BELOW-GROUND INTERACTIONS IN ECOLOGICAL PROCESSES

EDITED BY: Oren Shelef, Philip G. Hahn, Ana Pineda, Mysore V. Tejesvi and Ainhoa Martinez-Medina

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BELOW-GROUND INTERACTIONS IN ECOLOGICAL PROCESSES

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Aboveground interactions between plants and organisms have served as a foundation of ecological and evolutionary theories. Accumulating evidence suggests that interactions that occur belowground can have immense influence on eco-evolutionary dynamics of plants. Despite the increasing awareness among scientists of the importance of belowground interactions for plant performance and community dynamics, they have received considerably less theoretical and empirical attention compared to aboveground interactions. In this eBook we aim to highlight the overlooked roles of belowground interactions and outline their myriad ecological roles, from affecting soil health through impacting plant interactions with above-ground fauna. This eBook with 18 articles and an Editorial includes conceptual contribution together with original research work. The chapters are exploring the roles of belowground biotic interactions, in the context of ecological processes both below- and above-ground.

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Editorial: As Above So Below? Progress in Understanding the Role of Belowground Interactions in Ecological Processes

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Editorial on the Research Topic

As Above So Below? Progress in Understanding the Role of Belowground Interactions in Ecological Processes

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Biotic interactions that occur belowground involve all of the kingdoms of life and can have an immense influence on eco-evolutionary dynamics of the interacting organisms as well as the functioning of ecosystems (Lavelle et al., 2016; Orgiazzi et al., 2016; Coleman et al., 2017). For example, ecosystems are dependent on belowground diversity to regulate nutrient cycles and these interactions provide services critically important for the well-being of the biosphere, including food security, and carbon sequestration (Lal, 2004). Indeed, the importance and complexity of belowground interactions have long been recognized by ecologists and has even permeated into popular culture as illustrated in the quilt art "Mother Earth and Her Children" by Sieglinde Schoen Smith (von Olfers, 2007). While it may not represent a precise objective picture of nature, it does highlight the vital role of belowground interactions. Despite increasing awareness of the importance of belowground interactions and particularly how above- and belowground communities are linked through biotic interactions (Hooper et al., 2000; Wardle et al., 2004; Young and Crawford, 2004), belowground interactions have still received considerably less attention compared to aboveground interactions. This lack of attention is perhaps due to the complexity and many logistical challenges of working belowground (Shelef et al.) and remains a knowledge gap that severely hampers our ability to understand and predict the functioning of terrestrial ecosystems.

Exciting new discoveries published in this Research Topic provide state-of-the-art information on the belowground interactions. The 18 contributions published in this Research Topic explored interactions from a variety of taxonomic groups, such as fungi, bacteria, or invertebrates and include a mix of Mini Reviews, Reviews, Original Research Articles, and a Perspective. Through the development of novel conceptual frameworks and invigorated by modern molecular and chemical techniques, these articles shed new light on how belowground interactions affect ecological processes, from soil health through impacting plant interactions with above-ground fauna. Collectively, this body of work provides substantial progress in our understanding of ecology belowground and linkages with aboveground systems and has the potential to make a lasting

contribution to the field of ecology. These studies make a significant contribution on several themes in below- and aboveground ecology that are currently "hot topics," such as conceptual approaches to studying interactions belowground, ecological interactions in global change scenarios, patterns, and mechanisms of microbial effects on plant-insect interactions at multiple trophic levels, or factors affecting microbiome assembly belowground.

In a broad ecological context, the article by Shelef et al. highlights the importance of developing a conceptual framework for the study of belowground interactions. The authors indicate that many foundational ecological concepts (e.g., keystone species, island biogeography, trophic cascades) have been developed and tested mainly in aboveground systems and are sparsely referenced in the belowground literature. The authors suggest that while there are some fundamental differences in key ecological processes that occur above- vs. belowground, increased attention on belowground interactions using modern methodological approaches will ultimately help to integrate across the above- and belowground realms. Some of those methodological approaches to studying belowground microbial communities are thoroughly reviewed in the study of Mecado-Blanco et al. Here the authors highlight the importance of belowground microbes in forested ecosystems, as well as their role in mediating ecological processes, and how they influence the productivity of tree crops. They propose a strategy for deploying microbiota to combat not only belowground pests but also abiotic stressors that threaten tree crop production.

Abiotic stress can alter many fundamental ecological interactions and also is one of the predicted consequences of global change, the latter of which is a major societal concern. Hahn et al. examined how short- vs. long-term differences in soil moisture influences the effect of soil microbes on plant functional traits. While there were substantial differences in mycorrhizae communities found under spatially separated sites in the field, these different source communities did not differ in their effects on plant growth. Instead, plant growth responses to mycorrhizae were affected by short-term changes in soil moisture, where plant responses were positive under drought conditions but adverse under wet conditions. Focusing on other abiotic stresses that are predicted to increase in future climate scenarios, Johnson et al. show how silicon supplementation to the soil can maintain plant productivity of *Medico sativa* under conditions of elevated CO_2 and temperature, associated with an increase of root nodulation. This study shows how understanding the belowground interaction between microbes and plants can contribute to ameliorating the predicted impacts of climate change.

Other significant consequences of global change are the colonization of new areas by invasive or range expander species. Toward this end, Liu et al. examined how invasion by a non-native plant influences the composition of soil bacterial communities in wetlands. Using multivariate analyses of 16S rRNA gene sequences, they showed that plant invasion alters soil bacterial communities. Interestingly, plant invasion increased species richness but had more nuanced effects on community composition and functional diversity (Liu et al.). At another level, it is still unknown what mechanisms influence host preference for belowground organisms. In the context of species whose distribution change due to climate change, this question is of high importance to predict whether the native herbivores will attack a plant with a novel chemistry. Wilschut et al. address this question, by comparing pairs of three plant species, one range expander and a native one, in terms of volatile emissions and attractiveness to nematodes. Interestingly, the two pairs with the most distinct root volatile profiles between native and range expander, were also where nematodes preferred the native species. Climate change is imposing a plethora of new challenges that can only be faced when we understand the mechanisms by which plants interact with their new pests.

Plant phytohormones, such as jasmonic acid (JA) and salicylic acid (SA) orchestrate induced plant defenses to herbivores and pathogens. Nevertheless, how plants integrate their induced responses under multiple belowground and aboveground interactions remains obscure. Bakhtiari et al. explored how elicitation of root defenses by application of exogenous JA impacts shoot defenses and the resistance against aboveground herbivory. They found that the induction of the JA-pathway in roots altered the abundance and diversity of glucosinolates in the shoots, increasing the plant resistance against aboveground herbivory. The hormonal network regulating aboveground-belowground interactions seems to be highly influenced by the entity of the attacking organisms. Various paths of hormonal signaling may have a different regulating effect on the response to belowground nematodes. Van Dam et al. found that aphid infestation on plants previously infested with the cyst nematode *Heterodera schachtii* induced the SA-regulated pathway in aboveground tissues while the JA-regulated pathway was repressed. By contrast, aphid infestation on plants previously infested with the root knot nematode *Meloidogyne hapla* triggered the JA-regulated pathway. Interestingly, aphids performed worse in plants infested with the cyst nematode, while they performed better in plants infested with the root-knot nematode indicating impact of feeding strategy of the belowground attacker on the aboveground herbivores.

In addition to influencing plant growth, soil microbes can also affect insect herbivores aboveground through changes in plant chemistry. Bernaola et al. showed that inoculation with mycorrhizal fungi improved the growth of rice plants, but also made them more susceptible to insect and fungal pathogen pests. The authors found no difference in nutritional status between inoculated and uninoculated rice plants, suggesting that plant defenses may be responsible for differences in pest performance (Bernaola et al.). Meier and Hunter found that mycorrhizal fungi can increase levels of plant toxins (cardenolides) in several species of the genus *Asclepias* (Meier and Hunter). Interestingly, herbivores that fed on mycorrhizae-inoculated plants were able to sequester higher amounts of toxins in their own bodies, which may make them more resistant to their own predators. These studies demonstrate how belowground interactions between plants and soil microbes can have important effects on aboveground interactions between plants and insects, but also highlight the variability in the outcome of these interactions. Heinen et al. also point toward that variability and highlights that

most of the microbe-plant-insect research has been conducted in a laboratory and controlled environments. Heinen et al. then review and show the significant role of soil organisms in shaping plant-insect interactions in the field, similar to what has been shown in controlled conditions. Arbuscular mycorrhizal fungi (AMF) have both positive effects for specialist and adverse effects on generalist herbivores. Moreover, nematodes negatively affect herbivores. Some soil organisms may be promising agents for improving and protecting crop yields and may also influence community-level aboveground plant diversity, suggesting novel possibilities to recruit soil science to control aboveground communities of herbivores.

Examining community-level effects in more depth, a fascinating topic but still scarce in the literature is the impact of belowground communities on the functioning of multitrophic interactions aboveground. The review by Tao et al. summarizes recent literature on the mechanisms by which belowground mutualistic microbes affect predation and pathogen pressure on herbivores aboveground. They provide an excellent overview of how belowground mutualistic microbes influence predator attraction and foraging efficiency and the quality of the prey. However, certain predator species can also display herbivory feeding patterns, and in experimental work, Garantonakis et al. evaluated how the fungal root endosymbiont *Fusarium solani* affects the predatory bug *Nesidiocoris tenuis*, which can cause severe plant damage when prey insects are lacking. They show that damage to the plants by this zoophytophagous insect was reduced on *Fusarium*-inoculated plants (Garantonakis et al.). These studies nicely demonstrate the importance of including multiple trophic levels to fully understand ecosystem functioning and the ecosystem services of belowground communities.

Recently, more research is exploring how aboveground organisms trigger a series of physiological responses in the plant that systemically affect belowground communities, a direction much less studied than from below- to aboveground. Hoysted et al. demonstrates that aphid herbivory alters the root exudates of potato, with a negative effect on nematode egg hatching. Although sugars seemed to play a role, sugar addition did not recover the egg hatch whereas root exudates of uninfested plants did. Lee et al. show how whitefly herbivory suppresses the root bacterial pathogen *Ralstonia sonalacearum* in *Nicotiana benthamiana*. They are identifying two effectors (2G5 and 6A10) present in the whitefly salivary glands. It is fascinating that under attack aboveground, plants have developed defense mechanisms that protect them from belowground pathogens.

Belowground microbiomes also play important roles in terrestrial ecosystems and are one of the most cited fields in recent years. In microbiome research, a key issue is which factors drive microbiome community assembly and function. Ourry et al. showed that the presence of herbivore damage can influence the soil microbiome, associated with changes in the chemical composition of root exudates (Ourry et al.). An interesting applied benefit of understanding microbiome assembly is harnessing beneficial microbiomes to increase productivity of economically viable crops, which is still a challenge. Ma et al. applied the concept of plant-soil feedbacks to assess how inoculation of soil microbiomes cultured by 37 wild species of grass, forbs, and legumes, affect the growth of the cut flower chrysanthemum. Chrysanthemum grown in soil inoculated with grass legacies tended to be larger and less susceptible to soil-borne diseases than plants grown in soil cultured by forbs or legumes (Ma et al.), suggesting that we can apply this ecological concept to increase the sustainability of crop production.

CONCLUDING REMARKS

Soils are one of the most diverse habitats on Earth, with microbiota comprising a large portion of this diversity. One theme emerging from this review is the tremendous variation in soil microbial communities and the outcome of interactions between soil microbes and plants. In this Research Topic researchers highlight belowground interactions and outline their myriad ecological roles, from affecting soil health through impacting plant interactions with aboveground fauna. The studies here broadened the conceptual framework for the study of belowground interactions, explored interactions between plants and microbial communities in the soil, and the flow of Plant-mediated interactions between above- and belowground invertebrates. These studies are shedding light on missing details of the belowground complex "Mother Earth and Her Children" is illustrating.

AUTHOR CONTRIBUTIONS

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

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Coming to Common Ground: The Challenges of Applying Ecological Theory Developed Aboveground to Rhizosphere Interactions

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Accumulating evidence supports the importance of belowground interactions for plant performance, ecosystem functioning, and conservation biology. However, studying species interactions belowground has unique challenges relative to the aboveground realm. The structure of the media and spatial scale are among the key aspects that seem to strongly influence belowground interactions. As a consequence, our understanding of species interactions belowground is limited, at least compared to what is known about interactions aboveground. Here we address the general question: Do the ecological concepts that have been developed largely in aboveground systems apply to understanding species interactions in the rhizosphere? We first explore to what extent ecological concepts related to species interactions are considered in rhizosphere studies across various subdisciplines. Next, we explore differences and similarities above- and belowground for fundamental concepts in ecology, choosing topics that are underrepresented in rhizosphere studies but represent a swath of concepts: species diversity, island biogeography, self-organization and ecosystem engineering, trophic cascades, and chemical communication. Finally, we highlight to overcome major challenges of current methodologies to study rhizosphere interactions in order to advance the understanding of belowground interactions in an ecological context. By synthesizing literature related to rhizosphere interactions, we reveal similarities, as well as key differences, in how fundamental ecological concepts are used and tested in above- and belowground studies. Closing the knowledge gaps identified in our synthesis will promote a deeper understanding of the differences above- and belowground and ultimately lead to integration of these concepts.

Keywords: belowground, biodiversity, community ecology, ecological theory, rhizosphere, species interaction

INTRODUCTION

There has been a recent surge in studying species interactions that occur in the rhizospheres of plants (Brussaard, 1997; Hooper et al., 2000; Wardle et al., 2004; Orgiazzi et al., 2016). This surge has opened doors to new and exciting research questions related to how species interactions in the rhizosphere may influence various processes both above- and belowground, that integrate across many subdisciplines in ecology. For example, a recent list of 100 fundamental ecological questions includes several questions on how above- and belowground biodiversity interact and how biotic and abiotic feedbacks between plants and the soil influence plant growth (Sutherland et al., 2013). However, while substantial progress has been made in understanding both above- and belowground ecological processes as well as above-belowground linkages (e.g., Wardle et al., 2004; Lavelle et al., 2016; Coleman et al., 2018), the development of ecological concepts in above- vs. belowground systems have been somewhat disparate (Nobis and Wohlgemuth, 2004; Barot et al., 2007). The disconnect between concepts used in above- and belowground realms is perhaps because these realms differ in many important ways. The scale at which interactions operate between organisms belowground is often much smaller than in aboveground systems. Vertebrate herbivores may explore areas exceeding the size of 1 ha within a day, whereas most soil organisms do not explore more than 1 m^2 in their life time (van Der Putten et al., 2016). A further key difference lays in the provision of nutrients and energy, although root exudates provide a significant link of energy flow between the realms (Bais et al., 2006). Nevertheless, belowground, energy flows primarily through the detritus cycle rather than primary production on the terrestrial surface. Moreover, the soil medium differs from the aboveground environment, being a complex and heterogeneous matrix of interconnected spaces. Belowground interactions occur in darkness, where temperature fluctuations are low, relative humidity and CO_2 levels are high (Russell and Appleyard, 1915). Such specific characteristics belowground dictates dynamics of organism movement and sensory perception specific to the soil medium. For instance, arthropods adapted to live in deep soil layers show reduced body size, or loss of sight and flight capacity (Andújar et al., 2017). To summarize, fundamental differences between the above- and belowground realms suggest that patterns of interactions above- and belowground are, at least partially, governed by different mechanisms. Reconciling these differences in key ecological processes is critical to developing predictive theory and understanding responses to environmental changes (Wardle et al., 2004; Sutherland et al., 2013).

In this perspective, we address the general question whether ecological concepts that have been developed largely in aboveground systems apply to understanding species interactions in rhizospheres. To answer this question we first performed a literature search to evaluate how widely ecological concepts related to studying species interactions are used in studying belowground interactions. We looked at frequencies of studies related to ecological concepts in general ecology journals compared to sub-discipline journals. Next, we discuss several of the understudied concepts, focusing on concepts that are likely to function fundamentally different in above- and belowground realms. Finally, we outline challenges in studying rhizosphere interactions and some potential solutions. An improved understanding of how ecological concepts are used below- and aboveground should therefore improve research progress of rhizosphere interactions and ultimately enhance understanding of above-belowground linkages.

ADDRESSING THE USE OF ECOLOGICAL CONCEPTS IN SUBDISCIPLINES RELATED TO RHIZOSPHERE INTERACTIONS

To examine how widely ecological concepts related to studying species interactions in general ecology vs. sub-discipline journals, we focus on recent usage of ecological concepts in the literature by searching for articles published in the last two decades. We examined general ecology journals and four other subdisciplines related to rhizosphere interactions that cover ecological topics (**Supplementary Material 1**): soil science, botany, entomology, and microbial ecology. Within each of these five categories, we searched for keywords that represent fundamental concepts in ecology (see **Figure 1** and **Supplementary Material 1**). While our list of key concepts is not exhaustive, it is representative in that it covers topics dealing with species interactions in population, community, landscape, and ecosystem processes. We also included "soil" or "rhizosphere" and "species" to include only articles that were most likely to investigate rhizosphere interactions. Differences between the proportion of articles published in each subdiscipline vs. general ecology journals were tested using proportion tests, corrected for family-wise error rates (Newcombe, 1998).

As we expected, most ecological keywords were more highly referenced within ecology journals compared to other subdisciplines (**Figure 1**). Rarely, some of the terms were better represented in the sub-discipline journals. For example, compared to their use in ecology journals, "plant defense" is better referenced in the botany literature and 'species diversity' is better referenced in the microbial ecology literature. Some concepts also appear to not be well studied in the rhizosphere in any subdiscipline, such as "communication" or "island biogeography". Nevertheless, we found that most of the concepts were underrepresented in sub-discipline journals compared to ecology journals.

Clearly, there is a limited use of ecological concepts related to studying rhizosphere interactions in the focal subdisciplines. We posit that one reason for that is that researchers are still facing considerable technical challenges studying belowground biology. Hence, roots are still "the hidden half" (Waisel et al., 2002), the soil medium is "the final frontier" (Sugden et al., 2004), and soils are still viewed "through a ped darkly" (Coleman, 2011). Regardless of the reason, a disconnection of concepts among subdisciplines that investigate rhizosphere interactions may ultimately impede progress of understanding of general ecological processes in the rhizosphere and particularly above-belowground linkages.

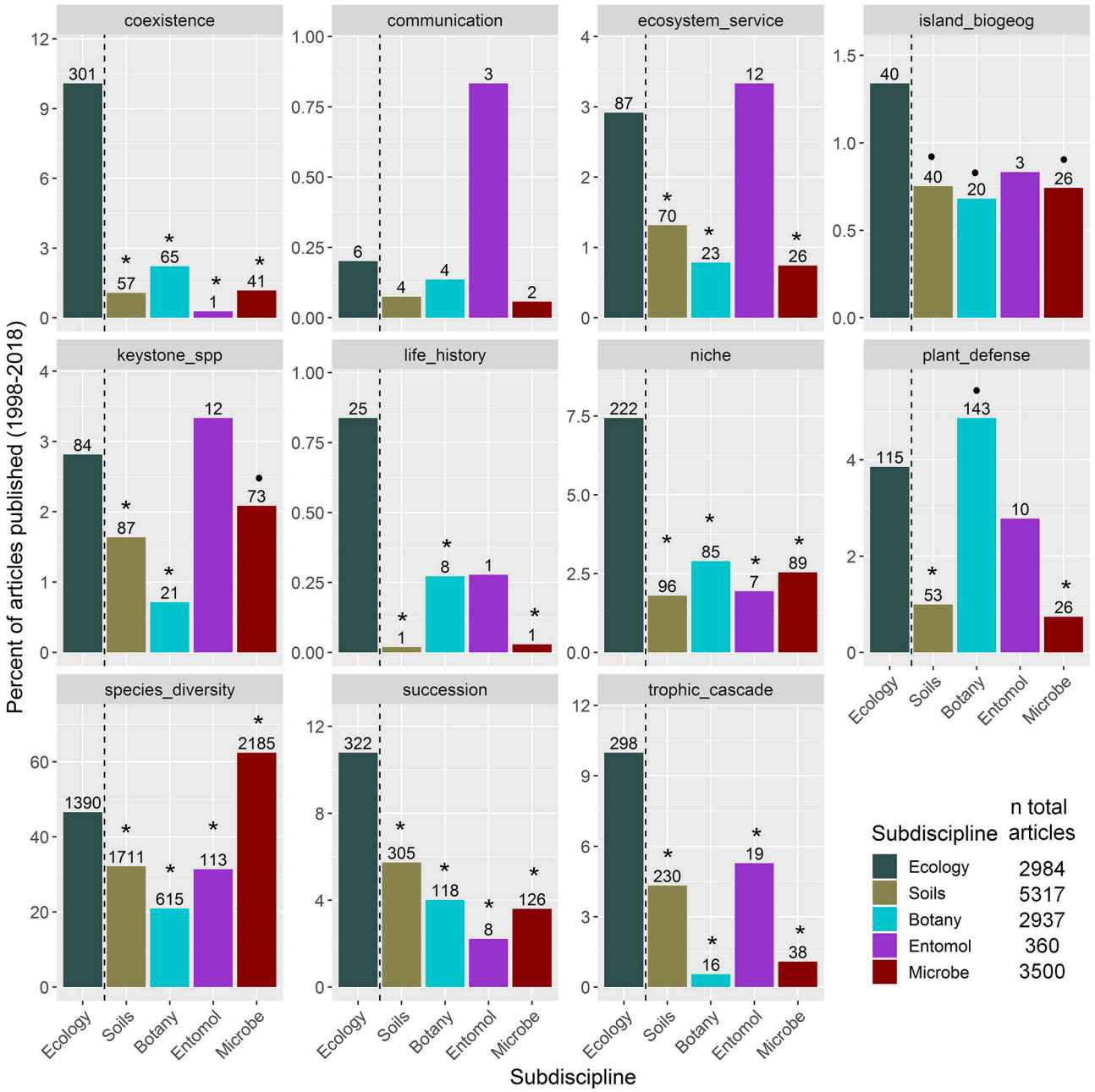

FIGURE 1 | Results of a literature search (1998–2018) for how frequently ecological concept keywords appear in articles that investigated rhizosphere interactions published in general ecology journals by subdiscipline journals. Bars represent the percent of articles related to each concept by subdiscipline. Numbers above the bars indicate the number of articles studying rhizosphere interactions within that subdiscipline that reference the concept, while the total number of articles that investigated rhizosphere interactions per subdiscipline is listed in the key. Subdisciplines that significantly differed in the percent of articles published compared to ecology journals based on pairwise proportion tests, corrected for family-wise error rates, are indicated as follows: •$P < 0.1$; *$P < 0.05$.

LIMITED UNDERSTANDING OF RHIZOSPHERE INTERACTIONS

According to the Global Soil Biodiversity Atlas (Orgiazzi et al., 2016) the soil is the most biodiversity rich habitat on Earth. It is estimated that 23% of terrestrial animals are soil invertebrates (Decaëns et al., 2006), of which 80% are insects and earthworms (Lavelle et al., 2006). Evidence shows that belowground biodiversity contributes to shaping the functioning of terrestrial ecosystems (Bardgett and Van Der Putten, 2014). Nevertheless, while conceptual frameworks are well developed (Hooper et al., 2000; Wardle et al., 2004; Lavelle et al., 2016) and some interactions are well studied (e.g., mycorrhiza), knowledge gaps remain regarding the diversity of other taxa that are

closely associated with plant roots. To demonstrate the paucity in understanding of rhizosphere biota, we pose a simple, but important question: how many of the organisms in a given ecosystem are associated with plant roots for a significant portion of their life cycles? This question is paraphrasing a broader question that several researchers tackled in the last decades: how many species are there on earth (May, 1988; Mora et al., 2011)? Similarly to estimating the total richness of species in an ecosystem based on extrapolations of specified community richness (Erwin, 1982), we could estimate the proportion of species engaging in rhizosphere interactions by looking only at beetles (**Supplementary Material 2**). The main problem with this approach is twofold: (1) Data scarcity. Exhaustive surveys and studies of belowground arthropod taxa are rare. (2) Conclusions are highly sensitive to model assumptions. Current theory and data are far from being able to provide a meaningful estimate to a fairly simple and fundamental question in ecological theory. It also highlights the gap of knowledge we still have in relation to belowground arthropod interactions as compared to microbes (Heinen et al., 2018). Another fundamental part of ecological research is related to the ways species are spatially arranged in the ecosystem.

ISLAND BIOGEOGRAPHY BELOWGROUND

The theory of Island Biogeography, first proposed by MacArthur and Wilson (1967), is a foundational concept in ecology that defined species richness on islands as the equilibrium between colonization and extinction. Due to the vast difference in scale, most soil organisms do not conform to the patterns of island biogeography on physical islands (Maraun et al., 2007; Ulrich and Fiera, 2009; Lavelle et al., 2016) or in "islands" created by habitat fragmentation (Mangan et al., 2004; Rantalainen et al., 2008). Ease of dispersal and low space requirements allow belowground biota to escape constraints on colonization faced by larger aboveground organisms (Griffin et al., 2002; Mangan et al., 2004; Rantalainen et al., 2008). However, there is some evidence that belowground communities do conform to the traditional theory of Island Biogeography when plant rhizospheres are considered as 'islands', likely because it is a more relevant scale (Peay et al., 2007, 2010; Glassman et al., 2017). The idea of plants as islands for herbivores was proposed as part of the theory of Island Biogeography and expanded to include plants as islands for microbes (Andrews et al., 1987; Martiny et al., 2006). However, studies are lacking assessments of temporal effects and cross taxa biodiversity when looking at plant islands. Thus, understanding belowground spatial ecology would benefit from long-term studies and examination of how species interactions might structure spatial patterns in belowground communities. Specifically, researchers asked how colonization after disturbance is different in rhizosphere interactions. Historically, the phrase 'succession' was used, but currently, a wider sense of this process is 'Self-Organization' (Lavelle et al., 2016).

SUCCESSION IN THE RHIZOSPHERE

Succession describes predictable and mainly linear shifts or development of communities through time and is one of the earliest ecological concepts (Cowles, 1899). In soil systems however, biological communities are often considered to self-organized across scales of time and space (Lavelle et al., 2016). The processes of soil community self-organization are generally consistent with patterns aboveground. Limited evidence suggests that an area is first colonized by autotrophs such as nitrogen fixing bacteria and algae, followed by the addition of simple heterotrophs, followed by larger and more complex animals and fungal structures (Ohtonen et al., 1999; Maharning et al., 2009). This turnover leads to increased network tightening and more efficient carbon uptake (Morriën et al., 2017). In abandoned agricultural fields, nematode communities shift from herbivorous to fungivorous species in later successional plots as plant productivity decreases and fungal biomass increases (Maharning et al., 2009). Arthropod community turn-over showed less clearly directional and progressive change. Many of the studies of soil self-organization focus on single clades or compare presence of large taxonomic groups, thus missing granular species diversity and turn-over (Maharning et al., 2009). Recent advances in sequencing will make it increasingly easy to monitor these processes in bacterial and fungal soil communities (Fierer et al., 2009, 2010; Hudson et al., 2017). Furthermore, network perspectives can shed light on the assembly and interaction of rhizosphere communities in successional processes (García de León et al., 2016; Morriën et al., 2017; Morriën and Prescott, 2018).

TROPHIC CASCADES AND TOP-DOWN EFFECTS ON SOIL FOOD WEBS

Trophic cascades, where predators indirectly benefit plants by consuming herbivores, is a fundamental concept in community ecology (Hairston et al., 1960; Pace et al., 1999). While the original concept was proposed mainly for aboveground systems, there has been very little consideration of whether trophic cascades might occur in belowground food webs (Denno et al., 2008). Carbon inputs belowground typically occur through detritus, and thus differ markedly from aboveground primary production by plants (Moore et al., 2004; Coleman et al., 2018). As such, soil food webs are considered to be self-organizing systems driven by bottom-up, mutually reinforcing processes (Lavelle et al., 2016). Nevertheless, secondary and tertiary consumers have long been recognized for their role in soil food webs, largely as ecosystem engineers and regulators of nutrient cycles (Lavelle et al., 2016; Coleman et al., 2018). Beginning around the turn of the century, researchers began probing a top-down perspective of soil food webs, asking whether top predators can regulate the abundance of lower trophic levels. While these early studies found evidence of top-down control on invertebrate communities, these impacts did not affect the basal microbial community (e.g., Mikola and Sktälä, 1998; Laakso and Setälä, 1999; Salamon et al., 2006). Other belowground studies, have documented top-down effects

of invertebrate predators (e.g., arthropods or nematodes) that have cascading effects on the productivity of plant roots and aboveground tissue (Preisser et al., 2006; Denno et al., 2008; Ali et al., 2013). Collectively, these small but representative examples from the literature suggest a mixture of evidence for belowground trophic cascades. Support for top-down control in belowground trophic interactions comes from cases largely involving the effects of insects and nematodes on plant productivity, whereas lack of support comes from systems with microbes or detritus at the base of the food web. Thus, predicting belowground trophic cascades may require knowledge of population growth rates of organisms interacting in the food webs. Understanding interspecies dynamics may further benefit from studying the way they interact and communicate belowground.

CHEMICAL COMMUNICATION IN THE RHIZOSPHERE

Communication among organisms is central to understanding any ecosystem, and the soil environment is no exception. Aboveground, vision and light sensing play an important role for organisms in many ecosystems (Doring, 2014; Kegge et al., 2015), but this option is lacking belowground. Thus, chemical communication is a more effective way for partners to communicate belowground (van Dam, 2009). The soil provides an environment that protects chemical compounds from degradation by oxygen and light, making belowground chemical signals more stable and possibly more reliable than aboveground (Karlovsky, 2008). Plant roots and soil microbes produce large arrays of volatile organic compounds (VOCs) that readily diffuse under atmospheric pressure and travel throughout the air- and liquid-filled pockets of the soil (van Dam et al., 2016). Therefore, they are believed to play important roles in soil interactions at multitrophic level (Schmidt et al., 2015; Venturi and Keel, 2016). Nevertheless, ecological functions of VOCs have mainly been studied for aboveground communities (Dicke and Baldwin, 2010). More recent studies on VOCs released by roots and soil bacteria and fungi found that similarly to the aboveground realm, VOCs also mediate species interactions in the rhizosphere (Rasmann et al., 2005; Martínez-Medina et al., 2017; Ossowicki et al., 2017; Schulz-Bohm et al., 2017). In addition, plants and microbes produce and secrete exudates containing an array of secondary metabolites, which can signal to and interfere with other soil organisms (Venturi and Keel, 2016). However, while the role of plant exudates has received significant attention (e.g., Steinkellner et al., 2007; Toussaint et al., 2012), the extent to which chemical communication in the soil affect interactions and dynamics of networks remains largely unknown.

RESEARCH METHODOLOGY OF RHIZOSPHERE INTERACTION

We briefly review important methodological gaps that are crucial to advance the understanding of rhizosphere interactions; we point readers to recent reviews for deeper reading into this topic (see McPhee and Aarssen, 2001; Forey et al., 2011). The challenge of studying biotic interactions is tackled by three types of methods: molecular/chemical techniques, field studies, and controlled conditions. Molecular and analytical chemistry tools are providing data on diversity of belowground microbial communities (Hudson et al., 2017). We can see the variation in broad taxonomic lines, but one of the outstanding challenges of the field is connecting species identity, or community composition, to function. Several methodologies such as large-scale and untargeted Gas Chromatography (GC)-mass spectrometry, liquid chromatography (LC)-mass spectrometry platforms, and nuclear magnetic resonance (H-NMR) are being used to decipher the chemical signaling in the rhizosphere (Oburger and Schmidt, 2016; van Dam and Bouwmeester, 2016; van Dam et al., 2016). The large-scale chemical analysis of the rhizomicrobiome, combined metagenomics, metatranscriptomics, metaproteomics and imaging mass spectrometry approaches (MSI) will help to understand the mechanisms involved in the communication between different members of the soil community (Oburger and Schmidt, 2016). Similarly, molecular databases such as Funguild and PICRUSt offer a promising framework to link taxa to function using 16S rRNA gene sequences (Langille et al., 2013; Tedersoo et al., 2014; Hahn et al., 2018). Given the spatial resolution required to investigate rhizosphere interactions (from cm to sub μm), the unpredictability of field conditions, and the instrumental limitations (e.g., immobility) most studies seek to simulate field conditions in semi-artificial experimental conditions (Oburger and Schmidt, 2016). Exploration of belowground organisms could be done with tools as simple as soil sampling and root excavations. For agricultural and plant physiology studies, Trachsel et al. (2011) coined the term "shovelomics" for high-throughput root phenotyping based on root crown architecture. We stress that continuing to apply the principles of shovelomics in ecological research will explore a lot of rhizosphere interactions. While it is difficult to sample soils with minimal disturbances, emerging techniques may allow for quantification of root traits and other belowground measurements using non-destructive techniques such as X-ray tomography (Bardgett et al., 2014). We propose that the integrated use of different molecular and metabolomic methodologies in semi-artificial and field conditions will be the most promising approach in shedding light onto the great number of yet unrevealed processes in the rhizosphere (Ferlian et al., 2018).

CONCLUDING REMARKS

The importance of belowground processes in ecology are being increasingly recognized (Wardle et al., 2004; Orgiazzi et al., 2016; Coleman et al., 2018). Still it is uncertain how ecological theories which were developed to describe aboveground interactions apply rhizosphere interactions. The literature shows some similarities between above- and belowground processes. For example, we can cautiously suggest that soil and plant heterogeneity is positively related to species diversity belowground (Kowalchuk et al., 2002). Yet, empirical evidence is still lacking (Scherber et al., 2009). Similarly, temporal development of belowground communities is somewhat predictable, much as it is aboveground (Maharning et al.,

2009). Evidence is mixed in other areas; trophic cascades seem to have an important role in community and population dynamics belowground, as they have aboveground. On the other hand, there are major differences between below- and aboveground conditions that affect trophic interactions and dynamics. For instance, the scale at which key ecological processes are examined, such as the strength of trophic cascade linkages, dispersal, and species area curves, all show differences between above- and belowground systems. Differences in media structure can influence the pattern of communication, which is based more on chemicals belowground as compared to a broader use of light perception in the aboveground communities. Furthermore, increased attention to rhizosphere interactions, particularly studies using modern methodological approaches, will allow for more robust tests of many ecological theories. In addition to advancing predictive ecological theory, an improved understanding of rhizosphere interactions will ultimately aid in preserving biodiversity and mitigating negative ecological impacts of global change (Wardle et al., 2004; Sutherland et al., 2013).

DATA AVAILABILITY

Data from the literature search are deposited in Figshare (10.6084/m9.figshare.7356551).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. OS initiated the process and lead the writing. PH lead the literature search and contributed to writing and editing. ZG-P and AM contributed to writing and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00058/full#supplementary-material

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Belowground Microbiota and the Health of Tree Crops

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Trees are crucial for sustaining life on our planet. Forests and land devoted to tree crops do not only supply essential edible products to humans and animals, but also additional goods such as paper or wood. They also prevent soil erosion, support microbial, animal, and plant biodiversity, play key roles in nutrient and water cycling processes, and mitigate the effects of climate change acting as carbon dioxide sinks. Hence, the health of forests and tree cropping systems is of particular significance. In particular, soil/rhizosphere/root-associated microbial communities (known as microbiota) are decisive to sustain the fitness, development, and productivity of trees. These benefits rely on processes aiming to enhance nutrient assimilation efficiency (plant growth promotion) and/or to protect against a number of (a)biotic constraints. Moreover, specific members of the microbial communities associated with perennial tree crops interact with soil invertebrate food webs, underpinning many density regulation mechanisms. This review discusses belowground microbiota interactions influencing the growth of tree crops. The study of tree-(micro)organism interactions taking place at the belowground level is crucial to understand how they contribute to processes like carbon sequestration, regulation of ecosystem functioning, and nutrient cycling. A comprehensive understanding of the relationship between roots and their associate microbiota can also facilitate the design of novel sustainable approaches for the benefit of these relevant agro-ecosystems. Here, we summarize the methodological approaches to unravel the composition and function of belowground microbiota, the factors influencing their interaction with tree crops, their benefits and harms, with a focus on representative examples of Biological Control Agents (BCA) used against relevant biotic constraints of tree crops. Finally, we add some concluding remarks and suggest future perspectives concerning the microbiota-assisted management strategies to sustain tree crops.

Keywords: tree crops, belowground microbiota, biological control agents, endophytes, mycorrhiza, phytoparasitic nematodes, plant-growth-promoting microorganisms, soil-borne pathogens

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INTRODUCTION

Tree crops are fundamental for human nutrition and warrant food security and stability of many farms. The surface covered by tree crops showed a growing trend in the last decade, approaching to a global acreage of 10 Mha for main fruit types with an ∼20% increase in productivity during the period 2004–2014 (FAOSTAT, *http://fenix.fao.org/faostat/beta/en/)* (**Figure 1**). Plants (like trees) as well as the environment (such as soil) consist of complex and diverse assemblage of myriads of microbial species closely associated with their host, either as epiphytes or as endophytes (Trivedi et al., 2016). The association established by a plant and its microbiota (Lederberg, 2006) can be either stable, transient or fluctuating, enduring along the host lifetime determines its development, fitness, and health (Kowalski et al., 2015). The belowground microbiota is mostly comprised of bacteria and fungi belonging to the second trophic level (i.e., decomposers, mutualists, pathogens, parasites, and root-feeders) of the soil food web (Ingham, 1999) (**Figure 2**). Because of their size, nematodes per definition are not part of the soil microbiota, although they can play important roles in shaping its structure, including not only species belonging to the second trophic level (root-feeder nematodes) but also those ones of the third level (i.e., shredders, predators, grazers), particularly nematodes feeding on fungi and bacteria. Despite their parasitic behavior, phytoparasitic nematodes spend a considerable part of their life-cycle in the soil and represent the first group of plant parasites present in the soil. Therefore, the fraction of microorganisms linked to them can be considered as a specific compnent of the plant-associated microbiota (Vandekerckhove et al., 2000; Haegeman et al., 2009).

The study of the belowground microbiota has gained attention during the last years. Many studies have investigated soil belowground microbiota focusing on key issues such as the composition, structure, and functioning of these microbial communities and how they are built up and influenced by a range of factors [e.g., changing environment, varying weather/climatic conditions, (diffuse) pollution, anthropogenic actions, plant genotype, plant signals, etc.] [see, for instance (Doornbos et al., 2012; Bakker et al., 2013; Bulgarelli et al., 2013; Mendes et al., 2013; Lakshmanan et al., 2014; Fierer, 2017)]. Structural and functional modifications in the soil/rhizosphere microbiota have a crucial impact on aboveground ecosystems. In the particular case of trees, the trophic interactions established between the host and its associated belowground microbiota could be assumed, at least *a priori*, as more durable than that occurring in short-living, herbaceous species. Indeed, due to their perennial, long-living nature, it could be envisaged that belowground microbial communities associated with tree crops may be shaped by more persistent changes than those taking place in annual crops. Trees provide, in a more long-lasting way, an energy flow through photosynthesis, mobilizing nutrients as part of a continuous process leading to their recycling via the organic matter accumulation and its eventual decay. Moreover, due to the absence of annual rotation and lack of soil tillage, perennial tree crops also represent a stable food source not only for building up consortia of beneficial microbial communities but also for many root pathogens or parasites. Direct effects, due to deposition of organic matter and nutrients, could be more constant while indirect effects through agricultural inputs (i.e., application of fertilizers, pesticides, etc., irrigation and soil labor) would potentially work in a similar way as in annual crops.

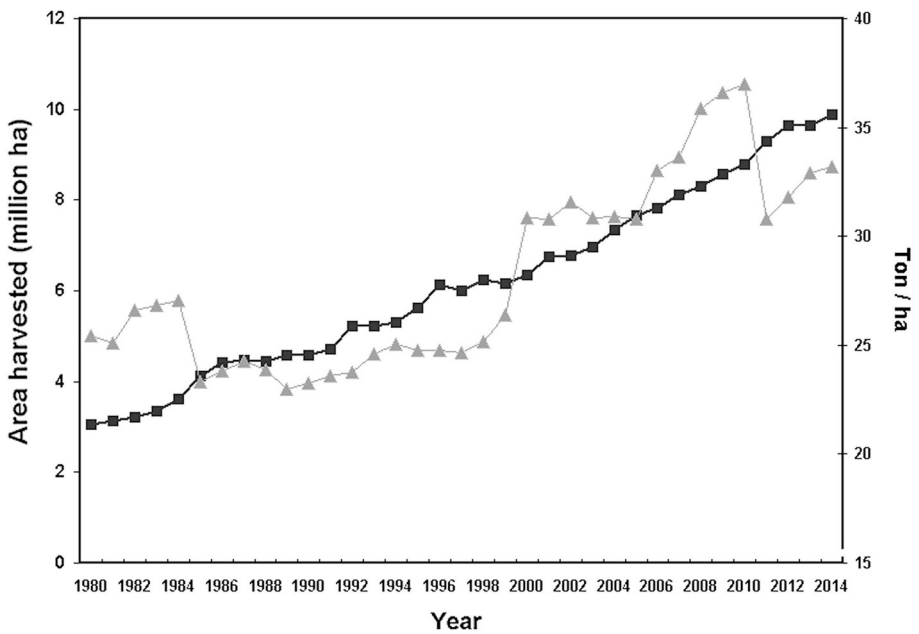

FIGURE 1 | Total world surface (triangles) and yield/hectar (solid squares) of main tree crops (citrus, fresh and tropical, pome and stone fruits) (source FAOSTAT: *http://fenix.fao.org/faostat/beta/en/)*.

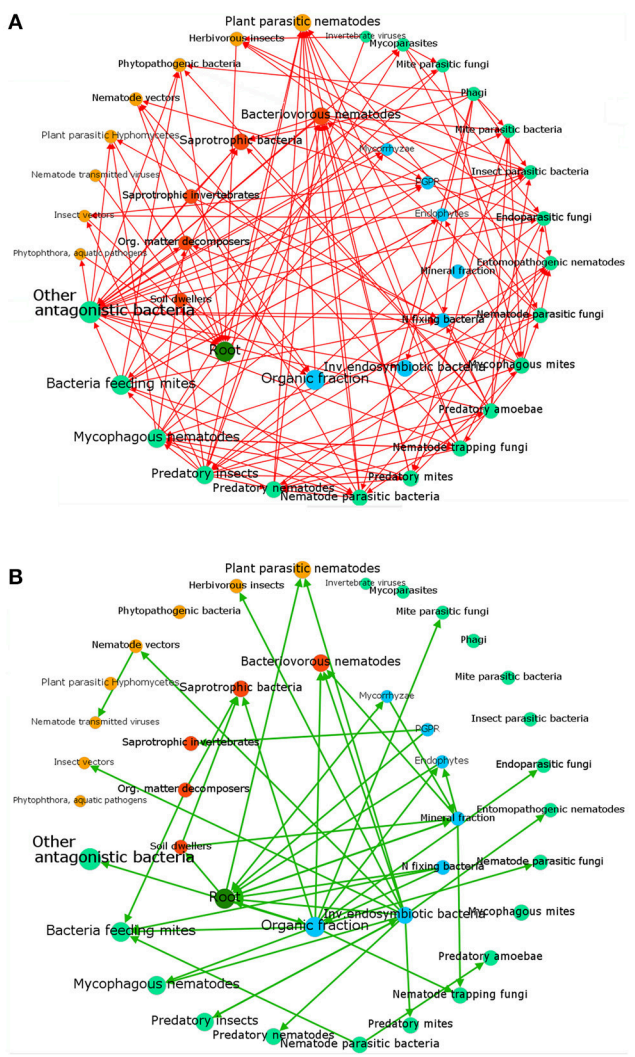

FIGURE 2 | A simplified food web describing main soil components and their relationships. The nodes are classified by roles as: primary root (dark green), beneficial soil components, organisms or promoters, including soil factors (blue), decomposers (brown), pathogens (orange) and biocontrol agents or antagonists (pale green). Arrows show negative effects **(A)**, such as predation, parasitism, pathogenicity or **(B)** positive links, such as growth promotion, symbiosis or alimentary provision. Indirect factors such as those related to abundance, competition or other density-dependent effects are not included. Node labels and sizes are proportional to their connection level (number of edges). Analysis produced with Gephi (Bastian et al., 2009).

Being present on a time scale of years, and having a persistent, deeper root system, the impacts of tree crops (e.g., on nutrients mobilization, organic matter accumulation, parasites, etc.) largely differ from annual crops and thus cannot be considered as comparable. This is well illustrated by the currently-available and powerful metagenomic approaches (Colagiero et al., 2017). Overall, the events taking place between a tree crop and its associated whole soil microbiota have not been widely investigated.

In this study, we consider a tree crop as a woody, perennial plant with a distinct trunk, such as fruit, nut, and timber trees of economic importance, grown in orchards or in planted forests. Therefore, we exclude from this definition any palm "tree" species (*Arecaceae* family) as well as any other herbaceous perennial monocots (e.g., *Musa* spp., *Dracaena* spp., *Poaceae* family representatives, etc.) showing arborescent growth, since from both botanical and anatomical point of view they are not true trees. Tree crop ecosystems are of immense importance since they provide a range of products and ecosystem services. An increased understanding of the links between soil microbiota and trees is certainly helpful for the development of more effective and sustainable tree crop management strategies. Here, we (i) summarize methodological approaches used to unravel belowground microbial communities, with emphasis on tree crops; (ii) review the composition, distribution, and multitrophic networks of soil and root-associated microbiota, including endophytes, and the way they influence aboveground ecosystems in tree crops; (iii) examine the benefits (productivity, development, health and fitness, stress alleviation) and harms (mainly biotic stresses) for tree crops and woody plantations upon interaction with indigenous and introduced soil-borne (micro)organisms; and (iv) recapitulate strategies implemented for tree crop growth promotion.

METHODOLOGICAL APPROACHES TO UNRAVEL THE COMPOSITION AND FUNCTION OF BELOWGROUND MICROBIOTA

Methods to assess the diversity, structure, and function of microbial communities can be categorized into three main groups, namely conventional, biochemical and molecular. Here, we summarize the advantages and limitations of main methodological approaches to study the composition and function of rhizosphere microbial communities, with emphasis on tree crops (**Table 1**).

Conventional and Biochemical Methods

Culture-based methods constitute a good complement to DNA-based approaches. However, they are extremely biased regarding the actual evaluation of microbial genetic diversity since only <1% of the total number of prokaryotic species present in the environment are culturable. Several improved procedures and media mimic natural environments in terms of nutrients, oxygen gradient, pH, etc. maximizing the cultivable fraction of soil-borne microbial communities (Gravel et al., 2007). In addition, the number of colony-forming units (CFU) is positively correlated with enzymes and respiratory activity. This approach may be applied to characterize the relative abundance of active microorganisms with certain functions or trophic requirements (Blagodatskaya and Kuzyakov, 2013). Even though culture-dependent methods are not ideal for evaluating the actual composition of natural microbial communities when used alone, they are useful for understanding growth habits, development,

TABLE 1 | Methods to study belowground microbial communities.

Methods	Advantages	Disadvantages	Crop examples	References
CONVENTIONAL AND BIOCHEMICAL				
Growth on culture media, Enrichment cultures,	Microorganism isolation, pest control purposes, etc.; fast and low cost	Useful exclusively for cultivable microorganisms	Maritime pine (*Pinus pinaster*), *Pinus densiflora* and *P. thunbergii*	Islam and Ohga, 2013; Proença et al., 2017a
Enzymatic measurements	Functional activity; soil quality bioindicators; early indication of changes in soil; ecosystem disturbance estimate	Not necessarily linked to real ecological functioning and activity	Poplar (*Populus* sp.)	Winding et al., 2005
Community level physiological profiling (CLPP), sole-carbon-source utilization (e.g., API and BIOLOG)	Versatile for bacteria, fungi and specific carbon sources (BIOLOG); fast, highly reproducible and relatively inexpensive	It reflects potential metabolic diversity of fast growing microorganisms and not of overall *in situ* diversity; sensitive to inoculum density	Grapevine (*Vitis* spp.), olive, *Picea abies* forest	Söderberg et al., 2004; Montes-Borrego et al., 2013; Salomon et al., 2014; Lladó and Baldrian, 2017; Ruano-Rosa et al., 2017
Phospholipid fatty-acid analysis (PLFA)/Total Ester-linked Fatty Acid (ELFA)/Fatty acid methyl ester (FAME) analysis	Indicator of active microbial biomass opposed to non-living biomass; fingerprint of overall microbial communities (Bacteria, Archaea, Fungi)	Limited number of microbial groups identified	Diverse	Hill et al., 2000; Söderberg et al., 2004; Winding et al., 2005; Bastida et al., 2008; Francisco et al., 2016
MOLECULAR				
Fluorescent *In situ* Hybridization (FISH)	Direct identification and visualization of microbial groups based on the hybridization with a high number copies of their rRNA; it reflects microbial activity and makes it possible to quantify cell number; DNA extraction from soil is not required	Cells with low activity are not identified; it requires skill and experience in the case of low probe penetration and autofluorescence; information obtained is dependent on the availability of designed probe(s)	*Cactaceae*, rosemary (*Rosmarinus officinalis*)	Hill et al., 2000; Barra Caracciolo et al., 2005, 2015; Lopez et al., 2011
Total microbial abundance (DAPI counts); cell viability	Detection of all microbial cells independently from their physiological state	Overestimation of cell number if they are just dead but with an intact DNA	Poplar, rosemary	Barra Caracciolo et al., 2015; Ancona et al., 2017
Genetic fingerprinting techniques (DGGE/TGGE, T-RFLP)	Sensitive to variation in DNA sequences; bands can be excised, cloned and sequenced for subsequent identification	Multiple bands for a single species can be generated due to micro-heterogeneity: can be used only for short fragments; complex communities may appear smeared due to a large number of bands; Inconsistencies (gel to gel variation)	*Citrus* spp., *Elsholtzia splendens* and *Commelina communis*, pine (*Pinus* sp.), cork tree (*Quercus suber*)	Hill et al., 2000; Araújo et al., 2002; Kuklinsky-Sobral et al., 2005; Winding et al., 2005; Singh et al., 2007; Sheng et al., 2008; Wang et al., 2008; Sun et al., 2010; Bevivino et al., 2014; Ng et al., 2014; Proença et al., 2017a
qPCR, RT-qPCR, dPCR	Quantitative and highly sensitive for species identification and functional genes; easy to implement and cheap; specific amplification confirmed by melting curve analysis	Can only be used for targeting of known DNA sequences; DNA impurities and artifacts may lead to false-positives or inhibit amplification	Poplar, switchgrass (*Panicum virgatum*)	Liang et al., 2014; Cai et al., 2015

(Continued)

TABLE 1 | Continued

Methods	Advantages	Disadvantages	Crop examples	References
Next-generation sequencing (NGS)	Rapid to assess biodiversity and abundance of many species/organizational taxonomic units simultaneously	Massive amount of sequencing data of DNA (genomic or PCR amplified fragments) or RNA error distribution within reads of a library; insertions or substitution errors; relatively expensive; replication and statistical analysis are essential; computational intensive; challenging in terms of data analysis	Maritime pine (*Pinus pinaster*)	Proença et al., 2017a
DNA sequence analysis of the internal transcribed spacer (ITS) region for mycorrhizal studies	Fast and accurate for the identification of mycorrhizal fungi and characterization of their distribution.	Relatively expensive, especially in case of metagenomic analyses	Ectomycorrhizas of poplar (*Populus nigra*×*maximowiczii*) and willow clone (*Salix viminalis*) cultivated as SRF, mycorrhizal fungi of willow (*Salix* spp. L.) from hydrocarbon-contaminated soils, AMF of *Acacia gerrardii* under salt stress	Hrynkiewicz et al., 2010; Hassan et al., 2014; Hashem et al., 2016;

A few illustrative examples of herbaceous plants are also given.

and potential functions of soil and rhizosphere microorganisms (VanInsberghe et al., 2013; Bevivino and Dalmastri, 2017).

Biochemical methods enable the assessment of soil microbiota activities of both the overall microbial community (e.g., dehydrogenase activity) and specific components (e.g., ammonia-oxidizing bacteria). The release of labile compounds, including enzymes, by living roots or lysis of root cells, stimulates microbial activity and growth in a similar way as rhizodeposits (Loeppmann et al., 2016). Consequently, localization of easily available C yields hotspots of microbial abundance and activities, frequently termed as the "rhizosphere effect" (Reinhold-Hurek et al., 2015; Thijs et al., 2016). Extracellular enzyme activities in the rhizosphere are higher compared to root-free soils, similarly to total microbial biomass and activity measured as respiration or growth rates (Allison and Vitousek, 2005; Ancona et al., 2017). Roots and associated mycorrhizal communities are known as major producers of β-glucosidases and acid phosphatases (Conn and Dighton, 2000). Despite soil enzymes being partly of plant origin, microorganisms constitute the main source of enzymes mediating the cycling of major nutrients (C, N, P, and S).

One approach to characterize the soil microbial communities is the Community Level Physiological Profiling (CLPP), in which species are identified based on utilization of different carbon sources with EcoPlateTM (Biolog, Inc.). CLPP yields information on both function and structure of part of a microbial community metabolically active under plate conditions (Garland and Mills, 1991). The BIOLOG® advantages include the identification of physiological profiles of a microbial community as a whole (Stefanowicz, 2006). However, most bacterial cells in natural ecosystems are inactive and the substrates available in BIOLOG® plates are not necessarily relevant from the ecological point of view, and do not reflect the diversity of substrates found in the environment (Konopka et al., 1998). This methodology has been applied to compare functional diversity of communities from rhizosphere and non-rhizosphere soils (Söderberg et al., 2004), from rhizospheres of different plant species (Grayston et al., 1998), and to link microbial functional diversity of olive rhizosphere soil to management systems in commercial orchards (Montes-Borrego et al., 2013). While limitations of this methodology for the characterization of whole communities are well known, it continues to be used in combination with molecular approaches to identify the copiotrophic, fast-growing fraction of the bacterial community of soil environments as those from coniferous forests, where oligotrophic taxa are usually dominant (Lladó and Baldrian, 2017).

Biochemical methods can also be used to assess microbial community structure and to perform a phenotypic fingerprinting of the main groups (Gram-positive and Gram-negative bacteria, fungi, etc.) in the rhizosphere. This is the case of the phospholipid-derived fatty-acid (PLFA) and the total ester-linked fatty-acid (ELFA) methods (Sharma and Buyer, 2015; Hinojosa et al., 2016). As the fatty-acid side chains are rather unique among the various life forms, these molecules are widely used as taxonomic and phylogenetic biomarkers to describe the structure and size of microbial communities in soil and rhizosphere samples (Debode et al., 2016; Francisco et al., 2016). Phospholipid fatty-acids are found exclusively in cell membranes

and not in other parts of the cell as storage products. This is important as cell membranes are rapidly degraded and the component PLFA is quickly metabolized following cell death. Consequently, phospholipids can serve as important indicators of active microbial biomass as opposed to non-living biomass. These methods are useful for assessing the structure of soil microbial communities and for determining effects of soil disturbances such as cropping practices, pollution, and changes in soil quality. For example, PLFA analysis was successfully used to investigate the impact of *Populus* spp. grown as short rotation coppice (SRC) on the microbial communities of arable soils (Baum et al., 2013).

Molecular Methods

Molecular methods have provided a more-in-depth understanding of the occurrence and phylogenetic diversity of soil microbial communities (Tiedje et al., 1999; Fakruddin and Mannan, 2013). Polymerase chain reaction (PCR)-based approaches are commonly used for phylogenetic assignments. Small subunit rRNA genes (for instance, the 16S small subunit ribosomal RNA [16S rRNA] for prokaryotic cells) are amplified from soil-extracted nucleic acids. Microbial rRNA gene sequences can then be sequenced and identified using appropriate databases (e.g., NCBI GenBank, EMBL, EzBioCloud, etc.) and compared with those of known microorganisms (Janssen, 2006). Similarly, the identification of soil fungi and fungal symbionts associated with previously selected and characterized mycorrhizas is based on sequence analysis of gene fragments from the large-subunit rRNA (LSU) or their internal transcribed spacer (ITS) regions (Porras-Alfaro et al., 2014). Taxonomic and phylogenetic affiliation of fungi can be based on widely available databases like the NCBI GenBank or on the stable and reliable platform UNITE, designed for sequence-based identification of ectomycorrhizal asco- and basidiomycetes.

Molecular-based approaches have revealed an extraordinary taxonomical and functional diversity of microorganisms. To study the population structures and dynamics of microbial communities, genetic fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) were developed (Muyzer et al., 1993). Nowadays, DGGE can be used as a first approach to visualize main differences in a given microbial community and subsequently high-throughput sequencing (HTS) can be applied to have a deeper understanding of the microbiota composition (Di Lenola et al., 2017; Proença et al., 2017a). This methodology has been implemented in different fields and it is very common in soil microbiology studies (Bevivino et al., 2014; Ng et al., 2014), or to assess the aboveground microbial structure of trees (e.g., maritime pine, *Pinus pinaster* Ait.) (Proença et al., 2017a). Other community profiling techniques include temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA), and amplified ribosomal intergenic spacer analysis (ARISA) (Anderson and Domsch, 1989; Anderson and Cairney, 2004). These methods can also provide detailed information about community structure in terms of richness, evenness and composition and permit to identify selected species and functional genes involved in specific processes. Nevertheless, these qualitative PCR-based methods do not provide information on the gene copy numbers. To achieve that, implementation of qPCR (quantitative PCR) is needed whereas RT-qPCR (reverse transcription qPCR) is informative about the expression of a specific gene (Stella, 2014). However, the phylogenetic characterization of prokaryotic cells based on DNA extraction from soil does not reflect the activity of rhizosphere microbial community, as DNA may also proceed from dead or inactive cells. Likewise, the analysis of biodiversity based on the molecular identification of single ectomycorrhizal roots or arbuscular spores, and the application of cloning for identification of arbuscular mycorrhizal fungi (AMF), have some limitations difficulting a reliable portrait of the microcosm environment condition. Thus, a novel sequence-based method was developed to describe AMF communities, coupling the previously established AMF-specific PCR primers that amplify a *c.* 1.5-kb long and AMF-specific pSSU-ITS-pLSU fragment with single molecule real-time (SMRT) sequencing (Schlaeppi et al., 2016). Finally, substantial progress has been also made to facilitate the quantitative detection of individual nematode taxa on the basis of small subunit ribosomal DNA-based (SSU-rDNA) monitoring of nematode assemblages (Vervoort et al., 2012). In complex environments, such as soil, the newly developed digital polymerase chain reaction (dPCR) has been recently applied to quantify the absolute concentration of DNA targets or functional genes in soil (Kim et al., 2014; Cavé et al., 2016). This technology represents a promising tool enabling to examine the dynamics of soil microorganisms and to target pathogen-derived nucleic acids in environmental samples (Farkas et al., 2017).

Epifluorescence Microscope-Based Methods

Epifluorescence microscope-based methods do not need DNA extraction from soil, enabling direct visualization of microbial cells/structures under an epifluorescence microscope. The total direct count, cell viability (live/dead) and Fluorescence *In situ* Hybridization (FISH) are reliable and commonly used methods. The total direct count allows assessing microbial abundance through a DNA fluorescent intercalant such as DAPI, which can detect all microbial cells in a rhizosphere sample regardless of their physiological state and metabolic activity (Lew et al., 2010; Barra Caracciolo et al., 2015). Similarly, two fluorescent dyes, SYBR™ Green II and propidium iodide, can be used to discriminate between viable and dead cells (Ancona et al., 2017). Finally, FISH enables phylogenetic *in situ* identification and quantification of soil and rhizosphere communities at different phylogenetic levels (from domain to species), by using fluorescent labeled rRNA-targeted oligonucleotide probes in single cells. rRNA-targeted probes that occur in a large copy number detect specific sequences of rRNA in single cells. Since only viable and active cells possess a sufficient number of undamaged ribosomes, they act as indicators of the physiological state of cells (Di Lenola et al., 2017). The detection of FISH-stained cells can be hampered by strong soil

background autofluorescence which is avoided by applying a density gradient centrifugation to extract the detachable bacteria from soil particles (Barra Caracciolo et al., 2005, 2010). FISH has been successfully applied in analyses of active microorganisms in the rhizosphere (Barra Caracciolo et al., 2015) including endophytes (Kutter et al., 2006; Lopez et al., 2011). The main limitations of this method are: (i) its inability to detect unknown species and those with low ability, or for which specific probes have not been designed yet, and (ii) probe's difficulty to enter into Gram-positive cells under specific conditions.

Meta-Omic Approaches

The recent development of HTS-based metagenomic analyses has further contributed to unveil either microbial or plant functioning in the rhizosphere, to yield a global view of the structure and diversity of the rhizosphere microbiota (Leveau, 2007; Barberán et al., 2012; Lindahl et al., 2013; Mendes et al., 2013; Hassan et al., 2014). The implementation of genomic methods to microbial assemblages is commonly used to describe communities overcoming biases inherent to PCR amplification of a single gene. The classical metagenomic strategy, as defined by Handelsman and colleagues (Handelsman et al., 1998), involves the following steps: DNA isolation, fragmentation and cloning, library screening, sequencing of interesting clones, and DNA comparison. Actually, three major and often overlapping directions can be recognized: the first trend aims at linking phylogeny to function; the second involves the discovery of genes or functions of interest; and the third is the mass sequencing of environmental samples which offers a more global (or systems-biology) view of the community under study (Steward Rappé and Rappé, 2007).

The HTS or next-generation sequencing (NGS) technology is experiencing a rapid development, providing wide and in-depth views in metagenomics. Several protocols and tools, including bioinformatic resources, are available for these studies. A number of HTS platforms have been developed and are widely used, including the Illumina (e.g., HiSeq, MiSeq), Roche 454 GS FLX+, SOLiD 5500 series, and Ion Torrent/Ion Proton platforms. Currently, the majority of microbial ecology studies implement HTS by focusing on either targeted gene sequencing with phylogenetic or functional gene targets or on shotgun metagenome sequencing (Pervaiz et al., 2017).

Most of the bacterial community studies have depended on a single gene, such as the hypervariable regions of the 16S rRNA gene, to assess taxonomic diversity and to determine which bacteria are present in a community. Other useful targets for bacterial community studies based on single amplicon sequencing include the type I chaperonins (*cpn60* gene) (Links et al., 2012). However, these "metabarcoding" methods (*sensu stricto* they cannot be considered as metagenomic approaches since they are just based on libraries of single amplicons) are limited by short read lengths, sequencing errors, differences arising from the different regions chosen, and difficulties in assessing operational taxonomic units (OTU). Shotgun metagenomics sequencing avoids many of the biases encountered in amplicon sequencing because it does not require amplification prior to sequencing (Fierer et al., 2012; Sharpton, 2014). Application of metagenomic analysis also paves the way for scientists to build fundamental knowledge on fungal communities in the environment. Actually, the metagenomics assessment of fungal diversity is common not only for soil but also for plant samples (mycorrhiza, endophytes), enabling detailed determination of all fungal trophic groups: saprophytic, pathogenic, endophytic, and symbiotic (Lindahl et al., 2013).

Further technologies such as the nanopore sequencing (with mini flow cells such as the MinIon™ by Oxford Nanopore™), or the PacBio™ sequencing based on ionic readings are gaining popularity due to their capability to sequence very long reads (up to several kilobases) in milliseconds and without amplification (Branton et al., 2008; Singer et al., 2016). Some of these novel approaches are promising, since they combine easy use and/or portability with a massive data production. They have the potential to sequence all the retrotranscribed rDNA molecules present in a sample, thus accounting for a direct identification of active species. In the light of experimental assays applied to plants, the information that may be gained through these studies are higher than the limits considered a few years ago, and often exceed the analytical potential of the bioinformatic resources eventually applied.

By using the above methodological approaches, the diversity, structure, and functioning of fungal and bacterial communities, endophytic and/or rhizospheric, were studied in tree species including *Populus deltodies* (Gottel et al., 2011; Shakya et al., 2013), native forest species (Buée et al., 2009), and conifers (Baldrian et al., 2012; Proença et al., 2017a). For instance, these studies were instrumental to link the so-called core (bacterial) microbiota to specific ecological niches in a given species and, more importantly, under field-grown conditions (Beckers et al., 2017). Based on sequencing data it is also possible to predict the function of a microbial community by using the bioinformatic tools PICRUSt (Langille et al., 2013) and tax4fun (Aßhauer et al., 2015).

Metatranscriptomics, in which total environment RNA is sequenced, is applied to reveal and compare active community members and metabolic pathways (Urich et al., 2008; Turner et al., 2013). Although the analysis of total rRNA has been widely used to profile microbial communities in soil (Carvalhais et al., 2012), the gene expression of microbes in the rhizosphere is much less studied due to the difficulty to obtain sufficient material under controlled conditions from a highly variable and irregular niche. Nevertheless, metatranscriptomics has been used to identify genes expressed by eukaryotes in forest soils, to study the fungal and bacterial responses to N deposition in two forests dominated by sugar maple (*Acer saccharum* Marsh), or to analyse ectomycorrhizal roots and the genes active in the *Piloderma–Pinus* symbiosis (Damon et al., 2012; Liao et al., 2014; Hesse et al., 2015). Finally, the sensitivity of current metabolomic platforms represents an important constraint showing that this approach cannot solve all rhizosphere-signaling relations such as chemical communications and interactions (van Dam and Bouwmeester, 2016).

FACTORS INFLUENCING BELOWGROUND MICROBIOTA ASSOCIATED WITH TREE CROPS

A long-living host may establish a durable interaction with its associated microbiota compared to that taking place in annual and/or herbaceous plants. Nevertheless, the composition and structure of the associated microbiota in any given tree crop undergo alterations along time and space due to factors such as environmental (sudden/long-term) changes, physical-chemical soil properties, anthropogenic actions, agronomical practices, climatic factors, plant developmental stage, (a) biotic stresses, etc. Depending on the tree crop under study, this range of factors may have either major or minor influence on the entire belowground microbial communities or on some of their specific components (Caliz et al., 2015).

Temperature and precipitation along with seasonal variations are among the main climatic/weather components controlling microbial growth and reproduction; therefore, these abiotic factors may substantially influence the soil microbiota of tree crop plantations and forests. Okada and colleagues found that autumn precipitation in the preceding year was a crucial factor influencing the biomass of ectomycorrhizal fungi (EMF) in a 40/50-year-old *Pinus densiflora* L. forest, while soil water availability for EMF and host plant roots in the growing season could positively impact ectomycorrhizal biomass in subsequent seasons (Okada et al., 2011). With the aim of simulating realistic future drought conditions, Felsmann and colleagues studied the effects of reduced precipitation for one growing season on the bacterial community of beech (*Fagus sylvatica* L.) and conifer forests (Felsmann et al., 2015). They found that moderate drought induced by the precipitation manipulation treatment significantly affected the active but not the total bacterial community, proposing that there is an adequate resistance of the soil microbial system over one growing season. In soils of a temperate beech forest, seasonality, resource availability and climatic factors (temperature and moisture) affected the community structure and abundance of Archaea and Acidobacteria indicating the high metabolic versatility and adaptability of these prokaryotic groups to environmental changes (Rasche et al., 2011). Finally, the effects of annual and interannual environmental variability of temperature, precipitation and chemical resources on soil fungi associated with an old-growth, temperate hardwood forest were investigated (Burke, 2015). Fungal communities were found to significantly vary by the season, sampling location, and depth with differences being consistent between years. Fungal species within the community were not consistent in their seasonality or preference for certain soil depths, but some of them were found to be consistently correlated with soil chemistry across the sampled years.

The soil properties are modified by a range of processes occurring during tree growth, which in turn affect rhizosphere microbial communities. Plant roots can influence the surrounding soil and inhabiting organisms (Lakshmanan et al., 2014). Roots release low-molecular-mass compounds (e.g., sugars, amino acids and organic acids), polymerized sugar, root border cells, and dead root cap cells. These rhizodeposits are used as carbon sources by soil microorganisms and can also contain secondary metabolites, such as antimicrobial compounds, nematicides, and flavonoids that are involved in establishing symbiosis or in warding off pathogens and pests, thereby acting as a crucial driving force for multitrophic interactions in the rhizosphere (Bais et al., 2006; Oldroyd, 2013). Experimental data from citrus crops parasitized by the insect pest *Diaprepes abbreviatus* in Florida showed that roots release specific volatile organic compounds (VOC) that attract entomopathogenic nematodes (EPN), with beneficial effects observable on the pest regulation. Also, plant-parasitic nematodes (PPN) revealed a positive tropism toward parasitized roots, mediated by one or more of the VOC components (Ali et al., 2010, 2011, 2013). This effect may be also significant for the microbiota associated with these nematode groups because several microbial species with a beneficial impact are passively dispersed by EPN and PPN. Soil pH, another important driver of soil microbial communities, can locally increase or decrease by up to two units in the rhizosphere due to the release and uptake of ions by roots (Hinsinger et al., 2009). Water uptake and root respiration affect soil oxygen pressure, thereby influencing microbial respiration. Soil nutrient availability can be modified in the rhizosphere by plant uptake and by the secretion of chelators, such as phytosiderophores, to sequester metallic micronutrients (Philippot et al., 2013).

The host plant can be considered as the primary biotic factor influencing the composition of soil microbiota associated with tree crops. The plant cover and crop types have an impact on the belowground microbial diversity, as shown by studies on soil metagenomes (Uroz et al., 2016; Colagiero et al., 2017). Structure and composition of fungal and archaeal communities proved to be dependent on the tree species, while bacterial communities differed between bulk soil and the rhizosphere but not between host trees. Similar results were obtained by Urbanová and collegues who demonstrated that fungal communities were strongly related to tree species while bacterial communities rather to root exudates (Urbanová et al., 2015). The composition of the nematode community in the rhizosphere soil is also influenced by the host genotype, as revealed by studies performed in olive (Palomares-Rius et al., 2012). Nematodes are also among the biotic factors influencing the composition of soil microbiota associated to tree crops, as shown by the differences induced on the AMF communities colonizing galls and roots of peach, *Prunus persica* (L.) Batsch, infected by the root-knot nematode *Meloidogyne incognita* (del Mar Alguacil et al., 2011).

Regarding anthropogenic factors, pollution caused by industrial and mining activities can shape microbiota associated with tree crops and timber trees. The effects of long-term metal pollution on soil microbial communities were evaluated along two soil gradients of forests with Scots pine, *P. sylvestris* L., and common beech as the dominant tree species (Azarbad et al., 2015). Metal pollution significantly affected bacterial community structure causing changes in the relative abundance of specific bacterial taxa resilient to metal pollution and increased frequency of certain metal-resistance genes, suggesting a link

between microbial community composition and their functional potential in long-term polluted forest soils. The activity of timber harvesting was also shown to exert a significant and persistent effect on soil bacterial and fungal communities in Northern coniferous forests via organic material removal and soil compaction (Hartmann et al., 2012). Among the components of microbiota, plant symbionts like EMF and saprobic taxa of bacteria and fungi were the most sensitive to harvesting disturbances. The diversity and structure of soil bacterial and fungal communities remained significantly altered by harvesting disturbances, even more than a decade after harvesting. A subsequent study (Hartmann et al., 2014) revealed that physical soil disturbance during logging-associated compaction induced profound and long-lasting changes in the forest soil microbiota and associated soil functions, significantly reducing bacterial and fungal abundance, increasing alpha diversity and persistently altering the microbiota composition with a maximum impact observed 6–12 months after compaction. Fungi were less resistant and resilient than bacteria, with ectomycorrhizal species detrimentally affected by compaction, while saprobic and parasitic fungi were proportionally increased. Bacteria capable of anaerobic respiration, including metal, sulfur, and sulfate reducers from Proteobacteria and Firmicutes, were found to be significantly associated with compacted soils. Agronomical management systems also greatly influence the structure and functioning of soil microbial communities associated with tree crops. For instance, Montes-Borrego et al. (2013) revealed in a comparative analysis of organic and conventional olive farming systems in southern Spain, how management practices affected the chemical and biological soil properties indicating that olive orchards under organic management exhibited higher microbial diversity compared to conventionally managed orchards. The structure and diversity of phytoparasitic nematode communities infesting olive orchards are also, but not exclusively, influenced by soil management practices (Palomares-Rius et al., 2015). Indeed, this study concluded that soil physicochemical factors such as texture, pH, and extractable K, the climatic parameters minimum and maximum temperatures, and olive cultivar as the key agronomic variable were factors driving the population levels and community structure of olive phytoparasitic nematodes. An advanced citrus production system with daily fertigation rates have been applied in Florida to contrast the bacterial disease huanglongbing, by shortening the trees production cycle. This system increased the densities of some microbial antagonists of PPN such as *Catenaria* or other parasitic fungi, associated to a higher root biomass. However, some effects were also found on the densities of EPN, which showed opposite responses for steinernematid or heterorhabditid species (Campos-Herrera et al., 2014).

BELOWGROUND MICROBIOTA AND TREE CROPS: BENEFITS AND HARMS

Beneficial soil/root microbiota can promote plant growth directly (i.e., biofertilization, phytostimulation) and/or indirectly (i.e., suppressing plant diseases and pests). Alleviation of stress due to environmental pollutants or heavy metals [i.e., (phyto)rhizoremediation)], drought or salinated soils, are mediated by the activity of the plant-associated microbiota. Trophic interactions established between the host plants and their associated microbiota at the root level provoke effects influencing aboveground ecosystems. Moreover, long-term associations (i.e., nodule-forming bacteria able to fix N_2, ecto- and endomycorrhizal symbioses, non-symbiotic plant-growth-promoting rhizobacteria [PGPR] and fungi [PGPF], endophytes, etc.) may influence aboveground ecosystems in ways other than direct plant growth promotion. Successful associations should be based on the capacity of the microbes to modulate the plant host immunity. The dialogue established between plants and (components of) their microbiota are likely variations of a common theme where the boundaries among symbiotic, pathogenic or endophytic associations are, indeed, fuzzy (Zamioudis and Pieterse, 2012; Mercado-Blanco and Lugtenberg, 2014). Responses triggered in the plant as a consequence of the interactions taking place at the root level have an effect on aerial parts. Induction of systemic defense responses is a clear example that may affect plant health by triggering an enhanced resistance status against a range of phytopathogens and/or pests (Pieterse et al., 2014). The challenge is to understand these responses and how they disturb aboveground ecosystems, individuals or specific plant organs.

Benefits

Mycorrhiza

Most of the known tree crops, i.e., fruit trees cultivated in orchards (e.g., olive, apple, *Malus domestica* L., pear, *Pyrus* sp., cherry, *Prunus* sp., plum, *P. domestica* L., peach, apricot, *P. armeniaca* L., etc.) or fast growing tree species cultivated in SRF systems for biomass production (e.g., willow, poplar, alder, *Alnus* sp., ash, *Fraxinus* sp., birch, *Betula* sp., eucalyptus, *Eucalyptus* sp., etc.) form stable symbioses with mycorrhizal fungi. Tree crops can form two types of mycorrhizas differing in morphology: ectomycorrhizas (EM) or arbuscular mycorrhizas (AM). Moreover, some tree crops can form dual EM/AM (e.g., willow, poplar), although a trend toward greater fractional colonization with EM and lower colonization with vesicular-arbuscular mycorrhiza (VAM) has been observed (Moyersoen and Fitter, 1999). Mycorrhizal fungi promote plant growth, aid nutrient uptake (reduced fertilizer requirement), increase yield, reproductive success and tolerance to abiotic (e.g., pollution, drought, salinity) and biotic (pathogens, herbivores, low microbial diversity in the soil) stresses, thereby improving field survival and establishment (Allen, 2006; Hrynkiewicz and Baum, 2012; Al-Karaki, 2013; Khabou et al., 2014; Manaut et al., 2015). Therefore, tree crops with well-established mycorrhizal symbiosis are characterized by increased adaptation level to edaphic parameters observed under unfavorable soil conditions. Direct and indirect beneficial effects of mycorrhizal fungi on plant growth and development are summarized in **Figure 3**.

Noteworthy, positive effects of mycorrhizal fungi on fruit tree growth can be detected only a few years after planting.

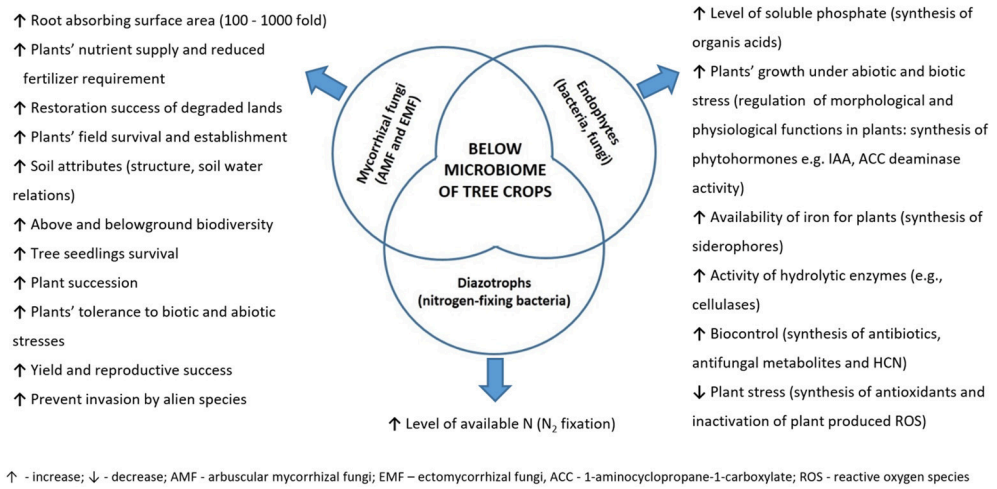

FIGURE 3 | Summary of the benefits that belowground microbiota (or some of their components) may confer to tree crops.

Indeed, during the first year of a tree growing in an orchard, it may happen that mycorrhizal fungi use some nutrients that could nourish the tree's own growth (Borkowska, 2002). In the case of ectomycorrhiza associated to *Salix viminalis*, a stronger growth of the plant can be already observed three months after EMF occurrence (Hrynkiewicz et al., 2012). Beneficial effects of mycorrhizal symbiosis may vary considerably between fungal and plant species, and with environmental conditions (e.g., physical-chemical soil parameters, climate, etc.).

Mycorrhizal associations of fast-growing trees play also a key role in host tolerance to unfavorable soil conditions, increasing phytoremediation efficiency of heavy metals and organic contaminants (Vervaeke et al., 2003; Baum et al., 2006). The most numerous group of EMF symbionts, along with the highest level of EMF colonization, observed in natural stands of tree crops, belong to orders Thelephorales (*Tomentella* sp.), Pezizales (*Tuber* sp., *Geopora* sp.) and Agaricales (e.g., *Hebeloma* sp., *Cortinarius* sp.). The mechanisms of action responsible for tolerance of EMF to adverse environmental conditions are not yet fully understood. Some results suggest that melanin or thelephoric acid present in the fungal mycelium can act as a protective interface between fungal metabolism and (a)biotic environmental stressors. Species of *Geopora* have been found to be the principal EMF symbionts of willows planted for restoration in fly ash, with high potential to survive under harsh environmental conditions (Hrynkiewicz et al., 2009; Gehring et al., 2014). Ectomycorrhizal associations, dominated by *Tomentella* sp., *Hebeloma* sp., *Geopora* sp. and *Helotiales* sp., were detected on the roots of willow and birch growing in saline soils (Hrynkiewicz et al., 2015), suggesting their importance in tolerance of host-plants to salinity. Yet, the mechanism by which mycorrhizal fungi improve salt resistance remains unclear. Positive effects of *Glomus* spp. on olive tree production and growth were confirmed by different studies (Khabou et al., 2014; Mechri et al., 2014). The cultivation range of this tree crop can be limited by water scarcity as well as ubiquitous gypsum in the soil, which is responsible for osmotic stress and the ion-specific toxicity for plants (Khabou et al., 2014). A number of studies have revealed that mycorrhizal symbiosis is important for improving plant growth and nutrient uptake under saline conditions, especially the uptake of immobile soil nutrients as P, Cu, and Zn (Berruti et al., 2015). Inoculation of olive plants with *Glomus* spp. improves growth and adaptation to arid areas, although AMF colonization did not improve tolerance to Verticillium wilt, one of the most important biotic constraints affecting olive cultivation (see below), under such conditions (Kapulnik et al., 2010).

Endophytes and Diazotrophic Bacteria

Beneficial endophytes, i.e., any microbe (mainly bacteria and fungi) isolated from asymptomatic plant tissue (Hardoim et al., 2015; Brader et al., 2017) represent another taxonomically and functionally highly diverse group of microorganisms associated with tree crops. Endophytes can promote plant fitness and growth through phytohormones synthesis, nitrogen fixation, phosphate solubilization, synthesis of siderophores or reduction of ethylene levels. Some endophytes can produce active substances with biotechnological potential such as antitumor and antifungal agents (Bhore et al., 2013; Mercado-Blanco and Lugtenberg, 2014; Hardoim et al., 2015). Endophytes of tree crops can also improve the host resistance to external stresses such as contaminants, temperature extremes, water and nutrient limitations, salt, and pathogens (Mei and Flinn, 2010). Thus, it has been demonstrated that some bacterial endophytes of poplar trees can show high tolerance to trichloroethylene (TCE) and potential for degradation of these toxic compounds, e.g., *Methylobacterium populum* BJ001 (Van Aken et al., 2004), *Pseudomonas putida* W619-TCE (Weyens et al., 2010), or

Enterobacter sp. PDN3 (Kang et al., 2012). Endophytic bacteria of willows from the phylum *Proteobacteria*, particularly the *Gammaproteobacteria*, increase considerably with cumulative contamination of soils with petroleum hydrocarbon (PHC) (Tardif et al., 2016). Finally, *Proteo-* and *Actinobacteria* from the root endosphere and from the rhizosphere of *Acer pseudoplatanus* L. show detoxifying ability in Trinitrotoluene (TNT)-contaminated soils (Thijs et al., 2014).

Diazotrophic bacteria (N_2-fixing bacteria) are ubiquitous in the rhizosphere or inside plant tissues of both herbaceous plants and tree crops, serving as significant sources of biologically available nitrogen for them (Bagwell et al., 2001; Kandel et al., 2015). The presence of diazotrophic bacteria in plant tissues of poplar, *P. trichocarpa* (Torr. & A.Gray ex Hook.) Brayshaw, and willow, *S. sitchensis* Sanson ex Bong., including species of *Burkholderia, Rahnella, Sphingomonas,* and *Acinetobacter*, was reported by Doty et al. (2009). Experiments confirmed that inoculation of poplar with diazotrophic bacteria increases the biomass over uninoculated control plants and the growth promotion is more pronounced with multi-strain consortia than with single-strain inocula (Knoth et al., 2014). The presence of these diazotrophic microorganisms may help to explain the ability of these tree crops to grow under nitrogen limitation.

Certain trees and woody shrubs from the orders Fagales (e.g., elder, *Sambucus* sp., from *Betulaceae*, and beefwood, *Grevillea striata* R.Br., from *Casuarinaceae*), Rosales and Cucurbitales are known as "actinorhizal plants," developing endosymbiotic relationships with filamentous, Gram-positive soil bacteria from the genus *Frankia* (*Frankiaceae*, Actinobacteria). These bacteria can fix nitrogen (N_2) both in their free-living form and as symbionts, that is, as beneficial endophytes in root nodules developed on their host plants (Santi et al., 2013), and many actinorhizal plants form mycorrhizal associations. The host plant–*Frankia*–mycorrhiza symbiotic interaction makes these trees and shrubs capable of adapting to flooded land, arid regions, contaminated soils, extreme pH and high salinity. They can, therefore, be used for revegetation of different landscapes or for preventing desertification (Dawson, 2008; Santi et al., 2013). For example, actinorhizal plants from *Casuarinaceae* (e.g., *Casuarina equisetifolia*) have been successfully used in African coastal and desert dunes for reclamation of salt-affected soils (Diem and Dommergues, 1990).

Nematodes

Soil nematodes have a number of beneficial and harmful associations with tree-crops, including trophic groups which provide fundamental services in the rhizosphere. Bacteriovorous species play a key role in recycling nutrients and in the dispersal of a number of bacterial groups, including rhizobia. Some bacteriovores in Diplogasteridae may also feed on insects, whereas some Rhabditidae evolved a specialized trophism, feeding on endosymbiotic bacteria that they inoculate on insect larvae, subsequently killed by the induced sepsis. EPN and associated insect-killing bacteria are involved in the natural regulation of many insect pests. Their practical and commercial exploitation as biological control agents (BCA) has been successfully achieved in many agroecosystems, including *Citrus* and other tree crops (Lewis et al., 2015; Stock, 2015). Most important associations involve two phylogenetically distant γ-Proteobacteria, *Xenorhabdus,* and *Photorhabdus*, that evolved a close necromenic and mutualistic association with two EPN genera, *Steinernema* and *Heterorhabditis*, respectively.

Some examples of metabolic or endosymbiotic interactions favoring trees are also available for plant-parasitic nematodes. *Pochonia chlamydosporia* (**Figure 4**) is a widespread hyphomycete found in soil as a facultative parasite of eggs of sedentary cyst and root-knot nematodes with a potential as a BCA. Isolates of this fungus showed different levels of adaptation to a wide range of nematode hosts, and in the ability to colonize the rhizosphere or act as root endophytes (Manzanilla-López et al., 2013). In fact, the egg parasitism seems to be correlated with *P. chlamydosporia* host preference, plant compatibility, and tolerance to abiotic factors (Vieira dos Santos et al., 2014). *Pochonia chlamydosporia* has an intimate metabolic link with roots (Rosso et al., 2014) and the potential of a *P. chlamydosporia* isolate combined with benzothiadiazole or cis-jasmonate against *M. incognita* has already been demonstrated (Vieira dos Santos et al., 2013). Studies on eggs degradation and root interactions showed changes of the fungus gene expression levels, in the transition from saprotrophic to the parasitic stage, affecting several metabolic functions. Genes activated after contact with eggs included a bZIP and a phytase-like gene. Sources of P such as phytic acid stimulated the fungal growth. Assays at varying levels of pH or glucose and NH_4^+ also showed early changes in the fungus metabolism (Rosso et al., 2011, 2014).

Data indicate that *P. chlamydosporia* plays a role in plant nutrition. Both nematode parasitism and nutrient mobilization are indicative of multiple potential benefits related to this fungus. Gene expression data on colonized barley, *Hordeum vulgare* L., revealed the production of many enzymes such as proteases, hydrolases and carbohydrate esterases (Larriba et al., 2014), suggesting a multilateral relationship with roots and nematodes. Considering the phylogenetic proximity of *P. chlamydosporia* to *Metarhizium* spp. (Larriba et al., 2014), with the ecology and metabolism of the latter species, some similarities may be inferred. In its endophytic phase, *Metarhizium* spp. provide to the plant nutrients subtracted by insects feeding on roots, when they are acting as entomopathogens, as shown using radio-labeled compounds (Behie et al., 2012). Although a similar behavior has not yet been demonstrated in *P. chlamydosporia*, it seems plausible that endophytism and parasitism may be part of a complex behavior, involving the transport of nutrients back to nematode-damaged roots. Further studies are needed to elucidate these patterns. In spite of the widespread occurrence of *P. chlamydosporia* in the rhizosphere of many perennial crops, no information exists on its role in the soil microbiota, either under controlled or field conditions. These studies would require long-lasting experiments on the changes in soil metagenome or root transcriptome, an effort not yet afforded.

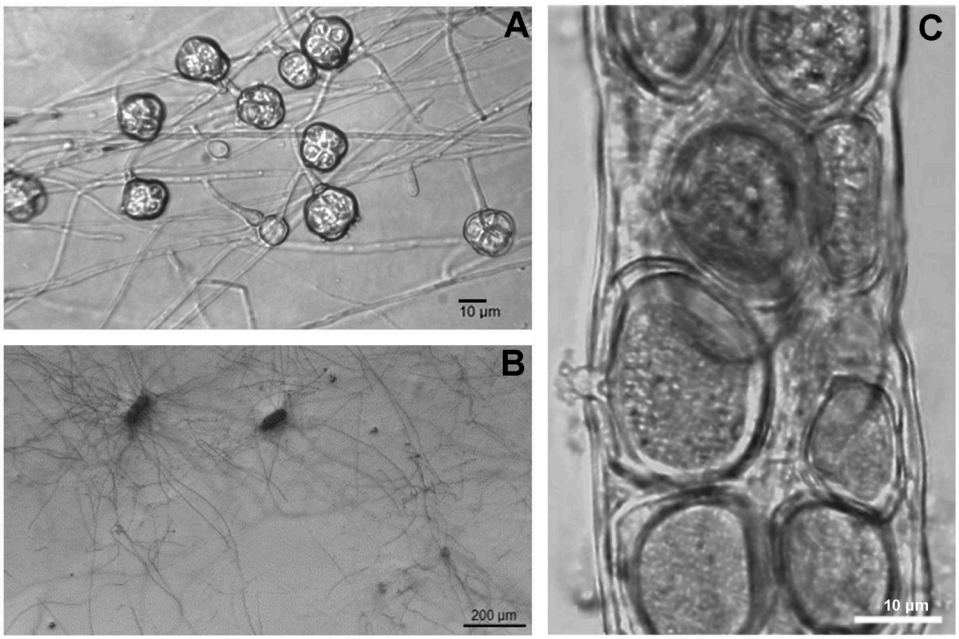

FIGURE 4 | Chlamydospores of the nematode parasitic and root endophytic hyphomycete *Pochonia chlamydosporia* showing their persistent cellular structure **(A)**. Hyphae emerging from killed root-knot nematode eggs, *in vitro* **(B)**. The aquatic fungus *Catenaria anguillulae* **(C)** is one of the most common parasites of nematodes (in the picture inside *Xiphinema* sp.) killing its hosts in a few hours. However, in spite of its ubiquity and polyphagy, and due to the zoospores dependence on water for host attachment, a persistent regulation of phytoparasitic nematodes is seldom observed.

Negative Effects

Although the belowground microbiota is crucial for the health of fruit, nut and SRF crops and timber trees, some members of soil microbial communities present in these agro-ecosystems have negative effects on their hosts (**Table 2**). On the one hand, the soil may contain inoculum sources of aboveground plant pathogens. On the other hand, the soil/rhizosphere microbiota also harbors a range of soil-borne plant pathogenic agents. Besides the prokaryotes *Rhizobium radiobacter* and *R. rhizogenes* (*Rhizobiaceae*, Rhizobiales, Proteobacteria, formerly known as *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively) capable of inducing tumor formation in many economically relevant tree crops (Hwang et al., 2015), the most important negative effectors of tree health in the soil microbiota are fungus-like organisms (i.e., oomycetes) and higher fungi. A brief overview of the most relevant is presented below.

Harmful Oomycetes

Phytophthora spp. are fungus-like microorganisms belonging to the *Pythiaceae* family of Peronosporales (Oomycetes, Heterokontophyta, Chromalveolata) and can reproduce both asexually by chlamydospores, or flagellated zoospores moving in soil water, and sexually in the form of oospores (Erwin et al., 1983). Most of the *Phytophthora* species are considered soilborne pathogens, and several representatives of the genus are known to cause devastating economic losses to various tree crops worldwide (Supplementary Table 1). *Phytophthora* species also cause significant damage in nurseries and can be spread from infested nursery stocks into tree plantations and forests (Jung and Burgess, 2009). *Phytophthora* spp. are known to cause various diseases (e.g., root and collar rot, stem canker, branch and foliar dieback) in natural and planted forests (pine, larch, *Larix* spp. Philip Miller, cypress, family *Cupressaceae*, oak, *Quercus* spp., beech, alder, etc.), fruit and nut crops including avocado, *Persea americana* Mill., apple, pineapple, *Ananas comosus* (L.) Merr., peach, citrus, cocoa, *Theobroma cacao* L., almond, *Prunus dulcis* (Mill.) D.A. Webb, pomegranate, *Punica granatum* L., fig, *Ficus carica* L., pistachio, *Pistacia vera* L., and cinnamon, *Cinnamomum verum* J. Presl (Supplementary Table 1). Species like *Ph. alni*, *Ph. lateralis* or *Ph. quercina* are more specialized, while others (e.g., *Ph. cinnamomi*, *Ph. niederhauserii*, *Ph. palmivora*, or *Ph. plurivora*) display a wide host range.

The genus *Pythium* from the *Pythiaceae* family, commonly occurring in forest nursery soils, also harbors important soilborne pathogens causing damping off of tree seedlings and root rot of mature trees. The life cycle of *Pythium* species is similar to that of *Phytophthora*. A study conducted on seedlings of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, demonstrated that besides *Py. aphanidermatum, Py. irregulare, Py. debaryanum, Py. sylvaticum,* and *Py. ultimum*, the species *Py. mamillatum* can also cause seedling damping-off, while others, e.g., *Py. dissotocum*, *Py.* aff. *macrosporum*, *Py.* aff. *oopapillum*, *Py. rostratifingens*, may be responsible for seedling loss (Weiland et al., 2013). *Pythium ultimum* and *Py. aphanidermatum* were also known to infect seedlings of tropical tree species (Augspurger and Wilkinson, 2007). The species

TABLE 2 | Examples for the most relevant microorganisms affecting tree crops as soil-borne pathogens.

Microbial pathogens	Host trees	Diseases	References
BACTERIA			
Rhizobium radiobacter (syn. *Agrobacterium tumefaciens*)	Various fruit trees	Crown gall disease (tumor formation)	Hwang et al., 2015
STRAMENOPILES			
Phytophthora spp.	Various fruit and nut crops, forest trees	Various diseases including stem, root and/or collar rot, ink disease, dieback	see Supplementary Table 1.
P. ultimum, P. aphanidermatum	Tropical tree species	Damping off of seedlings	Augspurger and Wilkinson, 2007
Pythium ultimum, P. vexans, P. irregulare, P. sylvaticum	Apple (*Malus domestica*)	Apple replant disease	Tewoldemedhin et al., 2011; Shin et al., 2014
P. vexans	Rubber tree (*Hevea brasiliensis*)	Patch canker	Zeng et al., 2005
P. undulatum	*Abies procera,* Douglas fir (*Pseudotsuga menziesii*)	Root rot	Weber et al., 2004
Various *Pythium* spp.	Douglas fir	Damping off of seedlings	Weiland et al., 2013
ASCOMYCETES			
Verticillium dahliae, V. albo-atrum	Cork tree (*Quercus suber*), cherry (*Prunus* sp.), elder (*Sambucus* sp.), elm (*Ulmus* spp.), maple (*Acer* spp.), oak (*Quercus* spp.), olive (*Olea europaea*), peppertree (*Schinus molle*), pistachio (*Pistacia vera*), plum (*P. domestica*), smoke tree (*Cotinus* spp.), walnut (*Juglans* spp.)	Vascular wilt disease	Berlanger and Powelson, 2000
Fusarium oxysporum f. sp. *passiflorae*	Passion fruit (*Passiflora* spp.)	Vascular wilt disease	Ploetz, 2006
Rosellinia necatrix	Apple, apricot (*Prunus armeniaca*), avocado (*Persea* Americana), citrus (*Citrus* spp.), pear (*Pyrus* sp.)	White rot	Pérez-Jiménez, 2006
Ophiostoma ulmi, Ophiostoma novo-ulmi	Elm	Dutch elm disease	D'Arcy, 2000
Cryphonectria parasitica	Chestnut (*Castanea* spp.)	Chestnut blight	Anagnostakis, 2000
BASIDIOMYCETES			
Armillaria mellea, *A. ostoyae*, *A. luteobubalina*	Conifers, fruit, and nut trees	Root disease	Baumgartner et al., 2011
Rhizoctonia spp.	Conifers	Root damage and damping-off of seedlings	Mazzola, 1997
R. solani	Apple	Root rot	Mazzola, 1997
Heterobasidion annosum	Conifers	Root and butt rot disease	Asiegbu et al., 2005

Py. ultimum, Py. vexans, *Py. irregulare* and *Py. sylvaticum* are associated with the worldwide occurring apple replant disease complex (Tewoldemedhin et al., 2011; Shin et al., 2014). *Pythium vexans* is a pathogen of rubber tree (*Hevea brasiliensis* Muell. Arg.) (Zeng et al., 2005), while *Py. undulatum* was identified as the causal agent of a devastating root rot disease of the Christmas tree *Abies procera* Rehd and Douglas fir [*Pseudotsuga menziesii* (Mirbel) Franco] in Northern Germany (Weber et al., 2004).

Deleterious Fungi Affecting Tree Crops

Among the higher fungi, important soilborne tree pathogens can be found both in Ascomycota and Basidiomycota. The most important ascomycetous soilborne pathogens causing wilt diseases of tree crops belong to the genera *Verticillium* and *Fusarium*. The economically most relevant member of the genus *Verticillium* (*Plectosphaerellaceae*, *incertae sedis*, Ascomycota) causing wilt diseases in tree crops is *V. dahliae* (Hiemstra and Harris, 1998; Berlanger and Powelson, 2000). Microsclerotia ensure the persistence of the fungus in soils for many years without susceptible hosts. In their presence, microsclerotia germinate in response to root exudates and the germinating hyphae penetrate the root, colonize the cortex and enter the xylem vessels, where the fungus is spread further by conidia (Pegg and Brady, 2002). Among many others, susceptible tree hosts of *V. dahliae* include elm, *Ulmus* spp., cork tree, *Quercus suber* L., elder, maple, *Acer* spp., oak, pepper tree, *Schinus molle* L., olive, smoke tree, *Cotinus* spp., cherry, plum, pistachio and walnut, *Juglans* spp. (Hiemstra and Harris, 1998).

Fusarium wilt is a vascular disease similar to Verticillium wilt. The disease is caused by members of the *F. oxysporum* species complex (FOSC, *Nectriaceae*, Hypocreales, Ascomycota), producing macro- and microconidia and chlamydospores allowing survival in the soil and plant debris. For instance, *F. oxysporum* f. sp. *passiflorae* causes wilt disease in passion fruit, *Passiflora edulis* Sims (Ploetz, 2006). Further important ascomycetous pathogens of trees include *Rosellinia necatrix* (*Xylariaceae*, Xylariales) causing white rot in several hosts including apples, apricots, avocados, pears and citruses (Pérez-Jiménez, 2006), *Ophiostoma ulmi* and

O. novo-ulmi (*Ophiostomataceae*, Ophiostomatales), the causal agents of the Dutch elm disease (D'Arcy, 2000) and *Cryphonectria parasitica* (*Cryphonectriaceae*, Diaporthales) causing the blight of chestnut, *Castanea* spp. (Anagnostakis, 2000).

Concerning the basidiomycete fungi, the most relevant soil-borne tree pathogens from an economical point of view are the honey mushrooms from the genus *Armillaria* (*Physalacriaceae*, Agaricales, Basidiomycota), causing root diseases in fruit trees (e.g., *Citrus*, *Malus* and *Prunus* species), nut crops (e.g., *Juglans* spp.) and timber trees (e.g., *Abies*, *Picea*, *Pinus,* and *Pseudotsuga* spp.) in both hemispheres of the world under temperate, boreal and tropical climates (Baumgartner et al., 2011). The most virulent species are *A. mellea*, *A. ostoyae,* and *A. luteobubalina*. Mycelia of *Armillaria* species are able to survive for several years in woody residual roots even after the removal of infected trees, which serve as inoculum for the infection of the next crop. During their infection cycle, *Armillaria* species can grow in contact with the host in the form of rhizomorphs - root-like multicellular structures of clonal dispersal enabling the achievement of immense colony sizes (Sipos et al., 2017)- which employ a combination of mechanical force and extracellular enzymes to penetrate root bark (Baumgartner et al., 2011). The mycelium is then colonizing the cambium of the living roots, killing the root tissues and utilizing them for nutrition. The fungus forms white, thick mats of mycelia beneath the bark of infected roots. Further symptoms of the diseased plants include dwarfed foliage, wilting, premature defoliation and stunted shoots in the case of conifer hosts, while dwarfed fruits can be observed in the case of fruit and nut crops. After the death of the host, *Armillaria* switches from parasitic to saprophytic phase and persists in the rhizosphere as a white-rotting fungus (Baumgartner et al., 2011). *Rhizoctonia* species (*Ceratobasidiaceae*, Cantharellales, Basidiomycota) are worldwide-distributed soil fungi with the capability to produce sclerotia overwintering in the soil. Members of this genus bear significant plant pathogenic potential and a wide host range including conifers, where the fungus may cause root damage and damping-off of seedlings (Hietala and Sen, 1996). *Rhizoctonia solani* is known to cause root rot in apple orchards (Mazzola, 1997). Relevant soil-borne basidiomycetous tree pathogens also include *Heterobasidion annosum* (*Bondarzewiaceae*, Russulales) causing root and butt rot disease of conifers (Asiegbu et al., 2005).

HARNESSING BENEFICIAL COMPONENTS OF BELOWGROUND MICROBIOTA TO SUSTAIN TREE CROPS

The soil targets for protection of tree crop plantations by means of biocontrol approaches include bacterial and fungal pathogens, nematodes and insect larvae (Cazorla and Mercado-Blanco, 2016). Root and rhizosphere microbiota of healthy fruit, nut, and timber trees are rich and powerful sources of BCA (Aranda et al., 2011). Below we present an overview of representative examples of BCA used against relevant biotic constraints of tree crops. Regarding biocontrol approaches implemented against soil-borne pathogenic bacteria infecting trees, the success of the non-pathogenic *R. radiobacter* strain K84 (formerly known as *Agrobacterium radiobacter* K84) to control crown gall caused by pathogenic *R. radiobacter* strains (formerly known as *A. tumefaciens*) in different agroecosystems worldwide has been impressive. Interested readers can consult, for instance, the reviews by Moore (1988) and Kerr (2016).

Biocontrol-Based Tools Against Deleterious Oomycetes

Due to the substantial economic damage caused by fungus-like organisms, there is an emerging need for large-scale screening efforts and the development of biocontrol strategies against oomycete tree pathogens. Among prokaryotes, the most promizing taxa with potential as BCA of oomycetes are within the genus *Pseudomonas* (Gammaproteobacteria, Pseudomonadales, *Pseudomonadaceae*) (Mercado-Blanco, 2015) and the order Bacillales (Firmicutes) (Borriss, 2015). Examples of bacteria-based biocontrol of woody crop diseases caused by *Phytophthora* spp. include field studies performed in citrus orchards against *Ph. parasitica* using *P. putida* 06909, a biocontrol strain capable of actively colonizing the hyphae of *Phytophthora* spp. (Steddom et al., 2002). Acebo and colleagues isolated 127 rhizobacteria from the rhizosphere of cocoa, identifying three strains of *P. chlororaphis* with both *in vitro* and direct antagonistic potential against the black pod rot pathogen *Ph. palmivora*. The biosurfactant viscosin was found to be crucial for the motility and biofilm formation of *P. chlororaphis*. Even though the involvement of viscosin in antagonism against *Phytophthora* was not demonstrated, its possible role in the bioprotection of *T. cacao* was suggested (Acebo-Guerrero et al., 2015). The ability of *Bacillus amyloliquefaciens* (Firmicutes, Bacillales, *Bacillaceae*) strain HK34 to induce systemic resistance in ginseng to *Ph. cactorum* suggests that this species may have potential also in the management of other tree diseases caused by the same pathogen (Lee et al., 2015).

Besides bacteria, the ascomycete *Trichoderma* (Hypocreales, *Hypocreaceae*) is also a powerful source of potential BCA against oomycete tree pathogens. Thus, the mycoparasitic activity of *T. virens* was shown to be involved in the control of *Pythium. ultimum* (Djonović et al., 2006), while the antagonistic potential of strains *T. virens* T7, *T. harzianum* T40, *T. asperellum* T54 and *T. spirale* T4 was demonstrated against *Ph. palmivora* (Mpika et al., 2009). *Trichoderma saturnisporum* was recently found to improve plant quality and showed biocontrol activity against *Phytophthora* spp., including *Ph. parasitica* (Diánez Martínez et al., 2016).

Biological Control of Soil-Borne Phytopathogenic Fungi Causing Vascular Diseases

Soil-borne fungi causing vascular diseases are also important threats to plants, including woody hosts. Pathogenic

representatives of *Verticillium* spp. pose a serious risk in many agro-ecosystems worldwide (Pegg and Brady, 2002; Inderbitzin et al., 2011). Verticillium wilts are among the most threatening biotic constraints for tree crops in many areas (Hiemstra and Harris, 1998). Biological control exerted by soil-borne beneficial microorganisms can be useful to confront the disease, particularly when applied as a preventive measure (Mercado-Blanco et al., 2004). One of the best examples in which effective BCA have been identified, characterized and successfully used is the case of Verticillium wilt of olive (VWO) caused by *V. dahliae* Kleb (López-Escudero and Mercado-Blanco, 2011). Strains of *Pseudomonas* spp. have been isolated from the olive rhizosphere (and elsewhere), and proved to suppress VWO in young, nursery-produced plants (Mercado-Blanco et al., 2004; Sanei and Razavi, 2011; Triki et al., 2012; Gómez-Lama Cabanás et al., 2018). One of the best known BCA against VWO is *P. fluorescens* PICF7 (Prieto et al., 2009; Martínez-García et al., 2015). This strain is a natural inhabitant of the olive rhizosphere and endophytically colonizes olive root tissues (Prieto and Mercado-Blanco, 2008; Prieto et al., 2011). While our knowledge about the traits of strain PICF7 involved in both endophytism and biocontrol is scarce (Maldonado-González et al., 2015), results have shown that olive root colonization by this bacterium triggers broad transcriptomic changes, both at local (roots) and systemic (aboveground tissues) level (Schilirò et al., 2012; Gómez-Lama Cabanás et al., 2017). Many of these changes are related to defense responses to different (a)biotic stresses and may shed light on why this endophyte is recognized by the host as a non-hostile colonizer and provide clues on the underlying mechanisms of its biocontrol activity. However, while aboveground defense responses are induced upon strain PICF7 root colonization, they are not effective to control another relevant olive pathogen, *Pseudomonas savastanoi* pv. *savastanoi* causing olive knot disease (Maldonado-González et al., 2013). Furthermore, where and when strain PICF7 is applied in the olive root system seems to be crucial for the effective suppression of VWO (Gómez-Lama Cabanás et al., 2017). Other soil-borne microorganisms have been studied and used as effective antagonists and/or BCA against *V. dahliae*, such as the bacteria *Serratia plymuthica* HRO-C48 (Müller et al., 2007) and *Paenibacillus alvei* K165 (Markakis et al., 2016), or the fungi *T. harzianum* CECT 2413 (Ruano-Rosa et al., 2016) and *T. asperellum* T25 and Bt3 (Carrero-Carrón et al., 2016). The report by Markakis et al. (2016) demonstrated for the first time an effective biocontrol of VWO under field conditions, a scenario not frequently explored in biocontrol research, particularly with trees (Cazorla and Mercado-Blanco, 2016). A recent review highlights all desirable traits that a BCA should have to confront pathogenic *Verticillium* spp., including those ones affecting tree crops. Similar requisites can likely be taken into account, when considering other soil-borne fungal phytopathogens (Deketelaere et al., 2017).

Additional prominent examples of biological control of tree pathogenic ascomycetes are the application of *V. albo-atrum* for the control of Dutch elm disease caused by *O. ulmi* and *O. novo-ulmi* (Scheffer et al., 2008; Postma and Goossen-van de Geijn, 2016), the exploitation of the hypovirulence phenomenon in the case of a dsRNA mycovirus-harboring strain of *C. parasitica* against chestnut blight (Milgroom and Cortesi, 2004) or the possibility of using fungi (*Trichoderma* species) or bacteria (*P. fluorescens*, *Bacillus subtilis*) for the control of avocado white root rot caused by *R. necatrix* (Sztejnberg et al., 1987; Cazorla et al., 2006, 2007; Ruano-Rosa and López Herrera, 2009).

Biological Control of Other Phytopathogenic Fungi

Amongst the soilborne basidiomycete pathogens of fruit and nut crops and timber trees, the main targets of biocontrol efforts are members of the genus *Armillaria*. BCA of *Armillaria* act through the limitation of the pathogen to—or elimination from—the already occupied substrate, and prevention of rhizomorph and mycelium development (Fox, 2003). Potential *Armillaria* antagonists include *Trichoderma* species: scanning electron microscopy studies revealed that some *Trichoderma* strains are able to attack and penetrate the outer tissue of the rhizomorphs, killing *Armillaria* hyphae after coiling and direct penetration (Dumas and Boyonoski, 1992; Pellegrini et al., 2012). Other fungi antagonistic to *Armillaria* include *Rhizoctonia lamellifera* that prevents the pathogen from colonizing tea roots, *Scytalidium lignicola* and its toxin scytalidin inhibiting *Armillaria* growth *in vitro*, *Phlebiopsis gigantea* and *Pleurotus ostreatus* capable of excluding *Armillaria* from its substrates, *Coriolus versicolor*, *Stereum hirsutum,* and *Xylaria hypoxylon* reducing the stump colonization by *Armillaria*, and cord-forming saprotrophs acting as competitive antagonists (Fox, 2003). The method based on isotope ratio mass spectrometry developed to study trophic interactions between *A. mellea* and fungal/bacterial antagonists is a promizing tool for the screening of further potential BCA (Pellegrini et al., 2012). Further examples for the biological control of tree pathogenic basidiomycetes are the application of forest soil-derived *Streptomyces* spp. or *P. gigantea* (Basidiomycota, Polyporales, *Phanerochaetaceae*) to control *H. annosum* causing root and bud rot of conifers (Lehr et al., 2008; Sun et al., 2009).

Biological Control Strategies Against Nematode and Insect Pests

Some specific and effective nematode antagonists such as *Pasteuria* spp. have been reported on tree crops, and their regulatory role described as well (Ciancio, 1995; Ciancio et al., 2016). As concerns the role of bacteria in nematode and insect management (see below), it is worth mentioning that our knowledge about several lineages is still very limited (Roesch et al., 2007).

In most cases, nematodes play different roles in soil food webs, acting as preys, predators, saprotrophs, or feeding on bacteria, fungi, roots or other invertebrates (**Figure 2**). Their association with tree roots and endoparasites, such as *Pasteuria* spp., can be monitored through the collection of time series data on host density and prevalence. *Pasteuria* spp. have a very narrow host specificity, due to an obligate parasitic behavior. Their persistence in soil is due to the presence of durable endospores, which are also the infective propagules. Through

this strategy these bacteria reduce their competition with other soil bacteria, confining their vegetative growth in the small microhabitat provided by the nematode body. This food web can persist for 20 years, as experimentally shown on a citrus grove in Southern Italy (Ciancio et al., 2016). In a different study on *Xiphinema diversicaudatum*-peach and *Pasteuria* sp. carried out in Piedmont, the food web persisted for at least 15 years. The nematode is a virus vector, and its population was also targeted by a predatory nematode (*Discolaimus* sp.), which in turn hosted a distinct *Pasteuria* sp. After trees have been removed from the parcel of study, the nematodes and *Pasteuria* associations were found 20 years later in other adjacent fields, suggesting a local endemism due to soil movement by farmers or water flows, and to the presence of natural reservoirs.

Until the late 1980's, many nematode pests were mostly managed by pesticides or soil fumigants. However, the use of nematicides raised several concerns for their potential harm to farmers, consumers, and damage to the environment (wildlife, water or soil pollution). Attention has thus been given to the effects of biological components of the rhizosphere on nematodes. Bacterial and fungal components of tree rhizosphere microbiota can also be exploited as BCA of phytoparasitic and soil-dwelling nematodes and insect larvae damaging forests and tree plantations. Predation and parasitism arose several times during the evolution of early eukaryotes and may be found among aquatic fungi, ascomycetes, and basidiomycetes. Aquatic fungi such as *Catenaria anguillulae* or *Myzocitium* spp. penetrate the nematode cuticle through motile zoospores that adhere to the host. After an encystation stage, colonization of the host body occurs through germinating thalli. While these species have specific parasitic habits and can regulate nematodes in a humid and wet soil environment, their regulatory potential appears, however, limited depending on high soil water content (Singh et al., 2007).

Many hyphomycetes like *Arthrobotrys* or *Drechslerella* spp. (Ascomycota, *Orbiliaceae*) produce hyphal traps or nets that actively capture and/or attract passing nematodes. This character arose through adaptive evolution in two distinct lineages, one trapping through constricting rings and the other by adhesive nets (Yang et al., 2007). Other parasitic strategies developed by hyphomycetes include the direct, passive adhesion of infective conidia to the nematode cuticle, with germinating hyphae penetrating the host to develop a lethal infection. These strategies are found in species such as *Hirsutella rhossiliensis* (anamorph of *Cordiceps* sp.), *Meria coniospora* or *Nematoctonus* spp., the latter a teleomorph of *Hohenbuehelia* (Basidiomycota, Agaricales, *Pleurotaceae*). *Nematoctonus* also shows the production of toxins by the germinating conidia, which reduce the host movement, thus lowering the probabilities of an early loss of the infective propagule (Giuma and Cooke, 1971). *Paecilomyces* (*Purpureocillium*) *lilacinus* may degrade nematode eggs and regulate their density, due to the activity of several chitinolytic and proteolytic enzymes. The latter provides the fungus a strong keratinolytic activity, a trait supporting its pathogenicity to superior animals, including man.

Pochonia chlamydosporia is also a root endophyte that may elicit several defensive pathways after colonization, without induction of any visible root damage (Maciá-Vicente et al., 2009; Ciancio et al., 2013; Rosso et al., 2014; Larriba et al., 2015). This behavior is indicative of a long-term evolutionary adaptation to the rhizosphere environment, exploiting strategies involving multitrophic relationships with the plants and other rhizosphere organisms.

Finally, pine wilt disease is caused by the pinewood nematode *Bursaphelenchus xylophilus*, leading to the death of susceptible pine trees. In order to control this disease, a few studies have been performed using chemical or biological compounds (Proença et al., 2017b). Several strains were reported to produce extracellular compounds with nematicidal activity, among which *Serratia marcescens* A88copa13 that produces an extracellular serine protease as the major key factor toward the nematode (Paiva et al., 2013).

Although most of the insect damage to fruit and nut crops and forest trees can be attributed to their herbivoural defoliating activity, a few of them are also important as soil-borne pests because their larvae feeding on the roots. An example of EPN impact and the regulatory role played in soil food webs is the biocontrol and management of *Diaprepes* sp. and other root-weevils infesting citrus and other perennial crops in Florida (Campos-Herrera et al., 2013, 2015). Other relevant examples are the larvae of May bugs (also known as white grubs), especially those of the forest cockchafer (*Melolontha hippocastani*), a species widely distributed in Eurasia. Besides EPN like Steinernematidae and *Heterorhabditis* spp. (Woreta, 2015), larvae of the forest cockchafer are subjected to infections by entomopathogenic fungi (e.g., *Beauveria brongniartii*) and bacteria (like *Bacillus popilliae* var. *melolonthae* or *B. thuringiensis*).

In the case of *B. brongniartii*, cereal grains infected with mycelia is the most frequent formulation used for the control of *M. hippocastani*. However, as summarized by Woreta (2015), the field performance of this biocontrol strategy revealed ambiguous results during several attempts since the 1880s in France, Poland, Italy, Switzerland, and Germany. This situation can be explained by difficulties of introducing and blending infected grains with the soil, especially around young trees where the abundance of cockchafer grubs is expected. Although it was shown that, under field conditions, grub population can be decreased to a harmless level by the application of an adequate *B. brongniartii* formulation thoroughly mixed with soil and applied at sufficient air temperature and humidity, *B. brongniartii* has not been authorized in the EU for use in commercial plant protection products (Woreta, 2015).

Among bacteria, *B. popilliae* var. *melolonthae*, the causal agent of the milky disease, has also been studied as a potential BCA of cockchafer grubs (Franken et al., 1996). The disease incidence increased when the grubs were infected simultaneously with *B. popilliae* and *B. brongniartii*, which is possibly due to synergistic effects between the two pathogens, suggesting the possibility of integrated biological control. Highly pathogenic *B. thuringiensis* subsp. *tenebrionis* and *B. weihenstephanensis* strains, isolated from larvae of the common cockchafer *M. melolontha* (Kati et al., 2007; Sezen et al., 2007), or *Serratia* species, causing feeding discontinuation of *M. hippocastani* larvae (Jackson and Zimmermann, 1996), may

be valuable as BCA of cockchafer white grubs damaging tree roots.

Inconsistencies and Risk Assessment in Biological Control of Tree Crops

Inconsistent field performance is one of the major challenges in the application of beneficial microorganisms as BCA and/or plant growth promoters (Weller et al., 1995). It is even more complex in the case of trees because of their own idiosincracy (Cazorla and Mercado-Blanco, 2016). Inconsistency can be the result of various abiotic and biotic factors (Meyer and Roberts, 2002). Physicochemical properties of the rhizosphere (temperature, pH, water availability, chemical composition) are parameters varying both in space and time, which have substantial influence on the performance of plant growth promoting and biocontrol microorganisms: an individual agent can have different activities in different soil environments. One of the possible approaches to counteract inconsistencies under different environmental conditions is the development of strategies based on more than just a single beneficial organism. The combined application of wide-spectrum BCA with efficient plant growth promoting microorganisms has the potential to reach the increased consistency of performance over a wider range of soil conditions. A recent example was presented by Imperiali et al. (2017), who applied *Pseudomonas* bacteria, AM fungi and EPN to improve wheat performance. Moreover, the application of entire, well-characterized, complex microbiota may further improve the efficiency of soil-borne pathogen management and other biotic constraints (Gopal et al., 2013; Berg et al., 2014; Kowalski et al., 2015). Other examples are the effect of chemically and microbiologically characterized vegetable compost in oak seedlings on decline caused by *Ph. cinnamomi* (Moreira et al., 2010), and the efficiency of organic amendments (yard waste and almond shells) to avocado crops in suppression of the white root rot fungus, *R. necatrix* (Bonilla et al., 2015). Based on their results these authors suggested that organic amendments can be useful cultural practices to reduce the impact of the pathogens.

Although sophisticated and ecologically "intelligent", many fungi acting as predators or parasites show a reduced biocontrol efficacy for pests such as root-knot (*Meloidogyne* spp.), cyst (*Heterodera* spp., *Globodera* spp.) or other nematode species, once applied to soil as bioformulations (Jaffee, 1992; Kluepfel et al., 2002; Castillo et al., 2010). The reasons for such low performance may depend on several factors, including the inhibition by the resident soil microflora, the evolution of low virulence traits allowing the maintenance of the host population, or the capacity of most fungi to grow on a wide range of substrates, using nematodes as additional food sources. Other factors are related to density-dependent relationships established with their hosts, as shown for *H. rhossiliensis* on *M. xenoplax* on peach or for other fungi parasitic on nematode eggs on kiwi (Jaffee et al., 1989; Roccuzzo et al., 1993). A further factor concerns the evolution of more complex adaptative behaviors, as in the case of the egg parasite *P. chlamydosporia* (**Figure 4**). This parasite produces specific enzymes allowing the lysis of the egg cuticle and vitelline layers, a step followed by the egg colonization through an appressorium and growing hyphae. This fungus has been reported as a highly-effective BCA, displaying specificity for the nematode species from which the isolates were obtained (Manzanilla-López et al., 2013).

Lastly, when planning the application of a biocontrol strategy, a thoroughly performed risk assessment is necessary. The EU policy support action REBECA (Regulation of Biological Control Agents) aims to review the possible risks of biocontrol agents (http://www.rebeca-net.de/?p=999). BCA may have negative effects on beneficial, non-target organisms (e.g. mycorrhizal fungi) or other crops. For example, although many *Trichoderma* species are considered as potential BCA for the protection of both herbaceous and woody plants, certain members of the genus, e.g., *T. aggressivum*,*T. pleurotum* and *T. pleuroti*, represent a risk to commercial mushroom production where they can cause green mold disease (Hatvani et al., 2008; Kredics et al., 2010) or to human health, with *T. longibrachiatum* as a potential opportunistic human pathogen (Hatvani et al., 2013). The application of these *Trichoderma* species for biocontrol purposes should, therefore, be carefully monitored.

Coping With Abiotic Stresses and Phytoremediation

Tree crops used in SRF aiming to biomass production (e.g., *Salix* spp. and *Populus* spp. and their hybrids) have been successfully used as sustainable solutions to recover contaminated soil (Licht and Isebrands, 2005; Zalesny et al., 2016). Phyto-assisted bioremediation, or phytoremediation, is an *in situ* treatment of contaminated soils, which relies on complex interactions established between roots and soil microorganisms in the rizhosphere (Wenzel, 2009). In this microhabitat, bacterial communities can respond promptly to pollutant occurrence, promoting organic contaminant degradation and/or inorganic phyto-containment (Simpson et al., 2009). Bioaugmentation of soils with selected microorganisms can significantly increase efficiency of phytoremediation (Złoch et al., 2017). The synergistic action between the tree root system and the natural belowground microbiota makes it possible to remove, convert, or contain toxic substances in soils.

Beyond the contaminant removal, an overall soil quality improvement is observable in terms of soil carbon sequestration, increased nutrient content, recycling and biomass production for energy purposes. Poplar is one of the most used tree crops for stimulating (e.g., through root exudates production, oxygen transport) bacterial degradation of persistent organic contaminants (e.g., polychlorinated biphenyls - PCB) and phyto-containment of inorganic ones (heavy metals) in the rhizosphere (Gamalero et al., 2012; Ancona et al., 2017). However, other tree species have been successfully applied for this purpose such as willow (*Salix* spp.), eucalyptus, black locust (*Robinia pseudoacacia* Simpson et al., 2009) and *Corylus* spp. for metal and metalloid phyto-containment (Radojevic et al., 2017). Although bacteria and archaea are the only groups within the plant microbiota able to transform and mineralize organic

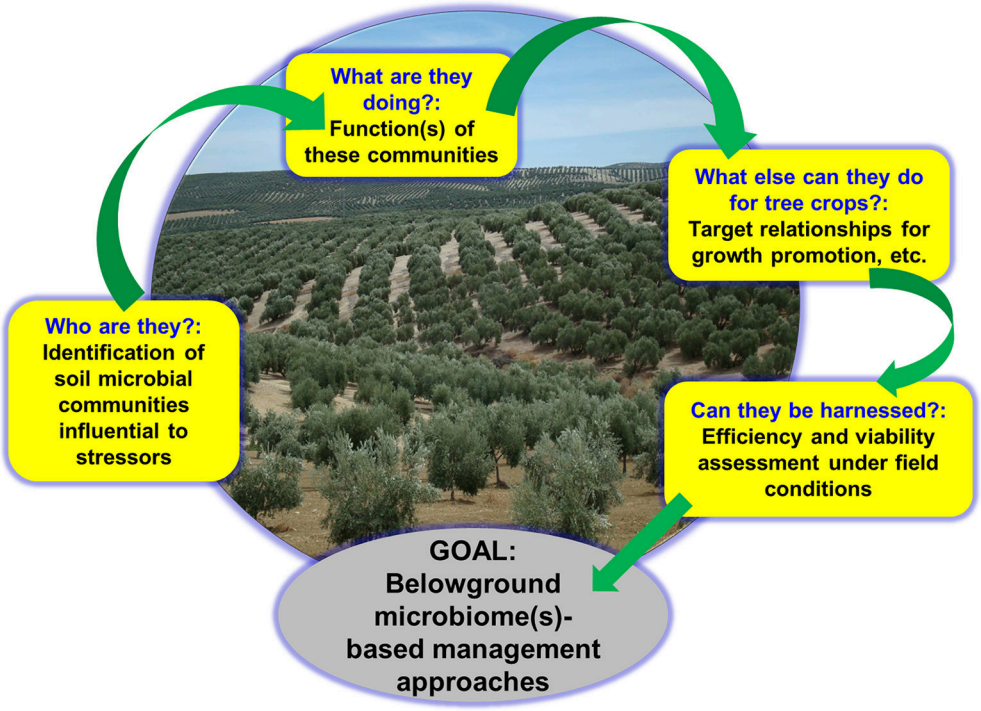

FIGURE 5 | A strategy to manage biotic constraints affecting tree crops (i.e., pathogens, pests, invasive species) based on the identification, characterisation and harnessing of soil/root microbiota [based on a conceptual framework by Kowalski et al. (2015)].

contaminants, their huge metabolic potential remains to be explored.

CONCLUDING REMARKS: TOWARD MICROBIOTA-ASSISTED MANAGEMENT STRATEGIES

Belowground microbial communities associated with tree crops are key factors for their growth, development, and health, particularly under non-favorable soil conditions. They decisively contribute to enhanced productivity, improve accessibility to low-abundant nutrients, cope with a range of (a)biotic stressors that affect their associated hosts, and also play an important role in phyto-assisted biodegradation of toxic compounds present in soils. Until now, how belowground microbiota contribute to the fitness of tree crop agro-ecosystems, remains largely unknown and only now it is starting to be unraveled in detail. The four fundamental questions to better understand these associations are: *who are there? what are they doing? who is active out there?* and *how do activities of these microorganisms relate to ecosystem functions?* (Amann, 2000; Leveau, 2007). The answers to these questions, based on an in-depth knowledge of the structure and functioning of belowground communities, will constitute the pillars to develop holistic management strategies aiming to cope with the range of (a)biotic constraints affecting tree crops (**Figure 5**). The relationship between soil-borne microbes and tree crops is delicate and complex and can have either positive or negative effects on the host. It can be assumed that benefits derived from the interaction of tree crops with beneficial belowground (micro)organisms are expected to yield similar outcomes in aboveground ecosystems than those observed, and more frequently investigated, in herbaceous, short-living species. Moreover, the associations established with trees are expected to be more stable, enduring along time, although variations in composition, structure, and functioning do occur, likely in a cyclic manner. These are subjected to a broad range of genetic, (a)biotic and environmental cues and factors. In this sense, integrated "omic" analyses, combining metagenomics, metatranscriptomics, metaproteomics, and metabolomics, are now providing a more accurate view of the activities and the physiological potential of belowground plant-associated microbiota (Zhang et al., 2010; Knief, 2014).

Studies on tree crop production and diseases have thus far historically relied on single microbe-based formulations or focused on single species (the pathogen), while little attention has been paid to the use of consortia of beneficial microorganisms or to investigate many other microorganisms most likely present in the infection sites. One way to assist tree crop production might be to integrate beneficial plant microbiota or use *ad hoc* tailored microbiota to target specific deleterious agents (Gopal et al., 2013; Kowalski et al., 2015; Pinto and Gomes, 2016; Berg et al., 2017; **Figure 5**). Due to the complexity of tree crop ecosystems—dominated by vegetal species displaying peculiarities such as large biomass, complicated anatomy, large root systems, longevity, and the large spatial domains and timescales over which tree crops are grown –management options such as soil amendments, intercropping and soil processing can be applied by farmers.

Once again, the currently-available multi-omic tools, combined with other methodological approaches, will provide a much better knowledge on the complex network of trophic interactions taking place at the soil/root level (Massart et al., 2015). A more-in-depth analysis of these interactions could be of crucial importance in designing new and effective microbial consortia for optimizing plant production and developing new strategies for disease control. In conclusion, a more holistic approach to tree crop agriculture is needed. Understanding the microbial diversity, distribution, activity, and function, and linking the microbial community structure with both environmental factors and ecosystem functioning, are major challenges for the soil/plant microbiology science in this century.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, wrote the review and gave approval to the final version. JM-B designed the study. AB critically reviewed the manuscript and supervised the manuscript drafting.

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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.2018.01006/full#supplementary-material

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Effects of Short- and Long-Term Variation in Resource Conditions on Soil Fungal Communities and Plant Responses to Soil Biota

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Soil biota can strongly influence plant performance with effects ranging from negative to positive. However, shifts in resource availability can influence plant responses, with soil pathogens having stronger negative effects in high-resource environments and soil mutualists, such as arbuscular mycorrhizal fungi (AMF), having stronger positive effects in low-resource environments. Yet the relative importance of long-term vs. short-term variation in resources on soil biota and plant responses is not well-known. To assess this, we grew the perennial herb *Asclepias speciosa* in a greenhouse experiment that crossed a watering treatment (wet vs. dry treatment) with a manipulation of soil biota (live vs. sterilized soil) collected from two geographic regions (Washington and Minnesota) that vary greatly in annual precipitation. Because soil biota can influence many plant functional traits, we measured biomass as well as resource acquisition (e.g., root:shoot, specific leaf area) and defense (e.g., trichome and latex production) traits. Due to their important role as mutualists and pathogens, we also characterized soil fungal communities in the field and greenhouse and used curated databases to assess fungal composition and potential function. We found that the experimental watering treatment had a greater effect than soil biota origin on plant responses; most plant traits were negatively affected by live soils under wet conditions, whereas responses were neutral or positive in live dry soil. These consistent differences in plant responses occurred despite clear differences in soil fungal community composition between inoculate origin and watering treatments, which indicates high functional redundancy among soil fungi. All plants grown in live soil were highly colonized by AMF and root colonization was higher in wet than dry soil; root colonization by other fungi was low in all treatments. The most parsimonious explanation for negative plant responses in wet soil is that AMF became parasitic under conditions that alleviated resource limitation. Thus, plant responses appeared driven by shifts *within* rather than *between* fungal guilds, which highlights the importance of coupling growth responses with characterizations of soil biota to fully understand underlying mechanisms. Collectively these results highlight how short-term changes in environmental conditions can mediate complex interactions between plants and soil biota.

Keywords: arbuscular mycorrhizal fungi, context-dependent, drought stress, intraspecific variation, plant-soil feedback, plant defense, plant traits, soil fungi

INTRODUCTION

Soil biota can strongly influence plant performance (Richardson et al., 2009; Berendsen et al., 2012; van der Putten et al., 2013). For example, ubiquitous arbuscular mycorrhizal fungi (AMF) colonize roots of approximately 75% of vascular plant species (Brundrett, 2009) and often benefit plants by facilitating their nutrient acquisition, protecting them from pathogen attack, or enhancing their drought tolerance (Smith and Read, 2008). However, colonization by AMF is not always a net positive for plant hosts, as these fungi can also act in a more parasitic fashion under certain circumstances (Johnson et al., 1997; Klironomos, 2003; Grman, 2012). Furthermore, some non-mycorrhizal fungi are well-known pathogens that can have strong negative impacts on their plant hosts (Raaijmakers et al., 2009). While these effects are well documented, it is also increasingly clear that the direction and magnitude of plant-soil biota interactions are extremely context dependent. A major challenge, therefore, is to understand what variables influence components of the soil biota community (e.g., AMF, fungal pathogens) and whether these changes affect plant responses to soil biota.

Resource availability in soils (e.g., nutrients or water availability) is thought to influence both attributes of the soil microbial community (Johnson, 1993; Leff et al., 2015) and how this community influences plants (Cook and Papendick, 1972; Johnson et al., 1997; Revillini et al., 2016). It is typically expected that mutualistic elements of soil biota are more beneficial and possibly more abundant in stressful, low-resource environments (Treseder, 2004; Johnson et al., 2010; Grman, 2012), because under these circumstances plants benefit by allocating more resources (carbon) to these symbionts which in-turn helps hosts acquire limiting resources or better tolerate various stressors. Soil pathogens, on the other hand, are thought to be more harmful in benign, high-resource environments, because they are more abundant under these conditions (Tompkins et al., 1992; Reynolds et al., 2003; Bell et al., 2006; Hersh et al., 2012; Veresoglou et al., 2012), and because plant hosts tend to be less defended against various antagonists in high-resource environments (Coley et al., 1985). Empirical support for these ideas comes from studies that have sampled soil biota from long-term fertilization plots and found them to be less beneficial to plants than soil biota collected from unfertilized soils (Johnson et al., 2010; Revillini et al., 2016).

Despite broad patterns in how nutrients may influence soil biota and plant responses, less is understood about how short-term changes in water availability, as occurs due to bouts of precipitation or drought, favor particular groups of soil biota and drive rapid shifts in their function. For example, AMF isolates from dry environments are better able to improve plant water relations than isolates from more mesic environments (Stahl and Smith, 1984), and a microbial community with previous exposure to drought is more beneficial to drought-stressed plants than a microbial community with no history of drought (Lau and Lennon, 2012). However, most studies that document effects of soil microbial communities and plant responses do so under either short-term or long-term conditions, but not both (e.g., Johnson et al., 2010; Lau and Lennon, 2012, but see Evans and Wallenstein, 2012; Zeglin et al., 2013; Kaisermann et al., 2017). Thus, an additional question concerns the relative importance of long-term differences in resource availability among locations, vs. short-term changes in resource conditions within sites on soil biota and their function.

In this paper, we used two complementary approaches to address these questions. First, we characterized fungal communities in soil in the top 15 cm from around the perennial herb *Asclepias speciosa* from two geographic regions that vary greatly in summer precipitation (Washington and Minnesota, **Figure 1**). We then grew *A. speciosa* in either live or sterilized soil from each of the two regions under well watered or drought conditions and measured plant functional traits and plant responses to soil biota depending on soil origin and water availability. We also characterized changes in the soil fungal community based on geographic origin of soil biota and watering treatment. We focused on soil fungi because they are one of the most important groups of soil-borne pathogens (Raaijmakers et al., 2009) and mutualists (Smith and Read, 2008). We predicted that fungal communities would differ between the two regions and that the relative abundance of pathogens would be greater in sites with higher precipitation. We also predicted that plants grown in wet soils in the greenhouse would experience more negative responses to soil biota than plants grown in dry soils, and that those negative responses would be greater in soil originating from wetter areas.

MATERIALS AND METHODS

Plant Material

Asclepias speciosa is a perennial herbaceous plant distributed throughout much of western North America. This plant is highly responsive to AMF (Busby et al., 2011); traits related to growth or resource acquisition (biomass, specific leaf area, root:shoot) and defense (latex and trichomes; Agrawal and Fishbein, 2006) all respond to AMF (Waller et al., 2018). Because *A. speciosa* traits vary among populations distributed across environments gradients (Waller et al., 2018), we collected seeds from five populations spanning the entire resource gradient over which we sampled soils to capture the range of traits values representative of this species (**Table S1**). Within each population, we haphazardly collected one seed pod (i.e., follicle) from 4 to 5 different ramets. All seeds were collected in September 2015. *Asclepias* are mostly self-incompatible and pollinated mainly by insects (mainly Hymenoptera). Pollen are transferred in packets (i.e., pollinium) such that all propagules within a fruit are full siblings.

Soil Collection for Fungal Community Characterization, Soil Nutrients, and Greenhouse Experiment

To assess whether soil fungal communities differed between the two regions, we identified three sites at the western end (two sites in Washington and one site in Montana) and three sites at the eastern end of *A. speciosa's* distributional range (two sites in Minnesota, and one in North Dakota, **Figure 1**). Within each

site, we collected soil (0–15 cm depth) from around 10 *A. speciosa* plants that were at least 5 m apart. Sites differ substantially in precipitation regimes (30-year averages), with sites in MN receiving about three times the amount of summer precipitation than sites in WA (**Figure 1** and **Table S1**). Approximately 10 mL of soil from each soil sample was placed in a small envelope and immediately dried using desiccant. This soil sample was ultimately used for soil fungal DNA extraction. The remaining soil samples were pooled within sites, sieved through a 2 mm sieve and air dried. Some of this soil was used for analyses of macro and micronutrients (Ward Laboratory Inc., Kearney, NE, United States). The rest was kept cool and ultimately used as inoculum in the greenhouse experiment (which was started within 2 weeks of soil collections). Soil inocula used in the greenhouse experiment were pooled across the three sites within a region, resulting in two sources of inocula. These pooled samples were either sterilized via autoclaving (3 sessions × 90 min per session) or not. We refer to these two regions as WA and MN for brevity hereafter, since the majority (2 of 3) of regional samples originated from these states.

Sampling design and analyses associated with assessing soil biota effects in plant-soil feedback experiments has received considerable attention recently (e.g., Reinhart and Rinella, 2016; Cahill et al., 2017; Gundale et al., 2017). Although pooling of samples reduces degrees of freedom and limits generalizations, we pooled our soil inocula within regions because we were primarily interested in testing the effects of short-term watering treatments on changes in soil biota community composition and responses of plant traits to live soil biota. As such, we considered the individual pot receiving the various soil inocula as the relevant replicate, not the individual soil samples collected within sites in the field. We recognize that this pooling approach limits our ability to robustly determine how soil origin influences plant responses to soil biota. However, we note that for comparisons of fungal community composition across sites, we relied on molecular analyses conducted on individual soil samples collected from the soils (0–15 cm deep) around 10 individual plants within each site (see below).

Greenhouse Experiment

On 13–20 July, 2016, we germinated *Asclepias* seeds in water and planted them in 550 ml Deepots (Stuewe and Sons, Inc., Tangent, OR, United States) in the University of Montana's greenhouse. We used between 1 and 28 individual plants from 2 to 6 full-sibling families from each of five populations, resulting in a total of 208 plants that survived until the end of the experiment. Pots contained 100 mL of sand topped with 400 mL of a 1:1:1 mixture of sand, turface and sterilized field soil mix

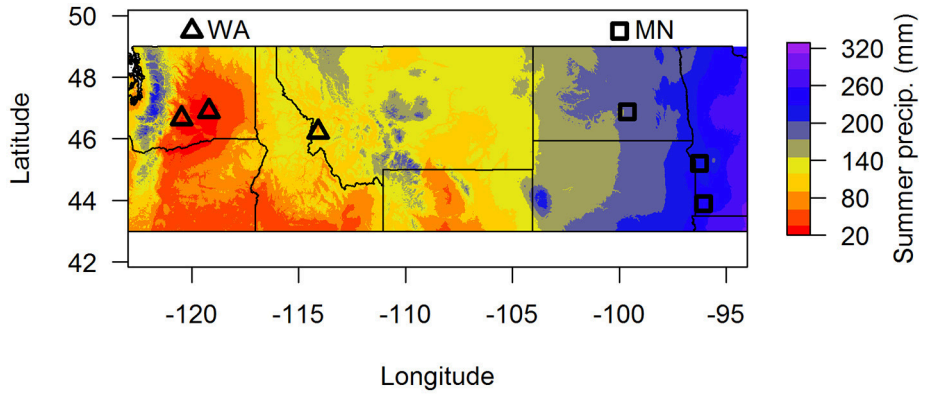

FIGURE 1 | Map of soil collection locations. Summer precipitation (mm) is 30-year averages from the BioClim database. Samples were pooled by region (dry = WA, wet = MN) for the greenhouse experiment.

TABLE 1 | Soil nutrient analysis from soil inoculate and background soil used in the greenhouse experiment.

Soil type	1:1 Soil pH	Organic matter LOI %	CEC/Sum of cations me/100 g	Nitrate-N ppm N	Potassium ppm K	Mehlich P-III ppm P
WA (Dry origin), Sterile pooled inoculate (10% Volume)	7.8	3.3	21.7	7.0	638	46
WA (Dry origin), Live pooled inoculate (10% Volume)	7.9	3.8	22.0	9.5	592	31
MN (Wet origin), Sterile pooled inoculate (10% Volume)	8.0	7.6	31.3	1.0	233	19
MN (Wet origin), Live pooled inoculate (10% Volume)	8.0	6.8	32.5	2.8	214	3
BACKGROUND Mix (90% Volume)	7.4	1.0	21.0	8.3	383	38

(see **Table 1** for soil characteristics). To each pot, we added 50 mL of either WA or MN live or sterile inocula, placed as a layer approximately 5 cm from the soil surface for a total of 25 replicates of each watering treatment (wet or dry) × soil biota (live or sterile) × inoculum origin (MN or WA) combination.

During the first 2 weeks of the experiment, plants received water every 1–2 days. Subsequently, we exposed plants to two watering treatments: wet (watered every 2–3 days) and dry (watered every 7 days) to field capacity. To minimize differences in nutrient availability due to soil sterilization (**Table 1**), and to ensure that plants were primarily limited by water, not nutrients, all plants received 20 ml of a 100 ppm 20N-2P-20K fertilizer on 28 July, 17 September and 3 October, 2016. All plants were destructively harvested on 18 Oct 2016. At this time we measured plant traits (described below) and also collected 10 mL of rhizoshere soil, which we sampled below the inoculum layer to ensure that we only characterized soil fungi that had proliferated during the experiment. We also collected a subset of fine roots for assessments of root colonization by AMF as well as other fungi (described below).

Plant Trait Measurements

We measured total biomass production plus five plant functional traits. Three traits were related to resource acquisition stem height, root:shoot ratio, and specific leaf area (i.e., leaf area per unit leaf dry mass; SLA). The other two traits were latex production and trichome density. Latex is a sticky substance exuded from specialized canals that run throughout the aboveground plant tissues that primarily functions as defense against herbivores (Agrawal and Konno, 2009). Trichomes can function as a defense trait, but also as a drought tolerant trait (Agrawal and Fishbein, 2006; Agrawal et al., 2009).

To measure leaf traits we harvested one of the top fully expanded leaves from each plant. Harvested leaves were refrigerated for <48 h and then scanned. Trichome density was counted under a dissecting scope in a 33 mm^2 area on the lower surface of the leaf and then the leaves were dried at 60°C for 48 h. Specific leaf area (SLA) was calculated as the area (cm^2) per unit mass (g). Immediately after individual leaves were harvested from each plant we captured exuded latex from the stem on a pre-weighed 1 cm diameter filter paper, which was then placed into a pre-weighed centrifuge tube. Centrifuge tubes were kept frozen and then weighed to the nearest 0.1 mg. Latex production was quantified as fresh weight (Agrawal and Fishbein, 2006; Waller et al., 2018). We then measured stem height on each plant from the soil to the apical meristem and harvested all above- and belowground biomass. Biomass was separated into above and belowground parts to allow calculation of root:shoot ratio, dried for at 60°C for 48 h, and then weighed.

Fungal Colonization of Roots

Fine roots (<1 mm diameter) were cleaned and stained in trypan blue (Phillips and Hayman, 1970; Brundrett et al., 1996) and fungal colonization was determined using the gridline intersect method based on approximately 50 intercepts per sample (McGonigle et al., 1990). Arbuscular mycorrhizal fungi were identified using morphological features associated with AMF, such as arbuscules, coils, vesicles and dichotmous branching patterns of mostly non-septate hyphae (Smith and Read, 2008). All other fungi (those staining blue as well as dark septate) not possessing these features were quantified as non-AMF (**Figure S1**). This approach remains the most commonly used method to assess AMF and parasitic fungal colonization (Smith and Read, 2008).

Molecular Characterization of Fungal Communities

DNA Extraction and PCR

We collected 10 soil samples from 0 to 15 cm deep around an individual *A. speciosa* plant per site from 6 sites across the moisture gradient for a total of 60 field soil samples. At the end of the greenhouse experiment, soil samples were also collected from 15 pots per live soil treatment (two inocula × two watering treatments) for a total of 60 greenhouse samples. Field and greenhouse soil was freeze-dried using Labconco Freezone benchtop freeze dry system (Labconco, Kansas City, MO, United States). Genomic DNA was extracted from ∼250 to 300 mg dried soil per sample using a PowerSoil™ DNA isolation kit (MoBio Laboratories, Inc., Solana Beach, CA, United States), following the manufacturer's instructions. Samples were then prepared for Illumina sequencing using a two-step PCR protocol to first amplify our target region and then attach unique sample identifiers. Detailed descriptions are in Bullington et al. (2018) and Lekberg et al. (2018). Briefly, the ITS2 region was amplified to target all fungi using general fungal primers, which included a mix of forward fungal primers flITS7 (Ihrmark et al., 2012) and flITS7o (Kohout et al., 2014) and the reverse primer ITS4 (White et al., 1990). Because general fungal ITS primers can sometimes result in poor amplification of AMF (Lekberg et al., 2018), we used the AMF-specific primers WANDA and AML2 (Lee et al., 2008; Dumbrell et al., 2011) targeting the small subunit (SSU) rRNA gene to characterize AMF communities. All PCR amplification was performed in a Techne TC-4000 thermocycler (Bibby Scientific, Burlington, NJ, United States). The second PCR reaction to attach sample-specific barcodes was the same for both SSU and ITS2 and followed Bullington et al. (2018). Resulting samples were pooled based on band intensities in a 1.5% agarose gel electrophoresis of PCR 2 product. Sequencing was done at the Institute for Bioinformatics and Evolutionary Studies (iBEST) genomics resources core at the University of Idaho (http://www.ibest.uidaho.edu/; Moscow, ID, United States). Amplicon libraries were sequenced using 2 × 300 paired-end reads on an Illumina MiSeq sequencing platform (Illumina Inc., San Diego, CA, United States).

Bioinformatics Analysis

Initial bioinformatics analyses were conducted using "Quantitative insights into microbial ecology 2" (QIIME2 version 2017.12; https://qiime2.org/; Caporaso et al., 2010). Sequence reads were demultiplexed using the q2-demux plugin (https://github.com/qiime2/q2-demux). Forward and reverse

reads were trimmed at 220 and 180 base pairs, respectively and paired for the ITS2 region only. Only forward reads were used for AMF, because the overlap between the forward and reverse reads is often too short to successfully merge the two without losing a lot of sequences. Restricting the AMF analyses to forward reads only should not influence our ability to identify AMF, because the forward read alone covers most of the highly variable region (Lee et al., 2008). Paired and unpaired sequences were quality filtered and de-replicated with the q2-dada2 plugin (Callahan et al., 2016), which simultaneously removes chimeras. The q2-dada2 plugin uses nucleotide quality scores to produce sequence variants (SVs), or sequence clusters with 100% similarity representing the estimated true biological variation within each sample. Although sequences are clustered at 100% similarity as opposed to the traditional 97% similarity, DADA2 produces fewer spurious sequences, fewer clusters, and results in a more accurate representation of the true biological variation present (Callahan et al., 2016). All SVs were assigned a taxonomic classification using the UNITE fungal ITS sequence database (Kõljalg et al., 2013) as a reference database for ITS2, and to a virtual taxon using MaarjAM (Öpik et al., 2010) as a reference database for AMF. The QIIME2 q2-feature-classifier (https://github.com/qiime2/q2-feature-classifier), a naive Bayes machine-learning classifier, which has been shown to meet or exceed classification accuracy of other existing methods (Bokulich et al., 2017), was used to assign taxonomy for ITS2 and SSU independently, using a confidence threshold of 0.94 as recommended for fungi in Bokulich et al. (2017). All non-fungal sequences were subsequently filtered out of each dataset before further analyses. Functional guild analysis of soil ITS2 data was performed according to Nguyen et al. (2016) using FUNGuild, which is an open access curated database that parses SVs into guilds based on taxonomic assignment. We focused this analysis on SVs that classified either as "AMF" or as "plant pathogens," with either "probable" or "highly probable" confidence as stated in FUNGuild. We used shifts in sequence numbers in various treatments to assess potential shifts in relative abundance of the different guilds. It should be noted, however, that some fungi do not fall exclusively into a single guild, but may present as multiple guilds depending on resource availability and life stage (Nguyen et al., 2016) and many fungal ITS2 sequences present in this study were not assignable to any guild.

Statistical Analyses

Analysis of Soil Fungal Communities

All statistical analyses associated with the fungal community composition in the field and greenhouse were conducted in R (R Core Team, 2017) using the vegan package (Oksanen et al., 2017) except where otherwise noted. All analyses were based on data rarefied to sequencing depth of 3300 for ITS2 data, 400 for field SSU data and 510 for greenhouse SSU data. These sampling depths were chosen based on saturation of species accumulation curves produced in QIIME2 (**Figure S2**). All samples were retained at these sequencing depths except in the SSU field data set where five samples were lost due to poor amplification. To assess if fungal community composition differed between the six sites and two regions or correlated with mean annual precipitation (field survey) or between the two pooled inocula (MN and WA) and watering treatments (greenhouse experiment), we performed permutation multivariate analyses of variance (Permanova) using the *adonis2* function in the vegan package in R with 999 permutations of Bray-Curtis distance matrices of Hellinger-transformed relative sequence abundance. For all field data analyses, site was used as a blocking factor nested within region. To visually assess patterns in soil fungal community composition between wet and dry regions in the field and treatments combination in the greenhouse, we used non-metric multidimensional scaling on the same distance matrices as the Permanova using the *metaMDS* function. NMDS results were plotted using the R package ggplot2 (Wickham, 2009). To compare richness (based on SVs) and relative sequence abundances of pathogens and AMF within our soil samples, we performed a two-way analysis of variance (ANOVA) using log or square-root transformed data where necessary to reduce variance heterogeneity. Correlations between pathogen and AMF richness and site-level precipitation were analyzed using Pearson's product-moment correlations.

Analysis of Plant Trait Responses to Soil Biota

To ensure that all variables were comparable we centered all variables to a mean of zero and scaled their standard deviation to one. To improve normality, height and SLA were natural-log transformed and latex was square-root transformed prior to standardization. To evaluate the response of individual traits to treatments, we conducted multilevel model ANOVA. The multilevel model is conceptually similar to MANOVA and quantitatively similar to redundancy analysis (Jackson et al., 2012). We evaluated the soil inocula (i.e., live vs. sterile soil) explicitly in order to avoid inflating Type I errors and to facilitate the use of more robust statistical contrasts than would be possible through analyzing ratios (i.e., live:sterile soil, Rinella and Reinhart, 2018). The predictor variables in our models were plant trait (i.e., six levels), soil inoculum origin (MN or WA), watering treatment (wet or dry), soil biota treatment (live or sterile soil), and all possible interactions. Plant population was included as a random effect along with a term that included all experimental treatments nested within population to account for the multiple traits measured on each individual plant.

Of interest to our hypotheses were the soil biota × soil inoculum origin term, which tests the hypothesis that plant responses to soil biota depends on soil inoculum origin, and the biota × watering treatment, which tests for plasticity in how plants respond to soil biota. The trait × soil biota interaction term tests whether the six plant traits respond differently to soil biota. The three-way interactions, trait × soil biota × soil inoculum origin or trait × soil biota × watering treatment, would further indicate that the geographic or plastic responses to soil biota differ among plant traits. We do not focus on potential interactions between the experimental treatment and plant populations (i.e., testing whether plant populations respond differently to the treatments), because in

a previous study we found no difference in responsiveness to AMF inoculations (Waller et al., 2018) and preliminary screening of our data showed no interactions with plant populations. We used *post-hoc* linear contrasts to evaluate how each trait responded to significant predictor variables and interaction terms.

We also tested whether fungal colonization of roots growing in live soil differed among the soil inoculum origin and watering treatments by quantifying percent colonization by AMF hyphae, arbuscules, and vesicles. We analyzed each of these separately, with fixed-effect predictor variables including soil inoculum origin (MN or WA), watering treatment (wet or dry), and their interaction. Plant population was included as a random effect. We also correlated percent AMF colonization with plant traits in treatments that were significantly affect by the microbial treatment in the dry and wet watering treatments.

Multilevel ANOVAs were run using the lmer function in the lme4 package (Bates et al., 2013). *F*- and *p*-values were estimated using the anova function in the lmerTest package, with the Satterthwaite approximation to estimate denominator degrees of freedom (Kuznetsova et al., 2016). *Post-hoc* contrasts were constructed using the lsmeans package in R (Lenth, 2013).

RESULTS

Soil Fungal Communities in Wet and Dry Regions

Targeting the whole fungal community, we recovered 5673 SVs from the six field sites compared to just 2393 SVs in greenhouse soil at the end of the experiment, with 456 SVs recovered from both greenhouse and field. SV turnover was higher in field samples than greenhouse samples, with only 21.4% of SVs found in more than 1 plant in the field compared to 42.0% in greenhouse samples. No SV was found in more than 25% of all field samples. Based on ITS2 sequences in UNITE, the most abundant fungal SVs in the field matched most closely to fungi in the genus *Mortierella* and unknown *Basidiomycota*, compared to *Chaetomium* (found in 75% of greenhouse samples) and *Spizellomyces* in the greenhouse.

Using site as a blocking factor, there were no differences in total fungal richness between wet (MN) and dry (WA) regions in the field (**Table 2C**). Richness did differ among individual sites (**Table 2C**), however, with the driest site in WA having lower fungal SV richness based on ITS2 data. Fungal community composition (all fungi, pathogens, and AMF) in the field differed between regions and sites (**Table 2E**) and was additionally related to mean annual precipitation ($F = 1.5$, $p = 0.001$, **Figure S3**).

According to FUNGuild, 8.2% of all ITS2 sequences in field soil were classified as "probable pathogens," and this abundance was higher in the dry than in wet region (**Figure 2A** and **Table S2**). In contrast to our hypothesis, the highest abundance of pathogens was observed in the site with the least mean annual precipitation, and pathogen abundance correlated negatively with mean annual precipitation across sites ($R = -0.31$, $p = 0.02$). The composition (**Table 2E**), but not richness (**Table 2C**), of fungal pathogen communities differed between regions as well (**Figure 2C**) and was also related to mean annual precipitation ($F = 1.6$, $p = 0.001$).

AMF were represented by 1.9% of total ITS2 sequences (**Table S3**). Relative abundance (**Table 2A**) and richness (**Table 2C**) of AMF differed across sites but not region (**Figure 2B**), and both were highest in the site in North Dakota. AMF community composition (based on SSU sequence data) also varied between the two regions (**Figure 2D**) and across all sites (**Table 2E**) and additionally related to mean annual precipitation ($F = 6.3$, $p = 0.001$).

Greenhouse Experiment

Fungal Community Differences Between the MN and WA Pooled Inocula and Responses to Soil Moisture

Each source of pooled inocula (WA and MN) had a higher relative abundance of pathogens in dry than in wet soils (**Table 2B** and **Figure 3A**), whereas AMF showed the opposite pattern and were more abundant in wet than dry soil (**Figure 3B**). Overall fungal richness did not differ between the WA and MN inocula or the two watering treatments (**Table 2D**), but composition did (**Table 2F**). Pathogens were represented by 17.3% of fungal sequences in the greenhouse. According to FUNGuild, 66% of pathogen sequences matched most closely to the genus *Spizellomyces*. AMF made up 8.7% of ITS2 sequences. The richness of pathogens and AMF was higher in soils inoculated with WA inoculum than MN inoculum, and was higher for pathogens in dry soil and higher for AMF in wet soils (**Table 2D**). The composition of pathogens differed between inocula, but not between moisture treatments (**Figure 3C**). AMF composition on the other hand, differed between both soil inocula and watering treatments (**Figure 3D**), but all communities tended to be dominated by Glomeraceae and Claroideoglomeraceae AMF (Hahn et al., 2018). For AMF, the extent of shift due to watering treatment depended on the inoculum source (**Table 2F** and **Figure 3D**).

AMF and Non-AMF Root Colonization

Colonization of roots by AMF hyphae and arbuscules was affected by the watering treatment [hyphae: $F_{(1, 49.3)} = 10.4$, $p = 0.002$ and arbuscules: $F_{(1, 49.3)} = 13.0$, $P < 0.001$] and was higher in wet than dry soil (**Figures 4A,C**) regardless of soil biota origin. Percent colonization by vesicles was affected by the watering treatment [$F_{(1, 49.2)} = 4.2$, $P = 0.043$] and soil inoculum origin [$F_{(1, 49.3)} = 7.7$, $P = 0.008$], and were three-times more abundant in the pooled WA soil inoculum (mean = 12.0%, $se = 2.3$) than the pooled MN inoculum (mean = 4.5%, $se = 2.3$, **Figure 4B**). The colonization by fungi other than AMF was low across all treatments (1.6% ± 0.36, mean ± se) and there were no effects of either soil biota origin or watering treatment.

Plant Responses

The two soil inocula (WA or MN) did not differ in their influence on plant responses and did not statistically interact with any other term (**Table 3**). The main effect of the watering treatment was highly significant (**Table 3**), with most traits increasing in wet vs. dry soils (**Figure 5**). The two-way interaction between soil biota treatment (i.e., live or sterile treatments regardless of soil

TABLE 2 | ANOVA tables for relative sequence abundance from the **(A)** field and **(B)** greenhouse; sequence variant (SV) richness in the **(C)** field and **(D)** greenhouse; and perMANOVA table for community composition in the **(E)** field and **(F)** greenhouse.

(A) Field data-relative abundance	*F*	*p*	(B) Greenhouse data-relative abundance	*F*	*p*
Pathogens∼Region/Site	**6.2**	**0.016**	**Pathogens∼Treatment**	**10.8**	**0.001**
Pathogens∼Site	**9.5**	**0.001**	Pathogens∼Inocula	1.4	0.258
AMF∼Region	3.3	0.076	Pathogens∼Treatment × Inocula	0.1	0.764
AMF∼Site	**6.3**	**0.001**	**AMF∼Treatment**	**57.2**	**0.001**
			AMF∼Inocula	0.0	0.874
			AMF∼Treatment × Inocula	2.9	0.094
(C) Field data - SV richness	***F***	***p***	**(D) Greenhouse data-SV richness**	***F***	***p***
All Fungi∼Region/Site	1.1	0.290	All Fungi∼Treatment	1.3	0.258
All Fungi∼Site	**11. 3**	**0.001**	All Fungi∼Inocula	0.01	0.894
Pathogen∼Region/Site	0.2	0.627	All Fungi∼Treatment × Inocula	2.4	0.126
Pathogens∼Site	**6.8**	**0.001**	**Pathogens∼Treatment**	**15.2**	**0.001**
AMF∼Region/Site	3.2	0.081	**Pathogens∼Inocula**	**13.8**	**0.001**
AMF∼Site	**17.4**	**0.001**	Pathogens∼Treatment × Inocula	0.7	0.396
			AMF∼Treatment	**87.0**	**0.001**
			AMF∼Inocula	**15. 4**	**0.001**
			AMF∼Treatment × Inocula	0.6	0.438
(E) Field data-composition	***F***	***p***	**(F) Greenhouse data-composition**	***F***	***p***
All Fungi∼Region/Site	**1.6**	**0.001**	**All Fungi∼Treatment**	**1.4**	**0.021**
All Fungi∼Site	**1.4**	**0.001**	**All Fungi∼Inocula**	**5.8**	**0.001**
Pathogens∼Region/Site	**1.7**	**0.001**	**All Fungi∼Treatment × Inocula**	**1.2**	**0.048**
Pathogens∼Site	**1.5**	**0.001**	Pathogens∼Treatment	1.2	0.203
AMF∼Region/Site	**10.2**	**0.001**	**Pathogens∼Inocula**	**10.4**	**0.001**
AMF∼Site	**6.6**	**0.001**	Pathogens∼Treatment × Inocula	1.1	0.246
			AMF∼Treatment	**5.350**	**0.001**
			AMF∼Inocula	**9.498**	**0.001**
			AMF∼Treatment × Inocula	**3.731**	**0.001**

Significant terms ($p < 0.05$) are bolded.

biota origin) and watering treatment was significant (**Table 3**). Comparing the trait responses to zero in each of the watering treatments, the average trait value (averaged across all plant traits) response to soil biota in the dry treatment was marginally positive (linear contrast: live-sterile in dry $= 0.19$, $se = 0.11$, $df = 188.5$, $t = 1.7$, $p = 0.090$), whereas the average trait value response in the wet treatment was significantly negative (linear contrast: live-sterile in wet $= -0.30$, $se = 0.11$, $df = 186.8$, $t = -2.75$, $p = 0.007$). Comparing the magnitude of trait responses between the dry and wet treatments, the effect of the soil biota treatment on plant traits (averaged across all six traits) was significantly more positive in the dry watering treatment compared to the wet treatment (linear contrast on [live-sterile in dry, estimate $= 0.19$]-[live-sterile in wet, estimate $= -0.30$] averaged across all plant traits $= 0.50$, $se = 0.16$, $df = 187.6$, $t = 3.14$, $p = 0.002$). There was also a significant two-way interaction between plant traits and soil microbes (**Table 3**), suggesting the traits responded differently to live vs. sterile soil. There was also a significant two-interaction between plant traits and watering treatment (**Table 3**), suggesting that the plant traits responded differently to the watering treatment.

To more fully understand how the individual plant traits responded to soil biota in wet vs. dry watering treatments, we performed two types of (*a priori*) linear contrasts specifically related to our hypotheses. First, we constructed contrasts to compare whether the response of soil biota for each trait (i.e., trait value in live-sterile) was significantly different than zero in each of the watering treatments. In the dry treatment, root:shoot ratio responded negatively to soil biota and trichomes responded positively to soil biota (**Figure 6** and **Table 4**). No other traits were significantly affected by soil biota in the dry treatment (**Figure 6** and **Table 4**). In the wet treatment, biomass and root:shoot ratio both responded negatively to soil biota (**Figure 6** and **Table 4**). No other traits were significantly affected by soil biota in the wet treatment. Second, we used linear contrasts to compare (the trait value for live-sterile in dry)-(the trait value for live-sterile in wet). The linear contrasts for biomass in live-sterile were significantly different (**Figure 6**, **Table 4**). The contrasts for

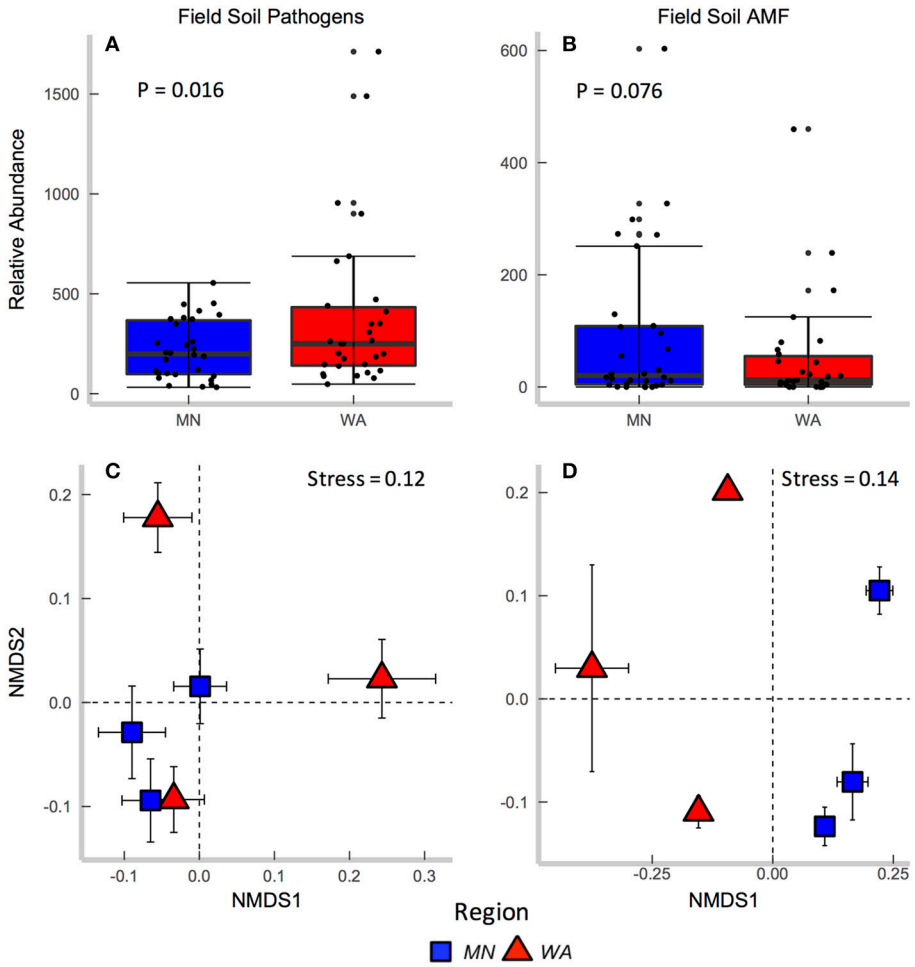

FIGURE 2 | Relative abundance of **(A)** fungal pathogens and **(B)** AMF in field collected soil. Centroids of ordinations of soil fungi community compositions for **(C)** pathogens and **(D)** AMF.

height and trichomes were marginally different (**Figure 6** and **Table 4**). Contrasts for the other traits did not differ (**Figure 6** and **Table 4**).

DISCUSSION

Our goal was to test how soil resource levels influenced soil fungal communities and plant responses to these communities. We were also interested in understanding whether soil biota and plant responses differed depending on soil biota origin across a resource gradient in the field. We show that AMF and fungal pathogen communities differed broadly between geographic regions that differ in precipitation. However, functionally we observed no difference in how plants responded to pooled inoculum from each region in the greenhouse. Thus, while long-term environmental conditions could have contributed to the regional differences in fungal communities we observed, these disparate fungal communities possessed high functional redundancy. Plant responses were more strongly driven by short-term resource availability, but the extent and direction of these responses depended on the specific plant trait. This highlights the complex relationships between resource availability and the outcome of plant-soil biota interactions.

Resource Supply Drives Trait-Specific Responses to Soil Biota

Water additions increased plant biomass, indicating that plants were resource limited in dry soils, either by water directly or via soil moisture-mediated effects on nutrient availability. Based on findings from work along fertility gradients (Johnson, 1993; Johnson et al., 1997; Leff et al., 2015), we predicted that soil biota would be beneficial when resources where limiting and detrimental when resources were abundant. Our results indicate that these relationships also apply along soil moisture gradients, because plant responses were neutral to positive under drought conditions, but negative in well-watered soil (**Figure 6**). This conditional response was especially strong for plant biomass; plants did not respond to soil biota in live dry soil, but responded negatively in live wet soil (**Figure 5**). In contrast, soil biota also

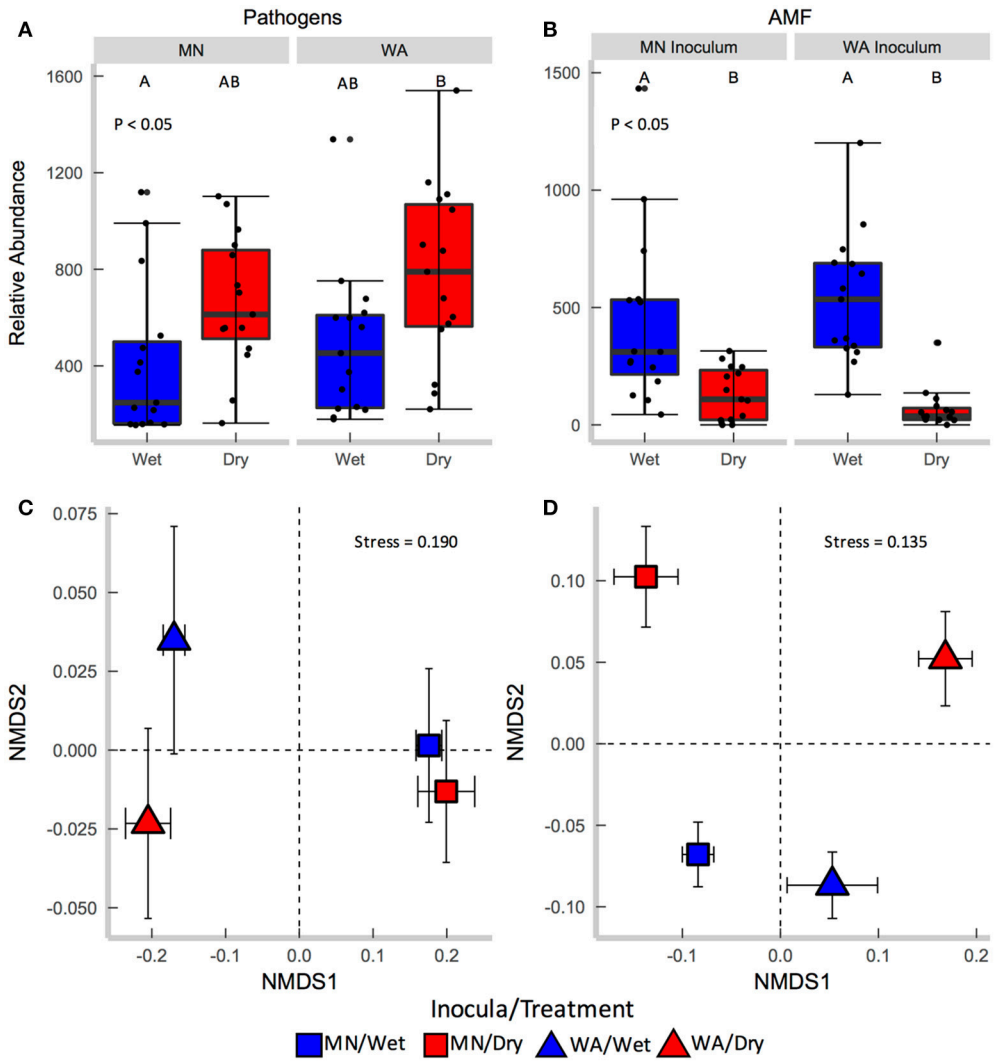

FIGURE 3 | Relative abundance of **(A)** fungal pathogens and **(B)** AMF in soil collected after the greenhouse experiment. Centroids of ordinations of soil fungi community compositions for **(C)** pathogens and **(D)** AMF.

influenced trichome density, but only in dry soil (**Figure 6**). This is perhaps not surprising since trichomes can increase drought tolerance (Farquhar and Richards, 1984; Agrawal et al., 2009) in addition to enhancing herbivore defense (Agrawal and Fishbein, 2006). We do not know which component of soil biota caused this effect, but inoculations with AMF alone have increased trichome density in a previous study (Waller et al., 2018) and all plants were highly colonized by AMF in our study (**Figure 4**). Given this high root colonization, the neutral or even negative plant responses to live soil were surprising, especially because *A. speciosa* and other *Asclepias* species generally benefit from AMF inoculations (Wilson and Hartnett, 1998; Busby et al., 2011; Tao et al., 2016; Waller et al., 2018).

One possible explanation for the above patterns is that there are negative correlations in the responsiveness of multiple traits, particularly between biomass and trichomes (Waller et al., 2018), or other unmeasured traits such as plant secondary metabolites (e.g., cardenolides; Vannette et al., 2013). Plants under dry conditions may have preferentially allocated resources to traits (i.e., trichomes) and soil biota that allow them to best cope with drought stress, which may not result in differences in biomass. It is also possible that strong, positive growth responses from AMF only occur when plants are limited by phosphorus (Smith and Read, 2008 and references therein), which, due to the high availability of this nutrient and repeated fertilizations (**Table 1**), was unlikely in this experiment. Interestingly, however, plants grown in live soil allocated less biomass to roots than plants grown in sterile soil, irrespective of watering treatment and soil biota origin (**Figure 5C**). Roots were also heavily colonized by arbuscules (**Figure 4**), which is where AMF deliver phosphorus to plants. Thus, it is possible that even though AMF did not promote growth and plants were not phosphorous-limited, fungal colonization prompted a shift in allocation patterns whereby AMF substituted for some root

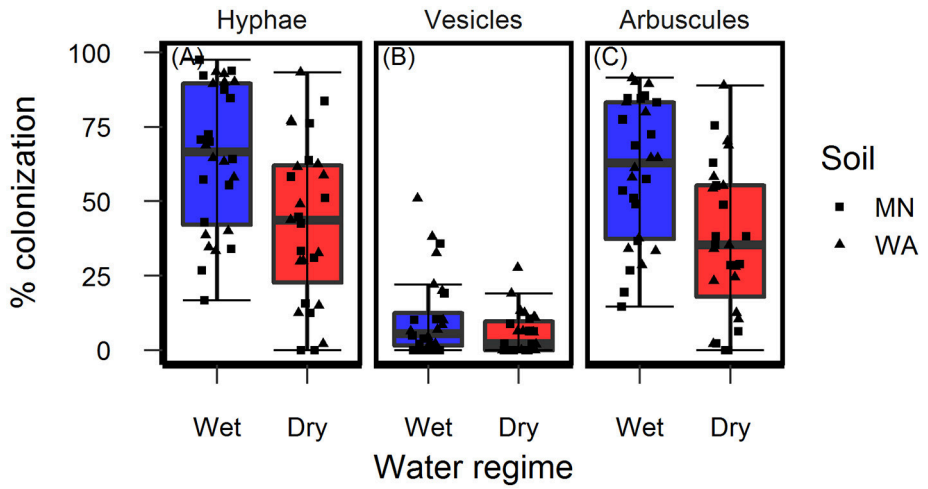

FIGURE 4 | Colonization of AMF **(A)** hyphae, **(B)** vesicles, and **(C)** arbuscules on *Asclepias speciosa* plants growing in live soil exposed to dry and wet watering treatments.

TABLE 3 | ANOVA table from the multilevel model of plant traits from the greenhouse experiment.

Effect	DF	*F*	*p*
Trait	5, 187.43	0.16	0.977
Soil inoculum origin	1, 187.55	1.54	0.216
Soil biota (live vs. sterile)	1, 187.74	0.50	0.480
Watering regime (dry or wet)	**1, 188.38**	**82.40**	**<0.001**
Trait × Inoculum	5, 187.43	0.21	0.958
Trait × Biota	5, 187.43	**3.53**	**0.005**
Inoculum × Biota	1, 187.54	0.79	0.374
Trait × Water	**5, 187.43**	**11.04**	**<0.001**
Inoculum × Water	1, 187.62	0.37	0.542
Biota × Water	**1, 187.54**	**9.87**	**0.002**
Trait × Inoculum × Biota	5, 187.43	0.42	0.831
Trait × Inoculum × Water	5, 187.43	0.41	0.840
Trait × Biota × Water	5, 187.43	0.95	0.449
Inoculum × Biota × Water	2, 187.57	0.53	0.469
Trait × Inoculum × Biota × Water	5, 187.43	0.47	0.800
†Plant population		3.34	0.070
†Nested term		**159.38**	**<0.001**

Significant terms ($p < 0.05$) are bolded. †Random effects; χ^2 values are shown in F column.

functions. Other work has found that AMF can be functionally important even in cases where growth is not affected (Smith, 2003). Alternatively, soil moisture may have shifted bacterial communities or function, which has been shown to influence plant performance (e.g., Letourneau et al., 2018). This study does not allow us to identify the underlying mechanisms of observed patterns. As such, measuring AMF-mediated phosphorous uptake, water use efficiency, shifts in allocation to various plant traits, or bacterial communities would be a productive future direction.

Plant Responses Driven by Shifts Within Rather Than Between Fungal Guilds

We predicted that plant growth responses to soil biota would be associated with the functional identity of the soil community. In other words, positive plant responses to soil biota should be associated with high mutualist to pathogen ratios whereas negative plant responses to soil biota should be associated with smaller mutualist to pathogen ratios. However, we found that root colonization by fungi other than AMF (which could include pathogens) was very low in all treatments. Furthermore, soil pathogen abundance (based on sequence abundance) was actually *higher* in dry than wet soil, which is inconsistent with the *less* negative plant responses in this treatment. The higher pathogen abundance in dry soil was unexpected, although some pathogens appear to thrive in dry soils (Cook and Papendick, 1972). Dry conditions could filter for fungi able to best tolerate desiccation and then reproduce quickly when wetted. It is also possible that these fungi experienced competitive release as a result of the lower AMF abundance (Borowicz, 2001), or that fungi classified as pathogens based on a match to the curated database FunGuild (Nguyen et al., 2016) may also function as saprotrophs. For example, *Fusarium*, which was recorded in our sequence data, is typically pathogenic to a narrow taxonomic host range, but saprotrophic strains can be broadly distributed across cultivated and native grassland soils (Gordon and Okamoto, 1989; Lozupone and Klein, 2002).

A more likely explanation for plant growth reductions in live, wet soil is that AMF were parasitic. AMF commonly function along a continuum from parasitism to mutualism (Johnson et al., 1997), and plant species, such as milkweed, that are very responsive to AMF under resource limiting conditions (Wilson and Hartnett, 1998; Busby et al., 2011) may also be more susceptible to parasitism when resources are not limiting (Grman, 2012). Indeed, AMF abundance in both roots and soil was higher in wet than dry soil (**Figure 4**), a pattern that has been

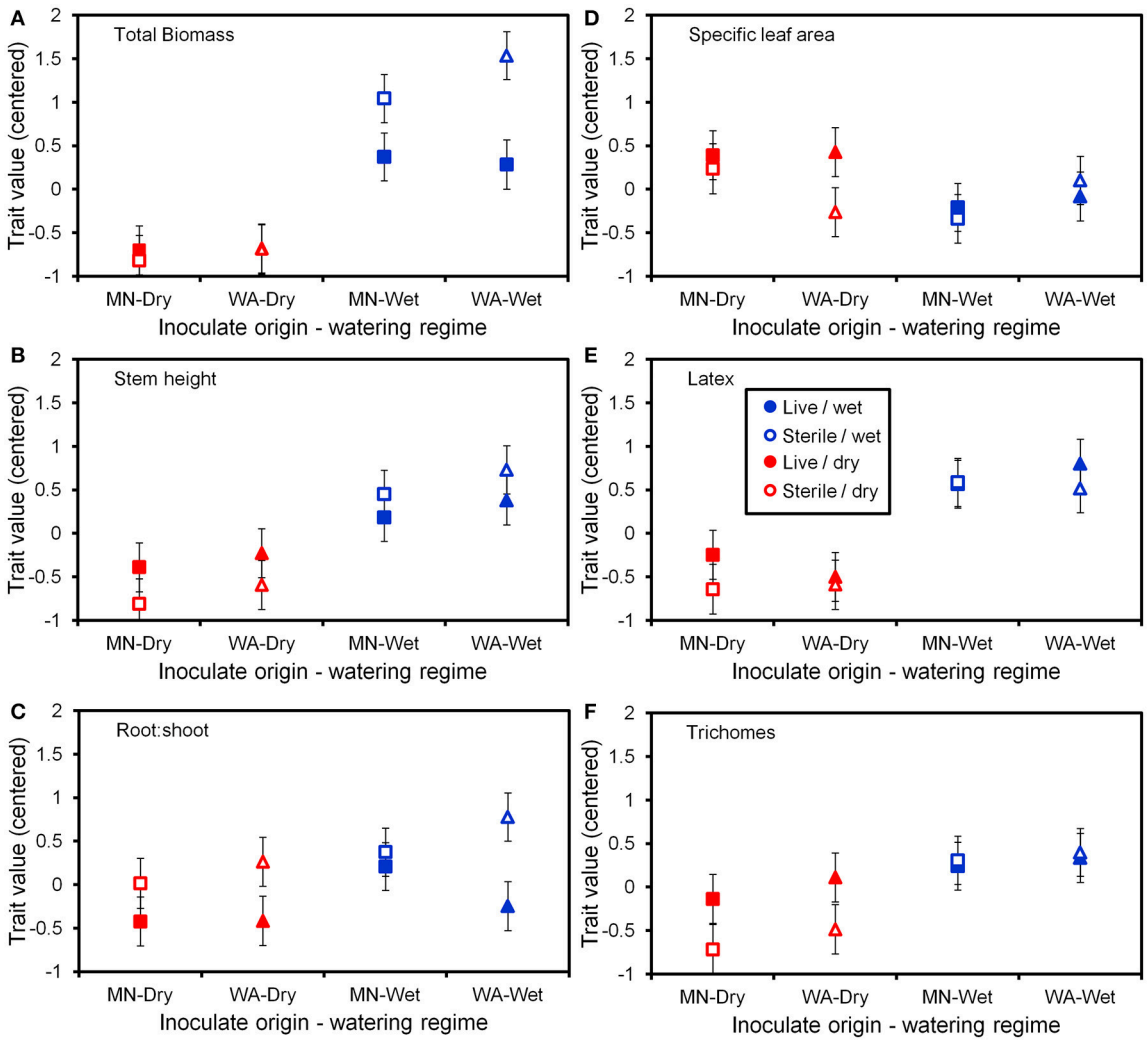

FIGURE 5 | Trait values (centered) for the six plant functional traits **(A–F)** measured at the end of the greenhouse experiment. Soil inoculates originated from wet field sites (MN) or dry field sites (WA). Bars are ±1 SE. Square symbols indicate soil inoculate originating from MN and triangles indicate soil inoculate originating from WA.

documented previously (Bell et al., 2014). This greater fungal biomass could have imposed an excessive carbon drain where the cost of associating with AMF exceeded the benefits derived under these particular conditions.

High Functional Redundancy Among Disparate Fungal Communities

We predicted that the origin of the soil inoculum would influence plant responses, such that soil biota sourced from wetter regions should have a stronger negative effect on plants when grown in wet soil than soil inoculum sourced from drier regions. This was not supported. Despite clear differences in both AMF and pathogen compositions in the field as well as in the pooled inocula in the greenhouse (**Figures 2**, **3**), these soils had similar effects on plants (**Figure 5** and **Table 3**). Because we pooled our inoculum from the gradient end points, we can only discuss the specific function of the pooled inocula, rather than making broad generalizations regarding regional differences in function. This pooling can also inflate Type I errors, i.e., falsely detecting statistically significant effects of soil inoculum origin (Reinhart and Rinella, 2016; Gundale et al., 2017; see discussions in Cahill et al., 2017). However, even with our liberal test, we found no apparent functional difference between the two soil inocula, despite clear differences in composition. This contrasts with some previous studies that have shown that soils experiencing drought can influence plant responses (Lau and Lennon, 2012; Kaisermann et al., 2017). However, while Kaisermann et al. (2017) found that soil microbial communities previously exposed to drought were less beneficial than those that had not experienced drought, Lau and Lennon (2012) showed that plants did better under drought when matched with a microbial community that had previously experienced drought. What explains these different results are unclear, but may be

related to the degree, nature and duration of stress imposed in various studies (Hawkes et al., 2011; Evans and Wallenstein, 2012). For example, the range of water availability experienced by plants in our experiment may not have been outside the natural variation that these fungi experience across seasons, or due to extreme weather events. Seasonal differences in microbial communities may exceed effects of severe experimental reductions in precipitation (Cregger et al., 2012). Natural variation in rainfall may result in a "storage effect" where fungi with wide environmental tolerances coexist and where subsets are favored based on current environmental conditions (Hawkes et al., 2011).

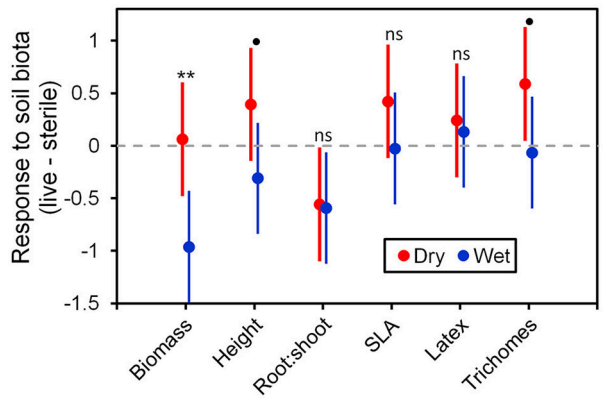

FIGURE 6 | Response to soil microbes, contrasts estimated as least-square means in live minus sterilized soil, for the six measured traits under wet and dry watering regimes. Bars are 95% confidence intervals. Error bars that do not overlap zero indicate that that response differed from zero (i.e., response $\neq$ 0). Different responses between wet and dry watering treatment are indicated as follows: $^{**}P < 0.01$; $^{\bullet}P < 0.1$.

The clear changes in AMF and pathogen communities in soil inocula experiencing drought indicate that soil moisture, or moisture-mediated shifts in either host plant status or nutrient availability can strongly influence fungal communities. Our results generally agree with previous studies that have shown shifts in fungal communities along precipitation gradients (Kivlin et al., 2011; Tedersoo et al., 2014; Zhang et al., 2016) and where soil moisture has been experimentally altered (Furze et al., 2017; Kaisermann et al., 2017; Meisner et al., 2018; Schmidt et al., 2018; She et al., 2018). These changes in composition may or may not result in altered fungal richness, which sometimes declines (Toberman et al., 2008; Gehring et al., 2017), show no difference (Schmidt et al., 2018; She et al., 2018), or even increase (Hawkes et al., 2011) with drought. We observed no regional difference in AMF richness, but a reduction in dry soils in the greenhouse, whereas pathogen richness was higher in the dry region and increased with experimental drought. Shifts in fungal richness can have functional consequences for plant growth and fitness as well as ecosystem processes (van der Heijden et al., 1998; Toberman et al., 2008; Lau and Lennon, 2011), but results to date suggest that responses to drought differ among studies and possibly fungal guilds.

Limitations and Future Directions

In this study, we collected soils from the endpoints of a precipitation gradient in order to understand how fungal communities might be influenced by differences in soil moisture. However, soil nutrient availabilities tended to be higher in drier sites (**Table S1**) and other factors that differ among sites might drive fungal community differences instead or in addition to soil

TABLE 4 | Contrasts from the multilevel mixed model from the greenhouse experiment.

Trait	Contrast	Estimate	SE	df	*t*	*P*
Biomass	Live–Sterile in "dry"	0.063	0.276	188.7	0.23	0.8206
Height	Live–Sterile in "dry"	0.394	0.275	186.1	1.43	0.1539
Root:shoot	**Live–Sterile in "dry"**	**−0.558**	**0.276**	**188.7**	**−2.02**	**0.0448**
SLA	Live–Sterile in "dry"	0.423	0.276	188.7	1.53	0.1271
Latex	Live–Sterile in "dry"	0.242	0.276	188.7	0.88	0.3828
Trichomes	**Live–Sterile in "dry"**	**0.589**	**0.276**	**189.7**	**2.13**	**0.0345**
Biomass	**Live–Sterile in "wet"**	**−0.962**	**0.271**	**187.2**	**−3.55**	**0.0005**
Height	Live–Sterile in "wet"	−0.309	0.270	183.2	−1.15	0.2530
Root:shoot	**Live–Sterile in "wet"**	**−0.594**	**0.271**	**187.2**	**−2.19**	**0.0296**
SLA	Live–Sterile in "wet"	−0.026	0.271	187.2	−0.10	0.9230
Latex	Live–Sterile in "wet"	0.133	0.271	187.2	0.49	0.6233
Trichomes	Live–Sterile in "wet"	−0.065	0.271	187.2	−0.24	0.8109
Biomass	**(LiveDry–SterileDry) – (LiveWet–SterileWet)**	**1.024**	**0.387**	**187.9**	**2.65**	**0.0088**
Height	*(LiveDry –SterileDry)–(LiveWet-SterileWet)*	*0.703*	*0.385*	*184.7*	*1.83*	*0.0696*
Root:shoot	(LiveDry –SterileDry)–(LiveWet–SterileWet)	0.036	0.387	187.9	0.09	0.9265
SLA	(LiveDry–SterileDry)–(LiveWet–SterileWet)	0.449	0.387	187.9	1.16	0.2467
Latex	(LiveDry –SterileDry) –(LiveWet–SterileWet)	0.108	0.387	187.9	0.28	0.7797
Trichomes	*(LiveDry –SterileDry)–(LiveWet-SterileWet)*	*0.653*	*0.387*	*188.4*	*1.69*	*0.0929*

Significant contrasts are bolded (P < 0.05) and marginally significant contrasts are italicized (P < 0.1).

moisture. As well, the limited overlap in fungal communities among sites within regions is consistent with taxa being dispersal limited, which could further obscure filtering based on soil moisture (Cottenie, 2005; Lekberg et al., 2007; Vellend, 2010). These factors could help explain the lack of functional differences observed between the two pooled inocula in the greenhouse. However, regardless of what shaped these communities in the field, our study shows that disparate fungal communities respond in similar ways to short-term differences in soil moisture and have high functional redundancy.

Whether or not responses observed in the greenhouse would also occur in the field is uncertain however, because greenhouse conditions tend to favor disturbance tolerant soil biota that may not be abundant in the field. Similar to previous work that has quantified this so-called "cultivation bias" effect (Sýkorová et al., 2007; Schmidt et al., 2018), we observed little overlap between field and greenhouse communities (**Figure S1**, Hahn et al., 2018). This reinforces the need to conduct experiments in the field, wherever possible (Lekberg and Helgason, 2018), because greenhouse experiments may poorly predict field responses (Heinze et al., 2016). For example, while AMF are not infrequently parasitic in the greenhouse (e.g., Klironomos, 2003), does this also happen under more natural conditions in the field? If so, it could have very important consequences for how we understand their role in structuring plant communities.

Summary

By linking changes in resource levels to shifts in composition and function of soil fungal communities, we show that distinct fungal communities that originate from disparate environments have similar directional responses and cause equivalent plant functional responses to short-term alterations in soil moisture. Overall, we found that plant trait responses to soil biota shifted from negative to neutral or slightly positive with declining resources (i.e., soil moisture) regardless of soil biota origin. Contrary to our predictions, however, the changes in plant responses were not driven by a shift *between* fungal guilds but rather *within* guilds. Furthermore, it is most likely AMF became parasitic in high-resource environments. Whether or not this also happens in the field is uncertain. Much could be learned from additional studies that jointly quantify how variation in resource availability in the field influences soil biota, and how this in-term affects plant responses.

DATA AVAILABILITY

The data will be made publically available via figshare at the time of publication (referenced as Hahn et al., 2018, doi: 10.6084/m9.figshare.5926378). Representative sequences from both target regions were submitted to GenBank and assigned the following accession numbers: ITS greenhouse: MH450315-MH452715; ITS Field: KBZH01000000; SSU greenhouse: MH453115-MH453369; SSU Field: MH452716-MH453114.

AUTHOR CONTRIBUTIONS

PH, JM, BL, and YL conceived the experiments. All authors contributed to designing the experiments and collecting data. PH analyzed plant and AMF data. LB analyzed molecular data. All authors contributed to interpreting the data. PH, JM, LB, and YL wrote the manuscript with input from the other co-authors.

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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.2018.01605/full#supplementary-material

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Benefits from Below: Silicon Supplementation Maintains Legume Productivity under Predicted Climate Change Scenarios

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Many studies demonstrate that elevated atmospheric carbon dioxide concentrations (eCO_2) can promote root nodulation and biological nitrogen fixation (BNF) in legumes such as lucerne (*Medicago sativa*). But when elevated temperature (eT) conditions are applied in tandem with eCO_2, a more realistic scenario for future climate change, the positive effects of eCO_2 on nodulation and BNF in *M. sativa* are often much reduced. Silicon (Si) supplementation of *M. sativa* has also been reported to promote root nodulation and BNF, so could potentially restore the positive effects of eCO_2 under eT. Increased nitrogen availability, however, could also increase host suitability for aphid pests, potentially negating any benefit. We applied eCO_2 (+240 ppm) and eT (+4°C), separately and in combination, to *M. sativa* growing in Si supplemented (Si+) and un-supplemented soil (Si−) to determine whether Si moderated the effects of eCO_2 and eT. Plants were either inoculated with the aphid *Acyrthosiphon pisum* or insect-free. In Si− soils, eCO_2 stimulated plant growth by 67% and nodulation by 42%, respectively, whereas eT reduced these parameters by 26 and 48%, respectively. Aphids broadly mirrored these effects on Si− plants, increasing colonization rates under eCO_2 and performing much worse (reduced abundance and colonization) under eT when compared to ambient conditions, confirming our hypothesized link between root nodulation, plant growth, and pest performance. Examined across all CO_2 and temperature regimes, Si supplementation promoted plant growth (+93%), and root nodulation (+50%). *A. pisum* abundance declined sharply under eT conditions and was largely unaffected by Si supplementation. In conclusion, supplementing *M. sativa* with Si had consistent positive effects on plant growth and nodulation under different CO_2 and temperature scenarios. These findings offer potential for using Si supplementation to maintain legume productivity under predicted climate change scenarios without making legumes more susceptible to insect pests.

Keywords: alfalfa, aphids, atmospheric change, climate change, global warming, silica, silicon

INTRODUCTION

Projected increases in atmospheric carbon dioxide (CO_2) have been shown experimentally to stimulate biological nitrogen fixation (BNF) in legumes (Soussana and Hartwig, 1996; Zanetti et al., 1996; Hungate et al., 1999; Edwards et al., 2006; Lam et al., 2012). These effects are strongest immediately after exposure to elevated CO_2 (eCO_2) (Hungate et al., 2004) and when other nutrients (especially phosphorus) are not limiting (Rogers et al., 2009). Elevated CO_2 (eCO_2) can promote BNF via several mechanisms, including larger numbers of N_2 fixing symbiotic bacteria in the rhizosphere (Schortemeyer et al., 1996), increased numbers of nodules which house N_2 fixing rhizobia bacteria (Ryle and Powell, 1992) and enhanced nitrogenase activity (Norby, 1987). Broadly speaking, eCO_2 allows legumes to increase rates of photosynthesis and allocate more carbon belowground to support increased root nodulation and therefore BNF (Aranjuelo et al., 2014).

Researchers are becoming increasingly aware of the importance of testing multiple environmental change factors because they are predicted to occur concurrently and often have either synergistic or antagonistic impacts on one another (Robinson et al., 2012; Johnson and Jones, 2017). Climate models predict, for instance, that air temperatures will increase in tandem with increases in atmospheric CO_2 and warmer temperature may negate any positive impacts of eCO_2 on plant growth (Newman et al., 2011). This may be particularly true in legume systems because higher temperatures can have inhibitory effects on BNF due to the relatively low tolerance of N_2-fixing bacteria to higher temperatures (Zahran, 1999; Whittington et al., 2013; Aranjuelo et al., 2014). The optimal temperature for root nodule symbiosis for temperate legumes is thought to be around 15–25°C, above which detrimental effects can become evident (Aranjuelo et al., 2014). Elevated temperature (eT) can directly hinder the development and functionality of root nodulation and accelerate nodule senescence (Piha and Munns, 1987; Aranjuelo et al., 2006). In addition, eT can inhibit nodulation via plant-mediated mechanisms, including reduced root hair formation, fewer nodulation sites and poorer adherence of bacteria to root hairs (Hungria and Franco, 1993; Hungria and Vargas, 2000; Aranjuelo et al., 2014).

Soil conditions play an important role in determining the extent to which eCO_2 and eT affect root nodulation in legumes (Aranjuelo et al., 2014). Several studies report that supplementation of soil silicon (Si) levels promotes growth in legumes (Horst and Marschner, 1978; Miyake and Takahashi, 1985; Guo et al., 2006; Johnson et al., 2017), though we know less about the functional role of Si in legumes compared to other plant families such as the Poaceae (Epstein, 1999; Cooke and Leishman, 2011). Moreover, Si supplementation can increase rates of root nodulation and symbiosis with nitrogen fixing bacteria (Nelwamondo and Dakora, 1999; Mali and Aery, 2008). However, how these positive effects of Si on nodulation are affected by eCO_2 or eT, alone or in combination, have not yet been addressed. If Si could maintain nodulation rates under future climate change scenarios, such as eT, which usually decrease it, then such supplementation could be important in the mitigation of climate change impacts on agriculture.

While rhizobial colonization promotes legume growth and vigor, this improved host quality can also increase susceptibility to belowground (Quinn and Hower, 1986; Gerard, 2001; Johnson and McNicol, 2010) and aboveground insect herbivores (Dean et al., 2009, 2014; Kempel et al., 2009; Katayama et al., 2010; Whitaker et al., 2014). Beneficial effects of rhizobia on herbivores most likely arise through increased provision of nitrogen, which is frequently limiting in insect herbivore diets (Mattson, 1980). Increased provision of nitrogen may, however, allow plants to invest in plant defenses with negative impacts on herbivores (Pineda et al., 2010; Brunner et al., 2015). While Si supplementation usually increases plant resistance to herbivores (mainly reported in the Poaecae; Reynolds et al., 2009), it may also indirectly increase susceptibility to herbivores via increases in legume growth and nutritional quality (Johnson et al., 2017).

The objective of this study was to determine how eCO_2 and eT, acting alone and in combination, affected root nodulation and plant growth in *Medicago sativa* in untreated (Si−) and Si supplemented (Si+) soil. We additionally aimed to establish whether these factors affected the abundance and colonization success of an insect herbivore (the aphid *Acyrthosiphon pisum*). We hypothesized that eCO_2 increases growth and root nodulation in *M. sativa* but eT negates these effects. Si supplementation increases nodulation, even under eT, and therefore maximizes plant growth regardless of CO_2 and temperature conditions. We hypothesized that aphid abundance would be positively linked to plant growth and nodulation, whether driven by Si supplementation or changes in CO_2 and temperature conditions.

MATERIALS AND METHODS

Insect Cultures and Plant Material

Four *A. pisum* cultures were established from a single parthenogenetic adult female collected from a pasture containing grasses and legumes, including lucerne, at the Hawkesbury Campus of the Western Sydney University, Penrith, NSW, Australia (latitude −33.608847, longitude 150.747016). Cultures were maintained on propagated lucerne (*M. sativa* L.) plants (Sequel cultivar) in each of the four CO_2 and temperature combinations (conditions below) for at least six generations (c. 7 weeks) prior to the experiment. For the experiment, *M. sativa* (Sequel) were grown from seed (Heritage Seeds Pty, Adelaide, SA, Australia) in glasshouse rooms receiving supplemental light (15:9 light:dark) under the same conditions. Plants were grown in 70 mm diameter pots containing c. 700g of soil excavated from the Hawkesbury campus of Western Sydney University (location as above). The soil is typified as low-fertility sandy loam in the Clarendon Formation (Chromosol) (Barton et al., 2010), which has low bioavailable Si content of 10–17 mg kg^{-1} (Johnson et al., 2017).

Growth Conditions and Experimental Procedures

Eighty lucerne plants were grown in each of four CO_2 and temperature-controlled glasshouse chambers (320 plants in total) using a fully factorial design of ambient CO_2 (aCO_2; 400 μmol mol^{-1}) and eCO_2 (640 μmol mol^{-1}) at ambient (aT) and elevated temperature (ambient + 4°C; eT). aT was set at 26/18°C day/night representing the average daily temperature (November to May) over the past 30 years for Richmond, NSW, Australia (Australian Bureau of Meteorology). eT (30/22°C day/ night) replicated the maximum predicted temperature increase for this region within this century (CSIRO, 2007–2016). Environmental conditions were monitored continuously throughout the experiment and temperature readings were verified with portable temperature loggers. To minimize 'chamber effects' associated with using four chambers, plants were circulated within each chamber every 5 days (apart from when plants were inoculated with aphids to avoid dislodgement of the insects) and chambers were swapped every c. 10 days by transferring plants between chambers and adjusting the environmental conditions accordingly. While this does not eliminate pseudoreplication, using this approach in these chambers has provided matching empirical results to fully replicated experiments, whether using multiple chamber replicates or multiple experimental runs (Johnson et al., 2016b).

Plants were irrigated with c. 70 ml of tap water (Si 3 ppm) three times a week. After growing for a further 2 weeks, half (40) of the plants continued to receive tap water (Si− plants or Si− soil hereafter) at the same intervals while the other half (selected at random) received 70 ml of 500 mg l^{-1} soluble silica in the form of $NaSiO_3.9H_2O$ three times a week (Si+ plants or Si+ soil hereafter). When plants were 6 weeks old, 20 of the plants receiving the Si supplementation and 20 of the plants receiving tap water (selected at random) were inoculated with two teneral adult *A. pisum*. White mesh (organza) bags (125 mm × 170 mm) were applied tightly around the rim of all pots confining aphids to their allocated plants. After 2 weeks, bags were removed aphids counted (including colonization success; at least one aphid being present). Plants were cleaned free of soil with water and the number of active (pink) root nodules quantified. Maximum rooting depth was also quantified to provide a rudimentary measure of nodule density in order to give an indication as whether changes in nodule abundance were a function of root growth or nodule density on the roots (i.e., nodules per unit of root growth). Plants were freeze dried for 48 h and weighed. Leaves were separated from the stems and ball-milled to a fine powder prior to analysis for Si concentrations.

Foliar Si Analysis

It was necessary to pool foliar samples (2–3 plants per sample), giving nine replicates of each treatment combination (CO_2, temperature, Si application and aphid inoculation). Foliar Si concentrations were analyzed with X-ray fluorescence spectrometry using the method described by Reidinger et al. (2012). In summary, plant material was ground to a fine powder and pressed into 13 mm-diameter pellets. Foliar Si concentration was determined using a Niton XL3t XRF analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, United States), for a measurement time of 30 s. Results we expressed as foliar Si concentration (as % of dry mass), calibrated against plant-certified reference material of known Si content (Garbuzov et al., 2011).

Statistical Analysis

Goodness-of-fit tests, using the 'goodfit' function in the vcd package (Friendly, 2000), were employed to determine which distributions best described the data. Plant dry mass and nodule density were transformed (logarithm and square-root, respectively) prior to analysis to meet model assumptions and give residual diagnostic plots which fitted a normal distribution and showed least heteroscedasticity. Plant dry mass and nodule density were analyzed using analysis of variance with CO_2, temperature, aphid presence, and Si supplementation included as fixed effects individually and in interaction with one another. Root nodule counts and aphid abundance were analyzed with generalized linear models with negative binomial error structures and log-link function using the same configuration of fixed effects as above. Aphid colonization success was analyzed in the same way but with binomial error structure and logit link function. Statistical tests of plant mass and nodulation were conducted on data collectively, before repeating the tests separately for Si− and Si+ plants since there were significant interactions between Si treatment and environmental treatments. Where non-significant effects were observed in full models (i.e., all factors included), non-significant factors were removed to determine whether this affected model inferences with more parsimonious models (e.g., fewer multi-way interaction terms were included in the model) – see Supplementary Table S1. All analysis was conducted in the R statistical package.

RESULTS

Plant growth was stimulated by eCO_2 and Si supplementation by 41 and 93%, respectively (**Figure 1** and **Table 1**). In contrast, eT and aphid presence depressed plant growth by 13 and 17%, respectively (**Figure 1** and **Table 1**). Temperature depressed plant growth in Si− soil (**Figure 1A**), but not in Si+ soil (**Figure 1B**), though there was an interactive effect of CO_2 and temperature in the latter, with eCO_2 promoting plant growth more at eT than under aT conditions (**Figure 1B**).

Root nodulation increased when plants grew under eCO_2 (+27%) and Si+ conditions (+50%) (**Figure 2** and **Table 1**), but eT caused significant declines in nodulation (−32%). In Si− soil, root nodulation patterns generally mirrored changes in plant growth (**Figures 1A**, **2A**, respectively). Levels of root nodulation were universally high in plants growing in Si+ soil and other factors (CO_2, temperature, and aphid presence) no longer had significant impacts (**Figure 2B**). This was particularly true for the negative impacts of eT, which was reversed under Si+ conditions, reflected by the significant interaction of these treatments (**Figure 2** and **Table 1**). Our rudimentary estimate of nodule density (nodules per unit of root depth) suggested this was not affected by CO_2 (other than the weak interaction

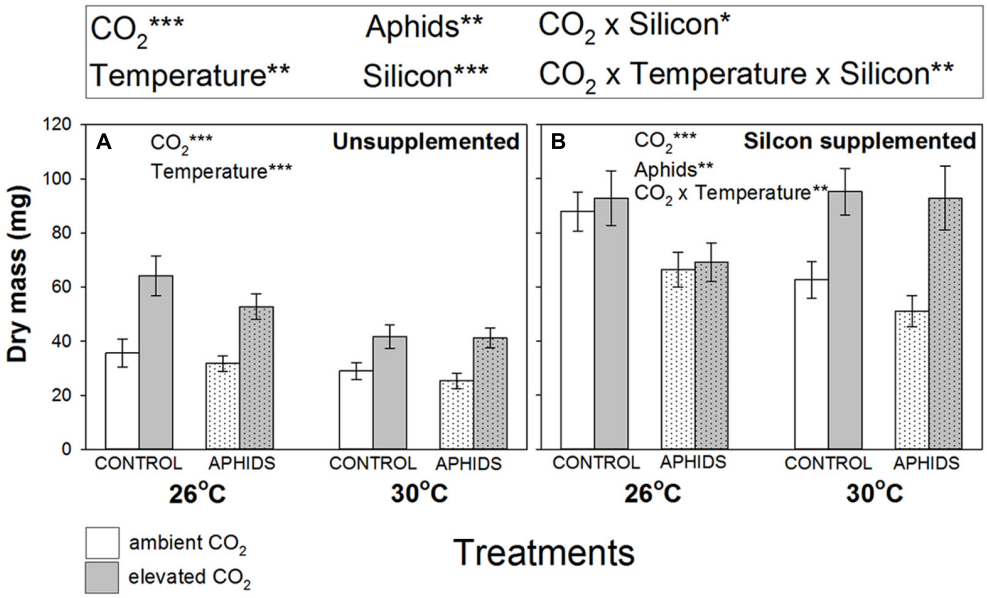

FIGURE 1 | Impacts of CO_2, temperature and aphid presence on dry mass of *Medicago sativa* when growing in **(A)** non-supplemented and **(B)** Si supplemented soil. Mean values ± standard error shown ($N = 20$) with statistically significant effects indicated $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Significant factors for the whole experiment given in the upper panel (see **Table 1** for full results) and for Si– and Si+ plants separately in the respective graphical panels.

described below) but declined by 25% under eT (Supplementary Figure S1 and Supplementary Table S1). Nodule density increased (c. +45%) under Si+ conditions and, like nodule abundance, there was a significant interaction between Si treatment and temperature, whereby negative effects of eT were reversed under Si+ conditions (Supplementary Figure S1 and Supplementary Table S1). There was a very weak interaction between Si, aphids, and CO_2.

Si concentration in the foliage was unaffected by CO_2, temperature and aphid presence, though unexpectedly there was a small but significant decline in foliar Si concentrations when growing in Si+ soil (**Figure 3** and **Table 1**).

TABLE 1 | Results of statistical tests examining the effects of CO_2, temperature (Temp), aphid presence and Si supplementation (Si) on plant growth, root nodulation, and foliar Si concentrations.

Plant response Model fixed effect	Dry mass[1]		Root nodules		Si concentration[1]	
	$F_{1,304}$	P	$RD_{1,304}$	P	$F_{1,94}$	P
CO_2	**51.86**	**<0.001**	**409.15**	**<0.001**	1.32	0.25
Temp	**10.28**	**<0.001**	**384.58**	**<0.001**	0.37	0.54
Aphids	**7.36**	**0.01**	384.57	0.90	1.17	0.28
Si	**150.55**	**<0.001**	**352.27**	**<0.001**	**15.22**	**<0.001**
CO_2 × Temp	3.19	0.07	351.81	0.50	2.09	0.15
CO_2 × Aphids	0.11	0.74	349.06	0.10	0.01	0.99
Temp × Aphids	0.63	0.43	345.60	0.06	1.03	0.31
CO_2 × Si	**4.06**	**0.04**	344.47	0.29	0.07	0.80
Temp × Si	2.00	0.16	**336.69**	**0.01**	0.01	0.92
Aphids × Si	1.83	0.18	336.66	0.86	0.15	0.70
CO_2 × Temp × Aphids	1.07	0.30	335.82	0.36	0.42	0.52
CO_2 × Temp × Si	**8.70**	**<0.001**	334.95	0.35	0.43	0.51
CO_2 × Aphids × Si	0.26	0.61	333.17	0.18	0.51	0.48
Temp × Aphids × Si	0.25	0.61	332.97	0.65	0.06	0.81
CO_2 × Temp × Aphids × Si	0.06	0.81	332.95	0.90	0.84	0.36

Statistically significant ($P < 0.05$) factors indicated in bold with Fisher's (F) or residual deviation (RD) given depending on the models used. Analysis conducted on transformed data as indicated.

[1]Log transformed. Degrees of freedom in each column apply to all effects.

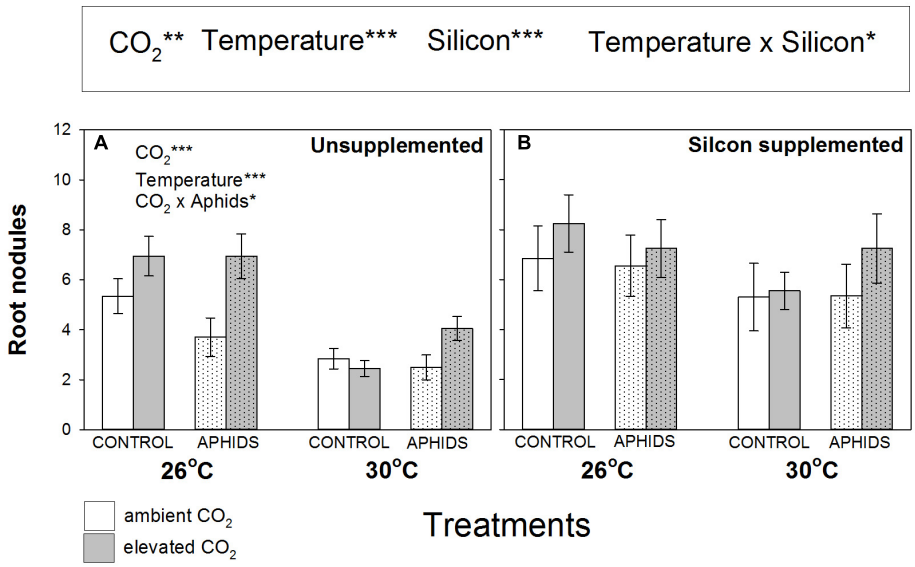

FIGURE 2 | Impacts of CO_2, temperature and aphid presence on root nodulation (number per plant) of *M. sativa* when growing in **(A)** non-supplemented and **(B)** Si supplemented soil. Mean values ± standard error shown (N = 20) with statistically significant effects indicated $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Significant factors shown as per **Figure 1** legend.

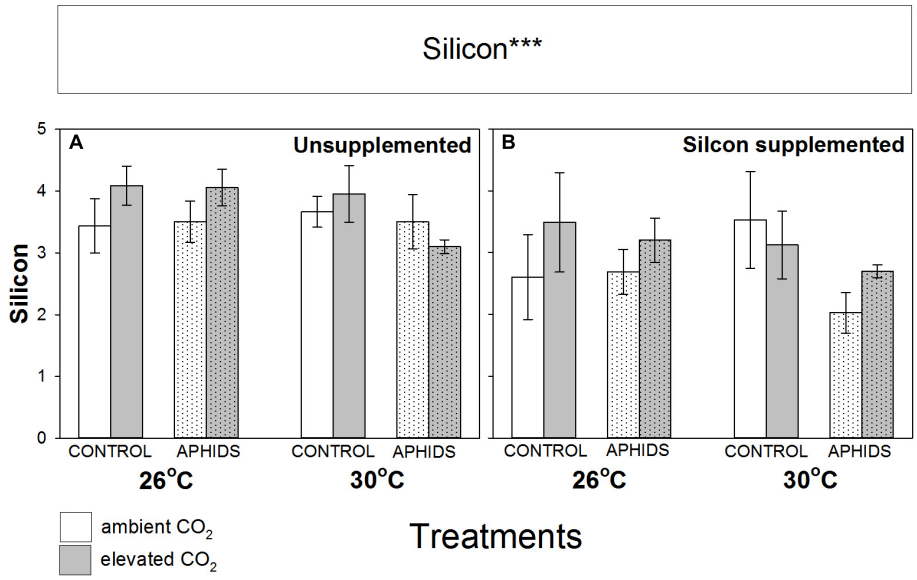

FIGURE 3 | Impacts of CO_2, temperature and aphid presence on Si concentrations of *M. sativa* foliage (% dry mass) when growing in **(A)** non-supplemented and **(B)** Si supplemented soil. Mean values ± standard error shown (N = 9) with statistically significant effects indicated $^{***}P < 0.001$. Significant factors shown as per **Figure 1** legend.

Aphid abundance was not significantly affected by eCO_2 (**Figure 4**), although colonization success increased by 14% under eCO_2 (**Table 2**). In contrast, eT caused substantial declines (−65%) in aphid abundance and reduced their ability to colonize plants, falling by 48 and 43% on Si− and Si+ plants, respectively (**Table 2**). Aphid populations at eT were similar regardless of Si treatments. In short, aphid abundance was always lowest at 30°C and Si promotion of plant growth and nodulation was decoupled from aphid performance, such that Si+ conditions led to increased nodulation (and potentially BNF) without increasing aphid numbers.

The key findings of this study are summarized in **Figure 5** which held true when non-significant terms were dropped from models for parsimony (see Supplementary Table S2). **Figures 5A–C** shows how aphid abundance mirrors patterns of nodulation and plant growth in non-supplemented

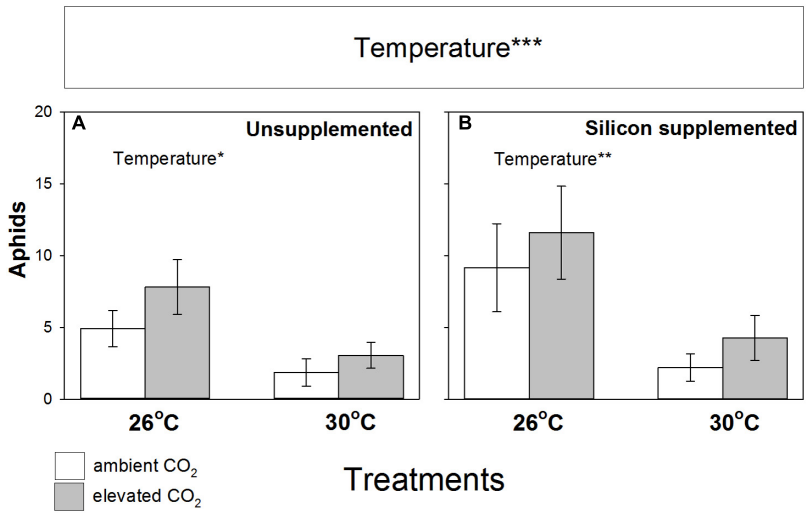

FIGURE 4 | Impacts of CO_2 and temperature on aphid (*Acyrthosiphon pisum*) abundance (number per plant) when feeding on *M. sativa* plants growing in **(A)** non-supplemented and **(B)** Si supplemented soil. Mean values ± standard error shown (N = 20) with statistically significant effects indicated ***P < 0.001. Significant factors shown as per **Figure 1** legend.

soils, but this becomes decoupled in Si+ soils, where Si supplementation restores the fertilizing effects of eCO_2 on *M. sativa* at higher temperatures without affecting aphid populations.

DISCUSSION

Results from this study suggest that Si supplementation may mitigate the negative impacts of eT on plant growth in *M. sativa* which was potentially due to stimulation of root nodulation, despite the reduction in nodulation at higher temperatures reported in previous studies (e.g., Ryalls et al., 2013b). Even more advantageously, this increased nodulation did not increase susceptibility to an aphid pest at eT, which had previously been observed for Si-induced increases in nodulation at ambient temperatures (Johnson et al., 2017).

TABLE 2 | Results of statistical tests examining the effects of CO_2, temperature and Si supplementation on aphid abundance and colonization success.

Plant response Model fixed effect	Aphid abundance		Aphid colonization	
	$RD_{1,159}$	P	$RD_{1,159}$	P
CO_2	177.62	0.16	**215.23**	**0.026**
Temp	**162.39**	**<0.001**	**197.21**	**<0.001**
Si	160.42	0.16	196.16	0.30
CO_2 × Temp	160.24	0.68	195.90	0.61
CO_2 × Si	160.24	0.93	195.90	0.99
Temp × Si	160.03	0.65	105.89	0.95
CO_2 × Temp × Si	159.91	0.73	194.50	0.24

Statistically significant (P < 0.05) indicated in bold with residual deviation (RD) given.

Aphid abundance was strongly suppressed by eT and this most likely explains why aphids did not benefit from increases in plant growth and nodulation that arose under Si+ conditions under eT. While aphid numbers often increase with higher temperatures via faster development and increased fecundity, this increase ceases abruptly over a certain temperature threshold because of the adverse effects on, for example, embryo development and maturation (Ryalls and Harrington, 2017). This temperature threshold depends on species, aphid biotype, and geographical region (Awmack and Leather, 2007). *A. pisum* has adapted to the warmer climate of Australia since introduction in the 1970s (Ryalls et al., 2013a). Some populations are able to function at temperatures above 35°C, although their optimum temperature is said to be c. 20–25°C (Ryalls, 2016) and temperatures above 28°C are likely to reduce aphid growth and development (Bieri et al., 1983; Lamb and MacKay, 1988; Mackay et al., 1993). Aphid biotypes with certain secondary bacterial endosymbionts may cope better with higher temperatures, however, since there have been several reports of endosymbionts alleviating the effects of heat stress (Montllor et al., 2002; Russell and Moran, 2005; Dunbar et al., 2007). To our knowledge, studies have not yet addressed how bacterial endosymbionts might change in response to eCO_2 *and* eT but endosymbionts could partially facilitate adaptation to climate and atmospheric change (Sun et al., 2016; Ryalls and Harrington, 2017).

Several studies using temperature gradient greenhouses have examined the impacts of eCO_2 and eT on legume performance, including root nodulation (Aranjuelo et al., 2006, 2008; Erice et al., 2006, 2007). These studies report a general trend for eCO_2 promoting nodulation, but only at the elevated experimental temperatures. This was probably because the elevated temperature range used in experiments (c. 24°C; Aranjuelo et al., 2008) was still within the optimal range (19–25°C) for nodulation in temperate legumes, so inhibitory

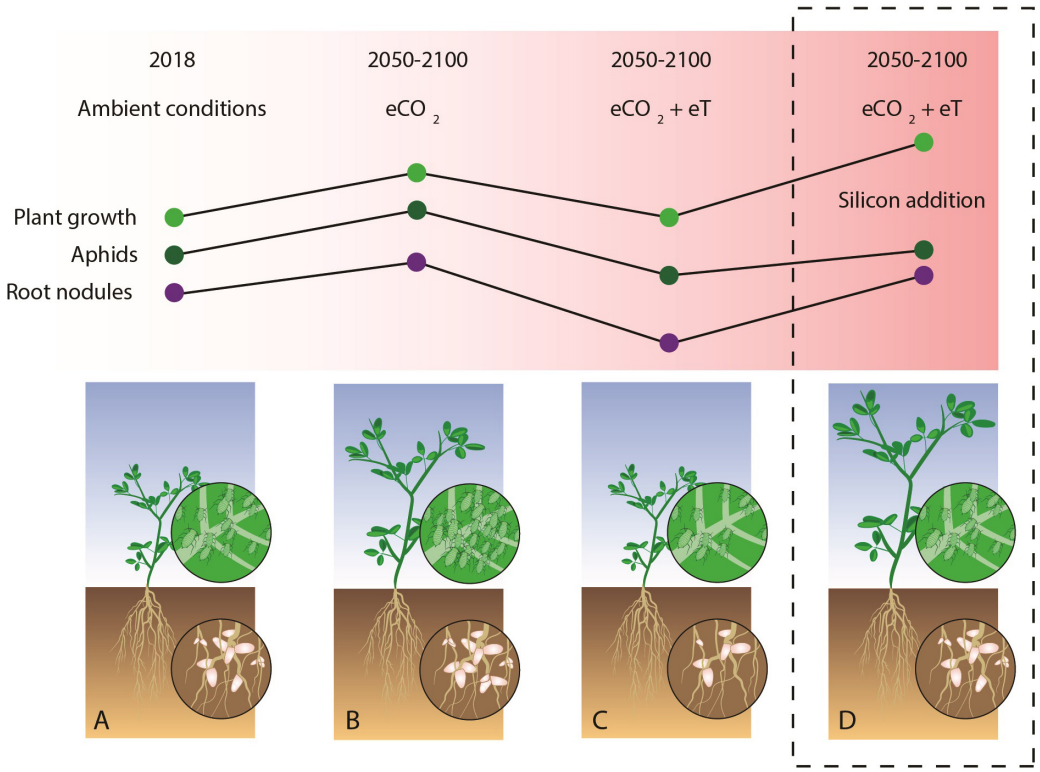

FIGURE 5 | Graphical summary showing how Si supplementation affects *M. sativa* growth, root nodulation and susceptibility to *A. pisum* in current and predicted changes to the climate and atmosphere. Compared with ambient CO_2 and temperature conditions **(A)**, eCO_2 had beneficial effects on plant growth and nodulation **(B)**, but these were negated when acting in combination with predicted increases in temperature **(C)**. Si supplementation **(D)** restored root nodulation to comparable levels as those seen under eCO_2 conditions (without warming) and stimulated plant growth beyond this. Aphid responses were decoupled from changes in nodulation and growth with Si supplementation.

effects of temperature on nodulation wouldn't necessarily have occurred (Aranjuelo et al., 2014). When temperature was elevated beyond 25–30°C, root nodulation in *M. sativa* has been reported to decrease by 22% under ambient CO_2 (aCO_2) and by 56% under eCO_2 (Ryalls et al., 2013b).

Despite increasing evidence that the effects of eCO_2 are often modified by eT, and *vice versa*, comparatively few studies manipulate both factors in tandem (Robinson et al., 2012). In the present study we established that positive impacts of eCO_2 on plant traits were not seen to the same extent when eT conditions were applied. This study therefore lends support to the notion that, wherever feasible, multiple environmental factors should be tested (Newman et al., 2011; Lindroth and Raffa, 2016). Crucially, Si supplementation had consistently stronger impacts on plant traits across a range of environmental conditions and regardless of whether plants were challenged by herbivores.

A counterintuitive finding of the study was that Si supplementation actually reduced concentrations of Si in the foliage. Si may have promoted plant growth to such an extent that Si became 'diluted' in foliage, or else had not had time to accumulate in plant tissues over the duration of the study. A similar trend in foliar Si was previously observed in this system however, associated with rapid plant growth, increases in root nodulation and synthesis of amino acids (Johnson et al., 2017). In addition to any increased nutritional value, the lower concentrations of Si in foliage of Si+ plants may explain why Si supplementation did not increase plant resistance to aphids.

Our results demonstrate conclusively the benefits of Si supplementation for root nodulation: root nodule abundance was always increased in plants growing in Si+ soil and other factors, whether CO_2, temperature and aphid presence no longer had significant impacts on nodule abundance. The mechanisms by which Si is so effective at promoting nodulation are not well-understood, but could include changes in soil conditions, increased root growth (and potential invasion sites), higher abundance of bacteroids and symbiosomes, together with the synthesis of compounds that upregulate nodulation genes (as discussed by Johnson et al., 2017). The increased nodule density reported in the present study tentatively suggests that greater nodule abundance was not merely a function of increased root growth. Further work is needed, but Si could provide a useful tool for mitigating some of the negative impacts of climate change on crop production – in this instance maintaining nodulation rates of *M. sativa* in warmer climates. Moreover, other studies suggest Si could redress negative effects of eCO_2 on plant–herbivore interactions. For example, herbivore damage to roots of sugarcane was exacerbated under eCO_2 conditions,

but application of Si reversed these effects and stimulated crop growth (Frew et al., 2017). Intervention strategies could include targeted application of Si (e.g., furnace slag), selection of plant lines that naturally take up large amounts of Si (McLarnon et al., 2017) and remediation of soils deficient in bioavailable Si (silicic acid) (Guntzer et al., 2012; Johnson et al., 2016a).

AUTHOR CONTRIBUTIONS

SJ, JR, AF, and AG: conceived the experimental design; SJ, JR, AF, and AG: acquired and processed data with SH undertaking Si analysis; JR: analyzed the data and all authors contributed to the interpretation and drafting of 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.2018.00202/full#supplementary-material

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ORIGINAL RESEARCH
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Invasion by Cordgrass Increases Microbial Diversity and Alters Community Composition in a Mangrove Nature Reserve

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Invasion by exotic plant species can alter ecosystem function and reduce native plant diversity, but relatively little is known about their effects on belowground microbial communities. Here we investigated the effects of exotic cordgrass (*Spartina alterniflora*) invasion on the distribution of soil bacterial communities in a mangrove nature reserve of the Jiulong River Estuary, southeast China using high-throughput sequencing of 16S rRNA gene and multivariate statistical analysis. Our results showed that *S. alterniflora* invasion altered soil properties, and significantly increased soil bacterial taxa richness, primarily by stimulating an increase in conditionally rare or rare taxa, and changes in community composition and function. Abundant, conditionally rare and rare subcommunities exhibited similar response patterns to environment changes, with both conditionally rare and rare taxa showing a stronger response than abundant ones. Habitat generalists were detected among abundant, conditionally rare and rare taxa, whereas habitat specialists were only identified among conditionally rare taxa and rare taxa. In addition, we found that vegetation was the key factor driving these patterns. However, our comparative analysis indicated that both environmental selection, and neutral process, significantly contributed to soil bacterial community assembly. These results could improve the understanding of the microbial processes and mechanisms of cordgrass invasion, and offer empirical data of use in the restoration and management of the mangrove wetlands.

Keywords: ***Spartina alterniflora*, biological invasion, bacterial diversity, community assembly, conditionally rare taxa, neutral process**

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INTRODUCTION

The human-mediated introduction of invasive plants has altered both the biodiversity and stability of ecosystems worldwide. These invasive plants can be increasingly expensive to control, particularly under pressures of global environmental change (Jackson et al., 2001; Wolfe and Klironomos, 2005; Bu et al., 2015; Hobbie, 2015). *Spartina alterniflora*, which is highly invasive species of cordgrass, as demonstrated by its successful introductions around the world, was first

introduced into China in 1979 (An et al., 2007; Yu et al., 2014b). Its distribution in Fujian province is more extensive than in other Chinese coastal provinces, and its expansion is replacing mangroves in the estuary of the Jiulong River at an alarming rate over recent years. This estuary is considered an important but fragile ecosystem, providing valuable ecosystem services to humans and other organisms (Wu et al., 2002; Wan et al., 2009; Yu et al., 2014b). Therefore there is an urgent need to understand the impacts of this invader on coastal ecosystem structure and function in this important area.

Although soil bacterial communities play important roles in ecosystem-level processes, most works on the effects of *S. alterniflora* invasions have focused on the plants properties, aboveground flora and fauna, abundant taxa and special soil microbial taxa associated with carbon, nitrogen and sulfur cycles (Wolfe and Klironomos, 2005; Liao et al., 2007; Zhou et al., 2009; Zhang et al., 2013b; Lin et al., 2015). Unfortunately, little is known about how *S. alterniflora* affects the rare bacterial subcommunities in the soils because it has been hard to study (e.g., by denaturing gradient gel electrophoresis (DGGE), clone library), the rare biosphere albeit its significant contribution to the cycling of particular elements such as nitrogen or sulfur (Pedrós-Alió, 2012; Hong et al., 2015). Currently, the development of high-throughput and deeper sequencing is allowing a much more direct identification, and increasing interest in, the rare biosphere community (Lekberg et al., 2013; Pholchan et al., 2013; Shade et al., 2014; Aanderud et al., 2015; Liu et al., 2015; Lynch and Neufeld, 2015). Recent studies indicated that abundant and rare taxa have similar spatial patterns, but do not contribute equally to the community variation because of differences in their ecological niches, role and intrinsic properties (Campbell et al., 2011; Liu et al., 2015; Chen et al., 2017; Dai et al., 2017). Other studies have provided detailed evidence of dynamic variation for rare taxa, implying that some rare taxa may be inactive or even permanently dormant, while others may conditionally bloom under favorable environmental conditions and conduct important ecological processes (Pedrós-Alió, 2012; Shade et al., 2014). However, the way rare bacterial taxa change after *S. alterniflora* invasion is currently unclear. In this study, we hypothesized that rare taxa do not respond in the same way as the abundant taxa to *S. alterniflora* invasion. The study of rare biosphere variation may give a better understanding of the influence of *S. alterniflora* invasion on soil bacterial community and ecosystem function.

Generally, bacterial communities are simultaneously influenced by both niche-based (e.g., environmental selection and niche partitioning) and neutral-based (e.g., ecological drift) processes. However, the relative importance of these processes in community variation remains difficult to resolve (Hanson et al., 2012; Logares et al., 2013). Several factors such as disturbance, habitat connectivity and size, predation, and resource availability have diverse and complex influences on the relative importance of niche-based vs. neutral-based processes in the assembly of bacterial communities (Zhou et al., 2014). Taxa with different relative abundances have been shown to be driven by different environmental factors (Pedrós-Alió, 2012; Liu et al., 2015). Other studies have provided evidence that taxonomic resolution, which can detect evolutionary forces, may influence the strength of community-environment relationship (Lu et al., 2016). Here, we hypothesized that factors affecting the different bacterial communities are not the same and that the impacts of niche divergence or niche conservatism based on observed evolutionary patterns or scales are different.

In this study, high-throughput sequencing of 16S rRNA gene (V3–V5 regions) was used to investigate the soil bacterial community in the mangrove nature reserve of the Jiulong River Estuary in Fujian province, southeast China. We aimed to compare the influence of *S. alterniflora* invasion on bacterial community composition and function, and determine the key factor for driving microbial community assembly at different relative abundances, different niche breadths and different taxonomic resolutions. Particularly, we aim to answer the following key questions: how do belowground bacterial community composition, diversity, function change after *S. alterniflora* invasion in mangrove wetlands? Which taxa are most sensitive to *S. alterniflora* invasion? Are the controlling factors, and their contribution to the variation of community, different based on the analysis of relative abundance and taxonomic resolution?

MATERIALS AND METHODS

Study Site and Sampling

This study was carried out in a mangrove nature reserve on the Jiulong River Estuary (117°53′-117°55′E, 24°25′-24°29′N) in Fujian province, southeast China (Figure S1). In this subtropical coastal wetland, the dominant plant is the mangrove *Kandelia obovata*, however *Spartina alterniflora* has invaded a large area over the past few decades. All sampling stations and study design also featured in our previous studies on other aspects of this system-biogenic elements (Yu et al., 2015) and microeukaryotic community (Yu et al., 2014b). In the current study, all sampling stations were located in the intertidal zones where sediments are not always covered with water. Sediments samples were collected from four different types of habitats, i.e., unvegetated bare mudflat, cordgrass invaded zone with *S. alterniflora*, ecotone area with *S. alterniflora* and mangrove growing mixed together in the same area and native mangrove zone in four seasons, spring (April 2010), summer (August 2010), autumn (November 2010), and winter (January 2011). All samples were collected from the top 0–5 cm layer in sediment using a polyvinyl chloride (PVC) pipe (7 cm in diameter) and transported to the laboratory. In total, 16 sediment samples were collected from four stations across four seasons. We treated four different seasons as replicates, because bacterial communities among four seasons could not be significantly distinguished and our previous study confirmed that the microbial communities were relatively stable over four different seasons based on three replicates for each sample (Yu et al., 2014b). Each sample was freeze-dried at −55°C, then homogenized, filtered through a 150 μm mesh and finally stored at −80°C until further analysis.

Measurement of Environmental Parameters

Salinity and pH of sediment porewater were measured by an ATAGO digital salt meter (Japan) and a Starter 2C pH meter (China), respectively. Total carbon (TC), total nitrogen (TN), total sulfur (TS), total phosphorus (TP), ammonium nitrogen (NH_4-N), nitrite and nitrate nitrogen (NO_x-N) concentrations were measured following standard methods used in our previous study (Yu et al., 2015). Detail information about environmental factors can be found in supplementary information (Figure S2).

DNA Extraction, PCR and 454 Pyrosequencing

Total DNA was extracted and purified from 0.5 g of dry sediment using the FastDNA SPIN Kit and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. All DNA samples were checked for quality using the agarose gel electrophoresis and quantified using the NanoDrop ND-100 device (Thermo Fisher Scientific, Waltham, MA, USA). The V3-V5 hypervariable region of 16S rRNA gene was amplified using the 357F (CCTACGGGAGGCAGCAG) and 926R (CCGTCAATTCMTTTRAGT) primer pair followed the protocol as described by Yu e al. (2014a). Each primer set used for PCR amplification contained an eight base DNA barcode for the multiplexing of samples in the pyrosequencing runs. In 50 μl reactions containing 1 μl of the primer set (10 μm each), 0.125 μl (5 U/μl) of Ex Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan), 2.5 μl of Ex Taq buffer (20 mM $Mgcl_2$), 2 μl of deoxyribonucleotide triphosphate mixture (2.5 mM each, Takara Bio, Otsu, Shiga, Japan) and 50 ng of DNA template, PCR was performed included an initial denaturation at 94°C for 4 min, 25 cycles of 30 s at 94°C, 45 s at 50°C, 1 min at 72°C, and a final elongation step for 8 min at 72°C. Pyrosequencing was performed with Genome Sequencer FLX Titanium emPCR Kit (Lib-L) on a Roche 454 GS FLX+ Instrument according to the manufacturer's protocols (454 Life Sciences, Branford, CT, USA).

Bioinformatics

Pyrosequencing flowgrams data were converted to sequence reads using the standard software provided by 454 Life Sciences. All the raw sequence data were processed in QIIME software packages (Caporaso et al., 2010). The sequences were quality-controlled using the split_libraries.py with following settings: 200 < sequence length < 1,000, mean quality > 25, ambiguous bases < 1, and homopolymer length < 6. Sequences were denoised using AmpliconNoise algorithm (shhh.seqs) in MOTHUR v.1.33.3 (Schloss et al., 2009; Quince et al., 2011; Liu et al., 2014). The sequences were then analyzed using pick_otus.py script (based on 97% sequence similarity). Chimeric sequences were checked by the ChimeraSlayer and then removed prior to further analysis (Schloss et al., 2009; Haas et al., 2011). Sequences were taxonomically classified using an 80% confidence threshold against the RDP Database (Yu et al., 2014a). All Archaea, Eukaryota, chloroplasts, mitochondria and unknown sequences and singleton OTUs were removed before the downstream analyses. Finally, in order to compare the community pattern between samples, the sequence data were normalized to 10,342 sequences per sample. The final cleaned sequence data set retained 165,472 reads and 9,749 OTUs at 97% sequence similarity level after trimming and quality filtering.

All sequence data, generated using Roche's 454 GS FLX+ system have been submitted to the Short Read Archive (SRA) database at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) under the BioProject number PRJNA415893 and the accession number SRP122000.

Definition of Rare, Conditionally Rare, and Abundant Taxa

Microbial communities normally consist of a few abundant, and many rare species (Pedrós-Alió, 2012). Defining the rare biosphere is of somewhat arbitrary (Lynch and Neufeld, 2015). In this study, the thresholds for rare, conditionally rare, and abundant taxa were defined based on relative abundance cut-offs, with reference to recent publications (Liu et al., 2015; Chen et al., 2017; Dai et al., 2017). Rare taxa were defined as the OTUs with a relative abundance always < 0.01% in all samples. Conditionally rare taxa were defined as the OTUs being rare (relative abundance < 0.01%) in some samples but never being abundant (relative abundance ≥ 1%). Abundant taxa were defined as the OTUs that do not fall in either rare or conditionally rare categories. To reduce the effect of arbitrary definition of abundant and rare OTUs, multivariate cutoff level analysis (MultiCoLA) was used to evaluate how our data sets were influenced by different definitions (Gobet et al., 2010).

Definition of Habitat Specialists, Generalists and Strict Habitat Specialists

The "Niche breadth" approach (Levins, 1968) was used to measure habitat specialization using the formula:

$$B_j = \frac{1}{\sum_{i=1}^{N} P_{ij}^2}$$

where B_j represents niche breadth and P_{ij} indicates the percentage of individuals belonging to species j present in a given habitat i. OTUs with mean relative abundances $\geq 2 \times 10^{-5}$ were considered in this study, as these taxa probably indicate specialized taxa (Pandit et al., 2009). Habitat generalists will have a higher B-value and be more evenly distributed along a wider range of habitats compared with habitat specialists. In this study, there are four types of vegetation zones with each type representing a given habitat. OTUs with $B > 3$ were arbitrarily defined as generalists, but OTUs with $B < 1.5$ were defined as specialists. $B > 3$ was selected because this value lies within the outlier area of the B distribution. $B < 1.5$ was chosen as it is close to 1, the smallest possible B-value.

To identify strict habitat specialists for each type of vegetation, we performed indicator species analysis (ISA). Data used in the ISA were similar to those used in the analysis of "Niche breadth," and samples were partitioned to mudflat, cordgrass, ecotone and mangrove as explained in the above section. Phylotypes with a P-value < 0.05 and both, a fidelity and specificity value ≥ 0.8,

were considered as a good threshold for strict habitat specialists (Dufrene and Legendre, 1997).

Analyses of Community Diversity

Rarefaction curves, ACE, Chao 1, Shannon-Wiener, Simpson and Pielou's evenness indices were calculated using MOTHUR v.1.33.3 (Schloss et al., 2009). One-way analysis of variance (ANOVA) was used to test the effects of vegetation and season on these indices by SPSS 20.0 (IBM Corp., Armonk, NY, USA).

Bray-Curtis similarity matrices within and between vegetation types were constructed using the bacterial community data based on relative abundance and database annotation, respectively. To compare the stability of habitats, coefficient of variation (CV) of Bray-Curtis dissimilarity across vegetation types or seasons was calculated. The non-metric multidimensional scaling (NMDS) ordination was performed using Bray-Curtis similarity matrices to investigate differences in bacterial community composition among samples using PRIMER 7.0 (Clarke and Gorley, 2015). Analysis of similarities (ANOSIM) was used to evaluate the significant differences between groups. No separation is indicated by $R = 0$, whereas $R = 1$ suggests complete separation (Clarke and Gorley, 2015). Spearman's rank correlations were used to determine the relationships between the Bray-Curtis similarity of bacterial community, Euclidean distance of environmental variables and the geographical distance of sampling sites, respectively.

Relationships between Community Composition and Environmental Variables

Preliminary detrended correspondence analysis (DCA) of bacterial community data showed that the longest gradient lengths were shorter than 3.0, thus redundancy analysis (RDA) was used for further analysis. All environmental variables except pH were log(x+1) transformed to improve normality and homoscedasticity. Monte Carlo permutation tests were applied to evaluate the effect of environmental variables on variations in the soil bacterial community. Environmental factors with variance inflation factors (VIF $>$ 20) were deleted to avoid collinearity. Variation partitioning analysis (VPA) was performed on the basis of RDA. We quantified the pure and shared influences of three groups of explanatory variables (environmental variables, season, and vegetation) on bacterial community composition variations. The residual fraction accounted for unexplained variation. DCA, RDA, and VPA were run in R using the vegan package (version 3.3.2) (R Development Core Team, 2015).

The distance-based redundancy analysis (Legendre and Anderson, 1999; Peres-Neto et al., 2006) was used to quantify the strength of community-environment relationships along taxonomic ranks by using the "capscale" function of "vegan" package in R (version 3.3.2) (R Development Core Team, 2015). Environmental factors used were similar to RDA analysis when we conducted distance-based redundancy analysis to equitably compare environmental influence on community composition along taxonomic ranks.

Predicted Functional Profiles

To predict bacterial functional responses to the *S. alterniflora* invasion, we used PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states; http://picrust.github.com; Langille et al., 2013) to generate a functional profile using our 16S rRNA gene data. We followed the suggested methods for OTU picking with Greengenes v. 13.5 using Galaxy (http://huttenhower.sph.harvard.edu/galaxy/). KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology group levels 2 and 3 of the predicted gene family abundances were compared using NMDS, ANOSIM and heat map, respectively.

Neutral Community Model

To determine the potential importance of neutral processes to the whole bacterial community, we used Sloan's neutral community model (NCM) to predict the relationship between OTU detection frequency and their relative abundance along taxonomic ranks (Sloan et al., 2006). This neutral model can incorporate the influences of demographic and random dispersal processes (Logares et al., 2013). In this model, the parameter R^2 determines the overall fit to the neutral model. The binomial proportion 95% confidence intervals around the model predictions were analyzed using the Wilson score interval in R using HMisc and minpack.lm packages (Logares et al., 2013).

RESULTS

Alpha Diversity of Bacterial Communities

A total of 165,472 high-quality sequences reads were obtained from all samples collected in the Jiulong River estuary, and were clustered into 9,749 operational taxonomic units (OTUs) based on 97% similarity level. The estimated species accumulation curves based on the pooled data set indicated that the majority of the bacterial taxa had been recovered from the studied sites, although none of single samples showed a full saturation in the rarefaction curve (Figure S3).

The one-way ANOVA indicated that vegetation types had significant effects on the number of OTUs, ACE, Chao 1, and Shannon-Wiener indices, but no significant difference of these alpha-diversity indices was found across four seasons (Table S1). Further, the community richness indices (number of OTUs, ACE, and Chao 1) in both partial and full cordgrass invasion zones were significantly higher than at the mudflat and mangrove stations, which indicates that cordgrass invasion may increase the alpha-diversity and richness of the soil bacterial community (**Figure 1**). However, Pielou's evenness showed no significant difference between the four types of vegetation zones or four seasons (**Figure 1** and Table S1).

Conditionally rare taxa comprised of 7,516 OTUs (77.10%) and 127,233 sequences (76.89%) were the most diverse and dominant among three categories (abundant, conditionally rare, and rare), whereas 96 (0.98%) OTUs with 32,364 sequences (19.56%) were defined as being in the abundant taxa category, and 2,137 (21.92%) OTUs with 5,875 (3.55%) sequences were defined as rare taxa (Table S2). The multivariate cutoff level analysis showed that our definitions of abundant (19.56%) and

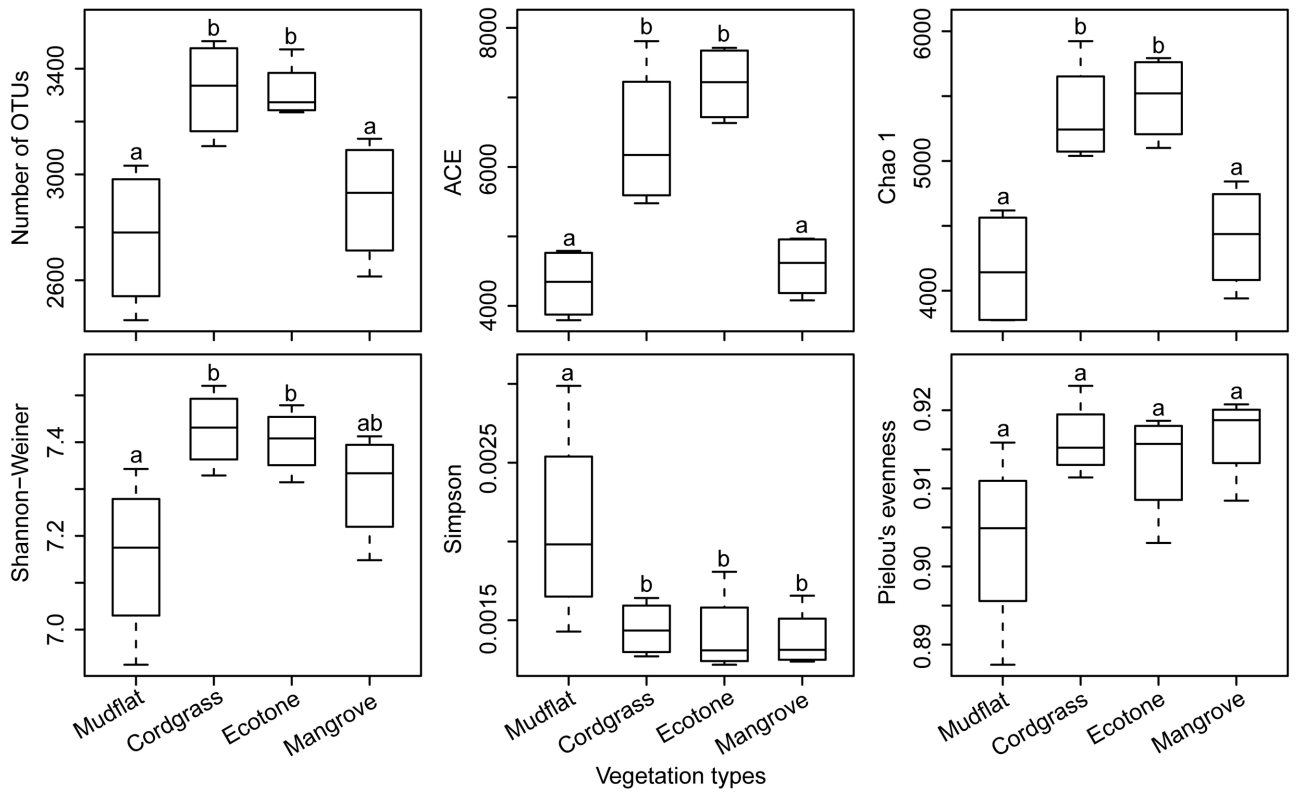

FIGURE 1 | Comparison of alpha-diversity of the soil bacterial communities among four different types of vegetation zones. Ecotone indicates a transition zone with the distributions of cordgrass and mangrove overlap. The operational taxonomic units (OTUs) were defined at 97% sequence similarity threshold. Significant differences ($P < 0.05$) between vegetation types are indicated by different letters of the alphabet. Statistical analysis is Student's *t*-test with Bonferroni correction ($n = 4$). The ends of the box represent the 25th and 75th percentiles, the whiskers represent minimum and maximum range, and the center lines represent the median.

rare (3.55%) bacteria are reasonable within the limitations of existing technology (Figure S4).

Variations of Bacterial Taxonomic Structure

Our results revealed that the differences between bacterial communities could be attributed to vegetation type rather than seasonality or spatial effect (**Figure 2**). The entire bacterial community showed a significant negative correlation ($r = -0.554$, $P < 0.01$) with the geographical distance, while the environmental variables did not show any significant relationship ($r = -0.074$, $P = 0.42$) with the geographical distance (Figure S5). The divergences of bacterial community were very high and highly variable at different relative abundances (abundant, conditionally rare and rare) and niche breadths (generalists, specialists, strict specialists), however it was relatively low and showed a gradual decrease along taxonomic ranks from species to phylum (**Figure 3**). In terms of relative abundance, the conditionally rare subcommunity showed a striking separation compared with abundant and rare assemblies, confirmed by the pairwise Bray-Curtis dissimilarity of bacterial communities among different vegetation types (**Figure 2C**), and the analysis of similarity (ANOSIM) comparisons between soil bacterial subcommunities (**Table 1**). At niche breadths level, we identified 1,336 OTUs (13.7%) as habitat generalists and 1,079 OTUs (11.1%) as habitat specialists. Interestingly, habitat generalists were present among abundant, conditionally rare and rare taxa, whereas habitat specialists were only present in conditionally rare and rare taxa (**Figure 4B**). Based on INDVAL analysis, 158 OTUs (1.6%) were identified as strict habitat specialists (**Figure 4**). The numbers of these strict specialist OTUs among vegetation types were 40 in mudflat zone, 6 in cordgrass zone, 29 in ecotone zone, and 83 in native mangrove zone, respectively. The taxonomic compositions of strict specialists were significantly different among four types of vegetation zones (**Figure 4C**).

Seven major phyla (Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Chloroflexi) were observed in this study. Proteobacteria (abundant 12.5 ± 0.5% vs. conditionally rare 35.7 ± 1.2% vs. rare 1.6 ± 0.1% based on the whole community) was the most dominant phylum in all subcommunities (Figure S6). At OTU level, 2,402 OTUs (24.6%) were shared among four different vegetation types, and most of the unique OTUs belonged to either conditionally rare taxa or rare taxa (Figure S7). All abundant taxa (96 OTUs) were shared among cordgrass, ecotone, and mangrove zones (**Figure 2B**).

The comparisons among the coefficient of variation (CV) of Bray-Curtis dissimilarity metrics showed that bacterial communities showed more stability between taxonomic ranks

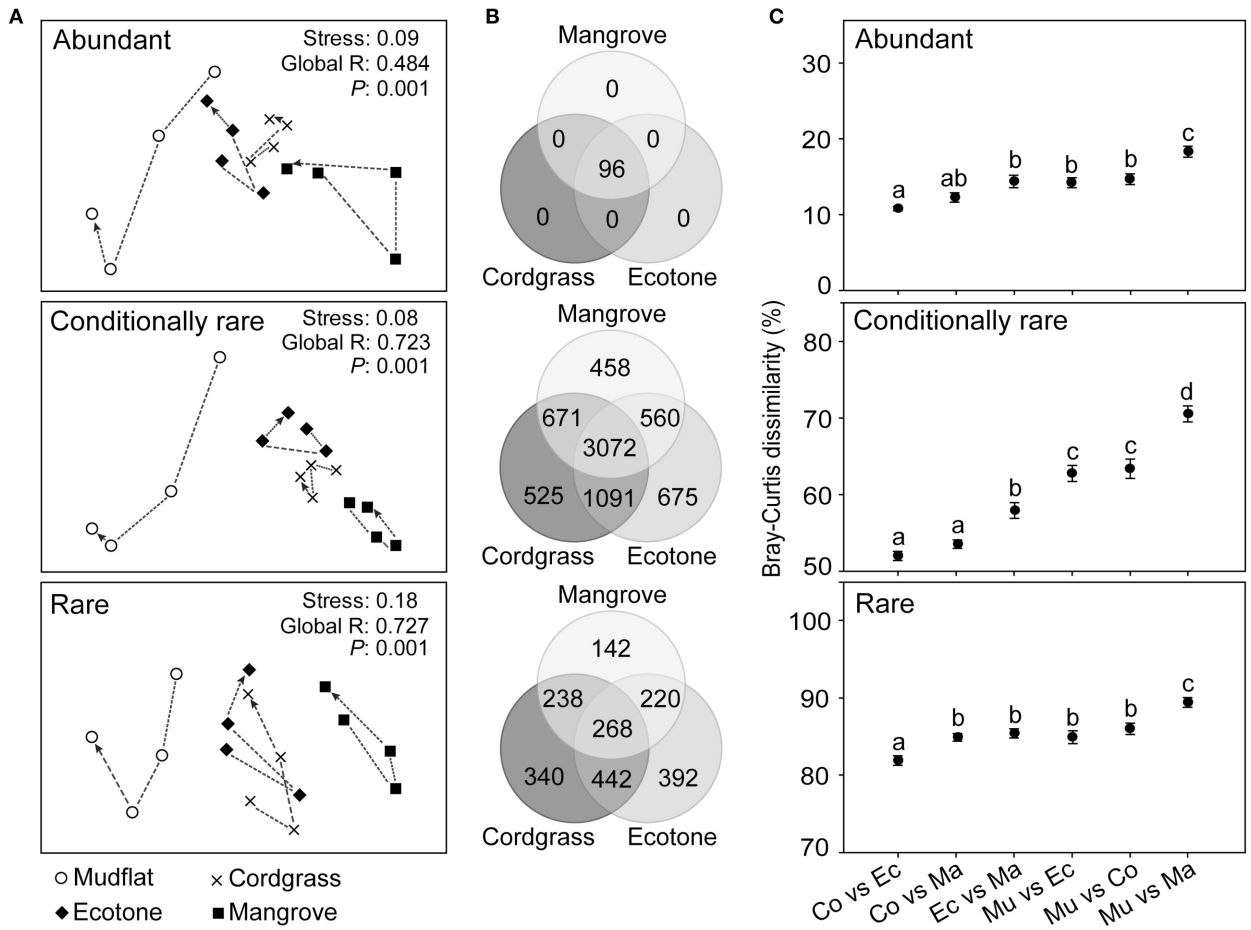

FIGURE 2 | Comparison of beta-diversity of the soil bacterial communities among different types of vegetation zones. **(A)** Non-metric multidimensional scaling (NMDS) ordination of soil bacterial communities based on the Bray-Curtis dissimilarity. Points are connected by dash lines according to the progression of time (from spring to winter). **(B)** Venn diagram showing the number of OTUs (96 abundant OTUs, 7,052 conditionally rare OTUs, 2,042 rare OTUs) that are unique and shared between three different types of vegetation zones. **(C)** Pairwise Bray-Curtis dissimilarity of bacterial communities among different vegetation types. Mu, mudflat; Co, cordgrass; Ec, ecotone; Ma, mangrove. Significant differences ($P < 0.05$) between vegetation types are indicated by different letters of the alphabet. Statistical analysis is Student's *t*-test with Bonferroni correction ($n = 16$). Data are means ± standard error (error bars).

than at different relative abundances (abundant, conditionally rare and rare) or niche breadths (generalists, specialists, strict specialists) (**Figure 3**).

Predicted Functions of Bacterial Communities

The predicted functional distribution was grouped roughly based on the vegetation types (Figure S8), indicating the strong influence of cordgrass (*Spartina alterniflora*) invasion on bacterial functional groups. The predicted bacterial community function in mudflat or ecotone zone was significant different from mangrove zone (Table S3).

The Factors Associated with Variation of Bacterial Community

The RDA ordination showed that the abundant, conditionally rare and rare taxa subcommunities were significantly correlated with total carbon (TC) and cordgrass (*Spartina alterniflora*) according to forward selection model ($P < 0.05$; **Figure 5**). However, interpretation of the first two RDA dimensions for community variability substantially decreased from abundant (58.6%) to conditionally rare (36.0%) and rare (20.1%) taxa subcommunities. Our variation partitioning indicated that the impact of environmental, vegetational, and seasonal factors contributed to the structure of abundant, conditionally rare and rare sub-communities to different degrees (**Figure 5**). The vegetation was the most important factor, followed by environmental and seasonal factors for these three bacterial subcommunities. In addition, simultaneous effects of environmental and vegetation factors or seasonal and vegetation factors jointly accounted for the variation in bacterial subcommunities, as calculated by the sum of the shared fractions.

Interestingly, the neutral model successfully described the frequency of whole bacterial taxa in the different vegetation zones across four seasons ($R^2 = 0.66$) (**Figure 6**). Further, the explained variation of neutral model and environmental selection

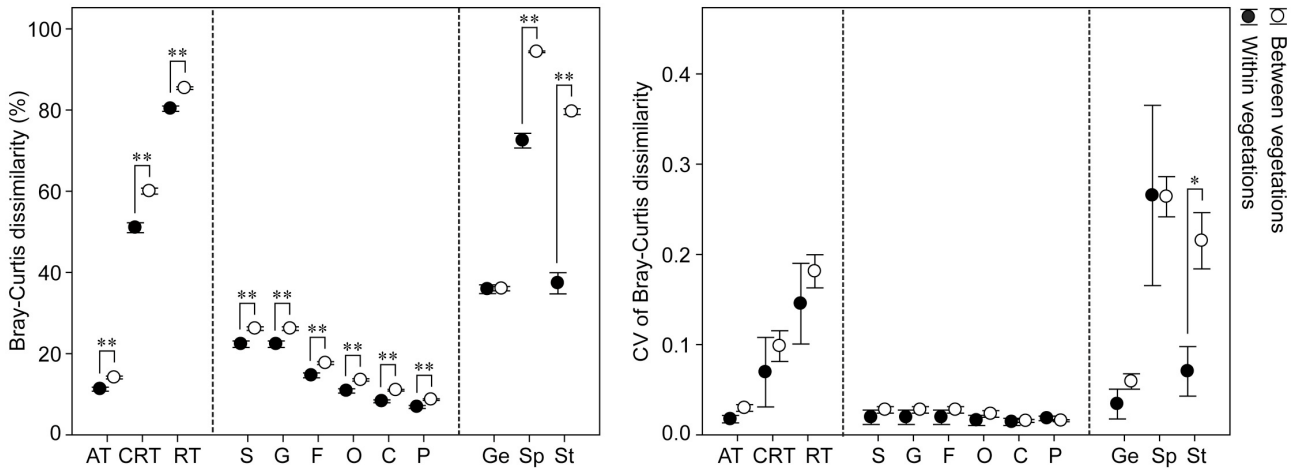

FIGURE 3 | Pairwise Bray-Curtis dissimilarity of soil bacterial community and its coefficient of variation (CV) measured at different relative abundances, different taxonomic resolutions and different niche breadths. The bacterial community analyses are conducted on four vegetation zones: mudflat ($n = 4$), cordgrass ($n = 4$), ecotone ($n = 4$) and mangrove ($n = 4$). AT, abundant taxa; CRT, conditionally rare taxa; RT, rare taxa. S, species; G, genus; F, family; O, order; C, class; P, phylum. Ge, generalists; Sp, specialists; St, strict specialists (indicator species). Significance is calculated by nonparametric Mann-Whitney *U*-test. $^*P < 0.05$, $^{**}P < 0.01$. Data are expressed as means ± standard error (error bars).

TABLE 1 | Analysis of similarity (ANOSIM) results for comparisons between soil bacterial communities in different types of vegetation zones.

Groups	Abundant taxa	Conditionally rare taxa	Rare taxa
Mudflat vs. Cordgrass	0.573*	0.729*	0.667*
Mudflat vs. Ecotone	0.500*	0.677*	0.688*
Mudflat vs. Mangrove	0.875*	0.906*	0.969*
Cordgrass vs. Ecotone	0.500	0.583*	0.396
Cordgrass vs. Mangrove	0.385*	1.000*	0.844*
Ecotone vs. Mangrove	0.635*	0.990*	0.990*

The operational taxonomic units (OTUs) were defined at 97% sequence similarity threshold.

Values show the R-value, and asterisks denote significant difference at the P < 0.05 level. The ANOSIM statistic compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups. An R-value close to "1" suggests dissimilarity between groups while an R-value near "0" suggests an even distribution of high and low ranks within and between groups, respectively.

tends to remain relatively constant along taxonomic ranks from fine to broad taxonomic levels, indicating that taxa within the same lineages generally show similar responses to environmental variations (**Figure 6**).

DISCUSSION

Invasion Effects on Alpha-Diversity of Bacterial Community

Our results clearly supported the view that cordgrass (*Spartina alterniflora*) invasion has positive effects on the alpha-diversity of soil bacterial community (**Figure 1**). So far, studies have suggested that the effects of cordgrass invasion on ecological diversity are complicated and inconsistent: having either negative, positive or no effect. For example, *S. alterniflora* invasion had negative effects on the alpha-diversity of macrobenthos (Wan et al., 2009), meiofauna (Lin et al., 2015), and nirS-containing denitrifiers (Zhang et al., 2013a), in contrast it had positive effects on the bacteria associated with the cordgrass roots (Hong et al., 2015) and nirK-containing denitrifiers (Zhang et al., 2013a). Several reasons could account for this positive effect on microbial diversity. First, the influence of *S. alterniflora* invasion on the diversity of the microbial community may depend on the community composition and structure. It has been shown that *S. alterniflora* invasion had negative, positive and insignificant effects on the diversity of methanogens, nirK-containing denitrifiers and sulfate-reducing bacteria, respectively (Zeleke et al., 2013; Zhang et al., 2013a; Yuan et al., 2016). Composition and structural variations within the entire bacterial community may offset each other. Second, different extents of invasion may lead to different results. It has been confirmed that severe plant invasions can increase mycorrhizal fungal abundance and diversity in a field experiment (Lekberg et al., 2013). This may imply that different invasion stages could result in distinct results. Also, the different sampling sites, such as endophytes vs. rhizoplane, or different latitudes, showed different results. Endophytes have been proven to be more sensitive to plant invasion than rhizosphere bacteria (Hong et al., 2015). In addition, rhizosphere bacterial diversity (Shannon-Weiner diversity index and number of DGGE bands) of *S. alterniflora* populations increased along a latitudinal gradient (Nie et al., 2010). It is important to note that the development of sequencing methods now allows a much more direct identification of the rare biosphere community in an environment (Pedrós-Alió, 2012). Conditionally rare and rare taxa, which are minor contributors to total community

abundance, had important contributions to the higher diversity in both partial and full invasions of cordgrass (Figure S6 and Table S2). Moreover, the diversity of soil bacterial communities has been linked to functionally significant processes (Wolfe and Klironomos, 2005; Wagg et al., 2014; Jing et al., 2015). Ecosystem multifunctionality is positively associated with the diversity of soil bacterial community (Jing et al., 2015) thus the increased diversity in our study may indicate a functional increase or change in *S. alterniflora* invasion zone.

Variations of Bacterial Community Composition and Functional Predictions

Our results suggest that the bacterial communities were highly variable at different relative abundances (abundant, conditionally rare, and rare taxa) and niche breadths (generalists, specialists, strict specialists), however community variability was relatively low and stable among the taxonomic ranks (**Figure 3**). Consistent with our current knowledges of the effects of *S. alterniflora* (Nie et al., 2010; Hong et al., 2015), we found that bacterial community significantly changed after invasion. However, within this general result there were also some interesting differences in the influence on the bacterial community. Our results help integrate previous studies that have been based on different relative abundant taxa (Liu et al., 2015; Dai et al., 2017), niche breadths (Levins, 1968; Logares et al., 2013), and taxonomic ranks (Lu et al., 2016), in response to *S. alterniflora* invasion. For differentially abundant OTUs, our results supported the hypothesis that taxa with different relative abundances do not respond equally to the *S. alterniflora* invasion. Conditionally rare and rare taxa had a stronger separation between groups than abundant taxa indicating a stronger influence of *S. alterniflora* invasion on these taxa (**Figure 2**). This difference in response could be explained in two ways. On the one hand, abundant taxa with high density had stronger probabilities of dispersal compared with the conditionally rare and rare taxa, thereby resulting in a widespread or ubiquitous distribution (Liu et al., 2015; **Figure 4B**). On the other hand, rare taxa-which were mainly structured by local environmental variables were more susceptive to the environmental variations (Pedrós-Alió, 2012; Shade et al., 2014; Lynch and Neufeld, 2015). For niche breadth, many habitat generalists (1,336 OTUs) and specialists (1,079 OTUs) were found in soil bacterial community (**Figure 4**), similar to what has been reported by Logares et al. (2013) for bacterioplankton. A total of 158 strict habitat specialists were found closely associated with different vegetation types. Invasion may increase habitat diversification, and the habitats are likely to be filled by a series of habitat specialists which can change the community composition and function. In brief, long-term *S. alterniflora* invasion could generate stable new niches that were filled by a series of new habitat specialists but not suitable for mangrove specialists. The relatively steady community variations along taxonomic ranks may plausibly be an artifact of limited annotation information in the database.

Cordgrass invasion not only changed the structure of bacterial community but also altered community function. Previous studies have indicated that the community structure of related functional microorganisms was transformed after *S. alterniflora* invasion, and the carbon and nitrogen cycles were influenced in the estuary ecosystem (Hong et al., 2015). In this study, the predicted functional communities were separated roughly based on the vegetation types (Figure S8). When contrasting bacterial community and predicted functional variability in the soil, higher community variability with relatively stable functional distribution was found (**Table 1** and Table S3). This is in accordance with a recent report on a marine system, where functional categories were found to be stably distributed across different zones, while community compositions varied significantly across zones (Sunagawa et al., 2015). Functional redundancy across different taxa in bacterial communities could explain this phenomenon and be regarded as a buffering capacity for an ecosystem resilience.

Community Assembly of Soil Bacteria

Several questions still remain unanswered in relation to the rare biosphere: particularly, which factors drive the variation in bacterial communities, and to what extent do these factors which influence the bacterial communities distributions vary with taxonomic resolutions (Logares et al., 2013; Liu et al., 2015; Lu et al., 2016).

It had already been shown that vegetation types, environmental factors, and seasons can drive microbial community structure in different types of ecosystems (Berg and Smalla, 2009; Zhang et al., 2013a; Yu et al., 2014b). Our variation partitioning analysis clearly showed the largest contribution to the variation in bacterial communities was from vegetation (**Figure 5**). This suggests that vegetation is the main force in shaping the soil bacterial community composition. Previous studies have shown that vegetation types can influence the bacterial community by several mechanisms-including complex effects such as the alteration of the properties of soil, litter quantity and quality, root exudates, transformation of the local microclimate or direct interactions with root-symbiotic microorganisms (Wolfe and Klironomos, 2005; Hui et al., 2017). In our study, *S. alterniflora* invasion had substantial effects on the properties of soil in the subtropical coastal wetland with the chemical characterization clustered by vegetation types (**Figure 5**; Yu et al., 2015). Some of the explanatory community variations were shared by both vegetation and environment. This may be due to the fact that vegetation can change the properties of the soil, so indirectly affect the bacterial community. In line with Sinha et al. (2009), we found that total carbon (TC) has a significant influence on the bacterial subcommunities (**Figure 5**). Vegetation can provide organic matter through leaf-litter inputs or through the release of root exudates into the soil environment (Wolfe and Klironomos, 2005).

However, the influence of environment (explanatory extent) on variations of bacterial subcommunities reduced from abundant, conditionally rare to rare taxa based on the partial RDA (**Figure 5**). One explanation for this may be the larger number of sequences of single specie for abundant taxa and their distinct ecological niches and different functions in ecosystem (Pedrós-Alió, 2012; Kim et al., 2013; Liu et al., 2015). Importantly, we found a constant strength for the

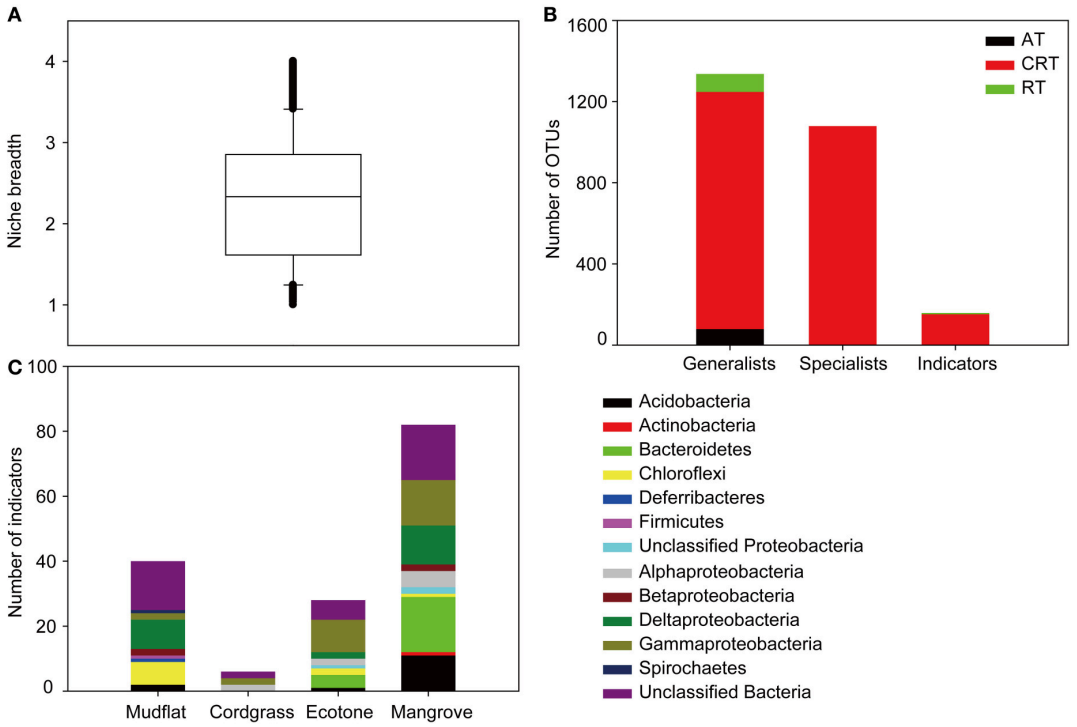

FIGURE 4 | Habitat specialization of different OTUs based on niche breadth and INDVAL (INDicator VALues) analysis. **(A)** Distribution of niche breadth values of all selected OTUs. **(B)** The number of generalists, specialists, and strict habitat specialists (indicators) belonged to abundant, conditionally rare and rare taxa. OTUs with niche breadth value >3 were arbitrarily defined as generalists, whereas those with niche breadth <1.5 were selected as specialists. For the indicators, phylotypes with a *P*-value < 0.05 and both, a fidelity and specificity value ≥0.8, were considered as a good threshold for strict habitat specialists (Dufrene and Legendre, 1997). AT, abundant taxa; CRT, conditionally rare taxa; RT, rare taxa. Indicators, strict specialists. **(C)** The number and taxonomic composition of strong indicator taxa for the specific vegetation zones. Four habitats were mudflat, cordgrass, ecotone, and mangrove.

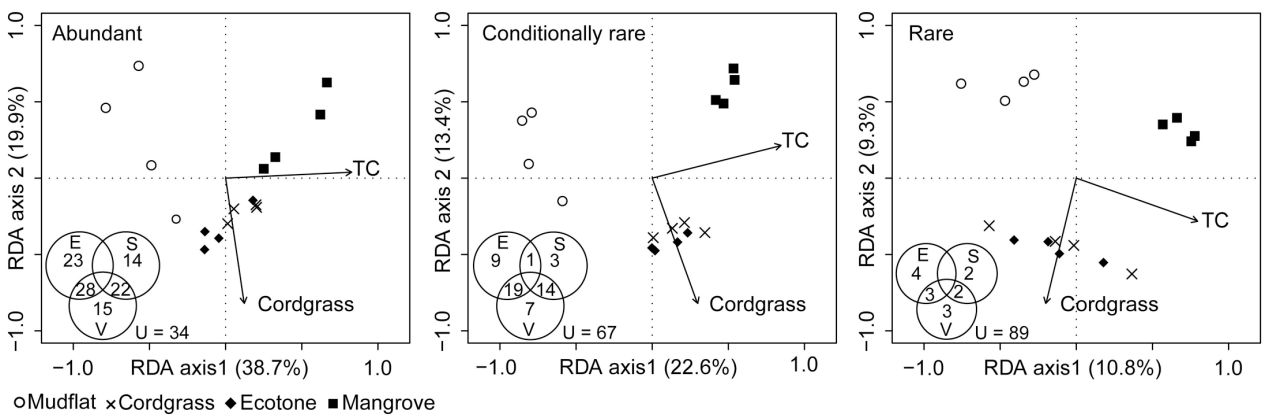

FIGURE 5 | RDA ordination showing the bacterial community composition in relation to significant vegetation and soil properties ($P < 0.05$). All environmental factors were used in this analysis except those with variance inflation factors higher than 20 (VIF > 20). Cordgrass represents for *Spartina alterniflora*. TC, total carbon. Inside Venn diagram showed results of variation partitioning analysis, illustrating the effects of environment (E), vegetation (V) and season (S) factors on the community composition of soil bacteria. Values indicate the percentage of variation explained by each fraction, including pure, shared explained and unexplained (U) variability. Note that the fraction of variation values <1% are not shown for simplicity.

community composition-environment relationships from fine to broad taxonomic levels (**Figure 6**), suggesting phylogenetic niche conservatism (the tendency of species to retain many of their ancestral ecological characteristics, Wiens and Graham, 2005). These findings further indicate that a broader taxonomic classification could strengthen niche-related signals, balance the

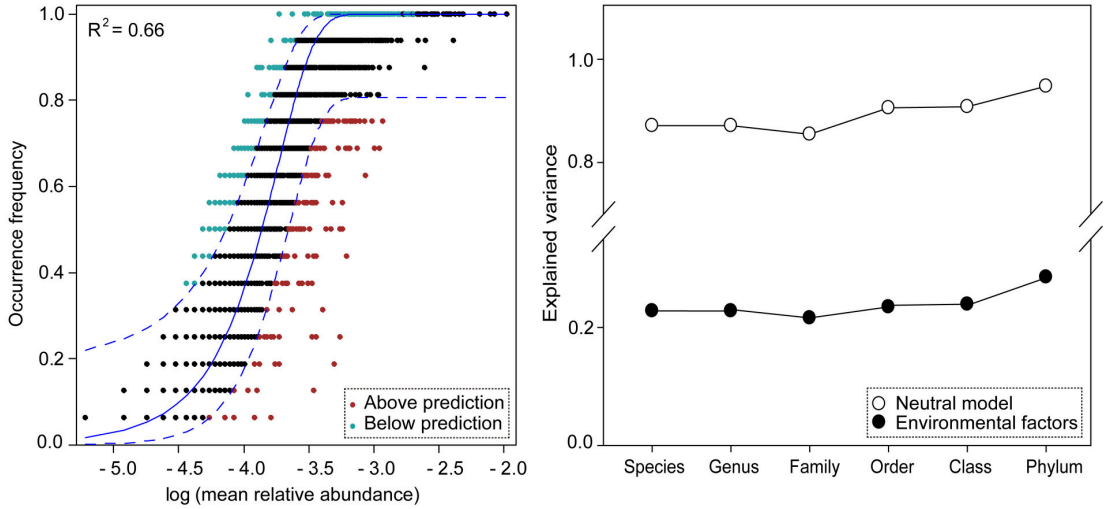

FIGURE 6 | The variation in soil bacterial metacommunity explained by neutral model and environmental variables, respectively. **(Left)** Fit of the neutral model based on the entire bacterial communities from all vegetation zones ($n = 9,749$). Frequency of occurrence of different OTUs as a function of mean relative abundance based on 10,342 reads per samples in all data sets. Dash lines represent 95% confidence intervals around the model prediction (blue line). OTUs that occur more or less frequently than predicted by the model are shown in different colors. **(Right)** Comparison of explained community variations between neutral model and environmental factors along the taxonomic ranks. Taxonomy-based compositional variation is calculated based on our database annotation from species to phylum. The soil bacterial community analyses are conducted on 16 samples from Jiulong River estuary.

distribution uncertainty associated with finer taxonomic units and support the recent view which suggest that same bacterial clades generally maintain similar ecological characteristics over evolutionary time (Martiny et al., 2015).

The unexplained community variation in variation partitioning analysis may be due to unmeasured abiotic variables (such as tide or irradiance) and biological variables (e.g., predator or virus). The tide can directly influence the bacterial species dispersal and could influence variations of physical and chemical parameters, which in turn influence microbial community dynamics (Yu et al., 2015). Moreover, based on neutral theory, ecological drift (stochastic processes of birth, death, colonization, and extinction) and evolutionary drift (stochastic genetic diversification) could also contribute to unexplained variation (Hanson et al., 2012; Zhou et al., 2014; Chen et al., 2017). Our results indeed showed that the neutral model successfully explained the 66% variations of bacterial community, indicating a strong role of stochastic processes (**Figure 6**). Altogether, community variation can also arise from an interaction mechanism between stochastic and deterministic processes due to the coexistence of multiple environmental gradients in the study areas.

CONCLUSIONS

Our results demonstrated that invasion by *Spartina alterniflora* had significant effects on the soil bacterial community composition, diversity and function in an estuarine system. Our results suggested that bacterial communities were highly variable at different relative abundances (abundant, conditionally rare, and rare taxa) and niche breadths (generalists, specialists, strict specialists), however community variation was relatively low and stable among the taxonomic ranks. Conditionally rare and rare bacteria subcommunities exhibited a stronger response to cordgrass invasion than abundant subcommunity, although the higher proportion of community variance was explained by cordgrass and total carbon for abundant taxa. *S. alterniflora* invasion may promote habitat diversification, which is likely to lead to a loss in mangrove specialists and an increase in cordgrass specialists among the bacteria. All habitat specialists and strict specialists were either conditionally rare or rare bacteria. Both environmental selection and neutral process play very important roles in the community assembly, while vegetation is the main force in shaping the soil bacterial community composition. Due to the existence of a large number of rare microbial species in natural ecosystems, future studies based on deeper high-throughput sequencing, longer time-series sampling strategy, more complete information about soil physiochemical profile and function genes analyses will be needed to improve our understanding of the invasive species and effects on the wetland ecosystem.

AUTHOR CONTRIBUTIONS

JY designed the research; ML, ZY, and XY performed the experiments; JY contributed the new reagents/analytic tools; ML and JY analyzed the data. All authors discussed the interpretation of the results and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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Belowground Plant–Herbivore Interactions Vary among Climate-Driven Range-Expanding Plant Species with Different Degrees of Novel Chemistry

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An increasing number of studies report plant range expansions to higher latitudes and altitudes in response to global warming. However, consequences for interactions with other species in the novel ranges are poorly understood. Here, we examine how range-expanding plant species interact with root-feeding nematodes from the new range. Root-feeding nematodes are ubiquitous belowground herbivores that may impact the structure and composition of natural vegetation. Because of their ecological novelty, we hypothesized that range-expanding plant species will be less suitable hosts for root-feeding nematodes than native congeneric plant species. In greenhouse and lab trials we compared nematode preference and performance of two root-feeding nematode species between range-expanding plant species and their congeneric natives. In order to understand differences in nematode preferences, we compared root volatile profiles of all range-expanders and congeneric natives. Nematode preferences and performances differed substantially among the pairs of range-expanders and natives. The range-expander that had the most unique volatile profile compared to its related native was unattractive and a poor host for nematodes. Other range-expanding plant species that differed less in root chemistry from native congeners, also differed less in nematode attraction and performance. We conclude that the three climate-driven range-expanding plant species studied varied considerably in their chemical novelty compared to their congeneric natives, and therefore affected native root-feeding nematodes in species-specific ways. Our data suggest that through variation in chemical novelty, range-expanding plant species may vary in their impacts on belowground herbivores in the new range.

Keywords: range-expanding plant species, novel weapons, plant–herbivore interactions, root chemistry, root-feeding nematodes, volatile organic compounds, *Centaurea stoebe*

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INTRODUCTION

One of the most evident ecological consequences of current climate change is the latitudinal and altitudinal range expansion of many plant and animal species (Walther et al., 2002; Parmesan, 2006; Le Roux and Mcgeoch, 2008). As not all species expand their range at similar rates (Berg et al., 2010), coevolved interactions between plants, aboveground and belowground organisms are likely

to become disrupted, whereas novel interactions can be developed in the new range (Lavergne et al., 2010; Van Der Putten, 2012). Range-expanding plant species might benefit from these new biotic conditions when they do not encounter coevolved natural enemies in the expanded range (De Frenne et al., 2014; Dostálek et al., 2015). At the same time, range-expanders will become exposed to non-coevolved natural enemies that are native to these new areas. The strength of the enemy release effect will be largely determined by the inability of the novel natural enemies to exploit the range-expanders and the ability of the range-expanders to successfully defend themselves (Verhoeven et al., 2009). The present study was initiated in order to examine how root herbivores in the new range respond to range-expanding plant species.

Range-expanding plant species could benefit from the lack of coevolved novel natural enemies when they produce chemicals to which these enemies are not adapted. Such novel chemicals make the plants either less attractive or less digestible. For intercontinental introductions of exotic plant species, this possibility has been investigated under the "novel weapon hypothesis" (Callaway and Ridenour, 2004; Schaffner et al., 2011). Several studies have shown that invasive exotic plant species produce more unique shoot compounds than native plant species in the invaded range (Cappuccino and Arnason, 2006; Macel et al., 2014), thereby negatively affecting the performance of native aboveground invertebrate herbivores (Macel et al., 2014). The strength of novel weapon effects could differ between introduced exotic plant species and intra-continental range-expanders as more natural enemies may be shared between the original range and the new range of intra-continental range-expanders than of intercontinentally introduced exotic species. Yet, aboveground herbivores that lack a co-evolutionary history with both the range-expanding and the related native plant species performed less well on some successful range-expanders than on related natives (Engelkes et al., 2008). This suggests a role for plant chemistry in the success of range-expanding plants. However, the novel weapon hypothesis so far has not been tested in studies on intracontinental range-expanding plant species. Moreover, there is a paucity of studies testing the effects of novel chemistry on belowground herbivores, both for introduced exotics and intra-continental range-expanders.

In their new range, successful range-expanding plant species on average are less negatively affected by soil communities than congeneric natives (Van Grunsven et al., 2007; Engelkes et al., 2008). This effect has been explained by the on average lower accumulation of soil-borne fungal pathogens (Morriën and Van Der Putten, 2013) and root-feeding nematodes (Morriën et al., 2012) on the roots of range-expanding plant species than on congeneric natives. However, there is considerable variation in the outcome of plant-nematode interactions among range-expanding plant species (Morriën et al., 2012; Viketoft and Van Der Putten, 2014; Wilschut et al., 2016). A likely explanation for this variation that has not yet been studied is the role of novel plant chemistry. Therefore, the aim of the present study was to examine how differences in plant-nematode interactions between range-expanding and native plant species relate to differences in root chemistries. We compared preference and reproductive performance of root herbivores on range-expanders with congeneric plant species that are native in the new range, in order to confound our tests as minimal as possible with general differences in plant chemistry.

We tested the hypotheses that native generalist root-feeding nematodes (1) are more strongly attracted to native than to range-expanding plant species, (2) prefer native plant species over range-expanding plant species and (3) show higher reproduction on native than on range-expanding plant species. We studied differences in nematode attraction to single plants of all tested plant species (hypothesis 1), differences in nematode preference between range-expanders and related natives (hypothesis 2) and differences in nematode performance between range-expanders and related natives (hypothesis 3) under both lab and greenhouse conditions. As root volatiles are known to influence attraction of entomopathogenic nematodes (Rolfe et al., 2000; Rasmann et al., 2005; Turlings et al., 2012), we examined volatile profiles of all six plant species as they also may explain patterns in root-feeding nematode attraction and preference. Together, our results will contribute to the understanding of how novel chemistry might affect belowground plant–herbivore interactions of range-expanding plant species.

MATERIALS AND METHODS

Plant Species and Seed Collections

We selected three plant species that recently expanded their range naturally from lower latitude areas to higher latitude areas in North–Western Europe and that have a related native species in their new range. Range-expanding plant species that were examined in the experiments were *Centaurea stoebe* L., *Geranium pyrenaicum* Burm. f., and *Rorippa austriaca* Crantz and their congeneric native species were *C. jacea* L., *G. molle* L., and *R. sylvestris* (L.) Besser. All six plant species now co-occur in riparian grassland areas in the eastern part of the Rhine-Waal area in The Netherlands. Therefore, these plant species are subjected to at least partly overlapping abiotic and biotic conditions. Range-expanding *R. austriaca* and *G. pyrenaicum* naturally established in the Netherlands at the end of the 19th century and are now widespread, while the first population of range-expanding *C. stoebe* in the Netherlands was recorded in the last decade of the 20th century (Floron, 2017). Seeds of all six plant species originate from natural areas in the Netherlands. Seeds of *C. stoebe*, *G. molle*, *R. austriaca,* and *R. sylvestris* were directly collected from single populations the field. Seeds of *C. jacea* were collected from mother plants that were grown in an outside experiment at NIOO-KNAW (Wageningen, The Netherlands) from seeds collected in a natural population. Seeds of *G. pyrenaicum* were delivered by the company Cruydthoeck (Nijeberkoop, Netherlands), that grows wild plants under field conditions from seeds that originate from natural field sites. For all experiments, seeds of *Centaurea* and *Geranium* species were surface-sterilized by washing for 3 min in a 10% bleach solution, followed by rinsing with demineralized water, after which they were germinated on glass beads. Due to their small size, seeds of both *Rorippa* species were not surface-sterilized, but directly

germinated on sterilized soil. Seeds were germinated in a climate cabinet at 20/10°C and 16 h light/8 h darkness.

Nematodes

We used cultures of two root-feeding nematode species, the ectoparasitic *Helicotylenchus pseudorobustus* Steiner (hereafter *Helicotylenchus*) and the sedentary endoparasitic *Meloidogyne hapla* Chitwood (hereafter *Meloidogyne*), originating from populations in The Netherlands. We selected these species as they both have a wide host range, are common and widely distributed throughout Europe (Bongers, 1988). Both used cultures were previously established in a greenhouse at NIOO-KNAW. The culture of *Helicotylenchus* on Marram grass (*Ammophila arenaria* L.) originates from nematodes collected from coastal dunes. The culture of *Meloidogyne* originates from nematodes collected from a field near Bovensmilde (Drenthe, Netherlands) which were subsequently cultured on tomato (*Solanum lycopersicum* L.).

Nematode Choice Experiments

To study differences in nematode attraction and preference, we performed choice experiments on agar and in soil, where nematodes could move to one of two opposing treatments. To examine nematode *attraction* to a plant species, we planted one seedling of a species at one side and left the other side unplanted. To examine nematode *preference* for either natives or range-expanders we planted single seedlings of congeneric native and range-expanding plant species at opposing sides of the test units. As a control for attraction and preference, we examined nematode movement in test units without seedlings. We calculated the percentage of nematodes moving to either one of the sides of the test units.

Choice Experiment on Agar

To examine nematode choice *in vitro*, we used Petri dishes of 9 cm diameter filled with 20 ml 0.5% microbial agar (Merck kGaA, Germany) (Piskiewicz et al., 2009). We used eight independent replicates for each treatment. We placed 20-days-old seedlings 4 cm from the center of the Petri dish. Thereafter, the Petri dishes were placed in a climatized chamber at 16/8 h light/dark and 20°C. After 2 days, 20 μl of tap water suspension containing 40 juveniles of either *Helicotylenchus* or *Meloidogyne* was pipetted at the center of the Petri dishes. Nematode choice was examined 2 days after inoculation by counting using a stereo-microscope (200× magnification). We considered a nematode to be significantly attracted to one treatment when it moved at least 0.5 cm into the half of the Petri dish oriented toward that treatment.

Choice Experiment in Soil

To examine nematode choices under more natural conditions than on agar, we performed a choice experiment in soil-filled Y-tubes (Van Tol et al., 2001; Piskiewicz et al., 2009) in a greenhouse at 16/8 h light/dark and 20/15°C. We used six independent replicates for each treatment. Each Y-tube consisted of a core piece and two removable arms (see Supplementary Figure 1A), which were all filled with gamma-sterilized soil (25 KGray, Syngenta bv, Ede, Netherlands). The soil originated from a former agricultural field (Beneden-Leeuwen, Netherlands; N51° 53.952, E05° 33.670) in a riparian system where all plant species can occur. Prior to sterilization, the field soil was homogenized with sand at a rate of 2:1 (w:w) in order to reduce the relative clay content. Seedlings of 20 days old were planted in the Y-tube arms. Soil moisture was adjusted to 10% (w:w) and maintained at this level until nematode inoculation. Five days after planting the seedlings, 2 ml of water suspension with 200 *Helicotylenchus* or *Meloidogyne* juveniles was inoculated 2 cm deep in both sides of the core piece, to have an equal distribution of nematodes throughout the core piece. Then, both units with the planted seedlings were placed on the Y-tube and for the remaining experimental time the arms were moistened daily with 5 ml of demineralized water. After that, nematodes could enter an arm in which the roots were growing. Four days after inoculation, the two arms of the Y-tube were separated and nematodes from each arm and the core piece were extracted by Cobb's decantation (Cobb, 1918) and counted using an inverted light microscope (200× magnification).

Nematode Reproduction Experiment

For each plant species, ten 12-days-old seedlings were planted separately in 11 cm × 11 cm × 12 cm pots filled with soil homogenized and sterilized as explained above. The pots were placed in a greenhouse in a randomized block design with five replicate blocks. After 12 days, pots were inoculated with 2 ml water suspension with either 200 *Meloidogyne* or 200 *Helicotylenchus* juveniles. During the subsequent 16 weeks the pots were watered twice a week and kept on the same weight of approximately 870 g, of approximately 15% (w:w) soil moisture content. Thereafter, roots and soils were separated and used for nematode extraction. All roots were washed in 200 ml tap water, after which the washing water containing nematodes that were present in the rhizosphere was stored. Nematodes of each individual replicate were combined into a single sample by extracting all nematodes from the wash and soil using an Oostenbrink elutriator (Oostenbrink, 1960). Roots collected from pots inoculated with the ectoparasite *Helicotylenchus* were dried at 70°C. Roots from pots inoculated with *Meloidogyne* were split and both halves were weighed fresh. One half of the roots was dried at 70°C until constant weight, whereas the other half was cut into pieces of 1–2 cm and placed for 4 weeks in a mistifier to extract nematodes from the inside of the roots (Funnel-spray method; Oostenbrink, 1960). Total dry root biomass was assessed using total fresh root weight and fresh/dry weight ratio of each sample. Nematode suspensions were harvested from the mistifier after 2 and 4 weeks, combined, and concentrated to 10 ml. Nematodes were counted using an inverted light microscope (200× magnification).

Root Volatile Analysis

To relate nematode attraction, preference, and performance to root chemistry, we analyzed root volatile profiles by Gas Chromatography Quadrupole Time of Flight (GC-QTOF) analysis.

Volatile Trapping

Four 20-days-old seedlings of each plant species were placed in individual 70 ml glass pots filled with sterilized soil (see choice experiment in soil). After 15 days, steel traps containing the volatile absorbants Tenax TA (150 mg) and Carbopack B (150 mg; Markes International Ltd., Llantrisant, United Kingdom) were attached at both sides of the glass pots (Supplementary Figure 1B). After 24 h of incubation the traps were removed, capped and stored at 4°C until GC-QTOF analysis.

GC-QTOF Analysis of Volatiles Compounds

The volatiles were collected from the traps using an automated thermos desorption unit (Unity TD-100, Markes International Ltd., Llantrisant, United Kingdom) at 210°C for 12 min (Helium flow 50 ml/min) and trapped on a cold trap at −10°C. The volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, CA, United States) by heating the cold trap for 3 min to 280°C. Split ratio was set to 1:10, and the column used was a 30 mm × 0.25 mm ID RXI-5MS, film thickness 0.25 μm (Restek 13424-6850, Bellefonte, PA, United States). The following temperature program was used: 39°C for 2 min, from 39 to 95°C at 3.5°C/min, then to 165°C at 6°C/min, to 250°C at 15°C/min and finally to 300°C at 40°C/min and 20 min at 300°C. The volatiles were detected by a mass spectrometer (MS) operating at 70 eV in EI mode. Mass spectra were acquired in full-scan mode (30–400AMU, 4 scans/s). GC-MS-data were collected and converted to a mzData file using the Chemstation B.06.00 (Agilent Technologies, United States). Data were further processed with MZmine 2.14.2 (Pluskal et al., 2010) with the tools mass detection (centroid mode, noise level = 1000), chromatogram builder (min time span = 0.05 min, min height = 1.5E03, m/z tolerance of 1 m/z or 5 ppm), and chromatogram deconvolution (local minimum search, chromatographic threshold = 40%, Min in RT range = 0.1 min, Min relative height = 2.0%, Min absolute height = 1.5E03, Min ratio of peak top/edge = 2, peak duration = 0.0–0.5 min). Detected and deconvoluted peaks were identified by their mass spectra using NIST MS Search and NIST 2014 (National Institute of Standards and Technology, United States) and aligned using Random Sample Consensus (RANSAC) aligner (mz tolerance = 1 m/z or 5 ppm, RT tolerance = 0.1, RT tolerance after correction = 0.05, RANSAC iteration = 10000, Min number of points = 60%, threshold value = 0.1). Processed data were exported for further statistical analysis as explained under 'Statistical analysis.' The identification of detected compounds was further evaluated using the software AMDIS 2.72[1] (Stein, 1999). The retention indexes were calculated for each compound and compared with those found in NIST 2014 and in-house databases.

Statistical Analyses

Differences in nematode attraction and preference were tested by pair-wise *t*-tests in SigmaPlot (Systat software, Inc.). Overall differences in nematode attraction between natives and range-expanders were tested using general linear models with origin as fixed factor and plant species as random factor (packages lme4 and lmerTest; Bates et al., 2014; Kuznetsova et al., 2015) using R studio (version 0.98.507; R Core Development Team, 2012). Differences in nematode numbers between plant species were tested for each nematode species separately using generalized linear models with a negative binomial error distribution (MASS package; Venables and Ripley, 2013) modeling fixed factors 'plant species' and 'experimental block'. Wald *post hoc* tests were then used to test for differences between plant species using the phia package (De Rosario-Martinez, 2013). Using Pearson correlation tests, we examined whether nematode reproduction corresponded with nematode attraction in the y-tubes. Analyses on volatile data were performed using MetaboAnalyst V3.0[2] (Xia et al., 2015). Prior to One-way ANOVA and multivariate analyses (PLS-DA) data were normalized via log-transformation and auto scaling. To identify mass features significantly differing between plant species, a one-way-ANOVA with *post hoc* Tukey HSD-tests was performed. Mass features were considered to be statistically relevant when *p*- and FDR-values were ≤ 0.05.

RESULTS

Nematode Attraction

First, we confirmed that the controls in the nematode attraction experiments were effective. Indeed, when the tests were performed in the absence of plants both on agar and in soil neither *Helicotylenchus* nor *Meloidogyne* showed significant movement away from the point of addition (**Figure 1** and Supplementary Figure 2).

Meloidogyne

On average, there was a trend of stronger attraction of *Meloidogyne* to natives than to range-expanding plant species on agar (natives: 25.3 ± 3.6%, range-expanders: 10.9 ± 3.9%; $F = 7.56$, $p = 0.051$), but this was not significant in soil (natives: 21.9 ± 4.4%, range-expanders: 9.0 ± 3.8%; $F = 4.86$, $p = 0.09$). On agar, all natives significantly attracted *Meloidogyne* away from the empty control (all *t*-values > 3.48, all *p*-values < 0.05; Supplementary Figure 2A), whereas none of the range-expanders did so (Supplementary Figure 2A). In soil, all three native species significantly attracted *Meloidogyne* away from the empty controls (all *t*-values > 6.65, all *p*-values < 0.01; **Figure 1A**). Both range-expanding *Geranium* and *Rorippa* also attracted *Meloidogyne* away from the empty control in soil (*t*-values > 4.84, *p*-values < 0.01; **Figure 1A**). Interestingly, the range-expanding *Centaurea* significantly repelled *Meloidogyne* toward the empty control in both agar and soil (*t*-values < −3.21, *p*-values < 0.05; **Figure 1A** and Supplementary Figure 2A). Thus, all natives significantly attracted *Meloidogyne*, whereas range-expanders either repelled *Meloidogyne* or attracted *Meloidogyne* only in one of the two test units.

[1]http://chemdata.nist.gov/

[2]www.metaboanalyst.ca

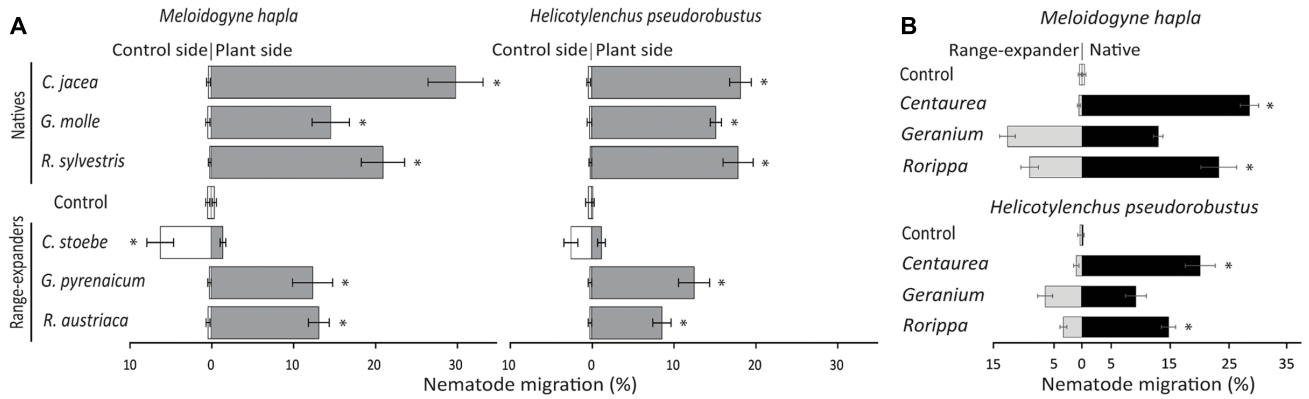

FIGURE 1 | (A) Attraction or repellence (% individuals migrated) of the nematode species *Meloidogyne hapla* and *Helicotylenchus pseudorobustus* by native and range-expanding plant species in sterilized soil. **(B)** Nematode preference between native plant species *Centaurea jacea*, *Geranium molle,* and *Rorippa sylvestris* and congeneric range-expanders *C. stoebe*, *G. pyrenaicum,* and *R. austriaca*. In both panels horizontal bars show averages ± standard errors and asterisks represent significant paired *t*-test values ($p < 0.05$) between empty control and plant **(A)** or between native and range-expanding plant species **(B)**.

Helicotylenchus

On average, native plant species did not attract *Helicotylenchus* more strongly than range-expanders on agar (natives: 21.9 ± 8.0%, range-expanders: 13.6 ± 2.4%; $F = 0.99$, $p = 0.38$), while they did so in soil (natives: 17.2 ± 0.8%, range-expanders: 7.4 ± 3.3%; $F = 7.83$, $p < 0.05$). Individually, all native plant species significantly attracted *Helicotylenchus* in both test units, when compared to empty controls (all *t*-values > 3.2, all *p*-values < 0.05; **Figure 1A** and Supplementary Figure 2A). On agar only range-expanding *Geranium* significantly attracted *Helicotylenchus* away from the empty control ($t = 4.34$, $p < 0.01$; Supplementary Figure 2A), while in soil both range-expanding *Geranium* and *Rorippa* did so (*t*-values > 6.57, *p*-values < 0.01; **Figure 1A**). Range-expanding *Centaurea* significantly repelled *Helicotylenchus* toward the empty control on agar ($t = -2.83$, $p < 0.05$; Supplementary Figure 2A), but not in soil ($t = -1.98$, $p = 0.10$; **Figure 1A**). Overall, native plant species always significantly attracted *Helicotylenchus*, whereas attraction and repellence by range-expanding plant were species-specific and depended on test unit.

Nematode Preference

Meloidogyne and *Helicotylenchus* preferred native *Centaurea* and *Rorippa* over their congeneric range-expanders (*t*-values > 3.68, *p*-values < 0.05; **Figure 1B** and Supplementary Figure 2B), although the preference of *Helicotylenchus* for native *Rorippa* was not significant on agar ($t = 1.47$, $p = 0.19$). Both *Meloidogyne* and *Helicotylenchus* did not show a preference for either native or range-expanding *Geranium* on either agar or in soil (all *t*-values < 1.59, all *p*-values > 0.15; **Figure 1B** and Supplementary Figure 2B). Therefore, our results show that two out of three native plant species were preferred over related range-expanding plant species by both nematode species, whereas in the third plant pair both nematode species did not show a preference for either the native or the range-expander.

Nematode Reproductive Performance

Meloidogyne reproduction differed significantly among plant species (explained deviance = 182.45, $p < 0.0001$). *Meloidogyne* numbers were higher on native *C. jacea* than on range-expanding *C. stoebe* ($\chi^2 = 251.94$, $p < 0.0001$; **Figure 2**) and higher on native *R. sylvestris* than on range-expanding *R. austriaca* ($\chi^2 = 12.18$, $p < 0.001$; **Figure 2**). However, in *Geranium*, *Meloidogyne* numbers were higher on the range-expander *G. pyrenaicum* than on the native *G. molle* ($\chi^2 = 5.87$, $p < 0.05$; **Figure 2**). *Helicotylenchus* numbers also differed significantly among plant species (explained deviance = 114.05, $p < 0.0001$; **Figure 2**). There were significantly more *Helicotylenchus* on native *C. jacea* than on range-expander *C. stoebe* ($\chi^2 = 10.10$, $p < 0.05$; **Figure 2**). However, *post hoc* analysis of the other two plant pairs did not reveal any significant differences in *Helicotylenchus* numbers between range-expanders and congeneric natives. *Meloidogyne* numbers per plant species strongly correlated with the attraction by these plant species in y-tubes ($R^2 = 0.92$, $p < 0.01$; **Figure 3A**), while this correlation was not significant for *Helicotylenchus* ($R^2 = 0.11$, $p = 0.52$; **Figure 3B**).

Root Volatiles

We detected 1964 putative volatile compounds in all samples, of which approximately 25% (491 volatile compounds) were produced by plants (Supplementary Figure 3). The other 1473 volatile compounds were detected in the tubes containing only gamma-sterilized soil. When the root volatiles of all six plant species were analyzed together, the strongest overlap between species was found within the pairs of congeneric species, indicating that chemistry varies more strongly between genera than within genera (Supplementary Figure 4). Within the *Centaurea* pair 21 volatile compounds were significantly different between the native and range-expander, resulting in a clear separation of their volatile profiles (**Figure 4A**). Five of these compounds were detected only in the headspace of *C. stoebe*: indene, tridecane and nonadecane (alkanes),

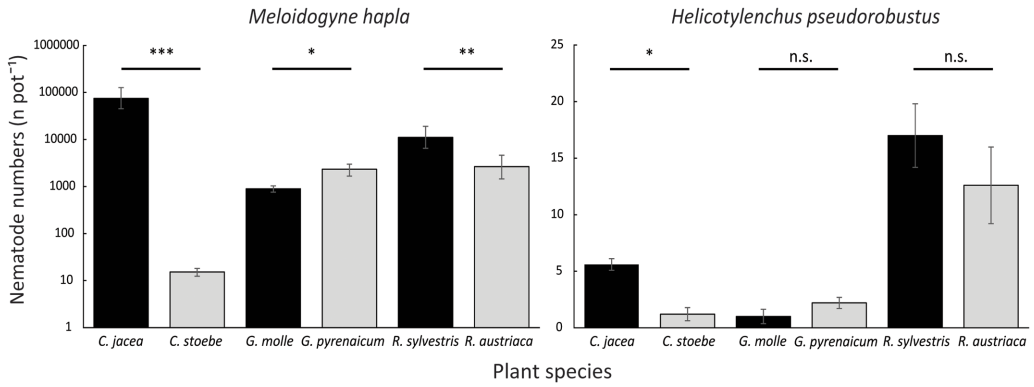

FIGURE 2 | Mean total numbers (N pot^{-1}) of root-feeding nematodes *M. hapla* (left; logarithmic scale) and *H. pseudorobustus* (right; linear scale) on range-expanding plant species *C. stoebe*, *G. pyrenaicum,* and *R. austriaca* (gray), and congeneric natives *C. jacea*, *G. molle,* and *R. sylvestris* (black). Vertical bars show means ± standard errors. Asterisks indicate levels of significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., not significant) of pairwise *post hoc* Wald tests within plant pairs.

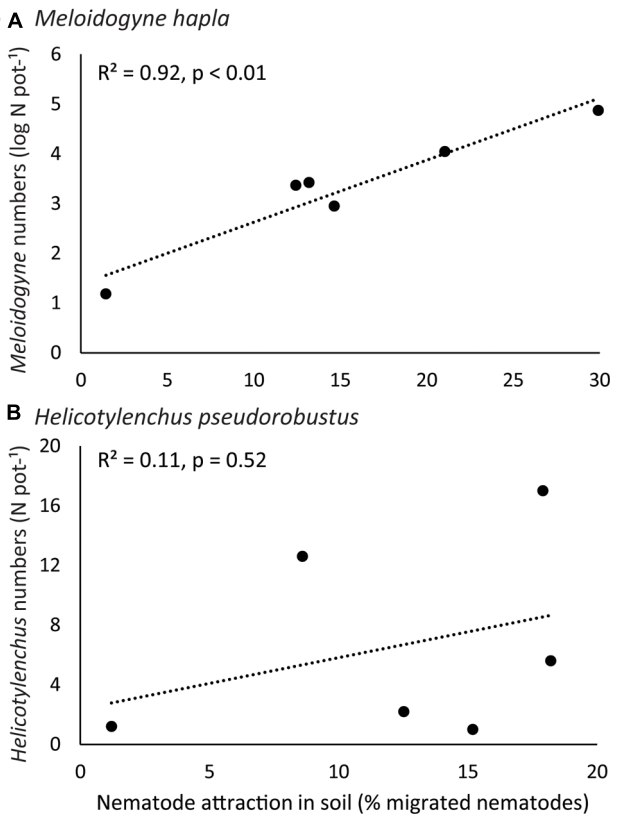

FIGURE 3 | Correlation plots between nematode attraction (*x*-axis) and nematode reproduction (*y*-axis), for root-feeding nematodes **(A)** *Meloidogyne hapla* and **(B)** *H. pseudorobustus*. Dots represent the six different plant species tested. R^2-values and *p*-values of the Pearson correlation tests are given.

1,2-benzisothiazole (benzenoids/ketone) and alpha-gurjunene (sesquiterpene), and three volatiles were detected only in the headspace of the native *C. jacea*: petasitene (sesquiterpene), benzophenone (benzenoids/ketone), and an unknown terpene (**Table 1**). Thirteen compounds where found in both *Centaurea* species, but in different abundances (**Table 1**). Volatile profiles from native and range-expanding *Geranium* and *Rorippa* were less clearly separated in the PLS-DA score plots, although samples from controls, native and range-expanding plants could still be divided into three distinct groups with 95% confidence intervals (**Figures 4B,C**). There were 11 volatiles that showed significant differences between the *Geranium* species and 6 between the *Rorippa* species (all *p*-values < 0.05). Native *G. molle* produced five unique volatile compounds, compared to four by range-expanding *G. pyrenaicum,* while two volatiles differed in production levels between the species. The native *R. sylvestris* produced four unique compounds compared to two unique compounds that were produced exclusively by the range-expander *R. austriaca*. Therefore, differences in volatile profiles between range-expanders and congeneric natives depended on the species pair; in two out of three cases, the range-expander produced fewer unique volatiles than the congeneric native.

DISCUSSION

Several studies have proposed that invasiveness of intercontinentally introduced exotic plant species can be enhanced by their novel chemistry, e.g., through allelopathy (Callaway and Aschehoug, 2000; Zheng et al., 2015), or by the suppression of the local natural enemies (Schaffner et al., 2011). Yet, little is known about the effects of novel chemistry of intra-continental climate-driven range-expanders on communities in the new range. Moreover, empirical studies testing novel chemistry effects on belowground plant–herbivore interactions in the novel range are lacking. Here, we show that root-feeding nematodes from the novel range were strongly attracted to native plant species, while, in support of our hypothesis, the average attraction by range-expanders mostly was less strong. Yet, we also found substantial differences in nematode attraction among range-expanding plant species: while the range-expanding

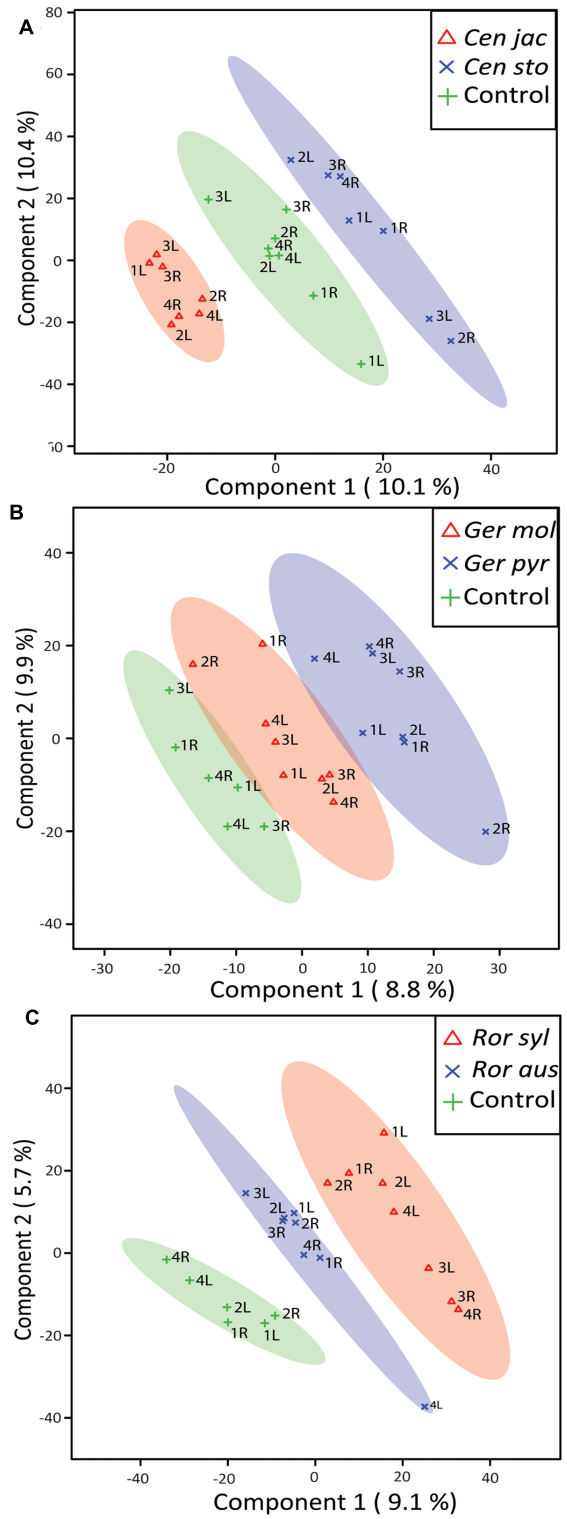

FIGURE 4 | Partial least square-discriminant analysis (PLS-DA) score plots of root volatile profiles measured with GC-QTOF-MS. The semi-transparent ovals outline the 95% confidence intervals of natives (red triangles), range-expanders (blue crosses) and sterilized control soils (green crosses) for *Centaurea* **(A)**, *Geranium* **(B)**, and *Rorippa* **(C)**. Sample numbers and position of the volatile trap (left or right) are given.

C. stoebe repelled both nematode species in at least one of the attraction experiments, range-expanding *G. pyrenaicum* and *R. austriaca* attracted nematodes. Therefore, we show that some range-expanding plant species will attract considerable amounts of root-feeding nematodes in their new range, while other species will repel them, potentially leading to profound differences in herbivore pressure between range-expanders in their new range.

In test units with both natives and congeneric range-expanders, both nematode species preferred native *Centaurea* and *Rorippa* over their congeneric range-expanders, while our hypothesis of stronger nematode preference for natives was not confirmed when comparing the *Geranium* species. In plant communities in the new range, the preference for native plant species could lead to apparent competition (Holt, 1977), when natives experience stronger herbivore pressure (Orrock et al., 2008), leading to indirect competitive benefits for the range-expanders. For *Meloidogyne,* reproduction strongly corresponded with the attraction to the different plant species, as we found that *Meloidogyne* reproduction was significantly higher in the roots of native *Centaurea* and *Rorippa* than in the roots of their congeneric range-expanders. Notably, the differences in *Meloidogyne* reproduction between the *Centaurea* species were more substantial than between the *Rorippa* species. This was especially due to poor nematode reproduction on the range-expanding *C. stoebe*, which is in line with a previous study (Wilschut et al., 2016). *Helicotylenchus* numbers did not fully

TABLE 1 | Volatile organic compounds produced by native *Centaurea jacea* and range-expanding *C. stoebe*.

Compound name	RT	ELRI	Plant
Sulfur dioxide	2.04	488	CJ
Dimethylsulfide	2.4	529	CS
Carbon disulfide	2.5	541	CJ
Furan, 2-methyl	2.9	583	CJ
1,3-dioxolane, 2-methyl-	3.4	639	CS
benzene 1,2 dimethyl	10.1	890	CJ
Dimethyl sulfone	10.9	916	CS
Dimethyl trisulfide	13.1	963	CS
Mesitylene	14.3	990	CJ
Indene**	15.7	1023	CS
Acetophenone	17.4	1062	CS
1,2-benzisothiazol**	23.9	1229	CS
Tridecane**	26.8	1299	CS
Petasitene*	30.1	1398	CJ
Alpha-gurjunene**	30.4	1407	CS
Unknown terpene*	32.73	1448	CJ
Phenyl maleic anhydride	34.29	1534	CJ
Benzophenone*	36.9	1620	CJ
Pentadecanoic acid	40.02	1867	CS
Nonadecane**	40.4	1901	CS
Diphenylsulfone	40.7	1934	CS

Tentative compound names are shown, which are based on retention time (RT) and ELRI (Experimental linear retention index) values, measured with GC-QTOF-MS. All compounds are significantly more produced by either C. jacea (CJ) or C. stoebe (CS). Compounds that are produced solely by C. jacea are indicated with '' and compounds produced solely by C. stoebe with '**.'*

correspond with the attraction to the different plant species. Although they were lower in the rhizosphere of range-expanding *Centaurea* than in that of native *Centaura*, no differences were found in the other two plant pairs. The overall very low *Helicotylenchus* numbers indicate that no – or hardly any – reproduction of this species took place in this experiment. While the species did show profound chemical attraction to some of the plant species, we could therefore not properly estimate differences in performance on these different plant species.

Contrary to our hypothesis, but in line with a previous study (Wilschut et al., 2016), the range-expanding *Geranium* hosted slightly higher numbers of *Meloidogyne* than the native *Geranium*, indicating that not all range-expanding plant species are poorer nematode hosts than congeneric natives. Depending on naivety of either the host plant species or the herbivore in a novel plant–herbivore novel interaction, herbivore performance can be found to be strong or weak (Verhoeven et al., 2009). We did not perform experiments using *Meloidogyne* and *Helicotylenchus* populations from the original range of the range-expanding plant species, so our data do not allow to draw conclusions on nematode preference and performance of the range-expanding plant species in their native range. However, as gene flow between soil-born nematode populations is expected to be low (Blouin et al., 1999), a certain degree of local adaptation is well possible, so that it may well be that the nematode populations in the new range differ, at least to some extent, from populations in the original range. The use of nematode populations originating from natural areas in the new range and the subsequent culturing on plant species that is phylogenetically unrelated to the examined plant species allowed a phylogenetically unbiased test of the effects of the natural co-evolutionary histories between the nematode and plant species on nematode attraction and performance.

We expected that the patterns in nematode attraction, preference and reproduction found in the present study would be caused by differences in root chemistry between native and range-expanding plant species. Indeed, the analyses of volatile compounds revealed that range-expanding *C. stoebe* produced more unique volatile compounds than native *C. jacea*. These results correspond with a study on aboveground herbivores, in which herbivore performance was also shown to be low on range-expanding and exotic plants with more unique chemistry than their related natives (Macel et al., 2014). In addition to higher numbers of unique compounds, our study also reveals differences in the production levels of several shared volatile compounds between the *Centaurea* species. Therefore, the nematode repellence and the poor nematode reproduction on the range-expanding *C. stoebe*, compared to the native *C. jacea,* might be explained by both the production of higher numbers of unique compounds and by different production levels of shared compounds. Interestingly, novel chemistry of *C. stoebe* has also been related to the poor performance of aboveground generalist herbivores in North America (Schaffner et al., 2011), where this plant species is invasive. In contrast to range-expanding *Centaurea*, both range-expanding *Rorippa* and *Geranium* produced fewer unique volatiles than their congeneric natives. Differences in volatile profiles were stronger in *Geranium* than in *Rorippa*, which was not reflected in the patterns of nematode preference and reproduction. Native *Rorippa* hosted higher nematode numbers and was more attractive to both nematode species than range-expanding *Rorippa*, while in *Geranium* there was no clear nematode preference for either the native or the range-expander, and nematode reproduction levels were higher in the range-expander than in the native. These results suggest that when unique volatile compounds play a role in nematode attraction or distraction, the identity, rather than the number of unique compounds may influence the outcome of plant-nematode interactions. Interestingly, but not unexpectedly, the differences in volatile profiles between all three pairs of congeneric native and range-expanding plant species were smaller than the differences among the three genera. This suggests that while root-feeding nematode species such as *Meloidogyne* have adapted to plant species with strongly different root chemistries, they may still perform poorly on range-expanding plant species that possess root chemistries slightly deviating from that of the plant species the nematodes are adapted to.

Our volatile analyses revealed, next to many plant volatiles, a large diversity of volatiles emitted by gamma-sterilized soils, which is in line with earlier studies (Schulz-Bohm et al., 2015; Kai et al., 2016). Possibly, the chemical background of the soil caused the differences in nematode attraction between the tests on agar and soil, namely the higher numbers of nematodes moving to the unplanted side on agar. Alternatively, this effect could be caused by a stronger diffusion of root metabolites in the Petri dishes than in the soil-filled Y-tubes, resulting in a more equal distribution of root metabolites throughout the Petri dishes. Based on the differences between the two choice experiments we therefore conclude that choice experiments with root-feeding nematodes should preferably be performed in soil.

The application of GC-QTOF for volatile analysis allowed to obtain the tentative identification of the measured root volatiles. We identified several volatile compounds that were only detected in range-expanding *C. stoebe*, and therefore could cause the nematode-repelling effect found for this plant species. Root-emitted volatiles are known to play versatile roles in long distance below-ground interactions (Erb et al., 2013; Van Dam and Bouwmeester, 2016) and some of the volatile compounds identified in the present study have been shown to negatively affect nematodes (Piluk et al., 1998). Future studies testing the identified metabolites in different combinations and ratios could reveal which compounds cause the nematode-repelling effect found in *C. stoebe*. Yet, pin-pointing of the observed effects to a single volatile compound can be complicated, because nematodes might react to a blend of volatiles, rather than to single compounds (Mccormick et al., 2012).

Successful range-expanding plant species have been shown to be better defended against naïve aboveground generalist herbivores than congeneric native plant species (Engelkes et al., 2008), indicating that they may possess superior defense mechanisms compared to related native species in the new range. Such defense mechanisms may especially be effective when they are novel to the natural enemies in the new range. Our results suggest that together with the release of soil enemies from

the original range (Van Grunsven et al., 2007), the possession of novel chemistry could explain why range-expanding plant species are less negatively affected by soil communities than related native plant species (Van Grunsven et al., 2007; Engelkes et al., 2008). As range-expanding plant species without closely related species in the new range are likely to possess the most unique root chemistries compared to native species present in the community, a phylogenetic approach (as in Strauss et al., 2006) may be considered to forecast which range-expanding plant species have the strongest potential to affect native communities in their novel range (Gilbert and Parker, 2016).

CONCLUSION

We provide evidence that novel belowground chemistry of the root system of range-expanding plant species may suppress root herbivores in the new range. A range-expander that had the most different root chemistry compared to its related native suppressed root-feeding nematodes more strongly than range-expanders with root chemistries that were more comparable to those of related natives. However, our study included six plant species from three genera. Therefore, while our results elucidate the variation in potential impact of range-expanding plant species on native communities in their novel range, further studies are needed in order to be able to generalize these results and predict which range-expanding plant species may have strong impacts on native communities in the future.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the study. Greenhouse experiments were performed by JS and RW. The volatile experiment was performed by PG and JS. Data analyses were performed by RW, PG, and JS. The manuscript was written by RW with the help of all other authors.

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SUPPLEMENTARY MATERIAL

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frontiers in Plant Science

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Root JA Induction Modifies Glucosinolate Profiles and Increases Subsequent Aboveground Resistance to Herbivore Attack in *Cardamine hirsuta*

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Alteration and induction of plant secondary metabolites after herbivore attack have been shown in almost all the studied plant species. Induction can be at the local site of damage, or systemic, such as from roots to shoots. In addition to immediate induction, previous herbivore bouts have been shown to "prime" the plants for a stronger and faster response only after a subsequent attack happens. Whereas several studies revealed a link between root herbivory and increased resistance against aboveground (AG) herbivory, the evidence of root defense priming against subsequent AG herbivory is currently lacking. To address this gap, we induced *Cardamine hirsuta* roots by applying jasmonic acid (JA), and, after a time lag, we subjected both control and JA-treated plants to AG herbivory by the generalist herbivore *Spodoptera littoralis*. We addressed the effect of root JA addition on AG herbivore resistance by measuring larval weight gain and tested the effect of root induction on abundance and composition of glucosinolates (GSLs) in shoots, prior, and after subsequent herbivory. We observed a strong positive effect of root induction on the resistance against AG herbivory. The overall abundance and identity of GSLs was globally affected by JA induction and by herbivore feeding, independently, and we found a significant correlation between larval growth and the shoot GSL profiles only after AG herbivory, 11 days after induction in roots. Contrary to expectations of priming, we observed that JA induction in roots altered the GSLs profile in the leaves that was maintained through time. This initial modification was sufficient to maintain a lower caterpillar weight gain, even 11 days post-root induction. Altogether, we show that prior root defense induction increases AG insect resistance by modifying and maintaining variation in GSL profiles during insect feeding.

Keywords: belowground-aboveground priming, glucosinolates, insect resistance, plant-mediated above-belowground interaction, plant chemical defenses, phytohormones

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INTRODUCTION

Resistance to herbivory in plants is mediated by pre-existing, or herbivore-inducible, physical and chemical barriers (Karban and Baldwin, 1997). Specifically, plants can enhance constitutive levels of defenses, or produce them *de novo*, upon herbivore damage (Agrawal et al., 1999). In addition, previous incidents of herbivory do not directly increase defenses but can "prime" plants

for a faster and stronger response against subsequent attackers (van Hulten et al., 2006; Ton et al., 2007; Pieterse et al., 2013). Plant defense orchestration is mediated by several plant hormones (Pieterse et al., 2014), of which salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the most important, but other phytohormones, such as abscisic acid (ABA), gibberellins, auxins, and cytokinins have more recently been described as important defense regulators as well (van Hulten et al., 2006; Giron et al., 2013). Generally, the plant hormone JA is a key player in the regulation of induced plant responses against chewing herbivores such as beetles and caterpillars (Farmer et al., 2003; Howe and Jander, 2008).

While previous studies of plant-mediated interactions with herbivores have mostly focused on locally infested tissues, it is now known that defense activation can spread systemically through the plant and can even cross the root–shoot divide (Bezemer et al., 2003; Bezemer and van Dam, 2005; Heil and Ton, 2008; Rasmann and Agrawal, 2008). Several studies have demonstrated the crucial role of JA in mediating below- and above-ground (BG and AG thereafter) interactions (Erb et al., 2008; Soler et al., 2013; Fragoso et al., 2014). For instance, exogenous JA exposure to BG or AG parts of a plant can systemically induce defense responses in roots or leaves, respectively (van Dam et al., 2004; van Dam and Oomen, 2008). Therefore, when specifically looking from the root to shoot, root herbivory could negatively affect the performance of leaf-chewing insects by inducing a systemic increase in secondary metabolites (Bezemer et al., 2003; Soler et al., 2005; van Dam et al., 2005; Staley et al., 2007; Erb et al., 2009a,b). BG insect herbivory, or JA application, in some studies, increased defense compound (e.g., glucosinolates (GSLs)) levels in shoots (Griffiths et al., 1994; van Dam et al., 2004; van Dam and Oomen, 2008; Qiu et al., 2009; Pierre et al., 2012, 2013). However, other studies demonstrated that BG induction resulted in a decrease (van Dam et al., 2005), or had no effect on secondary metabolites levels (van Dam and Raaijmakers, 2006; Pierre et al., 2012; Tytgat et al., 2013). This suggests that plant defense induction in the roots could reduce herbivore pressure AG by immediately increasing shoot defenses, (van Dam et al., 2001, van Dam et al., 2004), or by priming the plants for a subsequent stronger response induction only after the shoot herbivore is on the plant.

Stimuli such as previous herbivory, egg deposition, or volatiles from herbivore-infested adjacent plants have been shown to prime JA-mediated anti-herbivore defenses (Rasmann et al., 2012; Vos et al., 2013; Bandoly et al., 2015; Erb et al., 2015). Although, several studies indicate that root herbivory increases the resistance against shoot herbivores (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005), studies investigating the importance of JA-dependent priming through induction of GSLs in AG-BG context are scarce. For instance, it has been shown that root herbivory by *Delia radicum* primed *Brassica nigra* leaves against subsequent leaf herbivory by *Pieris rapae,* which resulted in stronger increase of AG chemical defenses compared to levels prior to leaf herbivory (van Dam et al., 2005). In contrast, Soler et al. (2005) found no clear effect of BG herbivory on chemical defenses in *B. nigra* leaves attacked by *Pieris brassicae*.

The aim of this study was to explore the JA-dependent root induction effect on subsequent AG herbivore attack. The idea being that root induction by JA would not result in immediate AG changes in secondary metabolites, but that AG priming of defenses – and subsequent increased plant resistance against the herbivore – would only be visible if, after a delay of few days, an herbivore would attack the plant (Martinez-Medina et al., 2016). We tested this hypothesis using a wild Brassicaceae species, the hairy bittercress *Cardamine hirsuta,* and a generalist noctuid butterfly caterpillar *Spodoptera littoralis*. In Brassicaceae plants, GSLs, sulfur- and nitrogen-containing plant secondary metabolites, are the main defensive compounds conferring plant resistance against insect herbivores (Howe and Jander, 2008). Induction by JA or herbivory has been shown to increase the concentration of GSLs in several systems (Papadopoulou and van Dam, 2016) and decrease the performance of generalist herbivores in particular (Bodenhausen and Reymond, 2007).

We specifically had the following questions: (i) does root induction by JA affect plants' resistance against subsequent shoot herbivory? (ii) does root JA application affect the amount and composition of GSLs in leaves prior and after subsequent shoot herbivory? (iii) is there a relationship between GSLs composition before and after herbivory and resistance to herbivory? We expected that root JA application would increase resistance against subsequent AG herbivore attack. We also expected that, in case of priming, JA application would not modify AG GSL composition, but JA effect would only be visible after AG herbivore application.

MATERIALS AND METHODS

Plant and Insect

To address the effect of root priming on AG plant defense and resistance, we used the hairy bittercress, *C. hirsuta* (Brassicaceae), a common weed growing in a variety of habitats in Europe (Pellissier et al., 2016). Seeds were collected from three different natural populations around Neuchâtel in Switzerland in 2016. Seeds from 26 half-sib families (pop A = 9 fam, pop B = 10 fam, and pop C = 7 fam) were germinated in Petri dishes lined with humid filter paper, and one week after germination, six seedlings per family (total of 156 plants) were transplanted independently into plastic potting pots (13 cm width × 10 cm height) filled with 500 ml of sieved soil (1 cm mesh size) mixed with sand in a 3:1 ratio. The soil/sand mixture was sterilized by autoclave. Plants were immediately transferred to a climate-controlled chamber and kept at 16 h/22°C - 8 h/16°C day-night and 50% relative humidity conditions. Plants received nutrients twice a week for three weeks until the beginning of experiment.

We used *S. littoralis* as generalist herbivore insects (obtained from Syngenta, Stein AG, Switzerland). Newly hatched larvae were reared on corn-based artificial diet until the beginning of the experiment. *S. littoralis* is a generalist herbivore, known to feed on species belonging to more than 40 families of plants (Brown and Dewhurst, 1975) and is widely used for performing plant resistance bioassays. In addition, *S. littoralis* has been shown to

activate JA-dependent defenses in *Arabidopsis thaliana*, a close relative of *C. hirsuta* (Bodenhausen and Reymond, 2007).

Experimental Set-Up

After 3 weeks of growth, plants were randomly assigned to two treatment groups. Half of the plants (three replicates per family, $n = 78$) were randomly assigned to the JA treatment, while the other half to the control treatment (three replicates per family, $n = 78$). JA-treated plants received 20 ml of JA solution in roots by adding the solution in the soil, 0.5 cm below the surface. The JA solution consisted of 2.4 μmol (500 μg) of JA (± - JA, Sigma-Aldrich, St Louis, IL, United States) per plant in 10 ml demineralized water and 0.5% EtOH (pH 4.0). The control group of plants received 20 ml of 0.5% EtOH in acid water (pH 3.7 with HCl) in roots for each plant. These amounts were chosen based on previous studies using other brassicaceous plants (van Dam et al., 2004; van Dam and Oomen, 2008).

Four days after the root treatment, two fully expanded new leaves per plant were collected, immediately frozen and stored at −80°C for further chemical analyses. Right after, two 7-day old *S. littoralis* larvae were added to the leaves of each plant. The combined weight of the insects per plant was measured and recorded. Plants were covered with gauze bags to prevent escape or cross-movement of insects between plants. After one week of herbivory (i.e., 11 days post JA treatment – hereafter "after herbivory"), bags were removed, the insects were retrieved from individual plants, and their weights were measured and recorded. We used the formula ln (final weight–initial weight) to determine the insects' weight gain and plant resistance (i.e., lower weight gain indicate that plants are more resistant). Two fully expanded herbivore-damaged leaves per plant were collected and immediately placed in −80°C for further chemical analyses. After the herbivore treatment, the plants were allowed to complete their life cycle. In the end of the life cycle, AG plant parts were separated from roots, weighted, oven-dried at 40°C for 48 h and weighted to determine their dry biomass.

Glucosinolate Extraction and Analysis

Plant leaves, harvested prior and after herbivore treatment, were ground to powder using mortars and pestles in liquid nitrogen, and a 100-mg aliquot was weighted in a 1.5-ml Eppendorf tube for glucosinolate extraction. 1.0 ml Methanol: H2O: formic acid (80:19.5:0.5, v/v) were added to the tubes along with 5 glass-beads and the tubes were shaken in a tissuelyser for 4 min at 30 Hz and centrifuged at 12,800 × *g* for 3 min. The supernatant was then transferred to an appropriate vial for liquid chromatography analysis. Glucosinolate identification and quantification was performed using an Acquity UPLC from Waters (Milford, MA, United States) interfaced to a Synapt G2 QTOF from Waters with electrospray ionization, using the separation and identification method as described in (Glauser et al., 2012). We acknowledge that we did not measure GSLs on a set of control plants that never experienced herbivory at time T2 to infer inducibility of GSLs. The reasoning for doing this was not to measure the specific inducibilities for each compound at time T2, but mainly to correlate what the larvae were experiencing at this time point, versus what the larvae initially experienced at time T1.

Statistical Analysis

All statistical analyses were carried out with R software (R Development Core Team, 2017). To address the priming effect of root JA addition to AG resistance against *S. littoralis*, as well as the total amount of GSLs, we ran linear mixed effect models with insect weight gain and total amount of GSLs as response variables, JA treatment (two levels) as fixed factor, plant biomass as covariate, and plant families nested within population as random factor using the function *lme* in the package *nlme* in R (Pinheiro et al., 2017).

To address how JA application in root would affect the abundance and composition of GSLs in the shoots, we first ran a full-factorial model including the individual GSLs abundance matrix as response variable and time after induction, JA treatment, and families nested within populations as factors using permutational analysis of variance (PERMANOVA) with the *adonis* function in the package *vegan* in R (Oksanen et al., 2017). To take into account the effect of measuring induction of GSLs on the same plants twice, we included plant IDs as "strata" in the *adonis* function. Finally, we also included plant biomass as covariate to control for potential direct effect of biomass on plant chemistry (Züst et al., 2015), as well as larval weight gain to take into account the effect of larval size, and indirectly, weight gain, on GSL production (Raubenheimer and Simpson, 1992; Horton and Redak, 1993). The Bray–Curtis metric was used to calculate a dissimilarity matrix of all compounds among samples for the PERMANOVA.

Finally, we analyzed the relationship between JA-induced GSLs and larval weight gain using the environmental fitting analysis [*envfit* function in *vegan* (Oksanen et al., 2017)] on the NMDS analysis of the chemical compounds (time = after

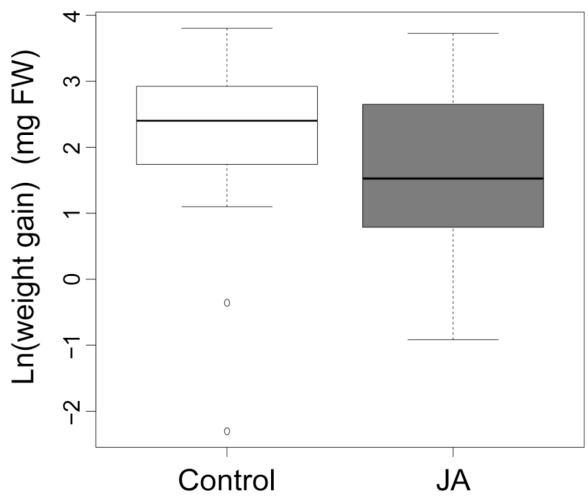

FIGURE 1 | Larval weight gain. The average weight gain of *Spodoptera littoralis* caterpillars feeding on plants that received jasmonic acid (JA) in the roots 4 days prior to the start of herbivory or received no JA in the roots (Control). Weight gain was calculated as the natural logarithm of the difference between final and initial fresh weight. The two boxplots are significantly different (ANOVA, $p < 0.05$).

herbivory). When applied to NMDS, the environmental fitting analysis can estimate the strength of the correlation of maximal correlation between the NMDS configuration and the weight gain variable. This approach can be used to indicate whether larval weight gain is associated with particular GSLs, as represented in the NMDS ordination.

RESULTS

Effect of JA Treatment on Resistance Against *S. littoralis*

Spodoptera littoralis larvae grew 55% less (absolute weight gain values) on JA-treated plants compared to control plants (**Figure 1**, $F_{1,76} = 9.67$, $p < 0.003$), indicating the significant effect of JA treatment in roots on AG herbivore resistance. We found no effect of plant biomass on larval weight gain ($F_{1,76} = 0.01$, $p = 0.93$).

Effect of JA Treatment on GSLs

The GSLs profile of the *C. hirsuta* leaves, harvested four (before herbivory) and 11 days after root induction (after herbivory), consisted of 28 GSL compounds: 15 aliphatic-GSLs, 8 aromatic-GSLs, 3 indole-GSLs, and 2 unknown-GSLs (**Figure 2** and **Supplementary Table S1**). Total levels of GSLs were only affected by herbivore damage over the 7 days period of feeding, in which, after herbivory, plants produced 10% more GSLs than 4 day post-induction (i.e., measures taken 4 and 11 days after JA treatment) (mixed effect model; Time effect: $F_{1,179} = 4.81$, $p = 0.02$). The PERMANOVA showed that the abundance and diversity of GSLs were globally affected by JA treatment and by one week of continuous damage by AG herbivores (**Figure 3A** and **Table 1**), however, we found no interaction between time and JA induction (**Figure 3A**). We also found that the maternal family background affected the GSLs production, indicating that the genetic background influences the magnitude of GSLs production in shoots after root JA induction and AG herbivory (**Table 1**). Finally, we found that overall; plant biomass was affecting GSLs production in shoots of *C. hirsuta* plants (significant at global GSL levels and significant for 25 of the individual compounds) (**Table 1** and **Supplementary Table S1**). Moreover, specifically, we found interaction between time and induction by JA in five of the individual GSL compounds (3 aliphatic and 2 aromatics), suggesting that despite the pattern observed at the global GSL

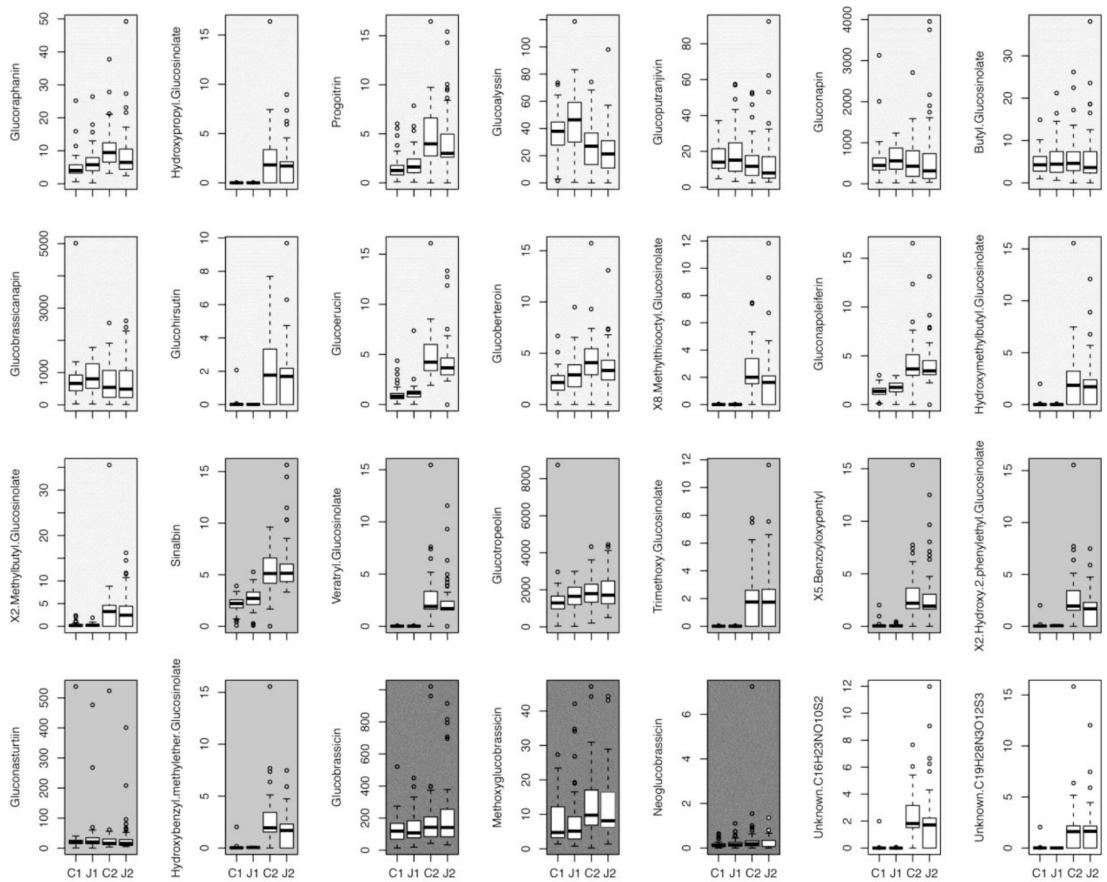

FIGURE 2 | Individual glucosinolate induction. Data show the effect of JA induction in the roots, at two different time points (J1 and J2) and no JA induction (C1 and C2), on individual glucosinolates (ng mg^{-1} FW) levels in the leaves of *Cardamine hirsuta* plants. C2 and J2 also represent 7 days of *Spodoptera littoralis* herbivore attack. Different shadings of gray indicate different classes of GSLs: from light to dark: unknown (white), aliphatic, aromatic, and indole.

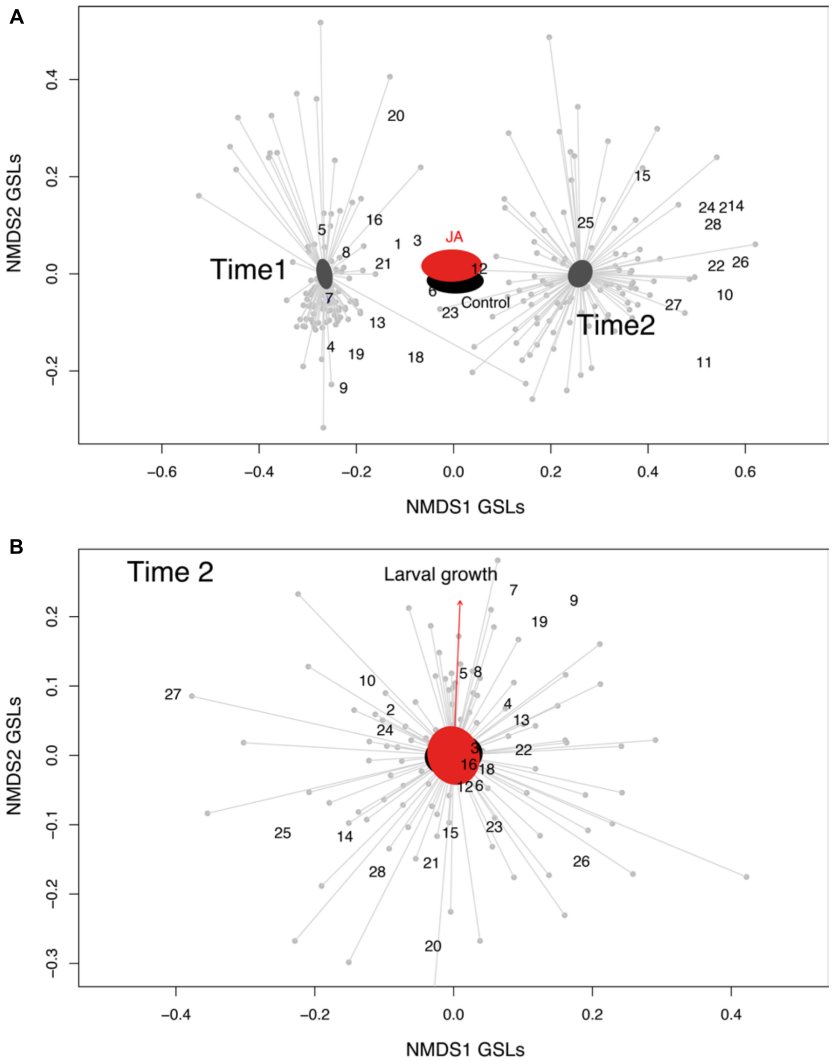

FIGURE 3 | Glucosinolates' ordination. **(A)** Representation of the non-multidimensional scaling (NMDS) indicating the glucosinolates found in *Cardamine hirsuta* leaves, and their 95% confidence interval ellipses based on the two treatments (root induction with JA, red polygon; and no-induction as the black polygon) at two-time points; Time 1 = 4 days after induction and Time 2 = 11 days after induction (stress value = 0.14, *k* = 2). **(B)** Effect of glucosinolates on larval growth. Representation of the non-multidimensional scaling (NMDS) indicating the glucosinolates found in *C. hirsuta* leaves, and their 95% confidence interval ellipses based on the two treatments (root induction with JA, pink polygon; and no-induction as the green polygon). The projection of the maximal correlation of the larval weight gain vector (from the envfit model) on the NMDS ordination is also shown (red arrow) (stress value = 0.26, *k* = 2). Glucosinolates are: 1 = Glucoraphanin, 2 = Hydroxypropyl-GSL, 3 = Progoitrin, 4 = Glucoalyssin, 5 = Glucoputranjivin, 6 = Sinalbin, 7 = Gluconapin, 8 = Butyl-GSL, 9 = Glucobrassicanapin, 10 = Veratryl-GSL, 11 = Glucohirsutin, 12 = Glucoerucin, 13 = Glucotropeolin, 14 = Trimethoxy-GSL, 15 = 5-Benzoyloxypentyl, 16 = Glucobrassicin, 17 = 2-Hydroxy-2-phenylethyl-GSL, 18 = Glucoberteroin, 19 = Gluconasturtiin, 20 = Methoxyglucobrassicin, 21 = Neoglucobrassicin, 22 = 8-Methylthiooctyl-GSL, 23 = Gluconapoleiferin, 24 = Hydroxymethylbutyl-GSL, 25 = 2-Methylbutyl-GSL, 26 = Unknown-GSL, 27 = Hydroxybenzyl-methylether GSL, and 28 = Unknown-GSL.

levels the production of these compounds between JA-treated and control plants depended on time (**Figure 2** and **Supplementary Table S1**).

Effect of GSLs Matrix and Time on Larval Growth

After correlating the larval growth with the GSLs ordination matrix (NMDS), we found that GSLs profiles of the shoots significantly correlated with larval growth only after herbivory (**Figure 3B**, envfit analysis, $r^2 = 0.07$, $p = 0.02$), while such a correlation was not present in time 4 days ($r^2 = 0.01$, $p = 0.44$).

DISCUSSION

Alteration and induction of plant secondary metabolites in response to herbivore attack have been shown in almost all the studied plant species. However, whereas several studies demonstrate that root herbivory results in increased resistance

TABLE 1 | Two-way permutation ANOVA table for measuring the effect of JA induction in roots and time after induction on the GSLs matrix of *Cardamine hirsuta* plants.

Factor	*df*	Mean SQ	*F* value	R^2	*p*
Time	1	0.57	7.29	0.03	0.001***
JA (induction treatment)	1	0.1	1.24	0.005	0.002**
Time × JA	1	0.15	1.85	0.008	0.180
Families	10	0.15	1.84	0.08	0.001***
Families/population	15	0.15	1.89	0.12	0.004**
Larval weight gain	1	0.07	0.83	0.003	0.001***
Plant biomass	1	0.38	4.87	0.02	0.002**
Residuals	175	0.08		0.74	

*Significance codes: ***p = 0.001, **p = 0.01, *p = 0.05.*

against AG herbivory (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005), the importance of root defense priming against subsequent AG herbivory has not been thoroughly investigated in this context. In this study, we expected a priming effect of JA application in the roots (**Figure 4A**); however, we observed that JA in roots induced an initial modification in the GSLs identity and quantity in the leaves that was maintained through time. This initial modification was sufficient to increase plants' resistance against AG herbivory, even 11 days post-root induction (**Figure 4B**). Altogether, these results indicate that root defense induction increases AG resistance to herbivory in *C. hirsuta*, by immediately modifying the GSL profiles in the leaves.

Effect of JA Treatment on Resistance Against *S. littoralis*

Jasmonic acid application in roots reduced *S. littoralis* weight gain. Overall, our results follows the general trend reported in the literature predicting that hormonal induction of BG tissues increases AG resistance against shoot herbivores (Erb et al., 2011; Papadopoulou and van Dam, 2016) and complement several other studies indicating that root herbivory results in increased resistance against AG herbivory (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005). For example, it has previously been shown that JA treatment of roots in *Brassica oleracea* negatively affected the growth and survival of a generalist *Mamestra brassicae* (van Dam and Oomen, 2008). This trend is however not universal. For example, JA treatment of roots have shown to be ineffective against *M. brassicae* in field-grown cultivated *B. oleracea* plants (Pierre et al., 2013), which could be explained by the differences between flowers' (the broccolis) and leaves' chemistry, and induction therein.

Although, in this study, we used JA to mimic the effect of BG herbivory, it has been clearly shown that JA-induced responses follow similar pattern of induction by BG herbivory. Indeed, the effect of BG herbivory on generating induced response in shoots has been amply demonstrated (van Dam et al., 2005; van Dam and Raaijmakers, 2006; Pierre et al., 2012, 2013), and several studies have shown the same induction pattern in roots caused also by application of JA in roots (van Dam et al., 2004; van Dam and Oomen, 2008; Pierre et al., 2012, 2013). Although, in one study, root infestation with *D. radicum* maggots resulted in weaker systemic responses than JA application (Pierre et al., 2013). Nevertheless, it should be noted that alterations in other plant chemicals, such as induced non-GSLs secondary metabolites, as well as reallocation of primary metabolites between root and shoots may contribute to the observed herbivore responses to induced plants (Jansen et al., 2008; van Dam and Oomen, 2008; Poelman et al., 2010; Pierre et al., 2012). Interestingly, we also found that plant biomass *per se* did not influence the insect weight gain, indicating the larval weight gain was independent of plant size, thus likely mainly mediated by plant defensive traits.

Effect of JA Treatment on GSLs

We found that the GSLs profiles were different between control and JA-treated plants before and after a week of herbivory. While ontogeny could play a strong role in affecting GSLs production (Barton and Koricheva, 2010), we observed that the total GSLs differences between treatments were maintained through the 7-day time difference. In contrast to that, we found high specificity in how the individual compounds responded to JA root induction and herbivory. Specifically, the production of five individual GSLs: glucoraphanin, glucoalyssin, glucoberteroin, 2-hydroxy-2-phenylethyl GSL, and hydroxybenzyl-methylether GSL across different treatments were significantly affected during herbivore feeding (i.e., significant JA × Time interaction in **Supplementary Table S1**). This suggests that JA induction had significant different effects on the amount of these compounds before and after AG herbivory. For the latter two compounds, we observed the both effect of time and induction as well as interaction between time and JA induction. These results suggest that changes in the complex combinatorial GSL matrix are driving variation in insect resistance, rather than the simple measure of total GSLs contents (**Figure 4C**). Our results are in line with the literature showing that while BG herbivory, or root induction by JA, results in increase in total levels of GSLs in shoots (Griffiths et al., 1994; van Dam et al., 2004; Soler et al., 2005; van Dam and Raaijmakers, 2006; van Dam and Oomen, 2008; Jansen et al., 2009; Qiu et al., 2009; Pierre et al., 2012), others have observed no changes in total GSLs when plants (broccoli) where induced in roots either by JA or *D. radicum* (Pierre et al., 2013). Therefore, both the total amount and the individual-level variation of GSLs could affect resistance against herbivores.

We found a significant effect of plant biomass on GSLs production in plant leaves, a common phenomenon when studying secondary metabolite production in plants (Traw, 2002; Glynn et al., 2003; Züst et al., 2015). We also found a significant effect of larval biomass on the glucosinolate matrix (**Table 1**), suggesting that the potential variation in insect weight gain (i.e., insects that grew more were also eating more) between treatments could potentially also drive the observed variation in the GSL matrix. Furthermore, the observed strong family level variation in induction of GSLs in shoots, after root induction and AG herbivory, is particularly interesting. Such results suggest a great potential for selection on BG-AG induction *per se*, which in turn set the stage for evolution of plant-mediated BG-AG interactions.

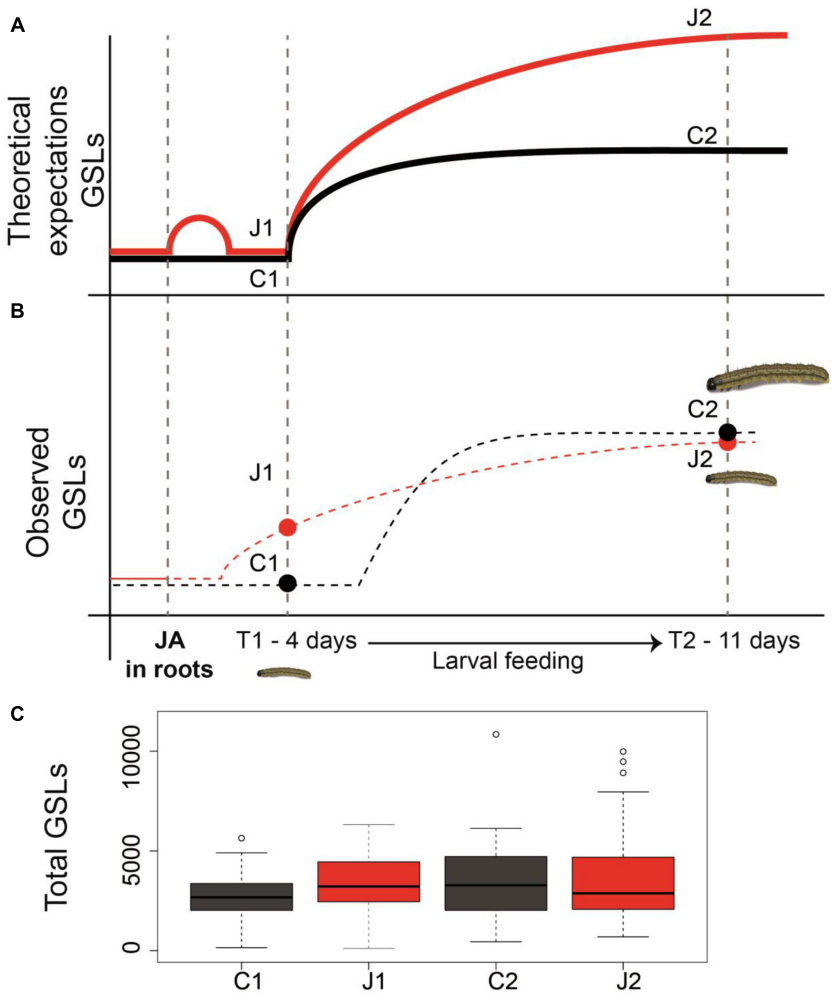

FIGURE 4 | Priming of defenses and resistance in plants. Panel **(A)** is showing the theoretical expectations of defense priming in plants based on the literature. Panel **(B)** shows the conceptual model of priming that was observed in this paper as well as the resistance bioassay with *Spodoptera littoralis* caterpillars. Dots represent the observed values of total GSL sampled at two time points. The dotted lines represent hypothetical GSL induction dynamics. Panel **(C)** shows how total glucosinolates levels (ng mg^{-1} FW) vary across time and based on the two treatments of JA induction in the roots (J1 and J2), and no JA induction (C1 and C2).

Effect of GSLs Matrix and Time on Larval Growth; Is It Priming?

The larval growth was affected by GSLs profile of the shoots only after herbivory, while such a correlation was not present in 4-day time. These results, while only correlative, point toward the possibility of priming for defense in BG-AG context which indicates that induction in one compartment should increase the resistance to subsequent herbivory in distant tissues (Erb et al., 2008). However, we take the evidence for potential priming with caution.

Despite the emerging evidence on the effect of root herbivory on enhanced resistance against AG herbivory, the importance of priming in BG-AG concept has generally investigated on local tissues. For example, priming by green leaf volatiles against leaf herbivory in maize plants (Engelberth et al., 2004; Ton et al., 2007), priming of feeding-induced defense triggered by ovipositioning against subsequent larval feeding (Bandoly et al., 2015, 2016), and priming of anti-herbivore defense by exposure of plants to volatiles released from feeding-damaged neighboring plants (Engelberth et al., 2004; Heil and Kost, 2006; Heil and Silva Bueno, 2007; Frost et al., 2008). Within the BG-AG framework, we have no clear evidence of priming, so far. Perhaps, the best example to date has shown that *D. radicum* attack of the roots resulted in lower initial GSL levels in the shoot of *B. nigra,* followed by a strong increase in leaf glucosinolate levels upon AG herbivory by *P. rapae*, suggesting that *B. nigra* leaves were primed for defense after root induction (van Dam et al., 2005).

As proposed by Martinez-Medina et al. (2016), in order to assess the presence of defense priming in plants, defense-primed plants should possess certain characteristic key features:(i) memory, (ii) more robust defense, and (iii) low fitness cost and better performance. In our study, in order to reveal whether the information of priming stimulus (JA induction)

was stored in plants, we applied two sequential incidents: a priming event followed by the AG herbivore challenge. In response to stressor, JA-treated plants (primed) exhibited higher resistance in a more robust manner compared to control plants (unprimed). As outlined in **Figure 4A**, the theoretical expectation of priming by induction suggests a slight and transient induction of defense traits, by priming stimulus, during the time between the perception of the priming stimulus and the triggering stress. This moderate induction should return to nearly basal levels prior to the triggering stress (see **Figure 4A**; Martinez-Medina et al., 2016). In line with this idea, we found a non-significant induction of total GSLs levels between JA-treated plants versus control plants at time T1. During the larval feeding, theoretically, primed plants should exhibit stronger defense response (**Figure 4A**; higher GSLs in this model); however, our results show no changes in GSLs between treated and non-treated plants (**Figures 4B,C**). This might be due to the fact that the allocation of defenses from root to shoots happened rather quickly upon induction in roots and root-induced plants invested their optimal defense energy quickly upon induction. Given such a scenario was in play; we could expect to observe such a decline at time T2. Perhaps if GSLs measurements were taken at rather earlier stage after AG herbivory, our results would deviate less from the theory expectations. Because priming often involves a faster reaction upon attack, it is crucial to take measurements at multiple time points to detect its occurrence (Engelberth et al., 2004; Ton et al., 2007). Nevertheless, decline of larval weight on JA-induced plants and the correlation between larval weight gain and GSL levels only at time T2 may suggest that the variation of GSL levels between the treatments were more pronounced prior to our measurement at time T2. Therefore, we suggest that the modification of the GSLs profiles upon subsequent AG herbivory and during larval feedings could explain the *S. littoralis* lower weight gain on induced plants. Interestingly, individual GSL induction was overall rather small (see **Supplementary Table S1**) compared to studies showing a clear link between GSL induction and resistance (see,e.g., knock-out mutant studies using *A. thaliana*) (Schlaeppi et al., 2008; Schweizer et al., 2013, 2017). However, other studies have shown weak-to-none GSL induction, while leading to strong induced resistance (Rasmann et al., 2012). Therefore, induction patterns of GSL are indeed informative but they can only give a partial picture of all the potential metabolic changes that happen during the priming phase, which eventually affect insect resistance.

Furthermore, although measuring the fitness cost of priming was outside of the intention of our study, we can argue that JA-treated (primed) plants performed better than control plants on a basis that larvae grew less, and potentially consumed less plant biomass. Our design could only partially address all the criteria for detecting the presence of priming, but the obtained results point toward this direction (Martinez-Medina et al., 2016). In order to evaluate the certainty of priming, further studies should take into consideration the fitness costs, plant lifetime performance, as well as molecular analysis to detect the primed state using molecular markers, such as measuring the expression of defense marker genes and hormone levels (Engelberth et al., 2004; Ton et al., 2007). Therefore, to step beyond the growing literature on plant-mediated BG-AG interactions that vary in space and time, we need to further develop novel model system that can be transposed in field situations.

AUTHOR CONTRIBUTIONS

MB performed the experiments, analyzed the data, and wrote the manuscript. SR supervised the experiment, analyzed the data, and wrote the manuscript. GG assisted with chemical analysis.

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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.2018.01230/full#supplementary-material

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Differences in Hormonal Signaling Triggered by Two Root-Feeding Nematode Species Result in Contrasting Effects on Aphid Population Growth

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Belowground feeding herbivores can affect their aboveground counterparts via systemic induced responses. Hormonal signaling pathways, such as the jasmonic acid (JA) and salicylic acid (SA) pathways, play a pivotal role in shaping such aboveground-belowground herbivore interactions. In this study, we analyzed the effects of two root-feeding nematode species, the cyst nematode *Heterodera schachtii,* and the root-knot nematode *Meloidogyne hapla*, on the preference and performance of cabbage aphid, *Brevicoryne brassicae*. The two sedentary nematodes differ in their feeding strategies and in which plant responses they trigger. We tested the hypothesis that differences in aphid preference and performance are governed by differences in systemic defense signaling triggered by the nematodes. When allowed to choose, aphids showed a lower preference for black mustard (*Brassica nigra)* plants infested with *H. schachtii* compared to uninfested plants. On these plants their population increase was reduced as well. Gene expression analyses revealed that aphid infestation on *H. schachtii*-infested plants strongly induced *PR1*, a marker gene for the SA-pathway. The expression of the JA marker genes *VSP2* and *MYC2* was repressed. On the other hand, *M. hapla* infestation increased aphid preference and population growth compared to those on control plants. Aphid feeding upregulated the expression of *VSP2* and *MYC2*, whereas *PR1* expression was not induced. Interestingly, aphid infestation on plants without nematodes did not activate any of the signaling pathways. This suggests that *H. schachtii* infestation systemically enhanced aphid induced-resistance via the SA pathway. In contrast, *M. hapla* infestation enhanced JA-pathway regulated responses. This may reduce SA-induced resistance to aphid infestation via negative JA-SA cross-talk. Based on our results, we conclude that the differences in the interactions of aphids with cyst and root-knot nematodes emerge from differences in the plant responses triggered by both nematodes. Our results show that aboveground herbivore performance on plants infested with different nematode species may be strongly associated with nematode feeding strategies.

Keywords: aboveground-belowground interactions, *Brevicoryne brassicae,* induced defense responses, *Heterodera schachtii*, hormonal cross-talk, gene expression, *Meloidogyne hapla,* plant-herbivore interaction

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INTRODUCTION

Plants have evolved sophisticated defenses to a wide range of above- and belowground herbivores and pathogens. Some of these responses are induced upon damage, and thus can be tailored to the type of attacker (Karban and Baldwin, 1997; Mithöfer and Boland, 2008; Danner et al., 2017). Induced responses are mainly governed by the phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Beckers and Spoel, 2006). The JA pathway is commonly induced by chewing herbivores and necrotrophic pathogens, which cause tissue damage (Verhage et al., 2011; Wasternack, 2014). Biotrophic pathogens and sap-sucking insects such as aphids and whitefly, on the other hand, induce the SA pathway (Walling, 2000; Moran et al., 2002). ET has a modulatory role and acts synergistically with JA in *Arabidopsis thaliana* (Adie et al., 2007; Leon-Reyes et al., 2009). Plants respond very specifically to the type of herbivore or pathogen that is attacking. This involves an intricate receptor and signaling network, which fine-tunes the response based on specific cues (Koornneef and Pieterse, 2008; Wasternack, 2014). This specificity is caused by a combination of chemical and mechanical cues. First, the plants may recognize herbivores based on salivary compounds they excrete while feeding (Mithöfer and Boland, 2008). Second, herbivores with different feeding strategies, for example sucking-piercing aphids and leaf chewing caterpillars, induce or suppress different signaling pathways (Bidart-Bouzat and Kliebenstein, 2011). Each herbivore and pathogen induces its own combination of JA, SA, and ET responses. Cross-talk between the signaling pathways results in a specific defense response (Pieterse, 2012; Mathur et al., 2013a).

Induced responses do not only occur in the affected areas, but also modify the defense status of undamaged organs (Dicke and Baldwin, 2010; Karban, 2011; Mathur et al., 2013b). Systemic responses are triggered by signals transported via the air or the plant's vascular system (van Dam and Heil, 2011). They may either cause Induced Systemic Resistance (ISR), or prime the plant systemically (van Dam and Oomen, 2008; Erb et al., 2009). ISR increases the resistance levels of undamaged plant parts. Priming, on the other hand, enhances the induced response to later arriving herbivores or pathogens (Martinez-Medina et al., 2016). Both ISR and priming may cause interactions between aboveground and belowground herbivores feeding on the same plant (Erb et al., 2011; Mathur et al., 2011; van Dam and Heil, 2011; van Geem et al., 2016). Consequently, aboveground herbivores may be confronted with plant defense responses activated by root herbivores, and *vice versa* (Kaplan and Denno, 2007; Wurst et al., 2008; Kafle et al., 2017; Papadopoulou and van Dam, 2017). The outcome of aboveground-belowground interactions may depend on the herbivore species that is feeding on either organ, as well as on the time and sequence of infestation (Erb et al., 2011; van Dam and Heil, 2011).

Plant parasitic nematodes are known to infect thousands of species, causing economic losses of more than $157 billion annually to global crop production (Abad et al., 2008). Sedentary cyst and root-knot nematodes are causing the greatest production losses (Jones et al., 2013). They parasitize plant roots by evading or suppressing host defenses (Sasser, 1989; Williamson and Kumar, 2006). Freshly hatched second stage juveniles (J2) migrate into the soil in search of a suitable host. By a combination of heavy stylet thrusting and release of cell wall degrading enzymes, the juveniles enter the root tissue close to the root tip at the elongation zone. Thereafter they migrate toward the vascular cylinder. Cyst and root-knot nematodes have different migration strategies which are essential for the interaction with their host plant. Cyst nematodes move intracellularly, thereby damaging root cells while moving to the vascular cylinder (Williamson and Gleason, 2003). Root-knot nematodes, on the other hand, move intercellulary through the cortex toward the root tip. In the root apex, they turn around, thereby damaging meristematic cells, and enter the vascular cylinder. In the vascular cylinder, they migrate again in a non-destructive way toward the differentiation zone (Williamson and Gleason, 2003). Both cyst and root-knot nematodes transform selected root cells into a permanent feeding site (Gheysen and Mitchum, 2011). Stylet secretions from the nematode pharyngeal glands are responsible for the induction of the feeding cell. Cyst and root-knot nematodes induce different feeding structures: cyst nematodes induce a syncytium, while root-knot nematodes induce the formation of giant cells (Gheysen and Mitchum, 2011). Throughout further development, the nematodes show a continuous cycle of alternate feeding on the cytoplasm and release of stylet secretions (Vanholme et al., 2004). In addition, nematodes manipulate hormonal signaling in their hosts in order to suppress defense responses and establish a sink for nutrients. As for aboveground herbivores, there is species-specificity among nematodes with regards to the hormonal pathways that are induced. This is also reflected in aboveground expression profiles. Plants infested with different nematodes show specific changes in the aboveground expression of signaling marker genes (Hamamouch et al., 2011). This suggests that, similar to aboveground herbivores, nematodes with different feeding strategies induce different signaling pathways in their host. In consequence, it can be postulated that nematodes with different invasion strategies, such as cyst and root-knot nematodes, have differential effects on aboveground feeding herbivores.

Like nematodes, aphids also feed directly on vascular tissue. When aphids arrive on their host plant they insert their stylets into the leaf tissue. On their way to the phloem, they puncture several mesophyll cells in which they inject salivary components or effectors (Hogenhout and Bos, 2011). The saliva of aphids contains enzymes such as peroxidases and β-glucosidases based on which plants may recognize aphids and respond accordingly (Miles, 1999; De Vos and Jander, 2009). As soon as the stylet is inserted into the phloem, aphids inject calcium-binding proteins to prevent blockage of the sieve elements (Hogenhout and Bos, 2011). Aphids are considered "stealthy feeders" (De Vos et al., 2005), because they elicit relatively few induced responses compared to chewing insects (Bidart-Bouzat and Kliebenstein, 2011; Danner et al., 2017). Next to causing little cell damage, they also inject effectors to reduce plant resistance responses (De Vos et al., 2005; Bidart-Bouzat and Kliebenstein, 2011; Hogenhout and Bos, 2011). Nevertheless, aphids can still be affected by (systemically) induced plant responses. For example, *B. brassicae*

is a well-adapted specialist on Brassicaceae which even sequesters its specific defense chemicals, the glucosinolates, for its own defense (Francis et al., 2001). Despite these adaptations, its population development can be affected by nematode feeding (Kutyniok and Müller, 2013; Hol et al., 2016).

We hypothesized that differences in nematode feeding strategies affect the preference and performance of shoot feeding aphids. More specifically, we postulated that this specificity in the interactions between nematodes and aphids is reflected in aphid-induced defense signaling observed in the shoots. We tested our hypothesis using *Brassica nigra* infested with *Heterodera schachtii*, a cyst nematode, or *Meloidogyne hapla*, a root-knot nematode. Both nematodes are generalist pests on many crop species (Jones et al., 2013). They also occur naturally on *B. nigra* in low numbers (Hol et al., 2016). We studied their effect on a common aboveground specialist aphid, *Brevicoryne brassicae*. To test our hypothesis, we set up a series of experiments. In all experiments, plants were infested with nematodes first. This mimics the natural sequence of events. Plant parasitic nematodes are amongst the first pests which an annual plant species, such as *B. nigra*, encounters. This is due to the fact that roots are the first tissues to emerge from the seed. Aphids generally arrive later in the life cycle of a plant, when sufficient leaf mass has formed (Kos et al., 2011). In natural environments, aphids are thus likely to encounter plants that are already infested by root nematodes. In our first experiment, we compared aphid population growth as affected by nematode infestation in a no-choice situation. In addition, we conducted a choice experiment in which aphids could choose between plants infested with a single nematode species and a control plant. We assessed both aphid preference within the first 48 h and long term aphid population development for up to 14 days. Finally, we designed an experiment to elucidate the signaling mechanisms underlying nematode-aphid interactions. Together, these three independent experiments allowed us to directly compare the ecological effects as well as underlying molecular mechanisms of the interactions between nematodes and the aphids.

MATERIALS AND METHODS

Biological Materials

Insect Culture

A starting colony of cabbage aphid, *Breviycoryne brassicae* (L.) was obtained from the Laboratory of Entomology, Wageningen University and Research Centre, Wageningen, the Netherlands. This colony was maintained on black mustard, *Brassica nigra*, plants in insect cages in a greenhouse facility at Radboud University, Nijmegen, the Netherlands. A cohort of nymphs were obtained by transferring adult aphids from the maintenance culture to aphid free *B. nigra* plants. On the following day, the adult aphids were removed from the plants and only the new-born nymphs were maintained. Winged aphids (alates), which were required for host preference test, were obtained by crowding and starving the colonies.

Plant Materials

Brassica nigra seeds (collected in 2004 from population in Wageningen, see Hol et al., 2016) were germinated on water soaked glass pearls in 15 × 10 cm plastic containers. The plastic containers were covered with transparent lids and kept in a climate chamber at a temperature of 20:16°C (day: night) and a photoperiod of 16: 8 h (light: dark). After 10 days, the seedlings were transplanted to 1.5 L pots filled with river sand. Each of the plastic pots was filled with 2,000 g of dry river sand and supplied with 200 mL of tap water. Directly after transplantation, the pots received 100 mL half-strength Hoagland solution with three times phosphorus (3P Hoagland, see van Dam et al., 2004). Twenty randomly selected pots were weighed every 2 days in order to monitor the moisture content of the pots. The pots were supplied with water or Hoagland solutions to maintain the moisture content of the sand at 15%. In cases where high variation in moisture content were observed among pots, the individual pots were weighed and supplied with water to bring the moisture content back to 15%. The plants were supplied with Hoagland solution every week. Developmental stages of *B. nigra* plants were determined following a universal BBCH scale (Lancashire et al., 1991).

Nematode Cultures

Second stage infective juveniles (J2s) of *Heterodera schachtii* and *Meloidogyne hapla* were purchased from HZPC Research and Development, Metslawier, the Netherlands. The nematodes were hatched in root exudates, then purified and shortly stored in tap water. The concentration of each nematode species was determined by counting the number of J2s per 1 mL of nematode suspension under a stereomicroscope.

Experiments

Aphid No-Choice Performance Experiment

Ten 4-weeks old *B. nigra* plants, each with two visibly extended internodes (BBCH code 32) were assigned to each of the following three treatment groups: Aphids only, Aphids + *H. schachtii*, Aphids + *M. hapla*. Prior to nematode inoculation, plants were supplied with Hoagland solution so that the plants were well watered at the time of nematode infection. Following this, each of the plants in the Aphids + *H. schachtii* and Aphids + *M. hapla* groups were inoculated with 3 mL water containing in total 750 J2s of *H. schachtii* or *M. hapla*, respectively. Plants in the first treatment group were mock inoculated with the same volume of water. The nematode suspension was injected into the sand mass close to the rhizosphere. After inoculation, 50 mL of water was supplied to each of the plants in order to facilitate the distribution of nematodes in the rhizosphere. On the seventh day after nematode inoculation, all plants were transferred to individual insect cages. Five 2-days old *B. brassicae* nymphs were released on the top three fully unfolded leaves of each of the thirty *B. nigra* plants. At this time point, the plants had four extended internodes (BBCH code 34). The performance of the aphids was determined over the next 28 days by counting aphids at day 7, 11, 14, 17, 20, 23, 26, and 28. At day 35, the plants were harvested and shoots were immediately freeze-dried to determine their biomass. The number of nematodes present on the roots of each plant were counted and the roots freeze-dried for biomass measurement.

Aphid Choice and Performance Experiment

Two separate choice experiments were conducted to study the preference of *B. brassicae* alates for *H. schachtii* and *M. hapla* infected *B. nigra* plants and their subsequent performance. In the first experiment, ten pairs of plants were kept in a cage. One plant was inoculated with 1000 J2s of *H. schachtii* in 4 mL water and the other was mock inoculated with 4 mL water. On the seventh day after nematode inoculation, 10 winged aphids (alates) were released in each cage in a plastic Petri dish placed equidistant from the two plants. In the second experiment, a similar set-up was used with only four pairs of plants, due to a paucity of plant materials. One was inoculated with 1000 J2s of *M. hapla* and the other mock inoculated. Seven days later, 20 alates were released in each cage. The preference of *B. brassicae* for nematode infected vs. nematode-free plants was assessed by counting the number of winged aphids that had landed on the plants at 16, 24, 40, and 48 h. After aphid preference assessment, the plants were maintained as pairs in the same cages. Aphid numbers were counted at 5, 8, 11, and 14 days after aphid release.

Gene Expression in Response to Nematode and Aphid Infestation

Four-weeks old *B. nigra* plants with two visibly extended internodes (BBCH code 32) were assigned to each of the following six treatment groups: nematode and aphid free plants (Control); only *H. schachtii* inoculated in the roots (Hs), only *M. hapla* inoculated in the roots (Mh), only *B. brassicae* aphids released on the shoot (BB), *H. schachtii* inoculated in the roots and aphids released on the shoot (Hs+BB), *M. hapla* inoculated in the roots and aphids released on the shoot (Mh+BB). Each of the plants receiving nematode treatments were inoculated with 750 J2s of the respective nematode species as above. On the seventh day after nematode inoculation, five developmentally synchronized *B. brassicae* nymphs were released on *B. nigra* plants in BB, Hs+BB and Mh+BB treatment groups. Plants were harvested for gene expression analyses on the third, seventh (just prior to aphid infestation) and sixteenth day (9 days after aphid release) of nematode inoculation. For each time point, ten plants were harvested from each treatment group.

Leaves of the harvested plants were snap frozen in liquid nitrogen, stored at −80°C and freeze-dried. The dried samples were ground with a Retsch Mixer Mill MM300 (Retsch GmbH, Rheinische, Germany) using stainless steel balls. Total RNA was extracted with Aurum™ Total RNA Mini Kit (Bio-Rad, Berkeley, USA) with an additional DNase treatment step included. The number of samples per treatment was reduced from ten to five by pooling two samples together in order to reduce biological variation. The RNA quality and absence of genomic DNA was checked on agarose gel. The concentration and quality of RNA was determined by Nanodrop (Thermo Fisher Scientific, Wilmington, U.S.A.). A 500 ng aliquot of total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Berkeley, USA). Prior to qPCR, the cDNA was diluted to 20-fold. To verify the absence of genomic DNA contamination, negative cDNA control samples were made by omitting the reverse transcriptase.

Expression levels of three plant defense-related marker genes: *PR1*, for the SA pathway (Fu and Dong, 2013), plus *MYC2* and *VSP2* as JA responsive genes (Pieterse et al., 2009; Verhage et al., 2011) were analyzed along with three *Brassica* internal control genes: *GAPC2, PP2A* and *SAND* (**Table 1**). Real-time amplification reactions were performed using SYBR Green detection method on 96-well plates with the Bio-Rad iCycler thermocycler (BIO-RAD, Hercules, CA, USA). Amplification reactions were performed in a 25 μL reaction solution comprising 12.5 μL of iQ™ SYBR® Green Supermix (BIO-RAD, Hercules, CA, USA), 0.75 μL (10 μM) of each of primer, 6 μL of nuclease free water and 5 μL of the template cDNA. A control reaction was run for each gene where the cDNA was replaced by nuclease free water. The reactions were run for 45 cycles at 95°C for 3 min, 95°C for 30 s, 60°C for 15 s (except for *VSP2* gene where the annealing temperature was 61°C) and 72°C for 15 s and followed by a melting curve analysis of 1 min at 95°C, 1 min at 55°C and 10 s at 55°C + 0.5°C each cycle for 80 cycles. For all target and reference genes, orthologous *Arabidopsis thaliana* locus numbers and primer sequences are shown in **Table 1**.

Data Analysis

No Choice Experiment

To detect differences in aphid population growth in the no-choice experiment, aphid numbers over time were analyzed using repeated measures ANOVA with a Greenhouse-Geisser correction to correct for lack of sphericity of the data.

Choice Experiment and Population Development

Aphid preference and performance data obtained in the choice experiments were analyzed using replicated *G*-tests (Sokal and Rohlf, 1995). This allowed us to analyze overall distribution of aphids over pairs of control and nematode infested plants (G_p, equivalent to Chi-square), as well as the total fit of the data to a 1:1 distribution (G_t). Gp or Chi-square values are based on overall numbers; the sums of rows and columns in the distribution table. The G_t value, however, takes into account that the experiment consisted of multiple replicates, in this case plant pairs. The *G*-test also allows to identify heterogeneity among the replicates (G_h; Sokal and Rohlf, 1995). For the longer term population analyses (5–14 days) the paired set-up of control and nematode infested plants was continued. The aphid counts over the experiment are thus a combination of per plant aphid population growth plus redistribution of aphids over the two plants. For this reason, the distribution of aphids at the end of the experiment (day 14) were also analyzed using *G*-tests.

Plant Biomass and Numbers of Galls/Cysts

Biomass data and numbers of cysts/galls were analyzed using ANOVAs per treatment group using SPSS 20.0 (SPSS, Chicago, IL, USA). *Gene expression data*: primer pair efficiencies were calculated using LinRegPCR (11.0) program (Ramakers et al., 2003). Expression levels of target genes were determined by normalizing over the expression levels of three reference genes (*GAPC2, PP2A and SAND*). The expression of the reference genes was computed using the average of mean

TABLE 1 | Sequences of the primers used for qPCR.

Gene	*A. thaliana* locus	Description/function	Sequence of forward primer	Sequence of reverse primer
GAPC2 (reference gene)	AT1G13440	Glyceraldehyde-3-phosphatedehydrogenase/Glycolysis	5′-AGTTGTTGACCTCACGGTTAGAC-3′	5′-TTCCTCCTTGATAGCCTTCTTG-3′
PP2A (reference gene)	AT1G13320	Protein phosphatase 2	5′-CATGCTCCAAGCTCTTACCTG-3′	5′-AATTTGATGTTTGGAACTCTGTCTT-3′
SAND (reference gene)	AT2G28390	SAND family protein	5′-TTTGAACTCTACGCAGCATTTG-3′	5′-ATCGCCATCGCCTTGTCT-3′
PR1 (target gene)	AT2G14610	Pathegenesis - Related Gene 1 (SA responsive gene)	5′-CTACGCCGACCGACTAAGAG-3′	5′-CTACTCCCGGCCAAGTTCTC-3′
VSP2 (target gene)	AT5G24770	Vegetative Storage Protein 2 (JA-responsive gene)	5′-TCTACGCCAAAGGACTTGCT-3′	5′-CCCGTATCCATATTGAGCGTA-3′
MYC2 (target gene)	AT1G32640	Transcription factor (JA-responsive gene)	5′-AGGTTGATGTCGGCGTTG-3′	5′-CGTTAACCACCGACATACTCG-3′

JA, jasmonic acid; SA, salicylic acid.

PCR efficiency and geometric mean of each reference gene (Vandesompele et al., 2002). Normalized expressions of the target genes were then calculated by dividing the expression of the reference genes by that of the target gene (Muller et al., 2002). The normalized expression values of the control groups were averaged. These averages were used to calculate Log2 expression data for each treatment group as follows: Log2 expression = Log2(ExpressionSample_norm/AverageControl_norm). For each treatment group, it was tested whether Log2 expression values deviated from 0 i.e., whether the gene was significantly up or down regulated, by a single sample *t*-test. To control for multiple comparisons we set alpha to 0.005. Data were checked for normality (Kolmogorov Smirnov test on residuals) and Homogeneity of Variance (HOV; Levene's test) and analyzed with ANOVA. When data did not meet requirements (e.g., *PR1* expression on day 16), the equivalent non-parametric Kruskall-Wallis analysis was applied. All gene expression data were analyzed using Statistica version 12.7 (StatSoft Europe, Hamburg, Germany). Tukey HSD tests were conducted to identify significant differences among treatments within harvest.

RESULTS

Aphid No-Choice Performance Experiment

Aphid population increase on nematode infected *B. nigra* plants was not significantly different from that on nematode-free plants (**Figure 1**; Repeated measures ANOVA, Treatment: $F = 1.199$, $p = 0.318$; Treatment × Time: $F = 2.119$, $p = 0.357$). However, on *H. schachtii* infected plants, aphid population numbers were consistently lower. The numbers of root cysts (adult females) and root galls on *H. schachtii* and *M. hapla* inoculated plants, respectively, did not significantly differ between plants with and without aphids (**Table 2**; $p = 0.436$). In the *H. schachtii* treatment group, the number of aphids per plant counted at the end of the experiment decreased with the number of cysts [**Figure 1**, **Table 2**; aphids = −24.94 ln(cysts) + 103.08, $R^2 = 0.3947$]. No correlation was found between aphids and root galls in the *M. hapla* treatment group. Herbivory by aphids and nematodes, alone or in combination, did not significantly affect plant total dry biomass (**Table 3**; ANOVA, $F = 2.214$; $df = 5$; $p = 0.066$), shoot ($F = 2.065$, $df = 5$; $p = 0.084$) or root dry biomass ($F = 1.098$, $df = 5$; $p = 0.372$).

Aphid Choice and Performance Experiment

We conducted a choice experiment to determine host preference of winged *B. brassicae*. This mimics the natural situation, where winged aphids (alates) select suitable host plants to establish and reproduce. When given the choice, significantly lower numbers of aphids were counted on *H. schachtii* infected plants at all time points, except for 40 h after aphid release, compared to nematode-free plants (**Figure 2A**; **Table 4**). In contrast, higher numbers of *B. brassicae* alates landed on *M. hapla* infected plants at 16 h after their release (**Figure 3A**).

The plant pairs were maintained in the same net cages and the population size of *B. brassicae* was counted at 5, 8, 11, and 14 days after aphid release. The average number of aphids on controls

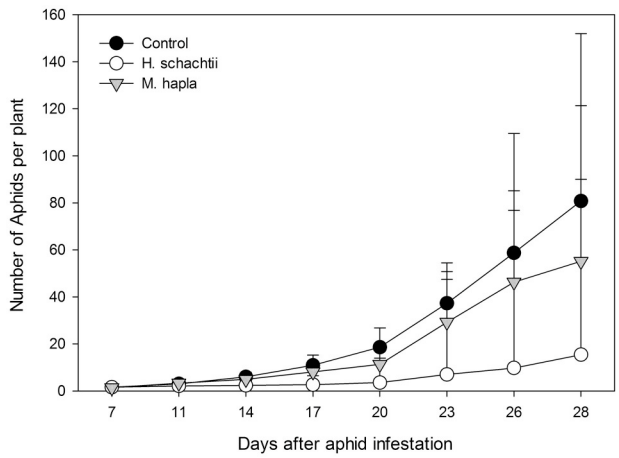

FIGURE 1 | Average numbers (±SEM) of *Brevicoryne brassicae* aphids found per plant from 7 to 28 days after five 2-day old nymphs were released on each plant (no-choice experiment; $n = 10$ per treatment group). Plants were either infested with *Heterodera schachtii* (white circles) or *Meloidogyne hapla* (gray triangles) or mock inoculated (black squares) 7 days before aphids were released.

TABLE 2 | Average number ± SEM of cysts (*Heterodera schachtii*) or root galls (*Meloidogyne hapla*) per plant at 16 days after infestation with nematodes on plants with and without 9 days of aphid (*Brevicoryne brassicae*) feeding.

	No aphids	With aphids (*B. brassicae*)
Heterodera schachtii	37.6 ± 5.6	37.8 ± 6.9
Meloidogyne hapla	28.6 ± 4.1	28.9 ± 4.4

N = 10 per treatment group.

was larger than on *H. schachtii* infested plants at each time point (**Figure 2B**). After 14 days, control plants supported 1.5 times more aphids than *H. schachtii*-infested plants (**Figure 2B**, *G*-test, Gt = 305.7, d.f. = 4, $p < 0.001$). The opposite pattern was observed for the control-*M. hapla* pairs; after 14 days *M. hapla* plants overall hosted about twice as many aphids as controls (*G*-test, Gt = 1409.15, d.f. = 10, $p < 0.001$). We found considerable variance in the numbers of aphids per plants (**Figures 2**, **3**), as well as significant heterogeneity in aphid distribution among the plant pairs (**Table 4**; *H. schachtii* pairs: Gh = 1181, d.f. = 9, $p < 0.001$; *M. hapla* pairs, Gh = 63.8,d.f = 3, $p < 0.001$).

Gene Expression in Response to Nematode and Aphid Infestation

To analyze how nematodes affect shoot defense responses to aphid infestation, we analyzed the expression of three marker genes before and after aphid infestation. We chose PR1 as a marker for the SA pathway. *MYC2* and *VSP2* served as marker genes for the JA signaling pathway. Both nematodes similarly affected *PR1* expression over time (**Figure 4A**). At 3 d.a.i., *PR1* expression levels in nematode-infested plants were similar to those in control plants. At 7 d.a.i. both nematode species reduced *PR1* expression, whereas they increased *PR1* expression at 16 d.a.i. Nine days of aphid infestation alone did not affect *PR1* expression (**Figure 4A**). However, when the aphids were feeding on plants infested with *H. schachtii*, the *PR1* expression in the shoots was significantly higher than that in plants with aphids only. In contrast, *PR1* expression in plants with aphids and *M. hapla* nematodes were close to control levels and significantly lower than on plants with *M. hapla* only (**Figure 4A**).

Early *MYC2* expression at 3 d.a.i. differed between nematode species; *H. schachtii* downregulated *MYC2*, whereas *M. hapla* increased its expression (**Figure 4B**). Interestingly, *VSP2*, which is downstream of *MYC2*, was significantly suppressed in both nematode treatments at the same time point (**Figure 4C**). At 7 d.a.i., the difference had disappeared and both nematode species downregulated *MYC2* and *VSP2* expression. This changed again at 16 d.a.i.; *H. schachtii* upregulated both *MYC2* and *VSP2*, whereas *M. hapla* downregulated both genes. Aphid feeding alone did not upregulate *MYC2* or *VSP2* over control levels (**Figures 4B,C**). However, when aphids were on plants with *H. schachtii*, the expression of both JA-marker genes was downregulated and lower than in plants with aphids or *H. schachtii* only (**Figures 4B,C**). In contrast, aphid feeding on plants with *M. hapla* upregulated the expression of *MYC2* and, even more so, of *VSP2*. This resulted in higher expression of these JA-markers than in plants with aphids or *M. hapla* only (**Figures 4B,C**).

DISCUSSION

Our results show that two species of nematodes with different feeding strategies affect the preference and performance of aboveground feeding aphids in opposite ways. The effects became most apparent when the aphids could choose between non-infected (control) and nematode-infected plants. Infestation by the cyst nematode *H. schachtii* had a negative impact on aphid preference and population growth. In contrast, *M. hapla* infestation attracted aphids and made *B. nigra* a more suitable host. Gene expression analyses revealed that these disparate effects are likely caused by differences in the systemically induced responses triggered by both nematodes. Nine days of aphid feeding more strongly upregulated *PR1* expression on plants infested with *H. schachtii* than on nematode-free plants. *M. hapla* feeding, on the other hand, reduced *PR1* expression, but upregulated the JA marker genes *VSP2* and *MYC2*. This means that *M. hapla* may suppress SA related responses triggered by aphids, most likely via negative cross-talk by enhancing the JA pathway. Together our results confirm the hypothesis that differences in nematode feeding strategies affect systemic effects on aboveground herbivores via differential elicitation of hormonal signaling pathways.

Systemic Responses to Nematode Infestation

Most studies analyzing aboveground-belowground interactions between defense responses analyze systemic responses to belowground insect herbivores or microbial pathogens (van Dam and Heil, 2011; Biere and Goverse, 2016). Compared to this large body of literature, relatively few studies consider how

TABLE 3 | *Brassica nigra* shoot, root and total plant biomass (g dry mass) ± SEM at 16 days after infestation with *Heterodera schachtii* or *Meloidogyne hapla* of plants with and without 9 days of aphid (*Brevicoryne brassicae*) feeding.

	No aphids			With aphids (*B. brassicae*)		
	Shoot	**Root**	**Total**	**Shoot**	**Root**	**Total**
Control	4.33 ± 0.43	1.01 ± 0.15	5.34 ± 0.44	4.77 ± 0.48	1.12 ± 0.23	5.88 ± 0.52
H. schachtii	4.77 ± 0.53	0.95 ± 0.16	5.73 ± 0.48	4.55 ± 0.44	1.09 ± 0.21	5.64 ± 0.43
M. hapla	4.34 ± 0.32	1.10 ± 0.18	5.45 ± 0.29	4.76 ± 0.44	1.10 ± 0.17	5.86 ± 0.48

N = 10 per treatment group.

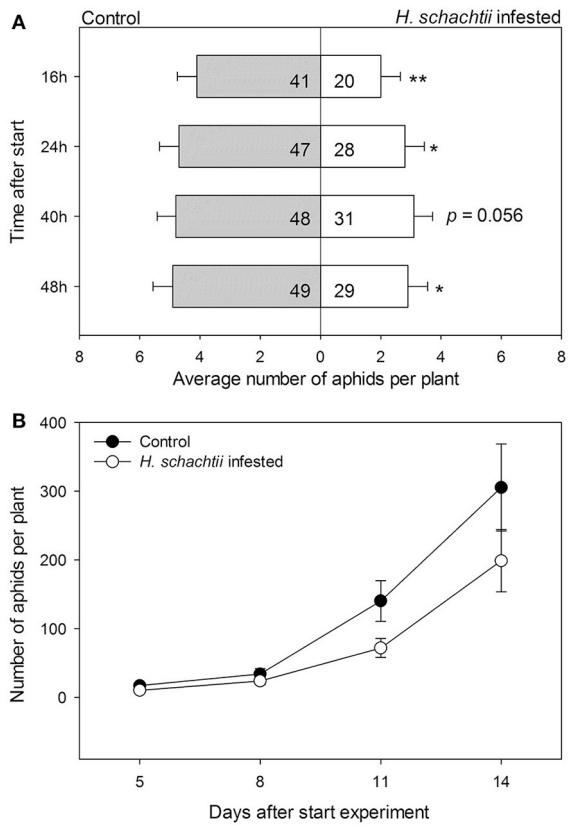

FIGURE 2 | Average numbers (± SEM) of *Brevicoryne brassicae* aphids found per plant. **(A)** Number of aphids at 16, 24, 40, and 48 h after 10 winged aphids were released in each cage ($n = 10$). The aphids were allowed to choose between a control plant (gray bars) and a plant infested with *Heterodera schachtii* nematodes (white bars) enclosed in a single cage. In the bars: sum of aphids out of 100 released in total found on plants in the respective treatment group. The remaining aphids were not found on any plant at the times the aphids were counted. The asterisks indicate whether the distributions of the aphids overall were deviating from a 1:1 distribution (replicated *G*-test per time point, Gpooled, d.f. = 1); $^{*}p < 0.05$, $^{**}p < 0.01$. **(B)** Numbers of aphids on either plant from 5 to 14 days after aphids were released. Black circles: control plants, white circles: *H. schachtii* infested plants. Both plants were left in the same insect cage over the course of the experiment; the resulting numbers thus are a combination of growth rates and redistribution of aphids.

nematode-infestation alters the expression of defense related genes in the leaves (Biere and Goverse, 2016). We found that the cyst nematode *H. schachtii* first suppresses (3–7 d.a.i), and then increases *PR1* expression at 16 d.a.i.. In line with our findings, the cyst nematode *Globodera pallida* increased endogenous SA concentrations 14 d.a.i. in *Solanum tuberosum* (Hoysted et al., 2017). In this study, the expression of three PR genes (PR1, PR2, and PR5) serving as markers for SA signaling (Fu and Dong, 2013) were analyzed. Only PR5 was significantly upregulated by cyst nematode infestation. Endogenous JA levels were not changed by *G. pallida* infestation (Hoysted et al., 2017). This contrasts with our observation that *H. schachtii* first downregulated (3 and 7 d.a.i) and then upregulated the JA marker genes *MYC2* and *VSP2* at 16 d.a.i.. *M. hapla* infestation caused a similar expression profile for *PR1* as *H. schachtii*. However, *M. hapla* mostly suppressed JA marker expression over the course of the experiment, with exception of *MYC2* at 3 d.a.i. The *M. hapla*-induced *PR1* expression in leaves contrasts with previous studies. Root-knot nematodes, especially *Meloidogyne* spp., generally suppress leaf defenses, independently of the response they induce in the root (Hamamouch et al., 2011). However, which pathways are affected, and how, varies among studies. In rice, *M. graminicola* infestation suppresses both SA and JA pathways in the leaves starting from 3 d.a.i. onwards (Kyndt et al., 2012b). Similarly, several SA and JA marker genes are suppressed in the leaves of *A. thaliana* infested for 5–14 days with *M. incognita* (Hamamouch et al., 2011). Direct measurements of the hormone concentrations in *S. lycopersicum* showed a lower endogenous SA, but higher JA concentrations in the leaves at 14 d.a.i. with *M. incognita* (Kafle et al., 2017). Due to a paucity of studies, it is currently not possible to identify general patterns. More detailed analyses of the effectors that the different nematodes excrete may shed more light on how they differentially manipulate their host's defense response (Vanholme et al., 2004; Abad et al., 2008; Haegeman et al., 2012).

Interactive Effects of Nematodes and Aphids

Once aphids were feeding on the nematode infected plants, we found a clear difference in the activation of signaling pathways between *H. schachtii* and *M. hapla* infested plants. *H. schachtii* infestation strongly enhanced *PR1* expression upon aphid feeding. A similar activation of the SA defense pathway, as indicated by an increase in endogenous SA concentration, was observed in *S. tuberosum* when plants were infected with the cyst nematode *G. pallida* and the aphid *Myzus persicae* (Hoysted et al., 2017). On the other hand, *MYC2* and *VSP2* expression

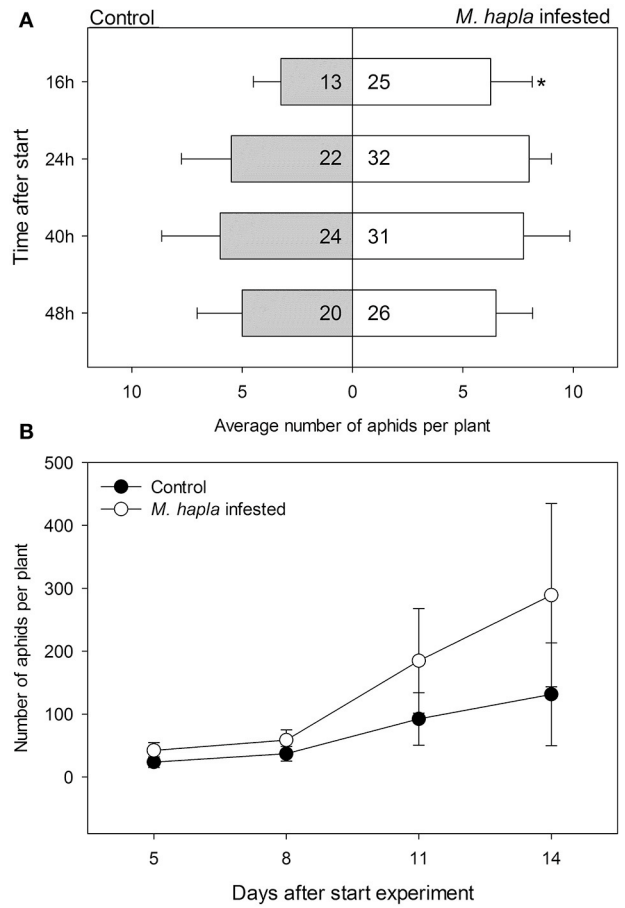

FIGURE 3 | Average numbers (± SEM) of *Brevicoryne brassicae* aphids found per plant. **(A)** Number of aphids at 16, 24, 40, and 48 h after 20 winged aphids were released in each cage ($n = 4$). The aphids were allowed to choose between a control plant (gray bars) and a plant infested with *Meloidogyne hapla* nematodes (white bars) enclosed in a single cage. In the bars: sum of aphids out of 80 released in total found on plants in the respective treatment group. The remaining aphids were not found on any plant at the times the aphids were counted. The asterisks indicate whether the distributions of the aphids overall were deviating from a 1:1 distribution (replicated *G*-test per time point, Gpooled, d.f. = 1); $^*p < 0.05$. **(B)** Numbers of aphids on either plant from 5 to 14 days after aphids were released. Black circles: control plants, white circles: *M. hapla* infested plants. Both plants were left in the same insect cage over the course of the experiment; the resulting numbers thus are a combination of growth rates and redistribution of aphids.

TABLE 4 | *G*-test test values for the *Brevicoryne brassicae* choice tests; short term distribution.

	Control vs. *H. schachtii*				Control vs. *M. hapla*		
	Gh	Gp	Gt		Gh	Gp	Gt
	9 d.f.	1 d.f.	10 d.f.		3 d.f.	1 d.f.	4 d.f.
16 h	229.52	7.36	236.88	16 h	14.49	3.85	18.33
P	< 0.001	0.0067	< 0.001	*p*	< 0.001	0.049	< 0.001
24 h	267.88	4.85	272.74	24 h	10.22	1.86	12.09
P	< 0.001	0.0276	< 0.001	*p*	0.017	0.172	0.017
40 h	279.22	3.64	282.86	40 h	22.30	0.89	23.19
P	< 0.001	0.0564	< 0.001	*p*	< 0.001	0.345	< 0.001
48 h	279.73	5.18	284.91	48 h	13.28	0.78	14.07
p	< 0.001	0.0228	< 0.001	*p*	< 0.001	0.376	0.007

d.f, degrees of freedom; Gh, G for heterogeneity among test replicates; Gp, G for pooled data (equivalent to Chi-square analysis on total numbers per treatment group); Gt, G for overall fit of the expected ratios (1:1); H. schachtii, plants infested with Heterodera schachtii nematodes; M. hapla, idem, with Meloidogyne hapla nematodes.

were suppressed on double infested plants, compared to plants infested with *H. schachtii* or the aphid alone. Interestingly, the aphid-induced suppression of *VSP2* was the strongest in the presence of *H. schachtii*, which on its own strongly increased *VSP2* expression. This indicates that the enhanced SA-response induced by aphids on *H. schachtii*-infested plants may suppress the JA-induced responses via negative cross-talk (Pieterse et al., 2009). In contrast, aphid feeding on *M. hapla*-infested plants induced the JA, but not the SA pathway. Indeed, it has been reported that aphid feeding triggers both SA and JA responses (Moran et al., 2002; De Vos et al., 2005; Cao et al., 2016). On *Arabidopsis thaliana*, *B. brassicae* feeding particularly increases the expression of genes in the SA pathway, and to a lesser extent in the JA pathway (Kuśnierczyk et al., 2008). Interestingly, *PR1* expression was found to be only upregulated at later time points (24–48 h after aphid infestation). *VSP2* expression was downregulated by aphid feeding in *A. thaliana*, despite the general upregulation of JA-related genes (Kuśnierczyk et al., 2008). In our study, we found that the specialist *B. brassicae* on its own induced very few defense responses, as indicated by marker gene expression. However, this does not preclude that plant defense levels are locally increased. Both *B. brassicae* and *My. persicae* feeding can up-or downregulate the levels of specific glucosinolates in leaves and phloem of *A. thaliana* and *Brassica* species (Kutyniok and Müller, 2012; Hol et al., 2013, 2016). Aphids, like nematodes, inject salivary components to manipulate their host's defense responses and to create a sink at their feeding site (De Vos and Jander, 2009). A reallocation of glucosinolates as a consequence of these manipulations, may require the action of glucosinolate transporters (Nour-Eldin et al., 2012), rather than the activation of glucosinolate biosynthesis genes via the SA or JA-signaling pathway.

Molecular Mechanisms Underlying Interactive Effects

The differential effects of the two nematodes species on aphid-induced responses in the shoots, may originate from differences in the specific plant-nematode interaction. As mentioned above, root-knot nematodes invade the plant causing little cell damage. Cyst nematodes, on the other hand, damage root cells while migrating to the vascular cylinder (Gheysen and Mitchum, 2011). The damage caused by *H. schachtii* in the early phases of the plant-nematode interaction, may have primed the plant to respond stronger to the later arriving aphids. When priming occurs, there may first be an initial response to the priming stimulus, in this case the nematode infestation (see Martinez-Medina et al., 2016). Indeed, *M. hapla* infested plants showed upregulated *MYC2* and, to a lesser extent *PR1,* expression

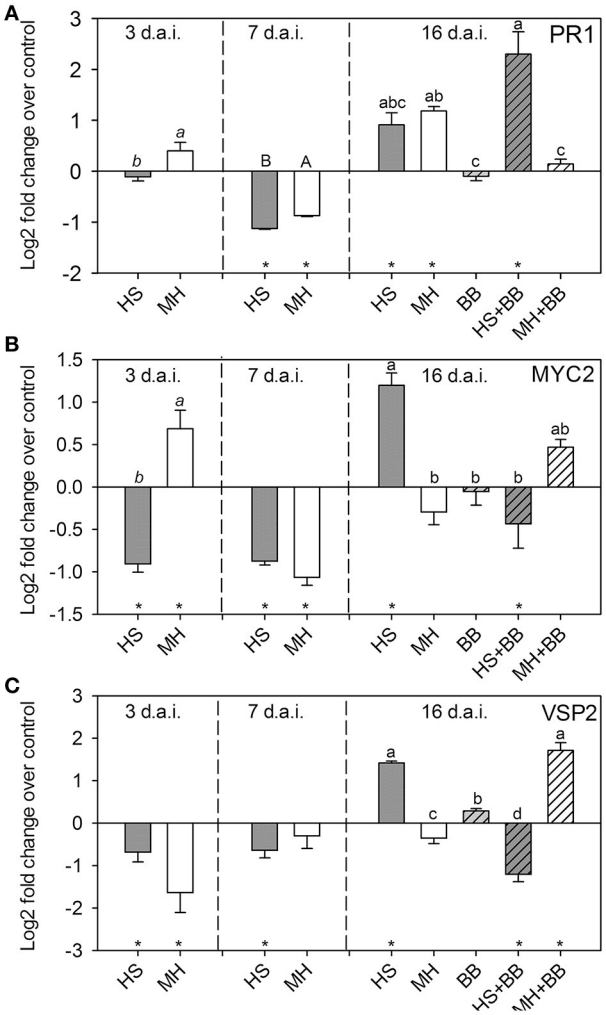

FIGURE 4 | Relative expression level (Log2) of **(A)** *PR1*, **(B)** *MYC2*, and **(C)** *VSP2* in *Brassica nigra* at 3, 7, and 16 days after infestation (d.a.i.) with either *Heterodera schachtii* (HS) or *Meloidogyne hapla* (MH) nematodes. At 7 d.a.i., a subset of the plants was infested with *Brevicoryne brassicae* (BB) aphids—the plants with BB harvested at 16 d.a.i. suffered 9 days of aphid feeding. The expression levels were calculated over the average expression levels in control plants (mock inoculated with water) taken at each time point. Stars on the X-axis indicate whether the treatment significantly up –or downregulated the gene (Log2 value larger or smaller than zero; Single sample *t*-test, $p < 0.005$ to correct for multiple comparisons). $N = 5$ biological replicates per treatment group. Different letters over bars indicate significant differences (3 and 7 d.a.i.: *t*-tests; 16 d.a.i.; *PR1*: multiple comparisons test after Krusall-Wallis ANOVA; *MYC2* and *VSP2*: Tukey HSD test after ANOVA) among treatments within harvest time point.

at 3 d.a.i. (**Figure 4**). *H. schachtii* already repressed defense marker genes at 3 d.a.i., despite the fact that feeding cell formation takes up to 5 d.a.i. (de Almeida Engler et al., 1999). Arguably, we may have missed the initial response to nematode invasion as these responses may have occurred at earlier time points (see references in Kyndt et al., 2014). However, our results obtained at 7 d.a.i, are in line with studies reporting that from this time point onwards, stress related genes, such as *LOX1, ERF2*, and genes coding for defenses, such as phytoalexins and protease inhibitors, were repressed in roots and shoots of nematode-infested plants (Kyndt et al., 2014). The suppression of plant immunity by nematodes may result in systemic induced susceptibility. For example, rice plants infected with *M. graminincola* become less resistant to the aboveground pathogen rice blast (Kyndt et al., 2017). Even though the effectors injected by root-knot and cyst nematodes greatly overlap, there may be essential differences affecting plant hormonal signaling upon invasion (Gheysen and Mitchum, 2011). For example, the establishment of the cyst nematode feeding cells involves ET signaling, whereas this is not the case for root-knot nematodes (Gheysen and Mitchum, 2011). ET in turn, can interact with both the JA and SA signaling pathways (Pieterse et al., 2009). Differences in the activation of the ET pathway thus can affect induced responses to aphids. Also after the feeding cell has been established, nematodes keep injecting effectors into the plant (Vanholme et al., 2004), thus maintaining the differences in chemical communication with their hosts.

Nematodes and Aphids May Compete for Nutrients

Our results provide evidence that interactions between defense signaling pathways may underlie the interactions between nematodes and aphids on *B. nigra*. However, this does not preclude that other processes play a role as well. Nematodes alter primary metabolite production and resource allocation within their host plant to enhance nutrient allocation to their feeding site (Kyndt et al., 2012a; Hol et al., 2013). Aphids also create a nutrient sink, enhancing amino acid and sugar concentrations in the phloem sap on which they feed (Cao et al., 2016; Hol et al., 2016). When nematodes and aphids feed on the same plant, it may also result in a "tug of war" for plant nutrients between the two herbivores. The outcome of this so-called "apparent competition" (Kaplan and Denno, 2007), may depend on the strength with which the first arriving herbivore, in this case the nematode, manipulates the source strength of its feeding site, the root. Further studies, analyzing transcriptomes, hormone and metabolome dynamics in roots, shoots and phloem are needed to identify how much defense responses and resource reallocation processes contribute to the observed effects.

Effect of Natural Insect Behavior

Our results also show the relevance of including natural herbivore behavior in experimental set-ups. Only when the aphids were allowed to choose, the effect of nematode infestations became evident. This is in line with the earlier observation that *Myzus persicae* aphids preferred nematode free *A. thaliana* over *H. schachtii*-infested plants (Kutyniok et al., 2014). Similar to our results, this study found that aphid preference correlated positively with their performance. As aphids are parthenogenetic, the number of foundresses initially colonizing the plants is greatly determining the final aphid load (Hol et al., 2016). Most studies do not allow aphids to choose among plants or even among leaves within the plant (by using clip cages, see Hoysted et al., 2017). In addition, most studies infest plants with unrealistically high numbers of aphids (e.g., 100 aphids on a single *A. thaliana*, De

Vos et al., 2005) in order to obtain a strong response. Differences in herbivore loads on the plant may also explain the variance in responses that are reported (Stewart et al., 2016). Additionally, it may also explain why results obtained in the greenhouse do not always translate to field situations.

Variation in Nematode Effects

There is ample evidence to suggest that belowground herbivory by nematodes negatively affects aphid performance (Bezemer and van Dam, 2005; Wurst and van der Putten, 2007; Kaplan et al., 2009, 2011; Hong et al., 2010). However, the reported outcomes vary considerably among studies, largely due to differences in experimental designs. For example, the numbers of J2 nematodes added to single plants ranges from 60 (Hamamouch et al., 2011) via a 500–1,000 (Hol et al., 2013; Kutyniok et al., 2014) to 10,000 (Hoysted et al., 2017). The first half of the range likely results in realistic infestation rates found in natural plant populations (Hol et al., 2013, 2016), whereas the latter may be more indicative for infestation levels in agricultural fields (Jones et al., 2013). In addition, the responses to nematode infestation and aphids, or combinations thereof, are assessed at different time points after infestation. These range from a few days to several weeks (Bezemer and van Dam, 2005; Wurst and van der Putten, 2007; Hol et al., 2013). Last but not least, the outcome of interactions between nematodes and aphids may be affected by nutrient availability (Kutyniok and Müller, 2013; Kutyniok et al., 2014). In our experiment, we controlled for most of these factors by directly comparing the responses to, and interaction between two species of nematodes and an aphid on their natural host plant, grown on plain sand with nutrient solutions. Nevertheless, in the field, *B. nigra* is colonized by a community of nematodes (Hol et al., 2016). Experiments with plant infested by multiple nematodes conducted under (near) field conditions can reveal whether the observed systemic defense responses also affect aphid preference and performance under natural conditions (see Vandegehuchte et al. (2010).

CONCLUSION

We found that the root feeding plant parasitic nematodes *H. schachtii* and *M. hapla* have contrasting effects on the aboveground phloem feeding aphid *B. brassicae*. The identity of the nematodes determined the outcomes of the plant-mediated effects on aphid preference and performance. Differences in hormonal pathways involved in induced plant responses were found to play a role. Our findings may be particularly relevant to agro-ecosystems, where usually one species of nematode is dominating pest in a crop. It is yet to be assessed how signaling pathways interact when multiple nematodes infest a plant, as is common in natural environments.

AUTHOR CONTRIBUTIONS

TT designed the study together with MW and NvD. MW carried out the experiments under direct supervision of TT. VM and NvD performed statistical analyses. MW wrote most of the Materials and Methods section. NvD, VM, and TT wrote the rest of the manuscript and prepared it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Belowground Inoculation With Arbuscular Mycorrhizal Fungi Increases Local and Systemic Susceptibility of Rice Plants to Different Pest Organisms

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Plants face numerous challenges from both aboveground and belowground stressors, and defend themselves against harmful insects and microorganisms in many ways. Because plant responses to biotic stresses are not only local but also systemic, belowground interactions can influence aboveground interactions in both natural and agricultural ecosystems. Arbuscular mycorrhizal fungi (AMF) are soilborne organisms that form symbiotic associations with many plant roots and are thought to play a central role in plant nutrition, growth, and fitness. In the present study, we focused on the influence of AMF on rice defense against pests. We inoculated rice plants with AMF in several field and greenhouse experiments to test whether the interaction of AMF with rice roots changes the resistance of rice against two chewing insects, the rice water weevil (*Lissorhoptrus oryzophilus* Kuschel, RWW) and the fall armyworm (*Spodoptera frugiperda*, FAW), and against infection by sheath blight (*Rhizoctonia solani*, ShB). Both in field and greenhouse experiments, the performance of insects and the pathogen on rice was enhanced when plants were inoculated with AMF. In the field, inoculating rice plants with AMF resulted in higher numbers of RWW larvae on rice roots. In the greenhouse, more RWW first instars emerged from AMF-colonized rice plants than from non-colonized control plants. Weight gains of FAW larvae were higher on rice plants treated with AMF inoculum. Lesion lengths and susceptibility to ShB infection were higher in rice plants colonized by AMF. Although AMF inoculation enhanced the growth of rice plants, the nutritional analyses of root and shoot tissues indicated no major increases in the concentrations of nutrients in rice plants colonized by AMF. The large effects on rice susceptibility to pests in the absence of large effects on plant nutrition suggest that AMF colonization influences other mechanisms of susceptibility (e.g., defense signaling processes). This study represents the first study conducted in the U.S. in rice showing AMF-induced plant susceptibility to several antagonists that specialize on different plant tissues. Given the widespread occurrence of AMF, our findings will help to provide a different perspective into the causal basis of rice systemic resistance/susceptibility to insects and pathogens.

Keywords: arbuscular mycorrhizal fungi, rice, root colonization, rice water weevil, fall armyworm, sheath blight, aboveground-belowground interactions

INTRODUCTION

Plants are active organisms capable of adapting to fluctuating environmental conditions; accordingly, they exhibit a high degree of phenotypic plasticity (Pozo et al., 2015). As an important example, plants respond to diverse biotic threats from above- and belowground herbivores and pathogens using a variety of direct and indirect defense mechanisms (Kessler and Baldwin, 2002; Robert-Seilaniantz et al., 2011). Because plant responses to herbivores and pathogens are both local and systemic, above- and belowground organisms may influence each other's fitness through changes in the shared host plant (Bezemer and van Dam, 2005; Soler et al., 2007; Soler et al., 2009; Ali et al., 2013). The presence of soilborne microbes in the rhizosphere plays a considerable role in ecosystem functioning by changing nutrient uptake by plants (thereby influencing quality of the host plant for herbivores), promoting plant growth, and altering plant defense pathways independently of plant nutrition (van der Heijden et al., 1998; Pozo and Azcon-Aguilar, 2007; Smith and Read, 2008). The interplay of these various changes controls the final impact of soilborne microbes on the structure of communities associated with plants.

Arbuscular mycorrhizal fungi (AMF) are well-known, essential components of soil biota within natural and agricultural ecosystems (Smith and Read, 2008). AMF form associations with the root systems of more than 85% of vascular plant species, including many important crops (Smith and Read, 2008). The symbiosis between AMF and plants results in a continuum of effects on plant growth and fitness, from highly mutualistic to antagonistic (Johnson et al., 1997; Smith and Read, 2008; Currie et al., 2011; Barber et al., 2013b). Most often, however, associations with AMF facilitate the acquisition by plants of essential nutrients such as nitrogen, phosphate, and water from the soil (Smith and Read, 2008). In exchange, the fungal partner receives photosynthetically fixed carbon, which is used to grow more mycelial networks that allow the root system to expand in the soil and absorb more nutrients (Parniske, 2008; Smith and Read, 2008; Bonfante and Genre, 2010). Although in agricultural ecosystems the association of plants with AMF often results in plant yield increases (Gosling et al., 2006), the effects of AMF can also vary markedly along a parasitism-mutualism continuum (Johnson et al., 1997; Paszkowski, 2006; Fesel and Zuccaro, 2016). Because AMF are important components of soil microbial communities and are a central part of agro-ecosystems, they can potentially provide benefits but also costs to farmers.

Colonization of plant roots by AMF has been shown to alter plant quality for both above- and belowground insect herbivores and pathogens (Goverde et al., 2000; Gange, 2001; Koricheva et al., 2009; Currie et al., 2011) and AMF can contribute to improved resistance or tolerance against abiotic (Ruiz-Sanchez et al., 2010; Maya and Matsubara, 2013) and biotic stresses, such as those caused by root and shoot herbivores and pathogens (Gange, 2001; Pozo and Azcon-Aguilar, 2007; Smith and Read, 2008; Campos-Soriano et al., 2011; Vannette and Hunter, 2011). However, the effects of mycorrhizal colonization on insect fitness or pathogen infection vary depending on the identity of both AMF and host plant, the insect or pathogen involved, and environmental factors (Gange and West, 1994; Gange, 2001, 2007; Gange et al., 2002; Bennett et al., 2006; Borowicz, 2009; Gehring and Bennett, 2009; Koricheva et al., 2009; Pineda et al., 2010; Campos-Soriano et al., 2011; Currie et al., 2011; Vannette and Hunter, 2011). It has been proposed that generalist herbivores and necrotrophic pathogens are usually negatively affected by the presence of AMF, whereas specialist herbivores and biotrophic pathogens are usually positively affected, performing better on mycorrhizal plants (Gange et al., 2002; Hartley and Gange, 2009; Koricheva et al., 2009; Currie et al., 2011; Borowicz, 2013). A meta-analysis of 34 studies showed that AMF predominantly have negative effects on the performance of generalist chewing herbivores, but positive effects on specialist chewing insects (Koricheva et al., 2009).

The mechanisms by which mycorrhizal colonization alters plant resistance, and the effects of agricultural practices on the presence and effectiveness of AMF symbiosis in crop plants, are not fully understood. Increases in plant growth and improvements in nutrient uptake resulting from mycorrhizal colonization might make plants more attractive or susceptible to herbivores and pathogens (Roger et al., 2013). Alternatively, evidence from tomato plants showed that mycorrhizal colonization may change plant resistance by altering plant defense such as the jasmonic acid pathways (Jung et al., 2012). A large body of evidence also shows that insect herbivores and plant pathogens frequently induce plant defense responses, but the indirect effects of AMF on these induced responses are not thoroughly understood. Importantly, agricultural practices often reduce the presence and effectiveness of AMF symbiosis in the soil (Barber et al., 2013b), which may reduce or delay colonization of the crop by AMF relative to herbivore infestation or pathogen attack. A better understanding of the changes in crop plants in response to root colonization by AMF in agricultural settings, principally in major crops, and how these changes affect plant-herbivore or plant-pathogen relationships, is urgently needed to more effectively utilize mycorrhizae in agriculture.

Cereal crops are an important group of plants that establish symbiotic associations with AMF (Sawers et al., 2008; Gutjahr et al., 2009; Vallino et al., 2009; Campos-Soriano et al., 2011; Gutjahr et al., 2015b). Rice (*Oryza sativa* L) is a staple for more than half the globe's population and represents a promising model system for studies of AMF interactions in general and plant-AMF-herbivore interactions in particular. The presence of AMF associations in rice roots has received increased attention in recent years (Gutjahr et al., 2009; Campos-Soriano et al., 2011; Edwards et al., 2015). In a recent study, a detailed characterization of the root-associated microbiomes of the rice plant revealed dynamic changes in these microbial communities as a function of geographical location, soil source, host genotype, and cultivation practices (Edwards et al., 2015). However, only a few studies have investigated the interacting effects of AMF symbiosis in rice plants and the implications of these interactions for insect herbivores or pathogens (Campos-Soriano et al., 2011; Cosme et al., 2011). For instance, mycorrhizal rice plants showed enhanced resistance to the rice blast fungus, *Magnaporthe oryzae* and this resistance appeared to rely on both the systemic activation of defense regulatory genes in the absence of pathogen

challenge and priming for stronger expression of defense genes during pathogen infection (Campos-Soriano et al., 2011).

The aim of the current study was to understand how AMF inoculation influences rice-herbivore and rice-pathogen interactions. We used as model organisms three important pests of rice in the southern U.S.: larvae of the rice water weevil (RWW; *Lissorhoptrus oryzophilus* Kuschel; Coleoptera: Curculionidae), larvae of the fall armyworm (FAW, *Spodoptera frugiperda* J.E. Smith; Lepidoptera: Noctuidae), and sclerotia of sheath blight (ShB, *Rhizoctonia solani;* Basidiomycete). Of these three study organisms, only the effects of AMF on rice water weevils have been previously investigated. Cosme et al. (2011) found, in a greenhouse experiment, that females of the grass-specialist RWW laid double the amount of eggs in AMF-inoculated rice plants, an effect they speculated was caused by AMF-mediated increases in plant nutrient concentrations. In light of these prior results with RWW, we explored the hypothesis that colonization of roots by AMF would reduce the resistance of rice to the RWW in the field and greenhouse experiments. Then, in light of new results, we addressed a second hypothesis that AMF colonization might reduce the resistance of rice to other pest organisms such as FAW and ShB under greenhouse conditions. We asked the following questions: (1) Does AMF inoculation reduce rice resistance against a root- and foliar-feeding herbivore in the field and greenhouse? (2) Does AMF inoculation affect resistance to a fungal pathogen? (3) Does AMF inoculation increase plant biomass? (4) Does AMF inoculation influence the nutritional status of rice plants? To answer these questions, we carried out a series of field and greenhouse experiments in rice by manipulating the availability of AMF (inoculated and non-inoculated plants) using a commercial inoculum containing six AMF species from the Glomeraceae family. We found that the performance of insects and the pathogen on rice was enhanced when plants were colonized by AMF, which was consistent with results from Cosme et al. (2011); however, this susceptibility was not correlated with changes in plant nutritional status.

MATERIALS AND METHODS

Study System: Plants, Fungi, and Insects

To study plant-AMF-herbivore and plant-AMF-pathogen interactions, we used two commercial varieties of rice as the host plant. 'Lemont' and 'Cocodrie' are high-yielding, early-maturing, conventional varieties developed at the Texas A&M University Agricultural Research and Extension Center (Beaumont, TX, United States) and the Louisiana State University Agricultural Center (LSU AgCenter) H. Rouse Caffey Rice Research Station (Crowley, Acadia, LA, United States), respectively (Bollich et al., 1985; Linscombe et al., 2000). 'Cocodrie' is a susceptible variety grown widely in the southern U.S. 'Lemont' is not widely grown currently but was chosen because it had been used in previous studies of rice-AMF interactions (Dhillion, 1992). Seeds of rice were kindly provided by the breeding and foundation seed program at the LSU AgCenter H. Rouse Caffey Rice Research Station. 'Lemont' was used for experiments in 2012 and 'Cocodrie' for experiments in 2013.

A commercial inoculum prepared *in vivo* to contain only AMF propagules (ECOVAM^{TM} VAM Endo Granular, Horticultural Alliance Inc., Sarasota, FL, United States) was used to promote and establish symbiosis with the host plants in the field and greenhouse experiments. The inoculum contained six species of AMF (*Rhizophagus irregularis*, *Funneliformis mosseae*, *Glomus deserticola*, *Rhizophagus fasciculatum*, *Sclerocystis dussii*, and *Glomus microaggregatum*) and consisted of spores, hyphae and colonized root fragments. All AMF species were originally obtained from the International Culture Collection of (Vesicular) AMF (INVAM, West Virginia University, United States). The AMF propagules were carried in an inert-like material consisting of a uniform mixture of zeolite, pumice, vermiculite, perlite, and attapulgite. According to the supplier, quantification of the number of spores per gram of inert material was accomplished by the wet sieving and decanting method of Gerdemann and Nicolson (1963) followed by sucrose gradient centrifugation according to the modification proposed by Schenck (1982). For the extraction of spores, 20 g of inert material was blended for 10 s in one liter of tap water. Counting was carried out under an optical microscope using a counting slide of 1 mL. The formulated material contained an average of 132 spores of AMF (all species) per gram, in addition to hyphae and colonized root fragments.

The RWW is the most destructive insect pest of rice in the United States (Stout et al., 2002; Tindall and Stout, 2003; Hamm et al., 2010). RWW adults feed on young rice leaves, producing longitudinal scars. However, this form of injury is not economically important; rather, the larvae have a strong impact on plant yields when they feed on roots of flooded rice (Cosme et al., 2011). Adult rice water weevils were collected from rice fields at the H. Rouse Caffey Rice Research Station 24 h prior to conducting greenhouse experiments. Field experiments relied on natural infestations of RWWs, which are abundant at the field site. Weevils were maintained in glass jars with freshly cut rice leaves and water until use. Before starting the experiment, weevils were captured in copula or sexed under a dissecting microscope in order to ensure equal numbers of males and females.

The FAW is a sporadic pest of rice that causes harm by consuming aboveground portions of rice with its chewing mouthparts. Adult female armyworms oviposit a large number of eggs on leaves, which give rise to larvae that begin to feed on leaves (Stout et al., 2009). Larvae of the FAW used in these experiments were obtained from a colony maintained continuously on meridic diet in a laboratory. The colony originated from larvae collected in rice fields near Crowley, LA, in 2011. Genetic variability and vigor of the colony were maintained annually with field-collected larvae. The diet used for rearing of larvae was a commercial formulation designed specifically for this species (Southland Products Incorporated, Lake Village, AR, United States). Pupae were placed in buckets containing vermiculite, wax paper as a substrate for oviposition, and two dental rolls soaked in a mixture of honey and beer (150 ml honey-150 ml beer- 300 ml water-12 g ascorbic acid) and covered with cheesecloth. After emergence, adults mated and females oviposited eggs onto the cheesecloth, which were collected daily and placed in 8-cell trays (Bio-Serv, Frenchtown,

NJ, United States) with a moistened cotton ball and sealed with lids. When neonates began to emerge, they were placed in cups supplied with artificial diet. Larvae were maintained on meridic diet until use for feeding assays. The colony was maintained under controlled environmental conditions (L14: D10, 28 ± 2°C, 38 ± 2% R.H).

Rhizoctonia solani (Basidiomycete), the causal agent of ShB of rice, is a soilborne pathogen with a wide host range. The disease caused by this organism in rice usually develops after the tillering stage of rice growth, and initial infection appears on the stem near the water line as oval lesions, which dry and turn tan (Lee and Rush, 1983). The fungal isolate LR172 of the ShB pathogen used in this study was originally isolated in 1972 from a naturally infected rice plant (cv. 'Lebonnet') in Louisiana. LR172 was generously provided by D. Groth (LSU AgCenter H. Rouse Caffey Rice Research Station) and maintained on potato dextrose agar (PDA). Mycelial growth and sclerotia production were typical of *R. solani*. The isolate of *R. solani* was examined for mycelial growth with a compound microscope (Olympus CH2, Pittsburgh, PA, United States). A verified isolate of *R. solani* was subcultured by placing sclerotia in the center of a 9-cm-diameter petri dish filled with PDA medium to produce active mycelia and grown at room temperature (22–25°C) under continuous light. These cultures were used to prepare agar blocks of 5-day-old cultures inoculation.

Experimental Design

Evaluating Effects of AMF on RWW Performance (Field Study)

To evaluate whether inoculation of rice plants with AMF affects the resistance of rice plants to *L. oryzophilus*, three small-plot field experiments were conducted during the 2012 and 2013 growing seasons at the LSU AgCenter H. Rouse Caffey Rice Research Station (Crowley, Acadia Parish, LA, United States). In 2012, one experiment, referred to as Experiment-1 (Exp-1) was conducted; in 2013, two experiments, Experiment-2 (Exp-2) and Experiment-3 (Exp-3) (**Table 1**), were conducted. Each experiment comprised three treatments. For the first treatment (F, fungicide) rice seeds were treated with a mixture of the fungicides Maxim 4FS (fludioxonil, 4.16 mg a.i. 300 g^{-1} of seeds; Syngenta Crop Protection, Greensboro, NC, United States), Apron XL 3LS (mefenoxam, 26.33 mg a.i. 300 g^{-1} of seeds; Syngenta Crop Protection, Greensboro, NC, United States) and Dynasty (azoxystrobin, 20.79 mg a.i. 300 g^{-1} of seeds; Syngenta Crop Protection, Greensboro, NC, United States) and planted in soil with sterilized AMF inoculum. Rice seeds were treated with a mixture of fungicides before planting to eliminate the presence of any fungi from experimental plots. For the second treatment (NM, nonmycorrhizal), rice seeds were sown in soil with sterilized AMF inoculum. The sterilized inoculum was used in nonmycorrhizal plots to control for the possibility that inert ingredients in the commercial inoculum altered soil properties. For the F and NM treatments, commercial inoculum was sterilized by autoclaving for 60 min at 120°C to destroy living AMF inoculum. For the third treatment (M, mycorrhizal), rice seeds were planted in soil inoculated with live AMF. For all three experimental treatments, rice plants were grown from seeds in the field; thus the soil was not sterilized and likely contained native AMF. Sterilized mock or live AMF inoculum was applied on the surface of the soil and gently raked in to incorporate the live or mock inoculum into the upper 2.5 cm of the soil. Experiments were laid out in a randomized complete block design (RCBD; in Exp-1) or in a completely randomized design (CRD; in Exp-2 and 3) with a total of eight and ten blocks (replications) per treatment per experiment for 2012 and 2013, respectively.

Rice was hand-seeded on the dates specified in **Table 1** at a rate of 10 g of seeds per plot. Plots measured 0.762 m × 0.762 m. A soil sample was collected from the plots before seeding in 2013 and sent for analysis to the LSU AgCenter Soil Testing & Plant Analysis Laboratory (STPAL, LSU, Baton Rouge, LA, United States). The principal chemical properties of the soil are reported in Supplementary Table S1. Each plot was inoculated with 1.5 kg (2012) or 2 kg (2013) of sterilized AMF inoculum (F and NM) or live inoculum (M). The inoculum amounts used in 2012 and 2013 corresponded to approximately 200 and 260 thousand AMF spores per plot, respectively. To avoid the spread of AMF inoculum from plot to plot during irrigation, plots were surrounded by an enclosure constructed of metal roofing flashing 20 cm high and held in place by pushing into the soil before planting. Plots were flushed with well water as necessary for the first month after seeding to establish stands of rice. We did not incorporate small filtrate aliquots of AMF inoculum into plots because we assumed that the large volumes of flooding water were sufficient to allow some homogenization among treatments in terms of water-soluble microflora, whereas the loose AMF spores, which are denser than water, were expected to remain precipitated. After allowing the plants to grow for approximately 1 month, a permanent flood was applied on the dates specified in **Table 1**. Plants possessed 4-5 leaves (early tillering) at permanent flooding. Metal flashing was removed after flooding. Plots in these experiments were not fertilized.

After natural infestation, densities of RWW larvae and pupae were determined by taking root/soil core samples from each plot (Stout et al., 2001). The core sampler was a metal cylinder with a diameter of 9.2 cm and a depth of 7.6 cm attached to a metal handle (Supplementary Figure S1). Core sampling was conducted twice for all experiments between 3 and 5 weeks after permanent flood. Dates of core samplings are shown in **Table 1**. For each sampling date, two (2012) or three (2013) core samples were taken from each plot. Core samples were placed into a 40-mesh screen sieve bucket to wash the soil and larvae from roots, buckets were placed into basins of salt water, and larvae and pupae were counted as they floated to the water surface (N'Guessan et al., 1994). RWW counts from two to three core samples per plot per sampling date were averaged to obtain an average number of larvae/pupae per core sample.

In order to confirm if the inoculum enhanced the abundance of AMF living in rice roots in Exp-2 and 3, the percentage of the root system containing AMF colonization was determined by observation of sub-sampled root fragments as described below. For Exp-2, the percentage of root fragments colonized by AMF was evaluated two times during plant development, before and

TABLE 1 | Planting and sampling dates for three field experiments conducted in 2012 and 2013 for evaluating the effects of arbuscular mycorrhizal fungi on the performance of rice water weevil in rice plants.

Year	Trial	Planting date	Flooding date	Larval sampling dates (cores)
2012	Experiment-1	17th April	30th May	15th June and 20th June
2013	Experiment-2	4th April	30th May	19th, 24th June and 2nd July
	Experiment-3	6th June	24th June	15th, 22th and 29th July

after flood. For Exp-3, this parameter was evaluated one time after the flood was established. On May 15th (41 dai) and June 7th (64 dai), 12 root samples from Exp-2 were randomly collected and analyzed from four plots of each treatment group per sampling date. The same number of root samples from Exp-3 were collected and analyzed from four plots of each treatment group on July 8th (32 dai). Sampling in Exp-2 and 3 was conducted by taking 9.2 cm diameter soil-root cores adjacent to plants. Each soil-root core (2–4 plants) was placed in plastic bags (one core per bag) and taken to the laboratory to be processed as described below for root staining. For the purpose of this study, one core represented one plant sample. A list of the experiments conducted in 2012 and 2013 are summarized in Supplementary Table S2.

Evaluating Effects of AMF on Plant Resistance to RWW (Greenhouse Study)

To further evaluate whether AMF inoculation alters the resistance of rice to *L. oryzophilus*, two choice experiments (RWW1 and RWW2) were conducted in the summer of 2013 in a greenhouse on the campus of Louisiana State University, Baton Rouge, LA, United States. For each experiment, two treatments were employed, namely mycorrhizal (M) and nonmycorrhizal plants (NM; control). All plants were grown in 2 liter round (15 cm diameter) plastic pots (Hummert International, Earth City, MO, United States) filled with a sterilized soil mix (2:1:1, soil: peat moss: sand), to which 50 g of AMF inoculum (corresponding to approximately 6500 AMF spores) or 50 g sterilized inoculum were added. For all greenhouse experiments, the soil substrate was sterilized by autoclaving for 60 min at 120°C to eradicate the indigenous AMF. The AMF inoculum was mixed with the soil, and rice seeds were sown directly into pots. Plants were maintained under greenhouse conditions with temperatures ranging from 25 to 35°C and ambient lighting. Plants were maintained in large wooden basins lined with heavy black plastic pond liner to hold flood waters when necessary as indicated in Stout and Riggio (2002). As for the field study, we assumed that flooding waters were suffice to allow some homogenization of water-soluble microflora. Approximately 10 days after planting, seedlings were thinned to a density of two or three plants per pot (RWW1 and RWW2, respectively). Experiments were conducted using 2-week-old plants (3-leaf stage). Because these experiments were conducted with rice at an early stage of growth, additional fertilizer was not necessary for adequate plant growth.

To initiate the choice experiments, two pots of each treatment were placed into each of seven (RWW1) or six (RWW2) infestation cages (Supplementary Table S2 and Supplementary Figure S2). Cages were set in the greenhouse basins and basins were flooded to a depth of ∼20 cm. Infestation cages were cylindrical wire frames (46 cm diameter × 61 cm tall) covered with a mesh fabric screening. After flooding, weevils were released into cages at a density of three weevils per plant (24 and 36 weevils per cage in RWW1 and RWW2, respectively) and allowed to feed, mate, and oviposit on plants of both treatments for 5 days. After that, pots were removed from cages and weevils were discarded.

The resistance of M and NM plants to *L. oryzophilus* was evaluated by counting first instars as they emerged from eggs laid in leaf sheaths of plants. Procedures for estimating larval densities were adapted from Stout and Riggio (2002). Briefly, after the 5-day adult infestation, plants for each pot were removed from the soil, washed free of soil, and placed individually in water in clean test tubes. Test tubes were labeled, arranged in a test tube rack, and placed in a growth chamber (30°C, 14:10 L:D). Using this method, weevils that infest plants hatch from eggs, emerge from leaf sheaths and settle on the bottom of the test tubes (Heinrichs et al., 1985). Larvae were removed by shaking roots free of larvae and then pouring water from test tubes into a petri dish for counting. After that, plants were placed back into the test tubes, and tubes were refilled with fresh water. Larva counts were started 3 days after placing plants in the tubes, and larvae were counted daily until no additional larvae were found for two consecutive days.

The percentage of root fragments colonized by AMF was measured in RWW2. Root samples from 5 plants of each mycorrhizal treatment were sampled on July 18th, 31 dai. A total of 10 plant samples were collected from this experiment.

Evaluating Effects of AMF on Plant Resistance to FAW (Laboratory Study)

To assess whether AMF inoculation influences resistance of rice to *S. frugiperda*, three laboratory feeding assays were conducted in 2012 (FAW1) and 2013 (FAW2 and FAW3). To this end, we cut leaf material from greenhouse-grown plants with or without AMF inoculum to determine *S. frugiperda* larval growth. 'Lemont' and 'Cocodrie' rice plants were grown under two treatments, namely M and NM. Plants were grown in the greenhouse as previously described. Six rice seeds were planted in each pot and thinned to three plants immediately before starting feeding assays for FAW1, FAW2, and FAW3 (Supplementary Table S2). Plants from which leaf material was taken were 3 weeks old and possessed three or four leaves. Because these experiments were conducted with rice at an early stage of growth, additional fertilizer was not necessary for adequate plant growth.

To initiate the assays, larvae of 4–5 days in age were selected from meridic diet and stage-synchronized at head capsule

slippage. Synchronized larvae were starved for 3 h to ensure that their guts were voided before their masses were determined using an analytical balance (model XS105, Mettler-Toledo LLC, Columbus, OH, United States). Larvae with similar masses were used in these experiments. Feeding assays were conducted in 9 cm plastic petri dishes lined with moistened cotton batting to maintain turgor in excised tissues (Supplementary Figure S3). Youngest fully-expanded leaves were removed from plants of each treatment group using scissors, transported on ice to the laboratory, cut into ca. 2 cm pieces and placed in petri dishes. Weighed larvae were placed together in petri dishes with foliage and allowed to feed on excised leaf material for 4 days (FAW1), 7 days (FAW2), or 10 days (FAW3). Larvae were observed daily to ensure they were not food-limited and leaves were changed every other day, but in later larval stage the leaves were changed daily. After ending the feeding assay, larvae were starved for 3 h to ensure that the larval gut was emptied before final mass was determined and recorded. For each experiment, 15 larvae (replicates) were used for each treatment for a total of 28, 30, and 30 observations for FAW1, FAW2, and FAW3, respectively (insects that died during feeding assays were excluded).

The percentage of root fragments colonized by AMF was measured in FAW2. To this end, root samples from 5 plants of each treatment were sampled on May 24th, 35 dai in 2013, and processed as described below. For the experiment FAW3 described here, RWW1 described above, and ShB1 described below, only one assessment of AMF colonization was conducted as these three experiments were planted at the same time and the inoculation success had been previously confirmed. From a total of 100 pots planted (50 M and 50 NM) in these three experiments, five M and five NM plants were sampled on Jun 27th, 36 dai in 2013. A total of 20 plant samples were collected from the four experiments.

Evaluating Effects of AMF on Plant Resistance to Rice Sheath Blight (Greenhouse Study)

To investigate whether AMF inoculation influences susceptibility of rice to infection by the fungus *R. solani*, two experiments (ShB1 and ShB2) were conducted in the summer of 2013. To obtain uniform disease development, rice plants at late tillering growth stage (approximately 8-weeks-old) were used for inoculation with *R. solani*. As in previous experiments, M and NM treatment plants were set up in the greenhouse filled with sterilized soil mix. Six rice seeds were planted in each pot and thinned to five and three plants immediately before pathogen inoculation for ShB1 and ShB2, respectively (Supplementary Table S2). Plants in each pot were collectively considered an experimental unit (replication). Fifteen pots of each treatment group were used for each experiment and arranged in a completely randomized design in greenhouse basins. Because these experiments were conducted with rice at late stage of growth, additional fertilizer was necessary for adequate plant growth. Urea (46% N) was applied at 0.5 g (134 kg N/ha) per pot in all pots (ShB1 and ShB2). Fertilizer was applied twice at 20 days and 40 days after planting.

Agar blocks (0.5 cm squares) of a 5-day-old culture of LR172 were cut from the outer growing area of culture plate using a pipette tip. Using forceps, one tiller of each plant, i.e., five or three tillers in each pot, was inoculated with *R. solani* by placing the mycelial agar block beneath the leaf sheath, ensuring that mycelia were in contact with the plant. The leaf sheath and agar block were covered immediately with aluminum foil as described by Park et al. (2008). Inoculated plants were maintained in the greenhouse, where relative humidity was favorable for the growth of ShB. When typical lesions started to appear 3 days after inoculation (dai), the aluminum foil was removed to allow for disease development (Supplementary Figure S4). Susceptibility of rice plants to ShB was evaluated 7 dai for each tiller by counting the number of lesions and measuring the lesion length of each inoculated plant. For each plant, measurements of lesion length were used to derive the maximum lesion length and the mean lesion length.

Processing and Quantification of Mycorrhizal Colonization

The trypan blue method of Koske and Gemma (1989) for root staining was used for quantification of mycorrhizal colonization with some modifications. Clearing and staining procedures require root samples to be washed from soil to remove all soil particles and then separating root and shoot tissues. For subsampling, roots of each plant were cut into 2-cm-long segments and placed in tissue processing cassettes (Ted Pella, Redding, CA, United States). At least 200 small root pieces per root sample were cleared in 10% KOH at 90°C for 20 min in a water bath. Clear pieces of roots were rinsed 5X with tap water to remove KOH, and roots were immersed in 2% HCl at room temperature for 10–15 min to ensure the roots were adequately acidified for staining. Cassettes containing roots were immediately stained with 0.05% trypan blue (Sigma-Aldrich, St. Louis, MO, United States) by incubation overnight and then transferred to vials containing lactoglycerol at 4°C to allow excess stain to leach out of the roots. Stained root samples were stored in destaining lactoglycerol solution for 48 h before being mounted in the same solution on a microscopic slide.

In order to quantify the abundance of AMF living in rice roots, the 2-cm-long root fragments were mounted after staining on microscopic slides as previously described (McGonigle et al. (1990). Five microscope slides, each containing ten stained randomly selected root fragments, were prepared from each plant sample. The random selection of root fragments is representative for the whole root system as it was often not possible to disentangle the root types. A total of 50 stained root segments per sample were examined with a compound microscope (Olympus CH2, Tokyo, Japan) at 40× magnification in order to confirm the levels of AMF colonization. Root fragments that contained blue-stained AMF structures such as intraradical aseptate hyphae linked to either fungal arbuscules or vesicles/spores were scored as colonized by AMF (Supplementary Figure S5) (DeMars and Boerner, 1996). Percent of root fragments with AMF colonization was averaged per treatment for the analyzed experiments. Photos of AMF structures on mycorrhizal colonized roots were taken using a microscope-mounted 5.0-megapixel digital camera (Leica DFC480, Cambridge, United Kingdom).

Evaluating Effects of AMF on Plant Biomass

To determine the effect of AMF on plant biomass, rice samples were collected from Exp-2 and from a separate greenhouse experiment (PB1) conducted in 2013 using previously sterilized field soil from the LSU AgCenter H. Rouse Caffey Rice Research Station. For PB1, NM and M treatments were established with 12 replications for each treatment as described previously (Supplementary Table S2). Entire plants were collected on June 18th from Exp-2 and on September 24th for PB1 at 75 and 30 dai, respectively. Pots for PB1 were not fertilized. Soil was washed from roots, and the shoots and roots were separated and blotted dry with a paper towel. Fresh weights of shoots and roots were recorded, and plant material was dried in an oven (60°C for 1 week) and reweighed (shoot and root dry weight) to calculate plant dry biomass as well as the ratio of root dry weight (RDW)/shoot dry weight (SDW).

Evaluating Effects of AMF on Plant Nutritional Status

To evaluate whether AMF inoculation affected the concentrations of nutrients in leaves and roots of rice, above- and belowground plant tissue samples from each of the treatments in Exp-1, Exp-2 and PB1 were collected on May 30th, June 18th, and September 24th at 43, 75 and 28 dai, respectively. Plant material was washed and transported to the laboratory. Samples were dried in an oven at 60°C for 1 week, ground in a Wiley mill (Thomas Wiley® Mini-Mill, Mexico) and submitted to the LSU AgCenter's Soil Testing & Plant Analysis Laboratory (STPAL, LSU, Baton Rouge, LA, United States) to determine nutrient concentrations in shoot and root tissues. The STPAL determined N and C concentrations by dry combustion using a LECO TruSpec™ CN analyzer (LECO Corp., St. Joseph, MI, United States), while the concentrations of the remaining nutrients (Ca, Mg, S, P, K, Al, B, Cu, Fe, Mn, Na, and Zn) were determined by inductively coupled plasma (ICP) analysis.

Statistical Analyses

Data were analyzed using SAS 9.4 (SAS Institute, 2014). The effects of AMF inoculation on rice plant responses for each experiment were analyzed separately by one-way analysis of variance (ANOVA) using PROC MIXED. For the RWW field experiments, effects of AMF inoculation on average number of larvae/pupae per core sample were analyzed as appropriate for a RCBD with treatment (F, NM, or M) as a fixed effect and block (replication) as a random effect for Exp-1 or CRD with treatment (F, NM, or M) as fixed effect for Exp-2 and Exp-3. For the RWW choice experiments, data were analyzed with treatment as a fixed effect and infestation cages (replication) as a random effect. For the FAW experiments, weight gain (final weight – initial weight) was the response variable, treatment was a fixed effect, and experiment was a random effect. For ShB experiments, disease ratings (lesion length and numbers of lesions) from five and three individual plants in each pot, respectively, were averaged as a single replication. The two experiments were analyzed independently with lesion length and number of lesions as dependent variables with treatment considered as a fixed effect. The data on AMF colonization were analyzed based on the percentage of root fragments colonized (see above) for Exp-2, Exp-3, RWW2, FAW2, and FAW3/RWW1/ShB1 experiments. Data for SDW and RDW were analyzed with the two treatments (M and NM) as fixed effects. For nutritional analyses, data for each nutrient (N, P, K, and C) were analyzed separately. Means were separated using the least significant difference (LSD) test in each of the experiments when there was a significant difference between treatments.

RESULTS

Root Colonization by AMF

The microscopic analyses of root fragments collected from M, NM or F treated rice plant samples in experiments Exp-2, Exp-3, RWW2, FAW2 and in a random sampling of FAW3, RWW1 and ShB1 combined (see section "Materials and Methods" above) confirmed that AMF inoculation significantly enhanced the percentage of root fragments colonized by AMF in relation to the non-inoculated controls. This was observed in greenhouse grown plants and in field grown plants (**Table 2** and Supplementary Figure S5); except in Exp-2 prior flooding at 41 dai, in which the enhanced percentage of root fragments colonized by AMF was only apparent in M plants compared with the non-inoculated plants. For both field experiments (Exp-2 and Exp-3), we detected a small percentage of fragments colonized by AMF in the non-inoculated plants or in the plants treated with fungicide (**Table 2**), probably due to native AMF already present in soil. Overall, although the percentages of root fragments colonized by AMF in rice were generally low, our data confirm that inoculation with AMF enriched the abundance of AMF living in rice roots grown under greenhouse and field conditions.

Effects of AMF Inoculation on RWW Performance in the Field

Under field conditions, the susceptibility of AMF-inoculated rice plants to RWW was measured by the densities of RWW larvae and pupae compared with that of rice plants treated with sterilized inoculum or with fungicides and sterilized inoculum (**Figure 1**). For Exp-1, we observed a significant positive impact of AMF inoculation on rice susceptibility to RWW larvae and pupae on both core sampling dates (June 15: $F_{2,14}$ = 7.45, P = 0.0063; June 20: $F_{2,14}$ = 21.06, P < 0.0001) (**Figure 1**). The highest immature densities were found in plots of plants inoculated with AMF on both sampling dates, whereas densities were lowest, at nearly equal numbers, in plots inoculated with sterilized inoculum or with fungicide and sterilized inoculum. Also, densities increased over time: weevil densities were lowest at 15 (core 1) days after permanent flood and highest at 20 (core 2) days after permanent flood. Increases in RWW densities in plots of AMF-inoculated plants ranged from 91.4% in core 1 (2.94 ± 1.01 to 0.25 ± 0.13, mean ± SE) to 94.3% in core 2 (7.75 ± 1.13 to 0.44 ± 0.19, mean ± SE) when compared to NM plants. For Exp-2, the AMF-mediated susceptibility of rice to RWW larvae and pupae was only significant in the first core sampling, while in the second and third core samplings the enhanced susceptibility was not apparent (June 19: $F_{2,18}$ = 4.15,

TABLE 2 | Percentage (%) of root fragments colonized by arbuscular mycorrhizal fungi (AMF) in rice plants.

Treatments	Root fragments colonized by AMF (%)		
Field 2013 (Mean of 4 samples each)	**Exp-2 (41 dai[1]) Mean ± SE**	**Exp-2 (64 dai) Mean ± SE**	**Exp-3 (32 dai) Mean ± SE**
Fungicide (F)	1.5 ± 0.95b	0.5 ± 0.50b	0.5 ± 0.50b
Nonmycorrhizal (NM)	4 ± 1.83ab	1.5 ± 0.95b	3 ± 1.29b
Mycorrhizal (M)	9 ± 2.08b	6 ± 2.16a	7 ± 1.29a
$F_{2,9}$	5.10	4.41	9.00
P-value	0.033	0.046	0.007
Greenhouse 2013 (Mean of 5 samples each)	**RWW2 (31 dai) Mean ± SE**	**FAW2 Mean ± SE**	**FAW3/RWW1/ ShB1 (36 dai) Mean ± SE**
Nonmycorrhizal (NM)	0.8 ± 0.49b	0.4 ± 0.40b	0 ± 0b
Mycorrhizal (M)	8.4 ± 2.48a	11.6 ± 1.72a	13.6 ± 1.72a
$F_{1,8}$	9.03	40.20	62.49
P-value	0.017	0.0002	< 0.0001

The percentage of colonized root fragments was determined from two field experiments (Experiment-2, Experiment-3), and from five greenhouse experiments (FAW2, RWW2, and from the combined experiments FAW3/RWW1/ShB1). Means ± standard errors are shown (n = 4 or 5 for field and greenhouse, respectively). Different letters indicate significant differences between mycorrhizal levels within each mycorrhizal treatments according to Least Significant Difference mean comparisons (P < 0.05; LSD). The F, NM, and M refer to AMF treatments of F: rice seeds + fungicides + sterilized AMF, NM: rice seeds + sterilized AMF, and M: rice seeds + live AMF. [1]dai, days after inoculation.

$P = 0.0331$; June 24: $F_{2,18} = 2.64$, $P < 0.0990$; July 2: $F_{2,18} = 1.26$, $P = 0.3074$). As in Exp-1, weevil densities in Exp-2 increased with sampling date, being lowest at 19 (core 1) days after permanent flood, intermediate at 24 (core 2) days, and highest at 32 (core 3) days after permanent flood (**Figure 1**). The increase in weevil densities in plots of AMF-inoculated plants in core 1 was 37% (5.70 ± 0.92–3.60 ± 0.52, mean ± SE) when compared to NM control plants. In second and third core samplings, increases were not meaningful with 24.2% (11.95 ± 1.72 to 9.05 ± 1.09, mean ± SE) and 12.3% (12.20 ± 1.60 to 10.70 ± 1.02, mean ± SE), respectively. In Exp-3, densities of RWW were significantly higher in AMF-inoculated plants in the first and third core samplings (July 15: $F_{2,18} = 4.32$, $P = 0.0293$; July 29: $F_{2,18} = 6.20$, $P = 0.0090$) but not in the second core sampling (July 22: $F_{2,18} = 1.11$, $P < 0.3497$), compared with both non-inoculated control treatments. Unlike previous experiments, weevil densities in Exp-3 decreased with sampling date: weevil densities were highest at 21 (core 1), intermediate at 28 days (core 2), and lowest at 35 (core 3) days after permanent flood. Increases in RWW densities in plots of AMF-inoculated plants ranged from 45% in core 1 (12.25 ± 2.20 to 6.75 ± 1.02, mean ± SE) to 36% in core 3 (3.65 ± 0.39 to 2.35 ± 0.45, mean ± SE) when compared to NM control plants. Overall, the inoculation of rice plants with AMF enhanced the susceptibility of rice to RWW in all three field experiments (Experiment-1: $F_{2,14} = 26.44$, $P < 0.0001$; Experiment-2: $F_{2,18} = 5.59$, $P = 0.013$; Experiment-3: $F_{2,18} = 7.00$, $P = 0.0056$).

Effects of AMF Inoculation on Plant Resistance to RWW in the Greenhouse

Arbuscular mycorrhizal fungi colonization can increase rice susceptibility to oviposition by RWW females (Cosme et al., 2011), but it was yet unclear whether this affects subsequent developmental stages. In order to address this question, we assessed the number of RWW first instars emerging from rice plants subjected to oviposition under controlled conditions. In two independent experiments (RWW1 and RWW2) inoculation with AMF of rice roots significantly increased the numbers of RWW first instars emerging from M treated rice plants (**Figure 2**; RWW1: $F_{1,48} = 6.99$, $P = 0.0110$; RWW2: $F_{1,65} = 13.66$, $P = 0.0005$). Numbers of RWW first instars emerging from M rice plants were 34 and 47% greater in RWW1 (12.39 ± 1.43 to 8.21 ± 0.95, mean ± SE) and in RWW2 (10.19 ± 1.11 to 5.44 ± 0.95, mean ± SE), respectively, compared to NM control plants. Therefore, AMF inoculation also has a positive impact on the performance of early stages of RWW.

Effects of AMF Inoculation on FAW Growth

To understand whether the increase in susceptibility of rice plants colonized by AMF is specific to RWW, we assessed the impact of inoculation with AMF on growth of FAW larvae. For all three FAW experiments, FAW larvae gained more weight when fed leaf material from plants inoculated with AMF compared with larvae fed leaf material from NM plants (FAW1: $F_{1,26} = 6.72$, $P = 0.015$; FAW2: $F_{1,28} = 16.82$, $P = 0.0003$; FAW3: $F_{1,28} = 159.24$, $P < 0.0001$) (**Figure 3**). Increases in larval growth on M rice plants ranged from 30.2% in FAW1 (0.053 ± 0.004 to 0.037 ± 0.003, mean ± SE), 31.4% in FAW2 (0.118 ± 0.004 to 0.014 ± 0.007, mean ± SE) to 75% in FAW3 (0.056 ± 0.003 to 0.014 ± 0.002, mean ± SE) compared with the NM control plants. These results show that the impact of AMF on rice susceptibility to herbivores affects aboveground herbivores as well as root feeding herbivores.

Effects of AMF Inoculation on Plant Resistance to Sheath Blight

In order to determine whether AMF-induced rice susceptibility also extends to pathogenic microorganisms, we analyzed the infection levels by ShB in rice stems. In two independent

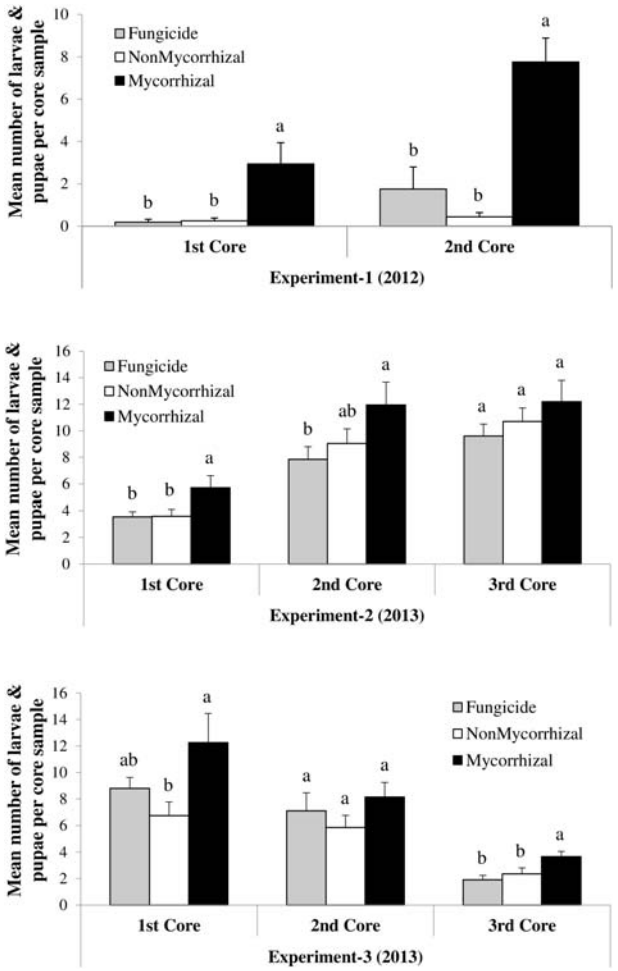

FIGURE 1 | Effects of arbuscular mycorrhizal fungi treatments on the densities (larvae and pupae per core sample) of *Lissorhoptrus oryzophilus* (± SE) in three field experiments (Experiment-1, Experiment-2, and Experiment-3) during 2012 and 2013. Fungicide: rice seeds + fungicides + sterilized AMF, NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

experiments, inoculation of rice roots with AMF significantly increased both measures of damage caused by ShB, i.e., lesion length (ShB1: $F_{1,28}$ = 11.83, P = 0.0018; ShB2: $F_{1,28}$ = 31.80, P < 0.0001) and numbers of lesions (ShB1: $F_{1,28}$ = 17.06, P = 0.0003; ShB2: $F_{1,28}$ = 34.27, P < 0.0001). Lesion length in M rice plants was 38% and 40% greater in ShB1 (3.86 ± 0.38 cm to 2.40 ± 0.20 cm, mean ± SE, n = 15) and ShB2 (10.85 ± 0.56 to 6.53 ± 0.52 cm, mean ± SE, n = 15), respectively, compared with lesion length in NM control plants. Similarly, the numbers of lesions in the two experiments were greater on M rice plants as compared to the NM plants (37% greater in ShB1: 3.67 ± 0.30 to 2.31 ± 0.14, mean ± SE, n = 15 and 38% greater in ShB2: 8.29 ± 0.39 to 5.16 ± 0.36, mean ± SE, n = 15). Leaves from M plants developed clear symptoms of infection at 3 days post-inoculation. At this time, only small necrotic spots were evident on NM plants. Lesions advanced aggressively on the leaves of mycorrhizal plants, and after 7 days post-inoculation these leaves were severely damaged (Supplementary Figure S4). Overall, these results show that AMF-induced rice susceptibility is also observed with an aboveground fungal pathogen (**Figure 4**).

Effects of AMF Inoculation on Plant Biomass

In Exp-2, the shoot biomass of M rice plants differed significantly from the shoot biomass of rice plants treated with sterilized inoculum (NM) or with fungicides and sterilized inoculum (F) ($F_{2,6}$ = 12.15, P = 0.008), ranging from 2.17 to 3.94 g (**Table 3**). The effect of AMF inoculation on root biomass and root-to-shoot ratio was not significant (**Table 3**). In 75-day-old rice plants, the SDW of M rice plants was 32.7% higher than the SDW of NM plants. In the PB1 experiment, M rice plants exhibited significantly higher shoot biomass than NM plants ($F_{1,11}$ = 6.53, P = 0.027) (**Table 3**), ranging from 0.88 to 1.09 g (**Table 3**). As in Exp-2, neither root biomass nor root-to-shoot ratio of rice plants differed among the different AMF treatments (**Table 3**). The SDW of the 30-day-old rice plants was 19.3% higher in M plants as compared to NM plants (**Table 3**).

Effects of AMF Inoculation on Plant Nutritional Status

No effects of AMF inoculation on concentrations of plant nutrients were found in either the field experiment, Exp-2, which showed low levels of AMF colonization in the non-inoculated controls, or in the greenhouse experiment (PB1), which had a nonmycorrhizal control without AMF (Supplementary Table S3). Therefore, the increases in shoot biomass and susceptibility to pests in AMF-inoculated plants were not accompanied by increases in concentrations of N, P, K or C. (Supplementary Table S3).

DISCUSSION

Interactions among AMF and plants can alter the suitability of plants for herbivores and pathogens. These effects have been investigated in a number of systems (Gange and West, 1994; Pineda et al., 2010; Currie et al., 2011) but have not been extensively investigated in rice, one of the most important crops not only in the United States but also worldwide (Campos-Soriano et al., 2011; Cosme et al., 2011). In this study, we used a commercial formulation of AMF containing multiple species from the Glomeraceae family to investigate the effects of inoculation with AMF on rice resistance against two important herbivores and one important pathogen. These biotic interactions were investigated in a wetland rice system. It is widely recognized for wetland systems that, although AMF can live through the year and occur in all plant developmental stages, flooding strongly suppresses levels of AMF colonization of roots (Solaiman and Hirata, 1995, 1996, 1997; Miller and Bever, 1999; Miller and Sharitz, 2000; Purakayastha and Chhonkar, 2001). Previously observed colonization levels in wetland rice under flooded conditions have ranged from 4% at 14 dai (Cosme et al., 2011),

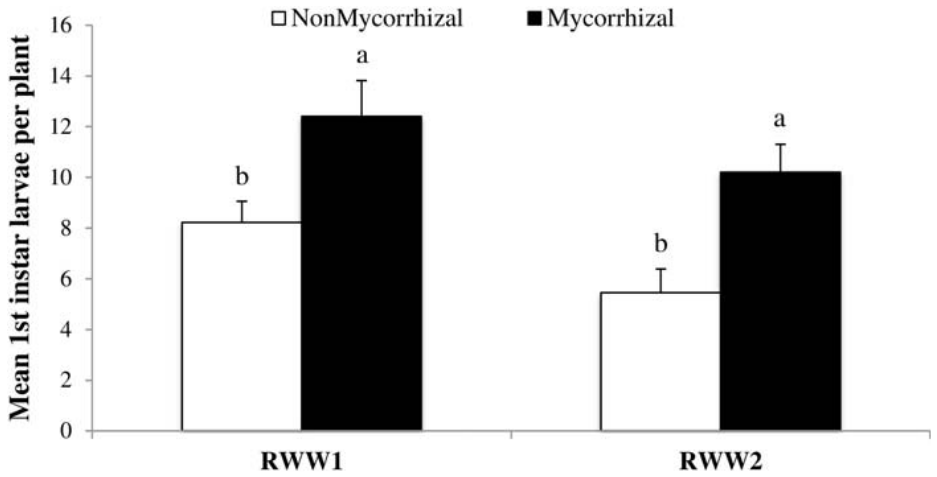

FIGURE 2 | Mean number of *Lissorhoptrus oryzophilus* larvae per plant (± SE) in a greenhouse experiment using mycorrhizal (M) and nonmycorrhizal (NM) rice plants of the variety 'Cocodrie.' Plants were infested with pairs of rice water weevil adults to feed on each plant for 5 days. NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

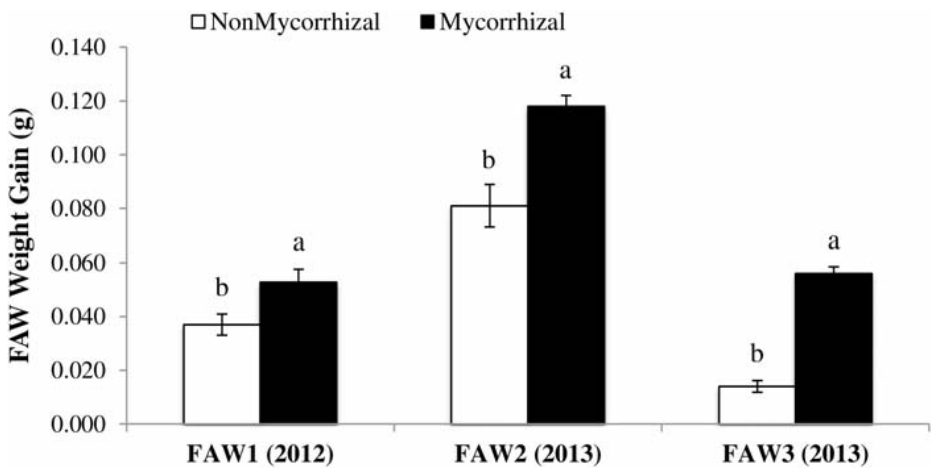

FIGURE 3 | Weight gain (g ± SE) of *Spodoptera frugiperda* larvae fed on rice leaves from nonmycorrhizal (NM) and mycorrhizal (M) plants in lab studies during 2012 and 2013. Feeding assays were performed for 4, 7, and 10 days with larvae of 4 to 5 days old. NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

5% at 30 dai (Campos-Soriano et al., 2010), 2–12% at 60 dai (Solaiman and Hirata, 1995), 14–29% at 40 dai (Purakayastha and Chhonkar, 2001), and > 30% at 75 dai (Solaiman and Hirata, 1997). Such low levels of colonization by AMF in wetland rice have nonetheless been associated with significant impacts on plant growth and nutrition (Solaiman and Hirata, 1995, 1996, 1997; Purakayastha and Chhonkar, 2001). In addition to the suppressive effects of flooding on AMF colonization, not all tissues of rice roots are susceptible to AMF colonization. Previous studies have shown that only large lateral roots of rice are substantially susceptible to AMF colonization, whereas crown roots are generally poorly colonized and fine lateral roots are never colonized (Gutjahr et al., 2009, 2015a). Such specialization in colonization dilutes the levels of colonization in the whole root system. Thus, the low levels of colonization of rice roots by AMF observed using the sampling and staining techniques described in this study were not surprising. Despite the low levels of colonization in our experiments, we detected significant impacts of AMF on susceptibility of rice to both below- and aboveground pest organisms. We found that AMF inoculation caused a strong positive effect on the performance of the leaf-feeding insect FAW and the root-feeding RWW, as well as on the severity of disease caused by a fungal pathogen. The increased susceptibility of rice to herbivores and a pathogen in AMF-inoculated plants was not associated with changes in plant nutrient concentrations but was associated with an increase in shoot biomass. Taken together, these results show that the interactions of rice roots with AMF caused a broad-spectrum reduction in resistance to pests of rice, perhaps by altering defense-related pathways.

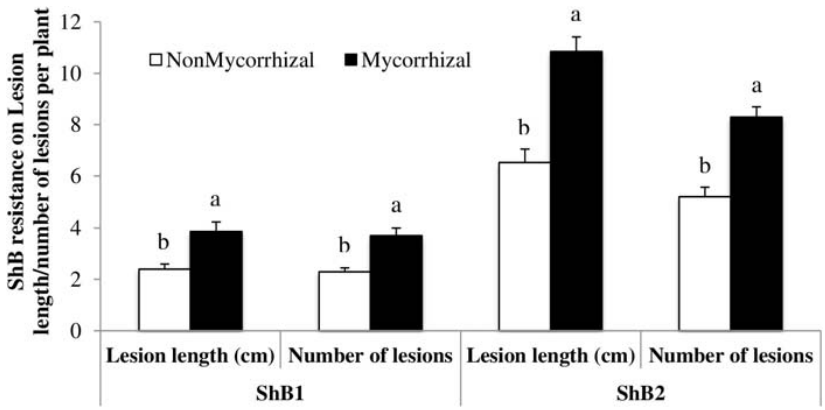

FIGURE 4 | Rice sheath blight disease variables (lesion length and number of lesions) measured after inoculation with isolate LR172 of *Rhizoctonia solani* in mycorrhizal and nonmycorrhizal rice plants in greenhouse experiments in the summer 2013. NonMycorrhizal: rice seeds + sterilized AMF, Mycorrhizal: rice seeds + live AMF. Bars and lower case letters at the column head indicate that means differ significantly (LSD, $P \leq 0.05$).

TABLE 3 | Results from one-way ANOVA on the effect of arbuscular mycorrhizal fungi (AMF) on the shoot and root dry weight biomass and root: shoot ratio of 75 and 30 day-old rice plants from a field (Exp-2) and a greenhouse experiment (PB1) in 2013.

Treatments	Shoot DW (g)	Root DW (g)	Root DW/ Shoot DW
Field 2013 (Exp-2)	**Mean ± SE**	**Mean ± SE**	**Mean ± SE**
Fungicide (F)	2.17 ± 0.38b	1.02 ± 0.08b	0.50 ± 0.07a
Nonmycorrhizal (NM)	2.65 ± 0.48b	1.19 ± 0.27a	0.45 ± 0.04a
Mycorrhizal (M)	3.94 ± 0.36a	1.25 ± 0.21a	0.34 ± 0.08a
	(32.7%)*	(4.8%)*	(−32.4%)*
$F_{2,6}$	12.15	0.38	2.15
P-value	**0.008**	0.699	0.198
GH 2013 (PB1)			
Nonmycorrhizal (NM)	0.88 ± 0.05b	0.51 ± 0.05a	0.57 ± 0.05a
Mycorrhizal (M)	1.09 ± 0.06a	0.60 ± 0.04a	0.56 ± 0.04a
	(19.3%)*	(15.0%)*	(−1.8%)*
$F_{1,11}$	6.53	2.46	0.02
P-value	**0.027**	0.145	0.901

*DW = Dry Weight. Mean values followed by different letters within columns indicate a significant difference among treatments by Least Significant Difference mean comparisons (P < 0.05; LSD). Significant P-values are in bold. The F, NM, and M refer to AMF treatments of F: rice seeds + fungicides + sterilized AMF, NM: rice seeds + sterilized AMF, and M: rice seeds + live AMF. *The relative change (%) in root, shoot and ratio was calculated by dividing the difference of AMF and non-AMF by AMF treatment.*

The increases in susceptibility to RWW in AMF-inoculated field plots, particularly in Exp-1, were greater than the differences in RWW densities typically observed among resistant and susceptible varieties of rice (N'Guessan et al., 1994; Stout et al., 2001), suggesting that the symbiotic status of rice plants might be a crucial component of susceptibility to RWW in the field. There was, however, some variability in the response of rice to AMF inoculation. In the second and third core samplings of Exp-2, and again in the second core sampling of Exp-3, densities of immature RWW did not differ between the M and NM treatments. The reasons for this variability in response to AMF inoculation are unknown. One possible reason is that sample and plot sizes might not have been sufficiently large to detect a weak effect of AMF inoculation among treatments, and it is interesting to note that all means in all core samplings trended in the direction of higher weevil densities in AMF-inoculated plants. Furthermore, experiments in 2012, when effects of AMF inoculation were large, and experiments in 2013, when effects were smaller, utilized different rice varieties ('Lemont' in 2012 and 'Cocodrie' in 2013), and were subject to different environmental conditions because they were conducted in different fields. With respect to the effect of rice variety, plant responses to AMF inoculation are known to vary among varieties within a plant species (Sawers et al., 2010).

The effectiveness of our experimental treatments in establishing AMF symbiosis was verified by quantifying AMF colonization in root samples in seven of our experiments. Although AMF colonization was not verified in all individual experiments, the substantial and statistically significant increases in colonization in response to commercial inoculants in the seven experiments in which colonization was assessed supports the postulation that addition of inoculum led to increased colonization in experiments in which mycorrhizal colonization

was not quantified. An unresolved question in our experiments is whether actual colonization of rice roots differed among the six species of fungi in our inoculum, as we did not examine changes in colonization by individual fungal species. Different species and combinations of AMF are known to have different effects on plant resistance to herbivores (Gange, 2001; Roger et al., 2013).

The effects of AMF colonization on plant-herbivore and plant-pathogen interactions have been variable in previous studies (Gange, 2001; Bennett and Bever, 2007; Hartley and Gange, 2009; Koricheva et al., 2009; Currie et al., 2011; Jung et al., 2012; Barber et al., 2013a). The effects of AMF colonization on herbivores and pathogenic microorganisms depend on numerous factors, including host plant species, AMF species, herbivores or pathogens involved, and environmental conditions (Pineda et al., 2010). Our study contributes to a growing body of evidence that the effects of AMF in plants do not always lead to priming of plant tissues for a more efficient activation of defense mechanisms (Pozo and Azcon-Aguilar, 2007). This study also extends a previous report of positive effects of AMF inoculation on RWW oviposition (Cosme et al., 2011) and shows that the positive effects of AMF inoculation on RWW are observed in different developmental stages of RWW. Furthermore, the oviposition preference of RWW for mycorrhizal over nonmycorrhizal plants (Cosme et al., 2011) coupled with the higher performance of RWW larvae on mycorrhizal plants (this study) provides support for the preference-performance hypothesis for belowground herbivores, which predicts that when insect herbivores have offspring with limited mobility, there will be strong selection pressure for adults to oviposit on plants that maximize offspring performance (Johnson et al., 2006).

As noted above, several previous studies have, like this one, found positive effects of AMF inoculation on herbivore performance. Currie et al. (2011) found colonization of clover plants by AMF increased on survival of larvae of the specialist clover root weevil (*Sitona lepidus*). Likewise, Goverde et al. (2000) reported that survival and larval weights of the common blue butterfly (*Polyommatus icarus*) were greater in larvae that fed on *Lotus corniculatus* plants colonized by AMF. Gange et al. (2002) demonstrated that AMF colonization increased the larval growth of the specialists lace border (*Scopula ornata*), mint moth (*Pyrausta aurata*), and redcurrant aphid (*Cryptomyzus ribis*) on plants in the Lamiaceae family. The stronger performance of RWW, an oligophagous insect that specializes on grasses, on AMF-inoculated rice is consistent with results of a meta-analysis (Koricheva et al., 2009) that noted a general pattern in which most specialist chewing insects, but not most generalist insects, perform better on plants colonized by AMF than on non-colonized plants. However, our results with the generalist FAW, which showed higher larval growth on AMF-inoculated rice plants, contradicts this general pattern. Gange et al. (2002), similarly found that AMF colonization had a positive effect on the growth of the generalist aphid (*Myzus persicae*), and Hoffmann et al. (2009) showed that females of the generalist two-spotted spider mite (*Tetranychus urticae*) preferentially resided and oviposited at a higher rate on common bean plants colonized by AMF.

The effects of AMF colonization on aboveground pathogenic microorganisms have also been investigated in several prior studies. In rice in particular, Campos-Soriano et al. (2011) found that AMF confers enhanced rice resistance against infection by the rice blast fungus. In our experiments with ShB, we found that mycorrhizal rice plants were more susceptible to infection by *R. solani* than nonmycorrhizal plants. Because flooded rice plants were used in our study, and non-flooded plants in the study by Campos-Soriano et al. (2011), it is possible that water regime might affect the impact of AMF on rice resistance to ShB, although other experimental differences may also have contributed to these contrasting results. Altogether, our results underscore the variability of the effects of AMF colonization in plant-insect and plant-pathogen interactions.

There are three major hypotheses to explain the increases in rice susceptibility when colonized by AMF in this study. First, the interaction of AMF with rice might increase susceptibility to pests by increasing plant quantity (biomass) with no change in plant quality. Bennett et al. (2006) refer to this hypothesis as the "nutritional quantity hypothesis." Second, AMF colonization might increase the quality of plant tissues for herbivores by improving plant nutrient status, which is referred by Bennett et al. (2006) as the "nutritional quality hypothesis." In our experiments, we found no support for the nutritional quality hypothesis; no significant differences in concentrations of P, N, K and C, the nutrients that are most frequently studied in plant-AMF experiments, were found among AMF-inoculated plants and non-inoculated controls. In a previous study using the same rice-RWW system, however, Cosme et al. (2011) found that increased oviposition preference of RWW adults on mycorrhizal rice plants was associated with increased N and P concentrations. The effects of AMF on plant nutritional status have been widely studied in other systems, particularly effects of AMF on P, where P deficiency in soil promotes mycorrhizal formation (Secilia and Bagyaraj, 1994; Cosme and Wurst, 2013; Babikova et al., 2014b). In contrast to the results for nutrient status, we observed that AMF inoculation increased shoot biomass of rice plants in field and greenhouse studies (**Table 3**), which is in agreement with previous studies (Campos-Soriano et al., 2010). This result is consistent with the nutritional quantity hypothesis for RWW first instars, FAW and ShB, which live on aboveground plant tissues. However, the relatively moderate increases in shoot biomass observed are unlikely to fully account for the substantial increases in susceptibility to pests found in greenhouse experiments. This is particularly true for the increase in FAW susceptibility, as the FAW assay used excised leaf tissue and insects were never food-limited.

A third major hypothesis to explain increases in rice susceptibility in this study involves AMF-mediated changes in the expression of plant defenses via modulation of phytohormone signaling and consequent reprogramming of defense-related gene expression and other processes (Jung et al., 2012; Gutjahr, 2014; Pozo et al., 2015). There is evidence that AMF colonization can prime or otherwise affect jasmonic acid (JA)- and salicylic acid (SA)-dependent pathways (Pozo and Azcon-Aguilar, 2007; Herrera-Medina et al., 2008; Koricheva et al., 2009; Jung et al., 2012), and that these changes in plant signaling can lead to

enhanced or decreased plant resistance against herbivores or pathogens (Campos-Soriano et al., 2011; Jung et al., 2012). Fontana et al. (2009) demonstrated that mycorrhizal symbiosis induced qualitative and quantitative changes in the production of volatile compounds of *Plantago lanceolata* plants when they were infested by caterpillars of *Spodoptera* spp. In another study, Jung et al. (2012) reported that AMF plants were usually more resistant to necrotrophs and chewing insects, which are affected by JA-dependent defense responses, and more susceptible to biotrophs (Jung et al., 2012). Thus, the evolution of plant-AMF interactions has apparently resulted in a repertoire of responses to AMF colonization that influence interactions with insects and pathogens (Gehring and Bennett, 2009; Gutjahr and Paszkowski, 2009; Kiers et al., 2010; Jung et al., 2012; Babikova et al., 2014a,b; Gilbert and Johnson, 2015; Pozo et al., 2015). However, the impact of AMF on plant defense hormone levels and gene transcription vary depending on the genotypes of the partners and other factors (Fernández et al., 2014).

In rice in particular, inoculation of unflooded roots with AMF induces a complex transcriptomic reprogramming, leading to enrichment of transcripts associated with phytohormones and secondary metabolism (Fiorilli et al., 2015; Gutjahr et al., 2015a). In our study, the fact that large effects of AMF inoculation on plant resistance were observed despite low levels of AMF colonization suggest that inoculation with AMF induced a systemic reprogramming of defense-related processes. However, the exact AMF-induced changes in JA and SA signaling and consequent changes in gene expression that influence the systemic susceptibility of wetland rice remain to be elucidated. Work is in progress to investigate expression levels of genes involved in the JA and SA signaling pathways of leaf tissues following AMF inoculation and FAW feeding using an RNA-seq and real time -PCR.

In summary, this study demonstrates that inoculation of rice plants with AMF rendered the plants more susceptible to pests without causing dramatic changes in plant nutrient concentrations. Our study highlights that AMF can compromise plant resistance and suggests that caution should be used when considering large scale applications of commercial AMF inoculant. However, despite the negative effects on plant resistance observed in this study, it would be premature to conclude that AMF does not have practical benefits for rice production. The higher shoot biomass of AMF-inoculated plants observed in two experiments in this study suggests that AMF inoculation may positively impact rice growth and perhaps yields under some circumstances. Moreover, the negative impact of AMF on plant resistance may not occur in all soil environments. Barber et al. (2013a), for example, found that the effects of AMF on plant nutrition vary with soil source and therefore soil characteristics may influence the effects of AMF colonization on herbivores. Although the effects of AMF on rice susceptibility were consistent in our study, the strength of these effects appeared to vary under the different conditions present in different experiments. Work is in progress to investigate whether different soil attributes, (e.g., soil P concentrations), alter the effects of AMF inoculation on the performance and growth of RWW and FAW in rice. Moreover, experiments are also being conducted to characterize the impacts of AMF inoculation on rice growth and yield when insects are not present. Responses to AMF provide a unique window for studying the traits or characteristics that make rice plants more susceptible or tolerant to insect and pathogen attack. A better understanding of the interactions of rice and other crops with AMF in the rhizosphere and with the different organisms they encounter both above and below ground may be a key to increasing plant productivity and improving pest management with less input of harmful chemicals.

AUTHOR CONTRIBUTIONS

LB, MC, and MS designed the experiments, which were then carried out by LB and MS. RS advised with the root staining technique, and LB improved it. LB collected the data and conducted the statistical analyses. LB, MC, and MS contributed to interpreting the results. LB wrote the first draft of the manuscript. All authors edited the manuscript and gave final approval for 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/fpls.2018.00747/full#supplementary-material

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Mycorrhizae Alter Toxin Sequestration and Performance of Two Specialist Herbivores

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Multitrophic species interactions are shaped by both top-down and bottom-up factors. Belowground symbionts of plants, such as arbuscular mycorrhizal fungi (AMF), can alter the strength of these forces by altering plant phenotype. For example, AMF-mediated changes in foliar toxin and nutrient concentrations may influence herbivore growth and fecundity. In addition, many specialist herbivores sequester toxins from their host plants to resist natural enemies, and the extent of sequestration varies with host plant secondary chemistry. Therefore, by altering plant phenotype, AMF may affect both herbivore performance and their resistance to natural enemies. We examined how inoculation of plants with AMF influences toxin sequestration and performance of two specialist herbivores feeding upon four milkweed species (*Asclepias incarnata, A. curassavica, A. latifolia*, *A. syriaca*). We raised aphids (*Aphis nerii*) and caterpillars (*Danaus plexippus*) on plants for 6 days in a fully factorial manipulation of milkweed species and level of AMF inoculation (zero, medium, and high). We then assessed aphid and caterpillar sequestration of toxins (cardenolides) and performance, and measured defensive and nutritive traits of control plants. Aphids and caterpillars sequestered higher concentrations of cardenolides from plants inoculated with AMF across all milkweed species. Aphid per capita growth rates and aphid body mass varied non-linearly with increasing AMF inoculum availability; across all milkweed species, aphids had the lowest performance under medium levels of AMF availability and highest performance under high AMF availability. In contrast, caterpillar survival varied strongly with AMF availability in a plant species-specific manner, and caterpillar growth was unaffected by AMF. Inoculation with AMF increased foliar cardenolide concentrations consistently among milkweed species, but altered aboveground biomasses and foliar phosphorous concentrations in a plant species-specific fashion. Increased herbivore sequestration of cardenolides followed AMF-mediated increases in foliar cardenolide concentrations. Aphid performance declined with increasing foliar cardenolide concentrations, while caterpillar survival increased with aboveground biomass. Our findings suggest that by altering plant phenotype, the availability of AMF in soil has the potential to influence both top-down (via sequestration) and bottom up (via plant defense and nutrition) forces that operate on herbivores.

Keywords: above-belowground interactions, sequestration, plant-herbivore interactions, plant-microbe interactions, arbuscular mycorrhizal fungi (AMF), *Asclepias, Danaus plexippus, Aphis nerii*

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INTRODUCTION

Multitrophic species interactions are governed by a mixture of top-down forces, such as predators and parasites, and bottom-up forces, such as resource availability (Hunter and Price, 1992; Schmitz et al., 2000). In terrestrial ecosystems, both top-down and bottom-up forces travel with ease across the traditional soil "boundary," with plants connecting the interactions that occur between above and belowground organisms (van der Putten et al., 2001; van Dam and Heil, 2011; Hunter, 2016). As a result, soil organisms that are associated with plant roots have the potential to affect herbivore populations above ground both by affecting plant quality for herbivores from the bottom-up (Hartley and Gange, 2009; Koricheva et al., 2009; Jung et al., 2012) and the resistance of herbivores to their natural enemies from the top down (Gange et al., 2003; Rasmann et al., 2017; Tao et al., 2017).

Arbuscular mycorrhizal fungi (AMF) engage in one of the most ubiquitous root-microbe symbioses in terrestrial ecosystems (Smith and Read, 2008), associating with over 80 percent of plant species globally (Wang and Qiu, 2006; Smith and Read, 2008; Soudzilovskaia et al., 2015). AMF provide nutrients to plants, such as phosphorous, in exchange for plant sugars (Smith and Read, 2008). In establishing and maintaining the symbiosis, AMF also interact with plant defensive signaling pathways, including the jasmonic acid and salicylic acid pathways (Jung et al., 2012; Cameron et al., 2013; Bucher et al., 2014; Gutjahr, 2014). As a result, AMF alter plant nutritive quality and a diversity of plant primary and secondary metabolites (Bennett et al., 2009; Roger et al., 2013; Vannette et al., 2013; Schweiger et al., 2014; Schweiger and Müller, 2015), affecting plant quality for insect herbivores substantially (Hartley and Gange, 2009; Koricheva et al., 2009).

The response of insect herbivores to AMF colonization of their host plants varies widely, from positive to neutral or negative (Koricheva et al., 2009). Much of this variation is explained by the degree of specialization and feeding mode of the herbivore (Hartley and Gange, 2009; Koricheva et al., 2009). For instance, both generalist and specialist phloem-feeding insects, such as aphids, generally benefit from AMF colonization of their host plants. Specialist chewing herbivores, such as caterpillars, also benefit, but generalist chewing herbivores are negatively affected by AMF colonization of their host plants (Hartley and Gange, 2009; Koricheva et al., 2009). Phloem-feeding insects may avoid AMF-mediated increases in plant defenses because phloem lacks or contains far lower concentrations of plant secondary metabolites than leaves (Züst and Agrawal, 2016a). In addition, phloem-feeding insects may benefit from AMF-mediated increases in the size of plant vascular bundles (Krishna et al., 1981; Simon et al., 2017). Generalist chewers may be more susceptible to AMF-mediated increases in plant defenses (Schoonhoven et al., 2005), while specialist chewers may benefit from increased nutritive quality of host plants colonized by AMF (Koricheva et al., 2009).

Even within these trends, there is large variation in herbivore responses to AMF, and we lack an understanding of what is driving this variation. For instance, aphids generally benefit from AMF colonization of their host plants; aphids are more attracted to plants colonized by AMF and have greater body masses, growth rates, and fecundity on host plants colonized by AMF (Gange and West, 1994; Gange et al., 1999, 2002; Koricheva et al., 2009; Babikova et al., 2014a,b; Simon et al., 2017). However, aphids have also been found to not respond to AMF colonization of their host plants (Pacovsky et al., 1985; Wurst et al., 2004; Colella et al., 2014; Grabmaier et al., 2014; Williams et al., 2014; Bennett et al., 2016) or to have reduced population growth on plants colonized by AMF (Gehring and Whitham, 2002; Hempel et al., 2009; Abdelkarim et al., 2011). Similarly, while some specialist chewers benefit from AMF colonization of their host plants (Borowicz, 1997; Goverde et al., 2000; Vannette and Hunter, 2013), others are unaffected (Laird and Addicott, 2008; Cosme et al., 2011). Some of this variation may be explained by the stage of the association between the plant and AMF; aphids, for example, tend to benefit only after at least a month of AMF establishment (Tomczak and Müller, 2017). This variation in herbivore responses to AMF may also be a consequence of plant species-specific responses of plant traits to the presence of AMF (e.g., Grman, 2012; Barber et al., 2013; Anacker et al., 2014; Tao et al., 2016a) and the density or identity of AMF inoculum available to the plant (Garrido et al., 2010; Vannette and Hunter, 2011, 2013; Barber et al., 2013).

In addition to being shaped by host plant quality, herbivore populations are also affected by their natural enemies. Root-associated microbes, such as AMF, affect herbivore-natural enemy interactions indirectly by altering plant phenotype (Rasmann et al., 2017; Tao et al., 2017). For instance, AMF increase the attractiveness of plants to natural enemies by changing the volatile emissions of their host plants (Guerrieri et al., 2004; Fontana et al., 2009; Hoffmann et al., 2011; Schausberger et al., 2012; Babikova et al., 2013). AMF also influence the searching efficiency of natural enemies, likely by changing plant size (Gange et al., 2003), and can improve natural enemy performance (Hempel et al., 2009; Bennett et al., 2016). AMF mediation of herbivore-natural enemy interactions can ultimately benefit host plants. For instance, AMF colonization increases herbivorous mite densities on *Phaseolus vulgaris* plants, yet improves plant productivity by enhancing the population growth of predatory mites and plant tolerance sufficiently to compensate for the increase in herbivores (Hoffmann et al., 2011).

Many specialist herbivores are able to resist their natural enemies by sequestering secondary metabolites from their host plants (Nishida, 2002; Opitz and Müller, 2009; Ode, 2013; Erb and Robert, 2016; Petschenka and Agrawal, 2016). The concentration and composition of secondary metabolites that herbivores sequester are tied closely with host plant secondary chemical profiles (Malcolm, 1990, 1994; Agrawal et al., 2015; Petschenka and Agrawal, 2015), and are affected by environmental factors, such as soil nutrient availability (Jamieson and Bowers, 2012; Tao and Hunter, 2015). Herbivores that sequester higher concentrations of secondary metabolites from their host plants are more toxic and deterrent to their natural enemies (Brower et al., 1968; Reichstein et al., 1968; Brower and Moffitt, 1974; Malcolm, 1992; Dyer and Bowers, 1996; Camara, 1997). Therefore, by increasing plant chemical

defenses, AMF may increase toxin sequestration by herbivores, thereby improving herbivore resistance to their natural enemies. Despite widespread recognition of sequestration as an integral component of host plant specialization and an important factor shaping ecological networks (Duffey, 1980; Lampert et al., 2014; Petschenka and Agrawal, 2016; Züst and Agrawal, 2016b), no study to date has considered how microbial root mutualists of plants, including AMF, affect herbivore sequestration of plant toxins.

Here, we evaluate how AMF affect toxin sequestration and performance of specialist herbivores of milkweed (*Asclepias*) species. Milkweed species provide an ideal system in which to address these questions because milkweed species produce a suite of resistance traits and are fed upon by specialized herbivores that can tolerate and sequester milkweed defenses. Milkweed tissues, including leaves and phloem, contain cardenolides, bitter tasting steroids that disrupt the functioning of sodium-potassium channels in animal cells by inhibiting an essential cation transporter, Na^+/K^+-ATPase (Agrawal et al., 2012; Pringle et al., 2014; Züst and Agrawal, 2016b). In response to leaf damage, milkweeds exude latex, a sticky isoprene polymer that gums up the mouths of chewing herbivores (Zalucki et al., 2001a; Agrawal and Konno, 2009). In addition, milkweed species vary in leaf toughness (Agrawal and Fishbein, 2006), which is tightly correlated with specific leaf mass (SLM) (Frost and Hunter, 2008).

We used two specialist herbivores of milkweed that vary in their feeding mode: oleander aphids (*Aphis nerii*; phloem-feeding) and monarch caterpillars (*Danaus plexippus*; leaf-chewing). Oleander aphids tolerate cardenolides through regulation of a narrow set of genes involved in canonical detoxification processes (Birnbaum et al., 2017). Monarch caterpillars, in contrast, have NA^+/K^+- ATPases that are insensitive to cardenolides (Dobler et al., 2012; Petschenka and Agrawal, 2015). Despite being able to tolerate cardenolides, both oleander aphids and monarch caterpillars exhibit reduced performance on host plants with high concentrations of cardenolides (Zalucki et al., 2001a; Agrawal, 2004, 2005; Rasmann et al., 2009; de Roode et al., 2011; Colvin et al., 2013; Tao et al., 2016b; Birnbaum et al., 2017). Furthermore, both oleander aphids and monarch caterpillars sequester cardenolides (Rothschild et al., 1970; Malcolm and Brower, 1989; Malcolm, 1990; Züst and Agrawal, 2016b), providing an effective defense against aphid predators (Pasteels, 1978; Malcolm, 1989, 1992; Pappas et al., 2007; Mooney et al., 2008) and monarch predators and parasites (Brower et al., 1968; Reichstein et al., 1968; Brower and Moffitt, 1974; Sternberg et al., 2012). Oleander aphids appear to sequester cardenolides passively through diffusion of non-polar (lipophilic) cardenolides (Malcolm, 1990; Züst and Agrawal, 2016b). In contrast, monarch caterpillars sequester polar cardenolides selectively (Malcolm and Brower, 1989; Petschenka and Agrawal, 2015; Tao and Hunter, 2015; Erb and Robert, 2016), likely through active translocation by transport proteins through gut membranes (Frick and Wink, 1995). Nonetheless, cardenolide sequestration by both oleander aphids and monarch caterpillars is closely correlated with their host plant cardenolides (Malcolm, 1990, 1994; Agrawal et al., 2015; Petschenka and Agrawal, 2015). Thus, AMF-mediated changes in plant cardenolide expression may influence aphid and caterpillar sequestration.

We performed a full-factorial experiment, manipulating oleander aphids and monarch caterpillars on four closely related milkweed species provided with different amounts of AMF inoculum. We expected herbivores to sequester higher concentrations of cardenolides on AMF-colonized plants due to AMF-mediated increases in the cardenolide concentrations of their host plants. Furthermore, we expected that AMF colonization would improve the performance of aphids and caterpillars by increasing plant nutritive quality and biomass, outweighing the negative effects of increased cardenolide concentrations on the herbivores. Because the outcomes of many AMF-plant associations are specific to the AMF and plant species (e.g., Grman, 2012; Barber et al., 2013; Anacker et al., 2014; Tao et al., 2016a), we expected the magnitude of the effects of AMF on herbivore sequestration and performance to vary among plant species and with the level of AMF inoculum available to the plant.

MATERIALS AND METHODS

Plants and Insects

We used four North American milkweed species (*Asclepias curassavica, A. latifolia, A. syriaca*, and *A. incarnata)* that show constitutive and AMF-mediated variation in milkweed defenses and nutritive quality (Vannette et al., 2013; Tao et al., 2016a). *Asclepias incarnata* and *A. syriaca* seeds were collected from naturally occurring populations in Livingston County, MI, and *A. latifolia* and *A. curassavica* seeds were purchased from commercial sources (Alplains and Butterfly Encounters Inc., respectively). We obtained fungal inoculum from Mycorrhizal Applications (Grants Pass, OR, USA), which was comprised of equal proportions of four AMF species including *Rhizophagus intraradices*, *Funneliformis mosseae*, *Glomus aggregatum*, and *Claroideoglomus etunicatum* (33 spores of each AMF species per gram of inoculum, www.plant-success.com). However, cloning and sequencing of the inoculum with AMF-specific primers (Krüger et al., 2009) revealed the mix to consist only of *F. mosseae* (details in Supplementary Material). Milkweeds grow in habitats that host a diversity of AMF taxa (Öpik et al., 2006), and can form associations with these cosmopolitan AMF species in natural and experimental populations (Landis et al., 2004; Vannette and Hunter, 2011; Vannette et al., 2013; Tao et al., 2015, 2016a). However, as with most systems, the frequency of these relationships is not known.

To assess how the availability of AMF inoculum influences the performance of herbivores, we used two specialist herbivores: oleander aphids (*A. nerii;* phloem-feeding) and monarch caterpillars (*D. plexippus;* leaf-chewing). All oleander aphids used in the experiment were clones derived from a single aphid collected in March 2014 from the Emory University greenhouses (Atlanta, GA) and reared indoors on *A. tuberosa*, which does not produce cardenolides, for 1 month prior the experiment. Monarch larvae were the second generation of outcrossed progeny of butterflies obtained from Shady Oak Farms (www.butterfliesetc.com), Mr. Butterfly (www.mrbutterflies.com),

and Butterfly Release Company (www.butterflyreleasecompany.com). Monarch larvae were raised on a combination of *A. syriaca*, *A. incarnata*, and *A. curassavica* in a growth room with photoperiod of 16:8 L:D and adults were reared on a 10% honey solution.

Experimental Protocols

After 6 weeks of cold, moist stratification at 4°C, we surface-sterilized seeds in 5% bleach and germinated them at room temperature (*A. curassavica* did not require stratification) in March 2014. We planted individual seedlings in conical deepots (D40H, Steuwe and Sons Inc., Corvalis, OR, USA) filled with 600 ml of a 3:1 mix of autoclaved soil (Metro-Mix 380; MetroMix Sun Gro Horticulture Canada CM Ltd., Vancouver, BC, Canada) and sand containing AMF inoculum. We manipulated the amount of live and autoclaved (dead) AMF inoculum available to experimental plants to generate zero, medium, and high levels of root colonization, which is possible because the amount of AMF inoculum available to milkweed plants affects the levels of AMF colonization of roots (Vannette and Hunter, 2011; Tao et al., 2015, 2016a). Specifically, we homogenized 4.20 g autoclaved AMF inoculum (zero treatment), 1.20 g live and 3.00 g autoclaved inoculum (medium treatment), or 4.20 g live inoculum (high treatment) in 200 ml of autoclaved soil, which was placed between 400 ml of autoclaved soil and sand to prevent the transfer of mycorrhizal spores or hyphae among treatments. To return the natural bacterial community of the potting soil to the autoclaved soil of each pot, we added 20 ml of bacterial solution made by suspending 100 ml potting soil in 1 L deionized water and filtering the suspension through an ultra-fine soil sieve (38 μm) to remove AMF hyphae and spores. Plants were grown at the Matthaei Botanical Gardens greenhouses (Ann Arbor, MI) with a photoperiod of 16:8 L:D for 3 months. Plants were watered *ad libitum* and fertilized biweekly with 90 ml of a low concentration (94 ppm) of 15-0-15 (N-P-K) dark weather fertilizer (JR Peters Inc., Allentown, PA). All experimental plants were exposed to colonization and damage by greenhouse thrips and sprayed monthly with a mixture of Enstar, Lucid, and MPede to minimize damage. No pesticides were sprayed for 3 weeks prior to the addition of herbivores; thrips were killed weekly by hand during this period.

In a fully factorial design, we placed oleander aphids, monarch caterpillars, or no herbivores on plants of each plant species x AMF treatment and allowed herbivores to feed for 6 days in June 2014. The 6 days of feeding represent approximately one generation for oleander aphids (Zehnder and Hunter, 2009) and 50% of the average larval period of monarchs under our rearing conditions (Vannette and Hunter, 2013). Effects of plant quality on monarch growth are most important during early instars (Zalucki et al., 2001b). All plants were covered with white, nylon mesh bags (5-gallon paint strainer bags) to prevent insect movement among experimental plants. Five reproductive, apterous oleander aphids were placed at the apex of 15 replicates of each plant species x AMF treatment and allowed to reproduce naturally for 6 days ($n = 180$). Dead or missing reproductive aphids were replaced on the second day. One newly hatched monarch caterpillar was placed on each of 20 replicates of each plant species x AMF treatment and allowed to feed for 6 days ($n = 240$). Missing or dead caterpillars were replaced on the second day. Twenty plants of each plant species × AMF treatment experienced no herbivory but were covered with white, nylon mesh bags to control for effects of mesh on plant traits ($n = 240$). We used these control plants to evaluate the effects of AMF on plant traits that may influence herbivore performance, and to determine the levels of AMF colonization of plant roots ($n = 240$). We could not use the plants upon which the herbivores fed, because aphid and caterpillar feeding alters milkweed defenses, nutritive quality, and levels of AMF colonization (A. R. Meier, unpublished data). Therefore, the traits measured in herbivore-damaged plants would not be representative of the initial plant quality experienced by aphids and caterpillars. We conducted this experiment in four temporal blocks separated by 1 day, with each treatment equally represented in each temporal block.

Analysis of Herbivore Traits

After the 6 days, aphids were counted and collected, allowed to void their guts for 24 h, frozen, lyophilized, and weighed. Caterpillars were also collected, allowed to void their guts for 24 h, frozen, dried at 50°C, and weighed. Simultaneously, control plants were harvested destructively to measure plant resistance and nutritive traits, biomass, and AMF colonization of roots. Aphid per capita growth rate per plant (r) was calculated by taking the natural log of the final aphid population size divided by the initial aphid population size (5 aphids) (Speight et al., 2008). Aphid individual mass was calculated by weighing each aphid population (i.e., all aphids present on one experimental plant) and dividing by the number of aphids in the population. Mean caterpillar growth rate per day was calculated by dividing the final, dry caterpillar mass by the 6 days for which it fed (Waldbauer, 1968). Leaves damaged by caterpillars were removed, scanned, and the area consumed by caterpillars (consumed leaf area, CLA) was determined with Image J (Schneider et al., 2012; Roger et al., 2013). To calculate the efficiency of conversion of ingested biomass (ECI) for caterpillars, we first determined the mass of leaves consumed by caterpillars. To do so, we calculated a mass/area ratio per plant by weighing and photographing two to three dried leaves from leaf pairs neighboring those consumed by caterpillars, and measuring the leaf area using Image J. Using this mass/area ratio, we calculated the mass of leaves consumed by caterpillars from the consumed leaf area that we measured. We calculated ECI per caterpillar as the final dry mass of the caterpillar divided by the dry mass of food it consumed (Waldbauer, 1968). Nine caterpillars that consumed flower buds in addition to leaves on *A. curassavica* plants were excluded from analyses of CLA and ECI. No other plant species produced flowers during the experiment.

After being dried and weighed, aphid populations and individual caterpillars were placed in 1 mL of methanol and stored at −10°C until cardenolide analysis. We assessed the cardenolides that herbivores sequestered following well-established methods (Zehnder and Hunter, 2007; Tao and Hunter, 2015). Aphids and caterpillars were ground for 3 min

in methanol, sonicated for 1 h, and then centrifuged for 6 min. The supernatant was evaporated under vacuum at 45°C until dry and resuspended in 150 μl methanol containing 0.15 mg ml^{-1} digitoxin as an internal standard. Samples were then separated by ultra-performance liquid chromatography (UPLC; Waters Inc., Milford, MA, USA) using a Luna 2.5 μm C18(2) column (50 × 2 mm, Phenomenex Inc., Torrance, CA, USA). Each 2 μl injection was eluted at a constant flow of 0.7 ml per min with a gradient of acetonitrile and water for the 9 min run, maintaining first at 20% acetonitrile for 3 min, increasing to 45% acetonitrile for 5 min, and then maintaining at 20% acetonitrile for 1 min. Peaks were detected by a diode array detector at 218 nm, and absorbance spectra recorded from 200 to 400 nm. Symmetric peaks with maximum absorbance between 217 and 222 nm were quantified as cardenolides. Cardenolide concentrations were calculated using the digitoxin internal standard and total cardenolide concentrations were calculated as the sum of individual peaks. The masses of some aphid populations were too small to obtain enough dried material to detect cardenolides, and those samples were not included in our analyses of cardenolide sequestration (Table S1). In total, we analyzed 107 aphid populations (=replicate plants) with masses from 1.0 to 13.3 mg.

Analysis of Plant Traits

To measure foliar traits, we punched three fresh leaf disks from each leaf of the sixth leaf pair (six hole punches, 424 mm^2 total) of each plant, placed the disks in 1 mL of methanol, and stored them at −10°C until cardenolide analysis. Foliar cardenolide concentrations were later assessed following the same procedure as for aphids and caterpillars (above). Latex that exuded from the hole punches was collected on pre-weighed cellulose disks, dried at 50°C, and weighed. Six additional leaf disks were taken from the same leaves, stored in glassine envelopes, and dried at 50°C. These leaf disks were weighed to estimate SLM and dry mass of foliar material used in cardenolide analyses. SLM was estimated by dividing the mass of dried leaf disks by the total disk area as a proxy for leaf toughness (Frost and Hunter, 2008). Additional leaves from neighboring leaf pairs were removed and dried at 50°C for subsequent carbon, nitrogen, and phosphorus analyses. Remaining plant material was dried at 50°C in paper bags and weighed to measure aboveground biomass after correcting for foliar tissue removed for chemistry sampling.

Carbon (C) and nitrogen (N) concentrations of foliar tissues were measured with a TruMac elemental analyzer (Leco Corporation, St. Joseph, MI, 49085, USA). Phosphorous (P) concentrations of foliar samples were determined by dry combusting ground samples in a muffle furnace at 550°C overnight, followed by persulfate digestion at 121°C for 60 min in an autoclave, and analysis by the molybdenum blue method on a PowerWave XS plate reader reading at 880 nm (Bio-Tek, Highland Park, Winooski, Vermont, 05404, USA). We calculated P concentrations of samples from a potassium phosphate standard curve and assessed quality control with NIST apple leaf standard analyzed with all samples. Only a subset of all experimental treatments were analyzed for nutritive traits, due to time and financial constraints (10 replicates of each plant species × AMF treatment, $n = 120$).

After washing the roots in deionized water, we stored 150 mg of 1 cm pieces of fresh fine root tissue in 60% ethanol at 4°C until we could quantify AMF colonization. We also took 400 mg of fresh fine root, dried it at 50°C, and reweighed it to calculate wet weight/dry weight ratios from which to estimate the dry mass of the subsample taken to quantify AMF colonization. We dried all remaining root tissue at 50°C and weighed its contribution to total root biomass. We analyzed a subset of roots of all experimental treatments (10 replicates of each AMF x plant species treatment, $n = 120$) due to time constraints in harvesting.

To quantify AMF colonization, roots were cleared with 10% KOH for 10 min, acidified using 2% HCl, and stained in 0.05% trypan blue in 1:1:1 water:glycerol:lactic acid (Vannette and Hunter, 2011). We mounted stained roots on slides and scored AMF colonization using the magnified gridline intersect method (McGonigle et al., 1990) with a Nikon compound microscope (Melville, NY, USA). A root intersection was considered colonized if hyphae, arbuscules, or vesicles were present. At least 100 root intersections were analyzed per plant.

Data Analyses

Some aphid populations did not sequester detectable concentrations of cardenolides on plants that contained cardenolides (Table S1), so we first determined whether the probability that aphids would sequester cardenolides was a function of plant species, AMF inoculum availability, or their interaction using a generalized linear mixed model with a binomial distribution and logit link function. Unlike aphids, all caterpillars sequestered cardenolides, except for those feeding on *A. incarnata*, so we did not evaluate the probability of caterpillar sequestration. For the aphid populations that did sequester cardenolides and all individual caterpillars, we used general linear mixed models to evaluate the effects of AMF inoculum availability and milkweed species on herbivore sequestration. In all models, temporal block was a random effect while milkweed species, AMF inoculum availability, and their interaction were fixed effects. For monarchs, we also included the family from which the caterpillar originated as a random effect. Using these models, we evaluated the effects of AMF inoculum availability on three measures of cardenolides sequestered by herbivores; total cardenolide concentration (sum of all cardenolide peaks), cardenolide diversity (using Shannon's index), and cardenolide polarity (relative representation of lipophilic cardenolides), calculated by summing the relative peak areas multiplied by each peaks' retention time (Rasmann and Agrawal, 2011; Sternberg et al., 2012). A greater diversity of cardenolides and more lipophilic cardenolides are considered more toxic than lower diversity or more polar mixes (Fordyce and Malcolm, 2000; Zehnder and Hunter, 2007; Sternberg et al., 2012). Because herbivores feeding upon *A. incarnata* rarely sequestered cardenolides (Table S1), they were excluded from all sequestration analyses.

For these and the following analyses, data were natural log- and log-transformed when necessary. In addition, we used Tukey's adjustment for multiple comparisons to identify significant differences among treatment means. We considered differences to be significant at $P < 0.05$, except when evaluating

differences among AMF treatments within plant species. For these analyses, we considered differences to be significant at $P < 0.1$ due to the reduced sample size of these analyses. All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA). Because several caterpillars died before the end of the experiment and several samples were lost during processing and chemical analyses, final sample sizes were smaller than initial (details in Table S2).

We also tested for differences in the composition (i.e., identity and relative abundance) of cardenolides sequestered by herbivores and present in leaves, among plant species, AMF treatments, and their interaction using permutational multivariate ANOVA (PERMANOVA; McCune et al., 2002). We used the adonis function in the vegan package (Oksanen et al., 2016) in R v 3.3.1 and calculated dissimilarities among samples using the Bray-Curtis metric for PERMANOVA. To evaluate how AMF influenced the composition of cardenolides in herbivore and foliar tissue, we used non-metric multidimensional scaling (NMDS) through the vegan package.

We also used general linear mixed models to compare the effects of AMF inoculum availability and milkweed species on aphid and caterpillar performance. As before, temporal block was a random effect while AMF inoculum availability, milkweed species, and their interaction were fixed effects. For monarchs, we included the family from which the caterpillar originated as a random effect. Each herbivore performance measure (aphid per capita growth rate, aphid mass per individual, caterpillar growth rate, ECI, CLA) was a dependent variable. Not all caterpillars survived through the 6th day of feeding, so we assessed the probability of caterpillar survival among treatments using a generalized linear mixed model with a binomial distribution and logit link function.

We used general linear mixed models to evaluate the effects of AMF inoculum availability and milkweed species on plant traits. In all models, temporal block was a random effect while milkweed species, AMF inoculum availability, and their interaction were fixed effects. Each plant trait (i.e., foliar defensive traits, foliar nutritive traits, aboveground biomass, and levels of AMF colonization of roots) was a dependent variable. *A. incarnata* produced no foliar cardenolides in this study, and were therefore excluded from analyses of foliar cardenolides.

To gain some insight into the phenotypic traits of plants through which AMF influenced herbivores, we assessed the effects of measured plant traits on herbivore performance and sequestration using multiple regression. However, because herbivore and plant traits were measured from different groups of plants (above), we could only assess relationships among average values for each plant species x AMF treatment, yielding only 8–12 data points for these analyses. Therefore, we present these analyses in the Supplementary Materials.

RESULTS

We summarize the effects of milkweed species, AMF inoculum availability, and their interaction on plant traits and herbivore traits (toxin sequestration and performance) in **Tables 1**, **2**, respectively. We describe key results in more detail below.

AMF Colonization

The proportion of roots colonized by AMF arbuscules was tightly correlated with root colonization by all fungal structures ($R^2 = 0.95$, $P < 0.0001$), so we report only the latter here. Inoculation with AMF led to successful root colonization, while control plants remained AMF-free [$F_{(2,106)} = 43.91$, $P < 0.0001$]. Analysis of plants inoculated with live AMF (medium and high AMF treatments only) illustrated that AMF colonization was not a simple function of inoculum availability. Rather, levels of colonization varied substantially among plant species [$F_{(3,70)} = 4.00$, $P = 0.011$; Figure S1], but were similar in medium and high AMF treatments [$F_{(1,70)} = 0.56$, $P = 0.4586$; Figure S1]. However, because herbivore performance varied substantially between medium and high AMF treatments (below), we conclude that the availability of inoculum had effects on plant phenotype beyond those observed by estimates of colonization alone. We have therefore continued to treat medium and high AMF treatments separately in all following analyses.

Herbivore Sequestration of Cardenolides

As expected (Malcolm, 1990, 1994; Agrawal et al., 2015; Petschenka and Agrawal, 2015), the concentration, diversity, polarity, and composition of cardenolides sequestered by aphids and caterpillars varied strongly among plant species, following plant species-specific differences in cardenolide expression (**Table 2**, PERMANOVA aphid: Plant species [$F_{(2,50)} = 22.2694$, $P < 0.001$]; caterpillar: Plant species [$F_{(2,110)} = 98.086$, $P < 0.001$]. For instance, aphids and caterpillars sequestered the highest cardenolide concentration and diversity, and most lipophilic (non-polar) cardenolides, when feeding upon the high cardenolide-containing *A. curassavica* and the least when feeding upon the low cardenolide-containing *A. syriaca.*

Importantly, the amount of AMF inoculum available to the milkweed hosts of aphids and caterpillars influenced the concentration of cardenolides that aphid populations and caterpillars sequestered (aphid: AMF [$F_{(2,48)} = 3.35$, $P = 0.0434$]; **Figure 1A**; caterpillar: AMF [$F_{(2,100)} = 4.05$, $P = 0.0203$]; **Figure 1B**). Across milkweed species, aphids sequestered, on average, 87% and 36% higher cardenolide concentrations when feeding upon plants under medium and high AMF availability, respectively, than when feeding upon plants without AMF (**Figure 1A**). Similarly, caterpillars sequestered 38 and 25% higher cardenolide concentrations when they fed upon plants under medium and high AMF inoculum availability, respectively, than caterpillars that fed upon plants without AMF (**Figure 1B**). The probability that aphid populations would sequester cardenolides did not vary among plant species or with AMF inoculum availability {Plant species [$F_{(2,93)} = 2.56$, $P = 0.0824$]; AMF [$F_{(2,93)} = 0.65$, $P = 0.5264$]}.

The availability of AMF inoculum also shifted the community of cardenolides that aphids and caterpillars sequestered {PERMANOVA aphid: AMF [$F_{(2,50)} = 2.2045$, $P = 0.047$];

TABLE 1 | Effects of plant species, arbuscular mycorrhizal fungi (AMF) inoculum availability, and their interaction on plant traits, including the proportion of roots colonized by AMF, natural log-transformed foliar cardenolide concentrations, foliar cardenolide diversity, foliar cardenolide polarity, leaf toughness (specific leaf mass, SLM; mg/cm^2), natural log-transformed latex exudation (mg), aboveground biomass (mg), foliar P concentration (%), foliar C concentration (%), foliar N concentration (%), foliar C/N ratio.

	Plant species		AMF		Plant species *AMF	
	F	***P***	***F***	***P***	***F***	***P***
Proportion AMF colonization	$F_{(3,70)} = 4.00$	0.011**	$F_{(1,70)} = 0.56$	0.4586	$F_{(3,70)} = 0.61$	0.6122
Foliar cardenolide concentration	$F_{(2,163)} = 251.05$	< 0.0001***	$F_{(2,163)} = 2.98$	0.0538*	$F_{(4,163)} = 0.46$	0.764
Foliar cardenolide diversity	$F_{(2,157)} = 351.18$	< 0.0001***	$F_{(2,157)} = 1.51$	0.2242	$F_{(4,157)} = 0.82$	0.5147
Foliar cardenolide polarity	$F_{(2,157)} = 18.75$	< 0.0001***	$F_{(2,157)} = 0.39$	0.6779	$F_{(4,157)} = 0.84$	0.5025
Leaf toughness (SLM)	$F_{(3,221)} = 113.58$	< 0.0001***	$F_{(2,221)} = 0.37$	0.691	$F_{(6,221)} = 1.36$	0.2324
Latex exudation	$F_{(3,218)} = 79.24$	< 0.0001***	$F_{(2,218)} = 0.62$	0.5381	$F_{(6,218)} = 1.4$	0.2167
Aboveground biomass	$F_{(3,215)} = 47.1$	< 0.0001***	$F_{(2,215)} = 1.8$	0.1681	$F_{(6,215)} = 2.69$	0.0155**
Foliar P concentration	$F_{(3,106)} = 12.57$	< 0.0001***	$F_{(2,106)} = 1.04$	0.3556	$F_{(6,106)} = 3.11$	0.0076**
Foliar C concentration	$F_{(3,106)} = 4.17$	0.0078**	$F_{(2,106)} = 0.90$	0.4112	$F_{(6,106)} = 1.04$	0.4067
Foliar N concentration	$F_{(3,106)} = 9.24$	< 0.0001***	$F_{(2,106)} = 0.34$	0.7141	$F_{(6,106)} = 0.39$	0.8866
Foliar C/N ratio	$F_{(3,106)} = 12$	< 0.0001***	$F_{(2,106)} = 0.05$	0.9535	$F_{(6,106)} = 0.16$	0.9864

*Numbers represent F-values and P-values from general linear mixed models. Final sample sizes per treatment are presented in Table S2 (see text for details). Note that because plants that received no experimental AMF inoculum remained free of AMF contamination, they were excluded from subsequent analyses of AMF colonization. Similarly, A. incarnata produced no foliar cardenolides in this study, and were therefore excluded from analyses of foliar cardenolides. ***P < 0.001, **P < 0.05, *P < 0.1.*

TABLE 2 | Effects of plant species, arbuscular mycorrhizal fungi (AMF) inoculum availability, and their interaction on measures of herbivore toxin sequestration and performance, including natural log-transformed cardenolide concentration sequestered by aphids (mg/g dry mass), diversity of cardenolides sequestered by aphids, natural log-transformed polarity of cardenolides sequestered by aphids, natural log-transformed cardenolide concentration sequestered by caterpillars (mg/g dry mass), diversity of cardenolides sequestered by caterpillars, natural log-transformed polarity of cardenolides sequestered by caterpillars, aphid per capita growth rate (r), individual aphid dry mass (μg), caterpillar growth rate (mg/day), log-transformed caterpillar efficiency of conversion (ECI) of ingested biomass, and log-transformed leaf area consumed (CLA) by caterpillars (cm^2).

	Plant species		AMF		Plant species *AMF	
	F	***P***	***F***	***P***	***F***	***P***
TOXIN SEQUESTRATION						
Aphid cardenolide concentration	$F_{(2,48)} = 9.24$	0.0004***	$F_{(2,48)} = 3.35$	0.0434*	$F_{(4,48)} = 0.08$	0.9879
Aphid cardenolide diversity	$F_{(2,48)} = 23.48$	< 0.0001***	$F_{(2,48)} = 0.01$	0.9868	$F_{(4,48)} = 1.2$	0.3221
Aphid cardenolide polarity	$F_{(2,48)} = 322.66$	< 0.0001***	$F_{(2,48)} = 0.1$	0.9028	$F_{(4,48)} = 2.12$	0.0934
Caterpillar cardenolide concentration	$F_{(2,100)} = 35.76$	< 0.0001***	$F_{(2,100)} = 4.05$	0.0203*	$F_{(4,100)} = 1.93$	0.1107
Caterpillar cardenolide diversity	$F_{(2,100)} = 36.49$	< 0.0001***	$F_{(2,100)} = 4.07$	0.02*	$F_{(4,100)} = 0.93$	0.4488
Caterpillar cardenolide polarity	$F_{(2,100)} = 351.27$	< 0.0001***	$F_{(2,100)} = 1.63$	0.2016	$F_{(4,100)} = 2.96$	0.0234*
PERFORMANCE						
Aphid r	$F_{(3,166)} = 9.10$	< 0.0001***	$F_{(2,166)} = 13.09$	< 0.0001***	$F_{(6,166)} = 0.49$	0.8154
Aphid individual mass	$F_{(3,159)} = 28.62$	< 0.0001***	$F_{(2,159)} = 8.74$	0.0003***	$F_{(6,159)} = 1.31$	0.2536
Caterpillar growth rate	$F_{(3,145)} = 8.18$	< 0.0001***	$F_{(2,145)} = 0.18$	0.8343	$F_{(6,145)} = 0.26$	0.9539
Caterpillar ECI	$F_{(3,137)} = 20.13$	< 0.0001***	$F_{(2,137)} = 1.12$	0.3284	$F_{(6,137)} = 1.62$	0.1448
Caterpillar CLA	$F_{(3,137)} = 6.06$	0.0007***	$F_{(2,137)} = 0.88$	0.4154	$F_{(6,137)} = 1.1$	0.3675

*Numbers represent F-values and P-values from general linear mixed models. Final samples sizes are presented in Table S2 (see text for details) No aphid populations and few caterpillars sequestered cardenolides when feeding upon A. incarnata (Table S1), so herbivores that fed upon A. incarnata were excluded from analyses of cardenolide sequestration. ***P < 0.001, **P < 0.01, *P < 0.05.*

caterpillar: [Plant species ∗ AMF $F_{(4,110)} = 2.022, P = 0.035$]}. In addition, caterpillars feeding on plants under high AMF availability sequestered more diverse communities of cardenolides, by an average of 23%, than did caterpillars feeding upon plants under zero or medium AMF availability {AMF [$F_{(2,100)} = 4.07$, $P = 0.02$; **Figure 1C**]}. There were also minor, plant species-specific effects of AMF on the polarity of cardenolides that caterpillars sequestered {Plant species∗AMF [$F_{(4,100)} = 2.96$, $P = 0.0234$]}. Caterpillars sequestered 22% more lipophilic (non-polar) cardenolides when feeding upon *A. syriaca* plants under high AMF availability than on *A. syriaca* plants under zero or medium AMF availability. However, the polarity of cardenolides that caterpillars sequestered was unaffected by the amount of AMF available to *A. curassavica*

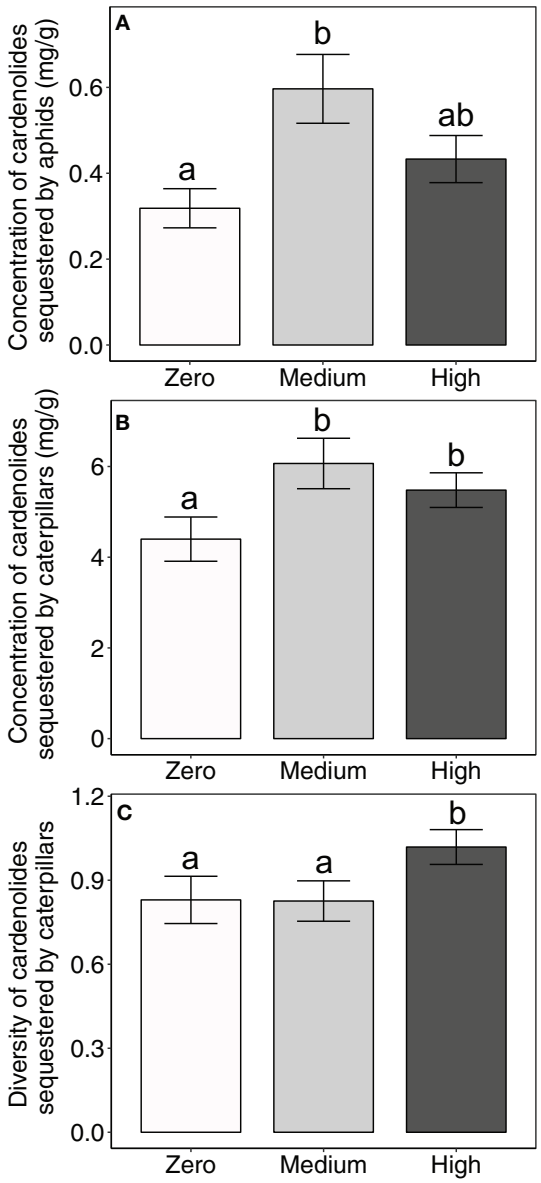

FIGURE 1 | Effects of AMF inoculum availability on the concentration of cardenolides sequestered by **(A)** aphid populations and **(B)** individual caterpillars, and on **(C)** the diversity of cardenolides sequestered by individual caterpillars reared on three milkweed species. Sample sizes range from 15 to 24 aphid populations (= replicate plants) for aphid cardenolide concentrations, 39–43 individual caterpillars (= replicate plants) for the concentration and diversity of cardenolides sequestered by caterpillars per AMF treatment. Bars display the mean ± 1 SE. Different letters indicate significantly ($P < 0.05$) different means (Tukey *post-hoc* test of the ANOVA).

and *A. latifolia*. AMF availability also did not influence the diversity or polarity of cardenolides that aphids sequestered (**Table 2**).

Herbivore Performance

Aphid performance varied non-linearly with increasing AMF availability; it was lowest on plants under medium AMF availability, but highest on plants under high AMF availability (**Table 2**, **Figure 2**). Specifically, aphid per capita growth rates were 19% greater under high AMF availability than under medium AMF availability, with intermediate per capita growth rates on plants without AMF [$F_{(2,166)} = 13.09$, $P < 0.0001$; **Figure 2A**]. Similarly, individual aphids were 24% heavier on plants under high AMF availability than were aphids on plants under medium AMF availability [$F_{(2,159)} = 8.74$, $P = 0.0003$; **Figure 2B**]. As expected from previous work (Agrawal, 2004), aphid per capita growth rates and masses varied among milkweed species {r: [$F_{(3,166)} = 9.10$, $P < 0.0001$; mass: $F_{(3,159)} = 28.62$, $P < 0.0001$]}.

The availability of AMF inoculum had striking effects on caterpillar survival, but those effects varied among milkweed species (Plant species∗AMF $\chi^2 = 14.1$, df = 6, $P = 0.0286$; **Figure 3**). For example, caterpillars feeding on *A. incarnata* and *A. syriaca* were 13 and 44% more likely to survive on plants without AMF than on plants with AMF, respectively. In contrast, caterpillars feeding on *A. latifolia* were 38% more likely to survive on plants grown under medium AMF inoculum availability than on plants without AMF. Caterpillars feeding on *A. curasssavica* were affected minimally by AMF inoculum availability (**Figure 3**). Caterpillar growth rates, efficiency of conversion of ingested biomass (ECI), and consumption of leaf area (CLA) varied widely among milkweed species, but were unaffected by the availability of AMF inoculum (**Table 2**).

Effects of AMF on Plant Traits

Consistent with the effects of AMF on cardenolide sequestration by herbivores (above), foliar cardenolide concentrations in milkweed plants under medium and high AMF availability were an average of 17 and 19% greater, respectively, than were concentrations in AMF-free plants {AMF [$F_{(2,163)} = 2.98$, $P = 0.0538$; **Figure 4**]}. As expected (Rasmann and Agrawal, 2011; Sternberg et al., 2012; Vannette et al., 2013), milkweed species varied in the diversity, polarity, and composition of cardenolides in their leaves, as well as in leaf toughness (SLM) and latex exudation {**Table 1**, PERMANOVA for composition [$F_{(2,160)} = 131.51$, $P < 0.001$]}. However, we observed no influence of AMF inoculum availability on any of these chemical or physical resistance traits {**Table 1**, PERMANOVA for cardenolide composition: AMF [$F_{(2,160)} = 1.62$, $P = 0.128$]}.

In contrast to their consistent effects on foliar cardenolide concentrations, AMF altered plant growth and nutritive traits in a plant species-specific fashion (**Table 1**, **Figures 5A,B**). AMF inoculation decreased the aboveground biomass of most milkweed species by 8–29%. The exception was *A. curassavica*, in which AMF inoculation increased aboveground biomass by an average of 28% {Plant species∗AMF [$F(_{6,215}) = 2.69$, $P = 0.0155$, **Figure 5A**]}. AMF inoculation increased foliar P concentrations in *A. curassavica* and *A. latifolia* by an average of 25 and 16%, respectively, but decreased P concentrations in *A. incarnata* and *A. syriaca* by an average of 8 and 13%, respectively {Plant species*AMF [$F_{(6,106)} = 3.11$, $P = 0.0076$; **Figure 5B**]}. In contrast, AMF inoculum availability did not affect foliar C or N concentrations, or foliar C/N ratios, although these traits did vary among plant species (**Table 1**).

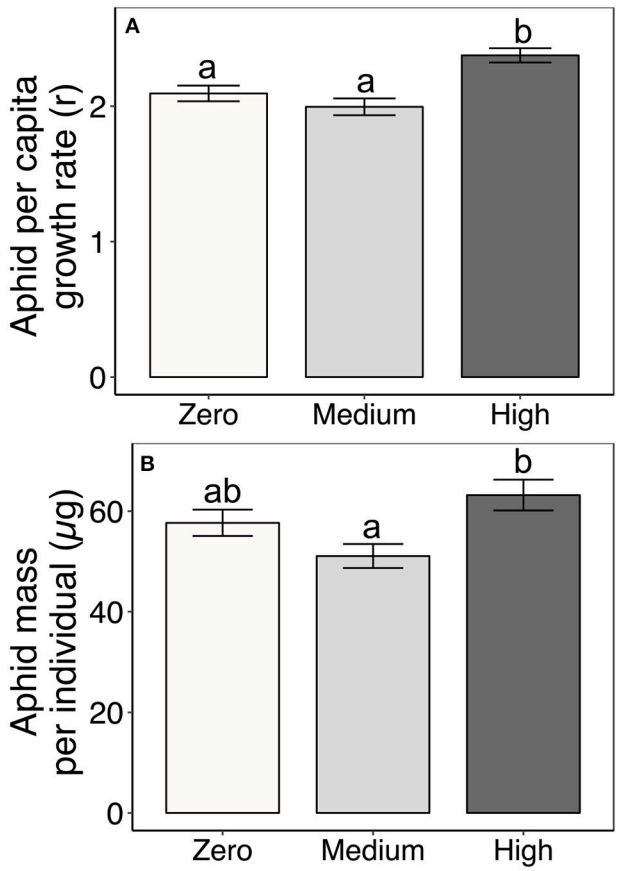

FIGURE 2 | Effects of AMF inoculum availability on **(A)** per capita growth rates of aphids (r over 6 days) and **(B)** average dry mass of individual aphids reared on four milkweed species. Sample sizes are 60 populations of aphids (= replicate plants) for aphid per capita growth rates and range from 55 to 59 populations for average individual aphid mass per AMF treatment. Bars display the mean ± 1SE. Different letters indicate significantly ($P < 0.05$) different means (Tukey *post-hoc* test of the ANOVA).

DISCUSSION

Our study is among the first to document the impacts of AMF on toxin sequestration by specialist herbivores, while measuring simultaneously effects on herbivore performance. We demonstrate that (1) aphids and caterpillars sequester higher concentrations of cardenolides from plants inoculated with AMF, following AMF-mediated increases in foliar cardenolide concentrations. (2) AMF availability influences the performance of both aphids and caterpillars on milkweed, though in different ways. On all milkweed species, aphid performance varies non-linearly with increasing AMF inoculum availability, with lowest performance under medium levels of inoculum availability and highest performance under high inoculum availability. In contrast, while caterpillar survival varies markedly with AMF inoculum availability, it does so in a plant species-specific manner, and caterpillar growth is unaffected by AMF. Our findings suggest that by altering plant phenotype, the availability of AMF in soil has the potential to influence both the top-down (via sequestration) and the bottom up (via plant defense and nutrition) forces that operate on milkweed herbivores.

Inoculation of plants with medium or high amounts of AMF inoculum resulted in equal levels of root colonization (Figure S1). Nonetheless, we observed that the availability of AMF inoculum (medium versus high) influenced herbivore performance and plant phenotype (**Tables 1**, **2**). Because the commercial AMF mix that we used was purported to consist of four AMF species, the different effects of AMF availability on herbivore performance may be a function of differential colonization by AMF species under medium and high AMF availability. AMF species vary in their relative trading of nutrients (Lendenmann et al., 2011; Thonar et al., 2014; Argüello et al., 2016) and effects on plant phenotype (Gehring and Bennett, 2009; Bennett et al., 2013) which can alter herbivore performance (Roger et al., 2013; Vannette and Hunter, 2013). However, cloning and sequencing of the AMF mix, and roots from milkweed plants grown under the same experimental conditions, with AMF-specific primers (Krüger et al., 2009) demonstrated that the AMF mix consisted only of *F. mosseae* (details in Supplementary Material).

Instead, the differential effects of medium and high AMF inoculum availability on herbivore performance and plant phenotype are more likely due to differential regulation of AMF colonization by plants under medium and high AMF availability. Although AMF colonization levels increase with increasing inoculum availability (Garrido et al., 2010; Vannette and Hunter, 2011), plants maintain a maximum level of AMF colonization of roots (Vierheilig et al., 2000a,b; Meixner et al., 2005) and suppress further colonization after reaching a critical level (Vierheilig, 2004). Plant regulation of AMF development in roots is controlled by the same plant hormones (Staehelin et al., 2011; Bucher et al., 2014; Gutjahr, 2014; and references therein) that are integral to the development of plant vascular tissues (Lucas et al., 2013) and the resistance responses of plants to insect herbivores (Pieterse et al., 2012, 2014). In our medium AMF treatment, there may have been sufficient inoculum to attain maximum levels of AMF colonization of plant roots. Therefore, under high AMF availability, plants may have suppressed AMF development in roots more strongly by altering phytohormone levels, resulting in the observed differences in herbivore performance and plant phenotype between medium and high AMF treatments.

Sequestration by Specialist Herbivores Is Altered by AMF Availability

Both aphids and caterpillars sequestered higher concentrations of cardenolides when feeding upon plants under medium and high AMF inoculum availability (**Figures 1A,B**), following AMF-mediated increases in foliar cardenolide concentrations (**Figure 4** and Figures S2A,B; Table S3). This is consistent with previous reports of tight links between aphid and caterpillar sequestration and host plant cardenolide concentrations (Malcolm, 1990, 1994; Agrawal et al., 2015; Petschenka and Agrawal, 2015). However, while AMF inoculum availability did not influence the composition of cardenolides in foliage, AMF did affect the composition of cardenolides sequestered by aphids and caterpillars. Sequestration of cardenolides by *A. nerii* occurs

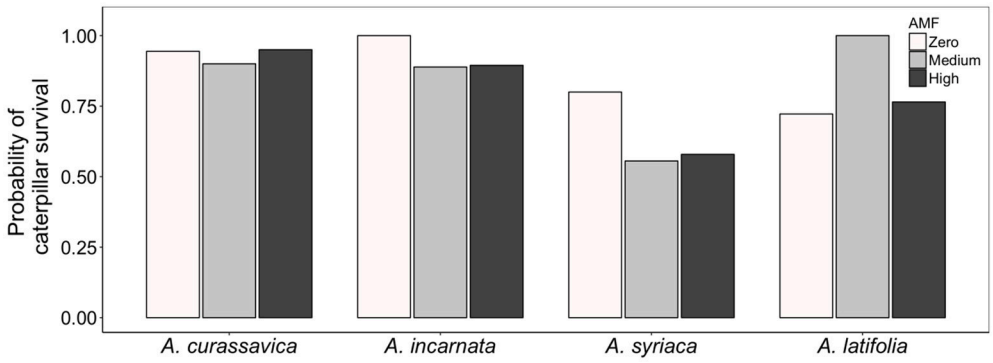

FIGURE 3 | Effect of AMF inoculum availability on the probability of caterpillar survival on four milkweed species. Sample sizes range from 17 to 20 caterpillars (= replicate plants) per plant species × AMF treatment.

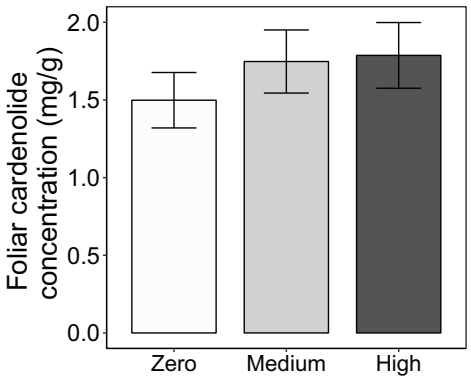

FIGURE 4 | Effects of AMF inoculation on foliar cardenolide concentrations of three milkweed species. Samples sizes range from 58 to 59 plants per AMF treatment. Bars display the mean ± 1SE. Foliar cardenolide concentrations vary among AMF treatments ($P = 0.0538$), but treatment means are not significantly different at $P < 0.05$ (Tukey *post-hoc* test of the ANOVA).

through passive diffusion (Malcolm, 1990; Züst and Agrawal, 2016b). Therefore, AMF-mediated changes in the composition of cardenolides sequestered by aphids may result from AMF changing the relative concentrations of cardenolides present in phloem, but not leaves. While milkweed phloem contains the same variety of cardenolides as leaves, the concentrations of specific cardenolides may vary between phloem and leaves (Züst and Agrawal, 2016b).

In contrast, monarch caterpillars may control the uptake of particular cardenolides and their amounts (Malcolm, 1994; Tao and Hunter, 2015) by sequestering cardenolides actively and selectively (Malcolm and Brower, 1989; Frick and Wink, 1995; Petschenka and Agrawal, 2015; Erb and Robert, 2016). AMF may have affected the composition of cardenolides sequestered by caterpillars, without affecting the composition of foliar cardenolides, by altering aspects of plant quality that may affect active sequestration, such as nutrient availability. We did not find correlations between foliar nutrient content and sequestration, potentially due to low sample sizes, but variation in soil N and P availability has been found to alter the efficiency of monarch caterpillar sequestration and the composition of cardenolides that monarch caterpillars sequester (Tao and Hunter, 2015). Alternatively, interactions between AMF and caterpillar feeding may have altered the composition of foliar cardenolides (Bennett et al., 2009; Agrawal et al., 2014; Wang et al., 2015), resulting in the observed, AMF-mediated differences in caterpillar sequestration. However, milkweed responses to monarch caterpillar feeding can take up to 5 days to occur (Agrawal et al., 2014) and monarch caterpillars fed on our experimental plants for only 6 days. Therefore, we think it unlikely that AMF-mediated changes in caterpillar sequestration were driven by interactions between AMF and caterpillar induction of foliar cardenolides.

AMF Abundance Alters Specialist Herbivore Performance and Survival

The availability of AMF inoculum had consistent, non-linear effects on aphid performance, regardless of milkweed species (**Figure 2**). Aphids had the lowest per capita growth rates and individual masses on plants under medium AMF availability, yet had the highest per capita growth rates and masses on plants under high AMF availability (**Figure 2**). Thus, we found within a single study the range of aphid responses to AMF from the literature, from positive to negative (Pacovsky et al., 1985; Gange and West, 1994; Gange et al., 1999, 2002; Gehring and Whitham, 2002; Wurst et al., 2004; Hempel et al., 2009; Koricheva et al., 2009; Abdelkarim et al., 2011; Babikova et al., 2014a; Colella et al., 2014; Grabmaier et al., 2014; Williams et al., 2014; Bennett et al., 2016; Simon et al., 2017; Tomczak and Müller, 2017). Our findings suggest that some of the previously found variation in aphid responses may result from differences in AMF inoculum availability among studies.

AMF may have affected aphid performance by altering foliar cardenolide concentrations; we found that aphid masses declined with increasing foliar cardenolide concentrations (Table S3, Figure S2d). Indeed, aphids had lower masses and per capita growth rates on plants under medium AMF

availability (**Figure 2**), which had greater foliar cardenolide concentrations than plants without AMF (**Figure 4**). Although *A. nerii* tolerate cardenolides, they are negatively affected by high cardenolide concentrations (Agrawal, 2004; de Roode et al., 2011; Birnbaum et al., 2017). Nonetheless, we interpret the regressions with caution due to low sample sizes and plant species-specific differences in traits. AMF-mediated increases in aphid performance under high AMF availability may also be a consequence of increased vascular bundle size; AMF colonization increases the size of vascular bundles in plants (Krishna et al., 1981), increasing aphid phloem feeding and reproductive success (Simon et al., 2017). Although aphids are often responsive to changes in amino acid content of phloem (Züst and Agrawal, 2016a), we think it unlikely that AMF influenced *A. nerii* performance by changing phloem soluble sugar or amino acid content because previous studies found no correlations among AMF-mediated changes in aphid performance and foliar or phloem nutrient content (Gange and West, 1994; Hempel et al., 2009; Grabmaier et al., 2014).

Although AMF colonization of plants has been found to increase the survival of specialist caterpillars (Goverde et al., 2000), we found that AMF inoculum availability improved, did not affect, or reduced the survival of a specialist caterpillar, depending on the plant species and density of AMF inoculum available to the plant (**Figure 3**). This breadth of responses of monarch caterpillars to AMF among plant species may result from plant species-specific effects of AMF on plant biomass (**Figure 5A**); caterpillar survival increased with increasing aboveground biomass (Tables S3, Figure S2e). Although caterpillars were never food limited in our study, AMF-mediated declines in plant biomass may have reduced caterpillar survival by decreasing the availability of young leaves because monarch caterpillars prefer younger leaves (Bingham and Agrawal, 2010). AMF-mediated increases in foliar cardenolide concentrations did not correlate with declines in caterpillar survival in this study, although high cardenolide concentrations often reduce monarch caterpillar performance and survival (Zalucki et al., 2001a; Agrawal, 2005; Rasmann et al., 2009; Tao et al., 2016b).

Interestingly, despite finding strong effects of AMF on monarch survival, we found no influence of AMF on monarch caterpillar growth rates (**Table 2**). Our findings confirm those for other specialist chewers, such as specialist beetle larvae and adult weevils (Laird and Addicott, 2008; Cosme et al., 2011), whose growth rates are also unaffected by AMF. However, our findings contrast with previous work that found monarch caterpillar growth rates to increase on milkweed plants under higher AMF inoculum availability (Vannette and Hunter, 2013). These conflicting findings may result from experimental milkweed plants being inoculated with different AMF species; individual AMF taxa and mixes alter plant phenotype differently (Bennett et al., 2009; Vannette and Hunter, 2011), affecting caterpillar performance (Goverde et al., 2000; Roger et al., 2013). Indeed, AMF-mediated increases

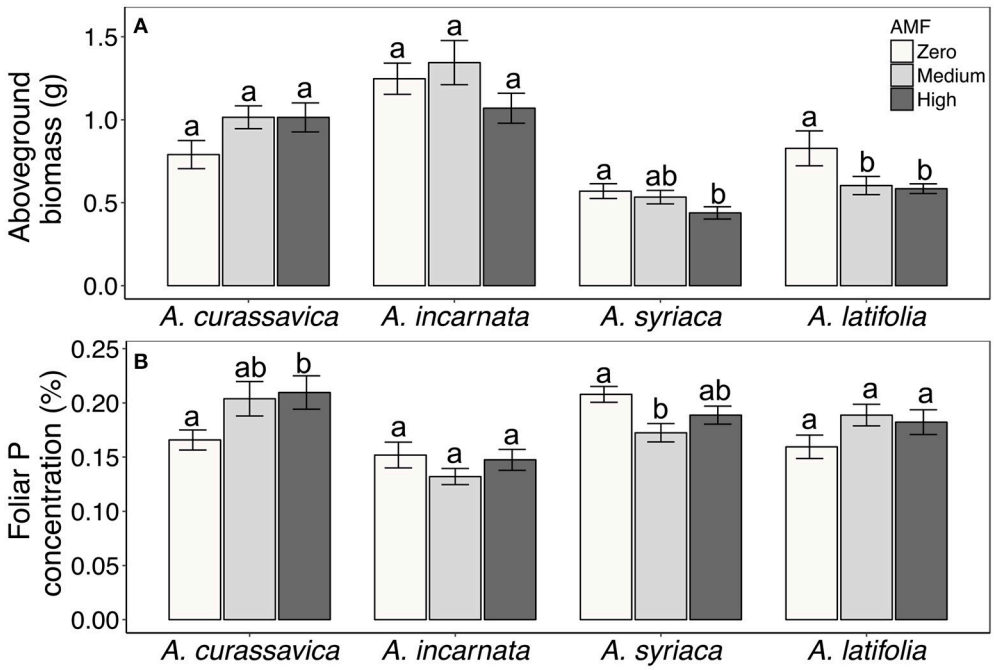

FIGURE 5 | (A) Aboveground biomass and **(B)** foliar phosphorus (P) concentrations of four milkweed species. Sample sizes range from 17 to 20 plants per treatment for aboveground biomass and 9–10 plants per treatment for P concentrations. Bars display the mean ± 1SE. Different letters indicate significantly ($P < 0.1$) different AMF treatment means within each plant species (Tukey *post-hoc* test of the ANOVA within plant species).

in monarch caterpillar growth rates were attributed to AMF-mediated declines in milkweed leaf toughness (SLM) and latex exudation (Vannette and Hunter, 2013) and we found no influence of AMF on these traits (**Table 1**). In addition, it is possible that our plants were already induced by thrip activity, whereas plants in previous studies were not. However, because plants of all treatments were attacked equally, we do not believe that the minor thrip damage altered the quality of our results.

Effects of AMF on Herbivore Performance and Toxin Sequestration May Have Community-Wide Consequences

Because the availability of AMF inoculum altered both toxin sequestration and performance of specialist herbivores, AMF may affect herbivore populations by altering both top-down and bottom-up factors. For instance, aphids that fed upon milkweeds under medium AMF availability sequestered nearly twice the concentration of cardenolides that they did when feeding upon AMF-free plants, potentially improving aphid resistance to natural enemies. Aphid predators exhibit high rates of mortality when fed oleander aphids from high cardenolide milkweeds, but experience low rates of mortality when fed aphids from low cardenolide milkweeds (Malcolm, 1992). Accordingly, in the field, oleander aphid populations are smaller and more influenced by predators when feeding on low cardenolide milkweed species than when feeding on high cardenolide milkweed species (Malcolm, 1992; Mohl et al., 2016). Similarly, monarch caterpillars that sequester higher concentrations of cardenolides are more toxic to their predators (Brower et al., 1968; Reichstein et al., 1968; Brower and Moffitt, 1974) and may be more resistant to their parasites (Lefèvre et al., 2010; Sternberg et al., 2012). Therefore, monarch caterpillars may be better protected against their natural enemies when their host plants are inoculated with AMF.

The strong effects of AMF on aphid per capita growth rates and caterpillar survival suggest that the availability of AMF in soil may also influence the population dynamics of herbivores by changing host plant quality. Furthermore, by altering aphid densities and individual masses, AMF may influence aphid-parasitoid interactions. Parasitism rates of *A. nerii* are density dependent (Helms et al., 2004), and parasitoids that develop in larger herbivore hosts have larger clutch sizes, bigger individual offspring, greater proportions of female offspring, and increased longevity (Hunter, 2003; Bukovinszky et al., 2008; van Veen and Godfray, 2012). AMF colonization of plants has been found to increase parasitoid attack rates, shorten parasitoid developmental times, and increase successful emergence of aphid parasitoids (Hempel et al., 2009; Bennett et al., 2016), even in the absence of plant-derived cues such as volatiles (Bennett et al., 2016). Our study suggests that AMF-mediated increases in aphid size may be a simple mechanism by which AMF improve parasitoid success. In support of this, communities of other belowground organisms, such as soil-dwelling nematodes, have been found to improve parasitoid performance, potentially by increasing aphid size (Bezemer et al., 2005).

CONCLUSION

In summary, we found that AMF inoculum availability influences strongly toxin sequestration and performance of two specialist herbivores, suggesting that AMF availability may substantially alter interactions among plants, herbivores, and their natural enemies. Furthermore, the availability of AMF inoculum, measured as infectivity and spore abundances, varies on small scales, such as centimeters (Wolfe et al., 2007) and meters (Carvalho et al., 2003). Therefore, plants within a single population may experience substantial variation in AMF availability in soils. This variation in AMF abundance may result in spatial variation in plant quality for herbivores, and herbivore quality for their natural enemies, ultimately affecting large scale population dynamics (Riolo et al., 2015). Future studies should consider how natural AMF abundances influence plant phenotype and the resulting herbivore and natural enemy population dynamics in the field.

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.8985578.

AUTHOR CONTRIBUTIONS

AM and MH: Conceived the ideas and designed methodology; AM: Collected the data; AM and MH: Analyzed the data; AM: Led the writing of the manuscript. Both authors contributed to the drafts and gave final approval for 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/fevo.2018.00033/full#supplementary-material

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REVIEW
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Effects of Soil Organisms on Aboveground Plant-Insect Interactions in the Field: Patterns, Mechanisms and the Role of Methodology

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Soil biota-plant interactions play a dominant role in terrestrial ecosystems. Through nutrient mineralization and mutualistic or antagonistic interactions with plants soil biota can affect plant performance and physiology and via this affect plant-associated aboveground insects. There is a large body of work in this field that has already been synthesized in various review papers. However, most of the studies have been carried out under highly controlled laboratory or greenhouse conditions. Here, we review studies that manipulate soil organisms of four dominant taxa (i.e., bacteria, fungi, nematodes, and soil arthropods) in the field and assess the effects on the growth of plants and interactions with associated aboveground insects. We show that soil organisms play an important role in shaping plant-insect interactions in the field and that general patterns can be found for some taxa. Plant growth-promoting rhizobacteria generally have negative effects on herbivore performance or abundance, most likely through priming of defenses in the host plant. Addition of arbuscular mycorrhizal fungi (AMF) has positive effects on sap sucking herbivores, which is likely due to positive effects of AMF on nutrient levels in the phloem. The majority of AMF effects on chewers were neutral but when present, AMF effects were positive for specialist and negative for generalist chewing herbivores. AMF addition has negative effects on natural enemies in the field, suggesting that AMF may affect plant attractiveness for natural enemies, e.g., through volatile profiles. Alternatively, AMF may affect the quality of prey or host insects mediated by plant quality, which may in turn affect the performance and density of natural enemies. Nematodes negatively affect the performance of sap sucking herbivores (generally through phloem quality) but have no effect on chewing herbivores. For soil arthropods there are no clear patterns yet. We further show that the methodology used plays an important role in influencing the outcomes of field studies. Studies using potted plants in the field and studies that remove target soil taxa by means of pesticides are most likely to detect significant results. Lastly, we discuss suggestions for future research that could increase our understanding of soil biota-plant-insect interactions in the field.

Keywords: soil, aboveground-belowground interactions, insects, field experiments, fungi, bacteria, nematodes, root herbivores

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INTRODUCTION

Soils are an important source of diversity of microbes worldwide (Ramirez et al., 2018), but soil is also home to various other higher taxa, such as nematodes, root feeding insects or even vertebrates (Bardgett and van der Putten, 2014). The role of soil biota in ecosystem functioning is widely recognized and the study of soil biota-plant interactions has developed into a very active and large field in ecology. Soil organisms fulfill key processes in the soil, such as decomposition and nutrient mineralization. Many microorganisms engage in mutualistic interactions with plant hosts, aiding in the uptake of nutrients and water (e.g., arbuscular mycorrhizal fungi, AMF), in exchange for photosynthates or other plant metabolites. Other groups of soil micro- and macro-organisms have antagonistic effects on plant health, for example via pathogenicity (e.g., pathogenic fungi) or herbivory (e.g., root herbivorous insects). It has been shown previously in studies carried out under artificial/controlled conditions that mutualistic and antagonistic players in the soil not only impact the growth (i.e., biomass production) of plants, but also lead to the alteration of various physiological processes in plant tissues, resulting in changes in tissue quality or palatability of the plant (e.g., Bezemer and van Dam, 2005). Through such mechanisms, soil biota can mediate interactions between the host plant and aboveground organisms, such as insect herbivores and pollinators. Despite all the attention that this subject has received, the majority of published studies have been conducted under more controlled conditions (hereafter "controlled studies"), such as in greenhouses or growth chambers. Hence, an important question is whether the results are a realistic representation of ecological processes that occur in natural systems.

Mechanisms through which soil organisms can affect aboveground insects in the field are mostly plant-mediated (**Figure 1**). Various organisms, most notably plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF), can boost plant growth (e.g., Saravanakumar et al., 2008; Gadhave et al., 2016), which has been hypothesized to increase plant palatability (i.e., the plant vigor hypothesis; Price, 1991; Cornelissen et al., 2008). On the other hand, plants under biotic or abiotic stress can also be more vulnerable to attack by herbivores (i.e., the plant stress hypothesis; White, 1969). Evidence for the former has been reported from field studies (e.g., for some AMF species in Wolfe et al., 2005; Ueda et al., 2013). Several studies also find support for the plant stress hypothesis (e.g., for nematodes in Alston et al., 1991; Vockenhuber et al., 2013). However, many field studies report plant-mediated effects of soil organisms on aboveground insects, without reporting any effects on plant vigor or stress, which suggests that other factors related to plant performance (see **Figure 1**) could play an important role in mediating aboveground plant-herbivore interactions.

Plant nutritional value (most importantly, nitrogen and sugar content) in the field can be positively affected by soil organisms (Gange and West, 1994; Gange et al., 2005a,b; Younginger et al., 2009; Moon et al., 2013; Brunner et al., 2015; Godschalx et al., 2015; Ryalls et al., 2016). Moreover, plant secondary defense metabolites, that play a role in the palatability of host plants, can be affected by soil organisms in the field (Wurst et al., 2008; Megías and Müller, 2010). Interactions with soil organisms can also sensitize the immune system of plants so that they can respond faster or more strongly to subsequent attack by antagonists (e.g., Pieterse et al., 2014). This process, better known as induced systemic resistance (ISR), can play an important role in plant-insect interactions in the field (Saravanakumar et al., 2008; Prabhukarthikeyan et al., 2014). Soil organisms can also interfere with plant volatile emissions, which are important cues for herbivores (e.g., for oviposition), as well as for many natural enemies, to detect host plants (Megali et al., 2015). Finally, several studies have shown that, for instance AMF can affect plant functional traits, such as flower size and stamen number (Gange and Smith, 2005; Gange et al., 2005a; Varga and Kytöviita, 2010).

In this review, we aim to answer three main questions. (1) What is the role of whole soil communities and plant-soil feedbacks in mediating aboveground plant-insect interactions in the field? (2) What is the role of the individual taxa of soil organisms in mediating aboveground plant-insect interactions in the field and how do potential patterns compare to those that are observed in controlled studies? (3) How does the experimental methodology used in the field affect the outcome of above-belowground studies? Furthermore, we will discuss potential applications and suggest future directions to advance this scientific field.

LITERATURE SEARCH METHODOLOGY

The scientific literature was searched using Web of Science for combinations of "soil 'faunal group'" AND "insect" AND "field," in which "faunal group" was replaced by; bacteria, fung*, nematod*, arthropod* or insect*, respectively. Furthermore, the literature was searched for combinations of "plant-soil feedback" AND "insects" AND "field". Suitable studies were selected first based on title and subsequently on abstract or full manuscript. Additionally, reference lists from suitable papers, as well as from recent reviews (Gehring and Bennett, 2009; Hartley and Gange, 2009; Koricheva et al., 2009; Pineda et al., 2010; Johnson et al., 2012; Soler et al., 2012; Wondafrash et al., 2013) on soil biota-plant-insect interactions were examined to detect additional publications. Lastly, for all suitable publications, the studies that cited these publications were scanned to detect additional studies that were published later.

In total, the literature search yielded 50 field studies, covering a total of 185 individual soil biota-plant-insect interactions (Supplementary Tables 1–4).

PLANT-SOIL FEEDBACK EFFECTS ON PLANT-INSECT INTERACTIONS IN THE FIELD

Plants are not only influenced by soil organisms, but they also play an active role in shaping the biome around their roots. Plant species typically manipulate the microbiome around their roots, e.g., via exudation of carbohydrates and other chemical

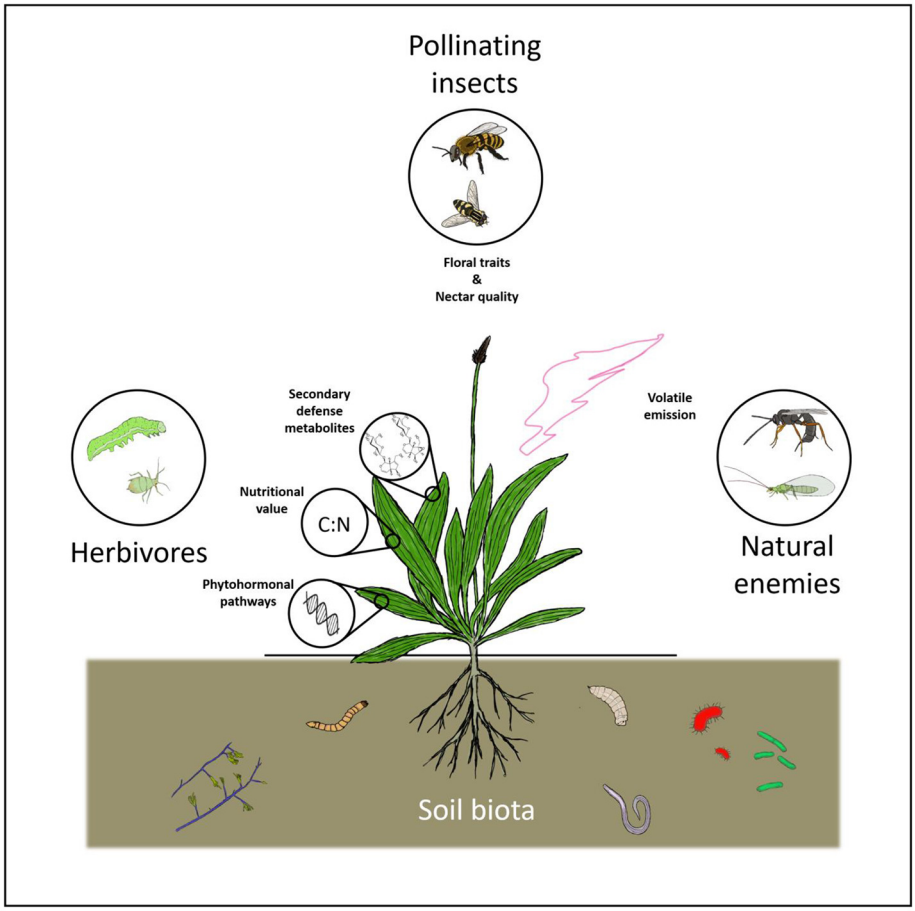

FIGURE 1 | A schematic overview of mechanisms through which soil organisms can affect plant phenotype and associated aboveground insects. Soil organisms can affect a variety of host plant traits, including nutritional quality and palatability, size, morphology and floral traits, as well as the activation of defense pathways and the emission of plant volatile organic compounds. Through these mechanisms they can influence insect herbivores, pollinators and natural enemies.

substances (Bais et al., 2006), resulting in specific microbial rhizosphere profiles (Lakshmanan et al., 2014). Such species-specific microbial profiles can influence the performance of other plants that grow later in the same soil (Kostenko et al., 2012; Bezemer et al., 2013; Kos et al., 2015; Heinen et al., 2018). This process is known as plant-soil feedback (Van der Putten et al., 2013) and can be an important driver of plant community dynamics (Kardol et al., 2006). In recent years, it has become evident that such changes in soil microbial communities, via plant-mediated processes, can affect the performance of aboveground organisms that interact with these plants. For example, several greenhouse studies have shown that soil legacy effects, the effects of earlier plant growth on the microbial community in the soil, can have strong effects on aboveground herbivores feeding on later growing conspecific plants in those soils (Kostenko et al., 2012; Kos et al., 2015). A recent study, for example, revealed that soil legacies left by grasses and forbs have contrasting effects on a chewing herbivore that fed on plant communities growing on soils with these legacies (Heinen et al., 2018).

Although most studies on the impact of whole soil microbiomes on plant-insect interactions have been performed in greenhouses and climate chambers, several studies have explored such relationships in the field. For example, in a field experiment, the proportion of ragwort (*Jacobaea vulgaris*) plants attacked by stem borers, leaf miners and flower feeders was much lower (up to 50%) for plants that were grown in soils with a ragwort legacy compared with plants grown in soils without this legacy, probably because of a soil legacy-induced reduction in plant size (Bezemer et al., 2006). Negative plant-soil feedback is generally seen as a result of the accumulation of pathogenic organisms (Nijjer et al., 2007; Van der Putten et al., 2013), and the effects observed in ragwort and their associated aboveground insects are likely caused by belowground pathogens (e.g., Van de Voorde et al., 2012). Another field study with the same plant species, found a positive correlation between the occurrence of seed feeding insects and colonization of ragwort roots by mycorrhizal arbuscules (Reidinger et al., 2012). These results indicate that soil legacies, most likely driven by soil organisms, can play a role in shaping plant-insect interactions

in the field. We have not been able to identify any manipulative studies that have, thus far, investigated plant-insect interactions in a plant-soil feedback framework. However, numerous studies have investigated the effects of the experimental manipulation of various groups of soil organisms on aboveground plant-insect interactions, and this area is discussed in more detail below.

SOIL BIOTA-PLANT-INSECT INTERACTIONS IN THE FIELD

Bacteria

Bacteria are a dominant group of organisms in the soil that can have strong effects on plant growth and quality. For example, nitrogen-fixing rhizobia that associate with leguminous plant species fix atmospheric nitrogen and thereby often increase nitrogen content in the plant tissues. On the other hand, plant-growth promoting rhizobacteria (PGPR) are known to have yield enhancing effects on plants, but also are known to induce systemic resistance by priming plants for the activation of defense pathways, which often results in negative effects on insect herbivores in controlled studies (Pineda et al., 2010).

The Effect of Nitrogen-Fixing Rhizobia on Aboveground Herbivores

One would expect that the increased plant quality resulting from plant mutualisms with nitrogen fixing bacteria would benefit aboveground insects. However, this is not necessarily the case, as rhizobia have been shown to also affect plant defense responses directly (e.g., Thamer et al., 2011) and indirectly (Godschalx et al., 2015). The latter is illustrated by a study with potted plants placed in the field that reported positive effects of the addition of *Rhizobium* sp. on plant protein levels in Lima bean, *Phaseolus lunatus*, but negative effects on extrafloral sugar content. This, in turn, led to 75% lower visitation numbers of the associated mutualist ant *Tetramorium caespitum*. Ants can act as natural enemies of herbivores and this study suggests that rhizobia can interfere with this indirect plant defense mechanism. In the presence of rhizobia, cyanogenesis (a chemical defense in legumes) is increased, and this may reduce the need for the plant to produce extrafloral nectar to attract ants (Godschalx et al., 2015).

The Effect of Plant Growth-Promoting Rhizobacteria on Aboveground Herbivores

Plant-mediated effects of the addition of PGPR on aboveground insects in the field are consistently negative in the studied systems. All interactions ($n = 17$) revealed from the literature search were negative for the aboveground herbivore, regardless of the insect feeding guild (**Figure 2A**, Supplementary Table 1, Zehnder et al., 1997; Commare et al., 2002; Saravanakumar et al., 2008; Gadhave et al., 2016). For instance, the addition of four different *Pseudomonas fluorescens* strains (individually, as well as in mixtures) to rice fields in India resulted in a ∼3 fold reduction of leaf rolling by the rice leaf roller *Cnaphalocrocis medialis* (Commare et al., 2002; Saravanakumar et al., 2008). These effects are most likely driven by ISR, as plants generally express higher levels of defense gene transcription after exposure to herbivory in plants that received bacterial treatments (Saravanakumar et al., 2008; Prabhukarthikeyan et al., 2014).

The Effect of Plant Growth-Promoting Rhizobacteria on Aboveground Natural Enemies

Inoculation with PGPR can also influence the performance or attraction of insects at higher trophic levels, such as predatory insects or parasitoids (Saravanakumar et al., 2008; Gadhave et al., 2016). It is difficult to elucidate clear patterns as from all interactions ($n = 18$), 50% reported negative effects while 44% of the studies reported positive effects (**Figure 2A**, Supplementary Table 1). For example, a study investigating the effects of inoculation with *Bacillus* spp. on field-grown broccoli (*B. oleracea*) reported consistently reduced numbers of the ladybug (*Coccinella septempunctata*) and various unidentified syrphid flies on plants that received bacterial inoculations, compared to control plants that did not receive additional bacteria (Gadhave et al., 2016). However, in the same study, the authors found that the percentage of cabbage aphids (*B. brassicae*) parasitized by the parasitoid wasp *Diaraetiella rapae* was two to three times higher in plants grown on soils treated with *Bacillus cereus* and *B. subtilis*, but not in those treated with *B. amyloliquefasciens* or a mixture of the species (Gadhave et al., 2016).

Fungi

Soil fungi are a diverse group of organisms and their role in above-belowground interactions has been studied for many years. The most studied taxa are mycorrhizal fungi that associate with the majority of plant species. Ectomycorrhizal fungi (EMF) generally form mutualistic bonds with trees, whereas AMF form mutualisms with plants throughout the plant kingdom. EMF have been poorly studied within the soil biota-plant-insect framework and hence they are only briefly discussed. Relationships between AMF and aboveground insects, mediated by plants, are commonly reported in literature, and these effects have already been summarized in various other reviews (e.g., Pozo and Azcón-Aguilar, 2007; Gehring and Bennett, 2009; Hartley and Gange, 2009; Jung et al., 2012) and a meta-analysis (Koricheva et al., 2009).

The Effect of Ectomycorrhizal Fungi (EMF) on Aboveground Herbivores

Studies on the influence of EMF on plant-insect interactions are limited, but the published reports suggest that they can also affect insects in different directions. One study showed that numbers of the sap sucking poplar aphid *Chaitophorus populicola* were five times higher on poplar trees (*Populus angustifolia* x *P. fremontii*) that were treated with the EMF *Pisolithus tinctorius* than in controls that did not receive EMF. However, another study showed that various insects, even of the same feeding guild, respond differently to EMF in the same study and more importantly, results differ strongly between the various methodologies used (Gange et al., 2005b), as will be discussed in more detail further onwards in this review.

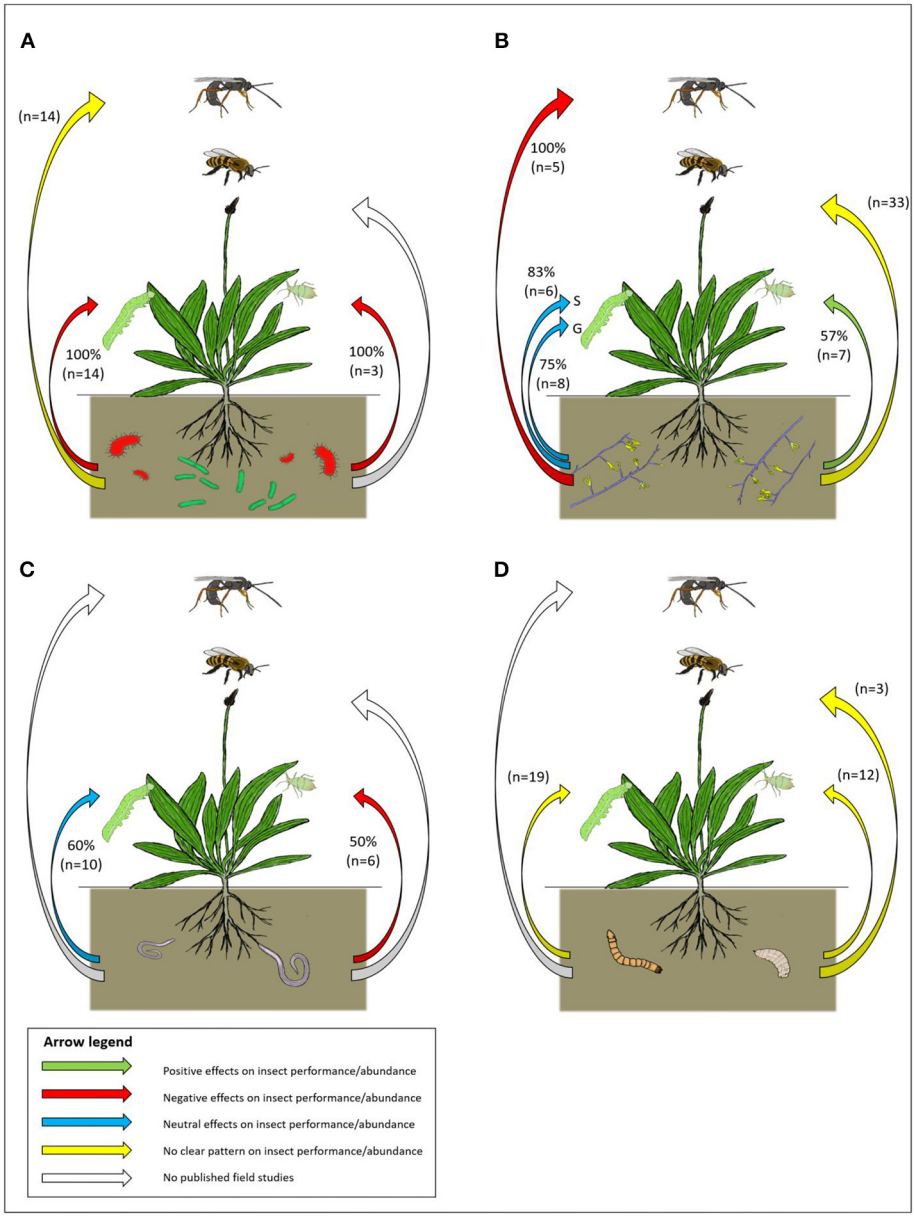

FIGURE 2 | A schematic overview of the effects of **(A)** plant growth-promoting bacteria, **(B)** arbuscular mycorrhizal fungi, **(C)** plant-parasitic nematodes and **(D)** soil arthropods on the most frequently reported aboveground plant-insect interactions (interactions between plants and chewing and sap sucking herbivores, pollinators and natural enemies, respectively). In **(B)** S, Specialist; G, Generalist. Arrows indicate plant-mediated effects of soil organisms on aboveground insects. Green arrows represent generally positive indirect effects on aboveground insects, red arrows represent generally negative indirect effects on aboveground insects, blue arrows represent generally neutral effects on aboveground insects. Yellow arrows indicate that effects are observed, but no clear patterns emerged and white arrows indicate that interactions have not been reported in literature. Percentages with the green, red and blue arrows represent the percentage of the total reported interactions that followed the pattern (sample size between brackets).

The Effect of Arbuscular Mycorrhizal Fungi (AMF) on Aboveground Herbivores

A general pattern that has emerged from controlled studies is that AMF negatively influence generalist chewers, while specialist chewers are positively affected by AMF (Hartley and Gange, 2009; Koricheva et al., 2009). From the interactions with generalist chewing herbivores revealed by our literature search ($n = 8$), 75% reported no effect and 25% reported negative effects of AMF on generalist chewers (**Figure 2B**, Supplementary Table 2, Gange and West, 1994; Vicari et al., 2002) or herbivore diversity (Guo et al., 2015) in the field. For example, in a field study on ribwort plantain, *Plantago lanceolata*, caterpillars of the highly polyphagous woolly bear moth, *Arctia caja,* were 25% smaller in plots with AMF than

in plots with AMF removed (Gange and West, 1994). On the other hand, from the interactions with specialist chewers ($n = 6$) 83% report neutral (Younginger et al., 2009), and 17% reported a positive plant-mediated effect on specialist chewers (**Figure 2B**, Supplementary Table 2, Barber et al., 2013). Plant-mediated AMF effects on chewing herbivores also differ between different plant functional groups. A recent study showed that AMF presence increased total levels of herbivory in tallgrass prairie plots, but at the plant functional group level herbivory levels only differed between AMF and control plots for C3 grasses, but not for C4 grasses or forbs (Kula and Hartnett, 2015).

In controlled studies, sap sucking insects generally benefit from the presence of AMF and the degree of specialization of the sap sucking insects does not appear to influence the effects of AMF (Hartley and Gange, 2009; Koricheva et al., 2009). From the interactions revealed from our literature search ($n = 7$), 43% were neutral (Colella et al., 2014) and 57% reported positive plant-mediated effects of AMF on sap suckers (**Figure 2B**, Supplementary Table 2, Gange and West, 1994; Ueda et al., 2013). For example, a recent field study reports more than tenfold higher numbers of *Aulacorthum solani* on soybean (*G. max*) inoculated with *Gigaspora margarita*, than on untreated control plants (Ueda et al., 2013), which is in line with the commonly observed patterns in controlled studies. Only one study reports that treatment with AMF led to two- to three-fold lower numbers of the poplar aphid *Chaitophorus populicola* on poplar trees, *Populus angustifolia* x *P. fremontii* that were placed in pots in the field (Gehring and Whitham, 2002). Why aphids responded negatively in this study is hard to pinpoint. The authors report no significant effects of AMF on plant performance, but they did not investigate effects on plant chemistry, which may have changed in response to the AMF interaction. AMF effects on plant-insect interactions may also differ among plant functional groups. Most previous studies have been performed with herbaceous species, thus studies on woody shrubs and trees may give contrasting results.

As discussed in Koricheva et al. (2009), patterns in AMF-plant-insect effects on insects belonging to feeding guilds other than leaf chewers and sap suckers, such as cell content feeders and leaf miners, are not straightforward to interpret. However, addition of AMF to plants in the field had neutral (Gange et al., 2003, 2005b; Colella et al., 2014) to positive effects on cell-content feeders, leaf miners and gall makers in several studies (Gange et al., 2003; Younginger et al., 2009; Moon et al., 2013; Ueda et al., 2013). Within the same study system, results may even vary between generations of insects. For instance, when AMF levels were reduced using iprodione, this did not at first affect proportions of leaves mined by the leaf-mining fly *Chromoatomyia syngenesiae* in ox-eye daisy, *Leucanthemum vulgare* (Gange et al., 2003). However, in a follow-up study, the authors report AMF species-specific differences in the proportion of *Leucanthemum* leaves mined by *C. syngenesiae*, and a 50% increase in pupal biomass of the leafminer in plots with higher levels of AMF. These significant effects were only found for the second generation of flies in the year of study (Gange et al., 2005a).

The Effect of Arbuscular Mycorrhizal Fungi (AMF) on Aboveground Natural Enemies

Several studies have incorporated higher trophic levels in the study of AMF-plant-insect interactions and in all of the studied interactions ($n = 5$) AMF presence had a negative effect on the performance or density of predatory insects (Ueda et al., 2013) or parasitoids (Gange et al., 2003; Moon et al., 2013). In one study on Sea myrtle, *Baccharis halimifolia*, parasitism rates of two species of co-occurring leafminers (*Amauromyza maculosa* and *Liriomyza trifolii*, respectively) and a gall making fly (*Neolasioptera lathami)* by parasitoid wasps were all negatively affected by AMF application (Moon et al., 2013). AMF colonization resulted in more leaves per plant, which also had higher nitrogen levels, subsequently leading to healthier and potentially more strongly defended hosts, negatively affecting the respective parasitoids (Moon et al., 2013).

The Effect of Arbuscular Mycorrhizal Fungi (AMF) on Aboveground Pollinators

AMF-plant interactions can have contrasting effects on pollinating insects in the field. From the interactions revealed by our literature search ($n = 35$), 34% were positive, 17% were negative and 49% reported no effects on pollinators (**Figure 2B**, Supplementary Table 2). Several studies report higher pollinator visitation or flower probing on plants that received AMF treatment (Gange and Smith, 2005; Wolfe et al., 2005; Cahill et al., 2008; Barber et al., 2013), whereas others report neutral or negative effects on pollinator visitation (Varga and Kytöviita, 2010). It is important to notice that effects of soil organisms on pollinating insects can vary between different levels of measurement (e.g., plot/community/species/pollinator taxa level). For example, in one study, levels of AMF were reduced by application of benomyl and the effects of AMF on six common forb species were investigated (Cahill et al., 2008). At plot level, plots with natural AMF levels showed an overall 67% higher number of pollinator visits per flowering stem, whereas the total number of visits per plot was not affected. AMF associations also led to a three-fold higher visitation by large-bodied bumblebees and a three-fold decrease in visitation by small-bodied pollinators such as bees and flies. At the plant species level, *Aster laevis* and *Solidago missouriensis* showed two to four times higher numbers of floral visits by pollinators in plots with higher AMF levels, whereas *Cerastium arvensis* showed a 80% decrease in total pollinator numbers in plots with higher AMF levels. Pollinator visitation of the herbs *Achillea millefolium*, *Campanula rotundifolia* and *Erigeron philadelphicus* was not affected by soil AMF levels (Cahill et al., 2008). More studies are needed to elucidate patterns for plant-mediated effects of AMF on pollinators in the field.

Nematodes

Nematodes are important soil dwelling organisms that belong to a range of trophic groups in the soil food web, and include bacterial feeders, fungal feeders, root feeders, and predators/carnivores. Their effect on host plants has been studied intensively, although fewer studies have focused on the indirect effects of nematodes on aboveground insects (reviewed

in Wondafrash et al., 2013). As the literature search for field studies only revealed studies of plant-parasitic nematodes on aboveground insects, only this group will be discussed here. It should be noted that other nematodes (e.g., fungal feeders, bacterial feeders) may, however, also indirectly affect plant-insect interactions by interacting with other soil organisms. Plant-parasitic nematodes, by feeding on the roots of shared host plants, can influence the defense status and nutritional quality of host plants, potentially leading to effects on herbivores (Bezemer et al., 2003; Bezemer and van Dam, 2005; Wondafrash et al., 2013; Biere and Goverse, 2016). Results from laboratory studies of the effects of plant-parasitic nematodes on aboveground insects are often variable for chewing insects, but generally show negative effects on either the performance or preference of sap sucking insects (Johnson et al., 2012; Wondafrash et al., 2013). As the number of field studies on plant-parasitic nematodes that describe effects on insect herbivores is rather low, we will treat plant-parasitic nematodes (PPNs) with different life styles (free-living, endoparasitic) as one group, and describe their effects on different types of insect herbivores. No studies that incorporated higher trophic levels or pollinating insects have been identified and therefore these are not discussed here.

The Effect of Plant-Parasitic Nematodes on Aboveground Herbivores

From the interactions revealed from our literature search ($n = 10$), 60% report neutral (e.g., Carter-Wientjes et al., 2004; Kaplan et al., 2009; Guo et al., 2016) and 40% report positive effects of PPNs on aboveground chewing herbivores (**Figure 2C**, Supplementary Table 3, Alston et al., 1991; Kaplan et al., 2009; Vockenhuber et al., 2013). For example, the addition of the root-knot nematode, *Meilodogyne incognita* to tobacco (*Nicotiana tabacum*) in field plots did not affect numbers of the specialist tobacco hornworn, *Manduca sexta*, or the growth of the generalist beet armyworm, *Spodoptera exigua*. In contrast, in the same experiment, nematode-treated plants had 30% higher numbers of chewing *Epitryx* flea beetles than untreated plants (Kaplan et al., 2009). Although correlative data should be interpreted with caution as they do not imply causation, numbers of free-living PPNs were also positively related to the levels of leaf consumption by chewing herbivores, although the observed correlations for PPNs were not significant for the three most abundant nematode genera *Tylenchorhynchus*, *Pratylenchus*, and *Xiphinema* (Kaplan et al., 2009).

From the interactions revealed from our literature search for nematode effects on sap suckers ($n = 6$), 50% reported no effects (e.g., Vandegehuchte et al., 2010; Heeren et al., 2012) and 50% reported negative effects (**Figure 2C**, Supplementary Table 3, Kaplan et al., 2009). In soy bean fields, *G. max*, the presence of the nematode *H. glycines* did not correlate with total aphid abundance in one study (Heeren et al., 2012), but was negatively correlated with the number of alates of the soy bean aphid *Aphis glycines* at the onset of the peak season in another study (Hong et al., 2011). It is important to note that in the former study, plant yield was also not affected, whereas yield also negatively correlated with the number of nematode eggs in the latter (Hong et al., 2011; Heeren et al., 2012).

Soil Arthropods

A relatively large number of studies have examined the effect of soil arthropods on aboveground plant-insect interactions. Soil arthropods are an abundant group of macro-invertebrates that can affect plants either directly, via root herbivory or indirectly, via decomposition of organic material. Although an increasing number of studies report on mechanisms through which root herbivory might impact aboveground plant-insect interactions (e.g., reviewed in Soler et al., 2012; Barber and Soper Gorden, 2014), most reviews remain inconclusive about the drivers behind the effects that are often observed. A meta-analysis showed that root herbivory by Diptera generally results in significantly negative effects on aboveground herbivores (Johnson et al., 2012), whereas herbivory by Coleoptera influences only aboveground Homoptera (positively) and herbivorous Hymenoptera (negatively), but has no significant effect on other groups.

The Effect of Root Herbivores on Aboveground Herbivores

From the interactions revealed by our literature search for root herbivore effects (regardless of taxa) on aboveground chewing herbivores ($n = 20$), 55% reported no effects, 10% reported positive effects and 35% reported negative effects.

Several studies in the 1990's investigated the effects of root herbivores on aboveground insects by means of reducing the total densities of soil arthropods with insecticides. In all of these studies, natural densities of soil arthropods had either no influence (Evans, 1991) or led to an increase (Evans, 1991; Masters et al., 1993, 2001; Masters, 1995) in aboveground herbivory. As there is little specificity in insecticide treatments, it is impossible to disentangle the effects of different soil arthropod taxa on plant-insect interactions from these older studies. Yet, they shed some light on the role of soil arthropods in shaping plant-aboveground insect interactions.

In field studies, plant-mediated effects of coleopteran root herbivores on aboveground chewing herbivores can be neutral (Hunt-Joshi et al., 2004; Barber et al., 2015; Borgström et al., 2017), positive (Wurst et al., 2008), or negative (White and Andow, 2006; Wurst et al., 2008; Megías and Müller, 2010, see **Figure 2D**, Supplementary Table 4). Interestingly, on ribwort plantain, *Plantago lanceolata* that were exposed to belowground herbivory by *Agriotes* spp., aboveground herbivory levels were three times lower on a high-iridoid glycoside (secondary defense metabolites in *Plantago*) producing lineage, compared to controls without root herbivores. In contrast, herbivory levels were nine times higher in response to the root herbivore on a low iridoid glycoside lineage (Wurst et al., 2008). This study illustrates that the genetic background of a plant can play an important role in determining plant-mediated effects of root insect herbivores on aboveground chewing insect herbivores. Although a meta-analysis (Johnson et al., 2012) concluded that dipteran root herbivores generally have negative plant-mediated effects on aboveground herbivores, there is no consistent support from field studies for this (see **Figure 2D**, Supplementary Table 4). For example, Cabbage root fly, *Delia radicum* negatively affected numbers of chewing *Phyllotreta* sp. leaf beetles (this genus

comprises mostly specialists and oligotrophs) in potted black mustard (*Brassica nigra*) in an experimental garden (Soler et al., 2009), but the addition of root flies had no plant-mediated effect on any lepidopteran chewers (Soler et al., 2009; Pierre et al., 2013).

There seems to be no pattern for the plant-mediated effects of coleopteran root herbivores on sap suckers in the field. From the interactions revealed by our literature search ($n = 22$), 54% reported no effects, compared to 23% that reported positive effects and 23% that reported negative effects (see **Figure 2D**, Supplementary Table 4). One study reports positive effects of root herbivory by coleopteran herbivory on aboveground sap suckers (Poveda et al., 2005). However, in other studies, the addition of coleopteran root herbivores had either no effect (Megías and Müller, 2010) or negative effects on sap suckers (Megías and Müller, 2010; Ryalls et al., 2016). For example, addition of larvae of a combination of the two beetle species *Morica hybrida* and *Cebrio gypsicola* on *Moricandia moricandioides* resulted in a more than three times lower number of aphids on the shared host plant, compared to controls. Similarly, in the same study, the addition of soil organisms resulted in a decrease in the total number of unidentified aphids on the plants, compared to controls, whereas the total number of planthoppers was not affected by the treatment with only *C. gypsicola*, but were 30% lower on plants that received only *M. hybrida* (Megías and Müller, 2010). This result could be driven by the fact that the latter is largely detritivorous and, thus, these two coleopteran soil arthropods may affect plant physiology in different ways. There is also no consistent effect of dipteran root herbivores on sap sucking herbivores in the field. Plants treated with root herbivores were found to have increased numbers of specialist aphid *Brevicoryne brassicae* (Pierre et al., 2013) and decreased numbers of the same species in another study (Soler et al., 2009). Numbers of the generalist aphid *Myzus persicae* were not affected by the presence of root herbivores in either of the two studies (Soler et al., 2009; Pierre et al., 2013).

As we identified only one study that described the effect of root herbivores on other feeding guilds, it is not possible to elucidate patterns. In this study, the abundance of the leafminer *Stephensia brunnichella* was 30% lower on Wild basil, *Clinopodium vulgare* plants that were infested with wireworms, *Agriotes* spp. than on controls without herbivores, whereas the size of the herbivores remained unaffected by the treatments (Staley et al., 2007).

The Effect of Root Herbivores on Aboveground Natural Enemies

The number of studies that have examined the effects of root-feeding insects on aboveground natural enemies in the field is limited. The available reports suggest that the presence of root feeding herbivores may have little effect on aboveground natural enemies in the field (e.g., Soler et al., 2009; Megías and Müller, 2010). Evans (1991) reported that soil arthropod reduction did not affect abundance of unspecified parasitic Hymenoptera, Arachnida and unspecified predatory and entomophagous insects in experimental field plots. In contrast, Megías and Müller (2010) found higher levels of parasitism by the braconid parasitoid *Cotesia kazak* in larvae of two pierid butterflies, *E. crameri* and *P. daplidice*, when soil dwelling larvae of the tenebrionid beetle *M. hybrida* were present in potted *M. moricandioides* plants. It is important to note that this beetle species is largely detritivorous and therefore may not directly affect plants, but its presence may influence plant-insect interactions by making nutrients available in the soil that may affect physiological processes in the plant.

The Effect of Root Herbivores on Aboveground Pollinators

The literature is inconclusive on the plant-mediated effects of root herbivores on pollinators. Soil arthropods often cause association-specific effects on their host plants, ranging from changes in flower number to flower size and nectar quality, which all may influence different types of pollinating insects (Barber and Soper Gorden, 2014). Likewise, there is no evident pattern for field studies (**Figure 2D**, Supplementary Table 4). Three studies investigated the effects of addition of root herbivores on pollinator visits in the field. In all cases, the plants were in pots in the field and the treatment was an addition of coleopteran root herbivores. Addition of wireworms, *Agriotes* spp. to charlock mustard, *Sinapis arvensis* consistently resulted in an increase in total pollinator visits (Poveda et al., 2003, 2005). However, in another study using cucumber plants, *C. sativus*, addition of larvae of the striped cucumber beetle, *Acalymma vittatum* resulted in half the number of pollinator visits, compared to untreated controls and pollinator visits showed a negative relationship with root herbivore density (Barber et al., 2015).

METHODOLOGY DETERMINES THE OUTCOME OF FIELD EXPERIMENTS

Although similarities between controlled studies and field studies can be found for some soil taxa, the field literature also shows considerable variation in responses and neutral effects are commonly observed for soil biota-plant-insect interactions. This may be at least partly due to the experimental methodologies applied in the field. Three main methodologies are widely applied; (1) Addition of soil organisms to potted plants that are placed in experimental outdoor areas; (2) Addition of soil organisms to plants that are grown in field plots; (3) Removal of specific soil organism taxa by application of pesticides (see **Figure 3**). Direct comparisons between potted plants and field grown plants were made in two studies. For instance, in Marram grass, presence of a PPN of the genus *Heterodera* had a negative effect on the aboveground aphid *Schizaphis rufula* in pots, but in the field this correlation was not significant (Vandegehuchte et al., 2010). In another study, when *Eucalyptus* trees were grown in pots in the field, addition of EMF had a negative effect on feeding by larvae of the chafer *Anomala cupripes*, but for trees growing directly in the field, no effect on chafer feeding was observed. Damage by geometrid moths was significantly increased under EMF treatment in the potted plants, whereas it was decreased in the field-grown *Eucalyptus*. However, the EMF treatment led to a reduction in leaf folding by *Strepsicrates* sp. in both potted plants in the field and in field-grown plants (Gange et al., 2005b).

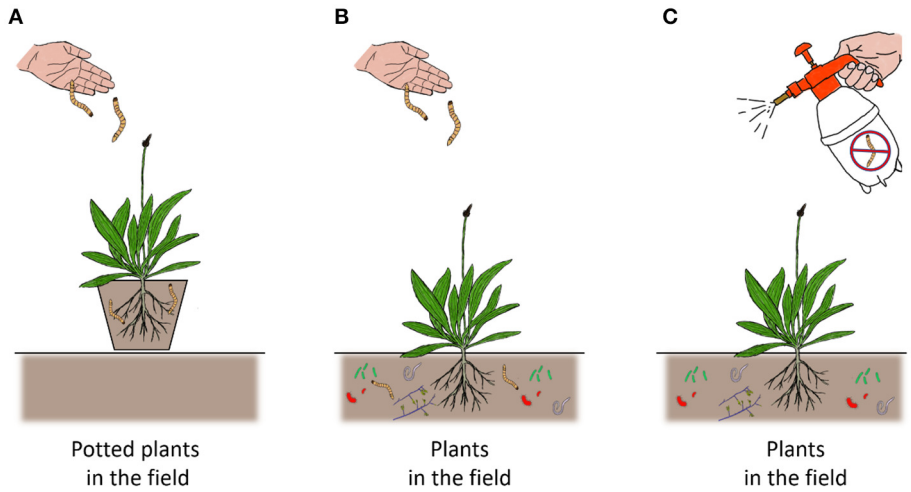

FIGURE 3 | A schematic overview of the three most widely used methodologies to investigate soil biota-plant-insect interactions in the field. In this representation we used additions of wireworms, *Agriotes* spp. to Ribwort plantain *Plantago lanceolata* as an example. **(A)** Potted plants, which are often grown in a greenhouse for a number of weeks, are placed in experimental fields or gardens after being treated with soil organisms. Interactions between the potted plants and natural herbivores or pollinators are then tested in the field. **(B)** Plants are planted in the field under natural conditions, including a resident soil community. Soil organisms are added to plots and thus in the treated plots the numbers of added soil organisms are augmented, compared to untreated control plots. **(C)** Plants are planted in the field under natural conditions, including a resident soil community. However, in this method, the soil organisms under investigation are reduced by means of application of a pesticide. Hence, the treated plots have reduced levels of soil organisms, compared to the control plots, which have natural (but higher) levels of the soil organism.

These two studies clearly illustrate that choice of methodology used in field experiments can strongly influence the outcome, and suggests that studies using potted plants are more likely to show significant effects of belowground organisms on aboveground insects than studies that examine plants grown directly in the soil in the field. This also emphasizes the need for standardized methodologies, in order to make comparisons between different field studies more powerful.

Interestingly, there is a strong difference between effects reported for the different methodologies among the studies compiled in this literature review (see **Table 1**). In the published literature, only for the taxa soil fungi and soil arthropods were there reports on all three methodologies used in the field (see **Figure 3**). When we compare methodologies within these two taxa, potted plant studies and field removal studies more often reported significant results (in either direction) than studies where soil organisms were added to field plots. For example, in the studies with fungi, 63% of the interactions studied in pots showed a significant plant-mediated effect (in either direction) on aboveground insects. Field removal studies also showed a significant plant-mediated impact in 73% of the studies, but only 25% of the field addition studies showed significant effects (see **Table 1**). A similar pattern emerges for the manipulation of soil insects. Here, 64% of the studied interactions resulted in significant plant-mediated effects on insect herbivores in pot experiments. Field removal studies showed significant plant-mediated effects in 70% of the studies, compared to only 33% in the field addition studies (see **Table 1**). These numbers suggest that there is a strong effect of methodology applied in the field, although it should be noted that publication bias may have also led to a bias toward studies that report significant results and in reality, the fraction of studies that report significant effects may be lower.

The use of pots comes with a range of disadvantages that may affect the study system, especially so in the field. First of all, studies often use sterilized soil or steamed potting soil, which excludes the interactions with resident soil organisms. Furthermore, pots not only impose a barrier to the root system, but also to the movement of the study organisms. Moreover, it prevents the influx of other soil organisms. Although pots may have the advantage of ensuring that the soil organisms are present at the root system, this methodology may be highly artificial compared to field plots. The barrier also inherently limits plant growth (i.e., pot limitation), leading to changes in plant growth and physiology (Poorter et al., 2012), which may either be beneficial or detrimental to insect performance. Lastly, abiotic conditions in pots can be quite different from conditions in soil. Placing pots (often of dark color, which absorbs more energy) on top of the soil, may increase soil temperature in the pot under warm conditions. Moreover, they may cool down more rapidly under cold conditions. We propose that pots can be extremely useful in studying soil organisms, both in laboratory and field conditions, but that they should be used with caution and that abiotic constraints should be countered as much as possible (for example by burying the pots, using large enough pots and including live soils into the design).

The use of pesticides in field experiments was a common approach in the early years of the development of this niche in ecology. However, this also comes with many obvious disadvantages. Several studies have shown that, although the pesticides are often rather specific and indeed reduce target organisms, there are also undesirable side-effects that influence

TABLE 1 | Comparison of the three most widely used field methodologies in studies investigating above-belowground interactions (potted plants placed in the field, inoculation of soil organisms in experimental plots, species removal by means of pesticides in experimental plots).

			Effect on herbivore		
Method	**Number of studies**	**Number of studied interactions**	**Percentage no effect**	**Percentage positive**	**Percentage negative**
FUNGI					
Pot	9	27	37.0	40.7	22.2
Field removal	4	11	36.4	45.5	18.1
Field inoculation	7	40	65.0	20.0	5.0
SOIL ARTHROPODS					
Pot	9	25	36.0	40.0	24.0
Field removal	5	10	30.0	60.0	10.0
Field inoculation	4	9	66.7	11.1	22.2

Shown are the total number of studies and the total number of organismal interactions for which relationships between soil organisms and aboveground herbivorous insects were investigated. The percentages were calculated for the studies that showed no significant effect on the herbivore, a significant positive effect on the herbivore or a significant negative effect on the herbivore. Only soil fungi and soil insect manipulation studies were included, since removal and pot studies were rare or non-existent in the other groups.

many other soil processes (e.g., Wang et al., 2004). We propose that addition of soil organisms to field plots may be the best methodology, as this allows for interactions of both the added soil organisms and the plant with resident soil communities. From an applied perspective, results from soil organism addition studies are perhaps also the most useful as these scenarios are most comparable to application of soil organisms (e.g., in Integrated Pest Management). However, it is very hard to standardize both the abiotic and biotic conditions of live field soils, and this can lead to considerable variation between or even within study sites. Introduced soil organisms may encounter antagonists, or effects may be "diluted" as field plots often do not have barriers and organisms may move away.

DISCUSSION AND FUTURE DIRECTIONS

In this review we have explored the scientific literature that discusses the effect of biotic manipulations of the soil on aboveground plant-insect interactions in the field. First, we asked if there is a role for soil organisms in shaping aboveground plant-insect interactions under field conditions. We searched the literature for studies that report on manipulations of the whole soil microbiome and how changes in soil community composition may affect aboveground insects in the field. It appears that there is ample evidence for effects of changes in whole soil communities on insect assemblages, but these findings are all correlative, not causative. This immediately highlights a first gap in the current scientific knowledge; how biotic "soil legacies" or plant-soil feedback (PSF) effects may influence aboveground insect communities in the field. To our knowledge, no studies thus far, have assessed these effects in a field setting. This is an important aspect of above-belowground ecology that deserves more attention in the future. We argue that introducing the PSF concept as a fourth applicable field method to shift soil communities in a certain direction would be less disruptive than the commonly used methodologies and would incorporate more ecological realism.

Our second question was whether the manipulation of specific taxa in the soil has the same effects on aboveground insects in the field as under more controlled conditions in greenhouses or growth chambers. Our survey indicates that this is true for most taxa except for soil arthropods. Bacterial inoculation in the field generally promotes plant growth and depresses abundance and performance of insects in the field, as they do in laboratory studies (e.g., Pineda et al., 2010). For AMF, the effects observed in laboratory settings have been thoroughly reviewed (Gehring and Bennett, 2009; Hartley and Gange, 2009; Koricheva et al., 2009) and the general patterns differ for insects from different feeding guilds and depend on the degree of specialization of the insects. Field studies, we show, report similar patterns; AMF negatively influences generalist chewers, but positively affect specialist chewing insects. AMF also generally benefit sap-sucking insects, regardless of their specialization. Under field conditions, nematodes affect chewing herbivores positively and sap suckers negatively and this is also in line with the general observations in laboratory studies (Wondafrash et al., 2013). Patterns in the effects of soil arthropods are less straightforward. In the current review of field literature, we have not been able to observe a clear pattern. One of the reasons for this could be the variation in abiotic and biotic conditions in the reported study systems. Furthermore, often only very few interactions are studied for each combination of taxa (both below and aboveground). Therefore there is currently a lack of relevant data and this makes it hard to compare the different results more thoroughly, e.g., in a meta-analysis. The same problem arises when we attempt to elucidate patterns for less abundant feeding guilds (such as leaf miners, gall makers or stem borers) or natural enemies and pollinators. Very few studies, so far, have investigated the effects of soil organism manipulations in the field on these less apparent aboveground feeding guilds and this is an area that requires further attention in order to better understand patterns in soil arthropod-plant-insect interactions.

Although we observed similarities between field and laboratory studies, in the field, it is also important to note

that a relatively large fraction of the studies that we detected reported neutral effects. We suggest that field methodology can drastically affect the outcome of above-belowground studies and that ecologists should be aware of this when designing experiments. Although there is a current lack of studies that compare the different field methodologies directly, the pattern is rather clear. In the case of pot experiments and removal experiments in the field, the likelihood of observing a statistically significant effect of any kind, are twice as high as those in field addition experiments. However, we argue that the latter is, to date, by far the most realistic and useful methodology to understand ecological processes. Clearly, there are opportunities to explore alternative ways to manipulate soil organisms, or steer soil communities in specific directions. For example through manipulation of soil via plant-soil feedback mechanisms where soils are manipulated in the field by plant species with specific effects on soil communities, or by inoculation of plots with soils that have been conditioned by specific plant species. Moreover, soil organisms can be manipulated via exclusion methods using variable mesh sizes that exclude certain soil taxa based on their sizes (e.g., Johnson et al., 2001, 2002), or via the addition of antagonistic organisms, that can impact specific groups of soil organisms.

Four aspects of the field of above-belowground ecology deserve further development. First, the response of insect species from less apparent feeding guilds (such as gall makers, stem borers, leaf miners and cell content feeders) has often been overlooked so far. In order to further elucidate patterns and more fully understand the ecological role of soil organisms in shaping plant-insect interactions, we need to use a more holistic approach that takes into account players from a broader range of guilds and trophic levels. Responses of natural enemies and pollinators aboveground have been studied infrequently, and are completely missing for certain types of soil manipulations, or soil taxa. The life history of the various natural enemies is quite diverse and their responses to soil biota-plant interactions may vary. Parasitoids and other flying natural enemies may respond more quickly than wingless, cursorial predators like spiders. Furthermore, parasitoids are affected by changes in the quality of their herbivore hosts, as their life cycles intimately depend on host ecophysiology (e.g., MacKauer, 1996; Harvey, 2000; Harvey et al., 2004). Moreover, when we searched for studies in the scientific literature, we could not detect any that focused on the effect of soil organisms, via plants, on interactions between plants and non-arthropod taxa, such as slugs, snails, but also higher vertebrates, such as grazers. As plants are the primary producers that support food chains, it is likely that other organisms will also be affected by belowground organisms.

Second, to increase our ecological understanding, it is important to also include more ecologically realistic model systems, as the current systems are often based on crops, as well as on insect species that are either crop pests or chosen for convenience, rather than based on ecological relevance (Chen et al., 2015). This could be accomplished, for example, by using a range of wild plant species that vary in functional traits, which could give better insight into what traits may predict certain plant responses. Studying their natural associated insect communities may also increase our understanding of which traits are important in mediating soil biota-plant-insect interactions. Future work could fill in these important gaps in our current knowledge.

Third, more emphasis should be placed on the role of time and space in these aboveground-belowground interactions in the field. It is currently unknown whether performing manipulations with the same soil organisms at different locations (e.g., differing in altitude and latitude, as well as abiotic conditions) will lead to differential effects on aboveground insects or not. Future studies should also focus on the temporal aspects of above-belowground interactions in the field. As soil communities are dynamic and species-specific soil communities accumulate over time (Diez et al., 2010; Flory and Clay, 2013; Van der Putten et al., 2013; Heinen et al., 2018), it is likely that these temporal dynamics will strongly influence the performance of aboveground insect communities over time. Various controlled studies have shown that the sequence of arrival of aboveground and belowground herbivores on the plant can greatly alter the outcome of soil biota-plant-insect interactions (e.g., Erb et al., 2011; Wang et al., 2014) and to some extent, this has also been shown in field studies (e.g., Gange et al., 2005a), although the link between temporally changing soil communities and temporal variation in aboveground insect communities has not been made. In the field, insect communities also change throughout the season. How soil treatments affect insects early compared to late in the season, and to what extent this is due to changes in plant-soil interactions or changes in plant-insect interactions is not known.

Fourth, most of the current research is focused on indirect effects that are mediated by shared host plants, but potential direct interactions should not be overlooked. There are various organisms, such as entomopathogens in the soil that can have direct impacts on aboveground insect performance. For instance, infection by entomopathogenic fungi, such as Beauveria bassiana and Metarhizium anisoplae can result in the quick death of many insect species (Meyling and Eilenberg, 2007; Vega et al., 2009, 2012), although its direct effects on aboveground insects in the field has been poorly documented. Interestingly, these fungi can also be endophytic in plants, and can influence both plant and herbivore performance (Meyling and Eilenberg, 2007; Vega et al., 2009, 2012; Senthilraja et al., 2010; Prabhukarthikeyan et al., 2014). Moreover, it has been shown for the fungus Metarhizium that it forms bridges between infected dead insects and plants, through which the fungus can provide the plant with extra nitrogen obtained from the insect bodies, which may also affect plant-insect interactions (Wang and St Leger, 2007; Behie et al., 2012; Sasan and Bidochka, 2012). Little is known about the extent to which aboveground insects pick up soil microorganisms and how this may affect their fitness, either through pathogenicity, or perhaps mutualistic interactions (e.g., in the gut microbiome), leaving an important gap in our current knowledge.

We conclude that there is strong support for a significant role of soil organisms in shaping plant-insect interactions in the field. With the exception of soil arthropods, we find that most field studies report effects that are similar to those of laboratory

studies. We argue that future studies should be carefully planned, as the methodology applied in the field strongly affects the chance of finding robust results. Nonetheless, there are ample opportunities to develop this research field further, especially in terms of exploring alternative and more realistic methods to steer soil biomes into a targeted direction. It should be emphasized that there is a large gap in our knowledge when it comes to less apparent insect herbivore taxa such as leaf miners, stem borers and others. There is virtually nothing known about the effects of soil organisms on a broad range of natural enemies (predators and parasitoids). However, as there are consistent reports of effects of soil organism addition in the field on aboveground insects, this opens up opportunities for the exploration of soil organism manipulation in agriculture or ecosystem restoration (e.g., Pineda et al., 2017). Some groups of soil organisms may be promising agents for crop yield enhancement and protection. Other groups of soil organisms may affect aboveground plant diversity at the community level and this gives rise to new opportunities to use soil organisms to "steer" the development of aboveground vegetation (Wubs et al., 2016), which may then subsequently affect aboveground insect communities. A challenge is to disentangle the drivers of soil organism manipulation effects on insects in the field. This will be an important step toward understanding how belowground organisms drive aboveground insect abundance, diversity and impacts in the field.

AUTHOR CONTRIBUTIONS

TB and RH conceived the idea for the literature review. TB, AB, and RH designed the structural framework of the review. RH performed the literature study. TB and AB provided several additional references. RH wrote the first version of the manuscript. TB, AB, JH, and RH contributed critically to later versions of the manuscript.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Microbial Root Mutualists Affect the Predators and Pathogens of Herbivores above Ground: Mechanisms, Magnitudes, and Missing Links

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Tri-trophic interactions among plants, herbivores, and natural enemies of herbivores are common in nature, and are crucial components of trophic cascades and the dynamics of community composition. Plant traits are key determinants of the interactions between herbivores and their natural enemies aboveground, which in turn are affected by soil organisms. Recent years have seen a surge in studies of the interactions between below- and aboveground biota, including descriptions of how microbial root mutualists influence plant traits and herbivore performance. However, concomitant effects on the natural enemies of herbivores remain relatively poorly understood. Here, we review the currently available literature to assess how and when mutualistic root microbes impose significant indirect effects on the performance of predators and pathogens of insect herbivores. We focus on how root microbes influence predator attraction, on-plant foraging efficiency, and the quality of prey tissues. We also consider the underappreciated effects of microbial root mutualists on the growth, transmission, and virulence of insect pathogens. We end by discussing missing links and important directions for future research.

Keywords: above- and below-ground interrelationships, tri-trophic interactions, soil ecology, disease dynamics, mycorrhizal fungi, soil biota, plant-herbivore interactions, microbial root mutualist

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INTRODUCTION

Understanding the mechanisms that underlie species interactions remains a central theme in ecology. Tri-trophic interactions among primary producers, herbivores and natural enemies of herbivores (predators, parasitoids, and pathogens) are common in nature, and are crucial mediators of trophic cascades, which can subsequently determine community dynamics, biodiversity, and ecosystem productivity (Hunter and Price, 1992; Polis et al., 2000; Borer et al., 2005). Therefore, understanding the factors that affect tri-trophic interactions is not only important for ecological research, but also critical for agricultural applications and conservation activities (Agrawal, 2000; Hunter, 2016). For example, natural enemies of herbivores are used extensively as agents of biological control, and understanding the factors that affect their efficiency can potentially optimize

pest management and crop yield (Symondson et al., 2002). Furthermore, human disruption of tri-trophic interactions can lead to substantial consequences for biodiversity management. In the southeastern United States for instance, over-harvesting of blue crabs, predators of plant-grazing snails, may result in extensive die-offs of plants in salt marshes, leading to substantial losses in biodiversity and primary production (Silliman and Bertness, 2002).

Plant traits, such as nutrient content, size, and secondary chemistry, are key determinants of the interactions between herbivores and their natural enemies (Price et al., 1980; Vet and Dicke, 1992; Cory and Hoover, 2006); these plant traits provide the mechanistic basis by which tri-trophic interactions occur. In turn, these same plant traits respond to the complex interactions that take place between plants and soil organisms belowground. Both root mutualists (e.g., rhizobia, mycorrhizal fungi, and detritivores) and root antagonists (e.g., herbivores, parasites) can strongly alter the fundamental plant traits that drive tri-trophic interactions above ground (Smith and Read, 2008; Chapin et al., 2011). As a consequence, recent work has begun to explore how belowground biota influence tri-trophic interactions aboveground through generating variation in plant traits (Hunter, 2016; Rasmann et al., 2017). Recent years have seen a surge in studies of the interactions between below- and aboveground biota, demonstrating that belowground organisms have major effects on aboveground ecological processes, including plant physical and chemical traits, plant performance, herbivore and pollinator performance, and their recruitment (Van der Putten et al., 2001; Wardle et al., 2004; Bezemer and van Dam, 2005; Schädler and Ballhorn, 2016). However, our understanding of the role of belowground interactions between plants and other organisms on aboveground tri-trophic interactions remains in its infancy (Rasmann et al., 2017). Most of the limited work to date has focused on microbial root mutualists, their impacts on plant traits, and how these traits generate tri-trophic interactions between plants, arthropod herbivores, and arthropod natural enemies. Here, we summarize this work, while adding in some recent studies of how the performance of the pathogens of herbivores responds to variation in plant traits introduced by microbial root mutualists. Our goals are (1) to summarize recent progress and identify the mechanisms by which belowground mutualists alter predation and pathogens pressure on herbivores aboveground; and (2) to point out missing links and important directions for future research. Under "mechanisms" we focus here on the plant traits that mediate the tri-trophic interactions. We also describe any associated changes that those plant traits engender in the behavior of herbivores or enemies that translate to modify herbivore mortality.

As we will show throughout the paper, this field is still in its infancy with a small number of published studies focusing on a handful of study systems. As a consequence, the mechanisms that we review here are by no means a complete accounting of the vast diversity and context-dependency of below-aboveground interactions. Rather, our main purpose is to demonstrate that belowground root mutualists can have major impacts on aboveground tri-trophic interactions through a variety of trait-based pathways, and that many exciting questions await future research.

FOCUS OF THE REVIEW

Our purpose here is not to review the myriad of ways in which microbial root mutualists influence plant traits and plant ecology. There are reviews, book chapters, and entire texts already available on these topics (Heath and Tiffin, 2007; Smith and Read, 2008; Hunter, 2016). Instead, we focus specifically on how root mutualists influence the tri-trophic interactions among plants, arthropod herbivores and their enemies (arthropod predators or parasitoids and agents of disease). Arthropod herbivores represent one of the largest and most diverse groups of metazoans on earth, and play essential roles in determining food web stability, community composition, diversity, and ecosystem functioning (Speight et al., 2008). Arthropod herbivores sustain a great diversity of enemies including predators, parasitoids and pathogens. At the same time, plants form associations with many organisms in the soil, from antagonistic organisms such as root herbivores and pathogens to mutualistic organisms including arbuscular mycorrhizal fungi (AMF), rhizobia, symbiotic bacteria, detritivores, and decomposers (Van der Putten et al., 2001; Wardle et al., 2004; Bezemer and van Dam, 2005; Pineda et al., 2013a; Schädler and Ballhorn, 2016). Here, we focus primarily on the beneficial microbes that associate with roots and summarize our current understanding of how they affect the impacts of predators and pathogens above ground. Again, the mechanistic basis underlying these tri-trophic interactions is generally the changes in plant traits mediated by plant associations with root microbes; we focus on the traits that dominate the literature on tri-trophic interactions, particularly primary and secondary metabolites and plant morphology. While we note briefly the simple but pervasive effects of mutualistic microbes on plant size and vigor, such effects have been reviewed recently (Rasmann et al., 2017) and are not a major focus here. Additionally, while belowground mutualists can affect the composition of enemy communities above ground (Schreck et al., 2013), we focus here on the performance and population dynamics of predators and pathogens because their links to chemical plant traits are much more firmly established. We first consider predators and parasitoids of arthropod herbivores, which belowground mutualists influence indirectly by changing the plant traits that determine long-distance plant attractiveness, on-plant foraging by enemies, and the nutritional quality of prey. Second, we consider how microbial root mutualists influence herbivore pathogens through their indirect effects on pathogen growth, transmission and virulence. We searched ISI Web of Science and Google Scholar using the keys words "belowground aboveground tri-trophic interactions" and their variants to find relevant publications. Subsequently, we read the literature cited by these papers to find and compile all other relevant studies. **Table 1** provides a full summary of existing studies and **Figure 1** provides three representative examples for a predator, parasitoid, and pathogen, respectively.

TABLE 1 | Published studies of the effects of belowground organisms on aboveground tri-trophic interactions.

Study	Type of belowground organism	Type of enemy	Response
PARASITOIDS AND PREDATORS: PREY LOCALIZATION			
Guerrieri et al., 2004	AMF	Parasitoid	(+) preference
Soler et al., 2007b	Root herbivore	Parasitoid	(−) avoidance
Hoffmann et al., 2011b	AMF	Predator	(+) preference
Schausberger et al., 2012	AMF	Predator	(+) preference
Battaglia et al., 2013	Non-AMF fungi	Parasitoid, Predator	(+) preference
Kruidhof et al., 2013	Root herbivore	Parasitoid	(−) avoidance in *C. glomerata;* (+) preference in *C. rubecula*
Pineda et al., 2013b	Rhizobia	Parasitoid	(−) avoidance
Godschalx et al., 2015	Rhizobia	Predator	(−) avoidance
Pangesti et al., 2015	Rhizobia	Parasitoid	(+) preference
PARASITOIDS AND PREDATORS: PREY QUALITY			
Masters et al., 2001	Root herbivore	Predator	(+) higher abundance
Gange et al., 2003	AMF	Parasitoid	(+, −, 0) on parasitism rate, depending on the fungal species
Bezemer et al., 2005	Soil microorganism and nematode	Parasitoid	(+) nematodes increase parasitoid survival, size and female ratio
Soler et al., 2005	Root herbivore	Parasitoid	(−) reduced size and increased development time
Hempel et al., 2009	AMF	Parasitoid	(+) increased parasitism rate and size, reduced development time
Megías and Müller, 2010	Detritivore and root herbivore	Parasitoid	(+) detritivores increased parasitism rate and abundance
Hoffmann et al., 2011c	AMF	Predator	(+) higher population growth rate
Johnson et al., 2011	Earthworm	Parasitoid	(+) higher abundance
Katayama et al., 2011	Rhizobia	Predator	(+) higher abundance and species richness
Wooley and Paine, 2011	AMF	Parasitoid	(+, 0) on abundance depending on AMF strain
Battaglia et al., 2013	Non-AMF fungi	Predator	(+) on development rate, (0) on birth rate
Moon et al., 2013	AMF	Parasitoid	(+, −) on percent parasitized depending on herbivore species
Kruidhof et al., 2013	Root herbivore	Parasitoid	(0) no effects on development time and adult weight
Pineda et al., 2013b	Rhizobia	Parasitoid	(−, 0) no effects on development time, survival or weight; negative effects on abundance
Schreck et al., 2013	AMF	Predator	(+) on predator density
Ueda et al., 2013	AMF	Predator	(+, −) on predator abundance depending on the sampling date
Pangesti et al., 2015	Rhizobia	Parasitoid	(0) on developmental time, survival, weight
PATHOGENS			
Tao et al., 2015	AMF	Pathogen	(+, −, 0) on parasite virulence and sporeload depending on plant species and AMF colonization level

(+), (−), and (0) signify positive, negative, and neutral effects of belowground organisms on traits of the enemies, respectively. AMF, arbuscular mycorrhizal fungi.

MUTUALISTIC MICROBES BELOW GROUND AFFECT PREDATORS AND PARASITOIDS OF HERBIVORES ABOVE GROUND

Here, we separate the interactions between herbivores and their parasitoids and predators into three stages: a prey localization stage, during which enemies actively forage for herbivores over long distances; an on-plant foraging stage, during which plant physical traits mediate enemy foraging efficiency; and an after-contact stage, when enemies consume and/or reproduce in herbivore tissues, the quality of which determines enemy performance. Critically, the mechanisms underlying all of these interactions are based on variable plant traits (morphology, chemistry, physiology), that are subject to modifications by microbial root mutualists. We encourage readers to explore a recent paper (Rasmann et al., 2017), which also considers how microbial traits (microbial volatiles) may influence tri-trophic interactions above ground, and (b) includes a review of indirect defenses below ground, which we do not consider here.

Prey Localization

Predators and parasitoids must locate their herbivorous prey before attacking those prey items. Prey location by invertebrate predators mainly occurs through visual and olfactory cues, which are strongly affected by plant morphology and chemical traits (the mechanisms). Plant size and architectural complexity are key determinants of foraging efficiencies of parasitoids, with increases in size and complexity reducing per capita foraging efficiency (Cloyd and Sadof, 2000; Gingras and Boivin, 2002), while increasing the diversity of alternative prey (Lawton, 1983; Fowler, 1985). Consequently, when oxeye daisies (*Leucanthemum vulgare*) grow larger through association with AMF, rates of parasitism of the leaf miner *Chromatomyia*

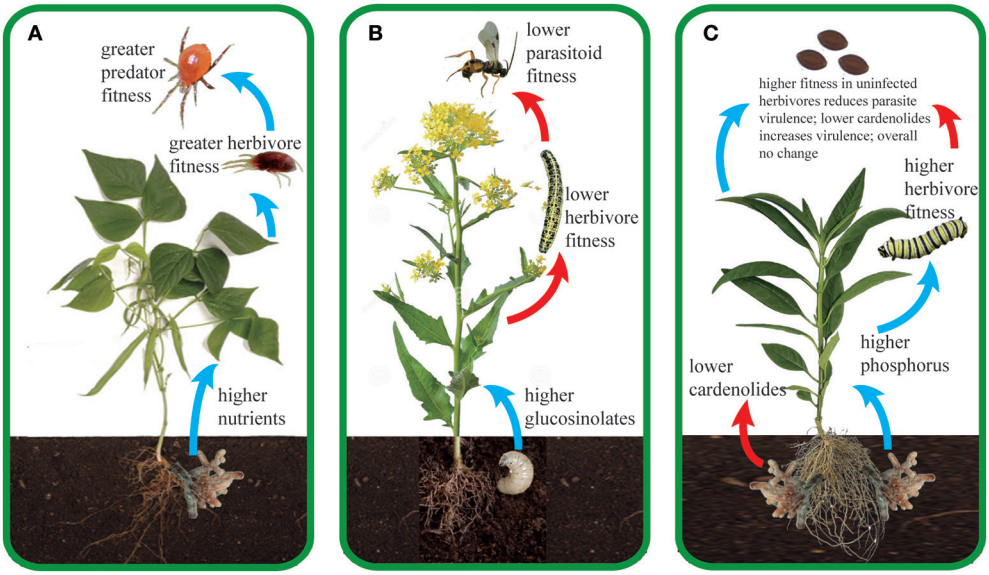

FIGURE 1 | Representative examples of how root mutualists **(A,C)** and root antagonists **(B)** can influence tri-trophic interactions aboveground. Blue and red arrows signify positive and negative effects on the next trophic level, respectively. **(A)** Associations with the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* can lead to higher macronutrient (phosphorus and potassium) content in bean plants (*Phaseolus vulgaris*), which subsequently results in greater oviposition rate, growth rate, and shorter doubling time of the herbivorous spider mite *Tetranychus urticae* (Hoffmann et al., 2009). Higher quantity and quality of *T. urticae* directly translate into higher fitness and population growth rate of the predatory mite *Phytoseiulus persimilis* (Hoffmann et al., 2011c). Female *P. persimilis* preferentially select eggs of *T. urticae* that are reared on AMF-associated bean plants due to their higher quality (Hoffmann et al., 2011b); additionally, they can "smell" AMF-associated plants because AMF increase the production of β-ocimene and β-caryophyllene in *T. urticae*-infested plants (Schausberger et al., 2012). Although AMF increase performance of the herbivore *T. urticae*, higher population growth of *P. persimilis* and higher plant tolerance compensate for the negative effects of the herbivores, leading to higher seed production in the bean plants (Hoffmann et al., 2011a). Additionally, *P. persimilis* increases root AMF colonization rates, which may create a positive feedback among AMF, plants and predators in this important agricultural system (Hoffmann et al., 2011a). **(B)** Infestation of black mustard (*Brassica nigra*) roots by cabbage fly larvae (*D. radicum*) increases sinigrin concentrations (a type of glucosinolate) in plant shoots, which negatively affects fitness of cabbage butterfly larvae (*P. brassicae*) and their parasitoid wasps *Cotesia glomerata* (Soler et al., 2005). As a result, both *P. brassicae* and *C. glomerata* have evolved to avoid plants with root herbivores (Soler et al., 2007a, 2010; Kruidhof et al., 2013), possibly through avoiding sulfur volatile compounds that are emitted by the plants after attack by the root herbivore (Soler et al., 2007a). Such avoidance behavior can increase foraging efficiency of *C. glomerata* on plants surrounded by root-infested plants, because the volatiles may provide a contrast within the background blends emitted by plants without root herbivory (Soler et al., 2007b). **(C)** AMF—milkweed *Asclepias* spp.—monarch butterfly *Danaus plexippus*—protozoan parasite *Ophryocystis elektroscirrha* For monarch butterflies (*Danaus plexippus*), milkweed (*Asclepias* spp.) secondary chemicals called cardenolides reduce growth of the protozoan parasite *Ophryocystis elektroscirrha* and increase the lifespan of infected butterflies (de Roode et al., 2008, 2011; Sternberg et al., 2012; Gowler et al., 2015). In both uninfected and infected butterflies, nutrients such as nitrogen and phosphorus positively affect monarch larval performance (Tao and Hunter, 2012; Tao et al., 2014, 2015). Since associations with AMF can significantly change cardenolide and nutrient concentrations across milkweed species (Vannette and Hunter, 2011; Vannette et al., 2013; Tao et al., 2015, 2016), they indirectly affect parasite virulence and monarch tolerance (Tao et al., 2015). For example, in *A. curassavica* (shown here), AMF increase foliar P concentrations while decreasing foliar cardenolide concentrations, yielding overall neutral effects on the lifespan of infected butterflies (Tao et al., 2015).

syngenesiae by the wasp *Diglyphus isaea* decline (Gange et al., 2003).

Plants recruit predators and parasitoids through indirect defense mechanisms, such as food rewards (e.g., extrafloral nectars), shelters (domatia), and volatile organic compounds (VOCs) (Dicke, 1999; Agrawal, 2000) that can attract predators and parasitoids over long distances. Because allocation to domatia and extra-floral nectaries depend on both nutrient status and plant size (Frederickson et al., 2012; Heil, 2015), microbial root mutualists are likely to influence the quality of such food and shelter rewards for predators and parasitoids (Heil et al., 2001; Radhika et al., 2008; Holland et al., 2009). Notably, microbial root mutualists receive sugar from their plant hosts, in exchange for mineral nutrients and water. The carbon costs associated with hosting microbial mutualists may explain why some plants reduce their rewards to enemies aboveground when associated with root microbes. For example, in *Vicia faba*, association with AMF reduces extrafloral nectaries, which may result in reduced protection by ants against herbivores (Laird and Addicott, 2007). Similarly, rhizobia reduce extrafloral nectar production by lima beans (*Phaseolus lunatus*), leading to fewer ants attracted to rhizobia associated plants (Godschalx et al., 2015). Here, the mechanistic basis underlying the tri-trophic interaction above ground appears to be a tradeoff in plant resource allocation between root mutualists and indirect defense rewards.

Compared to the other mechanisms described here, there is much more evidence in support of the hypothesis that microbial root mutualists alter the expression by plants of VOCs (Rasmann et al., 2017). For example, in sweet wormwood *Artemisia annua*, association with AMF increases emissions of the monoterpene limonene and artemisia ketone (Rapparini et al., 2008), which attract both herbivores and their natural enemies (Wei et al.,

2008; Rodríguez et al., 2011). Similarly, the root fungal endophyte *Acremonium strictum* changes the terpene composition in volatiles of tomato *Lycopersicon esculentum* (Jallow et al., 2008). In *Plantago lanceolata*, AMF reduce herbivore-induced sesquiterpenes, chemicals that recruit parasitoids after herbivory (Fontana et al., 2009). In short, since the composition of volatile compounds is key to predator/parasitoid attraction, these mutualist-induced changes in VOC emission likely represent dominant mechanisms by which microbial root mutualists influence tri-trophic interactions aboveground.

We should note that belowground mutualists may affect the composition of plant VOCs aboveground through multiple mechanisms. First, mutualistic microbes may alter the production and emission of plant VOCs by modifying nutrient availability. For example, higher nutrient (nitrogen, phosphorus and potassium) concentrations in *Eucalyptus tereticornis* increase emission of the volatile terpene 1,8-cineole, which subsequently attracts more predators and parasitoids (Low et al., 2014). Similarly, supplementing *A. annua* with phosphorus (P) largely mimics the effects of AMF on VOC production (Rapparini et al., 2008).

In contrast, greater attraction of aphids to beans, *V. faba*, associated with AMF is not due to changes in P availability (Babikova et al., 2014). Rather, microbial mutualists may influence expression of the jasmonic acid (JA), salicylic acid (SA), cytokinin, and abscisic acid (ABA) pathways (Pineda et al., 2013a), all of which influence the production of VOCs (Ballhorn et al., 2013; Pineda et al., 2013b). For example, by interfering with the JA pathway in *Arabidopsis thaliana*, rhizobia change the induction of plant VOCs by the aphid *Myzus persicae*, thereby reducing attraction of the parasitoid wasp *Diaeretiella rapae* (Pineda et al., 2013b). Moreover, mycorrhizal mycelia often connect the roots of neighboring plants (Francis and Read, 1984), such that mycorrhizal fungi may transmit signals among plants and thereby affect enemy attraction by neighboring plants (Song et al., 2010). For example, bean plants (*V. faba*) detect aphid herbivory of their neighbors through mycorrhizal fungal connections and alter their own production of VOCs (specifically increasing methyl salicylate), so that parasitoids are more attracted to them compared to plants without belowground mycelial connections (Babikova et al., 2013).

While evidence is accumulating that microbial root mutualists play an important role in mediating the production of VOCs and subsequent enemy foraging behavior, their impact is system-specific. Sometimes the effects can be large; in the tomato *L. esculentum,* for instance, association with the AMF *Glomus mosseae* results in a two-fold increase in parasitoid attraction, even in the absence of herbivores (Guerrieri et al., 2004). In other circumstances, effects are harder to detect. For example, attraction of the predatory mite *Phytoseiulus persimilis* to bean plants (*Phaseolus vulgaris*) appears unaffected by association with AMF, at least during the first 3 days of spider mite (*Tetranychus urticae*) infestation (Schausberger et al., 2012). This variation in effect sizes may be due to differences in responses of plants to their root mutualists, and/or interspecific variation in responses of enemies to plant VOCs (Leitner et al., 2010; Kruidhof et al., 2013). We return to this challenge of variability in the section on Missing Links (below).

We emphasized above that changes to plant chemical and physical traits provide the mechanistic basis underlying the effects of microbial root mutualists on the enemies of herbivores above ground. Consequently, when microbial root mutualists change more than one physical or chemical trait simultaneously, predicting the net outcome for tri-trophic interactions can be a major challenge. For example, with regard to the oxeye daisy-leaf miner-parasitoid interaction described previously, association with AMF increases plant size, which reduces parasitism of the leaf miner by the parasitoid due to lower prey location efficiency (Gange et al., 2003). However, in bean plants (*P. vulgaris*), AMF increase the emission of VOCs that can attract enemies (Schausberger et al., 2012). Thus, if both of these processes were to operate in the same system, their relative strength would determine whether the net effect of AMF was an increase or a decrease in parasitism. At this time, there are almost no data describing effects of root mutualists on tri-trophic interactions under multiple changes in plant traits. As we note under Missing Links (below) future studies are urgently needed to help understand the species specificity of plant responses to belowground mutualists, and incorporate simultaneously their effects on multiple plant traits that mediate herbivore-enemy interactions.

On-Plant Foraging Efficiency

After a predator or parasitoid successfully locates a plant with prey, it needs to find its prey on the plant and attack it. This foraging process is also influenced strongly by physical and chemical plant traits that can be altered by microbial root mutualists. For instance, glandular trichomes, hairs with secretory cells, can directly intoxicate parasitoids (Kennedy, 2003) and/or impede enemy walking speed (Krips et al., 1999), resulting in lower foraging efficiency. However, for some specialist predators, sticky trichomes may trap insect cadavers, thereby attracting more predators (Krimmel and Pearse, 2013). Similarly, plant epicuticular waxes can decrease attachment of predatory insects and parasitoids to the plant surface and disrupt their feeding (Eigenbrode, 2004). Critically, belowground mutualists affect the expression of both glandular trichomes and epicuticular waxes (Goicoechea et al., 2004; Copetta et al., 2006), providing additional mechanistic pathways by which microbial root mutualists may influence tri-trophic interactions. Unfortunately, to our knowledge, no study has explored explicitly the links among root mutualists, plant trichomes/waxes, and the efficiency of enemy foraging aboveground. Again, such work is urgently needed.

Prey Quality

Microbial root mutualists alter the nutrient and toxin concentrations of herbivore tissues (Hunter, 2016), providing an additional mechanistic pathway by which root mutualists mediate tri-trophic interactions aboveground. Prey quality is important in determining the fitness of predators and parasitoids. Compared to herbivorous insects, predatory arthropods and parasitoids have higher body nitrogen (N) and P contents, so

increases in plant nutrition can lead to greater performance of predators and parasitoids (Denno et al., 2002; Wurst and Jones, 2003; Maure et al., 2016). Therefore, by affecting plant nutritional status, root microbes belowground can indirectly affect predators and parasitoids aboveground. For example, in the presence of AMF, the predatory mite *P. persimilis* has a greater oviposition rate and shorter development time due to the higher quality of its prey, the two-spotted spider mite *Tetranychus urricae* (**Figure 1A**) (Hoffmann et al., 2011c). Additionally, plant nutrient status often affects herbivore size, which in turn influences predator and parasitoid performance (Hunter, 2016). When the aphid *Rhopalossiphum padi* feeds on plants infested with free-living and root-feeding soil nematodes, it grows significantly larger than when feeding on plants without soil nematodes, resulting in higher emergence success of its parasitoid *Aphidius colemani* (Bezemer et al., 2005).

Root associates may also alter the quality of prey for natural enemies by their impacts on plant secondary chemicals, which occur both passively in the hemolymph and midgut of herbivores, or may be sequestered in herbivore tissues (Nishida, 2002; Lampert et al., 2011). For example, plant glucosinolates occurring within herbivore prey negatively affect a wide range of parasitoids (Gols and Harvey, 2009). While we focus here on root microbial mutualists, we note that there is now abundant evidence of root-feeding herbivores influencing the chemistry of plant tissues above ground, with subsequent effects on herbivore and enemy performance (Hunter, 2016). For example, root-feeding cabbage fly larvae (*Delia radicum*) induce higher glucosinolate concentrations in *Brassica nigra*. In turn, higher glucosinolate concentrations lead to longer development time and smaller size of both cabbage butterfly caterpillars (*Pieris brassicae*) and their parasitoid wasps, *Cotesia glomerata* (**Figure 1B**) (Soler et al., 2005).

Importantly, plant secondary metabolites also affect herbivore immune defenses against predators and parasites (Smilanich et al., 2009; Lampert, 2012). High concentrations of plant secondary metabolites tend to reduce immune defenses, probably because of their negative effects on insect growth rate and reduced allocation to immune functions. For example, hydrolysable tannins in quaking aspen (*Populus tremuloides*) correlate negatively with immune defense in the autumnal moth *Epirrita autumnata* (Haviola et al., 2007), and high iridoid glycoside concentrations in *P. lanceolata* compromise immune responses in the common buckeye caterpillar *Junonia coenia* (Smilanich et al., 2009). Lower immune defense can lead to higher performance of parasitoids (Reudler et al., 2011; Kos et al., 2012). While microbial root mutualists affect the expression of these (and other) secondary chemicals (tannins, Beyeler and Heyser, 1997; iridoid glycosides, Bennett et al., 2009), to our knowledge, no study has directly explored effects of soil organisms on host insect immunity through changes in secondary chemistry.

SOIL ORGANISMS AFFECT PATHOGENS OF HERBIVORES

Many of the same mechanistic pathways (chemical and physical traits) by which microbial root mutualists impact the efficacy of predators and parasitoids may also affect the pathogens of herbivores. Herbivorous insects are host to a wide diversity of disease agents, including protozoans, bacteria, and viruses. As with parasitoids and predators, the performance of herbivore pathogens is affected by both plant nutritional and secondary chemicals, and therefore influenced by belowground root mutualists. However, the effects of increased concentrations of nutritional chemicals on pathogens are not as readily predicted as they are for other types of natural enemy. This is because increases in plant nutritional quality can result in increased resources for pathogens, but can also result in improved host immunity (Povey et al., 2009; Cotter et al., 2011). Therefore, when associations with soil mutualists result in higher plant nutrient concentrations, any subsequent increases in rates of pathogen replication may be counteracted by concomitant increases in host immunity.

With respect to secondary metabolites, multiple classes of chemicals inhibit insect pathogens (Cory and Hoover, 2006). For example, plant pyrrolizidine alkaloids reduce the production of entomopathogenic nematodes feeding within woolly bear caterpillars, *Grammia incorrupta* (Gassmann et al., 2010). Likewise, when chlorogenic acid in tomatoes is oxidized to chlorogenoquinone, it binds covalently to occlusion bodies of the baculovirus HzSNPV and reduces their infectivity in the corn earworm *Helicoverpa zea* (Felton and Duffey, 1990). Similarly, in monarch butterflies (*Danaus plexippus*), the growth of its specialist protozoan parasite (*Ophryocystis elektroscirrha*) correlates negatively with foliar concentrations of cardenolides, toxic secondary chemicals in milkweed host plants (de Roode et al., 2008, 2011; Sternberg et al., 2012). In addition, the lifespan of infected butterflies correlates positively with cardenolides, a result of reduced parasite growth as well as increased monarch tolerance of infection (de Roode et al., 2008, 2011; Sternberg et al., 2012; Gowler et al., 2015). Because AMF associations belowground change the composition and concentration of milkweed cardenolides aboveground, AMF have substantial effects on monarch-parasite dynamics across milkweed hosts (Tao et al., 2015), an interaction across four biological kingdoms (**Figure 1C**).

When the infective stages of pathogens are released on plants, many plant traits affect their survival and persistence. For example, plant architecture, leaf form and color affect the amount of UV that is reflected onto the leaf surface, and thereby affect the survival of insect baculoviruses, which are sensitive to UV light (Hunter-Fujita et al., 1998; Cory and Hoover, 2006). Additionally, phylloplane microclimate and physiochemical properties affect pathogen infectivity and persistence (Der Geest, 2000). Currently, there remains limited information on whether microbial root mutualists affect these physical plant traits; if they do, belowground mutualists may have significant indirect effects on pathogen survival and persistence prior to infection.

Intraspecific variation in plant nutritional and secondary chemistry induced by root mutualists can also affect the foraging and oviposition behaviors of insect herbivores, with implications for herbivore contact rates and disease transmission. For example, AMF-associated *Baccharis halimifolia* and prairie C_3 graminoids experience higher herbivory than do plants without AMF (Moon et al., 2013; Kula and Hartnett, 2015),

which may translate to higher rates of disease transmission among herbivores due to higher host density. Since transmission rate is fundamental to determining host-pathogen dynamics, understanding how microbial root mutualists affect disease transmission is important in both natural insect populations and in microbial biological control (Hunter, 2016). Although herbivore density and foraging behavior on individual plants clearly affect pathogen transmission (Parker et al., 2010), explicit links among root mutualists, plant traits, herbivore density, and disease transmission have yet to be made in the literature.

As we noted above for predators and parasitoids, soil mutualists affect multiple plant traits simultaneously, generating multiple mechanistic pathways by which root microbes influence tri-trophic interactions above ground. As with other enemies, the overall impact of root mutualists on disease dynamics will depend on the relative strength of each mechanistic pathway. Following the example of the monarch butterfly and its protozoan parasite described above, while cardenolides (secondary chemicals) reduce parasite numbers and increase the lifespan of infected butterflies (de Roode et al., 2008, 2011; Sternberg et al., 2012), macronutrients such as N and P also increase monarch performance (Tao and Hunter, 2012; Tao et al., 2014, 2015). Since associations with AMF change macronutrients and cardenolides simultaneously in milkweed leaves (Vannette and Rasmann, 2012; Tao et al., 2016), the net effects of AMF on butterfly-parasite interactions are best explained by the combined changes in milkweed P and cardenolide concentrations (**Figure 1C**) (Tao et al., 2015). Net effects on monarchs vary from positive, through neutral, to negative, depending on how particular milkweed species respond phenotypically to their root microbial mutualists.

MISSING LINKS

The last 15 years have seen an increase in the number of studies investigating indirect effects of belowground biota on aboveground tri-trophic interactions (Rasmann et al., 2017). As illustrated by our review, microbial root mutualists affect aboveground predators, parasitoids, and pathogens through a diverse set of mechanistic pathways, based on changes in the chemical and physical traits of plants engendered by root microbes. Specifically, chemical and morphological changes in plants alter the attractiveness of herbivore-infested plants to predators, the efficiency of their on-plant foraging behaviors, and the quality of herbivore tissues for enemy consumers. In addition, microbial root mutualists can change the efficacy of pathogens that attack herbivores above ground through their combined effects on plant morphology and plant nutritional and defensive chemistry.

However, the mechanisms of interaction documented to date (changes in plant nutritional quality, plant morphology, and plant secondary chemistry) represent a small subset of the potential pathways by which belowground biota more generally may influence tri-trophic interactions aboveground (van der Heijden et al., 1998; Smith and Read, 2008; Reinhart et al., 2012; Hunter, 2016; Rasmann et al., 2017). Unfortunately, the overall number of studies on this topic remains critically small, and most are focused on a few systems; as a result, significant knowledge gaps remain. Here, we highlight several of these gaps, in the hope that future studies will advance our understanding of these below-aboveground interactions.

(1) In reviewing this literature, we have been struck repeatedly by the difficulty of finding any generality in the magnitude and direction of effects of microbial root mutualists on tri-trophic interactions aboveground. One likely culprit is simply the small number of studies that have been conducted to date on this topic (**Table 1**); generality is hard to achieve when sample size is low. A major goal of this review is to support the call (Rasmann et al., 2017) for a concerted effort to understand how belowground organisms influence multi-trophic interactions aboveground. Pervasive effects of root biota on plant traits above ground are now well-documented (Hunter, 2016), but concomitant changes in the efficacy of predators and pathogens need much more attention.

Certainly, there is a clear need to look beyond effects mediated by mycorrhizal fungi and N-fixing symbionts, which still dominate the literature in this field. This is a two-part process: first documenting the diverse changes in plant physiology, chemistry, and morphology induced by different kinds of soil biota; second, linking explicitly these changes in plant traits to the expression of tri-trophic interactions. There has been substantial progress in the first of these, and minimal progress in the second. For example, evidence is accumulating that the rhizosphere is replete with other kinds of mutualistic microbe, including root endophytes and growth-promoting bacteria, which affect aboveground plant-herbivore interactions (Jaber and Vidal, 2010; Brunner et al., 2015). Similarly, soil macro-organisms, including dung beetles and springtails, are important ecosystem engineers that alter concentrations of the plant nutrients that are important to aboveground herbivores (Johnson et al., 2015c). Beyond root mutualists, there are well-characterized effects of root antagonists (root herbivores, pathogens, competitors) on plant phenotypic traits (Hunter, 2016), many of which are candidates for driving complex ecological interactions aboveground (Wyckhuys et al., 2017). Unfortunately, how these diverse soil biota influence tri-trophic interactions aboveground, either individually or interactively, remains largely unknown.

Beyond just a paucity of studies, a related barrier to generality is the apparent contingency in the responses of plant traits, and therefore tri-trophic interactions, to soil organisms (Barber et al., 2013). Within the microbial root mutualists, there have been several efforts to establish patterns among plant phenotypic responses based on plant phylogeny and life-history (Reinhart et al., 2012; Vannette et al., 2013). Unfortunately, the effects of microbial root mutualists on plant phenotype and herbivore performance seem to vary substantially among species of plant, species of microbe, species of herbivore, and environmental conditions (Garmendia et al., 2004; Gehring and Bennett, 2009; Grman, 2012; Grman and Robinson, 2012; Barber et al., 2013). To complicate matters further, the relative abundance of microbial root mutualists, and their degree of association with their hosts, also influences plant phenotype and herbivore performance (Garrido et al., 2010; Vannette and Hunter, 2011, 2013; Argüello et al., 2016). The unfortunate result is that, even within a single

genus of plants, the impacts of microbial root mutualists on tri-trophic interactions do not conform to any readily identifiable phylogenetic or life-history pattern (Tao et al., 2015).

The antidote to idiosyncrasy is additional work. Ultimately, phylogenetically-controlled experiments (Reinhart et al., 2012; Vannette et al., 2013) must be combined with realistic ecological treatments of density and diversity (Vannette and Hunter, 2011; Argüello et al., 2016) to establish generality. We will not make progress until we accumulate laboratory and field studies in diverse ecosystems that control phylogeny, identity, density, and environmental conditions of all the interacting partners. These experiments must also measure simultaneously the suite of plant traits that microbial root mutualists influence aboveground. It is increasingly clear that interactions among multiple plant traits will combine to determine the net outcome of tri-trophic interactions aboveground (Tao et al., 2015, 2016).

(2) Similarly, studies of how belowground biota influence tri-trophic interactions above ground are limited currently to a narrow range of natural enemies. Most studies have focused on parasitoids, and we found only four studies on predators and one on insect pathogens. To date, we have no information on effects of soil biota on other key groups of enemies, such as vertebrate predators (e.g., birds, bats, reptiles), insect baculoviruses, or macro-parasites (such as nematodes) that are ubiquitous and economically important across ecosystems. There is no *a priori* reason to suppose that effects on these groups of enemies should be uncommon. For example, evidence suggests that vertebrate predators can use plant VOCs as foraging cues (Seymour et al., 2010; Amo et al., 2013). Given that some VOC production is mediated by root microbes (above), those microbes may also influence the foraging of vertebrate predators.

Beyond vertebrates, we suggest that interactions among soil biota, plants, herbivores, and pathogens will provide particularly intriguing opportunities for further study. It is now abundantly clear that plant chemistry is a major driver of animal disease across diverse terrestrial ecosystems (de Roode et al., 2013). Plant nutritional and defensive traits influence host quality, host immunity, host behavior, and thereby disease transmission. Given that diverse soil organisms influence plant chemistry above ground (Hunter, 2016), incorporating soil biota more generally in studies of disease spread is vital in placing disease dynamics within a community ecology context (Johnson et al., 2015b).

(3) Another critical missing link is to understand the effects of belowground organisms on interactions among multiple enemies above ground. For example, most of the studies in **Table 1** describe the effects of microbial root mutualists on a single species of natural enemy. Yet there is abundant evidence in natural and managed systems of interactions among natural enemies that influence subsequent prey suppression (Cardinale et al., 2003; Johnson et al., 2013; Painter et al., 2015). We might expect that (a) not all enemy species will respond in the same fashion to a given plant trait-change induced by a root mutualist, and (b) multiple phenotypic changes induced simultaneously by root microbes may have differential effects on different enemies. We need detailed experiments, manipulating multiple enemy species simultaneously, to explore effects of microbial root mutualists on herbivore suppression in a community context.

Most intriguing among such interactions may be those between predators and agents of disease. The ecological and evolutionary dynamics of such interactions might be particularly fascinating because predators can have large impacts on disease transmission. Such effects may be density-mediated: for example, by selectively feeding on infected prey, predators can decrease overall parasite transmission (Packer et al., 2003). On the other hand, indirect effects of predators on disease transmission can also be trait-mediated, operating through changes in host behavior, physiology or immune defense. For example, female Trinidadian guppies *Poecilia reticulata* display strong shoaling tendency in the presence of predators, thereby increasing the transmission of *Gyrodactylus* parasites (Stephenson et al., 2015). In the snail *Lymnaea stagnalis*, anti-predator behavior (blood expulsion) reduces their immunocompetence, which also renders them more susceptible to pathogens (Rigby and Jokela, 2000). Critically, the trade-offs between anti-predator and anti-parasite traits can be affected by host resource-availability (Roff and Fairbairn, 2007), and in the case of invertebrate herbivores, the quality of their host plants. Although there has been no direct evidence of host plant quality mediating these traits in herbivores, the trade-offs between anti-predator behavior and growth rate in tobacco hornworms *Manduca sexta* are more prevalent on well-defended tomato plants (Thaler et al., 2014). Overall, we suggest that it will be particularly informative to link the effects of belowground biota on herbivore densities and traits with the interactions between parasites and predators. We recommend manipulative experiments that vary the densities of infected and uninfected herbivore hosts, in the presence and absence of predators, across a broad range of associations with microbial root mutualists.

(4) Almost all of the mechanisms that we documented above were based on changes in plant chemistry (nutrients, toxins, VOCs) mediated by microbial root mutualists. However, traits such as plant architecture, domatia, trichomes and surface waxes are all subject to influences from soil organisms. These same plant traits mediate predator and parasitoid recruitment and foraging efficiency (Speight et al., 2008), and affect the viability of insect pathogens (Cory and Hoover, 2006). To date, there has been no exploration of the extent to which belowground organisms affect the third trophic level through these critical plant traits. This is particularly important in agricultural systems, where the behavior and persistence of biological control agents determine in part the success of pest management.

(5) Future studies should quantify more thoroughly the effects of belowground organisms on the fitness of all partners in the aboveground tri-trophic interactions. For example, while root colonization by AMF affects the fitness of infected monarch butterflies, parasite growth remains unaffected (Tao et al., 2015). In this case, the effect of belowground organisms on the aboveground tri-trophic interaction would have been missed entirely if only parasite performance had been measured. Because the ecological and evolutionary consequences of species interactions depend on the fitness of all interacting species, we

urge researchers to quantify as many life history parameters of as many participants as possible.

(6) While a majority of studies has examined these complex interactions uni-directionally from a bottom-up point of view, the third trophic level can also impact plants and soil organisms and create important feedback loops. For example, increases in predation pressure on herbivores that result from mycorrhizal associations can subsequently feedback to increase plant fitness (Hoffmann et al., 2011a). Moreover, effects of root microbial mutualists that first "cascade up" to increase the abundance or efficacy of natural enemies can then "cascade down" again to influence the fitness of plants and their mutualists, as well as the availability of nutrients in soils (Hunter, 2016). Future studies should assess the general frequency and strength of feedback processes that link upper trophic levels aboveground with soil biota below.

(7) By influencing some species more than others, soil organisms can change the structure and composition of herbivore and enemy communities. For example, AMF colonization alters arthropod predator community composition on *Deinandra fasciculata* (Schreck et al., 2013) and on *Glycine max* (Ueda et al., 2013). These important studies suggest that we need a community perspective to understand and integrate complex species interactions below- and aboveground.

(8) Abiotic factors, such as nutrient and water availability, strongly regulate the diversity and composition of soil organisms and their interactions with plants (Johnson et al., 2015a). It remains an open and urgent question as to how above- and belowground multi-trophic interactions are shaped by environmental stresses and global environmental change.

(9) So far, most studies have focused on agricultural systems or model systems. While these provide a starting point for understanding the mechanisms in well-studied and/or economically important systems, we also need to study systems that are more diverse, such as wild herbs and woody plants, to explore the generality of effects. Even where natural systems have been used, it remains unclear how these interactions play out in the field. For example, in our monarch butterfly studies, we used commercially available mycorrhizal strains, and it remains unclear how natural milkweed-AMF interactions influence interactions aboveground under field conditions (Tao et al., 2015).

In conclusion, it is clear that belowground biota have important effects on aboveground tri-trophic interactions. However, this topic remains in its infancy and many questions remain unresolved. We hope that our review will provide some guidance in designing future studies to better understand interactions between below- and aboveground subsystems of the integrated whole.

AUTHOR CONTRIBUTIONS

LT and JdR: conceived the idea for the review; LT: conducted the initial literature review; JdR and MH: provided additional literature to the review. All three authors contributed to writing and editing the manuscript.

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Tomato Inoculation With the Endophytic Strain *Fusarium solani* K Results in Reduced Feeding Damage by the Zoophytophagous Predator *Nesidiocoris tenuis*

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Belowground symbiosis of plants with beneficial microbes is known to confer resistance to aboveground pests such as herbivorous arthropods and pathogens. Similarly, microbe-induced plant responses may also impact natural enemies of pests via the elicitation of plant defense responses and/or alteration of plant quality and growth. *Nesidiocoris tenuis* is a zoophytophagous predator and an efficient biological control agent of greenhouse pests. Its usefulness in plant protection is often hindered by its ability to damage plants at high predator population densities or when prey is scarce. In this study, we investigated the effect of *Fusarium solani* strain K (FsK), an endophytic fungal isolate that colonizes tomato root tissues, on the capability of *N. tenuis* to cause necrotic rings, an easily discernible symptom, on tomato stems and leaves. We found significantly less necrotic rings formed on FsK-inoculated plants for all tomato cultivars tested. FsK has been previously shown to confer ethylene-mediated tomato resistance to both foliar and root fungal pathogens; thus, the ethylene-insensitive *Never ripe* (*Nr*) and *epinastic* (*epi*) tomato plant mutant lines were included in our study to assess the role of ethylene in the recorded FsK-mediated plant damage reduction. The jasmonic acid (JA)-biosynthesis tomato mutant *def-1* was also used since JA is known to mediate major anti-herbivore plant responses. We show that ethylene and JA are required for FsK to efficiently protect tomato plants from *N. tenuis* feeding. No necrotic rings were recorded on FsK-inoculated *epi* plants suggesting that ethylene overproduction may be key to tomato resistance to *N. tenuis* feeding.

Keywords: biological control, endophyte, ethylene, jasmonic acid, plant damage, tomato, zoophytophagous predator

INTRODUCTION

Plants are associated with a vast diversity of microbes that exert beneficial effects on their performance. Soil-borne microbes in particular, such as endophytic fungi, plant growth promoting fungi and rhizobacteria as well as arbuscular mycorrhizae have long been recognized for their benefits to plant growth and nutrition (Smith and Smith, 2011; Hadar and Papadopoulou, 2012; Finkel et al., 2017). In addition, certain root colonizing microbes are known to antagonize soil-borne pathogens and/or prime plant defense against future attackers (Pineda et al., 2010; Pieterse et al., 2014).

Soil-borne beneficial microbes can affect aboveground herbivores both positively and negatively (Hartley and Gange, 2009; Shikano et al., 2017). Improved plant growth and/or nutrition by plant-growth promoting fungi and rhizobacteria have been shown to result in positive effects on herbivore performance (Pineda et al., 2010; Ahemad and Kibret, 2014). On the other hand, defense priming triggered by beneficial microbes, often referred to as Induced Systemic Resistance (ISR), can impact herbivores via direct or indirect defense elicitation (Pineda et al., 2010; Pieterse et al., 2014).

Microbe-ISR is mediated by phytohormones that also control plant defense against herbivores. In particular, ISR is mediated by priming of defense-related genes upon attack and involves an increased sensitivity to jasmonic acid (JA) and ethylene (ET) (Rosenblueth and Martínez-Romero, 2006; Van Wees et al., 2008). JA-mediated plant responses can be directly effective against chewing herbivores but also against phloem feeders, such as aphids and whiteflies, which normally activate the SA-signaling pathway to counteract JA-defenses via crosstalk (Walling, 2000; Schaller, 2008). Ethylene on the other hand is a modulator of the JA and SA signaling pathways in plant defense against pathogens and acts either by synergizing JA or by enabling SA antagonism with JA (Pieterse et al., 2012). To date, very little is known on the role of ET in plant responses against herbivores (Stahl et al., 2018).

Beneficial microbes can, also, impact the so-called plant's indirect defense. Upon herbivore attack plants normally emit a blend of volatiles that attract its natural enemies (Karban and Baldwin, 1997; Dicke and Baldwin, 2010). The JA signaling pathway is the key regulator of this process, suggesting that ISR in microbe-inoculated plants could modify the volatiles emitted in response to herbivory (Pineda et al., 2010). Indeed, selected soil beneficial microbes are capable of altering the composition or the emission rate of this blend and thus the attractiveness of the infested plant to certain predators and parasitoids (e.g., Fontana et al., 2009; Schausberger et al., 2012; Pineda et al., 2013). Nevertheless, besides behavior, plant-mediated effects of beneficial microbes on the performance of natural enemies have only been scarcely addressed so far (e.g., Battaglia et al., 2013; Prieto et al., 2017).

In this regard, zoophytophagous predators are of particular interest as they feed on both plant and prey. *Nesidiocoris tenuis* is one such predator and an efficient biocontrol agent of several plant pests. Nevertheless, it may also cause significant plant damage at high predator population densities or when prey is scarce (Sánchez and Lacasa, 2008; Sanchez, 2009; Arnó et al., 2010; Castañé Cristina et al., 2011) as it can feed on the plant i.e. shoots and petioles, specifically the phloem and neighboring parenchyma cells (Raman and Sanjayan, 1984). Necrotic rings are the externally visible symptoms around the stems and leaf petioles caused by the frequent stylet penetration and tissue sap feeding by *N. tenuis* along the stylet track, which result in wound response, cell necrosis and increased protein content at the feeding site. Besides necrotic rings on stems and leaves, flower abortion and punctures on fruits are the main symptoms related to *N. tenuis* feeding on tomato (Calvo et al., 2009; Arnó et al., 2010; Castañé Cristina et al., 2011).

In this study, we assessed the effects of *Fusarium solani* strain K (FsK) on *N. tenuis*, specifically its ability to cause necrotic rings on tomato plants. FsK is an endophytic fungus isolated from the roots of tomato plants grown on suppressive compost. It colonizes the roots, including vascular tissues but ingress ceases at the root crown area and fungal growth is not detected in aboveground tomato tissues (Kavroulakis et al., 2007, Skiada, unpublished data). FsK has been previously shown to confer resistance not only against root but also foliar plant pathogens in tomato. In addition, it was shown that an intact ethylene signaling pathway was necessary to confer resistance to foliar pathogens by FsK (Kavroulakis et al., 2007), indicating that FsK can induce systemic responses to the plant. We, thus, hypothesized that FsK mediates effectual tomato responses against arthropods that attack aboveground tissues of the plant, too. To explore putative defense mechanisms mediating the effects of FsK on the formation of necrotic rings by *N. tenuis*, ethylene and jasmonate plant mutant lines were used in parallel with their wild type progenitors.

MATERIALS AND METHODS

Fungal Strain

A *F. solani* strain FsK (Kavroulakis et al., 2007) routinely cultured on potato dextrose broth (PDB) at 25°C for 5 days in the dark was used in the experiments. Following removal of mycelium fragments by sieving, conidia were recovered by centrifugation at 4000 g, counted using a haemocytometer and suspended in an appropriate volume of 0.85% NaCl in order to achieve the desired inoculum concentration. Application of the inoculum of strain FsK with 10^4 conidia cm^{-3} of potting mix was performed as water drench 1 week after seed sowing.

Plants

Wild-type tomato (*Solanum lycopersicum*) cultivars Pearson, VFN8 and Castlemart and their mutant lines *Nr*, *epi,* and *def-1*, respectively as well as the commercial cultivar ACE55 were used in this study. The wild type cultivars are the progenitors of the mutant plant lines. *Nr* plants block ethylene perception (Lanahan et al., 1994) whereas *epi* is an ethylene overproducing tomato line (Fujino et al., 1988). *Def-1* plants are deficient in JA accumulation in response to wounding and systemin (Howe et al., 1996). Pearson, VFN8, *Nr* and *epi* seeds were obtained from the Tomato Genetics Resource Center (University of California,

Davis). Castlemart and *def-1* seeds were kindly provided by Greg Howe (Michigan State University).

Seeds were surface-sterilized in 2.5% NaOCl and sown directly into 10 cm diameter pots, each containing approximately 300 $cm^3$of peat blended with an NPK fertilizer (20-20-20) to a total concentration of 0.8 g l^{-1} of potting mix. The pots were placed in a climate room with a temperature of 25 ± 1°C, 65 ± 5% relative humidity (RH) and a 16L:8D photoperiod. Plants were regularly watered and once a week fertilized with a balanced nutrient solution which consisted of the following macronutrients (mM): $Ca(NO_3)_2.4H_2O$ (11.1); $NaH_2PO_4.2H_2O$ (0.0094); $Na_2HPO_4.12H_2O$ (0.006); K_2SO_4 (6.410); $MgSO_4.7H_2O$ (3,840); $CaCl_2.2H_2O$ (2); and micronutrients (μM): H_3BO_3 (69); $MnSO_4.4H_2O$(10.4); $ZnSO_4.7H_2O$ (1.2); $CuSO_4.5H_2O$ (1.7); $NaMoO_4.2H_2O$ (0.13); and FeEDDHA (0.3).

Predator

Nesidiocoris tenuis was reared on *Nicotiana tabacum* plants, which can support *N. tenuis* feeding (Calvo et al., 2012; Bueno et al., 2013; Sukhoruchenko et al., 2015). The rearing was initiated with nymphs and adults collected from *Solanum nigrum* plants in the area of Ierapetra, eastern Crete in the summer of 2012, and kept in wooden-framed muslin cages (100 cm length × 50 cm width × 70 cm height) in a climate room with 25 ± 1°C, 65 ± 5% RH and 16L:8D. Eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were provided *ad libitum* with a thin brush on the leaves of *N. tabacum* plants as supplemental food for the predator.

Plant Damage Assessment

Three to four weeks-old tomato plants of all plant cultivars were inoculated with FsK as described above and individually transferred in cylindrical net cages (30 cm length × 10 cm diameter). Control (uninoculated) plants received water only. A pair of *N. tenuis* adults (male and female, <1 week old) was introduced in each cage without food or prey so as to be forced to feed on the plant. Total number of necrotic rings on shoot and leaves as well as the number of live predators on each plant were recorded after 1 week. At this time period, no predator nymphs had hatched as anticipated (Martínez-García et al., 2016). All cages were kept in a climate room (25 ± 0.5°C, 65 ± 5% RH, and 16L:8D). Experiments with wild-type (WT) ($n = 10$–18) and mutant lines ($n = 14$–17) were carried out in parallel in two blocks in time.

Quantification of Fungal Colonization by qPCR

FsK colonization of root tissues was verified for all tomato genotypes both in control and *N. tenuis*-exposed plants. Root tissues were collected from four replicates of each treatment 1 week after exposure to *N. tenuis*. Samples were used for whole genomic DNA extraction using the "NuncleoSpin® Plant II genomic DNA extraction" kit (MACHEREY-NAGEL GmbH &Co.KG, Duren, Germany). FsK colonization of root tissues was assessed via qPCR, by using primers pair FFsITS (5′-TGGTCA TTTAGAGGAAGTAA-3′) and RFsITS (5′-GGTATGTTCACA GGGTTGATG−3′), specific for a ca 100 bp fragment of *F. solani* ITS region. An external standard curve was generated in order to quantify the copy number of ITS gene in total DNA extracted from root tissues of FsK-inoculated plants. The standard curve was generated as follows: ITS gene was amplified using FsK genomic DNA as template, the PCR product was purified and ligated into pGEM-T Easy vector (Promega, Madison, USA) and transformed to competent *Escherichia coli* DH5a cells. The recombinant plasmid was extracted again (NucleoSpin Plasmid, Macherey Nagel) and its concentration was determined via Qubit 3.0 Fluorometer. The copy numbers of the targeted gene were calculated from the concentration of the extracted plasmid DNA.

Serial 10-fold dilutions of the recombinant plasmid ranging from 5.9×10^0 to 5.9×10^8 copies/μl were subjected in triplicate to qPCR to construct the standard curve. qPCR amplification efficiencies for the under-study gene were 99.77%, with r^2 value of 0.998 and a slope of −3.327. Amplification occurred in a 10 μl reaction mixture containing Kapa SYBR FAST qPCR Master Mix (1x) Universal, 200 nM of each primer, and 1 μl of DNA, using the following thermocycling protocol: 3 min at 95°C; 45 cycles of 15 s at 95°C, 20 s at 58°C followed by a melting curve to check the specificity of the products. PCR products were furthermore analyzed on a 1.5% agarose gel in order to check for potential non-targeted amplifications.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to evaluate the effect of tomato cultivar and plant inoculation status (FsK inoculated/non-inoculated) and their interaction on the number of necrotic rings recorded on tomato plants when exposed to *N. tenuis*. Data were log(x+1) transformed to meet the criteria for parametric analysis. Pairwise comparisons by Student's *t*-test were used to compare the number of necrotic rings on wild-type tomato cultivars (ACE55, Castlemart, Pearson, and VFN8) and FsK inoculated or non-inoculated mutants (*def*-1, *Nr,* and *epi*) when exposed to *N. tenuis* as well as to compare FsK colonization levels between *N. tenuis* exposed wild-type plants and their mutants. In the cases homoscedasticity's assumption was not met, the non-parametric Mann-Whitney *U*-test was used. All statistics were performed in SPSS (SPSS, 2011).

RESULTS

Feeding Damage by *Nesidiocoris tenuis* Is Reduced on *Fs*K-Inoculated Plants Irrespectively of the Tomato Cultivar

Different tomato cultivars were used to assess putative cultivar-dependent effects of FsK on plant damage by *N. tenuis*. Plant feeding by the zoophytophagous predator for 1 week produced similar numbers of necrotic rings in all tomato cultivars [$F_{(3,105)} = 0.839$, $P = 0.475$] used in this study (**Figure 1**). Inoculating plants with FsK resulted in a significant reduction in the number of necrotic rings [$F_{(1,105)} = 82.128$, $P = 7.75E^{-15}$] in all cultivars compared to control (non-inoculated) plants (**Figure 1**). The interaction between cultivar and inoculation status (FsK-inoculated/non-inoculated) was not significant [$F_{(3,105)} = 0.013$,

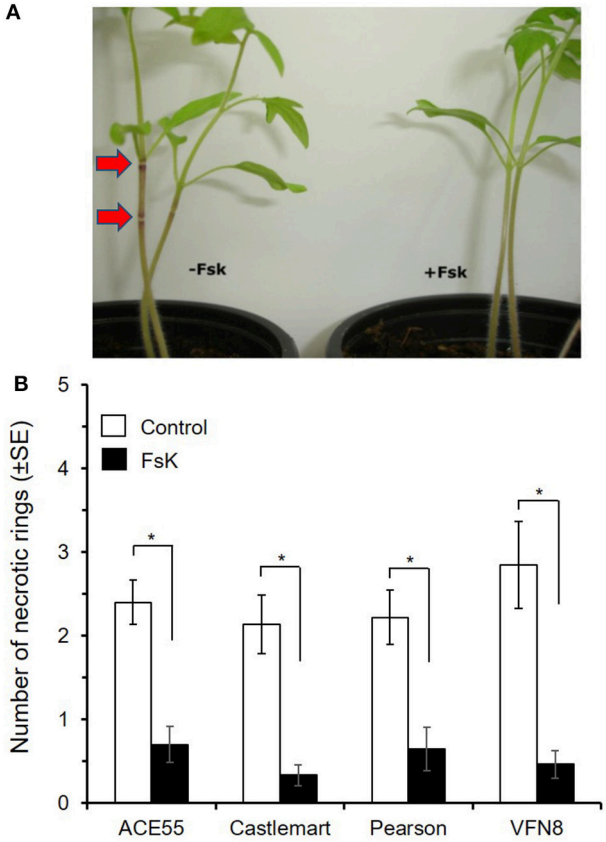

FIGURE 1 | Plant damage by *Nesidiocoris tenuis* is reduced on tomato plants inoculated with *Fusarium solani* strain K (FsK). Tomato FsK-inoculated (black bars, $N = 10$–17) or control (white bars, $N = 10$–18) plants were exposed to *N. tenuis* (one male, one female) for 1 week. **(A)** Necrotic rings (red arrows, left) caused by *N. tenuis* feeding on ACE55 control (-FsK) plants compared to FsK-inoculated (+FsK, right) plants where no symptoms are depicted **(B)** Mean (±SE) total number of necrotic rings on stems and leaves recorded for each tomato cultivar (ACE55, Castlemart, Pearson, VFN8) on day 7. Asterisks indicate significant differences within each cultivar after Student *t*-test ($P < 0.001$).

$P = 0.806$]. No effect was observed on the survival of the predators, which all remained alive at the end of the experiment (100% survival rate).

Ethylene and Jasmonic Acid Are Required for Plant Damage Reduction by *Nesidiocoris tenuis* on FsK-Inoculated Plants

We hypothesized that FsK-mediated tomato resistance to *N. tenuis* feeding may be linked to tomato JA-defenses since these constitute a major anti-herbivore defense (Howe et al., 1996; Karban and Baldwin, 1997; Walling, 2000). In addition, because FsK was previously shown to mediate tomato resistance against pathogens via the ethylene signaling pathway (Kavroulakis et al., 2007), we assumed ethylene might also be essential for FsK-mediated tomato resistance against *N. tenuis*. To test these, we investigated the effect of inoculating tomato mutant plant lines with FsK on the ability of *N. tenuis* to cause necrotic rings, to determine the involvement of the ethylene and jasmonic acid defense pathways in the FsK mode of action.

In the absence of FsK, we found that the numbers of necrotic rings recorded on both *Nr* and *def-1* plants were not significantly different compared to those on their wild-type relatives (Castlemart and Pearson, respectively) (**Figures 2A,B**), while significantly reduced number of rings were observed on *epi* mutant plants (**Figure 2C**). This indicates that although basal levels of ethylene or JA cannot protect tomato plants from the phytophagy, elevated ethylene levels may have a protective role against *N. tenuis* in tomato. In the presence of FsK, inoculated *Nr* and *def-1* plants displayed similar numbers of necrotic rings to non-inoculated mutants (**Figures 2A,B**). The plants of both mutant lines were not affected by the endophyte and the necrotic rings measured were significantly higher than those recorded on FsK-inoculated Castlemart ($t = -3.862$; $df = 28$; $P = 0.0006$) and Pearson ($t = 2.102$; $df = 32$; $P = 0.043$) wild-type plants, respectively (**Figures 2A,B**). In contrast, the presence of the endophyte further increased the response against *N. tenuis* feeding observed in *epi* mutant plants, resulting in significantly less necrotic rings on *epi* compared to wild-type VFN8 plants ($t = -2.744$; $df = 26$; $P = 0.011$). These results suggest that ethylene and jasmonate biosynthesis and signaling are essential for the expression of the FsK-mediated reduction of plant damage caused by *N. tenuis*.

To investigate the possibility that the differences observed in the activity of FsK in the various mutant plant lines could be attributed to a colonization efficiency of the FsK in these genotypes, we estimated by quantitative PCR the colonization levels of FsK in all tomato cultivars at the time of sampling. No significant differences were recorded in FsK colonization levels of tomato cultivars in all combinations [Pearson vs. *Nr*: $U = 7$, $P = 0.773$; VNF8 vs. *epi*: $t_{(5.96)} = -0.78$; $P = 0.465$; Castlemart vs. *def-1*: $t_{(3.74)} = 1.09$; $P = 0.341$]. Thus, the recorded reduction in the *N. tenuis*-caused plant damage could not be related to the colonization efficiency of FsK.

DISCUSSION

Microbes are considered capable of affecting plant-arthropod interactions (Hartley and Gange, 2009; Shikano et al., 2017). Induced plant responses by multiple biocontrol agents, such as zoophytophagous predators and soil-borne beneficial microbes may be mediated by interacting plant signaling pathways (Pappas et al., 2017). In this study, we report a mutualistic relationship between tomato and the fungal endophyte *F. solani* strain K (FsK), shown herein to mediate resistance to plant damage caused by the zoophytophagous predator *N. tenuis*. In addition, our data show that ethylene and jasmonic acid are required for the endophyte to effectively protect tomato, whereas ethylene overproduction results in null damage by *N. tenuis*.

Feeding intensity by *N. tenuis* is known to be affected by abiotic conditions (e.g., temperature) but also prey availability, with necrotic ring number increasing when prey is scarce, and vice versa (Arnó et al., 2006, 2010; Sanchez, 2008; Calvo et al.,

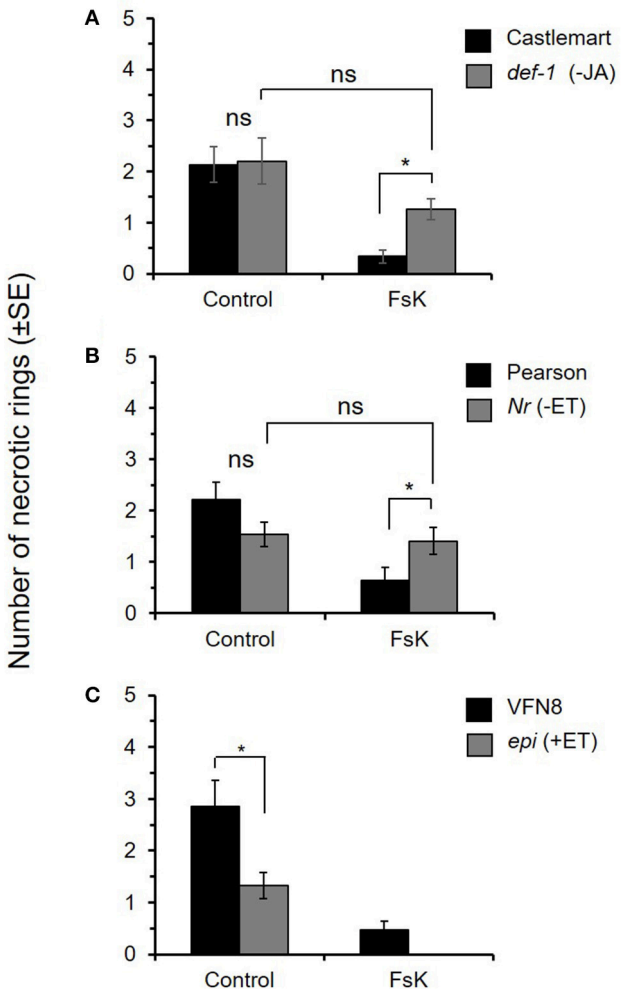

FIGURE 2 | Ethylene and jasmonate signaling is essential for the expression of *Fusarium solani* strain K (FsK) systemic effects on plant damage by *Nesidiocoris tenuis*. FsK-inoculated (FsK, $N = 14–17$) or non-inoculated (Control, $N = 13–18$) tomato plants were exposed to *N. tenuis* (one male, one female) for 1 week. Mean (±SE) total number of necrotic rings on stems and leaves on **(A)** Castlemart wild-type and JA-biosynthesis *def-1* mutant, **(B)** Pearson wild-type and ethylene-insensitive *Nr* mutant and **(C)** VFN8 wild-type and ethylene overproducing *epi* mutant. Bars depicting numbers of necrotic rings on WT plants (except ACE55) are the same as those shown in **Figure 1**. Asterisks indicate significant differences ($P < 0.05$); ns, not significant.

2009). In addition, specific tomato cultivars suffer more damage by *N. tenuis* than others (Pérez-Hedo and Urbaneja, 2016), suggesting that symptom intensity may also be related to plant traits. In our study, no cultivar-dependent difference on the symptoms developed was found and intensity of plant damage caused by *N. tenuis* was similar on all wild-type tomato cultivars tested. Moreover, FsK inoculation resulted in similar reduction in the number of necrotic rings across all wild-type tomato cultivars. In addition, no prey was available for the predators and experiments were conducted under controlled environmental conditions, suggesting that mainly plant-related factors should have affected *N. tenuis* ability to cause less necrotic rings. The fact that no differences were recorded between cultivars when FsK was present suggests the involvement of similar mechanisms mediating tomato resistance to *N. tenuis* feeding across all cultivars.

Reduction of feeding damage by *N. tenuis* on FsK-inoculated plants may be related with tomato resistance mechanisms. For example, antixenosis and/or antibiosis could be involved when *N. tenuis* is reluctant to feed on the plant due to the induction of plant defense-related responses or changes in plant nutritional quality by FsK. On the other hand, FsK-inoculated plants may display increased tolerance via accelerated healing of symptoms caused by *N. tenuis* feeding. The latter was shown for necrotic rings that completely disappeared after exposing tomato plants to *N. tenuis* only temporarily, for a few days (Arnó et al., 2006, 2010). Thus, antixenosis, antibiosis and/or tolerance may be involved in FsK-mediated tomato resistance to *N. tenuis* feeding but this needs to be further explored. In our study, all predators introduced to the control and the FsK-inoculated plants survived at the end of the experimental period, indicating that either the changes in plant response conferred by FsK have no direct impact on the predator or *N. tenuis* was not affected for the experimental period of this work. A more detailed investigation into the performance and feeding preferences of the predator will be needed to address the effects on the predator.

The prominent role of ethylene-mediated tomato responses in its interaction with *N. tenuis*, is clearly depicted by the significant reduction of necrotic rings in the ethylene overproducing *epi* mutant plants. This effect was evidently amplified by the presence of the endophyte and resulted in augmented tomato resistance against *N. tenuis,* since no rings were detected on FsK-inoculated *epi* plants. It is not known whether the endophyte is capable of inducing ethylene production in the plant and, thereby, further enhancing the positive impact of elevated levels of ethylene against *N. tenuis*. This putative mode of action resembles the reported induction of ethylene biosynthesis as a mechanism of plant protection against root-knot nematodes by *Trichoderma harzianum* (Leonetti et al., 2017). A focused study on the effect of FsK colonization on ethylene biosynthesis and signaling pathway, which would involve measurements of hormonal levels in plant tissues, will provide further insight on this mode of action.

On the other hand, both an intact ethylene and jasmonic acid pathway is shown to be essential for the expression of FsK-mediated resistance to *N. tenuis* feeding in this study. Our previous results show that FsK is able to colonize the root of tomato plants (Kavroulakis et al., 2007) and we have not been able to detect fungal ingress in the stems and leaves of the plant under our experimental conditions. Thus, a systemic effect of FsK on hormonal balance is anticipated. Hormone crosstalk is a well-established mechanism of plant resistance against pathogens and herbivores. Although for arthropods there is no general model that can describe the type of regulation exerted by the hormonal pathways and there is a strong influence of the feeding guild, JA signaling appears to be central to plant resistance against arthropods (Stahl et al., 2018). Ethylene, as a modulator of JA and SA signaling pathways has been shown to act by synergizing JA or enabling SA antagonism with JA and, thus, to variably impact arthropods studied so

far (Pieterse et al., 2012; Stahl et al., 2018). FsK inoculation did not increase resistance against *N. tenuis* feeding neither in the ethylene perception-deficient *Nr* nor the jasmonic-deficient *def-1* mutant plants. This indicates towards a synergistic role between ethylene and JA in this case. In this regard, ethylene involvement in SA antagonism to JA cannot be concluded by the present study; further studies including the SA-deficient transgenic *nahG* tomato line are needed to study the putative involvement of SA in this tripartite interaction. Finally, we have not observed any differences in the capability of FsK to colonize the various mutant genotypes when compared with the progenitor plant lines. This suggests that the endophyte triggers a systemic response in the plant, which is not related to its colonization level or to its physical presence and interaction with *N. tenuis*.

Zoophytophagous predators such as *N. tenuis* and *Macrolophus pygmaeus* are known to induce JA defenses in response to their phytophagy on tomato (Pappas et al., 2015, 2016; Pérez-Hedo et al., 2015a,b). Nevertheless, relatively little is known about the effects of JA- or SA-mediated plant responses on their performance and behavior. The expression of proteinase inhibitors, known to be induced by wounding, was recently shown not to affect the development and survival of *N. tenuis* in barley (Hamza et al., 2018). On the other hand, *Podisus maculiventris* preferred jasmonate-insensitive plants and their survival was higher on these compared to jasmonate-overexpressing plants (Thaler et al., 2015). Finally, *M. pygmaeus* development was shown to be positively affected by tomato inoculation with *Trichoderma longibrachiatum* strain MK1, which also increased plants attractiveness to this predator possibly via the involvement of both the SA and JA signaling pathways (Battaglia et al., 2013). To date, no study has ever explored JA/SA-mediated responses on *N. tenuis* feeding behavior nor the underlying mechanisms involved in zoophytophagous predator-plant-microbe interactions.

We conclude that inoculating tomato plants with FsK results in significant reduction in plant damage caused by *N. tenuis* feeding. In addition, we show evidence for the involvement of the ethylene and JA signaling pathways in FsK-mediated tomato resistance to *N. tenuis*. The ecological implications of these results are highly relevant to biological control because tomato association with FsK is shown to provide substantial benefits to the plant by conferring resistance not only to pathogens but also against arthropods. Plant damage caused by *N. tenuis* feeding poses an important limitation in the use of an otherwise highly efficient biocontrol agent, when prey is scarce or at high predator populations. The fact that FsK negatively affects *N. tenuis* feeding damage on plants is promising but needs to be further explored by considering effects on performance and predation efficiency, also in the presence of prey. In this regard, it is important to understand the regulatory mechanisms involved in FsK-mediated resistance to *N. tenuis* in tomato.

AUTHOR CONTRIBUTIONS

NK, KV, and KP designed the study. NK, KV, NG, and VS performed experiments. NK, KV, MP, GB, and KP analyzed data. NK, MP, KV, and KP wrote the paper, with contribution from all authors.

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Conflict of Interest Statement: Fusarium solani FsK is patented (20070100563/1006119, issued by the Industrial Property Organization to NK, KP).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aphid Colonization Affects Potato Root Exudate Composition and the Hatching of a Soil Borne Pathogen

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Plants suffer multiple, simultaneous biotic threats from both above and below ground. These pests and/or pathogens are commonly studied on an individual basis and the effects of above-ground pests on below-ground pathogens are poorly defined. Root exudates from potato plants (*Solanum tuberosum* L.) were analyzed to characterize the top-down plant-mediated interactions between a phloem-sucking herbivore (*Myzus persicae*) and a sedentary, endoparasitic nematode (*Globodera pallida*). Increasing inocula of the aphid, *M. persicae*, reduced the root mass of potato plants. Exudates collected from these roots induced significantly lower hatching of second-stage juveniles from *G. pallida* eggs over a 28-day period, than those from uninfested control plants. Inhibition of hatch was significantly positively correlated with size of aphid inoculum. Diminished hatching was partially recovered after treatment with root exudate from uninfested potato plants indicating that the effect on hatching is reversible but cannot be fully recovered. Glucose and fructose content was reduced in root exudates from aphid-infested potato plants compared to controls and these sugars were found to induce hatching of *G. pallida*, but not to the same degree as potato root exudates (PRE). Supplementing aphid-infested PRE with sugars did not recover the hatching potential of the treatment, suggesting that additional compounds play an important role in egg hatch. The first gene upregulated in the closely related potato cyst nematode *Globodera rostochiensis* post-exposure to host root exudate, *Neprilysin-1*, was confirmed to be upregulated in *G. pallida* cysts after exposure to PRE and was also upregulated by the sugar treatments. Significantly reduced upregulation of *Gpa-nep-1* was observed in cysts treated with root exudates from potato plants infested with greater numbers of aphids. Our data suggest that aphid infestation of potato plants affects the composition of root exudates, with consequential effects on the hatching and gene expression of *G. pallida* eggs. This work shows that an above-ground pest can indirectly impact the rhizosphere and reveals secondary effects for control of an economically important below-ground pathogen.

Keywords: aboveground–belowground interactions, aphids, fructose, glucose, plant-parasitic nematodes, root exudates

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INTRODUCTION

Plants are a primary source of nutrition for a wide range of organisms and are often subject to simultaneous attack from both above and below the ground (Wondafrash et al., 2013; Van Dam et al., 2018). Pest and/or pathogen attack can change the plant's phenotype, subsequently altering the attraction, behavior, performance, and abundance of other organisms on the same host (Sun et al., 2016). Interactions between spatially separated biota can be mediated systemically, as a result of a tight physiological integration of roots and shoots throughout the plant (Biere and Goverse, 2016).

Plant-feeding aphids and plant-parasitic nematodes (PPN) can be linked through host-mediated interactions (Kaplan et al., 2008; Kutyniok and Müller, 2012; Hoysted et al., 2017). Aphids use their stylet-like mouthparts to feed on photoassimilates found in the host's sap (Pollard, 1973; Blackman and Eastop, 2000). While feeding, aphids produce a gelling saliva that covers the stylet with a protective sheath and a watery saliva that is secreted into plant cells and the phloem (Miles, 1999; Tjallingii, 2006). Both salivas contain different proteins (Harmel et al., 2008; Hogenhout and Bos, 2011), which can induce or suppress plant defense responses (de Vos et al., 2007; Bos et al., 2010). If aphids are present in high populations, substantial reductions in yield can be observed (Kolbe, 1970) and the transmission of viral diseases by aphids can impose additional stresses (Dixon and Kindlmann, 1998; Foster et al., 2000). Nematodes constitute one of the most abundant phyla of the rhizosphere and many are phytophagous, feeding on the roots of plants (Jones et al., 2013; van Dam and Bouwmeester, 2016; Hewezi and Baum, 2017). Cyst nematodes, such as *Globodera pallida*, are a group of highly evolved sedentary endoparasites that are pathogens of temperate, subtropical, and tropical plant species (Nicol et al., 2011; Cotton et al., 2014). Second-stage juveniles (J2s) hatch in the soil in response to host root exudate, penetrate the root, and migrate intracellularly toward the vascular cylinder where each individual chooses an initial cell from which to form a highly metabolically active feeding site, termed a syncytium, from which the nematode extracts host resources (Lilley et al., 2005; Jones et al., 2013). At maturity the female is fertilized, her body swells, and the cuticle hardens to form a protective cyst that contains hundreds of eggs (Bohlmann, 2015; Moens et al., 2018).

Although aphids and cyst nematodes can share the same host, their attack on the plant is spatially separated: nematodes infect the roots of a suitable host, whereas aphids colonize above-ground biomass (Emden, 1969). The majority of studies on plant-mediated interactions between shoot herbivores and root-parasitic nematodes predominantly focuses on nematode-induced effects on herbivores rather than herbivore-induced effects on nematodes (Van Dam et al., 2005, 2018; Kaplan et al., 2008; Hofmann et al., 2010; Hong et al., 2010; Hol et al., 2013; Wondafrash et al., 2013; Hoysted et al., 2017). Although not as numerous, there have been examples of leaf feeding insects influencing the performance of PPN, however, feeding strategy of the above-ground pest played a role in the outcome of these interactions. Leaf-chewing herbivores (e.g., caterpillars) increased the abundance of PPN; however, sap-feeding insects (e.g., aphids) had a negative impact on the number of PPN present on tobacco (Kaplan et al., 2009). The specialist aphid, *Brevicoryne brassicae* had a negative effect on the abundance of the beet-cyst nematode *Heterodera schachtii* on *Arabidopsis thaliana*, with impaired development of *H. schachtii* possibly attributed to a significant reduction in individual glucosinolates in the roots (Kutyniok and Müller, 2012). Although top-down plant-mediated interactions between aphids and nematodes have been reported, these studies have focused on the indirect effects that above-ground pests can have on nematodes only after the nematode has parasitized its host. To our knowledge, no studies have elucidated the effects of aphids on the composition of plant root exudates and how these exudates may affect PPN.

Plants secrete a large array of compounds into the rhizosphere to facilitate interactions with their biotic environment (van Dam and Bouwmeester, 2016). The presence of certain compounds, termed hatching factors (Devine et al., 1996), in plant root exudates have been reported to stimulate the hatch of cyst nematode eggs from within their protective cysts (Perry, 1997). Hatching factors appear to alter the permeability of the eggshell membrane, causing trehalose to leak from the egg and water to move inward, resulting in rehydration of the J2 and contributing to the eclosion of the nematode (Perry and Beane, 1989). The hatching of some cyst nematodes displays a degree of host specificity, possibly mediated through differences in the structure of certain hatching factors, such as glycinoeclepin A in soybean (*Glycines max*) (Masamune et al., 1982) and solanoeclepin A in tomato and potato (*Solanum lycopersicum* and *S. tuberosum*, respectively) (Schenk et al., 1999). However, hatching of cyst nematodes (*Heterodera* and *Globodera* spp.) is probably much more complex than a simple reliance on a specific compound, as other chemicals such as picloronic acid, sodium thiocyanate, alpha-solanine, and alpha-chaconine (Byrne et al., 2001) can also stimulate hatch. In addition, spontaneous hatch for both *Heterodera* and *Globodera* spp. can occur in the absence of a suitable host crop (Been et al., 1995; Turner and Rowe, 2006). The compounds required for nematode hatch and the mechanisms behind eclosion remain poorly characterized. Additionally, the majority of genes involved in the hatching response has not been uncovered, however, a *G. rostochiensis* neprilysin gene (*Gro-nep-1*) was identified as the first transcript to be upregulated in eggs treated with host root exudate (Duceppe et al., 2017).

The compounds that are exuded by plant roots have been shown to change following attack by above-ground pests and/or pathogens (Rudrappa et al., 2008; Lakshmanan et al., 2012; Neal et al., 2012). Here, we investigated plant-mediated interactions between the generalist aphid *Myzus persicae* and the potato cyst nematode *Globodera pallida* by analyzing root exudates emitted from the potato crop (*Solanum tuberosum* cv. Désirée). Only a few studies have demonstrated the top-down effects of aphids on nematodes (Kutyniok and Müller, 2012, 2013), however, these focused on secondary metabolite changes in the plant caused by the above-ground herbivory. Using a combination of physiological, biochemical, and molecular techniques, we test the hypothesis that systemic changes in root exudates of the potato caused by the presence of *M. persicae* indirectly affect

the hatching of *G. pallida* eggs. We describe the composition of sugars contained within these exudates following aphid feeding and investigate the expression response of *Gpa-nep-1*, to study its link to hatching activity.

MATERIALS AND METHODS

Maintenance of Plants, Aphids, and Nematodes

Tuber cuttings of potato (*Solanum tuberosum* L. cv. Désirée) with one chit present were planted in 18 cm pots containing a mix of sand and loam topsoil (50:50). Growth took place in a glasshouse at 20–22°C under 16-h/8-h light/dark cycles for a total period of 3 weeks. Plants were watered every second day. Nymphs of the peach-potato aphid (*Myzus persicae* Sulzer) were obtained from the James Hutton Institute, Invergowrie, Dundee, Scotland. About 10 aphids, which were asexual clones of a wild population originally isolated in Scotland, and subsequently maintained on *S. tuberosum* in containment (Kasprowicz et al., 2008) were transported on leaves of *S. tuberosum* to Leeds in March 2017. Aphid colonies were maintained on potato plants, grown as described above, inside a mesh cage in a containment glasshouse. Cysts of *G. pallida* were extracted from soil of pure stock cultures using the Fenwick's (1940) method and stored dry at 4°C.

Preparation of Potato Root Exudates

The 11-day-old potato plants grown from chitted tubers in 50:50 sand/loam mix were infested with either 5, 50, 100, or 200 apterous (wingless) aphids 10 days prior to root harvest. No aphids were released on non-infested control plants. Each set (four plants per set) of aphid-infested plants and non-infested control plants was maintained inside a separate mesh cage to ensure there was no contamination across experiments. Roots of 3-week-old potato plants were excised intact from the bottom of the plant stem and washed to remove excess soil. Excised roots were soaked (80 g per liter tap water) in darkness for 24 h at 4°C. The resulting potato root exudate (PRE) was filter sterilized (0.22 μm) and stored at 4°C. PRE used in the hatching assays was combined from whole root systems obtained from four separate potato plants for each treatment or control.

Sugar Quantification in Root Exudates

Exudates were prepared from four individual root systems to provide four biological replicates per aphid treatment or control. The concentrations of glucose and fructose in the root exudates were quantified colorimetrically at 340 nm using Glucose (HK) and Fructose assay kits, respectively (Sigma–Aldrich, United States) according to the manufacturer's instructions provided with the kit. Each of the four biological replicate exudates from the five different treatments was assayed in technical triplicate to provide a mean concentration per replicate that was used for subsequent statistical analysis. Water was a negative control in each assay. Standards provided with the kits were used to construct calibration curves, to convert absorbance readings into μg/ml of glucose and fructose.

Hatching Assays

For each of the three experiments batches of five cysts (*G. pallida*; 10 replicates per treatment) were placed in wells of 12-well polypropylene plates. One milliliter of PRE from aphid infested plants, control potato plants or sugar solutions was added to each well ensuring the cysts were covered. All three cyst experiments were incubated at 20°C for the duration of the experiment. In the first experiment, PRE from aphid-infested plants was replaced with fresh PRE, and the number of hatched J2s was counted, every 4 days. After 18 days, the same cysts were washed and re-incubated in non-infested control PRE. In a second separate experiment, cysts which had been incubated in aphid-infested PRE were, after 18 days, washed and re-incubated in sugar replacement solutions. Sugar replacement solutions were prepared by adding glucose or fructose to each aphid-infested PRE to bring the concentrations equivalent to those found in non-infested control PRE (16.4 μg/ml glucose and 35.0 μg/ml fructose). Counting of hatched J2s for both the first and second experiment continued until day 28 when emergence of J2s had significantly declined in all treatments. In a third experiment, *G. pallida* cysts were treated with solutions of glucose (16.4 μg/ml), or fructose (35.0 μg/ml) or a combination of the two sugars at those concentrations for 28 days to assess the effect of sugars on *G. pallida* hatching. Cysts incubated in water provided a negative control and PRE was used as a positive control. At the end of each hatching experiment, cysts were opened and the numbers of unhatched J2s were counted, in order to express the data as a percentage of total potential hatch.

Analysis of *Gpa-nep*-1 Gene Expression

Groups of 10 *G. pallida* cysts (four reps per treatment) were treated with either root exudates from control or aphid-infested plants, sugar solutions, or water for 8 days. Total RNA was prepared using the E.Z.N.A®. Plant RNA Kit (Omega Biotek, United States) including a DNase treatment. First-strand cDNA was synthesized from 500 ng RNA using iScript cDNA Synthesis Kit (BioRad, United States) following the manufacturer's instructions. Quantitative reverse transcriptase (qRT)-PCR was carried out on the resulting cDNA using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) and a CFX Connect instrument (BioRad, United States). Expression of *G. pallida neprilysin-1* (GPLIN_000276000) was studied and normalized to the housekeeping gene *Elongation Factor 1*-α (Nicot et al., 2005). Primers Gpnep1F (5′-TCACGGCATCAGACAACATT-3′), Gpnep1R (5′-CCGTGTCACTTAGCCGATTT-3′), GpEF1aF (5′-AATGACCCGGCAAAGGAGA-3′), and GPEF1aR (5′-GTAGCCGGCTGAGATCTGTC-3′) were used for analysis of *G. pallida neprilysin-1* and *Elongation Factor 1*-α, respectively. Control reactions contained water instead of template. Each primer pair had an amplification efficiency of 97–101% and r^2 correlation coefficients for standard curves ranged between 0.94 and 0.99. Primer pair efficiencies were calculated using the BioRad CFX Manager 3.1 software. Gene expression analysis was performed on four biological replicates for all treatments and each reaction was carried out in triplicate. C_T values were determined using the BioRad CFX Manager 3.1 software. Relative expression between treatments was determined using

the $2^{-\Delta\Delta C_T}$ method as described in Livak and Schmittgen (2001).

Data Analysis

One-way ANOVA and Student-Newman-Keuls (SNK) *post hoc* tests were used to determine the significance of differences in potato root weight, final percentage hatch, sugar content of root exudates and gene expression data. All data were checked for normality using the Shapiro–Wilks test prior to statistical analysis. Pearson's correlation was used to measure the strength and direction of the relationship between inhibition of nematode hatch and size of aphid inoculum. SPSS v24 (IBM Corporation Armonk, New York, NY, United States) was used for all statistical analysis.

RESULTS

Increased Inoculum of *Myzus persicae* Reduces Below-Ground Tissue in Potato Plants

There was a significant reduction in both the fresh and dry root weights of potato plants that had been infested with at least 50 *Myzus persicae* individuals for 10 days compared to the roots of non-infested potato plants (**Figures 1A,B**; $P \leq 0.05$). Increasing inocula of aphids resulted in greater reductions in both fresh and dry weights of roots (**Figures 1A,B**; $P < 0.05$), with a significant dose-dependent correlation (Pearson's coefficient of $r = -0.727$, $P < 0.01$).

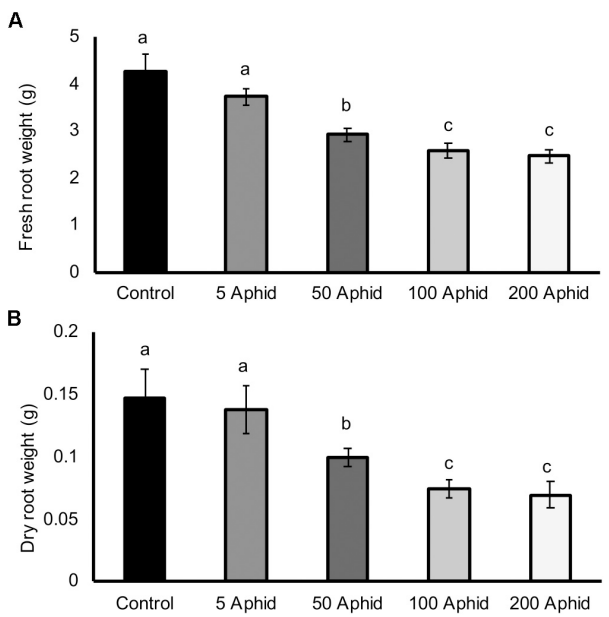

FIGURE 1 | Effect of *Myzus persicae* inoculum on the fresh **(A)** and dry **(B)** weight of potato roots (*Solanum tuberosum* cv. Désirée) 10 dpi. Values are means ± SEM from at least four replicates with different letters indicating significant differences between treatments ($P < 0.01$).

Root Exudate From Aphid-Infested Potato Plants Induces Diminished Hatching of *Globodera pallida*

In this study, we investigated the possible indirect effect that aphids may have on cyst nematodes via root exudate. Hatching of *G. pallida* was significantly reduced when cysts were incubated in PRE from potato plants infested with > 5 *M. persicae* compared to exudates from non-infested control plants (**Figures 2A,B**; $P < 0.05$). There was a significant positive correlation between the aphid inoculum level and the reduction of *G. pallida* hatching over 28 days (**Figures 2A,B**; Pearson's correlation $r = -0.792$, $P < 0.01$). Diminished hatching was partially recovered on day 20 after treatment with root exudate from uninfected potato plants, resulting in a second peak of hatching (**Figure 2A**). This indicates that the effect on hatching is reversible, however, hatching was not fully recovered to PRE control treatment levels (**Figure 2B**).

Increasing Inoculum of *M. persicae* Results in a Decreased Glucose and Fructose Content in Potato Root Exudates

Sugars are present in the honeydew of *M. persicae* implicating aphids in the translocation of sugars around the host plant (Hussain et al., 1974), therefore, we analyzed the amounts of

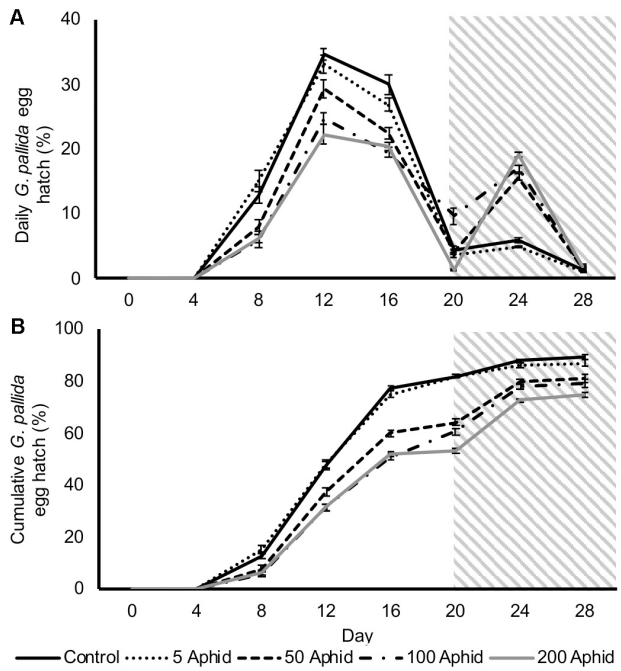

FIGURE 2 | Daily **(A)** and cumulative **(B)** *Globodera pallida* percentage egg hatch from cysts treated with root exudate from non-infested control and *Myzus persicae* infested potato plants (days 0–20). Initial inoculums of 5, 50, 100, and 200 aphids were applied to the leaves of potato plants for 10 days before collection of exudate. All cysts were treated with root exudate from non-infested potato plants (control) at day 20–28 (indicated by gray box). Values are means ± SEM from 10 replicates with five cysts per replicate.

glucose and fructose present in the control and treatment PREs. The concentrations of both glucose (**Figure 3A**) and fructose (**Figure 3B**) were significantly reduced in PRE of potato plants 10 dpi with *M. persicae* at any level of inoculum ($P < 0.05$). An increasing number of aphids resulted in a significant dose-dependent reduction of glucose and fructose in the root exudates (Pearson's correlation $r = -0.772$, $P < 0.001$ and $r = -0.843$, $P < 0.001$, glucose and fructose, respectively).

Glucose and Fructose Induce Hatching of *G. pallida*

In order to test if glucose and fructose directly stimulate hatching we incubated cysts in glucose and fructose solutions with concentrations equivalent to those detected in non-infested PRE. Treatment of *G. pallida* cysts with glucose and/or fructose induced egg hatch although peak hatching in sugar solutions occurred later than when cysts were treated with control PRE (**Figure 4A**). Total percentage egg hatch from cysts treated with sugars was greater than that from cysts treated with water but not as great as cysts treated with control PRE (**Figure 4B**; $P < 0.01$). Treatment with glucose and fructose combined resulted in significantly greater hatch than either single sugar but still significantly less than control PRE (**Figure 4**; $P < 0.01$).

Supplementing Root Exudate From Aphid Infected Potato Plants With Glucose and Fructose Does Not Rescue *G. pallida* Hatch

In order to test whether the reduced hatching rate in aphid-infested PRE was due to a reduction in fructose and glucose, we supplemented those exudates with sufficient sugars to restore the concentrations found in non-infested PRE and used this as the replacement exudate at day 20. However, the reduced hatch rates were not rescued by supplementation of exudates with glucose and fructose, nor was total hatch significantly different (**Figures 5A,B**).

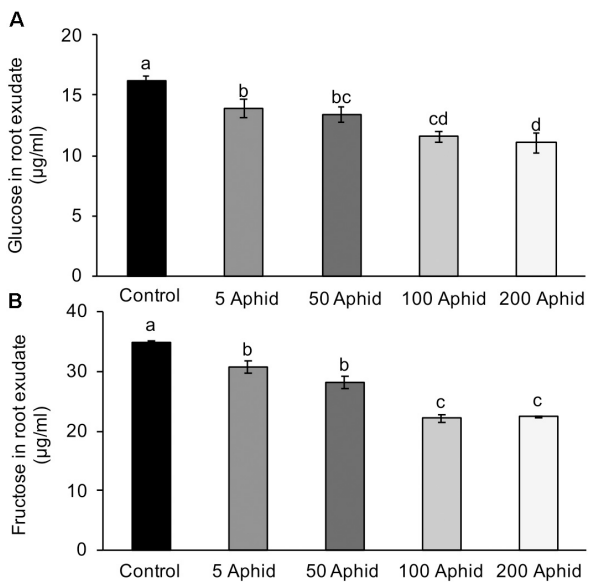

FIGURE 3 | Glucose **(A)** and fructose **(B)** content in root exudates from control and *Myzus persicae* infested potato plants. Values are means ± SEM at least four replicates with different letters denoting significance ($P < 0.05$ one-way ANOVA and SNK).

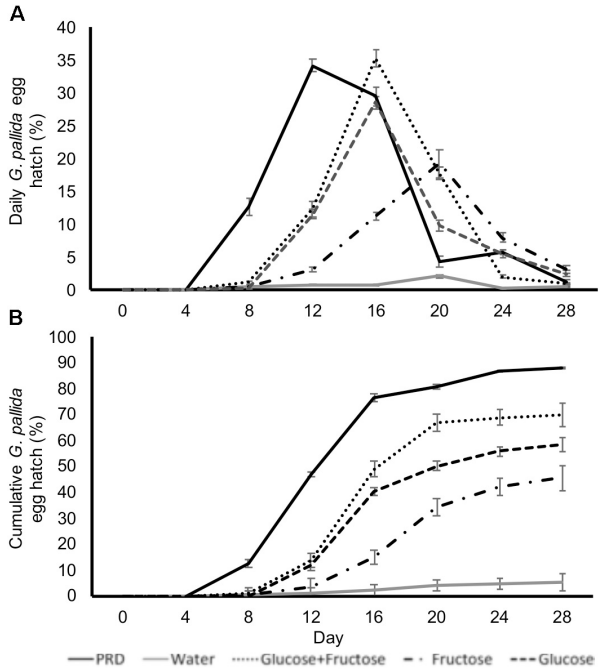

FIGURE 4 | Daily **(A)** and cumulative **(B)** *Globodera pallida* percentage egg hatch from cysts treated with water, potato root exudate (PRE), 16.4 µg/ml glucose (Glu), and/or 35.0 µg/ml fructose (Fru). These concentrations reflect the concentrations detected in PRE. Values are means ± SEM from 10 replicates with five cysts per replicate.

Induced Expression of *Gpa-nep-1* Varies in Response to Hatching Stimulants

A *Globodera* neprilysin gene has been detected as the first transcript to be upregulated in eggs treated with host root exudate (Duceppe et al., 2017), therefore we tested the expression of *Gpa-nep-1* in *G. pallida* eggs that had been incubated in non-infested control and aphid-infested PRE. There was a significant increase in the expression of *Gpa-nep-1* in unhatched *G. pallida* eggs 8 days post incubation in root exudates from non-infested control plants relative to eggs incubated in water (**Figure 6A**). Root exudates from aphid-infested potato plants significantly increased the expression of *Gpa-nep-1* in eggs but to a lower degree than non-infested control treatments ($P < 0.05$). There was also a significant increase in the expression of *Gpa-nep-1* in *G. pallida* eggs 8 days post incubation in glucose and/or fructose solutions relative to eggs in water ($P < 0.01$) (**Figure 6B**). Upregulation of *Gpa-nep-1* in response to the sugars was not as large as in eggs treated with PRE.

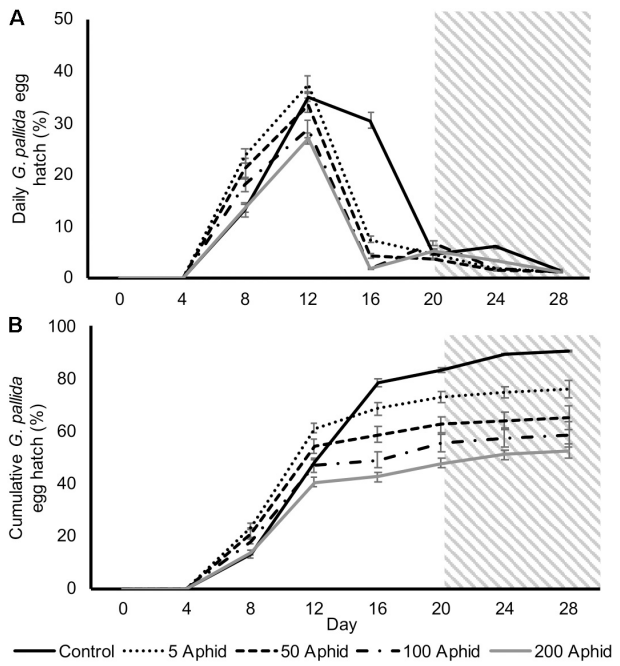

FIGURE 5 | Daily **(A)** and cumulative **(B)** *Globodera pallida* percentage egg hatch from cysts treated with root exudate from control and *M. persicae* infested potato plants (days 0–20). Initial inoculums of 5, 50, 100, and 200 aphids were applied to the leaves of potato plants for 10 days before collection of exudate. Root exudate from infested plants was supplemented with glucose and fructose for treatments on days 20–28 (gray box) to equate to concentrations found in root exudate from non-infested potato plants (16.4 and 35.0 μg/ml, respectively). Values are means ± SEM from 10 replicates with five cysts per replicate.

DISCUSSION

In this work, we demonstrate how the physiological response of the potato plant to attack by an above-ground herbivore, *Myzus persicae* can indirectly influence hatching of the soil-borne PPN, *Globodera pallida* through systemic changes in root exudates.

Below-Ground Plant Responses to Aphid Infestation

The top-down effect of shoot herbivory on below-ground biomass is relatively undescribed compared to the more direct effects of root herbivores (Masters et al., 1993; Bardgett et al., 1998; Wu et al., 1999; Soler et al., 2005; Van Dam et al., 2005; Gratwick, 2012; McKenzie et al., 2016). We found that the root mass of potato plants was reduced in the presence of increasing inocula of *Myzus persicae* (**Figure 1**). Above-ground foliar herbivory may affect the roots, and therefore soil biotic communities by altering root carbon allocation and/or patterns of root exudation (Bardgett et al., 1998). Annuals, such as potato, do not store a high proportion of primary productivity in the root system and are therefore more likely to divert the products to the shoot to maintain foliar growth upon herbivory, thereby decreasing biomass of the root system (Mooney, 1972).

Aphids feed from plant phloem tissue via their stylets (Dixon and Kindlmann, 1998) by removing water, ions, sucrose, and free amino acids, which are major sources of carbon and nitrogen and vital for plant growth (Girousse et al., 2005). Aphids have been implicated in the translocation of sugars through their host plant (Hussain et al., 1974). Translocation of substances can occur from root to shoot and *vice versa*. A proteinaceous salivary sheath is released from the aphid stylet during feeding and can move long distances throughout the plant, causing deleterious effects (Madhusudhan and Miles, 1998; Miles, 1999; Burd, 2002). Pea aphid (*Acyrthosiphon pisum*) feeding on alfalfa stems strongly reduces carbon flux and initiates translocation of amino acids from roots, leaves, and sink tissues (Girousse et al., 2005). This translocation of assimilates from the roots has an effect of decreasing the root C:N ratio, thereby suggesting that plants allocate most productivity into regrowth of foliar tissues rather than root (Seastedt et al., 1988).

Plant-Parasitic Nematode Responses to Root Exudation

The shift in root assimilates can modulate root exudation and can affect soil pathogens, such as rhizobacteria (Bardgett et al., 1998; Kim et al., 2016). Root exudates have traditionally been grouped into low- (amino acids, sugars, phenolics) and high (mucilage and proteins) molecular weight compounds. However, the complexity and chemical composition of root exudates from diverse plant species is unknown (Walker et al., 2003). Our results show that root exudates from aphid-colonized plants negatively affected nematode egg hatch, the initial stage of the life cycle, compared to exudates from non-infested control plants. Wounding of plants has been reported to elicit a defense response in roots (Savatin et al., 2014), however, all root exudates used in this study were prepared in the same way therefore the differences we observed between exudates reflect only the aphid infestations of the plants. Inhibition of hatch was positively correlated with size of aphid inoculum. This did not merely reflect the lower root mass of the aphid infested plants, which was taken into account during preparation of the exudate, suggesting that the composition of PRE may be indirectly changed as a result of the aphid feeding, in a dose-dependent manner. Aphid infestation has previously been reported to result in reduced infestation of *Arabidopsis* roots by pre-hatched J2s (Kutyniok and Müller, 2012). Compounds exuded by plant roots are known to stimulate the hatch of various cyst nematodes as well as affect stylet thrusting, attraction and transcription in other endoparasitic nematodes such as *Meloidogyne incognita* (Perry and Beane, 1989; Devine et al., 1996; Teillet et al., 2013).

Effect of Aphid-Infestation on Potato Root Exudate Composition

Simple sugars are known to attract some nematode species and induce their stylet activity but this is not the case for *G. pallida*, possibly due to its selective host nature (Kamilova et al., 2006;

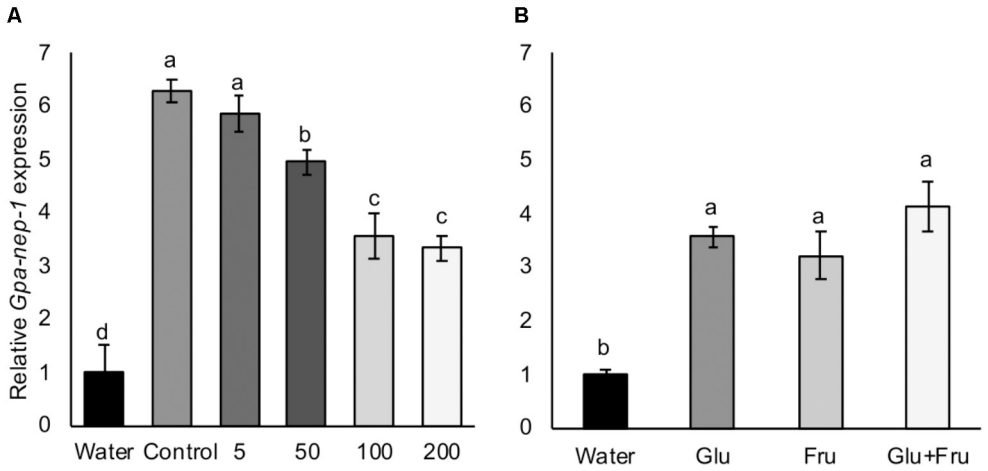

FIGURE 6 | Expression of a neprilysin gene (*Gpa-nep-1*) by RT-qPCR in five *Globodera pallida* cysts treated or 8 days with water or **(A)** root exudate from potato plants inoculated with 0, 5, 50, 100, or 200 *Myzus persicae*. **(B)** 16.4 μg/ml glucose (Glu) and/or 35.0 μg/ml fructose (Fru). These concentrations reflect the concentrations detected in non-infested potato root exudate. Expression was normalized to *Elongation Factor 1-α* and presented relative to expression in cysts treated with water. Values are means ± SEM from four replicates with five cysts per replicate. Letters denote significant differences between treatments ($P < 0.05$).

Warnock et al., 2016). Root exudates from aphid-infested plants had a reduced concentration of glucose and fructose, but an active role of sugars in stimulating nematode hatch has not been previously described. Our study found that both glucose and fructose, at concentrations present in our PREs, were sufficient to induce hatching of *G. pallida*. The effect of sugars on hatching also correlated with an increase in *Gpa-nep-1* transcript within the eggs. In a previous study, a role in hatching has been proposed for this gene as it is the first *Globodera* transcript to be upregulated post-treatment with root exudates from host plants (Duceppe et al., 2017). This study reinforces that proposed link as it correlates the hatching ability of the exudate with expression levels of the gene.

The hatching stimulation of glucose and fructose and their effects on *Gpa-nep-1* expression infer hatching of *Globodera* in exudates from non-host plants, as previously observed for *G. ellingtonae* (Zasada et al., 2013). The variance of egg hatch between host root exudates suggests varying concentrations of hatching stimuli or hatching inhibitors. Confirming either of these factors could direct a new pathway for manipulation of exudates to protect plants from nematode attack, not only for *Globodera* spp., but also for other PPN.

Effect of Aphid-Infestation on the Hatching of a Soil Borne Pathogen

Diminished hatching of *G. pallida* was partially recovered after treatment with root exudates from uninfested potato plants, indicating that the effect is reversible but cannot be fully recovered. The addition of sugars to exudates from aphid-infested plants did not increase their hatch stimulation. This suggests that as well as altering the sugar composition of exudant, aphid feeding may reduce the concentration of hatching stimuli and/or induce exudation of a factor/factors that can inhibit hatching. Exudates from control plants may reverse the effects of this compound, although not completely in some eggs, while sugars do not. Aphid feeding is known to induce systemic translocation and increased production of defense compounds, such as polyacetylenes (Wu et al., 1999), which can initiate defense pathways, such as the phytoalexin response (Flores et al., 1988) and play a role in resistance to nematodes (Veech, 1982). Additionally, genetic variation between individuals within a cyst could rationalize the portion of eggs that do not react to the hatching stimulant and are more susceptible to the inhibitory compound. Genetic variation is known to occur between individuals of *G. pallida* within a population (Eves-van den Akker et al., 2014) and could regulate the timeframe in which individual eggs hatch post-treatment with root exudate and in response to sugars. It would be of interest to determine variable loci, possibly *Gpa-nep-1*, in eggs with differential hatch under each treatment.

CONCLUSION

Our data reveal the systemic effects of aphid colonization on potato plants and how the compositional shift of root exudate can negatively impact the hatch and gene transcription of the potato cyst nematode *G. pallida.* We have determined for the first time that the sugars fructose and glucose, present in root exudate, can induce hatching of a cyst nematode and we suggest the presence of an unidentified compound that may inhibit the hatching stimulus. This insight will assist efforts to establish what determines host status of a plant and underpin the production of plants that do not exude hatch-inducing compounds. Although *G. pallida* infects the host plant soon after roots emerge, while *M. persicae* colonize the plant once there is sufficient biomass above-ground

(Emden, 1969), knowledge gained from the current study will be useful to inform management strategy for PPN, such as the beet and soybean cyst nematodes that can complete more than one generation in a cropping season (Alston and Schmitt, 1988).

AUTHOR CONTRIBUTIONS

GH, CB, CL, and PU designed the research. GH and CB performed the research. GH and CB analyzed the data. GH, CB, CL, and PU wrote the manuscript.

FUNDING

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Transient Expression of Whitefly Effectors in *Nicotiana benthamiana* Leaves Activates Systemic Immunity Against the Leaf Pathogen *Pseudomonas syringae* and Soil-Borne Pathogen *Ralstonia solanacearum*

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Infestation of plants with the phloem-feeding whitefly *Bemisia tabaci* modulates root microbiota and both local and systemic immunity against microbial pathogens. Specifically, aboveground whitefly infestation suppresses pathogen propagation and symptom development caused by the soil-borne pathogens *Agrobacterium tumefaciens* and *Ralstonia solanacearum* in the root system through systemic signal transduction. Therefore, we hypothesized that secreted protein(s)/non-protein factors from whitefly saliva (referred to as candidate effectors) might function as insect determinants that activate systemic acquired resistance (SAR) in the host plant. Here, we intensively screened a cDNA library constructed from mRNA from whitefly feeding on *Nicotiana benthamiana* leaves and selected three candidate effectors 2G4, 2G5, and 6A10, that appear to reduce disease development caused by the aboveground pathogen *Pseudomonas syringae* pv. *tabaci* and the soil-borne pathogen *R. solanacearum*. Transient expression of the three candidate effector cDNAs in leaves primed the expression of SAR marker genes *NbPR1a* and *NbPR2* in local and systemic leaves against *P. syringae* pv. *tabaci*, while leaf infiltration with 2G4 or 6A10 cDNA elicited strong defense priming of SAR markers following drench application of *R. solanacearum* on plant roots. *In silico* and qRT-PCR analyses revealed the presence of 2G5 and 6A10 transcripts in insect salivary glands. This is the first report of whitefly effectors that prime SAR against aboveground and belowground bacterial pathogens.

Keywords: whitefly, effector, *Nicotiana benthamiana*, *Ralstonia solanacearum*, transient expression

INTRODUCTION

Plants are constantly exposed to diverse insect pests and microbial pathogens (Agrios, 2005). To protect from these enemies, immune responses, including chemical and physical defense mechanisms, are activated in local and systemic plant tissues (Koornneef and Pieterse, 2008; Dangl et al., 2013). Plants have developed a sophisticated immune system against insect herbivory. Compared with our understanding of plant defensive responses against chewing insects, little is known about plant responses to phloem-feeding insects in the order Hemiptera (Van Oosten et al., 2008; Walling, 2008; Rodriguez-Medina et al., 2011; van Dam and Heil, 2011; Louis and Shah, 2013; Pitino and Hogenhout, 2013; Rao et al., 2013; VanDoorn et al., 2015). The detailed mechanisms of plant responses to this group of insects, such as aphids and whitefly (*Bemisia tabaci* Genn.), have only recently begun to be uncovered due to the small size and limited genetic and physiological information about these insects (Louis and Shah, 2013; Pitino and Hogenhout, 2013; VanDoorn et al., 2015).

For instance, aboveground (leaf) whitefly infestation increases plant immunity against soil-borne plant pathogens, indicating that systemic plant signaling is activated and translocated from leaf to root. Infestation of pepper leaves by whitefly increases systemic resistance against the soil-borne pathogen *Ralstonia solanacearum* (Yang et al., 2011; Lee et al., 2012). Further investigation revealed that infestation with this insect leads to the recruitment of beneficial rhizosphere bacterial species, which act as a biological trigger to elicit plant systemic defense responses against subsequent whitefly attack (Murphy et al., 2003). More recently, whitefly infestation was found to reduce *Agrobacterium tumefaciens* mediated crown gall formation on stems and roots (Song et al., 2015). Transcriptome and virus-induced gene silencing analyses demonstrated that whitefly-induced salicylic acid (SA) signaling attenuates *Agrobacterium* T-DNA transformation and gall formation. Root exudates that were collected from tobacco contained approximately 2.5-fold higher SA levels when whiteflies had infested leaf tissues (aboveground) compared to the uninfested control. Intriguingly, whitefly-elicited plant immunity in pepper activates both SA- and jasmonic acid (JA)-related gene expression in aboveground and belowground tissue, indicating that SA- and JA-dependent pathways are activated from leaf to root in response to whitefly feeding on leaves (Park and Ryu, 2014). Further investigation involving the fine-tuning of these signaling pathways following whitefly infestation using virus-induced gene knockdown of SA- and JA-responsive and biosynthesis genes revealed that SA is a major player in whitefly feeding-dependent signaling (Lazebnik et al., 2014; Song et al., 2015).

The whitefly determinant that confers resistance against soil borne pathogens in systemic plant tissues is still unknown. Our understanding of insect-mediated changes in the activation of plant immune responses is limited due to the lack of information on whitefly determinants that suppress or induce plant immune responses. To fill this knowledge gap, most studies on insect factors that modulate plant immunity have focused on the suppression of insect resistance rather than the induction of plant resistance responses such as systemic acquired resistance (SAR) (Kempema et al., 2007; Cooper et al., 2010; Su et al., 2012, 2015). The effector proteins from pathogenic bacteria and fungi induce and suppress plant immunity via a well-known process described by the zigzag theory (Jones and Dangl, 2006). Effectors are a group of proteins that translocate from microbes such as bacteria, fungi, and nematodes to host plants and animals (Elzinga and Jander, 2013). The major function of effectors is to modulate host immune responses though interactions with their counterpart proteins in the host plant. The outcomes of these interactions include effector-triggered susceptibility (ETS) and effector-triggered immunity (ETI) or immune reactions in the target plant, which occur in a protein-dependent manner.

Unlike microbial effectors, insect effectors have not been intensively studied. Recent studies explored effector proteins, primarily from sucking insects, and their role in plant immunity (Elzinga and Jander, 2013). Hemipteran and dipteran insect species, including phloem-feeding aphids and whiteflies, secrete certain proteins and translocate them into the cytosol of the host cell through their stylets (Kaloshian and Walling, 2015). These effectors play important roles in suppressing plant defense responses and helping the insect overcome plant immunity. The aphid effectors Coo2 and Armet, which were identified through transcriptome analysis of aphid glands, increase insect survival and host colonization (Mutti et al., 2006; Wang et al., 2015a). Another aphid effector, SHP (structure sheath protein), is primarily expressed in saliva and functions as a virulence factor. Interestingly SHP does not share any sequence homology with proteins from other insects, suggesting that it would be a good target for RNA interference-mediated insect control in SHP dsRNA-overexpressing transgenic plants (Abdellatef et al., 2015; Will and Vilcinskas, 2015). ACE2 (angiotensin-converting enzyme 2), SSGPs (secreted salivary gland proteins), and Mp10, Mp55, Me10, and Me23 are also candidates for this technique (Elzinga and Jander, 2013; Wang et al., 2015b; Zhao et al., 2015). However, the functions and molecular roles of effectors from whitefly have only recently been explored. Whitefly saliva is thought to contain proteins that modulate plant defense responses and facilitate feeding. Secreted whitefly laccase 1 (LAC1) and small RNAs have been identified and are thought to help the insect overcome plant immunity responses (van Kleeff et al., 2016; Yang et al., 2017).

In the current study, to extend our understanding of plant-microbe-insect tritrophic interactions, we focused on the following: (1) establishing a high-throughput screening system to screen whitefly effectors that elicit plant immune responses against aboveground virulent pathogens, (2) evaluating aboveground effector-mediated plant SAR against soil-borne pathogens, and (3) characterizing the identified effectors and confirming expression in the salivary gland. We used whitefly as a model insect and two microbial pathogens as model pathogens, including *Pseudomonas syringae* on local and systemic leaves (aboveground) and the soil-borne pathogen *Ralstonia solanacearum* on the plant root system (belowground).

The objective of the current study was to identify candidate whitefly effectors that activate plant immunity, as revealed

by the suppression of symptom development caused by virulent *Pseudomonas syringae* pv. *tabaci* or attenuation of the hypersensitive response (HR, a plant programmed cell death response) caused by avirulent *Pseudomonas syringae* pv. *syringae*. We hypothesized that pre-infiltration of *Nicotiana benthamiana* leaves with candidate whitefly effectors would delay or totally suppress lesion formation caused by the two *P. syringae* pathovars in the overlapping regions of leaves after cross-infiltration. Finally, we validated three putative effectors identified from high-throughput screening of an *Agrobacterium tumefaciens*-mediated transient expression system as candidate effectors that elicit plant systemic immunity (SAR) against a soil-borne pathogen *Ralstonia solanacearum*, prime plant SAR marker gene expression on root and confirmed their localization *in silico*. This study represents the first demonstration of whitefly effectors that trigger SAR against aboveground and belowground bacterial pathogens.

MATERIALS AND METHODS

Insect Culture and Plant Growth Conditions

Whiteflies (*Bemisia tabaci*) were grown and maintained in the KRIBB Greenhouse Facility in Daejeon, South Korea, as described previously (Yang et al., 2011; Park and Ryu, 2014). The whitefly was identified as Q biotype (data now shown). *N. benthamiana* was used as the model system, as described previously (Song et al., 2016). *N. benthamiana* seeds were surface-sterilized with 6% sodium hypochlorite, washed four times with sterile distilled water, and incubated in a growth chamber at 25 ± 2°C under fluorescent lights (light: dark 12: 12 h; c. 7000 lux light intensity). Seedlings were individually grown in plastic pots 9 cm in diameter at 25 ± 2°C under fluorescent lights in a controlled environment growth room (12 h/12 h day/night cycle, 7000 lux light intensity). Three-week-old *N. benthamiana* plants were used in the experiments. The experiments were repeated three times with 10 replications (one plant per replication and three leaves per plant).

Whitefly cDNA Library Construction

Whiteflies were collected from *N. benthamiana* leaves at mid-day and quickly ground in liquid nitrogen for RNA isolation using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNase-free DNase I (Promega). Purified total RNA was reverse-transcribed to cDNA using a Cloneminer II cDNA Library Construction Kit (Invitrogen) following the manufacturer's instructions. The cDNA was inserted into the pDONR222 Gateway vector. The initial titer of the library was determined through colony counts via plating on LB plates containing kanamycin (50 μg/mL). The average insert size was analyzed by restriction enzyme digestion with BsrG1 (New England Biolabs). The initial cDNA fragments were transferred to the pK7WG2 vector using LR recombinase (Invitrogen), and transformed into *A. tumefaciens* LBA4404 cells by electroporation and used for transgene expression in *N. benthamiana*.

5′ Rapid Amplification of cDNA Ends (RACE)

Sense cDNA was synthesized via 5′ rapid amplification of cDNA ends (RACE) adapter (5′-GCUGAUGGCGAUGAAUGA ACACUGCGUUUGCUGGCUUUGAUGAAA-3′) ligation with a First choice RLM-RACE Kit (Ambion). Antisense cDNA was synthesized by PCR using the 5′ RACE adaptor outer primer following the manufacturer's instructions. The amplified double-stranded cDNA was cloned into the pGEM-T Easy vector (Promega).

Intensive Whitefly Effector Screening via a Cross-Infiltration Assay

Agrobacterium-mediated gene transfer was conducted as described elsewhere with minor modifications (Win et al., 2011). The *A. tumefaciens* GV2260 culture was pelleted by centrifugation for 5 min at 4,000 rpm at room temperature (RT), and the cell pellet was washed three times with distilled water. The cells were re-suspended in Agro-induction medium (10 mM $MgCl_2$, 150 μM acetosyringone, pH 5.6). The concentration of the suspension was adjusted to $OD_{600} = 0.1$ prior to infiltration. To identify effectors from a total cDNA library prepared from whitefly during plant infestation, we reasoned that *Agrobacterium tumefaciens*-mediated transient transformation should delay or totally suppress lesion formation caused by *Pseudomonas syringae* pv. *tabaci* (*Pta*) and *P. syringae* pv. *syringae* (*Psy*) in the overlapping regions of leaves after cross-infiltration. Agro-infiltration assays were performed on the middle leaves of 3-week-old *N. benthamiana* plants. The two *P. syringae* pathovars (*Pta* and *Psy*) were selected on solid King's B medium containing 100 μg/mL rifampicin at 30°C for 2 days, scraped off the plates, and re-suspended in 10 mM $MgCl_2$ (King and Zeevaart, 1974; Song et al., 2015).The negative control was empty vector (pK7WG2). For *Pta* symptom evaluation, leaves were infiltrated with *Pta* ($OD_{600} = 0.01$) 3 days after agro-infiltration. To visualize HR symptoms, leaves were infiltrated with *Psy* ($OD_{600} = 0.1$) 3 days after agro-infiltration. The HR is normally apparent 24 h after infiltration.

P. syringae pv. *tabaci* Pathogenesis Assay

To investigate the impact of the effectors in detail, symptom development and bacterial numbers in local and systemic leaves at day were measured on days 0, 3, and 5 after pathogen challenge (**Figure 2A**). The foliar parts of 3-week-old *N. benthamiana* seedlings were infiltrated with 2 mL of a 10^6–10^7 cfu/mL suspension of *A. tumefaciens*. The positive control was 0.5 mM BTH (Syngenta, Durham, NC, USA), which elicits SAR to bacterial pathogens. The negative control was empty vector. *Pta* was selected on solid King's B medium containing 100 μg/mL rifampicin at 30°C for 2 days, scraped off the plates, and re-suspended in 10 mM $MgCl_2$ (King and Zeevaart, 1974; Song et al., 2015). For *Pta* symptom evaluation, leaves were infiltrated with *Pta* ($OD_{600} = 0.01$) at 3 days after agro-infiltration. To investigate the population size of *Pta* in leaves, *Pta* cells were counted at 0, 3, and 5 days after pathogen inoculation. Leaf discs (1 cm diameter) were ground in 10 mM $MgCl_2$, and serial dilutions of

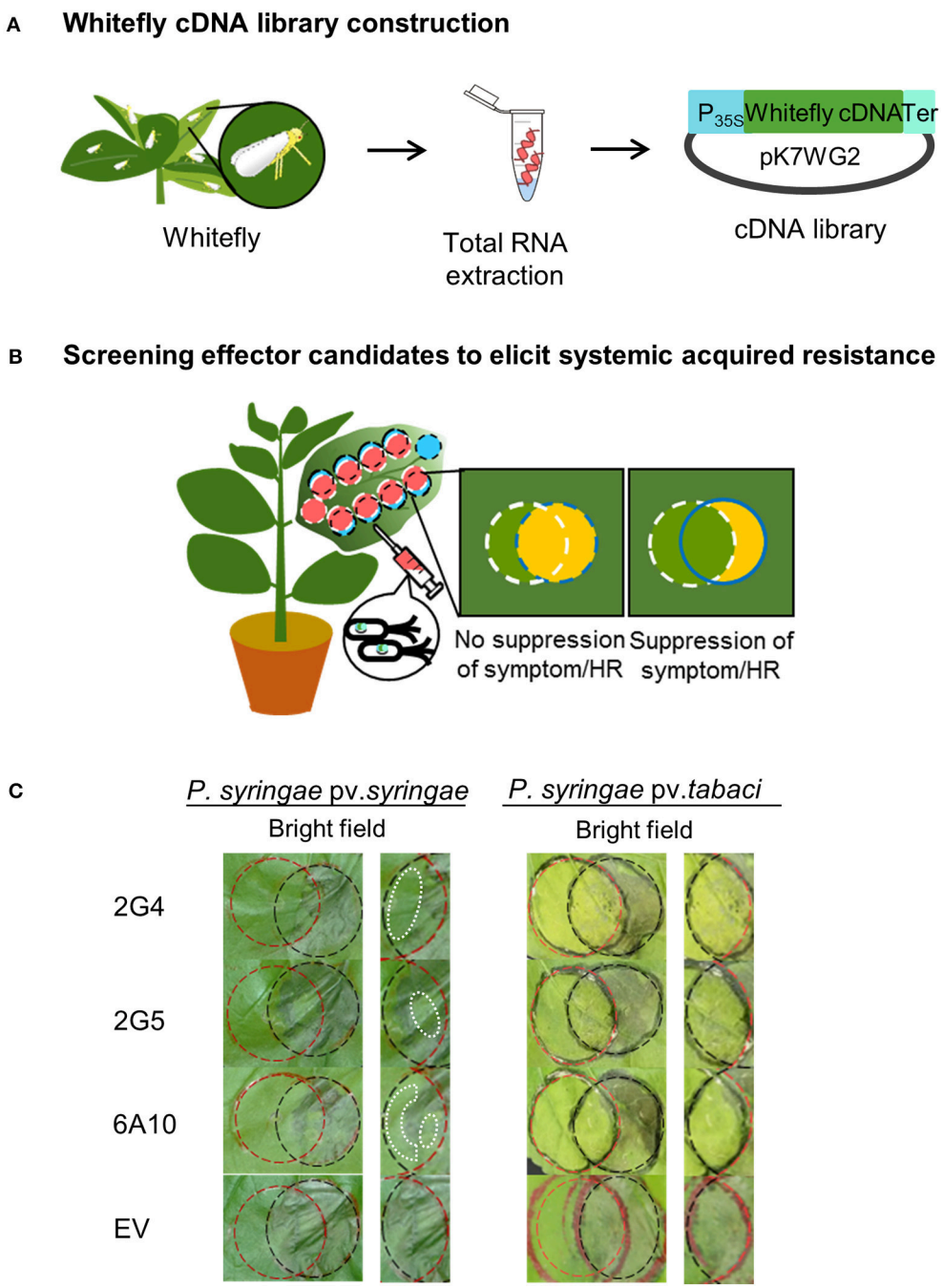


FIGURE 1 | Screening system to identify candidate whitefly effectors that induce plant immunity. **(A)** Whitefly cDNA library construction. Extraction of total RNA from whitefly during infestation of *Nicotiana benthamiana* leaves. The cDNA library from whitefly mRNA was constructed in *Agrobacterium tumefaciens* using a binary vector system. **(B)** Screening candidate effectors that elicit systemic acquired resistance against virulent *Pseudomonas syringae* pv. *tabaci* and avirulent (a non-host pathogen) *Pseudomonas syringae* pv. *syringae*. To identify whitefly effectors that activate plant pathogen immunity, cross-circle infiltration was performed with cDNA clones and pathogens, and the suppression of disease symptoms caused by *Pta* and the hypersensitive response (HR) caused by *Psy* at 1–5 days after pathogen infiltration were evaluated. **(C)** Suppression of the HR and symptoms by whitefly cDNA clones 2G4, 2G5, and 6A10. The white dotted lines in the left panel indicate the suppressed HR. The empty vector (EV) treatment showed no inhibition of the HR and symptom development in the intersecting area. The experiment was repeated three times with similar results.

bacterial solution were spread onto selection medium (King's B agar medium containing 100 μg/mL rifampicin) and incubated for 2 days in a 30°C growth chamber.

R. solanacearum Pathogenesis Assay

Ralstonia solanacearum was grown on Casamino acid-Peptone-Glucose (CPG) at 30°C for 1 day. The *R. solanacearum* culture was pelleted at RT for 5 min at 4,000 rpm and re-suspended in 10 mM $MgCl_2$ (Song et al., 2015). A freshly prepared 50 mL aliquot of *R. solanacearum* suspension at $OD_{600} = 1$ was used to drench the roots of *N. benthamiana* seedlings at 3 days after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector (EV) (Chandrasekaran et al., 2016). The severity of *R. solanacearum* symptoms was scored on a scale of 0–10 as follows: 0, no leaves wilted; 1, 1–5% of leaves wilted; 2, 6–20% of leaves wilted; 3, 21–35% of leaves wilted; 4, 36–50% of leaves wilted; 5, 51–65% of leaves wilted; 6, 66–80% of leaves wilted; 7, 81–95% of leaves wilted; 8, 96–100% of leaves wilted but stems intact; 9, 96–100% of leaves wilted and stems broken; and 10, 100% of leaves wilted and stems broken (Song et al., 2016; **Figure 4A**). The total number of *R. solanacearum* cells in the rhizosphere was counted at 10 days after drench application. Whole roots were collected from each plant without soil particles, placed in a flask containing 200 mL of sterilized distilled water, and incubated with shaking for 30 min at 30°C. The liquid from the flask was serially diluted and spread onto CPG agar plates. To test the effect of the candidate whitefly effectors on plant growth rates, root weights were measured 10 days after pathogen challenge as described previously (Yang et al., 2009). The experiment was repeated three times with 10 replications. Preparation of graphs were performed using the R studio (R-Studio, Boston, MA, USA).

GUS Staining

Histochemical GUS staining was performed as described previously (Brown et al., 2003). Three plants treated with candidate effectors were collected for GUS staining on days 0, 3, 5, and 7. The positive control was 0.5 mM BTH (Syngentay Crop Protection Inc., Greensboro, NC. USA), and the negative control was empty vector. Leaves were immersed in staining solution (2 mM X-Gluc in N,N-dimethyl formamide, 100 mM NaH2PO4, 10 mM Na2EDTA, 0.5 mM ferrocyanide, and 0.1% Triton X-100, pH 7.0) and incubated at 37°C overnight in the dark. The leaves were cleared of chlorophyll by treating them with 70% ethanol after staining at RT for 24 h. Stained samples were observed and photographed with a digital camera (Sony, Park Ridge, NJ, USA).

Extraction of Plant RNA, cDNA Synthesis, and Quantitative RT-PCR

For the leaf pathogen (*P. syringae*) experiment, following agro-infiltration, leaf tissue was collected 0, 12, and 24 h after agro-infiltration and used for total RNA isolation. Following inoculation with *Pta*, the leaf tissue was harvested at 0, 24, and 48 h after inoculation and used for total RNA isolation. Following agro-infiltration, root tissue was collected 0, 1, and 2 days after agro-infiltration and used for total RNA isolation. For the soil-borne pathogen, following inoculation with *R. solanacearum*, root tissue was harvested at 0, 1, and 2 days after inoculation and used for total RNA isolation. To validate candidate effector production in whitefly, whitefly adults were collected from *N. benthamiana* leaves at mid-day and used for total RNA isolation. Total RNA was isolated from tobacco leaf tissues using Trizol (Invitrogen) according to the manufacturer's instructions and as described in our previous study (Lee et al., 2012). First-strand cDNA synthesis was performed with 1 μg of DNase-treated total RNA, oligo-dT primers, and Moloney murine leukemia virus reverse transcriptase (Enzynomics, Daejeon, Korea). The qRT-PCR reaction mixtures consisted of cDNA, iQ™ SYBR® Green Supermix (Bio-Rad Inc., Hercules, CA, USA), and 10 pM each primer. The cycling parameters were as follows: initial polymerase activation for 3 min at 95°C, followed by 45 cycles of 30 s at 95°C, 60 s at 60°C, and 30 s at 72°C. Relative transcript levels were calculated using the $2\text{-}^{\Delta\Delta}$ CT method. The reference genes were *NbACT* mRNA (GenBank accession no. U60489) in tobacco plants, and *BtACT* mRNA (GenBank accession no. AF071908) in whitefly. For convenient comparisons, the expression levels were presented as fold change relative to those of the control (where empty vector or Lac1 is equal to 1). Gene specific primer sequences are listed in Table S1.

RNA Isolation, *De Novo* Assembly and Relative Expression Levels

Whiteflies (*B. tabaci* MEAM1_UvA) were reared on cucumber plants (*Cumumis sativus*, Ventura, RijkZwaan, the Netherlands) in a climatized chamber (Snijders, Tilburg, the Netherlands; 28°C, 16H light 150 μE $m^{-2}s^{-1}$, RH75%) as previously described (Bleeker et al., 2011).

The samples for RNA sequencing were obtained as follows: whitefly eggs and nymphs (1st, 2nd, and 3rd instar) were removed, between 10 a.m. and 6 p.m., from the cucumber leaf using an insect pin and transferred directly into 100% acetone for storage at RT. For whole body ($n = 1$), thorax ($n = 2$), and abdomen ($n = 1$) samples the whiteflies were collected at 9 AM by aspiration and transferred to 100% acetone at RT. The whiteflies were halved with a surgical knife while submerged in 100% acetone to obtain the thorax and abdomen samples. Salivary glands ($n = 1$) and midguts ($n = 1$) were dissected from adult whiteflies as described previously (Kliot et al., 2014).

RNA isolation was performed using the RNeasy mini kit from Qiagen (www.qiagen.com) according to manufacturer's protocol. RNA integrity was examined using the 2200 TapeStation System with Agilent RNA ScreenTapes (Agilent). RNA with RIN values greater than 7.4 were used for Illumina RNA sequencing (www.illumina.com, HiSeq 2000) except for salivary glands (RIN 5.4) and midgut (RIN 5.8). The Illumina reads were cleaned from adapter and ambiguous sequences by Trimmomatic 0.32 software (usadellab.org) (Bolger et al., 2014). The clean reads of thorax, abdomen, eggs and whole body (±20 million per sample) were used for *de novo* assembly using Trinity software (r20140313) [https://github.com/trinityrnaseq/trinityrnaseq/wiki; (Grabherr et al., 2011; Haas et al., 2013)] using the default settings. Trinity (r20140313) was used to realign reads to the *de novo* transcriptome contigs and to calculate the

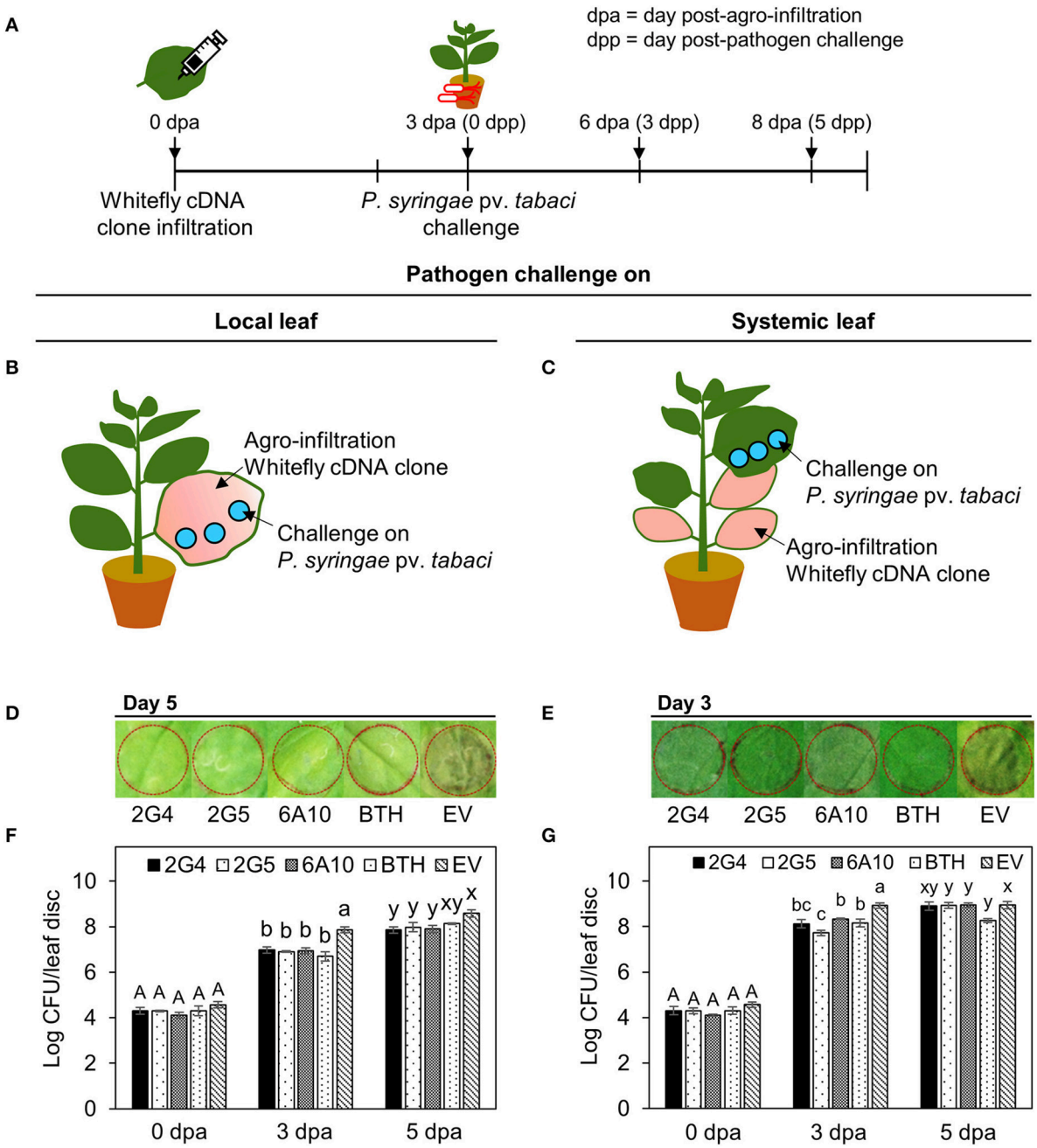


FIGURE 2 | Systemic acquired resistance against *P. syringae* pv. *tabaci* elicited by candidate whitefly effectors on local and systemic tobacco leaves. **(A)** Schematic diagram of the experimental design for investigating SAR against *Pta* after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10. **(B)** SAR against *Pta* in a local tobacco leaf. A suspension of *Pta* at $OD_{600} = 0.001$ was infiltrated into a tobacco leaf that had been pre-infiltrated with whitefly cDNA clones 2G4, 2G5, and 6A10 throughout the leaf at 3 days before pathogen challenge. **(C)** SAR against *Pta* on a systemic tobacco leaf. A suspension of *Pta* at $OD_{600} = 0.001$ was infiltrated into a whole tobacco leaf that had been pre-infiltrated with whitefly cDNA clones 2G4, 2G5, and 6A10 at 3 days before pathogen challenge using a needleless syringe. **(D)** Disease symptom development at 5 days after pathogen challenge on local leaf. **(E)** Disease symptom development at 3 days after pathogen challenge on systemic leaf. **(F)** Bacterial population size of local leaf were measured at 0, 3, and 5 days after *Pta* infiltration with a needleless syringe. **(G)** Bacterial population size of systemic leaf were measured at 0, 3, and 5 days after *Pta* infiltration. Bars represent the mean value ± SEM ($N = 10$). Infiltration with 1 mM BTH and *Agrobacterium* empty vector (EV) suspension was used as a positive and negative control, respectively. Different letters (a, b, and c; x and y) within day indicate statistically significant differences ($P < 0.05$).

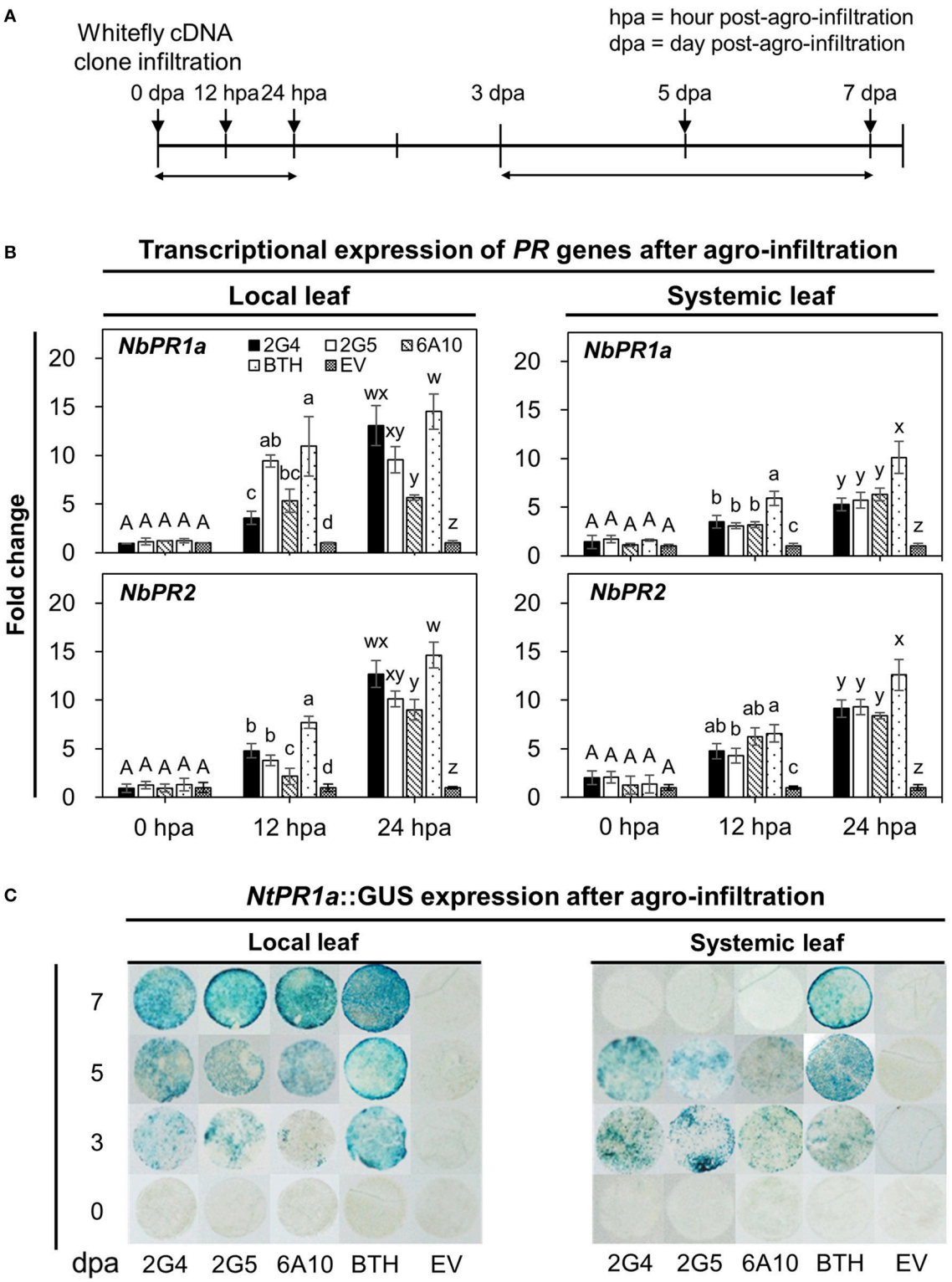


FIGURE 3 | Whitefly effector-mediated early and late expression of SAR marker genes in *N. benthamiana*. **(A)** Schematic diagram of the experimental design for investigating the expression of marker genes (*NbPR1a*, *NbPR2*) and the staining of leaf disc from *NtPR1a::GUS* transgenic tobacco after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector (EV). The samples used for the early gene expression were local and systemic leaves after whitefly cDNA clone leaf-infiltration at 0, 12, and 24 h post-*Agrobacterium* infiltration (hpa). The samples for GUS staining to late expression of a SAR marker gene were leaf disc collected at 3, 5, and 7 days post-*Agrobacterium* infiltration (dpa). **(B)** Quantification of defense priming of SAR marker genes *NbPR1a* and *NbPR2* in plants at 0, 12, and 24 h

(Continued)

FIGURE 3 | after inoculation with whitefly cDNA clones 2G4, 2G5, and 6A10 on local (left panel) and systemic (right panel) leaves by qRT-PCR. Transcription is shown relative to empty vector (expression level = 1) with the *NbActin* gene as an internal reference. Bars represent the mean value ± SEM ($N = 10$). Infiltration with a suspension of 1 mM BTH and *Agrobacterium* with empty vector (EV) was used as a positive and negative control, respectively. Different letters (a, b, c, and d; w, x, y, and z) within hpa indicate statistically significant differences ($P < 0.05$). **(C)** *NtPR1a* gene expression pattern following infiltration with candidate effectors using *NtPR1a::GUS* transgenic *Nicotiana tabacum* plants. GUS staining was conducted by incubating leaf tissues ($N = 30$) in X-gluc solution at 0, 3, 5, and 7 days post-*Agrobacterium* infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector. Infiltration with 1 mM BTH suspension was used as a positive control.

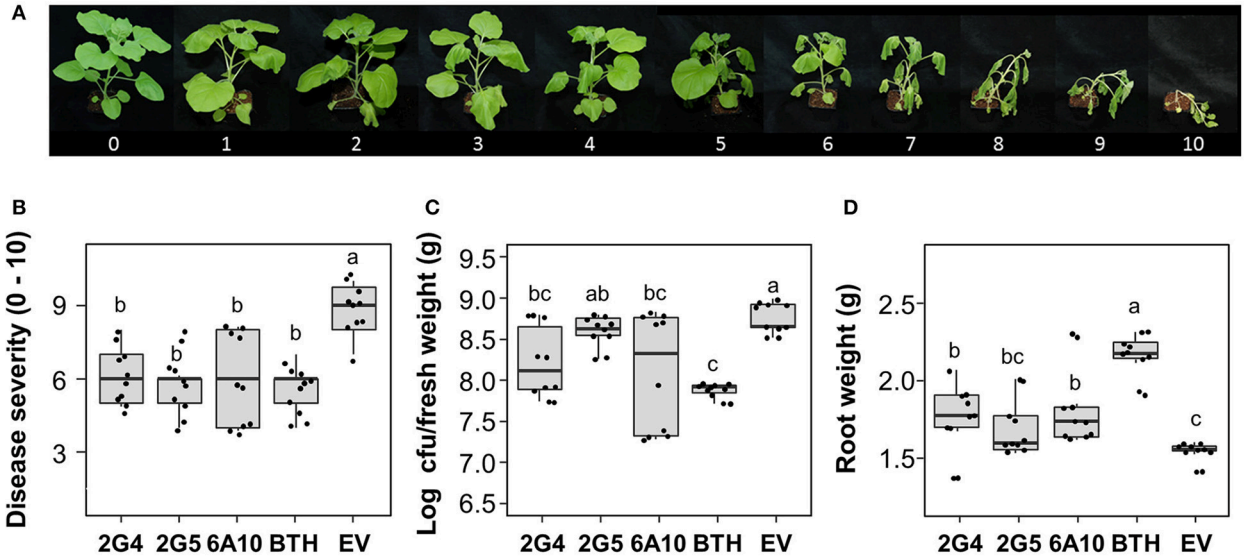


FIGURE 4 | Whitefly effector-mediated SAR against the soil-borne pathogen *Ralstonia solanacearum.* A freshly prepared 50 mL aliquot of *R. solanacearum* suspension at $OD_{600} = 1$ was used to drench the roots of *N. benthamiana* seedlings at 3 days after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector (EV). The disease severity (0–100) was measured at 10 days after pathogen challenge. **(A)** Disease scale (0–10). The disease severity of bacterial wilt caused by *R. solanacearum* was scored from 0 to 10 as follows: 0, no leaves wilted; 1, 1–5% of leaves wilted; 2, 6–20% of leaves wilted; 3, 21–35% of leaves wilted; 4, 36–50% of leaves wilted; 5, 51–65% of leaves wilted; 6, 66–80% of leaves wilted; 7, 81–95% of leaves wilted; 8, 96–100% of leaves wilted but stems intact; 9, 96–100% of leaves wilted and stems broken; and 10, 100% of leaves wilted and stems broken. **(B)** Quantification of disease severity, **(C)** pathogen population size, and **(D)** root fresh weight at 10 days after drench application of an *R. solanacearum* suspension at $OD_{600} = 0.01$ on 3 days after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 and empty vector. Infiltration with 1 mM BTH suspension in tobacco leaves was used as a positive control. Different letters indicate statistically significant differences ($P < 0.05$). Error bars represent mean ± maximum and minimum values ($N = 10$).

fragments per kilobase transcript length per million fragments mapped (FPKM) using RSEM (http://deweylab.github.io/RSEM; Li and Dewey, 2011), after which an normalization (Trimmed Mean of M) was performed across all whitefly samples using the abundance_estimates_to_matrix.pl script (Li and Dewey, 2011; Haas et al., 2013). The RNA-seq data are deposited at the European Nucleotide Archive (https://www.ebi.ac.uk/ena) which is a mirror site of NCBI (the project number: PRJEB26594).

Statistical Analysis

Data were subjected to analysis of variance using JMP software ver. 4.0 (SAS Institute Inc., Cary, NC, USA; www.sas.com). The significance of biological or chemical treatment effects was determined by the magnitude of the F-value at $P = 0{\cdot}05$. When a significant F-value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) test at $P < 0.05$. The results of repeated trials of each experiment outlined above were similar. Hence, one representative trial of each experiment is reported.

RESULTS

High-Throughput System Design and Identification of Potential Effectors

We developed a new screening method to assess the attenuation or suppression of the HR or symptom development caused two *P. syringae* pathovars (*Pta* and *Psy*) in the overlapping regions of *N. benthamiana* leaves after cross-infiltration with candidate whitefly effectors. In the first screening with usingthe two *P. syringae* pathovars in the overlapping regions of leaves after cross-infiltration, we selected 24 and 9 clones after *Pta* and *Psy* infiltration, respectively (**Figures 1A,B**). Of the 893 clones in the cDNA library, we ultimately selected three cDNA clones, 2G4, 2G5, and 6A10, due to their clear suppressive effects on lesion formation (Table S2).

Effector-Mediated Plant Immunity Against the Aboveground Pathogen *P. syringae* pv. *tabaci*

We evaluated whether the three candidate effectors would elicit plant immunity in local or systemic tissues of *N. benthamiana*.

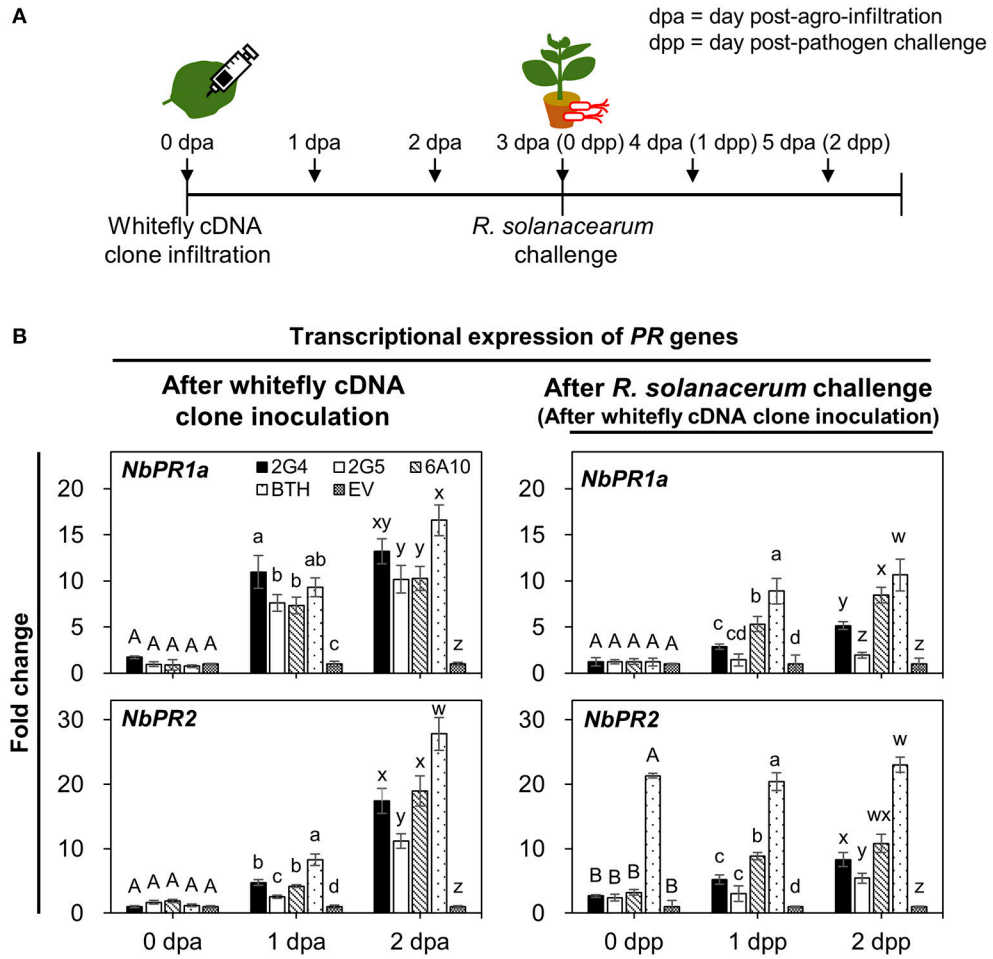
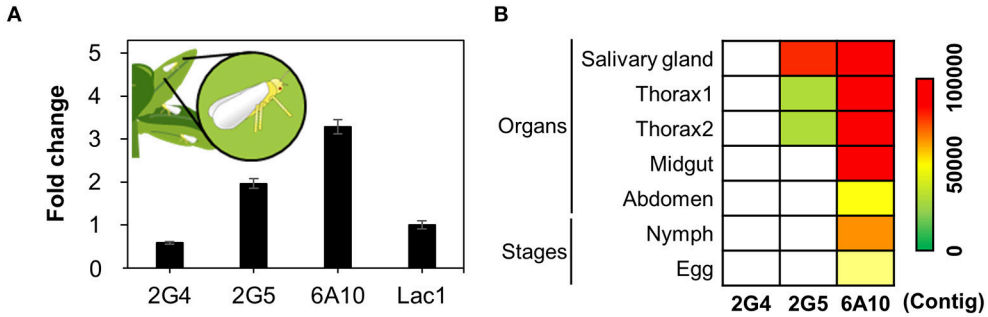


FIGURE 5 | Whitefly effector candidate-mediated defense priming of SAR marker genes in roots after challenge with *Ralstonia solanacearum*. **(A)** Schematic diagram of the experimental design for investigating the expression of SAR marker genes *NbPR1a* and *NbPR2* after leaf infiltration with whitefly cDNA clones 2G4, 2G5, and 6A10 into three whole leaves and on days 0, 1, and 2 after *R. solanacearum* challenge. The samples used to investigate whitefly effector candidate-mediated direct systemic activation of SAR marker genes were root tissues collected after leaf infiltration with whitefly cDNA at 0, 1, and 2 days post-*Agrobacterium* infiltration (dpa). The samples for the defense priming experiment were root tissues collected after pathogen challenge at 0, 1, and 2 day post-pathogen challenge (dpp).
(B) Quantification of the defense priming of SAR marker genes *NbPR1a and NbPR2* in roots at 0, 1, and 2 days after inoculation with whitefly cDNA clones 2G4, 2G5, and 6A10 (left panel) and after challenge with *R. solanacearum* (right panel). Transcription is shown relative to empty vector (expression level = 1) with the *NbActin* gene as an internal reference. Bars represent the mean value ± SEM ($N = 10$). Infiltration with 1 mM BTH and *Agrobacterium* empty vector (EV) suspension was used as a positive and negative control, respectively. Different letters (A, B, a, b, c, and d; w, x, y, and z) within dpa and dpp indicate statistically significant differences ($P < 0.05$).

We infiltrated each whitefly cDNA clone into one half of a leaf and the vector control into the other half. At 3 days after infiltration, we challenged the plants with *Pta* and measured bacterial numbers on days 0, 3, and 5. First, we confirmed the inhibition of symptom development by *Pta* using the overlay method after infiltration of the candidate effectors into whole leaves (**Figures 2B,C**). Plants pretreated with the three candidate whitefly effectors, 2G4, 2G5, and 6A10, showed significantly ($P < 0.05$) fewer (10-fold) bacteria number on days 3 and 5 than the empty vector control in local leaves (**Figure 2F**). On day 5 after pathogen challenge in leaves infiltrated with the three candidate effectors, the number of bacteria was not statistically different among systemic leaves while they differed compared to empty vector control on day 3 (**Figure 2G**), and the number of bacteria was similar to that of the BTH-pretreated positive control in local leaves (**Figures 2F,G**). The symptom in local and systemic leaves at day 5 after pathogen challenge are presented (**Figures 2D,E**).

Candidate Whitefly Effector-Elicited SAR Marker Gene Expression

We evaluated the short- and long-term elicitation of SAR by quantifying the expression of SAR marker genes *NbPR1a* and *NbPR2* at 0, 12, and 24 h and 0, 3, 5, and 7 days after infiltration with the candidate effector cDNAs (**Figures 3A,B**). To quantify the early expression of SAR marker genes, we evaluated the effects of pretreatment with the three candidate effectors, 2G4, 2G5, and 6A10, which induced the early expression of marker genes both locally and systemically (**Figure 3B**). Of the three clones, 2G4 had the strongest effects at 24 h to a level similar with that of positive control BTH treatment in local leaves (**Figure 3B**, left panel),



C

Contig	WF_genome	Annotation	Similarlity (%)
2G5	Bta04337	*Bemisia tabaci* uncharacterized LOC109034554	97.6
6A10	Bta15312	*Bemisia tabaci* large subunit ribosomal RNA	97.0

FIGURE 6 | Validation of candidate whitefly effectors via *in silico* and qRT-PCR analysis. **(A)** Transcript levels of candidate effector 2G4, 2G5, and 6A10 in whitefly feeding on tobacco as determined by qRT-PCR. The whitefly effector Lac1 was used as a positive control. The expression levels were presented as relative values compared to Lac1(expression level = 1). **(B)** Relative expression levels of the candidate effectors 2G4, 2G5, and 6A10 in different organs during whitefly feeding on cucumber. Colors indicate normalized expression levels within the whitefly RNAseq samples of salivary gland, thorax (salivary gland enriched), midgut, abdomen, 1st−3rd instar (nymphs) and eggs. **(C)** Annotation of the three candidate effector 2G4, 2G5, and 6A10. The detailed methodology is described in the Materials and Methods.

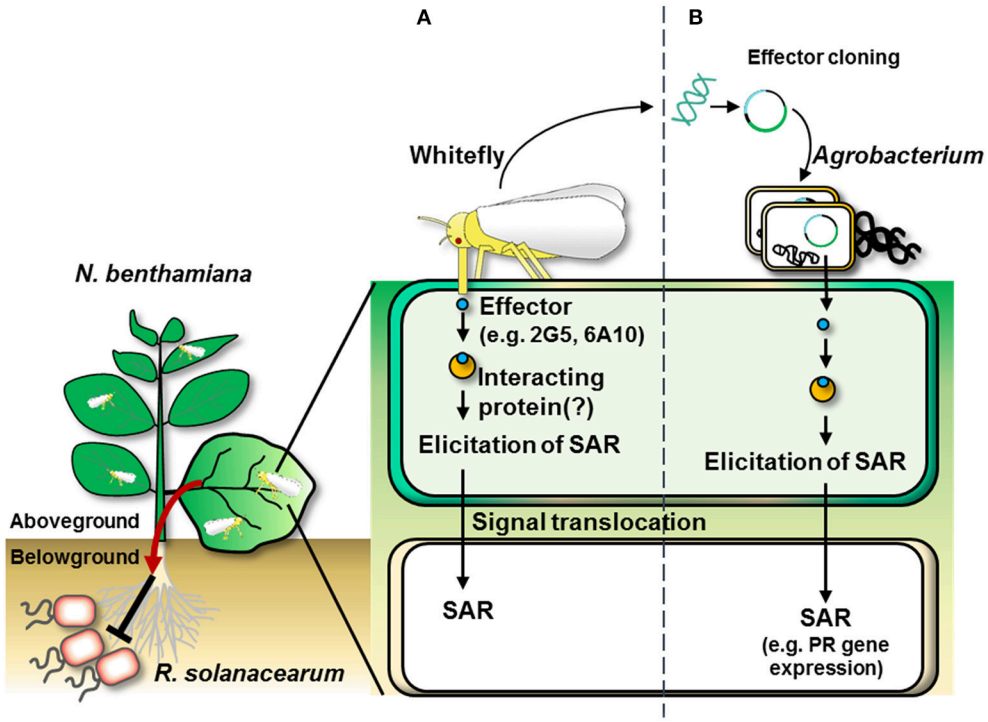


FIGURE 7 | Overall scheme of whitefly effector-induced plant SAR against aboveground and belowground pathogens. **(A)** The infestation of whitefly on the leaf delivers effectors into the plant. The whitefly effectors might interact with plant partner proteins to induce plant systemic immunity (referred to as "systemic acquired resistance") as indicated by the transcriptional activation of pathogenesis-related genes (e.g., *NbPR1a* and *NbPR2*). **(B)** Whitefly effector cloning. *Agrobacterium* -mediated transient expression of cDNAs of candidate effectors derived from total RNA from whitefly elicits SAR in *N. benthamiana*.

while all three clones increased *NbPR2* expression, and clones 2G4 and 6A10 more strongly increased *NbPR1a* expression in systemic leaves compared with the control (**Figure 3B**, right panel). At the same time, the transcriptional level of *NbPR1a* and *NbPR2* on the local leaf infiltrated with clone 2G5 was similar with 6A10 but higher than empty vector control (**Figure 3B**, left panel).

Next, to quantify the long-term expression of SAR marker genes, we evaluated their expression at 0, 3, 5, and 7 days after agro-infiltration. The activation of local and systemic plant immune responses was confirmed by examining the expression of *NtPR1a::GUS*, a representative SAR biomarker gene for plant immunity in tobacco (**Figure 3C**). GUS expression in tobacco leaves infiltrated with the three candidate effectors was first detected on day 3 and reached a maximized level on day 7 in local leaves, whereas the expression of this gene changed little in leaves pretreated with 1 mM BTH on days 3 to 7 (**Figure 3C**, left panel). The systemic expression of *NtPR1a* induced by the three whitefly effectors was detected only on day 3 and 5 but not on day 7 (**Figure 3C**, right panel). Maximum expression of *NtPR1a* in the positive BTH-treated control was detected on day 7 in local leaves and day 5 in systemic leaves (**Figure 3C**).

Whitefly Effector Expression in Aboveground Plant Tissues Activates Immunity Against the Soil-Borne Pathogen *R. solanacearum*

One important characteristic of plant immunity is "defense priming" (Song et al., 2015). Strong defense priming is generally detected at an early time point after pathogen challenge. To evaluate candidate effector-mediated SAR and defense priming against the soil-borne pathogen *R. solanacearum*, we measured bacterial wilt symptoms at 10 days after drench application of a 10^8 cfu/mL *R. solanacearum* suspension at 3 days after leaf infiltration with the three candidate effector cDNAs. Disease severity and the pathogen population were significantly reduced by 2G4 and 6A10 cDNA treatment (**Figures 4B,C**). We detected a 24, 12, and 27% reduction in symptom development in tobacco plants treated by leaf infiltration with 2G4, 2G5, and 6A10 cDNA, respectively (**Figure 4B**). The root fresh weight was 20% higher in plants pre-infiltrated with 6A10 than in the control (**Figure 4D**). By contrast, the bacterial number and root fresh weight in plants treated with 2G5 cDNA did not differ from those in the control (**Figures 4C,D**). However, the bacterial number in root system of plants treated with 2G4 cDNA was statistically lower than control treatment (**Figure 4C**).

To obtain further confirmation of candidate effector-mediated SAR against *R. solanacearum*, we performed two qRT-PCR experiments to evaluate the transcriptional expression of SAR marker genes *NbPR1a* and *NbPR2* in roots on days 0, 1, and 2 after cDNA infiltration and pathogen challenge (**Figures 5A,B**). First, we evaluated transcript levels of the two SAR marker genes after cDNA infiltration without pathogen challenge (**Figure 5B**, left panel). Compared with the control, the expression of *NbPR1a* was significantly different under all treatments on day 1. No significant difference was detected on day 2 across 2G4 and 6A10 treatments (**Figure 5B**, left and above panel). The maximum expression of *NbPR2* was detected in 2G4-, 2G5-, and 6A10-treated plants at 2 days (2 dpa) after leaf infiltration than in the control (**Figure 5B**, left panel). The expression of these genes was not different compared to control at the pathogen inoculation time point (3 dpa and 0 dpp) when *R. solanacearum* drench-applied 3 d after clone infiltration on the leaves (0 d for pathogen challenge in the roots) (**Figure 5B**, right panel).

Second, to evaluate the defense priming of SAR marker genes *NbPR1a* and *NbPR2*, we measured their expression at 0, 1, and 2 days after drench application of *R. solanacearum* on roots (**Figure 5B**, right panel). In plants subjected to leaf infiltration with 2G4 and 6A10 cDNA, *NbPR1a* and *NbPR2* were upregulated compared with the control at 2 days after pathogen challenge (**Figure 5B**, right panel). By contrast, pre-infiltration with 2G5 cDNA did not prime the expression of the two marker genes. The positive control treatment, 1 mM BTH, significantly increased *NbPR1a* and *NbPR2* expression after both direct infiltration and pathogen drench treatment (**Figure 5B**, right panel).

In Silico Analysis of Effector Expression in Whitefly

When whiteflies feed on phloem, they first produce saliva (Jiang et al., 1999; Jiang and Walker, 2003). This saliva, like the saliva of other herbivores, is thought to contain effector molecules produced by the salivary glands (Bos et al., 2010; Villarroel et al., 2016). From the *de novo* assembled RNA-seq data, the contig N50 number, median contig length, average contig length, and total assembled bases were 2445, 448, 1089.85, and 94604597 respectively. To validate candidate effector production in whitefly, we first performed qRT-PCR analysis to measure the expression levels of the three candidate genes from whitefly fed on tobacco (**Figure 6A**). All three candidates were expressed in whole whitefly adults (**Figure 6A**). Second, to confirm expression of the whitefly effectors in salivary glands, we generated RNA sequencing (RNA seq) libraries from whitefly salivary glands, thorax (salivary gland enriched), midgut, abdomen (midgut enriched), nymphs, and eggs from whiteflies collected from cucumber (**Figure 6B**).

Transcripts of 6A10 and 2G5 were detected in both whitelfy salivary gland and thorax tissue, indicating that they might be transferred in to plant tissue (**Figure 6B**). The 2G5 effector shows high expression in salivary glands and thorax compared to midgut, abdomen, nymph and egg. However, 2G4 expression is overall low (**Figures 6A,B**). These data indicate that 6A10 and 2G5 are indeed expressed in salivary glands, pointing to the possibility that they are transferred into plant tissue (**Figure 6B**). However, we did not detect any mRNA of 2G4 in the whitefly organs nor nymph/egg (**Figure 6B**). The candidate effector cDNAs encode proteins annotated as follows: 2G5, an unknown protein, and 6A10, large subunit ribosomal RNA (**Figure 6A**). The expression levels of these genes did not significantly differ from that of the positive control, *Lac1* (**Figure 6A**), encoding a recently identified whitefly effector (Yang et al., 2017). Collectively, of the three clones, only clones 2G5 and 6A10 represent solid candidate whitefly effectors.

DISCUSSION

Our study demonstrates that transient expression of putative whitefly effector cDNA induces plant systemic resistance against the soil-borne pathogen *R. solanacearum*, as well as the air-borne (aboveground) pathogen *P. syringae pv. tabaci (Pta)*, in *N. benthamiana*. These findings add to our previous finding that whitefly infestation elicits plant immunity in the root system through signal transduction from aboveground to belowground plant parts (Yang et al., 2011). Crosstalk between hormone signaling pathways is often detected in plants infested by chewing and phloem-sucking insects. In contrast to JA/ET-dependent signaling elicited by chewing insects, the infestation of Arabidopsis with sucking insects such as whitefly increases the expression of marker genes for the SA-response pathway (Park and Ryu, 2014).

In this study, we designed a new high-throughput screening protocol for isolating putative whitefly effectors that are translocated to the plant and activate systemic plant immunity. We performed *Agrobacterium*-mediated transient transformation of *N. benthamiana* leaf tissues with cDNA prepared from total RNA extracted from whitefly during infestation at mid-day (**Figure 1A**). Cross-inoculation with whitefly cDNA and virulent/avirulent pathogens allowed us to detect the induction of plant resistance responses (**Figures 1B,C**). The three selected candidate effectors suppressed avirulent pathogen-mediated HR responses and virulent pathogen-mediated symptom development. Indeed, we previously demonstrated bacterial effector-induced suppression of both the HR and pathogen-mediated symptom development, and transcriptome analysis revealed that whitefly infestation induced the expression of a large portion of a set of genes for plant immunity in leaves and roots (Park and Ryu, 2014). However, the identity of the determinants from whitefly that elicit SA signaling from leaf to root was previously unclear.

Our current results describing SA-responsive gene expression induced by candidate effectors in leaves are in agreement with previous investigations of SA marker genes in pepper and tobacco (Yang et al., 2011; Song et al., 2015). The induction of SA-responsive genes detected in the current study corresponded to the induction of plant immunity against *Pta* (**Figure 2**). More interestingly, the strength of whitefly effector-mediated SAR was more obvious in local leaves (transiently expressing elicitor) than in systemic (distal) leaves. The infiltration of all three candidate effectors was sufficient to attenuate pathogen growth in the intracellular spaces of local but not systemic leaves on day 5, but bacterial numbers were reduced on day 3 in both local and systemic leaves (**Figure 2**). The results of defense-related gene expression analysis support the differential induction of SAR in local vs. systemic leaves (**Figure 3**). The defense priming of SA-responsive biomarker genes *NbPR1a* and *NbPR2* was weaker in systemic vs. local leaves (**Figure 3A**). More importantly, *NbPR1a* expression was not detected systemically at 7 days after cDNA infiltration in the face of pathogen challenge (**Figure 3B**).

As expected, systemic plant immunity was induced by transient expression of the candidate whitefly effectors. Of the three candidates, 2G5 and 6A10 cDNA suppressed bacterial wilt symptom caused by *R. solanacearum* (**Figure 4B**). This decrease in 2G5 treatment severity could not be explained by defense priming of SA-responsive genes *NbPR1a* and *NbPR2* (**Figure 5B**). Pre-infiltration with 2G5 cDNA did not prime the response of the two SAR marker genes to *R. solanacearum* challenge at 1 dpp in roots (**Figure 5B**). These results might be due to the weak activation of defense priming by clone 2G5 (**Figure 5B**). The induction of SAR by clone 2G5 cannot be dependent of SA signaling but can be dependent some other defense signaling such as jasmonic acid or ethylene signaling (Hase et al., 2008; Baichoo and Jaufeerally-Fakim, 2017; Liu et al., 2017). In contrast, the pre-infiltration with 6A10 cDNA elicited defense priming in similar level of BTH treatment used as a positive control (**Figure 5B**). A previous study also showed strong defense priming when plants activated the SAR response (Song et al., 2015). Overall, these results represent the first demonstration that a single whitefly effector elicits SAR against aboveground and belowground microbial pathogens through systemic signal transduction.

The candidate effector cDNAs are annotated as encoding large subunit ribosomal RNA (6A10) and an unknown protein (2G5). Bioinformatics analysis revealed the presence of 2G5 and 6A10 in whitefly salivary glands. The identification of 6A10 as a large subunit ribosomal RNA of *B. tabaci* deserves further study. There are many examples of the secretion of effector proteins that modulate plant immunity in saliva from hemipterans including aphids and whitefly (Atkins et al., 2011; Will et al., 2013; Sharma et al., 2014; Su et al., 2015; Peng et al., 2016; Villarroel et al., 2016). For instance, the effector proteins C002, Mp1, and Mp2 from aphid promote fecundity, whereas Mp10 and Mp42 decrease fecundity (Bos et al., 2010; Pitino and Hogenhout, 2013).

We do not fully understand how whitefly rRNAs such as 6A10 are translocated to plant cells to elicit SAR. Besides protein effectors, non-protein salivary factors can also act as effectors (Su et al., 2015). Bacterial rRNAs and plant DNA were recently shown to elicit SAR (Bhat and Ryu, 2016; Lee et al., 2016). Destroying the structure of bacterial rRNA via sonication and RNase treatment greatly reduced its effect on inducing SAR, indicating that certain (structural or sequence) signatures of rRNA are required for full SAR elicitation. While this signature has not been identified, bacterial rRNA was successfully detected in plant cells. It appears that the plant recognizes whitefly rRNA as a non-self-molecular pattern. Also, three small RNAs from whitefly were detected in tomato leaf tissue through sequencing tomato phloem RNA from whitefly-infested plants and the nymphs themselves, and the translocation of these RNAs was confirmed using stem-loop qRT-PCR (van Kleeff et al., 2016). The translocation of sRNA has also been observed during plant-fungus interactions. The gray mold fungal pathogen *Botrytis cinerea* delivers its sRNA and suppresses host defense responses through silencing host mRNAs related to defense signaling (Weiberg et al., 2013). Like non-coding sRNAs that function as effectors from whitefly, the non-coding rRNA identified in the current study appears to function as a trigger of SAR, a notion that is currently under investigation.

In conclusion, we revealed a new function for whitefly effectors, i.e., eliciting systemic immunity from aboveground to belowground plant parts. Adding to our previous discovery

of whitefly-mediated SAR against aboveground and soil-borne plant pathogens, in the current study, we demonstrated that treatment with candidate whitefly effectors alone was sufficient to elicit plant immunity against microbial pathogens in local and systemic tissues (**Figures 7A,B**). *In silico* and qRT-PCR analyses confirmed that the candidate effectors were expressed. Both 2G5 and 6A10 were expressed in salivary glands and could be translocated into the host plant, resulting in defense priming of SAR-related marker genes, even in distal tissues. To our knowledge, this is the first report of whitefly effector-mediated induction of plant systemic immunity.

AUTHOR CONTRIBUTIONS

C-MR designed the study. H-RL, SL, and SP performed experiments. PvK performed the RNA-seq sampling and bioinformatic analysis. RC supervised PvK. C-MR contributed to scientific discussions that guided the project's direction. C-MR and H-RL wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00090/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Influence of Belowground Herbivory on the Dynamics of Root and Rhizosphere Microbial Communities

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Recent studies are unraveling the impact of microorganisms from the roots and rhizosphere on interactions between plants and herbivorous insects and are gradually changing our perception of the microorganisms' capacity to affect plant defenses, but the reverse effect has seldom been investigated. Our study aimed at determining how plant herbivory influences the dynamics of root and rhizosphere microbial community assemblages and whether potential changes in root metabolites and chemical elements produced during herbivory can be related to microbial community diversity. We conducted our study on oilseed rape (*Brassica napus*) and its major belowground herbivore, the cabbage root fly (*Delia radicum*). We further assessed the influence of initial soil microbial diversity on these interactions. Different microbial diversities based on a common soil matrix were obtained through a removal-recolonization method. Root and rhizosphere sampling targeted different stages of the herbivore development corresponding to different perturbation intensities. Root bacterial communities were more affected by herbivory than some rhizosphere bacterial phyla and fungal communities, which seemed more resistant to this perturbation. Root herbivory enhanced the phylum of γ-*Proteobacteria* in the roots and rhizosphere, as well as the phylum of *Firmicutes* in the rhizosphere. Herbivory tended to decrease most root amino acids and sugars, and it increased trehalose, indolyl glucosinolates, and sulfur. Higher abundances of four bacterial genera (*Bacillus*, *Paenibacillus*, *Pseudomonas,* and *Stenotrophomonas*) were associated following herbivory to the increase of trehalose and some sulfur-containing compounds. Further research would help to identify the biological functions of the microbial genera impacted by plant infestation and their potential implications in plant defense.

Keywords: *Delia radicum*, *Brassica napus*, soil microbial diversity, rhizosphere and root microbial communities, herbivory, metabolites, chemical elements

INTRODUCTION

Interactions between plants and herbivorous insects are known to influence the evolution of plant defense mechanisms such as defensive and toxic metabolites or volatiles attracting natural enemies of the pest (Fürstenberg-Hägg et al., 2013; Nishida, 2014). Plants constitute an important crossroad in biological interactions since they are also involved in interactions with microorganisms that can improve plant growth, nutrient acquisition, and protection from different plant bioagressors (Richardson et al., 2009; Schnitzer et al., 2011). Recent studies are unraveling the impact of microorganisms from the rhizosphere on plant-insect interactions and are gradually changing our perception of the microorganisms' capacity to affect plant defenses (Pineda et al., 2017). A single strain or a whole microbial community has been shown to increase plant defenses, which in turn can negatively impact insect fitness (Hol et al., 2010; Pangesti et al., 2015a,b; Lachaise et al., 2017). However, the reverse effect has seldom been investigated while herbivore attacks on plants could represent important perturbations for belowground microbial communities.

Perturbations are seen as major drivers of ecosystems stability (Milchunas et al., 1988). Since the 90's, the positive relationship between an ecosystem's stability and its diversity was addressed in different systems and particularly in grasslands facing different abiotic stresses (Ives and Carpenter, 2007). It seemed that the recovery of an ecosystem following a stress could be conditioned by the ecosystem's initial diversity prior to this stress. For example, an ecosystem with high plant diversity that suffers a drought will better recover than an ecosystem with a low plant diversity (Tilman and Downing, 1994; van Ruijven and Berendse, 2010). Effects of such stresses were investigated on resistance (the ability to sustain a perturbation or stress) and resilience (the capacity to come back to a stable state) of different soil processes such as nitrogen and carbon cycles, organic matter decomposition, or respiration (Griffiths and Philippot, 2013). Resistance and resilience of grassland communities toward biotic stresses such as herbivory has also been investigated (see review by Cingolani et al., 2005). However, these studies concerned either grazing mammals or insect herbivory in grasslands, with the latter having variable effects on mycorrhiza (Johnson and Rasmann, 2015). To our knowledge however, this question has never been addressed for microbial communities of agroecosystem plants confronted to insect herbivory.

Insect herbivory can modify plant physiology (Bolton, 2009) and alter metabolite concentrations in plant tissues (Griffiths et al., 1994; Hopkins et al., 1995; Ponzio et al., 2017) as well as nutrient uptake (Katayama et al., 2014; Aziz et al., 2016). It can also modify the release of organic matter by plant roots (rhizodeposition), a major driver of soil microbial communities (Singh et al., 2004; Paterson et al., 2007). Some studies showed that root herbivory can influence plant carbon sources and rhizosphere chemistry, which in turn modify the abundance of bacteria and fungi, and community physiological profiles in the rhizosphere (Grayston et al., 2001; Dawson et al., 2004; Treonis et al., 2005). Katayama et al. (2014) showed that herbivory could also alter chemical element uptakes by the roots as well as nitrogen concentration of microbial origin. So far, when the effect of herbivory on plant-associated microorganisms was covered, the experiments either neglected to take into account the plant metabolites and chemical elements or did not integrate the notion of perturbation dynamics, or cultivable methods and fluorimetry were used to study microbial communities.

Our study aimed at determining the impact of insect herbivory on the plant-microorganism interactions. We hypothesized that (i) the dynamics of root and rhizosphere microbial communities would be influenced by belowground herbivory, (ii) these dynamics would depend on root metabolites and chemical elements induced by herbivory, (iii) the initial soil microbial diversity would influence plant chemistry and hence the dynamics of microbial communities. We conducted our experiment on oilseed rape ("OSR," *Brassica napus*) and its belowground pest, the cabbage root fly ("CRF," *Delia radicum*) (Ahuja et al., 2010). This fly is a specialist of brassicaceous plants. Females lay their eggs aboveground, at the base of plant stems and, upon hatching, which occurs a few days later, larvae feed by tunneling into the roots for 2–3 weeks, before pupating in the nearby soil and emerging. Herbivory of the CRF on brassicaceous plants is known to change the concentration of primary (e.g., sugars such as glucose and sucrose) and secondary metabolites (e.g., indolyl glucosinolates) in the roots (Hopkins et al., 1999; van Dam and Raaijmakers, 2006; Pierre et al., 2012; van Geem et al., 2015). However, nothing is known about how CRF herbivory and resulting biochemical changes in the roots influence the belowground microbial communities which interact with the plant.

MATERIALS AND METHODS

Soil Preparation and Inoculation

A batch of soil was collected in November 2014 from the layer −10 to −30 cm deep of a field in Brittany (La Gruche, Le Rheu, France, 48°08′44″N, 01°47′97″ W) where wheat was cultivated for 20 years, and it was stored in containers at ambient temperature in the dark. After a year of storage in containers, the soil was homogenized, ground, sieved at 4 mm to remove the macrofauna and mixed with 1/3 silica. As described in Lachaise et al. (2017), this mixture was sterilized at 35 kGy and left 2 months to stabilize before inoculation while the remaining unsterilized soil with no silica was ground and sieved at 2 mm before being suspended in water. Following the detailed protocol of Lachaise et al. (2017), this suspension was then undiluted (10^0) or diluted at 10^6 before inoculating the sterilized soil, hence creating the two levels of soil microbial diversity used in our experiment: respectively "high" and "low" initial soil microbial diversities, also referred to as soil microbial modalities (**Figure 1**). This dilution-inoculation method was performed three times in order to obtain three soil biological replicates per soil microbial

Abbreviations: AA, Amino Acid; CPO, Carbohydrate, Polyol, and Organic Acid; CRF, Cabbage Root Fly; DAI, Days After Infestation; dbRDA, distance-based Redundancy Discriminant Analysis; DIABLO, Data Integration Analysis for Biomarker discovery using Latent cOmponents; FDR, False Discovery Rate; GSL, Glucosinolate; OSR, Oilseed Rape; RDA, Redundancy Analysis; SMCSO, S-Methyl Cysteine Sulfoxide.

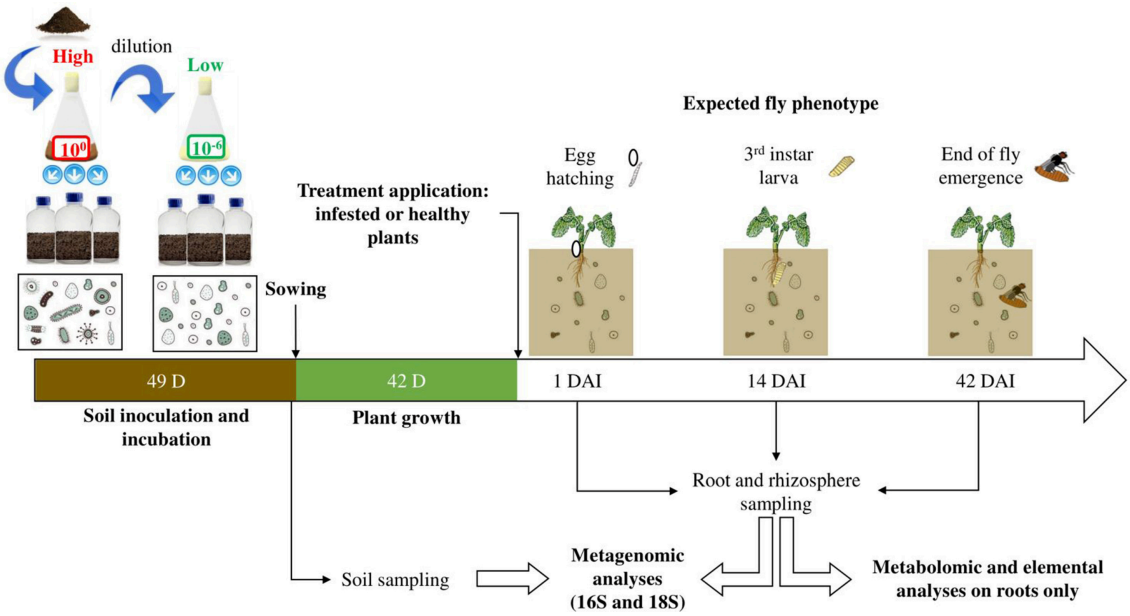


FIGURE 1 | Experimental design. Collected soil was suspended in water and diluted or not, to obtain two solutions of respectively low and high microbial diversities. Sterilized soil was inoculated with one of these two solutions and incubated for 49 days. After sowing, plants were cultivated for 42 days and then two treatments were applied, obtaining healthy and infested plants. The experiment started after the infestation (i.e., *D. radicum* eggs deposited on the plant crown) and sampling was carried out according to the fly biological cycle and important development stages: egg hatching, 3rd instar larva, end of adult emergence, occurring around 1 day after infestation (DAI), 14 and 42 DAI respectively. These sampling times also corresponded to the beginning, the peak and the end of herbivory. Metagenomics, metabolomics and elemental analyses were performed on the obtained samples.

modality. After inoculation, the soil was incubated in the dark for seven weeks at 18°C and 50% humidity. During this period, the bags containing the soil were opened weekly under sterile conditions using a laminar flow cabinet to homogenize the soil and facilitate microbial respiration and recolonization. This allowed the soil to reach optimal bacterial and fungal densities and similar abundances of Colony Forming Units between the two soil microbial modalities at the end of the recolonization period. A part of this soil was collected before sowing to evaluate initial soil microbial diversities ($N = 18$, 9 samples per soil microbial modality).

Insect Rearing

The population of cabbage root fly ("CRF," *Delia radicum*) used in our experiment was collected in the field in 2015 (Le Rheu, Bretagne, France). In the laboratory, flies were fed on sugar, milk powder and yeast (ratio 1:1:1) and they were reared on rutabaga roots (*Brassica napus* subsp. *rapifera*) in a climatic room (16:8 LD, 21 ± 2°C; 60% ± 10% RH) as described in Neveu Bernard-Griffiths (1998). Adult flies were left to oviposit on rutabaga roots for 24h and black-headed eggs (i.e., ready to hatch) were collected 3 days later for our experiment.

Plant Growth, Infestation, and Sampling

Seeds of *Brassica napus* L. (subsp. *oleifera* cv. Tenor) were sown in individual pots using a layer of pozzolan at the bottom and the soil previously obtained (characterized either with a "high" or "low" initial soil microbial diversity). Plants were watered twice a week by sub-irrigation with a nutritive solution based on Hoagland and Arnon (1950) during the whole experiment. This solution was obtained by blending three separate solutions: a macronutrient solution (3 mM of KNO_3, 0.5 mM of KH_2PO_4, 1 mM of $MgSO_4$ $7H_2O$, 2.5 mM of $Ca(NO_3)_2$ $4H_2O$), a micronutrient solution (10 μM of $MnSO_4$ H_2O, 1 μM of $ZnSO_4$ $7H_2O$, 0.5 μM of $CuSO_4$, 30 μM of H_3BO_3, 1 μM of Na_2MoO_4 $2H_2O$, 0.5 μM of $Co(NO_3)_2$ $6H_2O$), and a Fe EDTA solution (27 μM of Fe EDTA Na). Plants were cultivated during 6 weeks in a greenhouse under natural late 2016 winter photoperiod (mean temp. 15.3°C, min/max 5.5/25.6°C). Two treatments were applied on the plants: half of the plants were infested by depositing eight black-headed eggs on the crown per plant, the other half remained untreated. These two batches of plants were then referred to as "infested plants" and "healthy plants" respectively. All plants were then moved to a climatic chamber (photoperiod 16:8 LD and thermoperiod 20:18°C LD) for the rest of the experiment. Afterwards, healthy and infested plants were sampled at 1, 14, and 42 days after infestation ("DAI"), which respectively represented (i) the hatching stage which corresponds to the initiation of herbivory, (ii) the third larval instar which corresponds to the peak of herbivory, and (iii) the end of the fly emergence which corresponds to the end of herbivory (**Figure 1**). A total of 108 plants were harvested, with 9 plants per condition (2 soil microbial modalities, 2 treatments being healthy and infested, and 3 sampling times). Roots and rhizosphere were sampled as follows: the root fraction was collected, corresponding to the area from the crown to the

tip roots, and was washed twice in 20 mL of sterile permuted water before being transferred to a clean Falcon tube; the whole root bath (i.e., 40 mL in a Falcon tube) corresponded to the rhizosphere fraction; both tubes were immersed in liquid nitrogen and stored at −80°C before being freeze-dried; only roots were ground using glass beads. In order to have sufficient material for molecular, metabolomic and elemental analyses, roots from three different plants of the same treatment, cultivated on the same soil microbial diversity and biological replicates, were pooled to make one sample, hence obtaining a total of 36 root samples ($N = 3$ samples per condition). The rhizosphere fraction ($N = 36$ rhizosphere samples) was treated similarly.

Molecular and Bioinformatic Analysis

Initial bulk soil (i.e., before sowing), as well as root and rhizosphere samples collected during the experiment, were analyzed.

According to the protocol developed by the GenoSol platform (Dijon, France) and described in Plassart et al. (2012), soil DNA was extracted from 2 g of wet bulk soil (i.e., initial soil before sowing) or 1 g of freeze-dried rhizosphere respectively in 8 or 5 mL of lysis buffer containing 100 mM of Tris-HCl (pH 8), 100 mM of EDTA (pH 8), 100 mM of NaCl, 2% SDS, and ultrapure water. The following modifications were performed: tubes were vortexed at mid and at the end of incubation (i.e., bath at 70°C), then centrifuged at 3,500 rpm at 15°C for 10 min; during deproteinization, samples were centrifuged at 14,000 g at 4°C during 10 min; during DNA precipitation, tubes were placed at −20°C for 30 min. To obtain DNA pellets, tubes were centrifuged at 13,000 rpm at 4°C for 30 min and supernatant was discarded. DNA pellets were washed as follows: 400 μL of 70% ice-cold ethanol were added to samples, which were centrifuged at 13,000 rpm at 4°C for 5 min and supernatants were removed. Remaining traces of ethanol were removed by heating open tubes at 60°C for at least 15 min or more if needed. Pellets of DNA were resuspended with 100 μL of ultrapure water and the duplicated samples were finally pooled. Bulk soil and rhizosphere samples were purified twice. The first purification required Microbiospin (Biorad, Hercules, California, USA) columns of PVPP (PolyVinyl PolyPirrolidone, Sigma-Aldrich). To prepare the columns, their tips were removed and columns were placed in 2 mL Eppendorf tubes. Then, columns were washed with 400 μL of ultrapure water and centrifuged at 1,000 g and at 10°C during 2 min. After emptying the tubes, the previous step was carried out a second time, before centrifuging empty tubes at 1,000 g at 10°C for 4 min. Following Plassart et al. (2012), 100 μL of DNA were injected into the columns, previously transferred to a clean tube, however our samples were incubated on ice for 5 min, before a 4 min centrifugation at 1,000 g at 4°C. The obtained DNA (∼95 μL) was used for the second purification, performed using the Geneclean® Turbo kit (MP Biomedicals) with the following modifications: samples were centrifuged at 10,000 g for 10 s; after adding the GTE (GeneClean Turbo Elution Solution), samples were incubated on ice for 5 min and centrifuged at 10,000 g for 1 min; the GTE, incubation and centrifugation steps were repeated a second time, to finally obtain ∼60 μL of clean DNA.

Root DNA was extracted using the NucleoSpin® Plant II kit and protocol (Macherey-Nagel, Düren, Germany) with the following modifications: 30 g of freeze-dried root powder were used; (i) cell lysis was done with buffer PL1; (ii) incubation after adding RNase A lasted 30 min; (iii) the crude lysate was centrifuged before its filtration.

DNA quantification was performed using a Quantus fluorometer (Promega, Madison, Wisconsin, USA) and the Quantifluor kit (dsDNA: E2670).

PCR amplification and sequencing were performed at the GenoScreen platform (Lille, France) using the Illumina Miseq platform to a 2 × 250 bases paired-end version. We used PCR primer pairs 799F (5′-AACMGGATTAGATACCCKG-3′) and 1223R (5′-CCATTGTAGTACGTGTGTA-3′), and NS22B (5′-AATTAAGCAGACAAATCACT-3′) and SSU0817 (5′-TTAGCA TGGAATAATRRAATAGGA-3′) (Lê Van et al., 2017) to amplify 16S and 18S rDNA genes, respectively.

To manage mismatch between reads 1 and 2 in the overlap region, we used bases trimming at Q30 with PRINSeq, trimming of specific primers with Cutadapt, assembly with FLASH starting with trimmed reads 1 and 2, a minimum of 30 bases overlapping was required with a 97% homology between reads. Regarding 18S rDNA, only read 1 was analyzed because i) amplicon size did not allow a sufficient overlapping area between read 1 and read 2 and ii) read 1 was of better quality. The GnS-PIPE bioinformatical pipeline was used for the bioinformatical analyses of 16S rDNA and 18S rDNA (Terrat et al., 2012). Raw data sets were deposited on the European Nucleotide Archive database system under the project number (PRJEB25217). Bulk soil samples accession numbers range from ERS2281263 to ERS2281298, those of root and rhizosphere samples range from ERS2255945 to ERS2256016 for 16S rDNA and from ERS2256770 to ERS2256841 for 18S rDNA.

Metabolites and Chemical Elements Analysis

Primary Metabolites: Amino Acids, Carbohydrates, Polyols, and Organic Acids

Quantification of free amino acids (AAs), non-structural carbohydrates, polyols, and organic acids (CPOs) was based on the method described by Gravot et al. (2010) and performed using 9 to 12 mg of freeze-dried root powder, with the same methanol-chloroform-water-based extraction. Minor adjustments were made to this protocol: after adding 100% chloroform, tubes were rapidly vortexed and then agitated for 10 min at room temperature; after adding water, samples were vortexed for 20 s and centrifuged for 5 min at 12,000 g and 15°C.

For AA derivatization and profiling, 50 μL of methanol-water extract were vacuum-dried. The dry residue was resuspended in 50 μL of ultrapure water and the tubes were rapidly vortexed, put in an ultrasonic bath for 5 min and centrifuged for 5 s at room temperature. Derivatization of AAs was performed using the AccQTag Ultra Derivatization kit (Waters) with the following modified volumes: 5 μL of the resuspended sample, 35 μL of AccQTag Ultra Borate Buffer and 10 μL of AccQTag Reagent were placed in a new tube, which was vortexed and placed

in a water bath for 10 min at 55°C. The whole volume was transferred in a vial and derivatizated AAs were analyzed using liquid chromatography (Acquity UPLC-DAD system, Waters, Milford, MA, USA) according to Jubault et al. (2008). However, the column used for analyses was heated at 53°C and AAs were detected at 265 nm using a photodiode array detector. Identification of AAs was realized by comparison with a standard solution and quantification was made thanks to the internal standard BABA (3-aminobutyric acid).

Analysis of CPOs was performed by gas chromatography coupled to a flame ionization detector (GC-FID, Thermo Fisher Scientific, Waltham, MA, USA) and based on the protocol described by Lugan et al. (2009), which however required several modifications. The online derivatization for CPOs was performed with a Trace 1300 GC-FID (Thermo Scientific) equipped with a Tri Plus RSH (Thermo Scientific). Fifty microliters of the methanol-water extract were sampled in injection vials and vacuum-dried. This online derivatization was performed as follows: the dried extract was resuspended in 50 μL of pyridine containing 20 $mg.mL^{-1}$ of methoxyaminehydrochloride, under orbital shaking at 40°C for 90 min. Fifty microliters of MSTFA (N-methyl-trimethylsilyltrifluoroacetamide) were added before incubation at 40°C for 30 min. One microliter of the mixture was injected into the GC-FID with a split/splitless injector (split mode set to 1:20) at 260°C, on a TG-5MS column (30 m × 0.32 × 0.25 mm, Agilent Technologies) connected to a flame ionization detector at 300°C. The temperature gradient of the GC oven was: 4 min at 100°C followed by an increase of 10°$C.min^{-1}$ up to 198°C and maintained at this temperature for 2 min; an increase of 1°$C.min^{-1}$ up to 202°C; then an increase of 15°$C.min^{-1}$ ramp up to 268°C and held for 3 min followed by an increase of 1°$C.min^{-1}$ up to 272°C and raised to 210°C at 10°$C.min^{-1}$ maintained for 7 min. Identification of CPOs was realized by comparison with a standard solution, while quantification was achieved with adonitol as the internal standard.

Secondary Metabolites: Glucosinolates

Extraction and analysis of glucosinolates (GSLs) were performed based on the protocol from Hervé et al. (2014) with the following modifications: GSLs were extracted from 12 to 15 mg of freeze-dried root powder and tubes were centrifuged for sedimentation at 12,000 g and 15°C for 5 min. Analysis of GSLs was performed using liquid chromatography coupled with mass spectrometry (Acquity UPLC-TQD, Waters) with electrospray ionization in a negative mode. Chromatographic conditions, A and B-eluents and the gradient used are described in Hervé et al. (2014). Quantification of GSLs was realized using an external calibration with a standard solution containing glucoerucin, gluconasturtiin, and glucobrassicin in the range of 4 to 80 $\mu mol.L^{-1}$. These compounds were respectively used to quantify aliphatic, aromatic and indolyl GSLs.

Chemical Elements

Analysis of root chemical elements composition was performed according to Maillard et al. (2015). About 4 mg of freeze-dried root powder were used to measure total N and S contents, using a continuous flow isotope mass spectrometer (Nu Instrument, Wrexham, United Kingdom) linked to a C/N/S analyzer (EA3000, Euro Vector, Milan, Italy). For other elements such as K, Ca, P, Mg, Fe, Mn, Zn, Cu, Mo, Ni, and B, roots samples were submitted to microwave acid sample digestion (Multiwave ECO, Anton Paar, les Ulis, France) using 800 μL of concentrated HNO_3, 200 μL of H_2O_2 and 1 mL of Milli-Q water for 40 mg of root powder. All samples were previously spiked with two internal-standard solutions of Gallium and Rhodium, respectively, for a final concentration of 10 and 2 $\mu g.L^{-1}$. Mineralized samples were then diluted to 50 mL with Milli-Q water to obtain solutions containing 2.0% (v/v) of nitric acid, then filtered at 0.45 μm using a teflon filtration system (Filtermate, Courtage Analyses Services, Mont-Saint-Aignan, France). Samples were then analyzed by High Resolution Inductively Coupled Plasma Mass Spectrometry (HR ICP-MS, Thermo Scientific, Element 2^{TM}) and quantification of each element was performed using external standard calibration curves. Additional information about mineralization and the utilization of HR ICP-MS can be found in the annexes of Maillard (2016).

Statistical Analyses

Analyses were performed using the R software (R Core Team, 2016) and a 5% threshold for statistical significance.

Microbial Communities

When analyses were performed on microbial data, some samples had to be removed from the dataset for the following reasons: one root sample was lost before sequencing (from the low diversity-healthy plants-14 DAI condition), one rhizosphere bacterial sample (from the high diversity-healthy plants-1 DAI condition) showed an abnormal smaller total read count after normalization while one root fungal sample (from the high diversity-healthy plants-1 DAI condition) had abnormal phyla abundances compared to other samples.

Bacterial and fungal richnesses and diversities, represented by the number of observed Operational Taxonomic Units (OTUs) and the Shannon index (obtained with the diversity function in the "vegan" package) respectively, were obtained from non-rarefied OTU data and analyzed in roots and rhizosphere separately using a linear model. Models took into account the following factors: sampling time (1, 14, and 42 DAI), plant treatment (healthy vs. infested plants), soil microbial diversity (high vs. low diversity), and soil replicate, as well as paired interactions between the three first factors. Linear models were adjusted depending on the significance of interactions: interactions were all removed if none was significant while they were kept if at least one was significant or close, which was respectively the case of analyses on the rhizosphere and root compartments. A type II analysis of variance table was performed on the models, followed by comparisons based on least-squares means when possible.

In order to analyze the bacterial and fungal community structure, distance-based redundancy discriminant analysis (dbRDA, dbrda function in the "vegan" package) was performed on a Bray-Curtis dissimilarity matrix, obtained from OTU data,

which were filtered using a 1‰ threshold and log2-transformed. A type II permutation test for constrained multivariate analyses was performed on the dbRDA using the "RVAideMemoire" package (Hervé, 2016a,b) to evaluate the contribution of each factor (i.e., compartment, time, treatment, soil microbial diversity, and soil biological replicates) to microbial community structure. Quantified contributions expressed as r-squared, were obtained from the varpart function ("vegan" package).

Phyla analyses were performed according to the scripts provided by Bulgarelli et al. (2015) but *P*-values were corrected using the False Discovery Rate (FDR) method. Plotting was done with the "ggplot2" package.

Genewise negative binomial generalized linear models were conducted using the "edgeR" package (Anders et al., 2013) on filtered data to recover genera differences between plant treatments within sampling time. The obtained *P*-values were corrected with the FDR method. Using "ggplot2" package, genus count differences between infested and healthy (Xi – Xh) were plotted when the treatment was significant on one hand and when these differences exceeded 250 or −250 counts.

Metabolites and Chemical Elements

For each sampling time, two different analyses were performed on metabolites and chemical elements, taking into account the plant treatment (healthy vs. infested plants), the soil microbial diversity (high vs. low diversity), and the interaction between both factors, as well as soil biological replicates. Both analyses required the data to be transformed using the fourth root. First, a redundancy analysis (RDA), associated to a permutation significance test based on cross-validation, was performed to determine the influence of the previously mentioned factors on the root metabolomic and elemental profiles. Second, linear models were used to assess precisely which metabolites and elements were affected by the above factors. Finally, a type II analysis of variance was performed on the linear models and the obtained *P*-values were corrected with the FDR method. The content differences between infested and healthy (Xi – Xh) was plotted.

Relationships Between Root Microbial Communities and Root Chemistry

To assess the relationship between root microbial communities and root chemistry under herbivory, we applied the DIABLO (Data Integration Analysis for Biomarker discovery using Latent cOmponents) method on our data with the plant treatment (i.e., infested vs. healthy plants) defined as the grouping factor, using the scripts provided by Hervé et al. (2018) and two R packages ("mixOmics and "RVAideMemoire"). Only metabolites or chemical elements significantly impacted by the treatment at a given time were kept for this analysis at this same given time. Bacterial and fungal genera used in this analysis were also significantly impacted by the plant treatment and bacterial count differences respected the 250/−250 threshold mentioned above. Hence, a three block-DIABLO was performed at 14 DAI on one hand, using three datasets (i.e., absolute abundance (expressed in counts of OTUs) of microbial genera, metabolites, and chemical elements) while a two block-DIABLO was performed at 42 DAI on the other hand with two datasets: microbial genera and combined metabolites and elements. In the latter case, an analysis on three blocks was not possible using a single column dataset for chemical elements, which was why it was included in the metabolite dataset. Discrimination of the grouping factor was assessed with a permutation significance test based on cross-validation.

RESULTS

Our experiment was based on two soils of different bacterial and fungal alpha-diversities (Table S1) and phyla abundances (Figure S1).

Diversity and Structure of Microbial Communities

After 6 weeks of OSR culture in soils of different microbial diversities and 1 DAI, the bacterial and fungal alpha-diversities were similar in both root and rhizosphere compartments.

Bacteria

Root bacterial richness and diversity were significantly impacted by sampling time, initial soil microbial diversity and the interaction between time and treatment (**Table 1**). Diversity was also influenced by the interaction between time and initial soil microbial diversity.

At high initial soil microbial diversity, the bacterial richness (observed OTUs) of healthy plants increased with time, reaching a peak of 900 observed OTUs at 14 DAI, and then decreased at 42 DAI (**Figure 2A**). Herbivory reversed this trend and richness was significantly increased at 42 DAI. Richness was significantly different between healthy and infested plants both at 14 and 42 DAI. Root bacterial diversity (Shannon index) had a similar profile at high and low initial diversities whether or not plants were infested. The Shannon index was significantly lower in infested plants than in healthy plants at 14 DAI but it was higher at 42 DAI (**Figure 2A**).

At low initial soil microbial diversity, the bacterial richness (observed OTUs) of healthy plants did not change with time but increased significantly after herbivory (i.e., 42 DAI) (**Figure 2A**). At this low initial soil diversity, root bacterial diversity (Shannon index) increased in healthy plants at 14 DAI and remained stable at 42 DAI while infested plants displayed an opposite profile (Shannon index decreased at 14 DAI and increased at 42 DAI).

Rhizosphere bacterial richness and diversity were both influenced by sampling time and initial soil microbial diversity (**Table 1**, **Figure 2A**). Richness and Shannon index decreased over time in both healthy and infested plants, the latter being similar between 14 and 42 DAI. These indices showed higher values in plants grown on high soil microbial diversity than on low soil diversity.

A dbRDA confirmed that bacterial community structure was driven by compartment, time, treatment and initial soil microbial diversity (**Figure 3A**). Differences in bacterial community structures between healthy and infested plants were highly significant.

TABLE 1 | Statistical output of bacterial and fungal alpha-diversity indices in the root and rhizosphere compartments, associated to **Figure 2**.

		Factors	Roots			Rhizosphere		
			***F*-value**	**df**	***P*-value**	***F*-value**	**df**	***P*-value**
Bacteria	Richness (observed OTUs)	Sampling time	4.61	2	**0.020**	11.40	2	**<0.001**
		Treatment	0.80	1	0.370	0.16	1	0.680
		Soil microbial diversity	32.90	1	**<0.001**	95.80	1	**<0.001**
		Soil biological replicates	1.58	2	0.220	1.57	2	0.220
		Sampling time:Treatment	19.10	2	**<0.001**	_	_	_
		Sampling time:Soil microbial diversity	4.07	2	**0.030**	_	_	_
		Treatment:Soil microbial diversity	0.35	1	0.550	_	_	_
	Diversity (Shannon index)	Sampling time	9.34	2	**0.001**	4.94	2	**0.014**
		Treatment	0.006	1	0.930	4.06	1	0.053
		Soil microbial diversity	20.80	1	**<0.001**	39.80	1	**<0.001**
		Soil biological replicates	2.04	2	0.150	1.28	2	0.290
		Sampling time:Treatment	22.10	2	**<0.001**	_	_	_
		Sampling time:Soil microbial diversity	1.23	2	0.300	_	_	_
		Treatment:Soil microbial diversity	0.03	1	0.850	_	_	_
Fungi	Richness (observed OTUs)	Sampling time	9.19	2	**0.001**	1.50	2	0.230
		Treatment	0.07	1	0.780	0.56	1	0.450
		Soil microbial diversity	4.27	1	0.050	27.70	1	**<0.001**
		Soil biological replicates	0.91	2	0.410	3.58	2	**0.040**
		Sampling time:Treatment	3.28	2	0.056	_	_	_
		Sampling time:Soil microbial diversity	0.09	2	0.900	_	_	_
		Treatment:Soil microbial diversity	1.10	1	0.300	_	_	_
	Diversity (Shannon index)	Sampling time	9.25	2	**0.001**	4.20	2	**0.024**
		Treatment	0.70	1	0.410	2.34	1	0.130
		Soil microbial diversity	4.06	1	0.056	6.38	1	**0.017**
		Soil biological replicates	1.24	2	0.300	1.68	2	0.200
		Sampling time:Treatment	3.71	2	**0.040**	_	_	_
		Sampling time:Soil microbial diversity	0.05	2	0.940	_	_	_
		Treatment:Soil microbial diversity	0.88	1	0.350	_	_	_

The above table presents the outcome of variance analyses (F test) performed on linear models, based on richness (i.e., observed OTUs) or diversity (Shannon index) of each compartment, and taking into account sampling time (1, 14, and 42 days after infestation "DAI"), treatment (healthy vs. infested plants), soil microbial diversity (high vs. low soil diversity) and 2 × 2 interactions between these factors, as well as soil replicates. F-value, degrees of freedom (df), and P-value (in bold when significant) are given for each tested factor or interaction. Dashes indicate that all interactions in the rhizosphere were non-significant and have thus been removed from the models.

Fungi

Root fungal richness was four times lower than root bacterial richness and ranged from 150 to 300 observed OTUs. It was only influenced by sampling time (**Table 1**). The richness of healthy and infested plants at both soil diversities similarly increased over time and reached 250 OTUs at 42 DAI (**Figure 2B**).

Root fungal diversity (Shannon index) was impacted by time and by the interaction between time and treatment but not by soil microbial diversity (**Table 1**). Both richness of healthy and infested plants increased over time but the Shannon index was higher in infested plants at 14 DAI, hence during the peak of herbivory (**Figure 2B**).

Rhizosphere fungal richness was similar to root richness, with 250 to 350 observed OTUs, and it did not vary as much as bacterial richness. Rhizosphere fungal richness was impacted by initial soil microbial diversity and by the soil biological replicates (**Table 1**). Richnesses of healthy and infested plants remained similarly stable over time (**Figure 2B**). Richness was greater in plants grown on high soil microbial diversity.

Rhizosphere fungal diversity was influenced by both sampling time and initial soil microbial diversity (**Table 1**). The Shannon index of healthy and infested plants increased similarly over time and diversity was greater in plants grown on high soil microbial diversity (**Figure 2B**).

Fungal community structure drivers were compartment, time, and treatment, which were highly significant, as well as initial soil microbial diversity (dbRDA, **Figure 3B**).

Identification of Microbial Phyla and Genera Associated With Herbivory and Initial Soil Microbial Diversity

Relative Abundance of Microbial Phyla

The roots (**Figure 4A**) and rhizosphere (**Figure 4B**) contained four major bacterial phyla, of which *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the most abundant. The root bacterial

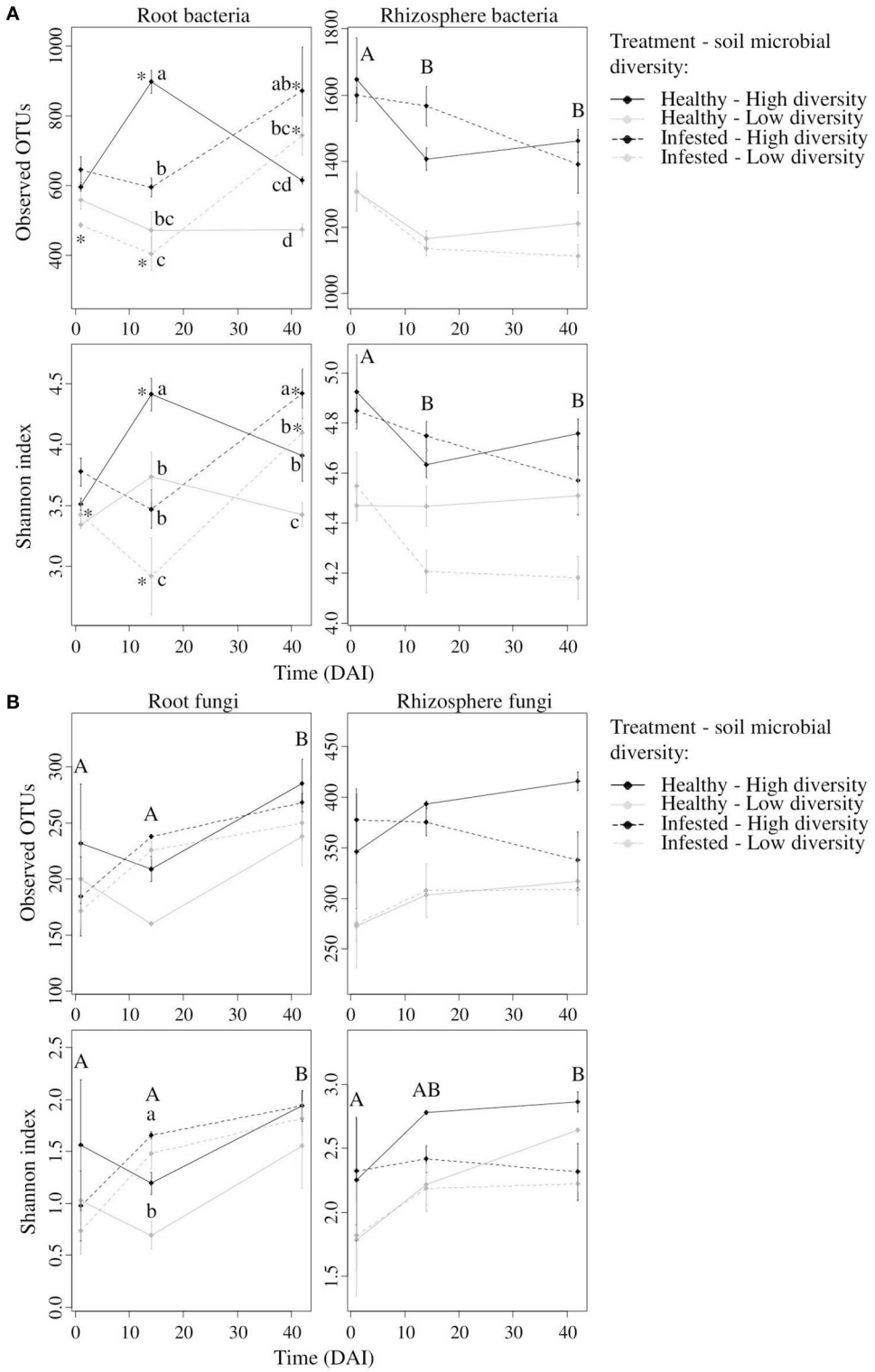
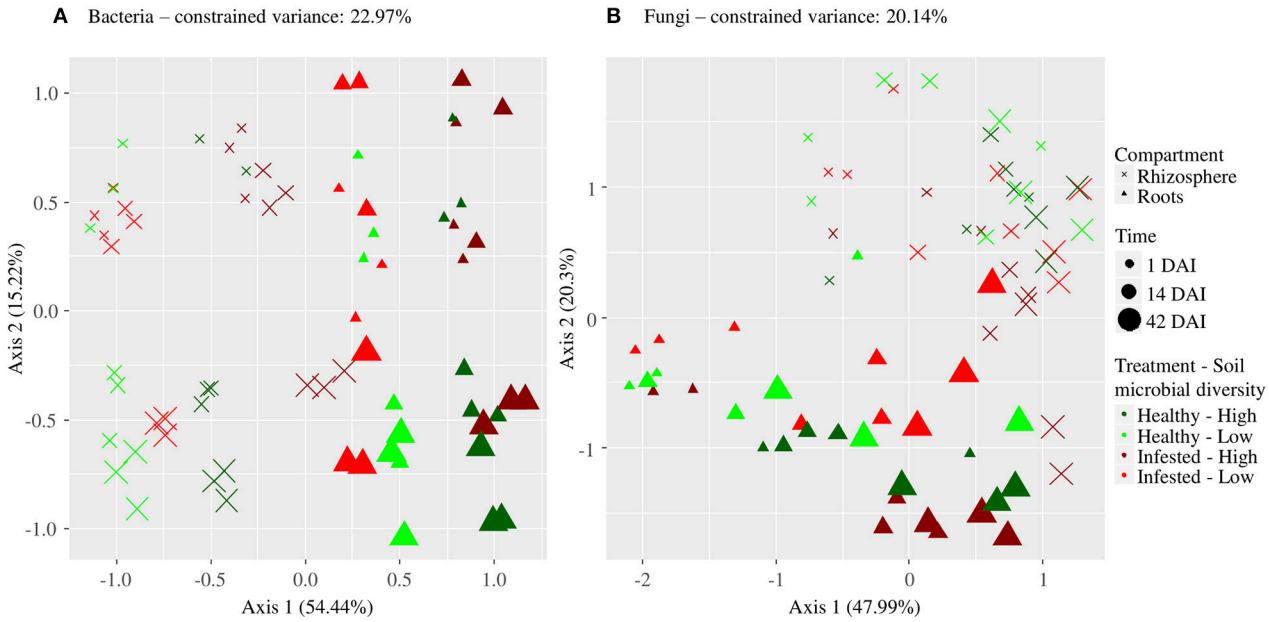


FIGURE 2 | Bacterial and fungal alpha-diversity indices in the root and rhizosphere compartments. Mean value (± se) of bacterial **(A)** and fungal **(B)** richness (i.e., Observed OTUs) and diversity (i.e., Shannon index) for the root (left column) and rhizosphere (right column) compartments at 1, 14, and 42 days after infestation (DAI). In the root compartment, lowercase letters represent significant differences between modalities at one given time while asterisks represent significant differences between times for one given modality. Regarding the rhizosphere compartment, capital letters represent significant differences between times for all modalities.



Factor	Variation	*F*-value	df	*P*-value
Compartment	3.34	2.81	1	**0.011**
Time	2.92	1.80	2	**0.030**
Treatment	13.5	8.38	1	**0.001**
Soil microbial diversity	2.51	2.34	1	**0.025**
Soil biological replicates	_	0.67	2	0.912

Factor	Variation	*F*-value	df	*P*-value
Compartment	7.44	3.84	1	**0.003**
Time	7.02	2.34	2	**0.033**
Treatment	7.22	3.69	1	**0.001**
Soil microbial diversity	3.06	2.17	1	**0.018**
Soil biological replicates	_	0.89	2	0.902

FIGURE 3 | Driving factors of bacterial **(A)** and fungal **(B)** community structure. Presented for each microbial kingdom is an ordination plot and the output of the type II permutation test performed on the dbRDA, using the Bray-Curtis dissimilarity index and OTU data set. It includes the variation (adjusted r-squared) explained by each factor, the *F*-value, the degrees of freedom (df), and the *P*-value (in bold when significant). Compartment refers to roots and rhizosphere, time refers to 1, 14, and 42 days after infestation (DAI), treatment refers to healthy and infested plants and soil microbial diversity refers to high and low soil microbial diversities.

phyla were mainly influenced by infestation but not at 1 DAI. Significant differences occurred during the peak of herbivory at 14 DAI: the phylum of *γ-Proteobacteria* increased under herbivory, while the phyla of *α-*, *δ-Proteobacteria*, *Actinobacteria*, *Bacteroidetes,* and "Others" decreased. At 42 DAI, infestation still influenced the phyla of *γ-Proteobacteria*, *Actinobacteria* and "Others." Initial soil microbial diversity impacted *β-Proteobacteria* at 14 and 42 DAI with a larger abundance in high diversity-infested plants while on the contrary *Actinobacteria* was more abundant in low diversity-infested plants at 42 DAI.

The bacterial phyla of the rhizosphere (**Figure 4B**) were also influenced by infestation as well as the initial soil microbial diversity and their abundances were in the same range as in the roots. Overall, the phyla of *Actinobacteria*, *β-*, *δ-*, *γ-Proteobacteria,* and "Others" differed between high and low microbial diversities at the different times. Infestation started to impact phyla at 14 DAI, with more *γ-Proteobacteria* and less *α-Proteobacteria* in infested plants. At 42 DAI, the phyla of α-*Proteobacteria* and *Firmicutes* were still affected by infestation and more abundant in infested plants.

In the roots and the rhizosphere, there were four major fungal phyla among which *Ascomycota* (divided into three sub-phyla), *Basidiomycota* and *Chytridiomycota* were the most abundant. Relative abundances of root fungal phyla (**Figure 4C**) were very variable. At 1 DAI, there was no difference between modalities. *Pezizomycotina* and *Basidiomycota* abundances increased in infested plants at 14 and 42 DAI respectively while *Taphrinomycotina* abundance decreased at 42 DAI. The phyla of *Basidiomycota* and *Chytridiomycota* were influenced by the soil microbial diversity at 42 DAI in infested plants.

Fungal phyla (**Figure 4D**) seemed to vary less in the rhizosphere than in the roots but proportions of *Chytridiomycota* seemed to decrease in favor of *Basidiomycota*, *Pezizomycotina,* and *Saccharomycotina*. There was no effect of herbivory on fungal phyla at 1 and 14 DAI, but *Chytridiomycota* abundance decreased in infested plants at 42 DAI. The phyla of *Blastocladiomycota* and "Others" were lightly influenced by the initial soil microbial diversity, with larger abundances in plants of high diversity.

Absolute Abundance of Microbial Genera

A total of 2,031 bacterial genera were detected in the roots and in the rhizosphere.

The root compartment presented 74 bacterial genera from nine different phyla, which varied significantly following

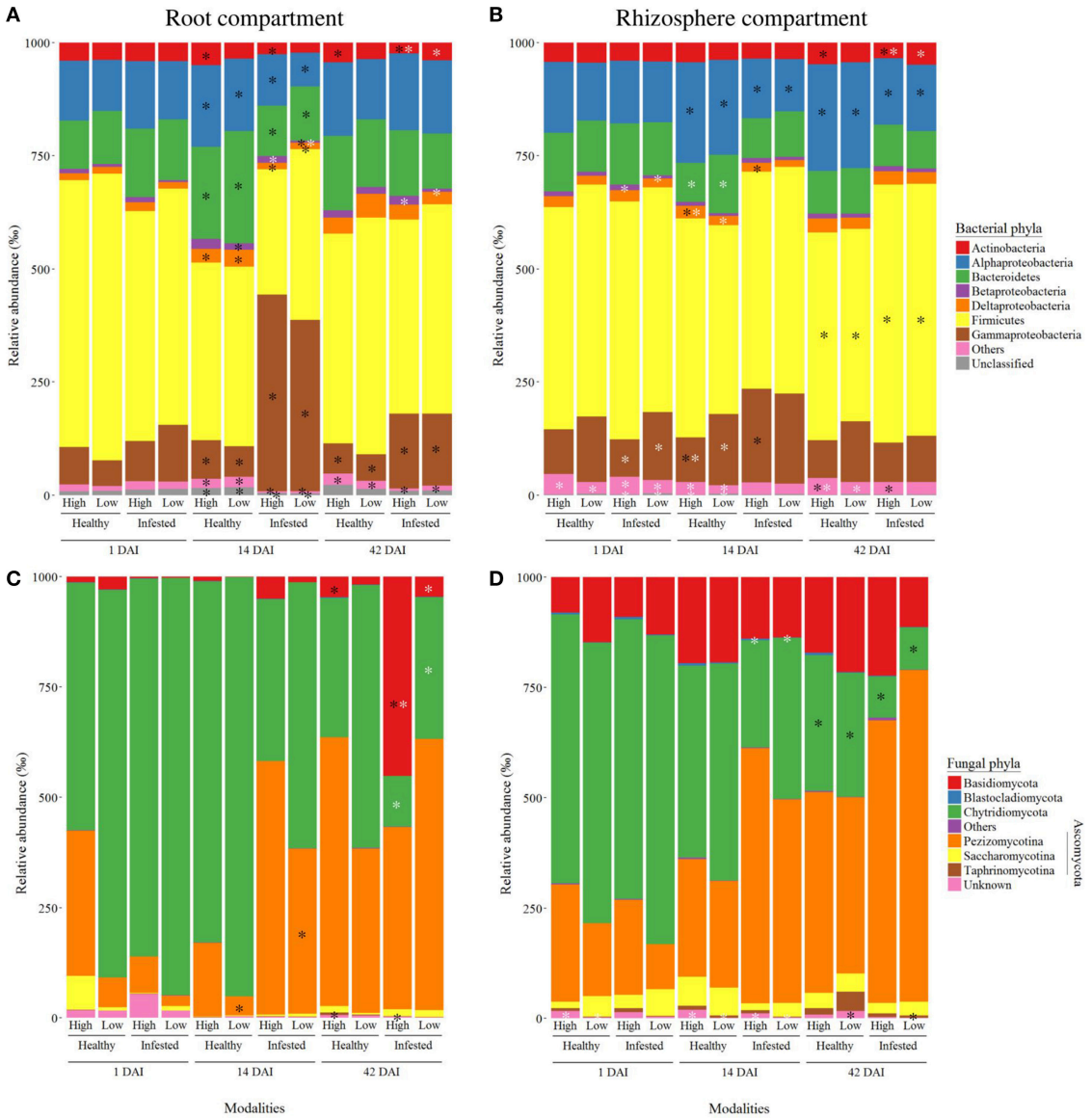


FIGURE 4 | Dominant bacterial and fungal phyla detected in the roots and rhizosphere. Mean relative abundance are presented for bacterial **(A,B)** and fungal **(C,D)** phyla from the root **(A,C)**, and rhizosphere **(B,D)** compartments. All modalities are represented, taking into account the sampling time (1, 14, and 42 days after infestation), the treatment (healthy and infested plants), and initial soil microbial diversity (high and low levels). Black asterisks indicate significant differences between healthy and infested plants at a given time and soil microbial diversity, while white asterisks indicate significant differences between soil microbial diversities at a given time and for a given treatment.

herbivory. Most of these genera showed a different abundance between healthy and infested plants at 14 and/or 42 DAI but not at 1 DAI (Table S2). Out of these 74 genera, *Bacillus*, *Clostridium*, *Paenibacillus*, *Pseudomonas,* and *Stenotrophomonas* were the most abundant genera influenced by herbivory (**Figure 5A**). *Bacillus* decreased in infested plants while the other four genera increased and none of them returned to an abundance similar to the one detected in healthy plants at 42 DAI. Abundance varied with the initial soil microbial diversity. At low soil diversity, the increase of *Paenibacillus* abundance following herbivory was greater than at high soil diversity while that of the other genera were similar between both diversities.

In the rhizosphere compartment, fewer bacterial genera (46 genera from 9 phyla) were impacted by the infestation at 14 and/or 42 DAI (Table S3). Compared to the roots, the range of bacterial absolute abundance in the rhizosphere was lower. This time, only *Bacillus*, *Clostridium*, *Paenibacillus,* and *Pseudomonas* were the most abundant genera influenced and increased by herbivory (**Figure 5B**). As previously shown in the roots, the rhizosphere was also characterized by a change of range in the

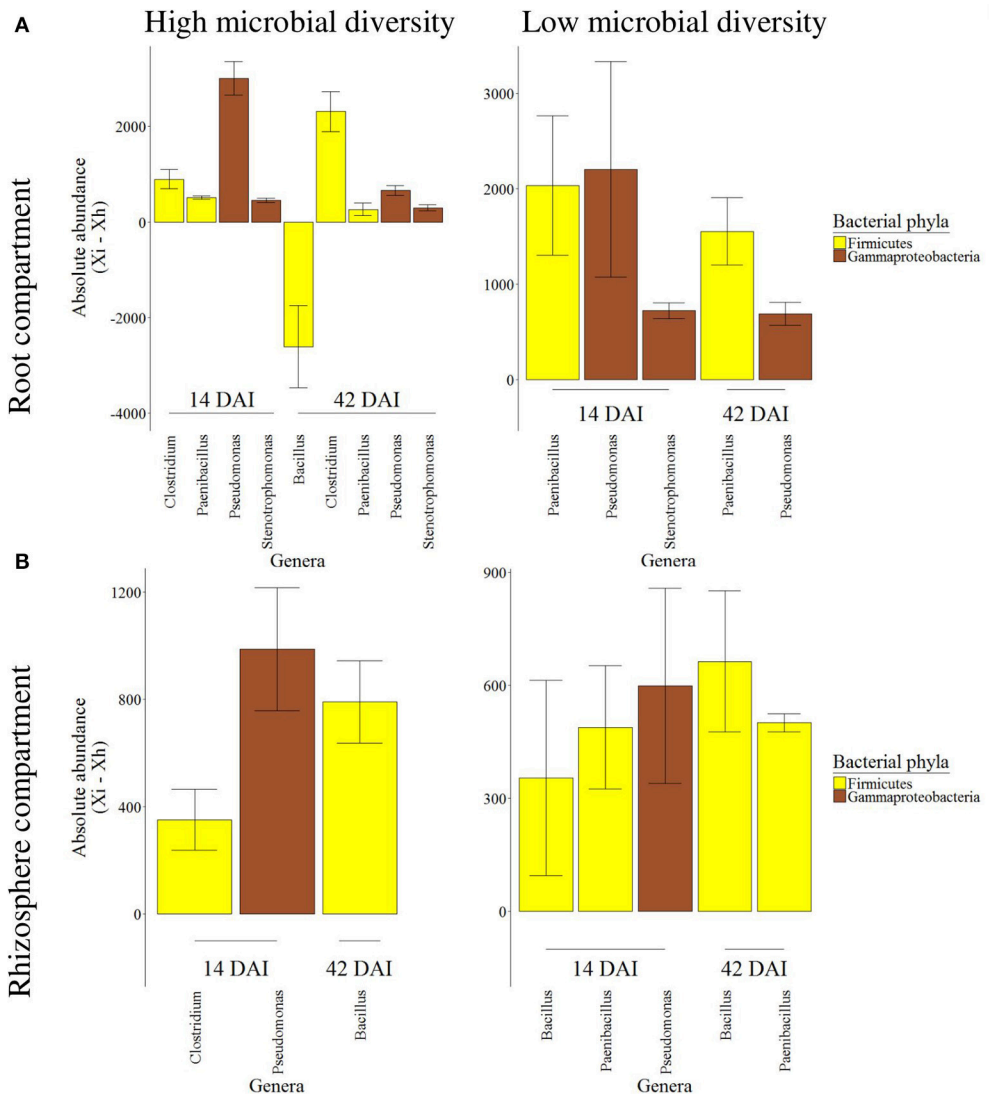


FIGURE 5 | Absolute abundance variation of root and rhizosphere bacterial genera between healthy and infested plants. Mean differential absolute abundance (± se) are presented for bacterial genera from the root **(A)** and rhizosphere **(B)** compartment, associated to either high or low soil microbial diversity, at 14 and 42 days after infestation (DAI). Genera represented in the figure were significantly impacted by the plant treatment (i.e., healthy vs. infested) at a given time and exceeded the 250/−250 threshold. Genus abundance is expressed as the difference between absolute abundance of infested plants (Xi) and absolute abundance of healthy plants (Xh).

differential abundance associated to the initial soil microbial diversity, where the range was lower at low diversity.

A total of 2,593 fungal genera were detected in the roots and in the rhizosphere. The fungal genera varied as much as their phyla (Table S4). In the roots, four out of the five herbivory-influenced genera were more abundant in infested plants, including *Ajellomyces* and *Filobasidiella* from the subphylum of *Pezizomycotina* and the phylum of *Basidiomycota* respectively. No herbivory-influenced genus was significantly impacted at low microbial diversity in the roots. In the rhizosphere, eight genera were influenced by infestation, *Torulaspora* and *Tuber* being more abundant in infested plants at 1 and 42 DAI respectively (Table S4).

Root Compounds Modulated by Herbivory

Primary and Secondary Metabolites

Metabolomic profile of OSR roots was significantly influenced by the treatment at 1, 14, and 42 DAI (**Table 2**) where infested and healthy plants showed two different profiles, associated to metabolite variations (Figure S2). Their profiles were also impacted by the initial soil microbial diversity but only at 14 DAI. The constrained variance (i.e., part of the variance explained by our variables) had the highest value (73%) at 14 DAI.

Overall, 33 metabolites (17 AAs, 9 CPOs, and 7 GSLs presented in **Figures 6A–C** respectively) were significantly affected by infestation at one or several moments of the plant-insect interaction and 2 metabolites were impacted by the

TABLE 2 | Statistical output of multivariate analysis performed on root metabolites and elements.

Compounds	Time	Total variance (%)	Constrained variance (%)	Factor	RDA test
Metabolites	1 DAI	Constrained = 67.28 Unconstrained = 32.72	Axis 1 = 67.96 Axis 2 = 16.73	Treatment	$F = 8.11$; df = 1; $P =$ **0.002**
				Soil microbial diversity	$F = 0.97$; df = 1; $P = 0.375$
				Soil biological replicates	$F = 1.07$; df = 2; $P = 0.354$
				Interaction Treatment-Soil microbial diversity	$F = 1.09$; df = 1; $P = 0.313$
	14 DAI	Constrained = 73.49 Unconstrained = 26.51	Axis 1 = 66.70 Axis 2 = 14.45	Treatment	$F = 9.95$; df = 1; $P =$ **0.001**
				Soil microbial diversity	$F = 3.22$; df = 1; $P =$ **0.039**
				Soil biological replicates	$F = 1.28$; df = 2; $P = 0.247$
				Interaction Treatment-Soil microbial diversity	$F = 0.87$; df = 1; $P = 0.463$
	42 DAI	Constrained = 61.18 Unconstrained = 38.82	Axis 1 = 52.32 Axis 2 = 23.88	Treatment	$F = 4.48$; df = 1; $P =$ **0.002**
				Soil microbial diversity	$F = 1.29$; df = 1; $P = 0.271$
				Soil biological replicates	$F = 1.45$; df = 2; $P = 0.184$
				Interaction Treatment-Soil microbial diversity	$F = 0.77$; df = 1; $P = 0.602$
Elements	1 DAI	Constrained = 54.38 Unconstrained = 45.62	Axis 1 = 46.08 Axis 2 = 39.89	Treatment	$F = 2.66$, Df = 1, $P = 0.055$
				Soil microbial diversity	$F = 2.00$, Df = 1, $P = 0.122$
				Soil biological replicates	$F = 0.80$, Df = 2, $P = 0.610$
				Interaction Treatment-Soil microbial diversity	$F = 0.87$, Df = 1, $P = 0.488$
	14 DAI	Constrained = 61.26 Unconstrained = 38.74	Axis 1 = 61.20 Axis 2 = 23.68	Treatment	$F = 5.20$, Df = 1, $P =$ **0.006**
				Soil microbial diversity	$F = 2.02$, Df = 1, $P = 0.105$
				Soil biological replicates	$F = 0.50$, Df = 2, $P = 0.897$
				Interaction Treatment-Soil microbial diversity	$F = 1.24$, Df = 1, $P = 0.301$
	42 DAI	Constrained = 55.99 Unconstrained = 44.01	Axis 1 = 55.54 Axis 2 = 27.07	Treatment	$F = 2.68$, Df = 1, $P =$ **0.045**
				Soil microbial diversity	$F = 1.16$, Df = 1, $P = 0.298$
				Soil biological replicates	$F = 0.73$, Df = 2, $P = 0.650$
				Interaction Treatment-Soil microbial diversity	$F = 2.31$, Df = 1, $P = 0.079$

The above table presents the outcome of permutation test performed on RDA, based on root metabolite and element contents, and taking into account the treatment (healthy vs. infested plants), soil microbial diversity (high vs. low soil diversity), soil biological replicates and the interaction between treatment and diversity. Total and constrained variances, F-value, degrees of freedom (df), and P-value (in bold when significant) are given for the tested factors and interaction, at each sampling time (1, 14, and 42 days after infestation "DAI").

soil microbial diversity (Table S5). Many of them differed at 1 and/or 14 DAI and most of these 33 metabolites were less abundant in infested plants except 2 AAs (β-alanine, SMCSO), 2 CPOs (trehalose, glycerate), and 2 GSLs (glucobrassicin, neoglucobrassicin), which production increased. Twenty-three of these metabolites reached back a stable state, close to the profile of healthy plants at 42 DAI. All GSLs varied due to infestation but six were still influenced at 42 DAI.

Chemical Elements

Elemental profiles of OSR roots were only impacted by the treatment at 14 and 42 DAI (**Table 2**, Figure S3). At 14 DAI, the profile of infested plants was highly modified compared to healthy plants. As for metabolites, the constrained variance was the highest at 14 DAI (61%).

Only 8 chemical elements (4 macroelements, 3 microelements, and 1 heavy metal) were significantly impacted by the infestation, while soil microbial diversity had no effect on chemical element content (**Figure 7**, Table S6). Six elements (Mg, K, Fe, Na, V, Cd) decreased in infested plants at 14 DAI while N content increased. All of these elements reached a state similar to healthy plants at 42 DAI. Sulfur also increased in infested plants, but only at 42 DAI.

Relationships Between Root Compounds and Microbial Communities

At the peak of herbivory (i.e., 14 DAI), the DIABLO method confirmed the significant differences between infested and healthy plants (CER = 0, $P = 0.002$) with the three score plots showing similar patterns (**Figure 8**). The axis 1 of microbiome data was positively correlated to axes 1 of metabolite and element data ($r = 0.86$ and 0.95, respectively) while metabolite data and element data were also positively correlated ($r = 0.84$). Infested plants seemed to be associated to (i) four bacterial genera (*Clostridium*, *Paenibacillus*, *Pseudomonas,* and *Stenotrophomonas*) and one fungal genus (*Torulaspora*), that were overexpressed, (ii) trehalose (CPO) and neoglucobrassicin (GSL), also overexpressed, (iii) nitrogen but to a lesser extent. The remaining AAs, CPOs, GSLs and seven elements were underexpressed in infested plants.

At 42 DAI, the discrimination between infested and healthy plants was also significant (CER = 0, $P = 0.002$) and axis 1 of microbiome data was highly positively correlated to axis 1 of combined metabolite and element data ($r = 0.95$), confirmed

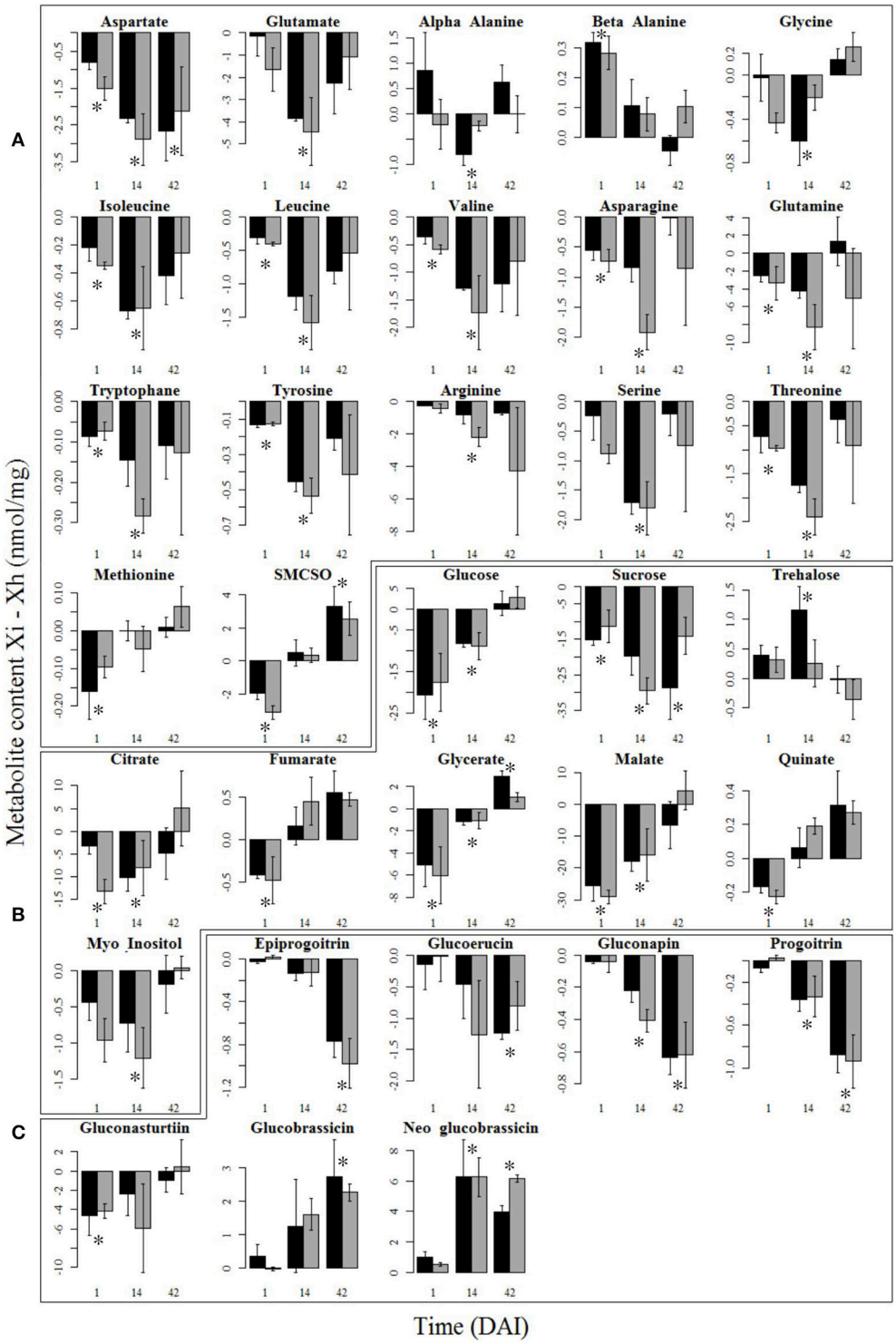


FIGURE 6 | Variation of root metabolite contents between healthy and infested plants. Mean differential content (± se) of the root amino acids **(A)**, carbohydrates, polyols and organic acids **(B)**, and glucosinolates **(C)** at 1, 14, and 42 days after infestation (DAI) that were significantly impacted by the plant treatment (i.e., healthy vs. infested), are represented in this figure. Metabolite content is expressed as the difference between content of infested plants (Xi) and content of healthy plants (Xh). Asterisks show significant differences ($P < 0.05$) between healthy and infested plants at a given time. High and low initial soil microbial diversities are represented in black and gray bars respectively. Metabolites are sorted out in the same order as in Table S5, with only the significant metabolites remaining.

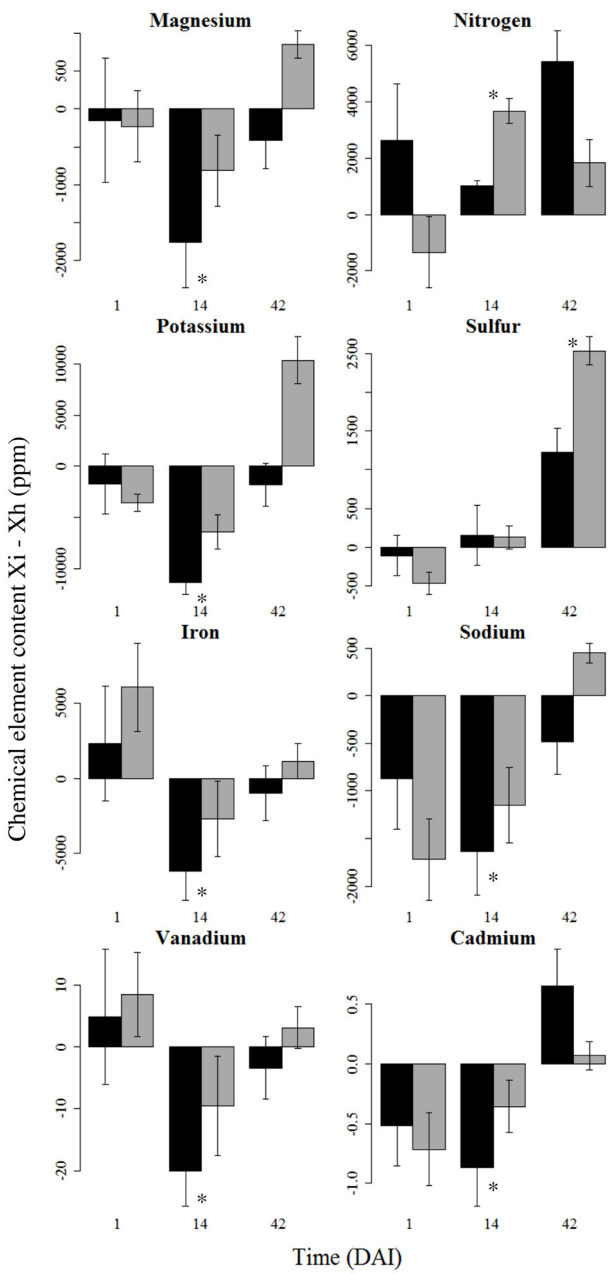


FIGURE 7 | Variation of root chemical element content between healthy and infested plants. Mean differential content (± se) of the macro- (Mg, N, K, S), microelements (Fe, Na, V), and heavy metal (Cd) at 1, 14, and 42 days after infestation (DAI) that were significantly impacted by the plant treatment (i.e., healthy vs. infested), are represented in this figure. Chemical element content is expressed as the difference between content of infested plants (Xi) and content of healthy plants (Xh). Asterisks show significant differences ($P < 0.05$) between healthy and infested plants at a given time. High and low initial soil microbial diversities are represented in black and gray bars respectively. Chemical elements are sorted out in the same order as in Table S6, with only the significant chemical elements remaining.

by similar patterns on the two score plots (**Figure 9**). At the end of herbivory, infested plants seemed to be associated to (i) four bacterial genera (*Clostridium*, *Paenibacillus*, *Pseudomonas*, *Stenotrophomonas*) and three fungal genera (*Ajellomyces*, *Filobasidiella*, *Torulaspora*), which were overexpressed and (ii) SMCSO (AA), glycerate (CPO), glucobrassicin and neoglucobrassicin (GSLs), and sulfur. Conversely, one bacterial and one fungal genus, as well as four metabolites were underexpressed in these infested plants.

DISCUSSION

Our study showed that infestation of OSR by a belowground herbivore increased root bacterial alpha-diversity, while it had no effect on rhizosphere bacteria and very little on fungi. Interestingly, herbivory was associated with an increase of γ-*Proteobacteria* and *Firmicutes* (and five of their most dominant affiliated genera: *Pseudomonas*, *Stenotrophomonas* and *Bacillus*, *Clostridium*, *Paenibacillus* respectively) as well as *Ajellomyces* and *Filobasidiella* from the fungal phyla of *Ascomycota* and *Basidiomycota*. Trehalose, sulfur-containing metabolites and sulfur contents increased in infested plants and could explain the variations observed in bacterial phyla and genera. Herbivory seemed to have only a short-term negative effect on the richness and diversity of the microbial communities, which were both restored when the perturbation ended. The chemical composition of roots matched this restoration. Initial soil microbial diversity itself had little impact on microbial communities of the root and its rhizosphere and did not significantly influence root chemistry.

Herbivory Influences Microbial Community Diversity and Composition

Our study showed that root herbivory decreased bacterial richness and diversity in roots at the peak of herbivory but globally increased both at the end of herbivory. Opposite trends were found in grassland plant communities, where plant diversity decreased after the end of herbivory (e.g., Collins et al., 1998; Pucheta et al., 1998) but microbial communities may be hard to compare with plant communities. Further from the roots, bacterial and fungal communities of the rhizosphere were respectively not affected and lightly affected by herbivory in our study. In other studies on the putative influence of herbivory on bacterial communities, herbivory marginally increased the abundance of soil nitrifying bacteria and archeae (Le Roux et al., 2008) and, (in contradiction with our own results) Kong et al. (2016) found that white fly herbivory significantly decreased bacterial richness in the rhizosphere of pepper. Differences between these two earlier studies may be due to differences in the biological models studied but also to the nature and length of the perturbation and type of experiments. Compared to bacteria, we observed that fungal communities were very variable and only lightly influenced by herbivory. These results are consistent with previous studies showing that fungi are quite resistant to herbivory, such as leaf mining in *Ageratina altissima* (Asteraceae), which did not influence the communities of endophytes colonizing leaves (Christian et al., 2016).

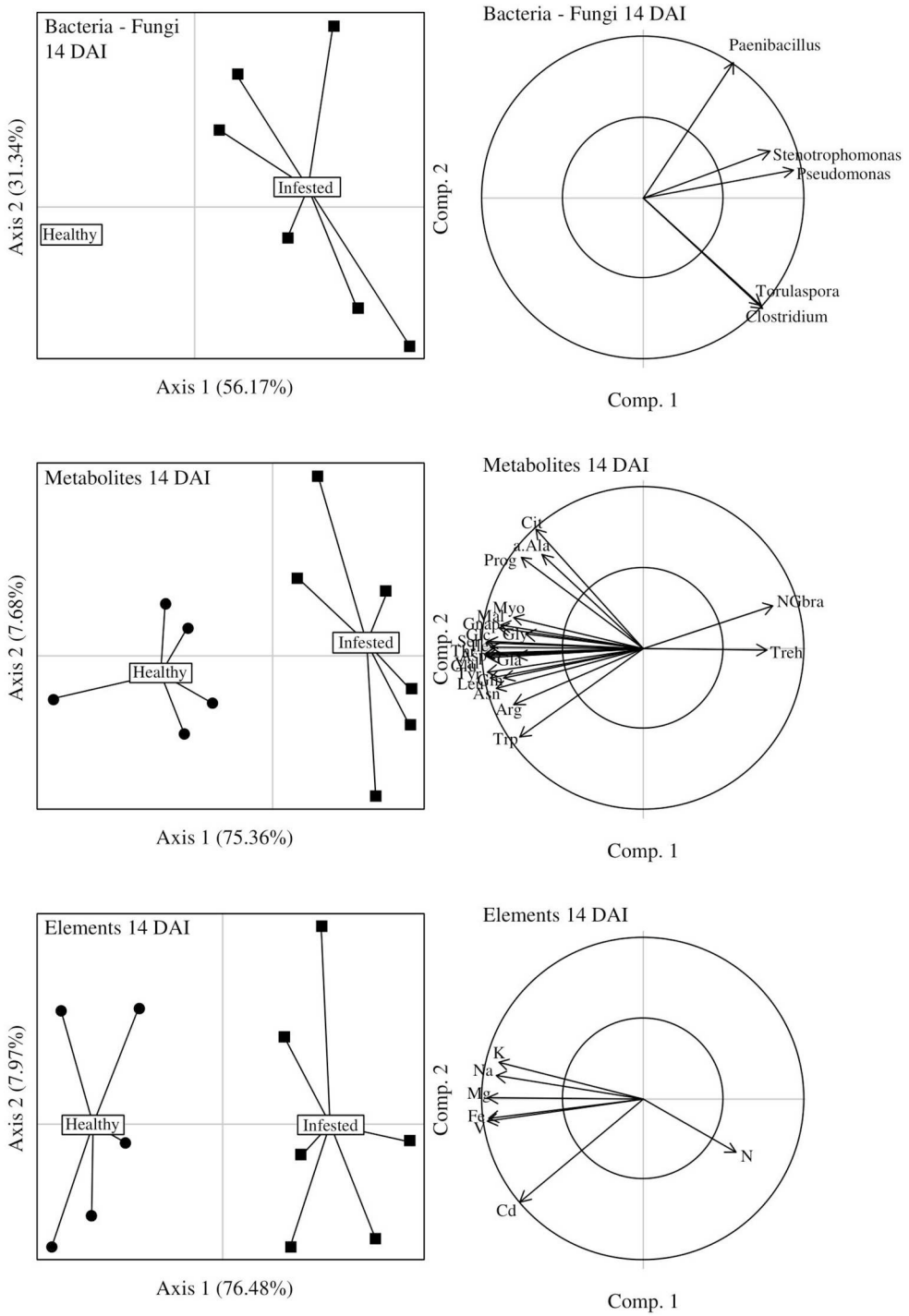


FIGURE 8 | DIABLO graphical outputs at 14 days after infestation (DAI). Score plots of microbiome, metabolite and chemical element datasets are positioned on the left side while the correlation circles associated to each of these score plots are positioned on the right side of the figure. Correlations between axes 1 (axes 2) of microbiome and metabolite datasets: 0.86 (0.86); of microbiome and chemical element datasets: 0.95 (0.60); of metabolite and element datasets: 0.84 (0.77).

Roots and rhizosphere microbial communities of healthy OSR were mainly composed of bacterial taxa *Actinobacteria*, *Bacteroidetes*, *Firmicutes,* and *Proteobacteria* (more specifically of *Bacillus*, *Paenibacillus,* and *Pseudomonas* at the genera level), while fungal taxa were mainly *Ascomycota*, *Chytridiomycota* and *Basidiomycota*. Our results on bacteria are in line with

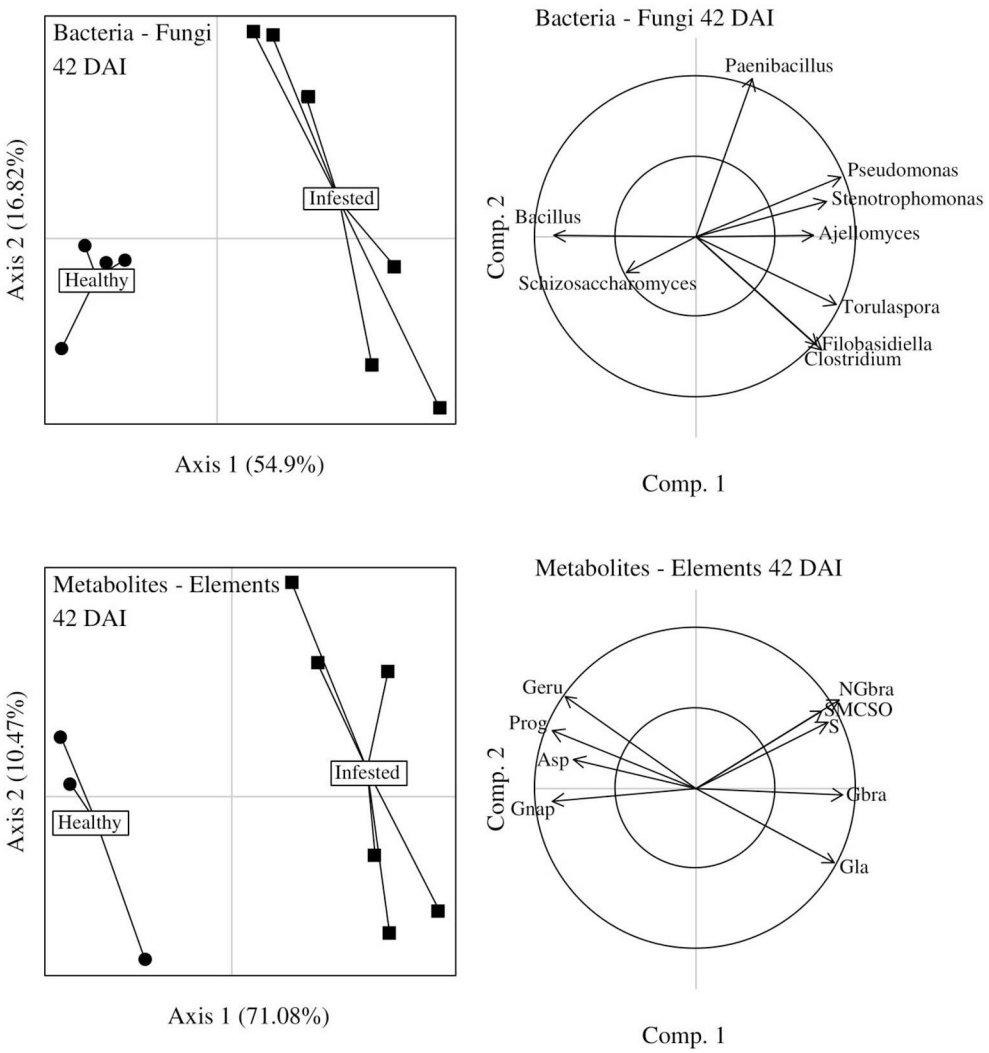


FIGURE 9 | DIABLO graphical outputs at 42 days after infestation (DAI). Score plots of microbiome dataset and combined metabolite and chemical element datasets are positioned on the left side while the correlation circles associated to each of these score plots are positioned on the right side of the figure. Correlations between axes 1 (axes 2) of microbiome and metabolite/chemical element datasets: 0.95 (0.90).

those of Bulgarelli et al. (2012) and Bodenhausen et al. (2013) in *Arabidopsis thaliana*. Regarding fungi, *Ascomycota* and *Basidiomycota* accounted for 96% of the community in *Microthlaspi* spp. populations, another brassicaceous plant (Glynou et al., 2018). A recent study on OSR showed that *Proteobacteria* and *Actinobacteria* as well as *Ascomycota* and *Basidiomycota* were the most abundant bacterial and fungal phyla respectively in the roots and rhizosphere, however with different dominant genera (Gkarmiri et al., 2017). While the phylum of *Chytridiomycota* was only present in roots in the previous study, it was part of the rhizosphere composition in ours. These differences in phyla and genera might be due to the use of isotopic labeling by Gkarmiri et al. (2017), representing only the active portion (i.e., assimilating labeled photosynthetates) of the plant communities. Furthermore, many drivers have been shown to shape plant microbial communities: plant root exudates (Turner et al., 2013; Tsunoda and van Dam, 2017); plant development (de Campos et al., 2013; Chaparro et al., 2014; Wagner et al., 2016); accession (Micallef et al., 2009); genotype (Wagner et al., 2016); species (Miethling et al., 2000); soil (Vandenkoornhuyse et al., 2015); and agricultural management practices (Hartmann et al., 2015; Rathore et al., 2017). It is therefore not surprising to find some differences in our study.

Root herbivory increased the abundance of *γ-Proteobacteria* in the roots and rhizosphere, and that of *Firmicutes* in the rhizosphere but it decreased abundance of *α-Proteobacteria* in both compartments and *δ-Proteobacteria* and *Bacteroidetes* in the roots only. These changes were accompanied by an increased abundance of *Bacillus*, *Paenibacillus*, *Pseudomonas,* and *Stenotrophomonas*. However, some of these phyla in infested plants at 42 DAI reached

back a stable state similar to that found in healthy plants at 42 DAI, hence highlighting the resilience of microbial communities after the end of insect herbivory. These results are consistent with those of Kong et al. (2016) on whitefly herbivory on pepper showing an increase in the abundance of *γ-Proteobacteria* and *Stenotrophomonas* in the rhizosphere and with those of Kim et al. (2016) where aphid herbivory increased the abundance of *Paenibacillus*. According to Card et al. (2015), *Bacillus*, *Paenibacillus*, *Pseudomonas,* and *Stenotrophomas* are either inhibitors or antagonists of plant-pathogenic bacteria or fungi, while *Bacillus* and *Paenibacillus* are known to be also entomopathogenous (Monnerat et al., 2009; Grady et al., 2016; Zhao et al., 2017).

Herbivory also affected fungal phyla and genera but to a lesser extent than bacterial taxa: it decreased the abundance of *Chytridiomycota* in the rhizosphere and increased the abundance of *Ajellomyces* (slightly) and *Filobasidiella* (strongly) in the roots. Tkacz et al. (2015) demonstrated an increase of a *Chytridiomycota* in the rhizosphere of *Brassica rapa* over time, while this phylum progressively colonized the roots during plant development, to finally dominate fungal communities (Lebreton, personal communication). Most fungi are oligotrophic and grow slowly, with limited carbon sources (Ho et al., 2017). The lesser effect of herbivory on fungal communities observed in our study might be due to the timeframe of our experiment, probably too short compared to the growth rate of fungi, which is based on their utilization of complex trophic resources.

Herbivory Influences Root Metabolite and Chemical Element Composition

Herbivory tended to decrease root AAs and CPOs, which is consistent with the results of Hopkins et al. (1999), van Leur et al. (2008), and Lachaise et al. (2017). Herbivory increased glycerate and trehalose, a sugar assumed to play a role in plant defenses against aphid infestation (Singh et al., 2011). Sucrose was not affected by herbivory, contrary to the study of Pierre et al. (2012). S-methyl cysteine sulfoxide (SMCSO), a toxic compound detectable in brassicaceous crops (Edmands et al., 2013) was increased by herbivory. We therefore hypothesize that both SMCSO and trehalose might play a role in OSR defense against root herbivory.

Our study showed that herbivory also modulated GSLs by increasing two indolyl (glucobrassicin and neoglucobrassicin) but decreasing four aliphatic and one aromatic GSLs consistently with van Dam and Raaijmakers (2006). This is also coherent with the same decrease of total GSL contents found by van Dam and Raaijmakers (2006) and Lachaise et al. (2017). A recent study demonstrated the detrimental effect of glucobrassicin on aboveground generalist and specialist herbivores: a lower larval weight and faster development time associated with an increased mortality (Santolamazza-Carbone et al., 2017). However, in another study, aliphatic GSLs affected larval weight in generalists but not in specialist insects (Arany et al., 2008). Glucosinolates are generally considered as defensive compounds against herbivores but their impact on insect life history traits appear very species specific and their influence on microbial communities is difficult to predict.

Herbivory increased sulfur and nitrogen contents. The increase of sulfur content in root tissues probably corresponded to higher amounts of indolyl GSLs (glucobrassicin and neoglucobrassicin) and SMCSO, which are all sulfur-containing metabolites. The increase in nitrogen could be linked to insect frass (Kagata and Ohgushi, 2011). These authors demonstrated that there was more nitrogen excreted by larva than ingested and they suggested that the excess of nitrogen might originate from the plant organic nitrogen (e.g., AAs or proteins), which could not be digested by the insect. These observations could explain our results, especially since the nitrogen increased occurred only at 14 DAI, the peak of herbivory when the CRF was still at the larval stage.

Relationship Between Bacterial Genera and Root Chemistry in Response to Herbivory: Focus on Dominant Genera and Compounds Increased by Herbivory

Root herbivory—obviously—generates root degradation, which creates habitat spatial heterogeneity and as a consequence could modify microbial communities. Changes in microbial diversity and composition following herbivory is often hypothesized to be based on modifications of the plant chemistry, such as plant exudates (Kim et al., 2016; Kong et al., 2016). However, variations in root metabolite and rhizodeposits occur during the plant's life cycle. In addition, bacteria and fungi are known to differ in their ability to use organic compounds (Boer et al., 2005). In our study, we focused on the vegetative stage of OSR, a stage when fungal communities show only limited changes (Mougel et al., 2006).

Increased trehalose following herbivory was associated with an overexpression of *Stenotrophomonas* and *Pseudomonas*. *Stenotrophomonas* appears to produce trehalose as an osmoprotective substance (Wolf, 2002) while *Pseudomonas* is able to use this compound as a carbon source for its growth (De La Fuente et al., 2007) as well as other sugars found in root exudates, a trait that makes this genus quite competitive in microbial communities (Lugtenberg et al., 1999). A modification of plant microbial communities mediated by plant chemistry was also found in Kim et al. (2016) where bacteria such as *Paenibacillus* were able to grow on medium-based root exudates, which came from aphid-infested plants. Moreover, glycerate was also associated with microbial changes under herbivory. This could be explained by the fact that glycerate-derived compounds are usually accumulated by microorganisms under abiotic stress and this process could be similar for biotic stress (see review on abiotic stress by Empadinhas and da Costa, 2011). We suggest that the root chemistry disturbed by CRF herbivory might be beneficial to colonization of microorganisms such as *Pseudomonas*, exhibiting

competitive traits to exploit trophic resources (Ho et al., 2017).

Following herbivory, enhancement of sulfur-containing compounds (i.e., SMCSO, glucobrassicin, neoglucobrassicin, sulfur) was associated to an overexpression of *Bacillus*, *Paenibacillus*, *Pseudomonas,* and *Stenotrophomonas*. Aziz et al. (2016) demonstrated that plant biomass loss caused by aboveground herbivory was lessened in presence of *Bacillus*. This decrease seemed mediated by an increase of indolyl GSLs. The fact that these bacteria are associated to sulfur-containing compounds might be linked to their potential role in the sulfur cycle. *Pseudomonas* for example dominates the arylsulfatase bacterial communities in the OSR rhizosphere (Cregut et al., 2009) and this enzyme (produced by microorganisms in order to mineralize organic sulfur) is present both in the roots and in the rhizosphere of this plant (Knauff et al., 2003). A plant undergoing herbivory could send external signals to recruit bacteria, which could stimulate the synthesis of plant defense compounds (e.g., GSLs). An increase of trehalose in the roots and rhizosphere via root exudates (Paterson et al., 2007) could represent an external signal, attracting beneficial symbionts and promoting bacterial growth. These bacteria could then enhance enzymatic activities (e.g., arylsulfatase) allowing sulfur mineralization and stimulation of GSL production.

Following herbivory, nitrogen was also associated with microorganisms but to a lesser extent. Nitrogen is a limiting factor for plant growth because of its low bioavailability in the soil, since eukaryotes are not able to fix atmospheric nitrogen (Grady et al., 2016). To have access to nitrogen sources, plants rely on nitrogen-fixing microorganisms (i.e., diazotroph). *Clostridium* and *Paenibacillus* are free-living diazotrophic species (Choudhary and Varma, 2016; Grady et al., 2016) while a nitrogen fixing trait was recently discovered in the genome of a *Pseudomonas* species (Yan et al., 2008). We suggest that plants stressed by herbivory might be able to select better microorganisms (i.e., in terms of fitness and cost in nutrients, such as carbohydrates, to the plant), which will provide more nitrogen and enable them to better resist this perturbation.

Soil Microbial Diversity Did Not Influence the Plant-Microorganism Interactions

Initial soil diversity had respectively little and no effect on root bacterial and fungal alpha-diversities. Only rhizosphere bacterial and fungal diversities were impacted by soil microbial diversity, but not by herbivory. When evaluating abiotic and biotic stresses (Kissoudis et al., 2014), some studies established a positive relationship between an ecosystem stability-resilience and its diversity, while others found opposite relationships or none at all (Griffiths and Philippot, 2013). In our study, the resilience of microbial communities did not seem to come from their initial diversity: microbial results were globally similar at high and low initial diversities and plant chemistry was not impacted by these levels of initial diversity either, as found by Lachaise et al. (2017). We hypothesize that our "low" microbial diversity condition might still have been too rich to properly assess the effect of soil diversity and that a stronger dilution may be necessary in future studies.

CONCLUSION

In summary, herbivory led to root chemical changes, involving carbohydrates and sulfur-containing compounds, which partly shaped belowground microbial communities and particularly the phyla of γ-*Proteobacteria* and *Firmicutes* and a couple of their affiliated genera. It indicates that a plant suffering from herbivory emits either defensive and/or nutritive compounds that influence the recruitment of soil microorganisms by the rhizosphere and the roots.

Our results encourage the determination of the precise identity and functions of microorganisms responding to herbivory, using more accurate primers and metatranscriptomic approaches, and the understanding of the feedback-loop existing between these identified microorganisms and the plant chemistry modified by herbivory. This future research could represent the next step toward the transition from correlation to causation in order to develop sustainable and innovative plant protection strategies.

AUTHOR CONTRIBUTIONS

MO, CM, and A-MC conceived and designed research. LL and A-YG-E prepared the soil used in the experiment. MO, CP, and VC conducted plant harvest. JL and CM conducted respectively the molecular and bioinformatics analyses. MO performed metabolomics analysis, advised by NM. MO did the statistical analyses and interpreted data, advised by LL, MH, AO, and DP. MO wrote the manuscript, which was commented and approved by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00091/full#supplementary-material

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Plant–Soil Feedback Effects on Growth, Defense and Susceptibility to a Soil-Borne Disease in a Cut Flower Crop: Species and Functional Group Effects

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Plants can influence the soil they grow in, and via these changes in the soil they can positively or negatively influence other plants that grow later in this soil, a phenomenon called plant–soil feedback. A fascinating possibility is then to apply positive plant–soil feedback effects in sustainable agriculture to promote plant growth and resistance to pathogens. We grew the cut flower chrysanthemum (*Dendranthema X grandiflora*) in sterile soil inoculated with soil collected from a grassland that was subsequently conditioned by 37 plant species of three functional groups (grass, forb, legume), and compared it to growth in 100% sterile soil (control). We tested the performance of chrysanthemum by measuring plant growth, and defense (leaf chlorogenic acid concentration) and susceptibility to the oomycete pathogen *Pythium ultimum*. In presence of *Pythium*, belowground biomass of chrysanthemum declined but aboveground biomass was not affected compared to non-*Pythium* inoculated plants. We observed strong differences among species and among functional groups in their plant–soil feedback effects on chrysanthemum. Soil inocula that were conditioned by grasses produced higher chrysanthemum above- and belowground biomass and less leaf yellowness than inocula conditioned by legumes or forbs. Chrysanthemum had lower root/shoot ratios in response to *Pythium* in soil conditioned by forbs than by grasses. Leaf chlorogenic acid concentrations increased in presence of *Pythium* and correlated positively with chrysanthemum aboveground biomass. Although chlorogenic acid differed between soil inocula, it did not differ between functional groups. There was no relationship between the phylogenetic distance of the conditioning plant species to chrysanthemum and their plant–soil feedback effects on chrysanthemum. Our study provides novel evidence that plant–soil feedback effects can influence crop health, and shows that plant–soil feedbacks, plant disease susceptibility, and plant aboveground defense compounds are tightly linked. Moreover, we highlight the relevance of considering plant–soil feedbacks in sustainable horticulture, and the larger role of grasses compared to legumes or forbs in this.

Keywords: chlorogenic acid, chrysanthemum, disease susceptibility, plant–soil feedback, *Pythium ultimum*, plant functional group, phylogenetic distance

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INTRODUCTION

Plants are the main primary producers in terrestrial ecosystems and as provider of resources, such as litter and root exudates, plants are important determinants of soil biota (Bever et al., 1997; Bardgett and Wardle, 2010). These effects of plants on the soil may differ greatly between plant species as plants vary in the quality and quantity of litter and in the chemical composition of root exudates (Wardle et al., 2003; Bais et al., 2006; Bardgett and Wardle, 2010). Moreover, via their effects on the soil, plants can influence other plants that grow later in the same soil, a phenomenon termed 'plant–soil feedback' (van der Putten et al., 2013). Plant–soil feedback effects can be positive, if the succeeding plant grows better in conditioned soil compared to a control soil, and negative, if the growth is reduced (van der Putten et al., 2013). Heterospecific plant–soil feedback (where one species influences the growth of another species) has been recognized as an important mechanism in plant competition and coexistence (Kulmatiski et al., 2008; van der Putten et al., 2013), and there is an increasing interest among ecologists to unravel the mechanisms and determine the generality of plant–soil feedback effects (van der Putten et al., 2013). Although negative conspecific feedbacks are the basis for crop rotation in agriculture, how heterospecific plant–soil feedback influences cultivated plant species is relatively poorly understood as most studies, so far, have focused on interactions among wild plant species (van der Putten et al., 2013; Dias et al., 2015; Detheridge et al., 2016).

Heterospecific plant–soil feedback effects may differ between plant functional groups such as grasses, forbs or leguminous plants (Bezemer et al., 2006; Kos et al., 2015). Legumes, as nitrogen fixers may increase nutrient availability for other plants, and thus may cause positive plant–soil feedback effects (Tilman et al., 1997; Harrison and Bardgett, 2010). Similarly, grasses which have highly branched roots may provide a more suitable habitat for root-associated microbes that have beneficial effects on other plants (Bessler et al., 2009; Pérès et al., 2013; Latz et al., 2015). Clearly, an increase in root surface area that is often found in grasses could also lead to an increase in the abundance of plant antagonists such as root pathogens, but root pathogens of grasses are specialized on monocots, and it is unlikely they will negatively influence plants from another functional group (Cortois et al., 2016) Instead, roots of forb species that typically have higher phosphate contents than grass species are more susceptible to soil-borne pathogens (Laliberté et al., 2015; Zhang et al., 2016). Hence, forbs often host more pathogens than grasses, and are thereby more likely to have a negative feedback effect on later growing plants (Rottstock et al., 2014). As closely related species are more likely to share the same natural enemies and resources (Webb et al., 2006; Gilbert and Webb, 2007), it is legitimate to hypothesize that heterospecific plant–soil feedback effects among closely related species are more negative than among more distantly related species (Brandt et al., 2009; Burns and Strauss, 2011; Anacker et al., 2014; Mehrabi and Tuck, 2015; Münzbergová and Šurinová, 2015).

By growing in the soil, a plant may cause an increase in the density of pathogens in the soil, but at the same time, it may also increase beneficial microbes such as bacteria and fungi that promote plant growth, suppress pathogens or induce resistance in plants against herbivore or pathogen attack (Haas and Défago, 2005; Pineda et al., 2010). Hence, plant–soil feedback effects could influence the susceptibility of a plant to soil pathogens or the disease or pest severity experienced by that plant. We are not aware of any work reporting how plant–soil feedback influences the susceptibility of a plant to soil pathogens, but several studies reported that conditioning of soil by a plant can influence the levels of aboveground herbivory experienced by another plant that grows later in that soil via the feedback effects on the composition and concentration of aboveground secondary compounds of the responding plant (Kostenko et al., 2012; Bezemer et al., 2013; Kos et al., 2015). Soil biota, such as root herbivores, nematodes, and (non-) pathogenic soil microbes can affect plant aboveground primary and secondary compounds (Bezemer et al., 2005; Soler et al., 2012; van de Mortel et al., 2012; Badri et al., 2013), and hence we may expect that plant–soil feedback effects on the susceptibility of a plant to soil diseases will also influence the concentration of aboveground defense compounds in that plant.

In the present study, we examine how plant–soil feedback effects of a wide range of plant species influence the growth and secondary chemistry of the commercial cut flower chrysanthemum and its susceptibility to the soil pathogen *Pythium ultimum*. *Pythium* causes damping off disease to a wide range of plants including chrysanthemum (Weller et al., 2002; Meghvansi and Varma, 2015). Several studies have shown that high abundance and diversity of soil microbes can suppress *P. ultimum* (van Os and van Ginkel, 2001; Yu et al., 2015). We examined in a greenhouse experiment the plant–soil feedback effects of 37 plant species belonging to three plant functional groups on chrysanthemum growth and disease susceptibility. We tested three hypotheses: (i) plant–soil feedbacks will not only influence plant growth, but also influence plant disease susceptibility and plant defense, (ii) soil conditioned by grasses and legumes will positively affect chrysanthemum growth and reduce disease severity relative to soil conditioning by forbs, (iii) species closely related to chrysanthemum will have a more negative effect on chrysanthemum growth than more distantly related species.

MATERIALS AND METHODS

Plant and Pathogen Material

The focal plant in our study is *Dendranthema X grandiflora* (Ramat.) Kitam. cv. Grand Pink [Chrysanthemum, syn. *Chrysanthemum* X *morifolium* (Ramat.) Hemsl., Asteraceae]. Chrysanthemum cuttings were provided by the breeding company FIDES by Dümmen Orange (De Lier, Netherlands). Chrysanthemum is one of the major cut flower crops that is cultivated in soil in greenhouses. In commercial chrysanthemum greenhouses, the soil is disinfected regularly with hot steam to circumvent soil diseases. However, this practice also eliminates the (beneficial) microbial community in the soil and pathogens

rapidly recolonize the soil after steaming (Thuerig et al., 2009; Tamm et al., 2010).

The soil–borne oomycete pathogen *Pythium ultimum* (Pythiaceae) was obtained from Wageningen UR Greenhouse Horticulture (Wageningen UR, Greenhouse Horticulture, Bleiswijk, Netherlands). *Pythium ultimum* was isolated from diseased chrysanthemum plants, and cultured on liquid V8 medium (200 ml of organic tomato suspension without added salt, 2 g $CaCO_3$, and 800 ml water) at room temperature for 2 weeks. Then, the *P. ultimum* culture was blended in a mixer and filtered to obtain a solution with only oospores based on a modified protocol of van der Gaag and Wever (2005). The oospores concentration was determined by counting (Fuchs-Rosenthal chamber) the oospore number in 1 ml liquid suspensions under the microscope.

Experimental Set-Up

The experiment consisted of two phases. In the first phase, the conditioning phase, we used 37 plant species to condition soil by growing them in monocultures. In the second phase, the test phase, we measured the effects of the species-specific conditioned soils as inocula on the performance of chrysanthemum plants with and without *P. ultimum* addition.

Phase I: Conditioning phase

For the conditioning phase, 300 Kg soil was collected (5–20 cm deep) in November 2014 from a semi-natural grassland that was previously used to grow maize and where agricultural activities ceased in 1995 (Mossel, Ede, Netherlands). The collected soil was homogenized and sieved (1 cm mesh size) to remove coarse fragments and all macro-arthropods. Pots (13 cm × 13 cm × 13 cm) were filled with a homogenized mixture of field soil and sterilized field soil in a 1:1 ratio (total 1.6 Kg soil per pot). Part of the soil was sterilized by gamma irradiation (>25 K Gray gamma irradiation, Isotron, Ede, Netherlands).

Thirty-seven plant species were selected to create conditioned soils (**Table 1**). The species were classified as grasses (9 species), forbs (21 species), or legumes (7 species) (**Table 1**). Most species were wild species that are typical of natural grasslands in Netherlands. *Tagetes minuta* is a domesticated species that was included because of its known disease suppressive properties (Hooks et al., 2010). Seeds of the wild species were obtained from a wild plant seed supplier (Cruydt-Hoeck, Assen, Netherlands) and *Tagetes minuta* seeds were obtained from a garden plant seed supplier (Vreeken seeds, Dordrecht, Netherlands). Seeds were surface sterilized in 3% sodium hypochlorite solution for 1 min, rinsed and germinated on sterile glass beads in a climate chamber at 20°C (16 h/8 h, light/dark).

Five 1-week-old seedlings were transplanted in monocultures in each pot (13 cm × 13 cm × 13 cm), with five replicate pots for each species. A set of five pots filled with field soil (without plants) was also kept in the greenhouse, and served as the "no plant" control for the test phase. In total, the conditioning phase comprised of 190 pots (monocultures of 37 plant species × 5 replicates + no plant pots × 5 replicates). The replicate pots of each species in the conditioning phase were kept separately throughout the experiment. Seedlings that died during the first week of the experiment were replaced. A few seedlings died after transplantation. Therefore, 2 week later, the number of seedlings in each pot was reduced to four. All pots were placed randomly in a greenhouse with 70% RH, 16 h 21° (day) and 8 h 16° (night). Natural daylight was supplemented by 400 W metal halide lamps (225 μmol s^{-1} m^{-2} photosynthetically active radiation, one lamp per 1.5 m^2). The pots were watered regularly. Ten weeks after transplanting, plants were clipped and the largest roots were removed from the soil as they may act as a source for re-growing plants. Finer roots were left in the soil as the rhizosphere may include a major part of the microbial rhizosphere community. The soil from each pot was homogenized and stored in a plastic bag at 4°C (1 bag for each pot) until used in the test phase. These soils are called "soil inocula" hereafter.

TABLE 1 | List of plant species used in the conditioning phase, their abbreviation used in the manuscript, family and functional group are also presented.

Species	Abbreviation	Family	Functional group
Agrostis capillaris	AC	Poaceae	Grass
Agrostis stolonifera	AS	Poaceae	Grass
Anthoxanthum odoratum	AO	Poaceae	Grass
Bromus hordeaceus	BH	Poaceae	Grass
Festuca filiformis	FF	Poaceae	Grass
Festuca rubra	FR	Poaceae	Grass
Holcus lanatus	HL	Poaceae	Grass
Lolium perenne	LP	Poaceae	Grass
Phleum pratense	PP	Poaceae	Grass
Carum carvi	CAC	Apiaceae	Forb
Achillea millefolium	ACM	Asteraceae	Forb
Arnica montana	ARM	Asteraceae	Forb
Centaurea jacea	CJ	Asteraceae	Forb
Crepis capillaris	CRC	Asteraceae	Forb
Hypochaeris radicata	HR	Asteraceae	Forb
Jacobaea vulgaris	JV	Asteraceae	Forb
Leucanthemum vulgare	LV	Asteraceae	Forb
Matricaria recutita	MR	Asteraceae	Forb
Tagetes minuta	TM	Asteraceae	Forb
Tanacetum vulgare	TV	Asteraceae	Forb
Taraxacum officinale	TO	Asteraceae	Forb
Arabidopsis thaliana	AT	Brassicaceae	Forb
Capsella bursa-pastoris	CB	Brassicaceae	Forb
Campanula rotundifolia	CR	Campanulaceae	Forb
Hypericum perforatum	HP	Hypericaceae	Forb
Prunella vulgaris	PV	Lamiaceae	Forb
Thymus pulegioides	THP	Lamiaceae	Forb
Plantago lanceolata	PL	Plantaginaceae	Forb
Rumex acetosella	RA	Polygonaceae	Forb
Galium verum	GV	Rubiaceae	Forb
Lotus corniculatus	LC	Fabaceae	Legume
Medicago sativa	MS	Fabaceae	Legume
Trifolium arvense	TA	Fabaceae	Legume
Trifolium pratense	TRP	Fabaceae	Legume
Trifolium repens	TR	Fabaceae	Legume
Vicia cracca	VC	Fabaceae	Legume
Vicia sativa	VS	Fabaceae	Legume

Phase II: Test phase

For the test phase, 1 L pots (11 cm × 11 cm × 12 cm; length × wide × height) were filled with a homogenized mixture of 10% soil inoculum (plant species-specific conditioned soil) and 90% sterile soil (see above). Two controls were included in the test phase: 100% sterile soil and 90% sterile soil mixed with 10% field soil that was kept without plants in the greenhouse during the conditioning phase ("no plant" inoculum). Two chrysanthemum cuttings (without roots) were planted in each pot as preliminary work showed that not all cuttings establish properly with this method. Prior to planting, the soil in each pot was well watered and 100 ml half-strength Hoagland nutrient solution was added. The pots were placed on trolleys, each trolley had 48 pots and was tightly covered with a thin transparent plastic foil for 10 days to create a closed environment with high humidity that favors rooting. After 10 days, one of the chrysanthemum cuttings was removed from each pot. Plants were fertilized following grower's practice: half-strength Hoagland nutrient solution for the first 2 weeks, and single strength Hoagland solution during the following 2 weeks. For the last 2 weeks, the strength was increased to 1.6 mS/cm EC (electrical conductivity). The density of pots on each trolley was reduced 2 weeks after the start of the second phase to 32 pots per trolley so that there was 10 cm space between each pot.

Five days after the transparent plastic foil had been removed, 3 ml of the oospore suspension (ca. 355000 oospores of *P. ultimum*) was added onto the soil next to the stem of each plant allocated to the disease treatment. For plants in the control treatment (non-*Pythium* inoculated), 3 ml water was added. In both treatments, there were two replicate pots for each soil from the conditioning phase. Hence, the feedback phase comprised of 780 pots [(37 plant specific soil inocula + no plant soil inoculum) × 2 disease treatments × 5 soil replicates × 2 replicate pots + 100% sterile soil × 2 disease treatments × 10 replicates]. All pots were randomly arranged in a greenhouse compartment and kept under the same conditions as described for the conditioning phase.

Plant Performance and Disease Susceptibility

Six weeks after disease inoculation, all plants were harvested. For each plant, the total number of leaves and the number of yellow leaves was recorded and plant yellowness was calculated as the proportion of yellow leaves. The third fully expanded leaf from the top of each plant was then clipped and stored at −80°C for chlorogenic acid analysis (see below). Plants were then clipped at soil level and roots were rinsed from the soil. Shoot and root biomass were oven-dried (60°C for 3 days) and weighed and the root/shoot ratio was calculated. The main symptom of *Pythium* infection is the reduced root system caused by root rot (Agrios, 2005), and thus plant root/shoot ratio is used as an indicator of plant susceptibility to *Pythium*.

Analysis of Chlorogenic Acid

Chlorogenic acid acts as an important resistance factor in chrysanthemum against plant attackers such as herbivorous insects (Leiss et al., 2009). Chemical analysis was performed using high performance liquid chromatography (HPLC) with UV diode array detection following the procedure outlined by Olszewska (2007). Leaves were freeze-dried and finely ground. Ten mg of ground leaf material was then used for chemical analysis. Each leaf sample was extracted twice. In the first extraction, 1 ml 70% MeOH was added to each sample, vortexed for 0.5 min, then ultrasonicated for 30 min at 20°C, centrifuged for 10 min at 10000 rpm, and labeled. The extraction was repeated so that each sample was extracted by 2 ml 70% MeOH. The extraction was filtered using a 0.2 μm PTFE syringe filter and stored at −20°C until analysis. A standard solution that contained 10 mg chlorogenic acid per 10 ml 70% MeOH was used to produce an external standard curve. In each sample chlorogenic acid was then quantified based on the standard curve. The concentration of chlorogenic acid was determined, and expressed per g leaf dry weight.

Phylogenetic Analysis

We constructed a phylogenetic tree of the 37 plant species, and chrysanthemum using the program Phylomatic (Webb and Donoghue, 2005), in which a taxon list is matched against a backbone 'metatree,' returning a pruned tree of genus-level relationships. The backbone tree is based on the recent phylogenetic hypothesis of the Angiosperm Phylogeny Group (R20120829 for plants). We used the BLADJ algorithm of the Phylocom version 4.1 software package (Webb et al., 2008) to get branch lengths scaled to time, based on clade ages according to Wikström et al. (2001).

Statistical Analysis

Prior to analyses, data from the two pots with the same soil inoculum replicate of the same disease treatment were averaged. Sterile soil came from the same homogenized source, and therefore these ten replicate pots were kept as 10 replicates. Before conducting analysis, data were checked for homogeneity of variance and normality was confirmed by inspection of the residuals. The overall effects of plant species-specific inocula and pathogen inoculation on chrysanthemum were analyzed using a linear mixed model. In the model, plant species-specific inocula and disease treatment were set as fixed factors, and soil replicate was set as random factor. In this analysis, sterile soil and no plant soil inocula were not included, as they are not species-specific soil inocula.

The pathogen effect was calculated for each soil replicate (including sterile soil and the no plant soil inoculum) as biomass in disease soil minus biomass in no disease soil. One-way ANOVA was used to determine the difference of pathogen effects between soils. A one sample *t*-test was then used to determine for each soil inoculum if the pathogen effect was significantly different from zero. The soil effects (including sterile soil and no plant soil) in the control treatment were compared using one-way ANOVA. *Post hoc* Dunnett tests were performed to compare each plant species-specific inoculum with sterile soil and with the no plant soil inoculum. The analyses described above were done for chrysanthemum aboveground biomass, belowground biomass, leaf chlorogenic acid and root/shoot ratio

(Supplementary Figure 1). Plant proportional yellowness was not normally distributed, and thus the analyses were done slightly different. The effects of plant species-specific inocula and pathogen inoculation on chrysanthemum yellowness were analyzed using a generalized linear mixed model with binomial distribution and logit link function, with plant species-specific inocula and pathogen inoculation set as fixed factors, and soil replicate as random factor. The pathogen effect was calculated for each soil replicate (including sterile soil and no plant soil inocula) as proportion yellowness in disease soil minus that in no disease soil. One-way ANOVA was used to determine the difference of pathogen effects between soils. A one sample *t*-test was then used to determine for each soil inoculum if the pathogen effect was significantly different from zero. The soil effects (including sterile soil and no plant soil inoculum) in the control treatment were compared using a generalized linear model. *Post hoc* Dunnett tests were performed to compare each plant species-specific inoculum with sterile soil and with the no plant soil inoculum. To quantify plant–soil feedback effects of a conditioning species on chrysanthemum, the plant–soil feedback effect was calculated as natural log of the (chrysanthemum biomass (aboveground biomass + belowground biomass) on soil conditioned by that species minus average chrysanthemum biomass on sterile soil or no plant inoculum). This calculation was done for both the control treatment and the pathogen treatment. Two-way ANOVA was used to determine the overall effects of conditioning species and disease treatment on plant–soil feedback effects. A one sample *t*-test was used to determine for each species inoculum, if the effect was significantly different from zero.

To compare functional groups of the conditioning plant species (grass, forb, or legume), linear mixed models were used with plant functional group and pathogen inoculation as fixed factors, and soil replicate nested in plant species identity as a random factor, so that each conditioning species was considered a replicate. In this analysis, the sterile soil and no plant soil inoculum were not included, as these treatments were not allocated to a specific plant functional group. *Post hoc* tests were conducted with the functions 'glht' (multcomp package) and 'lsm' (lsmean package) to assess pairwise comparisons between plant functional groups. The analyses described above were done for chrysanthemum aboveground biomass, belowground biomass, root/shoot ratio and leaf chlorogenic acid. For plant yellowness, a generalized linear mixed model was used (binomial distribution and logit link function), with plant functional group and pathogen inoculation as fixed factors, and soil replicate nested in plant species identity as random factor. The same *post hoc* tests were done for pairwise comparisons of different plant functional groups.

Linear regression analysis was used to test the relationship between the phylogenetic distance of the conditioning plant species to chrysanthemum, and chrysanthemum biomass (aboveground biomass + belowground biomass). Linear regression analysis was also used to determine the relationship between chrysanthemum leaf chlorogenic acid and chrysanthemum aboveground biomass for the control and disease treatment separately. All analyses were performed in R (version 3.0.1, R Development Core Team, 2013).

RESULTS

Above- and belowground biomass of chrysanthemum plants differed significantly between inocula and average root and shoot biomass varied more than threefold (**Figure 1** and **Table 2**). In the control treatment, aboveground biomass of chrysanthemum grown with soil inocula from 8 species (*Thymus pulegioides, Crepis capillaris, Tagetes minuta, Hypochaeris radicata, Centaurea jacea, Medicago sativa, Vicia Sativa,* and *Trifolium arvense*) was significant lower than that of chrysanthemum grown in sterile soil. Compared to the no plant inoculum this was observed for 19 of the 37 species-specific soil inocula (**Figure 1A**). Overall, pathogen addition did not significantly influence plant aboveground biomass, and did not modify the effects of the different soil inocula on chrysanthemum aboveground biomass (no interaction between disease treatment and soil inoculum, **Table 2**). However, chrysanthemum growing with soil inocula conditioned by *Lolium perenne* and *Vicia sativa* had significantly higher aboveground biomass with *P. ultimum* than without *P. ultimum* addition (**Figure 1A**).

Root biomass of chrysanthemum grown with inocula conditioned by *Centaurea jacea* and *Trifolium arvense* was significantly lower than that of plants grown in 100% sterile soil in the no-disease treatment (**Figure 1B**). Addition of 12 species-specific soil inocula resulted in lower chrysanthemum root biomass than no plant soil inoculum. Addition of *P. ultimum* caused a significant reduction in root biomass but the interaction between disease addition and soil inoculation was not significant (**Table 2**). Addition of *P. ultimum* in soil inoculated with *Agrostis stolonifera, Achillea millefolium, Tanacetum vulgare,* or *Tagetes minuta* soil resulted in a significant reduction in root biomass. Root/shoot ratios were significantly lower in soil with *P. ultimum* addition (Supplementary Figure 1) and the effects of *P. ultimum* addition differed between inocula resulting in a significant interaction between these two factors (**Table 2**). Grass species had neutral to positive plant–soil feedback effects on chrysanthemum, while forb and legume species had neutral to negative plant–soil feedback effects compared to sterile soil with or without *Pythium* addition (Supplementary Figure 2A). Most plant species had negative plant–soil feedback effects on chrysanthemum when compared with the no plant inoculum either with or without *Pythium* addition (Supplementary Figure 2B).

The proportion of yellow leaves differed significantly between soil inocula (**Figure 2A** and **Table 2**). In the control treatment, leaf chlorogenic acid concentrations of plants growing in soils with *Capsella bursa-pastoris*, *Centaurea jacea*, *Medicago sativa*, *Trifolium arvense*, *Trifolium pratense*, and *Vicia sativa* inocula were significantly lower than in sterile soil, and leaf chlorogenic acid concentrations in soil conditioned by *Centaurea jacea* was significantly lower than no plant soil (**Figure 2B**). With *P. ultimum* inoculation, leaf chlorogenic acid concentrations of

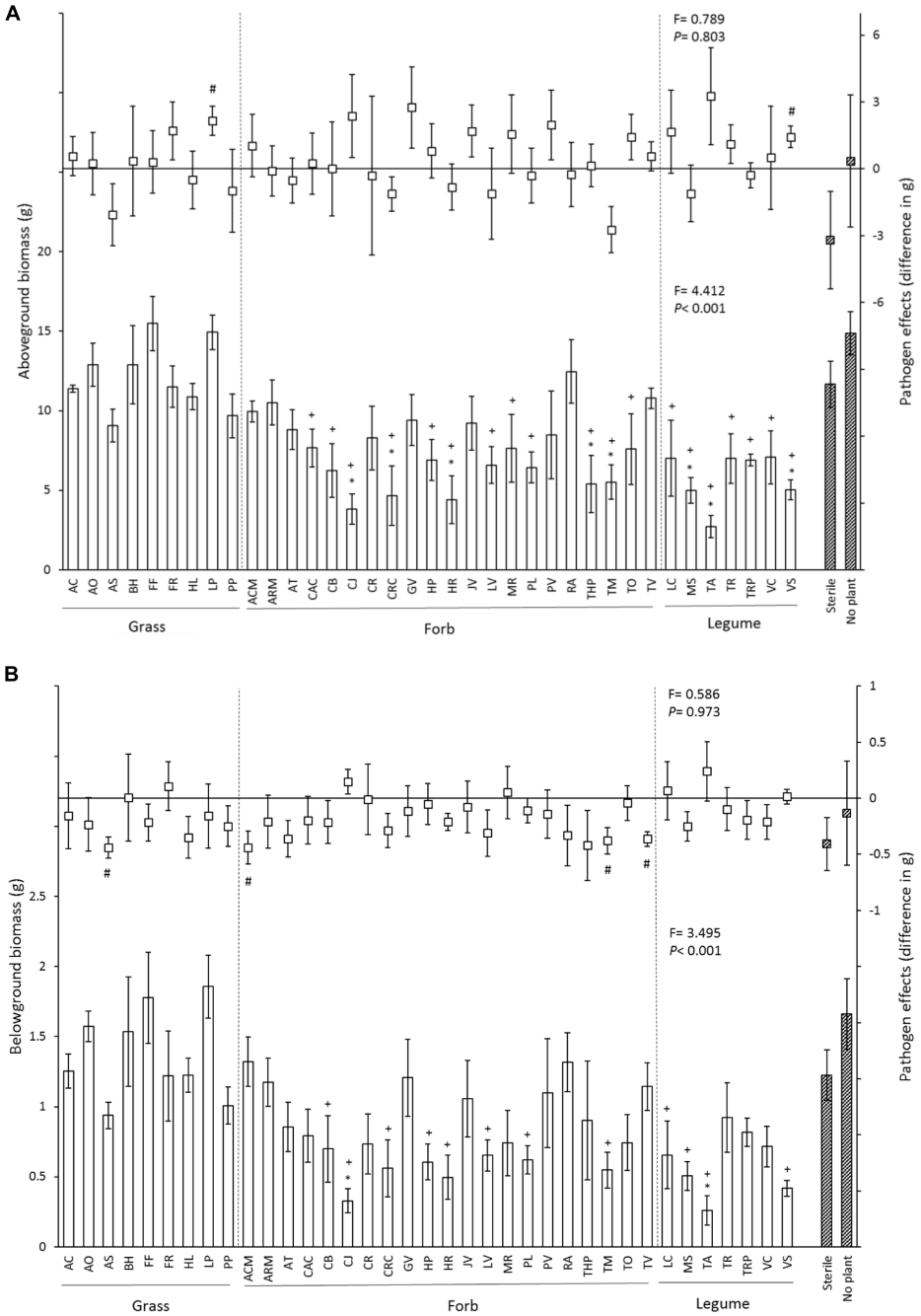

FIGURE 1 | Effects of 37 species-specific soil inocula, no plant inoculum and sterile soil on chrysanthemum aboveground biomass **(A)** and belowground biomass **(B)**. In each figure, bars represent chrysanthemum biomass (mean ± SE) of soil inocula in control soil, and squares represent the pathogen effect on plant biomass (biomass in *P. ultimum* soil – biomass in non-*Pythium* inoculated soil). Striped bars indicate controls. "*" Represents significant difference from the sterile soil ($P < 0.05$). "+" Represents significant difference from the no plant soil inoculum ($P < 0.05$), "#" represents significantly different from zero ($P < 0.05$). Dashed lines separate soil inocula into different functional groups. Species abbreviations are given in **Table 1**. Statistics presented in the lower part of each panel represent the effects of soil on chrysanthemum biomass in control soil, and statistics presented in the upper part of each panel indicate the effects of soil inocula on the disease severity of chrysanthemum biomass.

plants growing in soils with *Lolium perenne* and *Crepis capillaris* inocula were significantly lower than those in control treatment, while leaf chlorogenic acid concentrations of plants growing in soil conditioned by *Capsella bursa-pastoris*, *Centaurea jacea* were significantly higher than those growing in control soil (**Figure 2B**).

Both aboveground and belowground biomass of chrysanthemum differed significantly between functional groups of the conditioning plant species (**Figures 3A,B**). Addition of soil inocula created by grasses resulted in significantly higher above- and belowground biomass of chrysanthemum than addition of forb or legume inocula. The root/shoot ratio differed between functional groups of the conditioning plant species and disease treatment, there were interactions between functional groups and the disease treatment (**Figure 3C** and **Table 2**). Root/shoot ratios did not differ between grass, legume or forb inocula in control soil but in presence of *P. ultimum*, root/shoot ratios were significantly lower with forb than with grass inocula (**Figure 3C**).

The proportion of yellow leaves differed significantly between functional groups of the conditioning plant species (**Figure 3D**). *Pythium ultimum* inoculation did not significantly influence chrysanthemum yellowness. Addition of soil inocula created by grasses resulted in significantly lower chrysanthemum yellowness than addition of forb or legume inocula.

The concentration of chlorogenic acid was significantly influenced by the identity of the plant species that was used to create the inoculum but did not differ between plant functional groups (**Figure 3E** and **Table 2**). The concentration of chlorogenic acid significantly increased in response to *P. ultimum* addition (**Figure 3E** and **Table 2**). Chlorogenic acid concentrations were positively related with chrysanthemum aboveground biomass in both the no-disease and disease treatments (**Figures 4A,B**).

There was no significant relationship between phylogenetic distance and the effect of the inoculum on chrysanthemum growth (R^2 = 0.05, P = 0.11) (**Figure 5**). Topology of the phylogenetic tree is given in Supplementary Figure 3.

DISCUSSION

Our study shows that the identity of the plant species that conditioned the soil had a large effect on the plant–soil feedback effects on chrysanthemum growth and that plant functional group is a strong determinant of plant–soil feedback effects. When quantifying plant–soil feedback effects relative to sterile soil, most legume and forb species had negative plant–soil feedback effects on chrysanthemum biomass. In contrast, grass species had neutral to positive feedback effects on chrysanthemum biomass, and this became more apparent when *Pythium* was added. Moreover, addition of grass inocula led to more biomass and less yellowness than addition of legume or forb inocula, and led to less strong *Pythium* effects than addition of forb inocula. Importantly, and contrary to our initial hypothesis, addition of soil inocula that were created by legumes did not result in positive effects on chrysanthemum growth and did not reduce disease severity.

Inoculation with eight of the 37 soil inocula we tested negatively influenced chrysanthemum biomass compared with growth on sterile soil. Interestingly, plants grown with *Lolium perenne* inoculum that were exposed to *P. ultimum* had higher aboveground biomass than plants without *P. ultimum*. *Lolium perenne* has a highly diverse soil microbial community (Wardle et al., 2003; Clayton et al., 2005), and this species has been reported to cause increases in the density of bacteria that produce biocontrol compounds, such as 2,4-diacetylphloroglucinol, pyrrolnitrin and hydrogen cyanide (Latz et al., 2015). Thus, chrysanthemum plants grown with *Lolium perenne* inoculum may have been primed by these rhizobacteria, so that later when exposed to *P. ultimum*, the plants could respond better and faster to pathogen invasion (Pieterse et al., 2014). Pathogen infection can also lead to higher root colonization of beneficial bacteria (Rudrappa et al., 2008; Liu et al., 2014). This may explain why the biomass of chrysanthemum grown with *Lolium perenne* inoculum was larger in presence of *P. ultimum* than without the pathogen.

Chrysanthemum grown in soil with grass inocula sustained higher above- and belowground biomass than plants grown with inocula conditioned by legumes or forbs. This is partially in line with our hypothesis that grass and legume inocula have a more positive influence on chrysanthemum growth than forb inocula. Other studies with the same and with different soils have shown that the composition of the microbial community of grass-conditioned soil differs distinctly from legume-conditioned soil (Chen et al., 2008; Kos et al., 2015). Several other studies have shown that grasses in particular increase the abundance of soil bacteria, such as *Bacillus*, *Pseudomonas* and *Actinomyces*, which can act as antagonists of soil pathogens (Latz et al., 2012, 2016; Chen et al., 2016). Moreover, grasses can also increase the abundance of AM-fungi (De Deyn et al., 2010). These mechanisms may explain the better effects of grass inocula relative to legume or forb inocula in our study. Grass inocula

TABLE 2 | Overall effects of identity and functional group of the conditioning plant species, and of *Pythium* addition on aboveground biomass, belowground biomass, root/shoot ratio, proportion of yellow leaves and leaf chlorogenic acid concentrations in chrysanthemum.

	df	Aboveground biomass	Belowground biomass	Root/shoot ratio	Yellowness	Chlorogenic acid
Species	36, 148	6.01***	5.18***	1.94**	1.62*	2.05**
Pythium	1, 148	2.83	23.83***	115.15***	0.13	5.87*
Species × *Pythium*	36, 148	0.74	0.66	1.73*	1.40	1.47
Functional group	2, 34	14.30***	15.46***	5.89**	6.52**	2.71
Pythium	1, 182	2.97	25.57***	103.83***	0.01	5.20*
Functional group × *Pythium*	2, 182	0.53	1.20	3.86*	0.02	1.57

*Data presented are degrees of freedom (df) and F-values from the linear mixed models and generalized linear mixed model (only used for yellowness). Asterisks indicate significant effects at ***P < 0.001, **P < 0.01, *P < 0.05.*

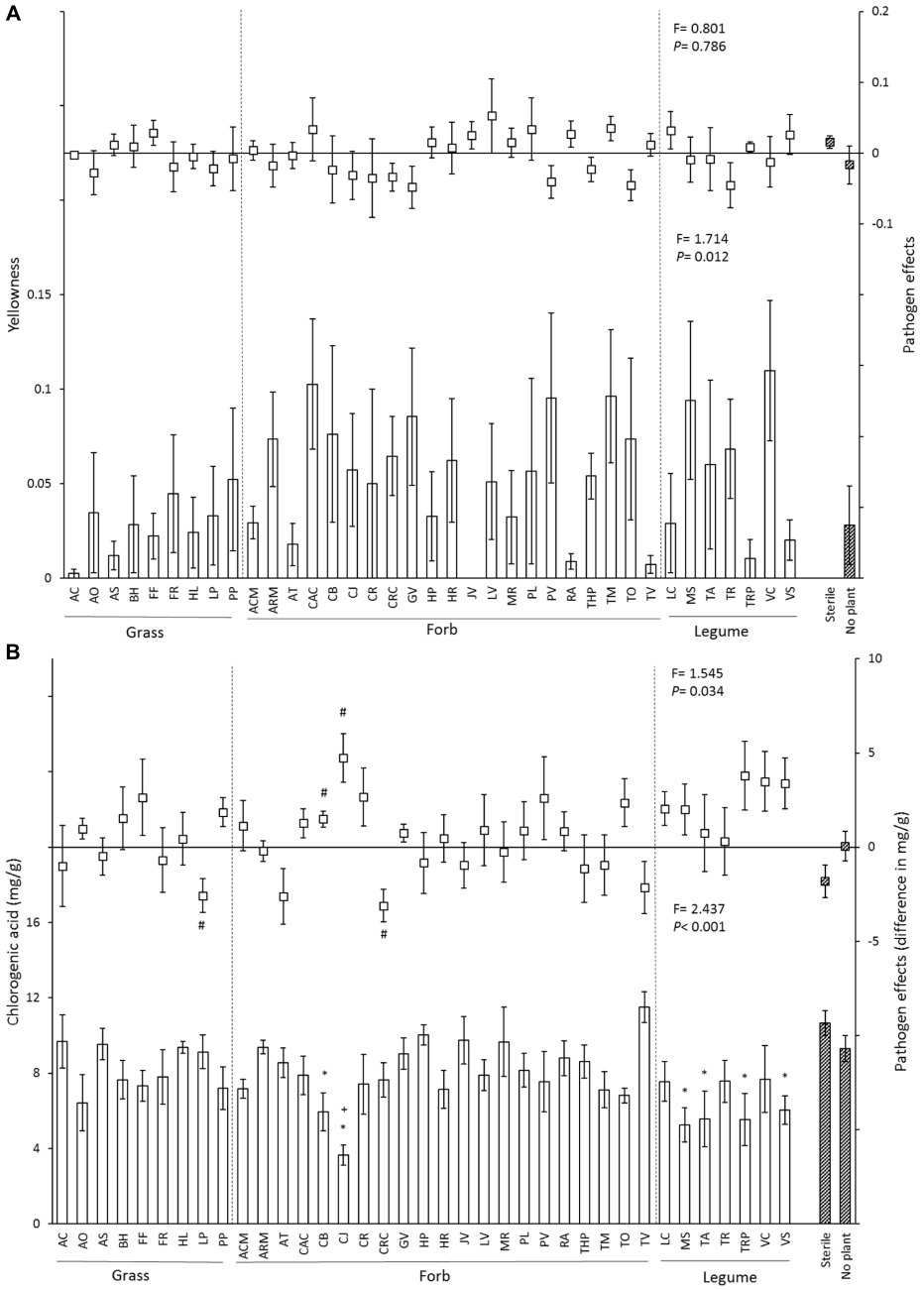

FIGURE 2 | Effects of 37 species-specific soil inocula, no plant inoculum and sterile soil on chrysanthemum yellowness **(A)** and leaf chlorogenic acid concentration **(B)**. In each figure, bars represent the mean (±SE) of each soil inoculum in control soil, and squares represent the pathogen effect (value in *P. ultimum* soil – value in non-*Pythium* inoculated soil). Striped bars indicate controls. "*" Represents significant difference from the sterile soil ($P < 0.05$). "+" Represents significant difference from the no plant soil inoculum ($P < 0.05$), "#" represents significantly different from zero ($P < 0.05$). Dashed lines separate soil inocula into different functional groups. Statistics presented in the lower part of each panel represent the effects of soil in control soil, and statistics presented in the upper part of each panel indicate the effects of soil inocula on the disease severity of chrysanthemum biomass.

also sustained lower chrysanthemum yellowness than forb or legume inocula, and grass inocula overall increased plant growth and health more than legume or forb inocula. Steaming soil can kill both beneficial and pathogenic microbes in the soil, and this can lead to the rapid build-up of soil pathogens. Although grass-conditioned soil inocula did not enhance chrysanthemum growth more than that of plants grown in sterile soil, our study shows that it can provide other benefits to plants, e.g., higher resistance to pathogen infection. For example, in presence of *Pythium*, addition *Lolium perenne* inoculum, resulted in

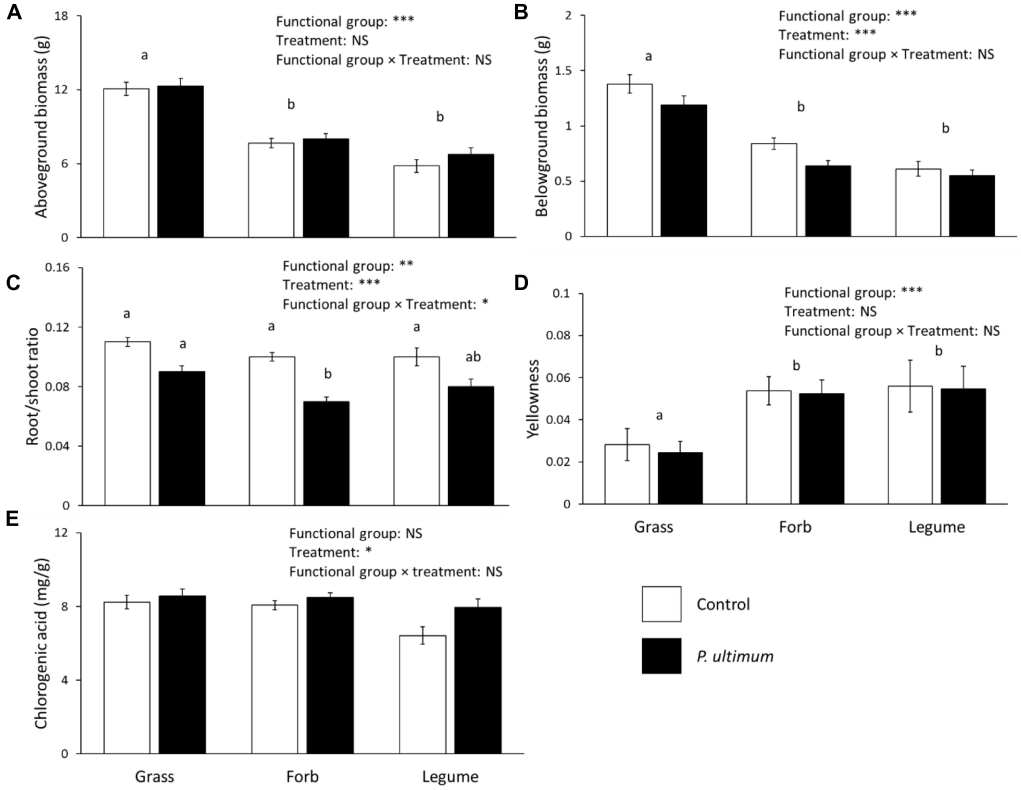

FIGURE 3 | Effects of plant functional group and pathogen addition on chrysanthemum aboveground biomass **(A)**, belowground biomass **(B)**, root/shoot ratio **(C)**, proportion of yellow leaves **(D)**, and leaf chlorogenic acid concentration **(E)**. Data show means ± SE, with white bars representing control soil, and black bars representing the *P. ultimum* treatment. Different letters indicate significant differences between functional groups ($P < 0.05$). For root/shoot ratio, different letters above bars indicate significant differences ($P < 0.05$). Full statistics are listed in **Table 2**.

higher chrysanthemum aboveground biomass. Further studies concerning the microbial interactions between soil pathogen addition and species-specific soil inocula are needed to unravel the mechanism behind this.

Surprisingly and in contrast to our hypothesis, chrysanthemum performance was worse overall with legume inocula. Legumes are often used in crop rotation to increase nitrogen content of soils (Drinkwater et al., 1998). Since in our experiments chrysanthemum plants were heavily fertilized, a nitrogen-mediated benefit of legume soil is unlikely. In contrast, the negative influence of soil inocula conditioned by legumes on chrysanthemum growth could be explained by the negative effects of legumes on certain beneficial soil bacteria (Latz et al., 2012, 2015). Legumes produce steroid saponins that act as antifungal and antibacterial compounds (Mahato et al., 1982). Moreover, the rhizobia have similar colonization strategies to both legume and non-legume plants, however, rhizobia refine their strategy to symbiosis when interacting with legumes (Soto et al., 2006, 2009). Thus, for the non-leguminous plant chrysanthemum, rhizobia would act like pathogens, explaining the reduction of plant growth in soils conditioned by legumes. Addition of soil inocula created by forbs overall also significantly decreased chrysanthemum growth. Chrysanthemum root/shoot ratios indicated plant susceptibility to *Pythium*, as *Pythium* infection reduces the root system and leads to root rot (Agrios, 2005). There were no significant differences between chrysanthemum root/shoot ratios in grass, forb or legume inocula without *P. ultimum* addition. However, with *P. ultimum* addition, chrysanthemum root/shoot ratios of plants growing with in forb inocula decreased significantly more than that of plants growing with grass inocula, suggesting poor plant resistance to *P. ultimum* attack when grown with forb inocula. Forbs generally allocate less carbon to roots and have overall less microbial activity and abundance in roots than grasses (Warembourg et al., 2003; Chen et al., 2016). Hence, we speculate that the microbial community of soil inocula from forbs was smaller or less active or diverse than the microbial community of grasses. Whether this is true remains to be tested.

Plant–soil feedback effects can also be due to the modification of abiotic conditions (Ehrenfeld et al., 2005). However, in our study, we inoculated 90% homogenized sterile soil with 10% conditioned soil, and thus we minimized the heterogeneity of abiotic factors (Kardol et al., 2006). More importantly, in the feedback phase, plants received a high dose of Hoagland fertilizer following common practice in commercial chrysanthemum greenhouses. Thus it is highly unlikely that inocula-related differences in nutrient availability influenced the results in

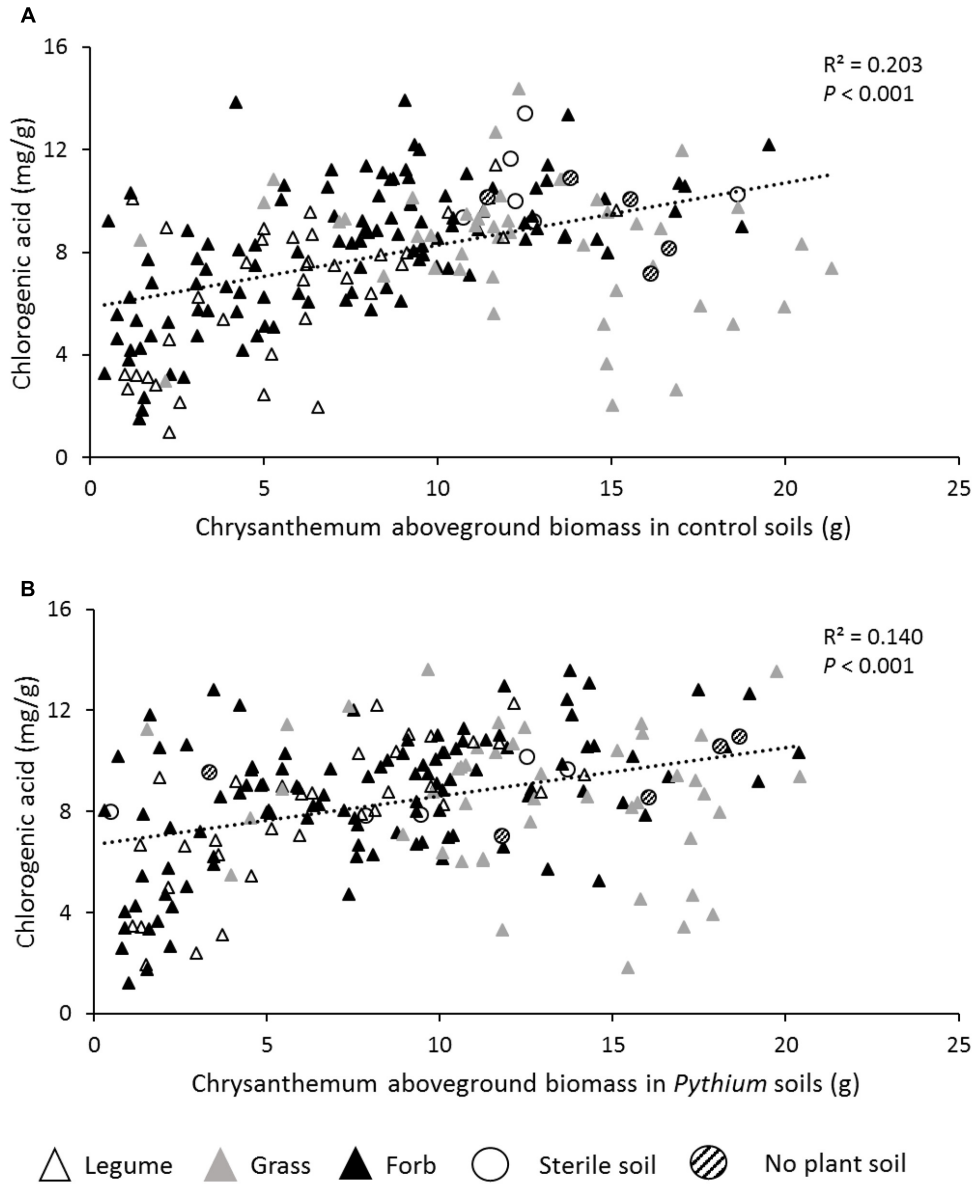

FIGURE 4 | Relationships between chrysanthemum leaf chlorogenic acid concentration and aboveground biomass in control soil **(A)**, and *Pythium* added soils **(B)**. Black triangles represent forb inocula; Gray triangles represent grass inocula; White triangles represent legume inocula; White circles represent 100% sterile soil; Striped circles represent no plant soil.

our study, and therefore we can assume that the different plant–soil feedback effects were due to differences in microbial communities. Nutrient-rich substrates are typically exploited by r-strategist species such as *P. ultimum*, and the suppression of *P. ultimum* can be difficult in soils with high nutrient levels (van Bruggen and Semenov, 2000). This may explain why the inocula were relative ineffective in suppressing *P. ultimum* infection.

Overall, the concentration of chlorogenic acid in chrysanthemum leaves differed significantly between the inocula. However, although the concentration of leaf chlorogenic acid was positively related with aboveground plant biomass, and grass inocula sustained significantly higher chrysanthemum aboveground biomass compared to forb inocula or legume inocula, the concentration of chlorogenic acid in grass inocula did not differ from those in legume inocula or forb inocula. The concentration of leaf chlorogenic acid was found to be positively correlated with plant carbon assimilation rates in sorghum (Turner et al., 2016). In our study, the levels of aboveground chlorogenic acid also increased with pathogen attack belowground compared to uninfected plants. Soil pathogens can increase aboveground plant defense even in absence of aboveground plant antagonists (Bezemer and van Dam, 2005). In chrysanthemum, chlorogenic acid is

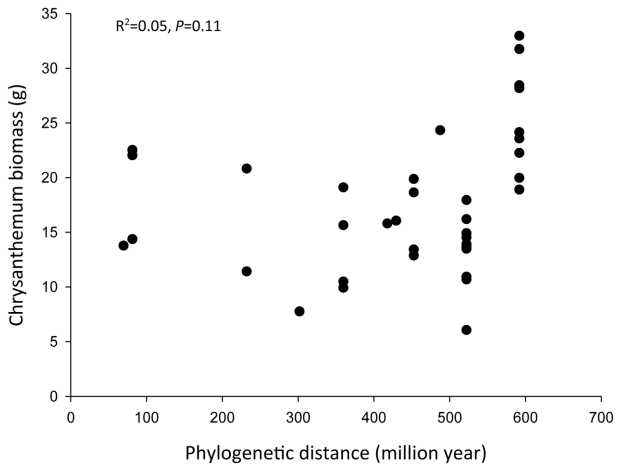

FIGURE 5 | Effects of phylogenetic relationships on the chrysanthemum biomass (aboveground biomass + belowground biomass). The phylogenetic distance is the distance of conditioning plant species to chrysanthemum. The phylogenetic relationship is based on a backbone tree of the recent phylogenetic hypothesis of the Angiosperm Phylogeny Group (R20120829 for plants). We used the BLADJ algorithm of the Phylocom to get branch lengths scaled to time, based on clade ages according to Wikström et al. (2001).

related to resistance against thrips (Leiss et al., 2009, 2011), as well as to other herbivores, such as leafminers and spider mites (Kos et al., 2014). Our work therefore suggests that soil inoculation but also the presence of soil pathogens can influence the resistance of chrysanthemum against aboveground herbivorous pests and that plant–soil feedback effects may influence pest severity and biocontrol in chrysanthemum cultivations.

In contrast to our hypothesis, the plant–soil feedback effect of species closely related to chrysanthemum was not more severe than that of distantly related species. It may be possible that beyond a certain threshold phylogenetic distance, effects do become apparent, as shown by the grass clade, which is the most distantly related one. To prove this, future studies should select species across large phylogenetic scales to test their plant–soil feedback effects. Our result is in line with an increasing number of studies with wild plant species showing that phylogenetic distance is a poor predictor of plant–soil feedback effects (Pavoine et al., 2013; Kelly et al., 2014; Mehrabi and Tuck, 2015; Mehrabi et al., 2015). Thus, although our study demonstrated species specific plant–soil feedback effects, these patterns may not correspond to mechanisms like shared pathogens or symbionts. Moreover, there is a growing awareness that the phylogenetic distance is a weak predictor of the dissimilarity of plant functional traits (Mouquet et al., 2012; Pavoine et al., 2013; Kelly et al., 2014). If for example, traits responsible for resource use or host susceptibility to natural enemies are not conserved, the plant species will influence or respond to the soil in a very different way even though they are closely related (Mehrabi and Tuck, 2015). Several recent studies have shown that PSF effects can be predicted from life history forms or plant traits such as root thickness or density or plant growth rate (Baxendale et al., 2014; Cortois et al., 2016; De Deyn, 2017). Therefor, plant traits instead of phylogenetic distance could be a good predictor of plant soil feedback effects.

CONCLUSION

In summary, we demonstrate that plant species through changes in the soil can influence the growth, disease susceptibility and the concentration of aboveground defense compounds of cultivated crop species, all in a species-specific manner. Our results further show clearly that these plant–soil feedback effects depend on plant functional groups of the species where the inocula are created from, with the highest chrysanthemum performance in soil with grass inocula. Our study with a cultivated plant species highlights that species-specific plant–soil feedback effects can also play an important role in deciphering interactions between plants and pathogens or herbivorous insects in horticulture. Disentangling the mechanisms of enhanced plant performance, and evaluating the consequences for plant yield in a real horticultural setting may allow us to implement the concept of plant–soil feedbacks in current greenhouse horticulture.

AUTHOR CONTRIBUTIONS

H-KM, AvdW, and TMB conceived the ideas and designed methodology; H-KM, CR, and TMB collected the data; H-KM, AP, and TMB analyzed the data; H-KM, AP, and TMB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for 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/fpls.2017.02127/full#supplementary-material

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