata* is a wood pathogen that causes foliar symptoms several years after infection. With the aim to classify the level of susceptibility of grapevine cultivars to the foliar symptoms caused by *E. lata*, artificial inoculations of Merlot, Cabernet Sauvignon, and Ugni Blanc were conducted over 3 years. Merlot was the most tolerant cultivar, whereas Ugni Blanc and Cabernet Sauvignon exhibited higher and differential levels of susceptibility. We took advantage of their contrasting phenotypes to explore their defense responses, including the activation of pathogenesis-related (*PR*) genes, oxylipin and phenylpropanoid pathways and the accumulation of stilbenes. These analyses were carried out using the millicell system that enables the molecular dialogue between *E. lata* mycelium and grapevine leaves to take place without physical contact. Merlot responded to *E. lata* by inducing the expression of a large number of defense-related genes. On the contrary, Ugni Blanc failed to activate such defense responses despite being able to perceive the fungus. To gain insight into the role of carbon partitioning in *E. lata* infected grapevine, we monitored the expression of plant genes involved in sugar transport and cleavage, and measured invertase activities. Our results evidence a coordinated up-regulation of *VvHT5* and *VvcwINV* genes, and a stimulation of the cell wall invertase activity in leaves of Merlot elicited by *E. lata*, but not in Ugni Blanc. Altogether, this study indicates that the degree of cultivar susceptibility is associated with the activation of host defense responses, including extracellular sucrolytic machinery

and hexose uptake during the grapevine/*E. lata* interaction. Given the role of these activities in governing carbon allocation through the plant, we postulate that the availability of sugar resources for either the host or the fungus is crucial for the outcome of the interaction.

Keywords: grape, eutypa dieback, *Eutypa lata*, cultivar susceptibility, pathogenesis-related (PR) proteins, stilbenes, sugar transporters, cell wall invertase

INTRODUCTION

Grapevine is a crop of great economic importance worldwide. Varieties used in all European vineyards belong to the *Vitis vinifera* species. In France, several grapevine varieties are imposed by French legislation, especially for the French AOC label (controlled designations of origin). For example, the main grapevine variety cultivated in the vineyard of Cognac (Charentes – France) is Ugni Blanc because of its high productivity trait and its lower susceptibility to the gray mold disease (personal communication, BNIC) (Cardot, 2017). This cultivar produces wines with low alcohol content and high total acidity, which is therefore ideal for the distillation of cognac spirits, but is highly susceptible to diseases, such as grapevine trunk diseases (GTDs; Péros, 1995; Dubos, 2002; Bertsch et al., 2013; Bruez et al., 2013). Collectively, the three main GTDs, esca disease, botryosphaeria dieback, and eutypa dieback, are major issues causing severe economic losses in vineyards (Sosnowski and McCarthy, 2017) by affecting plant vigor, reducing yield and quality of grapes, and premature plant death (Gramaje et al., 2018). For example, the cost of dead plant replacements due to these diseases is estimated to approximately 1.5 billion dollars per year worldwide (Hofstetter et al., 2012). GTDs have been associated with one or several fungal trunk pathogens, such as *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Eutypa lata* or members of the Botryosphaeriaceae, which colonize vivaceaous parts of the plant at all stages of growth (Fontaine et al., 2016). Symptoms, which include both foliage and vascular tissues, can overlap among different GTDs, making difficult the identification in the field (Gubler et al., 2005; Surico et al., 2006; Gramaje et al., 2018). Apart from prophylaxis methods, there is currently no effective treatment to combat wood diseases (Mondello et al., 2017). The severity of symptoms vary among cultivars (Gubler et al., 2005; Marchi, 2006; Travadon et al., 2013), however, no resistance locus against GTDs have yet been identified in *V. vinifera*. Together with the progressive restriction in pesticides use by the European Union, research efforts are needed to find effective solutions to this issue (Cardot, 2017).

Eutypa lata is the main fungus responsible for the eutypa dieback disease but it can be recovered from wood lesions in combination with other fungi that may be associated with processes leading to esca disease (Péros et al., 1999). This ascomycete develops in the trunk and spreads with the release of ascospores during rainy and windy periods and by infecting pruning wounds (Moller and Carter, 1965; Sosnowski et al., 2007; Rolshausen et al., 2008). Eutypa dieback symptoms, which mostly occurs several years after the infection, include sectorial necrosis in trunk, stunted and weak shoots with small chlorotic leaves. Sectorial necrosis cause the rupture of sap flow resulting in the dieback of infected grapevines, and conduct to the death of the vine within 3–5 years (Larignon, 2012). Because the fungus is restricted to the wood cane and not detected in the herbaceous part, symptoms observed on leaves and young stems are notably due to the secretion and the translocation of fungal toxins such as eutypine, glycoproteins or cell wall degrading enzymes (Deswarte et al., 1996; Mahoney et al., 2003; Octave et al., 2006, 2008; Rolshausen et al., 2008; Andolfi et al., 2011; Morales-Cruz et al., 2015).

Overall, *V. vinifera* cultivars are greatly affected by a large number of pathogens (fungi, bacteria, oomycetes or viruses). Grape, like other plants, attempt to respond by activating defense mechanisms, such as antioxidant system, phenylpropanoid pathway, pathogenesis-related (PR)-proteins and phytoalexin production (Armijo et al., 2016). However, this arsenal of defense deployed by the host against aggressors is frequently not sufficient to counteract the disease development. Depending on the pathogen lifestyle (necrotroph, biotroph or hemibiotroph), different hormone-mediated signaling pathways regulate a transcriptional reprogramming and more largely plant defense mechanisms (Oliver and Ipcho, 2004; Glazebrook, 2005; van Kan, 2006). Based on *Arabidopsis thaliana* studies, it is generally admitted that jasmonic acid (JA) and ethylene (ET) mediate defense responses to necrotrophic pathogen, while salicylic acid (SA) is required for the resistance against biotrophs and hemibiotrophs, with antagonistic relationships between both pathways (Glazebrook, 2005). The phytohormone network consisting of JA, ET, and SA, is required for the two modes of plant immunity, pattern-triggered immunity (PTI), and effector-triggered immunity (ETI) (Jones and Dangl, 2006; Cui et al., 2015; Peng et al., 2018).

Several PR proteins, belonging to different classes described by van Loon et al. (2006), are synthesized in grapevine following infection through the recognition of MAMPs (Microbe-Associated Molecular Patterns) or DAMPs (Damage-Associated Molecular Patterns) like oligosaccharide, lipid and proteinaceous elicitors (Kortekamp, 2006; Gomès and Coutos-Thévenot, 2009). Most of the PR-proteins have direct antimicrobial properties (e.g., osmotin and thaumatin) through hydrolytic activities on pathogen cell wall (e.g., glucanase and chitinase), and/or indirectly leads to the production of elicitors which trigger additional defense responses (van Loon et al., 2006). Numerous studies have described the selective expression of PR-protein-encoding genes in various grapevine cultivars following infection

with a wide range of pathogens, such as *Botrytis cinerea*, *Plasmopara viticola*, *Erysiphe necator* or *Pseudomonas syringae* pv *pisi* (Kortekamp, 2006; Chong et al., 2008; Armijo et al., 2016). Following infection with wood decay disease fungi, grapevine defense responses like PR-proteins synthesis and phytoalexins production are induced both at local (wood) and systemic (leaf, berry) levels [reviewed in Fontaine et al. (2016) and Bertsch et al. (2013)]. For example, Camps et al. (2010) reported an up-regulation of several genes encoding PR-proteins (thaumatin and osmotin, chitinase and β-1,3-glucanase) in leaves of infected rooted cuttings (Carbernet-Sauvignon) artificially infected with *E. lata*. This result has been further confirmed by the work of Mutawila et al. (2017) that showed that the elicitation of cell suspension culture of *V. vinifera* cv Dauphine with *E. lata* culture filtrate resulted in an induction of *VvPR2* (β-1,3-glucanase), *VvPR5* (thaumatin and osmotin-like proteins), *VvPR3* and *VvPR4* (chitinase), and *VvPR6* (protease inhibitor, PIN).

Induction of secondary metabolites are commonly associated with defense and pathogenic responses (La Camera et al., 2004; Piasecka et al., 2015). Secondary metabolism is strongly induced after infection of grapevine by various pathogens, including *E. lata* (Langcake, 1981; Adrian et al., 1997; Amalfitano et al., 2000; Jeandet et al., 2002; Armijo et al., 2016). Several reports have indicated an upregulation of phenylalanine ammonia-lyase (PAL), which encodes the first enzyme of the phenylpropanoid pathway, as well as genes coding for enzymes of the flavonoid and stilbenoid pathways, chalcone synthase (CHS) and stilbene synthase (STS), respectively (Rotter et al., 2009; Mutawila et al., 2017). Stilbenes, such as ε-viniferin and resveratrol, are major phytoalexins in grapes. Resveratrol limits *E. lata* mycelium growth *in vitro* (Coutos Thévenot et al., 2001), but it is uncertain whether the accumulation of resveratrol and phenolic compounds limits *in vivo* wood colonization (Mutawila et al., 2017).

According to current knowledge concerning grapevine/*E. lata* interaction, grapevine exhibits some of the typical responses of the PTI, such as PR-protein synthesis and secondary metabolites accumulation, suggesting that this fungus is perceived by the host (Rotter et al., 2009; Fontaine et al., 2016). However, most of *V. vinifera* cultivars are susceptible to *E. lata*, which indicates that defense responses are not sufficient to limit infection (Mutawila et al., 2017).

Alongside these so-called classical defense responses, responses to pathogens include important changes in host carbon metabolism, a reduction of photosynthesis and an accumulation of hexoses in the apoplast (Berger et al., 2007; Bolton, 2009). Furthermore, several recent studies indicate that regulation of carbon partitioning and competition for apoplastic sugars between the plants and the pathogen play a critical role in determining the outcome of the interaction [reviewed in Naseem et al. (2017), Bezrutczyk et al. (2017), and Oliva and Quibod (2017)]. Indeed, as heterotrophic for carbon, pathogens need photoassimilates produced by the host during the photosynthesis as energy sources for growth and life cycle completion. On the other side, defending plants require sugars for metabolic needs of individual sinks and to sustain the increased metabolism activity necessary to fuel plant defense. Depending on the mode of colonization, biotrophic, and necrotrophic pathogens use different strategies to retrieve sugars from host. As a consequence, heterotrophic pathogens may represent an additional sink competing with other sinks for carbohydrates, thereby modifying source-sink partitioning through the plant (Lemoine et al., 2013).

To gain access to carbohydrate at the plant-pathogen interface, both partners possess their own sugar transport machinery, including sugar transporters and cell wall invertases (Veillet et al., 2016, 2017). We and other previously reported the involvement of plant high-affinity sugar transporters in the recovery of hexoses from the apoplasm. Members of the Sugar Transport Protein (STP) family are induced in response to several fungal and bacterial pathogens, this include AtSTP13 in Arabidopsis and STP13 homologs in wheat (Lr67) and grapevine (VvHT5) (Hayes et al., 2010; Lemonnier et al., 2014; Moore et al., 2015; Yamada et al., 2016). In Arabidopsis, AtSTP13 is a component of the PTI and contributes to the fungal and bacterial resistance (Lemonnier et al., 2014; Yamada et al., 2016). Because sugars seem to be preferentially taken up in the form of hexoses, regulation of carbon fluxes across plasma membrane is also dependent on the sucrose cleaving activity of cell wall invertases (CWINs). Hence, coordinated activities of CWIN and STP proteins have been reported in several plant pathogen interactions (Ruan, 2014; Tauzin and Giardina, 2014; Veillet et al., 2016). For instance, Arabidopsis *AtSTP4*/*Atβfruct1* and grapevine *VvHT5*/*VvcwINV* are induced in response to biotrophic fungal infection and *AtSTP13*/*AtCWIN1*(*Atβfruct1*) are concomitantly induced by *B. cinerea* (Fotopoulos et al., 2003; Hayes et al., 2010; Veillet et al., 2016, 2017). Study of a loss-of-function mutation of *AtCWIN1* demonstrated that the corresponding protein was responsible for the *Botrytis*-induced apoplastic invertase activity in leaves (Veillet et al., 2016). To date, no information are available on the involvement of sugar transporters and invertases during infection with *E. lata*. Camps et al. (2010) showed that the most abundant genes in leaves that were regulated during the asymptomatic phase of *E. lata* infection were associated with energy metabolism, especially with the light phase of photosynthesis. Such alteration of the photosynthetic system probably contribute to the decline in plant growth and vigor and affect the carbon partitioning and the source/sink relationships.

In the present study, we compared the molecular responses of three *V. vinifera* cultivars after challenge with *E. lata*, the causal agent of eutypa dieback. We analyzed their levels of susceptibility 1 year after artificial inoculation of rooted cuttings with *E. lata* and investigated their specific molecular responses using the millicell *in vitro* infection system. We showed that the relative tolerance of Merlot is supported by the activation of a set of defense-related (PR, oxylipin, and phenylpropanoid) genes deployed by the host. Our study also underlines the probable involvement of the SA pathway and the coordinated role of a cell wall invertase and a hexose transporter in this process. This latter result suggests that active

extracellular sucrolytic machinery and hexose uptake may be advantageous for the host by promoting the availability of sugar resources.

MATERIALS AND METHODS

E. lata Culture

Eutypa lata strain BX1-10 was used to inoculate the plants materials. This strain was retrieved from the vineyard of Bordeaux in 1992 and characterized for its virulence and ability to induce important foliar symptoms on infected grapevine cuttings (Péros and Berger, 1994; Camps et al., 2010). Mycelium was cultured in Potato Dextrose Agar (PDA-Becton-Dickinton) medium at 22°C in the dark. Infections were performed using a 15 days-old culture old *E. lata* (Cardot, 2017).

Plant Materials, Growth Conditions, and Infection

Three grapevine cultivars, Merlot (clone 181), Cabernet Sauvignon (clone 169), and Ugni Blanc (clone 480) were used. Canes were collected in the dormant season and stored at 4°C until use. For re-hydration and disinfection, canes were immersed overnight in water with 2% bleach (2.6% of active chlorine). Canes were cut with a pruning shear to provide cuttings with 2–3 internodes (20 cm long) from which the lower bud was immediately removed. Cuttings were then placed in pot containing sand and installed on heating layers (20°C) to promote rooting for 6 weeks in a greenhouse. They were regularly water-sprayed to maintain a high humidity. After the root formation and the appearance of first three leaves (about 15 cm high), cuttings were transferred in potting soil (Peaty universal substrate n°4, Klasmann–Deilmann GmbH, Germany) and cultivated in a greenhouse thermoregulated with heating and cooling systems to maintain temperature between 19 and 24°C.

Infection of Grapevine Cuttings With *E. lata*

Sixty rooted cuttings (8 week old plants with 3–5 adult leaves) of each cultivar were used for the infection assay using a method previously developed by Chapuis (1995) and described by Camps et al. (2010) and Reis et al. (2019). Forty of these cuttings were infected with *E. lata* mycelium and twenty were inoculated with sterile water as control. Cuttings were drilled to create a 4 mm diameter hole at a 2 cm distance under the node. Infections were carried out by introducing a pellet of *E. lata* mycelium from a 15 days-old culture in PDA medium. The holes were then plugged with paraffin wax. Cuttings were maintained for 2 months in the greenhouse and then transferred in summer under a shelter-house until next spring. Foliar symptoms were assessed after bud break. A gradual scale of five classes has been determined using the following classification, which is similar to previous studies (Péros and Berger, 1994; Darrieutort and Lecomte, 2007; Camps et al., 2010; Cardot, 2017) (**Figures 1A–C**): asymptomatic plant (no typical symptom and normal growth), weak symptoms (sparse necrotic spots on one or few leaves), moderate symptoms (yellowing, spots on several leaves, reduced growth), severe symptoms (chlorosis, crispy leaves and short internodes, several spots on the whole plant) and dead plant or not bud break.

In vitro Interaction Assays Between Foliar Discs From Grapevine Cuttings and *E. lata* Mycelium

Plants of each cultivars growing in greenhouse (University of Poitiers, France) were used to carry out molecular and biochemical analyses using the method described in Veillet et al. (2017) with some modifications.

Eutypa lata mycelium culture was prepared 4 days prior infection as follow: *E. lata* mycelium from 15 days-old culture in PDA medium was harvested from petri plates with 100 μL of sterilized water and mixed with 50 mL of Gamborg G0210 medium (3.16 g L^{-1}, Duchefa biochemistry) containing sucrose (20 g L^{-1}). The mycelium suspension was filtered (0.86 mm-20 mesh) to obtain a homogenous mixture. Three mL of the mycelium/Gamborg medium mixture were placed in 6-well *in vitro* culture plate (Dutscher) and incubated for 4 days at 22°C under tridimensional shaking (50 rpm).

Leaves (3–5 foliar rank) from potted grapevine cultivar cuttings were collected, sterilized in a solution containing 5% NaOCl and 0.1% Tween 20 for 5 min and washed twice with sterilized water. Hydrophilic PTFE (Polytetrafluoroethylene) cell culture inserts (0.4 μm diameter pores, 30 mm diameter, Merck Millipore Ltd.) were placed in wells of 6-well culture plates containing growing mycelium. Three mL of fresh Gamborg/sucrose liquid medium were then added in each well. At time 0, a foliar disc of twenty-millimeter diameter was placed in the apical side of the millicell (treated condition) (**Figure 2A**). In the mock treatment (control condition), mycelium was omitted. Culture plates were incubated at 22°C under a 16 h (light)/8 h (dark) photoperiod with tridimensional shaking (50 rpm) during 3 days and leaf discs were collected, dried and frozen in liquid nitrogen before being grinded using a tissue LyserII (Qiagen).

For benzothiadiazole (BTH) treatments, foliar discs were prepared as described above and incubated for 3 days in presence of BTH at a final concentration of 0.59 mM in 3 mL of Gamborg culture medium into a 6-well culture plate.

Plant RNA Extraction and Reverse Transcription

Total RNA was extracted from frozen ground foliar discs. Leaf powder (100 mg) were mixed with extraction buffer (10 mL g^{-1} FW) containing 2% CTAB, 2% PVPP, 100 mM Tris–HCl pH 8, 25 mM EDTA, 2 M NaCl, 3.44 mM spermidine and 2% β-mercaptoethanol for 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24/1 v/v) was added and the mixture was centrifuged at 14,000 rpm for 10 min at 4°C. This step was repeated once and the

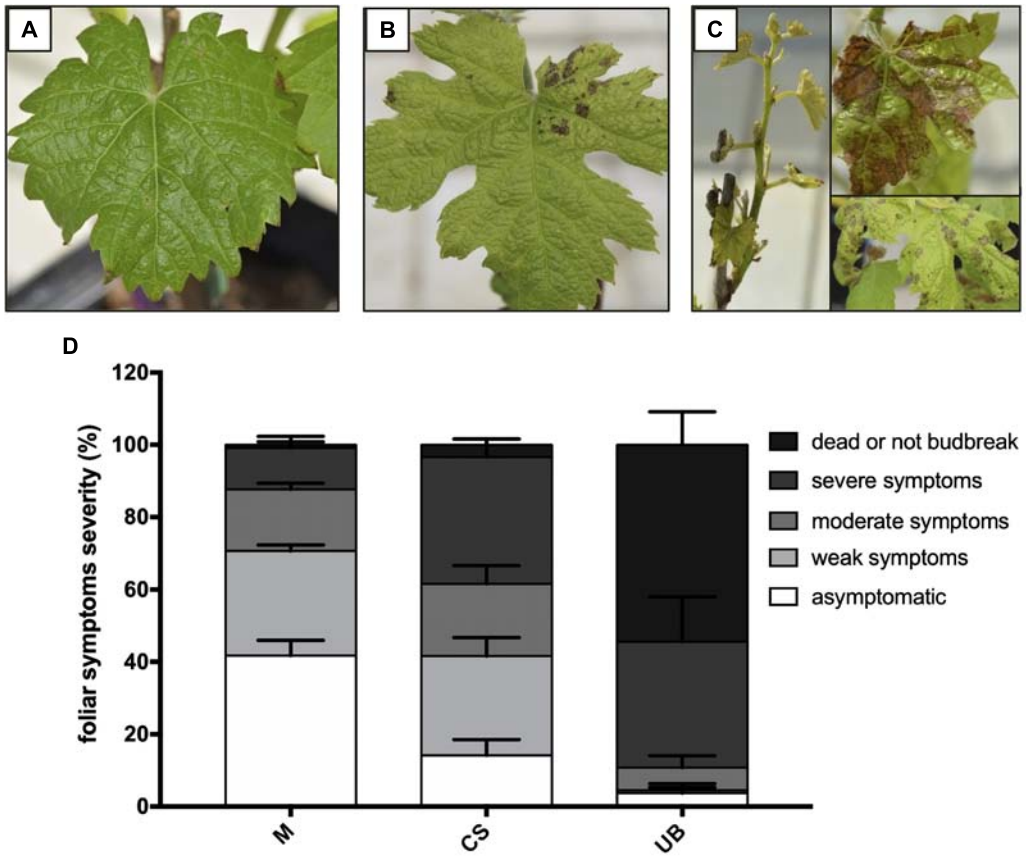

FIGURE 1 | Comparison of foliar symptom severity between three grapevine cultivars. Eutypa dieback symptoms on Merlot (M), Cabernet-Sauvignon (CS), and Ugni Blanc (UB) were recorded on potted cuttings cultivated in greenhouse 1 year post-infection with the *E. lata* strain BX1-10. Five symptoms classes were established: asymptomatic plants (no symptoms and a normal growth), **(A)** weak symptoms (one spot on the whole plant), **(B)** moderate symptoms (some spots on 2–3 leaves), **(C)** severe symptoms (chlorosis, slow growth, several spots on the whole plant) and dead plant (dead plant or not bud break). The analysis was carried out on 120 infected plants (40 per year, during 3 years), and **(D)** percentage of each class was calculated. Pathogenicity tests were conducted over three consecutive years. Data from each year have been considered as biological replicates. Data presented are the mean of the percentage of each class of foliar symptom (n = 3). Error bars represent the standard errors of mean (SEM). Plants inoculated with sterile water have been used to control the absence of cross inoculation during the procedure. Results of the statistical analyses are shown in the **Supplementary Table S1**.

aqueous phase was collected. RNA was precipitated with 0.25 volume of 10 M LiCl overnight at 4°C. RNA was pelleted by centrifugation at 15,000 rpm for 30 min at 4°C and resuspended with a water solution containing 0.5% SDS. Samples were extracted with one volume of phenol/chloroform/isoamyl alcohol (25/24/1 v/v/v) and centrifuged at 14,000 rpm for 10 min at 4°C. After an additional chloroform/isoamyl alcohol (24/1 v/v) extraction, 0.1 volume of sodium acetate 3 M and 2.5 of volume of ethanol (96%) were added to the supernatant and RNA were precipitated for 2 h at −20°C. After centrifugation at 15,000 rpm for 30 min at 4°C, RNA were washed with ice cold 70% ethanol, air dried and resuspended in 30 μL of sterilized water (Chang et al., 1993). RNA samples were quantified by spectrophotometry at 260 nm and their quality and integrity were verified by measuring the 260/280 nm ratio and by migration on agarose gel before DNase treatment (Sigma). For each sample, 2 μg of total RNA were reverse-transcribed in a total volume of 450 μL using M-MLV reverse transcriptase (Promega) following the manufacturer's instruction. The cDNA obtained were stored at −20°C (Cardot, 2017).

Real-Time Quantitative Reverse Transcription-PCR (qRT-PCR) Analysis

Expression levels of 16 genes were analyzed by qRT-PCR. This gene set included 12 defense-related genes and four genes involved in sugar transport or metabolism (**Supplementary Material S2**). Primers were designed using the Primer3 tool (Untergasser et al., 2012) and tested for their specificity and efficiency (>95% for all primers). Real-time quantitative PCR was carried out using the GoTaq qPCR MasterMix (Promega) and a Mastercycler realplex2 instrument (Eppendorf). In this work, four *V. vinifera* reference genes were tested, elongation factor EF1γ and EF1α, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin, described in several previous reports (Reid et al., 2006;

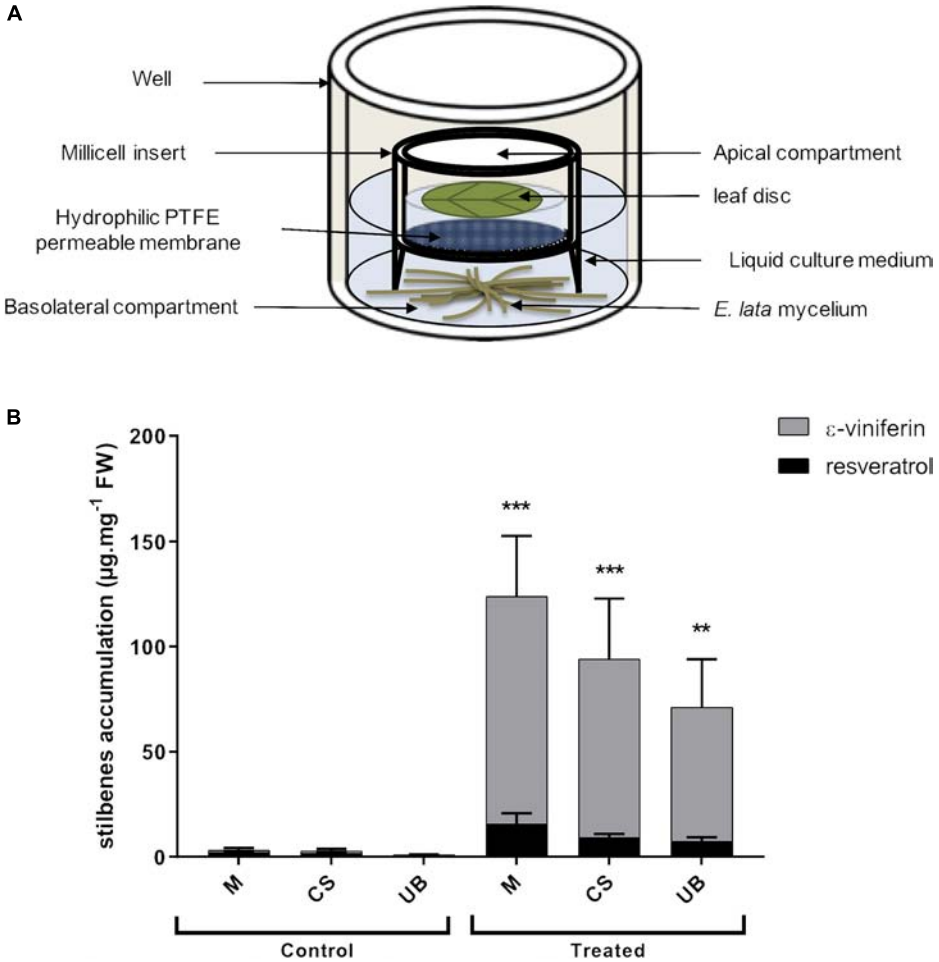

FIGURE 2 | Establishment of the molecular dialogue between *E. lata* and grapevine leaf disc using the millicell infection system **(A)** Molecular communication between leaf disc and fungal mycelium (*E. lata*) is enable through the permeable membrane (0.4 µm pore diameter). Molecules secreted by leaf and fungus can pass through the membrane. This *in vitro* infection system is adapted from Veillet et al. (2017). **(B)** Stilbenes quantification by HPLC analysis in leaf disc and treated or not with *E. lata* using the millicell infection system. Data represent mean (±SEM) of eight independent experiments. Statistical analysis was performed using GraphPad Prism 7, with a Sidak's multiple comparisons test between control and treated condition of ε-viniferin accumulation (**$p < 0.01$, ***$p < 0.0001$). No statistical difference has been determined between treated cultivars according to a Tukey's multiple comparison test used to compare cultivars.

Nicolas et al., 2013; Camps et al., 2014). The stability of all these genes was tested in our biological conditions. GAPDH was finally selected as the best reference gene. Gene expression results were normalized to GAPDH as an internal standard (**Supplementary Material S2**). Results were expressed as relative gene expression using the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008; Cardot, 2017). For all qRT-PCR analyses, the number of independent biological replicates is specified in the legend caption. For each independent experiment, three technical replicates have been performed.

Extraction and Quantification of Phenolic Compounds by HPLC

Stilbenes were extracted with 100% methanol (250 µl per 100 mg FW of foliar discs), and methanolic extract was centrifuged at 20,000 × *g* for 5 min. This extraction was repeated twice. The supernatant was passed through a Sep-Pack® C18 cartridge (Waters) to remove chlorophylls. Resveratrol and ε-viniferin were quantified by HPLC (Perkin Elmer 200 serie) according to a modified protocol of Coutos Thévenot et al. (2001). 180 µl of sample were loaded onto a silica C18 reverse phase column (Thermo Quest Hypersil Division, 5 µM, 250 × 4.6 mm) equilibrated with solvent A containing acetonitrile/TFA/H_2O (1/0.1/99 v/v/v) at a flow rate of 0.8 mL min^{-1}. Molecules are eluted with a step gradient of solvent B containing acetonitrile/TFA/H_2O (90/10/0.1 v/v/v). The gradient is formed according to the following steps: 0–10 min, 35% of solvent B; 10–20 min, 50% of solvent B; 20–30 min, 80% of solvent B. Elution is monitored at 305 nm that corresponds to the optimal absorption wavelength of stilbenic compounds identified from retention time analysis with commercial standards. Resveratrol

and ε-viniferin standards (Sigma) were used to establish the calibration curve as a function of integrated peak area. Results are expressed in μg of resveratrol or ε-viniferin g^{-1} fresh weight (Cardot, 2017).

Extraction and Quantification of Invertase Activities

Invertases were extracted from grapevine leaves and enzymes activities were measured using the method described in Veillet et al. (2016) with some modifications. A total of 220 mg of powder leaves were mixed with 1400 μl of ice-cold extraction buffer (50 mM HEPES, 1 mM EDTA, 5 mM DTT, and 1 mM PMSF). Soluble and insoluble fractions were separated by centrifugation at 20,000 × *g* for 15 min at 4°C. The supernatant contains cytoplasmic and vacuolar invertases, and the pellet contains cell wall invertases. The pellet was washed four times and resuspended in 700 μl of extraction buffer. To measure invertase activity, 50 μL of cell wall pellet suspension or soluble extract were mixed with 400 μl acetate buffer (100 mM) containing sucrose (100 mM v/v (pH 4 for apoplastic activity or pH 5 for vacuolar activity) or HEPES buffer (25 mM) containing sucrose (100 mM) (pH7 for cytoplasmic activity), and incubated 60 min at 30°C. To stop the reaction, 500 μl of DNSA reagent (1% 3.5-dinitrosalicylic acid, 0.5 M KOH and 1 M sodium potassium tartrate) were added and the mixture was placed in a boiling-water bath for 10 min and then cooled to room temperature. Tissue debris were removed by centrifugation at 20,000 × *g* for 5 min. Absorbance was read at 560 nm using a microplate reader (Multiskan GO, Thermo Fisher). Results were normalized to the fresh weight (Cardot, 2017).

Statistical and Computer-Assisted Analysis

Statistical and computer-assisted analyses were performed using the GraphPad Prism version 7.00 (GraphPad Software[1], La Jolla, CA, United States). Pathogenicity tests were conducted over three consecutive years. Results from each year has been considered as biological replicate. Data presented are the mean of the percentage of each class of foliar symptom (n = 3). Plants inoculated with sterile water have been used to control the absence of cross inoculation during the procedure. Because they were not inoculated with *E. lata*, they were not integrated in the statistical comparison of the symptom severity between cultivars. For each class of symptoms, percentages of foliar symptom severity of Merlot, Cabernet Sauvignon, and Ugni Blanc cultivars have been compared using a Sidak's multiple comparisons test.

For expression analyses, stilbene quantification and enzyme activities, comparisons between control and treated conditions have been analyzed using a Sidak's multiple comparisons test. For control or treated conditions, statistical differences between cultivars have been analyzed using a Tukey's multiple comparison test.

[1]www.graphpad.com

RESULTS

The Grapevine Cultivars Merlot, Cabernet Sauvignon, and Ugni Blanc Exhibit Different Levels of Susceptibility to the Fungal Pathogen *E. lata*

With the aim to classify the level of susceptibility of Merlot (M), Cabernet Sauvignon (CS), and Ugni Blanc (UB) cultivars to *E. lata*, we performed artificial inoculations of plants from rooted cuttings and followed eutypa dieback symptoms after 1 year. As seen in **Figure 1** and **Supplementary Table S1**, Merlot, Cabernet Sauvignon, and Ugni Blanc cultivars displayed contrasting disease susceptibility phenotypes, while none of the 60 control plants of each cultivar, that were not inoculated with *E. lata*, showed symptoms of eutypa dieback. In our conditions, the Merlot cultivar was the most tolerant to eutypa dieback, as 38% of inoculated plants were asymptomatic and a majority of symptomatic plants exhibited weak (30%) or moderate symptoms (17%) after 1 year (**Figure 1B**). Among the 120 inoculated plants, only 18 plants were severely affected by the disease and only one plant was dead. In contrast, Ugni Blanc cultivar exhibited a very high level of susceptibility to *E. lata*, as 87% of inoculated plants were dead or displayed severe symptoms (53 and 24%, respectively) after 1 year. Only one plant showed weak symptoms and five plants were asymptomatic. In comparison with Merlot and Ugni Blanc, Cabernet Sauvignon cultivar exhibited an intermediate phenotype (**Supplementary Table S1**). According to the disease scoring, this cultivar can be considered as susceptible, as most of the infected plants displayed symptoms with different degrees of severity and only few inoculated plants were asymptomatic (14%).

In order to correlate the presence of *E. lata* in the cane with foliar symptoms, *E. lata* was re-isolated *in vitro* from cane segments 1 year after the inoculation. Accordingly, the presence of *E. lata* was detected in all symptomatic plants of the three cultivars (data not shown), thus validating our infection procedure.

We were able to show that Merlot, Cabernet Sauvignon, and Ugni Blanc were differentially affected by *E. lata* in controlled conditions. We took advantage of the contrasting susceptibility of these three cultivars to explore their specific molecular responses.

Establishing the Molecular Dialogue Between *E. lata* and Grapevine Leaf Cells Using the Millicell *in vitro* Infection System

Because *E. lata* is a wood pathogen that is not found in leaves, it implies that long distance signals, released by the fungus in the wood, reach the leaves. To study such mechanisms, we developed the millicell system (Veillet et al., 2017), which allows the molecular dialogue between living organisms without physical contact. Here, the millicell insert physically separates growing *E. lata* mycelium (basolateral compartment)

and grapevine leaf disc (apical compartment), but enabled selective flow of molecules secreted by both partners across a membrane (**Figure 2A**).

To verify the molecular dialogue was established between *E. lata* and grapevine leaf discs after 3 days of interaction, we monitored the accumulation of ε-viniferin and resveratrol in all three grapevine cultivars described above. These stilbenes represent major forms of phytoalexins in grape and may be good markers of the fungus recognition by the host (Adrian et al., 1997; Coutos Thévenot et al., 2001; Jeandet et al., 2002, 2013; Mutawila et al., 2017). In untreated leaf discs (control), levels of resveratrol and ε-viniferin were below the detection threshold, indicating that leaf discs were not affected after 3 days in the millicell without *E. lata*. In contrast, the content of ε-viniferin, and to a lesser extent resveratrol, increased strongly upon elicitation by *E. lata* (treated) in all three cultivars (**Figure 2B**). Moreover, we observed that the content of stilbenes tends to decrease with the cultivar susceptibility even though the difference between treated cultivars were not statistically significant (**Figures 1B**, **2B**).

These results suggest that elicited leaf cells perceive the fungus through the release of pathogen or damage-associated molecular patterns (PAMPs or DAMPs) and trigger PAMP/DAMPs-induced responses, such as the accumulation of phytoalexins. Moreover, no symptom was visible in leaf discs after 3 days of interaction with *E. lata* secretome indicating that the millicell is a suitable system to monitor early molecular responses in grapevine elicited leaves.

PAMP/DAMPs-Triggered Responses of Grapevine Cultivars With Differential Susceptibility to *E. Lata*

We took advantage of the millicell system, which imposes strictly controlled conditions of elicitation, to compare general defenses of Merlot, Cabernet Sauvignon, and Ugni Blanc cultivars, previously described as differentially affected by *E. lata* (**Figure 1**). We analyzed the relative expression levels of seven genes encoding PR proteins in leaves, chosen as representative of different defense signaling pathways in grapevine (Jacobs et al., 1999; Robert et al., 2001; Aziz et al., 2003, 2004; Liu and Ekramoddoullah, 2006; Chong et al., 2008; Giacomelli et al., 2012; Spagnolo et al., 2017; Stempien et al., 2017).

In untreated leaves, basal transcript levels of all defense-related genes were low and relatively similar between cultivars (**Figure 3**). After 3 days of elicitation, comparison of expression patterns revealed that defense-related genes were differentially expressed among cultivars (**Figure 3**). Merlot challenged with *E. lata* exhibited a significant induction of 5 out of 7 *PR* genes, which belong to different classes, namely *VvPR2* (β-1,3-glucanase), *VvPR3* (chitinase class I), *VvPR5* (osmotin and thaumatin-like), *VvPR6* (protease inhibitor, PIN), and *VvPR10* (ribonuclease-like). These genes were induced 17, 3, 5, 7, and 10 times, respectively. Moreover, transcript levels were very high for *VvPR10* and relatively less abundant for *VvPR2* and *VvPR6*. In Cabernet Sauvignon, 4 *PR* genes were up-regulated by *E. lata*, i.e., *VvPR5*, *VvPR6*, *VvPR8* (chitinase class III) and *VvPR10*, whereas only *VvPR12* was down-regulated. Strikingly, the expression of all 7 *PR* genes was not significantly modified in the elicited Ugni Blanc compared to the control condition. The most tolerant cultivar, i.e., Merlot, responded to *E. lata* by inducing a large number of *PR* genes which belong to different classes, including proteins with antifungal activities (chitinase and glucanase) (van Loon et al., 2006). On the contrary, Ugni Blanc cultivar shown to be highly susceptible to *E. lata* was unable to activate such defense responses. Lastly, in agreement with its susceptibility phenotype, Cabernet Sauvignon exhibited intermediate responses.

To go further, we analyzed genes of the oxylipin pathway, *VvLOX9* and *VvLOX11*, that encodes lipoxygenases (Podolyan et al., 2010), and genes of the phenylpropanoid pathway, *VvPAL*, encoding the phenylalanine ammonia lyase, and *VvSTS1* encoding the STS responsible for the biosynthesis of resveratrol and stilbenoid compounds. In Merlot, *VvLOX9*, *VvLOX11*, *VvPAL*, and *VvSTS1* were overexpressed in treated leaves suggesting that both oxylipin and phenylpropanoid pathways were activated in this cultivar. Cabernet Sauvignon responded to *E. lata* by a significant increase of *VvLOX9*, *VvLOX11*, and *VvSTS1* transcript levels, but not *VvPAL*. No statistically significant change in expression was detected for these genes, except for *VvSTS1*, in Ugni Blanc upon challenge, which is in line with the absence of *PR* induction in this cultivar. It should be noted that *VvSTS1* is significantly induced in all cultivars, which is consistent with a transcriptional regulation of the stilbene production in infected leaf discs (**Figures 2B**, **4**).

Altogether, our results indicate that *E. lata* is detected by grapevine leaf cells in the millicell system without physical contact, which implies the release of PAMPs, DAMPs or effectors from the fungus. The perception by the host of such molecules leads to the activation of defense signaling pathways, especially in Merlot which exhibited a relative tolerance to eutypa dieback disease. By investigating the molecular responses of cultivars with differential levels of susceptibility to *E. lata*, we showed that the degree of tolerance is supported by the activation of clusters of defense-related genes. Our data reveal that UB was able to perceive the presence of the fungus that is exemplified by the induction of the STS pathway. However, this highly susceptible cultivar failed to trigger efficient defense responses, such as the activation of *PR* gene expression.

Involvement of Sucrose Cleavage and Hexose Transport Processes During the Grapevine/*E. lata* Interaction

Many studies suggest that pathogen infection leads to the establishment of a new sink competing with existing sinks (Lemoine et al., 2013). Cell wall invertases, which cleaves apoplastic sucrose into glucose and fructose, and plasma membrane hexose transporters are key regulators of sink strength by supporting sink demand for sugars and providing resources to cells responding to infections (Tauzin and Giardina, 2014). To gain insight into the putative role of the carbon partitioning in *E. lata* infected grapevine, we monitored the expression of *VvcwINV* and *VvHT5*, previously described as being involved in grapevine pathogenic responses, as well as

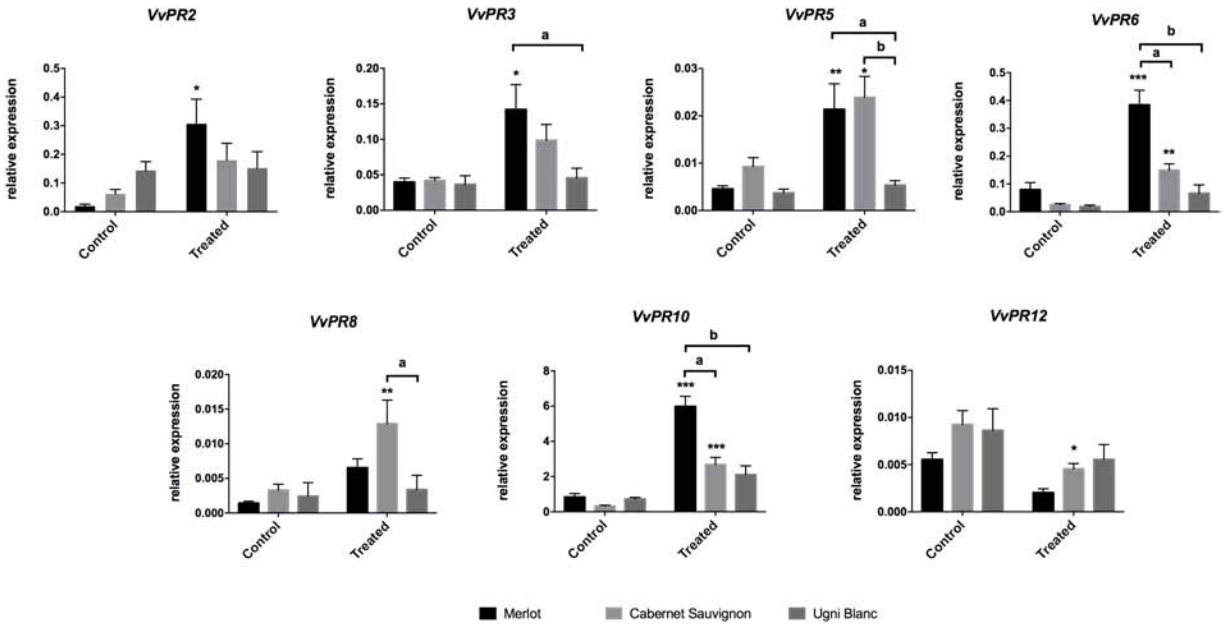

FIGURE 3 | PR-protein gene expression in response to *E. lata* infection in Merlot, Cabernet Sauvignon, and Ugni Blanc cultivars. Relative expression of *VvPR2* (β 1–3 glucanase), *VvPR3* (chitinase class I), *VvPR5* (thaumatin-like), *VvPR6* (protease inhibitor), *VvPR8* (chitinase class III), *VvPR10* (ribonuclease-like), and *VvPR12* (defensin-like) genes. Relative gene expressions were analyzed for the Merlot, Cabernet Sauvignon, and Ugni Blanc leaf discs in control and treated (elicited) conditions using the Millicell system. Data represent mean (±SEM) of six independent experiments. Statistical analysis was performed using GraphPad Prism 7. Stars represents significant difference between control and treated condition for each cultivar, with a Sidak's multiple comparisons test (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$). Letters represent results of a Tukey's multiple comparison test used to compare cultivars.

sucrose degrading activities in leaves (Hayes et al., 2010). As seen in **Figure 5**, the cell wall invertase *VvcwINV* and the hexose transporter *VvHT5* exhibited similar expression patterns. These genes were significantly induced in Merlot (fivefold) and Cabernet Sauvignon (fourfold and threefold, respectively) leaf discs challenged with *E. lata*. In contrast, transcript levels of both genes were not significantly different in treated Ugni Blanc compared to corresponding controls.

In addition to the study of transcriptional responses, insoluble fractions of protein extracts were assayed for cell wall invertase activities to explore the impact of *E. lata* infection on carbon fluxes in all three grapevine cultivars. Elicitation of Merlot leaves led to a significant stimulation of the cell wall invertase activity that was increased by 176% after 3 days, whereas in susceptible cultivars (CS and UB), cell wall invertase activities were not changed (**Figure 5C**). We noticed that transcript accumulation of *VvcwINV* did not fully correlate with enzymatic activity. This result is in accordance with the probable involvement of post-translational regulation of cell wall invertases as suggested by the work of Veillet et al. (2016).

Because intracellular sucrolytic activities, i.e., cytosolic and vacuolar invertases, are important in regulating the intracellular sugar homeostasis (Roitsch and Gonzalez, 2004), we monitored the transcript accumulation of genes encoding cytosolic invertase (*VvCIN2*) and vacuolar invertase (*VvGIN2*) and the related cytoplasmic and vacuolar invertase activities in elicited leaves. Results indicates that intracellular invertases were functional in all cultivars but their sucrolytic activities were relatively less important than cell wall invertase (**Supplementary Material S1** and **Figure 5C**). Ugni Blanc showed a very high vacuolar invertase activity, which may be due to its productive and vigorous metabolism. As shown in **Supplementary Material S1**, neither the level of transcript of *VvCIN2* and *VvGIN2*, nor the cytoplasmic and vacuolar invertase activities were modified upon elicitation.

Altogether, our results evidenced a coordinated transcriptional up-regulation of *VvcwINV* and *VvHT5* genes, accompanied by a stimulation of the cell wall invertase activity in leaves of merlot challenged by *E. lata*. It suggests that the enhanced extracellular sucrolytic machinery and hexose uptake may participate to the transition from source to sink upon infection.

BTH-Responsive Expression of Genes in Tolerant and Highly Susceptible Grapevine Cultivars

Many studies have reported that defense potentiators like β-aminobutyric acid (BABA), SA, methyljasmonate, laminarine sulfate or BTH, are effective against a broad spectrum of grapevine pathogens, such as *P. viticola* (downy mildew), *E. necator* (powdery mildew) or *B. cinerea* (gray mold) by strengthening plant defenses *via* the accumulation of PR proteins, phytoalexins, or phenolic compounds (Adrian et al., 1997; Jacobs et al., 1999; Aziz et al., 2003; Hamiduzzaman et al., 2005; Belhadj et al., 2008; Trouvelot et al., 2008; Dufour et al., 2013). BTH, an

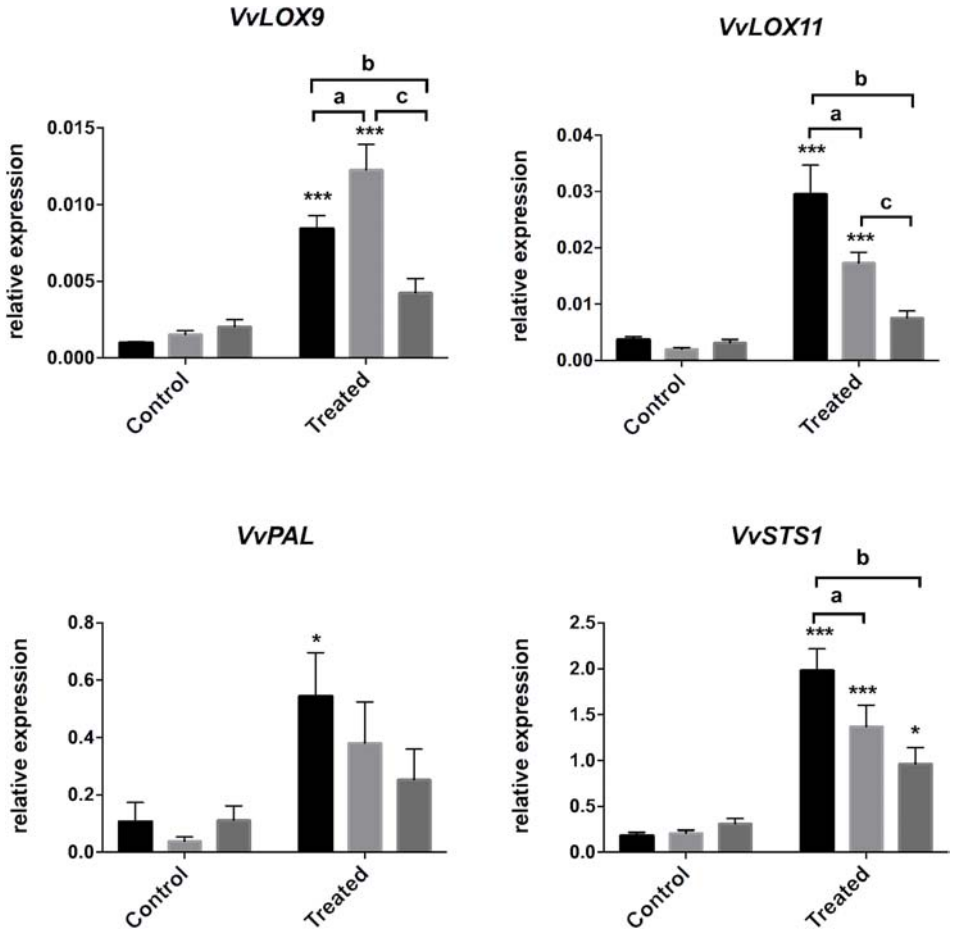

FIGURE 4 | Gene expression in response to *E. lata* infection in Merlot, Cabernet Sauvignon, and Ugni Blanc cultivars. Relative expression of genes of lipoxygenases 9 and 11 (*VvLOX9* and *VvLOX11*), phenyl alanine ammonia lyase (*VvPAL*), and stilbene synthase (*VvSTS1*). Relative gene expressions were analyzed in Merlot, Cabernet Sauvignon, and Ugni Blanc leaf discs in control and treated (elicited) conditions using the Millicell system. Data represent mean (±SEM) of six independent experiments. Statistical analysis was performed using GraphPad Prism 7. Stars represents significant difference between control and treated condition for each cultivar, with a Sidak's multiple comparisons test (*$p < 0.05$, ***$p < 0.0001$. $N = 6$, excepted for *VvSTS1*: $N = 10$). Letters represent results of a Tukey's multiple comparison test used to compare cultivars.

analog of SA, is a systemic acquired resistance (SAR) inducer that mimics the natural signaling activity of SA with no direct antifungal activity (Dufour et al., 2013; Bektas and Eulgem, 2014; Jiao et al., 2016). BTH treatment of Cabernet Sauvignon triggered the upregulation of several *PR* genes, *VvPAL*, *VvSTS1*, and *VvLOX9*, indicating that these genes were SA-inducible (Dufour et al., 2013). Because these defense-related genes were also induced by *E. lata*, we investigated whether the SA-inducibility of these genes were effective in leaf discs of Merlot and Ugni Blanc cultivars, described as tolerant and highly susceptible cultivars. In accordance, *VvSTS1*, *VvPAL*, *VvLOX9*, but also *VvPR2*, *VvPR8*, *VvPR10*, and *VvLOX11* were differentially expressed after BTH treatment in Merlot (**Figure 6**). Interestingly, BTH failed to induce all these genes in the leaves of Ugni Blanc. To go further, we monitored transcript levels of *VvcwINV* and *VvHT5* (**Figure 6**). Both genes followed the same expression patterns and were also differentially upregulated in Merlot (7 and 11 fold, respectively) but were not responsive to BTH in Ugni Blanc. *VvPR12*, encoding a defensin-like protein classically dependent on JA signaling (Penninckx et al., 1996; Manners et al., 1998; Giacomelli et al., 2012) displayed a significant induction by BTH treatment in UB but not in Merlot.

We showed that several defense- and sugar-related genes are induced by *E. lata* and are also responsive to SA analog in the tolerant Merlot, but not in Ugni Blanc. This result indicates that some components of the SA signaling, that are effective in Merlot, are deficient in the highly susceptible cultivar Ugni Blanc.

DISCUSSION

With the aim to explore biological processes involved in the mechanism of tolerance/susceptibility of *V. vinifera* against the wood decay fungus *E. lata*, we chose to undertake an analysis of molecular responses of three grapevine cultivars. To that end, grapevine cultivars with clear contrasting susceptibility to

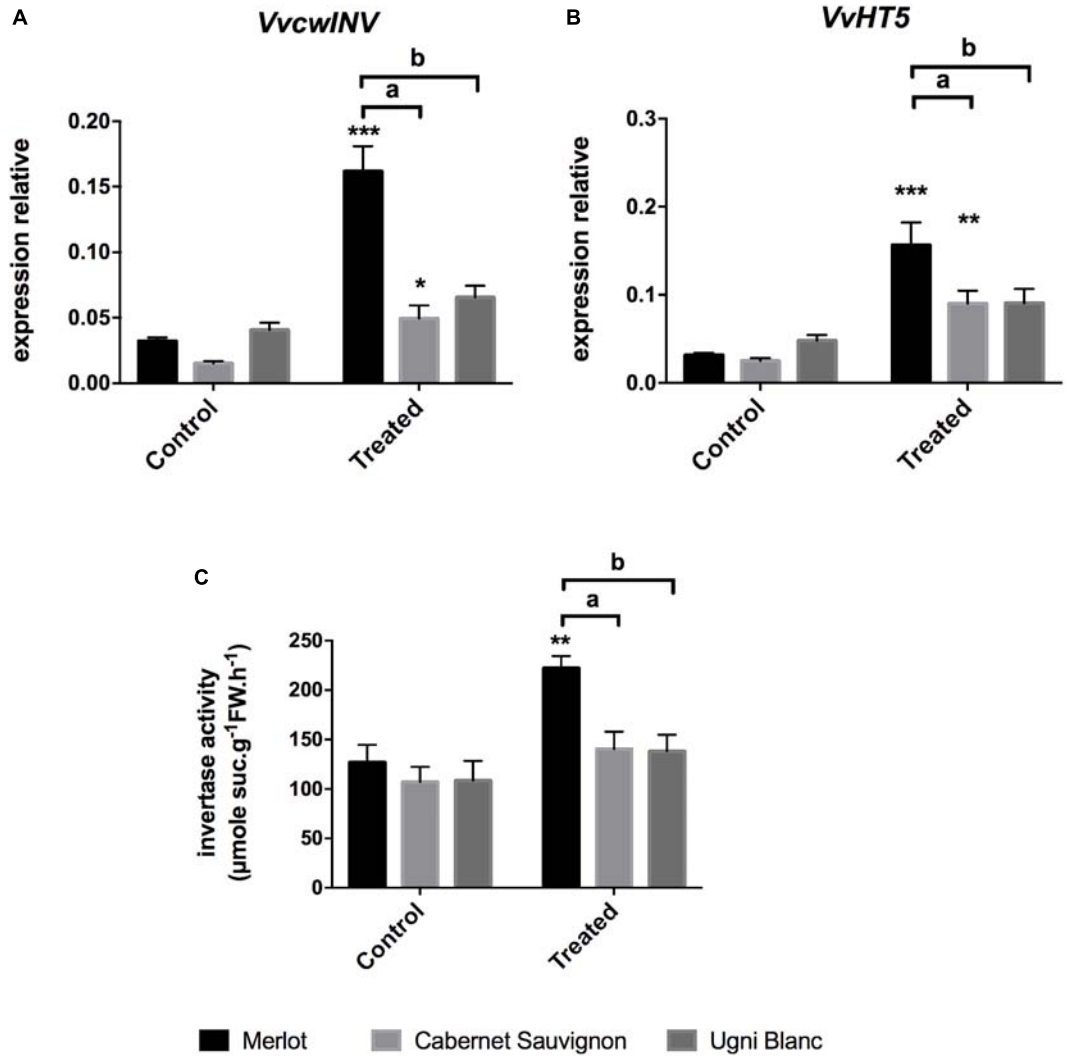

FIGURE 5 | Sugar cleavage and transport-related gene expression, and cell wall invertase activity in response to *E. lata* infection in Merlot, Cabernet Sauvignon, and Ugni Blanc cultivars. **(A)** Cell wall Invertase (*VvcwINV*) and **(B)** hexose transporter (*VvHT5*, relative gene expression were analyzed by RT-qPCR in Merlot, Cabernet Sauvignon, and Ugni Blanc leaf discs in control and treated (elicited) conditions using the Millicell system. **(C)** Cell wall invertase activity was studied in control and treated foliar discs using the Millicell system. Data represent mean (±SEM) of six independent experiments for the invertase activity analysis and 10 independent experiments for the gene expression analysis. Statistical analysis was performed using GraphPad Prism 7.00. Stars represents significant difference between control and treated condition for each cultivar, with a Sidak's multiple comparisons test (**$p < 0.001$, ***$p < 0.0001$). Letters represent results of a Tukey's multiple comparison test used to compare cultivars.

eutypa dieback were required. Available data on the susceptibility of *V. vinifera* cultivars to *E. lata* dieback are based on field observations by examination of foliar and/or wood symptoms and on artificial inoculations. Accordingly, cultivars have been ranked from tolerant to highly susceptible (Dubos, 1987, 2002; Péros and Berger, 1994; Chapuis et al., 1998; Fussler et al., 2008; Bertsch et al., 2013; Bruez et al., 2013). Such susceptibility scales have to be analyzed with caution, because susceptibility was designated through the leaf symptom intensity rather than the proportion of tissues killed by the fungus. However, besides varietal susceptibility, the degree of susceptibility of some cultivars can vary across studies because many other factors influence the disease development (region, climate, soil, grapevine age, strain genetic variations, presence of a other trunk pathogens. . .) (Munkvold, 1995; Péros, 1995; Péros et al., 1997; Chapuis et al., 1998; Sosnowski et al., 2007; Grosman and Doublet, 2012; Travadon et al., 2013; Lecomte and Bailey, 2015). For this reason, we have initiated a phenotypic analysis using rooted cuttings infections of three grapevine cultivars, Merlot, Cabernet Sauvignon, and Ugni Blanc, cultivated in semi-controlled conditions. These cultivars were chosen because they are representative of French regions of Bordeaux and Charentes. We found that Merlot, Cabernet Sauvignon, and Ugni Blanc exhibited clear contrasting phenotypes, with Merlot being relatively tolerant compared to Ugni Blanc, which was highly susceptible. Overall, these results are in accordance with the

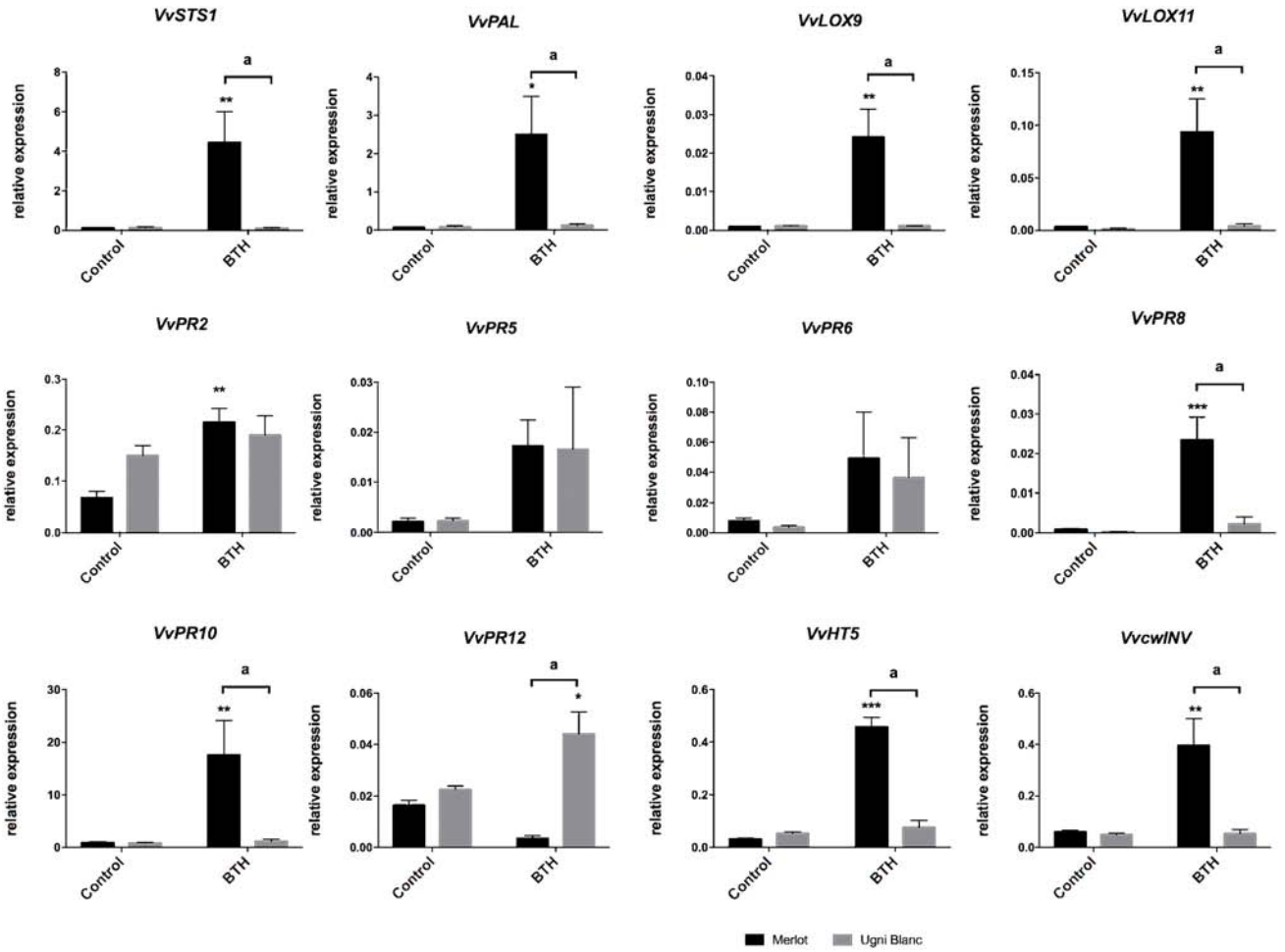

FIGURE 6 | Sugar metabolism and defense gene expression in response to treatment with the SA analog BTH in Merlot and Ugni Blanc cultivars. Stilbene synthase (*VvSTS1)*, phenylalanine ammonia lyase (*VvPAL*), lipoxygenase 9 (*VvLOX9*), lipoxygenase 11 (*VvLOX11*), *VvPR2* (β 1–3 glucanase), *VvPR5* (thaumatin-like), *VvPR6* (protease inhibitor), *VvPR8* (chitinase class III), *VvPR10* (ribonuclease-like), *VvPR12* (defensin-like) genes, hexose transporter (*VvHT5*), and cell wall Invertase (*VvcwINV*) relative gene expressions were analyzed by RT-qPCR in Merlot and Ugni Blanc leaf discs in control and BTH-treated conditions using the Millicell system. Data represent mean (±SEM) of three independent experiments. Statistical analysis was performed using GraphPad Prism 7.00. Asterisk represent significant difference between control and elicited condition for each cultivar, with a Sidak's multiple comparisons test (*$p < 0.05$, **$p < 0.05$, ***$p < 0.0001$). Letters represent results of a Tukey's multiple comparison test used to compare cultivars.

degree of susceptibility reported in previous studies. However, Cabernet Sauvignon was susceptible with an intermediate response, whereas it was rather classified as highly susceptible in previous reports (Péros and Berger, 1994; Péros, 1995; Travadon et al., 2013). The contrasting degrees of susceptibility of these grapevine cultivars to eutypa dieback represents a good opportunity to investigate their specific molecular responses in order to better understand underlying mechanisms governing the tolerance or susceptibility to this wood pathogen.

Early molecular responses to *E. lata* are difficult to study because experimental models (*in vitro*, greenhouse, or vineyard) are confronted with several technical issues. For example, infection process and environment are not controlled in vineyard and symptoms appear approximately after 1 year in artificially infected rooted cuttings. *In vitro* plants do not differentiate much woody tissue. Moreover, RNA isolation in woody tissues is hazardous. Cell suspension culture is a reproducible model system but responses of a single cell type-growing culture may be different from those of heterogenous tissues, such as leaf or stem tissues. However, previous reports, including such experimental models, brought valuable information on the transcriptomic responses of *V. vinifera* cv Cabernet Sauvignon during symptom expression and on the defense-related responses of *V. vinifera* cv Dauphine cell suspension to *E. lata* culture filtrate (Camps et al., 2010; Mutawila et al., 2017). Lastly, in wood decay diseases, the fungus is confined in the wood and is never found in leaves or herbaceous shoots, where symptoms are typically observed. This implies that interactions between the fungus and leaf cells occur through the systemic action of fungal secreted molecules or effectors. Consequently, we adapted the infection system described by Veillet et al. (2017), which enables a molecular dialogue between fungus and host cells

across a semi-permeable membrane without physical contact, to study early foliar responses to *E. lata* secretome. In a previous work, we successfully used the millicell system to unravel the molecular and biochemical responses during *A. thaliana* and *B. cinerea* interaction. The so-called millicell system provides highly controlled conditions and reproducible results enabling the comparison of the grapevine cultivars previously described as differentially affected by *E. lata*. In order to prove that leaf discs perceive signals from *E. lata*, we monitored the accumulation of stilbenic compounds, ε-viniferin and resveratrol, which is a classical PAMP-induced response in grapevine that plays key roles in plant resistance to a wide range of pathogens (Langcake, 1981; Adrian et al., 1997; Amalfitano et al., 2000; Coutos Thévenot et al., 2001; Belhadj et al., 2008; Chang et al., 2011). In numbers of reports investigating grapevine responses to various pathogens, stilbene contents are often correlated with disease resistance (Chong et al., 2009). Here, a strong induction of both ε-viniferin and resveratrol contents has been observed upon *E. lata* challenge. It indicates that all three cultivars were able to perceive the fungus, thus validating the millicell infection system using grapevine leaf discs. Interestingly, the expression of *VvSTS1*, which encodes the first enzyme of the stilbenoid pathway, was positively correlated with the level of tolerance, whereas the accumulation of the related stilbenes was not different among cultivars. Previous reports showed that an induction of *VvSTS1* gene expression and phenolic compound accumulation in grapevine cell culture treated with *E. lata* culture filtrate (Mutawila et al., 2017). Our results further suggest that stilbenes contribute to general defense to pathogens but do not have a crucial role in the tolerance of cultivars to *E lata*.

The successful use of the millicell infection system in studying the dialogue between grapevine foliar discs and *E. lata* mycelium open new perspectives. This method emphasizes the importance of fungal elicitors or effectors produced by the fungus. To our knowledge, information of molecules with such activity is scarce, whereas the secretome, proteome and toxins produced by *E. lata* and other grapevine trunk fungi have been extensively studied (Mahoney et al., 2003; Andolfi et al., 2011; Morales-Cruz et al., 2015; Masi et al., 2018). As an example, *E. lata* produces secondary metabolites, mainly acetylic and heterocyclic compounds. Toxins, such as eutypine, was determined as the main phytotoxins secreted by the fungus but the phytotoxicity of *E. lata* is probably caused by many structurally related compounds, which are responsible for leaf symptom development but have no eliciting activity (Bertsch et al., 2013). Because the millicell infection system allows the free diffusion of secreted molecules produced by both partners across a semi-permeable membrane, it opens up the possibilities to identify fungal elicitors or effectors, that are largely unknown so far, and to study temporal induced responses from either the host or the fungus. Many artificial infection methods have been developed to address questions and issues about esca disease, black dead arm and other wood decay diseases (Reis et al., 2019). The millicell system would be particularly well adapted to the identification of fungal effectors in such complex pathosystems.

Camps et al. (2010) and Mutawila et al. (2017) gave an overview of the expression patterns of genes related to defense (PR-proteins) and phenylpropanoid pathway in Cabernet Sauvignon and Dauphine cultivars, respectively, showing a differential induction of several *PR*-encoding genes, such as osmotin (*VvPR5*), β-1,3-glucanase (*VvPR2*), chitinases (*VvPR3*, *VvPR4*), and *VvPAL*. In the present study, we expanded this analysis to Merlot, Cabernet Sauvignon, and Ugni Blanc cultivars and compared the expression of different classes of *PR* and *VvPAL* genes using the millicell system. Strikingly, Merlot, which was relatively tolerant, responded to *E. lata* by inducing *VvPAL* expression and a large number of *PR* genes, belonging to different classes. In contrast, none of these defense-related genes were up-regulated upon challenge with *E. lata* in the highly susceptible cultivar Ugni Blanc. Cabernet Sauvignon exhibited intermediate responses in accordance with its moderate degree of susceptibility. Genes encoding PR proteins that were up-regulated in Merlot are described to have direct antifungal activities, such as osmotin, ribonuclease-like protein, or act through hydrolytic activities on pathogen cell wall (β-1,3-glucanase or chitinases), and represent important PR families classically induced in response to fungi (van Loon et al., 2006; Chong et al., 2008). Therefore, it may be hypothesized that the difference in susceptibly between the three cultivars could be explained, at least partly, by the differential expression of genes involved in antifungal defense and the activation of the phenylpropanoid pathway.

In *A. thaliana*, roles of SA, JA, and ET in regulating the pathogen-induced transcriptional reprograming are well known (Glazebrook, 2005). The resistance to biotrophic pathogens and the SAR is dependent on the SA pathway, which controls the expression of *PR1*, *PR2* (β-1,3-glucanase) and *PR5* (thaumatin and osmotin-like) genes. However, a functional JA- and/or ET-dependent pathway is required for the expression of the defensin *PDF1.2*, *PR3*, and *PR4* (chitinases) genes and the resistance to necrotrophic pathogens. In grapevine, while the induction of defense-related gene has been described in number of studies, the signaling pathways and the regulatory components controlling defense-related gene expression and pathogen resistance are not completely deciphered (Kortekamp, 2006; Chong et al., 2008; Dufour et al., 2013). In response to *E. lata*, Merlot, and Cabernet Sauvignon show an induction of lipoxygenase genes, *VvLOX9* and *VvLOX11* coding for enzymes of the biosynthesis of JA, and *VvPAL* gene, that encodes the first enzyme of the phenylpropanoid pathway involved in the biosynthesis production of SA. It indicates that both JA and SA signaling pathways may be activated in these cultivars. In grape, no marker genes of SA and JA/ET pathway have been clearly defined. However, parallel exists between *PR* gene expression in grape and *A. thaliana* homologs. Consequently, the up-regulation of class I chitinase (*VvPR3*), defensin (*VvPR12*), protease inhibitor (*VvPR6*), and 9-lipoxygenase (*VvLOX9*) on one side and the up-regulation of β-1,3-glucanase (*VvPR2*), osmotin and thaumatin-like (*VvPR5*), and class III chitinase (*VvPR8*) on the other side, suggests a much intense activation of JA and SA pathways in Merlot than in Cabernet Sauvignon, which probably promote the tolerance to *E. lata* (van Loon et al., 2006; Chong et al., 2008; Dufour et al., 2013; Mutawila et al., 2017). The induction of chitinase and glucanase genes in tolerant Merlot may result

in the hydrolysis of fungal cell wall components and the release of β-1,3 glucans and chitin fragments. These oligosaccharides are effective elicitors, which amplify plant defense responses, protecting susceptible grapevine cultivar against several diseases, such as downy mildew (*P. viticola*) and gray mold (*B. cinerea*) (Aziz et al., 2003; Trouvelot et al., 2008). According to our results, Ugni Blanc failed to trigger such defense responses, which is in correlation with its high susceptibility. It should be interesting to monitor the temporal accumulation of PR transcripts as well as stilbenic compounds to determine whether the kinetic of these responses is important (Lambert et al., 2013). It is difficult to presume that Ugni Blanc do not produce basal levels of JA or SA. Future works including the measurement of both hormones would help to have a better comprehension of the involvement of these pathways in the tolerance/susceptibility to *E. lata*. To go further with the regulation of molecular responses of the three cultivars, we used BTH, an analog of SA, known to be an inducer of SAR and to strengthen plant defense responses to several pathogens (Friedrich et al., 1996; Dufour et al., 2013). Our data indicate that all BTH-induced genes in Merlot are not responsive to this molecule in Ugni Blanc. These results further support the hypothesis that components of the SA signaling or perception are compromised in Ugni Blanc.

Recent reports have pointed out the importance of the plant carbon partitioning in various plant pathogen-interaction systems (Naseem et al., 2017). We investigated the involvement of sugar transport and cleavage processes in grapevine cultivars challenged with *E. lata*. We monitored the expression of three types of invertase, such as *VvcwINV*, which encodes for the only annotated cell wall invertase in grape genome, and genes encoding cytosolic invertase (*VvCIN2*) and vacuolar invertase (*VvGIN2*) (Hayes et al., 2010; Barnes and Anderson, 2018). We also studied the transcript accumulation of *VvHT5*, which encodes a high affinity hexose transporter of the plasma membrane (Hayes et al., 2007, 2010). There are six expressed *VvHTs* in grape displaying various expression patterns depending on specific organs and environmental conditions (Afoufa-Bastien et al., 2010; Lecourieux et al., 2013). Accordingly, we selected VvHT5 as candidate because it was the closest homolog of AtSTP13 (82% of sequence similarity). These homologs are both inducible by several pathogens and are coordinately expressed during pathogenesis with cell wall invertases *VvcwINV* and *AtCWIN1*, respectively (Hayes et al., 2010; Lemonnier et al., 2014; Veillet et al., 2016, 2017; Yamada et al., 2016). We showed that expression of cytosolic and vacuolar invertase genes, as well as their related activities, did not show relevant modification among cultivars. Transcript levels of *VvHT5* and *VvcwINV* under *E. lata* challenge were clearly correlated with the degree of susceptibility of all three cultivars. Accordingly, recent studies have reported that the hexose transporter of the plasma membrane AtSTP13 and the cell wall invertase AtCWIN1 participate to promote resistance to extracellular pathogens by retrieving apoplastic sugars at the plant/pathogen interface (Lemonnier et al., 2014; Yamada et al., 2016). We went further and analyzed the cell wall activities in *E. Lata*-elicited leaf disc extracts. The apoplastic sucrose-degrading activity was increased in Merlot only. Considering the role of cell wall invertase in regulating the sink strength (Ruan et al., 2010; Lemoine et al., 2013; Proels and Huckelhoven, 2014; Tauzin and Giardina, 2014; Veillet et al., 2016), we hypothesized that enhanced extracellular sucrolytic machinery and hexose uptake may participate to the transition from source to sink upon infection. During infection processes, *E. lata* is restricted to the trunk and may represent an additional sink and modify source/sink relationship through the plant. Capacity of the plant to face with the reduction of source activity (decrease of photosynthesis) and the probable re-routing of photoassimilates is a crucial issue for the outcome of the grapevine/*E. lata* interaction, particularly for the transition from the asymptomatic to the symptomatic phase. By regulating the partitioning of carbon, the expression/activity of cell wall invertases and sugar transporters may play important roles in this process. In Merlot, which is a relatively tolerant cultivar, such modification of the source/sink relationship and sugar fluxes throughout the host plant may affect the availability of sugar resources for either host and the fungus and impact positively, as energy and/or signaling molecule (Berger et al., 2007; Ruan, 2014), the outcome of the interaction toward the host. These hypotheses could be explored by monitoring carbon fluxes throughout the infected plants using radiolabeled sugars. Another challenge would be to decipher the specific roles of the recently discovered sugar transporters of the SWEET (Sugar Will be Eventually Exported Transporters) family. Members of this family are described as sugar efflux facilitators. Because they are targeted by pathogen effectors, they may contribute to sugar leakage toward the apoplast and may promote pathogen growth (Chen et al., 2010; Chandran, 2015).

In the present study, we analyzed the molecular responses of three *V. vinifera* cultivars after challenge with *E. lata* using the millicell infection system, which is a rapid, efficient and highly reproducible method. This work provides new insights into the mechanisms of tolerance/susceptibility of grapevine cultivars. Our results notably highlight the importance of the intensity of the induced-responses deployed by the host and the emerging role of sugar transport and cleavage in disease tolerance. In the future, this work will be extended to a genome wide gene expression analysis including the comparison of a larger number of cultivars and several fungal strains with different levels of virulence. Altogether, this work should identify a more comprehensive set of target genes and open new perspectives to improve the tolerance of *V. vinifera* to the eutypa dieback, and more largely to wood decay diseases, which cause severe economic losses in vineyards worldwide.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

PC-T, GF, SLC, CC, and GM conceived and designed the experiments. CC, GM, CG, and PL carried out the experiments.

SLC, PC-T, CC, GM, and CV analyzed the experiments. CC, CG, GM, CV, and PL contributed the materials and analysis tools. SLC, CC, PC-T, and CV wrote the manuscript. GF and PC-T acquired funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00991/full#supplementary-material

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Insight Into the Microbial Co-occurrence and Diversity of 73 Grapevine (*Vitis vinifera*) Crown Galls Collected Across the Northern Hemisphere

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Crown gall (CG) is a globally distributed and economically important disease of grapevine and other important crop plants. The causal agent of CG is *Agrobacterium* or *Allorhizobium* strains that harbor a tumor-inducing plasmid (pTi). The microbial community within the CG tumor has not been widely elucidated and it is not known if certain members of this microbial community promote or inhibit CG. This study investigated the microbiotas of grapevine CG tumor tissues from seven infected vineyards located in Hungary, Japan, Tunisia, and the United States. Heavy co-amplification of grapevine chloroplast and mitochondrial ribosomal RNA genes was observed with the widely used Illumina V3–V4 16S rRNA gene primers, requiring the design of a new reverse primer to enrich for bacterial 16S rRNA from CG tumors. The operational taxonomic unit (OTU) clustering approach is not suitable for CG microbiota analysis as it collapsed several ecologically distinct *Agrobacterium* species into a single OTU due to low interspecies genetic divergence. The CG microbial community assemblages were significantly different across sampling sites (ANOSIM global $R = 0.63$, p-value = 0.001) with evidence of site-specific differentially abundant ASVs. The presence of *Allorhizobium vitis* in the CG microbiota is almost always accompanied by *Xanthomonas* and *Novosphingobium,* the latter may promote the spread of pTi plasmid by way of acyl-homoserine lactone signal production, whereas the former may take advantage of the presence of substrates associated with plant cell wall growth and repair. The technical and biological insights gained from this study will contribute to the understanding of complex interaction between the grapevine and its microbial community and may facilitate better management of CG disease in the future.

Keywords: *Agrobacterium*, *Allorhizobium vitis*, amplicon sequence variants, crown gall disease, grape, microbiota, opine, vineyard

INTRODUCTION

Plant-associated microbial communities are complex and diverse. As with most microbial communities, there is a limited understanding of the factors and mechanisms that establish and stabilize plant-associated microbiotas. It is unclear how specific populations of microorganisms are established and maintained and what promotes the appropriate balance of different microbes (Ramey et al., 2004). It is widely accepted that greater than 99% of the microbes present in many environments are not readily culturable or *not-yet-cultured* and therefore not easily accessible for basic and applied research (Bidle et al., 2007). The species diversity in many unique environments has never been described. To more fully understand novel environmental niches, several DNA-based methods have been developed including 16S rRNA gene analyses and metagenomics (Whitman et al., 1998; Williamson et al., 2005). The former provides information about taxa present in an environmental sample while the latter offers insight into the functional roles of different microbes within a community (Handelsman, 2004; Riesenfeld et al., 2004).

The grapevine-associated microbiota has been a subject of several studies due to the importance of grape cultivation for the production of wine, fresh grapes, raisins, jelly, juice, jam and grape seed extracts, and oil. The tissue saps of grapevines are rich in nutrients that include organic acids, amino acids, sugars, and several inorganic compounds with a pH 5.7–6.9 (Roubelakis-Angelakis and Kliewer, 1979; Andersen et al., 1989; Glad et al., 1992; Prima-Putra and Botton, 1998). This nutrient-rich tissue environment supports the growth of several bacteria resulting in an epiphytic and endophytic population with several species including important pathogens such as *Agrobacterium* spp. and *Allorhizobium vitis* (*A. vitis*) (Bell et al., 1995; Szegedi and Bottka, 2002; Bulgari et al., 2009; Compant et al., 2011). *Agrobacterium* spp. or *A. vitis* often causes galls to develop at the crown of the vine; hence, the name crown gall (CG), but can also induce galls on the perennial stems where wounds are inflicted as a result of grafting or injury by freezing temperatures or farm implements (Burr and Otten, 1999). Interestingly, the natural occurrence of CG on young green grapevine shoots has not been documented. CG tumors first appear in early summer as soft masses of disorganized cells which are creamy white or light green in color. In autumn, they become dry and wood-like and turn brown to black, hence the origin of the alternative name "black knot of grapevine" (Süle and Burr, 1998). Bark cracking and peeling may be associated with gall development. Profuse gall development may cause girdling of the trunk which prevents the exchange of nutrients between root and shoot systems thus leading to the reduced vigor of CG-affected vines.

The causal agent of CG tumor disease is commonly referred to as *Agrobacterium vitis* which was recently reclassified to the genus *Allorhizobium* based on whole genome phylogeny (Mousavi et al., 2014; Gan et al., 2018). Although less common, some strains of tumor-inducing (Ti) plasmid-harboring *Agrobacterium tumefaciens* can also cause CG (Pu and Goodman, 1992; Abdellatif et al., 2013). The Ti plasmid (pTi) encodes genes for the processing, transfer, and stable insertion of the transfer-DNA (T-DNA) from pTi to the plant nuclear genome. The constitutive expression of T-DNA oncogenes in the plant genome results in the overproduction of plant hormones cytokinin and auxin which causes the unregulated proliferation of undifferentiated plant cells which manifest themselves as the tumorous outgrowth of CG. Additional T-DNA-encoded genes produce enzymes that synthesize novel low molecular weight class of compounds known as opines (Bevan and Chilton, 1982; Chilton et al., 1982; Zambryski et al., 1989). The specific opine-type produced in the tumor is characteristic of the virulent *Agrobacterium*/*Allorhizobium* strain. Common opines found in CG include octopine, nopaline, and vitopine. These metabolites produced by the transformed plant tumor cells are almost exclusively metabolized as an energy source by the virulent agrobacteria that have induced the CG (Dessaux and Faure, 2018; Kuzmanović et al., 2018). A physiologically active CG expands the assortment of nutrients utilizable by bacteria and fosters a rich niche for plant-associated bacteria to colonize, grow, and form complex ecological interactions (Barton et al., 2018; Dessaux and Faure, 2018). In one study, 138 culturable bacterial colonies were isolated representing distinct morphological groups from eight grapevine CG tumors that produced octopine, nopaline, or vitopine (E. Szegedi, unpublished data). All isolates were non-fluorescent on King's B medium indicating that none of them were fluorescent *Pseudomonas* species. On the basis of their morphological and physiological characters they could be allocated into three groups: (i) *A. vitis* type colonies (85), (ii) *A. tumefaciens* type colonies (8), and (iii) unidentified isolates which formed yellow colonies (45) (E. Szegedi, unpublished data). One of the unidentified yellow isolates (named Rr-2-17) was shown to accumulate large amounts of acyl-homoserine lactone quorum sensing signal molecules which can activate the *traR* promoter (used by the pTi for activation of pTi conjugation). This isolate was identified as a *Novosphingobium* sp. by full-length 16S rRNA gene sequencing and then verified via comparative genomics (Gan et al., 2009, 2012a). Further characterization of *Novosphingobium* sp. Rr2-17 showed the influence of the stringent response regulator, *rsh,* on the accumulation of the acyl-homoserine lactone quorum sensing signal (Gan et al., 2009).

Recent grapevine microbiota studies used Illumina 16S rRNA gene amplicon sequencing to investigate microbial communities associated with the grapevine organs such as leaf, fruit, cane, and root as well as soil surrounding the roots (Abdellatif et al., 2013; Pinto et al., 2014; Gilbert et al., 2014b; Belda et al., 2017; Manici et al., 2017; Morgan et al., 2017; Alaimo et al., 2018; Marasco et al., 2018; Wei et al., 2018). These studies provided intriguing insights into the effects of various environmental factors on the structure on the grapevine microbiota. Studies that focused on the microbiota of grapevine CG remain scarce with one of the first being conducted on samples collected from a single location in Germany across a temporal gradient (Faist et al., 2016). Using OTU clustering approach, Faist et al. (2016) showed that an OTU classified as *A. vitis* was the most common OTU in the CG affected graft unions followed by two OTUs belonging to *Enterobacter* and *Pseudomonas* with varying relative abundance from season to season. Recently, the amplicon sequence variant (ASV) approach is gradually gaining popularity as it can determine real biological sequences at single nucleotide

resolution albeit at the expense of higher false positives (Callahan et al., 2017; Nearing et al., 2018). The CG microbiota is an excellent model to test the utility of this approach since members of the genus *Agrobacterium* are known to exhibit strikingly high interspecies 16S rRNA gene similarity (Gan and Savka, 2018).

In this study, we investigated the CG microbiota of grapevines from seven different vineyards located in Hungary, Japan, Tunisia, and the United States through Illumina amplicon sequencing of the 16S V3–V4 rRNA gene region. First, we implemented a new primer design to limit amplification of the grape chloroplast 16S rRNA gene, thereby substantially improving the representation of reads belonging to bacterial 16S rRNA. Second, we implemented an ASVs method to improve recovery of *Agrobacterium* 16S rRNA gene sequences with ecological implications by only removing noise from sequencing instead of clustering sequences based on similarity into operational taxonomic unit (OTU). Third, we provide evidence that CG tumors have a small core microbiota and that the microbiota structure is dependent on the sampling site and/or climate. Finally, we show that the abundance of *A. vitis* in the CG is positively correlated with the abundance of at least three non-*A. vitis* groups, e.g., *Novosphingobium* sp., *Xanthomonas* sp., and Microbacteriaceae sp., suggesting the presence of a microbial CG "hub" in the CG tumor environment.

MATERIALS AND METHODS

Field Sampling and DNA Extraction

Crown gall tumor samples (1 tumor per grapevine) were collected from 73 grapevines in six vineyards from Hungary (two vineyards, $n = 37$), United States (two vineyards, $n = 13$), Tunisia (one vineyard, $n = 21$), Japan (one vineyard, $n = 2$) mostly in the summer or autumn (June–September) of 2013 and/or 2014 (see **Supplementary Table S1** for sample-specific detail). In the northern hemisphere, CG tumors start to develop in late May (Jackson, 2014), therefore the age of the tumor tissue collected in this study ranges from approximately 1-month to 3-month-old. Each sample was collected by using separate sterile or flamed surgical blades and stored on ice during transportation to the lab. The DNA extraction was performed from approximately 200 mg of homogenized CG tissue within a day of sample collection using a modified CTAB method (Xu et al., 2005) or the Qiagen DNAeasy Plant Minikit (Qiagen) according to the manufacturer's instructions (**Supplementary Table S1**). The extracted gDNA was sent to Monash University Malaysia for 16S amplicon library construction and Illumina sequencing.

Amplification of the 16S V3–V4 Region and Illumina Sequencing

Single-step polymerase chain reaction (PCR) amplification was performed using NEBNext High-fidelity 2X PCR MasterMix (New England Biolabs, Ipswich, MA, United States) and Illumina adapter-containing primers (Caporaso et al., 2012) targeting the V3–V4 region (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21) of the 16S rRNA gene (Klindworth et al., 2013). The cycling condition consisted of initial denaturation at 98°C for 1 min, 30 cycles of 98°C for 30 s, 58°C for 30 s, and 65°C for 1 min, followed by a final extension at 65°C for 5 min. However, due to heavy co-amplification of the chloroplast gene from an initial set of samples, a new reverse primer with a 3′-end base mismatch to the *V. vinifera* chloroplast sequence was designed to replace the reverse primer (**Figure 1A**). The phylum coverage of the reverse primer, when paired with the forward primer S-D-Bact-0341-b-S-17 was subsequently evaluated *in silico* using TestPrimer v1.0 (Klindworth et al., 2013). 16S rRNA amplicon from each sample was run on a 2% agarose gel and gel-extracted using E.Z.N.A. gel extraction kit (Omega Bio-Tek, Norcross, GA, United States). The gel-purified libraries were quantified using the KAPA library quantification kit Illumina (Kapa Biosystems, Cape Town, South Africa), normalized, pooled, denatured, and subsequently sequenced on a MiSeq Desktop Sequencer (Illumina, San Diego, CA, United States) located at Monash University Malaysia using the 2×250 bp run configuration.

Bioinformatics Analysis

Polymerase chain reaction primer sequences were trimmed from the raw paired-end reads using Cutadapt v. 1.16 with the default setting. Reads that failed to be trimmed due to significant sequence mismatch were discarded. The adapter-trimmed paired-end reads were merged and filtered with fastq_mergepairs (default setting) and fastq_filter (-fastq_minlen 380 – fastq_maxee 0.25) implemented by Usearch v10.0.24010 to retain only ultra-high quality merged reads for the generation of ASV or OTU representative sequences (Edgar, 2010). The merged reads were combined into a single fasta file and labeled according to their sample ID using the add_qiime_labels.py script implemented in QIIME v1.9 (Caporaso et al., 2010) followed by singleton and doubleton removal using the "fastx_uniques" command in Usearch v10 (Edgar, 2010). Error correction (ASV approach) and OTU clustering of the dereplicated sequences used UNOISE3 and UPARSE, respectively (Edgar, 2013, 2016). The ASV/OTU table was constructed by mapping the unfiltered merged reads at 97% nucleotide identity threshold with Vsearch v2.8.0 to the ASV or OTU sequences (Rognes et al., 2016). Taxonomic assignment of the ASV/OTU was carried out in QIIME1 using RDP trained on the greengene v 13.8 database (DeSantis et al., 2006). Grapevine chloroplast- and mitochondrial-derived sequences initially identified by RDP naïve Bayesian Classifier (Wang et al., 2007) were validated by blastN search against their respective reference sequences on GenBank (NC_012119.1 and NC_007957.1) and removed from subsequent analysis.

Microbiota Analysis

The chloroplast- and mitochondrion-filtered ASV table was rarefied to 28,364 sequences per sample and subsequently used to perform analysis of similarities (ANOSIMs) and to compute beta-diversity (Bray–Curtis distance matrix) and core microbiota in QIIME v1.9. For core microbiota computation, an ASV with $>0.05\%$ relative abundance is considered as "present" in a sample and the ASV needed to be present in at least 60% of the samples to be considered as part of the core microbiota. To identify ASVs that are significantly enriched in sites with more than

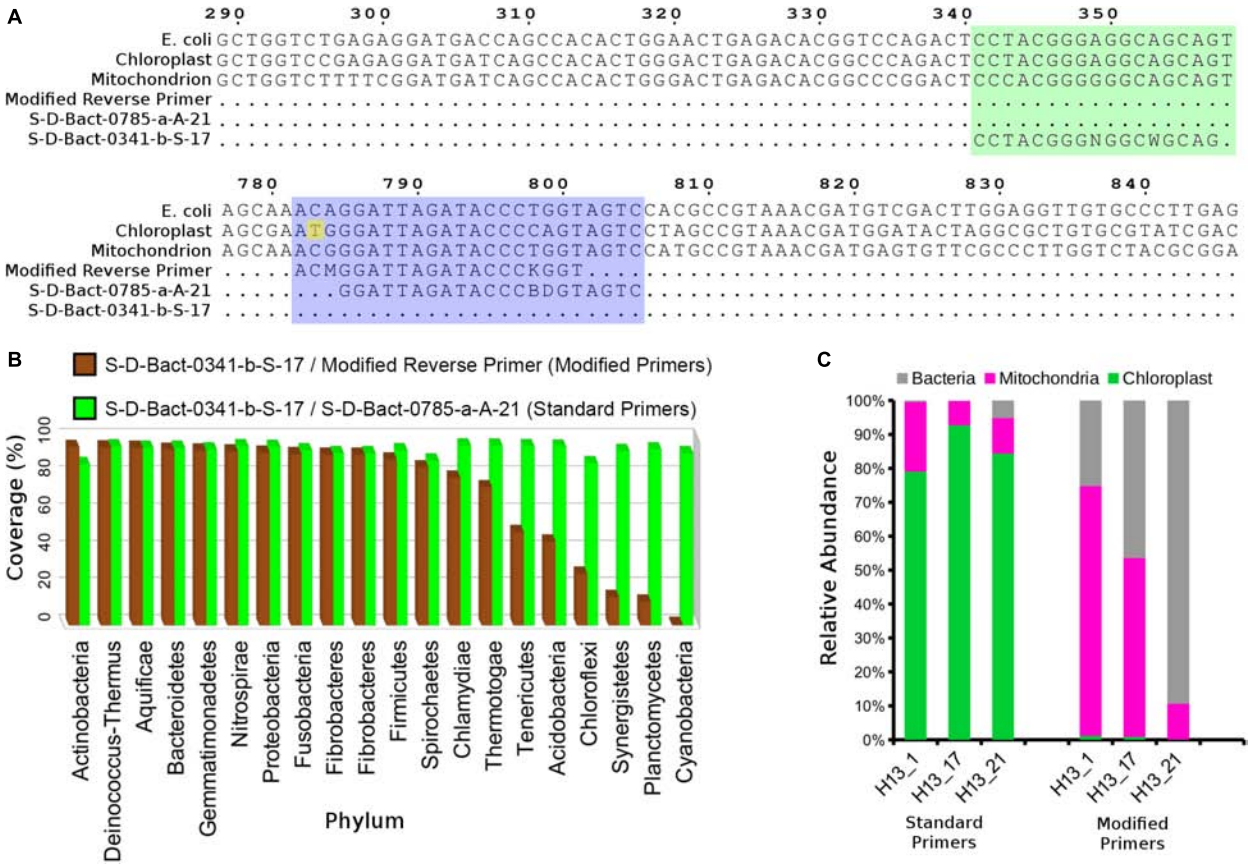


FIGURE 1 | Modification of the 16S V3–V4 reverse primer reduced co-amplification of grapevine rRNA gene. **(A)** Nucleotide alignment of standard and modified primers against the grapevine mitochondrial and chloroplast rRNA genes. Green and purple blocks indicate 16S rRNA gene regions containing the aligned forward and reverse 16S V3–V4 primers, respectively. Numbers above the alignment indicate base position on the *Escherichia coli* 16S rRNA gene sequence. **(B)** Phylum-level taxonomic coverage of the standard and modified V3–V4 primer pairs as assessed by SILVA TestPrimer 1.0 based on the SILVA SSU r132 RefNR database (maximum number of mismatches = 5; length of 0-mismatch zone at 3′-end = 5 bases). **(C)** Relative abundance of bacterial, grapevine mitochondrial, and chloroplast sequence for three 2013 Hungarian crown gall samples that were amplified using the standard and modified 16S V3–V4 primers.

five samples (biological replicates), we performed differential abundance analysis using the DESeq2 algorithm as implemented in the "differential_abundance.py" in QIIME v1.9 using the raw unrarefied OTU table (**Supplementary Table S2**) as the input.

Microbial Association Network Construction

Co-occurrences were calculated with SparCC using unrarefied ASV table (Friedman and Alm, 2012). Pseudo *p*-values were calculated based on 100 bootstraps. Correlations were subsequently filtered based on statistical significance (*p*-value < 0.001), correlation coefficient strength ($-0.5 < R < 0.5$), and percentage of total observation count ($> 0.1\%$). Construction of the association network based on the filtered correlations was performed using Gephi v0.9.2 (Bastian et al., 2009).

Phylogenetic Analysis

Amplicon sequence variants initially classified to the genus *Agrobacterium* by RDP and their corresponding exact OTU match were aligned with the 16S rRNA sequences of several described type strains belonging to the genera *Agrobacterium, Pararhizobium, Rhizobium*, and *Allorhizobium*. The alignment was performed using MAFFT v7.123b with the settings "–localpair –maxiterate 1000" (Katoh and Standley, 2013) and was subsequently trimmed with TrimAl v. 1.2 (Capella-Gutiérrez et al., 2009) retaining only the V3–V4 region to assess the accuracy of both RDP and 16S rRNA V3–V4 region in delimiting the genus *Agrobacterium*. Maximum-likelihood tree construction based on the trimmed alignment was performed using FastTree v2.1 and visualized in FigTree v1.4 (Price et al., 2010).

Opine Detection and PCR Detection of Phytopathogenic *Allorhizobium* (*Agrobacterium*) *vitis* and *Agrobacterium tumefaciens* From Hungarian Crown Gall Samples

For all 16 Hungarian CG samples that were collected in 2014 (**Supplementary Table S1** and **Table 1**), opine was extracted from approximately 100 mg of tumor tissue homogenized in distilled

TABLE 1 | Opine and molecular characterization of crown gall samples collected from Hungary in 2014.

Sample ID	Cultivar	Opine assay			PCR assay	
		Phenantrene-quinone	Silver-nitrate	Reversed silver-nitrate	PGF-PGR	VirD2A-VirD2E
H14_1	Lilla	Octopine	–	–	+	–
H14_2	Lilla	–	–	–	–	–
H14_3	Lilla	Octopine	–	–	–	–
H14_4	Lilla	Octopine	–	–	–	–
H14_5	Lilla	–	–	–	–	–
H14_6	Lilla	Octopine	–	–	–	–
H14_7	Medina	–	–	Vitopine	+	–
H14_8	Medina	–	–	Vitopine	–	–
H14_9	Medina	Octopine	–	–	–	–
H14_10	Teréz	–	–	–	–	–
H14_11	Teréz	Octopine	–	–	+	–
H14_12	Teréz	–	–	–	–	–
H14_13	Teréz	–	–	–	–	–
H14_14	Teréz	–	–	–	–	–
H14_15	Teréz	Octopine	–	–	–	–
H14_16	Teréz	Octopine	–	–	–	–

Presence of opine in the opine assay is indicated by the name of opine type detected by the specific test while minus signs indicate the absence of opine. Plus and minus signs in the PCR assay column indicate the presence and absence, respectively, of PCR products corresponding to the pehA gene of Allorhizobium vitis (PGF–PGR) or virD gene of A. tumefaciens (VirD2A–VirD2E) in the extracted crown gall DNA.

water as previously described (Szegedi, 2003). The extracted opine was separated with paper chromatography and stained using phenanthrene-quinone (for octopine and nopaline), silver-nitrate (for agropine and mannopine), or reversed silver nitrate (for vitopine) (Szegedi, 2003). PGF-PCR primers specific for the amplification of the polygalacturonase gene, *pehA*, from *A. vitis* were used to detect the presence of *A. vitis* in CG samples (Szegedi and Bottka, 2002). Detection of pathogenic *A. tumefaciens* strains used primers virD2A and virD2E which target the *virD* gene located on the Ti plasmid (Haas et al., 1995; Szegedi and Bottka, 2002). Purified gDNA of *A. vitis* strain Tm4 and *A. tumefaciens* A348 were used as positive controls for the PGF/PGR and VirD2A/VirD2E assays, respectively.

RESULTS

A Modified Illumina V3–V4 16S rRNA Primer Reduces Co-amplification of Grapevine Plastid DNA

A preliminary analysis of three Hungarian CG samples based on amplicons generated from primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 showed that nearly all of the entire sequencing reads originated from grapevine chloroplast (80–90%) and mitochondrial (5–20%) rDNAs (**Figure 1C**), rendering large-scale amplicon sequencing of CG microbiota potentially cost-ineffective using the standard primer pairs. A new reverse primer with a single base mismatch to the grapevine chloroplast 16S ribosomal DNA at its 3′-end was subsequently designed (**Figure 1A**). Based on *in silico* analysis, the newly designed primer exhibited high coverage across various phyla with a notable reduction in a few bacterial phyla such as Chloroflexi, Synergistetes, Planctomycetes, and Cyanobacteria (**Figure 1B**). A near 100% reduction in the coverage for the phylum Cyanobacteria is consistent with the relatedness of the chloroplast to this phylum. Amplicon sequencing of the three previously sequenced Hungarian samples using the modified primer resulted in nearly zero recoveries of chloroplast-derived reads, indicating the efficiency of the new primer in inhibiting the co-amplification of highly abundant chloroplast 16S rRNA gene from the CG samples. Although the modified primer still co-amplified the host mitochondrial 18S rRNA gene due to the lack of 3′-end mismatch (**Figure 1A**), we observed sufficient relative abundance of bacterial reads across the three samples (30–90%) to justify its use in subsequent microbiota analysis (**Figure 1C**).

A Small Crown Gall Core Microbiota

A total of 5,701 ASVs were generated from the de-replicated high quality overlapped paired-end reads of which 5,622 were inferred to be of bacterial origin (non-chloroplast and non-mitochondrial) (**Supplementary Data S1** and **Supplementary Table S2**). Relative abundance calculation at the family level indicates that >80% of the reads from each collection site could be classified into 14 core microbial families (**Figure 2** and **Supplementary Table S3**). The cumulative relative abundance of reads mapping to Rhizobiacea and Enterobacteriaceae is generally high across sites ranging from 35 to 90% (**Figure 2**). Some microbial families are only abundant at a particular site. For example, most reads assigned to Kineosporiaceae and Caulobacteraceae were found in samples collected from Hungary in September 2014. Ten ASVs were found to be present in >60% of the samples (**Figure 3**). ASV2 corresponding to *A. vitis* is the second most prevalent ASV with presence in >75% of the samples. This ASV is highly abundant in a majority of the Hungarian samples collected in July 2013 with an average 35% relative abundance (39% median relative abundance). On the contrary, the most prevalent ASV23 that was assigned to the genus *Agrobacterium* has a relative abundance of only <10% across all samples. Notably, the core microbiota matrix of most Tunisian samples is generally sparser with some of them even missing the prevalent ASV2 and ASV23 (**Figure 3**).

Low Occurrence of *Allorhizoium vitis* in Some Opine-Producing Grapevine Crown Galls

Ten out of 16 Hungarian CGs collected at the beginning of fall 2014 (**Supplementary Table S1**) contained either octopine or vitopine (**Table 1**). Of the 10 opine-containing Hungarian CGs, only 3 (H14_1, H14_7, and H14_11) were positive for *A. vitis* when tested with *A. vitis*-specific primers consistent with the high relative abundance of ASV2 (13–19%) in these samples (**Figure 3**). However, sample H4_14 that has a slightly lower ASV2 abundance (9.5%) in addition to a few samples with <1% ASV2 relative abundance were reported as *A. vitis*-negative by

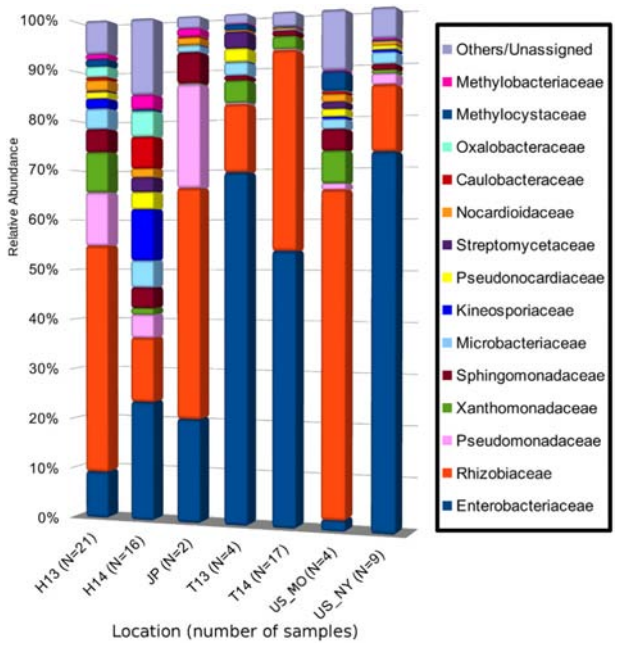


FIGURE 2 | Mean relative abundance of bacterial 16S rRNA gene sequences at the family level of taxonomic classification in each sampling site. Each bacterial family is represented as a different color in the bar chart. The combined relative abundances total to 100% for each site. H13, Hungary (Heves) samples collected in 2013; H14, Hungary (Bacs-Kiskun) samples collected in 2014; T13, Tunisia (Regueb) samples collected in 2013; T14, Tunisia (Regueb) samples collected in 2013; US_MO, United States (MO) samples collected in 2013; US_NY, United States (NY) samples collected in 2014; JP, Japan (Okayama) samples collected in 2004 and 2013. Numbers in brackets indicate the number of crown gall samples collected from each site.

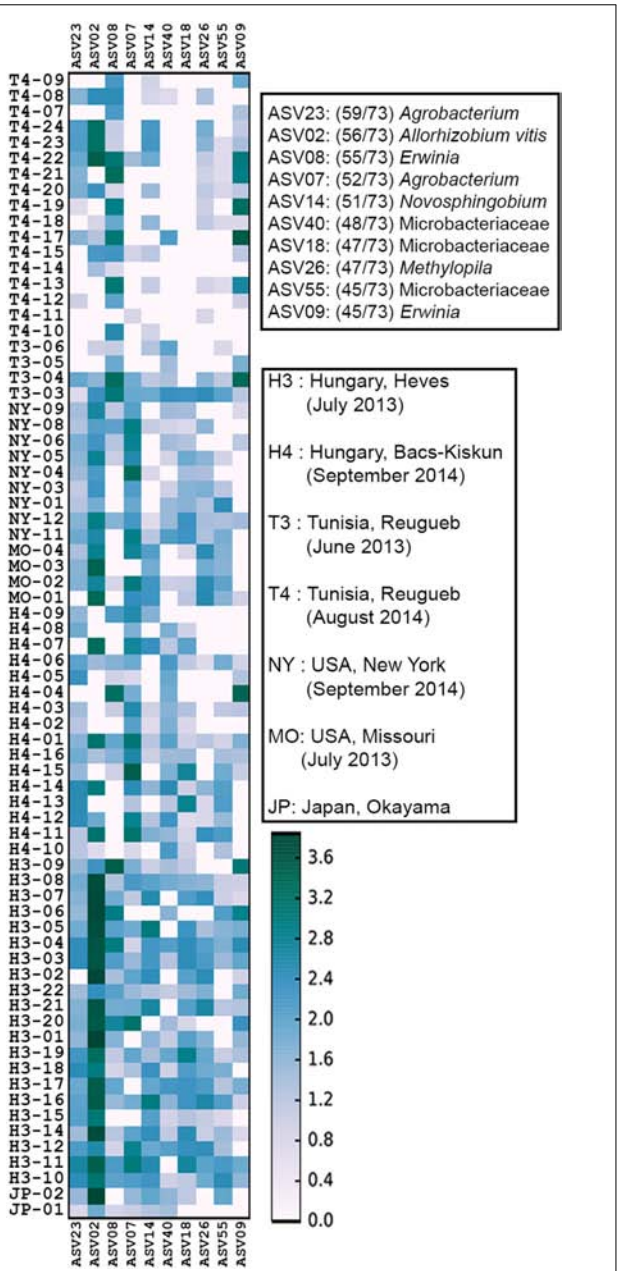


FIGURE 3 | Heat map of core microbiome abundance across 73 crown gall samples. The *x*-axis shows all 10 core ASVs in the order of decreasing prevalence. The prevalence (numbers in brackets next to each ASV) and classification of the 10 core ASVs to the lowest possible taxonomic rank were shown in the upper right legend. The heatmap scale indicates the abundance of ASV (normalized to 10,000 reads/sample) in a 10-based logarithmic scale.

the PCR approach. None of the samples were tested positive for *A. tumefaciens* that was occasionally identified as the causative agent of CG infection in grapevine (**Table 1**).

Crown Gall Microbiota Is Variable Across Sites

Analysis of similarities indicates that the microbial composition among sites is significantly different (ANOSIM global $R = 0.63$, p-value = 0.001). As expected, samples [represented by data points on the principal coordinate analysis (PCoA) plot] were broadly clustered based on their collection sites with some notable exceptions among the Hungarian samples collected in late September 2014 (H4 in **Figure 4**). Four of the H4 samples were positioned close to the New York cluster while two were observed in the upper left quadrant consisting mostly of 2013 samples from Hungary which represent the younger (approximately 2-month-old) CGs. On the other hand, the Tunisian samples were only found in the lower PCoA quadrants with a majority of them clustered along the vertical axis in the lower right quadrant. ASVs belonging to the genus *Pseudomonas* represent four out of five most significantly enriched ASVs among the 2013 samples from Hungary (**Table 2**). All five of the most significantly enriched ASVs in both New York and Tunisia sites were assigned to the family Enterobacteriaceae with one of them (ASV15) having an assignment at the genus level to the genus *Erwinia*. On the other hand, the differentially abundant ASVs in the 2014 samples from Hungary are more diverse, consisting of three Alphaproteobacteria, one Betaproteobacteria, and one Flavobacteria ASVs (**Table 2**).

Associations Between Crown Gall Microbes

Using SparCC, we generated a microbial interaction network capturing 194 significant associations (red and green lines in **Figure 5**) among 86 ASVs (nodes in **Figure 5**) across the 73 CG samples. The 86 ASVs mostly belong to nine microbial families with nearly half of them assigned to the families Enterobacteriaceae and Rhizobiaceae (purple and green nodes in **Figure 5**). ASV2 (*A. vitis*), the most abundant ASV, showed positive co-occurrence relationships ($R > 0.5$ and p-value < 0.001) with three Rhizobiaceae ASVs (ASVs 19, 173, and 1345), one *Xanthomonas* ASV (ASV6), one *Novosphingobium* ASV (ASV14), one Methylocystaceae ASV (ASV26), and one Microbacteriaceae ASV (ASV18). On the other hand, ASV3, the second most abundant ASV assigned to the genus *Agrobacterium*, exhibits mixed co-abundance relationships with 11 ASVs which proceed to form a complex interaction network (**Figure 5**). Furthermore, three small network clusters consisting exclusively of Enterobacteriaceae ASVs were also observed and may represent site-specific microbial interactions.

OTU Clustering Approach Underestimated *Agrobacterium* Diversity in CG Microbiota

Operational taxonomic unit clustering approach generated three OTUs with an exact match to ASV2, ASV3, and ASV119 (**Figure 6** and **Supplementary Data S2**). The other 11 non-matching ASVs exhibits >97% nucleotide similarity to the three OTUs, suggesting that biological sequences corresponding to these ASVs would have been removed/clustered and represented by only a few OTUs. Despite exhibiting high sequence similarity to their corresponding OTUs, phylogenetic clustering showed that most of these 11 non-matching ASVs formed a tight cluster with known type strains, indicating that these are likely *bona fide* biological sequences (**Figure 6**). ASV7 is identical to two different *Agrobacterium* species, *A. rosae,* and *A. bohemicum*, underscoring inability of the V3–V4 region to resolve some *Agrobacterium* species. It is worth noting that despite being initially classified as *Agrobacterium* by RDP, some of these ASVs are more closely related to *Rhizobium* (ASV76, ASV23, and ASV151) and *Neorhizobium* (ASV173). Although ASV33 and ASV1345 do not show identical match to any type strain, it is unlikely that they arose due to sequencing artifact given their relative abundance among samples collected from two distant North American sites (**Figure 6**).

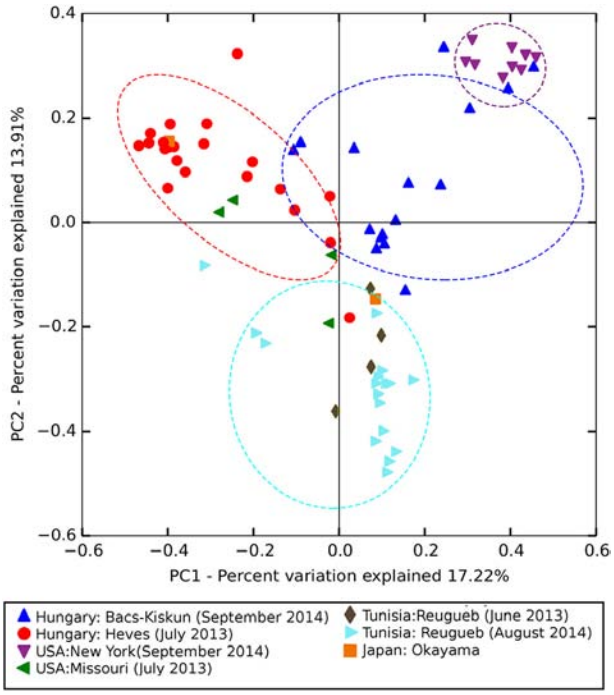


FIGURE 4 | Principal coordinate analysis (PCoA) ordination based on Bray–Curtis dissimilarity matrix showing significantly different (ANOSIM global $R = 0.63$, p-value = 0.001) microbial composition among sampling sites. Points in the PCoA plot represent crown gall samples each colored and shaped according to the sampling site. Points enclosed by dotted lines represent samples from major sampling sites ($N > 10$ samples).

DISCUSSION

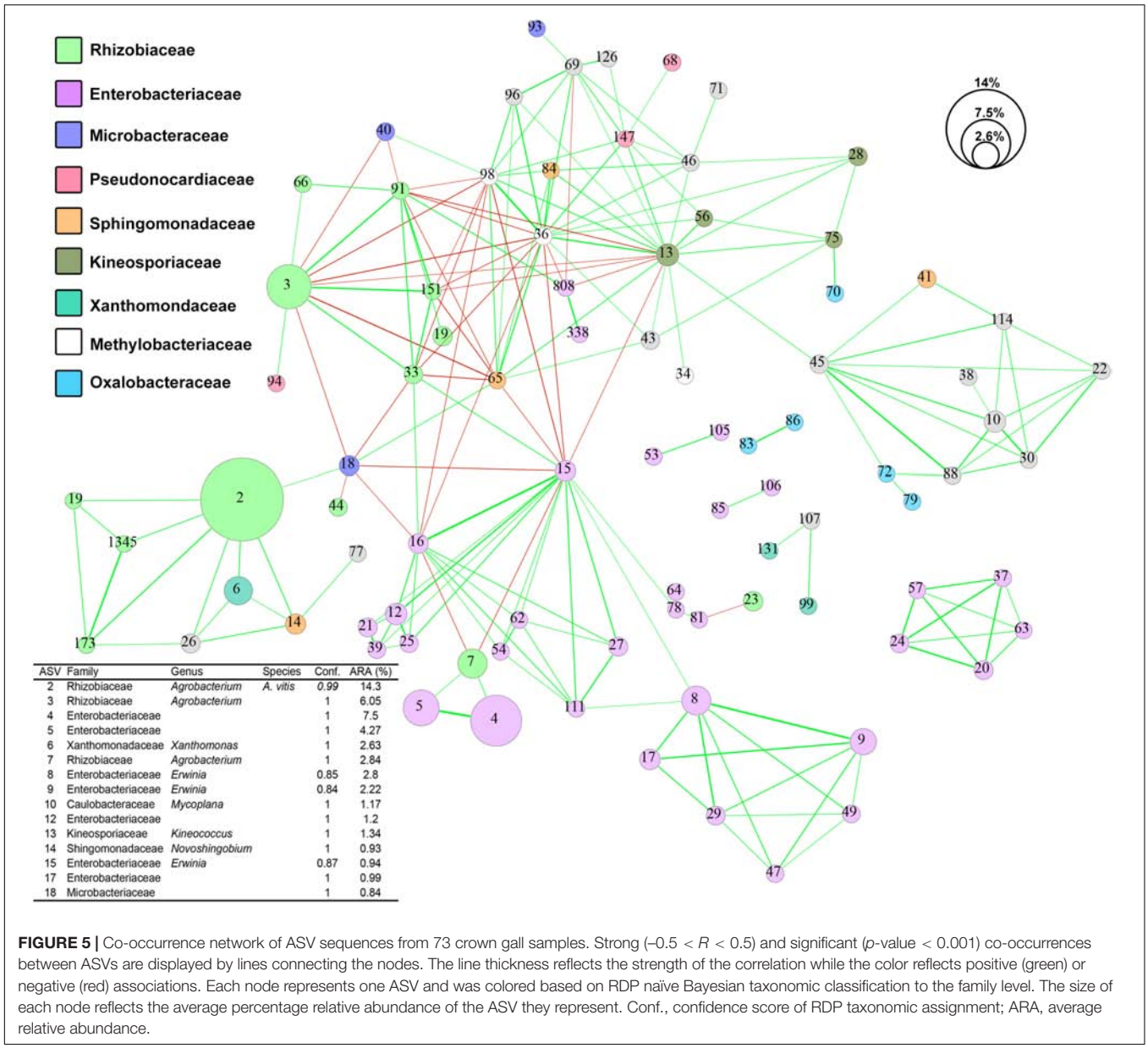
To define the microbiota of CG across, we sampled 73 CGs from various grape cultivars located in seven vineyards across four continents of the Northern Hemisphere. In contrast to the work done by Faist et al. (2016) who limited sampling to a single vineyard, our study provides a more comprehensive insight into the CG microbiota. Chloroplast contamination was dramatically reduced in this study using a modified reverse primer, albeit at the expense of reduced primer coverage for a few phyla which are not commonly plant associated (Hirsch and Mauchline, 2012; Turner et al., 2013; Schlaeppi and Bulgarelli, 2015). Similarly leveraging on primer mismatch to host plastid genome, a recent study has recommended the use of standard and unmodified primer pair "799F-1391R" targeting the V5–V7 region that was shown to dramatically reduce co-amplification of poplar plastid (Beckers et al., 2016). Given that a majority of Illumina-based microbiota studies target the V3, V4, or V3–V4 region (Klindworth et al., 2013; Gilbert et al., 2014a), protocol modifications directly aimed at overcoming co-amplification of host DNA may be beneficial. An attractive and potentially more cost-effective approach for future large-scale studies would be to design blocking primers that are complementary to the host chloroplast and mitochondrial rRNA gene (Hanshew et al., 2013; Beckers et al., 2016). A blocking primer will contain C3 spacer at its 3′-end that prevents extension during PCR when included into the standard PCR mix at an equal or higher concentration than the standard 16S primers (Arenz et al., 2015).

It is also worth noting that a majority of the sampling in this global study was performed between June and September, a critical period during which glucose concentration in the grape is most variable (Bauer et al., 1994). This may explain the variability in the microbiota composition across sample sites. It is also possible that the use of different DNA extraction methods

TABLE 2 | Top five significantly enriched ASVs by sampling sites.

ASV	Taxonomy	Group 1[a]	ARA (%)	Group 2	ARA (%)	*p*-value
ASV198	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas;	H13	0.31416	Non-H13	0.0002	1.67*E*−65
ASV404	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas;	H13	0.0995	Non-H13	0.00001	3.50*E*−49
ASV101	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas;	H13	0.30347	Non-H13	0.00083	2.04*E*−40
ASV11	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas;	H13	4.72383	Non-H13	0.2748	1.33*E*−34
ASV539	p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Sphingobacteriaceae;g_Pedobacter;	H13	0.01727	Non-H13	0.00034	1.88*E*−27
ASV277	p_Proteobacteria;c_Alphaproteobacteria;o_Caulobacterales;f_Caulobacteraceae;	H14	0.14546	Non-H14	0.00026	1.18*E*−102
ASV30	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Weeksellaceae;g_Chryseobacterium;	H14	1.02049	Non-H14	0.00026	2.94*E*−101
ASV181	p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_Novosphingobium;	H14	0.26148	Non-H14	0.00046	8.26*E*−99
ASV408	p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Bradyrhizobiaceae;g_Bradyrhizobium;	H14	0.08687	Non-H14	0.00015	1.39*E*−82
ASV485	p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Burkholderiaceae;	H14	0.06765	Non-H14	0.00007	1.50*E*−73
ASV2917	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	NY	0.08737	Non-NY	0.0001	1.89*E*−61
ASV3878	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	NY	0.12115	Non-NY	0.00025	3.38*E*−58
ASV828	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	NY	0.1007	Non-NY	0.0002	1.41*E*−55
ASV1502	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	NY	0.02389	Non-NY	0.00011	2.45*E*−42
ASV1154	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	NY	0.02789	Non-NY	0.00009	1.68*E*−40
ASV15	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Erwinia;	Tunisia	3.0591	Non-Tunisia	0.00036	3.29*E*−113
ASV16	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	Tunisia	2.70482	Non-Tunisia	0.00044	1.06*E*−92
ASV27	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	Tunisia	2.04908	Non-Tunisia	0.00323	2.38*E*−71
ASV12	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	Tunisia	3.91837	Non-Tunisia	0.00238	1.52*E*−69
ASV226	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;	Tunisia	0.29893	Non-Tunisia	0.00005	5.24*E*−67

Group 1 is the reference group (samples from a specific sampling site) that will be compared against Group 2 (all other samples that are not from Group 1 sampling site). ARA, average relative abundance. [a]H13, 2013 samples from Heves, Hungary (n = 20); H14, 2014 samples from Bacs-Kiskun, Hungary (n = 16), NY, 2013 samples from New York, United States (n = 9); Tunisia, 2013 and 2014 samples from Regueb, Tunisia (n = 21).



ASV	Family	Genus	Species	Conf.	ARA (%)
2	Rhizobiaceae	*Agrobacterium*	*A. vitis*	*0.99*	14.3
3	Rhizobiaceae	*Agrobacterium*		1	6.05
4	Enterobacteriaceae			1	7.5
5	Enterobacteriaceae			1	4.27
6	Xanthomonadaceae	*Xanthomonas*		1	2.63
7	Rhizobiaceae	*Agrobacterium*		1	2.84
8	Enterobacteriaceae	*Erwinia*		0.85	2.8
9	Enterobacteriaceae	*Erwinia*		0.84	2.22
10	Caulobacteraceae	*Mycoplana*		1	1.17
12	Enterobacteriaceae			1	1.2
13	Kineosporiaceae	*Kineococcus*		1	1.34
14	Shingomonadaceae	*Novoshingobium*		1	0.93
15	Enterobacteriaceae	*Erwinia*		0.87	0.94
17	Enterobacteriaceae			1	0.99
18	Microbacteriaceae			1	0.84

FIGURE 5 | Co-occurrence network of ASV sequences from 73 crown gall samples. Strong ($-0.5 < R < 0.5$) and significant (p-value < 0.001) co-occurrences between ASVs are displayed by lines connecting the nodes. The line thickness reflects the strength of the correlation while the color reflects positive (green) or negative (red) associations. Each node represents one ASV and was colored based on RDP naïve Bayesian taxonomic classification to the family level. The size of each node reflects the average percentage relative abundance of the ASV they represent. Conf., confidence score of RDP taxonomic assignment; ARA, average relative abundance.

may have contributed to the difference observed. However, given that the sampling was performed at multiple distant geographic regions at different time, the effect of such spatial and temporal variations on microbiota composition should outweigh the effect of variance in the DNA extraction method employed. Although examining the effect of extraction methodology on CG microbiota composition is beyond the scope of this study, similar studies have been conducted on non-CG samples with the general consensus that inter-sample variation always outweighed the variation in extraction method (Wesolowska-Andersen et al., 2014; Sinha et al., 2017).

The colony forming unit (CFU) of *A. vitis* from CG tumors of Riesling and Müller-Thurgau grapevine cultivars in Germany was previously shown to decrease dramatically after the month of June (Faist et al., 2016). Similarly, *A. vitis* was largely absent among Hungarian samples sampled in September 2014 but not July 2013 based on *A. vitis* PCR detection and amplicon sequencing assays. The absence of *A. vitis* in a majority of the opine-containing Hungarian CG samples suggests while opine serves as an attractant to *A. vitis*, there are additional biotic and/or abiotic factor(s) that can influence the population dynamics of *A. vitis* in CG tumors (Bhattacharya et al., 2010). On the other hand, high ASV3 to ASV2 ratio, indicating a strong dominance of *Agrobacterium* spp. over *A. vitis* was observed only in the Tunisian CGs. *Agrobacterium* spp. members were previously shown to be dominant in Tunisian soils based on culture-based method, an observation that was hypothesized to be due to the climatic and soil conditions of the country (Bouri et al., 2016). The diversity of *Agrobacterium* populations in Tunisian vineyards seems to be restricted to the genomic

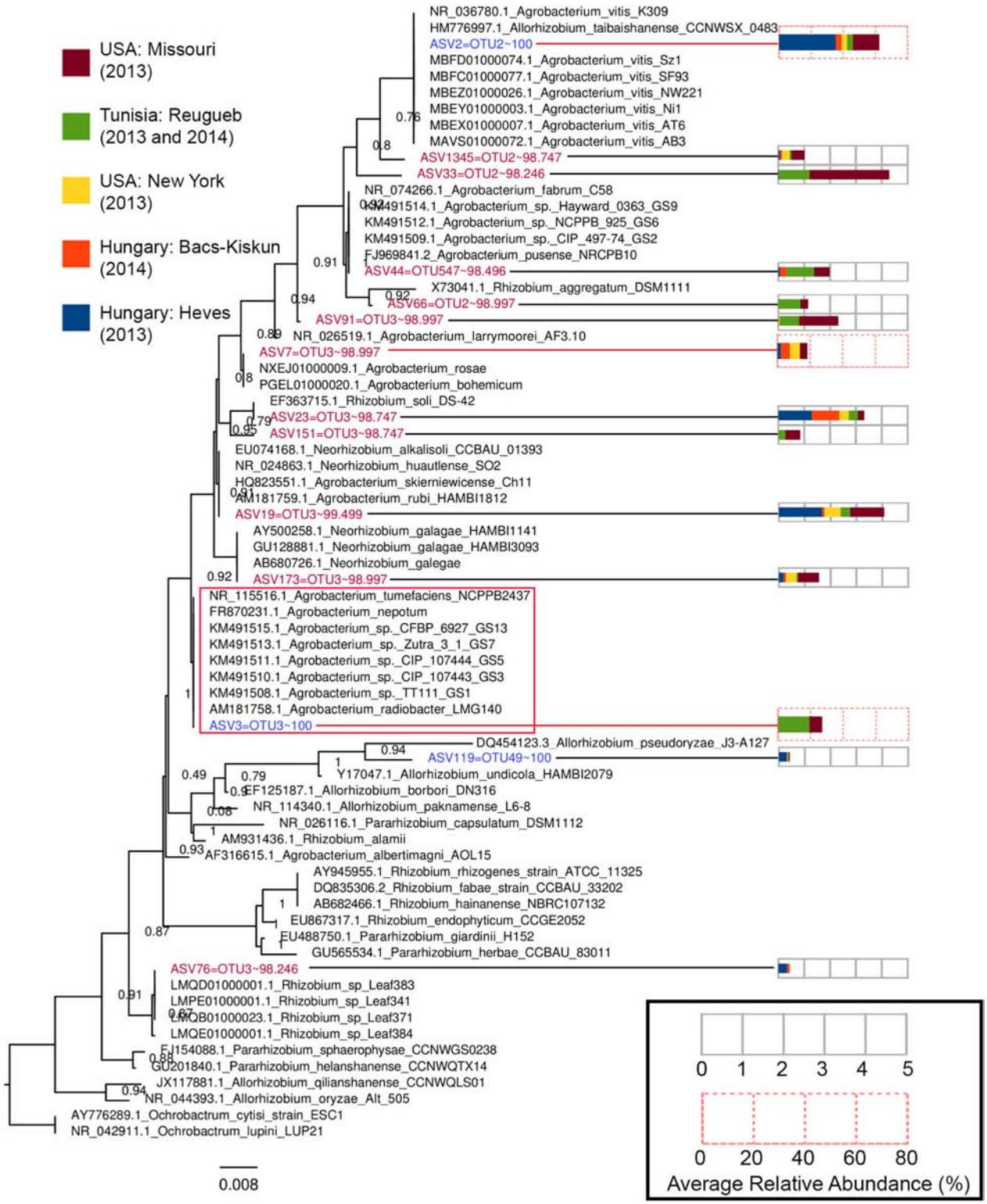


FIGURE 6 | A maximum-likelihood tree constructed based on the alignment of *Agrobacterium* and *Allorhizobium* ASVs to the V3–V4 16S rRNA gene region of *Agrobacterium, Allorhizobium*, *Pararhizobium*, and *Rhizobium* strains. The tree was rooted with members from the genus *Ochrobactrum* as the outgroup. Tips representing ASVs included in the tree construction were colored based on the percentage nucleotide identity (values after the tilde symbols) of an ASV to its best matching OTU, with blue and red indicating perfect match (100% identity) and near-perfect (98% $< x <$ 100% identity) match, respectively. Numbers at nodes indicate Shimodaira–Hasegawa (SH)-like local support values and branch lengths indicate the number of nucleotide substitutions per site. Codes preceding

(Continued)

FIGURE 6 | Continued
the genus name of each type strain are their NCBI accession numbers. The GS# labels present at the end of some *Agrobacterium* tip labels correspond their genospecies and the red box indicates an *Agrobacterium* clade consisting of multiple *Agrobacterium* genospecies with zero branch length or identical 16S rRNA V3–V4 gene sequence. Each ASV tip label associated with an abundance profile (right) that displays the average percentage relative abundance of the ASV in the major sampling sites.

species G4, G7, and G9. Since *Agrobacterium* genomic species G9 was represented by ASV44 instead of ASV3, 16S rRNA reads mapping to ASV3 in the Tunisian CG tumors may originate from *Agrobacterium* spp. belonging to genomic species GS4 and/or GS7. The lack of Tunisian tumorigenic strains associated with genomic species G7 as determined by PCR-based detection of Ti-plasmid (VCF3-VCR3 primers; *virC*) lends support toward the affiliation of ASV3 in Tunisian CG tumors to *Agrobacterium* spp. genomic species G4 (Bouri et al., 2016). It is important to note that such a correlation cannot be applied to non-Tunisian samples that are lacking background microbial genetic data.

In addition to *A. vitis* (OTU_0003), Faist et al. (2016) also identified two additional OTUs (OTU_0005, *Pseudomonas* sp.; OTU_0008, *Enterobacter* sp.) showing high abundance and prevalence in tissues sampled from vines containing CG tumors during spring and autumn. Although our study did indeed demonstrate the prevalence and substantial abundance of *A. vitis*-linked ASV in a majority of the CG tumor tissue samples, no ASVs corresponding to *Pseudomonas* could be found in the core microbiota. However, we do observe a significant enrichment of *Pseudomonas*-linked ASVs in the Hungarian 2013 CG tissue samples, which may suggest the positive association of members from this genus during early CG formation possibly due to the ability to utilize opine compounds as a growth substrate (Bell et al., 1990). For example, the genome of *P. kilonensis* strain 1855-344, a strain which can catabolize octopine, has recently been shown to contain an octopine-catabolic operon named *ooxAB* (Eng et al., 2015). Interestingly, despite being present in the core microbiota, none of the Enterobacteriaceae-linked ASVs formed a significant co-occurrence with *A. vitis* (ASV2). However, several Enterobacteriaceae-linked ASVs were shown to be differentially abundant among samples from New York and Tunisia. The occurrence of members of the Enterobacteriaceae has recently been associated with the native microbiota of viticultural regions, and has been proposed to impart distinct chemical composition and sensory characteristics of regional wines (Bokulich et al., 2016). It has been suggested that plant-associated members of Enterobacteriaceae also play a part in the complex interactions among the environmental, temporal, plant-genetics, human, and other factors which influence grapevine growth and development collectively referred as "terroir" (Fischer et al., 1999; López-Rituerto et al., 2012). Within the family Enterobacteriaceae, members from the genera *Pantoea* and *Erwinia* are common plant inhabitants. However, the evolutionary relationships of members from these two genera could not be confidently established using the nucleotide sequence of the 16S rRNA gene alone. This uncertainty may be responsible for our failure to assign several Enterobacteriaceae-linked ASVs to the genus level. Since *Erwinia* and *Pantoea* spp. are readily culturable on agar medium, it may be more appropriate to infer their roles in the CG microbiota using a culture-based approach followed by biochemical characterization, whole genome sequencing, and comparative genomics (Hong et al., 2012; Gan et al., 2014; Walterson and Stavrinides, 2015).

The strong co-occurrence of *Xanthomonas* and *Novosphingobium* with *A. vitis* as observed in this study is consistent with the affiliation of their members with grapevine and more specifically with CG tumor tissues. For example, an AHL-producing *Novosphingobium* sp. strain Rr 2-17 was isolated from a CG tumor and its quorum sensing AHL-signal production was shown via transposon mutagenesis to be regulated by the stringent response gene, *rsh* (Gan et al., 2009). The TraR protein associated with conjugal transfer of Ti plasmid reacts strongly to the AHL signals generated by strain Rr 2-17, suggesting that it may play a role in amplifying the quorum sensing signal required to disseminate Ti plasmid (via conjugal plasmid transfer) among *Agrobacterium, Allorhizobium*, and potentially additional compatible strains in the tumor environments (Zhu and Winans, 2001; Zhang et al., 2002; Lowe et al., 2009). *In silico* identification of quorum sensing synthase gene from *Novosphingobium* genomes indicates most members exhibit the genomic potential to produce AHL (Gan et al., 2013, 2015, 2016). Known for their ability to mineralize complex aromatic compounds, it is possible that *Novosphingobium* spp. might harbor homologous genes associated with the catabolism of opine compounds or intermediates which have a structural resemblance to oxygenase-cleaved aromatic compound (Wilcke, 2000; Gan et al., 2011, 2012b; Mallick et al., 2011). *Xanthomonas* spp. also have been reported to be commonly present on grapevine leaves and bark, but absent from grapes or grapevine-associated soils as determined through culture-dependent and culture-independent approaches (Martins et al., 2013). The strong co-occurrence of *Xanthomonas* with *A. vitis* in CG tumors can be explained by the presence of high concentrations of lignin, cellulose, *N*-glycosylated proteins, and other cell wall precursors as well as cell wall degradation products found in bark on developing CG tumors (Blanvillain et al., 2007; Boulanger et al., 2014).

The identification and delineation of agrobacteria even based on full-length 16S rRNA gene sequences has been problematic as indicated by polyphyletic clustering pattern and low bootstrap support values, as observed in various phylogenetic trees even with high taxon sampling. The lack of informative sites is presumably due to the slower evolutionary rate of 16S rRNA among agrobacteria (Costechareyre et al., 2010). Despite being able to resolve amplicon down to the single base resolution, the monophyletic clustering of ASV2 with multiple genomic species of *Agrobacterium* exhibiting diverse and distinct ecological niches strongly suggests that caution needs to be exercised when inferring ecological interactions from 16S rRNA dataset. An improved understanding of the microbial interactions within

such an environment can be gained through whole metagenome shotgun sequencing approach coupled with the ProxiMeta^{TM} Hi-C metagenome deconvolution method that can link plasmids to their hosts (Press et al., 2017). However, the challenges associated with strong host (grapevine) gDNA contamination will need to be addressed through increased sequencing depth and/or the selective removal of host methylated gDNA (Feehery et al., 2013).

CONCLUSION

The widely used Illumina standard V3–V4 16S rRNA primers are not suitable for grapevine microbiota studies as they exhibit perfect match to the grapevine plastid 16S rRNA gene. We report a new pair of V3–V4 16S ribosomal RNA gene PCR primers which prevent the co-amplification of this gene region and used this primer pair to investigate the microbial community of 73 CG tissue samples collected from multiple distinct geographic regions. The CG microbial community is diverse and varies significantly across samples and vineyards.

DATA AVAILABILITY

Sequencing data generated from this study have been deposited into the NCBI Sequence Read Archive under the BioProject code PRJNA490446.

AUTHOR CONTRIBUTIONS

HG, MS, and ES conceived the project, designed the experiments, and drafted the manuscript. ES, RF, SC, LK, AK, and TB collected the samples and performed the DNA extraction. HG performed the amplicon sequencing and conducted the bioinformatic analysis. All authors edited and contributed to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01896/full#supplementary-material

TABLE S1 | Detailed metadata of crown gall tumors collected from different sites.

TABLE S2 | Raw count data of bacterial ASVs and their taxonomic classification.

TABLE S3 | Relative abundance (%) of bacterial ASVs and their taxonomic classification.

DATA S1 | DNA sequences of unfiltered ASVs in fasta format.

DATA S2 | DNA sequences of unfiltered OTUs in fasta format.

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frontiers in Plant Science

REVIEW
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Recognition of Elicitors in Grapevine: From MAMP and DAMP Perception to Induced Resistance

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In a context of a sustainable viticulture, the implementation of innovative eco-friendly strategies, such as elicitor-triggered immunity, requires a deep knowledge of the molecular mechanisms underlying grapevine defense activation, from pathogen perception to resistance induction. During plant-pathogen interaction, the first step of plant defense activation is ensured by the recognition of microbe-associated molecular patterns, which are elicitors directly derived from pathogenic or beneficial microbes. *Vitis vinifera*, like other plants, can perceive elicitors of different nature, including proteins, amphiphilic glycolipid, and lipopeptide molecules as well as polysaccharides, thanks to their cognate pattern recognition receptors, the discovery of which recently began in this plant species. Furthermore, damage-associated molecular patterns are another class of elicitors perceived by *V. vinifera* as an invader's hallmark. They are mainly polysaccharides derived from the plant cell wall and are generally released through the activity of cell wall–degrading enzymes secreted by microbes. Elicitor perception and subsequent activation of grapevine immunity end in some cases in efficient grapevine resistance against pathogens. Using complementary approaches, several molecular markers have been identified as hallmarks of this induced resistance stage. This review thus focuses on the recognition of elicitors by *Vitis vinifera* describing the molecular mechanisms triggered from the elicitor perception to the activation of immune responses. Finally, we discuss the fact that the link between elicitation and induced resistance is not so obvious and that the formulation of resistance inducers remains a key step before their application in vineyards.

Keywords: *Vitis vinifera*, innate immunity, defense responses, Pattern Recognition Receptor (PRR), Microbe-Associated Molecular Pattern (MAMP), Damage-Associated Molecular Pattern (DAMP), Induced Resistance (IR)

INTRODUCTION

Vitis vinifera cultivars, cultivated worldwide for the production of table grape and wines, are susceptible to various pathogens, such as insects, viruses, bacteria, phytoplasmas, fungi, and oomycetes. The last two groups of microorganisms can rapidly and strongly impact the yield and the quality of the harvest. For example, the oomycete *Plasmopara viticola* and the ascomycete *Erysiphe*

necator, the causal agents of downy and powdery mildews, respectively, infect leaves but also inflorescences and young green berries, while *Botrytis cinerea*, a necrotrophic fungus causing gray mold, affects berries during ripening. These devastating diseases are currently managed mainly through repeated treatments with synthetic fungicides for grape protection. However, the intensive use of chemicals has a negative impact on environment and human health and contributes to the selection of resistant pathogenic strains (Casida, 2009; Nanni et al., 2016). Thus, in the context of the integrated pest management, the implementation of a sustainable viticulture requires alternative/complementary strategies to chemical treatments. Among them, a recognized approach for crop protection is the stimulation of natural plant defense to trigger induced resistance (IR) (Walters et al., 2013; Delaunois et al., 2014).

Plants detect pathogen attack through the perception of conserved microbial signatures, called microbe-associated molecular patterns (MAMPs) or host-derived damage-associated molecular patterns (DAMPs), thanks to plant plasma membrane pattern recognition receptors (PRRs) (Boutrot and Zipfel, 2017). PRRs are generally receptor-like kinases (RLKs) or receptor-like proteins (RLPs) with an extracellular domain for MAMP/DAMP recognition (Trdá et al., 2015). MAMPs can be microbial structural components such as bacterial flagellin, lipopolysaccharides (LPSs), peptidoglycans (PGNs), rhamnolipids (RLs), fungal chitin, or oomycete β-glucans but also secreted toxins or enzymes like fungal xylanase or endopolygalacturonase (**Figure 1**). DAMPs are endogenous danger signals, such as oligogalacturonides (OGs) or cutin monomers, released from degraded plant cell wall or cuticle, respectively (Boller and Felix, 2009). The MAMP/DAMP perception triggers a complex cascade of signaling events, including ion fluxes such as Ca^{2+} influx, the production of reactive oxygen species (ROS) and nitric oxide, and the activation of mitogen-activated protein kinases (MAPKs). These early events induce a massive transcriptional reprogramming of primary and secondary metabolisms. The induction of specific defense-related genes leads to the synthesis of (i) pathogenesis-related (PR) proteins, including hydrolytic enzymes (e.g., β-1,3-glucanases or chitinases), which degrade pathogen cell wall; (ii) antimicrobial compounds like phytoalexins; (iii) compounds involved in cell wall reinforcement; and, in some cases, (iv) proteins involved in the hypersensitive response (HR), a form of programmed host cell death (Garcia-Brugger et al., 2006). Altogether, these defense reactions represent the MAMP-triggered immunity (MTI), which is finely regulated by phytohormones (salicylic acid [SA], jasmonic acid [JA], and ethylene [ET]) to finally prevent or delay pathogen infection, thus ensuring a basal plant resistance to pathogens.

The possibility of activating plant immunity using MAMPs or DAMPs led to the development of IR strategy to protect crops against pathogens. Indeed, the application of elicitors mimics a pathogen attack, stimulating basal plant defenses and thus leading to plant resistance, at least in controlled conditions (Wiesel et al., 2014). Elicitors can be purified MAMPs or DAMPs, molecules displaying similar structures or even extracts derivate from

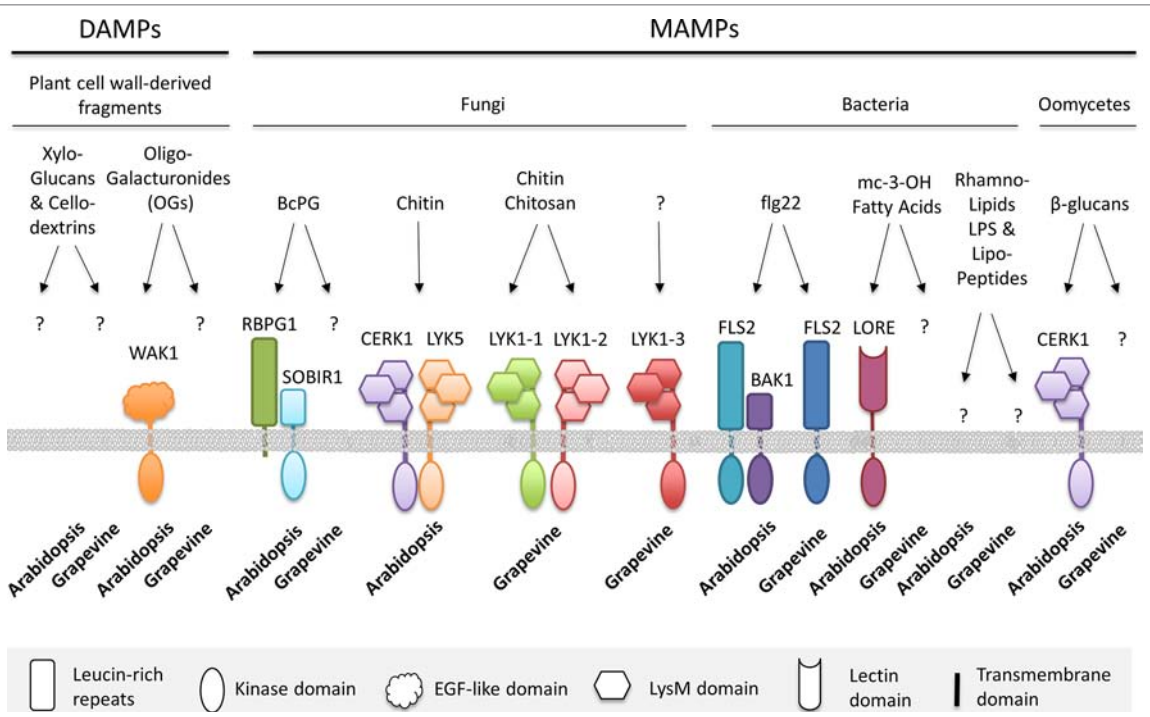

FIGURE 1 | Microbe- and damage-associated molecular patterns (MAMPs, DAMPs) recognized by grapevine or *Arabidopsis* pattern recognition receptors (PRRs), which trigger plant immunity. BAK1, brassinosteroid-insensitive 1 (BRI1)–associated receptor kinase 1; BcPG, *B. cinerea* polygalacturonase; CERK1, chitin elicitor receptor kinase 1; EGF, epidermal growth factor; FLS2, flagellin sensing 2; LORE, Lipooligosaccharide-specific reduced elicitation; LPS, lipopolysaccharide; LYK, lysin motif-containing receptor-like kinase; OGs, oligogalacturonides; RBPG1, responsiveness to botrytis polygalacturonases 1; SOBIR1, suppressor of BIR1; WAK1, wall-associated kinase1.

microorganisms or plants, and can have a biological or synthetic origin (Walters and Fountaine, 2009). For grapevine, several elicitors of various origins or structural patterns have been studied so far, and their mode of action has been well characterized (Delaunois et al., 2014). Some elicitors are able to induce a good level of resistance against pathogens in controlled conditions. For example, the β-glucan laminarin and its sulfated derivative (PS3) were shown to protect grapevine against *P. viticola* (Aziz et al., 2003; Trouvelot et al., 2008). Chitin and its deacetylated derivative chitosan act as MAMPs (Brulé et al., 2019) and induce grapevine resistance against *P. viticola*, *E. necator*, and *B. cinerea* (Trotel-Aziz et al., 2006; Aziz et al., 2007). Recently, it has been shown that a mixture of chitosan and OGs (COS-OGA) protects grapevine against powdery mildew in vineyards (van Aubel et al., 2014). Nevertheless, it is known that the efficacy of IR in the field is variable, depending on various biotic and abiotic factors (Adrian et al., 2012; Delaunois et al., 2014). Therefore, it is crucial to get a better understanding of IR mechanisms and to identify associated molecular markers.

In this review, we focus on the current knowledge about MAMPs/DAMPs which trigger immune responses in grapevine and also on their cognate PRRs in this plant. Moreover, we discuss the relation between the elicitation of immune responses and the actual IR, as well as the perspectives to improve the effectiveness of resistance inducers in a perspective of field application.

ACTIVE MAMPs AND DAMPs PERCEIVED BY *VITIS VINIFERA*

The *B. cinerea* Endopolygalacturonase 1 (BcPG1) and its Activity-Derived Products (OGs) Are Distinct Interconnected Elicitors That Activate Independently Grapevine Immune Responses

Bacterial or fungal necrotrophic phytopathogens secrete numerous plant cell wall–degrading enzymes (CWDEs) to break down the cell wall polymers, such as cellulose, hemi-cellulose, and pectin, and invade host tissues. The degradation of host cell wall then contributes to the development of soft rots or molds. Among necrotrophs, *B. cinerea*, an opportunistic plant pathogenic fungus able to cause rot in many plant tissues, including in grapevine, produces a variety of pectinases, including exo- and endopolygalacturonases, pectin methylesterases, pectin, and pectate lyases (ten Have et al., 2002; Zhang and van Kan, 2013). In particular, its genome encodes for six endopolygalacturonases (BcPG1-6), the expression of which varies depending on plant tissues (ten Have et al., 1998; ten Have et al., 2001). The biochemical characterization of their enzymatic activity and the production of deletion mutants of *BcPG1* and *BcPG2* demonstrated their capacity to produce tissue collapse and necrosis as well as their role in *B. cinerea* virulence on tomato, apple, broad bean, and *Arabidopsis thaliana* (hereafter *Arabidopsis*) (ten Have et al., 1998; Kars et al., 2005). The availability of a set of endoPGs with slightly different characteristics in terms of substrate specificity might enable the pathogen to hydrolyze a larger spectrum of pectin types, originating from different host species.

The degradation of pectin by polygalacturonases leads to the release of intermediate products, called OGs, which have been shown to act in vivo as DAMPs, protecting plants from infection by necrotrophic pathogens (Benedetti et al., 2015). OGs are α-1,4-linked galacturonosyl residues with different degrees of polymerization (DP) and esterification, which may influence their elicitor activity. Indeed, based on several transcriptomic studies, long OGs (DP > 10) have long been thought to be the only efficient pectin fragments for triggering plant defense responses (Moscatiello et al., 2006; Ferrari et al., 2007; Denoux et al., 2008). However, although short OGs suppress defense responses in wheat (Moerschbacher et al., 1999), they can also induce a strong defense gene expression in other plants, such as *Arabidopsis*, potato, and tomato (Simpson et al., 1998; Norman et al., 1999). Recently, a comparison of gene expression reprogramming, induced by both long (DP > 10) and short (DP3) OGs, revealed actually a great similarity of the response, in qualitative and quantitative terms, confirming that both long and short OGs are potentially biologically active, at least in *Arabidopsis* (Davidsson et al., 2017).

The formation of OGs during plant-pathogen interactions is promoted by polygalacturonase-inhibiting proteins (PGIPs) (Kalunke et al., 2015). These plant cell wall proteins selectively and partially inhibit PGs produced by pathogenic microorganisms allowing the accumulation of OGs, as intermediate reaction products, which are active elicitors of plant defense responses. In particular, expression of the grapevine VvPGIP1 in tobacco was shown to improve plant resistance against *B. cinerea*, through the partial inhibition of some fungal PGs, including BcPG1 (Joubert et al., 2006). On the other hand, OGs can be inactivated by berberine bridge enzyme (BBE)–like proteins, which act as specific OG oxidases (Benedetti et al., 2018). Through such *in vivo* OG oxidase (OGOX) activity, oxidized OGs become less active as defense inducers and less susceptible to hydrolysis by fungal PGs. It has been proposed that this mechanism may prevent the deleterious excessive OG accumulation, which may compromise plant growth and resistance through cell death induction, when reaching high concentrations (Cervone et al., 1987). Although OGOX activity has still to be identified in grapevine, it is reasonable to assume that OG turnover may be controlled in the same way to ensure grapevine growth-immunity trade-off.

Some strains of *B. cinerea* are unable to develop on grapevine, likely due to the induction of strong plant defense responses and the incapacity of the fungus to counteract such defense mechanisms (Derckel et al., 1999). Accordingly, an eliciting activity was found in the filtrate of the T4 strain of *B. cinerea*, nonpathogenic on grapevine, and, surprisingly, attributed to the protein BcPG1 (Poinssot et al., 2003). The protein BcPG1 was also more abundant in the nonpathogenic A336 mutant of *B. cinerea*, impaired in plant colonization capacity, and compared to the wild-type pathogenic strain Bd90, and this larger BcPG1 amount was correlated with the induction of a strong oxidative burst in grapevine cells (Kunz et al., 2006). It was thus assumed that the overproduction of BcPG1 may boost plant defense reactions against the mutant strain, thus contributing to its reduced pathogenicity. Interestingly, BcPG1 elicitor activity was not related to its enzymatic activity, i.e., from release of OGs from grapevine cell wall, but rather relied on the recognition of the protein itself (Poinssot et al., 2003). In particular,

besides the overall lower intensity of defense responses induced by OGs compared to BcPG1, grapevine cells pre-treated with OGs were not desensitized to the successive application of BcPG1, and vice versa, indicating the existence of two different receptors/pathways leading to defense induction (**Figure 2A**). Moreover, BcPG1 elicitor and enzymatic activities were discriminated by inhibiting selectively one or the other through different protein treatments (Poinssot et al., 2003). This study thus demonstrated that BcPG1 functions as a MAMP and that particular protein patterns may be responsible for defense induction, as previously reported for other CWDEs, such as the 22-kDa fungal protein ethylene-inducing xylanase (EIX), which *per se* functions as an elicitor, independently of its enzymatic activity (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Rotblat et al., 2002). The challenging aspect in the elucidation of plant defense mechanism induction is the identification of perception systems, which have been often characterized using the model plant *Arabidopsis*, allowing genetic studies. Wall-associated kinases (WAKs) have been proposed for a long time as candidates for the perception of OGs (**Figure 1**). These proteins possess a typical intracellular Ser/Thr kinase domain and an apoplastic domain containing several epidermal growth factor (EGF)–like repeats interacting with the cell wall. The role of WAK RLKs in plant immunity has been demonstrated in several plant species, including *Arabidopsis*, rice, maize, and more recently tomato and wheat (Diener and Ausubel, 2005; Zhang et al., 2005; Li et al., 2009; Rosli et al., 2013; Hurni et al., 2015; Zuo et al., 2015; Delteil et al., 2016; Harkenrider et al., 2016; Zhang et al., 2017; Saintenac et al., 2018; Yang et al., 2019). In *Arabidopsis*, WAKs are encoded by five tightly linked and highly similar genes and several reports demonstrated in particular the capacity of WAK1 to bind OGs *in vitro* through the N-terminal ectodomain (Wagner and Kohorn, 2001; Decreux et al., 2006; Cabrera et al., 2008). Moreover, *WAK*1 was the only member of the gene family to be up-regulated in response to OGs treatment (Denoux et al., 2008). To overcome the limits of reverse genetics due to redundancy and lethality of WAK silencing (Wagner and Kohorn, 2001), an elegant domain swap approach, using the construction of a chimeric receptor, demonstrated the function of WAK1 as a receptor of OGs and its role in defense response induction in *Arabidopsis* (Brutus et al., 2010). WAK1-mediated OG-induced responses were shown to be then negatively regulated by two WAK1 interactors, namely, the glycin-rich protein GRP-3 and a kinase-associated protein phosphatase (KAPP), which likely contribute to phase out the plant immune response (Gramegna et al., 2016).

On the other hand, by exploiting the capacity of *B. cinerea* PGs to induce different levels of necrosis formation in different *Arabidopsis* accessions, a map-based cloning strategy combined with comparative and functional genomics allowed the identification of the RLP RBPG1 (RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES 1) as endoPG receptor in *Arabidopsis* (Zhang et al., 2014). Lacking a cytoplasmic functional domain, the RBPG1 function as PRR relies on its interaction with the RLK SOBIR1 (for SUPPRESSOR OF BIR1). The formation of the RBPG1/SOBIR1 heterodimer, unaffected by the BcPG3 ligand, likely ensures intracellular signal transduction through SOBIR1 kinase activity (**Figure 1**). RBPG1 not only interacts with BcPG3 but also recognizes multiple active and inactive PGs from *B. cinerea*. Since the response of *Arabidopsis* to PGs appears different from that reported in other plant species (Kars et al., 2005; Joubert et al., 2007; Zhang et al., 2014), the identification of homologs of AtRBPG1, or other RLPs, should be carried out in *V. vinifera* to characterize its perception system for BcPG1.

Following OGs and BcPG1 perception, several studies reported a detailed description of the induced signaling events using pharmacological, biochemical, and genetic approaches. Both elicitors trigger an increase of cytosolic Ca^{2+} concentration

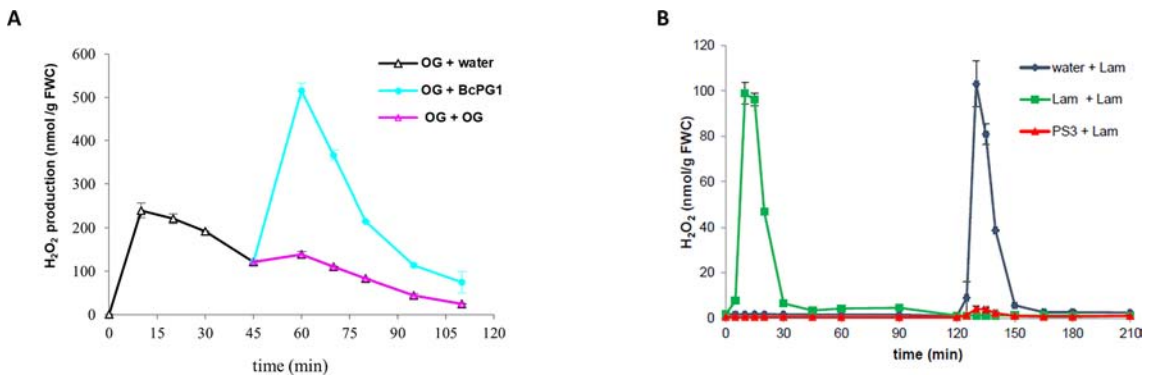

FIGURE 2 | Desensitization experiments can differentiate MAMPs/DAMPs perceived by independent or common receptors in grapevine. **(A)** Desensitization experiments reveals that BcPG1 and OGs are perceived differently by grapevine cells as the H_2O_2 production is abolished after two successive treatments with OG (pink) but not when BcPG1 (cyan) succeeds to a first treatment with OGs (black). Cells were first treated at time 0 with OGs (200 µg/ml; black), washed three times between 30 and 45 min with fresh medium, then treated a second time with OGs (200 µg/ml; pink), BcPG1 (0.2 µg/ml; cyan), or water (black). H_2O_2 production was determined using chemiluminescence of luminol as described by Poinssot et al. (2003). **(B)** Refractory state experiments revealed that the two β-glucan derivatives (PS3 and Lam) might be perceived by the same receptor in grapevine cells as a first treatment with PS3 abolishes the oxidative burst normally elicited by laminarin. Cells were first treated at time 0 with water (black), laminarin (Lam, 4 mg/ml; green), or PS3 (4 mg/ml; red); washed three times between 90 and 120 min with fresh medium; and then treated a second time with laminarin (4 mg/ml). H_2O_2 production was determined using chemiluminescence of luminol as described by Gauthier et al. (2014). Data are means ± SE of duplicates, representative of three independent experiments. FWC, fresh weight of cells.

($[Ca^{2+}]_{cyt}$) (Lecourieux et al., 2002; Vandelle et al., 2006). Ca^{2+} increase controls in turn the oxidative burst, which involves the activity of NADPH oxidases (Vandelle et al., 2006; Galletti et al., 2008) but also the production of nitric oxide (NO) (Vandelle et al., 2006; Rasul et al., 2012) by a NO synthase activity that still needs to be further characterized (Jeandroz et al., 2016). Independently of the above-mentioned physiological events, both BcPG1 and OGs activate two MAPKs related to AtMPK3 and AtMPK6 (Vandelle et al., 2006; Denoux et al., 2008; Galletti et al., 2011). Finally, as later events, both elicitors induce a set of defense-related genes and the production of phytoalexins, contributing to plant resistance.

The secretion of CWDEs by *B. cinerea* occurs constitutively. In particular, *Bcpg1* (together with *Bcpg2*) is among the earliest expressed fungal genes during the infection of tomato leaves, suggesting that the corresponding enzyme may play a role in early stages of the infection process (ten Have et al., 2001). Since the activation of PRR-mediated immunity aims at inhibiting pathogen development at early stages of infection (Melotto et al., 2006; Zeng and He, 2010), the early BcPG1 expression fits well with a possible rapid perception of the pathogen by grapevine cells. Moreover, its crucial role in pathogenicity makes it an indispensable element for the pathogenic fungus, and this supports further plant adaptation to recognize this protein that may remain stable in fungus genome to maintain its virulence, concept at the basis of the gene-for-gene/guard and decoy models (van der Hoorn and Kamoun, 2008). We could thus assume a scenario in which BcPG1 may represent the main elicitor of defense reactions. However, in case of perception failure by host cells, for instance in absence of the proper receptor, the enzymatic activity of BcPG1, regulated by PGIPs, would lead to the production of active OGs able to trigger grapevine immunity. Although less intense, this alternative defense layer may ensure the induction of a general mechanism of resistance to fight against *B. cinerea* infection and would be secured by the further inactivation of OGs by OGOXs once reached a concentration threshold.

The Cell Wall–Derived Xyloglucans and Cellodextrins Are New DAMPs That Elicit Immune Responses in Grapevine

It has been previously reported that xyloglucan oligosaccharides, the main component of hemicellulose, play a role in cell wall structure (Takeda et al., 2002; Whitney et al., 2006), in the development and the regulation of plant growth in different plant species (Fry et al., 1993; Vargas-Rechia et al., 1998) and can also act as a storage polysaccharide to provide energy for the emerging seedlings (dos Santos et al., 2004). Interestingly, xyloglucans were recently discovered as new DAMPs in grapevine and *Arabidopsis* (Claverie et al., 2018).

In grapevine, xyloglucans elicit a broad range of defense responses, including the activation of two MAPKs and the production of the phytoalexin resveratrol, correlated with the induction of the expression of defense genes, involved in particular in the phenylpropanoid pathway and resveratrol biosynthesis. Importantly, xyloglucans are able to protect grapevine detached leaves against *B. cinerea* (Claverie et al., 2018). Besides grapevine, xyloglucans also trigger the phosphorylation of MAPKs, defense gene expression, and callose deposition, through the callose synthase PMR4 in *Arabidopsis* (Luna et al., 2011), and confer resistance against *B. cinerea* and the biotrophic oomycete *Hyaloperonospora arabidopsidis*. The use of *Arabidopsis* mutants allowed to provide evidence that xyloglucan-mediated resistance against *B. cinerea* involves different defense pathways, including camalexin, SA, JA, and ethylene (Claverie et al., 2018). Compared to OGs, the immune responses induced by xyloglucans are strikingly different in terms of kinetics and strength of the response or activated immune responses. Indeed, xyloglucans do not elicit H2O2 production neither in grapevine nor in *Arabidopsis* cells, while OGs trigger resistance against *B. cinerea* independently of SA, JA, and ethylene (Ferrari et al., 2007). These results suggest that different DAMPs could operate through different signaling pathways.

Xyloglucan oligomers are characterized by a β-1,4 glucan backbone resembling that of cellodextrins (CDs). CDs and cellobiose are cellulose-derived oligomers from the cell wall that also act as DAMPs, eliciting $[Ca^{2}+]_{cyt}$ variations, defense gene activation, and protection against pathogens in *Vitis vinifera* and *Arabidopsis* (Aziz et al., 2007; Souza et al., 2017). As shown for OGs, the elicitor activity of CDs can be impaired by a member of the BBE-like protein family in *Arabidopsis*, namely, the cellodextrin oxidase (CELLOX), via the oxidation of their reducing end, likely as a general mechanism of BBE-like proteins to avoid oligosaccharide hyperaccumulation (Locci et al., 2019). Moreover, a poly(A)ribonuclease controls the cellotriose-based interaction between *Arabidopsis* and the plant-growth-promoting endophyte *Piriformospora indica* suggesting a major role of this enzyme in regulating the outcome of this beneficial interaction (Johnson et al., 2018).

The structure of the oligosaccharide elicitors also plays an important role in their biological activity. For example, xyloglucans used in our study were mainly constituted of a β-1,4-glucan backbone associated with xylosyl-, galactosyl-, and fucosyl-type branching, with a DP of 7 (Claverie et al., 2018). The order and the type of substituents depend on the plant species, the cell type, and the developmental state of the cell (Pauly and Keegstra, 2016). As indicated above for OGs, the biological activity of oligosaccharides is known to be dependent on their DP and decoration pattern. Accordingly, only CDs with a DP > 7 are able to induce a strong production of H2O2, the overexpression of defense genes associated with an increase in chitinase and glucanase activities, finally leading to an IR against *B. cinerea* (Aziz et al., 2007). The future comparison of immune responses triggered by xyloglucans possessing different carbohydrate decorations would therefore deserve further investigations and might lead to interesting applications for crop protection.

Overall, the activation of immune responses in grapevine and *Arabidopsis* strengthens the notion that these two plant species can perceive hemicellulose and cellulose–derived oligomers and that at least one cognate receptor exists. As *Arabidopsis* perceive xyloglucans and cellobiose, it should be envisaged to first isolate their PRR(s) through a genetic approach, and then identify their orthologs in *V. vinifera*.

The Grapevine LysM Receptors VvLYK1-1 and VvLYK1-2 Recognize Chitin and Chitosan Fragments to Elicit Similar Immune Responses

Chitooligosaccharides (COS), especially chitin and chitosan, are representative MAMPs known to induce immune responses in a wide range of plant species such as *Arabidopsis* (Miya et al., 2007; Cao et al., 2014) or rice (Hayafune et al., 2014). Chitin, a linear homopolymer of β-1,4 *N*-acetylglucosamine (GlcNAc), is a major structural component of fungal cell walls, Crustacean shells, and the exoskeleton of insects and nematodes. Chitosan, the deacetylated chitin derivative produced by chitin deacetylases, is also a naturally occurring polysaccharide, albeit less common. Chitosan can be notably found in some fungal species such as *Cryptococcus neoformans* (Baker et al., 2007). Since the 1980s, chitin and its deacetylated product chitosan have been used for crop farming as biopesticides, biofertilizers, for seed coating formulation, and agricultural film (El Hadrami et al., 2010; Hadwiger, 2013; Trouvelot et al., 2014). Both molecules are known to induce the biosynthesis of several antimicrobial compounds, namely, phytoalexins (Vasiukova et al., 2001), callose (Ren et al., 2001), and lignin (Barber et al., 1989). Many studies have reported that COS enhance plant defenses against bacteria (Rabea et al., 2003; Tikhonov et al., 2006), fungi (Park et al., 2002; Trotel-Aziz et al., 2006) and nematodes (Khalil and Badawy, 2012; Nunes da Silva et al., 2014).

Chitin hexa-, hepta-, or octamers are recognized by the lysin motif (LysM)-RLK chitin elicitor receptor kinase 1 (CERK1/LYK1) and LysM-containing receptor-like kinase5 (LYK5) in *Arabidopsis* (Miya et al., 2007; Wan et al., 2008; Petutschnig et al., 2010; Cao et al., 2014), by the complex OsCERK1/chitin elicitor-binding protein (OsCEBiP) in rice (Kaku et al., 2006; Shimizu et al., 2010) or by their ortholog complex MtLYK9/MtLYR4 in legumes such as *Medicago truncatula* (Bozsoki et al., 2017). Plant LysM domain proteins are required for chitin-binding and signaling and serve as modules mediating plant immunity and stopping pathogen infection. For instance, in line with AtCERK1 function in chitin recognition in *Arabidopsis*, the corresponding knockout mutant *Atcerk1/lyk1*, which completely lost its ability to respond to the elicitor, is more susceptible to the fungal pathogen *Alternaria brassicicola* and to the bacterial pathogen *Pseudomonas syringae* (Miya et al., 2007; Gimenez-Ibanez et al., 2009). In grapevine, a phylogenetic analysis highlighted three LysM proteins VvLYK1-1, VvLYK1-2, and VvLYK1-3, among 15, which are putative orthologues of the *Arabidopsis* AtCERK1/LYK1 and the rice OsCERK1 (Brulé et al., 2019). The functional complementation of the *Arabidopsis Atcerk1* mutant with the three grapevine orthologs of *AtCERK1/LYK1* demonstrated that the constitutive expression of *VvLYK1-1* or the inducible expression of *VvLYK1-2*, but not VvLYK1-3, restored COS-induced immune responses (Brulé et al., 2019). These results suggested that the same PRRs, VvLYK1-1, and VvLYK1-2 are involved in COS-triggered immunity in grapevine (**Figure 1**). Moreover, *VvLYK1-1* expression in the *Atcerk1* mutant background was demonstrated to restore non-host resistance against the grapevine powdery mildew *E. necator*.

Downstream *V. vinifera* receptor activation, chitosan elicits phytoalexins, chitinase, and glucanase activities leading to resistance against *B. cinerea* and *P. viticola* (Aziz et al., 2006; Trotel-Aziz et al., 2006). More precisely, hexamers of chitin and chitosan elicit the phosphorylation of MAPKs, phytoalexin production, and expression of defense genes, finally inducing resistance against the necrotrophic fungus *B. cinerea* and the obligate biotrophic oomycete *P. viticola* (Brulé et al., 2019). However, chitin hexamer do not induce any oxidative burst, contrary to what is observed in *Arabidopsis* (Miya et al., 2007).

The Flagellin-Derived Peptides Flg22 From Pathogenic or Beneficial Bacteria Recognized by the Grapevine VvFLS2 Receptor Do Not Elicit Similar Immune Responses

Flagellin, the main building protein of eubacterial flagella, is a potent defense elicitor in different plant species and is one of the best studied MAMPs. Plants perceive flagellin mainly through the N-terminal conserved epitope of 22 amino acids, called flg22. Flg22 peptide from *Pseudomonas aeruginosa* is a commonly used MAMP to study the responses to flagellin in a broad variety of plant species (Boller and Felix, 2009). Using *V. vinifera* cell suspensions, flg22 was shown to be also an active MAMP in grapevine (Trdá et al., 2014).

The plant PRR responsible for flagellin/flg22 perception is the leucine-rich repeat receptor like kinase (LRR-RLK) flagellin sensing 2 (FLS2) (Gomez-Gomez and Boller, 2000; Chinchilla et al., 2006). FLS2 was first identified in *Arabidopsis* (AtFLS2) (Gomez-Gomez and Boller, 2000), and functional FLS2 orthologues were further shown to be conserved within the plant kingdom (Boller and Felix, 2009). Among them, a clear orthologue of *AtFLS2* was identified in the grapevine genome, namely, *VvFLS2*, encoding a protein displaying a conserved domain structure. The functionality of VvFLS2 as a grapevine flagellin receptor (**Figure 1**) was demonstrated through the successful complementation of the corresponding *Arabidopsis* mutant *fls2*, which successfully restored flg22-induced H2O2 production (Trdá et al., 2014), as previously shown for the rice OsFLS2 or the tomato LeFLS2 (Takai et al., 2008; Mueller et al., 2012). At molecular level, the perception of flg22 in grapevine by VvFLS2 triggers common rapid and transient signaling responses, such as an increase in free $[Ca^{2+}]_{cyt}$, the phosphorylation of two MAPKs with relative molecular masses of 45 and 49 kDa and an oxidative burst. Later, flg22 perception activates the expression of a set of defense genes (e.g., genes encoding the acidic chitinase Chit4c and the protease inhibitor PR6). Finally, flg22-elicited responses leads to a partial resistance of grapevine against *B. cinerea* (Trdá et al., 2014).

Previous studies have reported different recognition specificities between FLS2 from tomato and *Arabidopsis* (Felix et al., 1999; Bauer et al., 2001; Sun et al., 2006; Robatzek et al., 2007; Mueller et al., 2012). Interestingly, expressing *VvFLS2* in the *fls2* background of *Arabidopsis* conferred a flagellin responsiveness profile specific to grapevine. Indeed, while different flg22 peptides, derived from different bacteria, exhibited comparable biological activities in *Arabidopsis*, they showed distinct eliciting activities in grapevine, and such species-specific differences in flg22 perception

were caused, at least in part, by the nature of *Arabidopsis* and grapevine FLS2 proteins. *Burkholderia phytofirmans* is a plant growth-promoting rhizobacterium (PGPR) naturally associated with grapevine (Ait Barka et al., 2000; Compant et al., 2005; Lo Piccolo et al., 2010) which modifies grapevine metabolism thus promoting an improved plant growth, a better tolerance to *B. cinerea* infection as well as to cold stress (Ait Barka et al., 2000; Ait Barka et al., 2006; Fernandez et al., 2012; Theocharis et al., 2012). Furthermore, it can also trigger grapevine defense responses, including extracellular medium alkalization and defense gene expression, suggesting that it is potentially perceived *via* MAMP detection (Bordiec et al., 2011). Accordingly, the flagellin purified from *B. phytofirmans* induces *PR1* gene expression in *Arabidopsis*. Similarly, flg22 from this endophytic bacterium (*Bp* flg22) triggers immune responses also in grapevine, but weaker than the responses triggered by flg22 peptides derived from the pathogenic bacteria *Xanthomonas campestris* (*Xc* flg22) (**Table 1**) or *P. aeruginosa* (*Pa* flg22) (Trdá et al., 2014). In addition, *Bp* flg22-triggered gene expression is very low and transient compared with the long-lasting effect of *Xc* flg22 and *Pa* flg22 treatment, and some genes—for instance, *PR6* or the grapevine SA marker gene *17.3* (Bordiec et al., 2011), are not significantly activated by *Bp* flg22 treatment. Finally, unlike *Xc* flg22, *Bp* flg22 does not significantly inhibit grapevine plantlet growth (Trdá et al., 2014). While *B. phytofirmans* is naturally colonizing grapevine, it can colonize *Arabidopsis* only under laboratory conditions (Poupin et al., 2013; Zuniga et al., 2013), and it was therefore proposed that alterations in the *Bp* flg22 sequence might represent a successful adaptation of *B. phytofirmans* to avoid recognition by the host VvFLS2 and activation of immune system.

Key amino acids described as crucial for flg22-eliciting activity (Felix et al., 1999; Bauer et al., 2001; Sun et al., 2006) are unchanged in *Bp* flg22. Nevertheless, three amino acid substitutions between the most active peptide in grapevine *Xc* flg22 and *Bp* flg22 are sufficient to explain this different sensitivity. The pathogenic bacterium responsible for crown gall disease, *Agrobacterium tumefaciens*, also possesses 12 mutations in flg22 avoiding the FLS2 recognition and the subsequent immune responses (**Table 1**). On the other hand, the fact that AtFLS2 and VvFLS2 do not similarly perceive different flg22 epitopes, especially *Bp* flg22, suggests that VvFLS2 has evolved to distinguish flagellin originating from the grapevine-associated PGPR *B. phytofirmans*. Therefore, VvFLS2 and/or flagellin from *B. phytofirmans* may have undergone evolutionary changes allowing the adapted endophytic bacterium to colonize its natural host plants without inducing a strong MTI. Of interest, not only *B. phytofirmans* evades perception by grapevine receptor, but it also seems to overcome flg22-induced MTI to efficiently colonize grapevine plants. Indeed, the application of the strong elicitor *Xc* flg22 to the roots of grapevine plantlets during the first stage of bacterization did not affect the process of plant colonization by *B. phytofirmans*. VvFLS2 thus represents the first example of a characterized receptor that differentially recognizes flg22 epitopes from a PGPR and plant-pathogenic bacteria, suggesting an evolutionary mechanism of grapevine innate immunity evasion by its beneficial endophytic bacterium *B. phytofirmans*.

Rhamnolipids, LPS, and Cyclic Lipopeptides Are Recognized by Grapevine and Trigger-Induced Resistance

Amphiphilic glycolipid and lipopeptide compounds have been recently identified as effective inducers of immune response in grapevine. Among them, bacterial RLs are the best characterized. Within the first hour after treatment, RLs from *P. aeruginosa* and *Burkholderia plantarii* trigger strong early signaling events in grapevine cell suspensions as well as defense markers, including genes encoding PR proteins or enzymes involved in oxylipin and phytoalexin biosynthesis pathways, and a programmed cell death reminiscent of animal apoptosis at high concentrations (Varnier et al., 2009). Furthermore, RLs potentiate defense responses induced by other elicitors and induce a local resistance of grapevine against the necrotrophic fungus *B. cinerea* (Varnier et al., 2009; Vatsa et al., 2010) but also an effective protection of *Brassica napus* and *Arabidopsis* toward fungal diseases (Sanchez et al., 2012; Monnier et al., 2018). In *Arabidopsis*, RL-mediated resistance involves different signaling pathways that depend on the type of pathogen. While JA is essential for the resistance to *B. cinerea*, the ET-mediated signaling pathway is involved in *Arabidopsis* resistance to the oomycete *H. arabidopsidis* and the bacterium *Pseudomonas syringae* pv. *tomato* (Sanchez et al., 2012). SA-dependent plant defenses are primed by RLs following pathogen infections suggesting that SA also participates to the restriction of these pathogens. How RLs are perceived by plant cells is still unclear. A recent report suggested that RLs could insert within the lipid bilayer of plant membranes leading to a moderate membrane destabilization influenced by the nature of the phytosterols. The resulting changes in lipid dynamics could have a direct impact on plant defense induction (Monnier et al., 2019). In grapevine, RLs trigger a very strong Ca^{2+} influx (Varnier et al., 2009), with concentrations of free cytosolic calcium reaching values higher than the concentrations detected

TABLE 1 | Intensity of the immune responses, in *Vitis vinifera* and *Arabidopsis thaliana*, following treatment with 1 μM of flagellin-derived flg22 epitopes derived from beneficial or pathogenic bacteria. ++, highly induced; +, induced; +/–, weakly induced; –, not induced; PGPR, plant growth-promoting bacteria. Results from Trdá et al., 2014.

Bacteria	Type	Flagellin epitope	*Vitis vinifera*			*Arabidopsis thaliana*		
			H_2O_2 production	Defense gene expression	Plant growth inhibition	H_2O_2 production	Defense gene expression	Plant growth inhibition
B. phytofirmans	PGPR	Bp flg22	+/–	+/–	+/–	++	++	++
X. campestris	Pathogen	Xc flg22	++	++	++	++	++	++
A. tumefaciens	Pathogen	At flg22	–	–	–	–	–	–

after the treatment of tobacco cell suspensions with the potent elicitor cryptogein (Lamotte et al., 2004). This Ca^{2+} signature seems to be very characteristic when compared with the signatures described for other elicitors (Garcia-Brugger et al., 2006) and could be related to the potential destabilization of grapevine cell membranes. Synthetic RL structures that induce a plant immune response are also able to interact with lipids from plant membrane models, suggesting that the perception of these molecules could involve a lipid-driven process (Nasir et al., 2017; Luzuriaga-Loaiza et al., 2018).

LPSs are complex amphiphilic glycolipids composed of a lipidic part (lipid-A), a conserved oligosaccharidic core, and an O-polysaccharidic part with variable length and composition (OPS) (Caroff and Karibian, 2003). They are present in the outer bilayer of the plasma membrane of most Gram-negative bacteria. While the lipid-A and the oligosaccharidic core are relatively well conserved, the OPS is chemically and structurally very heterogeneous among species. OPS is composed of polymers of several monosaccharide repeat units, and its variability depends on bacterial species (Lerouge and Vanderleyden, 2002). While the lipid-A is embedded in the outer membrane, the OPS is oriented outward of the membrane, thus being directly at the interface between the bacterium and the host during the interaction. The OPS plays an essential role in stress resistance, protecting bacteria against hostile environment and is considered as an important virulence factor for pathogenic bacteria during the colonization of their host (Clifford et al., 2013; Di Lorenzo et al., 2016). Phytopathogenic bacteria lacking OPS are generally considered less viable inside the host plant and are therefore expected to be non-virulent. For example, OPS mutants of *Erwinia amylovora*, *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. *citri*, or *Xylella fastidiosa* are significantly less virulent than their respective wild-type strains (Berry et al., 2009; Petrocelli et al., 2012; Clifford et al., 2013; Li et al., 2014). In contrast, host plants have developed perception systems of these LPSs leading to the induction of defense mechanisms to counter the invasion of pathogenic bacteria (Ranf et al., 2016). LPSs are considered as MAMPs and induce defensive responses, including ROS production, in several plant species such as *Arabidopsis*, tobacco, rice, or grapevine (Braun et al., 2005; Silipo et al., 2005; Desaki et al., 2006; Rapicavoli et al., 2018). In grapevine, *Agrobacterium vitis* and *Xylella fastidiosa*, two Gram-negative pathogenic bacteria, cause crown gall and Pierce's disease, respectively. *A. vitis* LPS extracts infiltrated into grapevine internodes protect the plant from tumor appearance following *A. vitis* infection (Alexandrova et al., 2000). Similarly, LPSs purified from *X. fastidiosa* induce a rapid production of ROS in grapevine leaf disks. Interestingly, OPS-deficient *X. fastidiosa* bacteria cause a higher production of ROS in vitro and in vivo compared to wild-type bacteria (Rapicavoli et al., 2018). Grapevine plants pre-treated with LPSs from the OPS-deficient *X. fastidiosa* strain showed a strongly attenuated development of the symptoms caused by *X. fastidiosa* in comparison with grapevine plants pre-treated with purified LPSs from wild-type *X. fastidiosa* strain (Rapicavoli et al., 2018). The presence of the OPS therefore delays the immune perception of *X. fastidiosa* by grapevine cells, probably by masking the MAMP motifs on the cell surface. The OPS is also likely involved in bacterial virulence, especially in biofilm formation and motility, and therefore related to its ability to enter and spread within the host (Kutschera and Ranf, 2019). In *Arabidopsis*, it was recently shown that the lectin receptor S-domain receptor kinase AtLORE (LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION, **Figure 1**) recognizes the medium chain 3-hydroxy fatty acid brick present in the lipid A part of LPSs but does not sense lipid A or LPSs themselves (Kutschera et al., 2019). Phylogenetically, this receptor seems to be specific of the Brassicaceae family (Ranf et al., 2015). Lipid-A from *Pseudomonas* seems also perceived by *Arabidopsis* as it induces a late oxidative burst, presumably not driven by the LORE receptor (Shang-Guan et al., 2018). This LORE-independent perception suggests that LPSs may be perceived by distinct perception mechanisms leading to the induction of the plant immune system. In rice, the perception of LPSs rather involves the OsCERK1 receptor, which also senses the lipid-A (Desaki et al., 2018). Bioinformatics and genetic approaches looking for homologous LORE-like or CERK1-like receptors might allow the identification of receptors involved in LPS perception by grapevine cells.

Lipopeptides produced by bacteria are also amphiphilic compounds perceived by plants as MAMPs (Ongena and Jacques, 2008). *Bacillus subtilis* produces three main families of cyclic lipopeptides (LPs), namely, surfactins, iturins, and fengycins. Purified surfactin, mycosubtilin (iturin family), and plipastatin (fengycin family) are sensed by grapevine plant cells (Farace et al., 2015). Although surfactin and mycosubtilin stimulate grapevine innate immune responses, they differentially activate early signaling pathways and defense marker genes. Plipastatin perception by grapevine cells only results in early signaling activation. Gene expression analysis suggested that mycosubtilin activates SA and JA signaling pathways, whereas surfactin mainly induces SA signaling. Interestingly, only surfactin and mycosubtilin treatments result in a local IR against *B. cinerea* in grapevine leaves. Challenge with specific *B. subtilis* strains overproducing surfactin and mycosubtilin also led to a slightly enhanced stimulation of grapevine defense responses compared with a wild-type strain of the bacteria (Farace et al., 2015). *B. subtilis* strain GLB191 confers protection to grapevine against downy mildew in controlled conditions and in the vineyard (Zhang et al., 2017). Recently, GLB191 supernatant was shown to be also highly active, with a double effect: oomycide and defense elicitor (Li et al., 2019). The supernatants of GLB191 mutants affected in the production of fengycin and/or surfactin loose partial or total activity, suggesting that both LPs contribute to the activity of GLB191 supernatant against downy mildew. As for RLs, the recognition mechanism of LPs is not fully understood. Mycosubtilin and fengycin are known to interact with biomimetic membrane lipids, in particular with phospholipids and sterols (Maget-Dana and Ptak, 1990; Deleu et al., 2005, Deleu et al., 2008).

Studies on surfactin showed that the LP structure strongly impacts the immune response (Jourdan et al., 2009). The reduced activity of some surfactin homologues indicates that surfactin perception is dictated by structural clues in both the acyl moiety and cyclic peptide parts. Surfactin immune signatures suggest that these molecules could interact with membranes without irreversible pore formation but in a way sufficient to induce disturbance or transient channeling in the plasma membrane. This phenomenon could, in turn, activate a biochemical cascade of molecular events leading to defensive responses (Jourdan et al., 2009). Surfactins with long chains are more effective than the shortest ones. Repeated challenges with surfactin do not result in a refractory state (desensitization), suggesting that the sensing of the molecule does not involve a high-affinity protein receptor (Henry et al., 2011). Moreover, surfactin is able to target the lipid fraction of the plant plasma membrane, and long-chain surfactins are more active. Altogether, as for RLs, it is proposed that the recognition of these amphiphilic elicitors could rely on a lipid-driven process, leading to the perturbation of the membrane structure, and resulting in the activation of the immune response. Interestingly, this lipid-driven process seems to be less specific than the perception by PRRs as it could take place in several plant species, including grapevine. However, a dichotomy between monocots and dicots cannot be excluded as it has been shown for the perception of the toxin necrosis and ethylene-inducing peptide 1–like protein (NLP) by sphingolipid receptors (Lenarčič et al., 2017).

Sulfation of the Laminarin β-Glucan Improves Its Resistance-Inducing Activity

The eliciting activity of the β-glucan oligosaccharides laminarin and its sulfated derivative PS3 have been studied in details. Laminarin is naturally produced by the brown algae *Laminaria digitata* and is a reserve carbohydrate of oomycetes (Bacic et al., 2009). It has an average DP of 25–33 glucose units and up to three single β-glucose branches at position 6 (Klarzynski et al., 2000). PS3 (phycarin sulfated 3) was obtained by chemical sulfation of laminarin and has a degree of sulfation of 2.4 (Ménard et al., 2004). In plants and mammals, the presence of sulfates is crucial for the biological activity of oligosaccharides, suggesting that chemical sulfation of oligosaccharides can improve their biological properties. Studies conducted with tobacco plants reported that, compared to laminarin, its sulfated derivative PS3 triggers a stronger immunity against tobacco mosaic virus (Ménard et al., 2004). Similarly, PS3 induces a stronger grapevine resistance against *P. viticola* compared to laminarin (Gauthier et al., 2014). In tobacco, Klarzynski et al. (2000) have shown that, while laminarin elicits phenylalanine ammonia lyase (PAL) activity, di-, tri-, and tetramers of β-1,3-glucan are inactive. In a structure-activity analysis, Ménard et al. (2004) demonstrated that the sulfate residues and a minimum β-1,3-glucan chain length (DP 5) are essential for PS3 activity in tobacco. Interestingly, PS3 and laminarin differ by their capacity to trigger (i) early grapevine defense responses and (ii) a stress-responsive transcriptome. Indeed, conversely to laminarin that elicits canonical early responses like cytosolic [Ca^{2+}] variations, H2O2 production, MAPK activation, and plasma membrane depolarization, PS3 only induces a long-lasting plasma membrane depolarization. Furthermore, transcriptional changes observed in response to both β-1,3-glucans present similarities but differ in the intensity of transcriptome expression. Indeed, several genes are up-regulated by both β-1,3-glucans, but PS3 triggers a variation in the global transcriptional reprogramming 3.9-fold higher (Gauthier et al., 2014). Among the genes induced by PS3, many genes encode proteins involved in carbohydrate catabolism providing energetic nucleotides (e.g., ATP) and reduced cofactors (e.g., NADH and NADPH). These cofactors are particularly important for the activity of enzymes involved in defense responses. For instance, the NADPH is used by the respiratory burst oxidase homologue (RBOH) to produce an intense ROS production at the site of pathogen infection (Gauthier et al., 2014). This observation might also explain why sulfation of laminarin improved laminarin-IR against *P. viticola* in grapevine, with a HR-like profile (Trouvelot et al., 2008). Moreover, our results revealed that, compared to laminarin, PS3-IR against *P. viticola* in grapevine was correlated with the priming of defense genes, SA and H2O2 productions, callose deposition, and HR-like cell death. These various responses have been shown to be dependent on anionic channel activity (Gauthier et al., 2014). This activity has already been demonstrated to play key role in the establishment of the HR in tobacco (Wendehenne et al., 2002; Gauthier et al., 2007).

To assess whether PS3 and laminarin are perceived by a common receptor, H2O2 production assay was used to determine whether successive additions of PS3 and laminarin would result in a desensitized state. As expected, grapevine cells treated twice with laminarin were unable to respond to the second treatment (**Figure 2B**). Interestingly, the pre-treatment with PS3 prevented grapevine cells to respond to the subsequent addition of laminarin (**Figure 2B**). These data suggest that PS3 and laminarin are recognized by a common receptor and that sulfation of laminarin elicits the activation of different signaling cascades, leading to an enhanced immunity correlated to a higher resistance. Recently, the non-branched β-1,3-glucan hexamer has been shown to trigger *Arabidopsis* immune responses which were impaired in the *cerk1* mutant, suggesting that CERK1 might be a co-receptor for the β-1,3-glucan perception in this plant species (Mélida et al., 2018).

DISCUSSION

Finding New VvPRRs and Investigating Their Spatiotemporal Expression in Grapevine

As described above, several elicitors of different nature, MAMPs or DAMPs, have been shown so far to efficiently induce immune responses in grapevine. Nevertheless, the discovery of grapevine PRRs began only recently and

some information are still missing to fully understand the mechanisms of perception of these elicitors by grapevine cells. Based on the actual knowledge in *Arabidopsis*, it is highly probable that a grapevine ortholog for BRASSINOSTEROID-INSENSITIVE 1–ASSOCIATED RECEPTOR KINASE 1 (VvBAK1) co-receptor might also exist in grapevine to form the functional receptor complex with the flg22 receptor VvFLS2 (**Figure 1**). Similarly, a co-receptor of the AtCERK1/LYK1 orthologues VvLYK1-1 and VvLYK1-2 might also exist in grapevine. However, the bioinformatics predictions are more complicated because the grapevine genome contains two putative genes encoding orthologs of the rice OsCEBIP. Moreover, grapevine possesses 13 other genes encoding putative VvLYKs, the function of which is still unknown and including two orthologues of the *Arabidopsis* AtLYK5, known as the high-affinity binding domain of the chitin receptor complex (Cao et al., 2014). Interestingly, the expression of *VvCEBIP1* is induced by chitin treatment in grapevine cells (Trdá, 2014), while two *VvLYK5* genes are induced by the necrotrophic fungus *B. cinerea* during berry infection (Brulé et al., 2019). Concerning the *B. cinerea* PGs, the production of recombinant BcPG1 in the heterologous system *Pichia pastoris* was not very efficient (Kars et al., 2005), thus rendering the identification of its cognate receptor in grapevine very difficult. Nevertheless, since the *Arabidopsis* receptor complex involves a RLP named RBPG1 and its co-receptor the RLK SOBIR1 (Zhang et al., 2014) and given the role of SOBIR1 as important co-receptor of many RLPs (Gust and Felix, 2014), the investigation of its grapevine ortholog definitively deserves attention. Lastly, the grapevine genome contains at least one clear orthologue gene of the OG receptor WAK1, which is induced during infection of grapevine flowers with *B. cinerea* (Haile et al., 2017). For all these PRR candidates, the development of genome editing by CRISPR-Cas9 could be a very useful technology to test the function of these putative orthologs through a loss-of-function approach in grapevine. The recent evidences that this technology can be efficiently used in this crop opens promising perspectives (Wang et al., 2018).

This fundamental knowledge will be important to better apply MAMPs/DAMPs to induce grapevine resistance, particularly in vineyards. Indeed, some biocontrol products are now homologated in the vineyards, such as the COS-OGA (van Aubel et al., 2014). However, are these elicitors actually perceived by the plant in natural conditions? This open question is of particular importance considering a perennial crop, which will require a deep investigation of the expression of the different immune receptors at different phenological stages and in different organs and taking plant aging into account. Since PRR-mediated immune responses vary during plant development (Zou et al., 2018), it would be interesting to know for instance if VvLYK1 and VvWAK1, two receptors involved in COS-OGA perception, are expressed in young leaves and flowers, two organs that are highly susceptible to filamentous microbes (**Figure 3**). Moreover, we must keep in mind the importance of plant genotypes (**Figure 3**) as the grapevine cultivars can differently respond to treatment with the same resistance inducers (Banani et al., 2014). In *Arabidopsis*, it has been shown that the existing natural mutations are sufficient to explain that some genotypes are

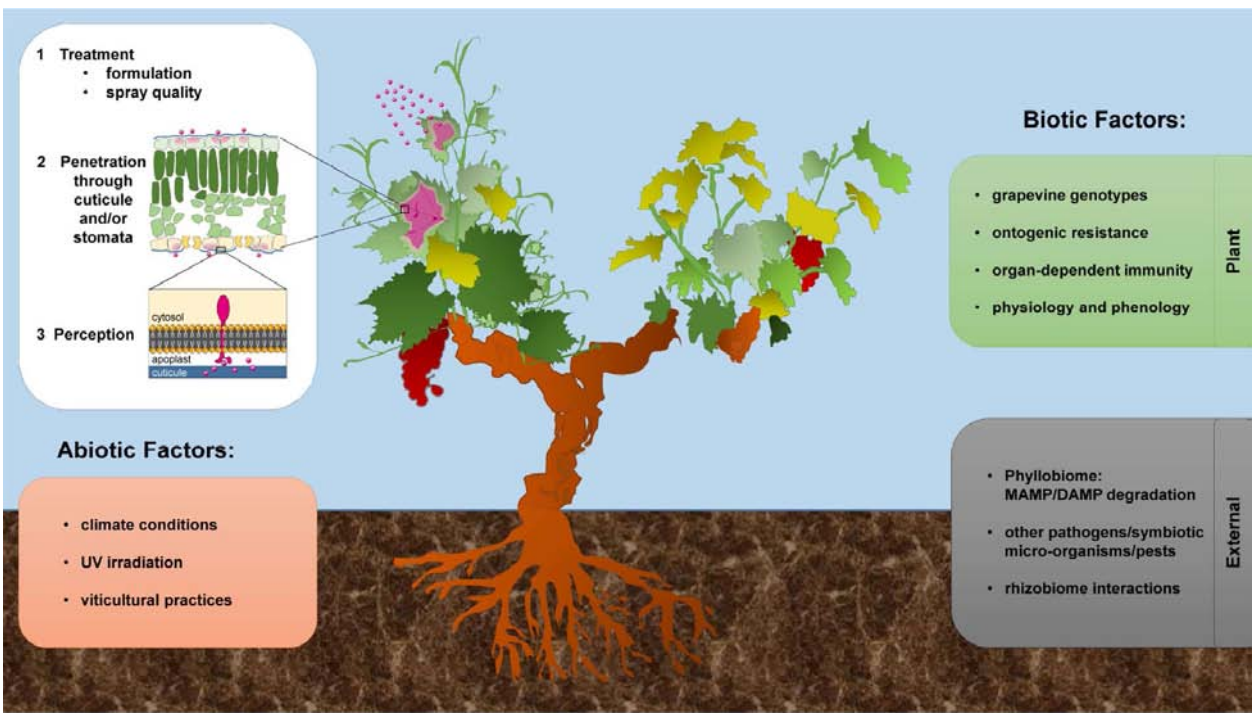

FIGURE 3 | Effect of different factors influencing grapevine induced resistance in vineyards.

totally unable to sense MAMPs as flg22 (Felix et al., 1999; Zipfel et al., 2004; Vetter et al., 2012). This supports the need for a better knowledge of the molecular mechanisms to ensure a broad and durable action of elicitors as resistance inducers in grapevine.

Searching for Effective IR Markers

Concerning MAMPs/DAMPs, the screening of active biomolecules must continue to identify new resistance inducers and develop their use in crop protection strategies. However, the stimulation of the immune system is not systematically correlated with IR. Hence, from an applicative point of view, it is necessary to identify effective IR markers. It was for this purpose that a comparative experiment was performed with two oligosaccharidic elicitors differing in their capacity of inducing resistance against *P. viticola*: one effective (PS3) and one non-effective (a short laminarin of DP 13 that triggers production of H2O2 without inducing any protection). On one hand, proteomics showed that only few proteins, including the 12-oxophytodienoate reductase (OPR-like) related to the OPDA pathway and an arsenite-resistance protein (serrate-like protein), were found as possible markers of PS3-IR (Lemaître-Guillier et al., 2017). On the other hand, metabolomics analysis highlighted erythritol phosphate, a phosphorylated polyol, as another possible marker of PS3-IR. In parallel, the microarray analysis showed that the PS3-induced transcriptomic profile includes genes encoding a SA methyl transferase and terpene synthases (Gauthier, 2009; Gauthier et al., 2014). Further investigation of the emission of volatile organic compounds (VOCs) in grapevine plants treated with PS3 showed the production of mono- and sesquiterpenes, notably trans-β-ocimene and (E-E) α-farnesene (Chalal et al., 2015). Altogether, these experiments revealed a rather limited number of putative IR markers, and we cannot rule out the possibility that IR markers would differ with other resistance inducers or in other genotypes. It will therefore be probably more reasonable to consider "metabolic signatures" of effective resistance markers rather than a specific one. In this context, the non-destructive monitoring of VOCs may become a convenient way to study the plant response to resistance inducers in the field.

Factors Influencing Efficient Grapevine IR in Vineyards

Polysaccharides and lipids are particularly interesting because many of them are approved for human feeding, and their costs of production are compatible with an application in viticulture. However, in field conditions, it is clear that only few elicitors, proved to be effective to induce resistance in controlled conditions, maintain their performances (Delaunois et al., 2014). Besides the above-mentioned lack of knowledge of elicitor recognition in natural conditions, the effectiveness of elicitor induced-resistance depends also on several biotic and abiotic factors (**Figure 3**). First, the active molecules must penetrate the plant tissues to elicit innate immunity. For oligosaccharidic elicitors (e.g., laminarin or PS3), the main barrier is represented by the cuticle, which is difficult to cross due to high hydrophobicity, and also generally thicker in field conditions. Using a fluorescently tagged PS3, it was shown how compound formulation is important for improving the bioavailability of certain elicitors (hydrophilic) and, consequently, their effectiveness to induce resistance (Paris et al., 2016). Such works also showed that the stomata, present in grapevine almost exclusively on the underside of the leaves, represent an important entry route for these treatments. Finally, it is nevertheless possible to improve the penetration of hydrophilic elicitors by (i) an *ad hoc* formulation with surfactants, and/or (ii) targeting a spray on the more permeable lower leaf surface (**Figure 3**).

The stability of the applied elicitors is another understudied aspect to consider for plant protection. Indeed, elicitors could be degraded after application, notably by plant microbiome (Trouvelot et al., 2014). For instance, laminarin is a substrate for β-1,3-glucanases. Thus, a basal activity of microbial or plant glucanases could degrade laminarin and, consequently, releases short inactive β-glucans. This biodegradation of elicitors might be very rapid in natural conditions and may partly explain the lower efficiency of these resistance inducers in vineyards (**Figure 3**). Interestingly, laminarin sulfation clearly protects the molecule from its enzymatic degradation, thus increasing its stability (Ménard et al., 2005) and probably explaining the higher resistance induced by PS3 compared to laminarin (Gauthier et al., 2014). In the same way, plant enzymes such as the BBE-like proteins play a crucial role in controlling the elicitor activity of oligosaccharides. According to their oxidase function dampening the excessive accumulation of active OGs and CDs (Benedetti et al., 2018; Locci et al., 2019), we could assume that such plant enzymatic activities may interfere with oligosaccharide stability as active elicitors. Moreover, in *Arabidopsis*, BBE-like proteins constitute a family of 28 members, and so far, only 5 of them have been demonstrated to be involved in the DAMP-homeostasis (OGs and CDs). The remaining uncharacterized members of this family might be responsible for the inactivation of "orphan" cell-wall-derived DAMPs, such as xyloglucans or the less characterized "Burdock" fructo-oligosaccharides (Sun et al., 2013). The identification of the BBE-like protein family in *Vitis vinifera* should also be investigated for a better knowledge of oligosaccharide defense induction in grapevine and their possible application. Other environmental factors must also be taken into account, such as molecule degradation by intense UV light, washing by rain falls or a rapid drying preventing the penetration of the elicitors through the cuticle (**Figure 3**). Therefore, the formulation of these new biocontrol products must be optimized to ensure molecule stability in changing conditions and allow their rapid penetration through cuticle and cell wall.

Finally, many other factors, such as viticultural practices or interactions with the plant-associated microbiomes, could impact the plant physiology which is determinant to mount an efficient IR in field conditions.

AUTHOR CONTRIBUTIONS

M-CH, MA, DB, JC, SC, XD, SD, AG, CL-G, JN, LT, ST, EV and BP contribute together to the writing of this review.

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Strengthening Grapevine Resistance by *Pseudomonas fluorescens* PTA-CT2 Relies on Distinct Defense Pathways in Susceptible and Partially Resistant Genotypes to Downy Mildew and Gray Mold Diseases

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Downy mildew caused by the oomycete *Plasmopara viticola* and gray mold caused by the fungus *Botrytis cinerea* are among the highly threatening diseases in vineyards. The current strategy to control these diseases relies totally on the application of fungicides. The use of beneficial microbes is arising as a sustainable strategy in controlling various diseases. This can be achieved through the activation of the plants' own immune system, known as induced systemic resistance (ISR). We previously showed that bacteria-mediated ISR in grapevine involves activation of both immune response and priming state upon *B. cinerea* challenge. However, the effectiveness of beneficial bacteria against the oomycete *P. viticola* remains unknown, and mechanisms underpinning ISR against pathogens with different lifestyles need to be deciphered. In this study, we focused on the capacity of *Pseudomonas fluorescens* PTA-CT2 to induce ISR in grapevine against *P. viticola* and *B. cinerea* by using two grafted cultivars differing in their susceptibility to downy mildew, Pinot noir as susceptible and Solaris as partially resistant. On the basis of their contrasting phenotypes, we explored mechanisms underlying ISR before and upon pathogen infection. Our results provide evidence that in the absence of pathogen infection, PTA-CT2 does not elicit any consistent change of basal defenses, while it affects hormonal status and enhances photosynthetic efficiency in both genotypes. PTA-CT2 also induces ISR against *P. viticola* and *B. cinerea* by priming common and distinct defensive pathways. After *P. viticola* challenge, PTA-CT2 primes salicylic acid (SA)- and hypersensitive response (HR)-related genes in Solaris, but SA and abscisic acid (ABA) accumulation in Pinot noir. However, ISR against *B. cinerea* was associated with potentiated ethylene signaling in Pinot noir, but with primed expression of jasmonic acid (JA)- and SA-responsive genes in Solaris, together with downregulation of HR-related gene and accumulation of ABA and phytoalexins.

Keywords: induced resistance, beneficial bacteria, *Pseudomonas fluorescens*, *Plasmopara viticola*, *Botrytis cinerea*, priming, *Vitis vinifera*

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is a perennial crop species that is sensitive to a large spectrum of pathogens. Downy mildew, caused by *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & de Toni, and gray mold, caused by *Botrytis cinerea* (Pers.), are important diseases in many of the world's viticultural areas. *P. viticola* is a biotrophic oomycete that attacks all green parts of the grapevine, while *B. cinerea* is a necrotrophic fungus that induces cell death to consume the host nutrients to spread (Dangl and Jones, 2001; Lenzi et al., 2016). The control of both diseases requires frequent fungicide applications to prevent significant crop losses. However, a growing concern about the impact of pesticides on the environment is motivating research for alternative strategies. The development of resistant cultivars is among the environmentally friendly alternatives to the use of chemicals. In this context, several resistance loci conferring resistance to *P. viticola* have been identified in *Muscadinia rotundifolia* (Bellin et al., 2009; Bouquet, 2011; Vezzulli et al., 2019), *Vitis riparia* (Marguerit et al., 2009), *Vitis amurensis* (Blasi et al., 2011; Schwander et al., 2012), and *Vitis cinerea* (Ochssner et al., 2016). However, *P. viticola* isolates are able to bypass or overcome resistance genes (Peressotti et al., 2010), thereby compromising the resistance sustainability in vineyards, while no source of resistance has been characterized against *B. cinerea* so far (Töpfer et al., 2011; Schwander et al., 2012).

Plant resistance can also be induced or strengthened after recognition of pathogenic or beneficial microbes through pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) by host-specific receptors. This resistance relies heavily on the ability of microorganisms to induce an immune signaling response known as PAMP-/MAMP-triggered immunity (PTI/MTI) (Jones and Dangl, 2006; Zipfel and Robatzek, 2010; Newman et al., 2013). Several beneficial bacteria, including *Pseudomonas* spp., have been shown to promote plant growth and to trigger activation of immune response at distant nonchallenged sites, thereby reducing plants' susceptibility to pathogens, a process termed "induced systemic resistance" (ISR) (Verhagen et al., 2010; Berendsen et al., 2012; Pieterse et al., 2014; Gruau et al., 2015; Aziz et al., 2016). Evidence is accumulating that molecular processes associated with ISR are diversified and depend on the beneficial microorganism and the plant/pathogen system considered (Pieterse et al., 2014). In most cases, researches support the idea that ISR is characterized by priming the plant for a more efficient activation of various cellular defenses upon subsequent pathogen infection (Pozo et al., 2008; Van der Ent et al., 2009; Perazzolli et al., 2011). Priming can save plant energy costs and provides plants with an enhanced capacity for rapid and effective activation of cellular defense responses only once primed plants are attacked by a pathogen or an insect (Van Hulten et al., 2006; Conrath, 2011), whereby plants memorize previous microbial recognition and can respond more robustly to and counteract the subsequent invader attack.

The priming state involves distinct well-characterized responses, including upregulation of defense-related genes (Verhagen et al., 2004; Gruau et al., 2015), release of reactive oxygen species (Iriti and Faoro, 2003; Verhagen et al., 2010), callose deposition, and hypersensitive response (HR) (Trouvelot et al., 2008; Palmieri et al., 2012), as well as biosynthesis of antimicrobial phytoalexins (Yedidia et al., 2003; Aziz et al., 2016). Major defense phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) take part in regulating the host immune response (Koornneef and Pieterse, 2008; Lopez et al., 2008; Bigeard et al., 2015). The effectiveness of phytohormones in induced resistance is mostly dependent on the pathogen's infection strategy. The effective defense against (hemi)biotrophic pathogens was generally assumed to be dependent on the SA signaling pathway and HR-like cell death of the host, whereas the resistance against necrotrophic pathogens was largely associated to JA and ET pathways (Glazebrook, 2005; Pieterse et al., 2009). These generalities are controversial, especially in grapevine as JA signaling has been implicated in resistance against the biotrophic oomycete *P. viticola* (Figueiredo et al., 2015). This is in line with the fact that SA and JA signaling pathways are interdependent and most reports indicate a mutually antagonistic and also synergistic interactions between SA- and JA-dependent signaling (Pieterse et al., 2014). ABA has also an important functional role in defense responses, although its involvement in induced resistance to pathogen remains less obvious compared to the other phytohormones (Ton et al., 2009). Based on *Arabidopsis*, *Pseudomonas fluorescens*-mediated ISR functions independently of SA but requires a functional response to ET and JA without any increase in their production (Pieterse et al., 2000; Van der Ent et al., 2009; Pieterse et al., 2014). However, other *P. fluorescens* strains can induce ISR *via* SA signaling and not *via* JA/ET pathways (Van de Mortel et al., 2012). Other beneficial microbes including *Bacillus* sp. (Niu et al., 2011) and *Trichoderma* sp. (Palmieri et al., 2012) can also trigger ISR against hemibiotrophic and necrotrophic pathogens by inducing the expression of SA and ET/JA signaling pathways.

In grapevine, various beneficial bacteria have been identified regarding their effectiveness to prime plant immune response and to induce ISR against *B. cinerea* (Magnin-Robert et al., 2007; Trotel-Aziz et al., 2008; Verhagen et al., 2010, Verhagen et al., 2011; Gruau et al., 2015; Aziz et al., 2016; Miotto-Vilanova et al., 2016). However, to date, nothing is known about the efficacy of these bacteria against the oomycete *P. viticola*. Some nonpathogenic fungi including *Trichoderma harzianum* T39 (Perazzolli et al., 2008; Perazzolli et al., 2011; Perazzolli et al., 2012) and natural molecules such as β-aminobutyric acid (BABA) (Hamiduzzaman et al., 2005), chitosan (Aziz et al., 2006), and laminarin and its sulfated derivative (Aziz et al., 2003; Aziz et al., 2004; Trouvelot et al., 2008; Gauthier et al., 2014), as well as thiamine (Boubakri et al., 2012), have been reported to induce efficient resistance in grapevine against downy mildew. The T39- and BABA-induced resistance against *P. viticola* seems to be linked to primed JA-related signaling (Hamiduzzaman et al., 2005; Perazzolli et al., 2008), while laminarin and its sulfated form prime both JA- and SA-dependent pathways upon *P. viticola* challenge (Aziz et al., 2003; Trouvelot et al., 2008; Gauthier et al., 2014).

Research performed on *P. fluorescens* PTA-CT2 isolated from vineyard provided evidence that this bacterium is capable of colonizing grapevine roots but not the aboveground plant parts,

inducing local and systemic immunity and boosting systemic resistance against *B. cinerea* (Trotel-Aziz et al., 2008; Verhagen et al., 2011; Gruau et al., 2015; Aziz et al., 2016). However, the molecular mechanisms governing this resistance have been only partially revealed, and the effectiveness of beneficial bacteria to control downy mildew caused by *P. viticola* remains to be deciphered. In this study, we focused on the ability of *P. fluorescens* PTA-CT2 to induce resistance in grapevine against *B. cinerea* and *P. viticola* by using two grafted grapevine cultivars differing in their susceptibility to downy mildew, Pinot noir as a sensitive cultivar and Solaris as a tolerant one, both grafted on the same rootstock under greenhouse conditions. Therefore, we investigated the capacity of grapevine plants to express basal and induced immune response at the systemic level upon interaction with *P. fluorescens* PTA-CT2, within a comparative context between susceptible and partially resistant cultivars. We especially evaluated the expression of defense-related genes by quantitative reverse-transcription PCR (RT-qPCR), including genes responsive to SA (*PR1*, *PR2*, and *GST1*) or involved in JA (*LOX9*) and ET biosynthetic (*ACO*) pathways, as well as HR-related (*HSR*) and phenylpropanoid-related genes (*PAL* and *STS*). Phytohormone and phytoalexin accumulations were also followed by liquid chromatography (LC)–tandem mass spectrometry (MS/MS) and ultra-performance LC (UPLC), and the consequence of the treatment on photosynthetic performance was evaluated by a pulse amplitude-modulated (PAM) fluorimeter. We also explored mechanisms underlying induced resistance triggered by PTA-CT2 upon challenge with the biotroph *P. viticola* and the necrotroph *B. cinerea* and addressed the similarities and differences in terms of phenotypic resistance and primed defense responses in both susceptible and partially resistant cultivars.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Two-year-old grapevine plants (*V. vinifera*), cultivar Pinot noir (PN114) as susceptible to *P. viticola* and Solaris 360 [Merzling × (Zaraya Severa × Muscat Ottonel)] as partially resistant and known to carry *Rpv3* and *Rpv10* loci, both grafted onto Kober 5BB rootstock, were individually planted in 4.5-L pots containing a compost–potting soil. Plants were grown in a greenhouse at a temperature of 24°C/day and 20°C/night, with 60% humidity and a photoperiod of 16-h light. Plants were watered twice a week. Three series of experiments were conducted over two consecutive years (2016 and 2017). After reaching the BBCH14 stage, plants were divided for each cultivar into three batches of five plants for the control and three batches of five plants for the bacterial treatment.

Bacterial Treatment

P. fluorescens PTA-CT2 (GenBank Nucleotide Accession No. AM29367) was isolated from healthy filed-grown grapevine cv. Chardonnay in Champagne Area, France (Trotel-Aziz et al., 2008). Bacterial preculture was started by adding 10 µl of glycerol stock to 10 ml of sterile lysogeny broth (LB) medium and was incubated at 26°C with 110-rpm overnight shaking. This preculture was used for a new culture on the next day, following the protocol described in Gruau et al. (2015). Bacteria were then collected in their exponential growth phase (18 h of culture) by centrifugation at 5,000 × *g* for 10 min. Pellets were washed once and resuspended in 10 mM of sterile $MgSO_4$. Concentration of the bacterial suspension was estimated by a spectrophotometer (450 and 650 nm) and was adjusted to 1×10^8 colony-forming unit (CFU) ml^{-1} in sterile $MgSO_4$. Three hundred milliliter of bacterial suspension was added at the root level of each pot, to reach a final concentration of 1×10^7 CFU g^{-1} of soil. Bacterial suspension was applied once per year for 2016 and 2017. Control plants were similarly drenched with 300 ml of $MgSO_4$. Both control and bacterium-treated plants were kept under greenhouse conditions mentioned above.

Determination of Photosynthetic Efficiency

Chlorophyll fluorescence emission was measured with a PAM fluorimeter (Heinz Walz GmbH). Control and bacterium-treated plants were dark adapted for 10 min to determine the minimal level of fluorescence (Fo) and the maximal fluorescence (Fm) after a saturating pulse (0–20,000 µmol m^{-2} s^{-1}). Leaves were then exposed to a halogen light source providing actinic light (0–3,000 µmol m^{-2} s^{-1}). Photosynthetic parameters were measured on the uppermost third and fourth attached leaves (two leaves per plant). The Fv parameter indicates the maximum capacity for photochemical quenching. It is calculated by subtracting the Fo value from the Fm value. The ratio Fv/Fm reflects the maximum quantum efficiency of photosystem II (PSII), and ΦPSII indicates the photochemical yield of PSII. ETR corresponds to the electron transport rate of PSII and calculated according to the equation [ETR = *Y*(II) × PAR × 0.5 × PAR absorptivity] (Genty et al., 1989). Data are the means ± standard deviations from three independent experiments, each in triplicates, and each replicate consisted of five plants per cultivar over two consecutive seasons (2016 and 2017).

Pathogen Growth Conditions, Infection, and Disease Evaluation

The *B. cinerea* strain 630 was grown on potato dextrose agar (PDA) medium and was incubated for 14 days at 22°C under continuous light for sporulation. Conidia were collected with 10 ml of sterile water as described in Aziz et al. (2003). The obtained suspension was filtered through sterile filter paper using a sterile syringe to remove the mycelium. Conidial concentration was determined by using a Malassez hemocytometer and adjusted with sterile water to 1×10^6 conidia per milliliter.

The *P. viticola* isolate used in this study was purified as fresh single spore from infected leaves of the susceptible Pinot noir. Sporangia were propagated on *V. vinifera* cv. Pinot noir leaves in a glasshouse. To obtain sporangia, plants with oil spot symptoms were placed overnight in the dark at 100% relative humidity (RH). Inoculum of *P. viticola* sporangia was prepared by washing the freshly sporulating lesions with sterile distilled water. The sporangial suspension was then adjusted to a concentration of 10^5 spores per milliliter using a Malassez hemocytometer.

Two weeks after the bacterial treatment, leaf disk assay was used for pathogen infection and disease evaluation. Leaf disks of 18-mm diameter were excised using the cork borer from the uppermost third and fourth leaves (two leaves per plant) of both control and bacterium-treated plants. The adaxial side of leaf disks was placed on a humidified Whatman paper in a Petri plate (12 disks per plate), and the abaxial side was inoculated with a 5-µl drop of a fresh suspension of *B. cinerea* (1×10^6 conidia per milliliter) or 20-µl drop of a fresh suspension of *P. viticola* (1×10^5 sporangia per milliliter). Inoculated leaf disks were placed in a growth chamber at 24°C with a 16- and 8-h photoperiod. Seven days after inoculation with *P. viticola*, all leaf disks were incubated overnight in the dark at 25°C with 95–100% RH.

Gray mold disease was evaluated at 7 days post infection (dpi) on 35–45 leaf disks from three independent experiments by scanning necrotic lesions caused by *B. cinerea* using Compu Eye, Leaf & Symptom Area (LSA) software (Bakr, 2005). Disease severity was also assessed at 7 dpi by scoring using a notation scale from 0 mm^2 to more than 40 mm^2 as follows: I, <5 mm^2; II, between 5 and 10 mm^2; III, between 10 and 20 mm^2; IV, between 20 and 40 mm^2; and V, >40 mm^2 of necrosis area.

For *P. viticola*, disease intensity was also assessed at 8 dpi on 35–45 leaf disks from three independent experiments by measuring the leaf area covered by sporulation and necrotic lesions. The sporangial density of *P. viticola* was also determined. Sporangia were collected by suspending leaf disks in sterile distilled water and shaking for 1 h. Sporangia concentration was determined by direct counting with a Malassez hemocytometer under a light microscope. Disease rating was also expressed as the fraction of leaf disks falling in the following four classes for *P. viticola* infection: I, <5 mm^2; II, between 5 and 10 mm^2; III, between 10 and 20 mm^2; and IV, >20 mm^2. Data are the means ± standard deviations of the three experiments for both 2016 and 2017 seasons.

Gene expression, phytohormones, and phytoalexins were analyzed in both cultivars at 0 and 48 h after pathogen inoculation. The 48-h post infection (hpi) point was chosen as an optimal time for the high expression of most of the defense-related responses in both susceptible and partially resistant cultivars. To this end, the diameter of the leaf disks was reduced to 12 mm using a cork borer to eliminate the area where defense responses to injury occurred. Samples were immediately frozen in liquid N_2 and stored at −80°C.

RNA Extraction and RT-PCR

Leaf disks collected from control and bacteria-treated plants at 0 and 48 hpi were ground in liquid nitrogen. For each point, three biological replicates were used. Total RNA was extracted from 50 mg of leaf ground powder following the protocol of a PlantRNA purification reagent according to manufacturer instructions (Invitrogen, Pontoise, France). RNA pellet was suspended in 20 µl of RNase-free water and was incubated for 2 h at −20°C for solubilization. Genomic DNA was removed by DNase treatment, according to the manufacturer's instruction (RQ1 RNase-free DNase, Promega). The concentration of the RNA was quantified using NanoDrop One by Thermo Fisher Scientific, and based on the obtained concentrations, all the samples were diluted to 100 ng μl^{-1}. First-stranded complementary DNA (cDNA) was obtained from 100 ng of the total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific).

The expression of eight genes known to be involved in defense was analyzed in both Pinot noir and Solaris plants. These genes encode glutathione-S-transferase (*GST1*), pathogenesis-related protein 1 (*PR1*), and β-1,3-glucanase (*PR2*) as markers of the SA pathway; 9-lipoxygenase (*LOX9*), an enzyme of the octadecanoid pathway; 1-aminocyclopropane carboxylic acid (ACC) oxidase (*ACO*) involved in ET biosynthesis; a HR-related gene (*HSR*) as a marker of cell death; phenylalanine ammonia-lyase (*PAL*), a key enzyme of the phenylpropanoid pathway; and stilbene synthase (*STS*), responsible for the biosynthesis of resveratrol. The primers for target genes were designed by Primer 3.0 software (Applied Biosystems) (Gruau et al., 2015) based on grapevine messenger RNA (mRNA) sequences deposited in GenBank. RT-PCR was performed with Absolute qPCR Mix, SYBR Green, ROX (Thermo Fisher Scientific), using a CFX96 system thermocycler (Bio-Rad). PCRs were carried out in 96-well plates to a final volume of 15 µl containing Absolute qPCR Mix, SYBR Green, ROX (*Taq* polymerase, dNTP, and SYBR Green Dye), 280 nM of forward and reverse primers (**Table S1**), and 30-fold diluted cDNA. Cycling parameters were as follows: 15 min of *Taq* polymerase activation at 95°C, followed by 40 two-step cycles composed of 10 s of denaturation at 95°C, and 45 s of annealing and elongation at 60°C. After the final cycle of the PCR, the specificity of each amplification was checked using melting analysis from 65°C to 95°C (**Figure S1**). Five commonly used reference genes, *EF1α* (elongation factor 1*α;* XM_002284888.3), *60SRP* (60S ribosomal protein L18; XM_002270599.3), *ADH2* (alcohol dehydrogenase; NM_001281154.1), *39SRP* (39S ribosomal protein L41-A; XM_002285709.4), and *UBE2* (ubiquitin-conjugating enzyme E2C; XM_002275879.4) (Gruau et al., 2015), were evaluated with Bio-Rad CFX Manager software v. 3.0 to select those with a stable expression in all tested conditions. *EF1* and *60SRP* genes were selected as the most stable reference genes in both grapevine cultivars and validated as internal controls for normalization for RT-qPCR analyses. PCR efficiency of primers was calculated by performing RT-PCR on serial dilutions of cDNA. All tested primer pairs were found to have amplification efficiencies of 90% to 103%.

Relative gene expression was determined with the formula fold induction: $2^{-\Delta\Delta Ct}$, using CFX Manager 3.0 software (Bio-Rad), where ΔΔCt = (Ct GI [unknown sample] – Ct GI [reference sample]) – (Ct GR [unknown sample] – Ct GR [reference sample]). GI is the gene of interest, and GR is the reference gene. Uninfected control of Pinot noir was considered as reference sample (1× expression level) for both cultivars. Integration of the formula for two reference genes was performed by the CFX Manager 3.0 software (Bio-Rad). The results presented correspond to the means of duplicate reactions of three independent biological replicates for the 2016 and 2017 seasons. The data were analyzed each year separately, since in 2016 the plants were treated once, while in 2017 the plants underwent a second treatment.

Quantification of Phytohormones by LC–MS

Phytohormones were extracted from 20 mg of ground leaf powder in 1 ml of cold solution of methanol/water/formic acid (70/29/1, v/v/v). The homogenates were stirred at room temperature for 30 min and then centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was evaporated under nitrogen, and the residue was dissolved with 1 ml of a 2% formic acid solution. The extracts were purified using a solid-phase extraction (SPE) EVOLUTE EXPRESS ABN 1 ml to 30 mg (Biotage, UK). The eluate was evaporated to dryness and dissolved in 200 µl of H_2O containing 0.1% of formic acid.

Phytohormones, SA, JA, and ABA were purchased from OlChemIm (Olomouc, Czech Republic), and the precursor of ET, ACC, was purchased from Sigma-Aldrich (Saint-Quentin, France). Phytohormones were analyzed by an ultra-high-performance LC (UHPLC)–MS/MS system. Phytohormone analysis was achieved using a Nexera X2 UHPLC system (Shimadzu, Japan) coupled to a QTrap 6500+ mass spectrometer (SCIEX, Canada) equipped with an electrospray ionization (ESI) source. Two-microliter aliquots of purified extract were injected into a Kinetex EVO C18 core–shell column (100 × 2.1 mm, 2.6 µm, Phenomenex, USA) heated at 40°C. The mobile phases composed of Milli-Q water (solvent A) and acetonitrile LC–MS grade (Fisher Optima, UK) (solvent B), both containing 0.1% formic acid (LC–MS grade), were used to elute phytohormones with a flow rate of 0.7 ml min^{-1}. The gradient elution started with 1% B, 0.0–5.0 min 60% B, 5.0–5.5 min 100% B, 5.5–7.0 min 100% B, 7.0–7.5 min 1% B, and 7.5–9.5 min 1% B. The ionization voltage was set to 5 kV for the positive mode and −4.5 kV for the negative mode, producing mainly [M+H]+ and [M−H]−, respectively. The analysis was performed in scheduled multiple-reaction monitoring (MRM) mode in positive and negative modes simultaneously with a polarity switching of 5 ms. Quantification was processed using MultiQuant software v. 3.0.2 (SCIEX, Canada). Data are the means ± standard deviations of the three independent experiments of the 2017 season.

Phytoalexins Analysis by UPLC

Phytoalexins were extracted from 120 mg of freeze-dried powder of leaf disks with 2 ml of a solution of methanol/water (85/15: v/v) as described by Aziz et al. (2006). Samples were incubated on a shaker in the dark at room temperature for 2 h and then centrifuged for 10 min at 12,000 × g. The supernatants were collected in hemolysis tubes, and the remaining pellets were resuspended in 1 ml of 100% methanol and incubated for 1 h more on the shaker at room temperature and then centrifuged. The supernatants were pooled together and dried with a speed vacuum. The remaining residues were solubilized in 1 ml of pure methanol of LC–MS grade, filtered with 0.22-µm polytetrafluoroethylene (PTFE) filters into 2-ml amber vials. Phytoalexin analysis was performed using the ACQUITY™ UPLC system (Waters Corporation). Two-microliter aliquots were injected onto an ACQUITY UPLC BEH C18 1.7-µm, 2.1 × 100-mm column heated at 40°C. A mixture of water (A) and acetonitrile (B) containing 0.1% formic acid was used to elute phytoalexins with a flow rate of 0.5 ml min^{-1}. Fluorescence was measured with an excitation wavelength of 330 nm and an emission wavelength of 375 nm using an ACQUITY fluorimeter (Waters). Signals were analyzed using Empower 2 software (Waters). Phytoalexins were quantified relative to retention time and calibration with external standards. Data are the means ± standard deviations of the three independent experiments for the 2016 season.

Evaluation of the Direct Effect of PTA-CT2 on Pathogen Growth

Bioassays were performed on the leaves of intact plants of the susceptible cultivar Pinot noir. The abaxial leaf surface was sprayed with a suspension of PTA-CT2 at a concentration of 10^7 CFU ml^{-1} using an atomizer, until homogenous coverage of the leaves was reached. Control leaves were sprayed with sterile water. Two hours later (a time supposed to be too early for the defense reaction to be active), leaf disks of 12-mm diameter were excised and placed with their adaxial side on a wetted filer paper and inoculated with 20 µl of *P. viticola* at 10^5 sporangia per milliliter. Development of *P. viticola* was assessed on 20–24 leaf disks from three independent experiments at 7 dpi, and sporangia density was quantified using a Malassez hemocytometer under a light microscope.

P. fluorescens PTA-CT2 was also tested for its potential ability to inhibit *B. cinerea* growth *in vitro*. PTA-CT2 grown in LB medium was inoculated (5-µl drop at 10^7 CFU ml^{-1}) at four spots away from the center of a Petri plate containing PDA medium with a sterilized toothpick. Plates were then incubated at 28°C in the dark, and 24 h later, fresh *B. cinerea* mycelium was co-inoculated in the center of PDA plates. Control is PDA plates with a 5-µl LB drop. Plates were incubated at 24°C, and *B. cinerea* development was assessed 7 days later.

Statistical Analysis

All experiments were repeated at least three times in both 2016 and 2017 seasons. For photosynthetic parameters, disease evaluation, direct effect, phytoalexins, and phytohormones, statistical analysis was performed using two-way analysis of variance (ANOVA) with the statistical program SPSS 20 software using *post hoc* Tukey's test honestly significant difference (HSD) to detect significant difference ($P < 0.05$) between the treatments. For RT-qPCR experiments, transcripts differentially expressed between control and bacteria-treated plants before and after pathogen infection in Pinot noir and Solaris were analyzed by comparing the expression means of duplicate reactions of three independent experiments in each treatment and cultivar over the 2016 and 2017 seasons.

RESULTS

In the Absence of Pathogen Infection, PTA-CT2 Does Not Elicit Any Consistent Change of Basal Defenses

In this study, we evaluated by RT-qPCR the expression of eight selected defense genes from the different functional categories: genes encoding a glutathione-S-transferase (*GST1*),

a pathogenesis-related protein 1 (*PR1*), and a β-1,3-glucanase (*PR2*) as markers of the SA signaling pathway; a 9-lipoxygenase (*LOX9*) involved in oxylipin synthesis as a marker of the JA pathway; a ACC oxidase (*ACO*) that catalyzes the conversion of ACC to ET; a HR-related (*HSR*) gene as a marker of cell death; a phenylalanine ammonia-lyase (*PAL*) catalyzing the first step in the phenylpropanoid pathway; and a stilbene synthase (*STS*) encoding a key enzyme of the resveratrol (a grapevine phytoalexin) biosynthesis. Data (**Figure 1A**) showed certain differences in the level of basal PR gene expression between the two grafted grapevine cultivars under greenhouse conditions. The partially resistant Solaris exhibited higher constitutive expression of PR2 transcripts compared to the susceptible Pinot noir. However, the application of PTA-CT2 at the root level does not induce any consistent change in gene expression at the systemic level in the absence of pathogen infection (**Figure 1A**). For most transcripts, the fold change ratio in PTA-CT2- over mock-treated plants was close to the corresponding control in both cultivars, except PR1, which was upregulated in Solaris.

Given the role of phytoalexins in plant defense and their known antimicrobial activity, we also investigated stilbene concentration in grapevine leaves after root treatment with PTA-CT2. In both cultivars, PTA-CT2 did not cause any significant production of resveratrol (3,5,4′-trihydroxystilbene) (**Figure 1B**) or its dimers ε-viniferin (**Figure 1C**) and δ-viniferin (**Figure 1D**). The basal level of these stilbenes remained also comparable between the two grape varieties.

PTA-CT2 Induces Differential Production of Phytohormones in Susceptible and Resistant Cultivars

Plant hormones have a critical role as cellular signaling molecules with a central function in the regulation of immune response. To assess whether PTA-CT2 impacts systemic changes in phytohormone production, the levels of SA, JA, ACC (ET precursor), and ABA were determined by LC–MS/MS in the leaves of both Pinot noir and Solaris upon treatment of the roots with PTA-CT2. Data (**Figure 2**) showed significant

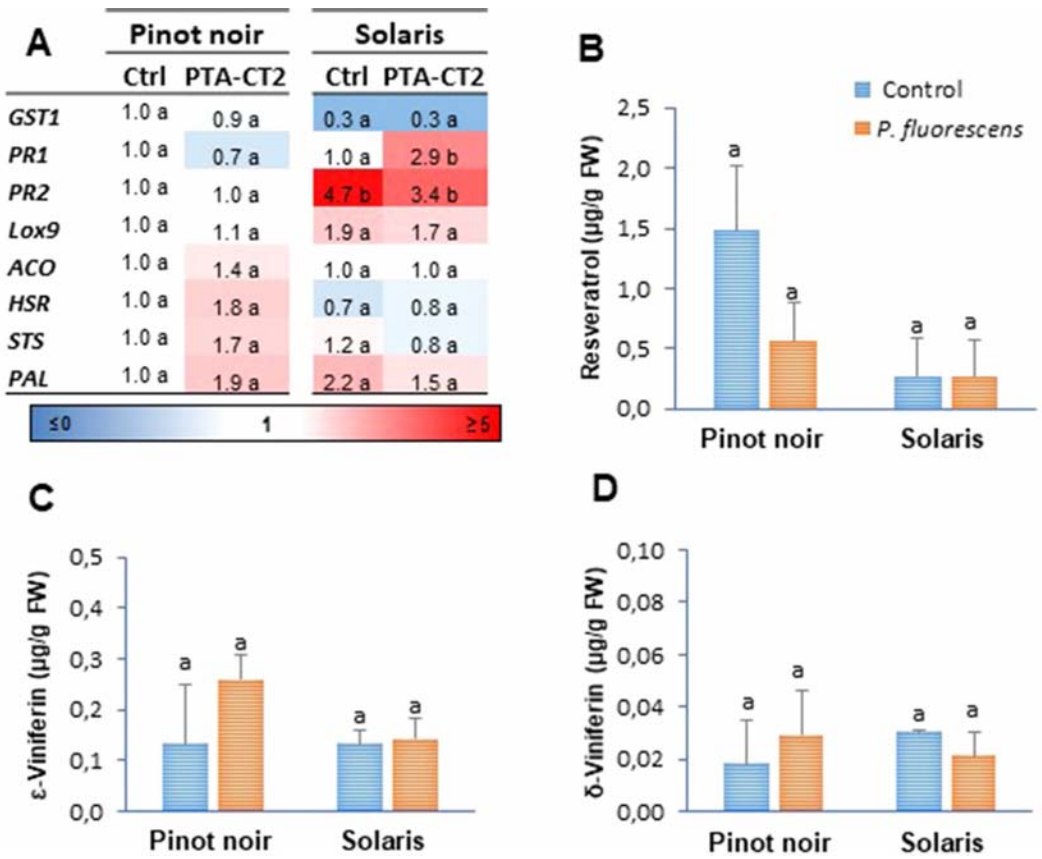

FIGURE 1 | *Pseudomonas fluorescens* PTA-CT2 has no effect on the basal expression of defense-related genes and phytoalexin production in the leaves of grapevine cultivars Pinot noir and Solaris. Plants from each cultivar were treated at the root level with *P. fluorescens* at 10^7 CFU g^{-1} of soil, and 2 weeks later, the transcript level of defense genes **(A)** and the amounts of resveratrol **(B)**, ε-viniferin **(C)**, and δ-viniferin **(D)** were quantified. For gene expression, uninfected control of Pinot noir was considered as the reference sample (1× expression level) for both cultivars, and heatmaps represent changes in transcript expression levels as indicated by the color shading. *GST1*, glutathione S-transferase 1; *PR1*, pathogenesis-related protein 1; *PR2*, β-1,3-glucanase; *LOX9*, lipoxygenase 9; *ACO*, 1-aminocyclopropane-1-carboxylic acid oxidase; *HSR*, hypersensitive related; *PAL*, phenylalanine ammonia-lyase; *STS*, stilbene synthase. Data are the means of three independent experiments performed in 2016, and vertical bars indicate standard deviation. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$).

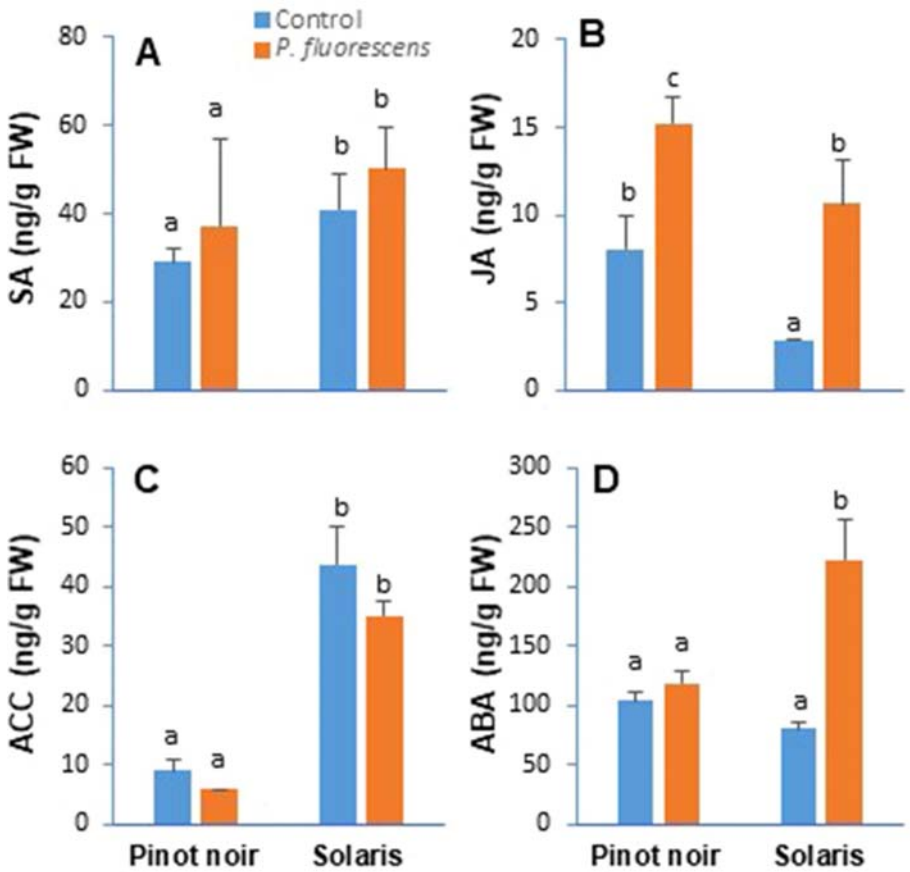

FIGURE 2 | *Pseudomonas fluorescens* PTA-CT2 induces differential production of phytohormones in the leaves of grapevine cultivars Pinot noir and Solaris. Plants were treated at the root level with *P. fluorescens* at 10^7 CFU g^{-1} of soil, and 2 weeks later, the amounts of salicylic acid **(A)**, jasmonic acid **(B)**, 1-aminocyclopropane-1-carboxylic acid **(C)**, and abscisic acid **(D)** were quantified by liquid chromatography (LC)–tandem mass spectrometry (MS/MS). Data are the means from three independent experiments performed in 2017, and vertical bars indicate standard deviation. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$).

cdifferences in the level of phytohormones between cultivars. Higher levels of SA (**Figure 2A**) and ACC (**Figure 2C**) were recorded in the leaves of nontreated Solaris compared to nontreated Pinot noir, which produced more JA (**Figure 2B**). However, any difference was observed between nontreated grape varieties regarding the level of ABA in the leaf tissues (**Figure 2D**). Upon treatment of the roots with PTA-CT2 bacteria, neither the SA content (**Figure 2A**) nor the level of ACC (**Figure 2C**) was altered in the leaves of both cultivars. However, a significant increase of JA level (**Figure 2B**) was observed in PTA-CT2-treated plants compared to their respective controls. The highest level of JA was recorded in the susceptible cultivar. The production of ABA in the leaves did not change in Pinot noir but strongly increased in Solaris after roots' treatment with PTA-CT2. These results indicate that grape cultivars displayed contrasting levels of phytohormones and that the bacterium does not impact SA and ACC levels, which were highly produced in the downy mildew-resistant Solaris. However, the production of JA and ABA was differentially increased by PTA-CT2 in grapevine cultivars.

PTA-CT2 Enhances Photosynthetic Capacity in Both Susceptible and Resistant Cultivars

Activation of plants defense responses is often accompanied by an increased energy supply deriving from photosynthesis (Shoresh et al., 2010). In order to evaluate the effect of PTA-CT2 on physiological status, photosynthetic parameters such as maximum photochemical efficiency of PSII (Fv/Fm), photochemical yield of PSII (ΦPSII), and relative photosynthetic electron transport (ETR) were measured on attached leaves from control and treated plants (**Figure 2**). Data revealed significant differences between controls of Pinot noir and Solaris in terms of the photosynthetic efficiency of PSII, especially in 2016 when Fv/Fm (**Figure 3A**) and ΦPSII (**Figure 3C**) were higher in Solaris than in Pinot noir. Clear differences were also recorded between PTA-CT2- and mock-treated plants in both Pinot noir and Solaris genotypes. PTA-CT2-treated plants showed a significant increase of photosynthetic efficiency as indicated by elevated values of Fv/Fm (**Figures 3A, B**), ΦPSII (**Figures 3C**, **D**), and ETR rate (**Figures 3E**, **F**) in both cultivars,

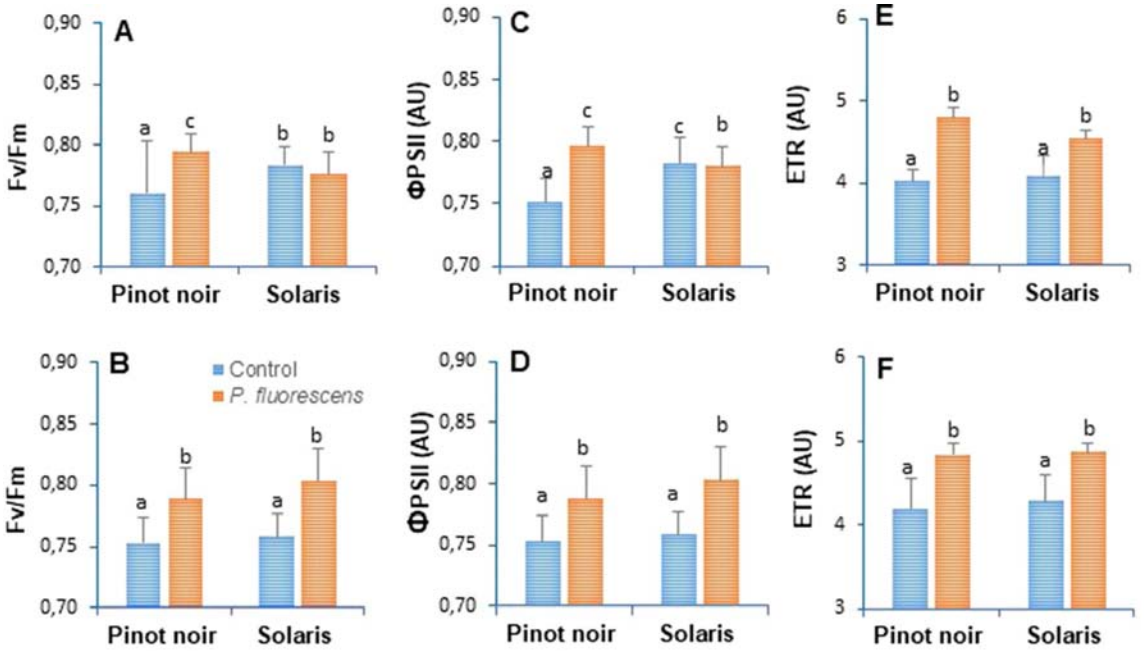

FIGURE 3 | *Pseudomonas fluorescens* PTA-CT2 stimulates photosynthetic efficiency in both grapevine cultivars Pinot noir and Solaris. Plants were treated at the root level with *P. fluorescens* at 10^7 CFU g^{-1} of soil, and 2 weeks later, the maximum photochemical efficiency of the photosystem II (PSII) (Fv/Fm, **A** and **B**), photochemical yield of PSII (*Φ*PSII, **C** and **D**), and relative photosynthetic electron transport (ETR, **E** and **F**) were determined by pulse amplitude-modulated (PAM) fluorimeter. Data expressed in arbitrary units (AU) of chlorophyll fluorescence are the means from three independent experiments performed in 2016 **(A, C, E)** and 2017 **(B, D, F)**, and vertical bars indicate standard deviation. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$).

highlighting an improvement of energy transfer within PSII after root treatment with PTA-CT2.

PTA-CT2 Induces ISR Against *P. viticola* and *B. cinerea* to Different Extents in Susceptible and Resistant Cultivars

The capacity of beneficial bacteria to trigger ISR in the susceptible cultivar Pinot noir and the partially resistant Solaris was assessed by drenching the soil with *P. fluorescens* PTA-CT2 at a concentration of 10^7 CFU g^{-1} of soil. Two weeks later, leaf disks were excised from control and treated plants and infected with *P. viticola* and *B. cinerea*. Disease symptoms were monitored 7 dpi by quantifying sporulation of *P. viticola* over the seasons of 2016 and 2017 (**Figures 4A**, **B**) and scanning necrotic lesions covered or not with spores (**Figures 4C**, **D**). Inoculated leaf disks were also ranked into four classes according to the severity of symptoms (**Figures 4G**, **H**). As expected, leaf disks of control Pinot noir were heavily colonized by spores of *P. viticola*, while no sporulation was observed in the partially resistant cultivar Solaris (**Figures 4A**, **B**). However, necrotic spots can be seen in the Solaris cultivar after 7 days of infection (**Figures 4E**, **F**). Based on sporulation symptoms in the susceptible cultivar Pinot noir, we can see that PTA-CT2 reduced sporulation density of *P. viticola* by about 54%. Based on necrotic lesions together with area covered with spores, data provide evidences that the lesion sizes in nontreated Pinot noir were larger than those in nontreated Solaris over the consecutive years (**Figures 4C**, **D**). Treatment with PTA-CT2 reduced significantly the disease severity caused by *P. viticola* in both 2016 and 2017. PTA-CT2 reduced development of *P. viticola* in the susceptible cultivar Pinot noir by about 80%, while in the partially resistant cultivar Solaris, necrotic lesions caused by *P. viticola* were reduced by about 55%. This protection seems to be more pronounced in both cultivars, which underwent a second treatment in 2017 (**Figure 4D**).

The difference between genotypes was also clear according to the severity of disease symptoms (**Figures 4G**, **H**). The number of leaf disks that displayed extensive lesions was much higher in the susceptible cultivar Pinot noir than in the partially resistant cultivar Solaris. In 2017 (**Figure 4H**), the disease rating caused by *P. viticola* in Pinot noir was higher, with approximately 92% of leaf disks heavily infected compared (**Figure 4G**) to 40% in Solaris (**Figure 4H**). However, the severity of the disease was strongly reduced in PTA-CT2-treated plants of both cultivars compared to the infected control in 2016. According to disease scoring in 2017, PTA-CT2 also reduced downy mildew severity by approximately 40% in both varieties. These results indicate that PTA-CT2 can induce resistance against *P. viticola* under greenhouse conditions by severely limiting downy mildew symptoms in leaf tissues.

Pinot noir and Solaris displayed also contrasting susceptibility phenotypes to *B. cinerea* (**Figure 5**). Over the two consecutive years, the nontreated Solaris showed very large necrosis caused by *B. cinerea* than the nontreated Pinot noir (**Figures 5A**–**D**).

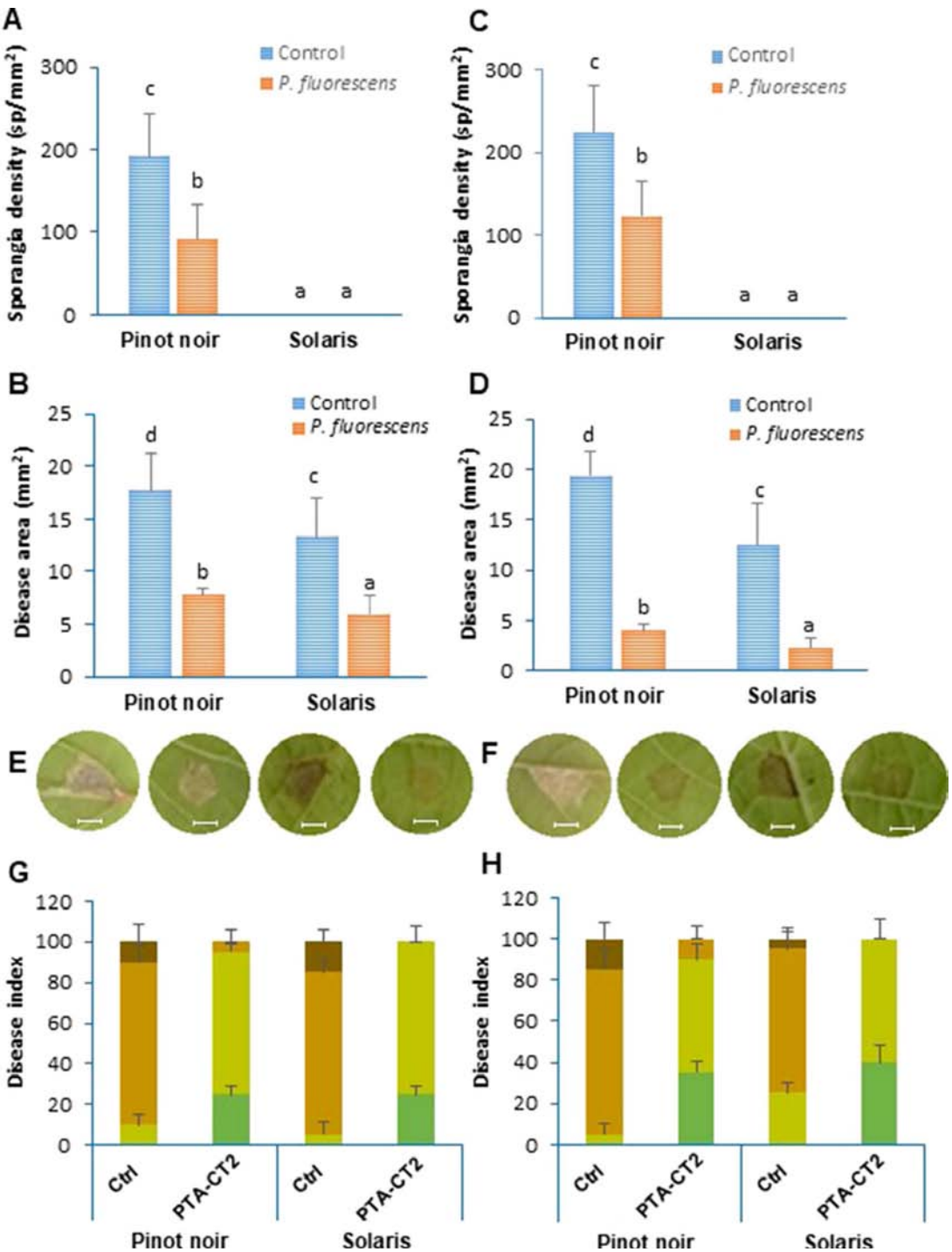

FIGURE 4 | *Pseudomonas fluorescens* PTA-CT2 strengthens systemic resistance against *Plasmopara viticola* in both susceptible cultivar Pinot noir and partially resistant cultivar Solaris. Plants were treated at the root level with *P. fluorescens* at 10^7 CFU g^{-1} soil, and 2 weeks later, leaf disks were excised from the upper third and fourth leaves and infected with 20 µl of 10^5 spores per milliliter of *P. viticola*. Sporangia density was quantified using Malassez hemocytometer at 7 days post infection (dpi) **(A, B)**, and necrotic lesions covered or not with spores were evaluated 7 dpi by Compu Eye, Leaf & Symptom Area (LSA) software **(C, D)**. Photographs **(E, F)** depict the representative disease symptoms on leaf disks of control and PTA-CT2-treated plants at 7 dpi. Bars = 4 mm. Disease index **(G, H)** was expressed as the fraction of leaf disks falling in the following classes: dark green, no visible symptom; light green, lesion covering 5–10 mm^2; light brown, lesion covering 10–20 mm^2; dark brown, lesion covering more than 20 mm^2 of the leaf disk area. Data are the means from three independent experiments, each experiment was performed with 30 leaves for each condition in 2016 **(A, C, E, G)** and 2017 **(B, D, F, H)**, and vertical bars indicate standard deviation. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$).

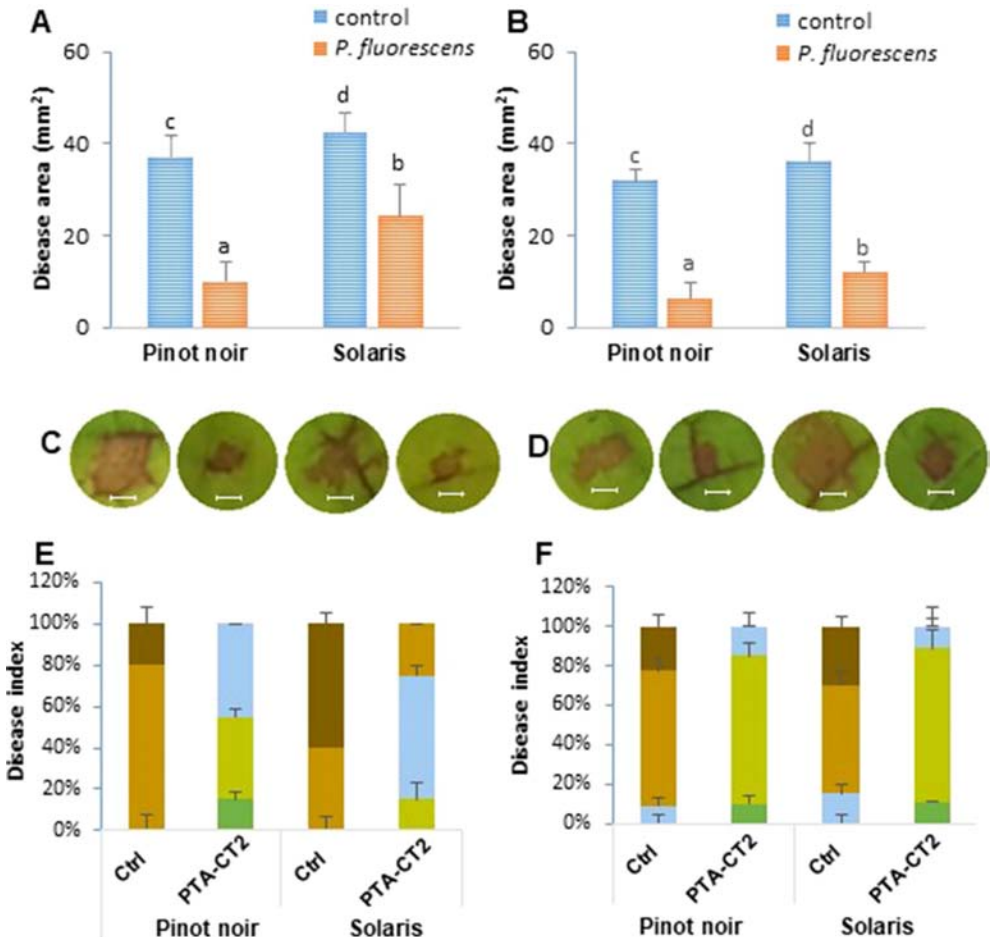

FIGURE 5 | *Pseudomonas fluorescens* PTA-CT2 induces systemic resistance against *Botrytis cinerea* in Pinot noir and Solaris cultivars. Plants were treated at the root level with *P. fluorescens* at 10^7 CFU g^{-1} of soil, and 2 weeks later, leaf disks were excised from the upper third and fourth leaves and infected with 5 µl of 10^6 conidia per milliliter of *B. cinerea*. Necrotic lesion sizes were evaluated at 7 dpi by Compu Eye, Leaf & Symptom Area (LSA) software **(A, B)**. Photographs **(C, D)** depict the representative disease symptoms on leaf disks of control and PTA-CT2-treated plants at 7 dpi. Bars = 4 mm. Disease index **(E, F)** was expressed as the fraction of leaf disks falling in the following classes: dark green, no visible symptom; light green, lesion covering 5–10 mm^2; blue, lesion covering 10–20 mm^2; light brown, lesion covering 20–40 mm^2; dark brown, lesion covering more than 40 mm^2 of the leaf disk area. Data are the means from three independent experiments, each experiment was performed with 30 leaves for each condition in 2016 **(A, C, E)** and 2017 **(B, D, F)**, and vertical bars indicate standard deviation. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$).

The difference of phenotypic susceptibility to *B. cinerea* was also apparent on a large number of leaf disks according to the disease symptoms (**Figures 5E**, **F**). However, in both cultivars, a limited progression of gray mold disease was observed after treatment with PTA-CT2. This bacterium appeared more efficient in Pinot noir than in Solaris, since the disease area was reduced by about 73% to 80% in Pinot noir over 2016 (**Figure 5A**) and 2017 (**Figure 5B**), respectively, while in Solaris the disease was reduced by 43% (**Figure 5A**) to 66% (**Figure 5B**). Similarly, according to the severity of disease symptoms of *B. cinerea* (**Figures 5E**, **F**), the number of leaf disks that displayed extensive lesions was much higher in nontreated Solaris than in nontreated Pinot noir. This phenotypic difference was especially apparent in 2016 than in 2017 year. Following bacterial treatment, inoculated leaf disks with *B. cinerea* showed weaker symptoms than the control in both cultivars (**Figures 5E**, **F**). The number of leaf disks with extensive spreading lesions was strongly reduced in the PTA-CT2-treated plants. Most of the leaf disks exhibited weak spreading lesions, with 5% to 10% disease area in Pinot noir and 10% to 20% in Solaris in 2016 (**Figure 5E**). This marked reduction of gray mold symptoms was similar in both cultivars in 2017 with a large proportion of leaf disks being weakly or not infected. This suggests that the mechanisms involved in mediated protection against *P. viticola* may differ from those involved against *B. cinerea*.

The direct effects of PTA-CT2 on *P. viticola* and *B. cinerea* were also evaluated in co-inoculation assays using intact leaves from Pinot noir and PDA medium, respectively. In both cases, we did not record any inhibitory effect of the bacterium compared to the control. The sporulation of *P. viticola* and mycelial development of *B. cinerea* were similar to that of the untreated control (**Figure S2**). Therefore, given the spatial separation of challenging pathogens

and the inducing bacterium, it can be inferred that the mediated protection against downy mildew and gray mold diseases resulted from an induced ISR in grapevine plants. We thus took advantage of the contrasting susceptibility and induced resistance phenotypes of both cultivars to explore mechanisms underpinning ISR against pathogens with different lifestyles.

PTA-CT2 Primes Differential Defensive Pathways Against *P. viticola* and *B. cinerea* in Susceptible and Partially Resistant Cultivars

To investigate the capacity of *P. fluorescens* PTA-CT2 to prime immune defense at the systemic level, the expression of defense-related genes responsive to different signaling pathways was monitored in both cultivars at 48 hpi with the biotrophic oomycete *P. viticola* and the necrotrophic fungus *B. cinerea*. As shown before, in the absence of pathogen infection, PTA-CT2 did not affect basal gene expression in either cultivar. This indicates that the onset of ISR is not associated with the change of defense responses before pathogen challenge but may involve the priming state of plants for more rapid and strong activation of immune defense upon pathogen inoculation. The relative expression of defense genes was then compared between *P. viticola*-, *B. cinerea*-, and mock-inoculated leaf disks of both Pinot noir and Solaris after pretreatment with PTA-CT2. As seen in **Figure 6**, the expression of targeted genes showed differential responsiveness between cultivars after challenge. After *P. viticola* inoculation, the expression of defense genes was generally weak in nontreated Pinot noir compared to nontreated Solaris in both 2016 (**Figures 6A**, **B**) and 2017 (**Figures 6C**, **D**), confirming the high basal defense level of the partially *P. viticola*-resistant Solaris. The control infected Solaris showed higher expression of genes responsive to SA, including *GST1*, *PR1*, and *PR2* in 2016 (**Figures 6A**, **B**) and *GST1*, *PR1*, and *HSR* in 2017

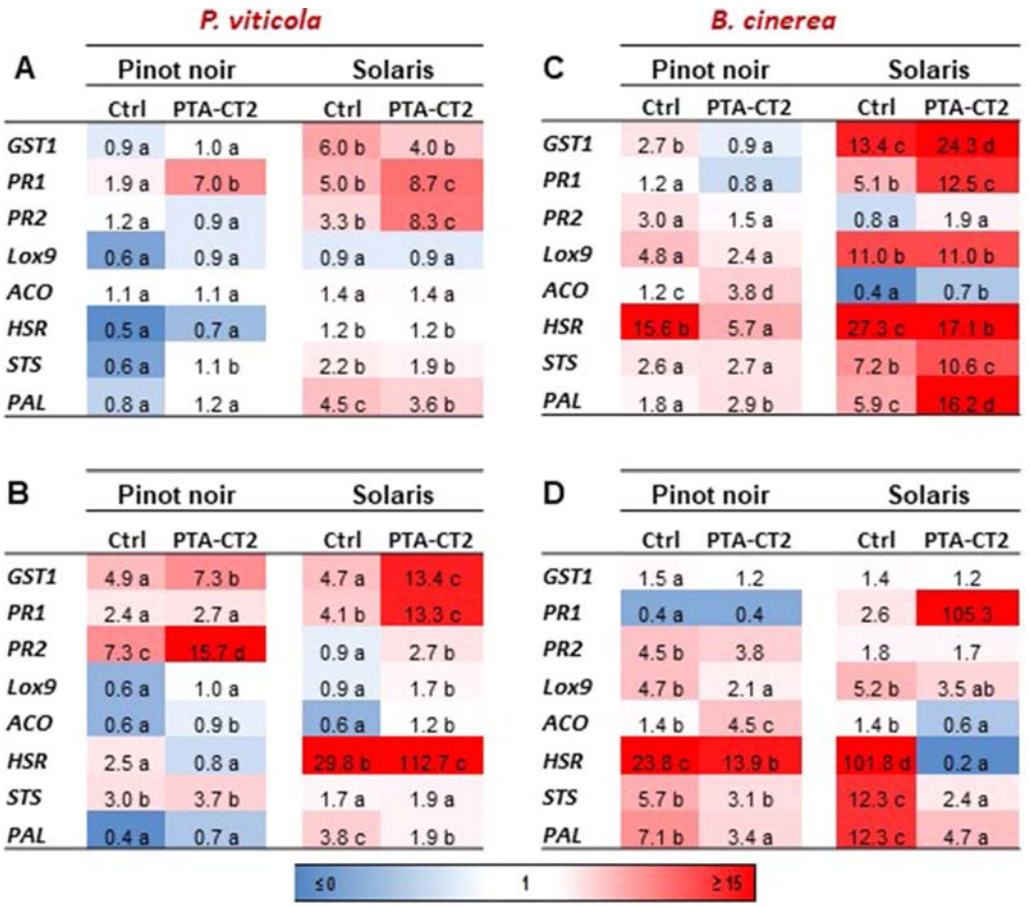

FIGURE 6 | *Pseudomonas fluorescens* PTA-CT2 primes differential expression of defense-related genes in leaf disks of Pinot noir and Solaris after *Plasmopara viticola* and *Botrytis cinerea* inoculation. Grafted plants were treated with *P. fluorescens* PTA-CT2 at the root level, and 2 weeks later, leaf disks were excised from control and treated plants and infected with 20 µl of 10^5 spores per milliliter of *P. viticola* **(A**, **B)** or 5 µl of 10^6 conidia per milliliter of *B. cinerea* **(C**, **D)**. The transcript level of defense-related genes was determined by quantitative reverse-transcription PCR (RT-qPCR) at 2 days post inoculation. Uninfected control of Pinot noir was considered as a reference sample (1× expression level) for both cultivars, and heatmaps represent changes in transcript expression levels as indicated by the color shading. Data are the means from three independent experiments performed in 2016 **(A**, **C)** and 2017 **(B**, **D)**. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$). *GST1*, glutathione-S-transferase 1; *PR1*, pathogenesis-related protein 1; *PR2*, β-1,3-glucanase; *LOX9*, lipoxygenase 9; *ACO*, 1-aminocyclopropane-1-carboxylic acid oxidase; *HSR*, hypersensitive related; *PAL*, phenylalanine ammonia-lyase; *STS*, stilbene synthase.

(**Figures 6C**, **D**) compared to Pinot noir. The expression of *PAL* was also higher in Solaris than in Pinot noir, suggesting that the basal resistance of Solaris to *P. viticola* can be linked to activated SA and phenylpropanoid defense pathways. However, after *P. viticola* inoculation, ISR-expressing plants by PTA-CT2 were primed for augmented expression of *PR1* in Pinot noir and *PR1* and *PR2* in Solaris in year 2016 (**Figure 6A**), while the transcript level of G*ST1*, *PAL*, and *STS* remained high in Solaris and comparable to control in 2016 (**Figure 6A**). The expression level of JA- and ET-responsive genes (*LOX9* and *ACO*) as well as that of *HSR* remained low in both cultivars and unchanged in the ISR-expressing plants compared to untreated plants. This highlights the prominent role of the SA signaling and phenylpropanoid pathways in the bacteria-mediated ISR against *P. viticola*.

Experiments performed in 2017 showed similar profiles with amplified gene expression in noninduced and ISR-expressing plants (**Figure 6B**). This demonstrates the ability of plants to respond and resist better after a second treatment in 2017. SA-related defense responses (*GST1*, *PR1*, and *PR2*) were much more expressed after *P. viticola* challenge. Interestingly, SA-responsive, but not JA- or ET-responsive, genes were activated in both cultivars after *P. viticola* challenge. Bacterium-mediated ISR against *P. viticola* is also accompanied by an impressive upregulation of *HSR* gene in Solaris, but not in Pinot noir. This result emphasizes the important role of the SA pathway in mediating ISR in both cultivars and further indicates that the HR, which is effective and strongly primed in Solaris, is deficient in the highly susceptible cultivar to *P. viticola*.

We also monitored transcript levels of the same genes after infection with the necrotroph *B. cinerea*. We showed that PTA-CT2 primed ET-responsive (*ACO*), but not JA- (*LOX9*) or SA-responsive genes (*GST1*, *PR1*, and *PR2*) in Pinot noir over the two consecutive years (**Figures 6C**, **D**). However, JA- and SA-responsive genes were highly upregulated in Solaris. PTA-CT2 treatment resulted also in a strong downregulation of *HSR*, a marker of cell death, in both cultivars after *B. cinerea* challenge (**Figures 6C**, **D**). These results suggest that the primed machinery of hypersensitive cell death by the beneficial bacterium may play a crucial role in strengthening resistance of Solaris against the biotrophic *P. viticola*, while induced resistance against the necrotrophic *B. cinerea* involves a decreased expression of *HSR* in both cultivars.

We also analyzed genes of the phenylpropanoid pathway *PAL* and *STS* that encode PAL and stilbene synthase involved in the biosynthesis of stilbenic phytoalexins. Both genes were significantly primed by PTA-CT2 in both cultivars upon *B. cinerea* inoculation, especially in 2016 (**Figure 6C**). The primed expression of PAL and STS was higher in Solaris than in Pinot noir. However, in both cultivars, the transcript level of PAL and STS remained low after renewing treatment with PTA-CT2 in 2017.

PTA-CT2 Primes Phytoalexin Accumulation After Pathogen Inoculation in Both Pinot Noir and Solaris

Stilbenoid compounds are important secondary metabolites of grapevine that represent central phytoalexins and therefore constitute an important element of basal immunity. In this study, the difference between induced resistance phenotypes of both cultivars was sought with respect to their output of stilbene accumulation. Results revealed significant differences in resveratrol, ε-viniferin, and δ-viniferin content between nontreated cultivars after pathogen inoculation (**Figure 7**). After *P. viticola* challenge, Solaris accumulated comparable amounts of resveratrol as Pinot noir did (**Figure 7A**), while the amounts of ε-viniferin (**Figure 7B**) and δ-viniferin (**Figure 7C**) were higher in Solaris than in Pinot noir. PTA-CT2 primed both ε- and δ-viniferin accumulation in ISR-expressing Pinot noir, while primed accumulation of resveratrol and δ-viniferin was recorded in Solaris in the 2016 season (**Figures 7B**, **D**). The primed δ-viniferin amount reached a higher level in challenged Solaris compared to the mock-infected plants and to Pinot expressing ISR (**Figure 7C**). Similar results were obtained over the 2017 year (data not shown), and no additional accumulation of these stilbenes was observed after *P. viticola* challenge. These results indicate that PTA-CT2-mediated ISR against *P. viticola* is tightly associated with priming stilbenoid compounds in the leaf tissues of both varieties.

Significant differences regarding the level of ε- and δ-viniferin were also recorded among cultivars after 48 h of *B. cinerea* infection (**Figures 7E**, **F**). Solaris exhibited a higher level of these stilbenoids than did Pinot noir, while both cultivars accumulated similar amounts of resveratrol (**Figure 7D**). PTA-CT2 treatment increased significant accumulation of resveratrol in both Pinot noir and Solaris after *B. cinerea* infection (**Figure 7D**). The beneficial bacterium also potentiated Pinot noir for enhanced accumulation of ε-viniferin (**Figure 7E**) and δ-viniferin (**Figure 7F**). No enhanced accumulation of these stilbenes was observed in Solaris, but their amount was maintained to a high extent in PTA-CT2-treated plants.

PTA-CT2 Primes Differential Accumulation of Phytohormones in Grapevine Cultivars Depending on the Pathogen Lifestyle

To gain further insight into the link between resistance phenotypes primed by PTA-CT2 and hormonal signaling, we monitored the amount of SA, JA, ACC (a precursor of ET), and ABA in Pinot noir and Solaris leaf disks after infection with *P. viticola* and *B. cinerea*. Results from the 2017 season (**Figure 8**) showed differential phytohormone accumulation between cultivars after pathogen challenge. After *P. viticola* inoculation, SA was accumulated to similar levels in both cultivars (**Figure 8A**), while JA (**Figure 8B**) and ACC (**Figure 8C**) levels were higher in Solaris than in Pinot noir. The control infected Pinot noir showed a higher level of ABA compared to Solaris (**Figure 8D**).

Pretreatment with PTA-CT2 induced significant accumulation of SA and ABA by 3- and 1.7-fold, respectively, compared to the infected control (**Figures 8A**, **D**). The increased accumulation of SA is consistent with the upregulation of SA-responsive genes *GST1* and *PR2* in the susceptible variety after *P. viticola* challenge (**Figure 6B**). This highlights the important role of SA level and signaling in ISR triggered by *P. fluorescens* against *P. viticola*. In PTA-CT2-treated Solaris, a nonconsistent change was observed

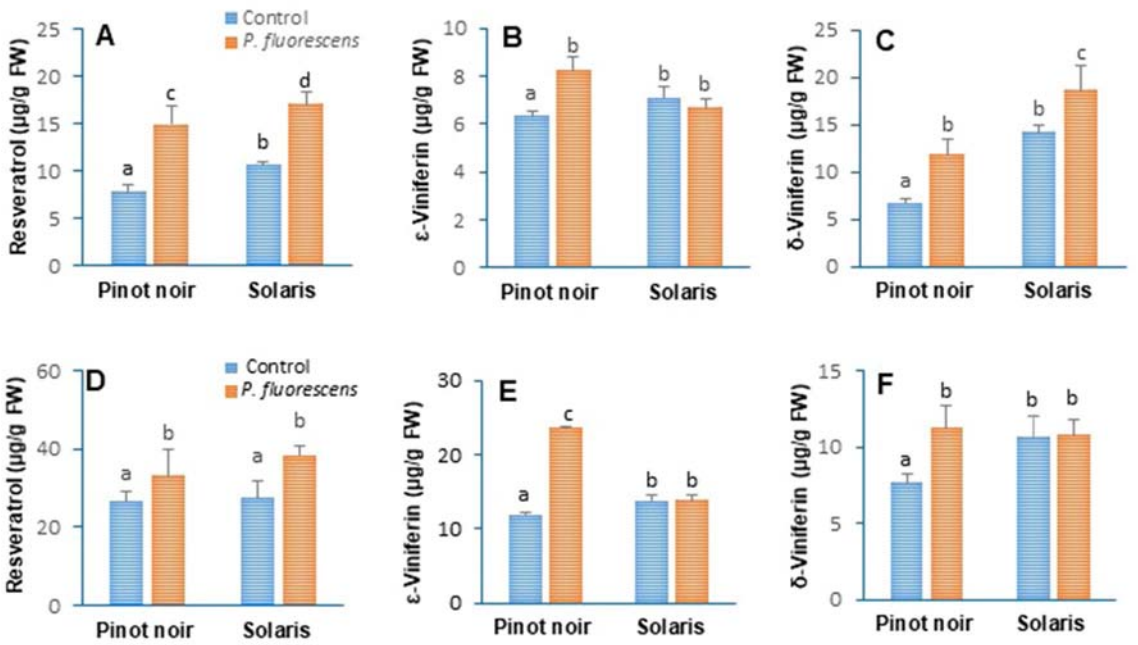

FIGURE 7 | *Pseudomonas fluorescens* PTA-CT2 primes phytoalexin accumulation in leaf disks of Pinot noir and Solaris after *Plasmopara viticola* and *Botrytis cinerea* inoculation. Grafted plants were treated with *P. fluorescens* PTA-CT2 at the root level. Two weeks later, leaf disks were excised from control and treated plants and infected with 20 µl of 10^5 spores per milliliter of *P. viticola* **(A–C)** or 5 µl of 10^6 conidia per milliliter of *B. cinerea* **(D–F)**. The amounts of resveratrol **(A, D)**, ε-viniferin **(B, E)**, and δ-viniferin **(C, F)** were determined by ultra-performance liquid chromatography (UPLC) at 2 days post inoculation. Values corresponding to uninfected control or PTA-CT2-treated plants are similar to those before inoculation (**Figure 1**), with less than 1 µg g^{-1} FW. Data are the means from three independent experiments performed in 2016, and vertical bars indicate standard deviation. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$).

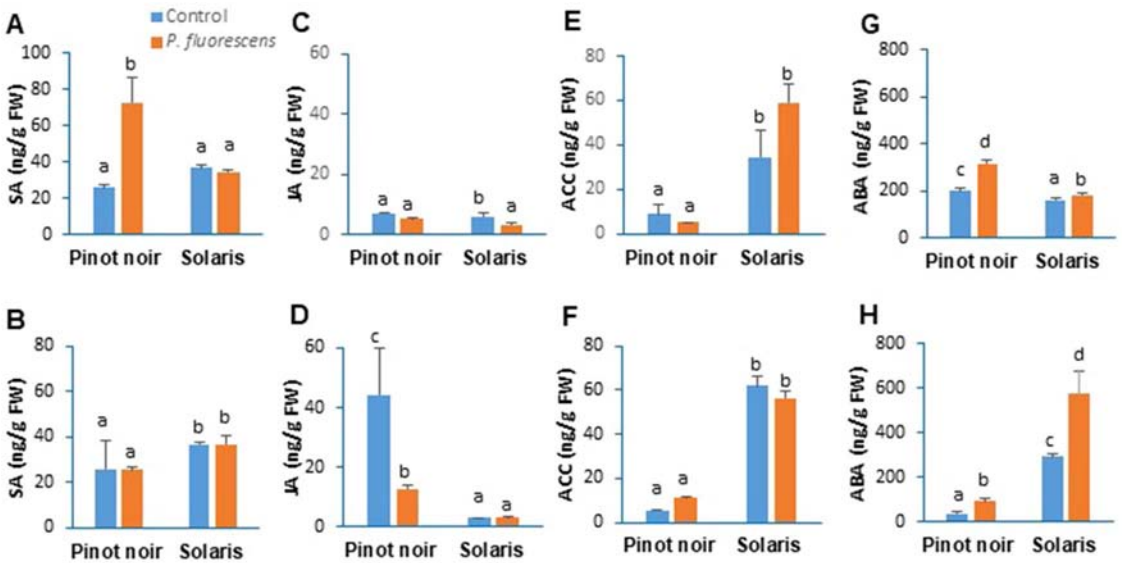

FIGURE 8 | *Pseudomonas fluorescens* PTA-CT2 primes phytohormone production in leaf disks of Pinot noir and Solaris after *Plasmopara viticola* and *Botrytis cinerea* inoculation. Grafted plants were treated with *P. fluorescens* PTA-CT2 at the root level. Two weeks later, leaf disks were excised from control and treated plants and infected with 20 µl of 10^5 spores per milliliter of *P. viticola* **(A, C, E, G)** or 5 µl of 10^6 conidia per milliliter of *B. cinerea* **(B, D, F, H)**. The amounts of salicylic acid **(A, B)**, jasmonic acid **(C, D)**, 1-aminocyclopropane-1-carboxylic acid **(E, F)**, and abscisic acid **(G, H)** were determined by liquid chromatography (LC)–tandem mass spectrometry (MS/MS) at 2 days post inoculation. Values corresponding to uninfected control or PTA-CT2-treated plants are similar to those before inoculation (**Figure 2**). Data are the means from three independent experiments performed in 2017, and vertical bars indicate standard deviation. Different letters indicate statistically significant differences between the treatments (ANOVA Tukey test, $P < 0.05$).

in SA (**Figure 8A**) and ACC (**Figure 8C**) content, which remained high after pathogen infection. However, a slight but significant accumulation of ABA was potentiated in the partially resistant Solaris (**Figure 8D**).

After *B. cinerea* infection, nontreated Solaris accumulated higher amounts of SA (**Figure 8E**), ACC (**Figure 8G**), and ABA (**Figure 8H**) compared to nontreated Pinot noir, which showed a greater accumulation of JA (**Figure 8F**). However, in PTA-CT2-treated Pinot noir, the amount of SA and ACC did not change, and only ABA accumulated after *B. cinerea* infection. Surprisingly, the JA content decreased after *B. cinerea* infection as compared to the infected control (**Figure 8F**). No additional accumulation of SA, JA, and ACC was observed in Solaris after infection. However, PTA-CT2 potentiated a strong accumulation of ABA in this cultivar after *B. cinerea* challenge.

DISCUSSION

In this study, we examined the immune responses triggered by *P. fluorescens* PTA-CT2 against biotrophic oomycete *P. viticola* and the necrotrophic fungus *B. cinerea* and compared these responses in two grafted grapevine cultivars with contrasting susceptibility to downy mildew, Pinot noir and Solaris, the former being susceptible and the latter partially resistant to infection. We provide evidence that *P. fluorescens* PTA-CT2 induces ISR against *P. viticola* and *B. cinerea* under greenhouse conditions by priming common and distinct defensive pathways depending on the basal immunity of genotype.

PTA-CT2 Does Not Affect Basal Defenses but Elicits Differential Production of Phytohormones and Photosynthetic Efficiency Before Pathogen Inoculation

Our study indicates that grapevine cultivars displayed contrasting levels of basal defenses and phytohormones. As reported for other cultivars (Figueiredo et al., 2012), some SA-related genes were preferentially expressed in Solaris as partially resistant to downy mildew. Similarly, higher amounts of SA and ACC (ET precursor) were recorded in healthy leaves of Solaris compared to Pinot noir, which produces more JA. This contrast could support the hypothesis that the resistance conferred by *Rpv10* or *Rpv3* in Solaris (Schwander et al., 2012; Vezzulli et al., 2019) could be linked to the constitutive expression of some defense genes and specific hormonal balance in favor of SA production. However, PTA-CT2 did not induce any consistent change of basal expression of defense genes or the amount of phytoalexins in either cultivar. PTA-CT2 did not impact either SA or ACC levels but elicited differential accumulation of JA and ABA in leaf tissues. It is therefore suggested that PTA-CT2 may confer a hormonal homeostasis for each cultivar and thus prime plants for enhanced resistance upon subsequent pathogen attack. Our results also revealed some differences with the study performed with Chardonnay vitroplantlets (Gruau et al., 2015), which showed activation of systemic defense responses including expression of transcription factors and *PR* genes, as well as the synthesis of stilbenic phytoalexins in response to PTA-CT2. This difference could probably be due to the intrinsic difference of the plant models or grapevine varieties. Furthermore, the mode of action of PTA-CT2 seems to be different to that of other inducers of grapevine resistance against *P. viticola*, such as *T. harzianum* T39 (Perazzolli et al., 2012), thiamine (Boubakri et al., 2012), laminarin (Aziz et al., 2003), and its sulfated form PS3 (Gauthier et al., 2014), that directly elicit some defense responses even before infection.

Clear differences were also recorded between cultivars with respect to photosynthetic efficiency as indicated by higher values of Fv/Fm, ΦPSII, and ETR rate in Solaris than in Pinot noir. PTA-CT2-treated plants showed a significant increase of photosynthetic efficiency in both cultivars, highlighting an improvement of energy transfer within PSII after root treatment with PTA-CT2. This effect appears to be a common aspect of plant growth-promoting rhizobacteria (PGPR)-mediated defenses (Zhang et al., 2008; Miotto-Vilanova et al., 2016), suggesting that PTA-CT2 may initiate plant immunity by regulating the photosynthetic machinery as an energy supplier. Furthermore, enhancement of electron transfer is well known to reduce susceptibility to necrotrophic pathogens (Kangasjarvi et al., 2012). Altogether, the apparent increase of JA and ABA content and photosynthetic performance suggests that PTA-CT2 may mobilize the central metabolism of host plants, which could be crucial for the outcome of plant immunity. Further investigations are clearly needed to identify bacterial traits that govern the photosynthesis efficiency in grapevine plants.

PTA-CT2 Induces ISR Against *P. viticola* and *B. cinerea* to Different Extents in Susceptible and Resistant Cultivars

As expected, Pinot noir and Solaris displayed contrasting phenotypic susceptibility to both *P. viticola* and *B. cinerea*. Over the two consecutive years, Pinot noir showed higher susceptibility to *P. viticola*, but it was more tolerant to *B. cinerea* compared to Solaris. These phenotypic differences could be attributed to contrasting pathogen infection strategies and host defenses against biotrophic and necrotrophic pathogens (Armijo et al., 2016). We also clearly demonstrated that PTA-CT2 strongly reduced downy mildew disease in both susceptible and partially resistant cultivars. To our knowledge, this is the first report characterizing effective protection of bacteria against *P. viticola* in *V. vinifera* genotypes. These effects are comparable to those induced by BABA in Chasselas (susceptible) and Solaris (tolerant) cultivars (Slaughter et al., 2008). PTA-CT2-induced resistance against gray mold disease was also confirmed in both Pinot noir and Solaris, consistent with our previous results using the Chardonnay cultivar (Verhagen et al., 2011; Gruau et al., 2015; Aziz et al., 2016). However, the level of induced protection was comparable between cultivars against *P. viticola*, while the efficiency of PTA-CT2 was greater in Pinot noir compared to Solaris. In both cases, the bacterium did not exert any direct effect on the pathogen growth or sporulation. Furthermore, it has been shown that PTA-CT2 can colonize grapevine roots, but not shoots (Gruau et al., 2015), suggesting that the protection mediated by this bacterium against both

necrotroph and biotroph pathogens resulted from an ISR in grapevine plants. Although additional proof is needed, the results raise the possibility that the efficacy of bacteria may rely on its root colonization capacity that could be influenced by basal defenses of genotype.

PTA-CT2 Primes Common and Distinct Defensive Pathways Against *P. viticola* and *B. cinerea* in Susceptible and Partially Resistant Cultivars

Our results clearly revealed an induced host priming state by *P. fluorescens*, with differential responsiveness between cultivars. After *P. viticola* inoculation, most responsive genes to SA (*GST1*, *PR1*, and *PR2*), *HSR*, and *PAL* were greatly upregulated, along with higher accumulation of JA and ACC in Solaris than in Pinot noir. Both cultivars accumulated the same amount of SA, but ABA content was higher in Pinot noir. This result can explain the contrasting phenotypes and supports the idea that the basal resistance of Solaris to *P. viticola* can be linked to an activated SA pathway, phytoalexin synthesis, and HR-like cell death, which are considered as key components differentiating genotypic resistance (Zyprian et al., 2016; Figueiredo et al., 2017; Vezzulli et al., 2019). According to our results, it has also been reported (Polesani et al., 2010; Malacarne et al., 2011) that *P. viticola* infection resulted in an induction of genes involved in ET biosynthesis more importantly in resistant than in susceptible cultivars. An important role of JA signaling in grapevine resistance against *P. viticola* has also been suggested in a study (Figueiredo et al., 2015) performed on two genotypes with different degrees of resistance to this pathogen. Similarly, the JA signaling pathway was shown to be implicated in induced resistance against powdery and downy mildews in grapevine by different elicitors (Hamiduzzaman et al., 2005; Trouvelot et al., 2008).

Interestingly, PTA-CT2 potentiated a high expression of SA-related genes and phytoalexin synthesis, while JA- and ET-responsive genes remained unchanged after *P. viticola* challenge. The primed responses seem to be enhanced by the second bacterial treatment in both cultivars, along with a strong expression of the HR-related gene in Solaris, indicating a common functional role of the SA signaling and stilbene accumulation in the PTA-CT2-induced resistance in both genotypes and more specifically of primed HR-like cell death in Solaris upon *P. viticola* inoculation. These results are at least partially in accordance with those reported with sulfated laminarin PS3 that primes SA- and HR-dependent defenses in both susceptible and tolerant cultivars (Trouvelot et al., 2008; Gauthier et al., 2014), but not with *T. harzianum* T39, which rather primes JA and ET pathways (Perazzolli et al., 2008; Palmieri et al., 2012) upon downy mildew infection. It has been shown that BABA-improved resistance of Solaris was accompanied by upregulation of genes involved in phytoalexin pathways (Slaughter et al., 2008). The upregulation of SA-related genes is consistent with the primed SA level, accompanied by ABA accumulation in the susceptible variety after *P. viticola* challenge. This suggests that ISR against *P. viticola* in the susceptible cultivar may be dependent on the coordinated action of both SA and ABA, rather than on HR-like cell death. The primed accumulation of ABA can be linked to its role in regulating stomatal closure (Ton et al., 2005) or callose biosynthesis and deposition (Flors et al., 2005), thus inhibiting pathogen spread and reducing disease infection.

However, after *B. cinerea* inoculation, Solaris plants showed enhanced expression of SA-related genes and accumulated higher amounts of SA, while Pinot noir rather accumulated JA. This can explain the contrasting susceptibility of Pinot noir and Solaris to the biotroph *P. viticola* and the necrotroph *B. cinerea*, respectively, as it is generally reported that JA and SA signaling cross-talk and have a mutual antagonistic effect on each other (Pieterse et al., 2009). Interestingly, PTA-CT2 primed ET gene expression and a high amount of JA in Pinot noir, while it potentiated high expression of JA- and SA-responsive genes and an increased level of ABA. This indicates that bacterium-mediated ISR to the necrotroph *B. cinerea* is dependent on JA/ET signaling and also on the basal immunity of each cultivar. The role of ABA in ISR needs further proof. PTA-CT2 treatment resulted also in an upregulation of *PAL* and *STS* genes and primed accumulation of resveratrol and viniferin in Pinot noir, whereas the amount of these stilbenes was maintained to a high extent in Solaris. These results confirm the prominent role of stilbenoids in strengthening resistance in grapevine plants against both biotrophic (Vezzulli et al., 2019) and necrotrophic pathogens (Gruau et al., 2015; Aziz et al., 2016). However, PTA-CT2 strongly reduced the expression of *HSR* in both cultivars after *B. cinerea* inoculation, suggesting that the primed machinery of hypersensitive cell death by the beneficial bacterium may play a crucial role in improving resistance of Solaris against the biotrophic *P. viticola*, while ISR against the necrotrophic *B. cinerea* seems to be associated with decreased HR-like cell death in both cultivars. This could be related to the necrotrophic nature of *B. cinerea*, which tends to trigger HR as a mechanism to provide the nutrient supply necessary for the infection process (Govrin and Levine, 2000).

Overall, we showed that grapevine cultivars displayed contrasting levels of basal defenses and phytohormones, as well as phenotypic susceptibility to *P. viticola* and *B. cinerea*. SA-related genes and accumulation of SA and ACC were preferentially expressed in the partially resistant Solaris, compared to the susceptible Pinot noir that required more JA signaling. The resistant cultivar also exhibited higher photosynthetic efficiency than the susceptible one. We also demonstrated that *P. fluorescens* PTA-CT2 induces ISR against *P. viticola* and *B. cinerea* by priming common and distinct defensive pathways depending on the basal immunity of genotype (**Figure 9**). Although PTA-CT2 seems to mobilize the host metabolism before infection, our results highlight an important role for SA-dependent defense and phytoalexin synthesis, but not JA and ET signaling in ISR against *P. viticola* in both susceptible and partially resistant cultivars. The priming state relies further on the strong expression of HR-like cell death in Solaris, while in the susceptible cultivar, it involves accumulation of ABA, rather than HR-like cell death. Interestingly, bacterium-mediated ISR against *B. cinerea* is dependent on JA/ET signaling and also on the accumulation of stilbenoids and weakening of hypersensitive cell death in both cultivars.

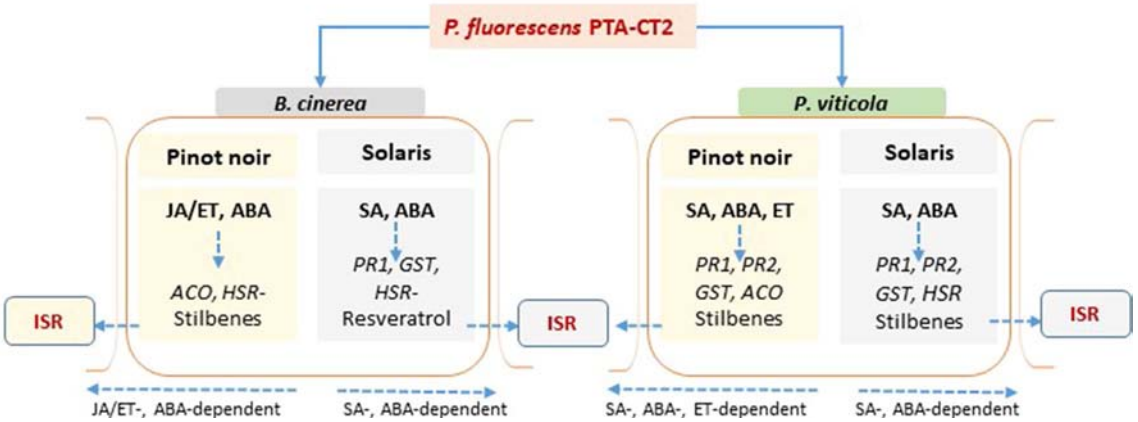

FIGURE 9 | Proposed model summarizing defensive pathways primed in *Pseudomonas fluorescens*-induced systemic resistance against *Botrytis cinerea* and *Plasmopara viticola* in the susceptible Pinot noir and the partially resistant Solaris grapevine genotypes. The phytohormones are in bold, the genes are in italic, and the sign (–) in front of the *HSR* gene means downregulated expression.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

AA, CC, and SL conceived and designed experiments; SL, FR, AS, and PT-A performed experiments; FR, AS, EN-O, CC, and AA gave their expertise for all steps of this work; AA, SL, FR, AS, and EN-O analyzed and discussed experiments; AA and SL wrote the paper with the contribution of all co-authors. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01112/full#supplementary-material

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Temporal Dynamics of the Sap Microbiome of Grapevine Under High Pierce's Disease Pressure

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Grapevine is a pillar of the California state economy and agricultural identity. This study provides a comprehensive culture-independent microbiome analysis from the sap of grapevine overtime and in a context of a vascular disease. The vascular system plays a key role by transporting nutrient, water and signals throughout the plant. The negative pressure in the xylem conduits, and low oxygen and nutrient content of its sap make it a unique and underexplored microbial environment. We hypothesized that grapevine hosts in its sap, microbes that have a beneficial impact on plant health by protecting against pathogen attack and supporting key biological processes. To address this hypothesis, we chose a vineyard under high Pierce's disease (PD). PD is caused by the xylem-dwelling pathogenic bacterium *Xylella fastidiosa*. We selected ten grapevines within this vineyard with a range of disease phenotypes, and monitored them over 2 growing seasons. We sampled each vines at key phenological stages (bloom, veraison, and post-harvest) and used an amplicon metagenomics approach to profile the bacterial (16S -V4) and fungal (ITS) communities of the sap. We identified a core microbiome of the sap composed of seven bacterial (*Streptococcus, Micrococcus, Pseudomonas, Bacteroides, Massilia, Acinetobacter* and *Bacillus*) and five fungal (*Cladosporium, Mycosphaerella, Alternaria, Aureobasidium*, and *Filobasidium*) taxa that were present throughout the growing season. Overall, the sap microbial makeup collected from canes was more similar to the root microbial profile. Alpha diversity metrics indicated a microbial enrichment at bloom and in vines with moderate PD severity suggesting a host-driven microbial response to environmental cues. Beta diversity metrics demonstrated that disease condition and plant phenology impacted microbial community profiles. Our study identified several potential taxonomic targets with antimicrobial and plant growth promoting capabilities that inhabit the grapevine sap and that should be further tested as potential biological control or biofertilizer agents.

Keywords: *Vitis vinifera* L., grapevine, sap, microbiome, Pierce's disease, *Xylella fastidiosa*, microbiome, xylem

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INTRODUCTION

The vascular system ensures several key biological functions in plants. The xylem and phloem elements are major vehicles for water and nutrient transportation and signaling routes between the below (i.e., root) and above (i.e., leaf, fruit) ground plant parts. The xylem and phloem saps differ in chemical composition and their profile is also influenced by the plant phenological stages

and environmental factors (Andersen et al., 1995; Lucas et al., 2013; Savage et al., 2016). In grapevine, the xylem sap is initially rich in sugars (especially glucose and fructose) and amino acids (especially glutamine) in the spring time right after the dormant season, because of the remobilization of nutrient reserves (e.g. starch) stored in the main permanent structure of the vine (Keller, 2010). This mobilization of nutrients is essential to support vegetative growth at bud-break until leaf becomes energy independent and transition from a sink to a source and export photosynthates and assimilates to the fruit via the phloem sap. To that end, research has focused on how irrigation and fertilization changed sap composition in order to maintain plant hydraulic function at maximum capacity and a balance between vegetative and reproductive growth (Peuke, 2000; El-Razek et al., 2011).

Several biotic and abiotic factors such as freeze damage, drought, and pathogen infection compromise the integrity of the grapevine vascular system and impair plant sap flux (Pouzoulet et al., 2014; Hochberg et al., 2017; Todaro and Dami, 2017). As a result the affected host shows a decrease in vigor and subsequently crop productivity and fruit marketability. One devastating vascular pathogen that plagues the California grapevine industry is Pierce's Disease (PD), caused by the gram-negative bacterium *Xylella fastidiosa* (Wells et al., 1987). Insect sharpshooters transmit *X. fastidiosa* to the host upon feeding events (Redak et al., 2004). Following infection, the bacterium systemically colonizes susceptible host and multiplies to high titer causing vascular occlusions and a loss of xylem conductivity (Deyett et al., 2019). As a result, grapevine decline rapidly displaying symptoms of canopy dwarfing and thinning as well as leaf scorching and berry raisining (Varela et al., 2001). *In planta*, *X. fastidiosa* resides exclusively in the lumen of the xylem vessel where the negative pressure, low oxygen and nutrient content selects for adapted microorganisms. However, very little is known about the microbial taxa that inhabit the grapevine sap, their population dynamic over a growing season and their interaction with *X. fastidiosa*. Wallis et al. (2013) showed that PD affected the grapevine sap chemical composition but no study has looked at microbial community shifts in response to pathogen infection and colonization of the host and during disease symptoms development.

Grapevine microbiome research has mainly focused on plant organ epiphytes (i.e., berry, leaf and root) because of its importance with grape production and specifically with regards to fruit and foliar diseases management as well as the biological significance of indigenous microbes with the regional signature of a wine (Bokulich et al., 2014; Perazzolli et al., 2014; Zarraonaindia et al., 2015). Identification of the microbial communities inhabiting the grapevine endosphere mocrobiome has been achieved using standard culture-dependent microbial techniques (West et al., 2010; Compant et al., 2011; Baldan et al., 2014; Dissanayake et al., 2018; Kraus et al., 2019). Culture-independent amplicon metagenomic approaches have recently been deployed to improve the microbial profiling of the grapevine woody organs including trunk and cane (Faist et al., 2016; Deyett et al., 2017; Dissanayake et al., 2018). Unraveling the microbiome of the grapevine sap would provide a comprehensive view of the microbes that systemically move throughout the plant and would establish a framework for targeting biological control agents (BCAs) of vascular pathogens or plant growth promoting agents. Identifying BCAs that can control *X. fastidiosa* through direct inhibition or niche displacement and/or commensal and symbiotic organisms that can support the host immune system could become an integrated alternative approach to the current insect vector management strategy. Our research was designed to address this gap in the knowledge by characterizing the bacterial and fungal taxa that shape the xylem sap microbial communities and determining how the host phenology and PD severity impact microbial profile.

MATERIALS AND METHODS

Sap Sample Collection

Shoot samples were collected from a commercial vineyard in Temecula, California, subjected to high insect sharpshooter (the vector of *X. fastidiosa*) pressure. Ten grapevines were selected in 2016 on the basis on PD symptoms, with five healthy vines showing no or mild symptoms and five vines showing intermediate to severe symptoms (**Figure 1**). Canes were later

FIGURE 1 | Pierce's disease (PD) rating scale; **(A)** No symptoms or mild PD symptoms with only a few canes (< 10% of the canopy) showing marginal leaf scorching; **(B)** Moderate PD symptoms with visual dwarfing and thinning on 10-50% of the canopy and/or canes displaying leaf scorching. The affected canes can also show green island and match-stick petioles and berry raisining late in the season. **(C)** Severe PD symptoms with 50–100% of the vine canopy displaying dwarfing, thinning, leaf scorching and wood dieback. The affected canes showed green island and match-stick petioles and berry raisining late in the season.

sampled in 2017 and 2018 at bloom (spring), veraison (summer) and post-harvest (fall) from the same grapevines. At each time point, three canes were pruned-off randomly along the grapevine cordons. In addition, grapevines were visually rated for PD symptoms at post-harvest using the same disease rating scale (no/mild, intermediate, or severe PD-symptoms; **Figure 1**). Canes were placed in a cooler with ice packs and brought back to the laboratory. On the same day of the sampling, canes were cut to 40 cm long, inserted in a Scholander pressure chamber (PMS Instruments, Albany OR) and pressurized to 400-550 PSI. Liquid sap was harvested from the end of the cane with a pipette and stored at 4°C until further processing. To obtain culturable sap microbiome, 20 µl of sap was plated onto each of the following media: lysogeny broth (LB), trypticase soy agar (TSA), and potato dextrose agar (PDA). TSA and LB plates were stored at 28°C for 3-5 days and PDA plates at room temperature for 7 days. The bacteria and fungi recovered were sub-cultured to obtain single colonies and pure strains, respectively.

DNA Extraction, PCR Condition, Library Preparation, and Sequencing

The DNA was extracted from 250 µl of sap using the ZymoBIOMICS DNA miniprep kit per manufacturer's protocol (Zymo Research). The DNA was quantified using a Synergy HTX multi-mode reader (Biotek Instruments, Winooski, VT) for nucleic acid quantification. In addition, the DNA was extracted from pure bacterial colonies and fungal strains using Qiagen blood and tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions for cultured cells with an initial extended incubation time of 30 min instead of 10 min.

The bacterial 16S and fungal ITS rRNA region was amplified for each DNA sample using the earth microbiome protocol and primers (http://www.earthmicrobiome.org/). Briefly, primers 515F and 806R with Caporaso barcodes were used for 16S amplification and ITS1f-ITS2 was used for fungal ITS amplification (Caporaso et al., 2010). Each sample was made in a 25 µl PCR reaction using 10 µl of Phusion hot start flex 2x master mix, 0.5 µl of primer (10 µm) and 2 µl of DNA. Thermal cycling parameters for ITS were 94°C for 1 min; 35 cycles of 94°C for 30 s, 52°C for 30 s, 68°C for 30 s; followed by 68°C for 10 min. Thermal cycling parameters for 16S amplification were 94°C for 3 min; 35 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s; followed by 72°C for 10 min. Each PCR was accompanied with a negative control to ensure that the mastermix and barcodes were not contaminated. Samples were checked against a 1% agarose gel for visualization of appropriate band size. PCR products were quantified using a Synergy HTX multi-mode reader for nucleic acid quantification. Libraries were prepared by combining equal quantities of DNA from each sample. Libraries were cleaned using AMPure XP PCR purification system (Beckman Coulter) per manufacturer's protocol. Final concentration of libraries was quantified using qPCR and bioanalyzer before being sequenced on the MiSeq instrument (Illumina, San Diego, USA) using 600-cycle (2X300 paired-end) V3 sequencing kit for fungal reads and the 500-cycle (2x250 paired-end) V2 sequencing kit for 16S at the UCR Genomics Core. Fungal and bacterial sequences were deposited in NCBI under the accession number PRJNA548584.

Culturable microbes were Sanger sequenced using the 27F/1392R primer pair of the 16S bacterial gene (Turner et al., 1999) and ITS1/ITS4 primer pair for fungal ITS region (White et al., 1990). Each sample was made in a 25 µl PCR reaction using 10 µl of Phusion hot start flex 2x master mix, 0.5 µl of each primer (10 µM) and 2 µl of DNA template. Thermal cycling parameters were 94°C for 2 min; 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min; followed by 72°C for 5 min. PCR product was visualized on a 1% agarose gel for appropriate band size, cleaned using Qiagen PCR Clean Up kit (Qiagen, Valencia, CA), quantified using Synergy HTX quantifier and submitted for Sanger sequencing at the UCR Genomics Core. Sequences were check for quality before using the NCBI BLAST database for identification.

Computational Analyses

Raw reads were first filtered for quality using sliding window 5:20 with trimmomatic (Bolger et al., 2014). Reads shorter than 125 bp were removed and low quality reads were filtered out. PhiX reads were then removed from the sequences. Due to the varying lengths of the fungal ITS reads, primers were removed using cutadapt version 1.18 and matching to primer sequences. Libraries were then demultiplexed using QIIME version 1.9.1 (Caporaso et al., 2010). DADA2 version 1.6.0 (Callahan et al., 2016) in R version 3.4.4 was used for the remainder of the processing. Within DADA2, sequences were filtered to contain no ambiguous base calling, and no more than two errors. 16S reads were fixed at lengths for 240 bp. Reads were then de-replicated, and further filtered using the sample inference algorithm and learned error rates within the DADA2 pipeline. Paired end reads were merged using standard arguments. Chimeric sequences were also filtered out. Taxonomy was assigned using the Unite database version 10.10.2017. DADA2 method creates ASV (amplicon sequence variant, similar to operational taxonomic units or OTUs, but is capable of resolving amplicons to a single nucleotide) and thus may make species level identification when 100% of sequences match to reference (Callahan et al., 2016). Bacteria identification was realized using the IDTAXA classification against the SILVA SSU r132 reference database. ClustalW and maximum likelihood parameters were used to create a bacterial tree for UniFrac metrics.

Data were then inputted into the phyloseq package version 1.22.3 (McMurdie and Holmes, 2013). Microbes unidentified at the kingdom and phylum level or classified as plant or other contaminants were removed for both datasets. Phyla that did not occur in at least 5 % of samples were removed as well as singletons, doubletons and taxa that only occurred in one vine. Samples that had too few reads were also removed. For analysis, comparing disease severity, only the post-harvest time points were used. For taxonomy charts, libraries were aggregated to the genus level and transformed to relative abundance. Fungal

and bacterial genera were filtered to the 0.1% level. SunburstR version 2.1.0 was utilized to visualize the taxonomy pie charts. Statistical differences between genera in the *Xanthomonadaceae* were computed using Kruskal-Wallis and pairwise comparisons where done with Wilcox pairwise test. Prevalence Venn diagrams was used to identify unique taxa that were more likely associated with the specific grapevine phenological stages at the different sampling time point and with specific disease condition. We set stringent filters with taxa needed to occur in at least 50% of the samples in a given category. UpSetR version 1.3.3 was used to create graphical representation of prevalence Venn diagrams.

Using the number of unique families as a proxy for alpha diversity, mean and standard error were calculated for each time point grouping. Error bars represent standard error of the mean. Statistics on count of family were determined by generalized linear model using Poisson regression and statistical significance on pairwise comparison were conducted through Tukey's test using the multcomp package version 1.4-8. To look at the difference between groupings, beta-diversity plots were created using weighted UniFrac and PCoA metrics for bacteria and Bray-Curtis dissimilarity matrix and NMDS ordination matrix for fungi using the vegan package version 2.4-5. Adonis test with 999 permutations were run to determine statistical differences among centroids. Pairwise comparisons were calculated with the RVAideMemoire package version 0.9-71. To find differentially abundant microbe associated with season or condition, DeSeq2 was used. Microbes that occurred in more than one sample and in more than one year were used in the analysis. Random Forests supervised machine learning algorithm was applied to the dataset to learn a function that relates predictors (ASVs) to classification, here phonological stage and disease condition (Breiman 2001). Random Forest creates a function using part of the dataset to train and validates that function by classifying the remaining subset of samples. ASVs are assigned with an importance score, which indicates the increase in the functions error rate if the taxa would be removed. In this way, microbes with a high importance score are valuable predictors for a classification. Random forest was carried out using package randomForest version 4.6-14.

RESULTS

Dada2 analysis revealed that there were 2,875 bacterial amplicon sequence variants (ASVs) and 2,694 fungal ASVs in 68 sap samples (two samples were removed based on poor quality sequences). After removal of contaminants, singletons, doubletons and taxa that did not occur in at least 5% of the samples, there were 168 fungal ASVs and 390 bacterial ASVs. Sap samples that did not have at least 500 reads were removed from the analyses. After filtering, we used a total of 61 samples from the bacterial dataset including 26 from post-harvest, 15 from bloom and 20 from veraison and a total of 65 samples from the fungal dataset including 29 from post-harvest, 16 from bloom, and 20 from veraison.

Phylogeny and relative abundance of fungal and bacterial taxa were represented in a Sunburst plot (http://rpubs.com/edeye001/487241). Results showed that fungal communities were dominated by the *Ascomycota* phylum (~92%) and the remaining was composed with the phylum *Basidiomycota* (~8%). *Cladosporium* represented over half (~56%) of the total fungal genera followed by *Mycosphaerella* (~16%) and *Alternaria* (~9%). *Filobasidium* (~6%) was the major genus among the *Basidiomycota*. The sap culturable mycobiome indicated that *Cladosporium* was also the main fungus recovered and also included the taxa *Filobasidium* and *Mycosphaerella*. The bacterial community composition was more diverse than the fungal community. *Proteobacteria* (~54%) was the major phylum, followed with *Firmicutes* (~24%), *Actinobacteria* (~12%), and *Bacteroidetes* (~8%). At the genus level an unidentified genus in the *Enterobacteriaceae* family was most abundant (~19%). *Streptococcus* represented (~10%) followed with *Bacteroides* (~6%), *Bacillus* (~6%), *Acinetobacter* (~4%) and *Pseudomonas* (~3%). The culturable bacteria were mostly in the genus *Bacillus*, but *Paenibacillus*, *Virgibacillus*, *Curtobacterium*, and *Micrococcus* were also re-isolated.

The *Xanthomonadaceae* (the family that *X. fastidiosa* belongs to) represented 3% of the overall dataset and was comprised of three genera, *Stenotrophomonas* (~2%), *Xylella* (~0.5%), and *Pseudoxanthomonas* (0.5%). *Xylella*, the causal agent of PD, was found at low frequencies and low incidence (11 samples total) and mostly at post-harvest (eight samples of the total 26 post-harvest samples = 31%). *Xylella* was also detected at bloom (one sample) and veraison (two samples). Relative abundance of *Xylella* at the post-harvest time point was only significantly higher ($P < 0.05$) in vines showing moderate PD symptoms compared to healthy vines (i.e., showing mild or no symptoms; **Figure 2**). *Stenotrophomonas* incidence and abundance was the highest in healthy vines although not statistically different from the other disease conditions.

Alpha-diversity showed that there were approximately twice as many bacterial than fungal families in grapevine sap (**Figure 3A**). Bacterial family richness increased significantly at post-harvest in aging grapevines between 2016-17 and 2018 ($P < 0.001$). In addition, bloom and veraison time-points displayed significant higher family richness compared to post-harvest in both years for bacteria ($P < 0.001$) and in 2018 for fungi ($P < 0.01$). Moreover, vine condition impacted microbial diversity (**Figure 3B**). Vines categorized as moderately PD symptomatic at post-harvest harbored significantly higher number of bacterial and fungal families compared to mildly symptomatic vines ($P < 0.05$). Severely symptomatic vines also had significantly fewer unique bacterial families compared to moderately symptomatic vines ($P < 0.05$).

Weighted UniFrac of bacterial beta-diversity plots (**Figure 4A**) indicated a significant clustering due to the year of sampling ($P < 0.001$) and the time of sampling in a given year ($P < 0.01$). Pairwise PERMANOVA revealed that bloom was distinct from the other two phonological stages ($P < 0.05$) and that 2018 was distinct from 2016 and 2017 ($P < 0.01$). Fungal Bray-Curtis beta-diversity plots (**Figure 4B**) indicated that both

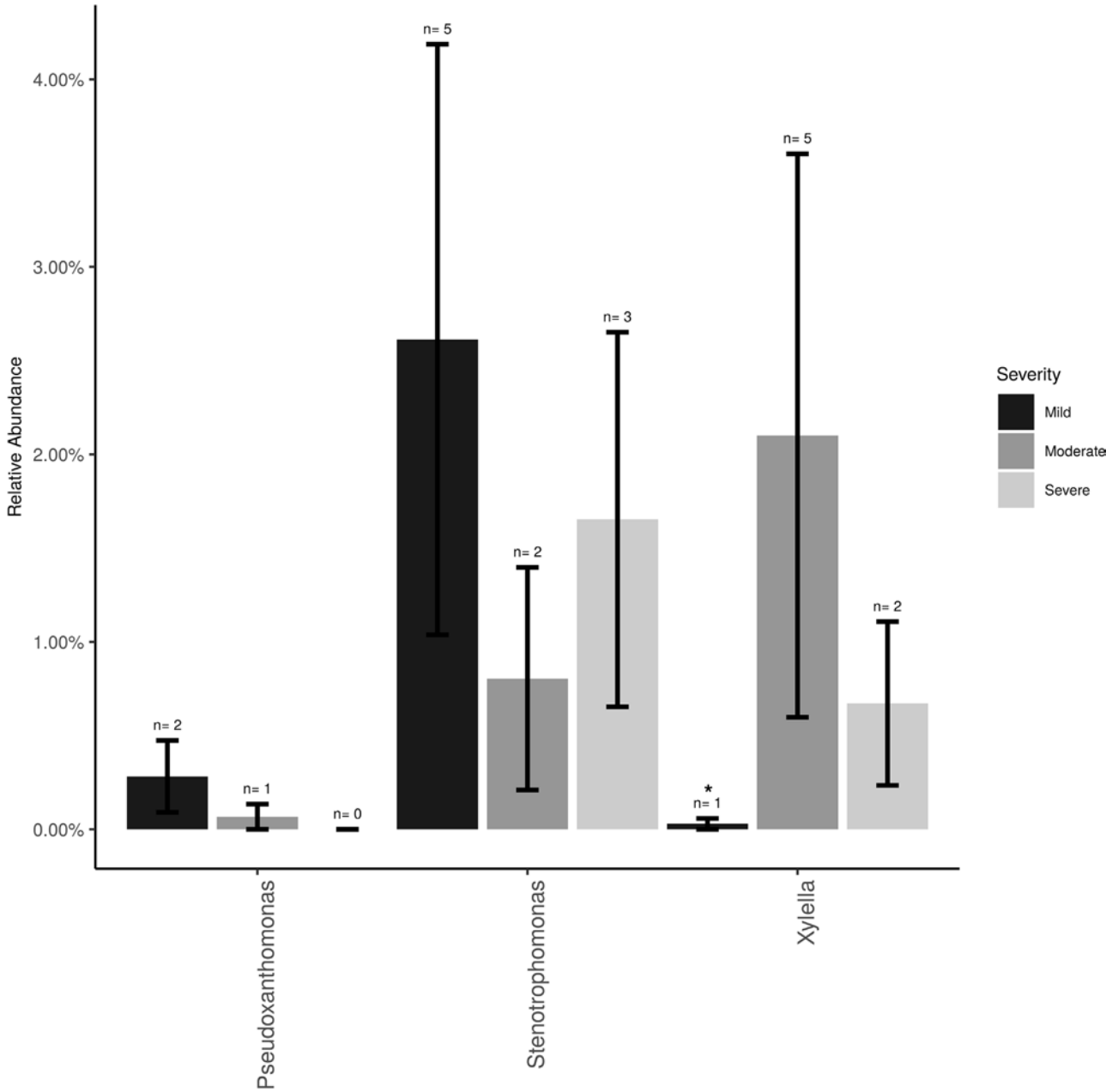

FIGURE 2 | Mean relative abundance and incidence for *Xylella*, *Stenotrophomonas and Pseudoxanthomonas* (Family *Xanthomonadaceae*) in the total sampled grapevines (n = 26) showing no/mild (n = 12), moderate (n = 7) or severe (n = 7) PD symptoms at post-harvest. Error bars represent standard error of the mean. n value above the bar represent the number of vines that tested positive for each bacterium.

year of sampling ($P < 0.01$) and time of sampling ($P < 0.01$) were significantly different in the clustering of fungal communities. There was a strong clustering of the samples collected at post-harvest for all three years in comparison to other sampling year and time. A pairwise PERMANOVA revealed that post-harvest was significantly different to both bloom and veraison ($P < 0.05$) and 2018 was significantly different from both 2017 and 2016, and that 2016 was significantly different than 2017 ($P < 0.05$). Prevalence Venn diagrams indicated that seven bacteria (*Streptococcus, Micrococcus, Pseudomonas, Bacteroides, Massilia, Acinetobacter* and *Bacillus*) and five fungi (*Cladosporium, Mycosphaerella, Alternaria, Aureobasidium*, and *Filobasidium*) were ubiquitous across season at all three plant phenological stages (**Figure 5**). In contrast, other taxa were more likely associated with specific seasonal time points likely contributing for the significant differences in alpha diversity (**Figure 3A**). Hence, *Sphingobium, Novosphingobium, Methylobacterium* and *Streptomyces* were more specific to

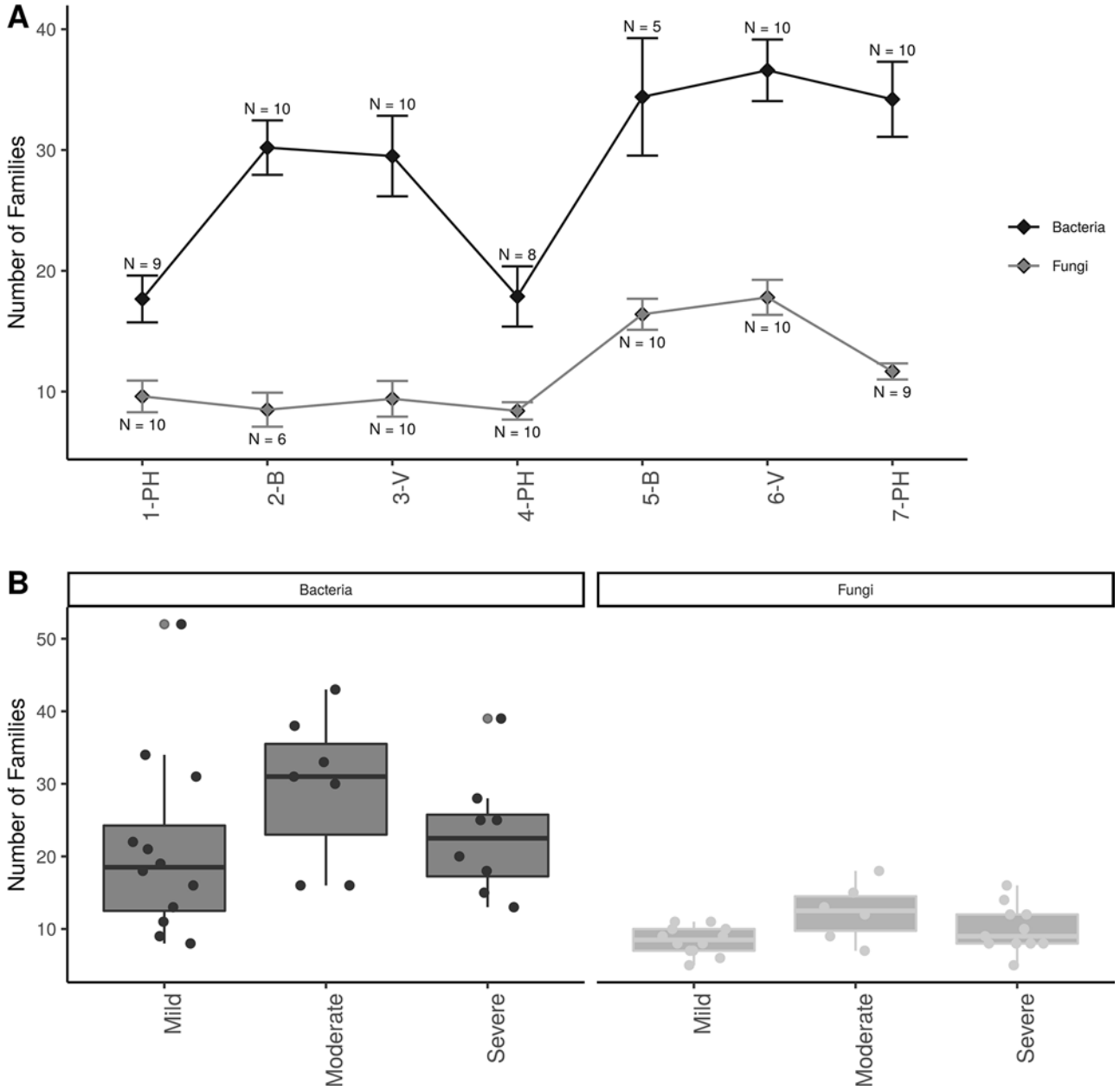

FIGURE 3 | Alpha diversity plots; **(A)** Familial richness in bacterial and fungal sap microbiomes across seasonal timepoints; 1-PH : post-harvest 2016, 2-B : bloom 2017, 3-V: veraison 2017, 4-PH : post-Harvest 2017, 5-B : bloom 2018, 6-V: veraison 2018, and 7-PH : post-harvest 2018. Timepoint scheme is the same across graphs. Error bars represent standard error of the mean. n values represented the number of samples for each time point. **(B)** Number families in bacterial and fungal within different disease conditions at the PH time point. Point represent individual samples.

bloom and *Actinomyces, Neisseria*, and *Hygrogenophilus* to veraison. We used Random Forest analyses to identify predictors (fungal and bacterial taxa) responsible for the clustering in the beta-diversity metrics (**Figure 4**) and identified that the most abundant taxa (*Alternaria, Cladosporium, Filobasidium, Mycosphaerella, Streptococcus, Micrococcus, Bacteroides* and unknown *Enterobacteriaceae*) were the likely drivers (**Figure 6**).

In order to evaluate the impact of disease on microbial communities, we only considered taxa from the post-harvest time points. Beta diversity weighted UniFrac metrics showed that bacterial community clustering was significantly affected by disease severity ($P < 0.05$; **Figure 7A**). In addition, Bray-Curtis metrics indicated that fungal clustering was not affected by disease condition of the vines but was rather attributed to year ($P < 0.05$; **Figure 7B**). Prevalence Venn diagrams showed that several microbes identified in the core microbiome were also found across all grapevines regardless of their disease condition (**Figure 8**). In addition, eleven bacteria (*Corynebacterium,*

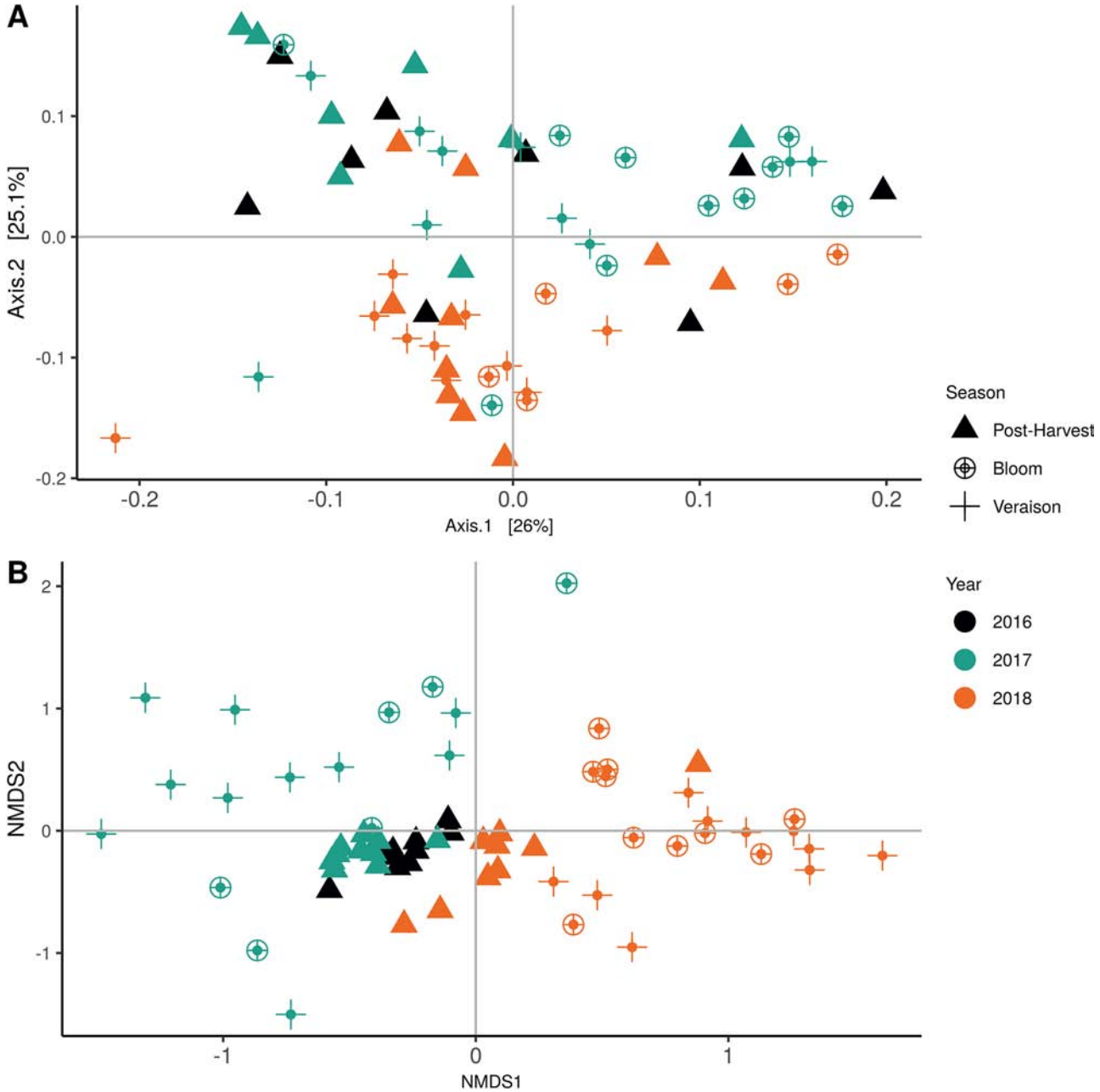

FIGURE 4 | Beta diversity plots for seasonal time points based on: **(A)** Weighted UniFrac distance for bacteria and **(B)** Bray-Curtis dissimilarity for fungi. Points represent one vine community. Point are colored by year, shaped by season.

Methylobacterium, Rothia, Actinomyces, Meiothermus, Neisseria, Ralstonia, Xylella, Hydrogenophilus, Gemella, and *Pseudomonas*) were specific to moderate PD symptoms severity likely contributing to the significant differences in alpha diversity (**Figure 3B**). Random Forest analysis suggested that the clustering in bacterial beta-diversity metrics (**Figure 7A**) was driven by the most abundant bacterial taxa (*Streptococcus, Pseudomonas, Micrococcus, Bacteroides* and unknown *Enterobacteriaceae*), disease condition-specific taxa (*Rothia* and *Meiothermus*) and the pathogen *Xylella* (**Figure 9**).

DISCUSSION

This culture-independent amplicon-based metagenomics microbiome study establishes a reference framework for the bacterial and fungal communities living in the xylem sap of grapevine and captures temporal microbial shifts during the annual cyclic changes of the host phenology. In addition, it provides insightful information about the impact of the xylem-dwelling pathogen *X. fastidiosa* on the microbiome profile of the grapevine sap.

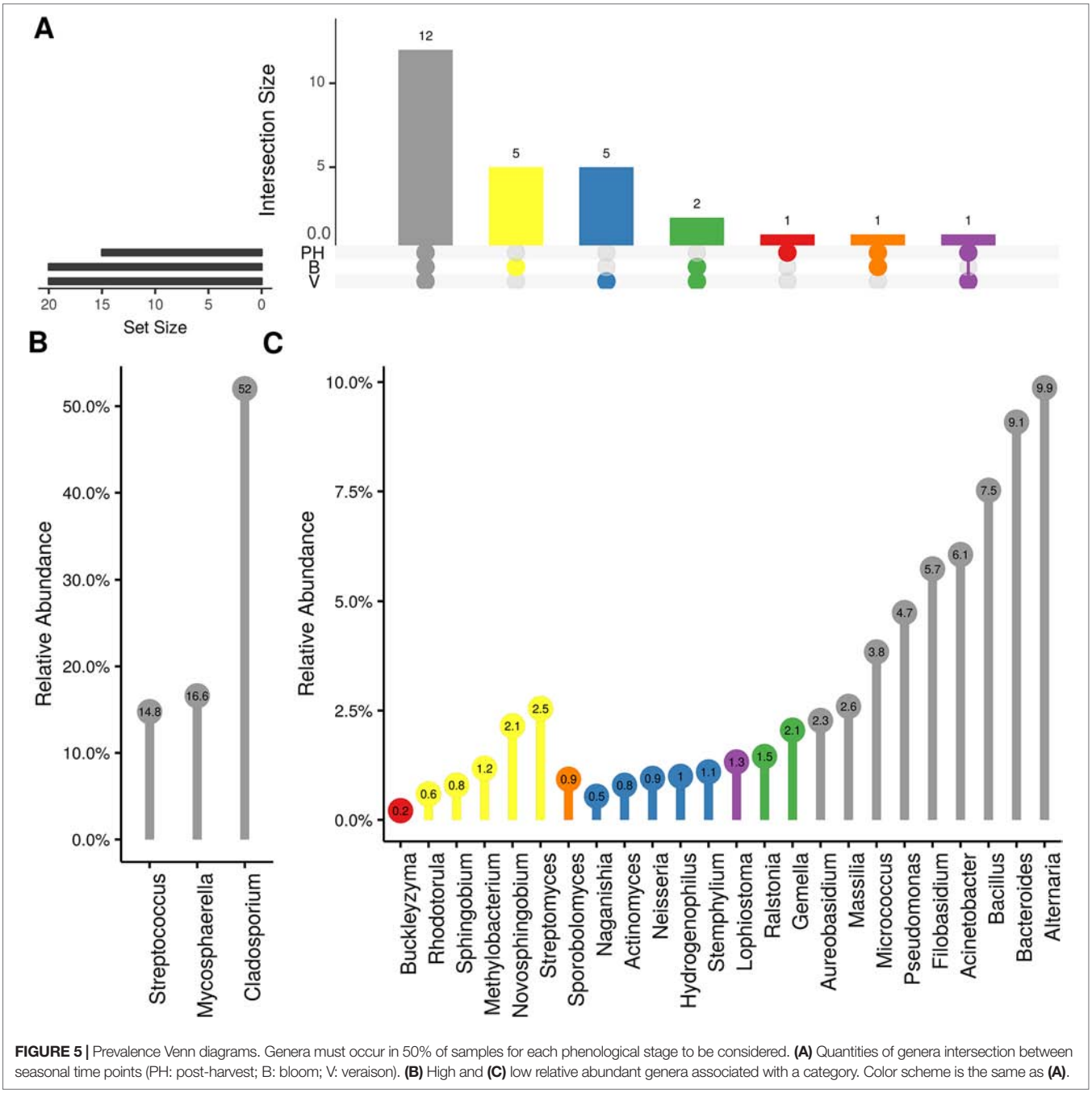

FIGURE 5 | Prevalence Venn diagrams. Genera must occur in 50% of samples for each phenological stage to be considered. **(A)** Quantities of genera intersection between seasonal time points (PH: post-harvest; B: bloom; V: veraison). **(B)** High and **(C)** low relative abundant genera associated with a category. Color scheme is the same as **(A)**.

The grapevine sap mycobiome was most exclusively composed of taxa in the *Ascomycota* in addition to a few yeasts from the phylum *Basidiomycota*. The sap bacteriome was similar to that of other biocompartments with a high abundance of *Proteobacteria*, and lower abundance of *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Zarraonaindia et al., 2015; Faist et al., 2016; Deyett et al., 2017; Marasco et al., 2018). Noticeably, no *Acidobacteria* were detected, whereas it was a significant member of the rhizosphere, and root endosphere community in other studies. However, the sap microbiome profile at the phylum level was more similar to the root than the cane, despite the fact that the sap was collected from the later tissue. In addition, we found evidence for the presence of many genera known to be associated with rhizosphere and root endosphere (*Rhizobium*, *Streptomyces*, and *Pseudomonas*) including several pathogenic fungi known to cause root diseases (*Campylocarpon*, *Ilyonectria*, *Fusarium*) (Brum et al., 2012; Cabral et al., 2012). Together these data support the evidence that the microbial makeup of the sap is of belowground origin. This result is in line with a report from another perennial crop (Fausto et al., 2018). However, one should also consider that some organisms, such as *X. fastidiosa,* can also be introduced to the host vascular system by insect feeding and

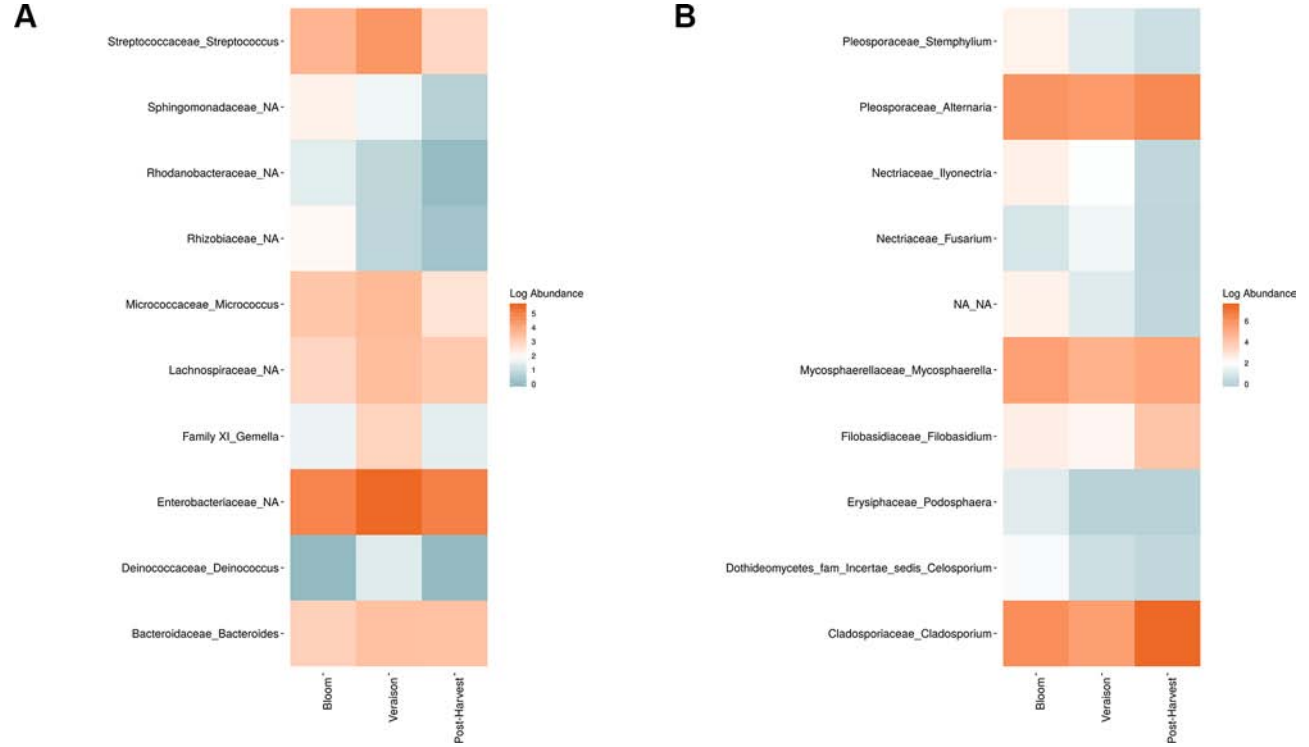

FIGURE 6 | Log abundance of the top 10 random forest important genera for **(A)** Bacteria and **(B)** fungal across seasonal time points.

become part of the sap microbiome (Redak et al., 2004; Lopez-Fernandez et al., 2017), while other airborne microbes may enter the plant endosphere through wounds, and plant surfaces *via* stomatas (Compant et al., 2011).

The core microbiome of grapevine sap, that was defined as the microorganisms that occurred in at least 50% of all samples regardless of seasonal time points, was composed of seven bacterial (*Streptococcus, Massilia, Bacteroides, Micrococcus, Pseudomonas, Acinetobacter* and *Bacillus*) and five fungal (*Cladosporium, Mycosphaerella, Alternaria, Aureobasidium*, and *Filobasidium*) taxa. Together, these make up over 51% and 92% of the bacterial and fungal datasets, respectively. *Pseudomonas*, *Bacillus, Micrococcus*, *Acinetobacter, Cladosporium, Aureobasidium Mycosphaerella, Filobasdium* and *Alternaria* have been identified in and/or on many plants organs of grapevine (West et al., 2010; Compant et al., 2011; Baldan et al., 2014; Brysch-Herzberg and Seidel, 2015; Deyett et al., 2017; Dissanayake et al., 2018; Kraus et al., 2019; Nigris et al., 2018). More surprising bacterial taxa included *Massilia*, *Bacteroides* and *Streptococcus. Streptococcus* is a facultative-anaerobe bacterium and a well-studied human pathogen. However, it was previously found to be associated with many grapevine biocompartments (soil, root, grape leaf) and across several viticulture areas (Zarraonaindia et al., 2015). *Massilia* is an aerobic bacterium found in a range of habitats (i.e., desert, glacier, water and soils) and was reported to colonize the rhizosphere of plants (Ofek et al., 2012). *Bacteroides* is an anaerobic bacterium that metabolize mono or polysaccharides and is known to colonize the guts of animals and survive in water habitats (Wexler, 2007). One should consider that the more unique bacteria (*Massilia*, *Bacteroides*), might be specific to the viticulture region, because of the water and soil properties. As previously demonstrated for grapevine epiphytes (Bokulich et al., 2014), each viticulture area possess unique indigenous microbial taxa that shape the characteristics of the region, and our data suggests that this paradigm also applies to endophytic microbes. For the other core microbiome taxa, the fact that several of them have been found in association with many grapevine biocompartments both as epiphytes and endophytes, suggest that those could use xylem (and perhaps phloem) as transport routes as previously suggested (Compant et al., 2010; Compant et al., 2011).

Our alpha and beta-diversity metrics showed that the sap microbiome assemblage was shaped by both the plant phenology and disease condition, although the later mainly affected bacterial communities. One should not rule out that the other vascular pathogens also detected in this study (i.e., *Campylocarpon*, *Ilyonectria*, *Fusarium*) may have increased background noise in our analyses. Interestingly we measured a peak in microbial richness during bloom, a key phenological stage for pollination and fertilization. Especially four taxa surfaced at this time point (*Streptomyces, Rhodotula, Sphingobium* and *Novosphingobium*), and those are known to possess plant growth promoting capabilities (El-Tarabily, 2004; Hahm et al., 2012; Seipke et al., 2012; Kim et al., 2019), suggesting that the plant could recruit microbes to participate in key physiological processes. Similarly, vines with moderate PD symptoms displayed higher microbial diversity than in either healthy or severely symptomatic vines. Plants are known to drive microbial assemblage in order to cope with biotic or

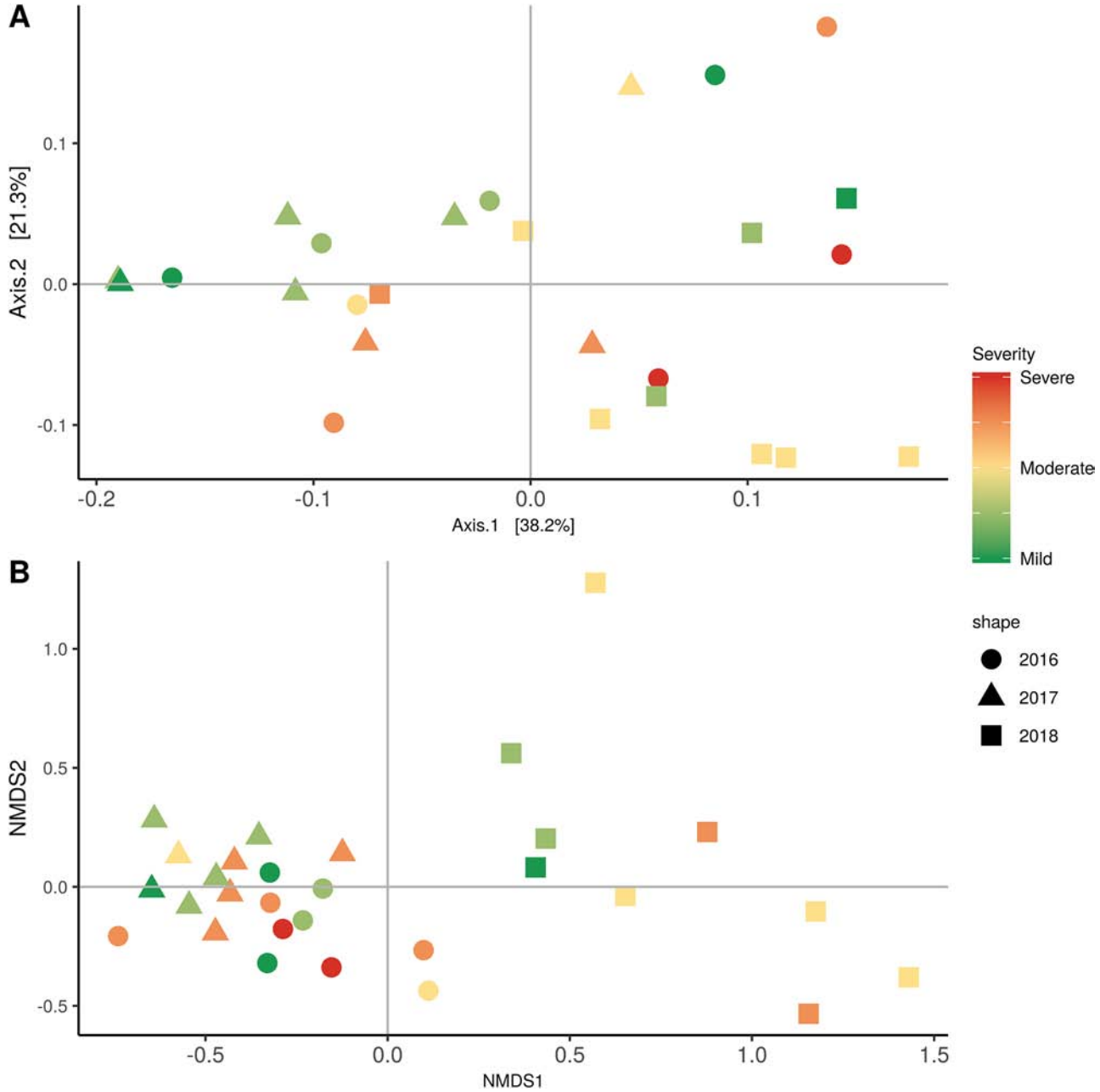

FIGURE 7 | Beta diversity for disease condition plots at post-harvest based on: **(A)** Weighted UniFrac distance for bacteria and **(B)** Bray-Curtis dissimilarity for fungi. Points represent one vine community. Point are colored by PD severity, shaped by year.

abiotic stresses and increase environmental fitness (Berendsen et al., 2012; Turner et al., 2013). Perhaps our data indicated a plant-driven microbial response to the pathogen infection. In contrast, in severely symptomatic vines, the toxic environment (e.g., occlusion of xylem vessel with tyloses and decrease of hydraulic conductivity; Deyett et al., 2019) is not conducive to microbial survival.

Asymptomatic grapevines at post-harvest displayed overall a lower pathogen incidence and abundance. The relatively low incidence of *X. fastidiosa* (~31%) measured in vines exacerbating PD symptoms was comparable to a previously published report (Deyett et al., 2017). These results can be attributed to the sampling design (only 3 canes per vine canopy), the heterogeneous distribution of *X. fastidiosa* in the vine canopy and the occlusion of vessels in severely symptomatic vines make it challenging to recover the bacterium (Deyett et al., 2019). Interestingly, *Stenotrophomonas* abundance was inversely proportional to that of *Xylella* in all three PD rating categories. *Stenotrophomonas is* a closely related bacterium to *Xylella* but is known to benefit grapevine by acting as a biofertilizer and biocontrol agent to nematodes (Aballay et al., 2011; Karagoz et al., 2012). *Pseudomonas*

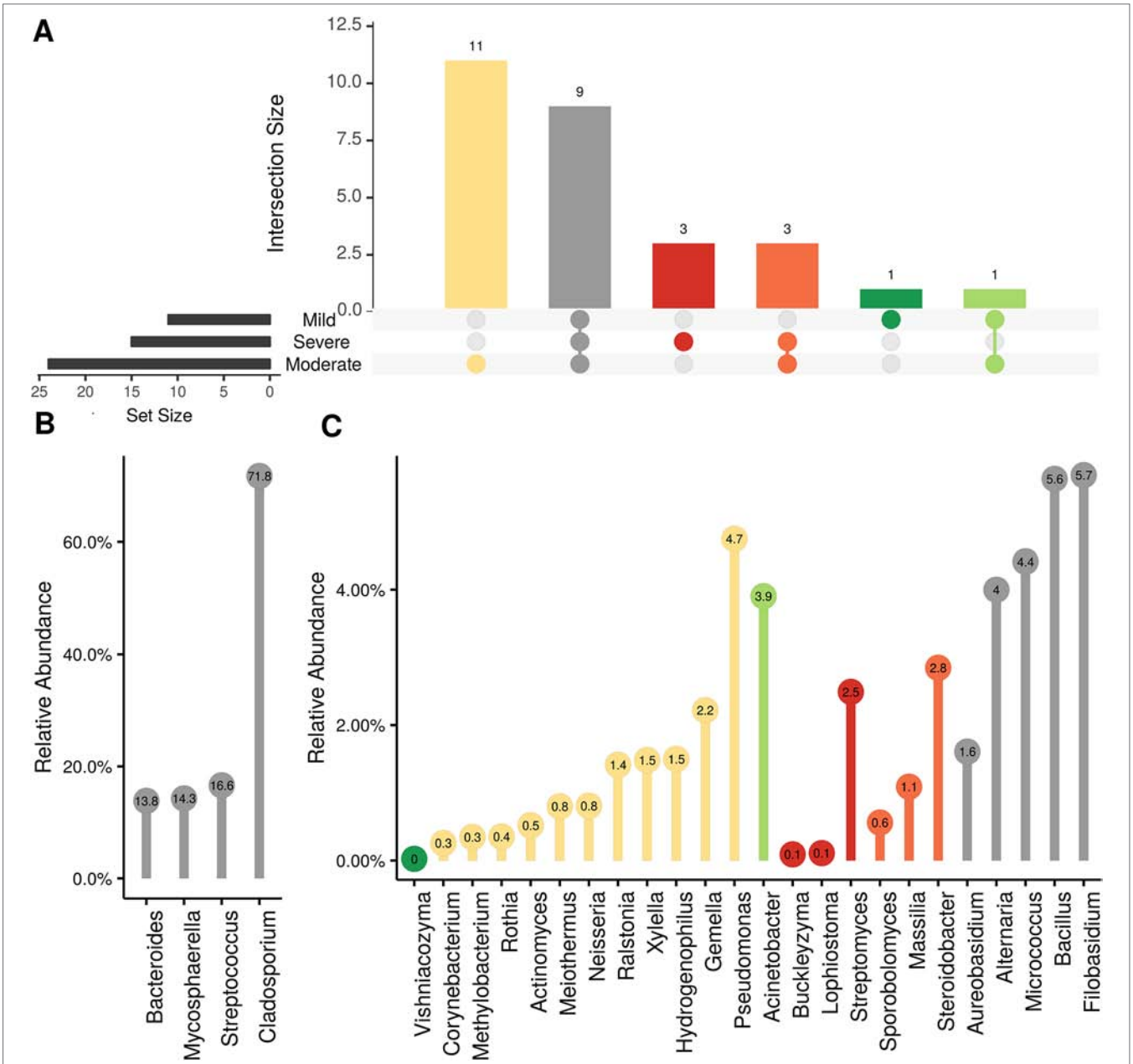

FIGURE 8 | Prevalence Venn diagrams. Genera must occur in 50% of samples for each disease condition at post-harvest to be considered. **(A)** Quantities of genera intersection between disease condition. **(B)** High and **(C)** low relative abundant genera associated with a category. Color scheme is the same as **(A)**.

was also found in vines with moderate PD symptoms, although this was a much lower abundance (~5%) than previously reported (~80%) from grapevine canes (Deyett et al., 2017; Faist et al., 2016). As previously discussed, *Pseudomonas* likely originates from the roots and colonizes the developing vegetative organs early in the growing season, but unlike most of the other microbial inhabitants of the sap, it appears to have the ability to become established in the lignified structural component of the grapevine vascular system. Deyett et al. (2017) showed that *Pseudomonas* correlated negatively with *Xylella* in PD-affected vines, suggesting that it could be used as a biocontrol agent given its known properties for secreting antimicrobial and plant growth promoting compounds (Khmel et al., 1998; Loper et al., 2012; Pieterse et al., 2014; Gruau et al., 2015). *Paraburkholderia phytofirmans* (formerly known as *Burkholderia phytofirmans*) has also been recognized as a potential biocontrol agent to grapevine PD because it has been identified as an endophyte of grapevine leaf and showed the ability to colonize the grapevine endosphere upon inoculation (root, vascular system and leaf) and prime host disease resistance pathways (Compant et al., 2005; Compant et al., 2008; Lo Piccolo et al., 2010; Baccari et al., 2019). Our data did not support the presence of this specific bacterium in the sap of grapevine but

FIGURE 9 | Log abundance of the top 10 random forest important genera for **(A)** Bacteria and **(B)** fungal across disease conditions at post-harvest.

identified several taxa from the family *Burkholderiaceae* (~5% abundance), including *Massilia* and *Ralstonia* that were a part of the core microbiome and a biomarker for vines showing moderate PD-symptoms, respectively. Finally, *Methylobacterium* also surfaced as a common bacteria in grapevines with moderate PD symptoms. Araujo et al., (2002) described that in the Citrus Variagated Chlorosis (CVC) pathosystem, *Xylella fastidiosa* subsp. *pauca* and *Methylobacterium* acted in synergy and caused an increase in disease severity. Our data support the evidence that *Methylobacterium* is an endophyte of grapevine and that perhaps similar synergistic mechanisms occur in the CVC and PD pathosystems.

This study highlights several potential fungal and bacterial targets with antimicrobial and plant growth promoting properties. Future studies should focus on isolating those potentially beneficial strains and test their biological functions *in vitro* and *in planta* bioassays. The broad host range of *X. fastidiosa* combined with the movement of plant material has led to the introduction of the pathogen to new agricultural areas and made it an emerging global threat (Rapicavoli et al., 2018; Sicard et al., 2018). PD management strategies has relied most exclusively on vector control and the breeding of PD-resistant varieties in grapevine (Krivanek et al., 2006; Daugherty et al., 2015). Few resources have been invested in developing commercial bioproducts that do not select for disease resistance or cause environmental pollution. We hope that this microbiome approach will lay the foundation for innovative research and lead to novel product development and marketing.

DATA AVAILABILITY STATEMENT

Fungal and bacterial sequences were deposited in NCBI under the accession number PRJNA548584.

AUTHOR CONTRIBUTIONS

ED designed the experiment, collected and analyzed the data, and co-wrote the manuscript. PR conceived the original project and research plans and co-wrote the manuscript.

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REVIEW
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The Role of the Endophytic Microbiome in the Grapevine Response to Environmental Triggers

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Endophytism within *Vitis* represents a topic of critical relevance due to the multiple standpoints from which it can be approached and considered. From the biological and botanical perspectives, the interaction between microorganisms and perennial woody plants falls within the category of stable relationships from which the plants can benefit in multiple ways. The life cycle of the host ensures persistence in all seasons, repeated chances of contact, and consequent microbiota accumulation over time, leading to potentially high diversity compared with that of herbaceous short-lived plants. Furthermore, grapevines are agriculturally exploited, highly selected germplasms where a profound man-driven footprint has indirectly and unconsciously shaped the inner microbiota through centuries of cultivation and breeding. Moreover, since endophyte metabolism can contribute to that of the plant host and its fruits' biochemical composition, the nature of grapevine endophytic taxa identities, ecological attitudes, potential toxicity, and clinical relevance are aspects worthy of a thorough investigation. Can endophytic taxa efficiently defend grapevines by acting against pests or confer enough fitness to the plants to endure attacks? What are the underlying mechanisms that translate into this or other advantages in the hosting plant? Can endophytes partially redirect plant metabolism, and to what extent do they act by releasing active products? Is the inner microbial colonization necessary priming for a cascade of actions? Are there defined environmental conditions that can trigger the unleashing of key microbial phenotypes? What is the environmental role in providing the ground biodiversity by which the plant can recruit microsymbionts? How much and by what practices and strategies can these symbioses be managed, applied, and directed to achieve the goal of a better sustainable viticulture? By thoroughly reviewing the available literature in the field and critically examining the data and perspectives, the above issues are discussed.

Keywords: *Vitis vinifera*, endophytes, plant growth-promoting bacteria, stress-tolerance, biocontrol

INTRODUCTION

Plant microbial endophytism represents a wealth of interactive relationships that are widespread in nature and often rely on mutual benefits due to plant growth-promoting (PGP) phenotypes. In some cases, the beneficial effect is directly related to microbial metabolism, such as nitrogen fixation, phytohormone production, phosphate solubilization, and pathogen suppression; or conversely, such benefits can be mediated by the stimulation of specific activities of the host plant, leading to increased enzymatic catalysis, and enhanced water and nutrient uptake or defense responses. Moreover, endophytic microorganisms occupy an ecological niche that overlaps that of many phytopathogens, and their exploitation as biocontrol agents (BCAs) represents a possible strategy to reduce the use of pesticides in vineyards (Compant et al., 2013). Much of the applicative potential of agriculture and biotechnology is derived from plant–microbe interactions, but some requisites are the isolation of *ex planta* and multiplication of useful strains. According to some authors, this condition appears proportionally frequent compared with the situation observed in bulk soil or water environments (Finkel et al., 2017). However, other authors report the opposite and note the considerable proportion of non-plate-culturable endophytes (Thomas et al., 2017). West et al. (2010) recorded a certain degree of difference between culturable endophytic communities and those obtained by culture-independent approaches, suggesting a non-negligible level of non-culturability among endophytes. This limitation has been confirmed by further studies, which testified a major issue of recalcitrance to cultivation in grapevine endophytic bacteria (Thomas et al., 2017). When amenable to growth, selected members of the inner microbiota can be exploited as inoculants to foster plant growth in agriculture, horticulture, and silviculture or used for soil or water decontamination, such as in phytoremediation applications (Santoyo et al., 2016).

The precise identification of endophytic microorganisms is necessary to determine their distribution in host plants and their beneficial effects. The traditional methods used for the identification of BCAs are based on isolation and culturing on artificial media, followed by biochemical, immunological, and molecular characterization; however, the techniques of fluorescence microscopy can be used to detect endophytes inside plant tissues (**Figures 1A**, **B**). Over the last few years, advanced molecular techniques have allowed researchers to investigate the biodiversity of grapevine endophytes, particularly endophytic species that are slow growing or cannot be grown in axenic culture. Culture-independent methods, including automated ribosomal intergenic spacer analysis (ARISA) (Pancher et al., 2012), denaturing gradient gel electrophoresis (DGGE) (West et al., 2010), and single-strand conformation polymorphism (SSCP) analysis (Rezgui et al., 2016), can provide a general overview of the grapevine endophytic microbiome composition, although metagenomic approaches based on massive DNA sequencing of prokaryotic 16SrDNA or eukaryotic ITS1-5.8S-ITS2 rDNA genes are the methods of choice to assess the composition of grapevine endophytic communities (Pinto et al., 2014; Morgan et al., 2017; Dissanayake et al., 2018).

BIODIVERSITY

Plant Influence on Endophytic Composition

Regarding the endophytic presence, a vast diversity of taxa has been documented in both cultivated (*Vitis vinifera* subsp. *vinifera*) and wild (*V. vinifera* subsp. *sylvestris*) grapevines. Different factors could shape the grapevine microbiome, as follows: seasonality, plant genotype, age, pedo-climatic features, surrounding wild plants, presence of pathogens, etc.

A survey of grapevine cultivar Glera plants from six different vineyards yielded 381 culturable bacterial isolates from the inner portions of surface-sterilized stems and leaves (Baldan et al., 2014). A large share of these bacteria (30%) belonged to the genus *Bacillus*, while the remainder included *Paenibacillus*, *Microbacterium*, *Staphylococcus*, *Micrococcus*, *Stenotrophomonas*, *Variovorax*, *Curtobacterium*, and *Agrococcus*. A certain degree of local specificity was reported as populations from different

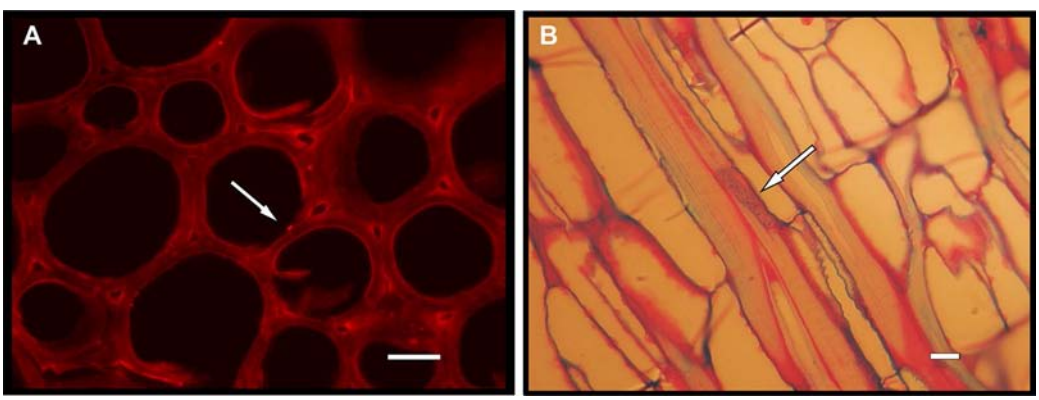

FIGURE 1 | Endophytes inside plant tissues detected trough fluorescence microscopy techniques. **(A)** Epifluorescence image on transverse sections of a *Centaurea horrida* stem stained by propidium iodide. Arrow: endophytic bacterium cell adhering to a vessel within the plant's vascular system. Bar = 15 µm. **(B)** Optical microscopy of basic fuchsin/astra blue double-stained longitudinal thin sections from a *Hedysarum glomeratum* root in proximity to the crown. Endophytic bacteria (indicated by the arrow) are distinguishable within a xylem vessel. Bar = 10 µm.

vineyards were rather different; in addition, seasonality was recorded, and differences were observed between the May and October samplings (Baldan et al., 2014).

In cultivated grapevine, the composition of bacterial communities is significantly associated with the rootstock genotype and shows different interactions; the nature of the rootstock was addressed as a possible variable explaining endophyte diversity. Marasco et al. (2018) investigated how different rootstocks could affect the recruitment of bacteria from the surrounding soil and found that bacterial community diversity and networking in the roots were profoundly influenced by the rootstock type. Interestingly, it has been observed that despite selecting different bacterial components, grapevine rootstock genotypes possess PGP traits similar to those exhibited by different bacteria that provide fundamental ecological services (Marasco et al., 2018).

The age of grapevine plants has been explored as a possible factor affecting endophytic community composition. Three-year-old and 15-year-old plants of the cv. Corvina were compared and found to be differentially invaded. Actinobacteria and Bacilli prevailed in the 3-year-old plants, while the older plants featured more Proteobacteria. Younger *Vitis* also have a higher diversity of taxa and a particular abundance of the *Rhizobium* genus, while the old plants contained higher shares of *Pantoea*. The phenotypes represented more in the 15-year-old plants were phosphate solubilization and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Andreolli et al., 2016).

Campisano et al. (2015) explored the difference between wild and cultivated grapevines in terms of endophytic communities; overall, 155 bacterial strains were isolated from 88 plants of the two types grown in the same climate. The species diversity resulted considerably higher in wild grapes than domesticated varieties with 25 genera versus six, respectively. In addition, regarding the multivariate ordination by phenotypes, the clustering was distinct to the isolates from the wild or cropped specimens, even when the bacteria belonged to the same genus.

An important question in this type of study is as follows: to what extent is a plant-associated microbiome specific to its host compared with the surrounding vegetation? The degree of overlap in bacterial communities isolated from grapevines and those found in weeds sharing the same vineyard has been analyzed (Samad et al., 2017). Assuming that the same soil offers the different coexisting plants a pool of microbes from which to choose, the authors identified the rhizosphere and inner root bacterial checklists of *V. vinifera* and those of four herbaceous plants, including three annual therophytes (*Stellaria media*, *Veronica arvensis*, and *Lamium amplexicaule*) and one perennial (*Lepidium draba*). The analysis was carried out using culture-independent 16S amplification and metabarcoding. The shared portion of the microbiome featured 145 operational taxonomic units (OTUs) in the rhizosphere, and only nine belonged to the truly endophytic contingent isolated from inside the roots. The bacteria associated with the weeds had a higher diversity in the rhizosphere than those related to the grapevine, but the endophytic subgroup displayed more diversity in the perennial plants (*Vitis* or *Lepidium*). The PGP traits were more represented in the isolates from the weeds. Overall, it appears that even plants sharing the same soil are characterized by significantly different microbiomes both at the rhizosphere level and inside the roots, but in the latter compartment, their differences are more pronounced.

The interaction between endophytes and plants occurs in different areas, including the root and foliar surfaces. Regarding the provenance of the endophytic microbiota and its way of entry into plants, it is generally assumed that the main route is from soil to roots with endophytes ascending to the epigeal parts or alternatively through gaps along the plant overall surface, including wounds or stomata (Compant et al., 2010). Once endophytes enter into the plant, they move systemically (Nigris et al., 2018), spreading in different tissues as shown in **Figure 2**, where green fluorescent protein (GFP)-tagged *Bacillus licheniformis* can be visualized within the stem of a grapevine-inoculated plant. Indeed, endophytes can colonize other tissues and organs, including reproductive organs, thus allowing their transfer through the vascular system (Lidor et al., 2018) or apoplastic spaces (Compant et al., 2011). An analysis of different aerial portions of grapevines was carried out and revealed differential colonization by endophytes; both *Pseudomonas* and *Bacillus* were found within the flower and ovules, while only the latter was present in the berries and seeds (Compant et al., 2011). Using DGGE and fatty acid methyl ester profiling, some authors have also documented the possibility that grapevine epiphytes originally dwelling on the external surface of leaves and stems could become endophytes when windows of opportunity for such internalization arise (West et al., 2010). However, there are documented exceptions that add complexity to the picture. In grapevines, the sap-feeding leafhopper pest *Scaphoideus titanus*, which is recognized as the vector for phytoplasma diseases, has also been found to be capable of transferring various species of endophytic bacteria from one grapevine plant (source plant) to another (sink plant) (Lòpez-Fernàndez et al., 2017); the acquired taxa included members of the phyla Proteobacteria, Actinobacteria, and Firmicutes. While the insect spends time exclusively on leaves or stems, in sink plants, the root endophytic communities acquired a composition very close to that found within the insect vector. These data suggest that the insect-mediated exchange of endophytes among plants could be an important and still overlooked component of the mechanisms underlying these plant–microbe interactions. However, an even more extreme example of the cross-transfer of microbes to plants concerns the taxon *Propionibacterium acnes*, which has been shown to have been acquired by grapevine in an inter-kingdom transmission from humans (Campisano et al., 2014a). Such occurrence is reminiscent of several cases in which bacterial host switches were traced from domestic animals to humans, but here, the case is particularly remarkable as it could involve the opposite direction and a member of the plant kingdom as a recipient sink.

A further factor conducive to differences in the endophytic communities of grapevine is the presence of pathogens, such as *Agrobacterium vitis*, which is the agent of grapevine crown gall disease (Faist et al., 2016). The authors have found the differences in the microbial community occurring at the graft union as discriminative. In particular, the microbial community from plants without crown gall disease showed a transient composition,

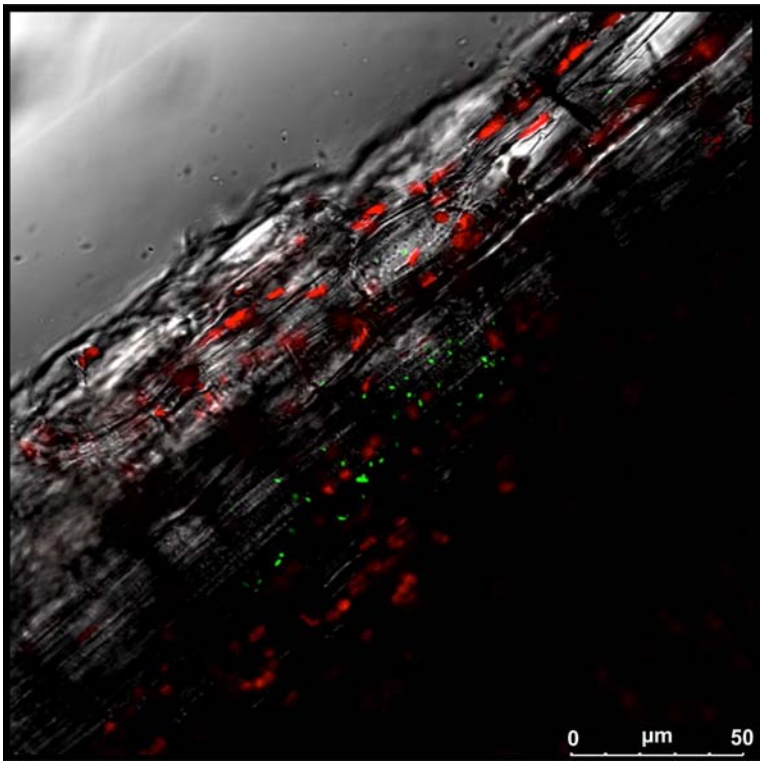

FIGURE 2 | *Bacillus licheniformis* colonizing a grapevine plant. Laser scanning confocal microscopy of stem sections of Glera cuttings inoculated with *Bacillus licheniformis* GL174::gfp2x. Overlay of green fluorescent protein (GFP) signal (green), chlorophyll (red), and bright field.

changing from across seasons, while that from plants with crown galls had a higher species diversity and regularly featured the dominance of the same three taxa, including *Pseudomonas* sp. *A. vitis* and a member of the Enterobacteriaceae family (Faist et al., 2016). The diversity and abundance of endophytes have been investigated and compared between healthy grapevine and plants affected by flavescence dorée (FD) and bois noir phytoplasmas while considering the seasonal variation (Bulgari et al., 2011). The authors sampled during three different time points and reported that independent of the sampling period, phytoplasmas can shape endophytic bacterial communities by enriching such communities with strains able to elicit plant defense responses. Genera associated with the infection process include some that have been reported to be associated with biocontrol action, such as *Burkholderia*, *Methylobacterium*, *Sphingomonas*, and *Pantoea*. Overall, the communities found in the healthy grapevine featured a higher diversity than those in the diseased plants. Endophytes and plant pathogens can indeed coexist within the same host and result in evolutionary paths that diverge at a certain stage. A study concerning grapevine invaded or not invaded by the black fungi *Aureobasidium pullulans* and *Epicoccum nigrum* reported that their presence did not result in detectable changes in the associated bacterial populations (Grube et al., 2011).

A different approach to studying endophytism relies on genomic data to search for genes that could distinguish endophytic strains from pathogenic strains belonging to closely related lineages. This type of survey was carried out in the genomes of seven endophytes isolated from grapevine, which were compared with members of the same genera but involved in pathogenic interactions (Lòpez-Fernàndez et al., 2015). Their comparison showed that endophytes and pathogens have a high number of common virulence-related genes in their core pangenome with a high degree of conservation in each genus regardless of the endophytic or pathogenic lifestyles. Therefore, the structural organization of endophyte genomes is reflective of the conservation of properties spanning over different attitudes, including pathogenicity. Through a bioinformatics approach, it is possible to identify particular genes in endophyte genomes strictly associated with the endophytic lifestyle, which can contribute to the peculiar recognition of beneficial microorganisms (Ali et al., 2014).

Environmental Influence on Endophytic Diversity

The microbial component in viticulture and oenology has been increasingly recognized to have a major imprint on the regional terroir. A complex of environmental variables is linked to the geographical origin and its ensuing endophytic community composition. Studies have shown that the microbiome involved during the early fermentation stages, which is partially determined by endophytic plant-borne yeasts and bacteria, complies with a well-delineated biogeography reflecting the signatures of different winegrowing regions with an additional but minor influence from

the grape variety and vintage year (Bokulich et al., 2014; Gilbert et al., 2014). Notwithstanding, the soil is a main reservoir for endophyte recruitment, and a strong selection process appears to be operative in favor of strains endowed with relevant PGP phenotypes, as the final community is not merely a mirror of the soil array of microbiota but rather a distinct sub-community (Novello et al., 2017). As the terroir concept implies, soil is ultimately regarded as a major environmental factor conditioning the microbial populations that are eventually associated with their host plants. This issue has been thoroughly addressed, and the factors particularly critical for the determination of grapevine inner microbiota include the soil pH, soil carbon, and C:N ratio; most taxa associated with grapevine organs originated in soil and had a marked degree of local vineyard scale specificity (Zarraonaindia et al., 2015). Among the environmental conditions, soil pH is generally recognized as a major driver shaping bacterial communities. Its effect in modelling those interacting with grapevine was addressed in plants grown in acidic or alkaline soils (Karagöz et al., 2012); in total, 27 genera were identified; overall, gram-negative taxa were dominant in both soil types, and *Pseudomonas* and *Bacillus* were recurring genera. The observed differences included that *Acidovorax delafieldii*, *Pseudomonas mendocina*, *Aeromonas ichthiosmia*, *Hafnia alvei*, *Raoultella terrigena*, *Paenibacillus alginolyticus*, *Arthrobacter aurescens*, *Kocuria kristinae*, *Curtobacterium flaccumfaciens*, *Flavobacterium johnsoniae*, and *Sphingobacterium spiritivorum* were isolated only from alkaline sites. In contrast, *Ralstonia eutropha*, *Citrobacter amalonaticus*, *Enterobacter hormaechei*, *Arthrobacter globiformis*, *Rhizobium rubi*, *Paracoccus denitrificans*, *Pseudomonas syringae*, *Citrobacter freundii*, *Serratia odorifera*, *Arthrobacter oxydans*, *Kocuria rosea*, and *Brevibacterium epidermidis* were isolated only from the acidic soils.

Other studies addressed pest management (organic vs. integrated) as a variable and determined its effect on the bacterial endophyte community of Merlot and Chardonnay grapevine cultivars (Campisano et al., 2014b). The results indicated that while *Ralstonia*, *Burkholderia*, and *Pseudomonas* were present in all samples, the relative abundance of the taxa defined the consistent differences, and the genera *Mesorhizobium*, *Caulobacter*, and *Staphylococcus* were found more frequently in the organically managed vineyards, while *Ralstonia*, *Burkholderia*, and *Stenotrophomonas* were abundant in the vineyards where integrated pest management was operative. In this respect, the differences related to the cultivar were minor compared with those ascribed to the management practices. Pancher et al. (2012) showed that the possibility of differentiating communities in relation to management or the cultivar also depends on the technique used. The fungal taxa detected in a parallel analysis of endophytic fungi in the same two grapevine cultivars included *Absidia glauca*, *Pennicillium restrictum*, *Alternaria* sp., *Botrytis cinerea*, *Fusarium* sp., *Trichoderma reesei*, *Fusarium oxysporum*, *Neurospora crassa*, *Pennicillium chrysogenum*, *Podospora anserina*, and *Davidiella tassiana*; the culture-dependent methods yielded overlapping results, while the molecular DNA-based approaches had sufficient power to resolve the differences between the organic and integrated pest managements.

BENEFICIAL EFFECTS OF GRAPEVINE ENDOPHYTES ON ABIOTIC STRESS TOLERANCE

Endophytes employ different mechanisms to exert beneficial effects on plant growth or biotic/abiotic stress resistance, and these mechanisms act either directly by releasing plant hormones, secondary metabolites (**Table 1**), biocides, or antibiotics (an example is shown in **Figure 3**) or indirectly by modifying the plant physiology and nutrient balance, leading to a reduced susceptibility to diseases or abiotic stresses (Lugtenberg and Kamilova, 2009; Compant et al., 2013).

Abiotic stresses, such as heat, salinity, and drought, trigger trehalose synthesis in plants, which can provide the plants stress endurance. Trehalose forms a gel during cellular dehydration, preventing excessive water loss due to its high stability against acids and heat. Inoculation with the *Paraburkholderia phytofirmans* strain PsJN has been observed to be conducive as a stress-preventive trait as plants bearing this endophyte began to accumulate trehalose and its intermediate trehalose-6-phosphate (T6P) at 26°C (Fernandez et al., 2012). In particular, it has been reported that *P. phytofirmans* can affect trehalose metabolism in grapevine plants through the stimulation of T6P synthesis or the inhibition of T6P degradation (Esmaeel et al., 2018). A deep analysis of 48 genomes of *Burkholderia* species revealed that 161 clusters involved in secondary metabolite synthesis were likely involved in signaling between grapevine and endophytic bacteria (Esmaeel et al., 2016). It has been demonstrated that *P. phytofirmans* sets grapevine metabolism to a state of alert, enabling rapid and intense resistance responses to subsequent stresses. In this case, the benefit provided by endophytes does not occur directly through the induction of stress-related genes but rather occurs by preparing the plant to activate a faster and stronger defense response upon stress (Esmaeel et al., 2016). In addition, *P. phytofirmans* can induce different *PR* genes [encoding chitinase, phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX), and glucanase], protecting the plant from low temperatures (Theocharis et al., 2012). In addition, the endophyte is able to induce genes coding enzymes involved in reactive oxygen species (ROS) scavenging; a decrease in the ROS concentration is important because it converts detoxification compounds into signaling molecules.

A different major factor of stress in most plants is drought. Soil microorganisms have different strategies to cope with limited water potential, including the accumulation of compatible solutes (glycine-betaine, proline, trehalose, and exopolysaccharide production) and exopolysaccharide production (Ngumbi and Kloepper, 2016). Their phenotypes have been shown to be transferrable to the plants that they can invade as endophytes (Mayak et al., 2004; Timmusk et al., 2014). Regarding grapevine, an extensive analysis of endophytes from different rootstocks and the cv. Barbera in relation to drought has been performed (Rolli et al., 2015). In this study, eight strains from the *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Delftia*, and *Sphingobacterium* genera were selected from over 510 total isolates and shown to be capable of colonizing different

TABLE 1 | Potential roles and mechanisms of main endophytes involved in biotic and abiotic stress tolerance.

BIOTIC STRESS

Pathogen	Endophytes	Mechanism associated with the tolerance	Reference
Botrytis cinerea	*Acinetobacter lwoffii* (PTA-113 and PTA-152), *Pseudomonas fluorescens* (PTA-268 and PTA-CT2), *Pantoea agglomerans* (PTA-AF1 and PTA-AF2), *Bacillus subtilis* (PTA-271)	Induced the activities of lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), β-1,3 glucanase, and chitinase. Induced the oxidative burst. Accumulated the stress-related metabolites phytoalexin (trans resveratrol and trans-ε-viniferin).	Magnin-Robert et al. (2007) *Eur. J. Plant Pathol*. 118, 43–57. Trotel-Aziz et al. (2008) *Environ. Exp. Bot*. 64(1), 21–32 Verhagen et al. (2011) *Phytophatol*. 101, 768–777.
	P. fluorescens PTA-CT2	Regulated the expression of defense-related genes in leaf and root, including those with transcriptional factor functions (*JAZ9*, *NAC1*, and *ERF1*), of secondary metabolism (*PAL*, *STS*, *LOX9*, *ACCsyn*, *GST*, *CHS*, *CHI*, *LAND*, and *ANR*), and PR proteins (*PR1*, *PR2*, *PR3*, *PR5*, and *PR6*).	Gruau, C. et al. (2015) *Mol. Plant Microbe Interact*. 28, 1117–1129.
	Ulocladium atrum	Enhanced chitinase activity.	Ronseaux et al. (2013) *Agronomy* 3, 632–647.
	Burkholderia phytofirmans PsJN	Induced callose deposition and H_2O_2 production. Primed the expression of *PR1*, *PR2*, *PR5*, and *JAZ* in bacterized plantlets after pathogen challenge. Modulated the carbohydrates metabolism.	Miotto-Vilanova et al. (2016) *Front. Plant Sci.* 7:1236.
	B. subtilis (BBG127, BBG131 Bs2504, and BBG125)	Treatment with *B. subtilis* strains non-producing lipopeptides, overproducing surfactin, overproducing plipastatin, and overproducing mycosubtilin differentially activated the plant innate immune response. Modulated genes encoding a chitinase (*chit4c*), a protease inhibitor (*pin*), a salicylic acid (SA)-regulated marker (*Vv17.3*), and a glucanase (*gluc*).	Farace et al. (2015) *Mol. Plant Pathol.* 16, 177–187.
	*Microbacterium imperiale Rz*19M10, *Kocuria erythromyxa* Rt5M10, *Terribacillus saccharophilus* Rt17M10 *Streptomyces anulatus* S37	Induced a systemic response that triggers increases on monoterpenes, sesquiterpenes, tocopherols, and membrane sterols (enhanced antioxidant capacity). Induced rapid and transient generation of H_2O_2, extracellular alkalinization, and an activation of two mitogen-activated protein kinase (MAPKs) followed by the expression of *LOX9*, *PAL*, *STS*, and *GST* genes in primed cells. Induced defenses modulated by Ca^{2+} signaling.	Salomon et al. (2016) *Plant Physiol. Biochem*. 106, 295–304. Vatsa-Portugal et al. (2017) *Front. Plant Sci.* 8:1043.
B. cinerea, Plasmopara viticola, Xiphinema index Nematodes	*Paenibacillus sp.* strain B2	Modulated the expression of defense-related genes *CHI, PAL, STS, GST*, and *LOX* and pathogenesis-related protein PR-6. Reduced nematode root infection associated with the expression of genes resistant to nematodes *Hero* and $Hs1^{pro-1.}$	Hao et al. (2017) *Biological Control* 109, 42–50.
Rhizobium vitis (Ti) VAT03-9 (tumorigenic)	*R. vitis* ARK-1	Co-inoculation of ARK-1 with a Ti strain VAT03-9 into grapevine shoots suppressed the expression of the virulence genes virA, virD3, and virG of VAT03-9.	Kawaguchi et al. (2019) *BMC Res. Notes.* 12:1.
Flavescence dorée phytoplasma	*Pseudomonas migulae* 8R6	Production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme helps the plant to regulate the level of the stress-related hormone ethylene	Gamalero et al. (2017) *Plant Biosyst.* 151, 331–340.
Diplodia seriata (strains F98.1 and Ds99.7) (botryosphaeria dieback)	*Aureobasidium pullulans* strain Fito_F278	Modulated genes encoded for plant defense proteins: PR protein 6 (*PR6*) and *β-1,3-glucanase* (*Gluc*); detoxification and stress tolerance: haloacid dehalogenase hydrolase (*Hahl*), α-crystalline heat shock protein (*HSP*), *β-1,3-glucanase* (*GST5*); phenylpropanoid pathway: (*STS*) cell wall (*fascAGP*) and water stress (*Pip 2.2*).	Pinto et al. (2018) *Front. Microbiol.* 9:3047.
Neofusicoccum parvum (botryosphaeria dieback)	*B. subtilis* PTA-271	Antagonized N. parvum by delaying its mycelial growth and detoxifying both (R)-mellein and (–)-terremutin. Primed defense genes including PR2 (a β-1,3-glucanase), NCED2 (involved in ABA synthesis), and PAL at systemic level after pathogen inoculation.	Trotel-Aziz et al. (2019) *Front. Plant Sci.* 10:25.
Phaeomoniella chlamydospora (trunk diseases)	*Paenibacillus sp.* S19 *Bacillus pumilus* S32	Induced resistance against trunk disease fungi: induced the expression of defense-related genes: *PR1, PR10, CHIT3, PAL, STS, CHS, ANTS, CALS, GST,* and *GLU*.	Haidar et al. (2016b) *Microbiol. Res.* 192, 172–184.

(Continued)

TABLE 1 | Continued

ABIOTIC STRESS			
STRESS	**Endophytes**	**Mechanism associated with the tolerance**	**Reference**
Chilling	*B. phytofirmans strain* PsJN	Elevated the stress-related metabolites (proline, aldehydes, malondialdehyde, and phenolics). Enhanced the rate of photosynthesis and starch deposition. Induced the expression of the defense genes: StSy, PAL, Chit4c, Chit1b, Gluc, and LOX. Induced the expression of trehalose-related genes *TPS1*, *TPPA*, and *TRE*. Accumulated T6P and trehalose.	Barka et al. (2006) *Appl. Environ. Microbiol.* 72, 7246–7252. Theocharis et al. (2012) *Mol. Plant Microbe Interact.* 25, 241–249. Fernandez et al. (2012) *Planta* 236, 355–369.
Drought stress	*Bacillus licheniformis* Rt4M10 *P. fluorescens* Rt6M10	Induced synthesis of monoterpenes and sesquiterpenes. Produced and induced synthesis of ABA.	Salomon et al. (2014) *Physiol. Plant.* 151, 359–374.
Salt or drought stress	*Bacillus amyloliquefaciens* SB-9	Secreted and produced melatonin and three intermediates of the melatonin biosynthesis pathway: 5-hydroxytryptophan, serotonin, and *N*-acetylserotonin. Increased the upregulation of melatonin synthesis, as well as that of its intermediates, but reduced the upregulation of grapevine tryptophan decarboxylase genes (TDCs) and a serotonin *N*-acetyltransferase gene (SNAT). Reduced the production of malondialdehyde and reactive oxygen species (H_2O_2 and O_2^-) in roots.	Jiao et al. (2016) *Front. Plant Sci.* 7:1387.
	Pseudomonas fluorescence RG11	Enhanced endogenous melatonin in plants. Regulated melatonin-related genes: *TDC1* (putative tryptophan decarboxylase-1) and *SNAT* (serotonin *N*-acetyltransferase).	Ma et al. (2017) *Front. Plant Sci.* 7:2068.
Arsenic contamination	*Micrococcus luteus, B. licheniformis, P. fluorescens*	Increased antioxidant enzyme activity (APX, ascorbate peroxidase; CAT, catalase; and POX, peroxidases activity). Reduced peroxidation of membrane lipids (reduced malondialdehyde content) and photosystems damage membrane damage in As presence.	Funes Pinter et al. (2017) *Appl. Soil Ecol.* 109, 60–68. éééé) *Agr. Ecosyst. Env.* 267, 100–108.
High temperature and drought stress	Bioradis Gel (Bioera SLU, Tarragona, Spain): mixture of five AMF fungi (*Septglomus deserticola, Funneliformis mosseae, Rhizoglomus intraradices, Rhizoglomus clarum, and Glomus aggregatum*), and a mixture of rhizobacteria belonging to the *Bacillus* and *Paenibacillus* genera	Under elevated temperature and deficit irrigation, inoculated plants reached higher berry anthocyanins and evidenced some modifications in berry ABA catabolism.	Torres et al. (2018) *Plant Sci.* 274, 383–393.

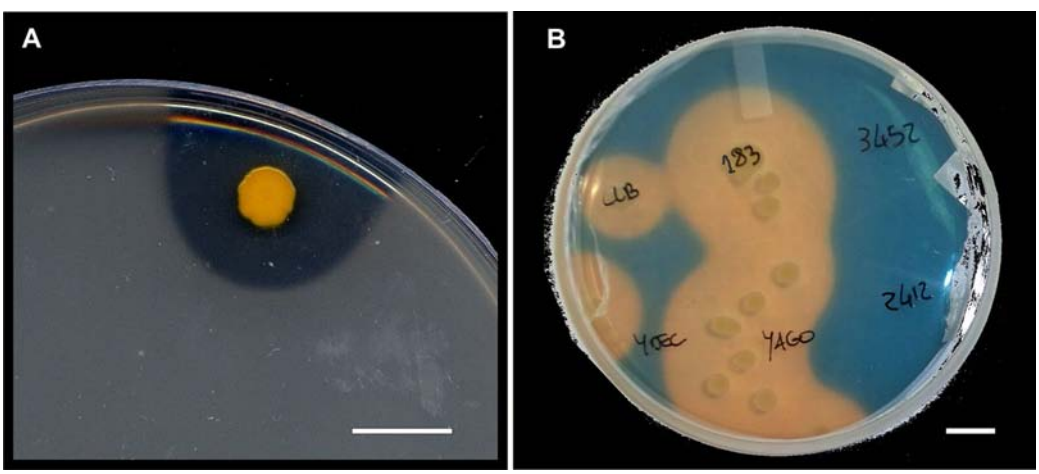

FIGURE 3 | Isolated grapevine endophytes. **(A)** Grapevine endophyte showing antibiosis production towards a layer of *Bacillus subtilis* on a petri dish. Bar = 1 cm. **(B)** Grapevine endophytes, including some showing positivity (discoloring to yellow background in the Chrome Azurol-S assay) to an iron-solubilization test for siderophores. Bar = 1 cm.

hosts, such as *Arabidopsis*. It has been demonstrated that different strains of the *Pseudomonas* and *Acinetobacter* genus and *Bacillus subtilis* could alleviate drought damage because of their root/shoot growth-promoting activities (Rolli et al., 2015; Rolli et al., 2017). These bacteria were characterized by the production of indole acetic acid (IAA), ACC deaminase activity, phosphate solubilization, and ammonia production *via* peptone mineralization. Using the same isolates, in pepper plants, the authors demonstrated that the PGP activity phenotypes related to drought were not constitutive but underwent activation *in planta* when the host was challenged with drought stress. Drought-sensitive rootstocks displayed the most enhanced effects. Three strains were further tested on grapevine under outdoor conditions and conferred higher root biomass to the plants (Rolli et al., 2015). The retardation of water loss in *Vitis vinifera* was also demonstrated by root isolates of *Bacillus licheniformis* and *Pseudomonas fluorescens*, whose effects, as measured from 30 days after the inoculation of *in vitro*-cultured plants, also extended to other measurable and possibly related parameters. These parameters included the accumulation of abscisic acid (ABA) (stimulated 76-fold over the untreated control by *B. licheniformis* and 45-fold by *P. fluorescens*) and synthesis of defense-related terpenes, such as α-pinene, terpinolene, 4-carene, limonene, eucalyptol, lilac aldehyde A, α-bergamotene, α-farnesene, nerolidol, and farnesol (Salomon et al., 2014). Regarding *sensu lato*, endophytic grapevine drought stress has also been shown to be highly alleviated by the presence of mycorrhizal fungi, especially in plants grafted on drought-sensitive rootstocks, such as 775P, 101-14Mgt, and 5BB (Nikolaou et al., 2003). A related stress factor in plants is soil salinity, and in this case, the recruitment of microorganisms under the endophytic condition is a critical aspect (Numan et al., 2018). The mechanisms by which internalized microbes could help alleviate salt stress appear to be linked to their production of plant hormones, including auxins, gibberellins, and ABA (Yanni et al., 2001; Jha and Subramanian, 2013).

Many endophytes have the ability to synthetize ACC deaminase, which alleviates plant stress consequences, as it contributes to lowering the ethylene level. Ethylene is an important plant hormone that is extensively studied as a mediator of plant stress response signaling. Ethylene is formed from methionine *via* *S*-adenosyl-L-methionine, which is converted into 1-aminocyclopropane-1-carboxylic acid by the enzyme ACC oxidase. The stress-induced accumulation of ethylene is usually deleterious to plant growth and health. Several endophytes, such as *P. fluorescens*, *P. phytofirmans*, and *Pseudomonas migulae*, possess the ability to produce the enzyme ACC deaminase, which, by cleaving the ethylene precursor, avoids ethylene accumulation, masking drought and salinity stress and, thus, improving stress tolerance and plant growth (Miliute et al., 2015; Gamalero et al., 2017).

Other molecules are released by endophytes that can intervene in ROS scavenging and, thus, help grapevine cope with different abiotic stresses, such as cold, drought, or salinity. *B. licheniformis* has been demonstrated to release several types of secondary metabolites, such as monoterpenes, exerting an antioxidant activity, and sesquiterpenes, showing antimicrobial properties (Salomon et al., 2014). The other secondary metabolites in the terpenoid biosynthesis pathway are produced by endophytic microorganisms that could be important for their beneficial activity in grapevine. Indeed, *B. licheniformis* produces carotenoids that could act as antioxidant species, which is particularly helpful under different stress conditions (Cohen et al., 2018). Notably, these compounds are precursors of ABA in plants, serving as the base of the drought resistance conferred by *B. licheniformis* (Salomon et al., 2016). It has also been reported that *B. licheniformis* and *P. fluorescens* are able to induce the expression of the genes coding components of ABA synthesis and signaling pathways in grapevine plants (Salomon et al., 2014). Both bacterial endophytes and endophytic fungi (*Septglomus deserticola*, *Funneliformis mosseae*, *Rhizoglomus intraradices*, *Rhizoglomus clarum*, and *Glomus aggregatum*) could modulate ABA metabolism in inoculated grapevine plants, giving an advantage over uninoculated plants under drought conditions (Torres et al., 2018). It has also been reported that in grapevine, *B. licheniformis* enhanced ascorbate peroxidase activity, while *Micrococcus luteus* and *P. fluorescens* augmented peroxidase activity, exerting strong antioxidant activity (Funes Pinter et al., 2017).

Actually, many endophytes produce protective molecules, such as melatonin, proline, and carotenoids, which play an active defense role against abiotic stress. The action is not only local; in fact, endophytes can also produce volatile organic compounds (VOCs), which, in addition to being involved in the first interaction/recognition by the plant (Liu and Brettell, 2019), can also be precursors of important signal molecules. Interesting examples include the release of carotenoids in the host plant as precursors of hormones (ABA) or detoxifying enzymes, such as ACC deaminase, that modulate the ethylene content and consequently the damage triggered upon stress. An interesting example is melatonin accumulation, which occurs in *Bacillus amyloliquefaciens*-inoculated grapevines, counteracting the negative effects of salt, drought, and cold stress (Jiao et al., 2016). The same bacterial strain reduced the upregulation of tryptophan decarboxylase and serotonin *N*-acetyltransferase transcription (Jiao et al., 2016). Indeed, melatonin is a strong antioxidant that increases CuZn superoxide dismutase (SOD), Fe-SOD, catalase, and thylakoid-bound ascorbate peroxidase activities (Baier et al., 2019). It has been observed that an enhancement of endogenous melatonin synthesis is triggered by various abiotic stress factors, which can also be due to endophytic bacteria, such as *P. fluorescens*, which is able to induce the transformation of tryptophan into melatonin in the roots of different grapevine cultivars (Ma et al., 2017). To achieve its beneficial effect, melatonin can be used in exogenous applications. However, melatonin-producing endophytes might have long-term effects on the endogenous melatonin levels in plants once they enter plant tissues. The presence of viruses in grapevine has been noted as a positive factor enhancing tolerance to water stress, shifting the role of some viruses from deleterious to mutualistic because of the long co-existence between the virus and its host (Gambino et al., 2012).

DEFENSE AGAINST BIOTIC AGENTS

General Aspects

Vitis vinifera can be affected by a numerous pathogens often associated with a severe reduction in yield and product quality (Armijo et al., 2016). To guarantee high-quality production, pesticides and fungicides are currently applied in vineyards, although the continuous use of chemicals may cause the emergence of resistant microorganisms, environmental pollution, and heavy consequences on human health (Pérez-Ortega et al., 2012; Damalas and Koutroubas, 2016).

Colonization by endophytic microorganisms places grapevine in a state of alert (primed) that allows a rapid and intense resistance response to subsequent stresses at a low fitness cost instead of wastefully activating defenses (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017). Experimental evidence showed that endophyte perception triggers a local immune response that is significantly weaker in intensity than that occurring during non-host interactions (Bordiec et al., 2011; Trdá et al., 2014). This type of resistance can provide protection to the entire plant against a broad spectrum of fungal, oomycete, bacterial, and viral pathogens and insects (De Vleesschauwer and Höfte, 2009; Van der Ent et al., 2009; Pineda et al., 2010).

Most endophytic bacteria are well known for their capability to produce secondary metabolites that have an inhibitory effect on a wide range of phytopathogens. Some compounds are important for protection because they can inhibit the growth of other bacteria and fungi (antimicrobial compounds) (Shameer and Prasad, 2018), but they also play a significant role in the mechanisms underlying signaling, defense, and gene regulation. These metabolites comprise phytoalexins (Gruau et al., 2015) and various biocide compounds (Verhagen et al., 2011; Esmaeel et al., 2018), such as HCN (Samad et al., 2017) and antibiotics (Nigris et al., 2018; Shameer and Prasad, 2018). A common trait in the group of *Pseudomonas* and *Bacillus* is HCN production (Shameer and Prasad, 2018). HCN can act as a biocide, suppressing the growth of different pathogens, although in grapevine, it has been mainly studied due to its property of breaking summer bud dormancy (Sudawan et al., 2016). Several VOCs produced by different endophyte strains exhibit antibacterial and/or antifungal activity because they can reduce fungal growth, impair fungal spores and hyphae, and/or promote plant growth (Haidar et al., 2016b). An important strategy that helps endophyte-colonized plants cope with pathogens is the ability of endophytes to modulate plant metabolism to restrict pathogen growth and invasion (Miotto-Vilanova et al., 2016). Considering that many factors can influence the effectiveness of BCAs, their efficacy must be confirmed through appropriate experimental procedures both *in vitro* and *in vivo* possibly using a multi-organ screening approach (Haidar et al., 2016a). The organ host and pathogen genotype/strain may considerably affect the antagonist efficiency. Haidar et al. (2016a) reported that the antagonist efficiency of *Pantoea agglomerans* (S2 and S3) and *Enterobacter* sp. (S24) on *Botrytis cinerea* in grapevine leaves was lower than that observed in unwounded berries. Moreover, some endophytic species active against one tested pathogen could stimulate other diseases as reported in *Bacillus* sp. S43, which improved the symptoms caused by *Neofusicoccum parvum* when applied to grapevine cuttings against *B. cinerea* (Haidar et al., 2016a).

Protection Against Bacterial Pathogens

Grapevine diseases caused by bacteria are characterized by a biotrophic host–pathogen relationship in which the microorganism interacts with the living plant without killing it. Crown gall caused by tumorigenic *Rhizobium vitis* is the most important bacterial disease of grapevine worldwide (Armijo et al., 2016). The biological control of crown gall disease in grapevine was achieved by the application of antagonistic endophytic bacteria, including *Enterobacter agglomerans*, *Rahnella aquatilis*, and *Pseudomonas* sp. (Bell et al., 1995), and the non-pathogenic *R. vitis* strains F2/5 (Burr and Reid, 1994) and ARK-1 (Kawaguchi, 2013). Moreover, endophytic *Pseudomonas fluorescens* 1100-6, *Bacillus subtilis* EN63-1, and *Bacillus* sp. EN71-1 isolated from *Malus domestica* were reported as potential BCAs of crown gall (Eastwell et al., 2006). The inhibition of tumorigenic *Rhizobium* spp. occurs through different mechanisms, such as the production of antibacterial compounds (Chen et al., 2007), quorum sensing, caseinolytic protease activation (Kaewnum et al., 2013), and suppression of virulence gene expression (Kawaguchi, 2015; Kawaguchi et al., 2019).

Xylella fastidiosa, which is the agent of grapevine Pierce's disease (PD), is a gram-negative, xylem-limited bacterium transmitted by leafhopper vectors (Kyrkou et al., 2018). Due to the impact of *X. fastidiosa* infections, several attempts to identify potential BCAs against this pathogen have been published. Virulent strains of *X. fastidiosa* acting as an antagonistic of the wild-type strain reduced the severity of PD symptoms in the grapevine cv. Carignan (Hopkins, 2005). In particular, the *X. fastidiosa* strain EB92-1 isolated from elderberry provided good control of PD in vineyard of cv. Flame Seedless and cv. Cabernet Sauvignon (Hopkins, 2005). Newman et al. (2008) demonstrated that the incidence and severity of PD were significantly reduced in grapevine co-inoculated with *X. fastidiosa* and *Pseudomonas* spp. strain G compared with those in plants inoculated with the pathogen alone. The disease reduction was dependent on endophyte interference with diffusible signal factor (DSF)-mediated signaling. Specifically, the highest suppression level was obtained using *carAB* mutants of *Pseudomonas* spp. strain G characterized by superior capabilities of DSF degradation (Newman et al., 2008). Lindow et al. (2005) confirmed the ability of endophytic species within the genera *Paenibacillus*, *Pseudomonas*, *Staphylococcus*, and *Bacillus* in reducing disease symptoms by altering *X. fastidiosa* DSF-mediated signaling. A promising result was obtained using the *Burkholderia phytofirmans* strain PsJN against *X. fastidiosa* in different grapevine cultivars co-inoculated or even inoculated 30 days after the pathogen (Lindow et al., 2017). *In planta* experiments suggested that the pathogen limitation could be due to not only molecular interference with *X. fastidiosa* quorum-sensing regulation and biofilm formation but also induction of the grapevine immune defense responses (Lindow et al., 2017). Deyett et al. (2017) found that endophytic *P. fluorescens* and *Achromobacter xylosoxidans* showed significant negative correlations with the *X. fastidiosa* titer; in particular, *P. fluorescens* emerged as a promising BCA of PD.

Phytoplasmas are phloem-limited plant-pathogenic bacteria transmitted by phloem-feeding insect vectors (Dickinson et al., 2013). Different "*Candidatus* phytoplasma" species can infect the grapevine to induce a complex of diseases commonly referred to as grapevine yellows (GYs) (Maejima et al., 2014). The contribution of endophytic *Pseudomonas migulae* 8R6 to inducing resistance to grapevine FD phytoplasma was demonstrated in the experimental host *Catharanthus roseus* by Gamalero et al. (2017). Specifically, the increased resistance was related to the bacterial ACC deaminase activity, which regulates the level of the stress hormone ethylene, leading to lower symptom expression (Gamalero et al., 2017). Bulgari et al. (2011, 2014) reported that the grapevine cv. Barbera recovered from FD showed a higher level of bacterial diversity than infected plants. In particular, some bacterial species associated with induced systemic resistance (ISR), such as *Burkholderia* sp., *Bacillus pumilis*, and *Paenibacillus pasadenensis*, were found only in the recovered but not in the infected grapevines (Bulgari et al., 2011). Moreover, the population dynamics of endophytic bacteria, such as *Burkholderia*, *Methylobacterium*, and *Pantoea*, were influenced by the presence of phytoplasma (Bulgari et al., 2014). The presence of ISR-inducing bacteria in the recovered plants could indicate the possible involvement of endophytes in recovery from GYs.

Protection Against Fungal Pathogens

Recently, several new potential BCAs were identified by culture-dependent techniques coupled with the *in vitro* assessment of fungal inhibition. Some endophytes isolated from Glera grapevine have been shown to produce biocontrol molecules active against phytopathogenic fungi; among them, *Bacillus licheniformis* GL174 was found to secrete cyclic lipopeptides (LPs) belonging to the surfactins and lichenisins families (Favaro et al., 2016; Nigris et al., 2018). Andreolli et al. (2016) isolated 11 bacterial strains within the genera *Bacillus*, *Brevibacillus*, *Lysinibacillus*, *Nocardioides*, *Stenotrophomonas*, *Microbacterium*, *Pantoea*, and *Pseudoxanthomonas* from field-grown grapevines cv. Corvina, showing *in vitro* antifungal activity against the necrotrophic pathogen *B. cinerea*, which is the agent of grapevine gray mold. The ability of endophytic *Bacillus* spp. to induce resistance against *B. cinerea* in grapevine was also reported by Farace et al. (2015), who showed that grapevine plant cells perceive three families of cyclic LPs from *B. subtilis* that differentially activate the plant innate immune response. Campisano et al. (2015) isolated 25 endophytic bacterial strains showing high inhibitory effects against *B. cinerea* from domesticated and wild grapevines in northern Italy. Among them, the most effective strains belonged to the genera *Bacillus* and *Pantoea*.

Several works proved the effectiveness of *Pseudomonas* spp. as BCAs against *B. cinerea*. *P. fluorescens* PTA-CT2 can elicit defense responses in grapevine against *B. cinerea* at both the local and systemic levels (Trotel-Aziz et al., 2008; Verhagen et al., 2011; Gruau et al., 2015). Although PTA-CT2 can colonize grapevine roots but not above-ground plant organs, a systemic defense response is activated by molecular signals transferred from the roots to distal leaves (Gruau et al., 2015). Distinct patterns of defense-related gene expression were found in grapevine roots and leaves, particularly in some genes associated with cell death and hypersensitive response (HR) (Gruau et al., 2015). Following *B. cinerea* infection in grapevine leaves, PTA-CT2-mediated ISR enhanced stilbene accumulation, glutathione 3-transferase gene expression, the downregulation of HR and cell death marker genes (Gruau et al., 2015). Verhagen et al. (2010) found that the resistance to *B. cinerea* in grapevine could be induced by *Pseudomonas aeruginosa* (7NSK2), *P. fluorescens* (strains CHA0, Q2-87 and WCS417), and *Pseudomonas putida* (WCS358) by the production of phytoalexins and stimulation of oxidative bursts.

Streptomyces anulatus S37 isolated from wild *V. vinifera* is an endophytic PGP rhizobacterium (PGPR) that confers resistance against different pathogens, including *B. cinerea* (Loqman et al., 2009). Vatsa-Portugal et al. (2017) showed that *S. anulatus* S37 perception by grapevine cells triggers early and late defense responses, such as ion fluxes, oxidative burst, extracellular alkalinization, activation of protein kinases, induction of defense gene expression, and phytoalexin accumulation. Moreover, *S. anulatus* S37-primed grapevine cells became refractory to infection by *B. cinerea*, showing a reduction in pathogen-induced cell death (Vatsa-Portugal et al., 2017).

The *Paraburkholderia phytofirmans* strain PsJN is an endophytic PGPR known for its antifungal activity against gray mold disease (Ait Barka et al., 2000). Following root inoculation, bacterial cells diffuse through grapevine xylem vessels, forming a biofilm at the leaf surface and exerting a direct antagonistic effect on *B. cinerea* (Miotto-Vilanova et al., 2016). Different works revealed that the grapevine responds to *P. phytofirmans* PsJN inoculation by the activation of a local immune response characterized by the accumulation of phenolic compounds, salicylic acid (SA) accumulation, ion fluxes, and defense gene regulation (Compant et al., 2005; Bordiec et al., 2011; Miotto-Vilanova et al., 2016). Moreover, following infection by *B. cinerea*, only the bacterized grapevine plantlets trigger an oxidative burst in leaf tissues, callose deposition in the stomata, the induction of SA and jasmonic acid (JA) pathogenesis-related (PR) genes (*PR1*, *PR2*, and *PR5*) and changes in leaf carbohydrate metabolism (Miotto-Vilanova et al., 2016). In particular, *P. phytofirmans* is associated with the ability to redirect carbohydrates in favor of fructose, which is actually not useful for the fungus (Miotto-Vilanova et al., 2016).

Grapevine trunk diseases (GTDs), such as Esca, Eutypiosis, and Botryosphaeriae diebacks, are associated with a complex of fungal species causing premature decline in vines, yield loss, and poor wine quality worldwide (Gramaje et al., 2018). Pruning and fungicide treatment applied to control GTDs have limited efficacy; moreover, these approaches are expensive and not environmentally sustainable. Eutypa dieback, which is caused by the Diatrypaceous fungus *Eutypa lata*, is a major trunk disease in grapevines associated with the heavy loss of production (Gramaje et al., 2018). Ferreira et al. (1991) reported that an endophytic strain of *B. subtilis* isolated from grapevine cv. Chenin Blanc inhibited *E. lata* mycelium growth and spore germination *in vitro* and significantly reduced fungal infection on pruning wounds. Several endophytic bacterial strains were investigated due to their inhibitory effect on *Phaeomoniella chlamydospora*

and *Phaeoacremonium aleophilum*, which are the agents of grapevine Esca disease colonizing the xylem tissues of vine plants (Gramaje et al., 2018). The *B. subtilis* strain AG1 was reported to inhibit mycelial growth of *P. chlamydospora* and *P. aleophilum in vitro* by producing antagonistic substances (antibiotics) stable at high temperatures and resistant to enzymatic degradation (Alfonzo et al., 2009). Another interesting example includes *Paenibacillus* sp. (S19) and *Bacillus pumilus*, which possess effective antagonistic activity against *P. chlamydospora* through the production of the volatile compound pyrazine, which inhibits mycelia growth (Haidar et al., 2016b). Similarly, two *Bacillus* spp. strains effective against Esca-associated fungi were isolated from field-grown grapevines cv. Corvina (Andreolli et al., 2016). More recently, the same research group identified a *Pseudomonas protegens* MP12 strain in a soil sample able to colonize inner grapevine tissues and is exhibiting *in vitro* inhibitory effects on mycelial growth of *P. chlamydospora* and *P. aleophilum* (Andreolli et al., 2019). This strain showed *in vitro* activity against several grapevine phytopathogens, such as *B. cinerea*, *Alternaria alternata*, *Aspergillus niger*, *Penicillium expansum*, and *N. parvum*, and *in vivo* antifungal activity against *B. cinerea* on grapevine leaves (Andreolli et al., 2019). Endophytic bacteria showing antagonistic activity against *N. parvum*, which is among the most virulent GTD-associated fungi, were isolated from grapevine wood tissues in Tunisia (Rezgui et al., 2016; Haidar et al., 2016a). Among 11 strains showing *in vitro* activities against *N. parvum*, *B. subtilis* B6 reduced the size of wood necrosis on young grapevines cv. Italia (Rezgui et al., 2016). Several endophytic fungi have also been proposed for the biocontrol of grapevine truncal diseases; these fungi include *Pythium oligandrum* as an inducer of plant resistance (Yacoub et al., 2016), *Aureobasidium* spp. (Gonzàlez et al., 2012), *Chaetomium* spp. (Spagnolo et al., 2012), *Fusarium lateritium* (Christen et al., 2005), *Trichoderma atroviride* (Pertot et al., 2016), and *Epicoccum layuense* (Del Frari et al., 2019)

Recently, Álvarez-Pérez et al. (2017) demonstrated the efficacy of Actinobacteria isolated from the grapevine root system as BCAs of GTDs. Field trials enabled the identification of the endophytic strain *Streptomyces* sp. VV/E1, which significantly reduced the infection rates of *Dactylonectria* sp., *Ilyonectria* sp., *P. chlamydospora*, and *Phaeoacremonium minimum* and is associated with a decline in young grapevines (Álvarez-Pérez et al., 2017).

Wicaksono et al. (2016) reported the potential role of endophytic bacteria from roots of *Leptospermum scoparium*, a New Zealand native medicinal plant, as BCA against botryosphaeriaceous species of fungi associated with GTDs. *In vitro* assays of 10 *Burkholderia* spp., *Serratia* sp. and *Pseudomonas* spp. showed that all isolates were effective against *Neofusicoccum* spp. by the production of antibiotic diffusible and/or volatile compounds (Wicaksono et al., 2017). *Pseudomonas* spp. isolates from *L. scoparium*, which are active against multiple botryosphaeriaceous species *in vitro*, showed evidence of specificity towards a particular pathogen species once inoculated *in planta* (Wicaksono et al., 2017).

Grapevine downy mildew caused by the oomycete *Plasmopara viticola* is a serious and persistent disease problem for the grapevine industry that is difficult to control through chemical and agricultural practices. Musetti et al. (2006) demonstrated the antifungal activity against *P. viticola* of diketopiperazines (DKPs) produced by an endophytic strain of *A. alternata* isolated from grapevine leaves showing anomalous downy mildew symptoms. The application of DKPs inhibited *P. viticola* sporulation and induced severe ultrastructural alterations on fungal mycelium (Musetti et al., 2006). *Acremonium byssoides* is an endophytic fungus naturally present in different grapevine varieties (Burruano et al., 2008). *A. byssoides* is able to actively parasitize the pathogen in grapevine leaves inoculated with *P. viticola*. Moreover, culture filtrates and a crude extract of an *A. byssoides* strain isolated from grapevine cv. Insolia completely inhibited sporangial germination of *P. viticola* (Burruano et al., 2008). In a recent study, *B. subtilis* GLB191 and *B. pumilus* GLB197 were identified among 239 bacterial endophytes isolated from grapevine leaves in China (Zhang et al., 2017). Their potential application as BCAs against downy mildew disease was demonstrated by leaf disk assays and under field conditions. The bacterium *Paenibacillus* sp. strain B2 secretes the peptide paenimyxin, which acts as a biopesticide against different grapevine pathogens. Hao et al. (2017) showed that *Paenibacillus* sp. strain B2 can inhibit the development of *P. viticola* and *B. cinerea in vitro* and affects the activity of the ectoparasitic nematode *Xiphinema index in vitro* and *in planta*.

Protection Against Insects

Finally, grapevine protection against piercing-sucking insects was achieved by the endophytic colonization of the entomophagous fungus *Beauveria bassiana* in both young potted grapevine plants in a greenhouse and mature plants in the vineyard (Rondot and Reineke, 2018). Following spray inoculation, endophytic *B. bassiana* was detected for at least 21 days inside the leaves of potted plants and up to 5 weeks after the final application in the field. Experimental trials demonstrated that the endophytic fungus can reduce the infestation rate and growth of the mealybug vector of leafroll and rugose wood viruses *Planococcus ficus* in leaves of potted grapevines and infestation of the grape leafhopper *Empoasca vitis* in the field (Rondot and Reineke, 2018).

INFLUENCE OF ENDOPHYTES ON PRODUCT QUALITY

Among the plant products, wine entails one of the most rewarding items in the current agricultural economy. Its market value attracts exceptional attention to the plant's overall conditions, including its inner microbiome. Since endophyte metabolism can contribute to that of the plant host and the biochemical composition of its fruits, the nature of grapevine endophytic taxa identities, ecological attitudes, potential toxicity, and clinical relevance are all aspects worthy of a thorough investigation.

While the beneficial effect of endophytes on host plants as growth promoters and stress resistance inducers has been reported in several papers, only a few studies addressed their influence on grape and wine quality and almost exclusively focused on fungal endophytes. Recent works demonstrated that

wine and its bouquet are also under the influence of endophytic colonizers of grapes. Yang et al. (2016) showed that the inoculation of eight fungal endophytes isolated from *Vitis vinifera* modified the physio-chemical status of field-grown grapevines in both leaves and berries during the ripening stage. In particular, fungal endophytes induced variations in the content of reducing sugar, total flavonoids, total phenols, trans-resveratrol, and activities of PAL in both tissues. Moreover, the inoculation of different strains of fungal endophytes led to different grape metabolite statuses with some strains, such as CXB-11 (*Nigrospora* sp.) and CXC-13 (*Fusarium* sp.), exerting greater promotion effects on grapevine metabolites (Yang et al., 2016). Huang et al. (2018) observed that dual cultures with different endophytic fungal strains were characterized by different metabolite compositions in grapevine flesh cells. In particular, the modification of metabolic profiles by fungal endophytes was fungal strain/genus specific. These works confirmed that endophyte–host metabolic interactions influence the introduction of specific metabolites in the host plant, supporting the possibility of using fungal endophytes to shape grape qualities and characteristics (Huang et al., 2018).

The capability of endophytic fungi to produce plant secondary metabolites or phytochemicals could be exploited for cost-effective large-scale production (Suryanarayanan et al., 2009). Over the last few years, different works reported the identification of endophytic fungal strains producing pharmaceutically valuable compounds with beneficial effects on human health, such as resveratrol. Grapevine inoculation with endophytic *Acinetobacter lwoffii*, *Bacillus subtilis*, and *Pseudomonas fluorescens* effective against *Botrytis cinerea* leads to the accumulation of host-synthesized stilbenic phytoalexins, especially trans-resveratrol (3,5,4′-tryhydroxystilbene) and its oligomer, trans-ε-viniferin, which, in turn, could contribute to the grape fruit metabolite composition (Verhagen et al., 2011). Recently, 36 endophytic fungal strains isolated in China from grapevine cv. Cabernet Sauvignon were assessed for their ability to produce resveratrol *in vitro* (Liu et al., 2016). The morphological and molecular analyses allowed the identification of the C2J6 strain of *Aspergillus niger*, showing stable high resveratrol production (Liu et al., 2016). Dwibedi and Saxena (2018) analyzed the resveratrol-producing potential of 53 endophytic fungi from different *V. vinifera* varieties in different regions in India. The resveratrol-producing isolates were assigned to the following seven genera: *Aspergillus*, *Botryosphaeria*, *Penicillium*, *Fusarium*, *Alternaria*, *Arcopilus*, and *Lasiodiplodia*. In particular, the highest resveratrol content was obtained from the culture of *Arcopilus aureus* isolate #12VVLPM (Dwibedi and Saxena, 2018).

In conclusion, far from being the ultimate framing of such a dynamic matter, this article describes the current picture of this entangling, paradigmatic, and multi-faceted example of a wealthy array of finely evolved plant–microbe interactions.

AUTHOR CONTRIBUTIONS

DP, AS, FC, and MZ wrote the first draft. DP, AS, DC, EB, FS, RM, FC and MZ made substantial, direct, and intellectual contributions to this work.

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Necrotic and Cytolytic Activity on Grapevine Leaves Produced by Nep1-Like Proteins of *Diplodia seriata*

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Many phytopathogenic fungi produce necrosis and ethylene inducing peptide 1 (Nep1-like proteins or NLP) that trigger leaf necrosis and the activation of defense mechanisms. These proteins have been widely studied in plant pathogens as *Moniliophthora perniciosa* or *Botrytis cinerea* between others, but little is known about their biological roles in grapevine trunk pathogens. Advances in the sequencing of genomes of several fungi involved in grapevine trunk diseases have revealed that these proteins are present in several copies in their genomes. The aim of this project was to analyze the presence of genes encoding NLP proteins in the *Diplodia seriata* genome and to characterize their putative role as virulence factors associated to grapevine trunk diseases. In this study, we characterized four NLPs from *Diplodia seriata*. All proteins showed highly similar amino acid sequences and contained the characteristic peptide motifs of NLPs. DserNEPs slightly reduced the viability of *Vitis vinifera* L. cell cultures. The cytolytic activity from DserNEP1 was stronger than that from DserNEP2, even at low concentrations. Purified DserNEPs also produced necrosis in leaves when they were inoculated into micropropagules of *V. vinifera* L. This is the first record of Nep1-like proteins from a fungus associated with grapevine trunk diseases and also from a member of the Botryosphaeriaceae family.

Keywords: Botryosphaeriaceae, NLP, RT-qPCR, grapevine trunk diseases, phytotoxicity, *Vitis vinifera*

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INTRODUCTION

Grapevines are one of the most important economic crops worldwide. Grapevine trunk diseases (GTDs) are a major threat for the wine sector, causing serious economic losses to the wine industry (Siebert, 2001; Gubler et al., 2005). This term encompasses different fungal pathologies such as Botryosphaeria dieback, Esca, Eutypa dieback, Petri disease, or Black foot, to cite the most relevant. The incidence of GTD have increased over the last decades, mainly due to the lack of effective strategies to fight these diseases (Chiarappa, 2000; Graniti et al., 2000; Spagnolo et al., 2017; Mondello et al., 2018). Botryosphaeria dieback has been reported since the 1970s as one of the main GTD. It is caused by several xylem-inhabiting fungi (Bertsch et al., 2013) which are primarily members of the Botryosphaeriaceae family such as *Diplodia seriata* De Not. (anamorph of *Botryosphaeria obtusa*, Shoemaker, 1964; Phillips et al., 2007), *Diplodia mutila* (anamorph of

Botryosphaeria stevensii, Shoemaker, 1964), and *Neofusicoccum parvum* (anamorph of *Botryosphaeria parva*, Crous et al., 2006).

D. seriata is one of the pathogens most frequently isolated from symptomatic grapevines in Spanish vineyards (Martín and Cobos, 2007) as well as French vineyards (Kuntzmann et al., 2010). It has been isolated from at least 34 different hosts (Punithalingam and Waller, 1976), mostly fruit trees, but also from other woody plants. *D. seriata* has been recognized as a wound pathogen and is associated with dieback symptoms and cankers (Larignon et al., 2001; Phillips, 2002; Van Niekerk et al., 2004). These pathogens attack the perennial organs of grapevines, although they have never been isolated from leaves (Larignon and Dubos, 1997). Therefore, it is supposed that symptoms observed in the berries and leaves might be caused by extracellular compounds produced by the fungi in colorless woody tissues of the trunk, which are then translocated to leaves through the transpiration system (Mugnai et al., 1999).

The toxicity of some extracellular metabolites produced by members of Botryosphaeriaceae family has been proven (Martos et al., 2008; Andolfi et al., 2011; Ramírez-Suero et al., 2014), and several phytotoxic metabolites have been identified (Bénard-Gellon et al., 2014; Abou-Mansour et al., 2015). However, little is known about putative proteins secreted by these fungi that could have any role in pathogenesis. Bénard-Gellon et al. (2014) detected some toxicity in an extracellular protein extract from *D. seriata* and *N. parvum*. However, to date no protein has been identified yet as a virulence factor in *D. seriata*, although in a previous work Nep1-like proteins were detected as putative virulence factors in the secretome of this fungus (Cobos et al., 2010). Among the host cell-death-inducing secreted proteins of plant pathogens are the necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs) that were first detected in culture filtrates of *Fusarium oxysporum* (Bailey, 1995). NLPs constitute a superfamily of proteins that are produced by phytopathogenic bacteria, fungi, and oomycetes (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006), which have been recognized as virulence factors in several phytopathogenic fungi such as *F. oxysporium* (Bailey, 1995; Bae et al., 2006), *Botrytis* spp. (Staats et al., 2007a; Staats et al., 2007b; Schouten et al., 2008), *Phytophthora megakarya* (Bae et al., 2005), *Moniliophthora perniciosa* (García et al., 2007; Zaparoli et al., 2009), *Mycosphaerella graminicola* (Motteram et al., 2009), or *Verticillium dahliae* (Zhou et al., 2012) among others.

NLPs have been proposed to have dual functions in plant–pathogen interactions, acting both as triggers of immune responses and also as toxin-like virulence factors (Qutob et al., 2006). NLPs are relatively small proteins of about 24 kDa that exhibit a high degree of similarity at amino acid sequence level, including the presence of two highly conserved cysteine residues that form an intramolecular disulfide bridge essential for NLP activities (Fellbrich et al., 2002; Qutob et al., 2006; Ottmann et al., 2009), and also a central hepta-peptide motif "GHRHDWE" that is part of the negatively charged cavity exposed at the protein surface. Both are necessary for plasma membrane permeabilization and cytolysis in plant cells (Ottmann et al., 2009). NLPs have been classified into type I and type II classes, depending on whether they contain two or four cysteine residues present at conserved positions, respectively (Gijzen and Nurnberger, 2006). A third type of NLP was described by Oome and Van Den Ackerveken (2014), but this type III only shares a central 50 amino acids with types I and II including the highly conserved heptapeptide motif.

The present study aims to analyze the presence of genes encoding NLP proteins in the *D. seriata* genome and to characterize their structure and their putative role as virulence factors associated to GTDs.

MATERIALS AND METHODS

DNA Isolation

The *D. seriata* strain used in this work was derived from a monosporic culture of *D. seriata* VS1 (Cobos et al., 2010). The strain was routinely maintained on potato dextrose agar (PDA; Scharlau Chemie S.A.). Genomic DNA was isolated from fresh mycelia following an adaptation of the method described by Möller et al. (1992). Briefly, 16 g of fresh mycelia was ground to a fine powder in liquid nitrogen, transferred to Falcon® tubes, and mixed with TES solution (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS); 120 μg/mL of Proteinase K was added and tubes were incubated for 1 h at 65°C with occasional gentle mixing. The salt solution was adjusted to 1.4 M with 5 M NaCl, and 1/10 volume 10% CTAB was added before incubating for 10 min at 65°C. The tubes were centrifuged at 10,000 rpm for 10 min. RNase (20 μg/mL) was added to the supernatant and incubated at 37°C for 1 h. The aqueous phase was extracted with 1 volume of phenol–CIA solution (phenol/chloroform/isoamyl alcohol; 25:24:1 v/v) mixed by inversion and placed on ice for 30 min. After centrifugation for 10 min at 8,000 rpm, and an additional extraction with 1 volume of CIA (chloroform:isoamyl alcohol; 24:1 v/v), the supernatant was mixed with 1/3 volume of 5 M NH4Ac, mixed gently, and placed on ice for 30 min. After centrifugation for 10 min at 8,000 rpm, the nucleic acids were precipitated with 1 volume of cold isopropanol. DNA was recovered by centrifugation and the pellet washed with 70% ethanol. DNA was dissolved in 500 μl of TE buffer and stored at −20°C. DNA concentration was estimated with a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Genomic Library Construction and Screening

Genomic DNA from *D. seriata* (12 μg) was partially digested with *Sau*3AI. DNA fragments (17–23 kb) were purified by ultracentrifugation in a sucrose gradient and ligated to Lambda DASH II *Bam*HI Vector Kit (Stratagene), followed by *in vitro* packaging. Degenerate primers NepFdeg (5′ GTRAATGGRTGCGTRCCATTCCC 3′) and NepRdeg (5′ CCTTCCCARTCGTGRCGGTGRCC 3′) were designed against conserved regions present in Nep1-like proteins (identified by *in silico* analysis of proteins deposited in GenBank database) that included the peptides previously identified by MASCOT from *D. seriata* NLPs (Cobos et al., 2010). These primers amplified a partial DserNEP sequence that was labeled with the DIG DNA labeling kit (Roche) and used as a hybridization probe to screen recombinant bacteriophage plaques of the genomic library.

PCR Amplification and Sequencing of *DserNEP* Genes

Primer pairs were designed to amplify the entire sequence of *DserNEP* genes from DNA and cDNA (**Table 1**). *DserNEP* genes were amplified by PCR. Each reaction contained 1× Kapa Hifi (KAPA BIOSYSTEMS), 300 μM of each dNTP, 0.3 μM of each primer, 0.5 U of Kapa Hifi polymerase, and 1 μl of template DNA. PCR amplifications were performed on a Mastercycler gradient (Eppendorf). The program consisted of an initial step of 2 min at 95°C, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 15 s, and elongation at 72°C for 30 s. A final extension was performed at 72°C for 3 min. DNA was sequenced by the dideoxynucleotide chain termination method using a BigDye Terminator cycle sequencing kit (Applied Biosystems). Signal peptide regions were predicted by using the Signal P3 program (Bendtsen et al., 2004).

Amino Acid Sequence Analysis

Sequences from GTD pathogens with significant similarity (value $1e^{-4}$) to DserNEP proteins were retrieved from the NCBI database and identified using BlastP (Altschul et al., 1990). Sequences in **Table 2** were aligned by ClustalW (Larkin et al., 2007) and then analyzed using the MEGA 5 phylogenetic package (Tamura et al., 2011). The phylogenetic tree was obtained using Neighbor analysis with 1,000 bootstrap replications.

Analysis of DserNLP Expression

A plug of mycelium of *D. seriata* VS1c grown on PDA was inoculated in Erlenmeyer flasks containing Czapeck liquid medium (control conditions) (Cobos et al., 2010) or Czapeck liquid medium supplemented with chips of grapevine wood as described by Paolinelli-Alfonso et al. (2016). Each condition was assayed in triplicate. After inoculation, flasks were incubated in an orbital shaker at 25°C and 100 rpm in darkness. Fungal mycelia were collected from each 24 hours during 6 days. All collected samples were immediately frozen in liquid nitrogen and stored at −70°C for RNA isolation. Total RNA was isolated with 1 mL of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was cleaned up with the RNeasy Plant Mini Kit (Qiagen) including the on-column DNase enzymatic treatment. RNA concentration and purity were measured with NanoDrop 2000 Spectrophotometer (Thermo Scientific). cDNA was synthesized from RNA with PrimeScript RT Master Mix (Takara). Transcript levels of NLPs were determined by quantitative real-time PCR (qRT-PCR, TB Green Premix Ex Taq; Takara). qRT-PCR reactions were carried out in triplicate in 96-well plates in a 20-μl final volume containing 1× TB Green Premix Ex Taq (Takara), and 400 nM forward and reverse primers (**Table 1**). Cycling parameters were 2 min of Taq polymerase activation at 95°C, followed by 40 two-step cycles composed of 20 s of denaturation at 95°C, and 20 s of annealing and elongation at 58°C. Melting curve assays were performed from 55 to 95°C, and melting peaks were visualized to check the specificity of amplification. The results obtained for each gene of interest were normalized to the expression of β-tubulin gene. Three biological with three technical replicates were used. Relative gene expression was determined with the formula fold induction: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = [Ct TG (US) – Ct RG (US)] – [Ct TG (RS) – Ct RG (RS)]. Ct (cycle threshold) value is based on the threshold crossing point of individual fluorescence traces of each sample, TG is target gene, RG is reference gene, US is unknown sample, and RS is reference sample. The genes analyzed were considered significantly up- or downregulated when changes in their expression were >2-fold or <0.5-fold, respectively.

TABLE 1 | Oligonucleotides used in this study.

Primer name	Primer sequence (5′–3′)
NepFdeg	GTRAATGGRTGCGTRCCATTCCC
NepRdeg	CCTTCCCARTCGTGRCGGTGRCC
DserNEP1F	ATGCTGTCCTCATCACTCTTCTGGCC
DserNEP1R	TCACAACGCAGCCTCAGCGAGGTT
DserNEP2F	ATGCCGCTCTCCATCCGCTAC
DserNEP2R	TCACAACGCCGCCTTGGCCAG
DserNEP3F	GCCCCCTTCACCCAGCAGCTGCACG
DserNEP3R	TCAAACCCACGCCTTATCCAAATTCCCC
DserNEP4F	GCCCCGGCAGCTGCCCCTGAGAG
DserNEP4R	TCAAAGGCAGGCCTTGTTAAGGTTG
qDsernep1F	ACGCTTTCGCCATCATGTAC
qDsernep1R	ACAATGCTCTCCCAGTCGTG
qDsernep2F	TACAACGTCTACCCCGTCAAC
qDsernep2R	TCTTTGAACGGCACATTGGC
qDsernep3F	GGTATGCGTTGCTGGATTGGGATGT
qDsernep4F	CGAGCTGCAGTTCAAGACCAGC
qtubulin Dser F	GAACGTCTACTTCAACGAGGT
qtubulin Dser R	GAGGACAGCACGAGGAACGT

Heterologous Expression in *Escherichia coli*

The cDNAs encoding for DserNEP1 and DserNEP2 proteins (without their putative signal peptides) were amplified and cloned into pET SUMO vectors and transformed into competent *E. coli* One shot Mach1-T1 cells. Recombinant clones were selected and sequenced to ensure that no erroneous nucleotide changes had resulted from PCR amplification. Expression of recombinant proteins in *E. coli* BL21 (DE3) strain was carried out by using the Champion pET SUMO Protein Expression System (Invitrogen) according to the manufacturer's instructions. Purification of His-tag fusion proteins from *E. coli* cell-free extracts was achieved by affinity purification with a Ni-nitrilotriacetic acid resin (Ni-NTA; Qiagen) balanced with buffer A (50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole; pH 8.0). After extensive washing, bound proteins were eluted with buffer B (50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole; pH8.0). Upon visual inspection in a SDS gel, DserNEP-containing fractions were pooled and dialyzed against a phosphate-buffered saline (PBS) solution (pH 7.4) at 4°C with a Slide-A-lyzer Mini Dialysis Float system (Pierce). The purity of the recombinant proteins (higher that 98%) was confirmed by SDS PAGE and quantified using the Bradford method (Bradford, 1976).

Grapevine *In Vitro* Cultures and Cellular Callus Production

For the development of *in vitro* cultures, grapevine shoots of Tempranillo cultivar, with three to four buds each, were treated with 16% (w/v) copper oxychloride fungicide (Cobre

TABLE 2 | Nep 1-like proteins used in the phylogenetical analysis.

Organism	Accession number	Plant pathogenicity of host	Reference
Diplodia seriata	KKY26562	Botryosphaeria dieback	Morales-Cruz et al., 2015
Diplodia seriata	KKY20654	Botryosphaeria dieback	Morales-Cruz et al., 2015
Diplodia seriata	KKY13781	Botryosphaeria dieback	Morales-Cruz et al., 2015
Diplodia seriata	KKY20647	Botryosphaeria dieback	Morales-Cruz et al., 2015
Diplodia seriata	AKQ49205	Botryosphaeria dieback	This study
Diplodia seriata	AKQ49206	Botryosphaeria dieback	This study
Diplodia seriata	MK978328	Botryosphaeria dieback	This study
Diplodia seriata	MK978329	Botryosphaeria dieback	This study
Neofusicoccum parvum	EOD44475	Botryosphaeria dieback	Blanco-Ulate et al., 2013b
Neofusicoccum parvum	EOD47698	Botryosphaeria dieback	Blanco-Ulate et al., 2013b
Neofusicoccum parvum	EOD48844	Botryosphaeria dieback	Blanco-Ulate et al., 2013b
Neofusicoccum parvum	EOD52252	Botryosphaeria dieback	Blanco-Ulate et al., 2013b
Neofusicoccum parvum	EOD44269	Botryosphaeria dieback	Blanco-Ulate et al., 2013b
Phaeoacremonium minimum	XP007911059	Esca disease	Blanco-Ulate et al., 2013c
Eutypa lata	EMR66076	Eutypa dieback	Blanco-Ulate et al., 2013a
Eutypa lata	EMR63075	Eutypa dieback	Blanco-Ulate et al., 2013a
Eutypa lata	EMR70921	Eutypa dieback	Blanco-Ulate et al., 2013a
Diaporthe ampelina	KKY35834	Phomopsis dieback	Morales-Cruz et al., 2015
Diaporthe ampelina	KKY36076	Phomopsis dieback	Morales-Cruz et al., 2015
Diaporthe ampelina	KKY37779	Phomopsis dieback	Morales-Cruz et al., 2015
Diaporthe ampelina	KKY30513	Phomopsis dieback	Morales-Cruz et al., 2015

Key-S; Químicas KEY S.A.). Buds were stimulated to sprout under culture room conditions maintained at 25 ± 2°C with a 16/8-hour light/dark cycle for 2 months. Two kinds of explants were used: double-node stem segments for *in vitro* plants and young leaves for calluses. Explants were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, and 0.4% (v/v) sodium hypochlorite solution with four drops of Tween 20 for 2 min, and then rinsed four to five times in sterilized water.

In vitro plants were obtained according to Sevillano et al. (2014). Double-node stem segment explants were cultured on Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 20 g/L sucrose, 1 mg/L benzyl adenine (BA), and 8 g/L agar, pH 5.8. Stems developed from nodal segments were multiplied through micro-cutting in order to obtain *in vitro* plants. All plant material was grown at 25 ± 2°C under 16/8-hour light/dark cycle and transferred to fresh medium every 2 months.

For callus induction, young sterilized grape leaves from Tempranillo cultivar were cultured on GB5 media (Gamborg et al., 1968) supplemented with 20 g/L sucrose, 1 mg/L 2,4-dichlophenoxyacetic acid (2,4-D), 0.1 mg/L BA, 0.5% (w/v) charcoal, and 8 g/L agar, pH 5.8. Calluses were maintained at 25 ± 2°C in darkness and transferred to fresh medium monthly. For establishment of liquid cell suspensions, 1 g of callus pieces was transferred into 150 mL flasks containing 50 mL liquid GB5 media supplemented with 20 g/L sucrose, 0.5 mg/L 2,4-D pH 5.8, placed in a rotary shaker (120 rpm) under 16/8-hour light/dark cycle, and routinely subcultured every 15 days. Manipulation of plant material was always performed on a clean bench and all instruments and growth media used were sterilized using dry heat or in autoclave.

In order to test the cultivar susceptibility, 1-year-old plants of four different cultivars (Chardonnay, Cabernet Sauvignon, Tempranillo, and Sauvignon Blanc) were potted in plastic pots and regularly irrigated by drip.

Necrosis Activity Assay

In vitro micropropagated *V. vinifera* plants from Tempranillo cultivar were inoculated with purified DserNEP1, DserNEP2, or PBS buffer (pH 7.4). We tested different protein concentrations ranging between 0 and 0.5 mg/mL. Protein application was made by dipping freshly cut micropropagules into DserNEP protein solution (100 µl), and the propagules were immediately transferred to fresh medium. Each concentration was assayed in three replicates and the experiment was repeated at least three times.

Application to potted plants was carried out by leaf infiltration of 20 µl of DserNEP1 and DserNEP2 proteins at 0.15 and 0.30 mg/mL, or PBS buffer (as negative control). Three plants for each cultivar and three leaves from each plant were assayed.

Necrosis quantification was carried out by performing an electrolyte leakage assay, as described previously (Sevillano et al., 2014), by using 50 mg of leaves that were removed and washed in 2 mL distilled water 4 days after propagule inoculation. After 5 min, the water was transferred to other tubes and electrolyte leakage was measured with a conductivity meter (Crison 522).

Fluorescein Diacetate Assay (FDA)

FDA assay was used to check cell viability. FDA is converted by non-specific esterases of vital cells to fluorescein, which produces a bright green fluorescence for at least 15 min. The polar fluorescein is trapped in cells with intact plasma membranes (Widholm, 1972). Cell suspensions were exposed to purified DserNEP1 (0.15 mg/mL) and DserNEP2 (0.60 mg/mL). Each concentration was assayed in triplicate. Cell viability was measured by staining with FDA [1:1 (v/v) dilution of 0.1 mg/mL FDA] 5 days after inoculation. After 2 min in darkness, cells were observed under a Nikon microscope equipped with epifluorescence irradiation. Cell viability was expressed as the

percentage of fluorescent cells with respect the total number of cells checked. The experiment was repeated three times.

Statistical Data Analysis

Conductivity and cell viability data were analyzed using a weighted least-square ANOVA test to determine if there were significant differences. When *F* ratios were statistically significant, *post hoc* tests (Tukey's honestly significant difference test) were performed to establish where the differences between groups were. Statistical analyses were performed using R Core Team 3.0.1 (2014) software (http://www.R-project.org). Error bars in graphs indicate SDs. Bars marked with the same letter do not differ at P = 0.05.

RESULTS

Cloning and Sequence Analysis of *DserNEP* Genes

Degenerate primers NEPdF and NEPdR were designed in order to amplify an internal fragment of *DserNEP* genes. These primers amplified a partial *DserNEP* sequence of 325 bp that was used as a probe to screen a *D. seriata* genomic library. Four bacteriophages containing the whole sequence of four different *DserNEP* genes were selected: phage λDASH-DsF1 contained a *DserNEP1* gene (NCBI GenBank database accession number AKQ49205), λDASH-DsF2 phage contained a *DserNEP2* gene (AKQ49206), whereas λDASH-DsF3 phage contained a *DserNEP3* gene (MK978328), and λDASH-DsF4 phage contained a *DserNEP4* gene (MK978329).

All the *DserNEP* genes were amplified by using specific primers (**Table 1**). *DserNEP1* gene consisted of an open reading frame (ORF) of 877 bp. *DserNEP2* gene consisted of an 858 bp ORF, whereas DserNEP3 and DserNEP4 ORFs had a size of 897 and 812 bp, respectively. Total RNA isolated from *D. seriata* was subjected to RT-PCR to obtain cDNAs of *DserNEP* genes. The resulting fragments were cloned and sequenced. Sequence analysis revealed that the *DserNEP1* gene contained a 142 bp intron to yield a cDNA of 729 bp encoding a 242 amino acid protein. The *DserNEP2* gene contained a 126 bp intron to generate a 735 bp cDNA encoding a 243 amino acid protein. The *DserNEP3* gene contained a 162 bp intron to generate a 735 bp cDNA encoding a 243 amino acid protein, whereas the *DserNEP4* gene contained a 59 bp intron to generate a 753 bp cDNA encoding a 250 amino acid protein. Signal peptides were predicted by using Signal P3 software (Bendtsen et al., 2004). This analysis suggested that *DserNEP* genes contain a typical signal peptide ranging from 18 to 22 amino acids (**Figure 1**).

Analysis of Amino Acid Sequences of *DserNEP* Proteins

According to sequence analysis, the DserNEP1 protein has an estimated isoelectric point (Ip) of 4.30 and a molecular mass of 25,384.99 Da; DserNEP2 has an estimated isoelectric point of 4.83 and a molecular mass of 25,647.41 Da; DserNEP3 protein has an estimated isoelectric point (Ip) of 5.59 and a molecular mass of 26,434.14 Da whereas DserNEP4 protein has an estimated isoelectric point (Ip) of 7.12 and a molecular mass of 27,482.45 Da.

D. seriata NEP proteins contain two conserved cysteine residues at positions 69 and 96 in DserNEP1, positions 64 and 90 in DserNEP2, positions 66 and 92 in DserNEP3, and positions 70 and 97 in DserNEP4. All of them also possessed the characteristic GHRHDWE central hepta-peptide motif starting at amino acid positions 132, 129, 130, and 136, respectively (**Figure 1**).

The publication of the *Diaporthe ampelina, D. seriata*, and *Phaeomoniella chlamydospora* genomes (Morales-Cruz et al., 2015), and the draft sequenced from *Eutypa lata* (Blanco-Ulate et al., 2013a), *N. parvum* (Blanco-Ulate et al., 2013b), and *Phaeoacremonium minimum* (Blanco-Ulate et al., 2013c) as well as the availability of the sequences posted at Joint Genome Institute (https://jgi.doe.gov/) revealed that there are no NLPs homologous in *P. chlamydospora* genome. *P. minimum* has two NLPs homologous (ID 1413 and 3642). *N. parvum* genome contains six NLPs homologous (ID 727, 928, 2549, 6217, 6314, and 7612). *E. lata* genome contains four NLPs homologous (ID 1995, 4041, 5290, and 8324) and *D. ampelina* has five NLPs homologous (ID 1057, 1426, 6910, 7604, and 9588), although some of them are truncated proteins.

A comparison of the amino acid sequences from NLPs of GTD pathogens was carried out (**Figure 2**). This analysis revealed that DserNEP1 exhibited a 100% sequence identity with a putative NPP1-domain type protein of *D. seriata* (KKY26562), 90.95% with a necrosis inducing protein from *Diplodia corticola* (XP_020133422), and 79.54% with a putative NPP1-domain type protein from *N. parvum* (EOD44475). DserNEP2 exhibited a sequence identity of 99.59% with a putative NPP1-domain type protein of *D. seriata* (KKY20654), 79.15% with a npp1 domain

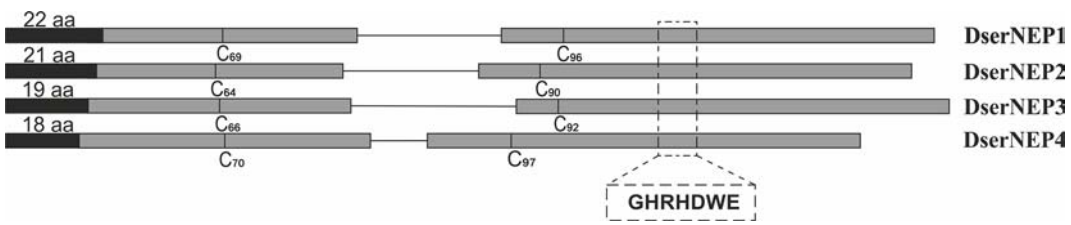

FIGURE 1 | Diagram shows the overall organization of *DserNEP* genes. The E1–E2 exons are represented in dark gray boxes. Both are separated by an intron (single line). The position (relative to the initial methionine residue) of the two conserved cysteine residues typical of type I NLP proteins is indicated. Signal peptides are indicated by black boxes. The relative location of the central hepta-peptide motif typical of NLP proteins in E2 is indicated (italics).

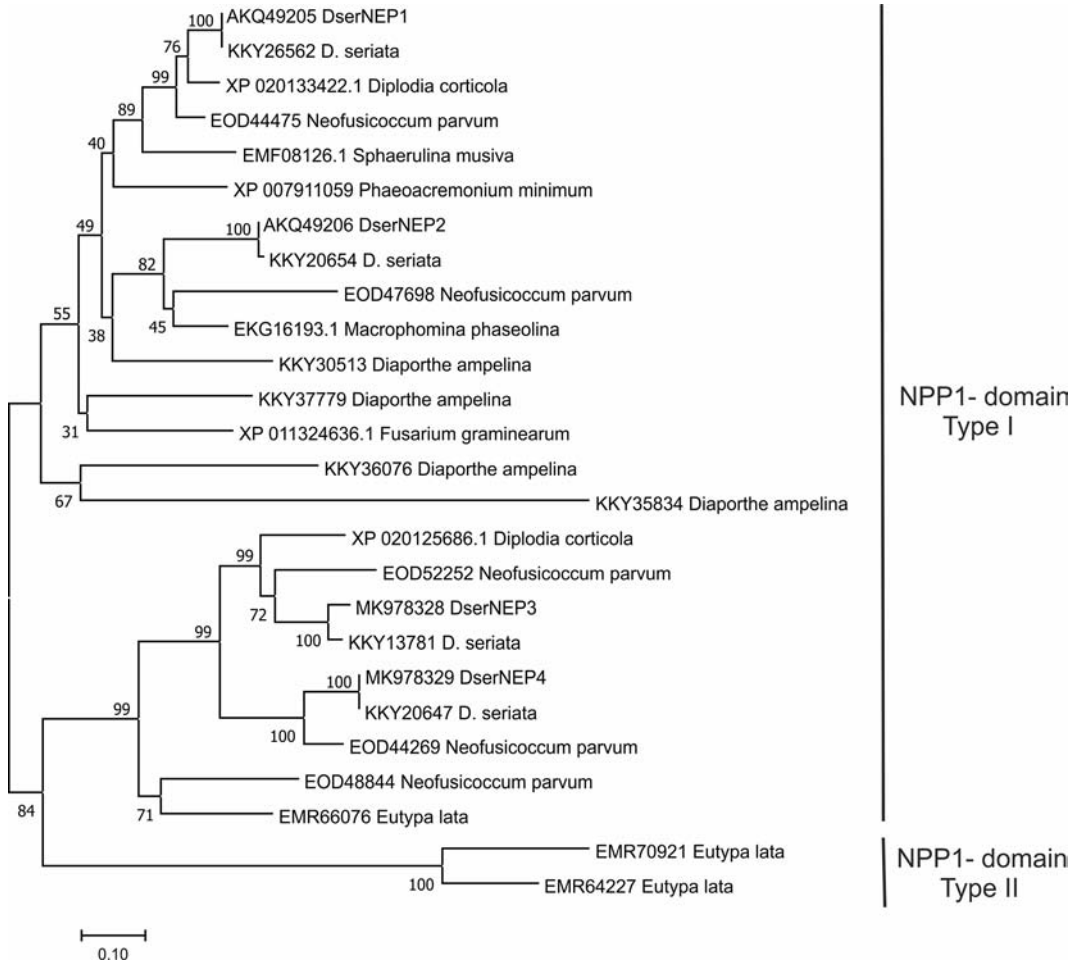

FIGURE 2 | Phylogenetic tree of *D. seriata* NEP proteins with necrosis and ethylene inducing peptide 1-like proteins from GTD-related fungi. The figure shows the result of a Neighbor analysis performed using the MEGA 5.0 package (Tamura et al., 2011). Percent bootstrap values (1,000 replicates) are shown above the forks. The scale bar represents 10% weighted sequence divergence.

protein (XP_020127860), and 76.13% with a hypothetical protein MPH_06630 from *Macrophomina phaseolina* (EKG16193.1), another Botryosphaeriaceae fungus, and 61.45% with a putative NPP1-domain type protein from *N. parvum* (EOD44475).

DserNEP3 exhibited a sequence identity of 100% with the hypothetical protein BK809_0004431 from *D. seriata* (OMP83050), 77.93% with a putative necrosis and ethylene inducing protein 1 precursor protein from *N. parvum* (EOD52252), and 75.00% with a necrosis and ethylene inducing peptide 1 from *D. corticola* (XP_020125686).

DserNEP4 exhibited a sequence identity of 100% with putative npp1 domain protein from *D. seriata* (KKY20647), 78.09% with a putative necrosis and ethylene inducing peptide 1 precursor protein from *N. parvum* (EOD44269), and 61.68% with necrosis and ethylene inducing peptide from *D. corticola* (XP_020125686).

Analysis of DserNLP Expression

Expression levels of genes encoding DserNEPs in *D. seriata* VS1 strain were analyzed in Czapeck liquid medium and compared to the expression levels observed in the same medium supplemented with chips of grapevine wood from Tempranillo cultivar to mimic a putative inductor effect carried out by some component present in the wood of grapevine plants. The obtained results showed that only *DserNEP1* and *DserNEP3* genes were upregulated in the assayed conditions *DserNEP1* was induced at 48 and 72 hours post inoculation, whereas *DserNEP3* gene was induced at 72 hours post inoculation. On the contrary, *DserNEP2* and *DserNEP4* genes were downregulated in the assay conditions (**Figure 3**).

Cytotoxic and Necrotic Activity of *D. seriata* NLPs

DserNEP1 and DserNEP2 were selected for further studies based on the fact that they had been the only two NEP proteins detected in the secretome of *D. seriata* in a previous work (Cobos et al., 2010). Genes encoding DserNEP1 and DserNEP2 proteins were cloned into the pETSUMO expression vector and expressed in *E. coli* BL21(DE3). Purified protein yields were 100 μg/mL for DserNEP1 and 500 μg/mL for DserNEP2

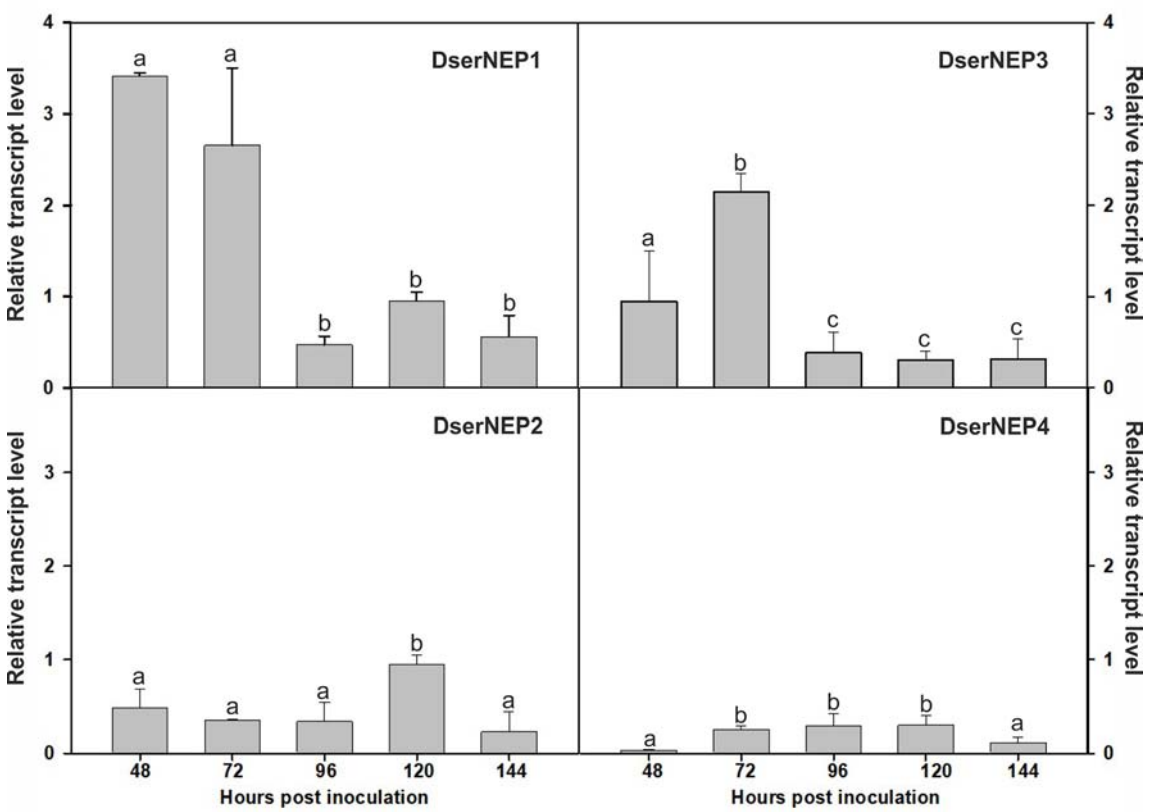

FIGURE 3 | Relative transcript levels of DserNEPs. The relative transcript levels of four *DserNEP* genes were determined by q-RTPCR using β-tubulin as reference gene. Samples were recovered at 48, 72, 96, 120, and 144 hours post inoculation. Data shown represent the mean ± SD from three independent experiments. The genes analyzed were considered significantly up- or downregulated when changes in their expression were > 2-fold or < 0.5-fold, respectively. Bars marked with the same letter do not differ at P = 0.05

under the tested assay conditions. The putative phytotoxicity of NEP proteins was first determined by dipping *in vitro* micropropagated *V. vinifera* plants into NEP suspensions. We tested different protein concentrations between 0 and 0.5 mg/mL. Necrosis symptoms were clearly visible 3 days after inoculation at 0.25 and 0.5 mg/mL (**Figure 4**). At lower concentrations (0.05 and 0.1 mg/mL), only the plants inoculated with DserNEP1 showed some symptoms of necrosis. The effect caused by DserNEP2 was less than that produced by DserNEP1. In fact, a DserNEP2 concentration of at least five times higher was required for detecting some necrotic activity as compared to the effect produced by DserNEP1. The necrotic symptoms first appeared on the leaf margin and then progressed through the center of the leaves. No symptoms were evident at either the lowest protein concentration tested or in the controls.

The quantification of the necrotic activity was carried out by infiltration of NEP proteins into *in vitro* micropropagated *V. vinifera* plants, followed by a typical electrolyte leakage assay. We tested the same protein concentrations that we had previously used in the immersion experiments (**Figure 5A**). The conductivity data obtained for inoculated leaves was indicative of some degree of cell permeability. DserNEP1 cytolytic activity was stronger than that detected for DserNEP2; this was also the case at lower concentrations. Cell viability was tested using the FDA assay. *V. vinifera* cell suspensions were exposed to purified DserNEP1 (0.15 mg/mL) and DserNEP2 (0.60 mg/mL) proteins. Five days after inoculation, nearly all cells in the control suspensions were alive whereas cell numbers in suspensions treated with the DserNEP1 protein were slightly reduced. Up to four times higher DserNEP2 concentrations were needed for a similar response to be observed, as compared with DserNEP1 (**Figure 5B**).

In order to test the effect of these proteins on adult plants, leaves of 1-year-old Chardonnay *V. vinifera* plants were inoculated with 0.15 and 0.30 mg/mL of DserNEP1 and DserNEP2 proteins by leaf infiltration. The necrotic symptoms were clearly observed after 2 days of infiltration (**Figure 6A**). The necrotic symptoms produced by DserNEP1 were quite similar at both concentrations, and the effect was greater than the effect produced by DserNEP2 at 0.15 mg/mL (**Figure 6B**). The highest necrotic effect was produced by DserNEP2 at 0.30 mg/mL and both proteins produced larger lesions than the control (wound inoculated with buffer). Although visually the necrotic activity of DserNEP1 seemed to be slightly higher, no significant differences between the two proteins were detected by performing an electrolyte leakage assay (**Figure 6B**).

Four different grapevine cultivars were assayed in order to test their susceptibility: Chardonnay, Cabernet Sauvignon,

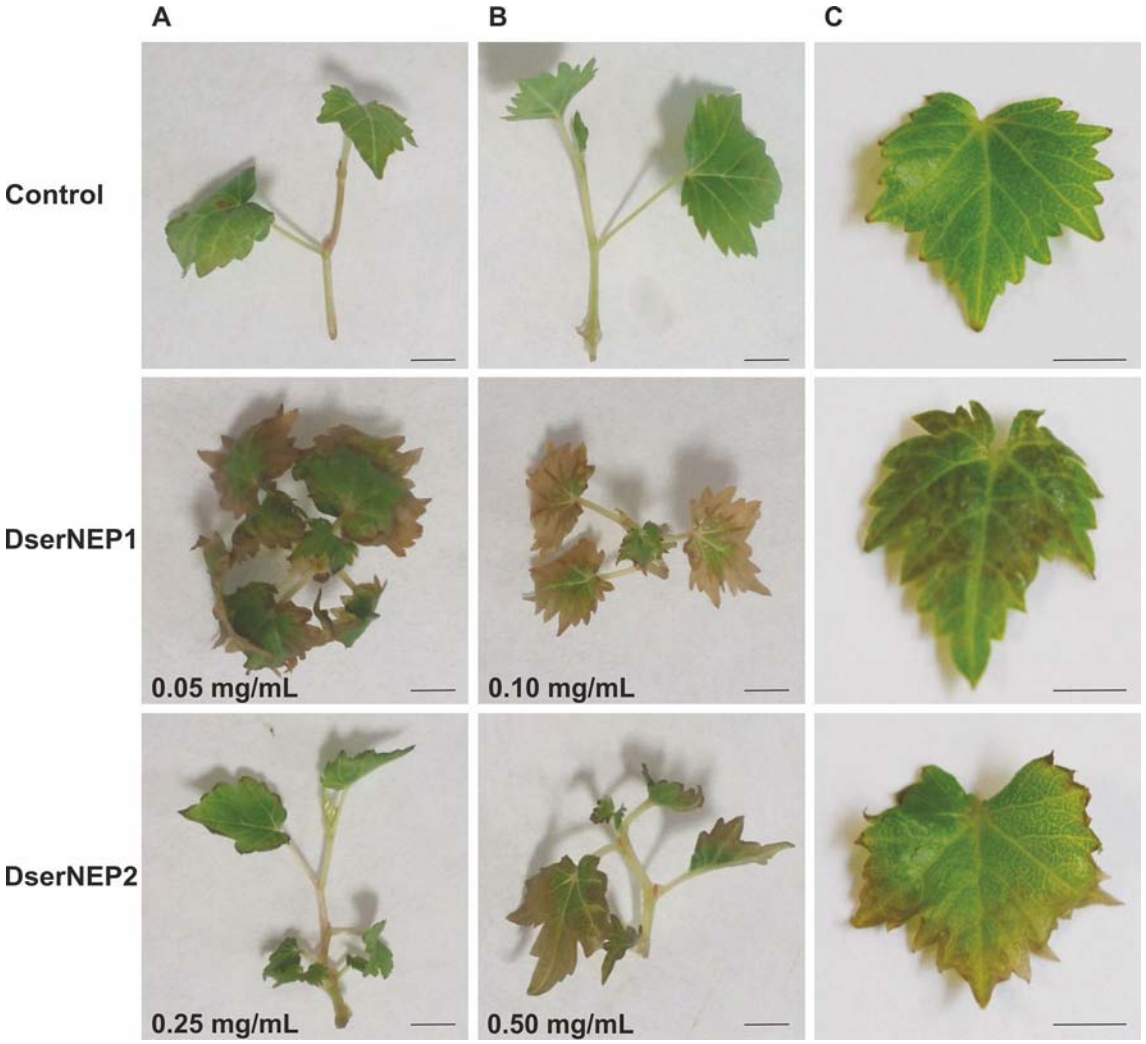

FIGURE 4 | Foliar symptoms caused by DserNEP proteins. Micropropagules of *V. vinifera* were inoculated by dipping into 100 µl of different concentrations of DserNEP1 **(A)**, DserNEP2 **(B)**, or buffer **(C)** as negative control. Pictures were taken 4 days after inoculations. The experiment was repeated three times, always using three independent samples for each treatment and three negative controls (dipped into buffer). The scale bar represents 1 cm.

Tempranillo, and Sauvignon Blanc. Three plants from each cultivar and three leaves from each plant were infiltrated with DserNEP1 at 0.15 and 0.30 mg/mL, and the conductivity of the necrotic tissues was measured 4 days after infiltration (**Figure 7**). As expected, the effect caused by DserNEP1 was different depending on the cultivar tested. The largest damage was produced by DserNEP1 at 0.30 mg/mL in Cabernet Sauvignon cultivar while the smallest was achieved in the Chardonnay cultivar. Again no significant differences were found in the susceptibility of this cultivar to the two protein concentrations tested.

DISCUSSION

In a previous work, we had detected three hypothetical proteins in the *D. seriata* secretome with significant similarity to necrosis and ethylene inducing proteins from *Nectria haematococca* and *Sclerotinia sclerotiorum* (Cobos et al., 2010). Peptides were identified by Mascot and used to design degenerated primers in order to amplify an internal fragment of *DserNEP* genes. Two different genes were amplified from *D. seriata* encoding proteins with high sequence similarity to NLPs, DserNEP1, and DserNEP2. The progression of the *D. seriata* genome sequencing suggests the putative existence of a small family of related genes in the genome of *D. seriata* since four homologous sequences could be detected (GenBank accession numbers KKY26562, KKY20654, KKY13781, and KKY20647). Similar results had been reported for other phytopathogenic fungi such as *Botrytis* species, which have two NLPs (Staats et al., 2007b; Schouten et al., 2008); *Moniliophthora perniciosa* with three NLPs (García et al., 2007), or *Verticillium dahliae* with up to nine NLP genes (Zhou et al., 2012).

The presence of a signal peptide in their sequences is in accordance with the extracellular location of the mature proteins

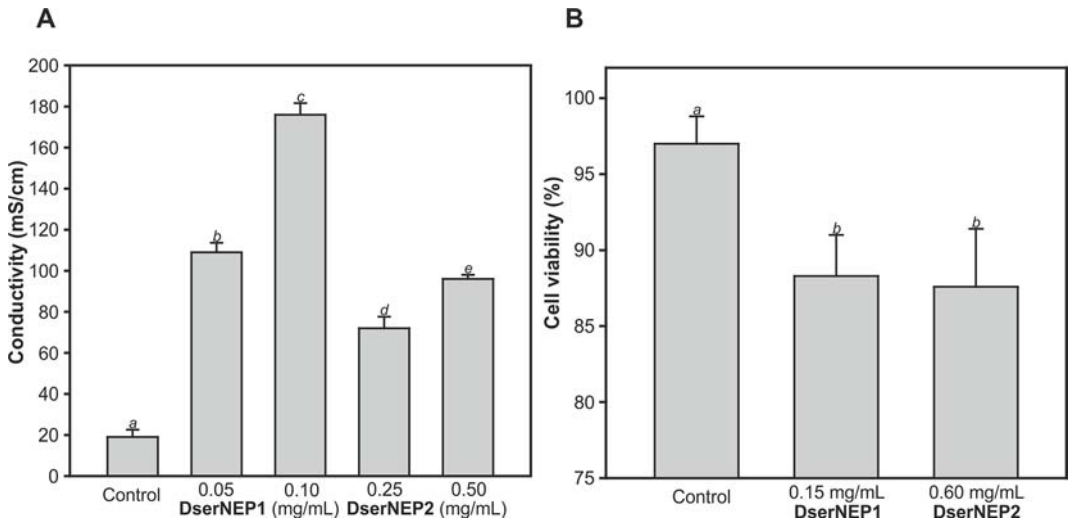

FIGURE 5 | Quantification of necrotic activity by electrolyte leakage assay induced by DserNEP proteins in micropropagated plants **(A)**, and viability of *V. vinifera* cell cultures treated with DserNEP proteins determined 5 days after protein exposition by staining with FDA **(B)**. DserNEP1 was assayed at 0.05 and 0.1 mg/mL, DserNEP2 at 0.25 and 0.5 mg/mL, and control leaves were inoculated with water. Data shown represent the mean ± SD from three independent experiments. Bars marked with the same letter do not differ at P = 0.05.

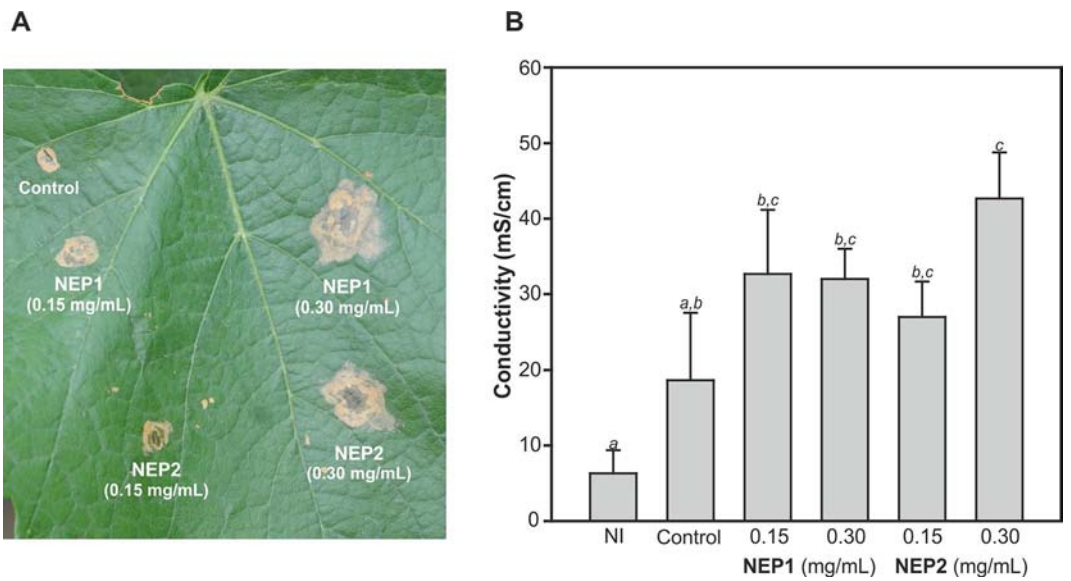

FIGURE 6 | Plant necrosis promoting activity of DserNEP proteins after infiltration of leaves of 1-year-old plants. Lesion formation 4 days after infiltration is shown **(A)**. Quantification of the necrotic activity by an electrolyte leakage assay induced by DserNEP proteins. Data shown represent the mean ± SD from three independent experiments. Bars marked with the same letter do not differ at P = 0.05. Control leaves were inoculated with water. Non-inoculated leaves are marked as NI **(B).**

previously reported (Cobos et al., 2010). The conserved NPP1 domain is typical for NLP proteins (PFAM domain PF05630) (Fellbrich et al., 2002). NLPs are classified into two groups, type I and type II, depending on the presence of two or four cysteine residues at conserved positions, respectively (Gijzen and Nurnberger, 2006). Accordingly, DserNEP proteins would belong to the type I group. DserNEP proteins also possessed the characteristic GHRHDWE central hepta-peptide motif (Pemberton and Salmond, 2004). This motif is part of a negatively charged cavity exposed at the protein surface that is supposed to be important for the biological activity of NEP proteins (Ottmann et al., 2009).

The publication of the *Diaporthe ampelina*, *D. seriata*, and *Phaeomoniella chlamydospora* genomes (Morales-Cruz et al., 2015), and the draft sequenced from *Eutypa lata* (Blanco-Ulate et al., 2013a), *N. parvum* (Blanco-Ulate et al., 2013b),

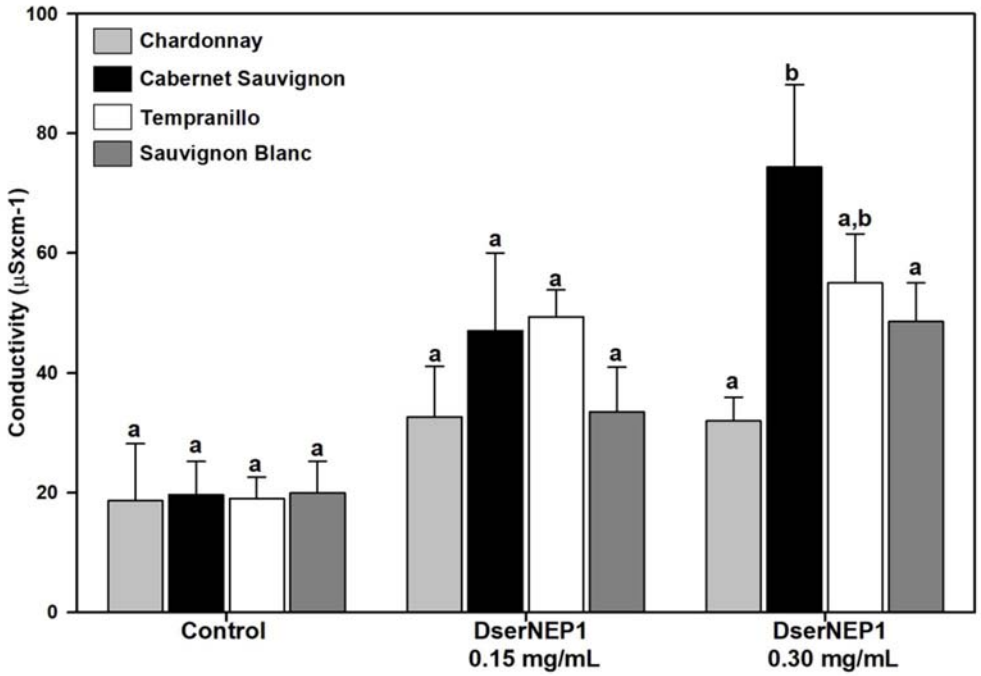

FIGURE 7 | Grapevine cultivar susceptibility was tested by leaf infiltration with DserNEP1 protein. Grapevine leaves from four different cultivars (Chardonnay, Cabernet Sauvignon, Tempranillo, and Sauvignon Blanc) were inoculated with DserNEP1 at 0.15 or 0.30 mg/mL. Control leaves were inoculated with water. Electrolyte leakage was measured for 4 days after infiltration. Data shown represent the mean ± SD from three independent experiments. Bars marked with the same letter do not differ at P = 0.05.

and *Phaeoacremonium minimum* (Blanco-Ulate et al., 2013c) have revealed that there are several NLP homologues in each fungal species, except in case of *P. chlamydospora*. Some of the detected sequences have an incomplete conserved hepta-peptide GHRHDWE motif, and sometimes they lack the upstream amino acid sequence, including the conserved cysteines, and the occurrence of premature stop codons suggests that some of these sequences could be pseudogenes (Gijzen and Nurnberger, 2006; Staats et al., 2007b).

The analysis of the amino acid sequence of DserNEPs allowed their location into two different branches of a putative phylogenetic tree, corresponding to type I (two conserved cysteines) and type II NLPs (four conserved cysteines). NLPs from GTDs pathogens belong mainly to type I group since solely *E. lata* contains NLPs from type I and type II groups. However, this analysis did not reflect the phylogenetic relationship among the fungal species checked, as it was unable to discriminate between different fungal classes. This could be an indication of an intense horizontal gene transfer between species as has been suggested by other authors (Gijzen and Nurnberger, 2006; Zaparoli et al., 2009). This hypothesis could explain the differences in G+C content observed between genes encoding DserNEP proteins and the *D. seriata* genome. While the *D. seriata* genome has a G+C content of 56.7%, this percentage is increased up to 60% in *DserNEP*1, 63% in *DserNEP*2, 64% in *DserNEP*3, and 60% in *DserNEP*4 genes, suggesting that these sequences could have been recently acquired by this microorganism and their maintenance could confer some evolutionary advantage (Pemberton and Salmond, 2004; García et al., 2007).

Little is known about how NLPs cause necrosis in plant cells, but several authors indicate that they may play as elicitors by manipulating cell death programs of the host (Fellbrich et al., 2002; Zhou et al., 2012) or acting like phytotoxins (Dong et al., 2012). The detection of DserNEPs during the fungal growth, as well as the overexpression of DserNEP1 and DserNEP3 in the presence of the wood chips, suggests a putative role of DserNEPs as virulence factors. Interestingly, in a previous work Cobos and colleagues (2010) detected that DserNEP1 and DserNEP2 proteins were upregulated in the presence of carboxymethylcellulose. These differences in the expression pattern of *D. seriata* NEP proteins could be due to the different experimental strategy used. Composition of trunk chips is much more complex than pure carboxymethylcellulose. We can speculate with the presence in trunk chips of compounds with an inducing effect on *NEP* gene expression, but we cannot rule out the presence of compounds with the opposite effect. The upregulation of NLP gene expression during infection has been described in many other plant pathogens like *Botrytis cinerea* (Arenas et al., 2010), *Magnaporthe oryzae* (Fang et al., 2017), or *Verticillium dahliae* (Zhou et al., 2012).

The necrotic activity of DserNEP1 and DserNEP2 proteins has been demonstrated. However, the differences in their toxicity could indicate differences in their mechanism of action. Both proteins can produce leaf injuries in both *in vitro* propagated and adult plants, although the effect of DserNEP1 was stronger than

that detected for DserNEP2. These results are in concordance with the data of NEP activity reported by Staats et al. (2007a) in *Botrytis elliptica*. The observed differences in the results obtained from *in vitro* plants of Tempranillo cultivar and those from Chardonnay 1-year-old potted plants suggest differences in cultivar susceptibility. These different susceptibilities could be related to the specific morphological characteristics or defense mechanisms of each cultivar. Moreover, the progress of foliar symptoms was quite similar to that observed in grapevines under field conditions. This fact deserves to be highlighted.

Taken together, these results suggest a putative role of DserNEPs in pathogenesis, especially in development of the leaf symptoms observed in *D. seriata* infected grapevines. Both proteins exhibited necrotic activity although DserNEP1 produced larger lesions than DserNEP2. However, little is known about the molecular mechanisms which produce the injuries detected. *D. seriata* is a vascular pathogen, but to our knowledge it has never been isolated from grapevine leaves, suggesting that DserNEP proteins may be able to reach leaves by some unknown mechanism. The increase of conductivity detected in infiltrated leaves suggests that NLPs induce plasma membrane disruption in the host. This could drive the release of host-derived molecules that would trigger damage *via* pathogen associated molecular patterns. Other authors suggest that NLPs could interact with targets in the plasma membrane. Indeed, Schouten et al. (2008) demonstrated that NLPs are associated with membranes and are accumulated in the cytosol, nuclear membrane, and the nucleolus. They proposed that NLPs might bind to, or associate with, specific plant lectins resulting in a loss of membrane integrity, possibly through the pore-forming activity of NLPs. Intracellular accumulation of NLPs could explain the high toxicity of these proteins which act as toxins, blocking transcription, interfering with chloroplast function, or inducing programmed cell death (Bae et al., 2006).

This is the first record of Nep1-like proteins from a fungus associated with GTDs and also from a member of the Botryosphaeriaceae family. Advances made in sequencing genomes of fungi associated with GTDs have revealed the presence of NLPs in most of them. Accordingly, it might be necessary to assay the role of these proteins in the development of GTDs, and particularly in the development of foliar symptoms. Further studies of all the DserNEPs present in the *D. seriata* genome and gene replacement assays might help to elucidate their mode of action and their role in plant–pathogen interactions. Unfortunately, the difficulty in developing an efficient method for genetic transformation of *D. seriata* is hampering these studies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Genbank: AKQ49205, AKQ49206, MK978328, MK978329.

AUTHOR CONTRIBUTIONS

RC and JC analyzed the data, interpreted the results, conceived and designed the experiments, and contributed materials, equipment, and analysis tools. PG-A and RC developed the grapevine *in vitro* cultures and cellular callus production. RC, CC, JÁ-P, AD-G, AI, and SG-G conducted the experiments. RC, JC, and JA wrote the manuscript. All authors reviewed the manuscript and approved the final version.

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Fungicides and the Grapevine Wood Mycobiome: A Case Study on Tracheomycotic Ascomycete *Phaeomoniella chlamydospora* Reveals Potential for Two Novel Control Strategies

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Phaeomoniella chlamydospora is a tracheomycotic fungus that colonizes the xylem of grapevines (*Vitis vinifera* L.), causing wood discoloration, brown wood streaking, gummosis, and wood necrosis, which negatively affect the overall health, productivity, and life span of vines. Current control strategies to prevent or cope with *P. chlamydospora* infections are frequently ineffective. Moreover, it is unclear how fungicides commonly applied in vineyards against downy and powdery mildew agents affect the wood mycobiome, including wood pathogens such as *P. chlamydospora*. In this study, we used next-generation sequencing to assess the effects of foliar spray of grapevines with inorganic (copper oxychloride and sulfur), synthetic (penconazole and fosetyl-aluminum), and natural (Blad) fungicides currently used against the downy and powdery mildews. The subjects of our investigation were (i) the resident wood mycobiome, (ii) the early colonization by a consortium of fungal wood endophytes (ACEA1), (iii) the wood colonization success of *P. chlamydospora*, and (iv) the *in planta* interaction between *P. chlamydospora* and ACEA1, under greenhouse conditions, in rooted grapevine cuttings of cv. Cabernet Sauvignon. The data obtained suggest that the resident mycobiome is affected by different fungicide treatments. In addition, the early colonization success of the endophytes composing ACEA1 varied in response to fungicides, with relative abundances of some taxa being overrepresented or underrepresented when compared with the control. The wood colonization by *P. chlamydospora* comported significant changes in the mycobiome composition, and in addition, it was greatly affected by the foliar spray with Blad, which decreased the relative abundance of this pathogen 12-fold (4.9%) when compared with the control (60.7%) and other treatments. The presence of the pathogen also decreased considerably when co-inoculated into the plant with ACEA1, reaching relative abundances between 13.9% and 2.0%, depending on the fungicide treatment applied. This study shows that fungicides sprayed to prevent infections of powdery and downy mildews have an effect on non-target fungi that colonize the endosphere of grapevines. We suggest two potential

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control strategies to fight *P. chlamydospora*, namely, the foliar spray with Blad and the use of ACEA1. Further studies to confirm these results are required.

Keywords: fungicides, *Phaeomoniella chlamydospora*, grapevine trunk diseases, mycobiome, microbial ecology

INTRODUCTION

Protecting grapevines (*Vitis vinifera* L.) from fungal pests by means of fungicides dates back to the late 1800s, with the accidental discovery that a mixture of copper sulfate and lime, sprayed on vine leaves, was not only able to deter thieves from stealing grapes but also able to prevent downy mildew infections (Morton and Staub, 2008). Since then, research on fungicides improved the use of copper and sulfur as contact fungicides and introduced additional synthetic broad-spectrum systemic fungicides (e.g., benzimidazoles, triazoles, fludioxonil, and organophosphorus compounds) during the mid-late 1900s (Morton and Staub, 2008). Overuse of fungicides in vineyards came at a cost. Several were shown to be phytotoxic to grapevines (Dias, 2012; Juang et al., 2012) and toxic to humans and the environment (Komárek et al., 2010), and some affected non-target organisms (La Torre et al., 2018), while others hampered beneficial insects (Thomson et al., 2000). In addition, the development of pathogen resistance pushed the search of ever more effective chemicals capable of dealing with increasingly resilient species (Verweij et al., 2009).

It was only in recent years, with the advent of molecular ecology and its tools, such as metagenomics and metabarcoding, that scientists have begun to understand how fungicides, along with herbicides and insecticides, impact the microbial ecology of environments with unpredictable medium- and long-term consequences (Komárek et al., 2010; Setati et al., 2012; Karlsson et al., 2014; Morrison-Whittle et al., 2017). The vineyard is an extremely diverse environment, characterized by complex interactions among plants, soil microbes, endophytes, and epiphytes, which are associated not only with grapevine health (Zarraonaindia and Gilbert, 2015; Jayawardena et al., 2018) but also with the concept of *terroir*, as it is a source of yeasts and bacteria especially important in winemaking (Pretorius et al., 1999; Setati et al., 2012).

Concerning plant health, grapevine trunk diseases (GTDs) are currently a major issue in viticulture, considered by some as "the new Phylloxera" due to their potential destructive power (Bruez et al., 2013). Early scientific reports on GTDs are over a century old (Surico, 2009), and the reasons underlying their recent worldwide outbreak have not yet been fully addressed. Unlike powdery and downy mildews agents (*Erysiphe necator* and *Plasmopara viticola*) and Phylloxera (*Daktulosphaira vitifoliae*), which were introduced from the Americas and found a susceptible host in *V. vinifera* (Jackson, 2014), GTD pathogens have presumably been co-evolving with grapevines for centuries, if not millennia (Mugnai et al., 1999).

Copper and sulfur fungicides were introduced approximately two decades before the first scientific reports on GTDs and the recent outbreak began some 30 years ago (Surico et al., 2004; Fontaine et al., 2016), approximately two decades after the massive introduction of synthetic broad-spectrum systemic fungicides in vineyards. To date, no study has investigated the effect of these fungicides, and that of other natural active ingredients, on the wood mycobiome and its possible implications in GTDs. To address this issue, we used metabarcoding (Illumina® next-generation sequencing, NGS) and tested four hypotheses. (1) Are fungicides commonly sprayed in vineyards, to fight powdery and downy mildews, capable of affecting the resident wood mycobiome and/or (2) an inoculated consortium of fungal wood endophytes? (3) Is *Phaeomoniella chlamydospora*, a wood pathogen associated with several syndromes in grapevine (Surico 2009), affected by such fungicides, when artificially inoculated in wood? (4) Is the fungal consortium inoculation potentially exploitable in the control of early infections by *P. chlamydospora*, independently of fungicide treatments?

MATERIALS AND METHODS

Experimental Setup

Plant and Fungal Material

The grapevines used in this assay were 1-year-old canes of cv. Cabernet Sauvignon, provided by the Viveiros VitiOeste nursery (Pó, Portugal), that were cut at three-bud-long size. The phytosanitary status of the propagation material was assessed by ensuring the absence of leaf symptoms and any visible wood symptomatology. The cuttings were rooted, potted in a mixture of peat and sand (1:1 v/v), and maintained under greenhouse conditions, at the temperature of 24 ± 5°C day/18 ± 5°C night.

The pathogenic ascomycete tested in this study was *P. chlamydospora* (CBS 161.90), from the CBS culture collection (Westerdijk Fungal Biodiversity Institute, Netherlands). Four grapevine endophytes, isolated from asymptomatic grapevine wood (cv. Cabernet Sauvignon), were used to produce the fungal consortium named "ACEA1," namely, *Alternaria* (*Al.*) *alternata* A101, *Epicoccum nigrum* E279, *Cladosporium* sp. C22, *Aureobasidium* (*Au.*) *pullulans* AU86. All fungi were maintained in Petri dishes with vents, on potato dextrose agar medium (Difco™), at 25°C, in the dark.

Inoculum Preparation and Delivery

Four different inocula were prepared for delivery into the plant wood, three of them consisting of a suspension of conidia and/or fungal cells and the fourth of a control. Inoculum (i) consisted of a conidia suspension of *P. chlamydospora*, which was prepared by flooding a 2-week-old colony. The conidia were dislodged from the mycelium with a sterile glass rod, and the suspension was filtered through a double layer of cheesecloth. The conidial concentration was determined using a hemocytometer and

adjusted to 1×10^5 conidia per milliliter with sterile distilled water (SDW). Inoculum (ii) consisted of ACEA1, which was prepared by joining conidia suspensions of ascomycetes *Al. alternata*, *E. nigrum*, *Cladosporium* sp., and cells of yeast *Au. pullulans*. Cultures growth conditions and conidia/cell suspension preparation occurred similarly to inoculum (i), where the final concentration of each one of the four fungi was 1×10^5 (conidia/cells) per milliliter. Inoculum (iii) was prepared by joining conidia suspensions of *P. chlamydospora* and ACEA1, in order to produce a solution with a final concentration of 1×10^5 (conidia/cells) per milliliter of each one of the five fungi. Inoculum (iv) consisted of SDW.

The delivery of the inocula into the rooted plants was performed as follows. Grapevine cuttings were surface disinfected with 70% (v/v) ethanol, and a wound in the wood was produced with the aid of a cork borer (4 mm diameter, 4 mm deep), approximately 1 cm below the green shoot (**Figure 1**). The four inocula were delivered with a micropipette by depositing 50 µl of solution in freshly made wounds, which were immediately sealed with Parafilm®. Each inoculum was delivered in 32 plants (**Figure 1**).

Treatments *via* Foliar Spray

Four treatments were tested for each inoculum type. The chemicals used and their application rate are reported in **Table 1**. The spray occurred every fortnight, starting from the seventh day post-inoculation and consisting in a total of six applications per treatment. Plants were sprayed on leaves and stem, with the exception of the control, which was sprayed exclusively on leaves. The chemicals were prevented from dripping onto the soil. All possible combinations of fungal inoculum and foliar treatment were tested, for a total of 16 combinations, each one consisting of eight biological replicates (**Figure 1**). The assay ended 1 week after the last spray (99 days post-inoculation).

Potassium permanganate ($KMnO_4$) was chosen as control treatment due to the need of dealing with natural infections of powdery and downy mildews, which normally occur under greenhouse conditions. This chemical is effective against these pathogens due to its strong oxidative properties (La Torre et al., 2004), and we used the assumption that, when sprayed exclusively on leaves, it would not interact in a significant way with the wood mycobiome. Concerning treatments "Copper-Sulfur" and "Systemic fungicides," the two active ingredients of each treatment were sprayed approximately 3 h apart from one another.

Assessment of the Plant Health Status

Three parameters were evaluated to assess the health status of the plants at the moment of sampling. First, a visual inspection of the leaves and green shoots to establish whether any symptomatology related to the pathogen or ACEA1 inoculation manifested ($n = 8$ for each combination inoculum/treatment). Second, the shoot length was recorded, to understand if inoculated fungi and/or treatments had an effect on the growth of the plants ($n = 8$

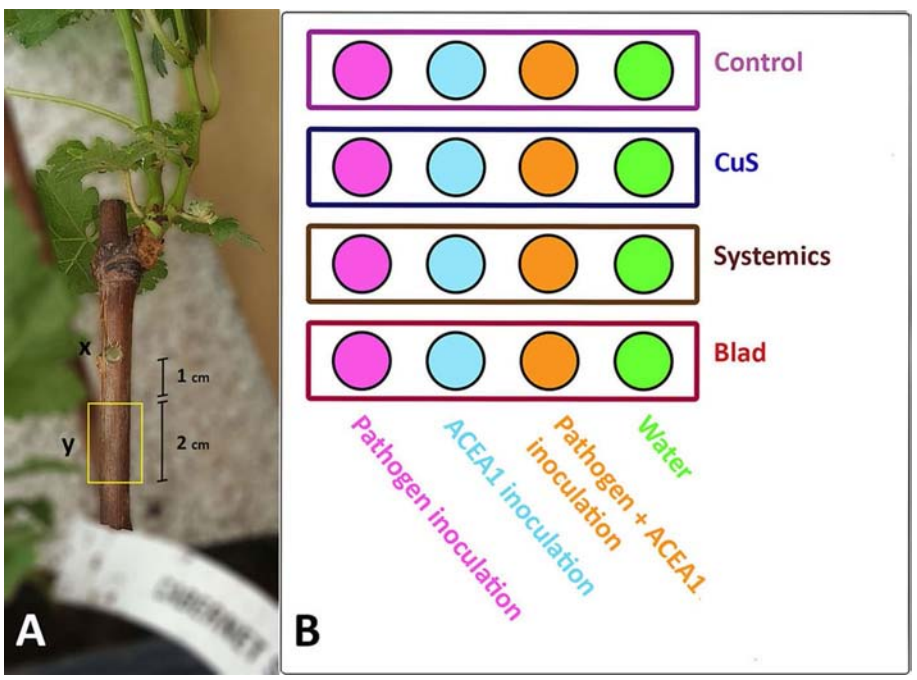

FIGURE 1 | (A) Grapevine rooted cutting of cv. Cabernet Sauvignon. (*x*) Inoculation point of a conidia suspension of *Phaeomoniella chlamydospora*, ACEA1, *P. chlamydospora* and ACEA1, or sterile distilled water. (*y*) Two centimeters of wood, located 1 cm below the inoculation point, was sampled 3 months post-inoculation, and the wood's mycobiome was examined through metabarcoding ($n = 5$ for each combination inoculum/treatment). **(B)** Experimental setup. Each circle represents a set of eight plants that underwent the same treatment. Same colors in the circles represent the same inoculum type, while rectangles represent different treatments. All possible combinations of inoculum and fungicide application by foliar spray are shown.

TABLE 1 | Chemical treatments sprayed fortnightly on grapevine rooted cuttings. Six applications were performed during 3 months. Each active ingredient was sprayed separately.

Treatment	Active ingredient	Trade name	Manufacturer	Formulation	Tested concentration
Control	Potassium permanganate[a]	Permanganato de potássio basi	Laboratórios Basi	Capsules 500 mg	1.0 g/L
Copper–sulfur	Copper oxychloride[b]	Cuprocol®	Syngenta	Copper oxychloride 36.5%	2.5 ml/L
	Sulfur[c]	Microthiol Special Disperss	Epagro	Sulfur wettable powder 80%	6.0 g/L
Systemic fungicides	Fosetyl-aluminum[b]	Aliette Flash®	Bayer	Fosetyl-aluminum 80%	2.5 g/L
	Penconazole[c]	Topaze®	Syngenta	Penconazole 10.5%	35 ml/100 L
Blad	Blad-containing oligomer[a]	Fracture®†	CEV/CONVERDE	Blad 20% Other ingredients 80%	2.0 ml/L

[a]*Treatments that target simultaneously powdery and downy mildews pathogens.* [b]*Treatments that target downy mildew.* [c]*Treatments that target powdery mildew.* †*Supplier: FMC Corporation, Agricultural Products Group, 175 Market Street, Philadelphia, PA 19103, USA. EPA Reg. No. 84876-1-279.*

for each combination inoculum/treatment). Lastly, for each combination of inoculum/treatment, three vines were subject to a destructive inspection to assess the presence and degree of internal wood symptomatology (e.g., brown streaking) visible in the stem of plants and starting from the inoculation point. Three categories of symptomatology degree were established: Category 1, absence of wood symptoms; Category 2, presence of brown streaking in proximity of the inoculation point; and Category 3, presence of brown streaking that extended several centimeters from the inoculation point (**Figure 2**).

Wood Sampling, DNA Extraction, and Amplification

Grapevines were uprooted and the stem processed under laboratory conditions (n = 5 for each combination inoculum/treatment). The bark was sterilized by dipping the stem in 70% (v/v) ethanol followed by flame sterilization and then removed with the aid of a sterile scalpel. Exactly 2 cm of wood was sampled, 1 cm below the inoculation point (**Figure 1**). Samples were frozen, freeze-dried, and stored at −80°C.

Wood samples were ground to dust using sterile mortars and pestles, aided by liquid nitrogen. An aliquot of ground wood (0.25 ± 0.01 g) of each sample was added to DNA extraction columns (FastDNA™ SPIN Kit for Soil, MP Biomediacals® LLC), and total DNA was extracted as described by the kit manufacturer. Three negative controls of the DNA extraction (no template) were included in this step and underwent the DNA extraction procedure.

The amplicon chosen for the metabarcoding analysis targets the internal transcribed spacer ITS2 region, and the primer set selected was ITS86F and ITS4R (Op De Beeck et al., 2014).

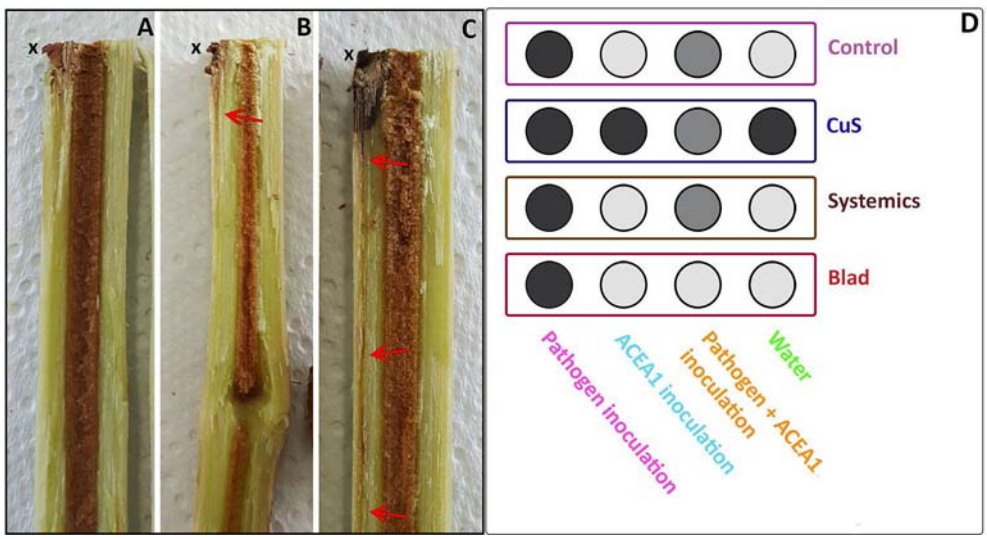

FIGURE 2 | Grapevine rooted cuttings were examined, revealing different degrees of brown wood streaking symptomatology. Category 1, absence of symptoms **(A)** departing from the inoculation point (x) downwards; Category 2, presence of mild symptomatology **(B)**; Category 3, extensive symptomatology **(C)**. **(D)** Each category is presented in a different scale of gray, as an average of the observations for each treatment/inoculum (n = 3), where Category 1 is shown in light gray, Category 2 in gray, and Category 3 in dark gray.

For building libraries, we used a double-step PCR approach as reported by Feld et al. (2016). The full primer sequences including Illumina overhangs are ITS86F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GTGAATCATCGAATCTTTGAA-3′) and ITS4R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-TCCTCCGCTTATTGATATGC-3′).

Every first PCR contained 12.5 µl of Supreme NZYTaq II 2x Green Master Mix™ (NZYtech™), 0.5 µl of forward and reverse primers from a 10 µM stock, 1.5 µl of sterile water, and 5 µl of template. Each reaction was pre-incubated at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 40 s; a further extension was performed at 72°C for 10 min. Negative controls of the PCRs were included alongside the samples. No-template DNA extracted samples also underwent the library preparation procedure.

A second PCR step for barcoding, MagBio bead fragment purification, and Qubit quantification were performed as reported in Gobbi et al. (2018). Final pooling was performed at 10 ng per sample. DNA sequencing was performed using an in-house Illumina MiSeq instrument and 2 × 250 paired-end reads with V2 Chemistry.

Bioinformatics

After sequencing, demultiplex was performed using an Illumina MiSeq platform, and the raw data were analyzed using QIIME 2 v. 2018.2 (Caporaso et al., 2010), using the same pipeline described in Gobbi et al. (2018); denoised reads were quality-trimmed by 15 bp on the left to remove adapters and primers by using the quality filter plugin implemented in QIIME 2 (Bokulich et al., 2013). Filtered reads were then analyzed using DADA2 with the amplicon sequence variant (ASV) methods (Callahan et al., 2017). To minimize barcoding noise, operational taxonomic unit (OTU) counts below 25 reads were filtered out across all samples. Taxonomic assignments were performed at 99% identity using QIIME feature-classifier classify-sklearn with a naïve Bayes classifier trained with UNITE (Nilsson et al., 2013) v. 7.2 for ITS. After taxonomy assignment, the dominant features assigned to high taxonomical ranks such as order, class, or family were further investigated using BLAST to refine the analyses (Bokulich et al., 2018). The raw data of this study are available in the European Nucleotide Archive (ENA accession number PRJEB32853).

Data Analysis

Shoot length data were subjected to analysis of variance (two-way ANOVA) to evaluate the effects of factors "fungicide treatment" and "inoculum type." Significant means were compared using the Tukey *post hoc* test at a 5% significance level (GraphPad Prism 7.05).

The frequency table and its taxonomy table were combined, converted to biom format in QIIME (Caporaso et al., 2010), then merged with a table of metadata into an S4 object, and analyzed in R (v. 3.4.3) using the following packages: phyloseq, v. 1.22.3 (McMurdie and Holmes, 2013); vegan, v. 2.5.2 (Oksanen et al., 2007); DeSeq2 v. 1.22.1 (Love et al., 2014); ggplot2, v. 3.0.0 (Wickham, 2016); metacoder, v. 0.2.1.9005 (Foster et al., 2017); adespatial, v. 0.1.1 (Dray et al., 2018); data.table, v. 1.10.4.3 (Dowle and Srinivasan, 2017). R code is publicly available at github.com/Marieag/EMG.

The alpha diversity was measured using the Shannon diversity index and Pielou's evenness and tested with one-way ANOVA with Tukey's honestly significant difference (HSD) *post hoc* to determine differences among treatments and inoculum types.

We analyzed the beta dispersion to measure between-sample variances in abundance, computing average distances of the individual samples. The resulting ordination was plotted using non-metric multidimensional scaling (NMDS) combined with a Jaccard index matrix. To assess overall inter-group variance, we performed a PERMANOVA, using a Jaccard distance matrix with 999 permutations. The *post hoc* test for the PERMANOVA (performed on the ASV counts) was done using the adonis function from the vegan package in R.

To investigate any systematic changes between treatments, we calculated the differential abundance of any taxon with a relative abundance (RA) greater than 0.1% using DESeq2. Twenty-four pairwise analyses were run for every combination of fungicide treatment per inoculum type; log2 fold changes were calculated for each taxon resolved to species or genus level and tested for significance using a Wald test on the negative binomial distribution of the pairwise treatments. The *P*-value threshold was set to 0.05, and log fold change threshold to 0.1. In order to illustrate the results and to factor in the RA of unresolved species, we created differential heat trees using MetacodeR. A Wilcoxon rank sum test was applied to test differences between the same species in different treatments, and the resulting *P*-values were corrected for multiple comparisons using FDR, as implemented in MetacodeR. *P*-value threshold was set to 0.05.

RESULTS

Evaluation of Plant Health Parameters

Visual inspection of grapevine cuttings, shoots length measurements, and wood examination occurred on the day of sampling. The visual inspection revealed no foliar symptomatology attributable to the wood pathogen *P. chlamydospora* or to the inoculated fungal consortium ACEA1.

The statistical analysis of the green shoot length measurements, performed as a two-way ANOVA, revealed no significant differences for the factor "inoculum type" ($P = 0.190$), meaning that shoot growth was not influenced by inoculation with the pathogen, ACEA1, or the combination of both. However, a positive effect was present when comparing the factor "fungicide treatment" ($P < 0.01$), revealing that Blad and/or systemic fungicides increased the shoot growth of inoculated plants (pathogen, ACEA1, and pathogen + ACEA1), when compared with one or both the two other treatments (**Figure 3**). No significant differences were detected from the interaction between the two factors under examination ($P = 0.136$).

The examination of the wood revealed different degrees of symptomatology, ranging from absence of symptoms to extensive brown streaking departing from the inoculation point, as shown

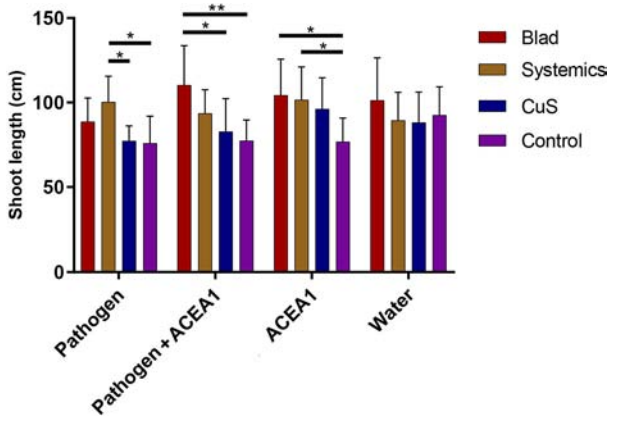

FIGURE 3 | Barplot showing the measurements of grapevine shoot length. Error bars represent the standard deviation. Grapevines were inoculated with water, *Phaeomoniella chlamydospora* (Pathogen), a consortium of wood endophytes (ACEA1), and a combination of both (Pathogen + ACEA1). Grapevines were also treated with a foliar spray of potassium permanganate (Control), copper oxychloride and sulfur (CuS), and fosetyl-Al and penconazole (Systemics), Blad. $n = 8$ for each combination inoculum/treatment. Statistical differences, according to Tukey's *post hoc* test, are shown by asterisk, where **P* 0.05 and ***P* 0.01.

in **Figure 2**. Plants inoculated with *P. chlamydospora* presented extensive wood discoloration (Category 3; **Figures 2C**, **D**), under all spray treatments, while mild to absent symptoms, such as those shown in **Figures 2A**, **B** were recorded when *P. chlamydospora* was inoculated along with ACEA1. Plants inoculated with only water or ACEA1 did not show brown wood streaking symptoms, with the exception of the copper–sulfur treatment, suggesting that either copper oxychloride or sulfur or the combination of both may contribute to this plant response, independently from the inoculation of the pathogen.

Sequencing Dataset Description

The dataset containing the NGS data produced and analyzed in this study accounts for 78 samples. These samples are represented by 6,454,075 high-quality reads distributed in 506 unique features. The average number of reads per sample is 82,744, which allowed an adequate sequencing depth to unravel the complexity of the grapevine cuttings wood mycobiome (alpha-rarefaction curves obtained with Shannon and observed OTUs are shown in **Figure S1**, results reported in **Table S2**). The raw ASV table (read counts) is available in **Table S3**.

Using BLAST to refine the taxonomic classification of the taxa composing ACEA1, taxon "Pleosporales incertae sedis" was assigned to *E. nigrum* in 98.12% of the reads. *Alternaria* sp. was retrieved in 16 sequence variants, with the dominant feature corresponding to 97.88% of the total. After blasting this dominant sequence, the assignment was refined to *Al. alternata*, with 100% identity. The genus *Cladosporium* sp. appears 12 times in our database for a total of 61,288 high-quality reads. Blasting the four dominant features, which cover 94.7% of the total amount of reads, they were all assigned to *Cladosporium* sp.

A Qualitative Overview of the Wood Mycobiome

The richness of fungal taxa, identified examining all grapevine cuttings ($n = 78$), amounts to 66 species or genera of both ascomycetes and basidiomycetes. Among these 66 taxa, 30 of them are represented in an RA greater than 0.1%, while the remaining 36 taxa are considered rare taxa (RA < 0.1%; **Tables 2** and **S1**). Among the 30 most abundant taxa, several genera of ascomycetes are known to be involved in GTDs (e.g., *Neofusicoccum*, *Diaporthe*, *Cadophora*, and *Fusarium*), while no known pathogenic basidiomycete was detected (**Table 2**). The most frequent (non-inoculated) taxa are *Debaryomyces* sp., *Cryptococcus* sp., *Malassezia restricta*, *Malassezia globosa*, *Diaporthe* sp., *Acremonium alternatum*, *Candida sake* and *Candida friedrichii*. Sixteen out of the 30 most abundant taxa (**Table 2**) were detected in less than 10% of the total number

TABLE 2 | Taxonomic classification of the most abundant taxa identified to genus or species level in the wood of grapevine rooted cuttings.

Phylum	Family	Species
Ascomycetes	-not available-	*Circinotrichum maculiforme*†
	Botryosphaeriaceae	*Neofusicoccum* sp.*†
	Cucurbitariaceae	*Pyrenochaeta* sp.†
	Davidiellaceae	*Cladosporium* sp.
		Cladosporium sphaerospermum
	Debaryomycetaceae	*Meyerozyma guilliermondii*†
	Diaporthaceae	*Diaporthe* sp.*
	Didymellaceae	Pleosporales incertae sedis (*Epicoccum nigrum*)
	Dothioraceae	*Aureobasidium pullulans*
	Helotiales	*Cadophora* sp.*†
	Herpotrichiellaceae	*Phaeococcomyces nigricans*†
		Phaeomoniella chlamydospora *
	Hypocreales	*Acremonium alternatum*
		*Ilyonectria destructans**†
	Nectriaceae	*Fusarium* sp.*†
	Pleosporaceae	*Alternaria* sp. (*Alternaria alternata*)
	Saccharomycetaceae	*Debaryomyces* sp.
		Candida friedrichii
		Candida parapsilosis†
		Candida sake
		Candida tropicalis†
Basidiomycetes	*Cystofilobasidiaceae*	*Cystofilobasidium capitatum*†
	Filobasidiaceae	*Naganishia* sp.†
	Malasseziaceae	*Malassezia* sp.†
		Malassezia restricta
		Malassezia globosa
		Malassezia sympodialis†
	Peniophoraceae	*Peniophora* sp.†
	Sporidiobolaceae	*Sporidiobolus* sp.†
	Tremellaceae	*Cryptococcus* sp.

The relative abundance of the listed taxa was equal to or greater than 0.1% of the total dataset. The taxonomic classification of species between parentheses was attributed using BLAST.

**Taxa associated with grapevine trunk diseases.*

†Taxa detected in less than 10% (n = 8 or less) of the total number of vines (n = 78).

of vines, and among these 16, eight of them were present only in a single rooted cutting. This observation highlights the high variability often encountered when comparing individual plants.

The metagenomic analysis detected *P. chlamydospora* in 28% of non-inoculated plants (RA > 0.1%), while the presence of wood endophytes that composed ACEA1, in non-inoculated grapevines, was revealed to be very low. *Alternaria* sp. and *Cladosporium* sp. were present in 8% of the plants, while *E. nigrum* (Pleosporales incertae sedis) and *Au. pullulans* in 3% of them (RA > 0.1%).

Alpha Diversity

The boxplots in **Figure 4** show the Shannon diversity and Pielou's evenness in grapevines treated with different fungicides and inocula. The Shannon diversity and evenness of the resident mycobiome of non-inoculated plants did not vary in relation to the fungicide treatment, according to Tukey's HSD ($P > 0.05$). On the other hand, the inoculation of *P. chlamydospora* comported changes in both diversity and evenness ($P < 0.05$) of the mycobiome, when vines were sprayed with Blad (**Figure 4**). This active ingredient induced a reduction in Shannon diversity when compared to control-treated plants, as well as a strong trend when comparing Blad-treated plants to those treated with systemic fungicides ($P = 0.058$). No diversity differences were significant among other treatments ($P > 0.05$). The evenness of the fungal communities significantly varied when comparing Blad-treated vines with copper–sulfur-treated vines (**Figure 4**), and a strong trend was detected when comparing the former treatment with systemic fungicide-treated vines ($P = 0.051$). The application of the different fungicides did not affect the Shannon diversity and evenness of vines inoculated with either ACEA1 alone or ACEA1 and the pathogen ($P > 0.05$).

Beta Dispersion

The Jaccard index, when visualized in an NMDS plot, shows a considerable overlap for different fungicide treatments and inoculum types (**Figure 5**). The PERMANOVA indicates a significant difference between groups ($P = 0.001$), but looking at the ordination, the difference seems to lie in the clustering of the observations, rather than any distinct difference in sample composition. For example, the fungal communities in Blad-treated plants cluster more tightly than the control-treated ones, and a similar trend applies to non-inoculated vines when compared with those inoculated with both pathogen and ACEA1.

Effect of Inoculum Type and Fungicide Treatments on Taxa Abundance

When examining the effect of fungicide treatments on the fungal communities of both non-inoculated and inoculated plants, no

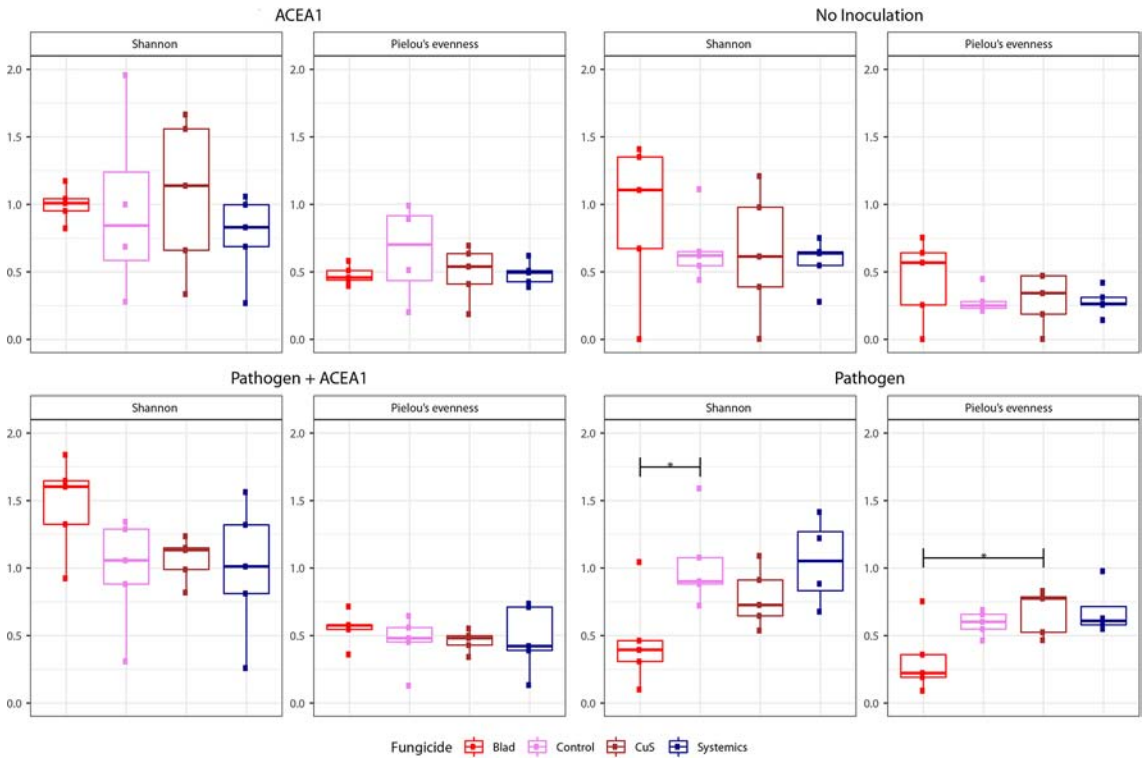

FIGURE 4 | Box plots of diversity indexes (Shannon, Pielou's evenness) of the fungal community present in grapevine cuttings added with water (No inoculation) or inoculated with *Phaeomoniella chlamydospora* (Pathogen), a consortium of fungal wood endophytes (ACEA1), or both *P. chlamydospora* and ACEA1. Inoculated plants were sprayed with either Blad or potassium permanganate (Control) or copper oxychloride and sulfur (CuS) or penconazole and fosetyl-aluminum (Systemics). $n = 5$ for each combination inoculum/treatment. The black, horizontal brackets at the top of the figures denote statistical comparisons of the two treatments at each end of the bracket, calculated using one-way ANOVA with Tukey's honestly significant difference (HSD) *post hoc*. Statistical differences are shown by asterisk, where *P 0.05.

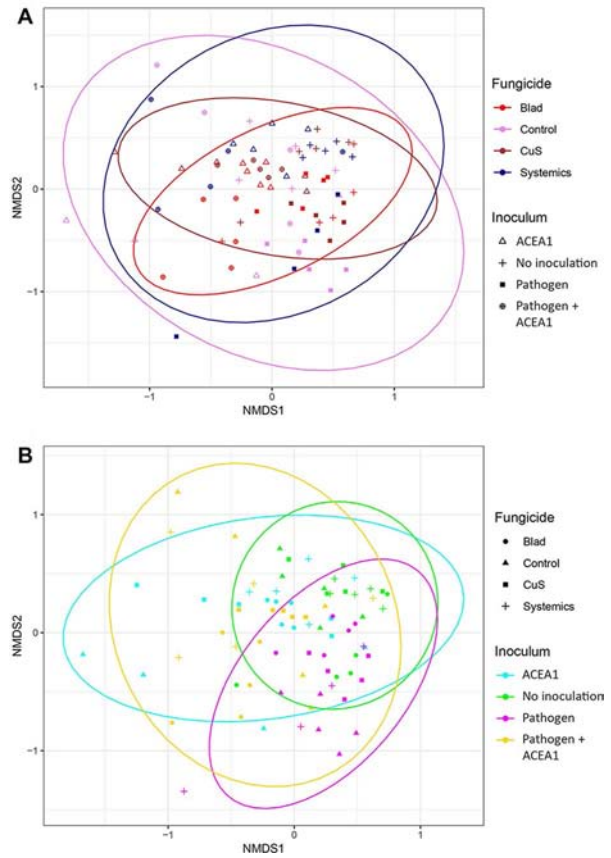

FIGURE 5 | Non-metric multidimensional scaling (NMDS) plots based on Jaccard's index. Fungal communities present in grapevine cuttings added with water (No inoculation), *Phaeomoniella chlamydospora* (Pathogen), a consortium of fungal wood endophytes (ACEA1), or both *P. chlamydospora* and ACEA1. Inoculated plants were sprayed with either Blad or potassium permanganate (Control) or copper oxychloride and sulfur (CuS) or penconazole and fosetyl-aluminum (Systemics). $n = 5$ for each combination inoculum/treatment. Ellipses illustrate the multivariate normal distribution of samples within the same fungicide **(A)** or inoculum **(B)** group.

differences are detected according to the Wilcoxon test ($P > 0.05$). However, several taxa were found to have significantly different abundances between treatments according to the Wald test on the pairwise treatment comparisons, and the combined MetacodeR and DESeq analyses revealed overrepresentation and underrepresentation of numerous taxa under different treatments (**Figures 6** and **7**). Among the taxa identified as differently abundant, we find some of the fungi present in less than 10% of the total number of plants (**Table 2**). This low frequency suggests that the detected differential abundance may not lie in the effect of the treatments, but instead in the uneven distribution (presence/absence) of these taxa in individual vines. For this reason, these taxa are not going to be further examined.

In non-inoculated vines, *Candida sake* and *C. friedrichii*, present in control-treated plants, are not detected in Blad-treated vines; similarly, *C. sake*, *Malassezia Restricta*, and *M. globosa* are present in control-treated plants but absent in systemic fungicide-treated vines; *Diaporthe* sp. was detected under all treatments with the exception of systemic fungicides. None of the most frequent taxa differs significantly when comparing control- with copper–sulfur-treated grapevines (**Figure 7**).

In inoculated vines, we assumed that a successful colonization only occurred when inoculated fungi were detected in at least 50% of the biological replicates (per treatment/inoculation combination). The inoculation with *P. chlamydospora*, whether alone or in combination with ACEA1, resulted in a successful wood colonization, 1 cm below the inoculation point, in 87% of the cases. In the pathogen-only inoculation, fungicide treatments affected the RA of *P. chlamydospora* (**Figure 7**, **Table 3**) and that of other taxa. The foliar spray with Blad significantly decreased the RA of this fungus when compared with the control and other treatments ($P < 0.05$).

In grapevines inoculated with ACEA1 and sprayed with potassium permanganate (control), only *E. nigrum* could successfully colonize the wood examined. The abundance of this ascomycete increased when plants were treated with Blad or systemic fungicides (**Table 3**). *Al. alternata* was absent (RA > 0.1%) in control-treated plants, while it was detected, in different abundances, under all other treatments (**Table 3**). The wood colonization by *Au. pullulans* was poor to absent, and it was never detected in 50% or more of the inoculated plants (**Table 3**). *Cladosporium* sp. was present exclusively in copper–sulfur-treated plants, although not detected in 50% or more of the inoculated plants (**Table 3**).

The simultaneous inoculation of *P. chlamydospora* and ACEA1 resulted in a lower abundance of the pathogen, when compared with the *P. chlamydospora*-only inoculum (**Table 3**), for all treatments except Blad. *Al. alternata* was detected under all fungicide treatments, except for the control-treated vines. *E. nigrum* exclusively colonized the wood when vines were treated with systemic fungicides or copper–sulfur. The Blad treatment facilitated the wood colonization by *Au. pullulans* and *Cladosporium* sp., which were not detected under any of the other treatments (**Figure 7**).

DISCUSSION

Current knowledge on the effects of fungicides on the grapevine microbiome is scarce. Perazzolli et al. (2014) examined the effect of penconazole and a biological control agent on the grapevine phyllosphere, finding that bacterial and fungal communities are only minimally affected by both treatments. However, studies that investigated the effect of vineyard management systems (organic or integrated pest management) on endophyte communities, discovered significant differences, in both fungi (Pancher et al., 2012) and bacteria (Campisano et al., 2014). These results suggest that fungicide treatments may be one of the factors driving such microbiome changes. In this study, we investigated the changes that occur in the wood mycobiome in response to the application of fungicides, sprayed on leaves, in young plants. Due to the numerous factors that may influence our understating of this interaction, we decided to perform the experiment under semi-controlled conditions (i.e., greenhouse) and with no experimental repetitions. It follows a brief list of factors known to play a role in shaping the wood mycobiome of grapevines.

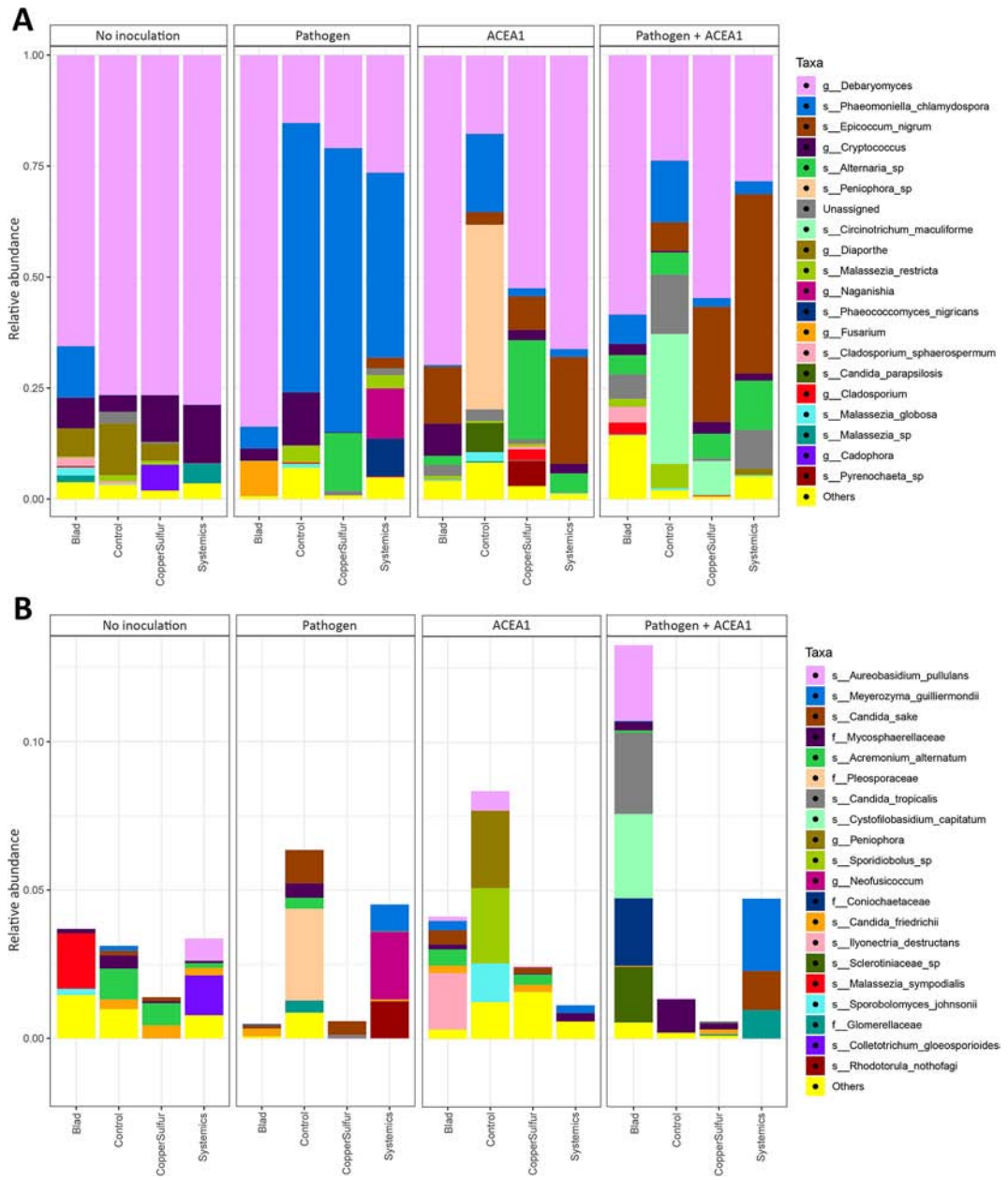

FIGURE 6 | Barplots of the relative abundance of the 20 most abundant taxa **(A)** identified to species (s_), genus (g_), or family (f_) level, found in rooted grapevine cuttings non-inoculated (No inoculation) or inoculated with *Phaeomoniella chlamydospora* (Pathogen) or a consortium of fungal wood endophytes (ACEA1) or a combination of both (Pathogen + ACEA1). Grapevines were treated with either Blad or potassium permanganate (Control) or copper oxychloride and sulfur (CopperSulfur) or fosetyl-aluminum and penconazole (Systemics). $n = 5$ for each combination inoculum/treatment. "Unassigned" are taxa identified to a lower taxonomic level than family or non-identified, "Others" are taxa not included in the 20 most abundant. **(B)** The 20 most abundant taxa within the "Others" group of **(A)** are shown.

(i) The age of the plants—Young grapevines (and canes) are known to host a lower richness in fungal taxa, when compared to adult vines (Dissanayake et al., 2018; Del Frari et al., 2019a).

(ii) The management of the vineyard—Fungal endophytic communities found in organic vineyards can significantly differ from those found in vineyards that use an integrated pest management strategy (Pancher et al., 2012).

(iii) The geographical location—In the same study by Pancher et al. (2012), significant differences in the wood mycobiome were detected when examining different vineyard locations.

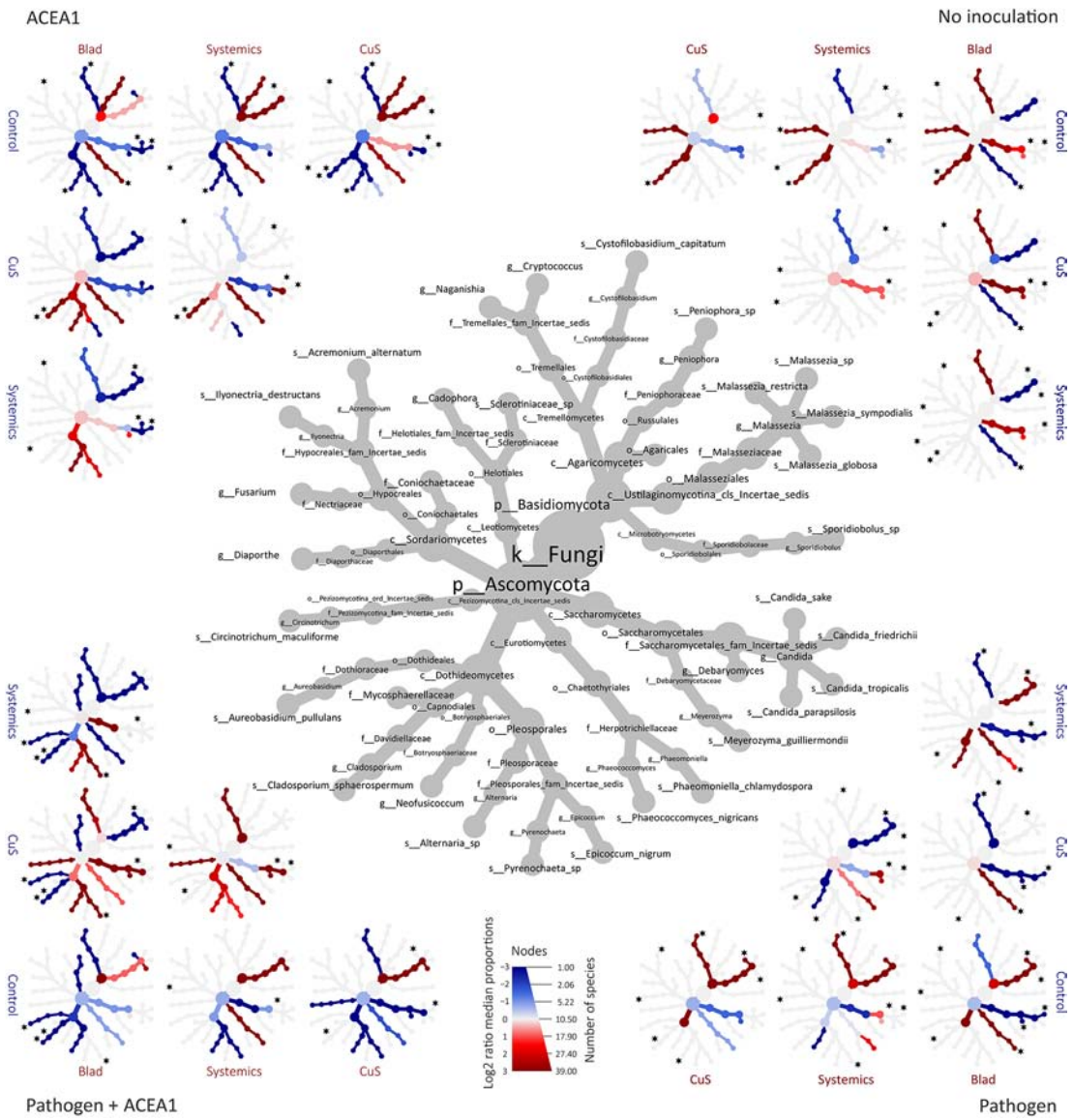

FIGURE 7 | Differential heat tree matrixes depicting the change in taxon abundance between different fungicide treatments, represented in the dataset with a relative abundance (RA) > 0.1% (n = 5 for each combination inoculum/treatment). The size of the individual nodes in the gray cladogram depicts the number of taxa identified at that taxonomic level. The smaller cladograms show pairwise comparisons between each treatment, with the color illustrating the log2 fold change: a red node indicates a lower abundance of the taxon in the treatment group stated on the abscissa than in the treatment group stated on the ordinate. A blue node indicates the opposite. A black star next to a node represents the statistical differences according to DeSeq2 (P 0.05), for the most frequent taxa (**Table 2**).

(iv) Grapevine cultivar—Jayawardena et al. (2018) proposed that grapevine cultivar, along with geographical location, is a determinant factor in the wood mycobiome composition. This observation is also supported by the results of a previous study by Travadon et al. (2016).

(v) Seasonality—In a study by Bruez et al. (2014), grapevine wood sampled in different seasons harbored diverse community structures.

(vi) The physiological status of grapevines may also influence their susceptibility to infection (e.g., biotic and abiotic stress; Gramaje et al., 2018).

Considering all these variables, it is reasonable to forecast that if any of the above factors changes, our understanding of the effect of fungicides on the wood mycobiome may vary. For this reason, the results obtained in this study should be considered as a case study, rather than a general understanding, and further investigation is required.

In order to assess the effect of fungicides on the early colonization success by wood endophytes, we selected four non-pathogenic fungi that are frequently found in grapevine wood and that are believed to hold potential in the biological control of wood pathogens (Pancher et al., 2012; Mondello et al., 2017;

TABLE 3 | Relative abundance of inoculated fungi in plants that were subject to different fungicide treatments.

	No inoculation				Phatogen inoculation				ACEA1 inoculation				Phatogen + ACEA1 inoculation			
	C	CuS	Sys	Blad	C	CuS	Sys	Blad	C	CuS	Sys	Blad	C	CuS	Sys	Blad
Phaeomoniella chlamydospora	–	–	–	11.6*	60.7*	64.0*	33.3*	4.9*	17.6	1.8	1.8	0.4	13.9*	2.0*	2.9	6.6*
Alternaria alternata (*Alternaria* sp.)	–	–	–	–	–	13.1	–	–	–	22.3*	4.3*	2.1*	5.0	5.5*	11.0*	4.3*
Epicoccum nigrum (Pleosporales incertae sedis)	–	–	–	–	–	–	2.0	–	2.8*	7.6*	24.1*	12.9*	6.4	25.9*	40.4*	–
Aureobasidium pullulans	–	–	0.8	–	–	–	–	–	0.7	–	–	0.2	–	–	–	2.5*
***Cladosporium* sp.**	–	–	–	0.3	0.3	–	0.1	–	–	2.5	–	–	–	0.2	–	2.7*

Grapevine cuttings were non-inoculated (No inoculation), inoculated with P. chlamydospora (Pathogen inoculation), or inoculated with a consortium of fungal wood endophytes (ACEA1 inoculation) or a combination of both (Pathogen + ACEA1 inoculation). Grapevines were treated with a control (potassium permanganate; C) or copper oxychloride and sulfur (CuS) or fosetyl-aluminum and penconazole (Sys) or Blad. Different colors represent different intervals of relative abundance (≤1; 1–5; 5–10; 10–20; >20). n = 5 for each combination inoculum/treatment.

(*) 50% or more of the biological replicates were successfully colonized by the inoculated fungus

Jayawardena et al., 2018; Pinto et al., 2018). The choice of using a consortium instead of single fungus springs from the two following ideas:

(i) To examine the effect of fungicide treatments on the early wood colonization success—The inoculation of multiple endophytes allows us to screen a higher number of fungi simultaneously.

(ii) To examine the effect of simultaneously inoculating multiple endophytes and *P. chlamydospora*—The antagonistic interactions among endophytes and pathogen become more complex when compared to the confrontation between a single antagonist and a single pathogen. We hypothesized that the competition for space and nutrients, as well as the chemical war that occurs in the wood (e.g., competition-induced fungal metabolites; Hardoim et al., 2015), would strongly reduce the likelihood of *P. chlamydospora* producing a successful infection.

Visual Inspection, Shoot Length, and Brown Wood Streaking

The absence of foliar symptoms in the vines inoculated with ACEA1 confirms the current understanding that none of the selected organisms composing ACEA1, when present in the wood, are pathogenic in grapevines (Jayawardena et al., 2018). Concerning *P. chlamydospora*, Sparapano et al. (2001) observed that this fungus induced foliar symptom appearance, albeit only several months/years post-inoculation; therefore, the absence of foliar symptoms at the end of the experiment was expected.

The differences in length of the green shoot, only observed in response to fungicide treatments, suggest that both systemic fungicides and Blad positively affect the growth of vines (**Figure 3**); however, their mechanisms of action remain to be assessed.

Wood discoloration, in the form of brown wood streaking, is a result of the oxidation and polymerization of phenolic compounds, through the action of phenolases, a plant response to pathogen infections (Agrios, 2012) but also to other stresses such as mechanical injuries (Pouzoulet et al., 2013). (i) The absence of brown wood streaking in water- and ACEA1-inoculated vines and in plants treated with potassium permanganate, systemic fungicides, and Blad confirms that none of the components of ACEA1 are pathogenic. On the contrary, copper–sulfur-treated plants manifested extensive brown wood streaking. A recent report revealed that wood necrosis caused by grapevine wood pathogen *Neofusicoccum parvum* was promoted by the presence of high doses of copper in soil (Bruez et al., 2017). Copper is also associated with fungal pigmentation production (Griffith et al., 2007), a virulence factor (e.g., fungal melanins; Jacobson, 2000); laccase synthesis, which is involved in wood degradation (Tychanowicz et al., 2006); and induction of phytotoxic effects, with changes in plant morphology, biochemistry, and physiology (Bruez et al., 2017). Overall, these clues suggest that copper may play a relevant role in the development of brown wood streaking, by interacting with plant and endophytes. (ii) The extensive symptomatology recorded in plants inoculated with *P. chlamydospora*, under all fungicide treatments, indicates that the plant readily responded to infection, and it did so independently of the abundance of *P. chlamydospora* in the wood (e.g., Blad treatment; **Table 3**). Wood pathogens are known to induce the appearance of brown wood streaking, but they are not always found in symptomatic wood, especially the farther from the inoculation point (Mugnai et al., 1999). This suggests that, under some circumstances, the extent of wood streaking may not be correlated with the abundance of the pathogen. Moreover, *P. chlamydospor*a has been previously reported in wood free from brown streaking symptoms (Rumbos and Rumbou, 2001; Abreo et al., 2011; Hofstetter et al., 2012), and its ecology requires further investigation. (iii) Moderate to absent wood symptoms were observed when co-inoculating *P. chlamydospora* and ACEA1, suggesting that the interactions among fungi resulted in a weaker or absent plant response to the infection. In fact, the reduction in wood streaking extent, due to the presence of fungal antagonists, has been previously reported in grapevines (Mondello et al., 2017; Del Frari

et al., 2019b). It is worth remembering that the observations related to the brown wood streaking symptomatology made in this work are preliminary, and further evidence is necessary to assess the relation among wood inhabiting fungi–fungicides–wood symptomatology.

Effect of Fungicides on the Resident Mycobiome and on the Early Colonization by Endophytes

Previous studies revealed that fungicide application can affect the mycobiome of grapevines and other crops (Karlsson et al., 2014; Morrison-Whittle et al., 2017), although none of them investigated the fungal communities present in the wood. This study shows that some fungal taxa composing the resident wood mycobiome of grapevine cuttings cv. Cabernet Sauvignon are affected by fungicide application (**Figure 7**). This observation suggests that active ingredients interacted with fungi either directly (e.g., copper oxychloride is known to be absorbed in the xylem of grapevines, when sprayed on the bark of vines; Di Marco et al., 2011) or indirectly, for example, stimulating plant defenses (García et al., 2003). Further studies are necessary to confirm this preliminary understanding, keeping into account (i) the variability in taxa composition that may characterize individual plants and (ii) that the DNA of dead fungal cells, supposedly affected by fungicides, may persist in the wood, during short-term experiments, hiding the magnitude of the treatment effect. It is also necessary to investigate whether the changes observed in taxa abundance are temporary or long-lasting, therefore determining the overall resilience of the endophytic communities that compose the resident mycobiome.

Multitrophic interactions involving plants and fungal and bacterial endophytes are still poorly understood (Hardoim et al., 2015). Current literature shows that host and microbe genotypes, as well as abiotic factors, can influence successful colonization by endophytes (Hardoim et al., 2015). In this study, we show that also fungicide application can affect the early colonization by fungal endophytes, in particular those composing ACEA1 (**Table 3**). Therefore, fungicides seem to have the potential of shaping the wood mycobiome by favoring/inhibiting the early wood colonization of specific fungi. For this reason, we propose to keep in consideration fungicide treatments when describing endophytic communities in grapevines.

Effect of Fungicides on the Early Colonization by *P. chlamydospora*

Numerous trials have been conducted, over the last 20 years, in order to find reliable means to control infections by wood pathogens, including *P. chlamydospora*. Chemical control, in the form of foliar spray or endotherapy, led primarily to negative or moderate results, while more promising ones have been achieved through pruning wound protection (Mondello et al., 2017). In this study, the copper–sulfur and systemic fungicide treatments did not affect the colonization success of the pathogen, within the timings of the experiment (**Figure 6**). A preliminary study in support of the neutral effect of copper oxychloride on the colonization success of *P. chlamydospora* was presented by Di Marco et al. (2011), although no literature is available on the influence of sulfur or other synthetic fungicides. Blad was the only active ingredient capable of significantly inhibiting the wood colonization success of *P. chlamydospora* (Del Frari et al., 2018; Monteiro et al., 2015). This is the first report of a foliar spray capable of reducing the wood colonization success of this pathogen.

Use of ACEA1 in the Biological Control of *P. chlamydospora*

As postulated, the wood colonization success of *P. chlamydospora* was greatly reduced when co-inoculated with a consortium of fungal wood endophytes (ACEA1) that are considered potential antagonists (**Figure 6**). Some of the possible explanations are found in the interactions that occur during conidia germination and mycelial growth (e.g., release of metabolites) and by increasing the competition for space and nutrients (Hardoim et al., 2015). Fungi composing ACEA1 are frequently isolated from grapevine wood; however, their exact niche (e.g., xylem vessels, tracheids) remains to be assessed. A histological examination of the wood may unveil the exact interaction that takes place among the components of ACEA1 and *P. chlamydospora*. Only minor changes in the abundance of the pathogen are observed with different fungicide treatments, making ACEA1 a potential tool to be exploited in biological control. Interestingly, the Blad treatment facilitated the wood colonization by both *Cladosporium* sp. and *Au. pullulans*, while inhibiting that of *E. nigrum*, in the presence of *P. chlamydospora*, when compared with the results obtained from ACEA1-only inoculation (**Table 3**). This observation suggests the occurrence of complex interactions in which not only the fungicide but also the pathogen play a primary role (e.g., competition-induced metabolites), resulting in selective wood colonization. Further investigation is necessary to confirm these results also from a quantitative point of view. It is also important to understand if the effect observed by the addition of ACEA1 is long-lasting, and it may be successfully applied to plants with an established infection, as a curative strategy.

CONCLUSION

This study shows that some common fungicides that have been applied in viticulture for decades (copper–sulfur and systemics) affect the resident mycobiome and/or the wood colonization success of some endophytes, while leaving nearly unaffected the wood pathogen *P. chlamydospora*. This has unpredictable consequences, in both the medium and long terms, on the wood mycobiome ecology and its influence on grapevine well-being. It is reasonable to wonder whether the wood mycobiome composition of grapevines before the fungicide era was considerably different from that of today. As the diversity of a biological system is positively correlated with its stability (McCann, 2000), a loss in endophytic species that antagonize GTD pathogens, such as *P. chlamydospora*, may be a possible explanation for the recent success of these pathogenic fungi. Another explanation may lie in the fungicide-driven increased likelihood of infection by some fungi, which creates an imbalance in the stability of the plant–mycobiome biological system.

This work addressed only four endophytes, although the wood mycobiome of adult plants is known to be potentially colonized by

hundreds of species, making this just the beginning of the research in this sense. In conclusion, enhancing the complexity of the wood mycobiome, for example, using multiple endophytic antagonists, may considerably increase our chances to control GTD-associated pathogens. While research on endophytic fungal consortia advances, Blad seems a promising active ingredient to confront early infections by *P. chlamydospora*, in young grapevines. This active ingredient is non-toxic for the environment and humans, has growth-promoting properties, and strongly antagonizes the wood colonization success of *P. chlamydospora*, making it a promising tool in viticulture.

DATA AVAILABILITY STATEMENT

The raw data of this study are available in the European Nucleotide Archive (ENA accession number PRJEB32853).

AUTHOR CONTRIBUTIONS

GF conceived the study, performed greenhouse experiment, performed wet-lab work, interpreted and discussed the results, and wrote the manuscript. AG performed wet-lab work, performed bio-informatics analyses, wrote the manuscript. MA performed statistical analyses and data visualization. HO, LH, and RF supervised the study and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01405/full#supplementary-material

TABLE S1 | Fungal taxa identified to genus or species level in the wood of grapevine rooted cuttings, at relative abundances (RA) inferior to 0.1 % or 0.01% of the total.

TABLE S2 | Rarefaction curves results based on richness, calculated on the number of amplicon sequence variants (ASVs), and on Shannon index.

FIGURE S1 | Rarefaction curves of alpha-diversity indexes; each line correspond to one sample in a range from 0 to 18000 reads. Each box plot represent the distribution of the selected alpha diversity metric for each sample at each 2000 reads of sampling depth. The lower and upper whiskers of the box plot correspond to the 9th and 91st percentiles of the distribution, while the lower and upper extents of the box correspond to the 25th and 75th percentiles of the distribution (respectively). The horizontal bar through the middle of the box is the median of the distribution (i.e., the 50th percentile). Outlier points of these distributions are not shown. The lines connect all the medians from the different. A) Rarefaction curves based on richness, calculated on the number of amplicon sequence variants (ASVs). B) Rarefaction curves based on Shannon index.

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REVIEW
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Emergent Ascomycetes in Viticulture: An Interdisciplinary Overview

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The reduction of pesticide usage is a current imperative and the implementation of sustainable viticulture is an urgent necessity. A potential solution, which is being increasingly adopted, is offered by the use of grapevine cultivars resistant to its main pathogenic threats. This, however, has contributed to changes in defense strategies resulting in the occurrence of secondary diseases, which were previously controlled. Concomitantly, the ongoing climate crisis is contributing to destabilizing the increasingly dynamic viticultural context. In this review, we explore the available knowledge on three Ascomycetes which are considered emergent and causal agents of powdery mildew, black rot and anthracnose. We also aim to provide a survey on methods for phenotyping disease symptoms in fields, greenhouse and lab conditions, and for disease control underlying the insurgence of pathogen resistance to fungicide. Thus, we discuss fungal genetic variability, highlighting the usage and development of molecular markers and barcoding, coupled with genome sequencing. Moreover, we extensively report on the current knowledge available on grapevine-ascomycete interactions, as well as the mechanisms developed by the host to counteract the attack. Indeed, to better understand these resistance mechanisms, it is relevant to identify pathogen effectors which are involved in the infection process and how grapevine resistance genes function and impact the downstream cascade. Dealing with such a wealth of information on both pathogens and the host, the horizon is now represented by multidisciplinary approaches, combining traditional and innovative methods of cultivation. This will support the translation from theory to practice, in an attempt to understand biology very deeply and manage the spread of these Ascomycetes.

Keywords: anthracnose, black rot, disease symptom phenotyping, genetic diversity, grapevine, powdery mildew, disease resistance loci, transcriptomics

INTRODUCTION

The earliest evidence of viticulture, namely grapevine cultivation and winemaking, was found in Iran, dating back to 7,400-7,000 B.C. (McGovern, 2004). Among the 60 hybridizing species ($2n = 38$) belonging to the *Vitis* genus, the Eurasian grapevine (*Vitis vinifera* L.) is the most extensively cultivated and of renowned worldwide economic importance, being used for the production of high quality wines, table grapes and raisins (Olmo, 1979). With the exception of a few recently explored *V. vinifera* accessions coming from the Caucasian cradle of grapevine domestication (e.g. Toffolatti et al., 2018), *V. vinifera* cultivars are generally highly susceptible to most fungal diseases, such as downy mildew, grey mold, powdery mildew (PM), black rot (BR), and anthracnose (AN) (Olmo, 1971). For this reason, the main strategy to prevent yield losses due to biotic adversities is the application of fungicide which is necessary to control the causal agents with inevitable negative impact on humans, animals and environment. Around 68,000 tons of fungicides per year are used in Europe to manage grapevine diseases, i.e. 65% of all fungicides used in agriculture, though viticulture encompasses only 4% of the arable land available in the EU (Muthmann and Nardin, 2007). Forecasts predict large increases in this trend, especially in viticulture, consistent with the worldwide data available (FAO, 2016).

A useful strategy to reduce the impact of pesticides towards a sustainable viticulture relies on breeding, by introducing resistance traits from wild species into domesticated varieties. Therefore, the change to these current varieties (cultivars) is now strongly advised. After initial difficulties, due to considerations regarding the quality of the wine produced with these varieties, resistant cultivars have recently been allowed by the EU Commission for the production of *PDO* (*Protected Denomination of Origin*) Dons wines in Denmark (Implementing Regulation 2018/606). In the last decade, it has been reported that the cultivation of new varieties resistant to downy mildew and PM, whose management needs less copper and sulphur-based treatments, favored BR diffusion (Harms et al., 2005; Töpfer et al., 2011). In fact, most cultivars which exhibit adequate resistance against mildews are highly susceptible to BR (Harms et al., 2005). In addition, considering BR is originally native to northern America, the appearance of BR symptoms is ongoing in a previously BR-free area (CABI, Crop Protection Compendium, 2018) (**Figure 1B**). Analogously, AN is sometimes problematic on highly susceptible interspecific cultivars, which typically receive only modest fungicide programs to control other diseases (Wilcox et al., 2017).

The transition to production of resistant cultivars, however, needs to be framed within the current climatic challenges. The two main players involved in the climate crises are temperature (global warming) and precipitation (including extreme phenomena) (Higgins and Scheiter, 2012). Besides having already extremely impacted natural phenology by limiting yields through drought and spring-frost damages, dramatic changes in climate will result in the accelerated reproductive cycles of biological organisms, destabilizing even further the precarious equilibrium among pathogens, pests and hosts (Salinari et al., 2006; Caffarra et al., 2012). Global surveys to identify the most relevant diseases and pests in many grape-growing regions worldwide provided preliminary results which allowed for the determination of the distribution of diseases by 2050 as a function of agroclimatic indicators. Upon these recent investigations, PM derived from northern America (**Figure 1A**), and AN which originated in Europe (**Figure 1C**), were also recently discovered in extremely diverse climatic conditions, including temperate regions with high rainfall, especially during spring months (Bregaglio et al., 2013; Bois et al., 2017; Wilcox et al., 2017).

Finally, in this challenging and dynamic viticultural context, we are witnessing the emergence of fungal diseases caused by Ascomycetes. The term "emergent disease" in this case is not to be interpreted in the strict sense of phytosanitary emergencies, but refers to diseases whose causative agents are already known, and for which control plans exist. These diseases however have manifested themselves or already conquered new regions, due to the recovery or the onset of favorable conditions, representing a real threat to worldwide viticulture.

DISEASE DESCRIPTION

Disease Symptom Assessment

Powdery Mildew

The causative agent of PM is the biotroph (obligate parasite) *Erysiphe necator* Schw. (asexual morph *Oidium tuckeri* Berk.) (**Figure 2**). PM is recognized by the appearance of a whitish-gray dusty layer on the grape which is caused by the spreading of mycelia and conidia onto green tissues (Pearson and Gadoury, 1992). Biological assays for the assessment of disease symptoms are fundamental to shed light onto host-pathogen interactions; conveniently, these can be carried out by observations in the field (e.g. Li, 1993; Wang et al., 1995; Pap et al., 2016), in greenhouses (e.g. Li, 1993; Amrine et al., 2015; Pap et al., 2016; Pessina et al., 2016), and *ex vivo* (e.g. Li, 1993; Wang et al., 1995; Staudt, 1997; Pap et al., 2016; Pessina et al., 2016).

In the field, observations are carried out in mid-summer, when the symptoms are more evident and it is possible to estimate the occurrence of the pathogen in vineyards treated and not-treated with fungicide (Li, 1993; Wang et al., 1995). Symptoms can be monitored studying artificial infections (inoculations) using amplified conidia (Pap et al., 2016). To standardize results, the age of the leaf must be recorded, since older leaves display ontogenic resistance (Gadoury et al., 2012). In a greenhouse setting, different strategies can be adopted: for instance Li (1993) and Pap et al. (2016) inoculated potted plants by spraying them with a suspension of *E. necator* conidia in aqueous and Tween solution using 5×10^5 conidia/ml and 0.7×10^5 conidia/ml respectively. Pessina et al. (2016) brushed the adaxial surface of older leaves with young leaves carrying *E. necator* sporulation. Under these growing conditions, disease severity can be assessed within 3 to 21 days post-inoculation (dpi). *Ex vivo* pathogenesis assays are conducted using detached leaves or leaf disks and different inoculation strategies have been used. Péros et al. (2006b) tested three different spots of inoculum on detached leaves, using a glass needle to transport 20-60 conidia a time. Alternatively, Staudt (1997) brushed leaf disks with conidia from the mycelium

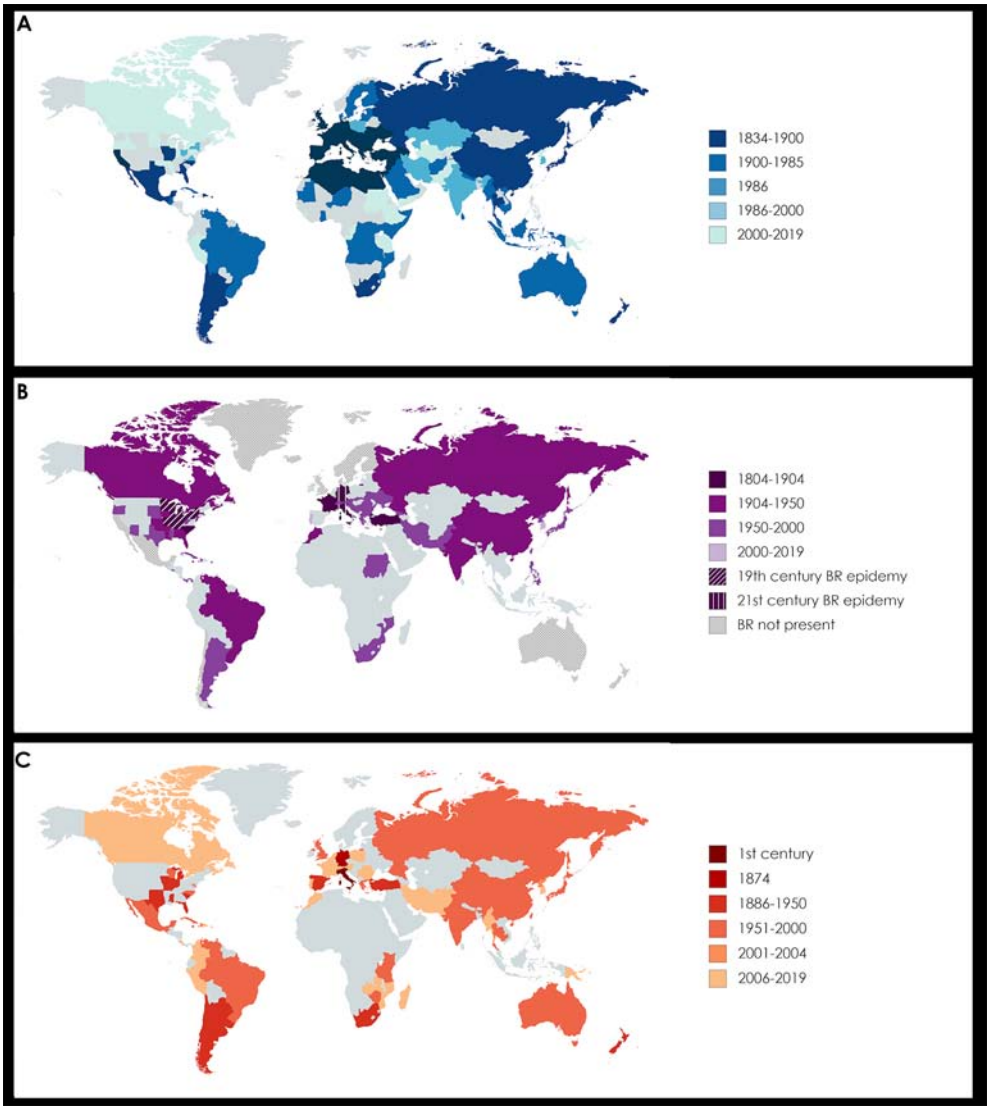

FIGURE 1 | Worldwide diffusion of powdery mildew (PM, panel A), black rot (BR, panel B), and anthracnose (AN, panel C). The relative bibliographic sources are reported in **Table S1**. **(A)** PM was first reported in northeastern America in 1834 by Schweinitz. In 1845 it was introduced in Europe and less than 10 years later was affecting all the wine producing country of the Mediterranean region. In 1986, Amano published an outstanding review listing the countries all over the world where fungi causing PM were present at that time in relation with their specific plant hosts, including grapevine. Today PM can be considered a "worldwide grapevine disease", since it afflicts vineyards all over the world. **(B)** BR is native of northeastern America. In 1804 it was noticed in Dufour's vineyard (Kentucky) and it became epidemic in the second half of the 19th century in all the Great Lake Region, where the entire yield in many fields was lost. The first occurrence in Europe was recorded in 1885 by Viala and Ravaz in Southern France, then it spread all around the world, although without a huge economic impact. In 1989, BR showed an increasing presence in Switzerland, but it was in the 21th century that a second outbreak afflicted Europe, starting from Germany, where the economic losses were severe, to the Alps area (Ticino, Switzerland; Friuli and Veneto, Italy), Hungary and Romania. Regions with an unfavourable climate, as Scandinavia for cold and Mexico for dry weather, are considered BR "free" today. Interestingly, also in Australasia and Chile BR remains absent. **(C)** AN is considered one of the oldest known plant disease, since reference to it were reported in ancient Rome by Theophrastus (in *De causis plantarum*) and by Pliny the Elder (in *Naturalis historia*), dating back to the first century of the Christian era. Its European origin was also confirmed by the first report of the modern era in 1874 by De Bary, in Germany. AN diffusion was not alarming until its arrival in Tropical areas, such as South America. Nowadays AN is again becoming a threat in Europe.

of *E. necator*, while Miclot et al. (2012) developed a ventilation method. Wang et al. (1995) and Pap et al. (2016) successfully sprayed conidia suspension on detached leaves and foliar disks (**Figures 3A–C**).

In these studies, symptom assessment was valued qualitatively–counting the ratio of organs infected over the healthy ones–and quantitatively–by measuring the percentage of organ surface affected by symptoms with respect to the total surface. Miclot et al. (2012) compared three different inoculation techniques to assess the best method for quantitative analysis of PM resistance in grapevine. Starting from dry (Cartolaro and Steva, 1990), wet (Yamamoto et al., 2000) and drop (Moyer et al., 2010) inoculations, they determined a semi-quantitative index, which integrates pathogen sporulation and mycelium

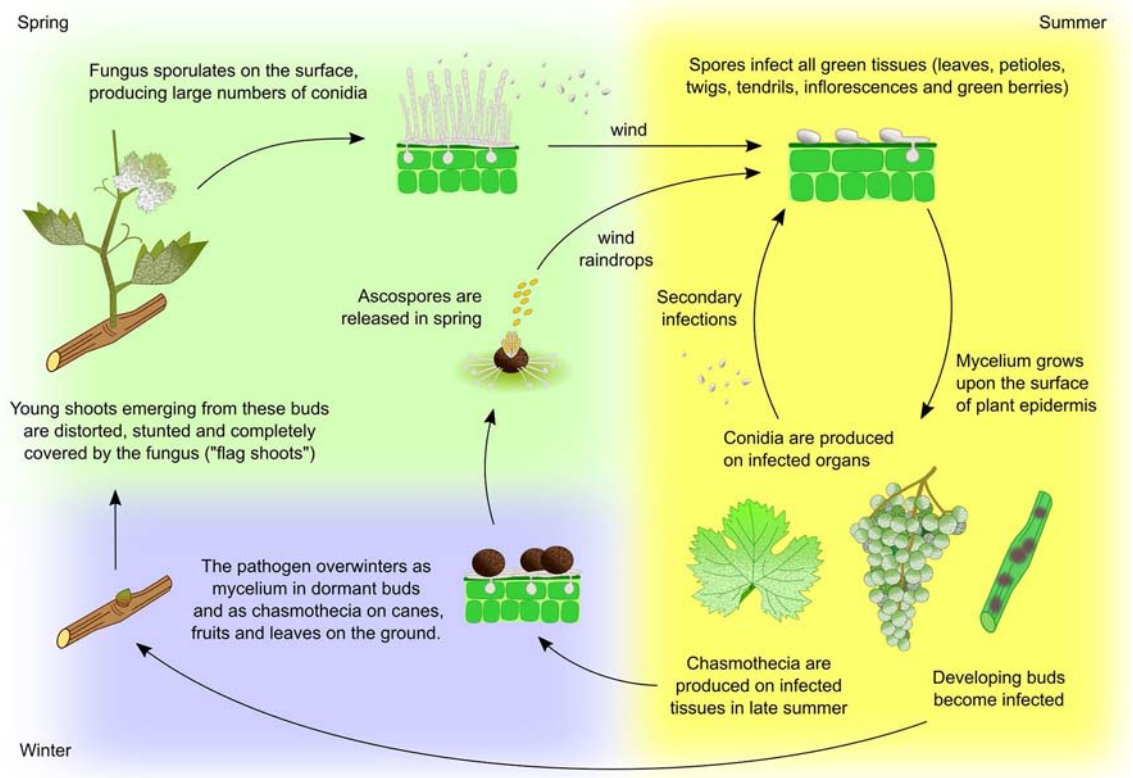

FIGURE 2 | *Erysiphe necator* life cycle. Two overwintering strategies have been observed in *E. necator*. In areas with relatively mild winters, the fungus commonly overwinters as mycelium on leaf primordia within dormant buds. In the following spring mycelium activity resumes, resulting in the production of heavily infected and deformed shoots, called 'flag shoots'. The fungus sporulates on these shoots, producing a large number of conidia that are carried by the wind to healthy plant tissues. Alternatively, the fungus can overwinter as chasmothecium (syn. cleistothecium, a former term for this structure that is still widely used) in bark, on canes, leftover fruit, and on leaves on the ground. Chasmothecia form on the surface of heavily diseased tissues from mid-summer to autumn. During spring rainfall the chasmothecia open and release ascospores, which are spread by wind or raindrops to infect the lower leaves near where the chasmothecia have overwintered. Although free water is necessary to release ascospores, continued wetness is not required for subsequent spore germination and infection. At each new infection site, conidia and ascospores germinate and form an appressorium. From its lower surface a penetration peg develops, piercing the cuticle and entering through an epidermal cell where a haustorium is formed. Mycelium grows upon the surface of the plant epidermis and new conidia are produced within a few days, completing the cycle. Repetition of this cycle continues throughout the growing season resulting in a rapid increase in disease incidence (Wilcox et al., 2017).

growth values. To normalize their results, data were converted into values according to the OIV 455 descriptor (Organisation Internationale de la Vigne et du Vin, 2009). This system relies on a rating of 1 to 9, where 1 represents the highest sporulated surface area (total susceptibility), 3 represents a strong infection, 5 indicates a medium infection, 7 a weak infection and 9 indicate that no symptoms are recognized (completely resistant). Within the EPPO (European and Mediterranean Plant Protection Organization, 2001) code, the two indicated parameters of quality (disease incidence) and quantity (disease severity) are available for symptom assessment. Improved accuracy in symptom assessment can be achieved through histochemical staining and microscopic analysis. Light microscopy can distinguish hyphae, appressoria, conidia and conidiophores, while haustoria cannot be monitored (Cadle-Davidson et al., 2010). Also confocal scanning electron microscopy (SEM) and low-temperature scanning electron microscopy (LTSEM) have been used to study *E. necator* (Carver et al., 1994; Cadle-Davidson et al., 2010; Gadoury et al., 2012; Ramming et al., 2012; Gao et al., 2016). Stainings successfully employed are: i) Coomassie blue, to monitor conidium germination and infiltration into host cells (Ramming et al., 2011; Ramming et al., 2012), ii) Trypan blue, to label the plant dead cells, following conidium germination, hyphae growth, and conidiophore emergence (Gao et al., 2016); iii) Aniline blue, to follow the infection using bright microscopy (Fekete et al., 2009; Gao et al., 2012a) as well as to localise the spores using fluorescence (Vanacker et al., 2000; Pessina et al., 2016).

Black Rot

The causal agent, *Guignardia bidwellii* (Ellis) Viala & Ravaz, is a hemibiotrophic pathogen [asexual morph *Phyllosticta ampelicida* (Engelm.) Aa]. BR can attack all the herbaceous expanding organs of the plant (leaves, shoots, tendrils, petioles and berries), with young shoots and fruits being extremely sensitive (Kuo and Hoch, 1996). The infection is characterized by a first symptomless phase and a second necrotic and

FIGURE 3 | Fungal morphological characteristics and symptoms of powdery mildew (PM, panel **A**-**C**), black rot (BR, panel **D**-**G**), and anthracnose (AN, panel **H**-**K**). **(A)** PM on grapes (field). **(B)** PM on leaves (greenhouse). **(C)** PM leaf disc infection under different magnification (above); conidiophores and conidia on a leaf surface (below-left); mature (black) and immature (yellow) chasmothecia (below-right). **(D)** BR field symptoms on leaves and grape cluster. **(E)** *G. bidwellii* pycnidia on petiole and leaf (above), on berry with detail of cirri development under humid conditions (below). **(F)** Detail of *G. bidwellii* pycnidia and conidia under different magnification. **(G)** *G. bidwellii* isolate growing on culture media (above) and leaf symptoms after artificial infection (below). **(H)** AN field symptoms on a grape cluster, leaf and young shoot, along with detail about the typical "shot-hole" lesions on old infected leaf. **(I)** AN symptom details on berries. **(J)** *E. ampelina* colony on culture media. **(K)** *E. ampelina* acervulus releasing conidia and detail of conidia.

damaging phase (Luttrell, 1974; Kuo and Hoch, 1996). On the adaxial surface of the leaves, the fungus causes the appearance of small circular spots that evolve into light brown lesions with darker borders. The central portion of the spot turns necrotic and pycnidia become visible as small black dots. On the fruits, the first occurrence is the appearance of small whitish dots that rapidly expand concentrically around the berry, forming a brown patch. Later, darker pycnidia develop as the berries rot and shrink, turning into black mummies (Ramsdell and Milholland, 1988).

BR is a polycyclic disease with repeated cycles of primary and secondary infections (**Figure 4**). As reviewed by Onesti (2015), the fruiting bodies bearing the ascospores (ascocarps) have been referred to as either perithecia or pseudothecia, since the wall of stromal tissue of the pseudothecium can be confused with the wall formed by the peridium in a simple perithecium; hence the term pseudothecia will be used throughout the manuscript. Ascospores and conidia are both released during precipitation events: Ferrin and Ramsdell (1978) found a positive correlation between BR infections and the magnitude of rainfall, the number of events and their duration, and the persistence of water on leaves. These observations were also confirmed recently (Onesti et al., 2018). As ascospores and conidia are both sensitive to desiccation, BR is not a prevalent disease in dry climates (Ferrin, 1976; Spotts, 1976; Ferrin and Ramsdell, 1977; Spotts, 1977), however, in the field, spores can germinate even after dry summer periods (Ferrin and Ramsdell, 1978; Besselat and Bouchet, 1984; Hoffman et al., 2004) and pycnidia can produce conidia even after three months of low humidity (Onesti et al., 2017a). Field experiments demonstrated that release dynamics of both types of spores are conserved: conidia are released approximatively at budburst stage, while for ascospores it occurs two weeks later; in the course of the fruiting season, when berries are pea-sized, both types reach their maximum (Onesti et al., 2018). For the ascospores, the first peak is registered between flower pre-blooming to anthesis (Ferrin and Ramsdell, 1977).

The most sensitive period for direct infection on berries is after flowering, from fruit onset to the beginning of bunch closure. A field-trial of artificial infection in a *V. labrusca* 'Concord' vineyard revealed that plants infected between mid-bloom and fruit onset present the highest lesion number on leaves and the greatest berry infection (Ferrin and Ramsdell, 1978). These data were confirmed

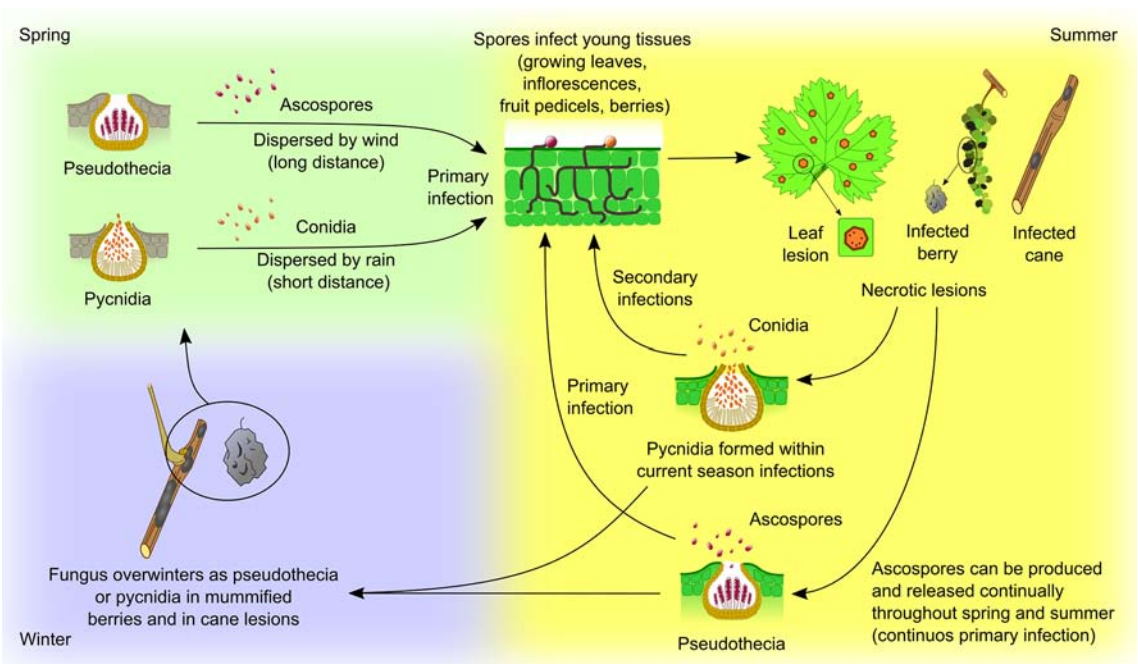

FIGURE 4 | *Guignardia bidwellii* life cycle. The fungus overwinters in mummified berries, retained on the vine or fallen to the ground, and on infected canes. Berry mummies host both pseudothecia, containing asci with ascospores, and pycnidia, with conidia, while canes and tendrils host pycnidia. Lesions capable of producing conidia can persist in the wood for at least two years. In spring, ascospores and conidia are released when pseudothecia and pycnidia become thoroughly wet; infection is therefore favored by frequent rainfall as the spores need water to be released and to germinate. Ascospores released from mummified berries are the most common form of primary inoculum. They are ejected actively from the asci during rainfall and are dispersed by wind currents (long distance dispersion). On the contrary, conidia are exuded from the pycnidium in a white, mucilaginous cirrus from which they can be splashed away by rain (short distance dispersion). Primary infection from ascospores or conidia takes place on young, rapidly growing green tissues (growing leaves, inflorescences, fruit pedicels, berries). Adult leaves and ripe fruits, that have become fully expanded, are not susceptible to infection. Pycnidia are produced rapidly within the necrotic lesions found on leaves, shoots and berries, and, once mature and dampened by rain, they release the conidia which serve as secondary inoculum throughout the season. When the weather is moist, ascospores may be produced and released continually throughout spring and summer from mummies retained in the canopy, providing continuous primary infection, although most of them are discharged in the spring. In late summer, the sexual cycle initiates on infected berries and pseudothecia are formed (Wilcox et al., 2017).

also in greenhouse conditions (Kuo and Hoch, 1996) (**Figures 3D–G**). Moreover, berries can be susceptible for a longer period to *G. bidwellii* compared to other relevant pathogens, for instance *E. necator* (Gee et al., 2008). Since the duration of phenological stages can differ among cultivars, windows of susceptibility (Hoffman et al., 2002) and the number of days after infection for symptom appearance (Roznik et al., 2017) are cultivar-specific.

Ontogenic resistance of plant hosts to fungi is widely documented (Populer, 1978), and for *G. bidwellii* it might be a defense mechanism able to counteract the pathogen, rather than a reduction in the germination ability of the fungus in older tissues (Kuo and Hoch, 1996; Molitor and Berkelmann-Löhnertz, 2011). Notably, the extent of the infection negatively correlates with leaf size, since smaller leaves display greater infected surface than larger, older leaves (Luttrell, 1948; Jabco et al., 1985). Kuo and Hoch (1996) suggest "expanding" and "non-expanding" organs to better describe the resistance displayed by aging tissue. With regards to the duration of pathogen incubation, Molitor et al. (2012) do not report differences on leaves of different ages. In contrast, at grape level ontogenic resistance under field conditions is responsible for the decrease of the number of infected berries and the increases of the incubation time (Hoffman and Wilcox, 2002; Hoffman et al., 2002). Roznik et al. (2017) suggested that the developmental stage of plant tissue is crucial for the results of the artificial tests, which could explain the incongruence of research group results. Finally, this issue is tightly linked to different inoculation conditions since constant temperature shortens the incubation period rather than fluctuations (Spotts, 1980), the required incubation time on leaves (Spotts, 1977; Molitor et al., 2012) and shoots (Northover, 2008), and the release of ascospores are temperature dependent (Rossi et al., 2015).

Cross-inoculation experiments between fungi collected from different species (*Parthenocissus tricuspidata, P. quinquefolia, Muscadinia rotundifolia, V. labrusca, V. bourquina, V. vinifera*) demonstrated that *G. bidwellii* includes three *formae speciales (f.sp.)*, named *parthenocissi, muscadinii* and *euvitis*, with different degree of pathogenicity on the different hosts (Luttrell, 1946; Luttrell, 1948). Greenhouse assays were later performed (Jabco et al., 1985) with *f.sp euvitis* and *muscadinii* exploring leaf and petiole infection to assess the specific resistance response of four grapevine classes: *vinifera*, French and American hybrids, *rotundifolia*. The latter class showed a medium to high susceptibility to f.sp. *muscadinii*, while it resulted highly resistant to f.sp. *euvitis*; in contrast, the other three classes displayed high to medium resistance to f.sp. *muscadinii*, but medium to high susceptibility to f.sp. *euvitis*, with *vinifera* class developping larger lesions. *In vitro* cultures were also used to clarify the life cycle of the homotallic *G. bidwellii* (Jailloux, 1992). Light triggers pseudothecia maturation and differentiation, but during the first phase of mycelial growth, it inhibits pseudothecial growth and it induces pycnidia production. The optimal temperature for mycelial growth and pycnidia development (25°C) is also adequate for the first phase of pseudothecial growth, however lower temperatures are necessary for the maturation and differentiation of the ascospores. Temperature was also found to influence the dynamic and the number of pycnidia production and conidial germination (Onesti et al., 2017b).

Anthracnose

The causal agent of AN is commonly attributed to the hemibiotrophic *Elsinoë ampelina* Shear, whose asexual morph is *Sphaceloma ampelinum* de Bary (**Figure 5**). Moreover, some authors reported also *Colletotrichum* spp., another ascomycete, associated with the disease symptoms, such as *C. nymphaeae*, *C. fructicola* and *C. gloeosporioides* (Sawant et al., 2012; Liu et al., 2016; Guginski-Piva et al., 2018), *C. goditiae* (Baroncelli et al., 2014; Zapparata et al., 2017) and a complex of species that were grouped as *C. viniferum* (Yan et al., 2015). However, according to another author, *Colletotrichum* spp. is the causal agent of "ripe rot" in grapevine (Huang, 2016; Wilcox et al., 2017). Therefore, this association between pathogen and disease is still controversial.

The symptoms reported for the disease are similar, regardless of the causal agent. The pathogen attacks all aerial green parts of the plant, including fruit stems, leaves, petioles, tendrils, young shoots, and berries, however lesions of the pathogen are more common and distinctive on young shoots and berries (Magarey et al., 1993a) (**Figures 3H–K**). Structures of the pathogen are also found in dead tissues of the host, such as branches and fruits, making it difficult to eliminate the initial inoculum source of the pathogen (Magarey et al., 1993b). Infection on branches is recognizable by small circular reddish spots, afterwards the spots enlarge, forming a depression with gray center and rounded or angular edges, and eventually becomes surrounded by reddish brown or violet edges. Later on, the lesions may coalesce, killing the infected tissues. In some cases, slightly raised edges surrounding the lesions are also visible (Magarey et al., 1993a). On berries, the symptoms appear similar, initially, reddish circular spots appear, which evolve in size, and normally become slightly sunken. As spots grow, the center of the lesion turns whitish-gray, while edges assume a reddish-brown to black color. At this stage the lesion resembles a "bird's eye", hence the popular name for the disease (Magarey et al., 1993a; Jang et al., 2011). Young leaves are more susceptible to infection than older leaves. The initial lesions are small, circular and chlorotic. The lesions become larger with gray centers and brown to black margins with round or angular edges. The center of the lesions becomes dry and ash, and often drops out, forming a "shot hole" appearance (Magarey et al., 1993a). The size of the lesions may vary with the degree of resistance of the host genotype. While bigger and circular spots are reported for susceptible cultivars, smaller and with irregular shape lesions were observed in resistant cultivars (Kono et al., 2012) (**Figures 3H–K**).

The identification of resistance sources to AN has been a major task for grapevine breeders, especially in Brazil and China. Screenings for AN resistance in fields with ongoing natural infections have effectively been applied for genetic improvement goals (Fennell, 1948; Mortensen, 1981; Wang et al., 1998; Li et al., 2008). However, in the field the disease is highly influenced by the climatic conditions, requiring some years of evaluation to produce robust data. This, in addition to the perennial nature of the host, makes the analysis time consuming and costly.

Therefore, many authors focused their efforts on establishing alternative methodologies based on artificial infection with conidia on potted plants in greenhouses and on detached cane, detached leaf or leaf piece assays. For these purposes, *in*

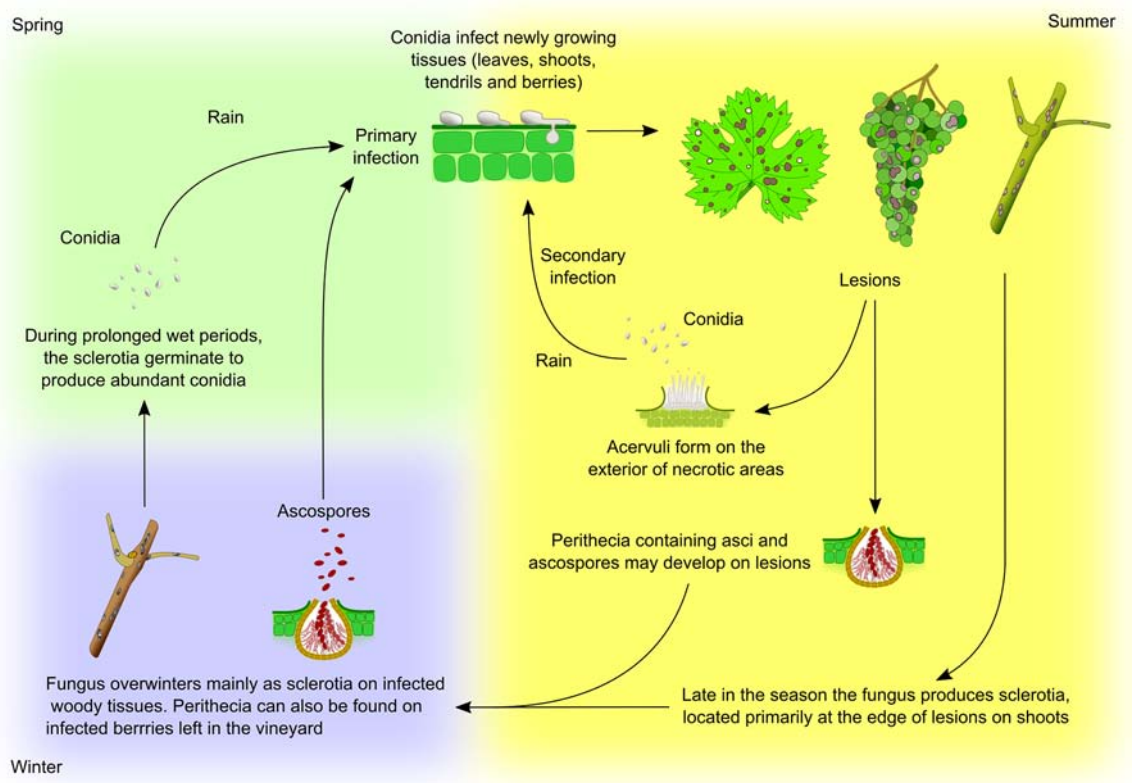

FIGURE 5 | *Elsinoë ampelina* life cycle. The fungus overwinters mainly in infected canes as sclerotia, dense mycelial masses which are formed in autumn at the edge of lesions on shoots. In spring, sclerotia develop stromata on which, under humid conditions, produce abundant conidia. These conidia are then carried by rain or free water to young, rapidly growing green tissues (leaves, shoots, tendrils and young berries) where they germinate causing the primary infection. Conidia are by far the most important source of primary inoculum in spring. The fungus can also overwinter on infected berries, left on the vine or vineyard floor, as conidia or ascospores, which may also cause primary infections. Once the disease is established, the pathogen produces lesions upon which acervuli form and produce conidia which, dispersed by raindrops, serve as secondary source of inoculum for the rest of the growing season. During periods of humid weather, conidia can be released the entire spring and summer providing continuous infection. Heavy rainfall and warm temperatures are ideal for disease development and spread. At times pseudothecia, containing asci and ascospores, develop on the lesions (Wilcox et al., 2017).

vitro production of conidia of *E. ampelina* proved to be a major challenge, due to its poor and unstable sporulation in culture. Even though Hopkins and Harris (2000) produced conidia from 3 to 4 week old cultures of *E. ampelina* maintained on potato dextrose agar (PDA), other researchers were unable to reproduce these results (Santos et al., 2018a; Santos et al., 2018b). In order to improve the yield of conidia, Yun et al. (2006) transferred the pathogen in Fries liquid medium and incubated it in a shaking incubator (140 rpm) at 28°C for 10 days. Cultures were then transferred to a V-8 juice agar medium and incubated at 28°C under a near ultraviolet lamp for two days to stimulate spore production. Kono et al. (2009) took a step forward and was the first to test the effect of culture conditions on conidia formation of *E. ampelina*. The study revealed the following three indispensable conditions for conidial production: (i) the density of colonies in pre-culture should be lesser than 2.5 colonies per cm^2; (ii) depending on colony density, colonies should grow for 4 to 9 days on PDA; and (iii) grown colonies should be cultured in water with shaking (150 rpm) for 8 to 10 h in the dark at 24°C. Santos et al. (2018a) used a similar protocol. Conidial suspensions were obtained by placing *E. ampelina* cultures in rainwater and shaking at 200 rpm in darkness for 7 days to induce sporulation. Mortensen (1981) set up greenhouse screenings for resistance to *E. ampelina*, however the protocol was deemed not reliable; later Hopkins and Harris (2000) optimized greenhouse screening by misting the seedlings with a suspension containing 10^6 conidia mL^{-1} and placing the inoculated seedlings in a moist chamber at 24°C for 48 hours, followed by 8 days on a greenhouse bench. However, consistent results were obtained at temperatures ranging from 20 to 28°C. Inoculations using conidia suspension were also performed on detached canes (Santos et al., 2018a), on detached leaves (Tharapreuksapong et al., 2009; Kono et al., 2012) and on leaf pieces (Poolsawat et al., 2012). After the inoculation, the tissues were maintained under high humidity at 25-27°C. The symptoms were assessed in variable times of incubation. The results obtained by these assays were consistent and were applied for pathogenicity analysis (Tharapreuksapong et al., 2009; Santos et al., 2018a), germplasm characterization (Kono et al., 2012) and genetic analysis (Tantasawat et al., 2012; Poolsawat et al., 2013).

As an alternative to artificial infection with conidia, Yun et al. (2006) developed a bioassay based on fungal cell-free culture filtrate (CFCF). In this case, the pathogen was cultivated in Fries

medium for 21 days at 28°C, after then the CFCF was obtained by centrifugation and sterilized through ultra-filtration (0.2 µm). The CFCF was applied on wounded young leaves. The results demonstrated that the bioassay with culture filtrates of the pathogen were consistent with those from pathogen inoculation in the greenhouse and screening in the vineyard. The methodology was further validated by Yun et al. (2007) and applied for germplasm characterization (Jang et al., 2011; Louime et al., 2011) and genetic analysis (Kim et al., 2008).

Powdery Mildew, Black Rot and Anthracnose Control Measures

Effective disease control encompasses the combination of sanitary as well as cultivation practices, the use of resistant or at least less susceptible grapevine varieties, the application of fungicides, and decision support systems (e.g. Wilcox, 2003; Hoffman et al., 2004; Molitor and Beyer, 2014). Cultivation and sanitation practices are essential to manage PM, BR and AN; for instance, adequate pruning and removal of leaves covering clusters provide conditions to reduce infections of PM. The removal of mummified bunches, infected canes and tendrils from the vineyard allows for the reduction of the primary inoculum of BR and AN, as well as covering mummies or infected berries on the ground by soil cultivation or mulching (Wilcox et al., 2017). Especially for BR, abandoned vineyards should be cleared since BR can build up high amounts of inoculum that can escape under favorable weather conditions and cause unexpected serious damages in the neighboring vineyards (Ullrich et al., 2009).

Due to the susceptibility of *V. vinifera* cultivars to PM, BR and AN, fungicide applications are necessary to control these diseases. In particular, thallus of *E. necator* develops almost completely outside of the infected tissues on the leaf and bunch surface, therefore the fungus is susceptible to topical applications of several contact active ingredients (Wilcox et al., 2017). Since the 19th century, sulfur remains the most widely used fungicide, due to its low cost and protectant-curative action (Wilcox et al., 2017). The persistence of sulfur efficacy relies on the lack of resistance development, depending on its multi-site mechanism of action by direct contact and vapour phase: respiration inhibition, chelation of heavy metals needed for biochemical pathways, and disruption of protein function (Oliver and Hewitt, 2014). It causes the damage of cellular membrane followed by loss of water and therefore death of the fungus by dehydration. Other than sulfur, several single-site synthetic fungicides are effective against PM, including contact, translaminar and systemic products, with specifically targeted mechanisms of action. Among them, mitosis and cell division inhibitors (e.g. benzimidazoles) and cell membrane synthesis alteration *via* ergosterol biosynthesis inhibitors (e.g. triazoles); different mechanisms concern respiration chain inhibition *via* quinon inhibitors (e.g. strobilurines) or succinate dehydrogenase inhibitors; and signal transduction inhibition (e.g. azanaftalenes) (Oliver and Hewitt, 2014; Wilcox et al., 2017). Alternation of active ingredients with different modes of action is important to avoid the development of pathogen resistance, especially towards single-site systemic fungicides. Regular plant protection with chemical fungicides is the strategy of choice to control BR. High efficacy was observed with the fungicide classes of demethylation inhibitors (DMI), quinone outside inhibitors (QoI) and dithiocarbamates (Molitor and Beyer, 2014). Analogously to PM and BR, AN control relies on prophylactic fungicide applications. The first measure against the disease is to reduce the inoculum source by the elimination of cultivation remains and dormant spray of lime sulfur (Magarey et al., 1993b). The initial development of the fungus occurs outside the green tissues, resembling the conditions described for PM. For chemical control of the AN disease, multi-site active fungicides based on copper (e.g. copper hydroxide, copper oxychloride, cuprous oxide and copper sulfate) and on the chemical groups dithiocarbamate, phthalimide, phthalonitrile and anthraquinon may be employed. Single-site synthetic fungicides are also effective acting on cell division (e.g. thiophanate), on extracellular inhibitor of quinone (e.g. methoxy-acrylate) and on sterol biosynthesis (e.g. triazoles) (FRAC, 2019).

Due to the potential negative impacts of fungicide application, non-synthetic chemicals and organic control measures are also used to regulate these three diseases. For organic viticulture, applications of copper and sulfur are recommended, but generally they are less effective in comparison to the synthetic active compounds (e.g. Loskill et al., 2009; Wilcox et al., 2017). Nowadays, organic management against PM can rely, other than sulfur, on non-toxic substances such as botanical oils and inorganic salts, acting by contact with the fungal thallus (Wilcox et al., 2017). The application of *Ampelomyces quisqualis* (hyperparasite fungus) at the time of chasmothecia formation can help in reducing the overwintering inoculum of *E. necator* (Pertot et al., 2017). Concerning organic BR control, the application of copper is insufficient but in combination with sulfur showed a clearly higher effect to reduce the disease impact (Loskill et al., 2009). Moreover, first attempts with plant extracts from *Primula* roots and *Hedera helix,* containing saponine as active compound, showed clear effects in greenhouse experiments. However, under field conditions the activity was low due to the high water solubility (Koch et al., 2013). Consequently, in organic farming, it is crucial to integrate adequate sanitation, new varieties with genetic resistance or high tolerance, application of forecast models and web-based decision support systems (Molitor et al., 2016; Onesti et al., 2016) for the optimization of the fungicide spray regime. Regarding AN, plant extracts from *Chaetomium cupreum* and *C. globosum*, antagonistic microorganisms such as *Trichoderma harzianum*, *T. hamatum*, *Penicillium chrysogenum* and the natural antibiotic substances Rotiorinol, Chaetoglobosin-C and Trichotoxin A50 have been tested and proved to reduce AN incidence on leaves, shoots and grapes (Soytong et al., 2005). Bacterial antagonists are also cited for organic control, like *Bacillus* species strains TS-204 and TL-171 (Sawant et al., 2016).

Ascomycete Resistance to Fungicide

To date, more than 50 modes of action have been identified for fungicides (FRAC, 2019); those ones that have a single-site mode of action are more problematic since resistance can rapidly evolve

by a single mutation (Brent and Hollomon, 2007). Pathogens show cross resistance to compounds with the same mode of action but not to the other ones (Hollomon, 2015).

Among the widely employed fungicides used to control PM are the sterol DMI and QoI. *E. necator* resistance to DMI was reported in the 80s from California, Portugal and Australia (Ogawa et al., 1988; Gubler et al., 1996; Steva and Cazenave, 1996; Ypema et al., 1997; Savocchia et al., 1999). The DMI resistance is a multigenic trait, but with one major mechanism involving a single mutation in the gene *CYP51* coding for the cytochrome P450 lanosterol C-14α demethylase. Studies on DMI fungicide resistance revealed several possibilities to confer reduced sensitivity: (i) mutation of *CYP51*; (ii) overexpression of *CYP51*; (iii) overexpression of transporter coding for efflux pumps and (iv) other unknown mechanisms able to confer weak resistance (Délye et al., 1997a; Délye et al., 1998; Hamamoto et al., 2000; Schnabel and Jones, 2001; Hayashi et al., 2002; Lupetti et al., 2002; Corio-Costet et al., 2003; Stergiopoulos et al., 2003; Wyand and Brown, 2005; De Waard et al., 2006; Ma et al., 2006; Leroux et al., 2007; Luo et al., 2008; Cannon et al., 2009; Kretschmer et al., 2009; Sombardier et al., 2010; Leroux and Walker, 2011; Cools and Fraaije, 2013; Frenkel et al., 2015). *E. necator* is one of the first fungi for which it was demonstrated that a point mutation in *CYP51* is associated with DMI resistance. A mutation in codon 136 converts tyrosine (Y) to phenylalanine (F), reducing the sensitivity to the fungicide (Délye et al., 1997a). Moreover, a nucleotide substitution in position 1119 (A1119C) increases the *CYP51* expression causing a comparable lower sensitivity to the fungicide (Frenkel et al., 2015). QoI fungicides inhibit mitochondrial respiration by binding to the cytochrome bc1 enzyme complex (complex III) at the Qo site, blocking the electron transfer to cytochrome c1, and preventing the synthesis of adenosine-5′-triphosphate (ATP). Several point mutations in the *cytochrome b* (*CYTB*) gene confer QoI resistance (Gisi et al., 2002). *E. necator* resistance to QoI was initially described in the United States (Wilcox, 2005; Baudoin et al., 2008; Miles et al., 2012) and it is mainly associated with a point mutation in the codon 143 of *CYTB* that converts glycine (G) to alanine (A) (Bartlett et al., 2002; Ma and Michailides, 2005; Dufour et al., 2011). Recently, the emergence of *E. necator* resistance to other fungicides was reported, such as metrafenone, a benzophenone of which mode of action is still not known, and boscalid, a fungicide that inhibits the activity of the enzyme succinate dehydrogenase (Kunova et al., 2016; Cherrad et al., 2018).

G. bidwellii resistance to fungicide is not well studied even if the first report of DMI fungicide reduced sensitivities dates back to 1986 in France (Thind et al., 1986). Different field experiments demonstrated that DMI and QoI are almost 100% efficient in the BR control (Lafon et al., 1984; Ellis, 1986; Wilcox and Riegel, 1996; Wilcox and Riegel, 1997; Wilcox et al., 1999; Wilcox, 2000; Harms et al., 2005; Loskill et al., 2009; Tomoiaga and Comsa, 2010). Moreover, sequence analysis pinpoints that *G. bidwellii* has a low risk to generate QoI resistance (Miessner et al., 2011).

Fungicide resistance in *E. ampelina* has not yet been characterized. So far just one report describes the reduced sensitivity of the pathogen to carbendazim, a methyl benzimidazole carbamate fungicide, that inhibits microtubule assembly during mitosis (Deokate et al., 2002).

GENETIC VARIABILITY

Molecular Marker Development

Erysiphe necator

Variations in the overwintering strategies have been observed in *E. necator* specimens in correlation to their geographic location. Given the scarcity of information about PM epidemiology, in the 90's Délye and co-workers (Délye et al., 1997b; Délye and Corio-Costet, 1998) carried out a number of studies based on the use of RAPD (Random Amplified Polymorphic DNA) molecular markers, as well as mutagenized *CYP51* which encodes a eburicol 14α-demethylase, a highly conserved cytochrome P-450 enzyme essential for sterol biosynthesis (Délye et al., 1999). Analyzing the genetic variation among populations of *E. necator* from Europe, Asia, North-Africa and Australia, the existence of two main genetic groups was identified: the flag-shoot (A) and the ascospores (B) biotypes. In contrast, Cortesi et al. (2004) using the Inter-Simple Sequence Repeat (ISSR) markers did not observe any correlation between overwintering strategies and genetic groups. To better understand the distribution of the two biotypes, more in-depth research was devised. Some studies employed tagging of specific sequences by PCR (e.g. Brewer et al., 2011; Oliveira and Cunha, 2015), while others reported the use of dominant markers such Amplified Fragment Length Polymorphisms (AFLPs) (Núñez et al., 2006) and Random Amplification of Polymorphic DNA (RAPDs) (Péros et al., 2005). Codominant markers, i.e. Sequence Characterized Amplified Regions (SCARs), Random Fragment Length Polymorphisms (RFLPs), and Single Nucleotide Polymorphisms (SNPs), have been adopted on *CYP51* and *Entub* genes with the same aim (Evans et al., 1997; Stummer et al., 2000; Hajjeh et al., 2005; Amrani and Corio-Costet, 2006; Miazzi et al., 2008). More recently, Single Sequence Repeats (SSRs) were proven to be the most effective markers because of their high polymorphism, co-dominance and reproducibility. SSRs are widely used but their application in fungi was limited due to scarce sources of genetic diversity; moreover microsatellites are less abundant and present a reduced number of repeats in fungi (Dutech et al., 2007). One of the first attempts to use SSR markers to distinguish genetic groups of *E. necator* was made by Péros et al. (2006a). Wakefield et al. (2011) used cDNA-AFLPs to investigate the transcriptional changes during pathogenesis stages. Currently, the leading study on this topic is Frenkel et al. (2012) which developed microsatellite markers to investigate population structure of the causal agent of grapevine PM in North America, using 11 transcriptome-based microsatellites. According to these studies—despite a higher amount of genetic group A isolates being usually found early in the growing season, slightly giving away to group B over the course of the epidemics (Amrani and Corio-Costet, 2006; Miazzi et al., 2008)—the separation between the two biotypes is not dependent on the primary source of inoculum (Núñez et al., 2006). Moreover, in Europe the distribution of the two biotypes seems to be linked to the geographic location and host cultivar though in some

cases both groups were detected in the same vineyard or even on the same plant (Amrani and Corio-Costet, 2006; Oliveira and Cunha, 2015).

Guignardia bidwellii

G. bidwellii-specific SSR markers appeared almost ten years later than *E. necator* markers. Only recently, Narduzzi-Wicht et al. (2014) developed 11 specific SSR markers, which were used to evaluate more than 1300 specimens, albeit without finding a relation between the species/cultivar of *Vitis* host, and the genotype of the infecting *G. bidwellii*. These 11 SSR markers were also used to analyze 37 strains of *G. bidwellii* and to assess the genetic variability of the fungus. This analysis revealed the presence of 56 haplotypes from 421 analyzed berries divided into four main subpopulations, pinpointing that the sexual reproduction is a crucial step in the progression of the epidemic in the vineyard (Rinaldi et al., 2017).

Elsinoë ampelina

The genus *Elsinoë* includes at least 75 species causing diseases on many plant hosts, including economically important crops. Hyun et al. (2001) performed a molecular characterization of several *Elsinoë* strains using RAPD markers, dominant markers with a limited application in genetic diversity studies. More recently, genome sequencing approaches allowed the revision of the fungus taxonomy, using both DNA sequences and published morphological data (Fan et al., 2017). As of today, codominant markers (SSRs or SNPs) are not available for genetic studies. Very little is known about *E. ampelina* genetic variability. First evidences of the pathogen variability were based on morphological characterization, which revealed polymorphism of colony size, colony color/appearance and conidial morphology (Poolsawat et al., 2009; Mathukorn, 2012). However, the morphological characteristics are too variable and do not necessarily reflect the genetic diversity (Poolsawat et al., 2009; Poolsawat et al., 2010). Therefore, molecular characterization is required to precisely differentiate the isolates. The use of the dominant RAPD markers confirmed the high pathogen diversity in Thailand (Tharapreuksapong et al., 2009; Poolsawat et al., 2010). It should be noted that, in this instance, genetically divergent isolates have shown different levels of pathogenicity. For example, Poolsawat et al. (2010) analysed five isolates, representing four genetically different groups, testing nine genotypes of grapevine. All isolates were pathogenic to susceptible genotypes: in particular, the host genotypes Wilcox321 and Illinois547-1 were highly resistant to all isolates. NY65.0550.04 was highly resistant to most isolates except Nk4-1. NY88.0517.01 and NY88.0507.01 were resistant only to some isolates and NY65.0551.05 was susceptible to most isolates except Nk5-1. These results suggest the presence of different resistance genes in the host and corresponding avirulent genes of the pathogen.

Within *Colletotrichum* spp., only *C. gloeosporioides* has 39 SSR markers identified (Penet et al., 2017) with a range of 2-29 alleles, enabling genetic diversity studies without the need of previous sequencing. However, there are no studies reporting the extensive use of these markers for isolate characterization. So far, the publications exploring *Colletotrichum* diversity and phylogeny involved the sequencing of genomic regions (Baroncelli et al., 2014; Guginski-Piva et al., 2018).

Barcoding and Genome Sequencing

Erysiphe necator

The first internal transcribed spacer (ITS) of ribosomal gene sequence from *E. necator* was released in 1999 (Saenz and Taylor, 1999): this enabled the identification of primers unique to *E. necator* to differentiate this fungus from other causal agents of PMs (Falacy et al., 2007). Since then several sequences have been deposited and nowadays there are 3380 nucleotide accessions available at the NCBI (National Center for Biotechnology Information), whereas 6681 genes are reported in the Ensembl Fungi database. However, since control of PM infection in the field is based on the use of chemical fungicides and canopy management, a deep focus on '-omics' approaches is fundamental to get a broad picture of the pathogen profile. Given the wealth of know-how and technologies becoming available, in the last decade this resulted timely and feasible and the genome of five *E. necator* isolates has been released in 2014 (Jones et al., 2014).

Causal agents of PMs (Ascomycota) are the most important biotrophic fungi together with rusts (Basidiomycota). In the last decade the genome of the most impacting pathogens belonging to Erysiphales were deciphered (Spanu et al., 2010; Wicker et al., 2013; Hacquard, 2014; Jones et al., 2014). Surprisingly the genome-size of these biotrophic pathogens (≈125Mb in *E. necator*) is 3-4 times larger than other Ascomycetes. Sanger sequencing of *Blumeria graminis* (Spanu et al., 2010) and shotgun approaches on *E. necator* (Jones et al., 2014) uncovered that genome expansion is a consequence of transposable element (TEs) and microsatellite accumulation, constituting more than the 60% of the total genome. Moreover, genes encoding enzymes involved in repeat-induced point mutations (RIPs), a natural mechanism to prevent TE accumulation, were lost. Genes, whose products participate in the synthesis of primary and secondary metabolites not required for biotrophy, have been lost too (Hacquard, 2014). This suggests that the high capacity of fungal genome to adapt to different environmental conditions is closely linked to their biotrophic life cycle (Bindschedler et al., 2016). Following the sequencing of five wild strains of *E. necator*, the data comparison confirmed the large number of repetitive elements and the difference in their copy number variations (CNVs) among isolates (Jones et al., 2014). This study demonstrated the adaptive role of CNVs in the establishment of resistance to DMI (sterol demethylase inhibitors) fungicides since structural variations are related to their target protein EnCYP51 (cytochrome P450 lanosterol C-14α-demethylase).

Guignardia bidwellii

The identification of *G. bidwellii* commonly relied on morphological analyses and on the observation of the symptoms on the affected plants (Kong, 2009), however these parameters are not sufficient for specific pathogen identification. Currently, a few hundred *G. bidwellii* reference sequences are available in public databases, most of them related to the ITS1 and ITS2 of ribosomal RNA genes (*18S* rRNA and *28S* rRNA), calmodulin

and beta-tubulin genes. Some of these sequences (ITS1-2 and calmodulin genes) were used to confirm that Boston ivy infections were due to *G. bidwellii* (Kwon et al., 2015) and to analyze *G. bidwellii* samples collected from several grapevine cultivars and ornamental plants suggesting a specificity at the host genus level (Wicht et al., 2012) (**Figure S1A**). Detailed phylogenetic trees were generated based on ITS, actin, TEF1A and GAPDH sequences (Zhang et al., 2013). The genetic relationship of *G. bidwellii* (asexual morph *P. ampelicida*) to other *Phyllosticta* species suggested to consider the *parthenocissi* form as a new species.

Currently, full genome sequencing of *G. bidwellii* is not yet available. This would greatly advance the wealth of biological information of this pathogen and it will certainly enhance the potential development of tools aimed to control infections.

Elsinoë ampelina

The recognition of AN mostly relies on the observation of symptoms on the plants and/or of fungal morphology by *in vitro* culture, as well as characterization of conidia (Mortensen, 1981; Fan et al., 2017). Recent studies have employed molecular tools to identify isolates using sequences of fungal barcode regions (Seifert, 2009; Schoch et al., 2012), which aim at the characterization and differentiation within the fungal species (Guginski-Piva et al., 2018; Santos et al., 2018a). Sequencing analysis of a selection of genes from *E. ampelina* is also quite recent; the sequencing of ITS, TEF1A and HIS3 in 39 *E. ampelina* isolates collected in 38 vineyards in southern and south-eastern Brazil revealed low genetic variability (Santos et al., 2018b). HIS3 sequence resulted to be the most informative, enabling the grouping of isolates into five haplotypes. Using the same genes, Santos et al. (2018a) compared the sequences of 18 isolates from Brazil and 17 isolates from Australia, where low levels of genetic variability were also detected. Remarkably, ITS and TEF sequences obtained from 35 isolates were identical. Polymorphism was observed only for HIS3 sequences, showing four distinct haplotypes. One of them was most predominant (82.9%) and was observed in both countries, other two were found exclusively in Brazil and one uniquely in Australia. The authors also found cultivation and conidial variation among the isolates, however no relationship was observed between the haplotype network structure and morphological characteristics of the isolates. Different levels of pathogenicity were observed, but this was not related to the origin of the isolates. Currently, there are no reports of complete sequencing, or scaffolding of the genome of *E. ampelina* in public databases. On NCBI 913 nucleotide sequences are available for this fungus, however 676 ESTs of them are related to the expression of host genes when this is challenged by the pathogen. Thus, only 236 deposited sequences are fragments of the genome of *E. ampelina*. Among these, ITS1 and ITS2 are the most representative with 78 and 79 deposited sequences, respectively, as well as the sequence for the 18S rRNA small subunit (SSU rRNA) with 36 sequences deposited and 42 sequences deposited as ssRNA, while for 5.8S and 28S, there are 78 and 36 deposited sequences respectively (**Figure S1B**).

Sequencing of the barcode regions was also used to identify *C. nymphaeae* as the causal agent of AN in China (Liu et al., 2016) and *C. godetiae* as the causal agent of AN in Italy (Zapparata et al., 2017) and the United Kingdom (Baroncelli et al., 2014). Among the various *Colletotrichum* spp. reported as causing AN, only *C. nymphaeae* and *C. fructicola* have scaffold sequences deposited on NCBI. With regards to *C. nymphaea* (access: JEMN01000491.1) 14,404 genes have been found within a 49.96 Mb genomic region, with 52.7% GC content. While for *C. fructicola* three sequencing projects are reported, one in the contig assembly phase and two with the scaffold of the genome; the bio design with greater detail of the genome (access: ANPB00000000.1) has a sequencing coverage of 55.61 Mb, with 53.5% GC content, where 15,469 genes and 88 pseudogenes were annotated, totaling 15,381 deduced proteins.

GRAPEVINE-ASCOMYCETE INTERACTION

Resistance Loci Identification

Powdery Mildew

Conidia of *E. necator* germinate on green tissues of living grapevine plants producing a infection vesicle that penetrates the surface and allows the mycelium to grow between the cells. To cope with the many microorganisms and potential pathogens on their surface, plants have evolved a system of basal immunity. This non-host resistance is based on the recognition of a pathogen-associated molecular pattern (PAMP), probably including chitin in PM (Qiu et al., 2015), sensed by an extracellular receptor-like kinase (RLK) (Dry et al., 2010). The response consists of the production of extracellular antimicrobial compounds and accumulation of molecules to reinforce cell wall as callose papillae. In grapevine-*E. necator* interaction, PAMP-triggered immunity (PTI) can be overcome by the secretion of fungal effectors, detected by the plant and able to trigger effector-triggered immunity (ETI) mediated by resistance (*R*) genes to reach programmed cell death (PCD) (Gadoury et al., 2012).

With regards to PM resistance, in the last decades, it emerged that American and Asian *Vitis* represent a valuable source of *R* genes, which are localized within *R*-loci or genomic intervals (**Table 1**). *Run1* (Resistance to *Uncinula necator* 1) is a single dominant locus on chromosome 12 known to confer high resistance to *E. necator* detected in *M. rotundifolia* (Bouquet, 1986; Barker et al., 2005). Introgressed into a *V. vinifera* background through marker-assisted selection (MAS) (Pauquet et al., 2001), it was found to co-segregate with the *Rpv1* (Resistance to *Plasmopara viticola* 1) locus and to encode full-length and truncated TIR-NBS-LRR (Toll/interleukin-1 receptor-nucleotide-binding site-leucine-rich repeat) resistance proteins (Feechan et al., 2013). Surveys on resistant cultivars showed that this locus is involved in the induction of PCD within penetrated cells at 24 and 48 hours post-inoculation (hpi) (Dry et al., 2010). Subsequently, the *Run2.1* and *Run2.2* loci variants (haplotypes) were identified on chromosome 18 in *M. rotundifolia* 'Magnolia' (Riaz et al., 2011), while *Ren5* (misnamed, actually *Run3*) was

TABLE 1 | *R*-loci associated with powdery mildew (*Run/Ren*) and black rot (*Rgb*) resistance [improved based on VIVC web source developed by Maul et al. (2012), Merdinoglu et al. (2018), and Hausmann et al. (2019)].

Locus	Chr	Origin of resistence	Genotype of origin	Resistence level	Associated marker	Reference
Ren1	13	*V. vinifera*	Kishmish vatkana	Partial	UDV-020 VMC9h4-2 VMCNg4e10.1	Hoffmann et al., 2008 Hoffmann et al., 2008 Hoffmann et al., 2008
Ren2	14	*V. cinerea*	Illinois 547-1	Partial	CS25	Dalbó et al., 2001
Ren3	15	American *Vitis*	Regent	Partial	UDV-015b VVIv67 ScORA7-760 VChr15CenGen02 GF15-28/VVIv67 ScORGF15-02 GF15-42	Welter et al., 2007 Welter et al., 2007 Akkurt et al., 2007 van Heerden et al., 2014 Zyprian et al., 2016 Zendler et al., 2017 Zendler et al., 2017
Ren4	18	*V. romanetii*	C166-043 C87-41	Partial	VMC7f2 SNPs	Riaz et al., 2011 Mahanil et al., 2012
Ren5	14	*M. rotundifolia*	Regale	Total	VMC9c1	Blanc et al., 2012
Ren6	9	*V. piasezkii*	DVIT2027	Total	PN9-057 PN9-068	Pap et al., 2016 Pap et al., 2016
Ren7	19	*V. piasezkii*	DVIT2027	Partial	VVIp17.1 VMC9a2.1 minor QTL of Ren6	Pap et al., 2016 Pap et al., 2016
Ren8	18	American *Vitis*	Regent	Partial	minor QTL of Ren3	Zyprian et al., 2016
Ren9	15	American *Vitis*	Regent	Partial	CenGen6	Zendler et al., 2017
Ren10	2	American *Vitis*	Seyval blanc		S2_17854965 Haploblock validation	Teh et al., 2017 Teh et al., 2017
Run1	12	*M. rotundifolia*	VRH3082-1-42	Total	VMC4f3.1 VMC8g9 49MRP1.P2 CB53.54	Barker et al., 2005 Barker et al., 2005 Feechan et al., 2013 Feechan et al., 2013
Run2.1	18	*M. rotundifolia*	Magnolia	Partial	VMC7f2 VMCNg1e3 VVIn16	Riaz et al., 2011 Riaz et al., 2011 Riaz et al., 2011
Run2.2	18	*M. rotundifolia*	Trayshed	Partial	VMC7f2	Riaz et al., 2011
Rgb1	14	*V. cinerea*	Boerner	Partial	Gf14-42	Rex et al., 2014
Rgb2	16	*V. cinerea*	Boerner	Partial	VChr16c	Rex et al., 2014

mapped on chromosome 14 in *M. rotundifolia* 'Regale' (Blanc et al., 2012). Resistance to *E. necator* due to PCD was also observed in 'Kishmish vatkana' and 'Dzhandzhal kara'. These related cultivars share the *Ren1* (Resistance to *E. necator* 1) locus carried on the chromosome 13 (Hoffmann et al., 2008; Coleman et al., 2009) and are an exception among the PM resistance donors since they belong to the *V. vinifera proles orientalis*. Very recently, a genome-wide characterization revealed role of NBS-LRR genes during PM infection in *V. vinifera* (Goyal et al., 2019).

In the same years, several Quantitative Trait Loci (QTL) analyses were carried out with the aim to identify new PM resistance loci. Partial resistance is conferred by major QTLs found on different chromosomes. *Ren2* on chromosome 14 confers race-specific resistance in *V. cinerea* (Dalbó et al., 2001; Cadle-Davidson et al., 2016). *Ren*3 on chromosome 15–derived from an undetermined American *Vitis* species–was localized in the variety Regent (Welter et al., 2007) and recently found to determine race-specific hypersensitive response by two different regions on that chromosome; in fact, Zendler et al. (2017) defined the *Ren3* limit and identified *ex novo* the distal *Ren9* locus. In addition, *Ren8* was mapped on chromosome 18 although with an uncertain origin (Zyprian et al., 2016). Besides the American sources, the wild Chinese species *V. romanetii* is donor of a non-race-specific and tissue-independent resistance conferred by the dominant locus *Ren4* on chromosome 18 (Riaz et al., 2011; Mahanil et al., 2012); this was introgressed into *V. vinifera* background to obtain vines able to prevent hyphal emergence from the PM agent (Ramming et al., 2011). Moreover, two major *R*-loci against *E. necator* were discovered in another Chinese species, *V. piasezkii*, *Ren6* and *Ren7* which are respectively localized on chromosome 9 and 19, and they both act in the post-infection stage bringing to PCD. The highest strength of (total) resistance is conferred by *Ren6*, even higher than *Run1*, while *Ren7* is responsible of a weak partial resistance to the pathogen (Pap et al., 2016). Finally, Teh et al. (2017) identified the new *Ren10* locus on chromosome 2 acting moderately against PM sporulation (**Table 1**).

Given the reported advances also on the molecular characterization of the studied Ascomycetes, we can assert that nowadays pathogen genetics can inform host genetics and host-pathogen interaction mechanisms. For instance, in the Eastern US, where the pathogen co-evolved with many mapped PM resistance genes, the *Ren2* locus has recently fully broken down and is no

longer detectable in the vineyard (Cadle-Davidson, 2018). Actually, in North America naturally occurring isolates displaying virulence on vines carrying the *Run* loci were already observed demonstrating that qualitative (vertical) resistance is strong, but since it is race-specific can be easily overcome (Feechan et al., 2015). By contrast, partial (horizontal) resistance–which typically is controlled by at least 4-5 QTLs–is usually more durable, particularly when it involves morphological or developmental changes in the plant, although might be prone to gradual loss (erosion) in the long term (Stuthman et al., 2007). Therefore, to achieve long lasting resistance, the combination of both types is needed; this process, named *R* gene pyramiding, relies on genetics built into vines.

Pyramiding entails stacking multiple genes leading to the simultaneous expression of more than one gene in a cultivar to develop durable resistance expression. Gene pyramiding is gaining considerable importance as it would improve the efficiency of plant (including grapevine) breeding, leading to the development of genetic stocks and precise development of broad spectrum resistance capabilities (Joshi and Nayak, 2010). Both wine and table grape breeding programs for PM resistance around the world are using this approach, providing endurance both in term of absolute disease resistance degree (e.g. Feechan et al., 2015) and of the degree fluctuation reduction during different years (e.g. Zini et al., 2019). According to the most updated information on MAS applications at European level, in France "ResDur" varieties presenting assorted combinations of *Rpv1, Rpv3, Rpv10* associated with DM and of *Run1, Ren3* and *Ren3.2* associated with PM resistance were obtained by breeding "Bouquet" varieties with American, Asian and wild *Vitis* backgrounds (Delmotte et al., 2018). In Italy, Vezzulli et al. (2019) were able to obtain pyramided genotypes carrying two or three *Run/Ren* loci, up to seven *R*-loci in total, while Foria et al. (2019) developed resistant genotypes derived from "elite" cultivars carrying *Rpv1, Rpv12* coupled with *Run1* and *Ren3.* Finally, besides increasing host diversity and complexity, to achieve higher durability, populations of biotrophic pathogens should be regularly monitored for their virulence frequencies and virulence combinations (Miedaner, 2016).

In order to indirectly dissect PM resistance, an alternative approach relies on the biological candidacy of susceptibility (*S*) genes. Unlike *R* genes, *S* genes are required for successful pathogen infection, and thus are considered essential for compatible plant-pathogen interactions. A state of the art about the investigation and the future application of grapevine *S* genes associated with PM susceptibility is reported in the **Text S1**.

Black Rot

In contrast to PM, for which several *R*-loci have already been discovered in various grapevine cultivars, very little is known about the genetic loci involved in BR resistance (**Table 1**). It has been observed almost a century ago that North American and French hybrids are more resistant to *G. bidwellii* infection, while *V. vinifera* is extremely susceptible (Demaree et al., 1937; Barrett, 1955; Hausmann et al., 2017). The American origin of this ascomycete explains the occurrence of genetic resistance in American *Vitis* species, due to their coevolution (Ramsdell and Milholland, 1988; Hausmann et al., 2017). Hints about the genetic segregation pattern of BR resistance were suggested in a 'Blue Lake' progeny by Mortensen (1977), but the first QTL analysis was performed much later by Dalbó et al. (2000). They studied the inheritance of BR resistance in the cross 'Horizon' ('Seyval' × 'Schuyler') × 'Illinois 547-1' (*V. rupestris* × *V. cinerea*) and constructed a genetic map based on RAPD markers for each parent. This analysis identified one QTL on chromosome 14 associated with the CS25b marker (Dalbó et al., 2000; Dalbó et al., 2001). Interestingly, the latter map interval was also associated with PM resistance though to different degrees (Dalbó et al., 2000).

Later Rex et al. (2014) carried out a QTL analysis using a cross between a cultivar that shows high resistance to BR (cultivar 'Börner', an inter-specific hybrid of *V. riparia* × *V. cinerea*) and a susceptible breeding line (V3125, 'Schiava grossa' × 'Riesling'). They performed six independent resistance tests in a climate chamber and one disease evaluation in the field, and identified two *R*-loci; the major QTL on chromosome 14, called *Resistance to G. bidwellii1* (*Rgb1*) co-mapped with the QTL identified by Dalbó et al. (2000). A second QTL on chromosome 16 (named *Rgb2*) was detected both in climate chamber and in field analysis, while the results on other minor QTLs were not reproducible. All these data suggest a polygenic nature of the resistance (Rex et al., 2014). The analysis of Sequence-Tagged Sites (STS) markers did not allow a more accurate delineation of the *Rgb2* locus position. For *Rgb1* locus, the fine mapping on chromosome 14 restricted the region of QTL to about 2.4 Mb in the reference grapevine genome and allowed the authors the development of a new marker, GF14-42, that showed a strong association with *Rgb1*. Some genes with a putative defense function were annotated in the genomic region of *Rgb1* (i.e. chitinase, RIN4-like protein, MAP kinase and F-box domain protein; Rex et al., 2014). The QTL site on chromosome 14 was also detected in another biparental mapping population derived from a cross between 'GF.GA-47-42' × 'Villard Blanc' with both parents being resistant to BR. This study revealed the presence of seven *R*-loci distributed over the genome. The major QTL on chromosome 14 in this mapping population was tightly linked to the SSR markers GF14-04 and UDV-095, both are located in the vicinity of the above mentioned markers CS25b and GF14-42 (Hausmann et al., 2017) (**Table 1**). All these data suggest that the major *R*-locus on chromosome 14, identified in three different biparental population, plays a leading role in conferring BR resistance. Finally, from the application point of view, nowadays some new bred varieties with a good field resistance to BR – carrying *Rgb* loci – are available (e.g. Töpfer and Eibach, 2016; Töpfer et al., 2018).

Anthracnose

In contrast to *E. necator* and *G. bidwellii* no QTL analysis has been published for *E. ampelina*. This is perhaps due to the fact that AN is not a major disease in the main grapevine production regions, such as Europe. However, the use of resistant cultivars, which reduces the amount of fungicide associated with the climatic break-down expected, might increase the incidence of this disease also in these regions. Therefore, the development of new varieties resistant to AN will be a major task worldwide in the near future and it is essential to develop molecular markers linked to AN resistance genes.

Resistance sources to *E. ampelina* have been identified through natural infection in field screenings (Fennell, 1948;

Mortensen, 1981; Wang et al., 1998; Yun et al., 2006; Li et al., 2008; Louime et al., 2011; Poolsawat et al., 2012), by artificial infection in greenhouse (Hopkins and Harris, 2000), by detached-leaf assays (Kono et al., 2012; Poolsawat et al., 2012) and filtrate culture (Yun et al., 2006; Kim et al., 2008; Jang et al., 2011; Louime et al., 2011). In the American genepool, variable levels of resistance to AN were found in different *Vitis* spp. Based on natural infections, Fennell (1948) reported that the majority of the American tropical grapevine species show moderate to good resistance to AN. Accessions of *V. gigas* (Fen), *V. rufotomentosa* (Small) and *V. smalliana* (Bailey) were classified as highly resistant. Similarly, Mortensen (1981) reported as sources of resistance to AN accessions of *M. rotundifolia* Michx., *V. aestivalis* ssp. *simpsonii*, *V. aestivalis* ssp. *smalliana, V. caribeae* DC (syn. *V. tiliafolia* Humb. & Bonpl.), *V. champini* Planch., *V. labrusca* L., *V. munsoniana* Simps, *V. rupestris* Scheele, *V. shuttleworthii* House and *V. vulpina* L. Field evaluations under natural infection of *E. ampelina* revealed that all the Asian *Vitis* species tested were classified as highly resistant or resistant to AN (Wang et al., 1998; Li et al., 2008). The species evaluated were *V. adstricta*, *V. amurensis*, *V. bashanica*, *V. betulifolia*, *V. bryoniifolia*, *V. davidii*, *V. flexuosa*, *V. hancockii*, *V. liubanensis*, *V. piazezkii*, *V. pseudoreticulata*, *V. qinlingensis*, *V. quinquangularis*, *V. romanetii*, *V. sinocinerea*, *V. wilsonae* and *V. yenshanensis*. In the same fields, the *V. vinifera* cvs. Cabernet Sauvignon, Carignane and Chardonnay were classified as susceptible, highly susceptible and highly susceptible, respectively.

Under natural conditions the *V. vinifera* cultivars evaluated (e.g. Alexandria, Cabernet Sauvignon, Cardinal, Carignane, Chardonnay, Chasselas Golden, Exotic, Lignan Blanc, Malaga, Muscat of Alexandria, Perlette, Red Muscat, Sultanina, Thompson Seedless) were classified as susceptible or highly susceptible to the disease (Fennell, 1948; Mortensen, 1981; Wang et al., 1998; Li et al., 2008). In contrast, a detached leaf assay with Exotic and Perlette cultivars possess moderate resistance (Kono et al., 2012). Fennell (1948) reported that, when tropical species were crossed to the highly susceptible *V. vinifera* cultivars, all the F_1 progenies were susceptible. The susceptibility was conditioned by two or three dominant genes, and the resistance transmitted as a recessive locus. A more detailed investigation to elucidate the inheritance of AN resistance of American *Vitis* species was performed by Mortensen (1981). His genetic strategy pinpointed that AN susceptibility/resistance is controlled by three genes, two dominants for susceptibility (*An1* and *An2*), and one dominant (*An3*) conferring resistance. The three genes segregate independently. When both dominant susceptibility alleles (*An1* and *An2*) are present, the phenotype is susceptible regardless of whether *An3* is present or no. If either *An1* or *An2* or both are not present, than *An3* conditions resistance and *an3* susceptibility. According to Mortensen (1981), most *V. vinifera* cultivars are homozygous dominant for both *An1* and *An2* (e.g. Cabernet Sauvignon; *An1An1*/*An2An2*/*an3an3*), complicating the introgression of AN resistance genes from American species into *V. vinifera* background. On the other hand, F_1 progenies derived from crosses between hybrids with variable levels of genetic composition from several American species (*V. cinerea*, *V. riparia*, *V. rupestris*, *V. labrusca*, and *V. lincecumii*, along with *V. vinifera*) and *V. vinifera* segregated for the resistance to AN (Kim et al., 2008; Poolsawat et al., 2013). The proportion of resistant and susceptible plants in the progenies obtained by Kim et al. (2008) suggests that the resistance is conferred by a single dominant gene. One example is the crossing between Concord (resistant) and Neomuscat (susceptible), for which a proportion of 1 resistant: 1 susceptible was obtained. The disease phenotyping was done by culture filtrates. In accordance, Poolsawat et al. (2013) reported high narrow sense heritability for AN resistance, suggesting the prevalence of additive over non-additive gene action. On the other hand, the crossing of a susceptible hybrid with a susceptible *V. vinifera* cultivar also segregated for AN resistance, suggesting the presence of susceptibility genes (Poolsawat et al., 2013).

The resistance present in the Asian species seems to be monogenic and dominantly inherited, without the presence of susceptibility genes. Wang et al. (1998) reported that all the progenies derived from the crosses between the Asian *Vitis* species *V. amurensis*, *V. davidii*, *V. piasezkii*, *V. pseudoreticulata*, *V. quinquangularis* and *V. romanetii* with *V. vinifera* were resistant to the disease, like their native parents used as resistant donor. The absence of susceptibility genes renders the Asian germplasm very interesting for breeding, since the segregation of resistance in the first generation saves time in the process of gene introgression. Finally, it is important to mention that in all progenies evaluated, a continuum variation of the AN symptoms were observed, suggesting also a quantitative inheritance of the disease resistance (e.g. Fennell, 1948; Mortensen, 1981; Yun et al., 2006). Taking all the data together, it may indicate that major genes–which explain the main variation observed–together with minor genes–confer the resistance to AN, as observed in other pathosystems. So far, only one work describes the genetic mapping of genes conferring resistance to AN (Kim et al., 2008). A RAPD marker closely linked to an AN-resistant gene was identified using bulked-segregant analysis (BSA) and was converted into a SCAR marker. The SCAR amplifies a specific band in resistant cultivars/hybrids with different American backgrounds (*V. berlandierii, V. champinii*, *V. labrusca, V. riparia, V. rupestris*) and no band in susceptible ones, mainly *V. vinifera* cultivars. However, no information was found about the use of the SCAR in routine breeding programs to assist the introgression of the resistant gene into *V. vinifera* germplasm. Recently, also RGA-SSCP markers have been found associated with AN resistance (Tantasawat et al., 2012).

Finally, AN resistance was also pursuit in an *in vitro* study carried out to select resistant cv. Chardonnay (*V. vinifera*) plants using *E. ampelina* culture filtrates (Jayasankar et al., 2000).

Transcript, Protein and Metabolite Exploration

Erysiphe necator

To date, several studies aimed to decipher the interaction between different *Vitis* genetic backgrounds and *E. necator* at different molecular levels, i.e. by analyzing transcripts, proteins and metabolites.

As an obligate biotroph, one of the most challenging steps for transcriptional characterization of *E. necator* is the harvest

of fungal tissue free of contaminating grapevine material. The technique for the isolation of fungal RNA developed by Cadle-Davidson et al. (2010) constituted a turning point. Taking advantage of it, Wakefield et al. (2011) investigated the genetic regulation of conidiation in *E. necator*. They identified new genes involved in conidiation, never found before in other filamentous fungi, probably related to the obligate biotrophic lifestyle. With the advent of next generation sequencing technologies additional progresses have been made. *De novo* transcriptomes were used to detect polymorphisms between different isolates of *E. necator* (Myers, 2012) as well as to identify candidate effector proteins specific to haustoria (Barnett, 2015). Transcriptome analyses, in addition to assisting in genome annotation (Jones et al., 2014), revealed the presence of several transcripts derived from transposable elements, indicating that they are transcriptionally active and can account for the observed genome expansion (Jones et al., 2014; Snyder, 2018).

Similarly, a large number of studies have been carried out to determine the genetic basis of resistance in *Vitis* species (**Tables 2A** and **S2A**). Plants react to pathogen attack with multiple common defense responses, such as cell wall reinforcements through callose and lignin synthesis at the site of attempted penetration and accumulation of antimicrobial secondary metabolites (phytoalexins) and of pathogenesis-related (PR) proteins (e.g. chitinases and glucanases, able to degrade fungal and oomycete cell walls). Pathogen recognition can also be followed by a burst of reactive oxygen species (ROS) culminating in a PCD at the site of invasion (Wilkinson et al., 2019). Moreover, plant defense responses are tightly regulated by hormone-mediated signaling pathways, with jasmonate (JA) and salicylic acid (SA) playing the main role (Berens et al., 2017). Genes implicated in PM resistance can be identified thanks to their activation or increased transcription during infection and/or different expression levels between resistant and susceptible species. First significant data on grapevine resistance mechanisms were showed by Jacobs et al. (1999), which observed the specific and local induction of some PR genes in susceptible *V. vinifera* cultivars in response to PM infection. Godfrey et al. (2007) characterized the *V. vinifera Germin-like proteins* (*GLP*) gene family, ubiquitous plant proteins known to be involved in the response to various stress conditions including plant defense against pathogens (Bernier and Berna, 2001), and found that *VvGLP3* was specifically and locally induced in the epidermal cells in response to pathogen attack, suggesting a role in the penetration-based defense response against PM infection.

A more comprehensive view of the networks and mechanisms mediating plant defense was obtained through high-throughput methods, able to monitor transcriptome and proteome-wide changes. Comparative transcriptomic studies revealed substantial variation in gene expression between *V. vinifera* and the resistant *V. aestivalis*, which showed a constitutively elevated expression of several PR genes, as well as higher endogenous SA levels (Fung et al., 2007; Fung et al., 2008). Moreover, the very weak PM-induced response occurring in *V. aestivalis*, unlike the major transcriptome remodelling observed in *V. vinifera*, suggests that the constitutive transcriptomic profile of the resistant genotype is already defense-oriented (Fung et al., 2008). Transcriptome and proteome changes observed in Cabernet Sauvignon during infection (Fekete et al., 2009; Marsh et al., 2010) demonstrated that the susceptible plant is able to initiate a basal defense response, since several defense-related transcripts and proteins, as well as SA, accumulate in infected leaves. This response however turns out to be insufficient to restrict fungal growth, thanks to the ability of the pathogen effectors to suppress the host defense system, as already observed in the barley (*Hordeum vulgare*)-PM agent (*Blumeria graminis* f. sp. *hordei*) interaction (Caldo et al., 2004; Caldo et al., 2006). Indeed, the expression levels of many grapevine defense-related genes reached a maximum at 12 hpi and then declined as the fungal infection became established (Fung et al., 2008).

In addition to differential transcriptional regulation, species- or cultivar-specific genes may contribute substantially to determine variable disease susceptibility among *Vitis* species (Da Silva et al., 2013; Venturini et al., 2013). Xu et al. (2009) identified several ESTs potentially associated with plant defense responses in *V. pseudoreticulata*. Gao et al. (2012a) focused on PM-induced gene expression changes in *V. quinquangularis*. Among the genes differentially expressed, a large part encoded for resistance and stress-related proteins, and some of them showed specific PM-induction only in the resistant genotype.

High-throughput transcriptomic analyses were performed more recently by next-generation sequencing (RNA-seq). Weng et al. (2014) investigated how global gene expression profile changes in response to PM infection in *V. pseudoreticulata*. They identified several genes and pathways that may contribute to PM resistance and found that the enhancement of JA pathway, systemic acquired resistance (SAR) and ROS-dependent hypersensitive responses as well as the accumulation of phytoalexins play a key role in the defense response mechanism. Jiao et al. (2015) *de novo* assembled the transcriptomes of three wild Chinese *Vitis* accessions and studied the differences with the reference genome. A large number of distinct transcripts were identified in the resistant accessions, which resulted to be highly enriched in genes involved in plant secondary metabolisms, such as biosynthesis of phenolic compounds, and defense responses. These studies, together with other research done in grapevine (e.g. Borges et al., 2013) and in several other plant-PM agent interactions (e.g. Xu et al., 2011; Fu et al., 2016; Zhu et al., 2018; Polonio et al., 2019; Tian et al., 2019), further highlight the contribution of secondary metabolites to the plant defense mechanisms. Amrine et al. (2015) studied wild and cultivated Central Asian *V. vinifera* accessions carrying a common *Ren1* locus but displaying significant variations in disease susceptibility. They identified several genes potentially involved in the *Ren1*-dependent resistance mechanisms, as well as genes whose expression levels correlated with the different levels of resistance observed among accessions. These transcriptomic data have recently been used by Goyal et al. (2019) to investigate the role played by the *NBS-LRR* genes. They identified a total of 63 PM-responsive *NBS-LRR* genes in different *V. vinifera* accessions, ranging from susceptible to partially resistant. Gene expression levels in response to PM infection changed greatly between cultivars. Some genes were either up-regulated

TABLE 2 | Transcriptomics, proteomics and metabolomics of grapevine-ascomycete interaction: *E. necator* (panel A) and *E. ampelina* (panel B).

A)

Gene or Metabolite	Description	Taxon	Genotype	Defense Response	Organ	Reference
PR proteins, e.g. chitinases (PR-2), glucanases (PR-3) and thaumatin-like (TL) proteins (PR-5)	Pathogenesis-Related protein	*V. vinifera* spp. *sativa*	Sultana, Cabernet Sauvignon	Susceptible	Leaves, berries	Jacobs et al., 1999
VvGLP3	Germin-like proteins 3	*V. vinifera* spp. *sativa*	Chardonnay	Susceptible	Leaves, berries	Godfrey et al., 2007
VvGLP4	Germin-like proteins 4					
Several gene families		*V. vinifera* spp. *sativa* *V. aestivalis*	Cabernet Sauvignon Norton	Susceptible Resistant	Leaves	Fung et al., 2007
Several gene families		*V. vinifera* spp. *sativa* *V. aestivalis*	Cabernet Sauvignon Norton	Susceptible Resistant	Leaves	Fung et al., 2008
Several gene families		*V. vinifera* spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Fekete et al., 2009
Several gene families		*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Xu et al., 2009
VaEDS1, VvEDS1	Orthologs of *Arabidopsis thaliana ENHANCED DISEASE SUSCEPTIBILITY1*	*V. aestivalis* *V. vinifera* spp. *sativa*	Norton Cabernet Sauvignon	Resistant Susceptible		Gao et al., 2010
VpWRKY1, VpWRKY2	WRKY domain transcription factor	*V. pseudoreticulata* *V. pseudoreticulata* *V. vinifera* spp. *sativa*	Baihe-35-1, Baihe-13, Baihe-13-1, 6-12-6, Guangxi-1 Guangxi-2, Hunan-1, 6-12-2, Shangnan-2, Baihe-35-2 Carignane	Resistant Susceptible Susceptible	Leaves	Li et al., 2010
Several gene families		*V. vinifera* spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Marsh et al., 2010
VpSTS	Stilbene synthase	*V. pseudoreticulata*	Baihe-35-1	Resistant		Xu et al., 2010a
VpPR10	Pathogenesis-Related protein 10	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Xu et al., 2010b
VpGLOX	Glyoxal oxidase	*V. pseudoreticulata* *V. pseudoreticulata*	Baihe-35-1 Guangxi-2	Resistant Susceptible		Guan et al., 2011
VpRFP1, VvRFP1	C4C4-type RING finger protein	*V. pseudoreticulata* *V. vinifera* spp. *sativa*	Baihe-35-1 Carignane	Resistant Susceptible	Leaves	Yu et al., 2011
STS	Stilbene synthase	*V. pseudoreticulata* *V. vinifera* spp. *sativa* *V. vinifera* spp. *sativa*	Baihe-35-1 Thompson Seedless Carignane	Resistant Susceptible Susceptible	Leaves	Xu et al., 2011b
STS8, STS13, STS16, STS17, STS22, STS23, STS27, STS31	Stilbene synthase	*V. aestivalis* *V. vinifera* spp. *sativa*	Norton Cabernet Sauvignon	Resistant Susceptible	Leaves, berries	Dai et al., 2012
Several gene families		*V. quinquangularis* *V. pseudoreticulata*	Shang-24 Hunan-1	Resistant Susceptible	Leaves	Gao et al., 2012a
VpALDH2B4	Aldehyde dehydrogenase	*V. pseudoreticulata*	Baihe-35-1	Resistant		Wen et al., 2012 (Wang et al., 2007)
VpWRKY3	WRKY domain transcription factor	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Zhu et al., 2012a
VpNAC1	NAC transcription factor	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Zhu et al., 2012b
Several gene families		*V. vinifera* spp. *sativa*	Touriga Nacional	Susceptible	Leaves	Borges et al., 2013
VpRFP1, VvRFP1	C4C4-type RING finger protein	*V. pseudoreticulata* *V. vinifera* spp. *sativa*	Baihe-35-1 Carignane	Resistant Susceptible		Yu et al., 2013a
VpEIRP1	E3 ubiquitin ligase *E. necator*-induced C3HC4-type Really Interesting New Gene (RING) finger protein 1	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Yu et al., 2013b

(*Continued*)

TABLE 2 | Continued

A)

Gene or Metabolite	Description	Taxon	Genotype	Defense Response	Organ	Reference
VpWRKY11	WRKY domain transcription factor	*V. pseudoreticulata*	Baihe-35-1	Resistant		
VpERF genes	Ethylene response factor	*V. pseudoreticulata*	Baihe-35-1	Resistant		Zhu et al., 2013
VvWRKY family	WRKY domain transcription factor	*V. aestivalis*	Norton	Resistant	Leaves	Wang et al., 2014
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible		
		V. pseudoreticulata	Baihe-35-1	Resistant		
VvNPF3.2	NITRATE TRANSPORTER1/ PEPTIDE TRANSPORTER FAMILY	*V. vinifera* spp. *sativa*	Cabernet Sauvignon	Susceptible		Pike et al., 2014
		V. aestivalis	Norton	Resistant		
trans-Resveratrol		*V. quinquangularis* Rehd.	Danfeng-2, Taishan-12, 83-4-96, Shangnan-24		Berries	Shi et al., 2014
		V. ficifolia Bunge	Weinan-3			
		V. amurensis Rupr.	Shuangyou, Zuoshan-1, Zuoshan-2			
		V. piasezkii Maxim.	Liuba-7			
		V. pseudoreticulata W.T.Wang	Hunan-1, Guangxi-1			
		V. thunbergii Sieb.et Zucc	Anlin-3			
		V. yeshanensis J.X.Chen	Yanshan-1			
		V. vinifera spp. *sativa*	Ugni Blanc, Pinot Noir, Carignane, Cabernet Sauvignon			
STS family; *trans*-Resveratrol		*V. quinquangularis*	Danfeng-2	Resistant	Leaves, berries	
		V. vinifera spp. *sativa*	Pinot Noir	Susceptible		
VpPR10-1	Pathogenesis-Related protein 10	*V. pseudoreticulata*	Baihe-35-1	Resistant		Xu et al., 2014
REN1-associated genes		*V. vinifera* spp. *sativa*	Carignane	Susceptible	Leaves	Amrine et al., 2015
		V. vinifera spp. *sativa*	Late Vavilov	Mid-Susceptible		
		V. vinifera spp. *sativa*	Husseine, Khalchili, Sochal	Mid-Resistant		
		V. vinifera spp. *sativa*	Karadzhandal	Resistant		
		V. vinifera spp. *sylvestris*	O34-16, DVIT3351.27	Resistant		
Several gene families		*V. pseudoreticulata*	Baihe-13-1	Resistant	Leaves	Jiao et al., 2015
		V. pseudoreticulata	Hunan-1	Susceptible		
		V. quinquangularis	Shang-24	Resistant		
VpCN	Disease resistance protein RxCC-like-NB-ARC	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Wen et al., 2015
VqSTS6	Stilbene synthase	*V. quinquangularis*	Danfeng-2	Resistant		Cheng et al., 2016
VpPR4-1	Pathogenesis-Related protein 4	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Dai et al., 2016
vvi-NewmiR2118 (*MIR2118*)	miRNA	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Han et al., 2016
VfMlo-like gene family	Powdery-mildew resistance locus o-like	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2016a
VpSTS, VvSTS	Stilbene synthase	*V. pseudoreticulata*	Baihe-35-1	Resistant		Jiao et al., 2016
		V. vinifera spp. *sativa*	Carignane	Susceptible		
Several gene families		*V. vinifera* spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Toth et al., 2016
VqDUF642	Domain of Unknown Function 642	*V. quinquangularis*	Danfeng-2	Resistant	Leaves, berries	Xie and Wang, 2016
VpUR9	RING-type ubiquitin ligase gene	*Vitis pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Dai et al., 2017
VqMAPKKK38	Raf-like Mitogen-activated protein kinase kinase kinase	*V. quinquangularis*	Danfeng-2	Resistant	Leaves	Jiao et al., 2017

(*Continued*)

TABLE 2 | Continued

A)

Gene or Metabolite	Description	Taxon	Genotype	Defense Response	Organ	Reference
VpEIFP1	F-box/Kelch-repeat protein	*Vitis pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Wang et al., 2017a
VpRH2	RING-H2-type ubiquitin ligase	*V. pseudoreticulata* *V. vinifera* spp. *sativa*	Baihe-35-1 Thompson seedless	Resistant Susceptible	Leaves	Wang et al., 2017b
VqWRKY52	WRKY domain transcription factor	*V. quinquangularis*	Shang-24	Resistant	Leaves	Wang et al., 2017c
VaSTS19	Stilbene synthase	*V. amurensis* Rupr.	Tonghua-3	Resistant	Leaves	Wang et al., 2017d
VpTNL1	TIR-NB-ARC-LRR R protein	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Wen et al., 2017
*VvTLP*gene family *VqTLP29*	Thaumatin-like protein (TLP)	*V. vinifera* *V. quinquangularis* *V. pseudoreticulata*	Red Globe Shang-24 Hunan-1	Susceptible Resistant Susceptible	Leaves	Yan et al., 2017
VqJAZ7	Jasmonate ZIM-domain (JAZ) transcriptional repressor	*V. quinquangularis*	Shang-24	Resistant	Leaves	Hanif et al., 2018
*NBS-LRR*family	Nucleotide Binding Sites-Leucine Rich Repeats proteins	*V. vinifera* spp. *sativa*	DVIT3351.27, Husseine, O34–16, Karadzhandal, Khalchili, Late vavilov, Sochal Carignane, Thompson seedless	From mid-susceptible to resistant Susceptible		Goyal et al., 2019

B)

Gene or Metabolite	Description	Taxon	Genotype	Defense Response	Organ	Reference
ATP Synthase, GS	Adenosine Triphosphate Synthase beta subunit, Glutamine Synthetase	*Vitis interspecific crossing* (Florida hybrids)	Lake Emerald, Blue Lake	Tolerant	Leaves	Vasanthaiah et al., 2009
Rubisco	Ribulose 1-5 bisphosphate-carboxylase	*Vitis interspecific crossing* (Florida hybrids) *Muscadinia (or Vitis) rotundifolia* (muscadine grape)	Blanc du Bois, Suwannee cv. Carlos	Susceptible Tolerant		
CHI *STS*	Chitinase Stilbene Synthase Transcription factor, Protein kinase, Sugar kinase genes	*Vitis interspecific crossing* (Florida hybrids) *Vitis interspecific crossing* (Florida hybrids)	Lake Emerald, Blue Lake Blanc du Bois, Suwannee	Tolerant Susceptible	Leaves	Vasanthaiah et al., 2010
STS, CHS, CHI, PGIP, LIP	Stilbene Synthase, Chalcone Synthase (CHS), Chitinase (CHI), Polygalacturonase Inhibiting Protein (PGIP), Lipid Transfer Protein (LIP)	*M. rotundifolia*	Derived selections	from susceptible to tolerant	Leaves	Louime et al., 2011
Several gene families		*V. coignetiae*		Resistant	Leaves	Choi et al., 2011
Several gene families		*V. quinquangularis* *V. vinifera*	Shang-24 Red Globe	Resistant Susceptible	Leaves	Gao et al., 2012b
Several gene families		*V. flexuosa*	VISKO001	Resistant	Leaves	Ahn et al., 2014a
VfGlu gene family	β-1,3-glucanase	*V. flexuosa*	VISKO001	Resistant	Leaves	Ahn et al., 2014b

(*Continued*)

TABLE 2 | Continued

B)

Gene or Metabolite	Description	Taxon	Genotype	Defense Response	Organ	Reference
VfRPS5-like gene family	Resistance to *Pseudomonas syringae* 5 (*RPS5*), member of the NBS-LRR family	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam et al., 2015a
VfRLK gene family	Receptor-like protein kinase	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam et al., 2015b
VfMlo-like gene family	Powdery-mildew resistance locus o-like	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2016a
VfCXE gene family	Carboxylesterase	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2016b
VfGST gene family	Glutathione S-transferase	*V. flexuosa*	VISKO001	Resistant	Leaves	Ahn et al., 2016
VfEDL1, VfEDL2, VfEDL3	Enhanced Disease Susceptibility 1 (EDS1)-like1	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2017
*VvTLP*gene family	Thaumatin-like protein (TLP)	*V. vinifera*	Red Globe	Susceptible	Leaves	Yan et al., 2017
VqTLP29		*V. quinquangularis* *V. pseudoreticulata*	Shang-24 Hunan-1	Resistant Susceptible		

A) Erysiphe necator; B) Elsinoë ampelina.

or expressed only in partially resistant cultivars. Moreover, at 5 dpi most of the genes were up-regulated in all the accessions, indicating a putative role in later stages of infection.

Since 1999, in different genetic backgrounds several genes that may contribute to PM resistance have been identified, such as *VpGLOX* (Guan et al., 2011); *VpPR-10.1* (Xu et al., 2010b; Xu et al., 2014); *VpRFP1* (Yu et al., 2011); *VpALDH2B4* (Wen et al., 2012); *VpNAC1* (Zhu et al., 2012b); *VpPR4-1* (Dai et al., 2016) (**Tables 2A** and **S2A**). Many of them are transcription factors (Zhu et al., 2012a; Zhu et al., 2012b), as expected since they are master regulators in controlling plant response to biotic stress (Amorim et al., 2017; Ng et al., 2018). In particular, *WRKY* transcription factors play an important role in plant defense (Pandey and Somssich, 2009) and WRKYs conferring resistance towards bacterial or fungal agents have been identified in several plants (Phukan et al., 2016). *WRKY* genes involved in response to fungal pathogens had been identified also in *V. vinifera*, i.e. *VvWRKY1* (Marchive et al., 2007) and *VvWRKY2* (Mzid et al., 2007). The entire *V. vinifera WRKY* gene family was later characterized by Wang et al. (2014). Interestingly, a significant induction of many *VvWRKY* genes occurred in the susceptible cv. Cabernet Sauvignon in response to PM infection, unlike the resistant cultivar Norton which however showed a constitutive higher level of *WRKY* genes expression, potentially confirming their role in defense even in resistant cultivars. Other *WRKY* genes implicated in PM resistance were identified in *V. pseudoreticulata* (Li et al., 2010; Zhu et al., 2012a; Yu et al., 2013b) and *V. quinquangularis* (Wang et al., 2017c).

Stilbenes, the grapevine phytoalexins, play a critical role in plant defense (Schnee et al., 2008). In grapevine stilbene synthase (*STS*) genes, the key enzyme in the biosynthesis of stilbenic compounds, are organized in an unusually large family (Parage et al., 2012; Vannozzi et al., 2012). Expression analyses performed in *V. vinifera* and *V. aestivalis* showed that individual *STS* genes are differentially expressed during leaf and berry development as well as in response to PM attack (Dai et al., 2012). PM-induction of *STS* genes was observed mainly in *V. vinifera*, whereas *V. aestivalis* showed constitutively higher transcript levels of some *STS* genes (Fung et al., 2008; Dai et al., 2012). Chinese wild grapevines are an important source of resistance genes related to stilbene production since several of them have a significantly higher resveratrol content than most European *V. vinifera* cultivars, and this accumulation correlates with *STS* genes expression. Indeed, both the endogenous and the PM-induced *STS* genes expression levels were much higher in the resistant *V. quinquangularis* than in *V. vinifera* (Shi et al., 2014). Comparative analyses of *STS* genomic regions and promoter activities in resistant and susceptible species revealed that the differential responsiveness of the *STS* genes is associated with differences in their promoter sequences, whereas the coding regions are highly conserved (Xu et al., 2010a; Xu et al., 2011b; Jiao et al., 2016). Jiao et al. (2017) identified in *V. quinquangularis VqMAPKKK38*, a PM-inducible Raf-like MAPKKK gene involved in the very complex network controlling activation of *STS* transcription.

Unlike the majority of the studies seen so far, which focus mainly on the early or midterm stages of infection, Borges et al. (2013) investigated the specific long term remodeling of the grapevine transcriptome operated by the pathogen to promote its survival, which requires uptake of nutrients and suppression of host defense responses. Interestingly none of the differentially expressed genes identified had correspondence to the ones detected by Fekete et al. (2009) in the early stages of infection. The mechanisms used by the pathogen to modulate host physiology were further investigated by Pike et al. (2014)

through the functional characterization of *VvNPF3.2,* a putative pathogen-inducible transporter in *V. vinifera* (Fung et al., 2008), and more recently by Toth et al. (2016), who identified genes induced by PM colonization but not by SA treatment.

Plant microRNAs (miRNAs) play pivotal roles in plant defense processes (reviewed in Niu et al., 2015; Islam et al., 2018). Han et al. (2016) used high-throughput sequencing of small RNAs to identify miRNAs potentially involved in PM resistance in *V. pseudoreticulata*. They showed that the expression of the highly accumulated vvi-NewmiR2118 miRNA, whose predicted targets are *NBS-LRR* type *R* genes, strongly and rapidly decreases following infection.

Guignardia bidwellii

Regarding the interaction between grapevine and *G. bidwellii* or only on the ascomycete itself, there are still no transcriptomic, proteomic or metabolomic data available. A possible reason of this delay could be attributed to the fact that research activities and investments from funding agencies mainly address major diseases (e.g. PM as well as downy mildew). Filling this gap of knowledge in the near future will be very helpful to understand not only the causal agent of BR, but also the molecular events which occur during the infection process.

Elsinoë ampelina

Unlike PM, few transcriptomic and proteomic studies have been conducted to elucidate the molecular interactions between *Vitis* species and *E. ampelina* (**Tables 2B**, **S2B**). A proteomic investigation identified several proteins differentially expressed between tolerant (mid-resistant) and susceptible cultivars, and between uninfected and infected leaves (Vasanthaiah et al., 2009). Two of them, the mitochondrial adenosine triphosphate synthase and glutamine synthetase, were newly expressed in tolerant cultivars upon *E. ampelina* inoculation. Conversely, several proteins, including ribulose-1,5-bisphosphate carboxylase (Rubisco), involved in photosynthesis, were suppressed in infected susceptible genotypes. These results highlighted the ability of the pathogen to significantly affect host physiology, especially in the susceptible cultivars. The tolerant genotypes had the ability to up-regulate and to induce new proteins in order to defend themselves from pathogen invasion and to maintain the normal physiological processes. In tolerant genotypes, a rapid and specific induction of various defense-related genes, including chitinase, stilbene synthase, chalcone synthase, putative proline-rich cell wall protein, thaumatin-like protein, pathogenesis-related (PR) protein 10 and other signal related genes were observed upon *E. ampelina* inoculation (Vasanthaiah et al., 2010; Choi et al., 2011; Louime et al., 2011; Gao et al., 2012b). Most of these genes were strongly induced by the pathogen only in the resistant accessions.

Ahn et al. (2014a) *de novo* assembled the transcriptome of the resistant Korean species *V. flexuosa* inoculated with the pathogen. This analysis led to the identification and subsequent characterization of many resistance related responsive genes to *E. ampelina* inoculation, such as β-1,3-glucanase genes (Ahn et al., 2014b), RPS5-like genes (Islam et al., 2015a), receptor-like protein kinase (RLK) genes (Islam et al., 2015b), Mlo-like genes (Islam and Yun, 2016a), carboxylesterase genes (Islam and Yun, 2016b), glutathione S-transferase genes (Ahn et al., 2016) and EDS1-like genes (Islam and Yun, 2017). Several *V. vinifera* thaumatin-like proteins (*VvTLP*) genes responsive to anthracnose were also recently identified (Yan et al., 2017).

DISCUSSION

Considering the combination of the three studied diseases, we have concluded that grapevine cultivars resistant to PM and downy mildew are mostly susceptible to BR and AN. This result could suggest a potential compromise of resistance to diseases, but instead can be attributed to the use of a limited source of genetic variation that confers resistance in breeding, particularly when compared with the genetic variability commonly found in grapevine germplasm collections. Secondly, positive selection of resistance to BR and AN is often overlooked, even when parental lines are resistant to one of these diseases in addition to PM. This implies the future proper use of multi-disease resistance sources, considering that potential interactions are not excluded. Indeed, it is currently unknown whether a common resistance mechanism is present in the genus *Vitis*, and if so at what biological scale, in light of the fact that the causal agents are all Ascomycetes. *R*-loci are polymorphic among species, and the upstream recognition phase is specific while a number of downstream mechanisms are common, highlighting that variability is present in the regulation, rather than the mechanisms themselves. In fact, heterologous expression of pathogen-responsive genes mostly confers broad-spectrum resistance, including in fungi of the same family, as well as bacteria. Focusing on single diseases, studies show that the pyramiding of *R*-loci provides an equal or higher degree of disease resistance, which confers long-term ability of overcoming pathogenic invasions.

Ultimately, it will be of paramount importance to characterize genes and pathways underpinning the quantitative PM, BR and AN resistance traits displayed by certain wild *Vitis* species. In order to prove conclusively that these genes do contribute to disease resistance, it will be necessary to demonstrate that the segregation of resistance is genetically linked to the inheritance of these candidate genes. This will also elucidate whether these genes are able to function in different genetic backgrounds i.e. in *V. vinifera*, which will be essential if they are to constitute the basis for any gene pyramiding strategy. Even nowadays, detailed insights about mechanisms underlying gene pyramids are missing–such as redundancy, additive effects or synergistic mechanisms (epistatic effects)–and expression and functional studies on pyramidized materials are very rare or lacking. The adoption of integrated strategies might facilitate the closing of the circle among grapevine/host disciplines (genomics, transcriptomics, and metabolomics).

Multidisciplinary approaches are also crucial and needed for a more comprehensive characterization of the ascomycete/pathogen. The identification of the described Ascomycetes commonly relied on the analysis of their morphological characters and on the observation of the affected plant symptoms, however these parameters are not sufficient for specific pathogen discrimination.

The recent advances in high-throughput sequencing technologies and in fungal genomics are opening new possibilities for fungi barcoding. Despite the economic importance of these pathogens, many aspects of their biology have not yet been fully explored because some (i.e. *E. necator*) are impossible to cultivate and propagate *in vitro* due to their obligate biotrophy lifestyle. In this respect, genomic studies, although complicated and challenging, are strongly advised to contribute to identify candidate effector genes involved in the pathogenicity mechanisms. On the other hand, more efforts and funding should be addressed to deepen our understanding of the mechanisms of biotrophy, shedding light onto its molecular and evolutionary basis. So far, to develop artificial media which resembles the host, only empiric strategies could be attempted to design media supplemented with (most probably several) plant molecules able to energetically support the fungus as well as to stimulate its growth.

To face the overall challenge, the vision is that each host discipline will inform the corresponding pathogen discipline, and *vice versa*, based on the practice of multidisciplinary culture.

AUTHOR CONTRIBUTIONS

CP, CM, and TCT searched for as well as organized literature and drafted the manuscript. MC searched for literature, drafted the manuscript, created figures and tables, and curated bibliography. PB searched for literature, created figures and tables, and curated bibliography. DP, LW, and LH searched for literature and integrated as well as revised the manuscript. EP, GA, and SM revised the manuscript. LZ searched for and organized literature. MS searched for literature. SV conceptualized as well as coordinated the writing work, searched for literature, and drafted as well as revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01394/full#supplementary-material

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Assessment of Resistance Components for Improved Phenotyping of Grapevine Varieties Resistant to Downy Mildew

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Grapevine varieties showing partial resistance to downy mildew, caused by *Plasmopara viticola*, are a promising alternative to fungicides for disease control. Resistant varieties are obtained through breeding programs aimed at incorporating *Rpv* loci controlling the quantitative resistance into genotypes characterized by valuable agronomic and wine quality traits by mean of crossing. Traditional phenotyping methods used in these breeding programs are mostly based on the assessment of the resistance level after artificial inoculation of leaf discs in bioassays, by using the visual score proposed in the 2nd Edition of the International Organization of Vine and Wine (OIV) Descriptor List for Grape Varieties and *Vitis* species (2009). In this work, the OIV score was compared with an alternative approach, not used for the grapevine-downy mildew pathosystem so far, based on the measurement of components of resistance (RCs); 15 grapevine resistant varieties were used in comparison with the susceptible variety 'Merlot'. OIV scores were significantly correlated with *P. viticola* infection frequency (IFR), the latent period for the downy mildew (DM) lesions to appear (LP50), and the number of sporangia produced per lesion (SPOR), so that when the OIV score increased (i.e., the resistance level increases), IFR and SPOR decreased, while LP50 increased. The relationship was linear for LP50, monomolecular for IFR and hyperbolic for SPOR. No significant correlation was found between OIV score and DM lesion size, sporangia produced per unit area of lesion, length of infectious period, and infection efficiency of the sporangia produced on DM lesions. The correlation between OIV score and area under the disease progress curve (AUDPC) calculated by using the RCs and a simulation model was significant and fit an inverse exponential function. Based on the results of this study, the measurement of the RCs to *P. viticola* in grapevine varieties by means of monocyclic, leaf disc bioassays, as well as their incorporation into a model able to simulate their effect on the polycyclic development of DM epidemics in vineyards, represents an improved method for phenotyping resistance level.

Keywords: *Plasmopara viticola*, *Vitis vinifera*, partial resistance, *Rpv*, OIV score

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INTRODUCTION

The ooomycete *Plasmopara viticola* originates from North America (Millardet, 1881) and is the causal agent of downy mildew (DM), one of the major diseases of *Vitis vinifera L.* worldwide. Its control still largely relies on fungicide treatments, which have environmental, social and economic impacts (Gessler et al., 2011). However, according to the Directive 2009/128/CE on the sustainable use of pesticides, the use of plant protection products has to be limited and alternative approaches to DM control are then needed (Rossi et al, 2012). The use of grapevine varieties showing partial resistance to DM represents an important tool for disease control (Töpfer et al., 2011) because it is compatible with other management options and does not have negative environmental impacts.

Wild grapevine species from North America and Asia, pertaining to the genera *Vitis* and *Muscadinia*, developed different mechanisms of resistance against *P. viticola* because of their coevolution with the pathogen (Kortekamp et al., 1997; Bellin et al., 2009; Casagrande et al., 2011; Gessler et al., 2011; Yu et al., 2012). The resistance response is conferred by quantitative trait loci (QTLs) named *Rpv* (meaning: "Resistance to *P. viticola*"); to date, 14 *Rpv* loci have been identified (Marino et al., 2003; Merdinoglu et al., 2003; Fischer et al., 2004; Wiedemann-Merdinoglu et al., 2006; Welter et al., 2007; Bellin et al., 2009; Marguerit et al., 2009; Peressotti et al., 2010; Blasi et al., 2011; Moreira et al., 2011; Di Gaspero et al., 2012; Schwander et al., 2012; Venuti et al., 2013; Ochssner et al., 2016; Zyprian et al., 2016; Sánchez-Mora et al., 2017). Depending on the *Rpv* locus and on the host genotype (Foria et al., 2018), the resistance responses to *P. viticola* infection involve different mechanisms, such as a hypersensitive response (Bellin et al., 2009; Venuti et al., 2013; Zyprian et al., 2016), callose and lignin accumulation (Dai et al., 1995; Kortekamp et al., 1997; Gindro et al., 2003), synthesis of stilbene phytoalexins (Pezet et al., 2004; Gindro et al., 2006), cell necrosis (Boso and Kassemeyer, 2008; Bellin et al., 2009; Zini et al., 2015), induction of peroxidase activity (Kortekamp et al., 1998; Toffolatti et al., 2012), and accumulation of phenolic compounds in the plant tissues surrounding the infection sites (Langcake, 1981; Alonso-Villaverde et al., 2011).

Since the discovery of the sources of resistance to *P. viticola* (Munson, 1909; Olmo, 1971), many breeding programs have been developed in order to obtain grapevine hybrids combining disease resistance genes from the wild grapevine species with the desirable agronomic and grape-quality traits carried by *V. vinifera* cultivars. From the beginning of the 20th century, several disease resistant varieties with a good level of grape quality and, in some cases, with a vinifera-like wine (Golodriga, 1978; Alleweldt, 1980; Bouquet, 1980; Kozma, 2002) have been selected and released by breeders. Some institutions are active in these breeding programs worldwide, including: Cornell University, UC Davis, and Florida AM University in the USA; several scientific institution in China (Lu and Liu, 2015); CSIRO in Australia; University of Krasnodar in Russia; JKI Gelweilerhof in Germany; INRA and IFV in France; University of Pécs in Hungary; and University of Udine, FEM, and CREA-VE in Italy (Bavaresco, 2019).

However, the use of resistant genotypes is still limited to few areas. Currently, resistant grapevine hybrids account for about 6% of the world grape-growing surface, with Kyoho (*V. labrusca* × *V. vinifera*), a Japanese table grape variety, being the most cropped one, especially in China (365,000 ha, OIV 2015 data). Resistant hybrids for wine production are mainly spread in America and Eastern Europe, specifically in Brazil (about 41,046 ha, 83% of the national viticultural surface), USA (about 11,980 ha, 5%), Moldova (about 11,656 ha, 13%), Russia (about 9,430 ha, 37%), Hungary (about 7,450 ha, 11%), Ukraine (about 3,251 ha, 6%), and Canada (about 2,680 ha, 27%) (Anderson, 2013). In the European Union (EU), the Regulation 1308/2013/EC states that resistant varieties can be grown to produce Table and PGI (Protected Geographic Indication) wines, but not PDO (Protected Denomination of Origin) wines (where only *V. vinifera* is allowed). In this respect, the situation is not uniform across the EU; for instance, Germany has classified the new disease resistant varieties as *V. vinifera*, unlike Italy and France.

The hybridization process using conventional breeding requires several years, but genetic engineering, marker assisted selection (MAS), genetic linkage maps, the availability of the *V. vinifera* genome sequence, and the knowledge of *Rpv* all help breeders in the selection of new resistant varieties. In addition to these methods, efficient phenotyping tools play a crucial role (Eibach et al., 2007; Töpfer et al., 2011; Schwander et al., 2012).

Traditional phenotyping is based on the evaluation of resistance after natural or artificial infection on leaves or leaf discs. Usually, the *in vitro* screening of partial resistance to DM is based on leaf disc bioassays (Bavaresco and Eibach, 1987; Staudt and Kassemeyer, 1995; Cadle-Davidson, 2008) and the assessment of the degree of resistance based on a visual score (from 1 = very low to 9 = very high), according to the 2nd Edition of the OIV Descriptor List for Grape Varieties and *Vitis* species (2009), i.e., the OIV 452-1 (hereafter referred to as OIV scale). In host-pathogen systems different from grape-*P. viticola* (Savary and Zadoks, 1989; Rossi et al., 1999; Rossi et al., 2000; Gordon et al., 2006; Burlakoti et al., 2010; Willocquet et al., 2011; Azzimonti et al., 2013; Schwanck et al., 2016), the measurement of resistance components (hereafter referred to as RCs) efficiently supports the evaluation of plant genotypes showing partial resistance, a type of resistance that affects several stages of the infection cycle, such as the resistance to *P. viticola*. Resistance components analysis is based on the phenotypic dissection of resistance into its components, which classically include: infection efficiency of spores, duration of latent period (i.e., the time from infection to the start of sporulation on lesions), lesion size, production of spores on lesions, and the duration of infectious period (i.e., the time a lesion continues producing spores) (Van der Plank, 1963; Zadoks, 1972; Parlevliet, 1979).

In a previous work, a resistance components analysis was conducted for 16 grapevine genotypes, some of them carrying one or more *Rpv* loci (Bove, 2018). In the present work, a comparison was made between the OIV scale and RCs with the aim of evaluating the relationships between the two phenotypic methods.

MATERIALS AND METHODS

Plant Material and Inoculation

The resistance response of different grapevine varieties was previously measured in a three-year study (2014 to 2016) after artificial inoculation of leaf discs in environmentally controlled conditions (Bove, 2018). Fifteen partially resistant varieties, some of them carrying one or more *Rpv* loci known to be involved in the resistance to *P. viticola*, and the *V. vinifera* 'Merlot', which is susceptible to downy mildew, were used (**Table 1**). Some of the resistant varieties consist of introgression lines developed in different European breeding institutes, such as the Julius-Kuehn Institute (JKI) and the Institute of Viticulture and Enology in Freiburg in Germany, and the University of Udine in collaboration with the Institute of Applied Genomics (IGA) in Italy.

The inoculation method is fully described in Bove (2018). In short, for each variety, fifteen fully developed leaves (specifically the fourth leaf from the shoot apex) were detached from five plants randomly selected at three growth stages (specifically at shoot growing BBCH 18, flowering BBCH 65 and fruit set BBCH 79; Lorentz et al., 1995) and immediately transported to the laboratory in a fridge at 5°C. Five leaf discs (21 mm diameter) were excised from each leaf using a cork borer. Leaf discs were repeatedly washed under tap water to remove superficial materials, dried under a laminar flow hood and finally placed, abaxial side up, in Petri dishes on filter paper wetted with 3 ml of sterile demineralized water. Each leaf disc was inoculated with four 10 µl drops of inoculum suspension containing a population of *P. v0iticola* at 5 × 10^5 sporangia ml^{-1} concentration. The inoculum suspension was prepared from freshly (2–3-day old) sporulating DM lesions obtained by inoculating the leaves of 'Merlot' plants grown in pots in a greenhouse by using a bulk of sporangia produced on field-grown leaves collected from different *V. vinifera* varieties in several untreated vineyards of northern Italy. Fresh sporangia produced on these leaves were collected, diluted in sterile water, adjusted to 5 × 10^5 sporangia ml^{-1}, and immediately used for the inoculation of leaf discs. After inoculation, leaf discs were incubated at 20°C with a 12-h photoperiod.

Phenotypic Assessment Using the OIV Scale

Leaf discs were observed both visually and with the help of a microscope (at tenfold magnification), in order to observe single sporangiophores of *P. viticola* (Schwander et al., 2012), at 11 days post inoculation (dpi), and assigned to one DM resistance score based on the OIV scale (OIV, 2009), as follows: 1—very little degree of resistance to *P. viticola* (dense sporulation at 100% of inoculation sites, on large lesions); 3—little (dense sporulation at 50 to 75% of inoculation sites, on medium to large lesions; 5—medium (sparse sporulation at 50% to 75% of inoculation sites, on medium size lesions); 7—high (sparse sporulation at 25% of inoculation sites, on small size lesions); 9—very high (no sporulation) (**Figure 1**). All the assessments were made by a single expert to minimize subjectivity.

Phenotypic Assessment Using the RCs

The following components of partial resistance were considered: i) infection frequency (IFR), ii) duration of latent period (LP50), iii) lesion size (LS), iv) number of sporangia per lesion (SPOR) and per mm^2 of DM lesion (SPOR'), v) infectious period (IP), iv) infection efficiency of the sporangia produced on inoculation sites and re-inoculated on the susceptible variety 'Merlot' (INF). Methods for the measurements of RCs are described in Bove (2018). Briefly, IFR was measured at 11 dpi as the proportion of the inoculation sites showing DM sporulation over the total inoculated sites (where 0 = no inoculation sites show sporulation to 1 = all inoculation sites show sporulation). LP50 was measured in degree days (DDs, base 0°C) cumulated between the inoculation and when 50% of the inoculation sites resulting in lesions at 11 dpi showed a DM

TABLE 1 | Grapevine varieties showing resistance to *Plasmopara viticola* used in leaf disc bioassays, their pedigree, the reference of the breeder, and *Rpv* loci.

Variety	Pedigree	Breeder	Loci
Bronner*	Merzling × Geisenheim 6494	Becker, N. (Freiburg)	*Rpv*10
Cabernet Volos*	Cabernet sauvignon × 20/3	Castellarin, S.D.; Cipriani, G.; Di Gaspero, G.; Morgante, M.; Peterlunger, E.; Testolin, R. (IGA)	*Rpv*12
Calandro*	Domina × Regent	Eibach, R.; Töpfer, R. (JKI)	*Rpv*3.1
Calardis blanc*	Geilweilerhof GA-47-42 × S.V. 39-639 KL.1	Eibach, R.; Töpfer, R. (JKI)	*Rpv*3.1, *Rpv*3.2
Felicia*	Sirius × Vidal Blanc	Eibach, R.; Töpfer, R. (JKI)	*Rpv*3
Fleurtai*	Tocai × 20/3	Castellarin, S.D.; Cipriani, G.; Di Gaspero, G.; Morgante, M.; Peterlunger, E.; Testolin, R. (IGA)	*Rpv*12
Johanniter*	Riesling Weiss × Freiburg 589-54	Zimmermann, J. (Freiburg)	*Rpv*3.1
Merlot Kanthus*	'Merlot' × 20/3	Castellarin, S.D.; Cipriani, G.; Di Gaspero, G.; Morgante, M.; Peterlunger, E.; Testolin, R. (IGA)	*Rpv*3
Merlot Khorus*	'Merlot' × 20/3	Castellarin, S.D.; Cipriani, G.; Di Gaspero, G.; Morgante, M.; Peterlunger, E.; Testolin, R. (IGA)	*Rpv*12
Palava	Traminer × Mueller Thurgau	Veverka, J. (Czeck Republic)	–
Reberger*	Regent × Lemberger	Eibach, R.; Töpfer, R. (JKI)	–
Regent*	Diana × Chambourchin	Alleweldt, G. (JKI)	*Rpv*3.1
Rkatsiteli	Unknown	-	–
Solaris*	Merzling × Geisenheim 6493	Becker, N. (Freiburg)	*Rpv*10
Villaris*	Sirius × Vidal blanc	Eibach, R.; Töpfer, R. (JKI)	*Rpv*3.1

The introgression lines (*) were developed in different European breeding institutes, such as the Julius-Kuehn Institute (JKI) and the Institute of Viticulture and Enology in Freiburg in Germany, and the University of Udine in collaboration with the Institute of Applied Genomics (IGA) in Italy (www.vivc.de).

FIGURE 1 | Scores assigned to grape leaf discs inoculated with *Plasmopara viticola* sporangia to assess the level of resistance based on the OIV 452-1 descriptor: 1—very little degree of resistance (dense sporulation at 100% of inoculation sites, on large lesions); 3—little (dense sporulation at 50 to 75% of inoculation sites, on large lesions); 5—medium (sparse sporulation at 50 to 75% of inoculation sites, on medium size lesions); 7—high (sparse sporulation at 25% of inoculation sites, on small size lesions); 9—very high (no sporulation). Inoculation was performed by placing on each leaf disc four 10μl drops of a suspension of 5×10^5 sporangia ml^{-1}.

lesion. For the measurement of LS, leaf discs were photographed at 11 dpi and the area (in mm^2) of each lesion was determined using the image analysis software Assess 2.0 (Lakhdar Lamari, APS PRESS, Saint Paul, Minnesota). SPOR was measured at 11 dpi as follows: sporangia produced on all the sporulating lesions of a leaf disc were carefully collected by using a sterile needle, suspended in 100 μl of sterile water, counted using a haemocytometer, and finally expressed as the number of sporangia per DM lesion. SPOR' was also calculated by dividing the number of sporangia per lesion by the lesion size, and expressed as the number of sporangia per mm^2 of DM lesion. IP was measured as the number of sporulating events on a DM lesion; after each sporulation, all the sporangiophores and sporangia were gently removed from the lesion using a sterile cotton swab, and this was repeated until the lesion no longer produced new sporangia. In order to measure INF, sporangia were collected at 11 dpi from leaf discs and then re-inoculated on new fresh leaf discs of 'Merlot', using the same procedure for inoculation and incubation previously described. INF was then measured as the proportion of inoculation sites showing DM sporulation on 'Merlot'. The measurement procedures, the statistical analyses and all the details concerning the assessment of partial resistance components are described in Bove (2018).

The following RCs: IFR, LP50, SPOR and IP, were incorporated into a model able to simulate downy mildew epidemics in partially resistant varieties. The model is described in Bove (2018). In short, the model captures the main features of the grapevine downy-mildew pathosystem, i.e. primary and secondary infections, physiology and dynamics of the host (crop growth, senescence and ontogenic resistance), and development of the disease on leaves and clusters. The parameterization of the model was performed according to the available literature (Bove, 2018) and considers the main environmental variables influencing the pathosystem (rain, wetness and temperature) by using a scenario approach. The model was developed by using the STELLA® software (Isee Systems, Inc, 2005). The model was operated in a favourable (i.e. not limiting) scenario for the disease, for each of the 16 varieties, by using the RCs measured in the leaf disc bioassay, so that the disease progress over time of the DM severity was simulated for each variety. The Area Under the Disease Progress Curve (AUDPC) (Campbell and Madden, 1990) was also calculated for comparing these epidemics.

Data Analysis

In order to compare the two phenotypic methods, a correlation analysis was conducted between OIV scores, each of the seven RCs, and the AUDPC, by calculating the Pearson's correlation coefficient, r. When correlations were significant at $P \leq 0.05$, a regression analysis was conducted by fitting different equations to the data, in which the OIV score was the independent variable and the RC or the AUDPC was the dependent one. All statistical analyses were all performed by using the SPSS software version 24 (SPSS, Chicago, IL).

RESULTS

Results on the phenotypic assessment of partial resistance components have been shown in detail in Bove (2018). Box plots of **Figure 2** show the distribution of OIV scores, components of resistance, and AUDPC in the 16 grapevine varieties. In **Figure 2**, original values of single varieties were standardized by dividing them by the average of the 16 varieties, to facilitate comparisons among variables that are expressed in different units.

Relationships between OIV score and the seven RCs measured for the 16 grapevine varieties are shown in **Figure 3**. OIV scores were significantly correlated with IFR ($r = -0.759$; $P = 0.001$), LP50 ($r = 0.710$; $P = 0.002$), and SPOR ($r = -0.763$; $P = 0.001$), so that when the OIV score increased (i.e., the resistance level increases), IFR and SPOR decreased, while LP50 increased (**Table 2**). The relationship was linear for LP50 (**Figure 3B**), so that the latent period increased by 2.22 degree-days for each unit of the OIV score (**Table 3**, Equation 2). For the infection frequency, the relationship fit a monomolecular equation (**Table 3**, Equation 1); based on this equation, IFR decreased at very slow rate when the OIV score increased from 1 to 5, at higher rates when OIV rated 5 to 7, and at a very high rate when the OIV score was >7 (**Figure 3A**). Conversely, the relationship was hyperbolic and inversely proportional for the production of sporangia on DM lesions (**Table 3**, Equation 3); therefore, the sporulation decreased sharply when the OIV score ranged 1 to 3, and then it decreased at lower and reducing rates (**Figure 3E**).

No significant correlation was found between OIV score and LS ($r = -0.393$, $P = 0.132$), SPOR' ($r = -0.142$, $P = 0.601$), IP ($r = -0.101$, $P = 0.709$), or INF ($r = -0.470$, $P = 0.066$) (**Table 2**).

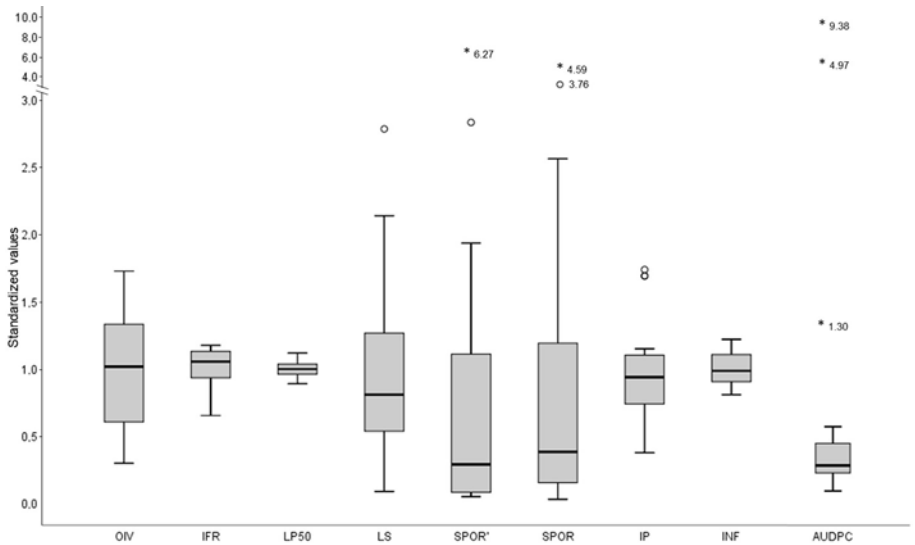

FIGURE 2 | Box plots showing the distribution of OIV scores, components of partial resistance, and AUDPC in the 16 grapevine varieties. Original values of single varieties were standardized by dividing them by the average of the 16 varieties, to facilitate comparison among variables that are expressed in different units. OIV is the OIV score (average 4.6); IFR is the infection frequency of sporangia (average 0.83, in a 0-1 interval); LP50 is latent period (average 102 degree-days); LS is the lesion size (average 7.6 mm^2); SPOR' is the number of sporangia per mm^2 lesion (average 344 sporangia × mm^{-2}); SPOR is the number of sporangia per lesion (1,582 sporangia × lesion); IP is the infectious period (average 2.84 sporulation events); INF is the infectivity of the sporangia produced on the varieties and inoculated on the susceptible 'Merlot' (average 0.77 in a 0–1 interval); AUDPC is the area under the disease progress curve calculated using a simulation model (average 42,679). The box representing the AUDPC is multiplied by 10 to visually appreciate its distribution. The thick line in the boxes is the median; the lowest value in each box represents the 1st quartile (25th percentile); the top part of each box represents the 3rd quartile (75th percentile). Circles and asterisks in the graph are outliers, i.e. values that are far or very far from the rest of the values, respectively.

The correlation between OIV score and AUDPC was negative and significant ($r = -0.633$; $P < 0.001$; **Table 2**). The relationship between these two variables fits an inverse exponential function, with $R^2 = 0.989$ (**Table 3**, Equation 4). Specifically, all the grape varieties with an OIV score between 3 and 9 showed similar epidemic patterns, with very low AUDPC values (**Figure 3H**).

DISCUSSION

This paper shows a comparison between two phenotypic methods used to assess partial resistance to *P. viticola* in grapevine trough an *in vitro* bioassay with artificial inoculation of leaf discs. Leaf disc inoculation is a well-established method to obtain reliable data for assessing grape resistance to *P. viticola* and many studies have revealed its strong correlation with data from naturally or artificially infected plants in the field or in pots (Stein et al., 1985; Eibach et al., 1989; Staudt and Kassemeyer, 1995; Brown et al., 1999; Boso et al., 2006; Boso and Kassemeyer, 2008; Bellin et al., 2009).

The phenotypic response of leaves in these bioassays mainly focused on the sporulation severity, assessed by means of the OIV 452-1 (Eibach et al., 2007; Bellin et al., 2009; Deglene-Benbrahim et al., 2010; Peressotti et al., 2010; Calonnec et al., 2012; Foria et al., 2018). For instance, Deglene-Benbrahim et al. (2010) scored leaf discs based on the severity of sporulation (abundant, dense, sparse, weak or absent) and its distribution (equally distributed or in large, small or very small patches) at 6 dpi. In epidemiological terms, the assessment of sporulation by the OIV scale accounts for the infection efficiency of the sporangia in causing infection (and then in causing DM lesions), for the density of sporangiophores and sporangia on lesions, and, indirectly, for lesion size. Results of the present work show that the OIV score is correlated with the number of sporangia produced on a DM lesion (SPOR). Similarly, a strong correlation between the number of sporangia per spray-inoculated leaf disc and the OIV score was found by Calonnec et al. (2013) and the number of sporangia per unit of leaf area and a modified OIV score by Bellin et al. (2009). However, the present work shows that this relationship is nonlinear. In addition, the OIV score is not related to the number of sporangia produced per mm^2 of DM lesion (SPOR'), which represents the real sporulation potential of *P. viticola* on leaf tissue. Therefore, the OIV scale does not represent adequately the quantitative differences in sporulation of resistant genotypes compared to a susceptible one ('Merlot' in this work). Concerning relevant periods of epidemics related to sporulation, the OIV score is linearly related to the time a DM lesion takes for starting to produce sporangia (i.e., the latent period, LP50), but it is not related to the time a lesion continues producing sporangia (i.e., the infectious period, IP), which is a key component for the development of polycyclic diseases in the vineyard, as DM is.

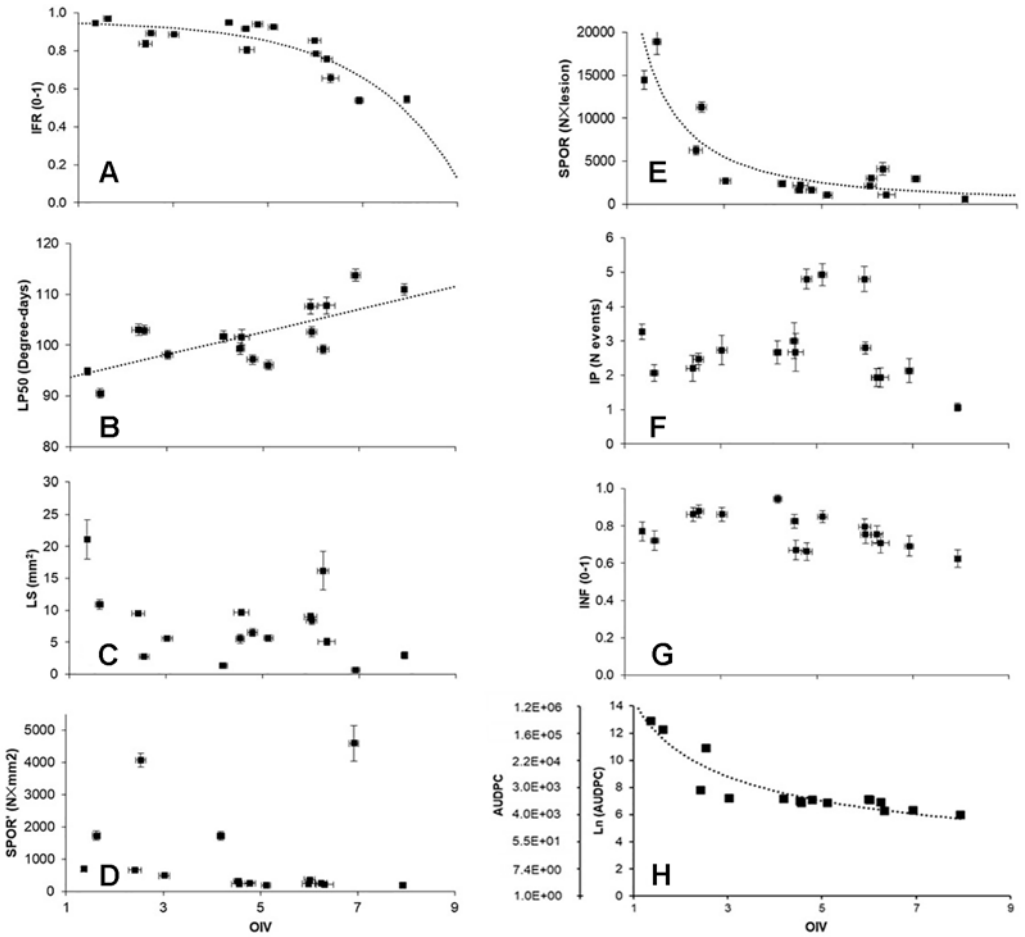

FIGURE 3 | Correlations between OIV score (see Figure 1), components of partial resistance (RCs) (panels A to G) measured for 16 grapevine varieties through artificial inoculation of Plasmopara viticola sporangia in leaf discs bioassays, and the area under disease progress curve (AUDPC) calculated by using the RCs and a simulation model (panel H). RCs are: infection frequency of sporangia (IFR; 0–1; panel A); latent period (LP50; degree-days; panel B); lesion size (LS; mm2; ;panel C); number of sporangia per mm2 lesion (SPOR'; panel D); number of sporangia per lesion (SPOR; panel E); infectious period (IP; number of sporulation events; panel F); infectivity of the sporangia produced on the varieties and inoculated on the susceptible 'Merlot' (INF; 0–1; panel G). For significant correlations (**Table 2**), data were fit by the equations described in **Table 3** (dotted lines).

TABLE 2 | Coefficients of correlation (r) between the OIV score assigned as in **Figure 1** and the components of partial resistance (RCs) to *Plasmopara viticola* measured for 16 grapevine varieties through artificial inoculation of *P. viticola* sporangia in leaf discs bioassays and the area under disease progress curve (AUDPC) calculated by using the RCs and a simulation model.

		RCs							AUDPC
		IFR	LP50	LS	SPOR'	SPOR	IP	INF	
OIV score	**r**	–0.759	0.710	–0.759	–0.759	–0.759	–0.759	–0.759	–0.759
	P-value	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001

RCs are: IFR, infection frequency of sporangia (0-1); LP50, latent period (degree-days); LS, lesion size (mm²); SPOR', number of sporangia per mm² lesion; SPOR, number of sporangia per lesion; IP, infectious period (number of sporulation events; INF, infectivity of the sporangia produced on the varieties and inoculated on the susceptible 'Merlot' (0–1).

Vezzulli et al. (2018) found a significant correlation between OIV 452-1 score and DM severity on spray-inoculated leaf discs, and supplemented the OIV descriptor by introducing the disease severity score, according to OEPP/EPPO, as follows: 1, corresponding to a leaf disc with more than 40% of the area occupied by sporulation, at 6 dpi (i.e., disease severity, DS > 40%); 2, DS ranging from 30 to 40%; 3, DS 25 to 30%; 4, DS 20 to 25%; 5, DS 15 to 20%; 6, DS 10 to15%; 7, DS 5 to 10%; 8, DS 1 to 5%; 9, DS < 1%. In epidemiological terms, the assessment based on disease severity accounts for infection frequency and lesion size. In the present work, the OIV score was correlated with the IFR, which is a measure of the infection efficiency

TABLE 3 | Parameters and statistics of the regression equations used for fitting the relationship between the OIV score assigned as in **Figure 1** (independent variable X), some components of partial resistance (RCs, dependent variable Y) to *Plasmopara viticola* measured for 16 grapevine varieties through artificial inoculation of *P. viticola* sporangia in leaf discs bioassays (specifically: IFR, infection frequency of sporangia, Equation 1; LP50, latent period, Equation 2; SPOR, number of sporangia per lesion, Equation 3) and the area under disease progress curve (AUDPC, depend variable Y) calculated by using the RCs and a simulation model (Equation 4).

Equations		Estimated parameters			R^2
		a	b	c	
(1)	$y = a - b \times c^{(-X)}$	0.960	0.008	0.601	0.763
		0.053[a]	*0.012*	*0.109*	
(2)	$y = a \times X + b$	2.222	91.511	–	0.504
		0.589	*2.921*		
(3)	$y = b \times X^{(-a)}$	1.537	29799.00	–	0.834
		0.221	*4662.48*		
(4)	$y = b \times X^{(-a)}$	0.447	14.393	–	0.848
		0.047	*0.866*		

[a]*Standard errors of the estimated parameters.*

of *P. viticola* inoculum of leaves, but it is not correlated with the LS. In addition, the relationship between OIV score and IFR is not linear. Therefore, the OIV scale does not accurately reflect the ability of resistance to reduce the disease severity of DM. Finally, the OIV scale does not clearly reflect the ability of the sporangia produced on resistant plants to cause new infections, which is an important driver of DM development in natural epidemics.

The OIV 452-1 descriptor considers only some aspects of partial resistance, in a monocycle. This approach may be reductionist and does not adequately represent the ability of plant resistance to reduce the disease development under natural vineyard conditions. This is confirmed by the nonlinear relationship existing between the OIV score and the AUDPC of the varieties considered in the present work, as determined through using a simulation model for monocycle concatenation during DM epidemics. The simulation model enabled the exploration of each component of partial resistance and of its effect in slowing down epidemic development, alone or in combination with other RCs (Van der Plank, 1963). In a previous work (Bove, 2018), the infection frequency showed the strongest effect in reducing the disease development because it affects both primary and secondary infections. The number of sporangia produced per lesion also had a strong effect in slowing the disease progress because the higher number of propagules produced per lesion caused a higher basic infection rate (R_c) (Zadoks and Schein, 1979). Therefore, resistant varieties showing low IFR and SPOR in monocyclic bioassay have great potential for slowing down DM epidemics in the vineyard. Varieties showing a shorter IP compared to susceptible ones also reduce epidemic development, because the number of secondary infection cycles originating from a lesion is lower. Similarly, the varieties showing a longer latent period slow down DM epidemics because the time at which they start producing sporangia for new infections is delayed.

The low accuracy of the OIV scale in representing the real effect of a resistant variety on slowing down DM epidemics in the vineyard is also confirmed by some contrasting results of previous works. In some works, a relationship was found between resistance levels from visual observation of leaf disc in bioassays and from field assessments (Sotolář, 2007; Boso et al., 2014; Zyprian et al., 2016; Buonassisi et al., 2018; Foria et al., 2018; Vezzulli et al., 2018). In other works, no relationship was found and this was imputed to the inoculation of a single *P. viticola* strain, which may be not representative of a field population (Cadle-Davidson, 2008), or to high inoculum concentration and optimal environmental conditions of the leaf discs bioassay, which may lead to an underestimation of the resistance level with the OIV scale (Calonnec et al., 2013). In addition (or as an alternative) to these argumentations, the inconsistent relationship between the resistance level of a variety in the bioassay assessed through the OIV scale and in the vineyard may be due to the fact that monocyclic experiments (i.e., leaf disc bioassays) and the OIV scale do not account for all the effects of partial resistance on each infection cycle and in the concatenation of infection cycles during the season as well. It might be also considered that in this study a mixture of isolates collected from different varieties in multiple vineyards were used for artificial inoculation of different resistant grapevine accessions carrying different resistant loci. It is known that some DM isolates are more aggressive than others (Delmotte et al., 2014), and each specific isolate/accession pair may have a unique scenario in term of disease severity and RCs.

Based on the results of this study, the measurement of the components of partial resistance to *P. viticola* in grapevine varieties by means of monocyclic leaf disc bioassays, as well as their incorporation into a model able to simulate their effect on the polycyclic development of DM epidemics in vineyards represents an improved method for phenotyping resistance level. In addition, the measurement of resistance components is a quantitative method, which is not influenced by the subjective bias of qualitative methods, like the OIV descriptor, and this would reduce bias of phenotyping outcomes. Therefore, resistance component analysis meets all the criteria for efficient phenotyping tools needed to grapevine breeders for the selection of new resistant genotypes (Töpfer et al., 2011).

DATA AVAILABILITY STATEMENT

Datasets are available on request: The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The authors declare that the present manuscript complies with the Ethical Rules of good scientific practice applicable for this Journal. The research does not involve humans or animals.

AUTHOR CONTRIBUTIONS

FB and VR mainly contributed to the conception and the design of the study. TC contributed to the analysis of results. FB and VR wrote the first draft of the manuscript. TC and LB contributed to the critical analysis of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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VvSWEET7 Is a Mono- and Disaccharide Transporter Up-Regulated in Response to *Botrytis cinerea* Infection in Grape Berries

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The newly-identified SWEETs are high-capacity, low-affinity sugar transporters with important roles in numerous physiological mechanisms where sugar efflux is critical. SWEETs are desirable targets for manipulation by pathogens and their expression may be transcriptionally reprogrammed during infection. So far, few plant SWEET transporters have been functionally characterized, especially in grapevine. In this study, in the *Botrytis*-susceptible variety "Trincadeira," we thoroughly analyzed modifications in the gene expression profile of key *SWEET* genes in *Botrytis cinerea*-infected grape berries. VvSWEET7 and VvSWEET15 are likely to play an important role during fruit development and *Botrytis* infection as they are strongly expressed at the green and mature stage, respectively, and were clearly up-regulated in response to infection. Also, *B. cinerea* infection down-regulated *VvSWEET17a* expression at the green stage, *VvSWEET10* and *VvSWEET17d* expression at the veraison stage, and *VvSWEET11* expression at the mature stage. VvSWEET7 was functionally characterized by heterologous expression in *Saccharomyces cerevisiae* as a low-affinity, high-capacity glucose and sucrose transporter with a K_m of 15.42 mM for glucose and a K_m of 40.08 mM for sucrose. VvSWEET7-GFP and VvSWEET15-GFP fusion proteins were transiently expressed in *Nicotiana benthamiana* epidermal cells and confocal microscopy allowed to observe that both proteins clearly localize to the plasma membrane. In sum, VvSWEETs transporters are important players in sugar mobilization during grape berry development and their expression is transcriptionally reprogrammed in response to *Botrytis* infection.

Keywords: sugar transporter, SWEET, biotic stress, grey mold, *Botrytis cinerea*, plant pathogens, grape berry, grapevine

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is prone to a wide range of pathogens that cause production and quality losses. Plant pathogens are separated in three broad classes according to their modes of nutrition. Biotrophs are pathogens that need a living host to survive, having developed strategies to obtain nutrients from the host without inducing plant defense mechanisms or cell death (Perfect et al., 1999; Glazebrook, 2005). For nutrient uptake and secretion of limited amounts of cell wall-degrading enzymes, they develop specialized structures, including haustoria (Mengiste, 2012). Necrotrophs obtain nutrients from dead cells, which are killed during the infection process. They cause necrosis secreting hydrolytic enzymes that degrade the cell wall (van Kan, 2006), secrete toxins (Govrin et al., 2006; Dalmais et al., 2011), and also hijack the plant enzymatic machinery, promoting programmed cell death (Cantu et al., 2009). The hemibiotrophs pathogens are able to switch lifestyles at specific developmental stages—they display an early biotrophic phase followed by a necrotrophic phase—or at different environmental conditions (Glazebrook, 2005; Kleemann et al., 2012). *Botrytis cinerea*, the causal agent of the grey mold disease in more than 200 plants (Elad et al., 2004), is one of the most important grapevine pathogens (Haile et al., 2017). It is a necrotrophic fungus with a short biotrophic phase (Veloso and van Kan, 2018). Usually, *B. cinerea* infection begins by airborne conidia that settle in the host superficial cells (Nair et al., 1995; Elmer and Michailides, 2004). In the vineyard, this pathogen is part of the microflora and infects primarily ripe berries; however, inoculation of *Botrytis* spores often occurs during the onset of the grape berry (McClellan and Hewitt 1973; Nair et al., 1995; Keller et al., 2003; Pezet et al., 2003). During development, grape berries suffer several modifications that decrease its natural resistance to the pathogen. The cuticle and the cell-wall suffer modifications that lower their mechanical resistance (promoting micro-fractures), the bunches become more compact, (Vail and Marois, 1991; Kretschmer et al., 2007), the sugar levels increase and the concentration of organic acids and several compounds related to biotic resistance decrease (Miedes and Lorences, 2007; Cantu et al., 2008; Cantu et al., 2009; Centeno et al., 2011; Blanco-Ulate et al., 2013; Prusky et al., 2013; Blanco-Ulate et al., 2015). During infection, *B. cinerea* secretes several compounds and enzymes to macerate and penetrate the host tissue (Dulermo et al., 2009). Additionally, the fungus can manipulate the host biological processes to its own gain, promoting the programmed cell death machinery (Veloso and van Kan, 2018) or hastening the ripening process. In infected immature grape berries, Agudelo-Romero et al. (2015) observed a large transcriptional activation of genes related to the maturation process and an accumulation of several compounds associated with maturation. A similar highjack of plant metabolism by pathogen agents was also observed in other plant species (Baker et al., 2012; Morkunas and Ratajczak, 2014). Different studies reported an increase of invertase activity in different plants in response to powdery mildew or other diseases (Ruiz and Ruffner, 2002; Roitsch et al., 2003; Kocal et al., 2008; Siemens et al., 2011). Also, up-regulation of sugar transporters was observed during pathogen infection in *Arabidopsis thaliana* and *Pinus pinaster* cultured cells (Truernit et al., 1996; Azevedo et al., 2006).

Recently identified and somewhat different from the common sugar transporting proteins, SWEETs transporters were characterized as bi-directional, low-affinity sugar carriers, probably operating by an uniport mechanism (Chen et al., 2010). Generally, in angiosperms, the SWEET family is composed of 20 members and have different physiological roles, typically related with sugar efflux mechanisms. In *Arabidopsis*, SWEET transporters are essential members in nectar secretion (Lin et al., 2014), phloem sugar loading (Chen et al., 2012), seed nutrient filling (Chen et al., 2015a), and pollen feeding and vacuolar fructose storage (Chardon et al., 2013; Klemens et al., 2013; Guo et al., 2014). In maize, ZmSWEET13a, b, and c are key components in apoplasmic phloem loading (Bezrutczyk et al., 2018); in rice, OzSWEET11 and 15 are crucial components in seed filling (Yang et al., 2018). Also, in *Arabidopsis*, AtSWEET13 and 14 are capable of transporting multiple forms of gibberellins, revealing an exciting plasticity of these transporters (Kanno et al., 2016). In the grapevine, the SWEET family is composed by 17 members, and only VvSWEET4 and VvSWEET10 were functionally characterized (Chong et al., 2014; Zhang et al., 2019).

Similar to their effect in other sugar transporters, pathogens are also able to alter the expression profile of different SWEET genes (reviewed by Chen et al., 2015b and Julius et al., 2017). In rice, some members of this family (*OsSWEET11*, *OsSWEET12*, and *OsSWEET14*) were up-regulated during *Xanthomonas oryzae* infection (Chu et al., 2006; Yang et al., 2006; Antony et al., 2010; Chen et al., 2010). In cassava (*Manihot esculenta*), *Arabidopsis*, citrus (*Citrus paradisi* and *Citrus sinensis*), barrel clover (*Medicago truncatula*), grapevine, and sweet potato (*Ipomoea batatas*), up-regulation of SWEET genes was also observed during pathogen attack (Chen et al., 2010; Chong et al., 2014; Cohn et al., 2014; Hu et al., 2014; Li et al., 2017). An increased number of sugar transporters can lead to sugar accumulation in the apoplast, which in turn is used as source of carbon and energy by the pathogen (Wright et al., 1995; Clark and Hall, 1998; Lemoine et al., 2013), but pathogens can also affect negatively the expression of SWEET genes. *Botrytis cinerea* infection in tomato cotyledons promoted a down-regulation of different members of the *SWEET* family (Asai et al., 2016). This form of regulation can lead to the disruption of sugar signaling pathways related to defense responses to biotic stress (Berger et al., 2007; Sade et al., 2013; Morkunas and Ratajczak, 2014).

Thus, considering the possible role of SWEET transporters either by accentuating or counteracting the infection, we aimed in this study to confirm the hypothesis that the infection by *B. cinerea* causes a transcriptional reprogramming of the expression of *SWEET* genes in grape berry tissues. Moreover, considering its naturally significant steady state transcript abundance and up-regulation by *B. cinerea* infection in berries, we functionally characterized VvSWEET7, unveiling its sub-cellular localization and sugar transport kinetics resorting to two heterologous expression models, tobacco and yeast, respectively.

MATERIALS AND METHODS

Biological Material

Clusters of Trincadeira grapes were infected by inoculation with a conidial suspension of *B. cinerea* at EL29 (peppercorn-size, early green stage) in very well-established and standardized conditions according to Agudelo-Romero et al. (2015) and Coelho et al. (2019). Inoculation was performed at the same time in multiple clusters in very similar conditions, particularly in size, appearance, exposure to light, canopy densities, and plant orientation between them and also identical to control clusters. Samples were harvested at three developmental stages: at green (EL32), veraison (EL35), and mature (EL38) (Coombe, 1995). For each treatment (infected and control) and ripening stage, three biological replicates were collected at around 10 a.m., each one constituted by a composite pool of at least 12 berries collected from different clusters from three different plants. Every collected infected berry had the same infection appearance and visual symptoms that were in fact similar between all infected clusters, as the inoculation was performed at the same time in all berry clusters. Thus, both control and infected collected grape berries were well representative of their physiological condition. The collected samples were frozen in liquid nitrogen and stored at -80°C. Prior to RNA extraction, the seeds of each of the three sampled biological replicates were removed and the remaining tissues were ground in liquid nitrogen to a fine powder. For all analyses performed on grape berry tissues in this work, each of the three biological replicates was used for a different RNA extraction and independent qPCR analysis, with each qPCR analysis having three internal technical replicates.

Cell suspension cultures of *V. vinifera* L. (Cabernet Sauvignon Berry - CSB) were freshly established from somatic callus that had been previously initiated from Cabernet Sauvignon berry pulp at Serge Delrot's lab (ISVV, Bordeaux) according to Calderón et al., 1994. They were maintained in 250 ml flasks at 25°C in the dark on a rotator shaker at 100 rpm on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; Decendit et al., 1996), supplemented with 2% (w/v) sucrose as carbon and energy source. The suspension-cultured cells were sub-cultured weekly by transferring 10 ml aliquots into 40 ml of fresh medium. *B. cinerea* growth and mycelia harvest were performed according to Azevedo et al. (2006). The fungus was cultivated in potato dextrose (PD) liquid medium at 25°C with agitation (150 rpm). Mycelia were harvested from 12-d cultures by centrifugation at 5,000×g for 5 min, followed by resuspension in sterile water. The mycelia were autoclaved and then lyophilized for 48 h and ground with a mortar and pestle to a fine powder. For elicitation experiments, grapevine suspension cells were harvested at the mid-exponential growth phase, centrifuged at 5.000 xg for 5 minutes, and resuspended in MS medium at a final density of 0.1 g F.W. mL^{-1}. *Botrytis* mycelia extract was added to the suspension cell culture at a final concentration of 2 mg mL^{-1}. Then, the control and elicited suspension cultures were incubated in the dark at 25°C on a rotatory shaker at 100 rpm. After 48 hours of incubation, cells were filtered through GF/C filters (Whatman) and samples were washed with deionized water and immediately frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. For both *Botrytis*-elicited and control suspension cell cultures, three biological replicates were used.

RNA Extraction

An initial amount of 200 mg of ground control or infected grape berry tissues from each of the three biological replicates sampled was used for total RNA extractions, as well as pulverized *Botrytis*-elicited and control suspension cell culture samples, following the method described by Reid et al. (2006) combined with in-column purification using the RNeasy Plant Mini Kit (Qiagen). After isolation and verification of RNA purity, treatment with DNase I (Qiagen) was performed and cDNA was synthesized from 1 μg of total RNA using the Xpert cDNA Synthesis Mastermix Kit (GRISP).

Gene Expression Analysis by qPCR

The expression profile of *VvSWEET* genes in all the studied samples (control and infected grape berries and control and *Botrytis*-elicited suspension cell cultures) was analyzed by real-time qPCR performed using cDNAs obtained from RNAs extracted from each of the three composite pools of grape berry samples that constitute the three biological replicates of each condition in this study. Real-time qPCR was performed with Xpert Fast SYBR Blue (GRISP) using 1 μL of diluted cDNA (1:10) in a total of 10 μL of reaction mixture per well. For reference genes, *VvACT1* (actin) and *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were used, as they are considered extremely adequate reference genes for gene expression normalization purposes in qPCR analyses in grapevine (Reid et al., 2006). Specific primers used for each studied gene are listed in the **Supplementary Table 1**. Melting curve analysis was performed for specific gene amplification confirmation. Stability of the reference genes was confirmed by the automatic M-value analysis performed by the Bio-Rad® CFX Manager 2.0 Software. For each gene, the relative gene expression values were obtained following calculation by the Bio-Rad® CFX Manager 2.0 Software. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with three internal technical replicates.

VvSWEET7 and *VvSWEET15* Molecular Cloning and Construction of Destination Plasmids

The putative sugar transporter genes, *VvSWEET7* and *VvSWEET15*, were cloned by Gateway® technology. Primers pairs, designed with the attB sequences (**Supplementary Table 1**) for site-specific recombination with the entry plasmid pDONR221, were used for PCR amplification of the target genes. Subsequently, recombination of the attB-containing target genes with the entry plasmid was performed using the BP clonase enzyme. The target genes carried in the entry plasmid were then recombined by the LR clonase enzyme into the *pH7WGF2* plasmid (containing the *egfp* gene) for sub-cellular localization and into the *pYES-DEST52* plasmid for heterologous expression in yeast. All constructs were confirmed by sequencing.

Sub-Cellular Co-Localization Studies in Tobacco Leaves

The N-terminally fused constructs *pH7WGF2-GFP-VvSWEET7* and *pH7WGF2-GFP-VvSWEET15* were introduced in *Agrobacterium tumefaciens* strain EHA105; transient transformation of tobacco leaves (*Nicotiana benthamiana*) was performed according to Sparkes et al. (2006). Transformed *Agrobacterium* cells were inoculated overnight in liquid LB medium with the appropriate antibiotic selection up to the exponential-stationary phase, and then diluted to OD_{600nm} = 0.1 with infiltration buffer (50 mM MES pH 5.6, 2 mM Na_3PO_4, 0.5% glucose, 100 µM acetosyringone). Cells were then incubated until the culture reached an OD_{600nm} = 0.2. Leaves of three different four-week-old tobacco plants were infiltrated with the *Agrobacterium* culture and, after 2 days, discs of the infected leaves were observed at the scanning confocal microscope (Leica TCS SP5IIE-Leica Microsystems). Data stacks were analyzed and projected using ImageJ 1.42m software (http://rsb.info.nih.gov/ij/). The plasma membrane marker used was the plasma membrane aquaporin AtPIP2;1 C-terminally fused to the fluorescent protein mCherry (AtPIP2;1-mCherry construct) (Nelson et al., 2007). This plasma membrane marker was co-expressed with either GFP-VvSWEET7 or GFP-VvSWEET15 constructs, allowing the observation of their co-localization at the plasma membrane revealed by the yellow fluorescence signal.

Heterologous Expression of *VvSWEET7* and *VvSWEET15* in *Saccharomyces cerevisiae*

The *S. cerevisiae* mutant strain EBY.VW4000 (Wieczorke et al., 1999) was used in this study to functionally characterize VvSWEET7 and VvSWEET15. This strain does not have the capacity to transport monosaccharides and sucrose due to multiple mutations in sugar-sensing and sugar transporter genes. The yeast was grown on rich medium supplemented with maltose (1% yeast extract, 2% peptone, 2% maltose). After transformation by the lithium acetate method (Gietz and Woods, 2002), with the constructions *pYES-DEST52-VvSWEET7* or *pYES-DEST52-VvSWEET15*, the yeast was grown in basic selective medium [0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 2% (w/v) carbon source] supplemented with maltose 2% (w/v) and without uracil for URA3-based selection. For control, the yeast cells were transformed with the empty vector.

Transport Studies in *S. cerevisiae* With Radiolabeled Sugars

EBY.VW4000 yeast cells, transformed with *pYES-DEST52-VvSWEET7* or *pYES-DEST52-VvSWEET15* (empty pYES-DEST52 for control) were grown in basic selective medium supplemented with 2% maltose at 30°C on a rotatory shaker at 220 rpm up to the exponential-stationary phase. To induce the expression of the target genes, the culture was washed twice in ice-cold sterile water and cultivated in fresh basic selective medium supplemented with 2% galactose at least during 4 h. Then, the cells were harvested by centrifugation and washed twice with ice-cold sterile distilled water and suspended in sterile water. To functionally characterize VvSWEET7 and VvSWEET15 and estimate the initial uptake rates of radiolabeled sugars, 30 µl of cell suspension were mixed with 15 µl of 100 mM KH_2PO_4 buffer at pH 5.0 in 1.5 mL microcentrifuge tubes. After 2 min of pre-incubation at 30°C in a thermoblock, the reaction was initiated by the addition of a volume of up to 15 µl of an aqueous solution of radiolabeled glucose (D-[^{14}C] glucose) or fructose (D-[^{14}C] fructose) with a specific activity of 150 dpm nmol^{-1}. Similarly, to determine sucrose initial transport rates, a volume of up to 15 µl of an aqueous solution of radiolabeled sucrose ([^{14}C]-sucrose) with a specific activity of 500 (for final concentrations between 7.5 and 50 mM) or 250 dpm nmol^{-1} (for final concentrations between 75 and 125 mM) was used. Potential competitive inhibitors or CCCP (carbonyl cyanide m-chlorophenylhydrazone) were added to the reaction mixture before the addition of the radiolabeled sugar for transport specificity and energetics assessment, respectively. After 3 min, the reaction was stopped by dilution with 1 mL of ice-cold water. Then, cells were washed twice with ice-cold water and 1 mL of scintillation fluid added for complete cell membrane disruption and radioactivity measurements. The radioactivity was then measured in a scintillation counter (Packard Tri-Carb 2200 CA). D-[^{14}C] glucose (287 mCi mmol^{-1}), D-[^{14}C] fructose (316 mCi mmol^{-1}), and [^{14}C] sucrose (592 mCi mmol^{-1}) were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). For every radiolabeled sugar transport experiment, three independent experimental repetitions, each one consisting of an independent VvSWEET7-overexpressing and respective control yeast growth and subsequent radiolabeled sugar uptake were performed. Also, each experimental repetition was performed with three technical replicates.

Measurement of Proton Pumping Activity of the Yeast Plasma Membrane ATPase

EBY.VW4000 yeast cells, transformed with *pYES-DEST52-VvSWEET7* and control ones (harboring the empty vector) were washed with deionized water and suspended in water (20 mg mL^{-1}) at room temperature under stirring for 3 h to induce starvation. For each experimented condition, 15 mg (D.W.) of yeast cell suspensions grown until OD_{600} = 0.8 was placed in a water-jacketed chamber with a total volume of 5 mL of non-buffered water. The suspension was mixed with a magnetic stirrer, and the temperature-regulated circulating water was at 30°C. Changes in pH were detected with a combination electrode (PHC-4000-8 RadioMeter) attached to a sensitive pH meter (PHM82 Standard pH Meter) and recorder (KIPP & ZONEN) with scale expander, as described by Serrano (1980). A concentration of 45 mM of different sugars (glucose, fructose, galactose, and sucrose) were used to activate the proton pump. Calibration was performed through the addition of 100 nmol HCl to the cell suspension. For proton pumping activity analysis, four experimental repetitions were performed, each one consisting of an independent VvSWEET7-overexpressing and control yeast growth and a subsequent sugar-induced pH variation analysis.

Statistical Analysis

To test if the data were normally distributed, the Shapiro-Wilk normality test was used, while the homogeneity of variances was confirmed using Bartlett's tests using Prism v. 6 (GraphPad Software, Inc.). Subsequently, the results were statistically verified by analysis of variance tests (one-way ANOVA) or Student's t-test using Prism v. 6 (GraphPad Software, Inc.). Post-hoc multiple comparisons were performed using the HSD Tukey test. Throughout the results, different letters denote statistical differences between columns and are presented in a progressive order from the highest to the lowest value, and asterisks indicate statistical significance.

RESULTS

Effect of *B. cinerea* Infection on the Expression Profile of Grapevine *VvSWEET* Genes

Grape berry clusters showed clear symptoms of infection at the green (EL32), veraison (EL35), and fully mature stages (EL38), which were very similar among infected clusters that were confirmed by amplification by qPCR of specific fungal genomic DNA as previously reported in **Figure 2** by Coelho et al. (2019). Also, visual symptoms of infection can be seen in **Supplementary Figure 1**.

Figure 1 shows that distinctive expression patterns along grape berry development were observed for each *VvSWEET* gene. While the transcript levels of *VvSWEET11* and *VvSWEET15* increased along development, the expression of *VvSWEET1*, *VvSWEET2b*, *VvSWEET4*, *VvSWEET7*, *VvSWEET17a*, and *VvSWEET17d* decreased from green to mature stage. The transcript levels of *VvSWEET10* peaked at veraison, and *VvSWEET2a* gene expression was similar during grape berry development. In each stage, the gene expression in whole berries was compared between control non-infected grapes (solid bars in **Figure 1**) and *B. cinerea*–infected berries (striped bars in **Figure 1**). As can be seen, *B. cinerea* infection up-regulated *VvSWEET2a* and *VvSWEET7* expression at the green stage and *VvSWEET15* expression at the mature stage, while down-regulated *VvSWEET17a* expression at the green stage, *VvSWEET10* and *VvSWEET17d* expression at the veraison stage, and *VvSWEET11* expression at the mature stage. Interestingly, down-regulation of *VvSWEET* genes occurred specifically in the developmental stages where the gene was most expressed in normal conditions. Expression of *VvSWEET1*, *VvSWEET2b*, and *VvSWEET4* was not modified by the infection. The expression of most *VvSWEET* genes, including *VvSWEET7*, in *Botrytis*-elicited grape berry cell suspensions originating from the pulp of berries from Cabernet Sauvignon berries, was modified in a similar way to the changes observed in infected grape berries from the field experiment when they occurred, with the exception of only *VvSWEET1* and *VvSWEET17a*, whose expression was repressed or unaltered, respectively (**Supplementary Figure 2**). These results confirm a similar *B. cinerea* effect also at more controlled conditions. The effect of *B. cinerea* infection on the expression profile of *VvSUC11* (grapevine sucrose transporter 11), *VvSUC12* (grapevine sucrose transporter 12), *VvSUC27* (grapevine sucrose transporter 27), *VvHT3* (grapevine hexose transporter 3), and *VvTMT1* (grapevine tonoplast monosaccharide transporter 1)—prominent members of the Major Facilitator Superfamily (MFS), is shown in **Figure 2**. As previously shown (Afoufa-Bastien et al., 2010), *VvSUC11* is mostly expressed in mature berries, while the transcript levels of *VvSUC12*, *VvSUC27*, and *VvHT3* are more abundant at the green stage. *VvTMT1* expression peaked at veraison. From all studied genes, only *VvHT3* was responsive to *B. cinerea* infection, which caused a 3-fold up-regulation at the mature stage.

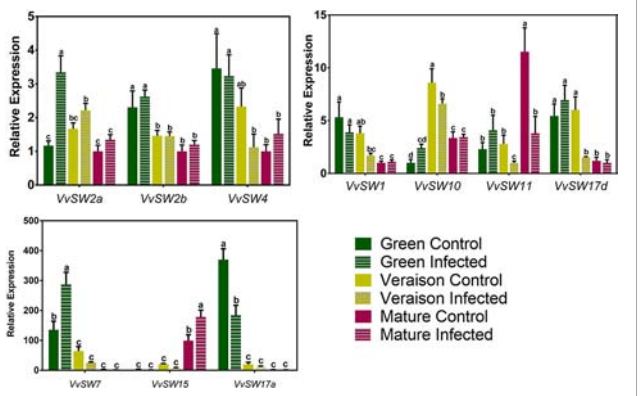

FIGURE1 | Expression profile of *VvSWEET* genes that are expressed in the grape berry, performed by real-time qPCR in infected (striped bars) and control (solid bars) berries, collected at three different developmental stages (green, veraison and mature). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with three internal technical replicates. Values are the mean ± SD. (one-way ANOVA with Tukey's post-test; different letters denote statistical differences between columns).

In Silico Characterization of *VvSWEET7* and *VvSWEET15*

The sequences of both *VvSWEET7* (GSVIVG01019601001) and *VvSWEET15* (GSVIVG01000938001) present two PFAM motif PF03083/MtN3_slv and are predicted to have seven transmembrane domains targeted to the plasma membrane. PLACE (Higo et al., 1999) and PlantPAN 3.0 databases (Chow et al., 2019) (**Supplementary Tables 2** and **3**) revealed that *VvSWEET7* promoter sequence (2kbp upstream) has several biotic stress-related cis-acting elements, such as WRKY71OS and GT1GMSCAM4; and also some sugar responsive elements as WBOXHVIS01, MYBGAHV, or SUR2STPAT21, a sucrose responsive element, a motif conserved among genes regulated by sucrose (**Supplementary Tables 2** and **3**). Also, several abiotic and hormone responsive cis-acting elements were localized. Several responsive elements that were identified in the *VvSWEET7* promoter region were also localized in the promoter region of the *VvSWEET15* gene, as the biotic stress

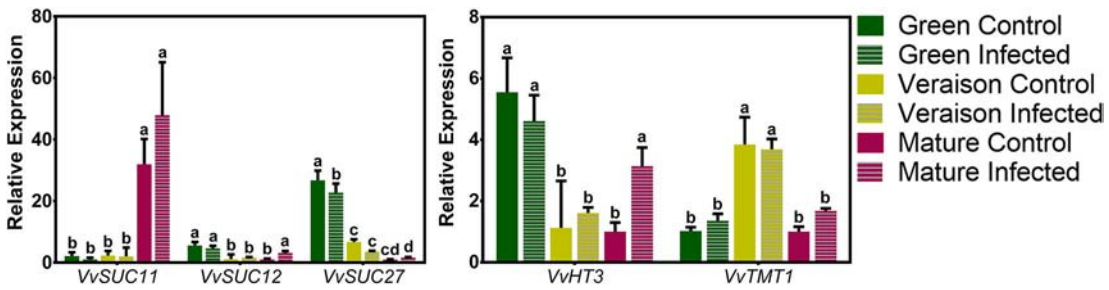

FIGURE 2 | Expression profile of some *VvSUCs* and *VvHTs* genes, highly expressed in the grape berry, performed by real-time PCR in infected (striped bars) and control (solid bars) berries, collected at three different developmental stages (green, veraison, and mature). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with three internal technical replicates. Values are the mean ± SD. (one-way ANOVA with Tukey's post-test; different letters denote statistical differences between columns).

related WRKY71OS and GT1GMSCAM4, or the abiotic stress related cis-acting element MYCCONSENSUSAT. However, only a few sugar responsive elements were identified in the promoter region of *VvSWEET15* gene, as the SBOXATRBCS element. When compared with other *VvSWEETs*, *VvSWEET15* presents fewer sugar responsive elements in its promoter region (**Supplementary Table 4**).

Sub-Cellular Localization of VvSWEET7 and VvSWEET15

VvSWEET7-GFP and VvSWEET15-GFP fusion proteins were transiently expressed in *Nicotiana benthamiana* epidermal cells and co-localization studies with the fusion protein AtPIP2.1-RFP, an aquaporin targeted to the plasma membrane, revealed that both VvSWEET7 and VvSWEET15 localize to the plasma membrane (**Figure 3**).

Functional Characterization of VvSWEET7 by Heterologous Expression in *Saccharomyces cerevisiae*

The *hxt*-null yeast strain EBY-VW4000 was transformed with pYES-DEST52 containing the cloned *VvSWEET7* cDNA under the control of the galactose-inducible *GAL1* promoter. The first evidence for the involvement of a sugar transport system was provided from the studies of the P-type ATPase activity after the addition of different sugars to suspensions of *VvSWEET7*-transformed cells (**Figure 4**). As can be seen, a clear acidification signal was recorded after addition of glucose, fructose, or sucrose to yeast cells harboring the construct *pYES-DEST52-VvSWEET7* that was not observed in suspensions of yeast cells transformed with the empty vector. The acidification signal after the addition of galactose was less evident. These results suggested that VvSWEET7 is capable of transporting both mono- and disaccharides that, once inside the cells, are catabolized into ATP that activates the proton pump.

The uptake of radiolabeled substrates was also performed and, as shown in **Figure 5**, both the initial uptake rates of 7.5-50 mM D-[^{14}C]-glucose and 7.5-125 mM [^{14}C]-sucrose followed Michaelis-Menten kinetics, suggesting carrier-mediated transport for both substrates. The kinetic parameters were as follows: K_m, 15.42 mM glucose and V_{max}, 7.4 nmol glucose mg D.W.$^{-1}$ min^{-1} and K_m, 40.08 mM sucrose and V_{max} 15.12 nmol sucrose mg D.W.$^{-1}$ min^{-1} (**Figures 5A**). Moreover, the addition of 50 μM of the protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) did not inhibit the uptake of 25 mM D-[^{14}C]-glucose at pH 5.0, suggesting the transport mechanism was not dependent on the proton gradient (**Figure 5**).

To assess the substrate specificity of VvSWEET7, the uptake rate of 25 mM of D-[^{14}C]-glucose was determined in the presence of putative competitive inhibitors of glucose transport, such as other monosaccharides, disaccharides, and polyols at a concentration 20-fold higher than that of D-[^{14}C]-glucose (**Figure 5**). Fructose inhibited radiolabeled glucose uptake by 79%, galactose by 56%, sucrose by 47%, mannitol by 29%, and sorbitol by 38%. These results suggest that VvSWEET7 has a broad transport capacity, including for sugar-alcohols.

Attempts were also made to assess the ability of EBY-VW4000 cells expressing VvSWEET15 to transport sugars (glucose, fructose, and sucrose), but no VvSWEET15-mediated sugar transport was detected in any case (data not shown), suggesting that VvSWEET15 may not have such a function.

DISCUSSION

The proteins encoded by *VvSWEET7* and *VvSWEET15* are likely to play important roles during fruit development and ripening. Our results, obtained in the variety Trincadeira, typically susceptible to *Botrytis* infection, are consistent with previous RNAseq results in Corvina cv. berries (Zenoni et al., 2010) regarding the expression profile throughout berry development, showing that *VvSWEET7* expression in berries peaks at the green stage and *VvSWEET15* at the mature stage. In the present study, we showed that the transcription of these 2 genes in response to *Botrytis* infection was up-regulated in those stages when the basal gene expression is high. The transcript levels of *VvSWEET2a* were also substantially increased at the green stage in response to *Botrytis* infection. In agreement with

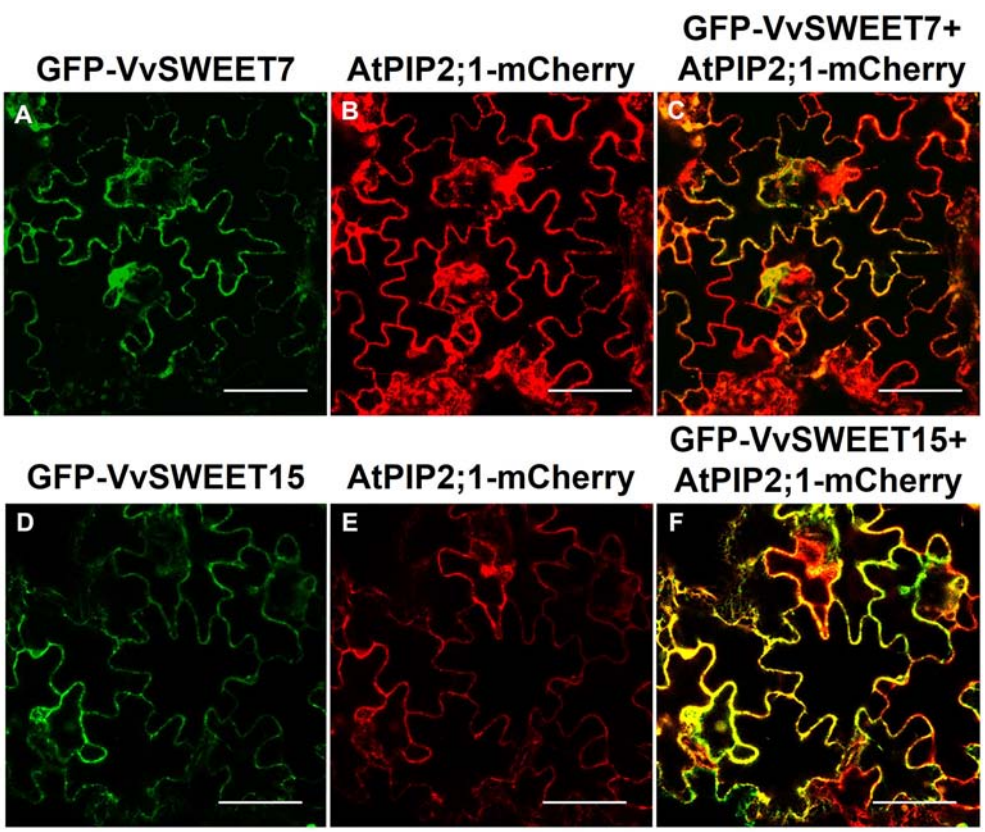

FIGURE 3 | Sub-cellular localization of VvSWEET7 (sub-figures **A–C**) and VvSWEET15 (sub-figures **D–F**) in tobacco leaves. Plasma membrane aquaporin AtPIP2;1 was used as a plasma membrane marker (positive control) (Nelson et al., 2007). Both GFP-VvSWEET7 and GFP-VvSWEET15 localize to the plasma membrane of leaf epidermis cells, as demonstrated by the yellow fluorescence signal observed by confocal microscopy resulting from co-localization of either GFP-VvSWEET7 or GFT-VvSWEET15 fusion proteins with the plasma membrane marker AtPIP2;1-mCherry. All pictures are representative of 3 different replicates. Bar = 100 μm.

this observation, the expression of *VvSWEET2a* and *VvSWEET7* in grape leaves increased 72 h after foliar inoculation with *Botrytis* (Chong et al., 2014). Still, no such response had been previously demonstrated in grape berry infection with *Botrytis*, and different plant tissues could, in theory, have different *SWEET* transcriptional responses to *Botrytis*.

Transcriptional reprograming of the expression of *SWEET* genes in response to *Botrytis* infection has also been reported in other plant species. The *Arabidopsis AtSWEET4*, *AtSWEET15* and *AtSWEET17* (Chen et al., 2010) and tomato *SlSWEET15* (Asai et al., 2016) are up-regulated by the infection. Other fungal pathogens such as *Golovinomyces cichoracearum* and mycorrhizal fungus as *Rhizophagus irregularis* (Ferrari et al., 2007; Chen et al., 2010; Manck-Götzenberger and Requena, 2016), as well as bacterial pathogens (Chen et al., 2010), are also known to modulate host *SWEET* gene expression. Transcriptional activator-like (TAL) effectors of *Xanthomonas oryzae* pv. *oryzae* induce rice *OsSWEET11*, *OsSWEET13,* and *OsSWEET14* expression (Chen et al., 2010; Zhou et al., 2014). Cassava *MeSWEET10a* and citrus *CsSWEET1* are also induced by *Xanthomonas* (Cohn et al., 2014; Hu et al., 2014). Other bacteria including *Pseudomonas syringae* induced several *AtSWEET* genes in infected leaves of *Arabidopsis* (Chen et al., 2010).

Induction of SWEET transporters by pathogens has been linked with higher susceptibility to pathogen-induced disease, as pathogens can explore these transporters to obtain sugars by SWEET-promoted leakage of sugars into the apoplastic space (Chen et al., 2010; Cohn et al., 2014). However, this correlation has not always been observed. In *Arabidopsis* roots, *AtSWEET2* gene expression was induced more than 10-fold during *Pythium* infection but *atsweet2*-knockout mutants were more susceptible to infection (Chen et al., 2015c). Also, gene expression of *IbSWEET10* was significantly up-regulated in sweet potato infected with *Fusarium oxysporum* and overexpression of the gene improved host resistance (Li et al., 2017). It has been proposed that sugar remobilization can trigger signaling cascades that activate defense mechanisms in plants (Gebauer et al., 2017). In this regard, glucose and sucrose-mediated induction of defense-related secondary metabolism has been reported (Xiao et al., 2000; Morkunas et al., 2005; Solfanelli et al., 2006; Dao et al., 2011; Kim and Hwang, 2014; Tonnessen et al., 2014). As the grape berry is more resistant to *Botrytis* attacks during its green stage (Goetz et al., 1999), it is reasonable to doubt that the overexpression of *VvSWEET2a* and

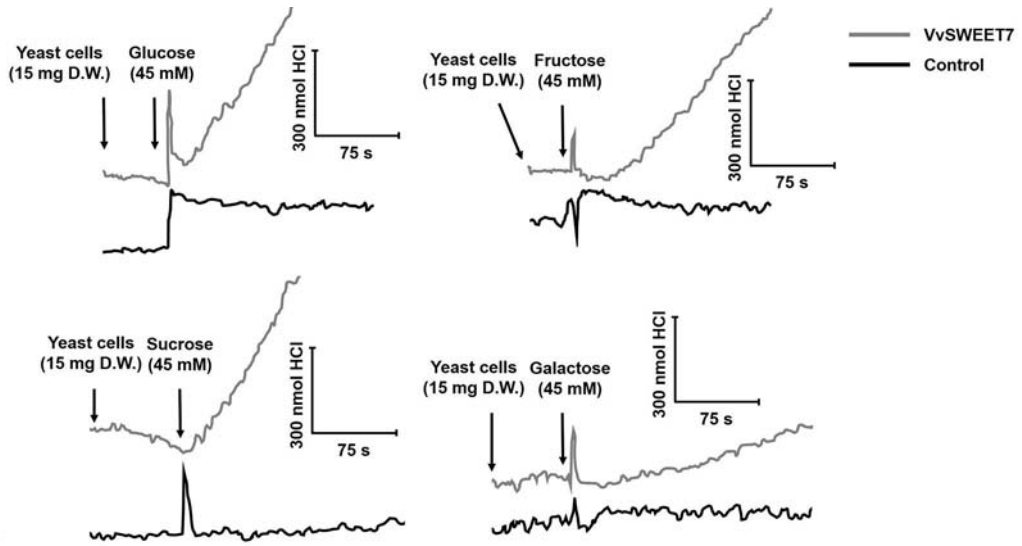

FIGURE 4 | Representative experiments of the activation of the plasma membrane H^+-ATPase in suspensions of VvSWEET7-expressing EBY.VW4000 yeast cells and controls (empty vector) induced by mono- and disaccharides. For proton pumping activity analysis, four experimental repetitions were performed, each one consisting of an independent VvSWEET7-overexpressing and control yeast growth and subsequent sugar-induced pH variation analysis. All illustrations are representative of 4 different replicates.

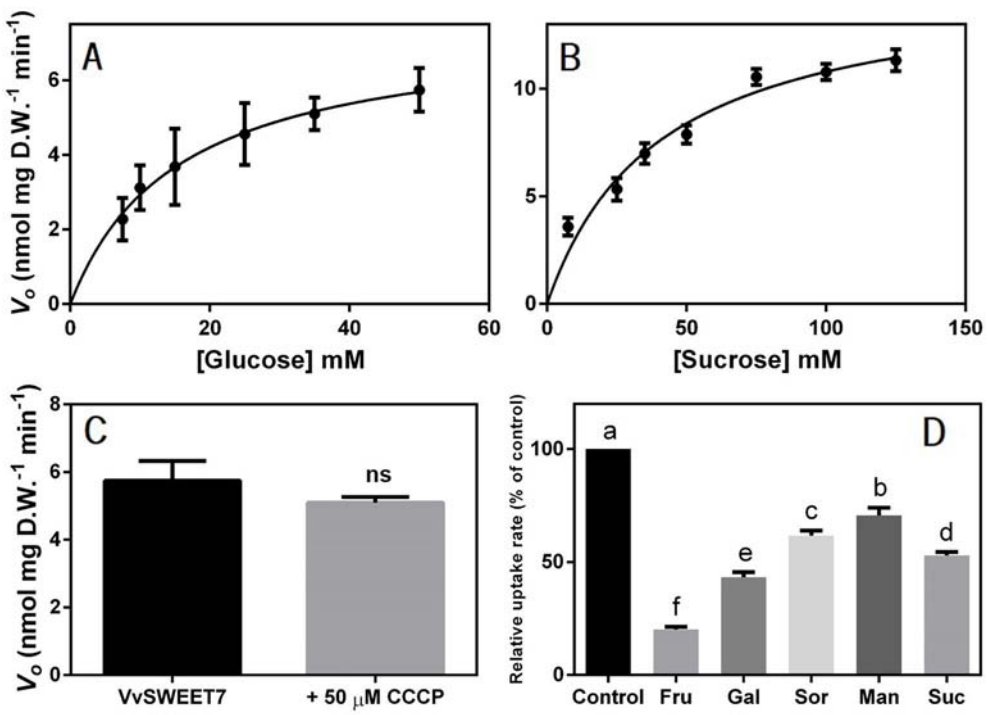

FIGURE 5 | Concentration dependence of the initial uptake rates of D-[^{14}C] glucose **(A)** and [^{14}C] sucrose **(B)** in VvSWEET7-expressing EBY.VW4000 cells at pH 5.0. **(C)** Effect of 50 μM of the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) on VvSWEET7-mediated uptake of 25 mM D-[^{14}C] glucose. **(D)** Competitive inhibition of VvSWEET7-mediated glucose uptake: substrate specificity of VvSWEET7 – fructose, galactose, sorbitol, mannitol, and sucrose at a concentration of 500 mM was added immediately before the addition of 25 mM of radiolabeled D-[^{14}C] glucose. The duration of all uptake experiments was 3 min. All values are the mean ± SD of three independent experimental repetitions, each one consisting of an independent VvSWEET7-overexpressing and control yeast growth and subsequent radiolabeled sugar uptake with three technical replicates. (one-way ANOVA with Tukey's post-test; different letters denote statistical differences between columns). ns, non significant.

VvSWEET7 is an exploitation by the fungus to facilitate infection, rather suggesting that their overexpression may be a defense-related mechanism.

More information regarding what stimuli, from sugar availability to pathogen attack or hormonal stimuli, could activate the promoters of grape berry SWEET genes can be thoroughly seen in **Supplementary Tables 2–4**, where the identification of cis-acting elements was performed initially using PLACE database (Higo et al., 1999) and subsequently confirmed following analysis using PlantPAN3 (Chow et al., 2019).

It has been hypothesized that if *SWEETs* transporters are exploited by the fungus to promote sugar leakage to the apoplastic space, plants can, as a response, induce secondary-active sugar transporters to retrieve that sugar (Fotopoulos et al., 2003; Lemonnier et al., 2014; Yamada et al., 2016). In *Arabidopsis*, the induction of hexose/H^+ symporters, such as STP1, 4, and 13 may counteract SWEET-mediated secretion induced by bacterial infection (Fotopoulos et al., 2003; Yamada et al., 2016). The sugar transporter AtSTP13 is phosphorylated after the interaction with the flagellin receptor AtFLS2 and its co-receptor receptor kinase 1 AtBAK1, which enhances AtSTP13 monosaccharide uptake activity. Hence, this transporter can compete with bacteria for extracellular sugars (Yamada et al., 2016). In the present study, out of the five secondary active transporters genes highly expressed in the grape berry (Lecourieux et al., 2014), only *VvHT3* was up-regulated at the mature stage in response to infection (**Figure 2**). This putative hexose transporter gene is the most highly expressed member of the *VvHT* family in the mature berry (Afoufa-Bastien et al., 2010), so it is tempting to speculate that it could indeed be recruited to retrieve sugar accumulated in the apoplast in response to infection, but this hypothesis needs further experimental clarification.

Results showed that some *SWEET* members were down-regulated in response to *Botrytis* infection. This was the case of *VvSWEET10*, *VvSWEET11*, *VvSWEET17a*, and *VvSWEET17d* whose transcript levels clearly decreased in the developmental stages where their expression was higher: *VvSWEET17a* at the green stage, *VvSWEET10* and *VvSWEET17d* at veraison, and *VvSWEET11* at the mature stage. It would be interesting to observe if this phenomenon also occurs in berries from grapevine varieties more resistant to *Botrytis*, but such results are so far lacking in the literature. Only a recent study reported a decrease in the expression of *SWEETs* genes upon infection, but in tomato. In *Botrytis*-infected cotyledons, 21 of the 31 tomato *SWEET* genes were down-regulated, including the tomato *VvSWEET10*, *VvSWEET11*, *VvSWEET17a*, and *VvSWEET17d* homologues (Asai et al., 2016). *Botrytis* is capable to silence *Arabidopsis* and tomato genes involved in immunity by producing and translocating small RNAs (sRNAs) that hijack the host RNA interference (RNAi) machinery (Weiberg et al., 2013). However, the significance of the down-regulation of *SWEET* genes during pathogen attack is still puzzling.

Clear-cut co-localization experiments revealed that VvSWEET7 and VvSWEET15 are plasma membrane-bound proteins and were heterologously expressed in an *S. cerevisiae* mutant to study their function. The yeast expressing VvSWEET7 showed the capacity to transport glucose and, remarkably, sucrose. In the presence of the protonophore, CCCP transport capacity of VvSWEET7 was not inhibited, demonstrating facilitated transport, in line with previous reports (review by Chen et al., 2015b). Fructose, mannitol, and sorbitol also inhibited glucose transport, suggesting that besides mono and disaccharides, VvSWEET7 may possibly mediate the transport of polyols. The affinity of SWEET transports has been reported in the mM range from ~9 (AtSWEET1) to ~70mM (AtSWEET12) (Chen et al., 2010; Chen et al., 2012), in line with the results of the present study. So far, in grapevine, only two SWEETs (VvSWEET4 and VvSWEET10) have been functionally characterized as sugar transporters following complementation studies in yeast; contrarily to the present work, kinetics analysis, kinetic parameter determinations, and substrate specificity studies were not performed (Chong et al., 2014; Zhang et al., 2019).

As previously mentioned, the SWEET family is divided into 4 different clades, but within each clade proteins may have different physiological roles (Eom et al., 2015). Considering the few characterized SWEETs, each clade appears to correlate well with the selectivity of each member towards monosaccharides versus disaccharides (Clade I and II prefer hexoses, clade III sucrose) (Chen et al., 2015b). However, in the present study, VvSWEET7 exhibited the capacity to transport both mono and di-saccharides, as previously reported for AtSWEET16 (Klemens et al., 2013).

The possible polyol transport capacity evidenced by VvSWEET7 is so far unique in the SWEET family. The polyol transporter AtPLT5, localized in the plasma membrane of *Arabidopsis*, is able to actively transport a broad-spectrum of substrates such as sorbitol, xylitol, erythritol, or glycerol and also different hexoses, such as glucose and pentoses including ribose, tetroses, and a sugar acid (Reinders et al., 2005; Klepek et al., 2005). Similarly, VvPLT1 (VvPMT5) has been characterized as a polyol transporter that is competitively inhibited by monosaccharides (Conde et al., 2015).

The observed broad range of transported substrates and its high expression in the green stage suggest that VvSWEET7 plays an important role in sugar partitioning during fruit development. At the green fruit stage, sucrose is predominantly translocated to the berry mesocarp cells *via* plasmodesmata (Zhang et al., 2006); however, apoplasmic transport through VvSWEET7 may be also involved. The *Arabidopsis* VvSWEET7 homologue functions as a glucose transport and is expressed mainly in the flower and seed (Chen et al., 2010); the cucumber CsSWEET7b transports glucose and, to a minor degree, mannose and galactose (Li et al., 2017). Interestingly, the tomato VvSWEET7 homologue (SlSWEET6) is also strongly regulated during the early phases of tomato fruit development (Shammai et al., 2018).

However, only a few sugar responsive elements were identified in the promoter region of the *VvSWEET15* gene, as the SBOXATRBCS element. When compared with other *VvSWEETs*, *VvSWEET15* presents fewer sugar responsive elements in its promoter region (**Supplementary Table 4**).

In our experimental conditions, we were not able to demonstrate that VvSWEET15 mediates sugar transport in grapevine, despite the fact that its *Arabidopsis* ortholog,

AtSWEET15, is well characterized as a sucrose transporter (Chen et al., 2012). In fact, *VvSWEET15* promoter region has fewer sugar-responsive cis-acting elements than other *VvSWEETs*, and together with the absence of sucrose responsive elements, suggests that VvSWEET15 might actually not be a sugar transporter. Contrarily, in *Arabidopsis*, AtSWEET15 appears to be involved in the remobilization of carbohydrates in senescent leaves as its expression increases by 22-fold during senescence (Quirino et al., 1999). Also, it regulates cell viability under high salinity (Seo et al., 2011) and is also involved, along with AtSWEET11 and AtSWEET12, in the sugar efflux required for seed filling (Chen et al., 2015a). In tomato, the not so well characterized SlSWEET15 showed a similar expression pattern to its grapevine homologue along with fruit development (Shammai et al., 2018), being more expressed in the mature stage.

CONCLUSION

Sugar metabolism and mobilization are important players that decide the fate of the ongoing battle between plant and pathogen during infection process. However, despite recent advances, the metabolic signatures and their regulatory nodes, which decide the susceptibility or resistance responses, remain poorly understood. In a variety of grapevine susceptible to *Botrytis* infection, grape berry infection with this pathogen promoted a transcriptional reprograming of the expression of *VvSWEET* genes in sink organs. *VvSWEET7* and *VvSWEET15* are likely to play an important role during fruit development and ripening as they are strongly expressed at the green and mature stage, respectively, and were clearly up-regulated in response to infection. VvSWEET7 was heterologously expressed in yeast and revealed a high-capacity, low-affinity glucose transport with a broad affinity to other substrates like disaccharides and polyols. Previous relevant studies have already addressed the role of key SWEET genes highly expressed in roots, stems, leaves, and nectary tissue (Chen et al., 2010; Chen et al., 2012; Chardon et al., 2013; Guo et al., 2014; Lin et al., 2014; Chong et al., 2014; Zhang et al., 2019), in some cases also demonstrating SWEET response to pathogen attack in such plant tissues; VvSWEET4 and VvSWEET10 have already been demonstrated, by yeast complementation, to be hexose transporters in grapevine. Still, the present study is relevant because: i) functional characterization revealed that VvSWEET7 transports both glucose and sucrose and the affinities for each of the substrates were successfully determined, together with substrate specificity assessment; and ii) the transcriptional reprogramming upon *Botrytis* infection was assessed in the grape berries and notably on a variety susceptible to infection with this pathogen. Considering the advances achieved in this work, together with what was previously known in the literature, the role of SWEETs in plants as friends or foes during pathogenic attack is still a matter of debate.

DATA AVAILABILITY STATEMENT

All datasets generated/analyzed for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

RB, AC, and HG conceptualized the work. RB conducted the experiments. DP and AF performed the sample treatment and harvest and the broad promoter analysis. CC and RB conducted the confocal microscope observations. RB, AC, and HG contributed to the analysis of the results. RB, AC, AF, AG, and HG wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:https://www.frontiersin.org/articles/10.3389/fpls.2019.01753/full#supplementary-material

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Photosynthetic Cost Associated With Induced Defense to *Plasmopara viticola* in Grapevine

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Downy mildew caused by *Plasmopara viticola* is one of the most destructive diseases of *Vitis vinifera* worldwide. Grapevine breeding programs have introgressed *P. viticola*-resistant traits into cultivated *V. vinifera* genotypes and launched interspecific hybrids with resistance against downy mildew. In general, pathogen infection affects primary metabolism, reduces plant growth and development and modifies the secondary metabolism toward defense responses, which are costly in terms of carbon production and utilization. The objective of this work was to evaluate the photosynthesis impairment by inducible defenses at the leaf level in *V. vinifera* cultivars resistant to *P. viticola*. Photosynthetic limitations imposed by *P. viticola* in susceptible and resistant grapevine cultivars were evaluated. Histochemical localization of hydrogen peroxide and superoxide and the activity of ascorbate peroxidase were assessed. Measurements of leaf gas exchange, chlorophyll fluorescence and the response of leaf CO_2 assimilation to increasing air CO_2 concentrations were taken, and photosynthetic limitations determined in cultivars Solaris (resistant) and Riesling (susceptible). The net photosynthetic rates were reduced (−25%) in inoculated Solaris plants even before the appearance of cell death-like hypersensitive reactions ("HR"). One day after "HR" visualization, the net photosynthetic rate of Solaris was reduced by 57% compared with healthy plants. A similar pattern was noticed in resistant Cabernet Blanc and Phoenix plants. While the susceptible cultivars did not show any variation in leaf gas exchange before the appearance of visual symptoms, drastic reductions in net photosynthetic rate and stomatal conductance were found in diseased plants 12 days after inoculation. Decreases in the maximum Rubisco carboxylation rate and photochemical impairment were noticed in Riesling after inoculation with *P. viticola*, which were not found in Solaris. Damage to the photochemical reactions of photosynthesis was likely associated with the oxidative burst found in resistant cultivars within the first 24 h after inoculation. Both chlorophyll degradation and stomatal closure were also noticed in the incompatible interaction. Taken together, our data clearly revealed that the defense response against *P. viticola* causes a photosynthetic cost to grapevines, which is not reversible even 12 days after the pathogen infection.

Keywords: gas exchange, downy mildew, *Vitis vinifera*, photosynthesis limitations, biotic stress

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INTRODUCTION

Downy mildew, caused by the biotrophic oomycete *Plasmopara viticola*, is one of the most devastating diseases of grapevine. *P. viticola* is native to North America and was considered to be of minor importance on *Vitis aestivalis*, in which it was first described in 1834 (Wilcox et al., 2015). However, this pathogen was inadvertently introduced into Europe in 1878 and now it is endemic in the main grapevine growing regions worldwide (Gessler et al., 2011). The European grapevine (*Vitis vinifera*) evolved in the absence of this disease and it is extremely susceptible to *P. viticola*. After *P. viticola* introduction the pathogen quickly spread throughout the European vineyards (Rossi et al., 2013). *P. viticola* affects grapevine leaves, inflorescences, berries and shoots with high disease severity causing defoliation and producing low-quality, unsightly or entirely damaged grapes. The pathogen can also cause weakening, dwarfing and death of young shoots (Agrios, 2005). When the weather is conducive to the disease and in the absence of effective disease control, downy mildew can easily reduce the grapevine yield up to 100% (Agrios, 2005; Wilcox et al., 2015). *P. viticola* zoospores penetrate the host through stomata and develop intercellular mycelium in the mesophyll of grapevine leaves, generating globose haustoria responsible for the oomycete nutrition. After infection and colonization periods, sporangiophores and sporangia emerge from the stomata (Ash, 2000; Agrios, 2005; Gessler et al., 2011; Kamoun et al., 2015; Armijo et al., 2016). Currently, downy mildew is of paramount importance in all humid parts of the world where grapevine is grown. In subtropical regions of South America, the occurrence of rainy periods at the time of budbreak and flowering can lead to severe epidemics of downy mildew. Consequently, high frequency of fungicide sprays is necessary to control downy mildew during the rainy season, i.e., from summer to autumn (Cappello et al., 2017).

Although European *V. vinifera* cultivars are highly susceptible to downy mildew, *Muscadinia* species and several American and Asian *Vitis* species exhibit varying levels of resistance to *P. viticola*. By the conventional breeding, *V. vinifera* has been crossed with grapevines showing resistance against *P. viticola*, and resistant interspecific hybrids have been subsequently found. In resistant accessions obtained by crosses with North American *Vitis* species, *P. viticola* completes its life cycle and releases less sporangia than on susceptible cultivars (Bellin et al., 2009). The genomic regions conferring resistance to downy mildew referred to as resistance to *P. viticola* (*Rpv*) are quantitatively inherited. To date, 27 quantitative trait loci (QTLs) with major effects on downy mildew resistance are known and described (Fischer et al., 2004; Welter et al., 2007; Bellin et al., 2009; Marguerit et al., 2009; Vezzulli et al., 2019). *Rpv1* and *Rpv2* are responsible for the resistance derived from *Muscadinia rotundifolia* (Peressotti et al., 2010) and map to chromosomes 12 and 18, respectively (Merdinoglu et al., 2003; Welter et al., 2007). The locus *Rpv3*, which was first described in cultivars Regent and Bianca, came from the American wild *Vitis* species and is located on chromosome 18 (Fischer et al., 2004; Welter et al., 2007; Bellin et al., 2009). The Asian resistance loci *Rpv8*, *Rpv10*, and *Rpv12* originated from *Vitis amurensis*. *Rpv8* and *Rpv12* map to chromosome 14 (Venuti et al., 2013) and *Rpv10* maps to chromosome 9 and is present in cultivar Solaris (Blasi et al., 2011; Schwander et al., 2012; Venuti et al., 2013). The table of loci traits in grapevine relevant for breeding and genetics with a complete description of associated markers, their chromosomal localization, and the donor genotype/species of *P. viticola* resistance is available online (www.vivc.de/loci).

The mechanisms by which grapevine cells reduce downy mildew intensity are complex and not fully elucidated (Kortekamp, 2006; Liu et al., 2015). These mechanisms comprise pre-existing chemical defenses and inducible structural and biochemical defenses. High constitutive levels of antimicrobial compounds, such as inositol and caffeic acid, are observed in healthy leaves of resistant cultivars (Figueiredo et al., 2008). The induced structural defenses include formation of callose (Kortekamp et al., 1997; Gindro et al., 2003) and lignification (Dai et al., 1995). The accumulation of stilbenes and pathogenesis-related proteins (Kortekamp, 2006; Richter et al., 2006; Slaughter et al., 2008), the generation of reactive oxygen species, the induction of peroxidases (Kortekamp and Zyprian, 2003), and finally, cell death-like hypersensitive reactions ("HR") represent induced biochemical defenses found in grapevine resistant cultivars after inoculation with *P. viticola* (Díez-Navajas et al., 2008).

Pathogen infection in incompatible interactions may affect the plant primary and also the secondary metabolism due to the induction of defenses (Berger et al., 2007). In the incompatible interaction of *Phytophthora nicotianae* and *Nicotiana tabacum,* photosynthesis is switched off few hours after pathogen inoculation, and defense mechanisms are initiated. This metabolic shift is related to an early blockage of intercellular sugar transport by callose deposition and increases in apoplastic invertase activity (Scharte et al., 2005). Both constitutive and inducible defenses are costly in terms of carbohydrate production and utilization. However, inducible systems seem to be less costly than constitutive systems in the absence of pathogens because inducible defenses require allocation of both energy and resources away from growth and reproduction (Cipollini, 1998; DeWitt et al., 1998; Purrington, 2000; Heil and Baldwin, 2002). The cost of inducible defenses is a widely discussed topic among breeders and pathologists and there are few papers conveying a significant piece of novel information on the drawbacks of plant disease resistance. The development of new resistant varieties to *P. viticola* is a promising way to control downy mildew and the better understanding of the effect of *P. viticola* in the leaf carbon assimilation of resistant plants is an important issue for the scientific community interested in grapevine-pathogen interaction.

Plasmopara viticola affects the photosynthesis of susceptible grapevine cultivars by reducing the diffusion of CO_2 in leaf mesophyll (Jermini et al., 2010; Nogueira Júnior et al., 2019). In diseased plants, the concentration of photosynthetic pigments in areas nearby lesions and the primary photochemistry are

reduced (Moriondo et al., 2005). Reductions and abundance in Rubisco activity have also been reported in susceptible cultivars of *V. vinifera* and *Vitis labrusca* infected by *P. viticola* (Gamm et al., 2011; Nogueira Júnior et al., 2019). However, the effect of downy mildew on photosynthetic capacity and leaf gas-exchange for resistant cultivars has not been characterized. Here, alterations in gas exchange, chlorophyll fluorescence and production of oxygen reactive species of leaves from cultivars with different resistance levels to downy mildew were examined. The objective of this work was to estimate the impairment of photosynthesis caused by inducible responses to the infection of *P. viticola* on downy mildew resistant cultivars and to elucidate the underlying limitations imposed by *P. viticola* to the photosynthesis of resistant and susceptible cultivars. The oxidative burst induced by *P. viticola* was also evaluated and related to the photosynthetic limitations in resistant cultivars.

MATERIALS AND METHODS

The characterization of photosynthetic cost associated with inducible defenses to *P. viticola* in grapevine was performed in three steps. The first step, which is described in section "Gas Exchange Variables in Cultivars Resistant and Susceptible to Downy Mildew," aimed to evaluate gas exchange in grapevine leaves of different cultivars, resistant and susceptible to downy mildew. The resistant cultivars Solaris, Cabernet Blanc and Phoenix and the susceptible cultivars Riesling, Niagara Rosada, Merlot and Moscato were used in this step. For the second step two representative cultivars, one resistant (Solaris) and one susceptible (Riesling) to *P. viticola* were selected. This step aimed to evaluate the diffusive, photochemical and biochemical limitations imposed by *P. viticola* on grapevine photosynthesis. The experiment at the last step aimed to characterize some induced responses in the cultivar Solaris and to correlate these responses with the early decrease on photosynthesis of leaves challenged with *P. viticola*.

Plant Material and Inoculum Maintenance

All experiments were performed with potted grapevine plants using the downy mildew resistant cultivars of *V. vinifera* Solaris, Cabernet Blanc and Phoenix; the susceptible cultivars Riesling, Merlot, Moscato; and the susceptible cultivar Niagara Rosada (*V. labrusca*). The cuttings were grown in pots containing sterilized substrate (manure, clay soil and sand at a ratio of 3:3:1) under greenhouse conditions where the average air temperature was 25 ± 5°C (error SEM), the RH was 60% and the photosynthetic active radiation (PAR) was 500 μmol m^{-2} s^{-1} with a 12-h photoperiod. The substrate was fertilized with 3 g of Osmocote® per gram of soil. After budbreak, the plants were conducted in a single stem and top pruned when they presented 6–7 fully expanded leaves. The plants received 200 mL of water daily and were fertilized monthly with 10 g of Hakaphos® Blau.

Leaves with symptoms of downy mildew were collected in vineyards, and sporangia of *P. viticola* were harvested, dried at room temperature and maintained at −25°C in Eppendorf tubes. Sporangia were rehydrated in 50 mL of bidistilled water and suspensions of the inoculum at a concentration of 10^4 sporangia mL^{-1} were obtained using a Neubauer chamber. One month after budbreak, potted plants of cv. Merlot were inoculated by spraying inoculum suspension in the abaxial side of leaves up to runoff. Immediately after the inoculation, plants were kept in an incubation chamber at 25°C and covered with a black plastic bag to keep leaves in darkness and high air relative humidity (>90%) for 24 h. Three drops of 50 μL each of the sporangia suspension used for inoculation were placed in a polystyrene dish and kept in the same incubation chamber of inoculated plants to evaluate the viability of sporangia. Viability of sporangia was determined by the observation at light microscope of the opened sporangium 24 h after incubation. Afterward, the plastic bags were removed and plants were kept in the same incubation chamber with a 12-h photoperiod and PAR of 400 μmol m^{-2} s^{-1}. The inoculum production was carried out by successive inoculations of *P. viticola* suspensions into new healthy potted plants every month.

Gas Exchange Variables in Cultivars Resistant and Susceptible to Downy Mildew

Seven experiments were performed, one per cultivar, with the three resistant and the four susceptible cultivars. Five plants of each cultivar were inoculated as described above and kept at humid chamber for 12 h. As a control, five plants were sprayed with distilled water. The experiments were performed in the full randomized design. Net photosynthetic rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration (E) were measured on the inoculation day (day 0) and 1, 4, and 12 days after the inoculation (dai) of *P. viticola* in all resistant cultivars, except for cv. Solaris in which the gas exchange was evaluated at 0, 1, 2, 5, and 12 dai. In the susceptible cultivars the gas exchange variables were measured at 0, 1, 2, 6, and 12 dai. The instantaneous carboxylation efficiency (k) was estimated as A/C_i (Machado et al., 2005) in each measurement of leaf gas exchange. The evaluations were performed with portable infrared gas analyzers (GFS3000, Heinz Walz GmbH, Germany; and Li-6400-XT, LI-COR Inc., United States) equipped with fluorometers (PAM 3055 and 6400-40, respectively). Cuvette temperature was set to 25°C and relative humidity was 60% at the inlet of the cuvette. The cuvette sizes were 2 and 4 cm^2, respectively for Li-6400-XT and GFS3000. CO_2 concentration was kept constant at 400 μmol mol^{-1}. A LED array provided PAR of 1000 μmol m^{-2} s^{-1}. A, g_s, C_i and E were recorded after fluxes had stabilized. The leaf areas evaluated for gas exchange were photographed at 4 and 12 dai and digital images were processed with Quant software (Vale et al., 2001) to estimate downy mildew severity. All experiments, except for cv. Phoenix, were repeated once. The average values of photosynthetic variables measured in healthy and diseased plants were compared by Student's t-test ($p \leq 0.05$) using STATISTICA 6.0 software (StatSoft Inc., Tulsa,

OK, United States). The normal distribution of data was verified by the Shapiro-Wilk's test and transformations were performed when necessary (**Supplementary Table S1**).

Photosynthetic and Photochemical Evaluations in Solaris and Riesling Cultivars

The photosynthetic limitations caused by *P. viticola* were evaluated in the grapevine cultivars Solaris and Riesling, which are resistant and susceptible to downy mildew, respectively. Three plants of each cultivar were inoculated, and three plants were sprayed with water as described previously. Six evaluations were performed in each experiment from the 5th day after inoculation onward (twice a day) using the GFS3000 (Heinz Walz, GmbH, Germany) equipped with a fluorometer (PAM 3055). Leaf CO_2 assimilation response to increasing air CO_2 concentration (C_a) was initially evaluated at 400 μmol mol^{-1}, which was gradually changed to 250, 200, 150, and 50 μmol mol^{-1} and then gradually increased to 400, 600, 800, 1400, and 2000 μmol mol^{-1}. Day respiration (R_d) was obtained through a linear regression between A (response variable) and C_i (predictor variable) under C_a <400 μmol mol^{-1} and corresponds to A when C_i is zero. Mesophyll conductance (g_m) was estimated as follows (Flexas et al., 2007):

$$g_m = A/(C_i - (\Gamma^*(J + 8(A + R_d)))/(J - 4(A + R_d))) \quad (1)$$

where Γ* is the photosynthetic compensation point, i.e., the CO_2 concentration at which the photorespiratory efflux of CO_2 is equal to the CO_2 photosynthetic assimilation rate; and J is the transport of electrons from chlorophyll fluorescence assessments. The calculated values of g_m were used to convert A-C_i curves into A-C_c (C_c is CO_2 concentration at carboxylation sites in chloroplasts) curves using the following equation (Flexas et al., 2007):

$$C_c = C_i - A/g_m \quad (2)$$

From A-Cc curves, the maximum Rubisco carboxylation rate (V_{cmax}) and maximum rate of electron transport driving regeneration of ribulose-1,5-bisphosphate (J_{max}) were estimated as follows (Farquhar et al., 1980; Flexas et al., 2007):

$$A = V_{cmax}(C_c - \Gamma^*)/(C_c + K_c((1 - (O/K_o))) \quad (3)$$

$$A = J_{max}(C_c - \Gamma^*)/4(C_c + 2\Gamma^*) \quad (4)$$

where K_c and K_o are the Michaelis-Menten constants of Rubisco for carboxylation and oxygenation (Bernacchi et al., 2003), respectively, and O is the internal O_2 concentration, which is considered equal to the external O_2 concentration. V_{cmax} and J_{max} were estimated by non-linear regressions using STATISTICA 6.0 software (StatSoft Inc., Tulsa, OK, United States).

The maximum leaf CO_2 assimilation (A_{max}) and the maximum stomatal conductance (g_{smax}) were also obtained in each A-C_c curve. The experiment was performed twice and the average values of photosynthetic variables measured in healthy and diseased plants were compared using Student's t-test ($p \leq 0.05$).

Detection and Quantification of Chlorophyll *a* Fluorescence, H_2O_2, O_2^- and APX Activity

Chlorophyll *a* fluorescence was measured using an Imaging-PAM Maxi (Heinz Walz GmbH, Germany) in cv. Solaris. The measurements were performed 6, 12, 30, 60, 120, and 288 h after *P. viticola* inoculation (hai). After dark-adapting leaves for 20 min, a saturation light pulse of 2700 μmol m^{-2} s^{-1} was applied and the maximum PSII quantum yield [$F_v/F_m = (F_m - F_o)/F_m$] was measured (Maxwell and Johnson, 2000). F_m and F_o denote the maximum and minimum fluorescence of dark-adapted samples, respectively.

The detection of H_2O_2 and O_2^- was performed in cvs. Solaris and Riesling and its quantification was only performed where H_2O_2 was detected. The samples were collected at 6, 12, 30, 60, 120, and 288 hai. For quantification of H_2O_2, five leaf discs of 0.46 cm^2 were removed with a cork borer approximately 2 cm away from the central vein of healthy and inoculated leaves. All five discs were immediately transferred to 1 mL of acetone acidified with 25 mM H_2SO_4 and frozen in liquid nitrogen, where they remained until measurements. For measurements, 50 μL of thawed samples were added to 1 mL of ferrous ammonium sulfate xylenol orange (FOX) solution that contained 250 μM ferrous ammonium sulfate, 100 mM sorbitol, 100 μM xylenol orange and 25 mM H_2SO_4. The samples were then incubated at room temperature for 30 to 45 min. H_2O_2 concentration was determined at 550 nm (EPOCH, BioTec, United States) and 850 nm (8453 UV–vis Spectroscopy System, Agilent, United States) using a standard curve ranging from 0 to 100 μM H_2O_2 (Wolff, 1994).

Leaf samples (0.5 g) were harvested and immediately frozen in liquid nitrogen. To assess ascorbate peroxidase (APX) activity, samples were homogenized in 5 mL phosphate buffer (pH 7.6) including 1% polyvinylpyrrolidone (PVP) and 0.1 mM EDTA and centrifuged for 20 min at 16,000 *g* at 4°C. The supernatant was collected and used as crude extract in the reaction mixtures of the enzyme activity assays. For the APX assay, the 0.3-mL reaction mixture contained 0.5 mM ascorbic acid, 50 mM phosphate buffer, 1 mM EDTA, 0.5 mM H_2O and 10–15 μL of the supernatant. The reaction was started by adding 10 μL of 15 mM of H_2O_2, and APX was assayed spectrometrically (EPOCH, BioTec, United States /8453 UV-VIS Spectroscopy System, Agilent, United States) following the decrease in absorbance at 290 nm (Nakano and Asada, 1981).

Histochemical detection of H_2O_2 and O_2^- was performed on leaf discs (3 cm^2) removed from the same leaves used for the estimation of H_2O_2 concentration. The samples were collected at 6, 12, 30, 60, 120, and 288 hai from each plant of each treatment. The samples were placed in glass vials containing 3,3′−diaminobenzidine tetrahydrochloride (DAB), pH 5.5, 1%; Sigma−Aldrich, or nitroblue tetrazolium (NBT), pH 6, 0.1%;

Sigma−Aldrich, and kept at 25°C for 2 h in darkness (Thordal-Christensen et al., 1997). After this period, the samples were cleared in 80% ethanol for 24 h, stored in glycerol solution (70%), and then observed with an optical microscope. The average values of H_2O_2 concentrations measured in healthy and diseased plants were compared by Student's *t*-test ($p \leq 0.05$).

RESULTS

Differential Gas Exchange Responses of Resistant and Susceptible Grapevine Cultivars to *Plasmopara viticola* Infection

The death-like hypersensitive reactions "HR" were observed 4 dai with *P. viticola* on the resistant cultivars Solaris, Cabernet Blanc and Phoenix (**Supplementary Figure S1**). In the susceptible cultivars, the symptoms of downy mildew and *P. viticola* sporulation were observed 6 to 7 dai. The visual area affected by "HR" did not change for cvs. Cabernet Blanc and Phoenix after 4 dai, and a slow progression (from 5.9% at 4 dai to 10.2% at 12 dai) was exclusively noticed in cv. Solaris. After 12 dai, necrotic areas accounted for 2.3 and 3.3% (on average) of Cabernet Blanc and Phoenix, respectively (**Supplementary Figures S1**, **S2**). Typical symptoms of downy mildew, i.e., oil spots on the adaxial leaf surface and abundant sporulation of *P. viticola* on the abaxial leaf surface (**Supplementary Figure S2**), were observed on all susceptible cultivars. Disease severity reached 47, 53, 46, and 34% in Riesling, Niagara Rosada, Merlot and Moscato, respectively.

On the day of inoculation with *P. viticola*, photosynthetic rates were similar for inoculated and non-inoculated leaves of resistant cultivars Solaris, Cabernet Blanc and Phoenix (**Figure 1**). However, significant reductions of leaf photosynthesis (*A*) were found at 2 dai in Solaris (**Figure 1B**) and at 1 dai for Cabernet Blanc and Phoenix (**Figures 1D,F**). At the appearance of "HR" and compared with healthy leaves, *A* values were 54, 35, and 77% lower on diseased leaves of Solaris, Cabernet Blanc, and Phoenix, respectively (**Figures 1B,D,F**). Then, the *A* values for inoculated leaves did not change significantly until 12 dai.

Photosynthetic rates ranged from 8.9 to 13.3 μmol m^{-2} s^{-1} on susceptible cultivars at the day of inoculation when no differences were found between inoculated and non-inoculated leaves (**Figures 1A,C,E,G**). When visual symptoms appeared, significant reduction of *A* was only observed in Niagara Rosada (**Figure 1C**). After 12 days of inoculation, *A* values on inoculated leaves were reduced by 56, 56, 51, and 41% compared with healthy leaves of Riesling, Niagara Rosada, Merlot, and Moscato, respectively.

No differences in C_i of inoculated and non-inoculated leaves were found before the appearance of "HR" in resistant cultivars (**Figure 2**), and this situation persisted until 12 dai for Cabernet Blanc and Phoenix. A slight increase (10.5%) was detected on C_i of inoculated leaves of Solaris 12 dai compared with non-inoculated leaves (**Figure 2B**). Similar C_i values were measured on inoculated and non-inoculated leaves of susceptible cultivars until symptoms appearance (**Figures 2A,C,E,G**). From this time, diseased leaves of Riesling and Niagara Rosada showed increases in C_i values of 14% compared with healthy leaves (**Figures 2A,C**). Similar to Cabernet Blanc and Phoenix (**Figures 2D,F**), C_i of Merlot and Moscato remained stable from 6 to 12 dai regardless of the treatment (**Figures 2E,G**).

Considering downy mildew resistant cultivars, g_s and *E* were always lower in diseased leaves than in healthy leaves from 4 dai (**Figures 3**, **4**). Differences in g_s between inoculated and non-inoculated leaves of susceptible cultivars were observed only at 12 dai in Riesling, Merlot, and Moscato (**Figures 3A,E,G**). Nevertheless, no reductions in *E* were found in susceptible cultivars with the exception of Riesling at 12 dai when the average *E* value of diseased leaves was reduced by 40% compared with healthy leaves (**Figure 4A**).

Significant reductions in the instantaneous carboxylation efficiency (*k*) due to downy mildew were measured from 1 dai for resistant cultivars and at 12 dai for susceptible cultivars (**Supplementary Figure S3**). In Niagara Rosada, reduction in *k* was also observed in diseased leaves at 6 dai (**Supplementary Figure S3C**).

Photosynthetic and Photochemical Evaluations in Solaris and Riesling Cultivars

Healthy plants were more responsive to increasing chloroplastic CO_2 concentrations when compared with *P. viticola* inoculated plants regardless of the cultivar (**Figure 5**). The maximum leaf CO_2 assimilation (A_{max}) was 18.7 and 12.7 μmol m^{-2} s^{-1} in healthy and diseased leaves of Solaris, respectively, and a reduction of 23% was noticed due to *P. viticola* infection. V_{cmax} was similar in healthy and diseased leaves of Solaris; however, J_{max} was reduced by 34% in diseased leaves (**Table 1**). The maximum stomatal conductance (g_{smax}) was reduced (−27%) in Solaris without any significant change in g_m (**Table 1**). In Riesling, A_{max} was 22% higher in healthy leaves than in diseased leaves. Unlike Solaris, biochemical limitations were higher in diseased leaves of Riesling, with plants showing low V_{cmax} and J_{max} (**Table 1**). In addition, no significant diffusive limitation was found in diseased leaves compared with healthy leaves of Riesling, as suggested by g_m and g_{smax} (**Table 1**).

Chlorophyll fluorescence from healthy and inoculated leaves of Solaris indicated similar maximum PSII quantum efficiency (F_v/F_m) at 12 hai (0.770 and 0.766). Reduced F_v/F_m in inoculated leaves was found at 5 dai (0.735) when "HR" symptoms were visible (**Figure 6**). In non-inoculated leaves, F_v/F_m was 0.752. Although the effects of *P. viticola* infection are clearly visible by imaging of chlorophyll *a* fluorescence, no significant difference was detected on any day (**Supplementary Figure S4**).

Oxidative Burst in the Resistant Cultivar Solaris

The presence of H_2O_2 as indicated by DAB staining was detected as brown spots close to the secondary veins of diseased leaves (**Figure 7B**). Brown spots were frequent and intense at 12 and 30 hai (**Supplementary Figure S5**). Necrotic tissue formed on diseased leaves from 120 hai, and no reaction to DAB staining was observed in diseased leaves at this time (data not shown). Brown

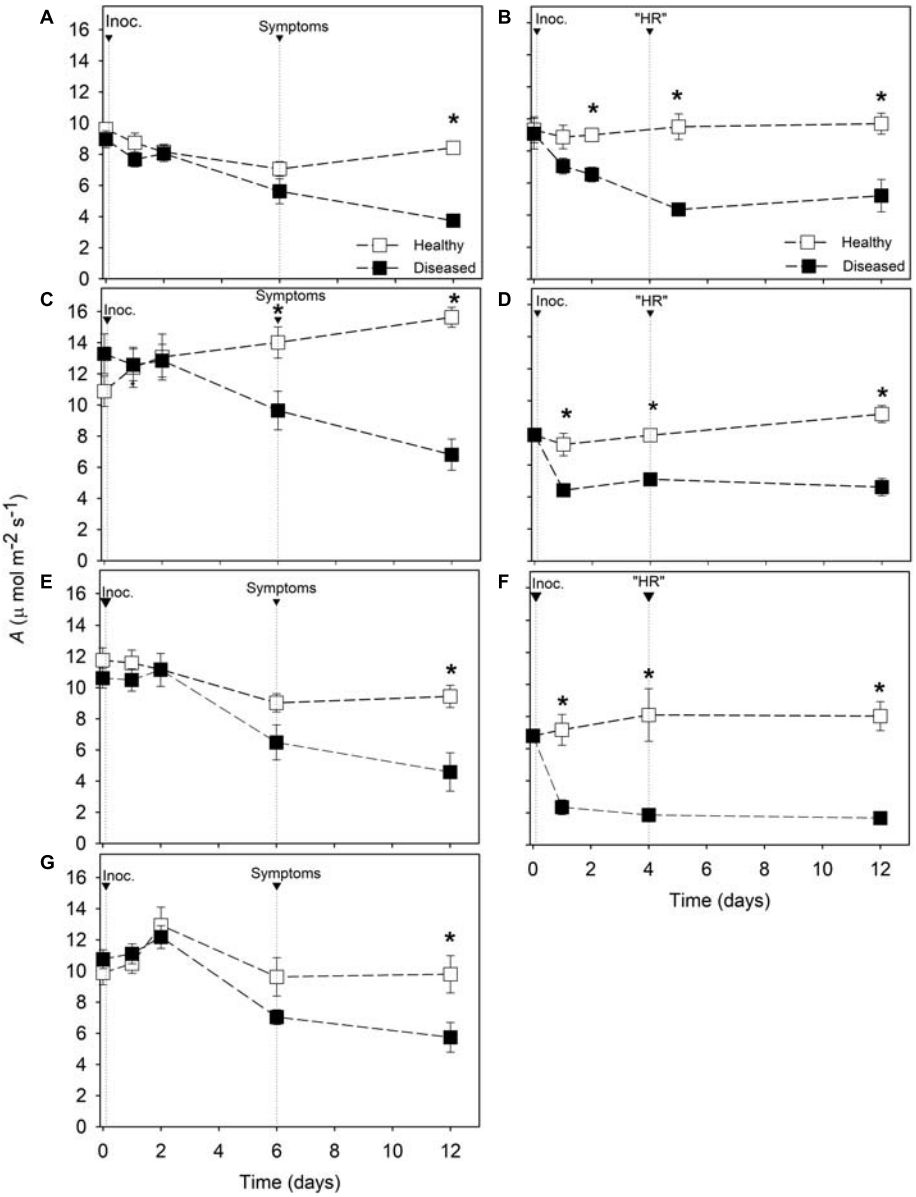

FIGURE 1 | Net photosynthetic rate *(A)* in *Vitis* spp. cultivars. Downy mildew symptoms were observed in Riesling **(A)**, Niagara Rosada **(C)**, Merlot **(E)** and Moscato **(G)** 6 days after inoculation. Cell death-like hypersensitive reactions "HR" were observed in Solaris **(B)**, Cabernet Blanc **(D)** and Phoenix **(F)** 4 days after inoculation (Inoc.). The average values of healthy and diseased leaves were compared using the Student's *t* test for each cultivar ($n = 6$, ± SE), and * indicates significant differences ($p < 0.05$).

spots were not detected in healthy leaves. H_2O_2 concentrations were lower than 0.5 μmol g^{-1} fresh mass (FM) in both healthy and diseased leaves of Solaris at 6 hai and from 120 hai. However, increases in leaf H_2O_2 concentration and data variability were noticed at 12 and 60 hai (**Figure 7**). O_2^- was detected as dark blue spots similar in shape to those spots observed in H_2O_2 detection and near secondary veins. Similar to H_2O_2, the frequency and intensity of blue spots were high at 12 hai (**Figure 7C**). No blue spots were observed at 30 hai in diseased leaves, and no O_2^- was detected in healthy leaves (data not shown). No significant difference was observed for APX activity between healthy and diseased leaves, although the highest APX activity (2.33 μmol H_2O_2 g^{-1} FM min^{-1}) was noticed in diseased leaves at 288 hai (**Supplementary Figure S6**).

DISCUSSION

The results of this study indicate that *P. viticola* infection is associated with a cost in terms of CO_2 assimilation per unit of leaf area, i.e., resistant cultivars reduced *A* earlier and more severely than susceptible cultivars. On average, 55% reduction in leaf CO_2

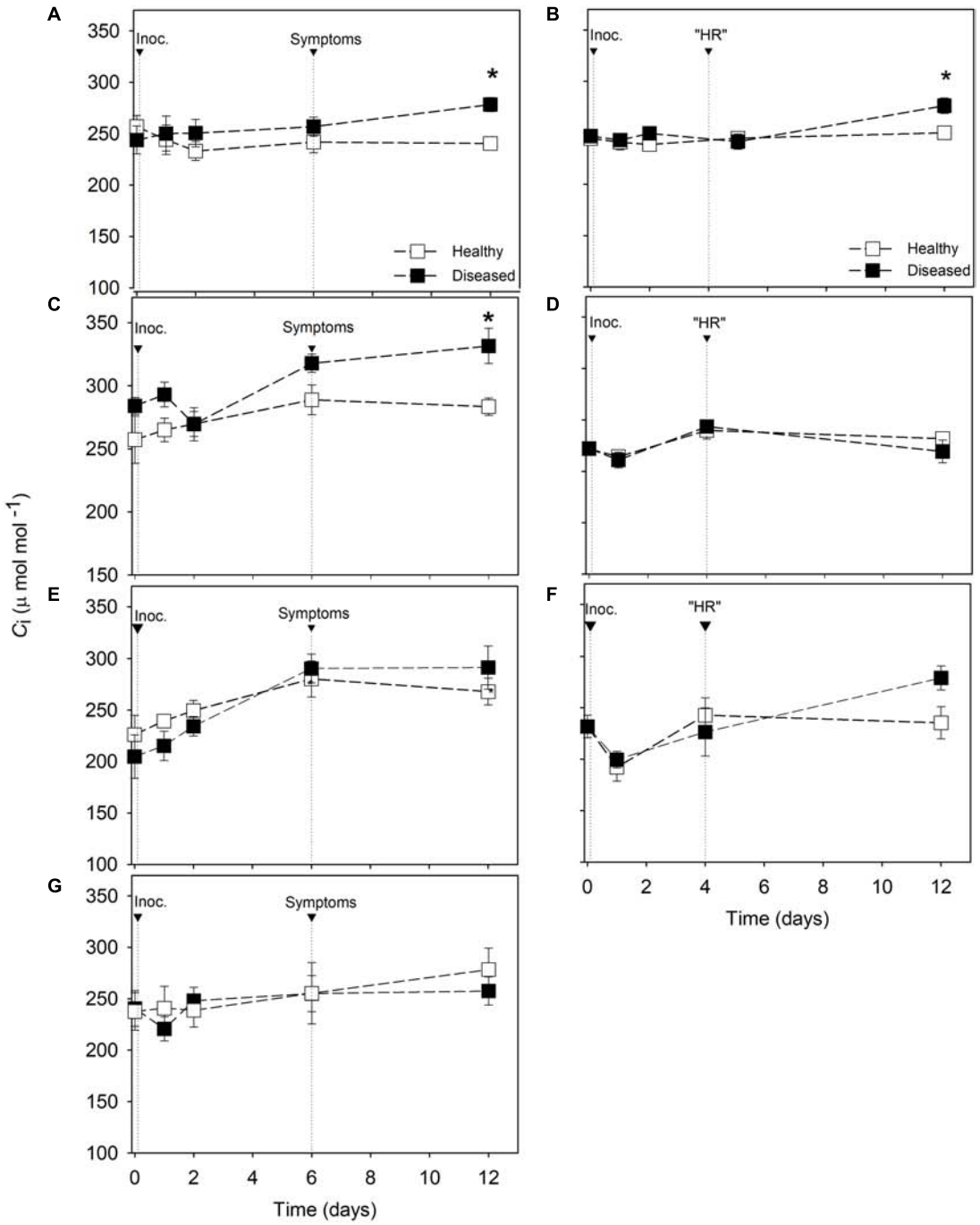

FIGURE 2 | Intercellular CO_2 concentration (C_i) in *Vitis* spp. cultivars. Downy mildew symptoms were observed in Riesling **(A)**, Niagara Rosada **(C)**, Merlot **(E)** and Moscato **(G)** 6 days after inoculation. Cell death-like hypersensitive reactions "HR" were observed in Solaris **(B)**, Cabernet Blanc **(D)** and Phoenix **(F)** 4 days after inoculation (Inoc.). The average values of healthy and diseased leaves were compared using the Student's *t* test for each cultivar ($n = 6$, ± SE), and * indicates significant differences ($p < 0.05$).

assimilation was noticed few hours after pathogen contact with its host, and such a decrease remained up to 12 days after *P. viticola* inoculation in resistant cultivars Solaris, Cabernet Blanc and Phoenix. This behavior could result from the downregulation of proteins related to photosynthesis and carbohydrate metabolism as previously reported in resistant grapevine cultivars (Figueiredo et al., 2017; Nascimento-Gavioli et al., 2017). A fast and massive transcription of signaling genes affected by *P. viticola* has also been reported on *Rpv*-bearing in grapevine cultivars (Fröbel et al., 2019). This response presumably is initiated by

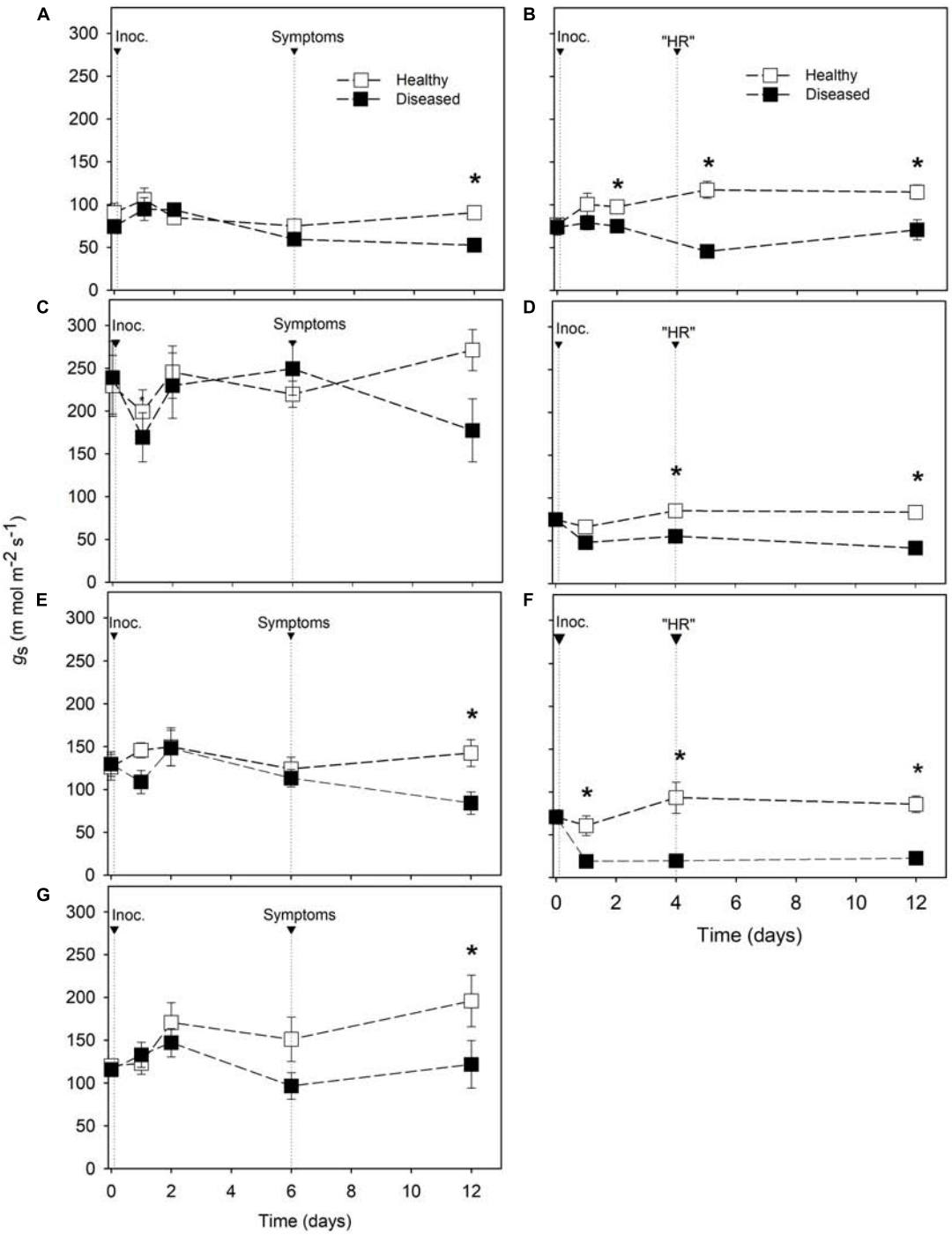

FIGURE 3 | Stomatal conductance (g_s) in *Vitis* spp. cultivars. Downy mildew symptoms were observed in Riesling **(A)**, Niagara Rosada **(C)**, Merlot **(E)** and Moscato **(G)** 6 days after inoculation. Cell death-like hypersensitive reactions "HR" were observed in Solaris **(B)**, Cabernet Blanc **(D)** and Phoenix **(F)** 4 days after inoculation (Inoc.). The average values of healthy and diseased leaves were compared using the Student's *t* test for each cultivar ($n = 6$, ± SE), and * indicates significant differences ($p < 0.05$).

a receptor gene that triggers the signal transduction cascade such as that noted in a pathogen-associated molecular pattern (PAMP, Fröbel et al., 2019). In contrast, no effect on leaf gas exchange was observed during the infection and colonization of *P. viticola* in susceptible cultivars. The net photosynthetic rate on susceptible cultivars was reduced only when symptoms appeared at approximately 6 days after pathogen inoculation. In the susceptible cultivar Trincadeira there was no reduction of

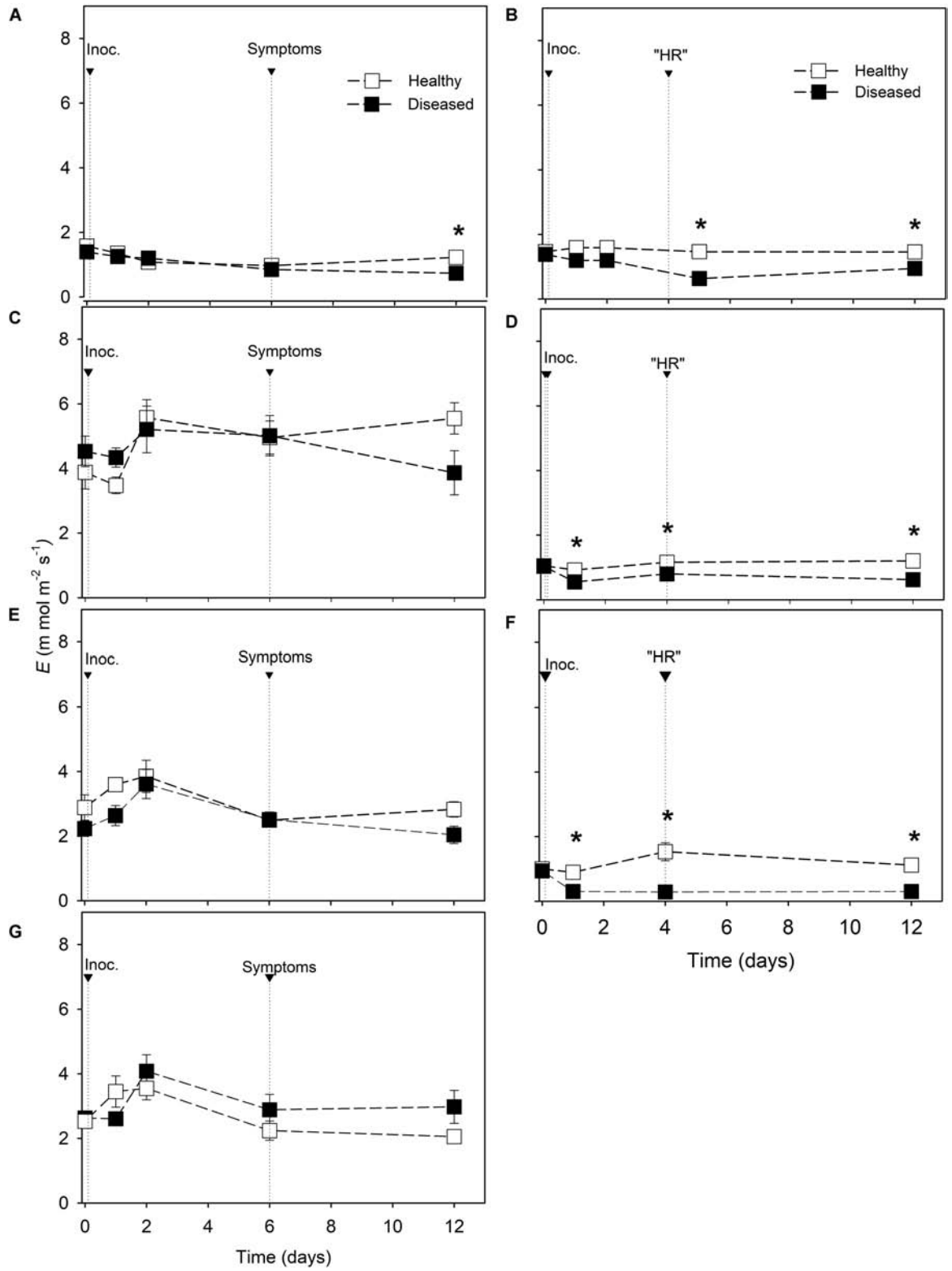

FIGURE 4 | Transpiration rate (*E*) in *Vitis* spp. cultivars. Downy mildew symptoms were observed in Riesling **(A)**, Niagara Rosada **(C)**, Merlot **(E)** and Moscato **(G)** 6 days after inoculation. Cell death-like hypersensitive reactions "HR" were observed in Solaris **(B)**, Cabernet Blanc **(D)** and Phoenix **(F)** 4 days after inoculation (Inoc.). The average values of healthy and diseased leaves were compared using the Student's *t* test for each cultivar ($n = 6$, ± SE), and * indicates significant differences ($p < 0.05$).

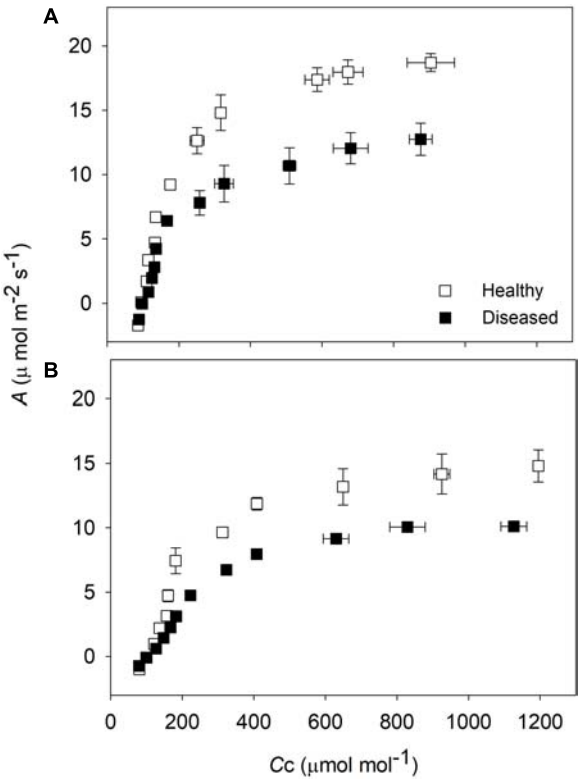

FIGURE 5 | Response of leaf CO_2 assimilation (*A*) to increasing CO_2 concentration at carboxylation sites in chloroplasts (C_c) of *Vitis vinifera* cv. Solaris **(A)** and Riesling **(B)**. Open symbols represent mean values (n = 3, ± SE) of healthy leaves, while filled symbols indicate diseased leaves.

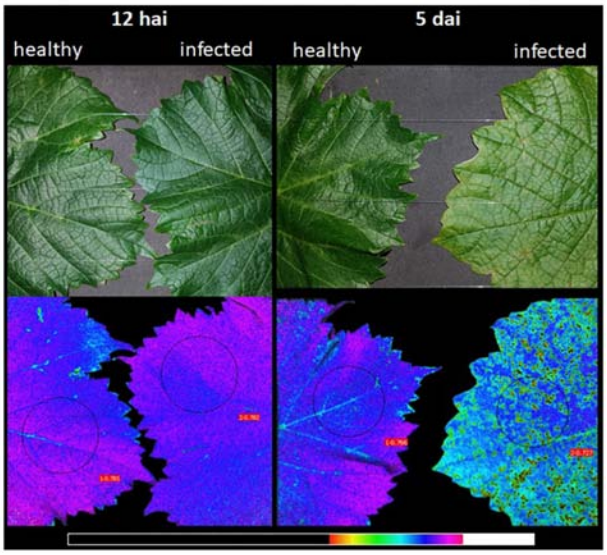

FIGURE 6 | Digital and chlorophyll fluorescence images of grapevine leaves of cv. Solaris after 12 h and 5 days after infection (dai) with *Plasmopara viticola*. Chlorophyll fluorescence images show the maximum PSII quantum efficiency (F_v/F_m). Circles indicate the area used for calculation of F_v/F_m. Leaves were dark-adapted prior to measurements. The false color code depicted at the bottom of images represents the range of 0 (black) to 1 (white).

TABLE 1 | Maximum Rubisco carboxylation rate (V_{cmax}), maximum rate of electron transport driving regeneration of ribulose-1,5-bisphosphate (J_{max}), mesophyll conductance (g_m), maximum stomatal conductance (g_{smax}), and maximum photosynthetic rate (A_{max}) in Solaris and Riesling cultivars as affected by downy mildew (*Plasmopara viticola*).

Variable	Healthy plants	Infected plants	*t*-test
Solaris			
V_{cmax} (μmol m^{-2} s^{-1})	83.6 ± 9.6	70.5 ± 11.1	ns
J_{max} (μmol m^{-2} s^{-1})	131.2 ± 9.2	86.4 ± 7.7	*
g_m (mol m^{-2} s^{-1})	0.11 ± 0.03	0.05 ± 0.02	ns
g_{smax} (mmol m^{-2} s^{-1})	143.5 ± 6.5	105.1 ± 5.6	*
A_{max} (μmol m^{-2} s^{-1})	18.7 ± 0.7	12.7 ± 1.3	*
Riesling			
V_{cmax} (μmol m^{-2} s^{-1})	58.4 ± 5.9	33.0 ± 0.8	*
J_{max} (μmol m^{-2} s^{-1})	88.1 ± 5.5	65.7 ± 0.2	*
g_m (mol m^{-2} s^{-1})	0.10 ± 0.02	0.08 ± 0.01	ns
g_{smax} (mmol m^{-2} s^{-1})	117.2 ± 12.6	90.5 ± 2.4	ns
A_{max} (μmol m^{-2} s^{-1})	13.1 ± 0.6	10.1 ± 0.2	*

*For t-test, * means statistical difference at $p \leq 0.05$ whereas ns means non-significant difference. Mean values (n = 3) ± SE.*

photosynthetic pigments content 24 h after *P. viticola* inoculation (Nascimento et al., 2019). This suggests that the photosynthetic rate remains unaltered on the first stages of the interaction where there are no visible lesions or symptoms (Nascimento et al., 2019). Similarly to our results, in the biotroph interaction between barley and powdery mildew (*Blumeria graminis*), no reduction of photosynthesis was observed in compatible interactions up to 3 dai. However, the photosynthesis in those leaves was progressively reduced up to 60% compared with healthy leaves at 7 dai (Swarbrick et al., 2006).

Plasmopara viticola infection increased stomatal closure and decreased transpiration rates of resistant cultivars from 4 dai. Stomatal closure is a mechanism related to grapevine resistance to downy mildew, impairing pathogen penetration and sporulation (Allègre et al., 2009). As soon as *P. viticola* zoospores are released from sporangia, they move toward stomata by chemotaxis, encyst and rapidly penetrate leaf tissue through the stomata in susceptible cultivars. Partial stomatal closure contributes to avoiding *P. viticola* penetration in resistant grapevine cultivars, although it is insufficient to limit infection (Allègre et al., 2009). Stomatal conductance was reduced only 12 days after inoculation in most susceptible cultivars and was less affected when compared with photosynthesis. In cv. Marselan, a susceptible cultivar to downy mildew, *P. viticola* affects guard cell function and interferes with stomatal opening, as observed for other oomycetes (Allègre et al., 2007). For instance, *Phytophthora infestans* can cause abnormal stomata functioning in potato leaves to facilitate the emergence of sporangiophores and sporulation in dark conditions (Farrell et al., 1969).

Although drastic alterations were observed in A, g_s and E of inoculated resistant grapevines, no variation was noticed in C_i of Cabernet Blanc and Phoenix leaves while it increased

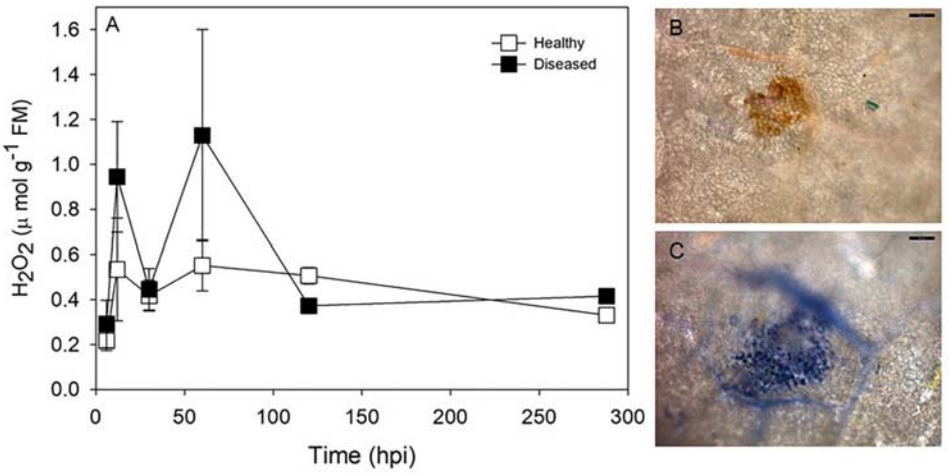

FIGURE 7 | Time-course of leaf H_2O_2 concentration **(A)** in healthy and *P. viticola* infected leaves of cv. Solaris after inoculation and visual localization of H_2O_2 **(B)** and O_2^- **(C)** in *P. viticola* inoculated leaves, 12 h after inoculation. Scale bar = 50 μm.

in inoculated leaves of Solaris at 12 dai. This was somehow unexpected since decreases in C_i are expected in leaves due to decreases in stomatal conductance. However, the low variation in C_i of resistant cultivars and the reduction in the instantaneous carboxylation efficiency after the infection (**Supplementary Figure S3**) in parallel to stomatal closure suggest that another physiological process controls intercellular CO_2 concentrations of *P. viticola*-infected leaves. A rapid increase in the respiration rate of barley leaves resistant to *Erysiphe graminis* was reported after pathogen contact, while such increase in respiration was found only 3 days after inoculation in compatible interactions (Smedegaard-Petersen and Tolstrup, 1985). A late increase in C_i after the development of disease symptoms was observed in downy mildew-susceptible cultivars, which is in agreement with the linear increase in C_i with increasing rust severity observed in *V. labrusca* infected with *Phakopsora euvitis* (Nogueira Júnior et al., 2017).

The activation of signal transduction pathways following the recognition of *P. viticola* by resistant grapevines leads to the production of reactive oxygen species (Aziz et al., 2003), such as O_2^- and H_2O_2 (Kortekamp and Zyprian, 2003). H_2O_2 production is one of the earliest detectable cytological responses to downy mildew in resistant cultivars, as noticed in Solaris 12 h after inoculation (our data and Trouvelot et al., 2008). The production of reactive oxygen species is associated with localized cell death and is crucial for delimiting the growth of pathogen into leaf tissues (Aziz et al., 2003). Reactive oxygen species cause oxidative damage to a variety of macromolecular targets, such as DNA, lipids and proteins, including enzymes of the Calvin–Benson Cycle and proteins located in the thylakoid membrane, leading to irreversible damage and ultimately tissue necrosis (Halliwell, 2006). Oxidative stress blocks key steps in chlorophyll biosynthesis by directly or indirectly inhibiting the enzymes involved in chlorophyll synthesis (Aarti et al., 2006). Herein, necrotic areas were observed 4 days after inoculation and ranged from 1.8 to 6.0% of total leaf area in resistant cultivars Solaris, Cabernet Blanc and Phoenix. Solaris inoculated with *P. viticola* showed low maximum PSII quantum efficiency 5 days after inoculation and low regeneration of RuBP driven by electron transport (**Figure 6** and **Table 1**). This photochemical limitation is presumably related to the production of H_2O_2 and the degradation of cell photosynthetic pigments. As shown in **Figure 6**, the occurrence of distinct spots indicative of low photosynthetic efficiency is clearly visible upon infection with *P. viticola*. These spots might contribute to overall reduction on photosynthetic performance. In grapevine leaves of cv. Marselan infected with *P. viticola*, chlorophyll concentrations were reduced by approximately 30% (Gamm et al., 2011). Measurements conducted in spots and adjacent areas without symptoms confirmed that F_v/F_m was reduced in the spots, but the asymptomatic adjacent areas had F_v/F_m values comparable to non-infected leaves (Gamm et al., 2011). In the present study, F_v/F_m tends to decrease at 5 dai (although not statistically significant). This might be due to the occurrence of necrotic spots that are present in the leaf area considered for calculation of F_v/F_m. Reduced F_v/F_m is an indicator for impaired PSII functionality due to damage to reaction center proteins by reactive oxygen species, and this finding is consistent with reduced assimilation rates. In susceptible cultivars, such as Riesling (this study), Sangiovese (Moriondo et al., 2005) and Niagara Rosada (Nogueira Júnior et al., 2019), the increase in photochemical limitations is also observed during downy mildew symptom expression. Low concentrations of photosynthetic pigments in the yellow halos surrounding *P. viticola* lesions are related with the reduction in photochemical reactions in these cultivars (Moriondo et al., 2005).

Expression of plant defenses is often assumed to be costly, requiring diversion of resources away from plant growth and development (Walters and Boyle, 2005), and *P. viticola* greatly enhances carbohydrate hydrolysis and represses photosynthesis-associated proteins in resistant cultivars

(Nascimento-Gavioli et al., 2017). In the incompatible interaction of barley and *Erysiphe graminis* f. sp. *hordei*, pathogen recognition occurs during haustoria development into host cells. After recognition, several histochemical and biochemical alterations are induced in hosts (Smedegaard-Petersen and Tolstrup, 1985). The cost to the net photosynthetic rate in barley leaves challenged by *E. graminis* f. sp. *hordei* is also similar to the cost observed in downy mildew-resistant grapevines. However, in the incompatible interaction of *Eucalyptus grandis* and *Puccinia psidii*, the host reacts immediately during pathogen appressoria formation. Consequently, only small flecks are observed in the host before pathogen penetration and photosynthetic activity is unaffected (Alves et al., 2011).

Although resistant cultivars decrease the photosynthetic activity when infected by *P. viticola,* its use provides a substantial contribution to the sustainability of viticulture while reducing pesticide applications (Buonassisi et al., 2017). *P. viticola* can complete its life cycle in resistant cultivars. However, low oomycete sporulation is noted compared with the sporulation in susceptible cultivars, and sporangiophores are abnormal. The reduced sporulation affects the secondary spread of the pathogen and reduces the rate of disease progression in field (Parlevliet, 1979). In the last 20 years, newly bred grapevine cultivars with disease resistance, desirable agronomic attributes and good enological characteristics have been introduced to the market (Buonassisi et al., 2017). These cultivars bear different *Rpv* resistance loci to downy mildew and several studies have been developed to identify the resistance loci on these materials. Locus *Rpv 3* is present in all resistant cultivars used in the present work. However, the cultivar Solaris has also the *Rpv10* locus[1]. In general, the effect of *P. viticola* on photosynthesis was similar for cvs. Solaris, Cabernet Blanc and Phoenix.

CONCLUSION

In conclusion, *P. viticola* differently affects leaf gas exchange in resistant and susceptible cultivars, with resistant cultivars exhibiting faster responses. While net photosynthetic rate and stomatal conductance of resistant cultivars are rapidly and negatively affected by *P. viticola*, susceptible cultivars only exhibit reduced leaf gas exchange at late stages of disease development. These results would suggest that the recognition followed by induced resistance responses is costly for grapevine carbon assimilation. In the absence of recognition, the pathogen colonizes the leaf tissue without affecting photosynthesis until the appearance of visual symptoms. Although the induction of defenses in resistant grapevine cultivars is costly, the use of genetic resistance to control downy mildew as an alternative to chemical control is highly desirable and complementary for a sustainable viticulture.

[1] www.vivc.de

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AN and LA devised the project and the main conceptual ideas. AN and MT performed the experiments. AN, LA, RR, and AT revised critically the project and the manuscript for important intellectual content. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00235/full#supplementary-material

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Diversity Profiling of Grapevine Microbial Endosphere and Antagonistic Potential of Endophytic *Pseudomonas* Against Grapevine Trunk Diseases

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Grapevine trunk diseases (GTDs) are a serious problem of grapevines worldwide. The microbiota of the grapevine endosphere comprises prokaryotic and eukaryotic endophytes, which may form varied relationships with the host plant from symbiotic to pathogenic. To explore the interaction between grapevine endophytic bacteria and GTDs, the endomicrobiome associated with grapevine wood was characterized using next-generation Illumina sequencing. Wood samples were collected from grapevine trunks with and without external symptoms of GTD (cankers) from two vineyards in the Hunter Valley and Hilltops, NSW, Australia and metagenomic characterization of the endophytic community was conducted using the 16S rRNA gene (341F/806R) and ITS (1F/2R) sequences. Among the important GTD pathogens, *Phaeomoniella, Phaeoacremonium, Diplodia* and *Cryptovalsa* species were found to be abundant in both symptomatic and asymptomatic grapevines from both vineyards. *Eutypa lata* and *Neofusicoccum parvum*, two important GTD pathogens, were detected in low numbers in Hilltops and the Hunter Valley, respectively. Interestingly, *Pseudomonas* dominated the bacterial community in canker-free grapevine tissues in both locations, comprising 56–74% of the total bacterial population. In contrast, the *Pseudomonas* population in grapevines with cankers was significantly lower, representing 29 and 2% of the bacterial community in Hilltops and the Hunter Valley, respectively. The presence of *Pseudomonas* in healthy grapevine tissues indicates its ability to colonize and survive in the grapevine. The potential of *Pseudomonas* spp. as biocontrol agents against GTD pathogens was also explored. Dual culture tests with isolated fluorescent *Pseudomonas* against mycelial discs of nine Botryosphaeria dieback, three Eutypa dieback, and two Esca/Petri disease pathogens, revealed antagonistic activity for 10 *Pseudomonas* strains. These results suggest the potential of *Pseudomonas* species from grapevine wood to be used as biocontrol agents to manage certain GTD pathogens.

Keywords: *Pseudomonas poae*, microbiome, endophyte, biocontrol, Botryosphaeria dieback, Eutypa dieback, Esca/Petri disease

INTRODUCTION

Grapevine trunk diseases (GTDs) are economically important in all grape growing regions and significantly impact grape production and the wine industry worldwide. These diseases are a major threat to the Australian wine industry and affect the long-term sustainability and productivity of many vineyards. Esca, Petri and black foot diseases, and Eutypa and Botryosphaeria diebacks are the major constraints in Australian vineyards potentially causing considerable economic loss to the industry (Wicks and Davies, 1999; Edwards and Pascoe, 2004; Whitelaw-Weckert et al., 2013; Billones-Baaijens and Savocchia, 2019). Once established, these diseases are often difficult to control and can cause establishment problems for young vines and the decline of established vines (Gramaje et al., 2018). The general symptoms of these diseases are wood necrosis characterized by brown streaking or cankers, foliar discoloration and drying. Symptoms of GTDs usually take several years to develop, making early detection difficult. Pruning wounds are the main point of entry for fungal spores (Slippers and Wingfield, 2007; Gramaje et al., 2018). Protection of pruning wounds is deemed essential to reduce infections from GTD pathogens (Mondello et al., 2018). The causal organisms grow, decay the wood and eventually kill the vines.

Botryosphaeria canker and dieback are caused by species of fungi in the *Botryosphaeriaceae* family. Many species associated with Botryosphaeria canker were grouped within the genus *Botryosphaeria*, hence, the disease is commonly referred to as Botryosphaeria dieback. However, current reclassification now includes the genera *Fusicoccum, Neofusicoccum, Diplodia, Lasiodiplodia, Dothiorella, Spencermartinsia,* and *Sphaeropsis* (Úrbez-Torres et al., 2015). Eutypa dieback is caused by *Eutypa lata* and other *Diatrypaceae* species including *Anthostoma, Cryptosphaeria, Cryptovalsa, Diatrype, Diatrypella,* and *Eutypella* (Trouillas et al., 2011; Luque et al., 2012). The etiology of Esca disease is still not fully understood. Although a number of unrelated microorganisms have been isolated from Esca-infected vines, their role and interaction with each other are not clear (Gramaje et al., 2018). However, *Phaeomoniella chlamydospora* and/or species of *Phaeoacremonium* are commonly associated with the disease. Although some authors claimed several basidiomycetous species belonging to the genera *Fomitiporia, Phellinus, Stereum, Fomitiporella, Inonotus,* and *Inocutis* to be requisites for the development of classic Esca symptoms (Cloete et al., 2015; Gramaje et al., 2018), others suggested that young esca is induced by *Phaeomoniella chlamydospora* alone (Edwards et al., 2001). Petri disease or black goo decline is primarily associated with *P. chlamydospora* (Gubler et al., 2015) although several species of *Phaeoacremonium*, *Pleurostoma*, and *Cadophora* have recently been linked to the disease (Gramaje et al., 2018).

Currently there are no reliable curative control measures against GTDs and there are no means, chemical or other, to eradicate the organisms once they become established within a vine. As eradication is problematic, disease control is mainly though disease prevention and mitigation (Úrbez-Torres, 2011). Management of GTDs is mainly though employment of cultural practices that include vineyard sanitation to reduce the inoculum density, remedial surgery wherein the infected parts of the vines are excised, and appropriate timing of pruning and training of vines (Gramaje et al., 2018). In Spain, anecdotal evidence has been presented for the use of copper nails placed in the scion parts of grapevine trunks, which slowed the progress of wood rot in infected plants, but this is yet to be scientifically validated (Mondello et al., 2018). Fungicides such as the benzimidazole carbamate group, tebuconazole, flusilazole, pyrimethanil, pyraclostrobin, and fluazinam are also used as pruning wound protection against Botryosphaeria and Eutypa dieback pathogens (Gramaje et al., 2018). For Esca and Petri disease pathogens, thiophanate-methyl and boron are found to be more effective pruning wound protectants (Rolshausen et al., 2010). However, the downside with fungicide usage is that some fungicides may only provide short-term protection. This may be problematic, since pruning wounds remain susceptible to *E. lata* for 2–4 weeks and up to 16 weeks to species of *Botryosphaeriaceae* (Munkvold and Marois, 1995; Úrbez-Torres and Gubler, 2011; Ayres et al., 2016). Furthermore, there are only limited fungicides that are commercially available to manage GTDs.

Biological control agents (BCAs) may be a potential strategy to supplement chemical methods. In recent years, the use of endophytic BCAs in the management of plant disease has gained popularity as an alternative to chemical application, driven in part by concerns for human and environmental safety with the use of fungicides (Chen et al., 1995; Ryan et al., 2008; Cabanás et al., 2014; Rybakova et al., 2015; Aydi Ben Abdallah et al., 2016; Hong and Park, 2016). Endophytes are microorganisms that spend at least parts of their life cycle inside the plant (Hardoim et al., 2015). They are ubiquitous in their host plant and may reside latently or actively colonize plant tissues (Hallmann et al., 1997). Most endophytes are commensals which reside within the plant tissues and live on metabolites produced by their host, often without any known effect on the plant (Hardoim et al., 2015; Brader et al., 2017). Another group of endophytes has mutualistic relationship with plants (Hardoim et al., 2015; Brader et al., 2017) and provide benefits to their host though promotion of plant growth, biocontrol of plant pathogens, enhancement of plant nitrogen fixation and phosphate solubilization (Hallmann et al., 1997; Iniguez et al., 2004; Ryan et al., 2008; Rybakova et al., 2015). Some endophytes may also exhibit pathogenicity when conditions become favorable (Hardoim et al., 2015; Brader et al., 2017).

Limited studies have been conducted on the grapevine endophytic microbial community, with most research focusing on the microbial composition of the grapevine phyllosphere (Leveau and Tech, 2011; Bokulich et al., 2013; Perazzolli et al., 2014; Pinto et al., 2014; Portillo et al., 2016). Moreover, most of the studies conducted on grapevine microbiota are focused on bacterial endophytes (Bell et al., 1995; Bulgari et al., 2009, 2011; West et al., 2010; Compant et al., 2011; Campisano et al., 2014, 2015; Andreolli et al., 2016) and much less on endophytic fungi. Deyett et al. (2017) conducted a comprehensive study of both the bacterial and fungal assemblages in the grapevine endosphere of a Californian vineyard and found a negative correlation between the Pierce's disease pathogen, *Xylella fastidiosa* and the endophytic bacteria *Pseudomonas fluorescens* and *Achomobacter xylosoxidans*. In Spain, Elena et al. (2018) characterized the fungal

and bacterial composition and diversity in GTD asymptomatic and symptomatic vines and found that there were no differences in the fungal communities between the healthy and diseased vines, while differences in the bacterial composition in non-necrotic and necrotic tissues were evident. The lone attempt to study the grapevine microbial profile in Australia was conducted by West et al. (2010), characterizing the diversity of culturable and non-culturable bacterial endophytes in grapevines. However, microbial distribution patterns may differ within and between geographical locations due to differences in environmental conditions (Bokulich et al., 2013). In Australia, Eutypa dieback and Botryosphaeria dieback are two of the most important GTDs (Scholefield and Morison, 2010; Billones-Baaijens and Savocchia, 2019). To our knowledge, studies on the use of grapevine endophytes to control Eutypa and Botryosphaeria diebacks in grapevines have not been conducted. There is a need therefore to investigate the potential of grapevine endophytic organisms as BCAs for dieback diseases, providing environmentally sustainable solutions to a major problem in the Australian wine industry. This study was conducted to identify the microbiome inhabiting the inner grapevine tissues and to examine the interaction between grapevine endophytic bacteria and GTD pathogens. Furthermore, the biocontrol potential of the most dominant endophytic bacterium against GTD pathogens was explored. Beneficial microorganisms that occur naturally within plant tissues have potential as effective BCAs as they are adapted to the plant host they were originally isolated from and may have greater potential to be sustained within the host.

MATERIALS AND METHODS

Plant Sample Collection

Samples were collected during the spring of 2016 from commercial vineyards in Hilltops and the Hunter Valley, which are the major grape-growing regions in New South Wales, Australia. These vineyards were selected as GTDs were previously reported to occur in these areas (Baaijens, personal communication). Vineyard surveys identified *Botryosphaeriaceae* and *Diatrypaceae* species as being present in these regions (Pitt et al., 2010; Qiu et al., 2011; Trouillas et al., 2011) while Esca/Petri disease pathogens were found in the Hunter Valley and inland NSW. For the Hunter Valley, 10 asymptomatic (no visible cankers) and 10 symptomatic (exhibiting cankers) Verdelho vines (18 years old) were sampled. For Hilltops, 20 years old Shiraz vines that had previously undergone remedial surgery (Savocchia et al., 2014) were sampled. These vines (10 each) exhibited internal necroses or were necroses-free when trunks were cut during surgery. The selected vines were debarked with a surface-sterilized knife and wood shavings (∼1 g) were obtained using an 8 mm auger bit driven by a CDL-018 cordless drill drive (Ozito Industries Pty. Ltd., Victoria, Australia). The drill bit was sprayed with ethanol for each vine sampled to prevent cross contamination. Wood shavings were placed in sterile plastic containers and transported to the laboratory on ice. One half of each sample was stored at 4°C prior to isolation of microorganisms, while the remaining half was stored at −80°C prior to DNA extraction.

DNA Extraction From Grapevine Wood Samples

DNA was extracted from 100 mg of ground wood shavings from both asymptomatic and symptomatic inner grapevine tissues following the methods of Pouzoulet et al. (2013) with slight modifications. Frozen samples were lyophilized for 24 h in a freeze drier (John Morris Scientific, United States) and ground at 20 Hz for 2 min in a TissueLyser II (Qiagen, United States) within 10 ml stainless-steel grinding jars, containing 20 mm stainless steel balls (Qiagen, United States). Total DNA was extracted from pulverized freeze-dried wood samples using the CTAB extraction buffer of Doyle and Doyle (1987). Pulverized wood samples were incubated in CTAB buffer at 65°C for 1 h, and then extracted using chloroform/isoamyl alcohol (24:1) (Sigma-Aldrich, United States) on ice for 5 min. The mixture was then centrifuged at 4°C for 10 min at 2300 × g. The lysate was then transferred to a QIAshredder spin column provided in the DNeasy Plant Mini Kit (Qiagen, United States). Following this procedure, the subsequent steps indicated in the DNeasy Plant Mini Kit were followed. DNA samples were eluted to a final volume of 100 μl using AE buffer (Qiagen, United States), and stored at −20°C. DNA concentration was determined using a Quantus Fluorometer (Promega, United States). The quality of DNA was further verified though gel electrophoresis and DNA samples (∼70 ng) were used for microbial diversity profiling analysis. Raw sequence reads are available from the NCBI's Sequence Read Archive (Bioproject PRJNA605898).

Sequencing of the 16S and ITS rRNA Genes

PCR amplification and sequencing was performed at the Australian Genome Research Facility (AGRF, Sydney, NSW, Australia). Amplicons for both 16S and ITS were generated using the primers and conditions outlined in **Table 1**. Thermocycling was completed with an Applied Biosystem 384 Veriti and using AmpliTaq Gold 360 (Life Technologies, Australia). Illumina indexing of the amplicons was achieved in a second PCR utilizing TaKaRa Taq DNA Polymerase (Clontech). Indexed amplicon libraries were quantified by fluorometry (Promega Quantifluor) and normalized. An equimolar pool was created and adjusted to 5 nM for sequencing on an Illumina MiSeq (San Diego, CA, United States) with a V3, 600 cycle kit (2 × 300 base pair, paired-end reads).

Isolation of Fluorescent *Pseudomonas* From Inner Grapevine Tissues

As the next generation sequencing data for microbial profiling revealed *Pseudomonas* to be the predominant endophytic bacterium in grapevine tissues, these were targeted for isolation from the collected grapevine wood samples. Approximately 0.5 g of wood shavings per sample were suspended in 6 ml of 3 × Ringer's solution (Surette et al., 2003). Samples were homogenized by incubating in a rotary shaker for 2 h at

TABLE 1 | Primers and PCR conditions for 16S and ITS amplicons.

Target	Cycle	Initial	Disassociate	Anneal	Extension	Finish
ITS1F–ITS2	35	95°C for 7 min	94°C for 30 s	55°C for 45 s	72°C for 60 s	72°C for 7 min
16S: V3–V4	29	95°C for 7 min	94°C for 30 s	50°C for 60 s	72°C for 60 s	72°C for 7 min

240 rpm under ambient temperature. A dilution series of up to 10^{-6} was prepared using 3 × Ringer's solution and plated on nutrient agar (NA, Oxoid Ltd., Hampshire, United Kingdom). Well separated and distinct bacterial colonies were subcultured onto King's B medium (King et al., 1954). Additional bacterial strains were further isolated from pruning stubs collected from Cabernet Sauvignon vines from the vineyard of Charles Sturt University, NSW, Australia.

The pure cultures obtained from the vineyards were observed for fluorescence on King's B media, under UV light at 365 nm after 3 days of incubation at 25°C in the dark. Fluorescent bacterial strains from single colonies were stored in 20% glycerol at −80°C.

In vitro Screening for Bacterial Antagonists Against GTD Pathogens

Bacterial Strains

A total of 57 fluorescent bacterial strains from inner grapevine wood tissues were tested for their antagonistic activity against the GTD pathogens: (a) *Diplodia seriata*; (b) *Neofusicoccum parvum*; (c) *Neofusicoccum luteum;* and (d) *Eutypa lata,* which are the most prevalent and virulent GTD pathogens in Australia. From this initial screen, 10 strains (**Table 2**) demonstrating inhibition of the GTD pathogens were further tested for their antagonistic activity against 14 GTD pathogens that are associated with Botryosphaeria dieback, Eutypa dieback, and Esca/Petri disease (**Table 3**).

Fungal Strains

Fourteen fungal strains associated with GTDs were obtained from the National Wine and Grape Industry Centre (Charles Sturt University, Wagga Wagga, NSW, Australia) culture collection (**Table 2**). Each of the selected strains had been stored on agar discs in 20% glycerol at −80°C prior to testing. All *Botryosphaeriaceae* strains were associated with Botryosphaeria dieback symptoms in Australian vineyards (Pitt et al., 2010; Qiu et al., 2011; Wunderlich et al., 2011), while all *Diatrypaceae* strains were associated with Eutypa dieback symptoms (Trouillas et al., 2011). The *Phaeoacremonium minimum* and *Phaeomoniella chlamydospora* strains were obtained from the culture collection of the South Australian Research and Development Institute (SARDI, Adelaide, Australia). All strains were previously identified to species level by DNA sequencing of the ITS region of the rRNA and β-tubulin genes (Pitt et al., 2010, 2013; Qiu et al., 2011; Wunderlich et al., 2011; Billones-Baaijens et al., 2018).

Dual Culture Assays

To study the ability of fluorescent *Pseudomonas* spp. (**Table 1**) to inhibit growth of the GTD pathogens (**Table 2**), dual plating assays were performed. For each pathogen, mycelial plugs 6 mm in diameter were placed on opposite sides of potato dextrose agar (PDA, Difco Laboratories, Maryland, United States) plates. A 10 μl *Pseudomonas* suspension (1 × 10^8 CFU/ml), grown for 24 h in nutrient broth (NB, Oxoid Ltd., Hampshire, United Kingdom), was then pipetted in the centre of the PDA plates. Petri plates containing mycelial plugs of the pathogens

TABLE 2 | Fluorescent bacterial strains with antagonistic activity against GTD pathogens *Diplodia seriata*, *Neofusicoccum parvum*, *N. luteum,* and *Eutypa lata.*

Bacterial strains	Identification based on 16S rRNA gene	GenBank accession no.
BCA11	*Pseudomonas poae*	MN480480
BCA12	*P. moraviensis*	MN480481
BCA13	*P. poae*	MN480482
BCA14	*P. poae*	MN480483
BCA15	*P. poae*	MN480484
BCA16	*P. poae*	MN480485
BCA17	*P. poae*	MN480486
BCA18	*P. poae*	MN480487
BCA19	*P. poae*	MN480488
BCA20	*P. poae*	MN480489

TABLE 3 | Fungal strains used for *in vitro* assays to test the antagonistic activity of *Pseudomonas* strains.

Fungal species	Isolate	Herbarium accession[a]	References
Botryosphaeria dothidea	BMV14	DAR79239	Pitt et al., 2010
Diplodia mutila	FF18	DAR79137	Pitt et al., 2010
D. seriata	A142a	DAR79990	Qiu et al., 2011
Dothiorella vidmadera	L5	DAR78993	Pitt et al., 2013
Lasiodiplodia theobromae	W200	DAR81024	Wunderlich et al., 2011
Neofusicoccum australe	SDW4	DAR79505	Pitt et al., 2010
N. luteum	BB175-2	DAR81016	Wunderlich et al., 2011
N. parvum	B22a	DAR80004	Qiu et al., 2011
Spencermartinsia viticola	L19	DAR78870	Pitt et al., 2010
Cryptovalsa ampelina	KC6		Trouillas et al., 2011
Eutypa lata	WB052		Billones-Baaijens et al., 2018
Eutypella citricola	WA06FH		Trouillas et al., 2011
Phaeoacremonium minimum	SMA 056		Billones-Baaijens et al., 2018
Phaeomoniella chlamydospora	SMA 006		Billones-Baaijens et al., 2018

[a] *DAR – Agricultural Scientific Collections Unit, NSW DPI, Orange, Australia.*

only served as controls. Three replicate plates were used for each bacteria-fungal pathogen treatment combination, and incubated at 25°C under continuous darkness. Mycelial growth of the fungi was measured after 7, 10, and 30 days to evaluate inhibition of the Botryosphaeria dieback, Eutypa dieback and Esca/Petri disease pathogens, respectively, by the antagonistic *Pseudomonas* species. Average fungal growth was determined by measuring the perpendicular diameter of the mycelial growth of the respective GTD pathogens. Percentage mycelial growth inhibition was calculated using the formula:

$$\text{Percentage mycelial growth inhibition} = \frac{(C - T)}{C} \times 100$$

Where C = colony diameter (mm) of the control
T = colony diameter (mm) of the test plate

In the case of *Ph. minimum*, the bioassays were conducted using a spore suspension of the fungi in the well dual culture. *Ph. minimum* readily produces abundant spores in PDA and spores were harvested from a 7 days old culture of the fungus. Spore concentration was adjusted to 1×10^6 spores/ml with a hemocytometer and 100 μl of the spore suspension was spread onto PDA plates. Four equidistant holes were created in the media using a flame-sterilized cork borer (4 mm) and 10 μl of the bacterial suspension was pipetted into each well. For the control plates, sterile distilled water was pipetted into the wells. Three replicate plates per treatment combination were used. The diameter of the zone of inhibition was measured around each well after 7 days of incubation under the conditions described above.

Identification of Selected Bacterial Strains by 16S rRNA Gene Sequencing

The 10 fluorescent bacteria that significantly inhibited the mycelial growth of the GTD fungal pathogens and 11 additional non-antagonistic fluorescent bacterial strains were selected for molecular identification. The bacterial growth conditions and DNA extraction method were as described by Niem et al. (2019), using the Gentra Puregene Bacterial DNA Extraction Kit (Qiagen, United States) following the manufacturer's instructions.

The bacterial species were identified though PCR amplification of 16S rRNA gene using the universal primers 8F and 1492 R (I) (Turner et al., 1999). PCR amplification was performed with 1 μl of genomic DNA template in a 25 μl reaction mixture containing 1× PCR buffer (Bioline, United Kingdom), 1.25 U of My Taq DNA polymerase (Bioline, United Kingdom) and 0.4 μM of each primer. PCR was performed with a thermal cycler (C100 Thermal Cycler, BIO-RAD Laboratories Pty. Ltd., United States) under the following conditions: initial denaturation for 3 min at 95°C; 35 denaturation cycles of 15 s at 95°C, annealing for 1 min at 56°C, extension for 1 min at 70°C; and a final extension of 8 min at 72°C. PCR products were purified with a FavorPrep Gel/PCR purification kit (Favorgen Biotech Corp, United States) and sequenced at the AGRF.

To identify the bacterial species, all DNA sequences and chromatographs were analyzed and trimmed using Chromas Lite Version 2.6.2 (Technelysium Pty. Ltd.) and DNAMAN Version 6.0.3.99 (Lynnon Biosoft) and compared with sequence data in GenBank[1] using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to determine the most probable identity for the 21 isolates examined. For phylogenetic analysis, published reference sequences for the 16S rRNA gene were obtained from the NCBI database and aligned with the 21 generated sequences, by Clustal W within MEGA 7 (Kumar et al., 2016). The aligned sequences were tested for their phylogeny (Tamura et al., 2004) and a neighbor joining tree was generated using MEGA 7 to determine the relationships between the reference and unknown sequences (Saitou and Nei, 1987).

Data Analysis

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR version 0.9.5 (Zhang et al., 2014). Primers were identified and removed, with trimmed sequences being processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 7.1.1090) (Edgar, 2010; Edgar et al., 2011), and UPARSE (Edgar, 2013) software. Within USEARCH, sequences were quality filtered, full length duplicate sequences removed, and reads sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtering using "rdp_gold" database as the reference. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned by QIIME using the Greengenes database (version 13_8 Aug 2013) (DeSantis et al., 2006). In the case of ITS sequences, these were clustered followed by chimera filtering using the "Unite" database as reference. To obtain number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned by QIIME using the Unite database (Unite Version7.1 2016-08-22) (Kõljalg et al., 2005). Unless mentioned, default settings were used for all procedures.

Microbial diversity profiling data were generated from nine and eight samples each from asymptomatic and symptomatic vines from Hilltops and the Hunter Valley, respectively. Only those taxa that represent more than 1% of the bacterial and fungal operational taxonomic units (OTUs) were included in the data set (Deyett et al., 2017). Data for 0.01–0.9% of the fungal OTUs were included as an inset to take into account fungal genera that were obscured by high relative abundance of the dominant genus. Chloroplast and mitochondrial sequences or OTUs with unidentified taxa were removed. Percent relative abundance of the bacterial and fungal taxa inhabiting the grape inner tissues was calculated. The compositional diversity of the fungal and bacterial endophytic communities were considered separately. Diversities were compared with alpha (Chao1 and Simpson) and beta (Bray-Curtis index) diversity statistics within the Marker Data Profiling module of MicrobiomeAnalyst (Dhariwal et al., 2017), implementing R version 3.6.1 (2019-07-05). Abundance data for bacteria and fungi were normalized by data transformation using centered log ratio, as recommended by Gloor et al. (2017). Differences in the Chao1 and Simpson index among factors (location and presence/absence of symptoms) were determined

[1] www.ncbi.nlm.nih.gov

via ANOVA when considering all factors, or *T*-tests for factors individually. For the beta diversity, differences were visualized using non-metric multidimensional scaling (NMDS). Permutational multivariate analysis of variance (PERMANOVA) was used to test which factors best explained the NMDS.

For the dual culture assay, all data were analyzed within GenStat (18th edition). Since mycelial growth rate differed between Botryosphaeria dieback, Eutypa dieback and Esca/Petri disease pathogens, assessments for each pathogen group were done at different periods and analyzed separately. Percent mycelial diameter inhibition for each pathogen group was initially tested for homogeneity using Levene's test at $P \leq 0.05$. Effects of treatments were determined by two-way analysis of variance (ANOVA). Pairwise comparison of treatment means was conducted using least significant differences (LSD) at $P \leq 0.05$.

RESULTS

Microbial Profile of Grapevine Endosphere

A total of 48,461 bacterial 16S rRNA gene sequences and 1,567,896 fungal ITS sequences were generated after removing mitochondrial and chloroplast sequences. These sequences were assigned to 573 bacterial and 244 fungal operational taxonomic units (OTUs).

Bacterial Community

Alpha diversity analysis indicated that significant differences existed in terms of richness (Chao1) within communities of bacteria considering all factors ($F = 7.13$, $P \leq 0.05$). Comparison between location suggested that the Hilltops region had significantly greater diversity in terms of OTU richness than the Hunter Valley ($t = 3.07$, $P \leq 0.05$; **Figure 1A**). Taking this in to account the differences between asymptomatic and symptomatic samples from within each location were determined. For both Hilltops and the Hunter Valley asymptomatic samples were lower in terms of OTU richness than symptomatic, however, the differences were not significant. For richness and evenness (Simpson) there were no significant differences between factors.

In regards to the composition of bacterial communities, non-metric multidimensional scaling and PERMANOVA analysis indicated that communities could be separated when considering all factors (R-squared = 0.24, $P \leq 0.05$; **Figure 1B**). This was attributable to compositional differences between location (R-squared = 0.14, $P < 0.01$) rather than expressed symptomology (R-squared = 0.07, $P > 0.05$).

When considering abundance at the phylum and genus level, in all samples, the dominant phylum among the prokaryotic community was Proteobacteria, accounting for 51–96% of the total reads for the given taxa followed by Actinobacteria with 3–44%. The phyla Bacteroidetes, Firmicutes, and Chloroflexi were detected in less than 10% of the reads. At the genus level, with the exception of symptomatic samples from the Hunter Valley at 2% relative abundance, the most abundant genus within the phylum Proteobacteria was *Pseudomonas* with 75 and 29% in asymptomatic and symptomatic samples, respectively, from Hilltops, and 56% in asymptomatic vines from the Hunter Valley. It is also noteworthy that in both locations, *Pseudomonas* had higher relative abundance in samples asymptomatic for GTDs (**Figures 2A**, **3A**) as compared to samples from symptomatic vines (**Figures 2B**, **3B**). Symptomatic vines harbored higher diversity of the most abundant genera accounting for 19 and 15 bacterial genera from Hilltops and the Hunter Valley, respectively. In contrast, asymptomatic samples host a total of nine genera in Hilltops and 10 in the Hunter Valley. The genera *Pseudomonas, Sphingomonas,*

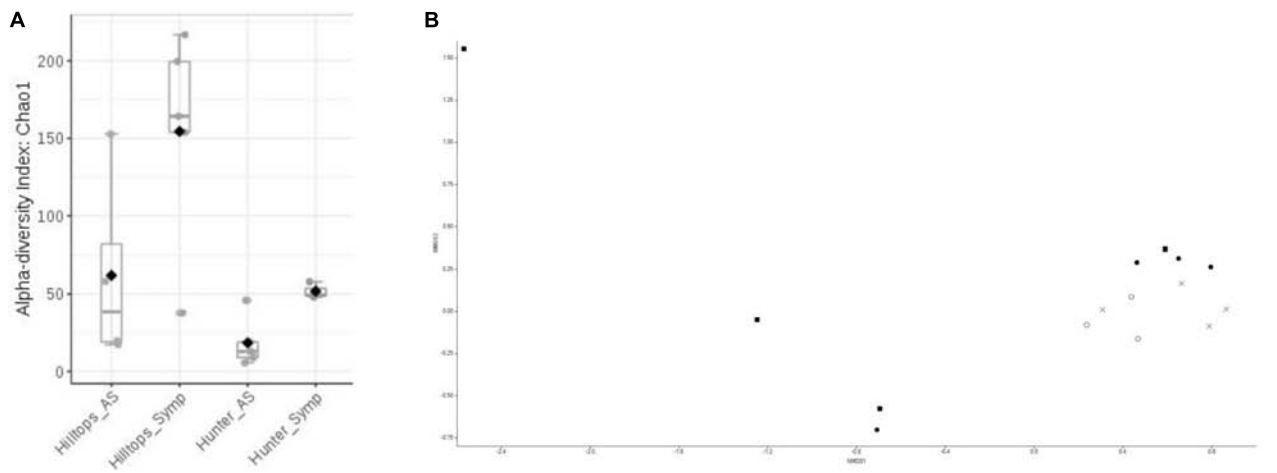

FIGURE 1 | Diversity analysis of bacterial endophytic communities sampled from GTD symptomatic and asymptomatic vines, in the Hilltops and Hunter Valley regions. Abundance of OTUs was determined following the sequencing of the V3–V4 region of 16s rRNA genes: **(A)** Alpha-diversity measure using Chao1 at the OTU level, with each boxplot representing the diversity distribution of each factor (location and symptomology); and **(B)** Ordination based upon beta diversity analysis using the Bray Curtis index. Closed circles = Hilltops, asymptomatic; closed squares = Hilltops, symptomatic; X = hunter Valley, asymptomatic; O = Hunter Valley, symptomatic.

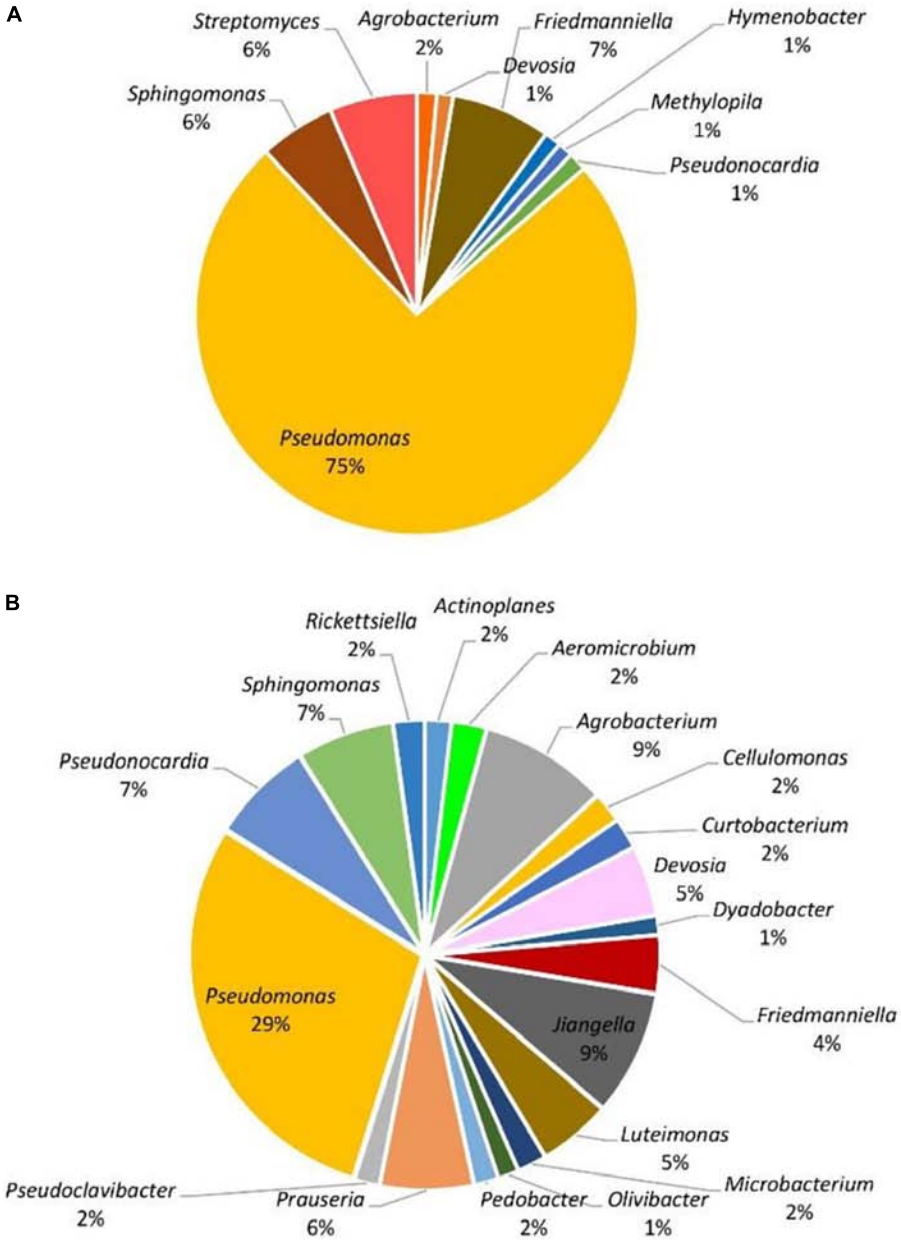

FIGURE 2 | Genera of grapevine endophytic bacteria from Hilltops, NSW and their relative abundance to grapevines that were: **(A)** asymptomatic; and **(B)** symptomatic to grapevine trunk diseases.

Agrobacterium, Deviosa, Friedmanniella, and *Pseudonocardia* were present in both asymptomatic and symptomatic samples from Hilltops. The composition of bacterial genera from the Hunter Valley appeared to be more unique with only *Pseudomonas, Rhodoplanes, Coprococcus,* and *Deviosa* shared between asymptomatic and symptomatic samples. Other agriculturally important bacterial genera that occurred in greater than 1% relative abundance were *Streptomyces* and *Agrobacterium. Streptomyces*, which was detected in asymptomatic samples from Hilltops (6%), is implicated in plant disease control and plant growth promotion. *Agrobacterium,* which causes crown gall in grapevines, was identified in 1–9% of the total bacterial reads.

Fungal Community

In terms of the fungal community, the richness of OTUs were significantly different across all factors ($F = 1.3$, $P \leq 0.05$), being driven by location with Hilltops greater than the Hunter Valley ($t = 2.12$, $p = 0.05$; **Figure 4A**). When richness and evenness were considered, no significant differences at the OTU level were present. The compositional analysis of fungal communities, analyzed for beta diversity, indicated that while communities

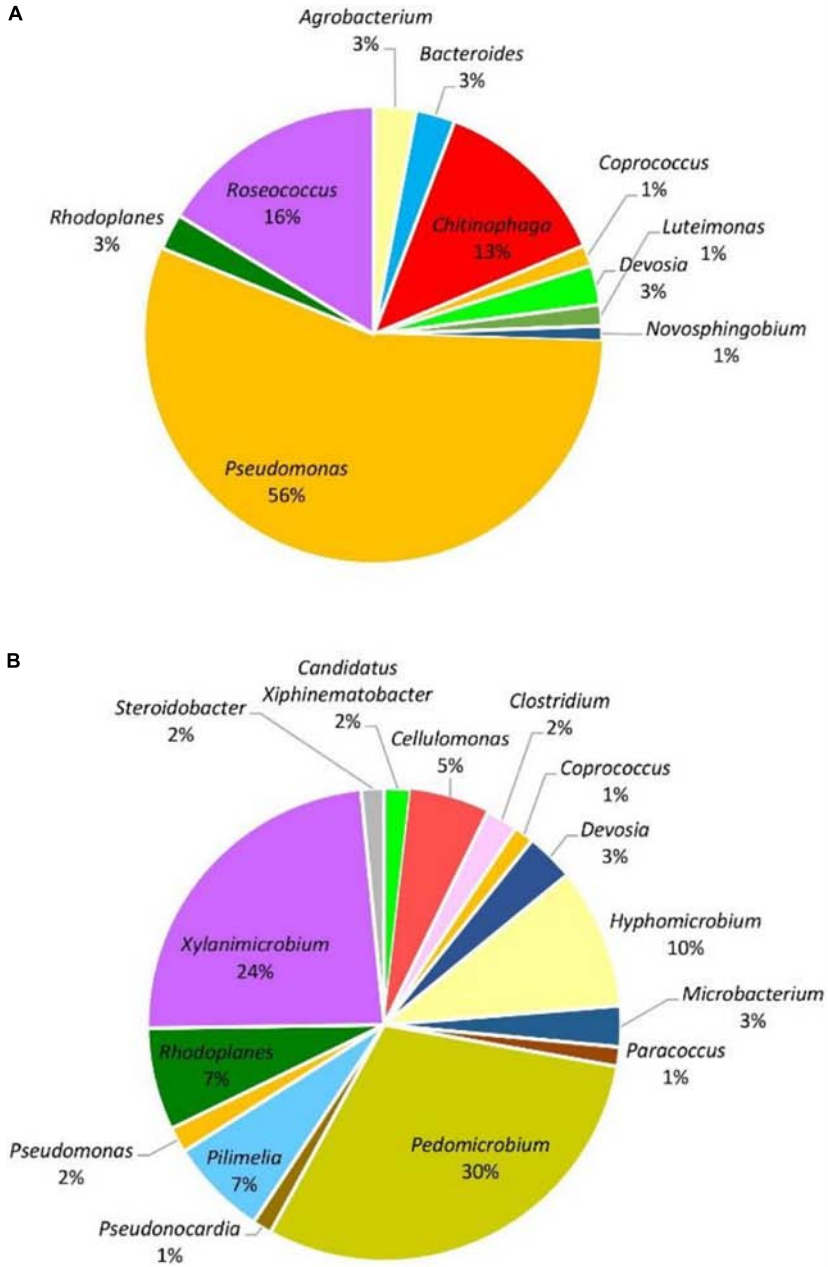

FIGURE 3 | Genera of grapevine endophytic bacteria from the Hunter Valley, NSW and their relative abundance to grapevines that were: **(A)** asymptomatic; and **(B)** symptomatic to grapevine trunk diseases.

could be clustered, these were not strict and no significant differences were evident ($R = 0.21$, $P > 0.05$; **Figure 4B**).

The eukaryotic microbiome was mostly characterized by a preponderance of the phylum Ascomycota with greater than 99% relative abundance from both asymptomatic and symptomatic samples from Hilltops and asymptomatic tissues from the Hunter Valley. The relative abundance of Ascomycota in symptomatic vines from the Hunter Valley was 83% while phylum Basidiomycota was 17%. At the genus level, the fungal community was predominated by *Phaeomoniella* (primarily *P. chlamydospora*), representing 59–89% of the total fungi detected (**Figures 5**, **6**). *Phaeomoniella* was more abundant in asymptomatic tissues accounting for 89 and 74% in Hilltops and the Hunter Valley (**Figures 5A**, **6A**), respectively as compared to symptomatic tissues with 78 and 59% relative abundance (**Figures 5B**, **6B**). This was followed by *Phaeoacremonium* species, specifically *Ph. iranianum* (5–21%) save for a divergence in asymptomatic vines from the Hunter Valley where *Inonotus* from the phylum Basidiomycota came in second at 18% relative abundance. *Phaeomoniella* and *Phaeoacremonium* species are

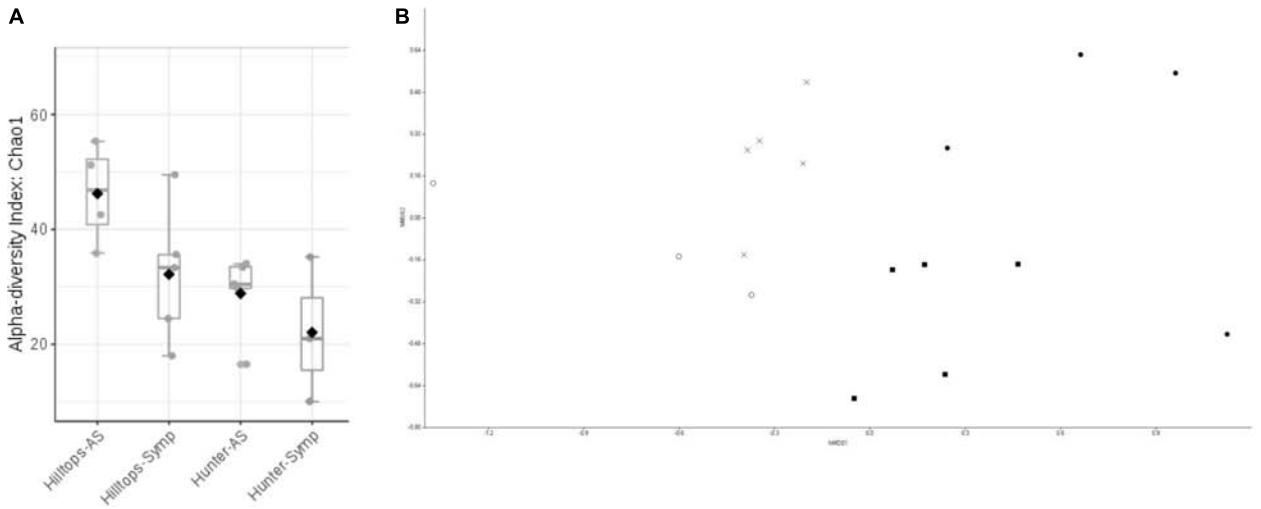

FIGURE 4 | Diversity analysis of fungal endophytic communities sampled from GTD symptomatic and asymptomatic vines, in the Hilltops and Hunter Valley regions. Abundance of OTUs was determined following the sequencing of the ITS regions of rRNA genes: **(A)** Alpha-diversity measure using Chao1 at the OTU level, with each boxplot representing the diversity distribution of each factor (location and symptomology); and **(B)** Ordination based upon beta diversity analysis using the Bray Curtis index. Closed circles = Hilltops, asymptomatic; closed squares = Hilltops, symptomatic; X = hunter Valley, asymptomatic; O = Hunter Valley, symptomatic.

GTD pathogens that are widely associated with Petri and Esca diseases of grapevines. *Inonotus* has also been isolated in some grapevines exhibiting Esca symptoms. Other trunk disease pathogens, *Cryptovalsa*, *Diplodia*, and *Neofusicoccum* were also detected in low abundance (0.01–1%). Some fungi implicated with various diseases of grapes and grapevines such as *Alternaria*, *Stemphylium*, *Phomopsis*, *Phyllostica*, *Penicillium*, *Phoma*, *Phialophora*, and *Aspergillus* were also noted in 0.01–2% of the eukaryotic population. Fungi with known biocontrol potential, including *Aureobasidium*, *Epicoccum*, and *Arthobotrys* were also found to inhabit the inner grapevine tissues.

Isolation of Fluorescent *Pseudomonas* From Inner Grapevine Tissues

A total of 200 bacterial endophytes from Hilltops and the Hunter Valley samples and 10 bacterial strains from the CSU vineyard were isolated. From these, 57 isolates resembling *Pseudomonas* fluoresced under UV light at 365 nm when grown in King's B medium. From among the 57 bacteria, 10 isolates exhibited antagonistic activity against the selected GTD pathogens (data not shown).

In vitro Screening for Bacterial Endophytes Antagonistic Toward GTD Pathogens

Antagonism Against Botryosphaeria Dieback Pathogens

The 10 *Pseudomonas* isolates showed varying degrees of antagonistic activity against Botryosphaeria dieback pathogens. Inhibition of mycelial growth was significantly affected by both BCAs and pathogens. Average mycelial inhibition caused by the different isolates of bacteria ranged from 12.0 to 43.1% with BCA11 and BCA13 having the highest inhibition (**Table 4**). BCA15 was the least inhibitory of the isolates examined. Sensitivity to BCAs was also significantly different ($P \leq 0.05$) among the nine Botryosphaeria dieback pathogens that were tested, with *Dothiorella vidmadera* being the most sensitive (52.3% average) (**Figures 7A,B**) and *N. parvum* and *N. luteum* (15.4–18.5%) being the least sensitive.

Antagonism Against Eutypa Dieback Pathogens

All 10 *Pseudomonas* BCA isolates showed antagonistic activity against Eutypa dieback pathogens, however, mycelial growth inhibition differed significantly between bacterial strains and pathogens. Average mycelial inhibition caused by the different bacteria ranged from 8.3 to 64.6% with BCAs 11, 13, 14, 18, 19, and 20 having the greatest inhibition (**Table 5**). BCA15 was the least inhibitory among the BCAs. Sensitivity to BCAs was also significantly different ($P \leq 0.05$) among the three Eutypa dieback pathogens that were tested, with *Cryptovalsa ampelina* (**Figures 7C,D**) and *Eutypella citricola* being more sensitive (53.8–51.0%) than *E. lata* (49.9%).

Antagonism Against Esca/Petri Disease Pathogens

The 10 *Pseudomonas* BCA isolates showed varying degrees of antagonistic activity against Esca/Petri disease pathogens. Mycelial growth inhibition was significantly affected by both BCAs and pathogens. Average mycelial inhibition caused by the different bacteria ranged from 9.7 to 44.9% with BCA11 and BCA13 having the highest inhibition (**Table 6**). BCA12 was the least inhibitory among the BCAs. Sensitivity to BCAs was also significantly different ($P \leq 0.05$) between the Esca/Petri disease pathogens that were tested, with *P. chlamydospora* being more sensitive (55.0% average) (**Figures 7E,F**) than *Ph.*

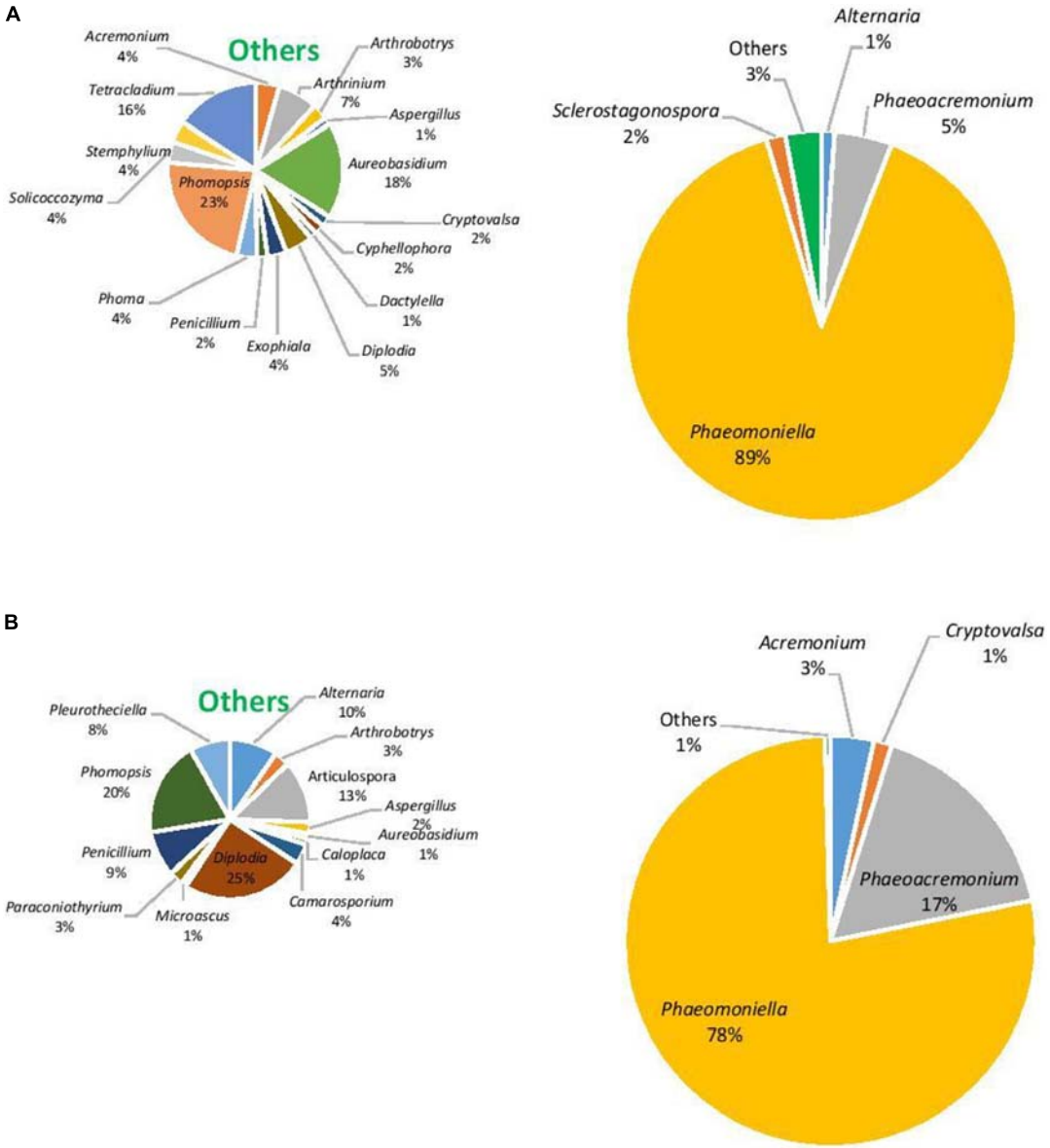

FIGURE 5 | Genera of grapevine endophytic bacteria from Hilltops, NSW and their relative abundance to grapevines that were: **(A)** asymptomatic; and **(B)** symptomatic to grapevine trunk diseases.

minimum (14.5%). However, although inhibition of mycelial growth by the BCAs was not as pronounced in *Ph. minimum*, an apparent change in the color along the edges of the hyphal colonies was observed (**Figures 7G,H**). Co-inoculation of *Ph. minimum* with the BCAs resulted in the disappearance of mycelial pigmentation along the margin indicating spore production was inhibited by the BCAs. Further tests on spore suspensions of *Ph. minimum* in a well dual culture assay showed the presence of zones of inhibition when cell suspensions of BCAs 13, 14, and 17 were present. Among the three BCAs, BCA11 produced the largest zone of inhibition at 21 mm (**Figure 8**). Average zones of inhibition produced by the three bacterial strains ranged from 19 to 21 mm and were significantly different from the control at $P \leq 0.05$ (**Figure 9**).

Identification of Selected Bacteria by 16S rRNA Gene Sequencing

Querying the Genbank database with the 16S rRNA gene sequences generated from the 12 selected bacterial isolates, and those from the CSU vineyard, indicated these belonged to the genus *Pseudomonas*. Phylogenetic analysis of the aligned sequences from the bacterial strains and reference sequences obtained from Genbank resulted in a neighbor joining tree with two major branches (**Figure 10**). The first main branch was

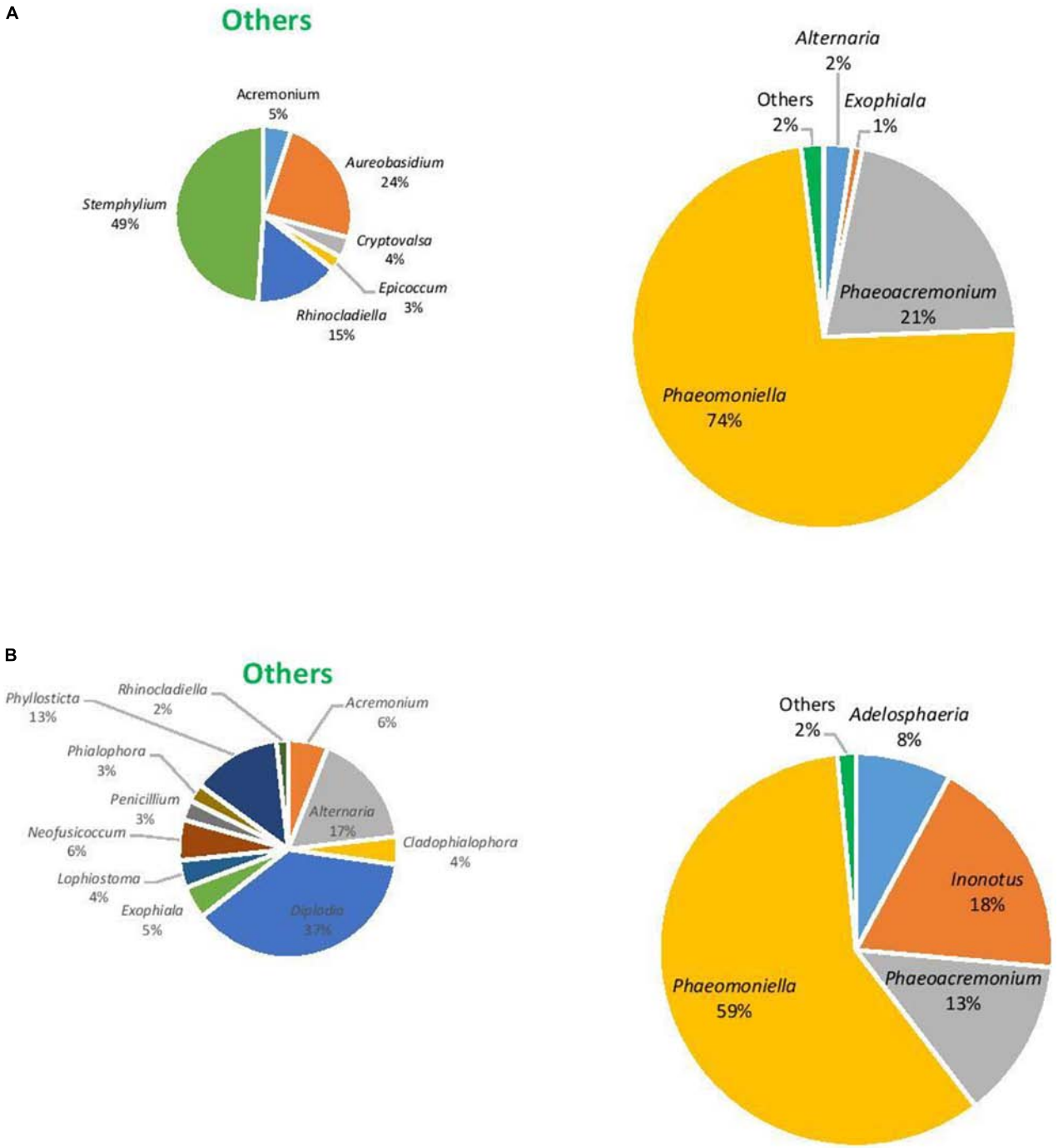

FIGURE 6 | Genera of grapevine endophytic fungi from the Hunter Valley, NSW and their relative abundance to grapevines that were: **(A)** asymptomatic; and **(B)** symptomatic to grapevine trunk diseases.

occupied by eight strains from CSU, five from Hilltops, and one from the Hunter Valley. This was further divided into two sub-branches. The first sub-branch consisted of eight strains from CSU and one strain each from Hilltops and the Hunter Valley, which clustered with *P. poae* reference strains. The second sub-branch was composed of four strains from Hilltops, which clustered with *P. azotoformans*. The second main branch was occupied by one strain from CSU, three from Hilltops, and three from the Hunter Valley and was divided into two sub-branches. The first sub-branch indicates a close relationship to *P. koreensis*. The second sub-branch is composed of one strain from CSU, two from Hilltops, and three from the Hunter Valley, which

TABLE 4 | Mycelial growth inhibition of Botryosphaeria dieback pathogens by *Pseudomonas* strains as potential biocontrol agents (BCAs).

BCA	Percent mycelial growth inhibition (%)[d]									
	BD[a]	*DM*[a]	*DS*[a]	*DoV*[a]	*LT*[a]	*NA*[a]	*NL*[a]	*NP*[a]	*SV*[a]	BCA mean[b]
11	70.4 a	26.3 abc	45.5 ab	66.5 ab	41.0 a	46.8 a	30.3 a	20.4 ab	40.9 b	43.1 A
12	58.1 a	0.0 d	47.6 a	0.0 d	38.5 a	0.0 d	0.0 c	10.1 ab	62.0 a	24.0 F
13	68.9 a	25.9 abc	37.6 abcd	53.5 bc	44.4 a	36.0 ab	29.5 a	22.2 a	58.1 a	41.7 A
14	64.1 a	29.4 ab	39.2 abc	45.8 c	23.5 b	37.8 ab	25.0 ab	21.0 ab	42.7 b	36.5 B
15	12.6 b	14.1 c	12.6 e	11.0 d	11.5 b	12.3 cd	12.0 bc	8.5 b	13.1 c	12.0 G
16	25.4 b	19.7 bc	28.1 cd	69.4 a	12.4 b	17.6 c	14.2 b	12.0 ab	40.9 b	26.6 EF
17	19.5 b	24.1 abc	25.5 de	72.8 a	13.3 b	15.6 c	14.9 b	12.6 ab	43.1 b	26.8 EF
18	22.9 b	29.2 ab	29.5 cd	77.6 a	13.8 b	17.8 c	12.6 bc	13.5 ab	44.6 b	29.0 DE
19	22.9 b	35.5 a	32.2 cd	79.1 a	22.0 b	17.6 c	20.8 ab	13.1 ab	45.0 b	32.0 CD
20	59.7 a	22.6 abc	33.8 bcd	47.6 c	24.0 b	32.4 b	25.4 a	21.0 ab	38.5 b	33.9 BC
Pathogen mean[c]	42.3 W	22.7 Y	33.1 X	52.3 V	24.4 Y	23.4 Y	18.5 Z	15.4 Z	42.9 W	

[a]*Means within a column followed by different letters are significantly different at P ≤ 0.05 LSD = 13.055 for BCA x pathogen interaction.* [b]*BCA means within a column followed by different letters are significantly different at P ≤ 0.05 LSD = 4.352 for BCA means.* [c]*Pathogen means within a row followed by different letters are significantly different at P ≤ 0.05 LSD = 4.128 for pathogen means.* [d]*BD, Botryosphaeria dothidea; DM, Diplodia mutila; DS, D. seriata; DoV, Dothiorella vidmadera; LT, Lasiodiplodia theobromae; NA, Neofusicoccum australe; NL, N. luteum; NP, N. parvum; SV, Spencermartinsia viticola.*

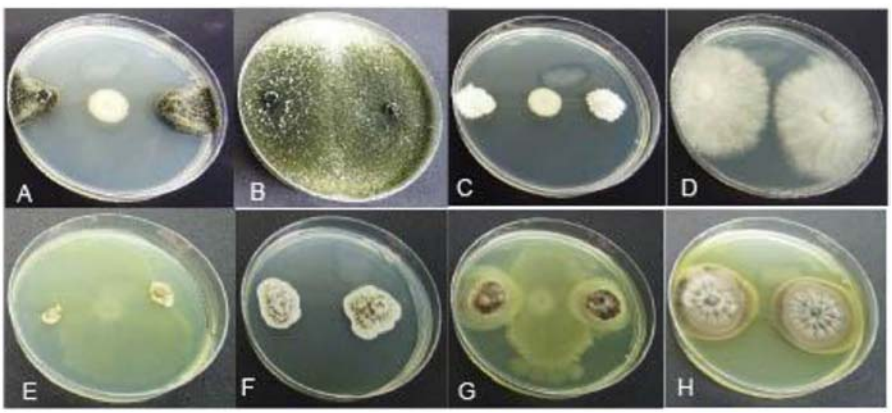

FIGURE 7 | Mycelial growth inhibition of representative GTD pathogens by antagonistic *Pseudomonas* BCA strains: **(A)** *Dothiorella vidmadera* + BCA19, **(B)** *Do. vidmadera* only, **(C)** *Cryptovalsa ampelina* + BCA14, **(D)** *C. ampelina* only, **(E)** *Phaeomoniella chlamydospora* + BCA11, **(F)** *P. chlamydospora* only, **(G)** *Phaeoacremonium minimum* + BCA13, **(H)** *Ph. minimum* only.

clustered with *P. moraviensis*. The fluorescent bacterial strains are all from the *P. fluorescens* complex (Garrido-Sanz et al., 2017). *P. aeruginosa* was included in the tree as an outgroup. Nine out of the 10 fluorescent bacterial strains (BCA11, BCA13–BCA20) that inhibited the mycelial growth of the GTD pathogens in the dual culture assay belong to the *P. poae* group. The remaining antagonistic strain, BCA12, is closely related to *P. moraviensis*.

DISCUSSION

This study investigated the diversity of the endomicrobiome associated with grapevines with and without symptoms of GTDs. Overall results showed that diversity of bacterial and fungal communities of grapevines with and without GTD symptoms differed between the regions examine, suggesting potential interactions between the beneficial and pathogenic organisms within a grapevine. To our knowledge, this study is the first attempt to investigate the microbiome of GTD symptomatic and asymptomatic wood tissues of grapevines in Australia, and the outcomes suggest that sampling of a number of agroecological zones would be beneficial to improve our understanding of endophytic communities. The microbial diversity data revealed a preponderance of the genus *Pseudomonas* in grapevine tissues that were asymptomatic to GTD in both locations, accounting for 56–75% relative abundance and in symptomatic tissues from Hilltops with 29% abundance. This concurs with the results of Bell et al. (1995), Campisano et al. (2015), Faist et al. (2016), and Deyett et al. (2017) who reported *Pseudomonas* to dominate the bacterial assemblage in grapevines in separate studies conducted in California, Germany, Italy, and Canada, respectively. Interestingly, in a study conducted in Australia, West et al. (2010) reported *Bacillus* to be the most frequently isolated bacterium in grapevine canes, comprising 55% of the total. *Pseudomonas* spp., *Curtobacterium* spp., *Burkholderia* spp., and *Streptomyces* spp. accounted for 34% of the total endophytic bacteria isolated. *Bacillus* was not detected in the current samples even though these were also collected from grapevines in

TABLE 5 | Mycelial growth inhibition of the Eutypa dieback pathogens by *Pseudomonas* strains as potential biocontrol agents (BCAs).

BCA	Percent mycelial growth inhibition (%)			
	***Cryptovalsa ampelina*[a]**	***Eutypa lata*[a]**	***Eutypella citricola*[a]**	**BCA mean[b]**
11	70.3 ab	54.8 abc	63.7 a	62.9 AB
12	6.5 d	49.8 c	27.3 c	27.9 D
13	70.6 ab	50.9 c	60.2 ab	60.6 ABC
14	77.1 a	51.8 c	65.0 a	64.6 A
15	6.2 d	1.8 d	16.8 d	8.3 E
16	56.5 c	53.4 bc	55.4 ab	55.1 C
17	59.3 c	57.8 abc	56.6 ab	57.9 BC
18	61.6 bc	62.6 ab	56.0 ab	60.1 ABC
19	59.3 c	65.2 a	53.1 b	59.2 ABC
20	70.8 ab	51.1 c	55.0 ab	58.9 ABC
Pathogen mean[c]	53.8 X	49.9 Y	51.0 XY	

[a]Means within a column followed by different letters are significantly different at P ≤ 0.05 LSD = 10.462 for BCA × pathogen interaction. [b]BCA means within a column followed by different letters are significantly different at P ≤ 0.05 LSD = 6.04 for BCA means. [c]Pathogen means within a row followed by different letters are significantly different at P ≤ 0.05 LSD = 3.308.

TABLE 6 | Mycelial growth inhibition of the Esca/Petri disease pathogens by *Pseudomonas* strains as potential biocontrol agents (BCAs).

BCA	Percent mycelial growth inhibition of Esca/Petri disease pathogens (%)		
	***Phaeomoniella chlamydospora*[a]**	***Phaeoacremonium minimum*[a]**	**BCA mean[b]**
11	70.3 a	17.3 abc	43.8 A
12	19.3 f	0.0 d	9.7 E
13	64.0 ab	25.8 a	44.9 A
14	53.7 cde	17.5 abc	35.6 BCD
15	60.7 abc	8.3 cd	34.5 BCD
16	59.7 bcd	12.6 bc	36.1 BCD
17	64.0 ab	13.6 bc	38.8 ABC
18	50.3 de	15.0 bc	32.7 C
19	47.7 e	15.2 bc	31.5 D
20	60.7 abc	19.3 ab	40.0 AB
Pathogen mean[c]	55.0 X	14.5 y	

[a]Means within a column followed by different letters are significantly different at P ≤ 0.05 LSD = 10.462 for BCA x pathogen interaction. [b]BCA means within a column followed by different letters are significantly different at P ≤ 0.05 LSD = 6.604 for BCA means. [c]Pathogen means within a row followed by different letters are significantly different at P ≤ 0.05 LSD = 3.308 for pathogen means.

New South Wales, Australia. The difference in the microbial composition may be explained by the difference in the age of the vines as West et al. (2010) sampled from relatively younger vines (10 years old) as compared to the ≥20 years old grapevines sampled in this study. Similar observations were reported by Bruez et al. (2015) where *Bacillus* sp. and *Pantoea agglomerans* were the most commonly isolated bacteria from 12 years old grapevine wood samples either symptomatic or asymptomatic for esca-foliar symptoms. Andreolli et al. (2016) identified age of the vine as an important factor affecting the composition of bacterial endophytes in grapevines, as well as genus richness, with greater genus richness observed in younger vines. The results obtained in this current research indicated higher diversity of bacterial genera in symptomatic vines, hosting 15–19 genera as compared with 9–10 for asymptomatic vines. While these were not found to be significant differences, these were used to provide the indicator for identifying organisms contributing to the effect. Other factors may also influence the composition of the grapevine microbiome. Campisano et al. (2015) found bacterial endophytic communities to be affected by the pest management strategy employed in the vineyard, and revealed differences in microbial composition in grapevines cultivated using organic production as opposed to those using integrated pest management (IPM). The health status of the vine (Bulgari et al., 2011; Bruez et al., 2014; Faist et al., 2016), the growing region, cultivar and climate (Bokulich et al., 2013; Bruez et al., 2014; Portillo et al., 2016) may also shape the community of microorganisms within the grapevine. The results presented in this current research concur with past findings as location was the influencing factor for differences in endophytic diversity.

Higher relative abundance of *Pseudomonas* in asymptomatic tissues observed in this study may impart a suppressive effect on GTD pathogens, and suggests a plausible antagonistic role of *Pseudomonas* in inhibiting symptoms of GTDs. In a similar study, Deyett et al. (2017) found a decrease in the relative abundance of *Xylella fastidiosa*, a xylem-limited bacterium causing Pierce's disease in grapevines, in the presence of *Achomobacter xylosoxidans* and *P. fluorescens*. The presence of grapevines asymptomatic for Pierce's disease in vineyards with high disease pressure was attributed to the microbial community that inhabits the grapevine vascular endosphere.

This study also showed that the eukaryotic community was dominated by the genus *Phaeomoniella* in both asymptomatic and symptomatic grapevines, representing 74–89% and 59–78% of the total fungi, respectively. *Phaeoacremonium* came in second, with 5–21% relative abundance except in asymptomatic vines from the Hunter Valley where *Inonotus* had a higher relative abundance at 18%. *P. chlamydospora* and *Ph. aleophilum* (syn. *Ph. minimum*) have been implicated in two GTDs: Petri disease (previously known as black goo decline) and Esca (Crous and Gams, 2000; Mostert et al., 2006b). *Inonotus* has also been reported in grapevines exhibiting white heart rot, a symptom that is also generally associated with Esca (Edwards and Pascoe, 2004; Fischer et al., 2005; González et al., 2009; White et al., 2011). Occurrence of these three dominant fungi in grapevines showing symptoms of Petri disease and Esca has previously been reported in Australian vineyards. Petri disease was found to be expressed in 82% of the diseased samples (from a total of 124 symptomatic samples) while only 18% were diagnosed with Esca (Edwards and Pascoe, 2004). Moreover, *P. chlamydospora* was found to be widely distributed in Australia and was detected in Western Australia, South Australia and Victoria in 98% of the samples while *Ph. minimum* was isolated in only 15% of the samples. The current fungal diversity profile indicates *P. chlamydospora* to be the dominant fungal species but *Phaeoacremonium* is from the species *Ph. iranianum* in contrast to *Ph. minimum* that was previously reported in Australia.

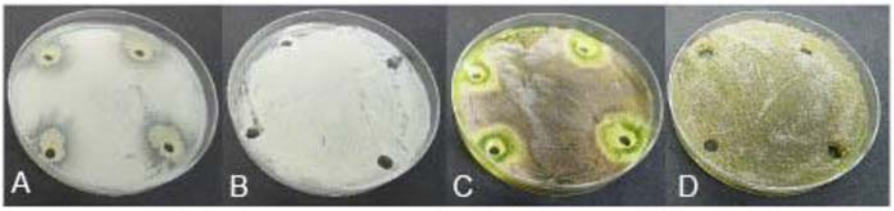

FIGURE 8 | Antagonistic effect of *Pseudomonas* BCA strains on Esca/Petri disease pathogens: **(A)** *Phaeomoniella chlamydospora* + BCA17, **(B)** spores of *P. chlamydospora* only, **(C)** *Phaeoacremonium minimum* + BCA17, **(D)** spores of *Ph. minimum* only.

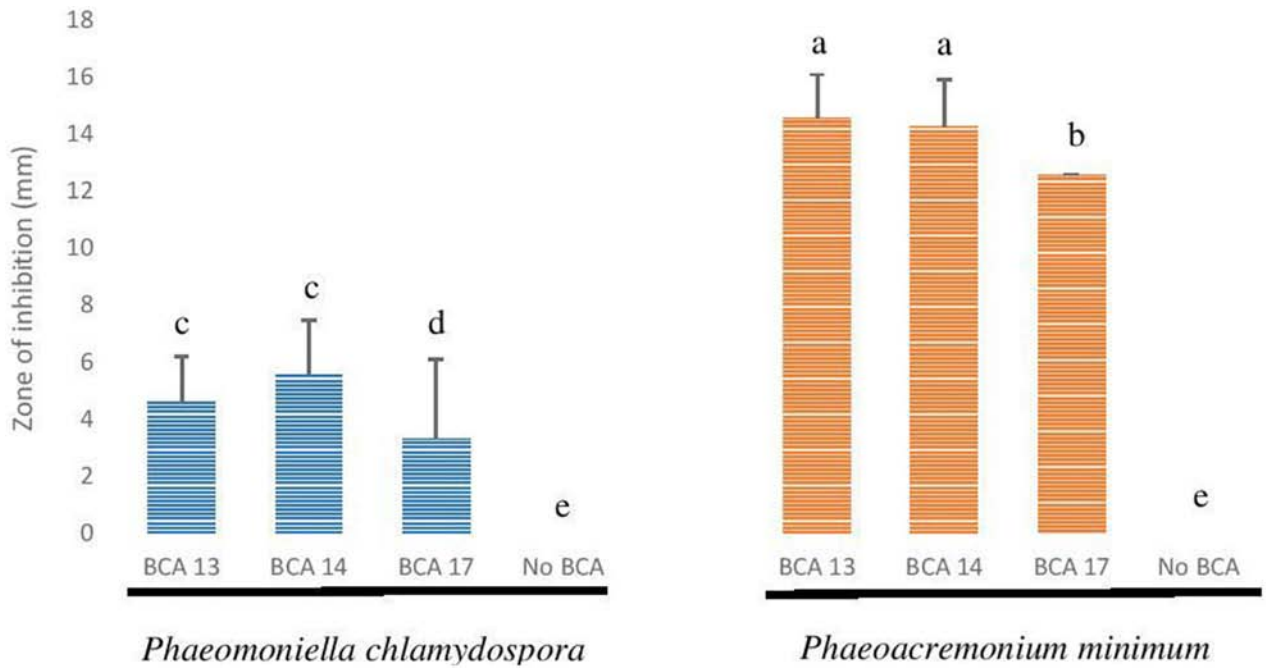

FIGURE 9 | Antagonistic effect of selected *Pseudomonas* BCA strains on *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*.

Ph. iranianum, which has been reported to be isolated from *Vitis vinifera* in Iran, Italy and South Africa (Mostert et al., 2006a; White et al., 2011) is phylogenetically and morphologically close to *Ph. minimum* and can only be differentiated by the predominance of type III phialides and by its subcylindrical type II phialides (Mostert et al., 2006a). Its presence indicated here by diversity profiling would need to be confirmed by these conventional techniques.

The diversity profiling revealed a higher abundance of *Phaeomoniella* in asymptomatic grapevines than in the symptomatic tissues. In France, Bruez et al. (2016) was able to recover the Esca pathogens, *Ph. minimum* and *P. chlamydospora* and the white rot fungi, *Fomitiporia mediterranea* and *Stereum hirsutum,* consisting 50% of all the fungal taxa isolated, from 42 years old vines that did not express symptoms of grapevine trunk disease. Edwards and Pascoe (2004) were also able to isolate *P. chlamydospora* in symptomless grapevines, as did Mugnai et al. (1999). They postulated that the fungus may initially behave as an endophyte or latent pathogen, only becoming pathogenic when the grapevines are stressed, after which Petri disease starts to manifest. Furthermore, symptoms of Esca are erratic and may not show every year (Edwards et al., 2001). Hofstetter et al. (2012) also found the same taxa of presumed Esca-associated fungal pathogens, including *P. chlamydospora* and *Phaeoacremonium* spp., in both diseased and healthy grapevines that were 15–30 years old. This indicates that these fungi are part of the normal mycota associated with adult grapevines. Ferreira et al. (1999) reported water stress to be a predisposing factor for *P. chlamydospora* infection and slow dieback of grapevines in South Africa. Plant genotype (Marchi, 2001), age (Mugnai et al., 1999), and climate (van Niekerk et al., 2011) are also factors that may affect the susceptibility of the grapevines to the disease. Environmental changes may also elicit various plant responses (i.e., development of thicker wax layers on leaves or changes in stomatal densities) that could affect infection and expression of symptoms (Fischer and Peighami Ashnaei, 2019).

Abundance of the *Pseudomonas* spp. in asymptomatic grapevines led to the further investigation of the antagonistic

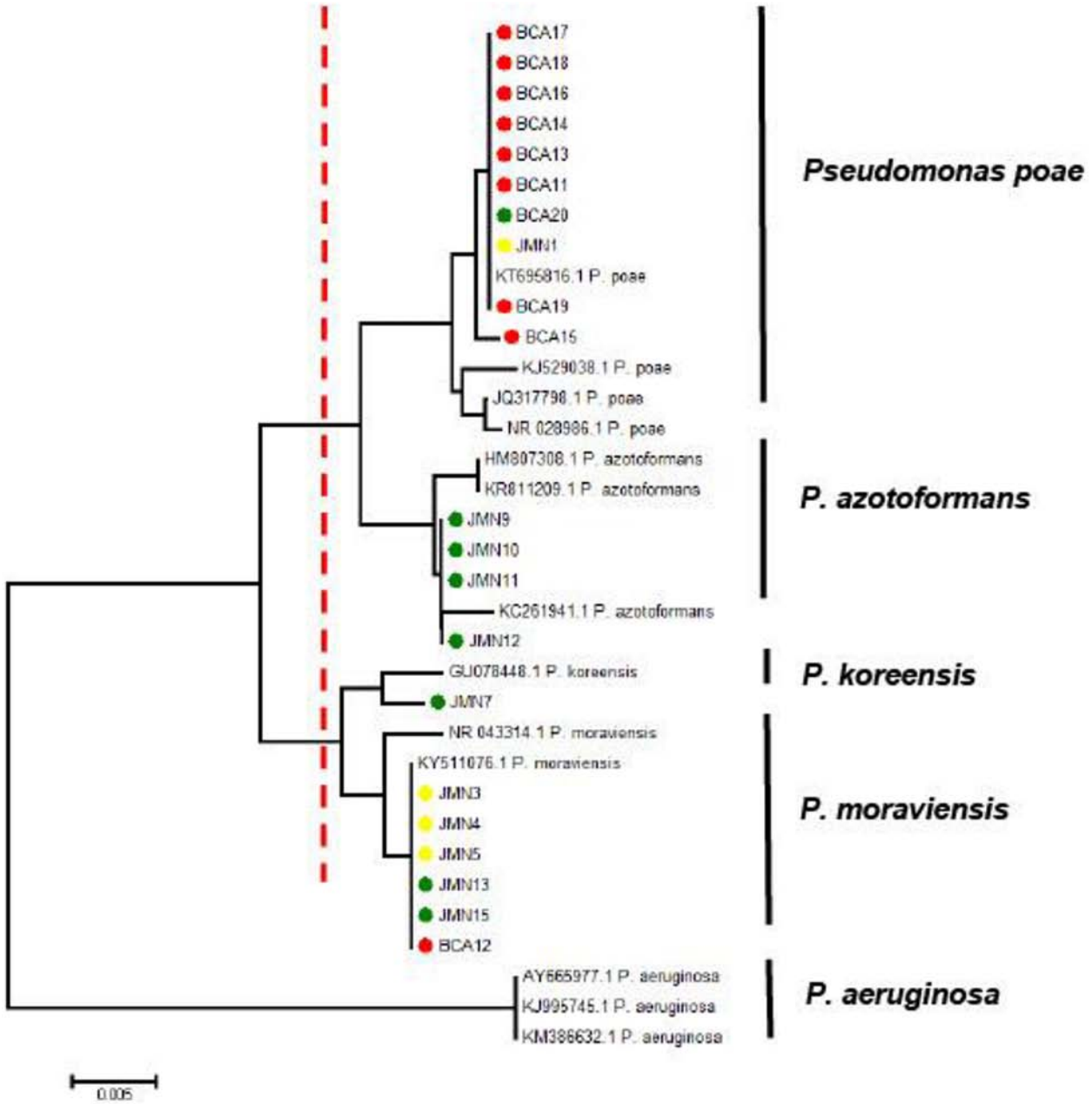

FIGURE 10 | Neighbor joining tree obtained from the 16S rRNA gene sequence data of fluorescent bacteria isolated from inner grapevine tissues. The red dotted line indicates the main branches of the tree. Strains with red symbols are from the CSU vineyard, green from Hilltops, and yellow from the Hunter Valley. Reference sequences obtained from the NCBI data base are indicated by their accession numbers. The tree is rooted to the outgroup *P. aeruginosa.*

activity of these bacteria against GTDs. The results of the dual culture assays revealed 10 strains of grapevine endophytic *Pseudomonas* with antagonistic activity against Botryosphaeria dieback, Eutypa dieback and Esca/Petri disease pathogens based on their ability to inhibit mycelial growth in culture. *Pseudomonas* spp. also appeared to affect spore production of *Ph. minimum* as demonstrated by the presence of a zone of inhibition between the bacterium and the spores of the pathogen in a well dual culture assay. This suggests that the *Pseudomonas* spp. are capable of producing potent antifungal agents that leached into the agar and inhibited the growth of the fungal pathogens. Of the *Pseudomonas* strains identified, nine out of the 10 were closely related to *P. poae*. *P. poae*, originally isolated from the phyllosphere of grasses, has been reported as an endorhiza of sugar beet (Behendt et al., 2003; Müller et al., 2013; Xia et al., 2019). It is used as a BCA and is applied as a seed treatment to suppress late root rot in sugar beet (Zachow et al., 2010). The other antagonistic

bacterium, BCA12, belongs to the same clade as *P. moraviensis* which has been reported as a soil bacterium implicated in the suppression of Rhizoctonia diseases of potato (Tvrzová et al., 2006; Mrabet et al., 2013; Miller et al., 2016). However, the *in vitro* assay conducted in the current study indicated that BCA12 was less inhibitory compared to the other nine BCAs from the *P. poae* group.

Although *P. poae* has been identified to have biocontrol potential, the exact mechanism of control is yet to be elucidated. Unlike other species of *Pseudomonas* that are more popularly known to produce 2,4 diacetylphloroglucinol, phenazine 1-carboxylic acid and its derivatives, and the siderophores pyoluteorin and pyrrolnitrin, which are antifungal metabolites identified with *Pseudomonas*, *P. poae* lacks the ability to produce these compounds (Mavrodi et al., 2012). Instead, it was found to be a good rhizosphere colonizer with exoprotease activity (Mavrodi et al., 2012). Niem et al. (2019) identified putative biocontrol gene clusters in the genomes of BCAs 13, 14, and 17 (*P. poae*) responsible for the biosynthesis of the secondary metabolites Poaeamide, Rhizomide and Rhizoxins. Gene clusters for production of lipopeptide antibiotics, siderophores, proteases, detoxification, lipopolysaccharide, multidrug resistance, microbe-associated molecular proteins (MAMPS), and biofilms were likewise detected (Niem et al., 2019). Production of other antifungal compounds, possibly not previously reported in other *Pseudomonas* species, requires further investigation. Furthermore, the efficacy of *P. poae* to suppress trunk disease infection *in planta* should be investigated to ascertain its efficacy as a biocontrol agent.

The results of this study indicate that some grapevine endophytic bacteria such as *P. poae* may be potential BCAs which could be used to control GTD pathogens. Further tests are currently being conducted to assess the bioactivity of these BCAs *in planta*.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Genbank Accession numbers: MN480480-MN480489. Raw sequence data for the diversity profiling is available through BioProject PRJNA605898.

AUTHOR CONTRIBUTIONS

JN conducted the research, analyzed the data, and wrote the manuscript. RB-B, SS, and BS contributed to the direction of the research and advised on analysis and interpretation of the data. All co-authors revised the manuscript.

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