INDUCING PLANT RESISTANCE AGAINST INSECTS USING EXOGENOUS BIOACTIVE CHEMICALS: KEY ADVANCES AND FUTURE PERSPECTIVES

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PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-124-1
DOI 10.3389/978-2-88976-124-1

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Citation: Sobhy, I. S., Lou, Y., Bruce, T. J. A., eds. (2022). Inducing Plant Resistance Against Insects Using Exogenous Bioactive Chemicals: Key Advances and Future Perspectives. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-124-1

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frontiers | Frontiers in Plant Science

EDITORIAL
published: 18 April 2022
doi: 10.3389/fpls.2022.890884

Editorial: Inducing Plant Resistance Against Insects Using Exogenous Bioactive Chemicals: Key Advances and Future Perspectives

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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: 07 March 2022
Accepted: 28 March 2022
Published: 18 April 2022

Citation:
Sobhy IS, Lou Y and Bruce TJA (2022) Editorial: Inducing Plant Resistance Against Insects Using Exogenous Bioactive Chemicals: Key Advances and Future Perspectives. Front. Plant Sci. 13:890884. doi: 10.3389/fpls.2022.890884

Keywords: induced plant defenses, tritrophic interactions, priming, insect herbivores, crop protection

Editorial on the Research Topic

Inducing Plant Resistance Against Insects Using Exogenous Bioactive Chemicals: Key Advances and Future Perspectives

Due to the constraints and hazards of using insecticides such as development of insect resistance, severe decline in availability of conventional pesticides and off-target effects on beneficial insects (Desneux et al., 2007), there is an urgent need to develop the underpinning science to protect crop harvests from insect pests in the face of rising demand for food (Savary et al., 2019). Given the recent advances in our understanding of plant-insect interactions, it is proposed that boosting the overall plant immunity could provide novel alternative control tactics. Constitutively increasing defense could have a negative trade-off with growth or yield (Huot et al., 2014) and therefore inducing resistance could be a more attractive prospect.

During their coevolution with insects, plants have evolved a complex arsenal of defense mechanisms against antagonistic herbivores while also attracting beneficial insects (Bruce, 2015). Some plant defenses are constitutive (i.e., always present in the plants) while others are induced after plant perception of stimuli associated with insect herbivory (Erb et al., 2012). Such inducible defenses against insect herbivores are regulated by the signaling of two major phytohormones, i.e. salicylic acid (SA) and jasmonic acid (JA) (Thaler et al., 2012). Typically, JA is associated plant defenses against chewing insects while SA induces resistance against piercing/sucking insects but there is considerable variation between different insect-plant systems (Erb et al., 2012).

The induction of both JA and SA pathways can also be achieved by applying bioactive chemicals that act as inducers of plant resistance against herbivores (Pickett and Poppy, 2001). An accumulated body of studies, from the last three decades, has explored inducing plant defense *via* the application of bioactive chemicals as a sustainable and ecologically sound approach to control insect pests in agriculture (Stout et al., 2002; Sobhy et al., 2014). Even though there has been mounting attention to the potential of using defense inducers, their exploitation in agricultural practice is still at its infant stage and therefore further development is required (Yassin et al., 2021). Given that, the objective of this Research Topic is to highlight recent progress, key advances, and future perspectives in manipulating plant defense against insect pests using bioactive chemicals. Fourteen articles were published in this Research Topic, and from these we only focus our editorial

on ten papers covering the following research themes: (1) defense inducers impact on plant growth and yield; (2) dual effect of plant inducers on pathogen and insect pests; (3) genotypic variation in elicitor -induced defense; and (4) exploiting these chemicals as defense inducing or priming agents.

Little is still known about the possible negative trade-off effects of defense inducers on plant growth and yield under field conditions (Yassin et al., 2021). Four papers in the Research Topic address the impact of defense inducers on plant growth and yield. Bhavanam and Stout found that when seed treatment with JA and methyl jasmonate (MeJA) enhanced resistance of rice plants to rice water weevils but also reduced seedling emergence, plant height, and filled grain mass. Under field conditions, Yoshida et al. observed that whereas Japanese radish treatment with Prohydrojasmon (PDJ) induced direct and indirect defense against several insect pest species (e.g., aphids, leaf-mining fly larvae, vegetable weevils, and thrips), the biomass of both aboveground and belowground parts of PDJ treated plants was significantly lower than untreated plants. Similarly, Chen et al. provide another field evidence that MeJA significantly slowed down the growth of Conifer seedling relative to control. In contrast, Mouden et al. reported that strawberry growth was not affected by MeJA application as were fruit yield and quality whereas leaf damage by thrips was lower on treated plants. These four papers therefore support the idea that negative impact on plant overall growth may impede agricultural exploitation of plant defense inducers (Walters and Heil, 2007).

To prioritize one response over the other, plants under natural conditions exhibit a crosstalk between plant hormonal signaling pathways which may result in either synergistic or antagonistic effects (Spoel and Dong, 2008). Exogenous application of elicitors of pathogen resistance could lead to increased susceptibility to one or more attacking herbivores due to negative crosstalk between the SA and JA pathways (Sobhy et al., 2012; Thaler et al., 2012). Two papers in the Research Topic address SA and JA crosstalk following plant treatment with defense elicitors. Puentes et al. found that MeJA treatment not only increased resistance to the pine weevil *Hylobius abietis* but also enhanced the Norway spruce (*Picea abies*) resistance to the necrotrophic blue-stain fungus *Endoconidiophora polonica*. Using a trophic system comprising of cranberries, the phytoplasma that causes false blossom disease, and two herbivores—the blunt-nosed leafhopper (*Limotettix vaccinii*), the vector of false blossom disease, and the non-vector gypsy moth (*Lymantria dispar*), Rodriguez-Saona et al. evaluated the treatment effect of four commercial elicitors, including three that activate mainly SA-related plant defenses (Actigard, LifeGard, and Regalia) and one activator of JA-related defenses (Blush) on cranberries defense induction. They found that phytoplasma infection and elicitor treatment had positive effects on *L. vaccinii* and *L. dispar* performance in cranberries, likely *via* enhancement of plant nutrition and changes in phytohormone profiles, suggesting that the studied elicitors did not improve herbivore resistance or reduce phytoplasma infection in cranberries.

To advance practical use of defense activators, more consistent and repeatable responses to treatment are required. Plant responses to elicitor chemicals are variable due to differences in plant genotypes and environmental conditions they are deployed in, which is a main reason why it is challenging to use them widely in agriculture (Bruce, 2014). In this article collection, when Puentes et al. examined genotypic variation between nine clones of *P. abies* in MeJA-induced responses, they found that MeJA treatment increased resistance to *H. abietis* damage and *E. polonica* infection, but effects varied among clones depending on their constitutive resistance levels. In addition, using a model system comprising the generalist herbivore fall armyworm (FAW) *Spodoptera frugiperda* and three economically important plant species with differential ability to uptake silicon: tomato (non-Si accumulator), soybean, and maize (Si-accumulators), Acevedo et al. found that FAW herbivory and Si supply increased peroxidase (POX) activity and trichome density in tomato, and the concentration of phenolics in soybean suggesting variations in defense inducibility between plant species. The same pattern was also reported by Ali et al. who investigated the effect of treating a range of Brassica cultivars with the defense activators *cis*-Jasmone (CJ) on tritrophic interactions with *Myzus persicae* aphids and their parasitoid *Diaeretiella rapae*. They found that CJ treatment made plants less attractive to and less suitable for *M. persicae* but more attractive to *D. rapae* in certain brassica cultivars due to variation of emitted volatile profiles upon CJ treatment.

Defense priming allows plants to deploy induced defenses more rapidly and robustly when subsequently challenged by future insect attacks (Conrath et al., 2015) and can be triggered using certain compounds called priming agents (Sobhy et al., 2018). In this collection, two papers addressed this phenomenon under greenhouse and field conditions. Shiojiri et al. conducted a field experiment for 2 years in which rice seedlings were exposed to artificially damaged weed volatiles and then leaf damage was observed. They found that total number of damaged leaves in volatile-exposed plants was significantly lower but their grain weight per bunch was significantly higher, indicating a significant increase of grain numbers and thereby yield production. Testing another cereal plant, Ninkovic et al. exposed barley plants to methyl salicylate (MeSA) and then investigated its biological effects on the bird cherry-oat aphid *Rhopalosiphum padi* after different time intervals. They found that aphid settlement and behavior were negatively affected on MeSA-exposed plants due to subsequent metabolic changes in the released volatiles and phloem content.

In conclusion, this Research Topic identifies possible reasons why practical application of defense inducers, on farms for crop protection, is still low and highlights that more research under field conditions is still needed in future to further develop defense inducers. This would facilitate their adoption in IPM programs as environmentally safe and IPM-compatible agrochemicals to manage insect pests. The demand for alternatives is increasing due to restrictions in availability of conventional pesticides. To this end, the effects of defense inducers on (i) plant growth, productivity and yield, (ii) multiple plant antagonists, and (iii) effectiveness across a range of crop germplasm, still needs to be explored.

AUTHOR CONTRIBUTIONS

IS, YL, and TB contributed to organizing this Research Topic. IS wrote the first draft of this editorial based on the contributed articles. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank the Frontiers in Plant Science for such opportunity to edit this Research Topic and the Journal Editorial Office for their technical support which facilitate our task. We thank also all the authors for their contribution to our Research Topic and the reviewers for their critical reviewing of the manuscripts.

REFERENCES

Bruce, T. J. A. (2014). Variation in plant responsiveness to defense elicitors caused by genotype and environment. *Front. Plant Sci.* 5:349. doi: 10.3389/fpls.2014.00349

Bruce, T. J. A. (2015). Interplay between insects and plants : dynamic and complex interactions that have coevolved over millions of years but act in milliseconds. *J. Exp. Bot*. 66, 455–465. doi: 10.1093/jxb/eru391

Conrath, U., Beckers, G. J. M., Langenbach, C. J. G., and Jaskiewicz, M. R. (2015). Priming for enhanced defense. *Annu. Rev. Phytopathol.* 53, 97–119. doi: 10.1146/annurev-phyto-080614-120132

Desneux, N., Decourtye, A., and Delpuech, J.-M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol*. 52, 81–106. doi: 10.1146/annurev.ento.52.110405.091440

Erb, M., Meldau, S., and Howe, G. A. (2012). Role of phytohormones in insect-specific plant reactions. *Trends Plant Sci*. 17, 250–259. doi: 10.1016/j.tplants.2012.01.003

Huot, B., Yao, J., Montgomery, B. L., and He, S. Y. (2014). Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Mol. Plant* 7, 1267–1287. doi: 10.1093/mp/ssu049

Pickett, J. A., and Poppy, G. M. (2001). Switching on plant genes by external chemical signals. *Trends Plant Sci*. 6, 137–139. doi: 10.1016/S1360-1385(01)01899-4

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nat. Ecol. Evol*. 3, 430–439. doi: 10.1038/s41559-018-0793-y

Sobhy, I. S., Bruce, T. J. A., and Turlings, T. C. J. (2018). Priming of cowpea volatile emissions with defense inducers enhances the plant's attractiveness to parasitoids when attacked by caterpillars. *Pest Manag. Sci.* 74, 966–977. doi: 10.1002/ps.4796

Sobhy, I. S., Erb, M., Lou, Y., and Turlings, T. C. J. (2014). The prospect of applying chemical elicitors and plant strengtheners to enhance the biological control of crop pests. *Philos. Trans. R. Soc. B Biol. Sci*. 369:20120283. doi: 10.1098/rstb.2012.0283

Sobhy, I. S., Erb, M., Sarhan, A. A., El-Husseini, M. M., Mandour, N. S., and Turlings, T. C. J. (2012). Less is more: treatment with BTH and Laminarin reduces herbivore-induced volatile emissions in maize but increases parasitoid attraction. *J. Chem. Ecol*. 38, 348–360. doi: 10.1007/s10886-012-0098-6

Spoel, S. H., and Dong, X. (2008). Minireview making sense of hormone crosstalk during plant immune responses. *Cell Host Microbe* 3, 348–351. doi: 10.1016/j.chom.2008.05.009

Stout, M. J., Zehnder, G. W., and Baur, M. E. (2002). Potential for the use of elicitors of plant resistance in arthropod management programs. *Arch. Insect Biochem. Physiol*. 51, 222–235. doi: 10.1002/arch.10066

Thaler, J. S., Humphrey, P. T., and Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci*. 17, 260–270. doi: 10.1016/j.tplants.2012.02.010

Walters, D., and Heil, M. (2007). Costs and trade-offs associated with induced resistance. *Physiol. Mol. Plant Pathol*. 71, 3–17. doi: 10.1016/j.pmpp.2007.09.008

Yassin, M., Ton, J., Rolfe, S. A., Valentine, T. A., Cromey, M., Holden, N., et al. (2021). The rise, fall and resurrection of chemical-induced resistance agents. *Pest Manag. Sci*. 77, 3900–3909. doi: 10.1002/ps.6370

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 11 February 2021
doi: 10.3389/fpls.2021.631824

Silicon-Mediated Enhancement of Herbivore Resistance in Agricultural Crops

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Silicon (Si) is a beneficial mineral that enhances plant protection against abiotic and biotic stresses, including insect herbivores. Si increases mechanical and biochemical defenses in a variety of plant species. However, the use of Si in agriculture remains poorly adopted despite its widely documented benefits in plant health. In this study, we tested the effect of Si supplementation on the induction of plant resistance against a chewing herbivore in crops with differential ability to accumulate this element. Our model system comprised the generalist herbivore fall armyworm (FAW) *Spodoptera frugiperda* and three economically important plant species with differential ability to uptake silicon: tomato (non-Si accumulator), soybean, and maize (Si-accumulators). We investigated the effects of Si supply and insect herbivory on the induction of physical and biochemical plant defenses, and herbivore growth using potted plants in greenhouse conditions. Herbivory and Si supply increased peroxidase (POX) activity and trichome density in tomato, and the concentration of phenolics in soybean. Si supplementation increased leaf Si concentration in all plants. Previous herbivory affected FAW larval weight gain in all plants tested, and the Si treatment further reduced weight gain of larvae fed on Si accumulator plants. Notably, our results strongly suggest that non-glandular trichomes are important reservoirs of Si in maize and may increase plant resistance to chewing herbivores. We conclude that Si offers transient resistance to FAW in soybean, and a more lasting resistance in maize. Si supply is a promising strategy in management programs of chewing herbivores in Si-accumulator plants.

Keywords: silicon, plant resistance, plant defenses, *Spodoptera frugiperda*, tomato, maize, soybean

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *21 November 2020*
Accepted: *18 January 2021*
Published: *11 February 2021*

Citation:
Acevedo FE, Peiffer M, Ray S, Tan C-W and Felton GW (2021) Silicon-Mediated Enhancement of Herbivore Resistance in Agricultural Crops. Front. Plant Sci. 12:631824. doi: 10.3389/fpls.2021.631824

INTRODUCTION

Silicon (Si), one of the most abundant elements on earth is ubiquitously present in the soil, but mainly in forms unavailable for plant uptake (Tubaña and Heckman, 2015). Si in the form of silicic acid is absorbed by a diverse number of plant families and stored as hydrated silica (SiO_2nH_2O) in roots and shoots (Hodson et al., 2005; Trembath-Reichert et al., 2015). All rooted plants interact with Si in the soil, but there is great variation in their ability to accumulate Si in their tissues. The concentration of Si in shoots can range from 0.1 to 10% of dry weight among different plant species (Ma and Yamaji, 2015). A large number of studies have reported the benefits of Si in alleviating the effects of a stunning number of plant biotic and abiotic stresses, including drought, salinity, metal

toxicity, nutrient deficiency, heat, cold, pathogens, and insect herbivores (Cooke and Leishman, 2016; Imtiaz et al., 2016; Debona et al., 2017; Luyckx et al., 2017; Etesami and Jeong, 2018; Hall et al., 2019; Zhu et al., 2019; Singh et al., 2020; Vaculík et al., 2020). Evidence that Si ameliorates diverse stresses in plants led to its classification as a beneficial substance by the International Plant Nutrition Institute (IPNI) in 2015 (http://www.ipni.net/publication/nutrifacts-na.nsf/0/A7B4AB4D35C153BF85257ECE006E0E34/\protect\T1\textdollarFILE/NutriFacts-NA-14.pdf). However, Si is still considered a non-essential element because it is not involved in plant metabolism (Vaculík et al., 2020). Despite the documented benefits of Si in plants, its use in agriculture remains poorly adopted.

Based on their ability to accumulate Si, plants have been empirically classified as low, intermediate and high Si accumulators. However, this classification has changed due to the discovery of Si transporters that provided a better understanding of the mechanisms by which plants accumulate Si (Ma et al., 2007). Si uptake by the roots is mediated by aquaporin-like channels named LSi (Ma et al., 2007; Gaur et al., 2020). In rice, LSi1 is involved in the passive transport of Si from the soil to the root plant cells; the element is then moved inside the plant by the efflux transporter LSi2 and deposited in the form of silica in various plant structures (Ma et al., 2007; Ma and Yamaji, 2015). Furthermore, there is a specific number and amino acid sequences in aquaporin proteins necessary for Si permeability and plant uptake (Deshmukh et al., 2015). Based on these discoveries, Deshmukh et al. (2015) proposed a new classification of plants as either Si accumulators or Si excluders. More recently, Coskun et al. (2019) proposed that plants could be classified as accumulators or non-accumulators based on the presence of Si-specific aquaporin channels with functional Si permeability (Mitani and Ma, 2005; Deshmukh et al., 2015; Ma and Yamaji, 2015). The physical deposition of Si in plants has been linked with protection against insects and pathogens (Singh et al., 2020). However, Si supplementation also enhances physical and biochemical defenses in Si-accumulators and non-Si-accumulator plants (Alhousari and Greger, 2018; Singh et al., 2020).

Plants have a variety of defense mechanisms to protect themselves against herbivores; these defenses are classified as physical or chemical, which can be constitutive or inducible (War et al., 2018). Physical defenses are trichomes, thorns, lignin, waxes tough leaves, laticifers (latex), and mineral depositions (War et al., 2012). Chemical defenses include secondary metabolites (e.g., terpenes, phenols, alkaloids, sulfur, and nitrogen-containing compounds), antinutritional proteins, and enzymes (polyphenol oxidase, peroxidase, protease inhibitors, etc) (Mithöfer and Boland, 2012). Plant physical and chemical defenses may be constitutively expressed or induced by herbivory (War et al., 2018). Herbivore-induced defenses begin with the plant's recognition of damage, herbivore, and/or microbe-associated molecular patterns (DAMPS, HAMPS, MAMPS, respectively) followed by the activation of downstream reactions that occur within hours of initial herbivory (Fürstenberg-Hägg et al., 2013; Santamaria et al., 2018). Early recognition events lead to the biosynthesis of plant hormones that may include jasmonic acid (JA), salicylic acid (SA), and/or ethylene (ET), and the upregulation of gene transcripts involved in the synthesis of defensive compounds (Santamaria et al., 2018). The expression of late defense genes coding for proteinase inhibitors is activated by hydrogen peroxide and the antinutritional products are accumulated in plant tissues within days of initial herbivore damage (Fürstenberg-Hägg et al., 2013). Yet another set of late defense responses are changes in trichome density and leaf mineral accumulation that often occur in newly grown leaves several days or weeks after herbivory (Massey et al., 2007; Dalin et al., 2008). However, studies on the model grass, *Brachypodium distachyon* suggest that Si deposition in leaves starts as early as 6 h upon treatment with methyl jasmonate (Waterman et al., 2020). Plant defenses can affect herbivores directly by compromising their survival, growth, development, reproduction, oviposition, and behavior, etc. (Howe and Schaller, 2008). Biochemical defenses in the form of volatile blends and extrafloral nectar can also affect herbivores indirectly by attracting natural enemies (Karban, 2011; War et al., 2018).

Silica depositions increase the strength and rigidity of plant tissues and constitute a physical barrier for insect feeding (Bowen et al., 1992; Massey et al., 2007; Ma and Yamaji, 2015). Si accumulation enhances the abrasiveness of plant tissues causing wear of insect mouthparts and may reduce food intake and digestibility (Massey and Hartley, 2009; Leroy et al., 2019). Si can also increase callose deposition reducing food intake by sucking insects (Hao et al., 2008; Yang et al., 2018). In addition to the mechanical protection, upon damage, Si may enhance host plant resistance by increasing production of secondary metabolites and defensive proteins and enzymes in some plant species (Reynolds et al., 2016; Alhousari and Greger, 2018). Si also modifies the production of plant volatiles and enhances recruitment of herbivores' natural enemies (Kvedaras et al., 2010; Liu et al., 2017; Leroy et al., 2019). Insects exposed to Si-treated plants have exhibited reduced growth and development (Frew et al., 2017; Yang et al., 2018), alterations of feeding behavior (Assis et al., 2013), changes in oviposition preference (Peixoto et al., 2011), increased mortality (Han et al., 2015), lower fecundity (Alvarenga et al., 2017), and increased susceptibility to insecticides (Wang et al., 2020).

Induced defenses are key in host plant resistance against herbivores (Karban, 2011) but often have a metabolic cost. Plants may allocate resources to defenses that would otherwise be used for growth and other processes (Cipollini et al., 2014). It is known that Si enhances herbivore-induced defenses in different plant species, but the majority of studies have been done in wild and cultivated Poaceae plants (Alhousari and Greger, 2018; Singh et al., 2020). Moreover, most studies have measured defense responses at a single time point. It is currently unknown if the plant defense boots mediated by Si supplementation is transient or long-lasting. The temporality of plant defense induction has implications for plant resistance and possibly fitness due to the metabolic cost of those defenses. In this study, we investigated the effects of Si supplementation and insect herbivory on the induction of physical and biochemical plant defenses, foliar Si accumulation, and herbivore growth at early and late time

points after initial herbivore exposure. We hypothesized that (a) Si supplementation enhances herbivore-induced defenses and foliar Si deposition in crops that accumulate different amounts of Si; (b) the timing and duration of herbivore-induced plant defenses varies for different plant species; and (c) herbivore growth is reduced when feeding on Si-supplemented plants previously exposed to herbivores. To test these hypotheses, we used the generalist herbivore fall armyworm (FAW) *Spodoptera frugiperda* and three crop species with differential ability to uptake Si: tomato (non-Si accumulator), soybean, and maize (Si-accumulators) as our model system. The results of this study contribute to a better understanding of the beneficial effects of Si in plant protection.

MATERIALS AND METHODS

Plants

Tomato (*Solanum lycopersicum*, cv Better boy), and soybean (*Glycine max* cv *FS HiSOY*® HS33A14-98SB132B) plants were grown in Promix potting soil (Premier Horticulture, Quakertown, PA, U.S.A.). Seeds were first germinated and then transplanted into individual 10 cm square pots (Dillen, Griffin Greenhouse Supplies, Morgantown PA, USA). Each plant was fertilized once with 3 g of the slow-release fertilizer Osmocote plus (15–9-12, Scotts, Marysville, OH, USA) at the moment of transplant. Subsequently, plants were watered every other day with a 50 ml aqueous solution of either 5 mM potassium silicate (SiO_2K_2O, Alfa Aesar^{TM}) or 5 mM potassium chloride (KCl, EMD millipore) (pH. 6.8). Each plant received a total of 500 ml of either solution. KCl was used to replenish the amount of potassium in non-Si-supplemented control plants. Tomato and soybean plants were exposed to insect herbivory when their 5th leaf was fully extended. Maize (*Zea mays*) seeds of the herbivore susceptible genotype TX601 (inbred line) were obtained from W. P. Williams from Mississippi State University and the USDA-ARS, (Mississippi State, MS), and germinated in Promix potting soil (Premier Horticulture Quakertown, PA, USA). The seedlings were transplanted 10 d after germination into 3.78-l pots (C400 Nursery Supplies Inc. Chambersburg, PA, USA) containing Hagerstown loam soil and Promix potting soil mixed in relation 2:1. Each 3.78-l pot contained either 3 g of calcium silicate ($CaSiO_3$, Alfa Aesar^{TM}) or 3 g of lime ($Ca(OH)_2$, Alfa Aesar^{TM}) mixed with the soil. Lime was used to replenish the amount of calcium in control plants. The amounts of calcium silicate and lime applied to the soil mixture were calculated to raise the pH to 6.0–6.5 which is considered appropriate for maize growth (Unagwu et al., 2013). Maize plants were fertilized once with 3 g of Osmocote plus at the moment of transplant and were exposed to herbivory at their V7–V8 physiological stage (7–8 leaf collar). All plants were grown under glasshouse conditions (14 h light: 10 h dark) at the Pennsylvania State University, University Park, PA.

Insects

Fall armyworm, *S. frugiperda* eggs were purchased from Benzon Research (Carlisle, PA, USA) and reared in laboratory at 25°C in 16:8 light:dark conditions. Larvae were reared individually in 30 ml cups (DART 100PC, Mason, MI, USA) containing ∼5 ml of a wheat germ and casein-based artificial diet (Chippendale, 1970; Peiffer and Felton, 2005).

Herbivore Treatment and Plant Defense Responses

Herbivore-induced plant defense responses were measured by quantifying the activity of plant defensive proteins, the expression of plant defensive genes, the concentration of total phenolics, and the number of trichomes in leaves. In tomato and soybean, we measured the enzymatic activities of three known herbivore-induced antinutritional proteins: polyphenol oxidase (PPO), peroxidase (POX), and trypsin protease inhibitor (trypsin PI). PPO and POX were measured at early (48 h in tomato, and 72 h in soybean) and late (15 d) time points following FAW herbivory, whereas trypsin PI was only measured at the early time points. In maize plants, the expression of a maize proteinase inhibitor gene (*mpi*) was measured 24 h (early time point) after FAW exposure. The concentration of total phenolics, the number of trichomes, and foliar Si content were quantified 15 d after FAW herbivory in tomato, soybean, and maize plants.

For early time point experiments, a set of plants supplemented and non-supplemented with Si were exposed to actively feeding last-instar FAW larvae enclosed in click cages (polypropylene with metallic micromesh screen, 23 mm diameter and 18 mm height) to standardize the amount of damage between treatments. FAW larvae were randomly assigned to the treatments and removed from plants after consuming the entire 415.48 mm^2 of leaf tissue contained in the cage. The plant tissue surrounding the feeding sites was harvested 24, 48, and 72 h later for maize, tomato, and soybean, respectively. For late time point experiments, a separate set of Si-supplemented and non-Si-supplemented plants were exposed to heavy defoliation by FAW. Each tomato and soybean plant were exposed to three last-instar FAW larvae individually enclosed in cages (5.5 cm diameter, 1.5 cm high, 23.76 cm^2 area) built with two plastic petri dish bottoms (60 × 15 mm, VWR, West Chester, PA, USA) held together with aluminum hair clips (Acevedo et al., 2018). These cages allowed larvae to feed on leaves attached to plants while preventing their spread in the greenhouse. The cages also helped standardize the amount of damage to about 90% per plant. Each maize plant was infested with one FAW larva placed at the whorl and allowed to eat freely for 3 days. Fifteen days after herbivore exposure, the new regrowth leaves were harvested for analyses. The fresh tissue excised from plants at early and late time points was immediately weighed, frozen in liquid nitrogen, and stored at −80°C for further analyses. We collected 50 mg of fresh tissue for enzymatic assays, 20 mg for quantification of phenolics, and 70 mg for RNA extractions. The remaining leaves were used for quantification of trichomes and for larval bioassays. Leaves from the late time point were also used for Si quantification; these were placed inside paper bags, dried at 60°C until constant weight, and ground to powder in a Udy cyclone mill (Thomas Scientific, Swedesboro, NJ. USA).

Activity of Defensive Enzymes

The activities of PPO and trypsin PI were measured following the procedure described by Chung and Felton (2011). Trypsin PI activity was calculated as described previously (Acevedo et al., 2017). POX activity assays followed a previously described protocol (Acevedo et al., 2017). The activity values from the enzymatic assays were normalized by the amount of protein (mg/ml) contained in each sample.

Concentration of Phenolics

The concentration of total phenolics in leaf samples was quantified following the Folin-Ciocalteu protocol (Ainsworth and Gillespie, 2007). The content of phenolics was expressed as mM of gallic acid equivalents per gram of fresh tissue.

Proteinase Inhibitor (*mpi*) Gene Expression

The procedures for RNA extraction, cDNA synthesis, and quantitative qPCR were carried out as previously described (Acevedo et al., 2017).

Density of Leaf Trichomes

Fifteen days after plants were exposed to insect herbivory, the newer fully expanded leaf was harvested from each plant to count the number of trichomes under a dissecting stereoscope (Olympus SZ30). In tomato, we counted the number of glandular trichomes type VI in an area of 0.24 mm^2. In soybean, we registered the number of non-glandular type V trichomes in a 16 mm^2 area. For maize, the number of non-glandular macro hairs or long trichomes was counted in an area of 2.84 cm^2. For each plant species, two samples were taken per leaf, and their average number of trichomes was used for the statistical analyses. For imaging, leaf pieces of 0.24 mm^2 were excised and immersed in a fixative solution (2.5% glutaraldehyde, 1.5% formaldehyde in 0.1M sodium cacodylate buffer pH. 7.4) for 48 h; subsequently, the samples were dehydrated through ethanol series and critical point dried with liquid CO_2. The samples were then mounted in aluminum stubs with carbon tape and imaged in a Scanning Electron Microscope (SEM) at the Penn State Microscopy Facility (University Park, PA). The type VI trichomes in tomato are characterized for having four glandular cells connected to a short stalk, whereas the type V trichomes in soybean consist of a hollow long stalk cell with a multicellular base (Glas et al., 2012; Li et al., 2018). Maize macro hairs are long single cell stalks present in the adaxial epidermis of the leaves (Nelson et al., 2002). These trichome types are abundant, relatively easy to count and some have been associated with plant herbivore resistance (Glas et al., 2012).

Si Quantification

Total Si was extracted with hydrofluoric acid (HF) and quantified with the molybdenum blue method reported by Diogo and Wydra (2007). Briefly, 30 milligrams of grounded tissue were placed in a 2 ml plastic tube to which 1 ml of extraction solution (1.5 M HF, 0.6 M HCl) was added; these tubes were then agitated overnight inside a fume hood. Samples were then centrifuged at 10,000 g for 10 min. Twenty microliters of the supernatant were transferred into a new 1.5 ml tube to which 230 µl of 3.2% boric acid (H_3BO_3) were added; tubes were agitated overnight. Subsequently, 250 µl of the color solution [1:1 mixture of 0.08 M H_2SO_4 and 20 mg/ml of $(NH_4)_6$ Mo_7^* $4H_2O$] were added and incubated for 30 min. Then, 250 µl of 33 mg/ml of tartaric acid and 250 µl of 4 mg/ml of ascorbic acid were added. Lastly, 200 µl of the mixture were used to measure absorbance at 811 nm in a spectrophotometer (SpectraMax 190, Molecular Devices, San Jose, CA, USA). The amount of Si in the samples was determined using a blank-corrected standard curve constructed with different concentrations of a commercial Si [$(NH_4)_2SiF_6$] standard (Cat. # 10M50-4F, High-Purity standards, Charleston, SC, USA).

Si Quantification in Maize Trichomes

To quantify the amount of Si deposited in maize macro hairs (trichomes), we excised leaves from either Si-supplemented or non-Si-supplemented plants. Those leaves were immediately taken to the lab and frozen in liquid nitrogen; long trichomes were extracted from frozen leaves using a scalpel (# 10). The trichomes were dried to constant weight for Si extraction and quantification following the procedure described in section Si Quantification. The deposition of Si in these trichomes was also analyzed with Energy Dispersive x-ray Spectroscopy (EDS) following the procedure below.

Energy Dispersive X-Ray Spectroscopy and Elemental Mapping

For microscopic observation of Si bodies, leaf pieces of 0.24 mm^2 were excised and dehydrated in serially diluted ethanol solutions. These samples were critical point dried with liquid CO_2 and mounted in aluminum stubs with carbon tape. Backscattered electron images (BSE), and EDS were carried out at the Penn State Materials Research Institute (University Park, PA) in an FEI Quanta 200 ESEM equipped with a 10 mm Si drift detector and the Aztec software version 2.3 (Oxford Instruments). The instrument was operated under low vacuum conditions of 70 Pa, at a voltage of 20 kV, and a working distance of 12.5 mm.

Bioassays

Effect of Plant Si Supplementation and Insect Herbivory on Larval Weight Gain

FAW larval neonates were fed on detached leaves from either tomato, soybean or maize untreated plants until their second or third instar. Afterwards, larvae were weighed on a precision scale (Sartorius BP 61S, precision: 0.1 mg) to obtain their "initial weight" before being exposed to the corresponding treatments. Each larva was then individualized in 30 ml plastic cups (DART 100PC, Mason, MI, USA) containing detached leaves from either tomato, soybean or maize plants previously exposed to the different soil amendments (potassium silicate/potassium chloride or calcium silicate/lime) and herbivore treatments (FAW larval fed or undamaged control plants) described in the sections of Plants and Herbivore treatment and plant defense responses. Each cup had a 3 ml bottom layer of 2% agar to prevent dehydration of the leaves. Larvae were grown under laboratory conditions (25°C, 75% RH, and photoperiod of 16 h light: 8 dark) with constant

food supply for 4–5 d, time at which their "final weight" was obtained. Larval weight gain was calculated as the difference between their final and their initial weights. Larvae were randomly assigned to each of the treatments in a complete randomized design.

Effect of Si on Larval Weight Gain and Mandible Wear

To test the effect of Si on FAW larval weight gain, freshly hatched neonates were individually placed inside 30 ml plastic cups (DART 100PC) containing a wheat germ and casein-based artificial diet (Chippendale, 1970; Peiffer and Felton, 2005) supplemented with either 0, 0.5, 1, 2, 5 or 10% Si dioxide (SiO_2, SIGMA). Larvae were weighed 5 days later on a precision scale (Sartorius BP 61S). Thirty larvae were randomly assigned to each of the Si concentrations in a complete randomized design. To investigate the effect of Si on mandible wear, larvae from FAW were grown on non-Si-supplemented artificial diet (described in section Insects), for their first five instars. Newly molted six-instar larvae were then transferred to new cups containing the same type of diet supplemented with either 0, 2.5 or 5% Si dioxide (SiO_2, SIGMA). After 3 d of feeding, the larval mandibles were dissected and placed in fixative solution (2.5% glutaraldehyde, 1.5% formaldehyde in 0.1 M sodium cacodylate buffer pH. 7.4). The samples were then washed with 0.1 M sodium cacodylate buffer, dehydrated through ethanol series and critical point dried with liquid CO_2. The samples were mounted in aluminum stubs with carbon tape and imaged in an SEM at the Penn State Microscopy Facility.

Effect of Maize Trichomes on Larval Weight Gain

Leaves from maize plants (V7-V8) supplemented with Si, as indicated in section Plants, were detached and taken to the lab. The mid-portion of the leaves was cut out, the midvein was removed, and the non-glandular macro-hairs from one side (left or right from the midrib) of each leaf were excised using a razor blade under a stereoscope. The other side of the leaf was left intact. The leaf pieces with or without trichomes were used to feed FAW larvae. FAW neonates were grown on wheat germ diet (described in section Effect of Si on Larval Weight Gain and Mandible Wear) until they reached an average of 27 mg. Then, larvae ($n = 20$) were weighed and placed individually inside 30 ml plastic cups containing leaf pieces with or without trichomes. Two days later, the larvae were re-weighed to determine their weight gain. Each cup had a 3 ml bottom layer of 2% agar to prevent dehydration of the leaves. To determine if trichomes would break down in the larval gut, frass pellets from larvae fed on leaves with trichomes were placed in fixative solution, critical point dried, and photographed in an SEM.

Experimental Design and Statistical Analyses

We used a two-factor factorial design that combined two Si treatments (Si treated and Si untreated) with two herbivore treatments (insect-fed and undamaged controls). The experimental units (either plants or insects) used in our experiments were randomly assigned to each of the treatments in a complete randomized design. We used two-way ANOVA for testing the effects of Si (Si supplemented vs. non-Si-supplemented plants) and herbivore treatments (larval fed vs. undamaged controls) on the activity of defensive enzymes (PPO, POX, and trypsin PI), the concentration of phenolics, the expression of *mpi*, the density of trichomes, larva weight gain, and the amount of Si in leaves. When the interaction factor was significant, a one-way ANOVA was used to elucidate the differences among treatment combinations. Differences between treatment means were obtained using either the Tukey's Honestly Significant Difference (HSD) test or the Tukey-Kramer test (for balance and unbalance replications, respectively) at alpha $= 0.05$; when needed, data were transformed to meet the assumptions of normality and equal variance before doing ANOVA. A two-sample *t*-test was used to identify differences in the amount of Si deposited in maize trichomes, and the effect of siliceous trichomes in larva weight gain. One-way ANOVA was used to test for differences in larva weight gain grown in diet supplemented with Si. ANOVAs and *t*-tests were conducted using the software Minitab 18 (Minitab Inc., State College, PA, USA). All graphs were generated in R version 3.6.6 (Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Si Supplementation and Insect Herbivory Induced Defense Responses in Si-Accumulator and Non-Si-Accumulator Plants

In tomato, the enzymatic activities of polyphenol oxidase (PPO), peroxidase (POX), and trypsin protease inhibitor (trypsin PI) were greater in insect-fed plants compared with undamaged controls 48 h after larval exposure irrespective of the Si treatment ($P < 0.05$; **Figure 1**; **Table 1**). At this early time point, the activity of POX was higher in Si-supplemented plants compared with non-Si-supplemented controls (**Figure 1B**; Tukey HSD $t = 3.72$, $P = 0.001$). Fifteen days after larval damage, the activity of PPO was greater in plants exposed to herbivory compared with the undamaged controls [ANOVA $F_{(3,36)} = 19.06$, $P = 0.00$], irrespective of the Si treatment (**Figure 1D**). The effects of the herbivory and Si treatments on PPO activity at 15 d were analyzed with a one-way ANOVA due to the significance of the interaction factor obtained in the two-way ANOVA (**Table 1**). At this late time point, the activity of POX and the concentration of total phenolics were not different among treatments (**Figures 1E,F**). The number of glandular trichomes in leaves was higher in plants supplemented with Si and exposed to insect herbivory compared with undamaged controls and non-Si-supplemented plants [**Figure 2A**; ANOVA $F_{(3,20)} = 34.23$, $P = 0.00$]. The effects of the herbivory and Si treatments on the number of trichomes were analyzed with a one-way ANOVA due to the significance of the interaction factor obtained in the two-way ANOVA (**Table 1**).

In soybean, the enzymatic activities of POX and Trypsin PI, and the concentration of total phenolics were affected by insect feeding and Si treatment (**Figure 3**). The activity of POX was higher in both Si-supplemented and non-Si-supplemented plants fed on by FAW within 72 h of larval exposure compared with

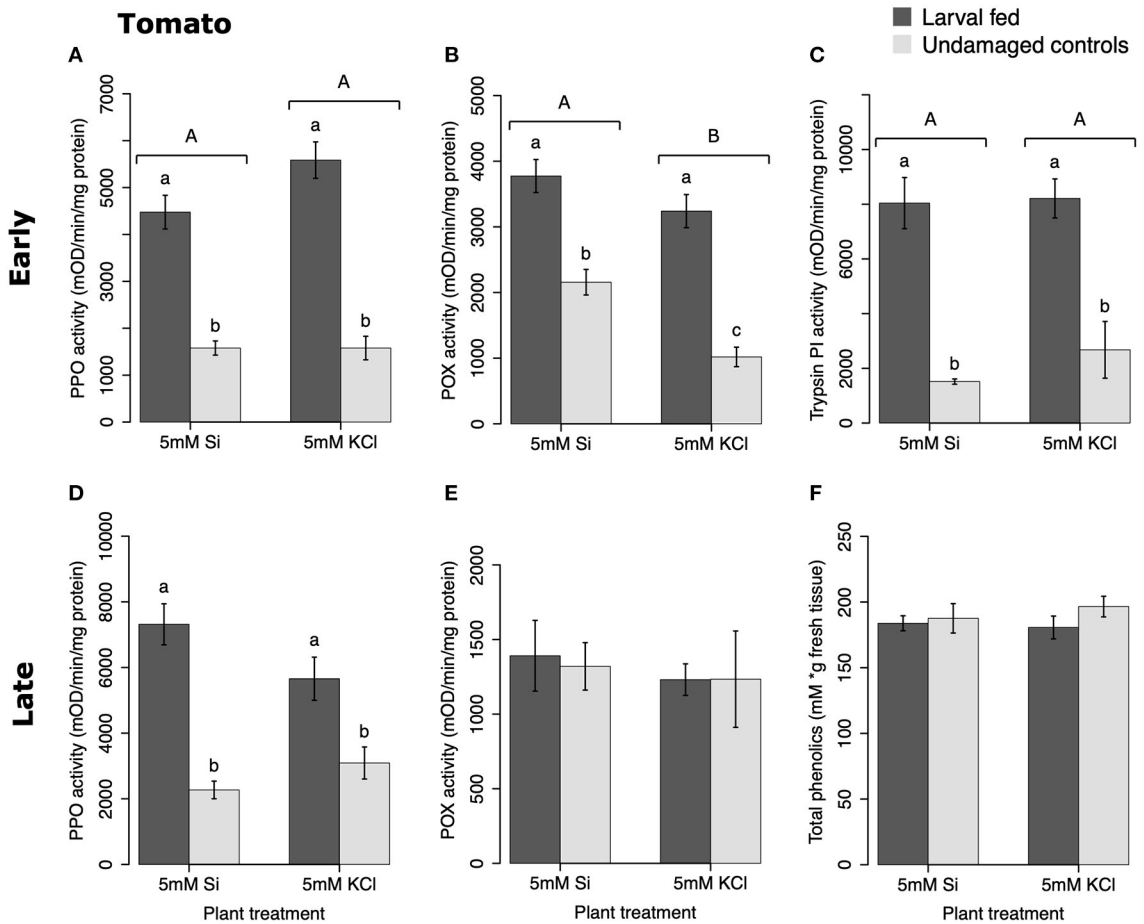

FIGURE 1 | Herbivore-induced defense responses in tomato plants supplemented with either silicon (5 mM K_2SiO_3) or potassium chloride (5 mM KCl) and exposed to fall armyworm herbivory. Early and late defense responses are those measured 48 h and 15 d after herbivore exposure, respectively. The activity of plant defensive enzymes is represented in: **(A,D)** polyphenol oxidase (PPO), **(B,E)** peroxidase (POX), and **(C)** trypsin protease inhibitor (Trypsin PI). **(F)** The concentration of total phenolics is expressed as mM of gallic acid equivalents per gram of fresh tissue. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$. Capital letters represent differences between the Si treatment and potassium chloride while lowercase letters indicate differences between treatments of herbivory and undamaged controls. In **(D)**, letters denote differences among treatment means obtained with the Tukey HSD test after one-way ANOVA.

undamaged controls (**Figure 3B**). However, 15 d after insect treatment, the activity of POX had an opposite trend being greater in undamaged controls when compared with insect-fed plants (Tukey-Kramer $t = -2.11$, $P = 0.042$); no effect of the Si treatment was found at this late time point (**Figure 3E**). The activity of Trypsin PI was higher in Si-supplemented plants exposed to herbivory compared with Si-supplemented undamaged controls and non-Si supplemented larval fed plants [**Figure 3C**; ANOVA $F_{(3,35)} = 5.77$, $P = 0.003$]. The effects of the herbivory and Si treatments on Trypsin PI activity were analyzed with a one-way ANOVA due to the significance of the interaction factor obtained in the two-way ANOVA (**Table 1**). Fifteen days after insect treatment, the concentration of total phenolics was higher in plants supplemented with Si compared with non-Si-supplemented controls; their concentration was also greater in undamaged controls compared with larval fed plants (**Figure 3F**; **Table 1**). The activity of PPO was not different among treatments in either early or late time points (**Figures 3A,D**). The number of trichomes was higher in soybean plants exposed to herbivory, but there was no effect of the Si-treatment (**Table 1**; **Figure 2B**).

In maize, the gene expression of *maize proteinase inhibitor (mpi)* was greater in insect-fed plants 24 h after herbivory compared with undamaged controls (**Figure 4A**). Likewise, the concentration of total phenolics in maize was higher in insect-fed plants 15 d after herbivory compared with intact controls (**Figure 4B**; **Table 1**). There was no effect of Si supplementation on either *mpi* expression or total phenolics (**Figure 4**). The density of long trichomes in maize plants was not affected by the treatments of herbivory or Si supplementation (**Table 1**; **Figure 2C**).

Si Supplementation and Herbivory Affected Leaf Si Accumulation

All Si-supplemented plants accumulated greater amount of this element in their tissues than non-Si-supplemented controls (**Figure 5**). Tomato plants treated with Si had on average 0.72

TABLE 1 | Two way ANOVA results of the effects of silicon (Si) (Si/KCl) and insect treatments (fed/undamaged controls) on the enzymatic activities of polyphenol oxidase (PPO), peroxidase (POX), and trypsin protease inhibitor (Trypsin PI) in tomato and soybean plants.

Plant	Variable	Time	Effect	df (trt, error)	F	*p*-value	Figures
Tomato	PPO	Early	Si treatment	1,38	3.02	0.09	**1A**
			Insect treatment	1,38	117.19	0.000*	
			Interaction	1,38	3.02	0.09	
		Late	Si treatment	1,36	0.62	0.437	**1D**
			Insect treatment	1,36	51.14	0.000*	
			Interaction	1,36	5.42	0.026*	
	POX	Early	Si treatment	1,42	13.84	0.001*	**1B**
			Insect treatment	1,42	73	0.000*	
			Interaction	1,42	1.81	0.186	
		Late[a]	Si treatment	1,36	0.48	0.492	**1E**
			Insect treatment	1,36	0.17	0.680	
			Interaction	1,36	0.05	0.826	
	Trypsin PI	Early[a]	Si treatment	1,37	0.64	0.428	**1C**
			Insect treatment	1,37	67.62	0.000*	
			Interaction	1,37	0.32	0.573	
	Total phenolics	Late	Si treatment	1,36	0.11	0.738	**1F**
			Insect treatment	1,36	1.31	0.260	
			Interaction	1,36	0.50	0.484	
	Number of glandular trichomes	Late	Si treatment	1,20	16.5	0.001*	**2A**
			Insect treatment	1,20	57.09	0.000*	
			Interaction	1,20	29.10	0.000*	
	Leaf Si content	Late	Si treatment	1,23	50.95	0.000*	**5A**
			Insect treatment	1,23	1.7	0.205	
			Interaction	1,23	0.98	0.333	
	Larval weight gain	Early[a]	Si treatment	1,42	0.2	0.654	**7A**
			Insect treatment	1,42	401.66	0.000*	
			Interaction	1,42	0.83	0.367	
		Late	Si treatment	1,74	0.37	0.546	**7D**
			Insect treatment	1,74	18.15	0.000*	
			Interaction	1,74	0.13	0.723	
Soybean	PPO	Early	Si treatment	1,26	0.87	0.360	**3A**
			Insect treatment	1,26	0.85	0.365	
			Interaction	1,26	6.25	0.019	
		Late[b]	Si treatment	1,26	0.06	0.805	**3D**
			Insect treatment	1,26	0.18	0.672	
			Interaction	1,26	1.46	0.237	
	POX	Early	Si treatment	1,31	0.02	0.882	**3B**
			Insect treatment	1,31	19.74	0.000*	
			Interaction	1,31	0.63	0.434	
		Late	Si treatment	1,35	0.11	0.743	**3E**
			Insect treatment	1,35	4.47	0.042*	
			Interaction	1,35	0.00	0.962	
	Trypsin PI	Early	Si treatment	1,35	5.27	0.028*	**3C**
			Insect treatment	1,35	0.18	0.675	
			Interaction	1,35	12.14	0.001*	
	Total phenolics	Late	Si treatment	1,36	21.68	0.000*	**3F**
			Insect treatment	1,36	38.72	0.000*	
			Interaction	1,36	0.12	0.732	
	Number of trichomes	Late	Si treatment	1,20	0.64	0.434	**2B**
			Insect treatment	1,20	12.53	0.002*	
			Interaction	1,20	4.22	0.053	

(Continued)

TABLE 1 | Continued

Plant	Variable	Time	Effect	df (trt, error)	F	*p*-value	Figures
	Leaf Si content	Late	Si treatment	1,24	234	0.000*	**5B**
			Insect treatment	1,24	24.99	0.000*	
			Interaction	1,24	2.13	0.157	
	Larval weight gain	Early	Si treatment	1,36	4.18	0.048*	**7B**
			Insect treatment	1,36	15.14	0.000*	
			Interaction	1,36	0.42	0.519	
		Late	Si treatment	1,103	0.31	0.576	**7E**
			Insect treatment	1,103	12.62	0.001*	
			Interaction	1,103	1.04	0.310	
Maize	mpi gene expression	Early[b]	Si treatment	1,20	2.21	0.153	**4A**
			Insect treatment	1,20	129.47	0.000*	
			Interaction	1,20	3.15	0.091	
	Total phenolics	Late[a]	Si treatment	1,32	0.69	0.411	**4B**
			Insect treatment	1,32	18.17	0.000*	
			Interaction	1,32	3.76	0.061	
	Number of long macro hairs	Late	Si treatment	1,19	2.53	0.128	**2C**
			Insect treatment	1,19	1.14	0.298	
			Interaction	1,19	1.43	0.246	
	Leaf Si content	Late	Si treatment	1,24	211.84	0.000*	**5C**
			Insect treatment	1,24	5.50	0.028*	
			Interaction	1,24	0.36	0.556	
	Larval weight gain	Early[a]	Si treatment	1,46	53.28	0.000*	**7C**
			Insect treatment	1,46	16.31	0.000*	
			Interaction	1,46	0.17	0.682	
		Late	Si treatment	1,32	7.51	0.010*	**7F**
			Insect treatment	1,32	0.08	0.783	
			Interaction	1,32	0.45	0.509	

The table also depicts the results of the effects of Si and insect treatments on the relative expression of a maize proteinase inhibitor gen (mpi), and on the concentration of total phenolics, the number of trichomes, leaf Si content, and larval weight gain in tomato, soybean, and maize plants. The effects of Si and insect treatments on plant defensive compounds and larval weight gain were tested at different time points for each plant species: 48 h (early response) and 15 d after treatment (late response) in tomato; 72 h (early response) and 15 d after treatment (late response) in soybean; and 24 h (early response) and 15 d after treatment (late response) in maize plants. Asterisks denote significant p-values (*) at $\alpha = 0.05$*. df, degrees of freedom; trt, treatment.*

[a] *Squared root transformed.*

[b] *Log transformed.*

$\pm$ 0.022 (95% CI) mg/g (dry tissue) of leaf Si content, whereas non-Si treated plants contained 0.52 $\pm$ 0.05 mg/g of this element. The foliar Si concentrations in soybean were 2.6 $\pm$ 0.18 and 1.42 $\pm$ 0.11 mg/g in Si-supplemented and non-Si-supplemented plants, respectively. In maize, there was 4.77 $\pm$ 0.36 mg/g of Si in Si-treated plants, and 2.05 $\pm$ 0.15 mg/g in Si-untreated controls. Herbivory also influenced the accumulation of Si in soybean and maize plants, but not in tomato (**Figure 5**). Soybean plants fed on by fall armyworm larvae accumulated less Si than undamaged controls (**Figure 5B**). In contrast, fall armyworm herbivory induced higher accumulation of Si in maize plants non-supplemented with Si but growing in soil with Si content (**Figure 5C**). Si-supplemented tomato plants had higher Si content than non-Si supplemented controls (**Figure 5A**), but we were unable to detect Si structures in leaves through BSE or EDS (**Figures 5D,G**). Si accumulation in soybean was found in trichomes and leaf epidermal compartments at the base of those trichomes (**Figures 5E,H**). Maize plants accumulated Si in trichomes and silica cells (**Figures 5F,I, 6**). Although the density of long trichomes in maize leaves was not affected by Si-supplementation or insect herbivory (**Figure 2C**), the amount of Si contained in those trichomes was higher in Si-supplemented plants compared to non-Si-supplemented controls (**Figure 6A**; $t = -9.85$, $P = 0.000$, $n = 4$). In maize, Si deposition occurred in the whole trichome from base to tip (**Figures 6B,C**).

Bioassays

Plant Si Supplementation and Former Insect Herbivory Affected Larval Weight Gain

Larvae fed on tomato plants previously exposed to herbivory gained less weight at the early and late time points than those fed on undamaged controls; plant Si-Supplementation did not affect larva weight gain (**Table 1**; **Figures 7A,D**). In soybean, former herbivory influenced larval weight gain at early and late time points, but Si-supplementation only had an effect at the early time point (**Table 1**). Larvae fed on soybean leaves detached from

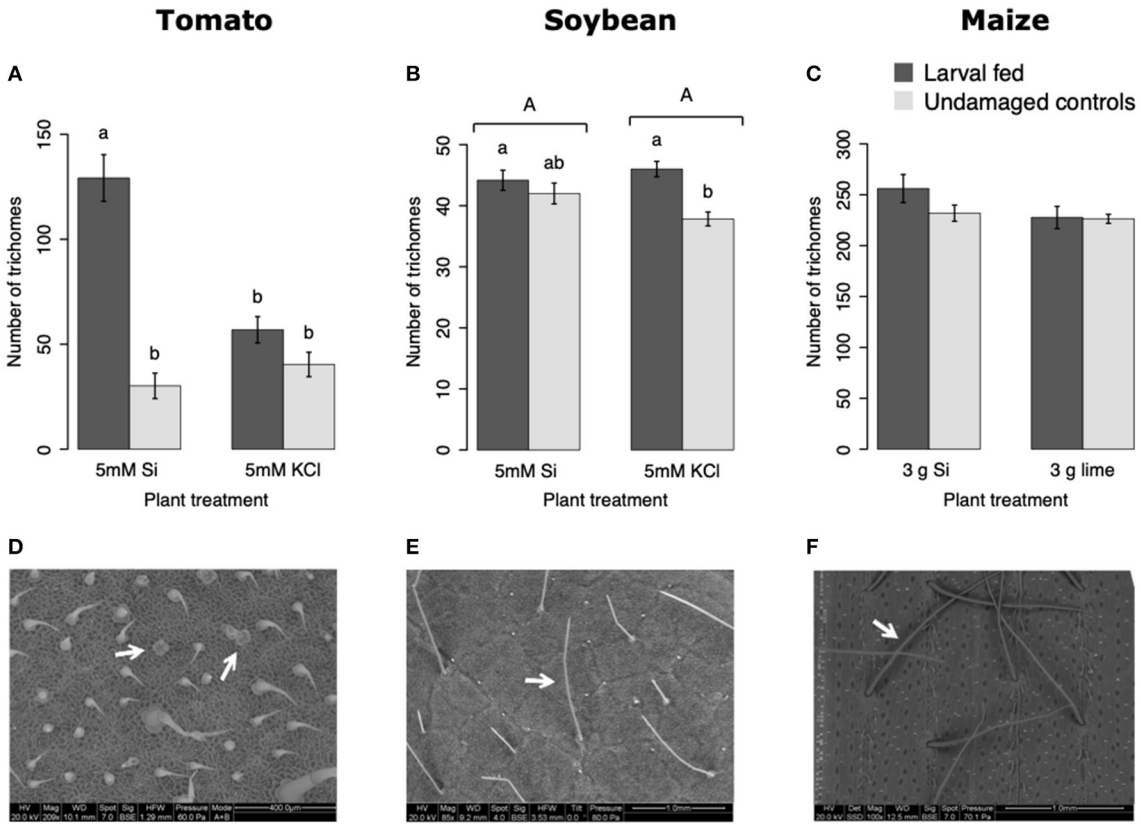

FIGURE 2 | Number of trichomes in tomato **(A)**, soybean **(B)**, and maize **(C)** plants either supplemented with silicon or non-supplemented controls exposed and non-exposed to fall armyworm herbivory. Scanning electron images (SEM) of tomato **(D)**, soybean **(E)**, and maize **(F)** trichomes; white arrows indicate the type of trichomes counted in this study. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$. Capital letters represent differences between Si treated and Si-untreated plants while lowercase letters indicate differences between treatments of herbivory and undamaged controls. In **(A)**, letters represent differences among treatment means obtained with the Tukey HSD test after one-way ANOVA.

plants exposed to herbivory 72 h earlier gained less weight than those fed on undamaged plants (**Figure 7B**). Conversely, young FAW larvae gained more weight when fed on leaves detached from soybean plants exposed to herbivory 15 d earlier than those fed on undamaged control plants (**Figure 7E**). In maize, the Si treatment resulted in lower larval weight gain at both early and late time points, whereas previous herbivory reduced larval weight gain only at the early time point (24 h) (**Table 1**; **Figures 7C,F**).

Si-Supplemented Artificial Diet Induced Larval Mandible Wear but Did Not Affect Weight Gain

Si-containing diets enhanced larval mandible wear. FAW larvae fed on the artificial diet supplemented with SiO_2 had greater mandible wear than larvae fed on the diet without Si. Greater mandible wear was observed in larvae fed on the diet supplemented with 5% SiO2 than in those fed with 2.5% of Si (**Figure 8**). Contrarily, Si-supplemented diet had no effect on larval weight gain [$F_{(5,172)} = 0.77$, $P = 0.570$; $n = 28$–30].

Siliceous Maize Macro-Hairs Reduced FAW Larval Weight

FAW larvae fed on maize leaves with macro hairs gained less weight than those fed on leaves from which trichomes were removed (**Figure 9A**; $t = 2.18$, $df = 35$, $P = 0{,}036$, $n = 20$). Trichomes did not break down in the larval gut; rather they appear to have been excreted almost intact (**Figure 9B**).

DISCUSSION

In this study, we investigated if Si supplementation would increase herbivore resistance in crop plants with differential ability to accumulate this element. Our results show that herbivory and Si supply augmented the levels of some plant biochemical and physical defenses at early and late time points in a species-specific manner. Additionally, Si supplementation increased leaf Si content in all plants. Former herbivory affected FAW larval weight gain in all plants tested, and the Si treatment further reduced weight gain of

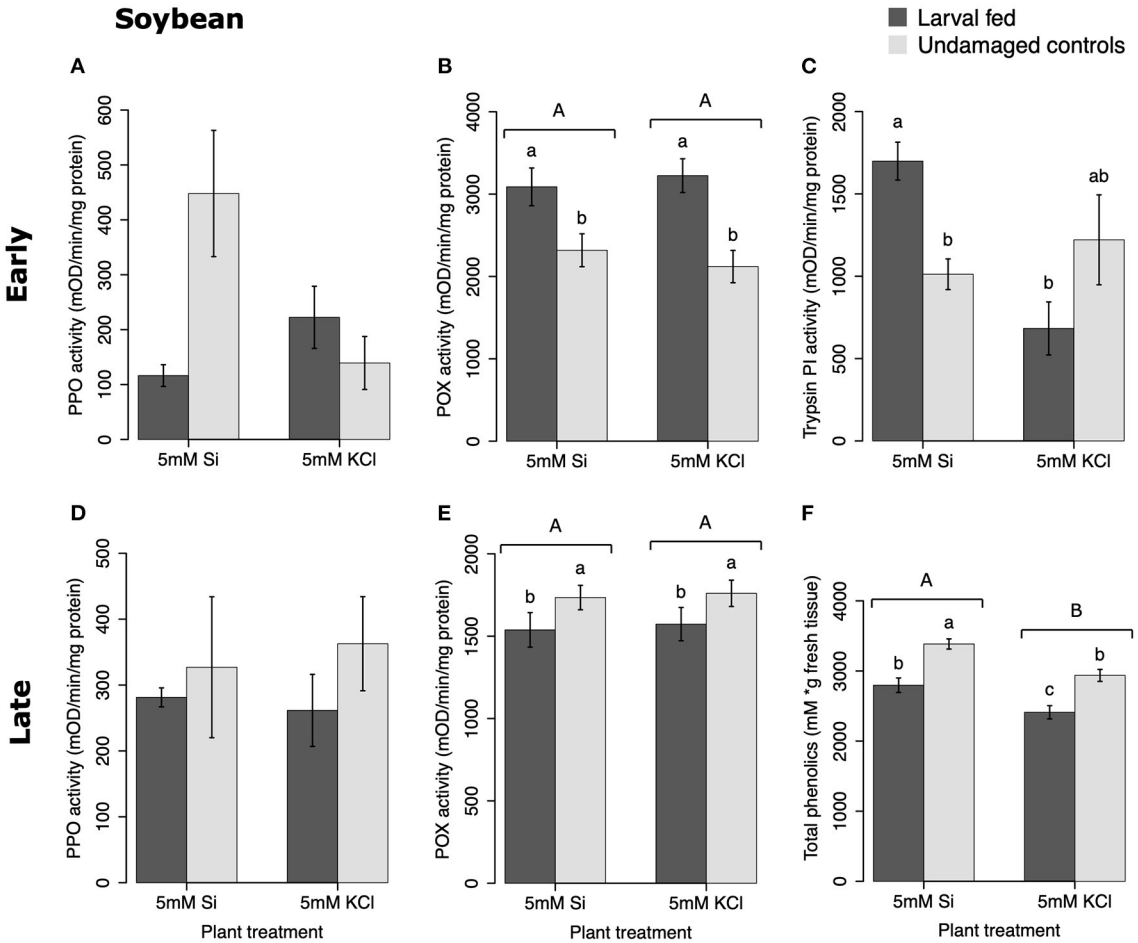

FIGURE 3 | Herbivore-induced defense responses in soybean plants supplemented with either silicon (5 mM K_2SiO_3) or potassium chloride (5 mM KCl) and exposed to fall armyworm herbivory. Early and late defense responses are those measured 72 h and 15 d after herbivore exposure, respectively. The activity of plant defensive enzymes is represented in: **(A,D)** polyphenol oxidase (PPO), **(B,E)** peroxidase (POX), and **(C)** trypsin protease inhibitor (Trypsin PI). **(F)** The concentration of total phenolics is expressed as mM of gallic acid equivalents per gram of fresh tissue. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$. Capital letters represent differences between the Si treatment and potassium chloride while lowercase letters indicate differences between treatments of herbivory and undamaged controls. In **(C)**, letters represent differences among treatment means obtained with the Tukey-Kramer test after one-way ANOVA.

larvae fed on Si accumulator plants. Notably, our results strongly suggest that non-glandular trichomes are important reservoirs of Si in maize and may increase plant resistance to chewing herbivores.

Si Supplementation and Insect Herbivory Influence Plant Defense Responses

In tomato plants, FAW herbivory increased PPO, POX, and trypsin PI activities within 48 h of larval exposure (**Figure 10**). The Si treatment further enhanced POX activity at this early time point (**Figure 1B**). PPO was still upregulated 15 d after herbivore exposure, but POX and total phenolics were not different among treatments at the late time point. Furthermore, the herbivore treatment combined with Si supplementation increased the number of glandular trichomes in tomato leaves 15 d after herbivory (**Figure 2A**). The effect of Si on herbivore-induced defenses in tomato has been scarcely studied, but induction of plant resistance has been reported (Faraone et al., 2020). There is also evidence that Si enhances resistance of tomato plants against pathogens. For example, Diogo and Wydra (2007) found less incidence of the bacterial wilt disease, *Ralstonia solanacearum* in tomato plants treated with Si. This enhancement of resistance appears to be correlated with higher activities of the defensive proteins POX, PPO, phenylalanine ammonia lyase (PAL), and lipoxygenase as well as higher total soluble phenolics (Jiang et al., 2019). Likewise, the Si treatment reduced the severity of soil-borne diseases in tomato caused by *Fusarium oxysporum* (Huang et al., 2011). Previous studies have demonstrated that insect feeding induces the number of glandular trichomes in tomato plants (Tian et al., 2012); interestingly, our results indicate that

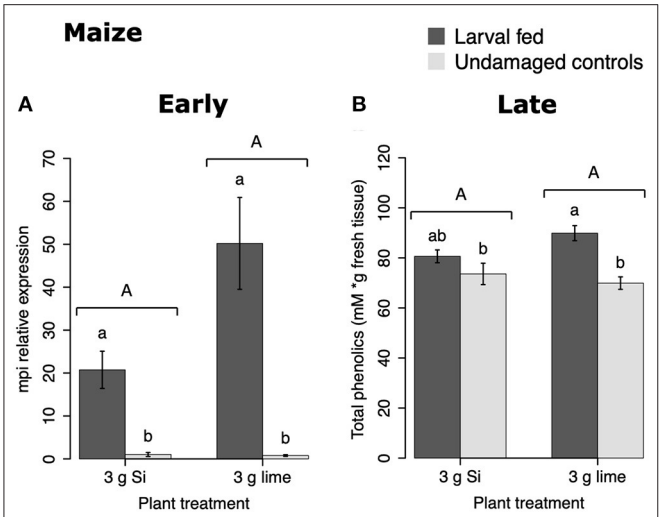

FIGURE 4 | Herbivore-induced defense responses in maize plants supplemented with either silicon (3 g Ca_2SiO_4) or lime [3 g Ca $(OH)_2$] and exposed to fall armyworm herbivory. Early and late defense responses are those measured 24 h and 15 d after herbivore exposure, respectively. **(A)** Relative expression of a maize proteinase inhibitor gene (mpi), and **(B)** concentration of total phenolics is expressed as mM of gallic acid equivalents per gram of fresh tissue. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$. Capital letters represent differences between Si treated and Si-untreated plants while lowercase letters indicate differences between treatments of herbivory and undamaged controls.

the number of trichomes is enhanced by Si supplementation in the presence of herbivory. These results suggest that Si supplementation enhances some biochemical and physical defenses in tomato despite the low capability of this species to accumulate Si.

In soybean, insect feeding induced greater production of trichomes irrespective of the Si treatment (**Figure 2B**; **Table 1**). The combination of Si and FAW herbivory induced higher activity of trypsin PI at 72 h compared with undamaged Si-supplemented controls and herbivore-treated plants not supplemented with Si (**Figure 3C**). The Si treatment induced greater accumulation of total phenolics than those found in non-Si-supplemented plants 15 d after herbivore exposure (**Figure 3F**; **Table 1**). In contrast to our results, a previous study reported a decrease in total phenolics in non-insect infested soybean plants treated with Si (Ferreira et al., 2011). The discrepancy of these results may be explained by differences in herbivore treatments and sampling time points between studies or genetic variation among soybean cultivars. FAW herbivory induced higher activity of POX 72 h after treatment. But 15 d later, the POX activity levels and the concentration of total phenolics were downregulated in herbivore-treated plants with respect to untreated controls. Shifts in the activity patterns of defensive enzymes in response to herbivory have been previously reported in soybean. Locateli et al. (2019) found higher activity of PAL in soybean plants 96 h after treatment with *Bemisia tabaci*, but at 168 h the PAL activity was lower than that of untreated controls. Speculatively, it may be possible that herbivore-derived effectors change the initial plant response over time. Si supplementation appears to enhance some biochemical defense responses in this plant species.

In maize, FAW feeding induced early (*mpi* expression) and late defense responses (total phenolics). But Si addition did not affect the expression of *mpi*, the concentration of total phenolics or the density of leaf macro hairs in maize plants (**Figures 2C**, **4**, **10**). Si-mediated increase in maize resistance to herbivores has been reported previously (Sétamou et al., 1993; Boer et al., 2019; Moise et al., 2019) but the mechanisms have not been elucidated. Further RNA-seq or quantitative proteomic studies may help reveal the means by which Si enhances herbivore resistance in maize.

Si Supplementation Enhances Si Accumulation in Plants

The Si treatment increased foliar Si content in Si-accumulators and non-Si accumulator plants regardless of herbivory. Si supplementation in tomato, soybean, and maize increased the foliar content of this element by 38.4, 83, and 132.7%, correspondingly, with respect to the Si content in non-Si-supplemented plants (**Figure 5**). Increase in shoot Si content in response to Si supply has been previously reported in these plant species (Huang et al., 2011; Boer et al., 2019; Johnson et al., 2020b). Some studies have also shown that herbivory induces Si deposition in Si-accumulator plants (Massey et al., 2007; Johnson et al., 2020b); in our studies, there was a slight significant increase of Si in maize plants exposed to FAW herbivory but not in tomato or soybean. The Si content was higher in maize leaves followed by soybean and tomato which can be explained by the differential ability of these plants to uptake Si (Deshmukh et al., 2015; Coskun et al., 2019). Maize and soybean contain functional influx and efflux transporters, whereas tomato only contains Si influx, but not functional Si efflux transporters (Deshmukh et al., 2015; Sun et al., 2020). Variable Si accumulation in maize and soybean plants may also be associated with differences in the density and possibly size of silica cells and non-glandular trichomes. Si-containing cells are present in row arrangements as well as spread out in the leaf epidermis of maize and other grasses, whereas in soybean, silica was found in the basal cells of non-glandular trichomes (**Figures 5E–I**). Moreover, the maize genotype used in this study contained three times more macro-hairs per squared mm than soybean (**Figures 2B,C**). Notably, non-glandular trichomes appear to be important structures for Si deposition, leaf macro-hairs extracted from Si-supplemented maize plants had 116.5% more Si than those extracted from non-Si supplemented controls (**Figure 6**). Accumulation of Si in the tips of trichomes has been observed in grasses, but there are no previous reports of their actual Si concentration (de Melo et al., 2010; Andama et al., 2020). Si may increase the rigidity of trichomes and physically harm the digestive system of chewing herbivores. Trichomes are often ingested along with leaf tissue and excreted almost intact in larval frass (**Figure 9**).

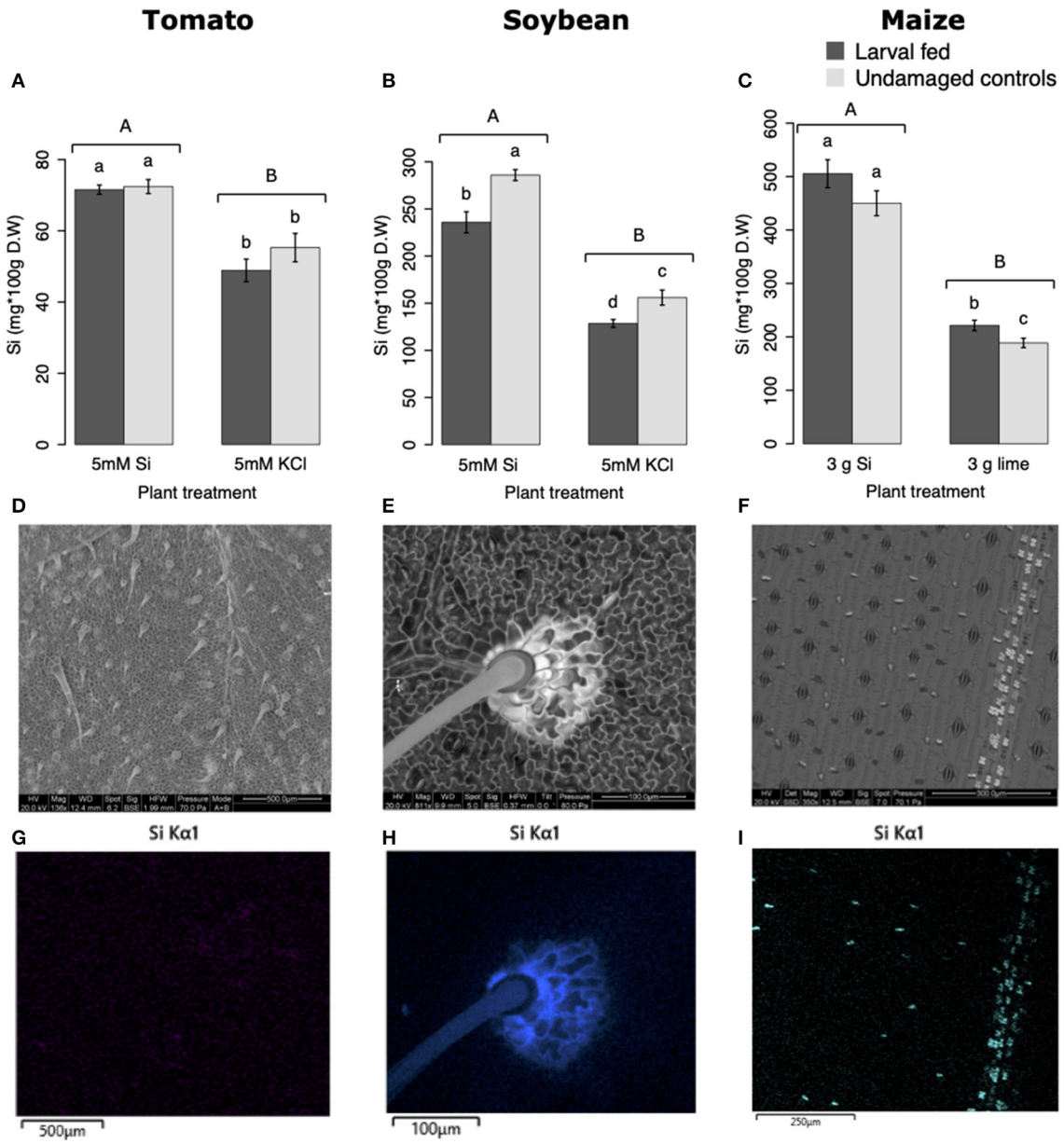

FIGURE 5 | Silicon accumulation in: **(A)** tomato, **(B)** soybean, and **(C)** maize leaves; plants were either supplemented with silicon or non-supplemented controls exposed and non-exposed to fall armyworm herbivory. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$. Capital letters represent differences between Si treated and Si-untreated plants while lowercase letters indicate differences between treatments of herbivory and undamaged controls. Backscattered electron images (BSE) of tomato **(D)**, soybean **(E)**, and maize **(F)** leaves show mineral deposition in leaf epidermis and trichomes (white structures). Si distribution in tomato **(G)**, soybean **(H)**, and maize **(I)** leaves obtained with energy-dispersive X-ray spectroscopy (EDS).

Si Supplementation and Insect Herbivory Influence FAW Larval Weight Gain

Larval weight gain was affected by the Si and herbivore treatments in a specific way for each plant species. FAW larvae gained less weight when fed on tomato, soybean, and maize plants previously exposed to herbivory (**Figure 7**). In tomato, reduction in larval weight gain at early and late time points was exclusively associated with former herbivory and was negatively correlated with higher activity levels of PPO. In soybean, former herbivory and the Si treatment reduced larval weight gain at the early time point, but there was no correlation with the activity of defensive enzymes measured in this study. Contrarily, at the late time point, FAW larvae gained more weight when fed on soybean plants previously exposed to herbivory; this was negatively correlated with POX activity and the concentration of total phenolics. Si supplementation to soybean plants has been shown to reduce the growth of *Helicoverpa punctigera* larvae within 6 days of herbivore exposure, and to increase mortality

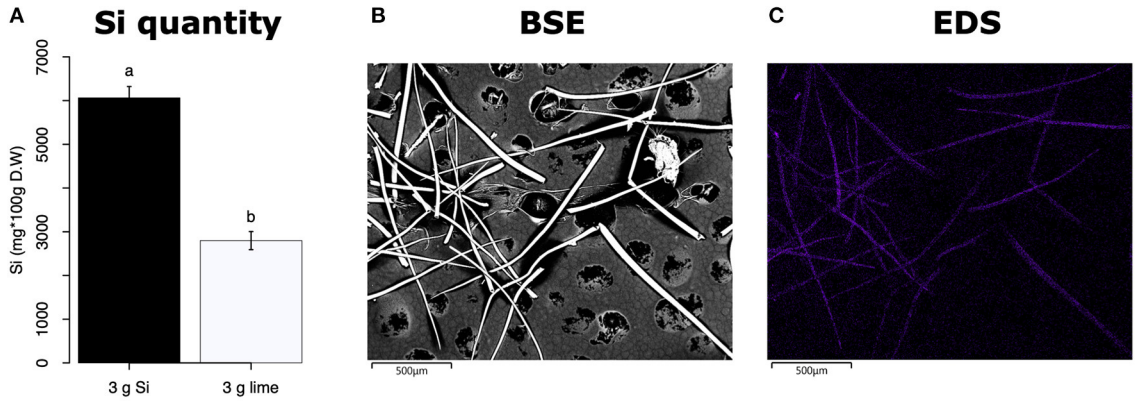

FIGURE 6 | (A) Si content in maize trichomes extracted from plants supplemented with either Si (3 g Ca_2SiO_4) or lime [3 g Ca $(OH)_2$]. **(B)** Backscattered electron (BSE) images of long macro hairs extracted from maize leaves. **(C)** Si distribution in maize trichomes obtained with energy-dispersive X-ray spectroscopy (EDS), the purple coloration represents accumulation of Si.

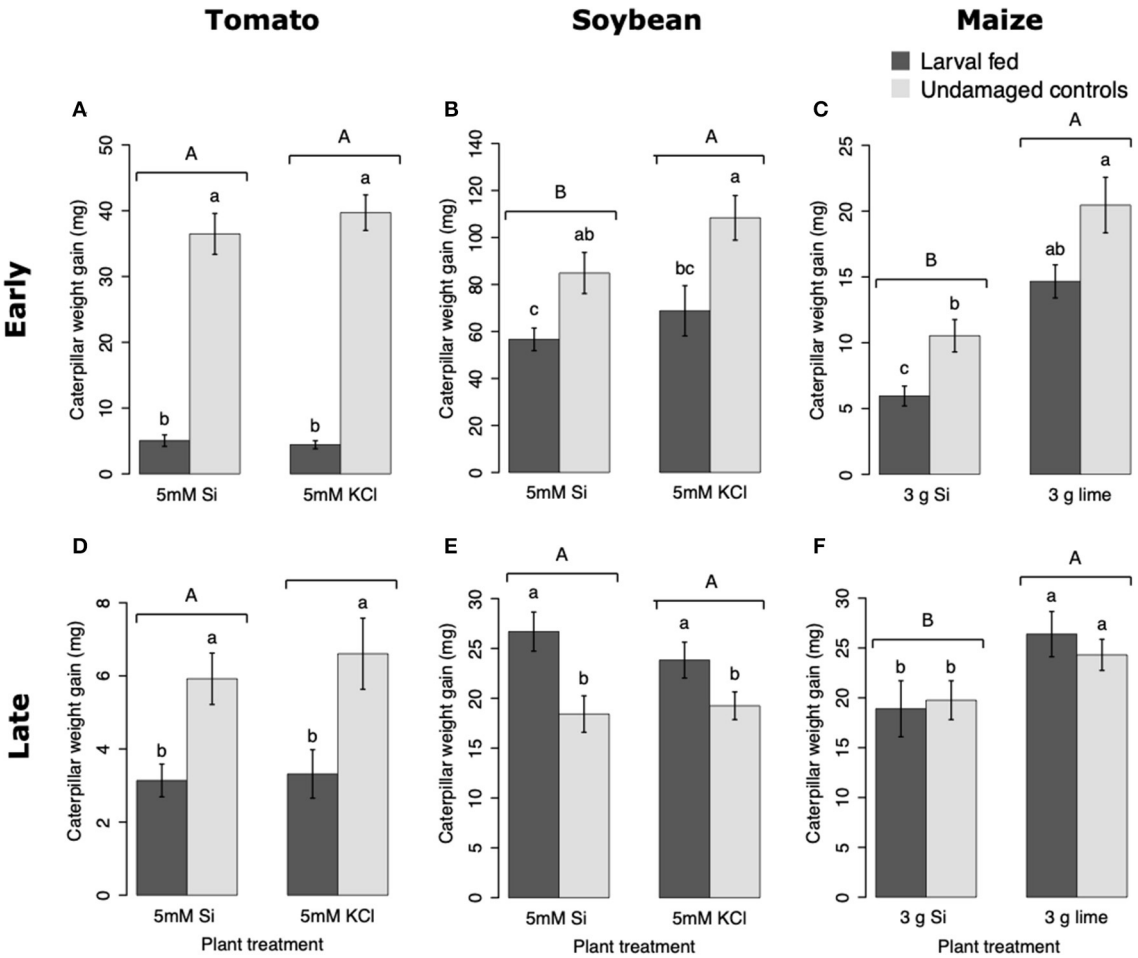

FIGURE 7 | Weight gain of fall armyworm (FAW) larvae fed on tomato **(A,D)**, soybean **(B,E)**, and maize **(C,F)** plants either supplemented or non-supplemented with Si with or without previous exposure to herbivory. Early and late, correspond to different time points at which the effects of Si plant supplementation and previous insect herbivory were tested on FAW larval weight gain. For tomato, early represents 48 h, and late represents 15 d after plants were treated with FAW larvae. In Soybean, early and late refers to 72 h and 15 d after herbivore treatment, respectively. For maize, early denotes 24 h whereas late indicates 15 d after herbivory. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$. Capital letters represent differences between Si treated and Si-untreated plants while lowercase letters indicate differences between treatments of herbivory and undamaged controls.

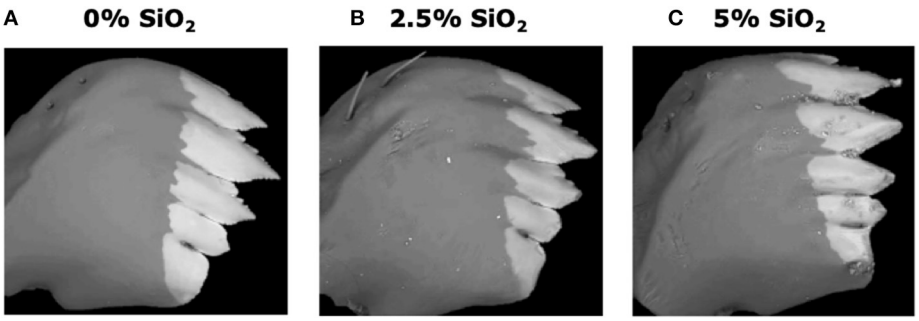

FIGURE 8 | Backscattered electron images of six-instar fall armyworm (FAW) mandibles extracted from larvae fed on artificial diet containing various amounts of silicon dioxide: **(A)** 0% SiO_2, **(B)** 2.5% SiO_2, and **(C)** 5% of SiO_2. White areas represent the distribution of mineral accumulation (mainly zinc).

of whitefly nymphs fed on intact plants (Ferreira et al., 2011; Johnson et al., 2020b). Therefore, the potential of Si to enhance plant protection may differ with time and depend on specific herbivore-plant interactions. In Maize, there was a decrease in larval weight gain associated with previous herbivory and Si treatments at the early time point. But the reduction in larval weight at the late time point was only present in Si-treated maize plants which may be negatively correlated with the presence of tougher trichomes. Si, by itself, did not decrease herbivore weight, but trichomes with higher Si content appear to affect larval growth (**Figure 9**). Indeed, other studies have shown that leaf macro hairs reduce the relative growth rate of chewing herbivores (Johnson et al., 2020a). Si is also likely to affect other herbivore parameters not measured in this study. For example, Si treatment of maize plants reduced fecundity of FAW moths (Alvarenga et al., 2017), reduced growth rate of the stemborer *Busseola fusca* (Juma et al., 2015), and increased mortality of the lepidopteran larvae *Pseudaletia unipuncta* and *Sesamia calamistis* (Sétamou et al., 1993; Moise et al., 2019). Herbivory and Si treatments are likely to trigger various plant responses, including changes in gene expression, protein abundance, and nutritional quality. Although our ability to draw conclusions are limited to the plant responses tested in this study, the topic deserves further investigation using more comprehensive omics techniques.

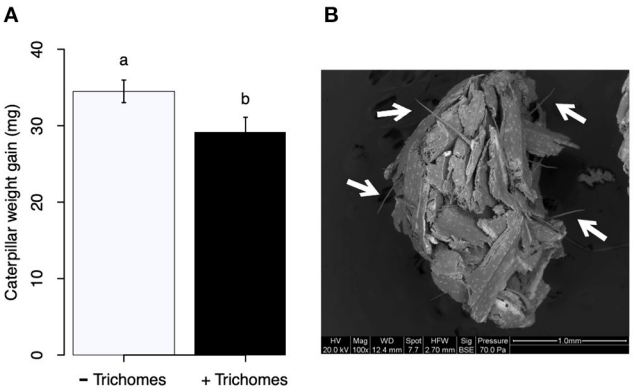

FIGURE 9 | (A) Weight gain of fall armyworm (FAW) larvae fed on maize leaves without (–Trichomes) or with (+Trichomes) siliceous macro hairs. **(B)** Frass pellet from FAW larvae fed on leaves with macro hairs. White arrows point to trichomes that appear to be intact after their passage through the larval gut.

Mechanisms of Si-Mediated Plant Resistance Against Insect Herbivores

Si increases resistance to herbivores through physical and biochemical mechanisms (Alhousari and Greger, 2018). The role that physical depositions of Si (e.g., increase in toughness and abrasiveness) play on plant protection against herbivores is widely accepted, but the mechanisms by which Si mediates induction of biochemical defenses is currently under debate. Biochemical compounds that confer resistance to herbivory are regulated by plant hormones such as JA, SA, and ET (War et al., 2012). Some studies have reported an increase in JA in response to Si treatment, but others have found lower levels of this hormone (Hall et al., 2019). Due to the inability of Si to interact biochemically with the cell machinery, Coskun et al. (2019) proposed the so-called "apoplastic obstruction hypothesis." In addition to the mechanical protection conferred by silica deposition in plant tissues, this model proposes that Si protection against herbivores is attributed to the physical movement obstruction of herbivore effectors within cells mediated by Si deposition in the cell apoplast. Although compelling, this argument contradicts numerous reports of systemic induction of plant biochemical responses triggered by herbivores in Si-treated plants. Alternatively, Si physical deposition may increase plant stress and result in upregulation of defensive pathways (Ye et al., 2013; Hall et al., 2019). In this case, upregulation of plant defenses would also be found in plants not exposed to herbivory. In line with the physical role of Si in mediating plant protection, Hall et al. (2019) proposed that Si-accumulator plants with constitutive antiherbivore defenses exhibit lower JA levels because of their existing physical protection. Our results indicate that Si enhances physical plant defenses in the form of Si accumulation in leaves and trichomes, and augment some JA-dependent biochemical defenses. The proposed models aim to promote further investigation to identify the underlying mechanism by which Si confer plant resistance to biotic and abiotic stresses

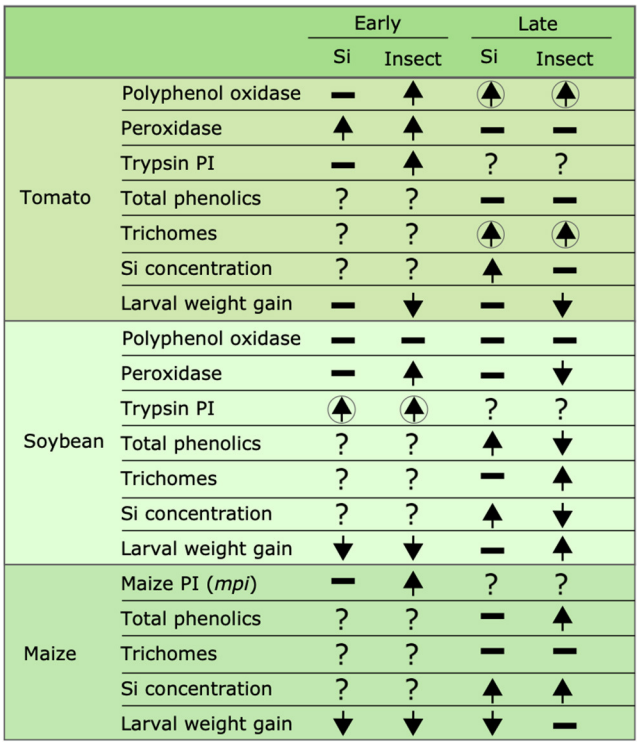

FIGURE 10 | Summary of the effects of Si supplementation and insect herbivory on plant defense responses and larval weight gain at early and late time points in tomato, soybean, and maize. Upward arrows represent a significant increase, downward arrows represent significant reduction, hyphens represent no significant effects, and question marks indicate that the effect was not tested. Arrows enclosed in circles indicate that the combination of both Si supplementation and insect herbivory had a significant effect. Early effects were measured 24, 48, and 72 hrs after treatment in maize, soybean, and tomato, respectively; late effects were measured 15 d after treatment in all plant species.

(Coskun et al., 2019; Hall et al., 2019). These mechanisms may vary based on the plant' ability to accumulate Si and the nature of the stress.

Conclusions

Our results show that FAW herbivory induces production of biochemical and physical defenses in tomato, soybean, and maize plants. The Si treatment enhanced some of these defenses, but resistance to herbivory measured as a reduction in larva weight gain was only observed in soybean at the early time point and in maize at early and late time points (**Figure 10**). Si alone did not reduce larva weight gain, but Si deposited in non-glandular trichomes did reduce weight gain. This study and others (Andama et al., 2020; Johnson et al., 2020a) emphasize the potential role of silicified trichomes in increasing plant resistance against chewing herbivores. We conclude that Si offers transient resistance to FAW in soybean and a longer duration of resistance in maize. Further studies are needed to assess the effect of transient and long-term defense responses in plant fitness under single and multiple herbivore events. Si supply appears to be a promising strategy in management programs of chewing herbivores in Si-accumulator plants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FA and GF designed the study. FA and MP conducted the experiments. SR and C-WT helped with bioassays and plant treatments. FA analyzed the data and wrote the manuscript. All authors read, contributed to revisions, and approved the manuscript.

FUNDING

We greatly appreciate the financial support provided by the following funding agencies: The Northeast Sustainable Agriculture Research and Education (NE-SARE) program (grant number GNE15-093 awarded to FA and GF), the United States Department of Agriculture AFRI program (2017-67013-26596 awarded to GF), and the hatch Project Grants PEN04576 and PEN04757 (accession No.1024573).

ACKNOWLEDGMENTS

We thank Ju-Che Lo and Dean Taylor for their assistance with greenhouse work and tissue grinding. We thank Julie Anderson from the Penn State Materials Research Institute for her guidance and assistance with the EDS and SEM techniques. We also thank Scott DiLoreto for glasshouse management. Special thanks to Dr. W. P. Williams for supplying the TX601 maize seeds, and to Dr. Dawn Luthe for use of the qPCR machine and plant tissue grinder. Many thanks to the three reviewers that helped improve the quality and presentation of the manuscript.

REFERENCES

Acevedo, F. E., Peiffer, M., Ray, S., Meagher, R., Luthe, D. S., and Felton, G. W. (2018). Intraspecific differences in plant defense induction by fall armyworm strains. *New Phytol.* 218, 310–321. doi: 10.1111/nph.14981

Acevedo, F. E., Peiffer, M., Tan, C.-W., Stanley, B. A., Stanley, A., Wang, J., et al. (2017). Fall armyworm-associated gut bacteria modulate plant defense responses. *Mol. Plant Microbe Interact.* 30, 127–137. doi: 10.1094/MPMI-11-16-0240-R

Ainsworth, E. A., and Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nat. Protoc.* 2, 875–877. doi: 10.1038/nprot.2007.102

Alhousari, F., and Greger, M. (2018). Silicon and mechanisms of plant resistance to insect pests. *Plants (Basel)* 7:33. doi: 10.3390/plants7020033

Alvarenga, R., Moraes, J. C., Auad, A. M., Coelho, M., and Nascimento, A. M. (2017). Induction of resistance of corn plants to *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae) by application of silicon and gibberellic acid. *Bull. Entomol. Res.* 107, 527–533. doi: 10.1017/S0007485316001176

Andama, J. B., Mujiono, K., Hojo, Y., Shinya, T., and Galis, I. (2020). Nonglandular silicified trichomes are essential for rice defense against chewing herbivores. *Plant Cell Environ.* 43, 2019–2032. doi: 10.1111/pce.13775

Assis, F. A., Moraes, J. C., Auad, A. M., and Coelho, M. (2013). The effects of foliar spray application of silicon on plant damage levels and components of larval biology of the pest butterfly *Chlosyne lacinia saundersii* (Nymphalidae). *Int. J. Pest Manag.* 59, 128–134. doi: 10.1080/09670874.2013.779049

Boer, C. A., Sampaio, M. V., and Pereira, H. S. (2019). Silicon-mediated and constitutive resistance to *Rhopalosiphum maidis* (Hemiptera: Aphididae) in corn hybrids. *Bull. Entomol. Res.* 109, 356–364. doi: 10.1017/S0007485318000585

Bowen, P., Menzies, J., Ehret, D., Samuels, L., and Glass, A. D. M. (1992). Soluble silicon sprays inhibit powdery mildew development on grape leaves. *J. Am. Soc. Hortic. Sci.* 117, 906–912. doi: 10.21273/JASHS.117.6.906

Chippendale, G. M. (1970). Metamorphic changes in fat body proteins of the southwestern corn borer, *Diatraea grandiosella*. *J. Insect Physiol.* 16, 1057–1068. doi: 10.1016/0022-1910(70)90198-8

Chung, S. H., and Felton, G. W. (2011). Specificity of induced resistance in tomato against specialist lepidopteran and coleopteran species. *J. Chem. Ecol.* 37, 378–386. doi: 10.1007/s10886-011-9937-0

Cipollini, D., Walters, D., and Voelckel, C. (2014). "Costs of resistance in plants: from theory to evidence," in *Annual Plant Reviews: Insect-Plant Interactions*, eds C. Voelckel and G. Jander (Oxford: Wiley Blackwell), 263–307.

Cooke, J., and Leishman, M. R. (2016). Consistent alleviation of abiotic stress with silicon addition: a meta-analysis. *Funct. Ecol.* 30, 1340–1357. doi: 10.1111/1365-2435.12713

Coskun, D., Deshmukh, R., Sonah, H., Menzies, J. G., Reynolds, O., Ma, J. F., et al. (2019). The controversies of silicon's role in plant biology. *New Phytol.* 221, 67–85. doi: 10.1111/nph.15343

Dalin, P., Ågren, J., Björkman, C., Huttunen, P., and Kärkkäinen, K. (2008). "Leaf trichome formation and plant resistance to herbivory," in *Induced Plant Resistance to Herbivory*, ed A. Schaller (Dordrecht: Springer Science and Business Media), 89–105.

de Melo, S. P., Monteiro, F. A., and De Bona, F. D. (2010). Silicon distribution and accumulation in shoot tissue of the tropical forage grass *Brachiaria brizantha*. *Plant Soil* 336, 241–249. doi: 10.1007/s11104-010-0472-5

Debona, D., Rodrigues, F. A., and Datnoff, L. E. (2017). Silicon's role in abiotic and biotic plant stresses. *Annu. Rev. Phytopathol.* 55, 85–107. doi: 10.1146/annurev-phyto-080516-035312

Deshmukh, R. K., Vivancos, J., Ramakrishnan, G., Guérin, V., Carpentier, G., Sonah, H., et al. (2015). A precise spacing between the NPA domains of aquaporins is essential for silicon permeability in plants. *Plant J.* 83, 489–500. doi: 10.1111/tpj.12904

Diogo, R. V. C., and Wydra, K. (2007). Silicon-induced basal resistance in tomato against *Ralstonia solanacearum* is related to modification of pectic cell wall polysaccharide structure. *Physiol. Mol. Plant Pathol.* 70, 120–129. doi: 10.1016/j.pmpp.2007.07.008

Etesami, H., and Jeong, B. R. (2018). Silicon (Si): review and future prospects on the action mechanisms in alleviating biotic and abiotic stresses in plants. *Ecotoxicol. Environ. Saf.* 147, 881–896. doi: 10.1016/j.ecoenv.2017.09.063

Faraone, N., Evans, R., LeBlanc, J., and Hillier, N. K. (2020). Soil and foliar application of rock dust as natural control agent for two-spotted spider mites on tomato plants. *Sci. Rep.* 10:12108. doi: 10.1038/s41598-020-69060-5

Ferreira, R. S., Moraes, J. C., and Antunes, C. S. (2011). Silicon influence on resistance induction against *Bemisia tabaci* biotype B (Genn.) (Hemiptera: Aleyrodidae) and on vegetative development in two soybean cultivars. *Neotrop. Entomol.* 40, 495–500. doi: 10.1590/S1519-566X2011000400014

Frew, A., Allsopp, P. G., Gherlenda, A. N., and Johnson, S. N. (2017). Increased root herbivory under elevated atmospheric carbon dioxide concentrations is reversed by silicon-based plant defences. *J. Appl. Ecol.* 54, 1310–1319. doi: 10.1111/1365-2664.12822

Fürstenberg-Hägg, J., Zagrobelny, M., and Bak, S. (2013). Plant defense against insect herbivores. *Int. J. Mol. Sci.* 14, 10242–10297. doi: 10.3390/ijms140510242

Gaur, S., Kumar, J., Kumar, D., Chauhan, D. K., Prasad, S. M., and Srivastava, P. K. (2020). Fascinating impact of silicon and silicon transporters in plants: a review. *Ecotoxicol. Environ. Saf.* 202:110885. doi: 10.1016/j.ecoenv.2020.110885

Glas, J. J., Schimmel, B. C. J., Alba, J. M., Escobar-Bravo, R., Schuurink, R. C., and Kant, M. R. (2012). Plant glandular trichomes as targets for breeding or engineering of resistance to herbivores. *Int. J. Mol. Sci.* 13, 17077–17103. doi: 10.3390/ijms131217077

Hall, C. R., Waterman, J. M., Vandegeer, R. K., Hartley, S. E., and Johnson, S. N. (2019). The role of silicon in antiherbivore phytohormonal signalling. *Front. Plant. Sci.* 10:1132. doi: 10.3389/fpls.2019.01132

Han, Y., Lei, W., Wen, L., and Hou, M. (2015). Silicon-mediated resistance in a susceptible rice variety to the rice leaf folder, *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae). *PLoS ONE* 10:e0120557. doi: 10.1371/journal.pone.0120557

Hao, P., Liu, C., Wang, Y., Chen, R., Tang, M., Du, B., et al. (2008). Herbivore-induced callose deposition on the sieve plates of rice: an important mechanism for host resistance. *Plant Physiol.* 146, 1810–1820. doi: 10.1104/pp.107.111484

Hodson, M. J., White, P. J., Mead, A., and Broadley, M. R. (2005). Phylogenetic variation in the silicon composition of plants. *Ann. Bot.* 96, 1027–1046. doi: 10.1093/aob/mci255

Howe, G. A., and Schaller, A. (2008). "Direct defenses in plants and their induction by wounding and insect herbivores," in *Induced Plant Resistance to Herbivory*, ed A. Schaller (Dordrecht: Springer Science and Business Media), 7–29.

Huang, C.-H., Roberts, P. D., and Datnoff, L. E. (2011). Silicon suppresses Fusarium crown and root rot of tomato. *J. Phytopathol.* 159, 546–554. doi: 10.1111/j.1439-0434.2011.01803.x

Imtiaz, M., Rizwan, M. S., Mushtaq, M. A., Ashraf, M., Shahzad, S. M., Yousaf, B., et al. (2016). Silicon occurrence, uptake, transport, and mechanisms of heavy metals, minerals, and salinity enhanced tolerance in plants with future prospects: a review. *J. Environ. Manage.* 183, 521–529. doi: 10.1016/j.jenvman.2016.09.009

Jiang, N., Fan, X., Lin, W., Wang, G., and Cai, K. (2019). Transcriptome analysis reveals new insights into the bacterial wilt resistance mechanism mediated by silicon in tomato. *Int. J. Mol. Sci.* 20:761. doi: 10.3390/ijms20030761

Johnson, S. N., Hartley, S. E., Ryalls, J. M. W., Frew, A., and Hall, C. R. (2020a). Targeted plant defense: silicon conserves hormonal defense signaling impacting chewing but not fluid-feeding herbivores. *Ecology*. e03250. doi: 10.1002/ecy.3250

Johnson, S. N., Rowe, R. C., and Hall, C. R. (2020b). Silicon is an inducible and effective herbivore defence against *Helicoverpa punctigera* (Lepidoptera: Noctuidae) in soybean. *Bull. Entomol. Res.* 110, 417–422. doi: 10.1017/S0007485319000798

Juma, G., Ahuya, P. O., Ong'amo, G., Le Ru, B., Magoma, G., Silvain, J.-F., et al. (2015). Influence of plant silicon in *Busseola fusca* (Lepidoptera: Noctuidae) larvae–*Poaceae* interactions. *Bull. Entomol. Res.* 105, 253–258. doi: 10.1017/S000748531500005X

Karban, R. (2011). The ecology and evolution of induced resistance against herbivores. *Funct. Ecol.* 25, 339–347. doi: 10.1111/j.1365-2435.2010.01789.x

Kvedaras, O. L., An, M., Choi, Y. S., and Gurr, G. M. (2010). Silicon enhances natural enemy attraction and biological control through induced plant defences. *Bull. Entomol. Res.* 100, 367–371. doi: 10.1017/S0007485309990265

Leroy, N., de Tombeur, F., Walgraffe, Y., Cornélis, J.-T., and Verheggen, F. J. (2019). Silicon and plant natural defenses against insect pests: Impact on plant volatile organic compounds and cascade effects on multitrophic interactions. *Plants (Basel)* 8:444. doi: 10.3390/plants8110444

Li, C., Wang, P., Lombi, E., Cheng, M., Tang, C., Howard, D. L., et al. (2018). Absorption of foliar-applied Zn fertilizers by trichomes in soybean and tomato. *J. Exp. Bot.* 69, 2717–2729. doi: 10.1093/jxb/ery085

Liu, J., Zhu, J., Zhang, P., Han, L., Reynolds, O. L., Zeng, R., et al. (2017). Silicon supplementation alters the composition of herbivore induced plant volatiles and enhances attraction of parasitoids to infested rice plants. *Front. Plant Sci.* 8:1265. doi: 10.3389/fpls.2017.01265

Locateli, B. T., da Cruz, M. P., Dalacosta, N. L., Oligine, K. F., Bertoldo, E., Mazaro, S. M., et al. (2019). Elicitor-induced defense response in soybean plants challenged by *Bemisia tabaci*. *J. Agric. Sci.* 11:p251. doi: 10.5539/jas.v11n2p251

Luyckx, M., Hausman, J.-F., Lutts, S., and Guerriero, G. (2017). Silicon and plants: current knowledge and technological perspectives. *Front. Plant Sci.* 8:411. doi: 10.3389/fpls.2017.00411

Ma, J. F., and Yamaji, N. (2015). A cooperative system of silicon transport in plants. *Trends Plant Sci.* 20, 435–442. doi: 10.1016/j.tplants.2015.04.007

Ma, J. F., Yamaji, N., Mitani, N., Tamai, K., Konishi, S., Fujiwara, T., et al. (2007). An efflux transporter of silicon in rice. *Nature* 448, 209–212. doi: 10.1038/nature05964

Massey, F. P., Ennos, A. R., and Hartley, S. E. (2007). Herbivore specific induction of silica-based plant defences. *Oecologia* 152, 677–683. doi: 10.1007/s00442-007-0703-5

Massey, F. P., and Hartley, S. E. (2009). Physical defences wear you down: progressive and irreversible impacts of silica on insect herbivores. *J. Anim. Ecol.* 78, 281–291. doi: 10.1111/j.1365-2656.2008.01472.x

Mitani, N., and Ma, J. F. (2005). Uptake system of silicon in different plant species. *J. Exp. Bot.* 56, 1255–1261. doi: 10.1093/jxb/eri121

Mithöfer, A., and Boland, W. (2012). Plant defense against herbivores: chemical aspects. *Annu. Rev. Plant Biol.* 63, 431–450. doi: 10.1146/annurev-arplant-042110-103854

Moise, E. R. D., McNeil, J. N., Hartley, S. E., and Henry, H. A. L. (2019). Plant silicon effects on insect feeding dynamics are influenced by plant nitrogen availability. *Entomol. Exp. Appl.* 167, 91–97. doi: 10.1111/eea.12750

Nelson, J. M., Lane, B., and Freeling, M. (2002). Expression of a mutant maize gene in the ventral leaf epidermis is sufficient to signal a switch of the leaf's dorsoventral axis. *Development* 129, 4581–4589.

Peiffer, M., and Felton, G. W. (2005). The host plant as a factor in the synthesis and secretion of salivary glucose oxidase in larval *Helicoverpa zea*. *Arch. Insect Biochem. Physiol.* 58, 106–113. doi: 10.1002/arch.20034

Peixoto, M. L., Moraes, J. C., Silva, A. A., and Assis, F. A. (2011). Effect of silicon on the oviposition preference of *Bemisia tabaci* biotype B (Genn.) (Hemiptera: Aleyrodidae) on bean (*Phaseolus vulgaris* L.) plants. *Ciênc. Agrotecnol.* 35, 478–481. doi: 10.1590/S1413-70542011000300006

Reynolds, O. L., Padula, M. P., Zeng, R., and Gurr, G. M. (2016). Silicon: potential to promote direct and indirect effects on plant defense against arthropod pests in agriculture. *Front. Plant Sci.* 7:744. doi: 10.3389/fpls.2016.00744

Santamaria, M. E., Arnaiz, A., Gonzalez-Melendi, P., Martinez, M., and Diaz, I. (2018). Plant perception and short-term responses to phytophagous insects and mites. *Int. J. Mol. Sci.* 19:1356. doi: 10.3390/ijms19051356

Sétamou, M., Schulthess, F., Bosque-Pérez, N. A., and Thomas-Odjo, A. (1993). Effect of plant nitrogen and silica on the bionomics of *Sesamia calamistis* (Lepidoptera: Noctuidae). *Bull. Entomol. Res.* 83, 405–411. doi: 10.1017/S000748530002931X

Singh, A., Kumar, A., Hartley, S., and Kumar Singh, I. (2020). Silicon: its ameliorative effect on plant defense against herbivory. *J. Exp. Bot.* 71, 6730–6743. doi: 10.1093/jxb/eraa300

Sun, H., Duan, Y., Mitani-Ueno, N., Che, J., Jia, J., Liu, J., et al. (2020). Tomato roots have a functional silicon influx transporter but not a functional silicon efflux transporter. *Plant Cell Environ.* 43, 732–744. doi: 10.1111/pce.13679

Tian, D., Peiffer, M., Shoemaker, E., Tooker, J., Haubruge, E., Francis, F., et al. (2012). Salivary glucose oxidase from caterpillars mediates the induction of rapid and delayed-induced defenses in the tomato plant. *PLoS ONE* 7:e36168. doi: 10.1371/journal.pone.0036168

Trembath-Reichert, E., Wilson, J. P., McGlynn, S. E., and Fischer, W. W. (2015). Four hundred million years of silica biomineralization in land plants. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5449–5454. doi: 10.1073/pnas.1500289112

Tubaña, B. S., and Heckman, J. R. (2015). "Silicon in soils and plants," in *Silicon and Plant Diseases*, eds F. Rodrigues and L. Datnoff (Cham: Springer International Publishing), 7–52.

Unagwu, B. O., Asadu, C. L., and Ezeaku, P. I. (2013). Residual effects of organic and NPK fertilizer on maize performance at different soil pH levels. *J. Agr. Vet. Sci.* 5, 47–53. doi: 10.9790/2380-0554753

Vaculík, M., Lukačová, Z., Bokor, B., Martinka, M., Tripathi, D. K., and Lux, A. (2020). Alleviation mechanisms of metal (loid) stress in plants by silicon: a review. *J. Exp. Bot.* 71, 6744–6757. doi: 10.1093/jxb/eraa288

Wang, J., Xue, R., Ju, X., Yan, H., Gao, Z., Esmail Abdalla Elzaki, M., et al. (2020). Silicon-mediated multiple interactions: simultaneous induction of rice defense and inhibition of larval performance and insecticide tolerance of *Chilo suppressalis* by sodium silicate. *Ecol. Evol.* 10, 4816–4827. doi: 10.1002/ece3.6235

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., et al. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signal. Behav.* 7, 1306–1320. doi: 10.4161/psb.21663

War, A. R., Taggar, G. K., Hussain, B., Taggar, M. S., Nair, R. M., and Sharma, H. C. (2018). Plant defence against herbivory and insect adaptations. *AoB Plants* 10:ply037. doi: 10.1093/aobpla/ply037

Waterman, J. M., Hall, C. R., Mikhael, M., Cazzonelli, C. I., Hartley, S. E., and Johnson, S. N. (2020). Short-term resistance that persists: rapidly induced silicon anti-herbivore defence affects carbon-based plant defences. *Funct. Ecol.* 35, 82–92. doi: 10.1111/1365-2435.13702

Yang, L., Li, P., Li, F., Ali, S., Sun, X., and Hou, M. (2018). Silicon amendment to rice plants contributes to reduced feeding in a phloem-sucking insect through modulation of callose deposition. *Ecol. Evol.* 8, 631–637. doi: 10.1002/ece3.3653

Ye, M., Song, Y., Long, J., Wang, R., Baerson, S. R., Pan, Z., et al. (2013). Priming of jasmonate-mediated antiherbivore defense responses in rice by silicon. *Proc. Natl. Acad. Sci. U.S.A.* 110, E3631–E3639. doi: 10.1073/pnas.1305848110

Zhu, Y.-X., Gong, H.-J., and Yin, J.-L. (2019). Role of silicon in mediating salt tolerance in plants: a review. *Plants (Basel)* 8:147. doi: 10.3390/plants8060147

Conflict of Interest: 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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 May 2021
doi: 10.3389/fpls.2021.678959

Variation in Methyl Jasmonate-Induced Defense Among Norway Spruce Clones and Trade-Offs in Resistance Against a Fungal and an Insect Pest

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OPEN ACCESS

Edited by:
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Reviewed by:
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Nadir Erbilgin, University of Alberta, Canada

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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *10 March 2021*
Accepted: *28 April 2021*
Published: *24 May 2021*

Citation:
Puentes A, Zhao T, Lundborg L, Björklund N and Borg-Karlson A-K (2021) Variation in Methyl Jasmonate-Induced Defense Among Norway Spruce Clones and Trade-Offs in Resistance Against a Fungal and an Insect Pest. Front. Plant Sci. 12:678959. doi: 10.3389/fpls.2021.678959

An essential component of plant defense is the change that occurs from a constitutive to an induced state following damage or infection. Exogenous application of the plant hormone methyl jasmonate (MeJA) has shown great potential to be used as a defense inducer prior to pest exposure, and could be used as a plant protection measure. Here, we examined (1) the importance of MeJA-mediated induction for Norway spruce (*Picea abies*) resistance against damage by the pine weevil *Hylobius abietis*, which poses a threat to seedling survival, and infection by the spruce bark beetle-associated blue-stain fungus *Endoconidiophora polonica*, (2) genotypic variation in MeJA-induced defense (terpene chemistry), and (3) correlations among resistance to each pest. In a semi-field experiment, we exposed rooted-cuttings from nine different Norway spruce clones to insect damage and fungal infection separately. Plants were treated with 0, 25, or 50 mM MeJA, and planted in blocks where only pine weevils were released, or in a separate block in which plants were fungus-inoculated or not (control group). As measures of resistance, stem area debarked and fungal lesion lengths were assessed, and as a measure of defensive capacity, terpene chemistry was examined. We found that MeJA treatment increased resistance to *H. abietis* and *E. polonica*, but effects varied with clone. Norway spruce clones that exhibited high constitutive resistance did not show large changes in area debarked or lesion length when MeJA-treated, and vice versa. Moreover, insect damage negatively correlated with fungal infection. Clones receiving little pine weevil damage experienced larger lesion lengths, and vice versa, both in the constitutive and induced states. Changes in absolute terpene concentrations occurred with MeJA treatment (but not on proportional terpene concentrations), however, variation in chemistry was mostly explained by differences between clones. We conclude that MeJA can enhance protection against *H. abietis* and *E. polonica*, but the extent of protection will depend on the importance of constitutive

and induced resistance for the Norway spruce clone in question. Trade-offs among resistances do not necessarily hinder the use of MeJA, as clones that are constitutively more resistant to either pest, should show greater MeJA-induced resistance against the other.

Keywords: *Ceratocystis polonica*, conifer resistance, *Endoconidiophora polonica*, forest pest, genetic correlations, *Hylobius abietis*, resistance trade-offs, terpene chemistry

INTRODUCTION

Plant resistance to biotic threats is mediated through defensive traits that are present at all times (constitutive defense), but also through those triggered once damage or infection is perceived (induced defense). Constitutive defenses provide a first line of protection that can deter or stop attackers, while induced defenses strengthen these effects making plants unsuitable hosts and decrease the likelihood of further attack (Karban and Myers, 1989; Agrawal, 1999). In conifers, quantitative and qualitative changes to constitutive conditions are rapidly initiated upon mechanical wounding, insect feeding and fungal infection. Interestingly, defenses can even be mobilized earlier and prior to damage, as for example recently shown for the effects of insect egg deposition on *Pinus sylvestris* (Bittner et al., 2019). These responses include secondary resin production and traumatic resin duct formation, synthesis of new phenolics, lignification of fibers, and initiation of wound periderm (Franceschi et al., 2005). The difference between the constitutive and induced state is referred to as "inducibility," and represents a key functional trait of defensive investment and effective resistance to specific enemies (Cipollini and Heil, 2010). Indeed, the essential contribution of induced responses to effective conifer resistance has been demonstrated for bark-beetle-fungi complexes (e.g., Krokene, 2015; Raffa et al., 2017), infection by fungal pathogens (e.g., Danielsson et al., 2011; Ganthaler et al., 2017) as well as weevil pests (e.g., Zas et al., 2014; Whitehill et al., 2019). Host colonization by many of these attackers impairs water and nutrient transport, and result in tree death if induced responses fail to hamper damage levels (Kolosova and Bohlmann, 2012). Thus, understanding the extent of inducibility is essential for improving tree survival, for example within forest protection, and to reveal ecologically-relevant tree properties that mediate interactions.

The extent of effective resistance against coniferous pests, achieved through constitutive and/or induced resistance, has been largely examined separately for most pest organisms without consideration on how they affect each other. Resistant individuals are those that can stop or deter attackers, and thus effectively reduce their damage or infection levels relative to susceptible individuals. In particular, little is known about the relationship between resistance to insects and fungal pathogens that are not mutually associated (i.e., those that do not co-occur and depend on each other for host colonization), and if the relative importance of defense components varies or shifts with respect to each pest species (Eyles et al., 2010; Raffa et al., 2020). Given that effective resistance against fungal and insect pests may or may not involve different defense pathways (e.g., jasmonic acid or salicylic acid signaling pathways), the consequences of responses induced by these pests can be antagonistic or complementary to each other (Rostás et al., 2003; Beckers and Spoel, 2006; Eyles et al., 2007). For example, responses to feeding by the jack pine budworm (*Choristoneura pinus pinus*) have been shown to also mediate effective resistance against a fungal pathogen (*Grosmannia clavigera*) in *Pinus banksiana* (Colgan and Erbilgin, 2010). On the other hand, attack by the silver fir wooly adelgid (*Dreyfusia nordmannianae*) makes *Abies nordmanniana* more susceptible to infection by the fungus *Neonectria neomacrospora* (Xu et al., 2018). Thus, identifying potential correlations among defense components to different pests is essential to understanding the outcome of interactions.

The presence or absence of trade-offs among defense components against specific pests, and the consequences for effective resistance, can vary depending on plant genotype or species in question (e.g., Koricheva et al., 2004; Erb et al., 2011; Moreira et al., 2014; Hahn and Maron, 2016; Shikano et al., 2017). For instance, not all genotypes respond equally to attack by the same pest in conifers, as they can vary, e.g., in their allocation to constitutive and induced defense (e.g., Ott et al., 2011; Sampedro et al., 2011; Villari et al., 2014; Moreira et al., 2016; Pimentel et al., 2017; Reglinski et al., 2019). Moreover, the relative importance of constitutive and induced defense is also dependent on whether the species examined is fast- or slow-growing, or its degree of competitive ability (Endara and Coley, 2011; Kempel et al., 2011; Moreira et al., 2014). Differences in allocation to defense strategies can result in trade-offs among constitutive and induced resistance (e.g., Rasmann et al., 2011). Depending on which strategy is most important for deterring the attacker in question, this could in turn result in negative correlations among resistances to each pest. Understanding such variation in resistance trade-offs is especially important for conifer breeding programs and novel plant protection tools aimed at enhancing intrinsic tree defenses. Without knowledge of such correlations, breeding may select for genotypes that exhibit opposing susceptibility to, e.g., fungal and insect pests. Thus, uncovering genetic relationships among defenses is crucial to producing improved tree material that can effectively resist various pests (Telford et al., 2015).

From a plant protection perspective, exogenous application of phytohormones such as jasmonic acid and its methyl ester (methyl jasmonate) has been proposed as a tool to prime or induce defenses associated with conifer resistance to insects and fungal pests (Holopainen et al., 2009; Zas et al., 2014; Mageroy et al., 2020a). Treatment with methyl jasmonate (MeJA), for example, induces the formation of

traumatic resin ducts and synthesis of terpene and phenolic-based compounds (Krokene et al., 2008; López-Villamor et al., 2020). These MeJA-mediated changes have been shown to increase resistance against bark-feeding insect pests like the pine weevil *Hylobius abietis* (Heijari et al., 2005; Zas et al., 2014; Fedderwitz et al., 2016; Chen Y. et al., 2020), and the spruce bark beetle-blue stain fungus complex *Ips typographus-Endoconidiophora polonica* (Zhao et al., 2011; Schiebe et al., 2012). However, responses to MeJA can vary with plant genotype (Zeneli et al., 2006; Moreira et al., 2013; López-Goldar et al., 2018), with some genotypes showing greater or lesser levels of inducibility relative to their constitutive state. Therefore, to tackle challenges posed by multiple pests it is not only of interest to identify trade-offs in defense, but also those genotypes that respond strongly to elicitors such as MeJA and effectively increase their resistance. This knowledge allows us to better predict and understand the outcome of conifer interactions with various pests, and implement long-lasting and sustainable plant protection strategies.

In this study, we conducted an experiment to examine genotypic variation in defense inducibility, in terms of terpene chemistry, and the importance of induction for insect and fungal pathogen resistance in Norway spruce (*Picea abies*). We examined variation between clones in MeJA-induced responses, and to damage by the pine weevil (*H. abietis*) and a virulent blue-stain fungus (*E. polonica*) associated with the spruce bark beetle. The pine weevil represents the main threat to newly planted conifer plants in Europe (Långström and Day, 2004) as it can feed on the phloem and bark of seedlings, causing stem girdling and high mortality rates. The necrotrophic blue-stain fungus *E. polonica* plays a key role in mediating tree death following spruce bark beetle attacks, which are a major threat to mature Norway spruce in Europe (Krokene, 2015; Hlásny et al., 2019). Thus, the pine weevil and blue-stain fungus pose large threats to tree survival but at different developmental stages. Resistance to these economically-important threats has been examined separately, and the relationship among resistance to insect damage and fungal infection is not known. Being resistant to the pine weevil early in life, for example, could come at the cost of being susceptible to fungal infection later in life and vice versa. Such trade-offs are important from an ecological perspective, but also from a plant breeding perspective as there are ongoing efforts to produce genetically improved tree material that is resistant to various pests. Here, we aimed to investigate the correlation among constitutive and induced resistance to an herbivorous insect and a pathogenic fungus. More specifically, we aimed to answer:

1. Does MeJA treatment influence the terpene chemistry of Norway spruce and its resistance against the pine weevil *H. abietis* and the blue-stain fungus *E. polonica*?
2. Does the effect of MeJA treatment on terpene chemistry and resistance against *H. abietis* and *E. polonica* vary among different clones of Norway spruce?
3. Does resistance against the pine weevil *H. abietis* and blue-stain fungus *E. polonica* exhibit trade-offs?

To answer these questions, we used a clonal set-up where we compared chemical defenses (terpenes) and resistance of MeJA-induced and non-induced plants of the same Norway spruce clones. Resistance to the insect and fungus pests were investigated separately (in different individuals of the same clones) in a semi-field experiment. We quantified stem area debarked by the pine weevil and fungal growth (lesion length) as measures of plant resistance, with resistant plants being those receiving little to no insect damage or shorter lesion lengths. We quantified terpene chemistry as a measure of defense, and refer to it as defense or defensive chemistry throughout. The use of clones facilitates correlations among resistances to be estimated, and to tease apart the relative importance of constitutive and induced resistance against each attacker.

MATERIALS AND METHODS

Plant Material and Methyl Jasmonate Treatments

In August 2011, cuttings were made from nine clones (clone identification numbers: 1 to 9 hereafter) of Norway spruce [*P. abies* (L.) Karst.]. These clones originated from the clonal archive material produced by The Forestry Research Institute of Sweden (Skogforsk, series S21K0420-), as part of their breeding trials for Norway spruce. Cuttings were individually planted in 6.5 cm-sized pots, and allowed to grow at the growing facilities in Skogforsk (Ekebo, 55.9°N; outside of Svalöv in southern Sweden) first, and later at the Swedish University of Agricultural Sciences (SLU, Uppsala, 59°49′N, Sweden) until the start of the experiment in the summer of 2014. By then, plants had reached an average height of 32.6 cm (standard error: ±0.4), and a diameter of 6.3 mm (±0.08; **Supplementary Figure 2**).

To examine constitutive and MeJA-induced responses in Norway spruce clones, plants from each clone were assigned to either a control or a MeJA treatment group. According to the Krutzsch index (Krutzsch, 1973), clones were at a similar developmental stage (between 0 and 3, **Supplementary Table 1**) when MeJA was applied. We chose two concentrations of MeJA (25 and 50 mM) based on previous studies in our group and on the size of the plants. Smaller Norway spruce plants (average height: 20 cm, average diameter: 3 mm) have often been treated with concentrations ranging from 5 to 15 mM MeJA (Fedderwitz et al., 2019; Chen Y. et al., 2020). Larger and thicker plants (average heights above 25 cm, and diameters above 5 mm) as those used in our experiment, have been treated with higher concentrations (25 mM MeJA, Lundborg et al., 2016b; 50 mM MeJA, Fedderwitz et al., 2016). For the pine weevil experiment, 20 replicates per clone (n = 9 clones) were included in each of the three treatments (0, 25 and 50 mM MeJA). However, clone number 5 had three individuals less than all the other clones from the beginning, and plants in the 50 mM MeJA treatment for clone number 1 were not planted in the field (see Semi-field experiment description). The total sample sizes for 0, 25, and 50 mM MeJA treatments were 180, 177, and 160 plants, respectively. For the fungus-inoculation experiment, 5 plants per clone were included in each of the three treatments. Clone numbers 3 and 6 had

one less plant, and clones 4 and 7 had two less plants from the beginning, and for clone number 1 plants in the 50 mM MeJA were not planted in the field. The total sample sizes for 0, 25, and 50 mM MeJA treatments were 43, 45, and 36 plants, respectively.

Treatment of Norway spruce plants with MeJA followed Zas et al. (2014). Briefly, MeJA (95%, Sigma-Aldrich, ref. 392707) was first dissolved in ethanol, then deionized water was added to this mixture to achieve a final ethanol concentration of 2.5% (v:v). The solution was shaken vigorously until a uniform milky emulsion was obtained, and then transferred to a spraying bottle (0.5 L plastic bottle; Part No. 62526011, Canyon, United Kingdom). Spraying was conducted so that the solution reached and covered the entire plant in each pot. Each plant received approximately 1 ml of either 25 or 50 mM MeJA solution. The control group (0 mM MeJA) was treated in the same way but only with carrier solution (deionized water and ethanol). Plants were treated twice, once on April 28th and then again on May 14th, 2014. To avoid any potential defense induction of neighboring non-treated controls, MeJA-treated and control plants were kept separately from the first application of MeJA until planting occurred.

Semi-Field Experiment

To examine constitutive and MeJA-induced resistance against the pine weevil and the blue-stain fungus, a semi-field experiment was set up. On May 21st, 2014 plants were planted on a 1-year-old clear-cut situated in a forest dominated by Scots pine (*P. sylvestris*), located near Uppsala, Sweden. The plants were randomly assigned to positions in rows with a spacing of 0.1 m between the plants in the same row, and 0.1 m between the rows. Planting was done all in 1 day and plants were planted with their entire soil plug in the clear-cut (see **Supplementary Figure 1** for the soil plug). Planting was done using a standard cylindrical planting tube (Pottiputki, Finland, 75 mm). To minimize planting stress, we kept soil plugs in a bucket with water before planting them. Once the planting hole was made with the planting tube, we poured water into the hole to moisten the soil and minimize the risk of drought stress. Plants in all treatments and enclosures were treated the same way.

Plants intended for exposure to pine weevils were planted separately from plants intended for fungal inoculations. In the same clear-cut, four enclosures (see description below) were built for the pine weevil experiment, enclosing 127, 130, 130, and 130 plants each, and one enclosure with two sub-plots for the 250 plants included in the fungal inoculation experiment. All MeJA treatment × clone combinations were represented in each enclosure, but replicate numbers for each combination varied across enclosures. However, the plants from the 50 mM treatment of clone 1 were never included in the experiment due to MeJA-treatment-related damage, i.e., needles turning brown before the start of the field experiment. Plant height, basal stem diameter and top shoot length were measured for each plant before the start of the experiments.

Pine Weevil Experiment

To investigate resistance to pine weevil damage, weevils were released in the enclosures containing the group of plants intended for this purpose. Approximately 300 pine weevils were released on July 1st, 2014 in each of the four enclosures (48 days after the second MeJA treatment occurred). The enclosures were about 0.2 m high, and prevented pine weevils from escaping and reaching the experimental plants intended for fungal inoculations. Each enclosure consisted of a wooden framework placed around each group of plants (but open across the top). The inner edges of the wooden enclosure were covered with a plastic film, and this plastic film was painted with polytetrafluoroethylene (Fluon®, Blades Biological Ltd., Cowden Edenbridge, Kent, United Kingdom). This created a slippery surface preventing the weevils from climbing over the enclosure. The pine weevils that were released into these enclosures had been collected at the same clear-cut earlier in the spring, and kept in rearing boxes with access to food (freshly-cut conifer branches) and water until the start of the experiment. After release, damage inflicted by pine weevils on each plant was measured as the stem area debarked (cm^2), and was recorded during July 23rd–24th, 2014. Area debarked was measured as the sum of the areas of each wound inflicted by the pine weevil. A template with different area sizes illustrated on millimeter paper, was used for calculating the area of each wound. Only the area, and not the depth of the wound, was measured. Enclosures were included as blocks in the statistical analyses.

Blue-Stain Fungus Experiment

The strain of the blue stain fungus *E. polonica*, which was used for inoculations, was NFLI 1993–208/115. It was obtained from the culture collection of the Norwegian Institute for Bioeconomy Research in Ås, Norway. The strain was isolated from a Norway spruce log inoculated with the bark beetle *Polygraphus poligraphus* L. (Krokene and Solheim, 1996). This *E. polonica* isolate has been used in many of our previous studies (e.g., Zhao et al., 2010, 2011; Axelsson et al., 2020), it grows well on Norway spruce and has shown consistent virulence across our studies. The fungal strain was maintained on malt agar (2% malt, 1.5% agar) at 4°C, and transferred to fresh malt agar and cultivated at 25°C in darkness for 7-10 days, before the start of the experiment.

To investigate resistance against the blue-stain fungus, plants in the different MeJA treatments (0, 25 or 50 mM) were further assigned to two treatments: fungal or no fungal inoculation (control) group. On July 1st and 2nd, 2014 (48-49 days after the second MeJA treatment occurred) the lower part of the stem of plants in the fungal inoculation group was inoculated with *E. polonica*. Using a 4 mm cork borer, a phloem plug (about 1.5-2.0 mm in depth) was removed from each plant and an agar plug with *E. polonica* inoculum was in turn introduced. For plants in the control group, an agar plug not containing *E. polonica* was introduced instead. The *E. polonica* inoculum or the non-infected agar plug was fixed to the stem with Parafilm®. On August 29th, 2014 plants were harvested for analyses, i.e., about 8 weeks after the inoculation. The blue-stain fungus experiment was ended at a later date than the pine weevil experiment, given that the insect feeds at a faster pace than fungal lesions occur. Lesion lengths of *E. polonica* in the phloem were assessed by removing the outer bark upward from the inoculation point. A surgical knife was

used for the removal of the bark and the length of the lesions was measured using a ruler. Only for plants in the 0 and 50 mM MeJA treatments, we cut the plants into parts and kept only ten centimeters of the entire stem (which included the inoculated area), and froze these stem pieces in an −80°C freezer until chemical analyses were conducted.

Chemical Analyses

The terpene chemistry of plants in the blue-stain fungus experiment (*E. polonica*-inoculated and control group) which had received 0 and 50 mM MeJA treatment, was quantified to investigate the effect of MeJA on plants' terpene production. Note that for clone 1, plants in the 50 mM were never included in the experiment, so for this clone plants in the 25 mM MeJA treatment were used instead. No plants in the insect enclosure experiment were harvested for chemical analyses.

Stem pieces were taken from the freezer after 5 months, and two phloem samples were removed from stems using a surgical knife. To examine the chemical effects of the MeJA treatment *per se* (non-fungus inoculated MeJA treated vs. untreated plants), phloem was collected from a "control zone" located 5 cm below and on the opposite side of the stem from the "reaction zone" (inoculation area). Further, to also be able to analyze the induced defense caused by fungus inoculation, phloem was collected from directly below the inoculation area. This method of comparing tissue from the "inoculation area" and a "control area" from the opposite side of the stem, is usually used in older trees with thicker trunks (e.g., Axelsson et al., 2020). Since we used thinner trees in our experiment, we cannot be completely sure that fungal infection does not affect the opposite side of the stem bark. Samples of approx. 1.5 cm^2 in size were chopped into smaller pieces (following standard protocol as described in Persson et al., 1993; Axelsson et al., 2020), and each was immediately placed (using tweezers) in a 2-ml glass vial with solvent. Phloem samples were extracted in 0.5 mL n-hexane (VWR, Ref no. 601-037-00-0) containing 0.05 mg mL^{-1} of internal standard (pentadecane; Lancaster synthesis, Alfa Aesar). Extraction time was 48 h, and after this time, extracts were transferred to new 2-ml glass vials. These were stored at −30°C until analysis using a gas chromatograph-mass spectrometer (GC-MS) was conducted. The dry weight of the extracted phloem pieces was measured after 5 h at 80°C.

The separation and identification of volatiles was made on a 2DGC-MS Agilent instrument (7890A GC; 5975C MS), equipped with a DB-5 column followed by a Cyclodextrin-β column (both Agilent; 30 m, ID 0.25 mm, and film thickness 0.25 μm). Samples were injected splitless into an injector temperature of 250°C, isothermal, and with a purge time of 1 min. The GC oven program started at 40°C, and was kept isothermal for 3 min, followed by a temperature ramp of 3°C min^{-1}, up to 100°C, followed by a second ramp of 5°C min^{-1} up to 250°C, and then isothermal for 1 min. The transfer line temperature to the second GC was isothermal at 40°C.

The temperature program in the second GC was isothermal at 58°C for 50 min, then ramped from 100°C min^{-1} up to 200°C, and kept for 3.5 min. On the second column, the enantiomers of α-pinene, β-pinene and limonene were separated. The first two substances were cut from the first to the second column between 8 and 14 min, and evaluated on m/z 93 (their most abundant fragment). On the first column, the coeluting limonene and β-phellandrene were quantified on the amount of m/z 68 compared to limonene standard (β-phellandrene does not give this fragment). These were in the same run as the other chiral compounds, cut from 15 to 18 min on GC1, for limonene to be evaluated on m/z 68 on GC2.

The terpene hydrocarbons were identified by comparing retention times and mass spectra with available authentic standards, or by comparing retention indexes (RIs) and mass spectra with Massfinder 3 (Hochmuth Scientific Consulting, Germany) and the reference libraries of NIST (National Institute of Standards and Technology, United States). The absolute amounts of terpenes were calculated relative to the internal standards, and expressed as μg g^{-1} dry wt. The relative amounts of terpenes were calculated as the ratio of the area of each peak to the sum of all the areas of terpene hydrocarbons in a defined GC fraction, and expressed as percentages.

Statistical Analyses

All analyses were conducted in R version 4.0.0 (R Core Team, 2020) using R studio version 1.1.463 (RStudio Team, 2020), and figures were plotted using *ggplot2* (Wickham, 2016). To examine constitutive and MeJA-induced defense and resistance against *H. abietis* and *E. polonica*, and if this varies among clones, we fitted various linear models using either the *lm* (The R stats package, R Core Team, 2020) or the *glmmTMB* functions in R (*glmmTMB* package, Brooks et al., 2017). For pine weevil damage (response variable: stem area debarked), we fitted a *glmmTMB* model with a negative binomial distribution that included MeJA treatment (3 levels: 0, 25, and 50 mM), clone (n = 8, since clone 1 had no individuals in the 50 mM MeJA treatment), and the interaction of MeJA and clone, with enclosure (n = 4) and plant height (continuous covariate) as fixed factors. Model fit was explored with the *DHARMa* package (Hartig, 2020) and no data transformations were necessary. Significance of main effects and interactions was tested using analysis of deviance with the *Anova* function (*car* package, Fox and Weisberg, 2019). For fungal infection (response variable: lesion length), an *lm* model that included MeJA treatment (3 levels: 0, 25, and 50 mM), clone (n = 8), the interaction of MeJA and clone, and sub-plot (n = 2) within one enclosure as fixed factors. Lesion length was log-transformed to meet model assumptions. Significance of main effects and interactions was tested using an F-test with the *Anova* function. Contrasts among treatment means were conducted using the *emmeans* function (*emmeans* package, Lenth et al., 2020). Note, however, that we present visually results for models that include only two MeJA levels (0 and 50 mM, and 0 and 25 mM for clone 1, n = 9 clones) for both area debarked and lesion length (see motivation in Results section).

To examine any potential trade-offs between resistance to insect and fungal damage, we conducted Pearson's product-moment correlations using the *cor.test* function in R (The R stats package, R Core Team, 2020). To examine the correlation between constitutive resistances, we correlated estimated means for each clone (from the models described above) for area

debarked and lesion length in the control group (0 mM MeJA). To examine the correlation in MeJA-induced resistance, we correlated the coefficients of the change between 0 and 50 mM (i.e., slope of the reaction norms) for each clone, from the models described above for area debarked and lesion length. Using these estimates, we also conducted Pearson's product-moment correlations between terpene compounds and insect damage or fungal infection. Mean estimates of area debarked or lesion length were correlated to the average absolute terpene amounts of each compound (or the sum of all terpenes) per clone, both under the constitutive and induced state and (0 and 50 mM MeJA respectively) but without fungal inoculation. These bivariate correlations were conducted separately for each terpene compound and area debarked or lesion length.

For analysis of the chemical data, terpenes were selected with a 2% limit in relative amounts, in at least 3 replicates and then normalized to 100%. In our experiment, Norway spruce clones previously treated with 0 or 50 mM MeJA were subsequently inoculated with an agar plug (no fungus) or *E. polonica*. Thus, both MeJA treatments are represented in the agar and fungus-inoculated plant groups. Since we were interested in examining the effect of MeJA separately from that of fungal infection on terpene chemistry, we conducted individual analyses for those plants receiving an agar plug or *E. polonica*. The analyses described below were conducted for each of these two plant groups. The effects of MeJA treatment (0 or 50 mM), Norway spruce clone (n = 9), and their interaction, were examined with a multivariate analysis of variance (MANOVA; *adonis* function, *vegan* package version 2.5-6, Oksanen et al., 2019) on the absolute amounts of terpene compounds. We also fitted the same model but using instead relative amounts as a response variable (amount of each terpene compound was divided by the sum of total terpenes for that sample). In other words, this model examined proportional changes in terpene chemistry. To visualize the effect of treatment and clone on the chemical profile, dimensions of the dataset acquired from the GC-MS analysis were reduced by Principal Component Analysis (PCA), also using the *vegan* package in R. Loadings were obtained to examine the contribution of each compound to the PCAs first and second axes of variation.

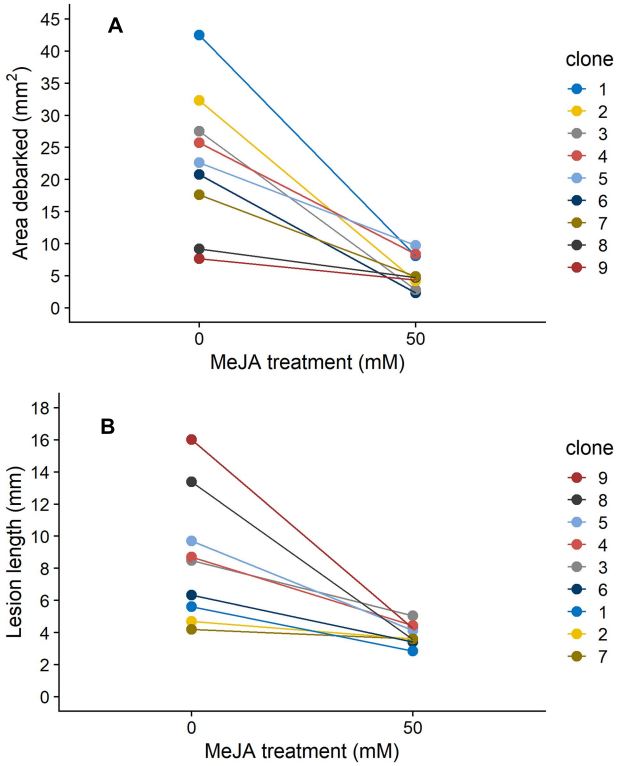

FIGURE 1 | Reaction norms depicting the change in **(A)** area debarked (mm^2) by pine weevils (*H. abietis*) and **(B)** lesion lengths (mm) inflicted by the blue-stain fungus (*E. polonica*) on Norway spruce (*P. abies*) clones (1–9) treated with 0 or 50 mM methyl jasmonate (MeJA). Estimated means (from models presented in **Table 1**) for each clone are shown. For clone 1, plants in the 50 mM were not included in the experiment (see section "Materials and Methods"), so results for the 25 mM MeJA treatment are presented instead. The order in which each clone's reaction norm appears in the graph is reflected in the clone number legend for each panel. Estimates ± standard error for area debarked and lesion length, per clone and treatment combination, can be found in **Supplementary Table 3**.

RESULTS

We found that MeJA treatment decreased the extent of pine weevil damage and blue-stain fungus infection on Norway spruce clones, which were separately exposed to these two pests. Plants treated with 25 or 50 mM MeJA showed significantly lower levels of insect damage and shorter lesion lengths relative to control plants (**Supplementary Table 2**). However, clones responded similarly to 25 and 50 mM MeJA, with no significant differences among these treatments for insect or fungal damage (**Supplementary Table 2**). Given this lack of difference and that samples for terpene chemistry were only taken from Norway spruce clones receiving 0 and 50 mM MeJA, we present results based on models that included only these two levels of MeJA. Note that for Norway spruce clone number 1, plants in the 50 mM MeJA group were never planted in the field experiment due to needle browning (see section "Materials and Methods"), so results for the 25 mM MeJA treatment are presented instead.

Norway spruce plants that were treated with 50 mM MeJA received on average 76% less pine weevil damage, and experienced 55% shorter lesion lengths compared to those in the control group (**Figure 1**). However, the decrease in insect damage and fungal infection observed for plants in the MeJA treatment tended to differ among clones (**Figure 1**). For area debarked, there was a statistically significant MeJA × clone interaction (**Table 1**), while for lesion length it was close to significant at the $P < 0.1$ level (**Table 1**). Overall, there was greater variation among clones in the amount of pine weevil damage and fungal infection when plants were not induced (0 mM MeJA) relative to when they were MeJA-induced, especially for lesion length (**Figures 1A,B**, compare 50 mM MeJA for the two variables). In other words, clones responded more similarly to MeJA treatment in terms of lesion length than area debarked. Nonetheless, some clones such

TABLE 1 | Analysis of deviance (Chisq, Chi-square Wald statistic; DF, degrees of freedom; and *P*, *p*-value) and Analysis of variance (SS, Sum of Squares; DF, degrees of freedom; *F*, *F*-value; and *P*, *p*-value) results from models examining the effects of MeJA (MJ: 0, 50 mM) on area debarked (mm^2) by pine weevils (*H. abietis*) or lesion lengths (mm) inflicted by the blue-stain fungus (*E. polonica*) on Norway spruce (*P. abies*) clones.

Variable	Source	Chisq	DF	*P*	Variable	Source	SS	DF	*F*	*P*
Area debarked (mm^2)	MJ	101.3	1	**<0.0001**	Lesion length (mm)	MJ	10.8	1	50.1	**<0.0001**
	Clone	39.2	8	**<0.0001**		Clone	5.8	8	3.4	**0.003**
	MJ × clone	18.5	8	**0.02**		MJ × clone	3.1	8	1.8	0.08
	Plant height	11.9	1	0.0006		Block	0.1	1	0.5	0.5
	Block	52.6	3	<0.0001		Residuals	13.9	65		

Models included the main effect and interaction of MeJA (MJ) and clone, experimental enclosure or sub-plot for lesion length (Block), and plant height as a continuous covariate (for area debarked only). Significant effects are in bold (P < 0.05).

as numbers 2 and 7 showed little change in lesion length when treated with MeJA (**Figure 1B**).

We also found that insect damage and fungal infection exhibited a negative relationship to each other, when estimates for each clone were compared. For plants that were not induced (0 mM MeJA), mean estimates of area debarked and lesion length for each clone negatively correlated with each other (**Figure 2A**; Pearson's product-moment correlation coefficient: −0.71, 95% Confidence intervals: −0.93, −0.08, t = −2.7, DF = 7, and P = 0.033). Likewise, we found a negative correlation between the effect of MeJA treatment on lesion length and area debarked (**Figure 2B**; Pearson's product-moment correlation coefficient: −0.70, 95% Confidence intervals: −0.93, −0.06, t = −2.6, DF = 7, and P = 0.037). In other words, we found a negative relationship between the estimates of the change in area debarked and lesion length that occured from the 0 to the 50 mM MeJA treatment (reaction norms observed in **Figure 1**). Norway spruce clones showing the largest reduction in area debarked following MeJA treatment, showed a smaller reduction in lesion length and viceversa. We also found a few negative bivariate correlations between mean area debarked per clone and each teperne compound, but only when plants had been treated with 50 mM MeJA (**Supplementary Table 7**). Moreover, mean estimates of lesion lengths per clone positively correlated with some terpene compounds, but only in the constitutive state (0 mM MeJA) (**Supplementary Table 7**). In line with other studies, we found that MeJA negatively affected plant growth in terms of apical shoot length, total plant height and stem diameter (**Supplementary Figure 2** and **Supplementary Table 4**). On average, the the apical (top) shoot length decreased with 22%, the total plant height with 3%, and the stem diameter with 2% when plants were MeJA-treated relative to controls.

In addition to changes in resistance to pine weevil damage and blue-stain fungus infection, we also found differences in terpene chemistry among non-treated and MeJA-treated plants. A total of 15 terpene compounds were identified, including monoterpenes [(+)-α-pinene, (−)-α-pinene, (−)-β-pinene, (+)-β-pinene, (+)-3-carene, (−)-β-phellandrene, (−)-limonene, (+)-limonene, camphene, and myrcene], sesquiterpenes (calarene) and a few diterpenes (neocembrene, thunbergene, thunbergol, and geranyllinalool). Total terpene chemistry (absolute amounts) was significantly different between plants treated with 0 and 50 mM MeJA (**Table 2**). Treatment with MeJA shifted terpene chemistry mostly across the first axis of variation (PC 1), with larger increases in the amounts of compounds such as (−)-α-pinene, (−)-β-pinene, (+)-limonene, β-phellandrene, camphene, myrcene, and calarene (**Figure 3** and **Table 3**). An increase in (+)-3-carene was observed across the second axis

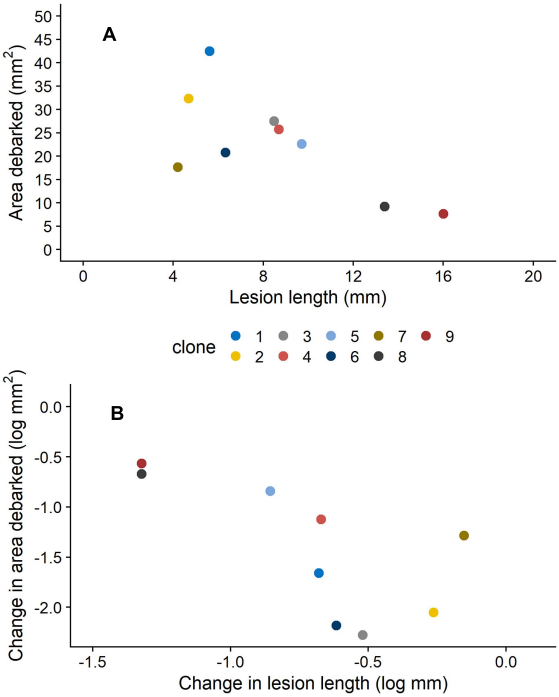

FIGURE 2 | Relationship between **(A)** estimated means (from models presented in **Table 1**) for area debarked (mm^2) by pine weevils (*H. abietis*) and lesion lengths (mm) inflicted by the blue-stain fungus (*E. polonica*) on Norway spruce (*P. abies*) clones (1–9) treated with 0 mM methyl jasmonate (MeJA), and **(B)** the change in area debarked (log mm^2) and lesion length (log mm; i.e., slope coefficients of reaction norms) that occurred for each clone between the 0 and 50 mM MeJA treatments. Clones in the 0 mM MeJA that received little pine weevil damage experienced longer fungal lesion lengths, and vice versa [panel **(A)**]. Clones that exhibited a minor difference (i.e., decrease) in area debarked between the 0 and 50 mM MeJA treatment, experienced a greater change (i.e., decrease) in fungal lesion lengths between these two treatments [panel **(B)**]. For clone 1, plants in the 50 mM were not included the experiment (see section "Materials and Methods"), so results for the 25 mM MeJA treatment are presented instead. Estimates ± standard error for area debarked and lesion length, per clone and treatment combination, can be found in **Supplementary Table 3**.

TABLE 2 | Multivariate analysis of variance (MANOVA) results (DF, degrees of freedom; SS, Sum of Squares; MS, Mean Squares; *F*, *F*-value; R^2, adjusted *R*-squared; and *P*, *p*-value).

	Source	DF	SS	MSS	*F*	R^2	*P*
No fungus exposure	MJ	1	31.83	31.83	3.37	0.02	**0.01**
	Clone	8	584.64	68.58	7.25	0.42	**0.001**
	MJ × clone	8	62.44	7.80	0.83	0.05	0.7
	Residuals	70	662.08	9.46		0.51	
	Total	87	1305.00			1.00	
Exposed to fungus	MJ	1	33.92	33.92	3.50	0.03	**0.01**
	Clone	8	501.60	62.70	6.48	0.39	**0.001**
	MJ × clone	8	86.44	10.81	1.12	0.07	0.28
	Residuals	69	668.06	9.68		0.52	
	Total	86	1290.00			1.00	

Models examined the effect of Norway spruce (P. abies) clone, MeJA treatment (MJ: 0, 50 mM) and their interaction, on absolute terpene amounts when plants had not been (No fungus exposure) or had been exposed to E. polonica (Exposed to fungus) following MeJA treatment. Significant effects are in bold (P < 0.05).

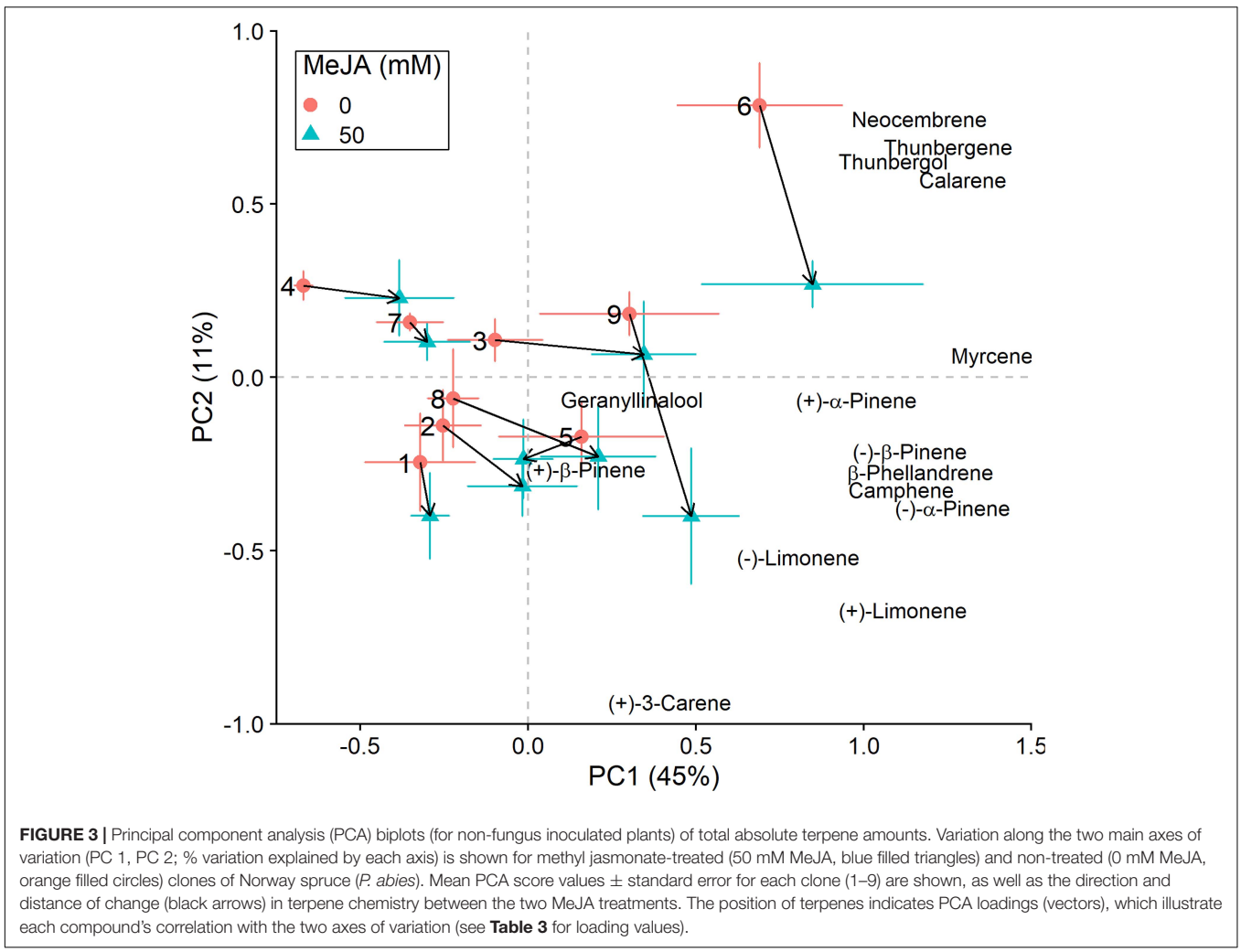

FIGURE 3 | Principal component analysis (PCA) biplots (for non-fungus inoculated plants) of total absolute terpene amounts. Variation along the two main axes of variation (PC 1, PC 2; % variation explained by each axis) is shown for methyl jasmonate-treated (50 mM MeJA, blue filled triangles) and non-treated (0 mM MeJA, orange filled circles) clones of Norway spruce (*P. abies*). Mean PCA score values ± standard error for each clone (1–9) are shown, as well as the direction and distance of change (black arrows) in terpene chemistry between the two MeJA treatments. The position of terpenes indicates PCA loadings (vectors), which illustrate each compound's correlation with the two axes of variation (see **Table 3** for loading values).

of variation for some clones (e.g., clones 6 and 9, **Figure 3**). MeJA resulted mostly in quantitative rather than qualitative changes in terpene chemistry, as it had a non-significant effect on the relative proportions of terpene compounds (**Supplementary Figure 3** and **Supplementary Tables 5**, **6**). Even though changes in chemistry following MeJA treatment were similar among clones (non-significant MeJA × clone interaction, **Table 2** and **Supplementary Table 5**), total terpene levels differed significantly among clones irrespective of treatment (**Figure 3**, **Supplementary Figure 3**, **Table 2**, and **Supplementary Table 5**).

Among-clone differences explained most of the variation in terpene chemistry rather than MeJA treatment (**Table 2** and **Supplementary Table 5**).

We conducted the same analyses as described above also for plants that had received inoculation with *E. polonica*. Treatment with MeJA had a signficant effect on the total concentration of terpenes (**Table 2**), and it influenced the clones' positions along the axes of variation differently than when plants were not inoculated with the fungus (**Figures 3**, **4**). Both increases and decreases along PC1 and PC2 were observed depending on the clone. Similar to those not inoculated with the fungus, compounds like (−)-α-pinene, (−)-β-pinene and myrcene increased following MeJA, but changes in compounds such as geranyllinalool and (+)-3-carene were more important for plants responses to the fungus (**Figure 4** and **Table 3**). Again, differences among clones explained most of the variation in terpene chemistry (**Table 2**). Unlike the effects observed when MeJA occurred alone (without fungal inoculation, **Supplementary Figure 3**), fungal infection caused qualitative changes in terpene chemistry as indicated by changes in the relative amounts of terpenes (**Supplementary Figure 4** and **Supplementary Table 5**). These relative changes varied per treatment and clone (MeJA × clone interaction, **Supplementary Table 5**).

DISCUSSION

Our study showed that treatment with the plant hormone methyl jasmonate (MeJA) increased the resistance of Norway spruce (*P. abies*) clones that were separately exposed to pine weevil (*H. abietis*) damage and blue-stain fungus (*E. polonica*) infection.

TABLE 3 | Loadings for each compound in Principal components 1 and 2 (PC 1, PC 2) from the PCAs that examined variation in total absolute terpene chemistry (see **Figures 3**, **4**) among Norway spruce (*P. abies*) clones treated with 0 and 50 mM MeJA.

Compound	No fungus exposure		Exposure to fungus	
	PC1	PC2	PC1	PC2
(−)-α-Pinene	0.31	−0.20	0.33	−0.15
(+)-α-Pinene	0.24	−0.04	0.22	−0.13
Camphene	0.28	−0.16	0.22	−0.14
(+)-β-Pinene	0.04	−0.14	0.06	0.33
(−)-β-Pinene	0.28	−0.14	0.31	−0.05
Myrcene	0.34	0.03	0.34	−0.09
(+)-3-Carene	0.10	−0.48	0.22	−0.27
(−)-Limonene	0.20	−0.27	0.26	−0.18
(+)-Limonene	0.28	−0.35	0.31	−0.27
β-Phellandrene	0.29	−0.15	0.31	−0.05
Thunbergene	0.31	0.32	0.26	0.47
Calarene	0.32	0.32	0.27	0.34
Neocembrene	0.29	0.38	0.19	0.36
Geranyllinalool	0.08	−0.04	0.06	0.40
Thunbergol	0.27	0.32	0.29	0.10

Separate analyses were conducted for plants not exposed (No fungus exposure) and those exposed to E. polonica (Exposure to fungus).

Treatment with MeJA also changed the terpene chemistry of plants relative to those that were untreated, however, most of the variation in chemistry was explained by differences between Norway spruce clones. Most interestingly, we found that resistance against the pine weevil and blue-stain fungus exhibited a trade-off when comparing the estimates of area debarked and lesion lengths for each clone. Clones that received little pine weevil damage exhibited larger fungal lesion lengths, and vice versa, both constitutively and when treated with MeJA. To our knowledge, this is the first report of such a trade-off.

Treatment with MeJA enhanced Norway spruce resistance to insect and fungal damage when clones were separately exposed to these two pests. On average, plants treated with 50 mM MeJA received 76% less *H. abietis* damage and experienced 55% shorter *E. polonica* lesion lengths relative to untreated control plants. Clones receiving 25 mM MeJA also experienced similar reductions (78% less pine weevil damage and 56% shorter lesion lengths compared to controls). Our results are in line with previous studies on MeJA-induced resistance in *P. abies* and other conifers species. Decreases in pine weevil damage ranging from 50 to 70%, for example, have been shown to occur after consecutive treatment with 10 mM MeJA in Norway spruce seedlings (Chen Y. et al., 2020). For other species such as *Pinus pinaster*, pine weevil damage was reduced by 80% after treatment with 100 mM MeJA (Moreira et al., 2009), and reduced by 30–60% for *P. pinaster*, *P. radiata*, *P. sylvestris,* and *P. abies* seedlings treated with 25 mM MeJA (Zas et al., 2014). MeJA can change the pattern of pine weevil feeding, with weevils inflicting fewer and smaller scars on induced seedlings (Fedderwitz et al., 2016), and resulting in less damage overall. Similarly, treatment with 10 mM MeJA has also been shown to reduce feeding by the bark-feeding beetle *Monochamus alternatus* in *Pinus massoniana* roughly by half compared to non-treated plants (Chen R. et al., 2020). For *E. polonica*, Norway spruce seedlings treated with 100 mM MeJA have been reported to experience 51% shorter lesion lengths than control plants (Krokene et al., 2008). Even mature trees treated with 50 mM MeJA have shown decreased blue-staining of the sapwood (caused by *E. polonica* infection) compared to non-treated trees (15% vs. 70% staining, respectively; Zeneli et al., 2006). Plant resistance to other pathogens, such as *Sphaeropsis sapinea* and *Pythium ultimum* in *P. radiata* and *P. abies,* respectively, has also been shown to be enhanced by exogenous application of MeJA (Kozlowski et al., 1999; Gould et al., 2008).

Even though overall plant resistance was improved, we found that MeJA-mediated changes in resistance occurred to a greater or lesser extent depending on the Norway spruce clone examined. Some clones exhibited high levels of resistance (i.e., received less insect damage or fungal infection) constitutively (0 mM MeJA), and for these clones, MeJA treatment did not result in a large change in area debarked or lesion length (flatter reaction norms from 0 to 50 mM MeJA in **Figure 2**). Likewise, clones that were less resistant (i.e., received greater insect damage or fungal infection) constitutively, experienced large reductions in pine weevil feeding and shorter fungal lesions (i.e., became more resistant) when treated with MeJA (steeper reaction norms in **Figure 2**). Thus, not all clones responded equally to MeJA treatment and their degree of

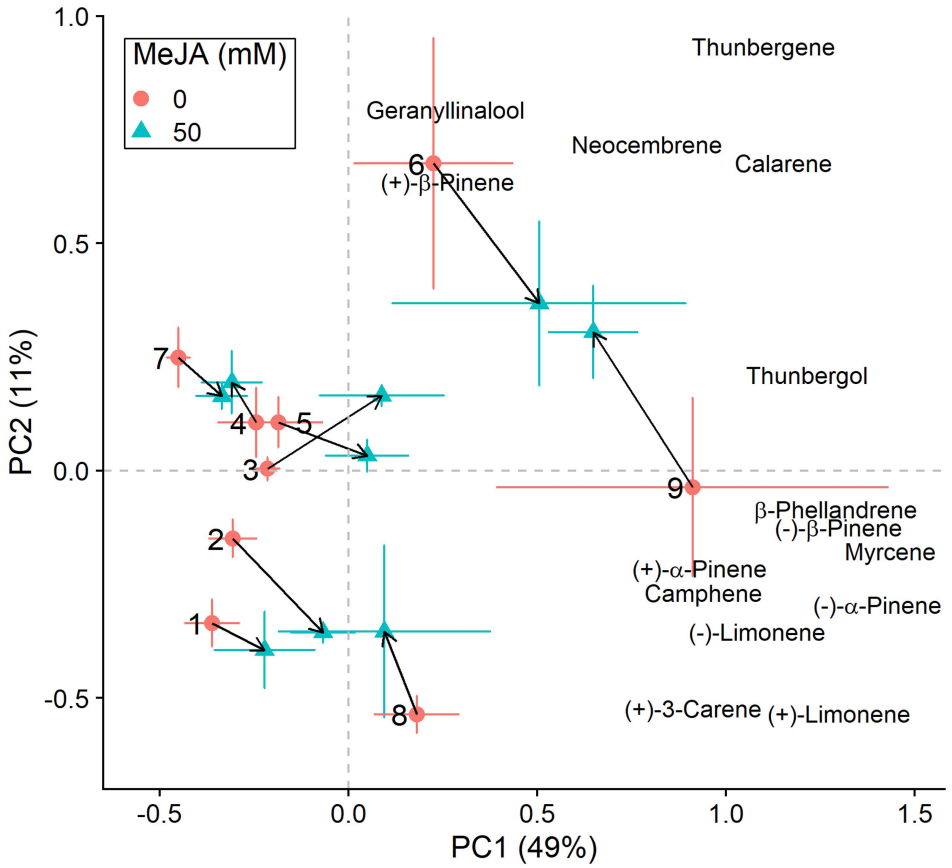

FIGURE 4 | Principal component analysis (PCA) biplots (for *E. polonica*-inoculated plants) of total absolute terpene amounts. Variation along the two main axes of variation (PC 1, PC 2; % variation explained by each axis) is shown for methyl jasmonate-treated (50 mM MeJA, blue filled triangles) and non-treated (0 mM MeJA, orange filled circles) clones of Norway spruce (*P. abies*). Mean PCA score values ± standard error for each clone (1–9) are shown, as well as the direction and distance of change (black arrows) in terpene chemistry between the two MeJA treatments. The position of terpenes indicates PCA loadings (vectors), which illustrate each compound's correlation with the two axes of variation (see **Table 3** for loading values).

MeJA-mediated inducibility differed. In line with these findings, other studies have also found that conifer clones, genotypes, families, provenances, and even different species can vary in their responses to MeJA (Zeneli et al., 2006; Heijari et al., 2008; Semiz et al., 2012; Moreira et al., 2013, 2014; López-Goldar et al., 2018). However, a recent meta-analysis (Howe et al., 2020) found that inverse relationships between constitutive and inducible levels of defense (i.e., individuals with high constitutive defense do not show large changes when induced) against bark beetle damage are often detected at the genotype/family level in common gardens, yet these relationships do not hold at the forest population level. Thus, the scale at which genetic correlations are examined is relevant for their detection and implications. If MeJA is to be implemented as a tool in plant protection, it is important that this variability among individuals or genotypes is considered and quantified. For example, screening for variation in the degree of inducibility in material from tree breeding populations or from various provenances, should be conducted.

In addition to the effects of MeJA treatment on overall resistance, its effect on terpene chemistry was also investigated. Plants treated with MeJA exhibited an increase in the amounts of terpenes such as α-pinene, β-pinene, limonene, β-phellandrene, camphene, calarene, myrcene and even (+)-3-carene. Terpene accumulation following MeJA treatment has been previously described to occur in Norway spruce (Franceschi et al., 2002; Martin et al., 2002; Erbilgin et al., 2006; Zulak et al., 2009; Zhao et al., 2010), and also in other conifer species (e.g., Hudgins et al., 2003; Erbilgin and Colgan, 2012; Pham et al., 2014; Lundborg et al., 2019; Chen R. et al., 2020). In line with our findings, Zhao et al. (2010) and Lundborg et al. (2016b) found that α-pinene and limonene respond strongly to treatment with MeJA in Norway spruce. Similarly, myrcene and β-pinene have been shown to increase after treatment with MeJA in mature Jack pine trees (*Pinus banksiana*) and Chinese white pine (*Pinus armandi*) saplings (Erbilgin and Colgan, 2012; Pham et al., 2014). We also found that MeJA treatment resulted in changes in absolute terpene amounts, but did not have a significant effect on their relative proportions (**Supplementary Table 5**). Thus, MeJA resulted in quantitative rather than qualitative changes in terpene chemistry, which is also in line with previous findings (Zhao et al., 2010).

For plants that were inoculated with *E. polonica* instead of an uninfected agar plug, we observed similar effects of MeJA treatment, yet more variation in responses among clones (**Figure 4**). Also, qualitative changes in terpene chemistry occurred (**Supplementary Table 5**). Compounds such as (+)-3-carene and geranyllinalool varied more strongly among clones (across the first axis of variation) when MeJA treatment and fungal infection co-occurred. This is consistent with previous studies which have found that (+)-3-carene is often induced following fungal infection (Croteau et al., 1987; Fäldt et al., 2006; Zhao et al., 2010), and that both compounds appear to play a role in differential susceptibility of Norway spruce to *E. polonica* and *Heterobasidion parviporum* infection (Danielsson et al., 2011; Axelsson et al., 2020). Moreover, (+)-3-carene has also been associated with conifer resistance against insects. For example, it is important for Sitka spruce (*Picea sitchensis*) resistance against the white pine weevil (*Pissodes strobi*; Robert et al., 2010), in *P. sylvestris* against the pine sawfly *Diprion pini* (Pasquier-Barre et al., 2001), and in lodgepole pine (*Pinus contorta*) against the Douglas-fir pitch moth (*Synanthedon novaroensis*; Rocchini et al., 2000). Lundborg et al. (2016b) also found that (+)-3-carene appears to be a feeding deterrent to the pine weevil (*H. abietis*), however, it seems to be induced by MeJA to a lesser extent in *P. abies* compared to *P. sylvestris* (Lundborg et al., 2016a). Overall, the changes we observed in terpene chemistry are consistent with ours and others' previous work, and corroborate that MeJA induces changes in defensive chemistry that likely influence Norway spruce resistance to insect damage and fungal infection.

Despite the significant effect of MeJA on terpene compounds, treatment explained little variation in plant defensive chemistry relative to clone (2% vs. 42%, respectively, **Table 2**). Norway spruce clones responded in a similar way to MeJA treatment (non-significant MeJA × clone interaction, **Table 2**), however, differences among clones were most important for determining terpene composition. This in contrast to the study by López-Goldar et al. (2018) where MeJA treatment explained 31% of the variation in defensive compounds, while family explained 2%, in *P. pinaster*. Yet, it is in line with other studies in which variation in defensive chemistry has been to a large extent explained by among family (or genotype/clone) differences (e.g., Havill and Raffa, 1999; Ott et al., 2011; Axelsson et al., 2020). Our results from terpene chemistry analyses are consistent with our results on insect damage and fungal lesion lengths, for which we also found that differences among clones had a strong effect on the response variables (**Table 1**). All in all, if MeJA is to be implemented as a plant protection tool, these findings reiterate firstly the importance of quantifying among-clone (or family) variation in defensive responses. And secondly, evaluating the relative importance of constitutive and MeJA-induced responses among clones, and the relationship with actual resistance (i.e., change in damage or infection levels).

Lastly, but most interestingly, we also found that damage by *H. abietis* negatively correlated with fungal infection by *E. polonica* both when treated with 0 and 50 mM MeJA. Clones that were constitutively more resistant to *H. abietis*, exhibited lower resistance against *E. polonica* and vice versa when clones were separately exposed to these two pests. Likewise, those genotypes showing a high degree of MeJA-inducibility for insect damage (greatest change in area debarked from 0 to 50 mM MeJA) showed little change in fungal infection when induced. Thus, there is a trade-off between resistance to *E. polonica* and *H. abietis*. Few studies have examined the relationship between resistance to both insect and fungal pests in conifers, and using the same clones. To our knowledge, this is the first time a trade-off between pine weevil damage and a fungal infection has been reported. Relationships among different fungal pathogens are more often studied, and in these studies resistance or susceptibility to fungal infection have been shown to correlate positively or negatively with each other (e.g., Bonello et al., 2008; Xu et al., 2018; Hurel et al., 2019). For instance, infection by *Heterobasidion annosum* in the basal stem of *Pinus pinea* reduced the concentration of total terpenoids in the shoots in response to *Diplodia pinea* inoculation relative to those found in healthy shoots (Bonello et al., 2008). Lower concentrations of terpenes were found in shoots more susceptible to *D. pinea* (i.e., those with greater lesion sizes), indicating that higher resistance to *H. annosum* came at the cost of resistance to *D. pinea* (Bonello et al., 2008). In our study, MeJA treatment increased resistance to both insect and fungal damage, thus it is less likely that a negative correlation among resistances is due to different defense pathways being involved. Also, as described above, many of the terpene compounds that reduce fungal infection have also been shown to play a role in deterring insect feeding (e.g., Zhao et al., 2010; Lundborg et al., 2016b). Note, however, that we evaluated resistance to each pest separately in clonal individuals (i.e., infection and pine weevil damage did not co-occur). It is possible that the clones examined differed in other unmeasured traits (morphological, physiological or chemical), which confer high constitutive resistance to insect or fungal damage. We are currently unable to discern underlying mechanisms behind this trade-off, but it certainly deserves further attention.

Even though pine weevil damage and infection by *E. polonica* do not co-occur naturally, a negative correlation among resistance to each of these pests could potentially constrain the availability of suitable Norway spruce genotypes for tree breeding programs. Current breeding programs in Sweden and other Nordic countries are interested in identifying clones or families with resistance to pine weevil damage, but also resistance to attack by the spruce bark beetle and infection by *E. polonica* (Steffenrem et al., 2016; Zas et al., 2017; Puentes et al., 2018; Axelsson et al., 2020). Yet, the negative correlation documented in our study does not necessarily pose a hinder for selection of such families. Clones that are constitutively more resistant to *E. polonica* should show high MeJA-induced resistance to *H. abie*tis damage and vice versa. In other words, Norway spruce clones that are constitutively more resistant to one of the pests, should show greater induced resistance against the other. One alternative could be the selection of clones/families that exhibit high MeJA-mediated resistance against pine weevil damage and are constitutively more resistant to fungal infection. Protection against pine weevil damage is necessary for only a few years after planting, since pine weevils cause high mortality only at the seedling stage. In terms of applicability, several studies have shown that treating seedlings with MeJA can be compatible with

nursery practices (Fedderwitz et al., 2019; Chen Y. et al., 2020). Infection by *E. polonica* occurs when mature trees are attacked by the spruce bark beetle, and it would be important for these trees to be constitutively more resistant to infection. Note that we have identified this negative correlation among resistances at an early plant stage, in separate clonal individuals and with a limited number of clones. This relationship could, thus, be different for mature Norway spruce trees that were damaged early in life by pine weevils and later become infected by *E. polonica*. Nonetheless, if breeding for increased resistance is of interest, this trade-off should be taken into consideration.

So far, we have discussed our results assuming that MeJA acts as an elicitor that results in defense induction, which has been supported by previous studies (e.g., Hudgins et al., 2003; Krokene et al., 2008). It is important to note that recently, MeJA was also found to be able to act as a priming stimulus and need not always result in full direct induction of defenses. Mature Norway spruce trees treated with MeJA did not show any change in terpene levels 14 days after treatment, yet they experienced increased resistance to spruce bark beetle attack relative to untreated controls, 65 days after treatment (Mageroy et al., 2020a). MeJA treatment can lead to the formation of an immunological-like memory, and a second stimulus (wounding, feeding, and infection) allows plants to recall this memory and super-induce defenses (Mageroy et al., 2020a,b). In our study, we observed both changes in terpene chemistry and enhanced resistance following MeJA treatment. Treatment occurred in April/May and plants were exposed to the insects/fungus in July, while samples for chemical analyses were taken in August. The effects of MeJA on terpenes and resistance can change with time, but can still be observed at 1 month and even 1 year after treatment (Martin et al., 2002; Miller et al., 2005; Zhao et al., 2010; Zas et al., 2014). Thus, our experimental timings are in line with when effects can be detected. However, since our study design was not intended for discerning the underlying mechanisms of MeJA treatment, we are unable to say whether priming or direct induction of defenses occurred.

MAIN CONCLUSION

We conclude that MeJA treatment can change Norway spruce terpene chemistry, and increase resistance to insect damage by *H. abietis* and fungal infection by *E. polonica*. However, for Norway spruce genotypes that are constitutively more resistant to insect damage or fungal infection, treatment with MeJA may not result in larger additional changes in resistance. Differences among clones appear to be most important for changes in defensive chemistry and degree of MeJA-mediated responses. Interestingly, we also found a trade-off between resistance to insect and fungal damage when examining estimates per clone, but this does not necessarily entail negative consequences for overall plant defense or hinder selection of the most resistant trees. Norway spruce clones that are constitutively more resistant to one of the pests, should show greater induced resistance against the other. Thus, treatment with MeJA should result in enhanced resistance to one of the pests, without compromising resistance to the other. Future studies should quantify the relative importance of constitutive vs. induced responses to various types of pests, and examine the generality of negative correlations among resistances in a larger number of clones, as well as in other conifer species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

AUTHOR CONTRIBUTIONS

A-KB-K, TZ, NB, and LL conceived and planned the experiments. NB, TZ, and LL conducted the experiments and collected the data. TZ and LL performed chemical analyses. AP conducted the statistical analyses of the data and wrote the manuscript with input from co-authors. All authors contributed to the article and approved the submitted version.

FUNDING

Financial support was provided to LL from the Swedish Foundation for Strategic Research SSF; part of the "PaReTree" project, coordinator: Stefan Jansson, PI: A-KB-K and the Royal Institute of Technology (KTH), Department of Chemistry. Support was also provided by the research council for sustainable development FORMAS to TZ (project 229-2011-890), by the Skogssällskapet foundation to NB (project 1213-111/159-9), and by the Swedish Research Council (project 2018-05389) and FORMAS (project 942-2016-37) to AP.

ACKNOWLEDGMENTS

We are grateful to Bo Karlsson at Skogforsk for providing the Norway spruce clones and Paal Krokene at the Norwegian Institute for Bioeconomy Research for supplying us with the strain of *E. polonica* and Anders Molin for help with planting the clones.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.678959/full#supplementary-material

REFERENCES

Agrawal, A. A. (1999). "Induced plant defense: evolution of induction and adaptive phenotypic plasticity," in *Inducible plant defenses against pathogens and herbivores: biochemistry, ecology, and agriculture*, eds S. Agrawal, S. Tuzun, and E. Bent (St. Paul, MN: American Phytopathological Society Press), 251–268.

Axelsson, K., Zendegi-Shiraz, A., Swedjemark, G., Borg-Karlson, A.-K., and Zhao, T. (2020). Chemical defence responses of norway spruce to two fungal pathogens. *Forest Pathol.* 50:e12640.

Beckers, G. J. M., and Spoel, S. H. (2006). Fine-tuning plant defence signalling: salicylate versus jasmonate. *Plant Biol.* 8, 1–10. doi: 10.1055/s-2005-872705

Bittner, N., Hundacker, J., Achotegui-Castells, A., Anderbrant, O., and Hilker, M. (2019). Defense of Scots pine against sawfly eggs (*Diprion pini*) is primed by exposure to sawfly sex pheromones. *Proc. Natl. Acad. Sci.* 116, 24668–24675. doi: 10.1073/pnas.1910991116

Bonello, P., Capretti, P., Luchi, N., Martini, V., and Michelozzi, M. (2008). Systemic effects of *Heterobasidion annosum ss* infection on severity of *Diplodia pinea* tip blight and terpenoid metabolism in Italian stone pine (*Pinus pinea*). *Tree physiol.* 28, 1653–1660. doi: 10.1093/treephys/28.11.1653

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., et al. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J.* 9, 378–400. doi: 10.32614/rj-2017-066

Chen, R., Huang, K., Pan, S., Xu, T., Tan, J., and Hao, D. (2020). Jasmonate induced terpene-based defense in *Pinus massoniana* depresses *Monochamus alternatus* adult feeding. *Pest. Manage. Sci.* 77, 731–740. doi: 10.1002/ps.6068

Chen, Y., Bylund, H., Björkman, C., Fedderwitz, F., and Puentes, A. (2020). Seasonal timing and recurrence of methyl jasmonate treatment influence pine weevil damage to norway spruce seedlings. *New Forests* 52, 431–448. doi: 10.1007/s11056-020-09803-4

Cipollini, D., and Heil, M. (2010). Costs and benefits of induced resistance to herbivores and pathogens in plants. *Plant Sci. Rev.* 5, 85–100.

Colgan, L. J., and Erbilgin, N. (2010). The ecological interaction of the mountain pine beetle and jack pine budworm in the boreal forest. *Forestry Chronicle* 86, 766–774. doi: 10.5558/tfc86766-6

Croteau, R., Gurkewitz, S., Johnson, M. A., and Fisk, H. J. (1987). Biochemistry of oleoresinosis: monoterpene and diterpene biosynthesis in lodgepole pine saplings infected with *Ceratocystis clavigera* or treated with carbohydrate elicitors. *Plant physiol.* 85, 1123–1128. doi: 10.1104/pp.85.4.1123

Danielsson, M., Lundén, K., Elfstrand, M., Hu, J., Zhao, T., Arnerup, J., et al. (2011). Chemical and transcriptional responses of Norway spruce genotypes with different susceptibility to *Heterobasidion* spp. infection. *BMC Plant Biol.* 11:154. doi: 10.1186/1471-2229-11-154

Endara, M. J., and Coley, P. D. (2011). The resource availability hypothesis revisited: a meta-analysis. *Funct. Ecol.* 25, 389–398. doi: 10.1111/j.1365-2435.2010.01803.x

Erb, M., Balmer, D., De Lange, E. S., Von Merey, G., Planchamp, C., Robert, C. A., et al. (2011). Synergies and trade-offs between insect and pathogen resistance in maize leaves and roots. *Plant Cell Environ.* 34, 1088–1103. doi: 10.1111/j.1365-3040.2011.02307.x

Erbilgin, N., and Colgan, L. J. (2012). Differential effects of plant ontogeny and damage type on phloem and foliage monoterpenes in jack pine (*Pinus banksiana*). *Tree Physiol.* 32, 946–957. doi: 10.1093/treephys/tps047

Erbilgin, N., Krokene, P., Christiansen, E., Zeneli, G., and Gershenzon, J. (2006). Exogenous application of methyl jasmonate elicits defenses in norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. *Oecologia* 148, 426–436. doi: 10.1007/s00442-006-0394-3

Eyles, A., Bonello, P., Ganley, R., and Mohammed, C. (2010). Induced resistance to pests and pathogens in trees. *New Phytol.* 185, 893–908. doi: 10.1111/j.1469-8137.2009.03127.x

Eyles, A., Chorbadjian, R., Wallis, C., Hansen, R., Cipollini, D., Herms, D., et al. (2007). Cross-induction of systemic induced resistance between an insect and a fungal pathogen in austrian pine over a fertility gradient. *Oecologia* 153, 365–374. doi: 10.1007/s00442-007-0741-z

Fäldt, J., Solheim, H., Långström, B., and Borg-Karlson, A.-K. (2006). Influence of fungal infection and wounding on contents and enantiomeric compositions of monoterpenes in phloem of *Pinus sylvestris*. *J. Chem. Ecol.* 32:1779. doi: 10.1007/s10886-006-9109-9

Fedderwitz, F., Björklund, N., Anngren, R., Lindström, A., and Nordlander, G. (2019). Can methyl jasmonate treatment of conifer seedlings be used as a tool to stop height growth in nursery forest trees? *New Forests* 51, 379–394. doi: 10.1007/s11056-019-09737-6

Fedderwitz, F., Nordlander, G., Ninkovic, V., and Björklund, N. (2016). Effects of jasmonate-induced resistance in conifer plants on the feeding behaviour of a bark-chewing insect, *Hylobius abietis*. *J. Pest Sci.* 89, 97–105. doi: 10.1007/s10340-015-0684-9

Fox, J., and Weisberg, S. (2019). *An R companion to applied regression*, Third Edn. Thousand Oaks, CA: Sage.

Franceschi, V. R., Krekling, T., and Christiansen, E. (2002). Application of methyl jasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. *Am. J. Bot.* 89, 578–586. doi: 10.3732/ajb.89.4.578

Franceschi, V. R., Krokene, P., Christiansen, E., and Krekling, T. (2005). Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol.* 167, 353–375. doi: 10.1111/j.1469-8137.2005.01436.x

Ganthaler, A., Stöggl, W., Kranner, I., and Mayr, S. (2017). Foliar phenolic compounds in norway spruce with varying susceptibility to *Chrysomyxa rhododendri*: analyses of seasonal and infection-induced accumulation patterns. *Front. Plant Sci.* 8:1173. doi: 10.3389/fpls.2017.01173

Gould, N., Reglinski, T., Spiers, M., and Taylor, J. T. (2008). Physiological trade-offs associated with methyl jasmonate-induced resistance in *Pinus radiata*. *Can. J. Forest Res.* 38, 677–684. doi: 10.1139/x07-193

Hahn, P. G., and Maron, J. L. (2016). A framework for predicting intraspecific variation in plant defense. *Trends Ecol. Evol.* 31, 646–656. doi: 10.1016/j.tree.2016.05.007

Hartig, F. (2020). *DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.3.1.* Available Online at: https://CRAN.R-project.org/package=DHARMa.

Havill, N. P., and Raffa, K. F. (1999). Effects of elicitation treatment and genotypic variation on induced resistance in *Populus*: impacts on gypsy moth (lepidoptera: lymantriidae) development and feeding behavior. *Oecologia* 120, 295–303. doi: 10.1007/s004420050861

Heijari, J., Nerg, A. M., Kainulainen, P., Viiri, H., Vuorinen, M., and Holopainen, J. K. (2005). Application of methyl jasmonate reduces growth but increases chemical defence and resistance against *Hylobius abietis* in scots pine seedlings. *Entomol. Exper. Appl.* 115, 117–124. doi: 10.1111/j.1570-7458.2005.00263.x

Heijari, J., Nerg, A. M., Kainulainen, P., Vuorinen, M., and Holopainen, J. K. (2008). Long-term effects of exogenous methyl jasmonate application on scots pine (*Pinus sylvestris*) needle chemical defence and diprionid sawfly performance. *Entomol. Exper. Appl.* 128, 162–171. doi: 10.1111/j.1570-7458.2008.00708.x

Hlásny, T., Krokene, P., Liebhold, A., Montagné-Huck, C., Müller, J., Qin, H., et al. (2019). *Living with bark beetles: impacts, outlook and management options*. Joensuu, Finland: European Forest Institute.

Holopainen, J. K., Heijari, J., Nerg, A. M., Vuorinen, M., and Kainulainen, P. (2009). Potential for the use of exogenous chemical elicitors in disease and insect pest management of conifer seedling production. *Open Forest Sci. J.* 2, 17–24. doi: 10.2174/1874398600902010017

Howe, M., Mason, C. J., Gratton, C., Keefover-Ring, K., Wallin, K., Yanchuk, A., et al. (2020). Relationships between conifer constitutive and inducible defenses against bark beetles change across levels of biological and ecological scale. *Oikos* 129, 1093–1107. doi: 10.1111/oik.07242

Hudgins, J. W., Christiansen, E., and Franceschi, V. R. (2003). Methyl jasmonate induces changes mimicking anatomical defenses in diverse members of the pinaceae. *Tree Physiol.* 23, 361–371. doi: 10.1093/treephys/23.6.361

Hurel, A., de Miguel, M., Dutech, C., Desprez-Loustau, M. L., Plomion, C., Rodríguez-Quilón, I., et al. (2019). Genetic basis of susceptibility to *Diplodia sapinea* and *Armillaria ostoyae* in maritime pine. *bioRxiv* 2019:699389.

Karban, R., and Myers, J. H. (1989). Induced plant responses to herbivory. *Ann. Rev. Ecol. Syst.* 20, 331–348. doi: 10.1146/annurev.es.20.110189.001555

Kempel, A., Schädler, M., Chrobock, T., Fischer, M., and van Kleunen, M. (2011). Tradeoffs associated with constitutive and induced plant resistance against herbivory. *Proc. Natl. Acad. Sci.* 108, 5685–5689. doi: 10.1073/pnas.1016508108

Kolosova, N., and Bohlmann, J. (2012). "Conifer defense against insects and fungal pathogens," in *Growth and defence in plants*, eds R. Matyssek, H. Schnyder, O. Wolfgang, D. Ernst, and J. C. Munch (Berlin: Springer), 85–109. doi: 10.1007/978-3-642-30645-7_4

Koricheva, J., Nykänen, H., and Gianoli, E. (2004). Meta-analysis of trade-offs among plant antiherbivore defenses: are plants jacks-of-all-trades, masters of all? *Am. Nat.* 163, E64–E75.

Kozlowski, G., Buchala, A., and Métraux, J. P. (1999). Methyl jasmonate protects norway spruce [*Picea abies* (L.) Karst.] seedlings against *Pythium ultimum* trow. *Physiol. Mol. Plant Pathol.* 55, 53–58. doi: 10.1006/pmpp.1999.0205

Krokene, P. (2015). "Conifer defense and resistance to bark beetles," in *Bark beetles: biology and ecology of native and invasive species*, eds F. E. Vega and R. W. Hofstetter (Cambridge,MA: Academic Press), 177–207. doi: 10.1016/b978-0-12-417156-5.00005-8

Krokene, P., Nagy, N. E., and Solheim, H. (2008). Methyl jasmonate and oxalic acid treatment of norway spruce: anatomically based defense responses and increased resistance against fungal infection. *Tree Physiol.* 28, 29–35. doi: 10.1093/treephys/28.1.29

Krokene, P., and Solheim, H. (1996). Fungal associates of five bark beetle species colonizing norway spruce. *Can. J. Forest Res.* 26, 2115–2122. doi: 10.1139/x26-240

Krutzsch, P. (1973). *Norway spruce development of buds. Internal report*. Vienna, Austria: IUFRO.

Långström, B., and Day, K. R. (2004). "Damage, control and management of weevil pests, especially *Hylobius abietis*," in *Bark and wood boring insects in living trees in Europe: a synthesis*, eds F. Lieutier, K. R. Day, A. Battisti, J. C. Grégoire, and H. F. Evans (Dordrecht, Netherlands: Kluwer Academic Publishers), 415–444. doi: 10.1007/1-4020-2241-7_19

Lenth, R., Singmann, H., Love, J., Buerkner, P., and Hervé, M. (2020). *Estimated Marginal Means, aka Least-Squares Means*. Available online at: https://cran.rproject.org/web/packages/emmeans/emmeans.pdf.

López-Goldar, X., Villari, C., Bonello, P., Borg-Karlson, A.-K., Grivet, D., Zas, R., et al. (2018). Inducibility of plant secondary metabolites in the stem predicts genetic variation in resistance against a key insect herbivore in maritime pine. *Front. Plant Sci.* 9:1651. doi: 10.3389/fpls.2018.01651

López-Villamor, A., Zas, R., Pérez, A., Cáceres, Y., Nunes Da Silva, M., Vasconcelos, M., et al. (2020). Traumatic resin ducts induced by methyl jasmonate in *Pinus* spp. *Trees* 2021:35. doi: 10.1007/s00468-020-02057-9

Lundborg, L., Fedderwitz, F., Björklund, N., Nordlander, G., and Borg-Karlson, A. K. (2016a). Induced defenses change the chemical composition of pine seedlings and influence meal properties of the pine weevil *Hylobius abietis*. *Phytochemistry* 130, 99–105. doi: 10.1016/j.phytochem.2016.06.002

Lundborg, L., Nordlander, G., Björklund, N., Nordenhem, H., and Borg-Karlson, A. K. (2016b). Methyl jasmonate-induced monoterpenes in scots pine and norway spruce tissues affect pine weevil orientation. *J. Chem. Ecol.* 42, 1237–1246. doi: 10.1007/s10886-016-0790-z

Lundborg, L., Sampedro, L., Borg-Karlson, A.-K., and Zas, R. (2019). Effects of methyl jasmonate on the concentration of volatile terpenes in tissues of maritime pine and monterey pine and its relation to pine weevil feeding. *Trees* 33, 53–62. doi: 10.1007/s00468-018-1757-1

Mageroy, M. H., Christiansen, E., Långström, B., Borg-Karlson, A.-K., Solheim, H., Björklund, N., et al. (2020a). Priming of inducible defenses protects norway spruce against tree-killing bark beetles. *Plant Cell Environ.* 43, 420–430. doi: 10.1111/pce.13661

Mageroy, M. H., Wilkinson, S. W., Tengs, T., Cross, H., Almvik, M., Pétriacq, P., et al. (2020b). Molecular underpinnings of methyl jasmonate-induced resistance in norway spruce. *Plant Cell Environ.* 43, 1827–1843. doi: 10.1111/pce.13774

Martin, D., Tholl, D., Gershenzon, J., and Bohlmann, J. (2002). Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of norway spruce stems. *Plant physiol.* 129, 1003–1018. doi: 10.1104/pp.011001

Miller, B., Madilao, L. L., Ralph, S., and Bohlmann, J. (2005). Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiol.* 137, 369–382. doi: 10.1104/pp.104.050187

Moreira, X., Mooney, K. A., Rasmann, S., Petry, W. K., Carrillo-Gavilán, A., Zas, R., et al. (2014). Trade-offs between constitutive and induced defences drive geographical and climatic clines in pine chemical defences. *Ecol. Lett.* 17, 537–546. doi: 10.1111/ele.12253

Moreira, X., Sampedro, L., and Zas, R. (2009). Defensive responses of *Pinus pinaster* seedlings to exogenous application of methyl jasmonate: concentration effect and systemic response. *Environ. Exper. Bot.* 67, 94–100. doi: 10.1016/j.envexpbot.2009.05.015

Moreira, X., Sampedro, L., Zas, R., and Pearse, I. S. (2016). Defensive traits in young pine trees cluster into two divergent syndromes related to early growth rate. *PLoS One* 11:e0152537. doi: 10.1371/journal.pone.0152537

Moreira, X., Zas, R., and Sampedro, L. (2013). Additive genetic variation in resistance traits of an exotic pine species: little evidence for constraints on evolution of resistance against native herbivores. *Heredity* 110, 449–456. doi: 10.1038/hdy.2012.108

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *Vegan: community ecology package. R package version 2.5-6*. Available Online at: https://CRAN.R-project.org/package=vegan.

Ott, D. S., Yanchuk, A. D., Huber, D. P., and Wallin, K. F. (2011). Genetic variation of lodgepole pine, *Pinus contorta* var. *latifolia*, chemical and physical defenses that affect mountain pine beetle, *Dendroctonus ponderosae*, attack and tree mortality. *J. Chem. Ecol.* 37:1002. doi: 10.1007/s10886-011-0003-8

Pasquier-Barre, F., Palasse, C., Goussard, F., Auger-Rozenberg, M.-A., and Géri, C. (2001). Relationship of scots pine clone characteristics and water stress to hatching and larval performance of the sawfly *Diprion pini* (hymenoptera: diprionidae). *Environ. Entomol.* 30, 1–6. doi: 10.1603/0046-225x-30.1.1

Persson, M., Borg-Karlson, A.-K., and Norin, T. (1993). Enantiomeric composition of six chiral monoterpene hydrocarbons in different tissues of *Picea abies*. *Phytochemistry* 33, 303–307. doi: 10.1016/0031-9422(93)85508-o

Pham, T., Chen, H., Zhang, R., Dai, L., and Vu, T. (2014). Changes of monoterpenes in stem of chinese white pine (*Pinus armandi*) saplings following treatment with methyl jasmonate. *Forestry Stud.* 60, 69–81. doi: 10.2478/fsmu-2014-0006

Pimentel, C. S., Gonçalves, E. V., Firmino, P. N., Calvão, T., Fonseca, L., Abrantes, I., et al. (2017). Differences in constitutive and inducible defences in pine species determining susceptibility to pinewood nematode. *Plant Pathol.* 66, 131–139. doi: 10.1111/ppa.12548

Puentes, A., Högberg, K.-A., Björklund, N., and Nordlander, G. (2018). Novel avenues for plant protection: plant propagation by somatic embryogenesis enhances resistance to insect feeding. *Front. Plant Sci.* 9:1553. doi: 10.3389/fpls.2018.01553

R Core Team. (2020). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Raffa, K. F., Bonello, P., and Orrock, J. L. (2020). Why do entomologists and plant pathologists approach trophic relationships so differently? identifying biological distinctions to foster synthesis. *New Phytol.* 225, 609–620. doi: 10.1111/nph.16181

Raffa, K. F., Mason, C. J., Bonello, P., Cook, S., Erbilgin, N., Keefover-Ring, K., et al. (2017). Defence syndromes in lodgepole – whitebark pine ecosystems relate to degree of historical exposure to mountain pine beetles. *Plant Cell Environ.* 40, 1791–1806. doi: 10.1111/pce.12985

Rasmann, S., Erwin, A. C., Halitschke, R., and Agrawal, A. A. (2011). Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *J. Ecol.* 99, 16–25. doi: 10.1111/j.1365-2745.2010.01713.x

Reglinski, T., Taylor, J. T., Northcott, G. L., Ah Chee, A., Spiers, M., and Wohlers, M. (2019). Growth environment and seedling age affect constitutive and inducible defence in radiata pine. *Plant Pathol.* 68, 1481–1492. doi: 10.1111/ppa.13068

Robert, J. A., Madilao, L. L., White, R., Yanchuk, A., King, J., and Bohlmann, J. (2010). Terpenoid metabolite profiling in Sitka spruce identifies association of dehydroabietic acid, (+)-3-carene, and terpinolene with resistance against white pine weevil. *Botany* 88, 810–820. doi: 10.1139/b10-049

Rocchini, L. A., Lindgren, B. S., and Bennett, R. G. (2000). Effects of resin flow and monoterpene composition on susceptibility of lodgepole pine to attack by the douglas-fir pitch moth, *Synanthedon novaroensis* (Lep., Sesiidae). *J. Appl. Entomol.* 124, 87–92. doi: 10.1046/j.1439-0418.2000.00449.x

Rostás, M., Simon, M., and Hilker, M. (2003). Ecological cross-effects of induced plant responses towards herbivores and phytopathogenic fungi. *Basic Appl. Ecol.* 4, 43–62. doi: 10.1078/1439-1791-00132

RStudio Team. (2020). *RStudio: Integrated Development for R*. Boston, MA: RStudio.

Sampedro, L., Moreira, X., and Zas, R. (2011). Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. *J. Ecol.* 99, 818–827. doi: 10.1111/j.1365-2745.2011.01814.x

Schiebe, C., Hammerbacher, A., Birgersson, G., Witzell, J., Brodelius, P. E., Gershenzon, J., et al. (2012). Inducibility of chemical defenses in norway spruce bark is correlated with unsuccessful mass attacks by the spruce bark beetle. *Oecologia* 170, 183–198. doi: 10.1007/s00442-012-2298-8

Semiz, G., Blande, J. D., Heijari, J., Işık, K., Niinemets, Ü, and Holopainen, J. K. (2012). Manipulation of VOC emissions with methyl jasmonate and carrageenan in the evergreen conifer *Pinus sylvestris* and evergreen broadleaf *Quercus ilex*. *Plant Biol.* 14, 57–65. doi: 10.1111/j.1438-8677.2011.00485.x

Shikano, I., Shumaker, K. L., Peiffer, M., Felton, G. W., and Hoover, K. (2017). Plant-mediated effects on an insect–pathogen interaction vary with intraspecific genetic variation in plant defences. *Oecologia* 183, 1121–1134. doi: 10.1007/s00442-017-3826-3

Steffenrem, A., Solheim, H., and Skrøppa, T. (2016). Genetic parameters for wood quality traits and resistance to the pathogens *Heterobasidion parviporum* and *Endoconidiophora polonica* in a Norway spruce breeding population. *Eur. J. Forest Res.* 135, 815–825. doi: 10.1007/s10342-016-0975-6

Telford, A., Cavers, S., Ennos, R. A., and Cottrell, J. E. (2015). Can we protect forests by harnessing variation in resistance to pests and pathogens? *Forestry Int. J. Forest Res.* 88, 3–12. doi: 10.1093/forestry/cpu012

Villari, C., Faccoli, M., Battisti, A., Bonello, P., and Marini, L. (2014). Testing phenotypic trade-offs in the chemical defence strategy of scots pine under growth-limiting field conditions. *Tree Physiol.* 34, 919–930. doi: 10.1093/treephys/tpu063

Whitehill, J. G., Yuen, M. M., Henderson, H., Madilao, L., Kshatriya, K., Bryan, J., et al. (2019). Functions of stone cells and oleoresin terpenes in the conifer defense syndrome. *New Phytol.* 221, 1503–1517. doi: 10.1111/nph.15477

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Xu, J., Budde, K. B., Hansen, O. K., Thomsen, I. M., Ravn, H. P., and Nielsen, U. B. (2018). Do silver fir woolly adelgids (*Dreyfusia nordmannianae*) facilitate pathogen infestation with *Neonectria neomacrospora* on christmas trees (*Abies nordmanniana*)? *Forest Ecol. Manage.* 424, 396–405. doi: 10.1016/j.foreco.2018.05.006

Zas, R., Björklund, N., Nordlander, G., Cendán, C., Hellqvist, C., and Sampedro, L. (2014). Exploiting jasmonate-induced responses for field protection of conifer seedlings against a major forest pest, *Hylobius abietis*. *Forest Ecol. Manage.* 313, 212–223. doi: 10.1016/j.foreco.2013.11.014

Zas, R., Björklund, N., Sampedro, L., Hellqvist, C., Karlsson, B., Jansson, S., et al. (2017). Genetic variation in resistance of norway spruce seedlings to damage by the pine weevil *Hylobius abietis*. *Tree Genet. Genom.* 13: 111.

Zeneli, G., Krokene, P., Christiansen, E., Krekling, T., and Gershenzon, J. (2006). Methyl jasmonate treatment of mature norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. *Tree Physiol.* 26, 977–988. doi: 10.1093/treephys/26.8.977

Zhao, T., Borg-Karlson, A. K., Erbilgin, N., and Krokene, P. (2011). Host resistance elicited by methyl jasmonate reduces emission of aggregation pheromones by the spruce bark beetle, *Ips typographus*. *Oecologia* 167, 691–699. doi: 10.1007/s00442-011-2017-x

Zhao, T., Krokene, P., Björklund, N., Långström, B., Solheim, H., Christiansen, E., et al. (2010). The influence of *Ceratocystis polonica* inoculation and methyl jasmonate application on terpene chemistry of norway spruce, *Picea abies*. *Phytochemistry* 71, 1332–1341. doi: 10.1016/j.phytochem.2010.05.017

Zulak, K. G., Lippert, D. N., Kuzyk, M. A., Domanski, D., Chou, T., Borchers, C. H., et al. (2009). Targeted proteomics using selected reaction monitoring reveals the induction of specific terpene synthases in a multi-level study of methyl jasmonate-treated norway spruce (*Picea abies*). *Plant J.* 60, 1015–1030. doi: 10.1111/j.1365-313x.2009.04020.x

Conflict of Interest: 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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 01 July 2021
doi: 10.3389/fpls.2021.695908

Elicitor Application in Strawberry Results in Long-Term Increase of Plant Resilience Without Yield Loss

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For a first step integrating elicitor applications into the current IPM strategy increasing plant resilience against pests, we investigated repeated elicitor treatments in a strawberry everbearer nursery and cropping cycle under glass. During nursery methyl-jasmonate (MeJA), testing induction of defenses with plant bioassays was applied every 3 weeks. Thrips damage and reproduction by spider mites, whitefly and aphids were strongly reduced upon elicitor treatment. Subsequently, we applied MeJA every 3 weeks or based on scouting pests during a whole cropping cycle. Thrips leaf bioassays and LC-MS leaf metabolomics were applied to investigate the induction of defenses. Leaf damage by thrips was lower for both MeJA application schemes compared to the control except for the last weeks. While elicitor treatments after scouting also reduced damage, its effect did not last. Thrips damage decreased from vegetative to mature plants during the cropping cycle. At the end of the nursery phase, plants in the elicitor treatment were smaller. Surprisingly, growth during production was not affected by MeJA application, as were fruit yield and quality. LC-MS leaf metabolomics showed strong induction of vegetative plants decreasing during the maturation of plants toward the end of cultivation. Concurrently, no increase in the JA-inducible marker PPO was observed when measured toward the end of cultivation. Mostly flavonoid and phenolic glycosides known as plant defense compounds were induced upon MeJA application. While induced defense decreased with the maturation of plants, constitutive defense increased as measured in the leaf metabolome of control plants. Our data propose that young, relatively small plant stages lack constitutive defense necessitating an active JA defense response. As plants, mature constitutive defense metabolites seem to accumulate, providing a higher level of basal resistance. Our results have important implications for but are not limited to strawberry cultivation. We demonstrated that repeated elicitor application could be deployed as part of an integrated approach for sustainable crop protection by vertical integration with other management tactics and horizontal integration to control multiple pests concurrently. This approach forms a promising potential for long-term crop protection in greenhouses.

Keywords: induced defense, metabolomics, thrips, methyl-jasmonate, greenhouse horticulture, strawberry cultivation, everbearer

OPEN ACCESS

Edited by:
Islam S. Sobhy,
Keele University, United Kingdom

Reviewed by:
Maria L. Pappas,
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *15 April 2021*
Accepted: *07 June 2021*
Published: *01 July 2021*

Citation:
Mouden S, Bac-Molenaar JA, Kappers IF, Beerling EAM and Leiss KA (2021) Elicitor Application in Strawberry Results in Long-Term Increase of Plant Resilience Without Yield Loss. Front. Plant Sci. 12:695908. doi: 10.3389/fpls.2021.695908

INTRODUCTION

Strawberry production has globally increased over the last 10 years, both in acreage and yield per hectare [Mezzetti et al., 2018; FAOstat (http://www.fao.org/faostat/en/#search/strawberries)]. However, cultivation is under pressure in nurseries and fruit production due to stricter regulations increasingly restraining pesticide use, demanding new ways of pest and disease control. Integrated pest management (IPM) using different pest management approaches compatible with each other is adamant. A promising way in this regard is developing resistant cultivars (Mangandi et al., 2017). However, this remains a challenge in a polyploid crop like strawberry and requires a substantial time investment of at least several years (Mezzetti et al., 2018). Instead, using the natural potential of plants to defend themselves appears a promising way forward. By inducing their defense, plants become more resilient, making it more difficult for pests and diseases to establish and develop in the crop.

In Northern Europe, strawberries are increasingly cultivated under glass, allowing year-round, high-quality fruits (Mezzetti et al., 2018). Besides, the production of strawberries in tabletops protected with rain covers is quite popular. Strawberry nurseries are mostly outside still, but the most innovative frontrunner-growers are starting to experiment with protected plant nurseries under glass. Protected cultivation indoors or on covered tabletops is greatly advantageous for pest control. While keeping out pests and diseases allows for the introduction of natural enemies, both preventive and curative. The use of biological control in greenhouses is quite successful in practice but still has some challenges, particularly in the cultivation of strawberries requiring a relatively low-temperature regime compared to other greenhouse crops. This, together with the low amount of natural light in winter, hampers the establishment of the population and the development of most natural enemies (Sampson and Kirk, 2016; Clymans et al., 2017; Vervoort et al., 2017; Sampson, 2018). On the other hand, relatively high temperatures in summer allow for the rapid development of pest populations in the outdoor tabletops so that natural enemies cannot always keep up. This is especially true for strawberry summer-bearers with a relatively short cropping cycle. Induction of plant defenses may overcome these problems supporting and maintaining an equilibrium between pests and natural enemies (Pappas et al., 2017).

The induction of plant defense is regulated through plant hormones (Pieterse et al., 2012). Jasmonic acid and ethylene are generally associated with activation of defenses against pests, necrotrophic pathogens, and nematodes (Turner et al., 2002; War et al., 2012; Okada et al., 2015), while salicylic acid is associated with the activation of defenses against biotrophic pathogens (Palmer et al., 2017). Jasmonic acid is an important regulator of plant defenses. However, an important hurdle is the growth–defense trade-off (Koo, 2018). Increased plant defense usually comes at a fitness cost to growth. Nevertheless, in a recent meta-analysis of different studies, it appeared that induction through herbivore infestation reduced plant growth, photosynthesis, and reproduction upon feeding various insect guilds, but this depended on the plant development stage (Garcia et al., 2021). Negative effects on plant growth and development were only reported in the vegetative plant stage compared with the reproductive one.

Exogenous application of the active form of jasmonic acid, methyl jasmonate (MeJA), is known to induce plant defenses, resulting in increased plant resilience against herbivorous insect pests (Yu et al., 2018). In strawberry fruits, such application, at pre- and postharvest, resulted in accelerated fruit ripening as well as changes in levels of primary metabolites such as sugars and secondary metabolites such as anthocyanins and polyphenols, leading to improved fruit quality and shelf life (Giné-Bordonaba and Terry, 2016; Saavedra et al., 2016; Han et al., 2019; Zuñiga et al., 2020). Strawberry fruits infected with *Botrytis cinerea* experienced an up-regulation of marker genes associated with the induction of defenses (Valenzuela-Riffo et al., 2020). Less is known about the metabolome of strawberry leaves. So far, only total phenol content, antioxidant capacity, and organic acids have been described for some cultivars (Giné-Bordonaba and Terry, 2016). Concerning plant defense, strawberry leaf phenols were associated with lower feeding rates of spider mites (Luczynski et al., 1990; Wang and Lin, 2000), whereby elevated levels of phenols were reported to be observed upon feeding of this pest on strawberry leaves (Golan et al., 2017). Induction of defenses holds great potential as part of integrated pest management (IPM) strategy by enhancing the natural ability of a plant to defend itself against pests and diseases, i.e., plant resilience. However, little is known about the practicality of modulating plant resilience by elicitors during the whole strawberry cultivation cycle.

Therefore, this study investigated the induction of plant defenses, using the elicitor MeJA, against pests during the entire cropping cycle of greenhouse cultivated strawberries in a practice-like setting. We primarily focused on western flower thrips (WFT, *Frankliniella occidentalis*), one of the major pests in strawberry cultivation worldwide (Rahman et al., 2010; Sampson and Kirk, 2013; Strzyzewski et al., 2021). Moreover, we evaluated the potential of MeJA to induce plant defenses against spider mite as another cell-feeder and against phloem-feeding whiteflies and aphids. In general, direct thrips damage on leaves can potentially cause loss of photosynthetic capacity and crop yield in heavy infestations and indirect damage of virus transmission (Reitz et al., 2020). Specifically, in strawberries, thrips cause leaf and flower damage and bronzing and russeting of strawberry fruits leading to diminished shelf-life and fruit appearance (Rahman et al., 2010; Sampson and Kirk, 2013). Furthermore, the potential to modulate plant defenses by inducing host plant resistance to thrips has been stressed by Mouden and Leiss (2021). Therefore, to avoid thrips damage, it is important to inhibit thrips population build-up early in the vegetative leaf stages. We, therefore, concentrated on induced leaf defense, particularly looking into the following questions:

1. Does the exogenous application of MeJA induce plant leaf defense in both vegetative plants during nursery and mature plants during the production phase?

2. If so, is the application of MeJA concerning increased pest risk, as recorded by regular scouting, sufficient to decrease thrips damage?
3. Does induced leaf defense cause trade-offs such as reduced plant biomass, diminished fruit yield, and quality?
4. Which leaf metabolites are related to induction of defense by MeJA?
5. Which leaf metabolites are related to constitutive defense?

MATERIALS AND METHODS

Effect of MeJA Application on Plant Resilience in the Plant Nursery, Greenhouse Experiment 2019

Cultivation

Strawberry seeds from the cultivars Delizzimo, Rowena, and Elan (ABZ Seeds, Andijk, The Netherlands) were germinated and grown in rockwool plugs at Beekenkamp Plants (Maasdijk, The Netherlands) until the two-leaf stage. Then, plugs were transferred to a greenhouse compartment at the experimental site of Wageningen University in Bleiswijk on the 29th of March. Upon arrival, plugs were transferred into nutrient solution saturated rockwool blocks (4 × 4 × 4 cm). When blocks reached 50% of the fully saturated weight, blocks were saturated again with nutrient solution applied from the bottom (eb-flood system).

One week after the experiment, plants were sprayed preventatively against mildew with Serenade [*Bacillus amyloliquefaciens* (str. QST 713)].

The climate conditions, such as temperature and relative humidity, realized during nursery are described in **Supplementary Data 1**.

Plant Resilience Treatments and Measurement of Plant Development and Growth

Plants were randomly assigned to three blocks. Per cultivar, blocks contained an equal number of plants. For each cultivar in each block, half of the plants were treated with MeJA (Sigma-Aldrich; 1 mM in 1% EtOH), the other half, as control, was mock-treated with 1% EtOH without MeJA Solutions were sprayed onto the plants until small droplets were visible on all leaves. Spraying was applied in the 1st, 2nd, and 3rd weeks of the experiment. In the 4th week, part of the plants was used to determine growth parameters, like length of the longest leaf stem, fresh and dry weight of the canopy. Another part was used to perform leaf bioassays with thrips (*Frankliniella occidentalis*), and the remaining plants were transferred to cages to perform whole-plant bioassays with thrips, spider mites (*Tetranychus urticae*), greenhouse whiteflies (*Trialeurodes vaporariorum*), or Buckthorn aphids (*Aphis nasturtii*). Each cage contained eight MeJA-treated and eight control plants of the same cultivars randomly placed.

Bioassays

Thrips, spider mites, and whiteflies were taken from our standard rearing on chrysanthemums, cucumber, and tomatoes. Rearing has occurred in small greenhouse compartments. Additional lighting (son-T) was given on dark days, but the day length was not extended. Relative humidity was set at 70%. Compartments were heated when the temperature dropped below 18°C. Compartments did not have windows, so cooling was not possible. Rearing of aphids occurred on strawberry plants in cages placed in the compartment used for the strawberry nursery (climate conditions in **Supplementary Data 1**). For each rearing, host plants were refreshed regularly.

The whole plant bioassay with thrips was conducted with 10 adult thrips per plant released into each cage. After 1 week, thrips damage on the leaves was visually evaluated using a score from 0 to 5; with 0: no thrips damage, 1: small spot on one leaf, 2; several spots on one leaf, 3 several spots on several leaves, 4 large spots, several leaves; 5 severe damage, all leaves with thrips damage (Bac-Molenaar et al., 2019).

The whole plant bioassay with spider mites was performed, attaching a piece of cucumber leaf with seven adult female spider mites to the largest leaf of each strawberry plant. After 1 week, the number of eggs was counted on all leaves of the plant using a binocular microscope.

The whole plant bioassay with whiteflies was executed by releasing five adults per plant into the cage. No sex was determined for these individuals, but in our rearing, most whiteflies are female. After 3 weeks, the number of eggs and number of larvae/nymphs (all stages together) was determined per plant.

The whole plant bioassay with aphids was conducted, attaching a piece of a leaf containing 10 aphids (mix of life stages) to the largest leaf of each strawberry plant. After 2 weeks number of individuals (all life stages) was counted on each plant. The establishment of aphids was only successful on the cultivar Delizzimo.

For the detached thrips leaf bioassay, the protocol was adapted from the dual-choice assay (Leiss et al., 2009a). A leaflet of a fully developed leaf from the middle of the strawberry foliage was placed on 1% water-agar in a pot with its abaxial surface up. After adding 10 adult thrips, the pot was closed with a lid partly containing a fine-mesh to prevent condensation. Thrips feeding damage was visually assessed in mm^2. Per treatment, 10 replicates were used.

Repeated Application of MeJA During Cropping Cycle—Greenhouse Experiment 2019–2020

Cultivation

Elicitor treatments to increase plant resilience of the everbearer strawberry cultivar Delizzimo, were evaluated during a whole cropping cycle (**Figure 1**) in 2019–2020. Although the dimensions of our trial compartment were relatively small in comparison to commercial greenhouses in the Netherlands, we aimed at achieving conditions as close as possible to real practice. Strawberry seeds were germinated on the 11th of November 2019 (week 46) as described above, transferred and transplanted in 5 × 5 × 5 cm rockwool blocks in week 51 with a density of 100 plants per m^2. On the 30th of January 2020 (week 5), plants were transplanted in larger rockwool blocks (10 × 10 × 10 cm) with a density of 25 plants per m^2. Until week nine, runners

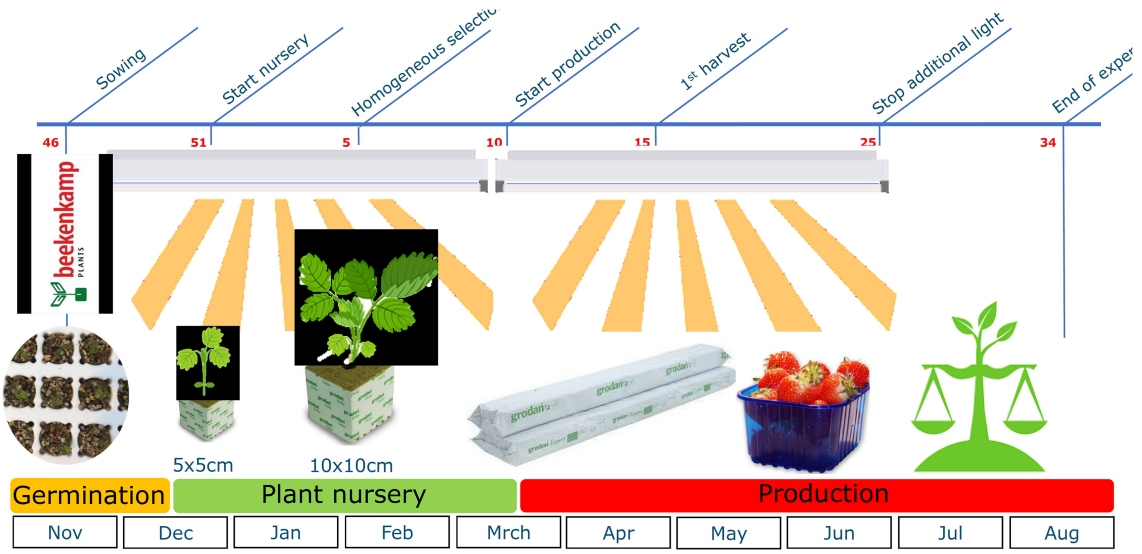

FIGURE 1 | Strawberry cropping cycle 2019–2020.

and trusses were removed regularly. On the 3rd of March 2020 (week 10), selected plants in comparable developmental stages were transferred to the production greenhouse. These five plants per running meter were placed, with a distance between two gutters of 1.20 m, resulting in a plant density of 4.5 plants per m^2. Additional light (120 $\mu mol/m^2/s$, Son-T) was provided until week 25 to obtain a day-length of 16 h and reduce differences in perceived light between the left and right sides of the compartment due to shadowing from the neighboring compartments. The climate conditions, such as temperature and relative humidity, realized during the cropping cycle are described in **Supplementary Data 1**.

A standing army of preventatively introduced natural enemies was chosen as a basis pest management strategy. Plants were scouted weekly to optimize pest management. A list of natural enemies that were released in the greenhouse is presented in **Supplementary Data 2**. In addition, we had to use Xentari (active compound, *Bacillus thuringiensis,* Bayer, Germany) once against caterpillars of the Turkish moth (*Chrysodeixis chalcites*). Also, diseases were scouted weekly. We applied several products to control powdery mildew. A list of products applied is provided in **Supplementary Data 2**.

Measuring Plant Development and Growth

In week 10, at the end of the nursery phase, 10 plants per block were sampled for destructive measurements. We determined the number of leaves for each plant, the number of trusses, length of the longest leaf stem, total leaf area using a leaf area meter, and plant dry weight after 36 h of drying at 50°C. The root index was established as a score of the amount of roots at the bottom of the rockwool on a scale from 0 to 5.

Light interception, as an approximation of photosynthesis and plant growth during the cultivation cycle, was measured three times each in weeks 18, 25, and 29. These measurements were performed on cloudy days, ensuring small variation in light intensity over time.

Measuring Fruit Production, Fruit Shelf Life, and Fruit Taste

The first fruits were harvested on the 6th of April (week 15). From then on, fruits were harvested twice a week until the 19th of August (week 34). Each harvested fruit was classified according to quality: Class I large: diameter >27 mm, good shape, no fungi. Class I small diameter <27 mm, good shape, no fungi. All other fruits which did not have any fungi and were of reasonable size were categorized as Class II. Fruits with fungi were discarded. In weeks 22, 27, 30, and 33, fruit shelf life was determined by storing Class I fruits for 4 days of 4°C and an additional day at room temperature. One box of fruits was stored for each block, resulting in 8 boxes per treatment. After storage, fruit quality was determined by scoring fruit damage and fruit rot in each box. In weeks 21, 24, 29, and 31 taste of the strawberries was determined as the two most important components for sweetness: soluble solids concentration (°Brix) and acid contents (titratable acidity) in the juice of strawberry fruits. In week 21, fruits of all blocks of the same treatment were pooled. In the other weeks, two pools were made for each treatment, each containing fruits from different blocks per treatment. A hand refractometer was used to determine the total soluble solids in each sample of blended pulp and is reported as the mean value of triplicate analyses. To determine the total titratable acidity levels, the potentiometric endpoint titration was applied. The blended pulp (5 g fresh weight) and distilled water (50 mL) were added to titrate with aqueous NaOH (0.1 M) to obtain pH 8.2. Total acid content was determined in mmol H_3O^+/100 g fresh weight.

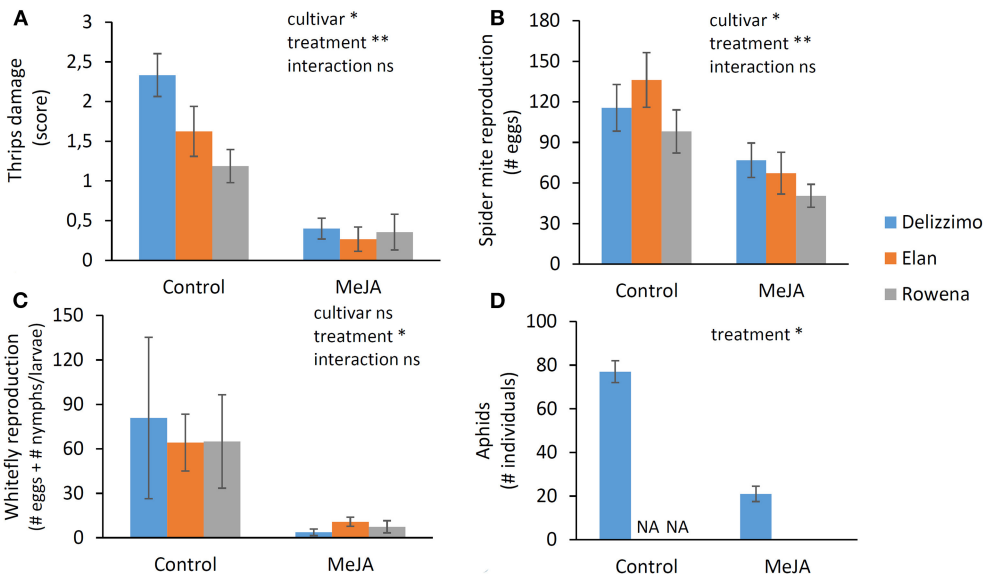

FIGURE 2 | Effect of MeJA treatment on plant resilience against the four major pests during strawberry plant nursery as measured in whole-plant bioassays. **(A)** Thrips (*Frankliniella occidentalis*) leaf damage, scale score: 0 (*no damage*) to 5 (*severe damage*), **(B)** spider mite (*Tetranychus urticae*) reproduction (number of eggs per plant), **(C)** greenhouse whitefly (*Trialeurodes vaporariorum*) reproduction (number of eggs and number of larvae/nymphs per plant), **(D)** Buckthorn aphids (*Aphis nasturtii*) reproduction (number of individuals (all stages) per plant). Data present mean ± SEM ($n = 8$). Significant differences are indicated as $^{*}p \leq 0.01$, $^{**}p \leq 0.001$, based on GLM using a Wald chi-squared test.

Elicitor Treatments Inducing Plant Resilience

A completely random block design was used to study the effect of MeJA on plant resilience. Plant nursery was carried out on six tables. Each table was divided into three blocks, labeled "Control," "Scouting," and "MeJA." In the production phase, each gutter (eight in total) was divided into three blocks, which were treated accordingly. Plants in each block were sprayed every 3 weeks (starting week 51 of 2019) as first observations pointed out that the effect of MeJA lasted about 3 weeks. "MeJA"-blocks were sprayed with 1 mM MeJA dissolved in 1% EtOH whereas plants in "Control"-blocks were sprayed with the corresponding mock solution of 1% EtOH. Blocks labeled as "Scouting" were sprayed with 1 mM MeJA upon scouting when pest pressure was increasing and not yet in balance with its natural enemies (**Supplementary Data 1**). This occurred in weeks 2, 5, 23, and 26. In the remaining weeks, plants in the "Scouting"-blocks were sprayed with the mock solution.

Bioassays

Starting in week 3, 1 week after each spraying, we performed a detached leaf bio-assay with thrips in the way described above with slight modifications: The leaflet was rinsed in MilliQ water and, upon drying, carefully checked for the presence of natural enemies and eggs of *Amblyseius limonicus* and *cucumeris* to avoid thrips predation. Subsequently, each leaflet was embedded in 1% water agar and infested with five adult thrips. For each treatment, 10 replicates were used. Thrips feeding damage was visually assessed in mm^2 after 5 days. In weeks 19, 24, and 33, detached leaf bio-assays were also performed for *Botrytis cinerea*. The fungus was reared from a frozen stock culture of the strain BC 700 on a half-strength potato dextrose agar medium. For the bioassay, the same leaflet for the thrips bioassay was used, using 10 replicates per treatment. Two drops of 4 uL spore suspension containing 10^{-6} spores were placed at both sides of the main leaf vein ~2 cm apart from each other. The diameter of the developing lesions was determined after 6 d, averaging the diameters of both spots.

In weeks 9, 15, and 24, leaf material was sampled for metabolomics by flash-freezing it in liquid nitrogen and storage at −80°C. Since leaves of strawberry are composed of three leaflets, one leaflet each was used for the thrips and botrytis bioassays described above and one for metabolite analysis. In addition, a leaflet of an equivalent leaf was used for the analysis of polyphenol oxidase (PPO), a jasmonic acid defense-related marker protein (Mouden et al., 2020).

Polyphenol Oxidase (PPO)

Polyphenol oxidase (PPO) activity was determined toward the end of cultivation in week 24, as previously described by Mouden et al. (2020). One week after spray applications, 150 mg of fresh leaf material was sampled, flash-frozen in liquid nitrogen, and stored at −80°C until analysis. Leaf material was ground to a fine powder and extracted with 1.25 mL ice-cold potassium phosphate buffer (0.1 M, pH 6.8) containing 7% (w:v) polyvinyl polypyridine. To this homogenate, 0.4 mL of a 10% solution of Triton X-100 was added. Plant extracts were vortexed for 2 min and centrifuged at 11,000× *g* for 10 min at 4°C. The resulting supernatant was used directly as an enzyme source

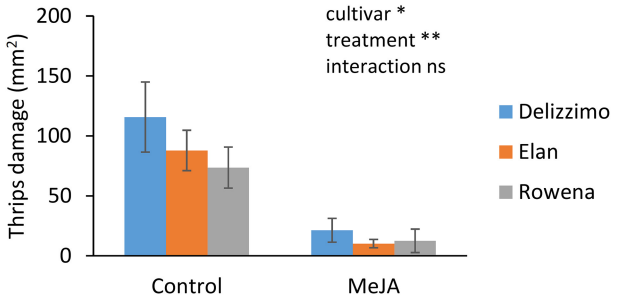

FIGURE 3 | Effect of MeJA treatment on plant resilience at the end of a strawberry nursery measured by a thrips (*Frankliniella occidentalis*) detached leaf bioassay. Damage was visually estimated in mm^2. Data present mean ± SEM ($n = 10$). Significant differences are indicated as $^*p \leq 0.01$, $^{**}p \leq 0.001$, based on GLM using a Wald chi-squared test.

using chlorogenic acid as a substrate. The reaction mixture consisted of 5 μL enzyme extract and 1 mL of 2.92 mM chlorogenic acid dissolved in 0.1 M potassium phosphate buffer at pH 8.0. The rate of change of absorbance of this mixture was spectrophotometrically measured at 470 nm for 1 min (UV-1800 UV–VIS spectrophotometer, Shimadzu Europe GmbH, Duisburg, Germany). PPO activities were calculated from the linear slope and reported as changes in absorbance values per min per gram of fresh weight. Per treatment, 10 replicates were used.

Statistical Analyses

Most data collected on plant growth, plant development, plant resilience, fruit quality, fruit shelve life, and fruit taste was ordinal or continuous, in most cases not normally distributed, even after transformation. Therefore, if one grouping factor was tested, we used a non-parametric Kruskal–Wallis test with subsequent pairwise comparisons by a Mann–Whitney *U* test. If two factors were tested, we performed generalized linear models (GLM) using Wald chi-squared tests with subsequent Mann–Whitney *U post hoc* tests. All statistical analyses were conducted with SPSS v. 26 software (IBM; SPSS Inc., Chicago, IL, United States).

Metabolomics of Endogenous Semi-polar Metabolites

For untargeted analysis of semi-polar leaf metabolites, 20 mg of lyophilized leaf tissue was extracted with 500 μL 75% MeOH [0.125% formic acid (FA)] according to De Vos et al. (2007). After sonication and centrifugation, 10 μL aliquots were taken from each sample and combined for quality control (QC) sample analyzed multiple times throughout the randomized sample sequence.

Extracts were analyzed by accurate mass LC-MS as described previously by Jeon et al. (2021) with minor adaptations. An UltiMate 3000 U-HPLC system (Dionex, Sunnyvale, CA, USA) was used to create a 45 min linear gradient of 5–75% acetonitrile in a 0.1% FA water a flow rate of 0.19 mL min^{-1}. Of each extract, 5 μL was injected, and compounds were separated on a Luna C18 column (2.0 × 150 mm, 3 μm; Phenomenex) at 40°C. A Q-Exactive plus-Orbitrap FTMS mass spectrometer (Thermo Fisher Scientific), operating at a resolution of 35.000 with scan-to-scan switching between negative (2.5 kV) and positive (3.5 kV) electrospray ionization (ESI) mode both over the m/z range 95–1,350, was used to detect eluting compounds. The QC sample was also analyzed in MSMS mode, in positive and negative mode, respectively.

Raw data files of –ESI mode LCMS analysis were subsequently processed in an untargeted manner using XCMS (https://xcmsonline/scripts.edu), including unbiased peak picking, alignment, and assembling of mass signals likely derived from the same metabolite. This unbiased processing resulted in putative compounds characterized by a specific in-source mass spectrum, including the putative molecular ion, isotope(s), and possible adducts and in-source fragments, at a specific retention time. All masses in the in-source spectra were searched for the presence of the calculated exact masses of the molecular ions $[M–H]^-$ of all possible target compounds and their hexose and malonyl-hexose conjugates, allowing a mass deviation of 7 ppm. Annotation of selected compounds was performed manually by checking the relative intensities and accurate mass differences between m/z signals in the reconstructed full scan ESI-MS spectra. Processed mass signals were kept for further analysis when they were present in at least five out of six biological replicates per treatment.

Multivariate Analysis

We used six biological replicates for each treatment/time. Each biological replicate consisted of a pool of five plants. The preprocessed data were log-transformed, scaled and a one-way ANOVA per single metabolite was carried out using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) determine which metabolites were different between conditions. Metabolites showing significance in the one-way ANOVA test were followed up by a Tukey's HSD *post hoc* test (alpha = 0.05) and used to perform principal component analysis (PCA) and hierarchical cluster analysis (HCA), reducing the dimensionality of the data to explore specific patterns of change in the metabolome as a result of plant developmental stage and MeJA applications. The HCA was performed using Pearson's correlation coefficient and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). In addition, partial Least Squares Discriminant Analysis (PLS-DA) models were generated for each developmental stage to identify variable importance in projection (VIP) metabolites.

Metabolite Identification

Annotation of differentially regulated metabolites was performed based on matching the accurate masses of selected pseudomolecule ions to compound libraries, including Metlin (http://metlin.scripps.edu/), Metabolomics Japan (www.metabolomics.jp), the Dictionary of Natural Products (http://dnp.chemnetbase.com), and KNApSAcK (http://kanaya.naist.jp/KNApSAcK), using a maximum deviation of observed mass from the calculated mass of 15 ppm.

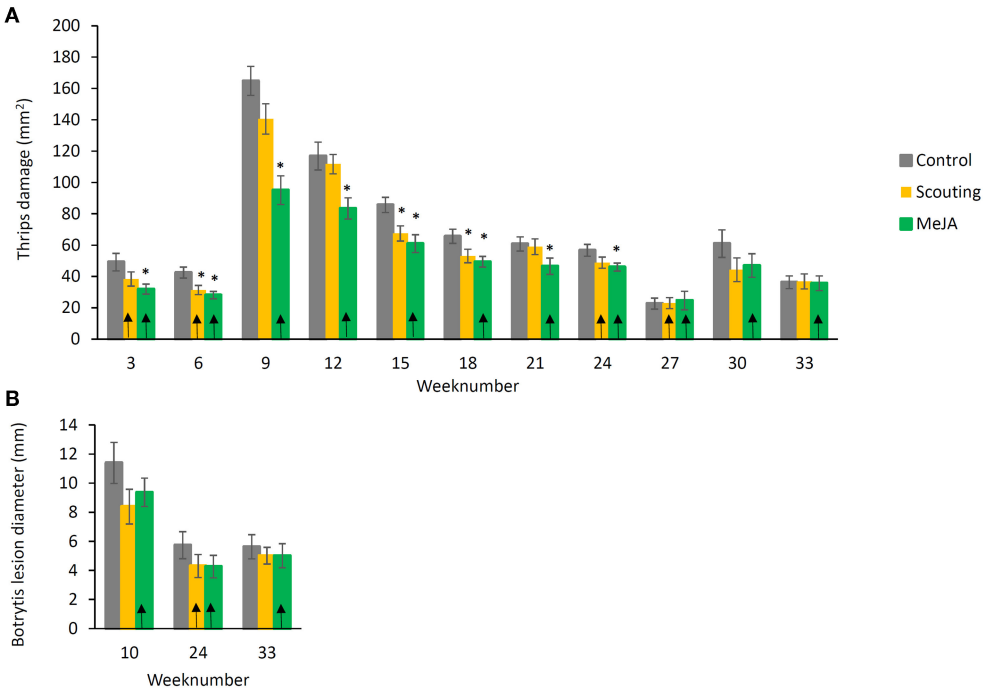

FIGURE 4 | Plant resilience during the strawberry cropping cycle. **(A)** Thrips damage and **(B)** Botrytis lesions in detached leaf bioassays. Plant resilience against thrips was evaluated every 3 weeks. Plant resilience against Botrytis was evaluated in weeks 10, 24, and 33. Control: leaves from mock-treated plants, Scouting: leaves from plants treated with 1 mM MeJA when this was deemed necessary based on scouting, occurring in weeks 2, 5, 23, and 26. MeJA: leaves from plants that were treated with MeJA every 3 weeks. Upward arrows (↑) indicate that plants received MeJA treatment in the previous week. Data present mean ± SEM ($n = 10$). Significant differences are indicated as $p \leq 0.05$ * based on a Kruskal–Wallis test.

RESULTS

MeJA Negatively Impacts the Performance of the Four Major Pests in Strawberry Cultivation

The elicitor effect of MeJA on plant resilience against pests of different feeding guilds was evaluated in different strawberry cultivars in the nursery phase. While there was a significant cultivar effect ($p \leq 0.05$) for thrips and spider mites, indicating that cultivars differed in their susceptibility to these pests, MeJA treatment affected all cultivars ($p \leq 0.01$). Thrips and spider mites as cell feeders and greenhouse whiteflies and Buckthorn aphid as phloem feeders were negatively affected by MeJA treatment. In a whole-plant bioassay, thrips damage was 5 to 10 times lower in MeJA treated plants than the control plants (**Figure 2A**). The number of eggs laid by spider mites was 34–50% reduced in MeJA treated plants concerning the control (**Figure 2B**). Settlement of greenhouse whiteflies on MeJA treated plants was significantly lower ($p \leq 0.05$) than the control, although the standard errors in the control treatment were relatively high (**Figure 2C**). Especially oviposition was strongly decreased. The aphid population on control plants showed a significantly stronger ($p \leq 0.05$) 7-fold increase (from 10 to 77 individuals) in contrast to MeJA treated plants with a doubled population (from 10 to 21 individuals) (**Figure 2D**).

We used a detached leaf bioassay to obtain data on plant resilience at the end of the nursery phase. In agreement with the whole plant assay, a significant effect ($p \leq 0.01$) of MeJA treatment was observed for the three cultivars, leading to a more than 80% reduction of thrips damage compared to the control (**Figure 3**).

Repeated Application of MeJA Results in Long Term Increase of Plant Resilience

Plant resilience was evaluated every 3 weeks during the cropping cycle using detached leaf bioassays. Significantly fewer thrips damage ($p \leq 0.05$) was observed for the "MeJA" treatments compared to the "Control" for all bioassays except the last three (**Figure 4A**). Thrips damage strongly increased in week 9, whereafter it steadily declined toward the end of the cropping cycle. In treatment "Scouting," MeJA was applied when this was deemed necessary based on pest pressure. Thrips damage on leaves from "Scouting" plants was significantly lower ($p \leq 0.05$) than "Control" plants in weeks 6, 15, and 18. In weeks 9 and 12, the same trend was observed but was not significant. As a marker of JA plant defense responses, the polyphenol oxidase (PPO) activity was measured in week 24. One week before, plants had been treated with MeJA, and we observed a significant reduction in thrips feeding damage ($p \leq 0.05$). However, this response was not accompanied by an increase in PPO levels

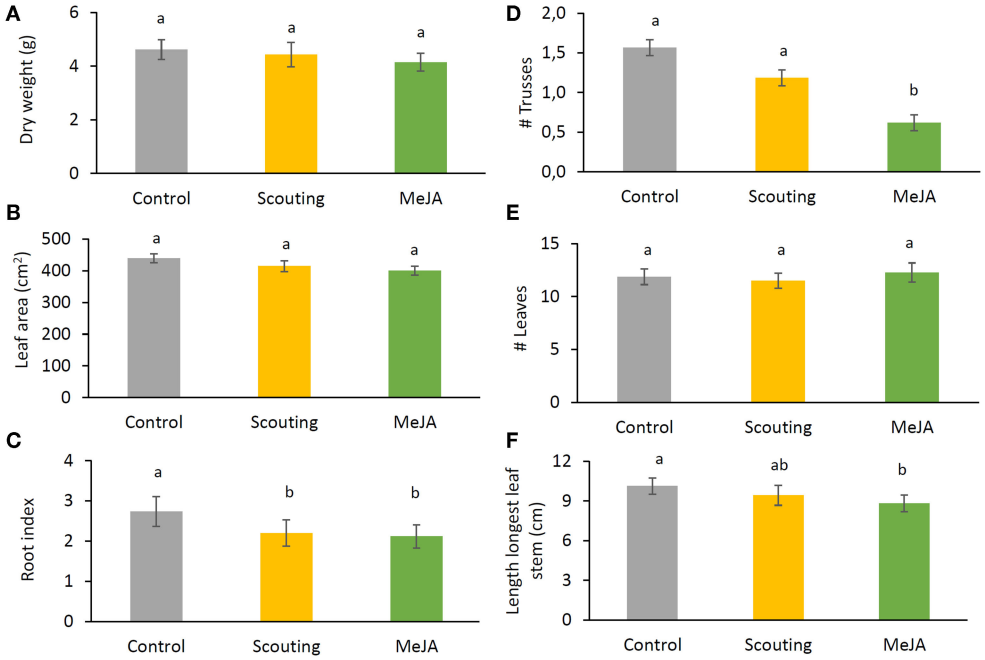

FIGURE 5 | Effect of MeJA treatment on plant growth and development traits at the end of the nursery phase of the strawberry cropping cycle. **(A)** The dry weight of aboveground plant parts; **(B)** total leaf area; **(C)** root index. Score of the amount of roots at the bottom of the rockwool block on a scale from 0 to 5; **(D)** number of trusses; **(E)** number of leaves; **(F)** length of the stem of the longest leaf. Data present mean ± SEM ($n = 10$). Letters indicate significant differences between treatments at $p \leq 0.05$ based on Kruskal–Wallis and subsequent pairwise comparisons using as Mann–Whitney *U* test.

(**Supplementary Figure 1**). Detached leaf bioassays with Botrytis did show less fungal growth on leaves of plants treated with "MeJA" or "Scouting," but significance levels were not reached (**Figure 4B**).

An Increase in Plant Resilience Does Influence Growth, but Not Production

At the end of the nursery phase, plant growth and development were determined. No differences between treatments for aboveground dry-weight (**Figure 5A**) nor leaf area (**Figure 5B**) or number of leaves (**Figure 5E**) were observed. The length of the stem of the longest leaf was shorter ($p \leq 0.05$), and the number of trusses was lower ($p \leq 0.05$) for the MeJA treatment compared with the control (**Figures 5D,F**). The root index was lower ($p \leq 0.05$) for plants in both treatments, "MeJA" and "Scouting" concerning control plants (**Figure 5C**). Thus, fewer roots grew through the rockwool block, reaching the bottom. Although light interception was increasing over time, no significant differences were observed between treatments (**Figure 6**).

The weekly production of fruits did not differ between treatments. Also, the numbers of first and second-class fruits and their weight were not different between treatments (**Figures 7A,B**). Fruit damage ($p < 0.05$) was increasing over time (**Figure 8A**), and fruit rot was lower in weeks 27 and week 30 compared to week 33 (**Figure 8B**). While no differences between treatments were observed concerning fruit rot, fruit damage was significantly increased ($p \leq 0.05$) in the scouting treatment,

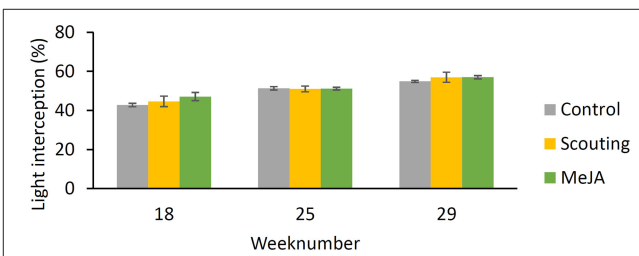

FIGURE 6 | Light interception (%) during the strawberry production phase as determined in weeks 18, 25, and 29. Data present mean ± SEM ($n = 3$).

but the control did not differ from MeJA treatment. Brix and acidity, objective determinants of fruit sweetness, increased over time ($p \leq 0.01$) (**Figures 8C,D**). Acidity did not differ between treatments (**Figure 8D**), but fruits of the "Scouting" treatment showed lower Brix levels than the other treatments (**Figure 8C**). However, the Brix of MeJA treated fruits did not differ from control fruits. These observations indicate that repeated application of MeJA did not affect fruit production, fruit quality, or fruit taste.

Leaf Metabolome Is Changed During Plant Development

To obtain an overall view of adaptations of the leaf metabolomes upon MeJA treatment, we choose to use an unbiased LC-MS

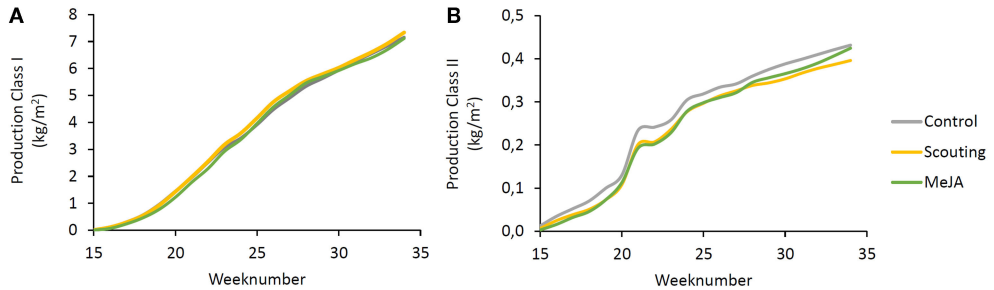

FIGURE 7 | Strawberry fruit production over time. Cumulative weight of **(A)** first class and **(B)** second class fruits per week.

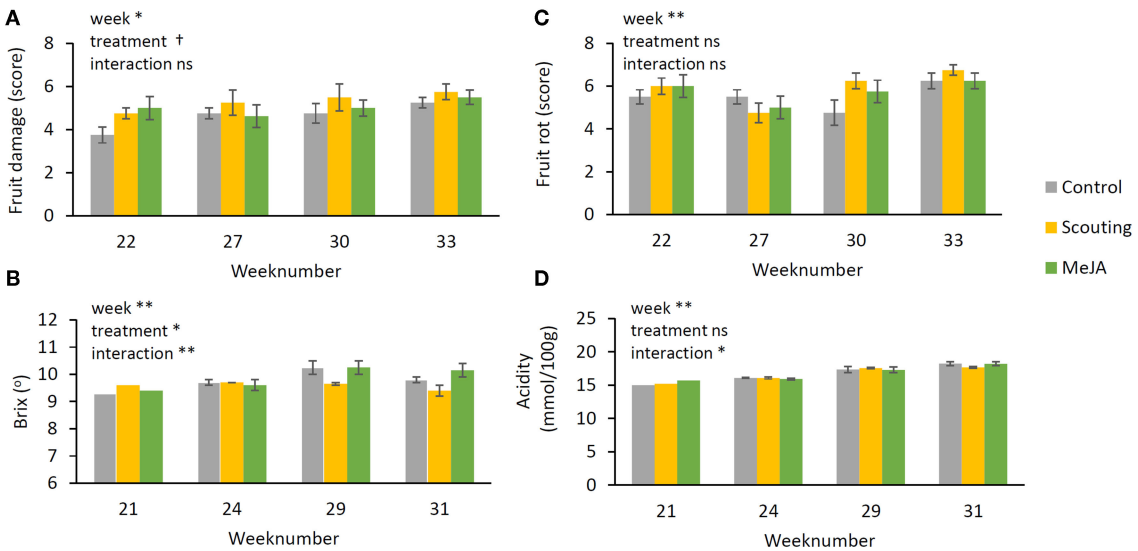

FIGURE 8 | Shelf lives and tastes of harvested strawberry fruits during the production period. Shelf life was scored **(A)** fruit damage and **(B)** fruit rot. Scores are three: not acceptable, five: acceptable, seven: good. Data present mean ± SEM ($n = 8$). Taste was evaluated by determining the two most important components for sweetness **(C)** Brix (°) and **(D)** acidity (titratable acid in mmol/100 g fresh weight). Data present mean ± SEM (taste $n = 1$ for week 21 (mix of fruits of all eight blocks per treatment), $n = 2$ for weeks 24, 29, 31 (mix of fruits of 4 blocks per treatment). Significant differences are indicated as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, based on GLM using a Wald chi-squared test.

approach being inherently more sensitive above NMR, as we expected that changes in specific metabolite abundances might be small. LC-MS data were processed for negative and positive ionization mode separately, resulting in 1,627 and 378 mass signals. Technical reproducibility was high as Quality Control (QC) samples showed low variation. As biological variation within replicates was smaller than between experimental groups, we considered six replicates sufficient to capture a proper view of the metabolomes (**Supplementary Figure 2**).

Leaf metabolomes of strawberry plants differed during the developmental stage as visualized by unbiased PCA based on all detected mass signals normalized to the sum and $\log_2$ transformed (**Figure 9**). About 30–40% of the total variation in the dataset could be explained by the first two PCs for negative and positive ionization mode, respectively, separating leaf metabolomes from 9-week-old and 24-week-old plants among the first PC, while the second PC was additionally distinguishing 9-week-old plants from 15-week-old ones.

MeJA Induced Impact on the Strawberry Leaf Metabolome Is Associated With Changes in Phenolics and Flavonoids Content

Considering the developmental variations in the leaf metabolomes of strawberry plants, we investigated the effect of MeJA applications on leaf metabolomes in weeks 9, 15, and 24 separately. The data were subjected to ANOVA with correction for multiple testing (Benjamini–Hochberg), and metabolites that were significantly different ($P < 0.05$ and fold change >2) between at least two treatments within each developmental stage

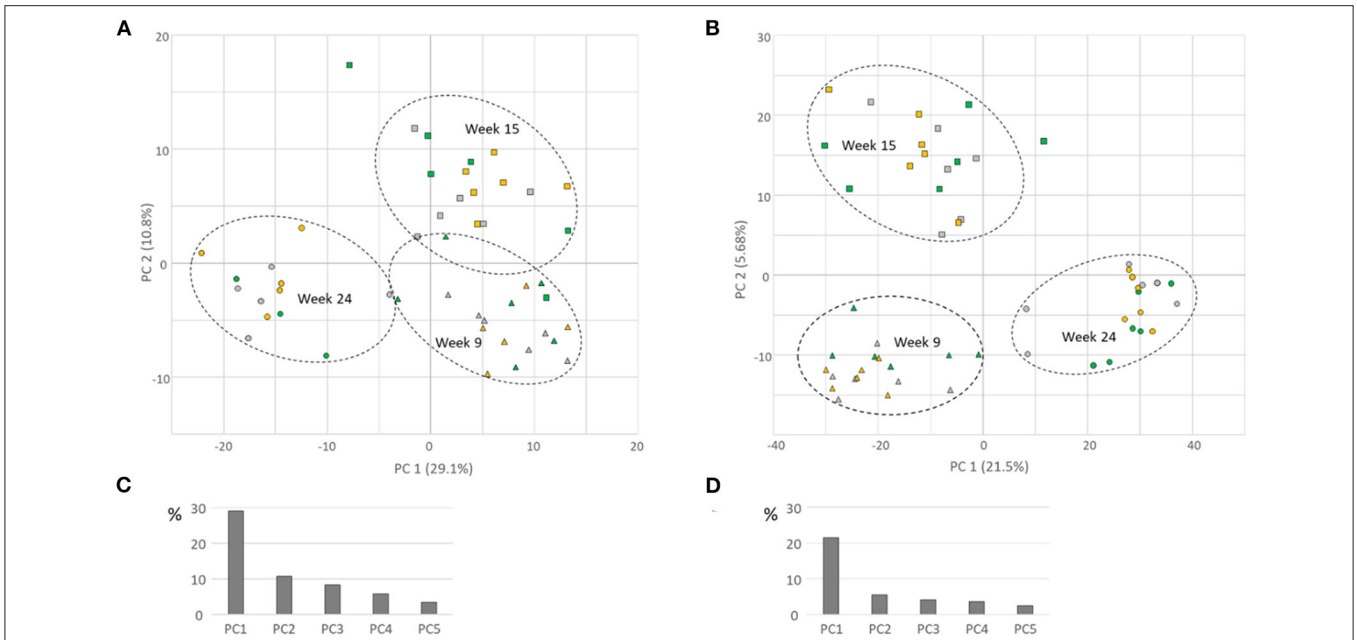

FIGURE 9 | Leaf metabolomes of strawberry plants in week 9 (triangles), 15 (squares), and 24 (circles). Principal Component Analysis (PCA) of mass signals obtained in positive ionization mode **(A)** and negative ionization mode **(B)**. Bar diagrams show the variance that can be explained by the first five components (PCs) for positive **(C)** and negative **(D)** ionization mode. Gray symbols: mock-treated control plants; green symbols: MeJA-treated every 3 weeks; yellow symbols: MeJA treatment upon scouting.

TABLE 1 | Number of mass features included in the PLS-DA models and the qualitative descriptors of the PLS-DA models in **Figure 10**.

	# features [$P < 0.05$; >2-fold]	ANOVA (% of total features)	# components for max accuracy	R^2	Q^2
Week 9	1683	258 (15.33%)	5	0.99	0.89
Week 15	897	5 (0.56%)	2	0.98	0.73
Week 24	1200	13 (1.08%)	4	0.98	0.81

were used to generate partial least squares discriminant analysis (PLS-DA) models (**Table 1**).

Of the 8,685 features detected by LC-MS, 3,780 (43.5%) were significantly different between at least two treatments in either developmental stage. A PLS-DA model of the selected metabolites present in 9-week-old plants demonstrated distinct clustering of the samples based on MeJA application, with latent variables 1 and 2 explaining 38.3% of the total variation (**Figure 10A**). Similarly, PLS-DA models for metabolomes of 15-week and 24-week-old plants indicated 29.1 and 24.2% of the variation, respectively, could be explained by the two dominant latent variables (**Figures 10B,C**).

Next, we selected the features that contributed to the variables in projection for each of the PLS-DA models with a cut-off value >1.5 and used these to create a heatmap using Z-scores (**Figure 11**). Cluster features were associated with sample differences due to metabolites that were intrinsically more abundant in MeJA treated plants, while cluster II features were more abundant in mock-treated control plants. After removing adducts, tentative metabolite identification (**Supplementary Table 1**) showed that metabolites that were more abundant in MeJA treated plants than mock-treated control plants predominantly included glycosylated compounds derived from the phenylpropanoid pathway, including phenolic acids and flavonoid compounds such as flavanes, flavanones, isoflavones, and anthocyanidins. The most induced compound was a hepta-methoxy-flavanone ($[M-H]^-$ 433.1508; MetLIN ID 53117) which was 111-fold more abundant in MeJA-treated leaves than control leaves. Also, catechin 3-O-rutinoside (flavan-3-ol) and pelargonidin 3-(6″-caffeylglucoside) ($[M-H]^-$ 593.1305; MetLIN ID 46799, anthocyanidin) were significantly induced in MeJA-treated leaves compared to non-treated control leaves (**Supplementary Table 1**). Furthermore, multiple glycosylated phenolic acid compounds were more abundant in MeJA-treated leaves.

Leaf Metabolites Related to Constitutive Defense Increase With Plant Maturity

A PLS-DA model based on all detected metabolite features constitutively present in control plants of different ages

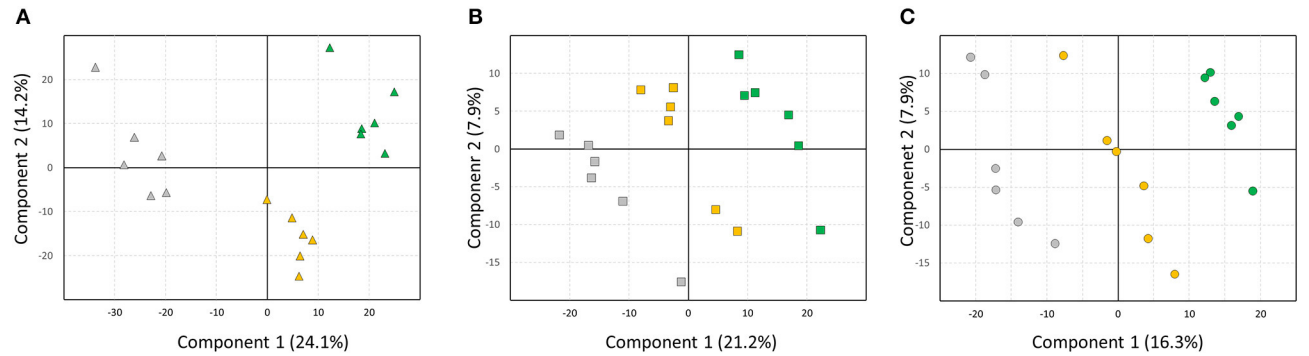

FIGURE 10 | Partial least squares discriminant analysis (PLS-DA) based on methyl jasmonate-mediated changes in leaf secondary metabolites in strawberry plants in different developmental stages. **(A)** 9-week-old leaf metabolomes; **(B)** 15-weeks old; **(C)** 24-weeks. Gray symbols: mock-treated control plants; green symbols: MeJA-treated every 3 weeks; yellow symbols: MeJA treatment upon scouting. Cross-validation of the models: $Q^2 = 0.8$, 0.75, and 0.75, respectively.

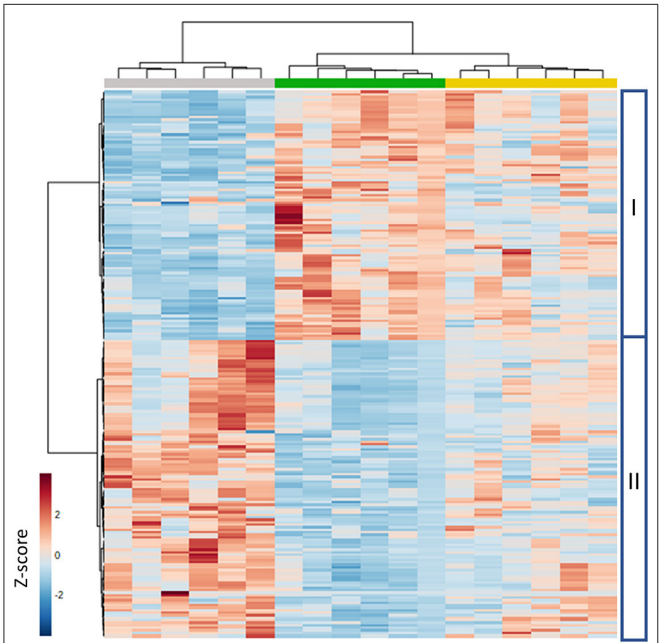

FIGURE 11 | Hierarchical cluster analysis of selected (ANOVA, $P < 0.05$; fold change > 2) semi-polar metabolites in strawberry leaf metabolomes. A color-coded matrix represents the Z-score of the metabolite intensity in six biological replicates of strawberry leaves. I and II represent clusters of mass features induced (I) or reduced (II) upon MeJA application. Characteristics of the underlying metabolites are presented in **Supplementary Table 2**. Gray: mock-treated plants; green: MeJA-treated every 3 weeks; yellow: MeJA treatment upon scouting.

demonstrated distinct clustering of the samples, and latent variables one and two explained 36% of the total variation (**Figure 12A**). Of the 25 metabolites that were most explanatory for the model (high variable in projection score), 18 showed increased abundance during development (**Figure 12B**). Metabolites relating to the developmental stage originate from different biochemical classes, including some flavonoid- and diterpene glycosides (**Supplementary Table 2**).

DISCUSSION

Modulation of plant defenses to increase host plant resistance becomes increasingly important within IPM (Mouden and Leiss, 2021). However, despite an extensive knowledge base, not much of existing basic research on natural plant defense strategies have been translated into applications that have been put into agricultural practice. To make the first step toward integrating elicitor application in current IPM practice, methyl jasmonate (MeJA) was either applied every 3 weeks or based on scouting of pests during the cultivation cycle of a strawberry everbearer. The present study is the first to report repeated pre-harvest foliar applications with the elicitor MeJA enhanced plant resilience against thrips and against the remaining major pests in strawberry: spider mite, whitefly, and aphids. Notably, we demonstrated that during production, these applications did not constrain fruit yield nor quality.

Artificial manipulation of Jasmonic acid (JA)-associated defenses has been reported to increase resistance against herbivores of multiple arthropod taxa and has been described extensively in several agri- and horticulturally important species (Dicke and Hilker, 2003; Miyazaki et al., 2014; Escobar-Bravo et al., 2017; Chen et al., 2018; Warabieda et al., 2020). While MeJA application has been described to induce resistance against the two-spotted spider mite (Warabieda et al., 2005), most studies concerning elicitor use in the Rosaceae family frequently evaluated postharvest effects of elicitor treatments rather than defense responses during cultivation. Congruent with the reported effects of JA-mediated plant-induced defenses on thrips in tomato (Chen et al., 2018, 2020) and chrysanthemum (Escobar-Bravo et al., 2017) showed that thrips feeding damage was significantly reduced upon repeated MeJA foliar sprays during the nursery phase. The three commercial cultivars varied inherently in thrips susceptibility, with the cultivar Delizzimo being more susceptible than the cultivars Elan or Rowena. Similar results were observed for spider mites where MeJA negatively affected reproduction. Moreover, MeJA sprays significantly reduced whitefly as well as aphid reproduction in comparison to control plants. Based on their feeding guild, spider mite and

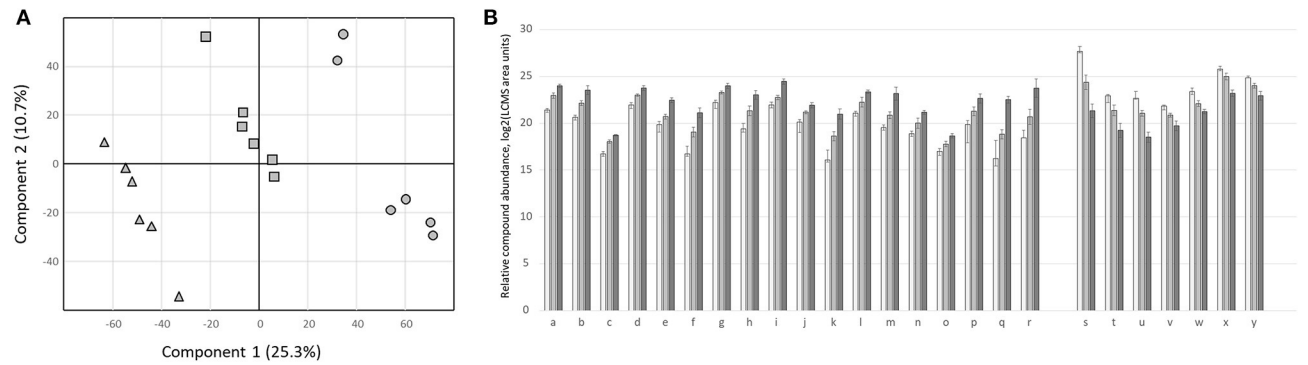

FIGURE 12 | Leaf metabolome changes of control plants during development. **(A)** Partial least squares discriminant analysis (PLS-DA) based on secondary metabolites in control treatments of strawberry leaves at different developmental stages. Triangles 9-week-old leaf metabolomes; squares, 15-week-old; circles 24-week-old. Cross-validation of the model: $Q^2 = 0.9$. **(B)** Relative abundance of metabolite features with highest variable in projection (VIP) score. White bars abundance in 9-week-old leaves; light-gray bars 15-week-old; dark-gray bars 24-week-old. Bars represent $\log_2$ values of means ± SD $N = 6$. Letters indicate different compounds, for details see **Supplementary Table 3**.

thrips are considered cell-content feeders whereas, aphids and whiteflies are phloem suckers. We thus demonstrated that both feeding types were negatively influenced by MeJA application in strawberries. While it is generally stated that insects activate the JA defense pathway, there are arguments that different insect feeding guilds elicit different plant hormonal signaling pathways, mediated by the phytohormones salicylic acid (SA) or JA. Chewing and cell-content feeding insects such as thrips predominantly induce JA-mediated defenses, whereas the SA-signaling pathway is induced by phloem-feeding insects (Pieterse et al., 2012; Lazebnik et al., 2014). The SA-pathway is considered to be the predominant signaling pathway during green peach aphid (*Myzus persicae*) infestation (Rodriguez et al., 2014), while JA-responsive genes were repressed (Kerchev et al., 2013). However, several authors suggest a role for both pathways in activating defenses against aphids (Cooper et al., 2004; Boughton et al., 2005; Selig et al., 2016). Similarly, the whitefly *Bemisia tabaci* has been shown to promote induction of SA-defenses while suppressing JA defense responses (Zarate et al., 2007; Walling, 2008; Estrada-Hernández et al., 2009; Puthoff et al., 2010). It is plausible that the antagonistic or synergistic function of the JA and SA defense pathways depends on the timing and level of the interaction between host plant and aphid species (Pieterse et al., 2009; Cao et al., 2014). Nonetheless, exogenously applied JA has been reported to decrease reproduction in whitefly (Shi et al., 2017; Esmaeily et al., 2020) and even virus transmission (Escobar-Bravo et al., 2016).

While JA treatment is known to induce defenses against necrotrophic fungi such as Botrytis in tomato (Bruce et al., 2017; Courbier et al., 2020), we did not observe any such effect in our leaf bioassays. However, although using lower concentrations compared to the ones we used, the effects described in the literature were obtained for 3-week-old vegetative plants and seedlings, respectively, while our first botrytis bioassay involved plants already 10 weeks old. Thus, MeJA application in older strawberry plants was not effective in inducing plant defenses against Botrytis. Young plants or tissues are frequently more responsive to defense elicitors than older plants or tissues (Cipollini and Redman, 1999; Köhler et al., 2015; Chen et al., 2018).

On the contrary, induction of plant defenses led to reduction of thrips damage of young plants in the nursery phase and older plants in the production phase, when MeJA was applied every 3 weeks. Also, incidental MeJA application after scouting reduced thrips feeding damage in the cultivation cycle. The positive effect, however, diminished over time when these plants were not repeatedly sprayed. In order to maintain durable protection, repeated MeJA applications during the cropping cycle are required. We observed a long-term increase of plant resilience from plant nursery until week 24 in the production. A long-term increase of plant resilience is particularly important in greenhouse horticulture, with usually relatively long cultivation cycles. Intriguingly, for an unknown reason, we observed a reduction in thrips feeding damage in non-treated scouting plants in weeks 15 and 18 while only plants from MeJA-plots were elicited. Moreover, we observed that thrips feeding symptoms were significantly higher in week 9 and decreased over time. From week 9 onwards first trusses became visible, indicating that the plant switched from the vegetative to the generative phase. In the generative phase, trusses, flowers, and fruits demand relatively many assimilates, possibly rendering plants more nutritious to thrips. Sugars may stimulate thrips feeding in plants (Scott Brown et al., 2002).

Strikingly, during the last 6 weeks of the production phase, no increase in plant resilience could be detected anymore. We assume that this may be the result of the age-dependent effects of the MeJA application. Induced plant responses have been reported to decline with age (Mao et al., 2017). Flower and fruit production over a longer period requires a substantial request for resources. Investment in growth comes at the expense of plant defense, as expressed by the growth-defense trade-off theory (Koo, 2018). At the same time, we showed that repeated elicitor applications resulted in shorter leaf length and less plant dry weight in the nursery. No reduction of plant growth nor fruit

yield, or quality during production was observed. This is in line with the conclusions of a meta-analysis of induced defense costs by Garcia et al. (2021) stating that negative effects on growth occur during the vegetative but not during the reproductive plant stages. It is hypothesized that when the reproductive stage is reached, the plant prioritizes growth against the defense to guarantee a proper seed set. We did measure a decrease in defense metabolites induction from vegetative plants at the beginning to mature plants at the end of the cropping cycle. This is in line with our observation that polyphenol oxidase (PPO), an important JA-inducible marker, did not increase after MeJA application in week 24. Doan et al. (2004) also reported that PPO induction by foliar application of JA depended on plant developmental stage, indicating active defense of young tissues and potential shifts in defensive strategy during plant transition from growth to reproduction. However, we did not observe a concurrent increase in susceptibility to thrips. It has been proposed that in the early plant stages, when plants have relatively little biomass and potentially insufficient amounts of constitutive defense compounds, and active JA response is required for defense. As plants mature, constitutive defense metabolites seem to accumulate, providing a higher level of basal resistance (Barton and Koricheva, 2010; Mao et al., 2017). Indeed, the metabolome of the control plants embodying constitutive defenses present was significantly different between young and mature plants, as revealed by PLS-DA analysis. Changes were mainly dominated by increased abundance in flavonoids and diterpene glycosides, supporting a role in the reprogramming of constitutive leaf-based resistance in control plants over time. Indeed, diterpene glycosides (capsianosides) were negatively correlated with thrips preference and damage in an untargeted metabolomic profiling approach in Capsicum (Macel et al., 2019; van Haperen et al., 2021). Nevertheless, from week 25 onwards, we experienced caterpillar damage by the Turkish moth at the end of the cropping cycle—the only incidence during the whole cropping cycle we had to use insecticides. Concurrently, plants were infected with powdery mildew. It is generally stated that the two main plant defense pathways, JA and SA, work antagonistically (Pieterse et al., 2012). Thus, mildew infection in the MeJA treatment may be expected to be more severe. However, no differences in the percentage of infected fruits between treatments were observed (**Supplementary Figure 3**).

Exogenous application of MeJA has frequently been shown to alter the metabolome of plants by increasing the production of secondary metabolites, often in a defense-related context (Tugizimana et al., 2018). Partial Least Square-Discriminant analysis (PLS-DA) permitted to classify of leaf metabolomes based on MeJA treatment, exploring the effects per given timepoint. Heat map analysis of the differential metabolites revealed two clusters containing metabolites that were either induced or repressed upon foliar treatment with MeJA. Treatment with MeJA showed strong induction of metabolites compared to both scouting plants that were only incidentally treated with MeJA and control plants. Furthermore, multiple sugar-conjugated phenolic acid compounds were more abundant in MeJA-treated leaves, suggesting that treatment with MeJA shifted the glycosylation patterns of flavonoids. Many plant secondary metabolites in nature occur as glycosides (Bartnik and Facey, 2017). In general, glycosylation is known to affect the stability, storage, transport, availability, reactivity, and bioactivity of sugar acceptors. More importantly, glycosylation plays an important role in reducing phenylpropanoid toxicity, explaining the widespread occurrence in plant development and resistance/tolerance to major biotic and abiotic stresses (Bowles et al., 2005; Le Roy et al., 2016).

We tentatively identified 30 major potential metabolites induced upon MeJA application. Accordingly, main compound groups induced by MeJA were attributed to phenylpropanoid biosynthesis and included several flavonoid groups such as flavanes, flavanones, isoflavones, and anthocyanidins well as phenolic acids. Phenolic secondary metabolites are a major group directly involved in host plant resistance to insects and pathogens (Daayf et al., 2012; Mouden et al., 2017). In addition, they are involved in host plant resistance to thrips in different hosts such as carrot (Leiss et al., 2013) and tomato (Bac-Molenaar et al., 2019) as vegetables and chrysanthemum as an ornamental (Leiss et al., 2009b).

Among these metabolites, MeJA-triggered metabolic changes were largely characterized by a significant increase in levels of the annotated flavonoids, catechin 3-O-rutinoside, and pelargonidin 3-(6″-caffeylglucoside). The most abundant glycosylated forms in plants occur commonly as O-glycosides (Bartnik and Facey, 2017). The flavan-3-ol, catechin 3-O-rutinoside contains a rutinoside (6-O-α-L-rhamnosyl-D-glucosides) linkage bond via oxygen. Catechin itself is the major antioxidant compound in strawberry leaves (Buričová et al., 2011) and is considered among the major end products of the biosynthetic pathway in many plant species (Dixon et al., 2004). Catechins have been shown to play an important role in inducible defenses against foliar pathogen infection (Ullah et al., 2017; Wang et al., 2017) as well as herbivory (Kundu et al., 2018). In strawberry specifically, catechins are known to play a role in both constitutive (Yamamoto et al., 2000) and induced resistance to foliar pathogens (Feucht et al., 1992). Moreover, in buckwheat, catechins have also been reported to be induced upon JA treatment (Park et al., 2019). Pelargonidin 3-(6″-caffeylglucoside) belongs to a common class of organic anthocyanidins. Besides their role as natural pigments, glucoside derivatives which have putatively been implied as resistance related secondary metabolites (Karre et al., 2019). Another discriminatory marker with increased abundance in response to MeJA treatment was hepta-methoxy-flavanone. Although this poly methoxylated flavanone has not been previously linked to plant defenses, Roland et al. (2014) reported that six-methoxyflavanone acted as an antagonist of dietary bitter compounds.

CONCLUSIONS

Integration of repeated application of methyl jasmonate in an IPM strategy, thus vertical integration of pest control measures, enabled increased plant resilience during a complete strawberry cropping cycle under glass, controlling thrips without reduction in fruit yield nor quality. In addition, reducing spider

mites, whitefly and aphids indicates the potential of horizontal integration of elicitor application, controlling several different pests concurrently. While induced defense decreased with the maturation of plants during the cropping cycle, constitutive defense increased as measured in the leaf metabolome of control plants. Our data propose that when plants are relatively small in the early plant stages, a potential lack of constitutive defense compounds necessitates an active JA response for defense. As plants, mature constitutive defense metabolites seem to accumulate, providing a higher level of basal resistance. The results obtained have important implications for the cultivation of strawberry and other long-term cultivation crops in practice, showing that regular application of methyl jasmonate and potentially other elicitors during the crop cycle forms a very promising management tool for pest control a sustainable IPM strategy in the production greenhouses in general.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JB-M, SM, EB, and KL: concept and design of experiments. JB-M and SM: execution of the experiments and analysis of data. IK: performance and analysis of metabolomics. JB-M, SM, IK, and KL: writing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Results were obtained within the project Green crop protection: re-designing strawberry cultivation under glass, funded by the Dutch Ministry of Agriculture, Nature, and Food Quality.

ACKNOWLEDGMENTS

We thank Ge Bentvelsen from ABZ seeds for the provision of the strawberry cultivars. Jan Janse was thanked for cultivating strawberries and Hessel van der Heide to manage natural enemies during strawberry nursery and production. We are grateful for the technical assistance of Marianne Noordam, Ariyati Persijn, Renata van Holstein-Saj, and Chantal Bloemhard for MeJA applications and conductance of bioassays and Bert Schipper for technical assistance with LCMS analysis. Trudy van Twist is thanked for fruit sampling and measurements of fruit yield and quality.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.695908/full#supplementary-material

REFERENCES

Bac-Molenaar, J. A., Mol, S., Verlaan, M. G., Van Elven, J., Kim, H. K., Klinkhamer, P. G. L., et al. (2019). Trichome independent resistance against western flower thrips in tomato. *Plant Cell Physiol.* 60:18. doi: 10.1093/pcp/pcz018

Bartnik, M., and Facey, P. C. (2017). "Glycosides," in *Pharmacognosy: Fundamentals, Applications and Strategy*. (London: Elsevier Inc.), 101–161. doi: 10.1016/B978-0-12-802104-0.00008-1

Barton, K. E., and Koricheva, J. (2010). The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. *Am. Nat.* 175, 481–493. doi: 10.1086/650722

Boughton, A. J., Hoover, K., and Felton, G. W. (2005). Methyl jasmonate application induces increased densities of glandular trichomes on Tomato, Lycopersicon esculentum. *J. Chem. Ecol.* 31, 2211–2216. doi: 10.1007/s10886-005-6228-7

Bowles, D., Isayenkova, J., Lim, E. K., and Poppenberger, B. (2005). Glycosyltransferases: managers of small molecules. *Curr. Opin. Plant Biol.* 8, 254–263. doi: 10.1016/j.pbi.2005.03.007

Bruce, T. J. A., Smart, L. E., Birch, A. N. E., Blok, V. C., MacKenzie, K., Guerrieri, E., et al. (2017). Prospects for plant defence activators and biocontrol in IPM – concepts and lessons learnt so far. *Crop Prot.* 97, 128–134. doi: 10.1016/J.CROPRO.2016.10.003

Buričová, L., Andjelkovic, M., Cermáková, A., Réblová, Z., Jurček, O., Kolehmainen, E., et al. (2011). Antioxidant capacity and antioxidants of strawberry, blackberry, and raspberry leaves. *Czech J. Food Sci.* 29, 181–189. doi: 10.17221/300/2010-cjfs

Cao, H. H., Wang, S. H., and Liu, T. X. (2014). Jasmonate- and salicylate-induced defenses in wheat affect host preference and probing behavior but not performance of the grain aphid, Sitobion avenae. *Insect Sci.* 21, 47–55. doi: 10.1111/1744-7917.12023

Chen, G., Klinkhamer, P. G. L., and Escobar-Bravo, R. (2020). Constitutive and inducible resistance to thrips do not correlate with differences in trichome density or enzymatic-related defenses in chrysanthemum. *J. Chem. Ecol.* 46, 1105–1116. doi: 10.1007/s10886-020-01222-1

Chen, G., Klinkhamer, P. G. L., Escobar-Bravo, R., and Leiss, K. A. (2018). Type VI glandular trichome density and their derived volatiles are differently induced by jasmonic acid in developing and fully developed tomato leaves: implications for thrips resistance. *Plant Sci.* 276, 87–98. doi: 10.1016/j.plantsci.2018.08.007

Cipollini, D. F., and Redman, A. M. (1999). Age-dependent effects of jasmonic acid treatment and wind exposure on foliar oxidase activity and insect resistance in tomato. *J. Chem. Ecol.* 25, 271–281. doi: 10.1023/a:1020842712349

Clymans, R., Trekels, H., Boonen, M., Craeye, S., Hanssens, J., Smagghe, G., et al. (2017). "Matching commercial thrips predating phytoseids with the highly diversified climatic conditions of different strawberry production systems," in *Acta Horticulturae (International Society for Horticultural Science)*, ed Y. Desjardins (Belgium), 863–870. doi: 10.17660/ActaHortic.2017.1156.127

Cooper, W. C., Jia, L., and Goggin, F. L. (2004). Acquired and r-gene-mediated resistance against the potato aphid in tomato. *J. Chem. Ecol.* 30, 2527–2542. doi: 10.1007/s10886-004-7948-9

Courbier, S., Grevink, S., Sluijs, E., Bonhomme, P., Kajala, K., Van Wees, S. C. M., et al. (2020). Far-red light promotes *Botrytis cinerea* disease development in tomato leaves via jasmonate-dependent modulation of soluble sugars. *Plant. Cell Environ.* 43, 2769–2781. doi: 10.1111/pce.13870

Daayf, F., El Hadrami, A., El-Bebany, A. F., Henriquez, M. A., Yao, Z., Derksen, H., et al. (2012). "Phenolic compounds in plant defense and pathogen counter-defense mechanisms," in *Recent Advances in Polyphenol Research*, eds V. Cheynier, P. Sarni-Manchado, S. Quideau (Oxford: Wiley-Blackwell), 191–208. doi: 10.1002/9781118299753.ch8

De Vos, R. C. H., Moco, S., Lommen, A., Keurentjes, J. J. B., Bino, R. J., and Hall, R. D. (2007). Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat. Protoc.* 2, 778–791. doi: 10.1038/nprot.2007.95

Dicke, M., and Hilker, M. (2003). Induced plant defences: From molecular biology to evolutionary ecology. *Basic Appl. Ecol.* 4, 3–14. doi: 10.1078/1439-1791-00129

Dixon, R. A., Xie, D.-Y., and Sharma, S. B. (2004). Proanthocyanidins - a final frontier in flavonoid research? *New Phytol.* 165, 9–28. doi: 10.1111/j.1469-8137.2004.01217.x

Doan, A.-T., Gary, E., and Gary, F. (2004). Temporal effects on jasmonate induction of anti-herbivore defense in *Physalis angulata*: seasonal and ontogenetic gradients. *Biochem. Syst. Ecol.* 32, 117–126.

Escobar-Bravo, R., Alba, J. M., Pons, C., Granell, A., Kant, M. R., Moriones, E., et al. (2016). A jasmonate-inducible defense trait transferred from wild into cultivated tomato establishes increased whitefly resistance and reduced viral disease incidence. *Front. Plant Sci.* 7, 1732. doi: 10.3389/fpls.2016.01732

Escobar-Bravo, R., Klinkhamer, P. G., and Leiss, K. A. (2017). Induction of jasmonic acid-associated defenses by thrips alters host suitability for conspecifics and correlates with increased trichome densities in tomato. *Plant Cell Physiol.* 58:622. doi: 10.1093/pcp/pcx014

Esmaeily, S., Amin Samih, M., and Izadi, H. (2020). Induced eggplant resistance against Trialeurodes vaporariorum triggered by jasmonic acid, abscisic acid, and Nesidiocoris tenuis feeding. *Bull. Entomol. Res.* 110, 285–292. doi: 10.1017/S0007485319000646

Estrada-Hernández, M. G., Valenzuela-Soto, J. H., Ibarra-Laclette, E., and Délano-Frier, J. P. (2009). Differential gene expression in whitefly bemisia tabaci-infested tomato (Solanum lycopersicum) plants at progressing developmental stages of the insect's life cycle. *Physiol. Plant.* 137, 44–60. doi: 10.1111/j.1399-3054.2009.01260.x

Feucht, W., Treutter, D., and Christ, E. (1992). The precise localization of catechins and proanthocyanidins in protective layers around fungal infections. *Zeit. Pflanzenkrankheiten Pflanzenschutz,* 404–413. Available online at: https://agris.fao.org/agris-search/search.do?recordID=DE92U0698 (accessed April 13, 2021).

Garcia, A., Martinez, M., Diaz, I., and Santamaria, M. E. (2021). The price of the induced defense against pests: a meta-analysis. *Front. Plant Sci.* 11:615122. doi: 10.3389/fpls.2020.615122

Giné-Bordonaba, J., and Terry, L. A. (2016). Effect of deficit irrigation and methyl jasmonate application on the composition of strawberry (Fragaria x ananassa) fruit and leaves. *Sci. Hortic. (Amsterdam).* 199, 63–70. doi: 10.1016/j.scienta.2015.12.026

Golan, K., Sempruch, C., Górska-Drabik, E., Czerniewicz, P., Łagowska, B., Kot, I., et al. (2017). Accumulation of amino acids and phenolic compounds in biochemical plant responses to feeding of two different herbivorous arthropod pests. *Arthropod. Plant. Interact.* 11, 675–682. doi: 10.1007/s11829-017-9522-8

Han, Y., Chen, C., Yan, Z., Li, J., and Wang, Y. (2019). The methyl jasmonate accelerates the strawberry fruits ripening process. *Sci. Hortic. (Amsterdam).* 249, 250–256. doi: 10.1016/J.SCIENTA.2019.01.061

Jeon, J. S., Carreno-Quintero, N., van Eekelen, H. D. L. M., De Vos, R. C. H., Raaijmakers, J. M., and Etalo, D. W. (2021). Impact of root-associated strains of three Paraburkholderia species on primary and secondary metabolism of Brassica oleracea. *Sci. Rep.* 11:2781. doi: 10.1038/s41598-021-82238-9

Karre, S., Kumar, A., Yogendra, K., Kage, U., Kushalappa, A., and Charron, J. B. (2019). HvWRKY23 regulates flavonoid glycoside and hydroxycinnamic acid amide biosynthetic genes in barley to combat Fusarium head blight. *Plant Mol. Biol.* 100, 591–605. doi: 10.1007/s11103-019-00882-2

Kerchev, P. I., Karpińska, B., Morris, J. A., Hussain, A., Verrall, S. R., Hedley, P. E., et al. (2013). Vitamin C and the abscisic acid-insensitive 4 transcription factor are important determinants of aphid resistance in arabidopsis. *Antioxidants Redox Signal.* 18, 2091–2105. doi: 10.1089/ars.2012.5097

Köhler, A., Maag, D., Veyrat, N., Glauser, G., Wolfender, J. L., Turlings, T. C. J., et al. (2015). Within-plant distribution of 1,4-benzoxazin-3-ones contributes to herbivore niche differentiation in maize. *Plant Cell Environ.* 38, 1081–1093. doi: 10.1111/pce.12464

Koo, A. J. (2018). Metabolism of the plant hormone jasmonate: a sentinel for tissue damage and master regulator of stress response. *Phytochem. Rev.* 17, 51–80. doi: 10.1007/s11101-017-9510-8

Kundu, A., Mishra, S., and Vadassery, J. (2018). Spodoptera litura-mediated chemical defense is differentially modulated in older and younger systemic leaves of Solanum lycopersicum. *Planta* 248, 981–997. doi: 10.1007/s00425-018-2953-3

Lazebnik, J., Frago, E., Dicke, M., and van Loon, J. J. A. (2014). Phytohormone mediation of interactions between herbivores and plant pathogens. *J. Chem. Ecol.* 40, 730–41. doi: 10.1007/s10886-014-0480-7

Le Roy, J., Huss, B., Creach, A., Hawkins, S., and Neutelings, G. (2016). Glycosylation is a major regulator of phenylpropanoid availability and biological activity in plants. *Front. Plant Sci.* 7:735. doi: 10.3389/fpls.2016.00735

Leiss, K. A., Choi, Y. H., Abdel-Farid, I. B., Verpoorte, R., and Klinkhamer, P. G. L. (2009a). NMR Metabolomics of Thrips (Frankliniella occidentalis) resistance in senecio hybrids. *J. Chem. Ecol.* 35, 219–229. doi: 10.1007/s10886-008-9586-0

Leiss, K. A., Cristofori, G., van Steenis, R., Verpoorte, R., and Klinkhamer, P. G. L. (2013). An eco-metabolomic study of host plant resistance to Western flower thrips in cultivated, biofortified and wild carrots. *Phytochemistry* 93, 63–70. doi: 10.1016/j.phytochem.2013.03.011

Leiss, K. A., Maltese, F., Choi, Y. H., Verpoorte, R., and Klinkhamer, P. G. L. (2009b). Identification of chlorogenic acid as a resistance factor for thrips in chrysanthemum. *Plant Physiol.* 150, 1567–1575. doi: 10.1104/pp.109.138131

Luczynski, A., Isman, M. B., and Raworth, D. A. (1990). Strawberry foliar phenolics and their relationship to development of the twospotted spider mite. *J. Econ. Entomol.* 83, 557–563. doi: 10.1093/jee/83.2.557

Macel, M., Visschers, I. G. S., Peters, J. L., Kappers, I. F., de Vos, R. C. H., and van Dam, N. M. (2019). Metabolomics of thrips resistance in pepper (*Capsicum* spp.) reveals monomer and dimer acyclic diterpene glycosides as potential chemical defenses. *J. Chem. Ecol.* 45, 490–501. doi: 10.1007/s10886-019-01074-4

Mangandi, J., Verma, S., Osorio, L., Peres, N. A., van de Weg, E., and Whitaker, V. M. (2017). Pedigree-based analysis in a multiparental population of octoploid strawberry reveals QTL alleles conferring resistance to phytophthora cactorum. *G3 Genes Genomes Genet.* 7, 1707–1719. doi: 10.1534/g3.117.042119

Mao, Y. B., Liu, Y. Q., Chen, D. Y., Chen, F. Y., Fang, X., Hong, G. J., et al. (2017). Jasmonate response decay and defense metabolite accumulation contributes to age-regulated dynamics of plant insect resistance. *Nat. Commun.* 8, 1–13. doi: 10.1038/ncomms13925

Mezzetti, B., Giampieri, F., Zhang, Y. T., and Zhong, C. F. (2018). Status of strawberry breeding programs and cultivation systems in Europe and the rest of the world. *J. Berry Res.* 8, 205–221. doi: 10.3233/JBR-180314

Miyazaki, J., Stiller, W. N., Truong, T. T., Xu, Q., Hocart, C. H., Wilson, L. J., et al. (2014). Jasmonic acid is associated with resistance to twospotted spider mites in diploid cotton (Gossypium arboreum). *Funct. Plant Biol.* 41, 748–757. doi: 10.1071/FP13333

Mouden, S., Kappers, I. F., Klinkhamer, P. G. L., and Leiss, K. A. (2020). Cultivar variation in tomato seed coat permeability is an important determinant of jasmonic acid elicited defenses against western flower thrips. *Front. Plant Sci.* 11:1724. doi: 10.3389/fpls.2020.576505

Mouden, S., Klinkhamer, P. G. L., Choi, Y. H., and Leiss, K. A. (2017). Towards eco-friendly crop protection: natural deep eutectic solvents and defensive secondary metabolites. *Phytochem. Rev.* 16, 935–951. doi: 10.1007/s11101-017-9502-8

Mouden, S., and Leiss, K. A. (2021). Host plant resistance to thrips (Thysanoptera: Thripidae) – current state of art and future research avenues. *Curr. Opin. Insect Sci.* 45, 28–34. doi: 10.1016/j.cois.2020.11.011

Okada, K., Abe, H., and Arimura, G. I. (2015). Jasmonates induce both defense responses and communication in monocotyledonous and dicotyledonous plants. *Plant Cell Physiol.* 56, 16–27. doi: 10.1093/pcp/pcu158

Palmer, I. A., Shang, Z., and Fu, Z. Q. (2017). Salicylic acid-mediated plant defense: recent developments, missing links, and future outlook. *Front. Biol. (Beijing).* 12, 258–270. doi: 10.1007/s11515-017-1460-4

Pappas, M. L., Broekgaarden, C., Broufas, G. D., Kant, M. R., Messelink, G. J., Steppuhn, A., et al. (2017). Induced plant defences in biological control of arthropod pests: a double-edged sword. *Pest Manag. Sci.* 73, 1780–1788. doi: 10.1002/ps.4587

Park, C. H., Yeo, H. J., Park, Y. E., Chun, S. W., Chung, Y. S., Lee, S. Y., et al. (2019). Influence of chitosan, salicylic acid and jasmonic acid on phenylpropanoid

accumulation in germinated buckwheat (Fagopyrum esculentum moench). *Foods* 8:153. doi: 10.3390/foods8050153

Pieterse, C. M. J., Leon-Reyes, A., Van Der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Pieterse, C. M. J., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Puthoff, D. P., Holzer, F. M., Perring, T. M., and Walling, L. L. (2010). Tomato pathogenesis-related protein genes are expressed in response to trialeurodes vaporariorum and bemisia tabaci biotype B feeding. *J. Chem. Ecol.* 36, 1271–1285. doi: 10.1007/s10886-010-9868-1

Rahman, T., Spafford, H., and Broughton, S. (2010). Variation in preference and performance of Frankliniella occidentalis (Thysanoptera: Thripidae) on three strawberry cultivars. *J. Econ. Entomol.* 103, 1744–53.

Reitz, S. R., Gao, Y., Kirk, W. D. J., Hoddle, M. S., Leiss, K. A., and Funderburk, J. E. (2020). Invasion biology, ecology, and management of western flower thrips. *Annu. Rev. Entomol.* 65, 17–37. doi: 10.1146/annurev-ento-011019-024947

Rodriguez, P. A., Stam, R., Warbroek, T., and Bos, J. I. B. (2014). Mp10 and Mp42 from the aphid species myzus persicae trigger plant defenses in nicotiana benthamiana through different activities. *Mol. Plant-Microbe Interact.* 27, 30–39. doi: 10.1094/MPMI-05-13-0156-R

Roland, W. S. U., Gouka, R. J., Gruppen, H., Driesse, M., van Buren, L., Smit, G., et al. (2014). 6-methoxyflavanones as bitter taste receptor blockers for hTAS2R39. *PLoS ONE* 9:e94451. doi: 10.1371/journal.pone.0094451

Saavedra, G. M., Figueroa, N. E., Poblete, L. A., Cherian, S., and Figueroa, C. R. (2016). Effects of preharvest applications of methyl jasmonate and chitosan on postharvest decay, quality and chemical attributes of Fragaria chiloensis fruit. *Food Chem.* 190, 448–453. doi: 10.1016/j.foodchem.2015.05.107

Sampson, C. (2018). Sustainable management of the western flower thrips in strawberry crops. *Outlooks Pest Manag.* 29, 180–184. doi: 10.1564/v29_aug_08

Sampson, C., and Kirk, W. D. J. (2013). Can mass trapping reduce thrips damage and is it economically viable? Management of the western flower thrips in strawberry. *PLoS ONE* 8:e80787. doi: 10.1371/journal.pone.0080787

Sampson, C., and Kirk, W. D. J. (2016). Predatory mites double the economic injury level of Frankliniella occidentalis in strawberry. *BioControl* 61, 661–669. doi: 10.1007/s10526-016-9747-y

Scott Brown, A. S., Simmonds, M. S. J., and Blaney, W. M. (2002). Relationship between nutritional composition of plant species and infestation levels of thrips. *J. Chem. Ecol.* 28, 2399–2409. doi: 10.1023/A:1021471732625

Selig, P., Keough, S., Nalam, V. J., and Nachappa, P. (2016). Jasmonate-dependent plant defenses mediate soybean thrips and soybean aphid performance on soybean. *Arthropod. Plant. Interact.* 10, 273–282. doi: 10.1007/s11829-016-9437-9

Shi, X., Pan, H., Xie, W., Wang, S., Wu, Q., Chen, G., et al. (2017). Different effects of exogenous jasmonic acid on preference and performance of viruliferous *Bemisia tabaci* B and Q. *Entomol. Exp. Appl.* 165, 148–158. doi: 10.1111/eea.12635

Strzyzewski, I. L., Funderburk, J. E., Renkema, J. M., and Smith, H. A. (2021). Characterization of frankliniella occidentalis and frankliniella bispinosa (Thysanoptera: Thripidae) injury to strawberry. *J. Econ. Entomol.* 114, 794–800. doi: 10.1093/jee/toaa311

Tugizimana, F., Mhlongo, M., Piater, L., and Dubery, I. (2018). Metabolomics in plant priming research: the way forward? *Int. J. Mol. Sci.* 19:1759. doi: 10.3390/ijms19061759

Turner, J. G., Ellis, C., and Devoto, A. (2002). The jasmonate signal pathway. *Plant Cell* 14, S153–S164. doi: 10.1105/tpc.000679

Ullah, C., Unsicker, S. B., Fellenberg, C., Constabel, C. P., Schmidt, A., Gershenzon, J., et al. (2017). Flavan-3-ols are an effective chemical defense against rust infection. *Plant Physiol.* 175, 1560–1578. doi: 10.1104/pp.17.00842

Valenzuela-Riffo, F., Zúñiga, P. E., Morales-Quintana, L., Lolas, M., Cáceres, M., and Figueroa, C. R. (2020). Priming of defense systems and upregulation of MYC2 and JAZ1 genes after botrytis cinerea inoculation in methyl jasmonate-treated strawberry fruits. *Plants* 9:447. doi: 10.3390/plants9040447

van Haperen, P., Voorrips, R. E., van Kaauwen, M., van Eekelen, H. D. L. M., de Vos, R. C. H., van Loon, J. J. A., et al. (2021). Fine mapping of a thrips resistance QTL in Capsicum and the role of diterpene glycosides in the underlying mechanism. *Theor. Appl. Genet.* 1, 3. doi: 10.1007/s00122-021-03790-6

Vervoort, M., Melis, P., Hanssens, J., Craeye, S., Pisman, M., Smagghe, G., et al. (2017). "Thrips control with predatory mites *A. limonicus* and *A. swirskii* in different strawberry cultivation systems," in *Acta Horticulturae (International Society for Horticultural Science)*, ed Y. Desjardins (Leuven: ISHS (International Society for Horticultural Science)), 833–842. doi: 10.17660/ActaHortic.2017.1156.123

Walling, L. L. (2008). Avoiding effective defenses: strategies employed by phloem-feeding insects. *Plant Physiol.* 146, 859–866. doi: 10.1104/pp.107.113142

Wang, L., Ran, L., Hou, Y., Tian, Q., Li, C., Liu, R., et al. (2017). The transcription factor MYB115 contributes to the regulation of proanthocyanidin biosynthesis and enhances fungal resistance in poplar. *New Phytol.* 215, 351–367. doi: 10.1111/nph.14569

Wang, S. Y., and Lin, H. S. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J. Agric. Food Chem.* 48, 140–146. doi: 10.1021/jf9908345

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., et al. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signal. Behav.* 7, 1306–1320. doi: 10.4161/psb.21663

Warabieda, W., Markiewicz, M., and Wójcik, D. (2020). Mutual relations between jasmonic acid and acibenzolar-S-methyl in the induction of resistance to the two-spotted spider mite (Tetranychus urticae) in apple trees. *Exp. Appl. Acarol.* 82, 59–79. doi: 10.1007/s10493-020-00539-6

Warabieda, W., Miszczak, A., and Olszak, R. W. (2005). The influence of methyl jasmonate (JA-Me) and B-glucosidase on induction of resistance mechanisms of strawberry against two-spotted spider mite (Tetranychus urticae Koch.). *Commun. Agric. Appl. Biol. Sci.* 70, 829–836.

Yamamoto, M., Nakatsuka, S., Otani, H., Kohmoto, K., and Nishimura, S. (2000). (+)-Catechin acts as an infection-inhibiting factor in strawberry leaf. *Phytopathology* 90, 595–600. doi: 10.1094/PHYTO.2000.90.6.595

Yu, X., Zhang, W., Zhang, Y., Zhang, X., Lang, D., and Zhang, X. (2018). The roles of methyl jasmonate to stress in plants. *Funct. Plant Biol.* 46, 197–212. doi: 10.1071/FP18106

Zarate, S. I., Kempema, L. A., and Walling, L. L. (2007). Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. *Plant Physiol.* 143, 866–75. doi: 10.1104/pp.106.090035

Zuñiga, P. E., Castañeda, Y., Arrey-Salas, O., Fuentes, L., Aburto, F., and Figueroa, C. R. (2020). Methyl jasmonate applications from flowering to ripe fruit stages of strawberry (Fragaria × ananassa 'Camarosa') reinforce the fruit antioxidant response at post-harvest. *Front. Plant Sci.* 11:538. doi: 10.3389/fpls.2020.00538

Conflict of Interest: 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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 July 2021
doi: 10.3389/fpls.2021.692924

Field-Grown Rice Plants Become More Productive When Exposed to Artificially Damaged Weed Volatiles at the Seedling Stage

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It is known that undamaged plants that have been exposed to volatiles from damaged con- or heterospecific plants become more resistant against herbivores. This is one of the plants' induced resistant responses against herbivores. To test whether this response can be used for rice production, we conducted the following experiments over 2 years (2012 and 2013). Rice seedlings were first planted in the rice seedling bed for 2 weeks in early May. There, half of the rice seedlings were exposed to artificially damaged weed volatiles three times for 12 days (treated plants). Weeds were randomly collected from the areas that were >100 m away from the seedling bed and the rice paddy fields. The remaining seedlings were not exposed (control plants). In the middle of May, bunches (ca. three seedlings per bunch) were transplanted to the rice paddy field. In July, leaf damage was observed. The total number of leaves in the treated and control plants was not significantly different. In contrast, the total number of damaged leaves in the treated plants was significantly lower than that in the control plants. In September, rice grains were harvested. The average weight of a rice grain from the treated and control plants was not significantly different. However, the weight of grains per bunch of treated plants was significantly higher than that of control plants; this indicated a significant increase of the number of grains by 23% in 2012 and by 18% in 2013 in the treated plants compared to that in the control plants. The volatiles emitted from the weeds included monoterpenoids (40.4% in total), green leaf volatiles (46.5%), short-chain alcohols (5.3%), short-chain ketone (5.4%), short-chain acetate (0.5%), short-chain aldehyde (1.1%), and hydrocarbon (0.7%). These results suggest that exposure of volatiles from artificially damaged weeds to rice seedlings has the potential to increase rice production.

Keywords: rice, artificially damaged plant volatiles, weeding, green leaf volatiles, terpenoids, *Oryza sativa* subsp. *japonica*

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Metabolism and Chemodiversity, a section of the journal Frontiers in Plant Science

Received: *09 April 2021*
Accepted: *18 June 2021*
Published: *12 July 2021*

Citation:
Shiojiri K, Ozawa R, Uefune M and Takabayashi J (2021) Field-Grown Rice Plants Become More Productive When Exposed to Artificially Damaged Weed Volatiles at the Seedling Stage. Front. Plant Sci. 12:692924. doi: 10.3389/fpls.2021.692924

INTRODUCTION

In response to damages caused by herbivorous arthropods, plants start emitting herbivory-induced plant volatiles (HIPVs) (Takabayashi and Shiojiri, 2019). When uninfested conspecific plants received HIPVs, they become more defensive against herbivores [for review see Karban et al. (2014) and Yoneya and Takabayashi (2016)]. Besides responding to HIPVs, plants also respond to volatiles

from artificially damaged plants. These phenomena are called "priming of plant resistant by plant volatiles." Under laboratory conditions, green leaf volatiles (GLVs), which are one of the common volatiles emitted from herbivore-damaged and artificially damaged plants, have been shown to induce resistant responses in *Arabidopsis thaliana*, *Citrus jambhiri* (rough lemon), *Zea mays* (corn), and *Nicotiana attenuata* (wild tobacco) (Bate and Rothstein, 1998; Gomi et al., 2003; Engelberth et al., 2004; Farag et al., 2005; Kishimoto et al., 2005, 2006; Paschold et al., 2006; Yamauchi et al., 2018). When undamaged pyrethrum plants (*Chrysanthemum cinerariifolium*) were exposed to a blend of volatiles (GLVs and a sesquiterpene) emitted from artificially damaged conspecific leaves, the amount of pyrethrin in the exposed plants increased significantly compared to that in plants that were not exposed (Kikuta et al., 2011). Under field conditions, sagebrush plants (*Artemisia tridentata*) exposed to volatiles from artificially damaged conspecific plants suffered less damage compared to unexposed conspecifics (Karban et al., 2006). The priming is also observed between heterospecific plants (Farmer and Ryan, 1990; Oudejans and Bruin, 1995; Karban et al., 2003). Under laboratory conditions, exposure of undamaged cucumber plants (*Cucumis sativus*) to HIPVs from spider mite-infested Lima bean (*Phaseolus lunatus*) plants resulted in the attraction of predatory mites that preyed on spider mites (Oudejans and Bruin, 1995). Under field conditions, Karban et al. (2003) showed an induced resistance in tobacco when sagebrush neighbors were clipped either with scissors or damaged with herbivores.

Weeding is a common practice in agricultural fields. During this practice, a large amount of artificially damaged weed volatiles is spread over the surrounding areas, including agricultural fields, thus exposing the surrounding crops to artificially damaged weed volatiles. After weeding, the exposed crops are expected to be more resistant than non-exposed crops to herbivores (priming of plant resistance by volatiles from heterospecific plants). Recently, this possibility was demonstrated in field-grown black soybeans and yellow soybeans (*Glycine max*); when young soybean plants were exposed to artificially damaged goldenrod volatiles, both plants and their grains became more resistant against herbivores (Shiojiri et al., 2017, 2020).

Sticky rice (*Oryza sativa* subsp. *japonica*) is an important food source worldwide. In Japan, weeding around rice paddy fields is conducted in spring to remove Gramineae weeds, which host many insect pests of rice plants. This weeding results in the rice seedlings being exposed to weeding-related volatiles. In the present study, we investigated whether such exposure affected rice grain production in pesticide-free rice paddy fields. We discussed the potential of using weeding-related volatiles in yield control in rice production.

MATERIALS AND METHODS

Experimental Conditions

Field experiments were carried out during 2012 and 2013 in pesticide-free commercial rice paddy fields owned by a farmer in Makino, Shiga, Japan. Rice plants (*O. sativa* subsp. *japonica* var. Koshihikari) were cultivated in eight paddy fields. Approximately 15,000 rice bunches (2–3 plants per bunch) were planted in each paddy field (20 m × 50 m). Four paddy fields (A, B, C, and D) were set together, while the other four (E, F, G, and H) were set at another site; the two sites were located ca. 200 m away from each other. At each site, we established treatment and control fields. A seedling bed (1 m × 15 m) was used to grow rice seedlings before transplanting them to the paddy fields.

Odor Sources

Several species of weeds growing in areas that were >100 m away from the rice paddy fields and from the rice seedling bed were used as odor sources. In both years, we observed weeds belonging to more than six families (including Asteraceae, Equisetaceae, Caryophyllaceae, Fabaceae, Poaceae, and others) and identified the following four major species: *Picris hieracioides* var. *glabrescens* (Asteraceae; Cichorieae), *Artemisia indica* Willd. var. *maximowiczii* (Asteraceae), *Equisetum arvense* (Equisetaceae), and *Trifolium repens* (Fabaceae; Trifolieae). We randomly collected weeds (5 kg) in the areas and divided them equally into ten portions. Each portion was placed in a mesh bag (45 cm × 35 cm). The composition of weed species used for the experiments was the same during the 2-year experiments. The used weeds were perennial plants and no herbicides were used in two consecutive years.

Experimental Procedures

To facilitate the exposure experiments, we conducted experiments in a rice seedling bed, because plant defenses for future growth are often developed at the seedling stage (Barton and Koricheva, 2010). We divided the seedling bed into two parts (the control area and the exposed area) with a polycarbonate board (ca. 1 m high and 2 m long) to prevent weed volatiles from reaching the untreated seedlings. When the seedlings in the bed were ca. 3–5 cm high, half of them (ca. 150,000 seedlings) were exposed to weeding-related volatiles. Ten mesh bags containing cut weeds (ca. 500 g per bag) were hung equidistantly in the exposed area of the seedling bed. The cut weeds were replaced with new ones every 4 days for 12 days. In 2012, after the exposure, the treated and control rice seedlings (ca. 10 cm high) were transplanted into rice paddy fields: the exposed seedlings were planted in A and H fields, and the control seedlings were placed in D and E fields. B, C, F, and G fields were used for other experiments. In 2013, the exposed seedlings were placed in B, D, E, and G fields, and the control seedlings were placed in A, C, F, and H fields.

Approximately 50 days after the transplantation, we collected the leaves from 15 treated and 15 control rice bunches randomly selected [except for field B (treated) in 2013, $N = 14$] to evaluate the number of damaged leaves on each paddy field.

In the rice paddy field used in this study, we observed commonly found herbivorous arthropods, such as rice leaf beetles (*Oulema oryzae*), rice leaf folders (*Cnaphalocrocis medinalis*), rice green caterpillars (*Naranga aenescens*), and Japanese

grasshoppers (*Oxya yezoensis*). Rice leaf beetles and rice leaf folders made similar fed-edge leaving leaf veins after herbivory. Rice green caterpillars and Japanese grasshoppers made different fed-edge because they did not leave leaf veins. In the field observation, we could not identify the herbivore species based on the fed-edges on damaged leaves. The two types of fed-edges were evaluated together as damaged leaves.

After harvest, we measured the weight of rice grains from 15 treated, and 15 control rice bunches randomly selected. We weighted 100 grains per bunch. Because each bunch had approximately 8,000–10,000 grains, we were unable to count the number of grains per bunches on site. The detailed procedure of the experiments is presented in **Supplementary Table 1**.

Chemical Analysis

On May 12, 2013, in order to analyze the chemical structure of weeding-related volatiles, we used ca. 100 g of cut weeds per mesh bag from five randomly selected mesh bags that were used for the exposure experiments. After volatile collection, we returned them to the mesh bag. We placed the samples in polyethylene terephthalate (PET) bags (180 mm × 250 mm) (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). To collect volatiles, we sent air (100 mL/min airflow) into each PET bag containing weeds. At the outlet of each bag, we set a tube containing Tenax-TA (3.0 mm internal diameter, 160 mm long with 100 mg, GL Sciences Inc., Tokyo, Japan) to collect volatiles for 1 h. We analyzed the collected volatiles by gas chromatography/mass spectrometry (GC/MS) (GC: Agilent 6890 with an HP-5MS capillary column: 30 m long, 0.25-mm i.d., and 0.25-μm film thickness; MS: Agilent 5973 mass selective detector, 70 eV with helium as the carrier gas; Agilent, Santa Clara, CA, United States) equipped with a thermal desorption cold trap injector (TCT; CP4010; Chrompack, Middelburg, Netherlands). We programmed the oven temperature of the

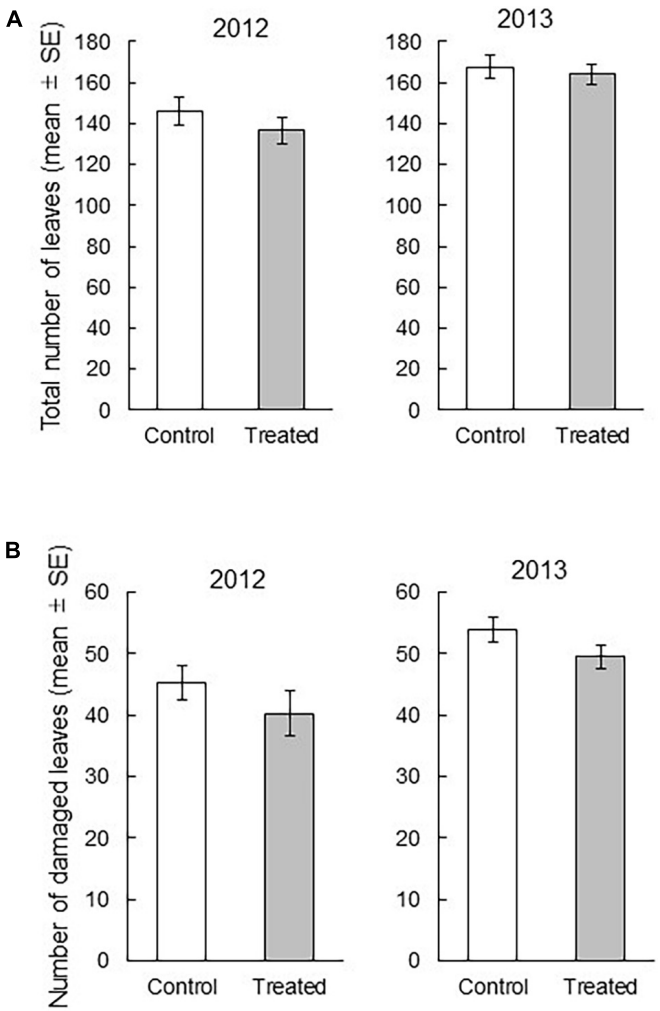

FIGURE 1 | (A) Comparison between the total number of leaves in a treated bunch and that in a control bunch (treatment $P = 0.3147$, year $P = 0.0001$, treatment × year $P = 0.7884$), and **(B)** the comparison between the total number of damaged leaves in a treated bunch and that in a control bunch (treatment $P = 0.0468$, year $P = 0.0003$, treatment × year $P = 0.8564$). The effects of treatment, year, and their interaction on the number of leaves were analyzed with the least square mixed model after Box-Cox transformation. $N = 15$, except for the data of one field in 2013 ($N = 14$).

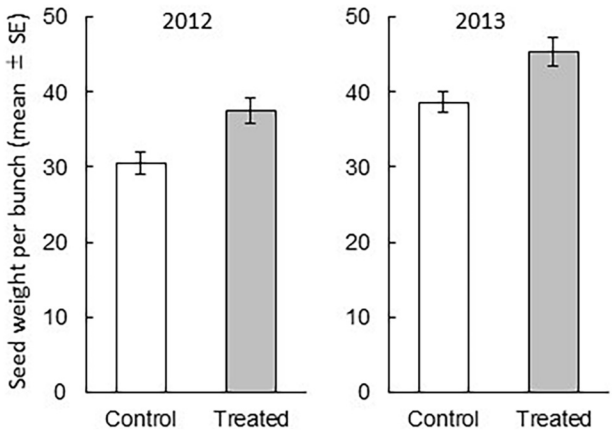

FIGURE 2 | Comparison between the rice grain weight (mean ± SE) from a treated bunch and that from a control bunch (treatment $P = 0.0073$, year $P < 0.0001$, treatment × year $P = 0.9126$). The effects of treatment, year, and their interaction on grain weight per bunch were analyzed with the least square mixed model after Box-Cox transformation. $N = 15$, except for the data of one field in 2013 ($N = 14$).

gas chromatograph to increase from 40°C (5 min hold) to 280°C at 15°C/min. We identified the compounds by comparing their mass spectra with those of compounds from the Wiley7N database (John Wiley & Sons Inc., Hoboken, NJ, United States) and their retention times and mass spectra with those of synthetic compounds.

Statistical Analyses

The effects of treatment, year, and their interaction on the number of leaves and grain weight per bunch were analyzed with the least square mixed model after Box-Cox transformation in JMP version 14.2.0 (SAS Institute, 2018). The rice paddy field was a random effect in all models. The weight of 100 grains was analyzed with a *t*-test in JMP version 14.2.0.

RESULTS

Leaf Damage

We found that the treatment and the interaction of treatment × year did not significantly influence the total number of leaves per bunch (treatment: df = 1, 159.6, $F = 1.0171$, $P = 0.3147$; interaction: df = 1, 7.138, $F = 0.0777$, $P = 0.7884$), whereas the year had highly significant influence on the total number of leaves per bunch (df = 1, 159.6, $F = 15.2639$, $P = 0.0001$) (**Figure 1A**). In contrast, we found that the treatment and year had significant influence on the number of damaged leaves per bunch (treatment: df = 1, 173.2, $F = 4.008$, $P = 0.0468$, year: df = 1, 173.2, $F = 13.3238$, $P = 0.0003$), whereas their interaction did not (df = 1, 6.763, $F = 0.0354$, $P = 0.8564$). These results indicated that over 2 years, the number of damaged leaves was significantly lower in the exposed rice plants than in the control rice plants (**Figure 1**). The relative decrease of the damaged leaves in the exposed plants compared to that in control plants of respective year was 11.1% in 2012 and 9.8% in 2013.

Grain Weight

We found that the treatment and year had significant influence on the grain weight per bunch (treatment: df = 1, 174, $F = 7.3799$, $P = 0.0073$, year: df = 1, 174, $F = 16.2277$, $P < 0.0001$), whereas their interaction did not (df = 1, 5.838, $F = 0.0131$, $P = 0.9126$) (**Figure 2**). In addition, we measured the weight of 100 grains to obtain the weight of one rice grain. In both years, the weight of a grain from treated bunches (mean ± SE) was not significantly different from that from the control bunches (2012: the control bunch = 2.683 ± 0.026; the treated bunch = 2.659 ± 0.028, $P = 0.476$; 2013: the control bunch = 2.684 ± 0.024; the treated bunch = 2.633 ± 0.033, $P = 0.251$). These results indicated that over 2 years, the weight of rice grains per bunch was significantly higher in the treated rice plants than in the control rice plants. The increase in grain weight per bunch was explained by the increase of the number of grains. The relative increase of the number of grains in the exposed plants were 23% in 2012 and 15.9% in 2013 compared to that in control rice plants of respective year.

Chemical Analyses

The headspace of volatiles emitted from the five odor sources was analyzed separately. We found 26 compounds in all the odor sources (**Table 1**). The compounds were classified into monoterpenoids (12 compounds, 40.4% in total), green leaf volatiles (eight compounds, 46.5%), short-chain (C5) alcohols (two compounds, 5.3%), short-chain (C5) ketone (5.4%), short-chain (C5) acetate (0.5%), short-chain (C9) aldehyde (1.1%), and hydrocarbon (0.7%). The major compounds were (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, α-pinene, camphene, β-pinene, β-myrcene, 1,8-cyneole, and pentene-3-one.

DISCUSSION

Artificially damaged weeds set in the seedling bed emitted 26 volatile compounds, including green leaf volatiles (GLVs), monoterpenoids, short-chain (C5) alcohol/ketone/acetate, a short-chain (C9) aldehyde, and a hydrocarbon (**Table 1**). The exposure of rice seedlings to these volatiles resulted in a significant increase in the number of rice grains (23% increase in 2012 and 18% increase in 2013 compared to that in control rice plants). It is important to note that the exposure was only three times with an interval of 4 days at the seedling stage (from ca. 5 cm to ca. 10 cm plant height), indicating that the exposure at the rice seedling stage affected the rice grain production.

One of the possible reasons for the increase would be the reduction of the leaf damages in the exposed plants. Although the damaged leaves of the exposed rice plants were significantly lower than that of the control rice plants, the differences were relatively small (11.1% reduction in 2012 and 9.8% reduction in 2013). In this study, we did not evaluate the behavior of

TABLE 1 | The volatile organic compounds in the headspace of artificially damaged weed.

Compounds	Peak area/gFW ($\times 10^{-3}$)
Alcohols	
1-penten-3-ol	3, 086 ± 244
(*Z*)-2-penten-l-ol	492 ± 60
(*Z*)-3-hexen-l-ol	14, 963 ± 1, 381
(*E*)-2-hexen-l-ol	2, 146 ± 598
hexan-1-ol	1, 004 ± 109
Aldehydes	
(*Z*)-3-hexanal	3, 760 ± 800
Hexanal	713 ± 44
(*E*)-2-hexenal	1486 ± 414
nonanal	731 ± 126
Acetates	
(*Z*)-2-penten-1-yl acetate	364 ± 39
(*Z*)-3-hexen-1-yl acetate	6, 692 ± 634
(*E*)-2-hexen-l-yl acetate	722 ± 95
Terpenoids	
tricyclene	538 ± 155
α-thujene	373 ± 52
α-pinene	6, 336 ± 1, 222
camphene	5, 408 ± 1, 368
β-pinene	5, 975 ± 1, 570
β-myrcene	2, 280 ± 557
α-phellandrene	534 ± 250
α-terpinene	826 ± 175
limonene	1, 478 ± 284
1,8-cineole	2, 300 ± 830
γ-terpinene	955 ± 463
terpinolene	357 ± 69
Others	
pentan-3-one	3, 670 ± 429
1-octene	494 ± 216

Data are represented by mean ± standard error.

the herbivorous insects observed in the rice paddy fields. The mechanisms involved in the reduction of the damage made by herbivores insects remains necessary.

The differences in the rice grain production between the control and treated plants might also be explained by other factors, such as effects of fungi/viruses, growth, nutrition intake, photosynthesis, which might be affected by the volatiles either positively or negatively. As we did not find disease symptoms on leaves, and the farmer did not mention to us disease problems during the experimental periods, the involvement of fungi and viruses was unlikely. Whether other factors were involved in the increase of performance in rice plants is worth evaluating, as previous studies showed that volatiles affect plant physiology. (*E*)-2-Hexenal acts as signal chemicals that strongly induce the gene expression of abiotic-related transcription factors in *Arabidopsis* (Yamauchi et al., 2015). Inhibitory effects on the growth of volatile compounds have been reported in *Arabidopsis*, for example, on root length in *Arabidopsis* (Bate and Rothstein, 1998; Mirabella et al., 2008; Scala et al., 2017). Horiuchi et al. (2007) also reported that monoterpenoids, borneol, and bornyl acetate reduced the root length of *Arabidopsis*.

The origins and the principal compounds involved in the increased rice grain production remained unknown. Among them, GLVs are commonly found in green plants whose leaves underwent mechanical damage. GLVs have been reported as signaling molecules in the elicitation of priming in several plant species (Bate and Rothstein, 1998; Gomi et al., 2003; Engelberth et al., 2004; Farag et al., 2005; Kishimoto et al., 2005, 2006; Paschold et al., 2006; Yamauchi et al., 2018). Volatile terpenoids are also involved in the priming. Soybean plants previously exposed to a blend of α-pinene, β-myrcene, and limonene became more defensive against common cutworms (*Spodoptera litura* larvae) (Shiojiri et al., 2017). 1.8-Cineol, constitutively released from mint plants (*Mentha* × *piperita*), was suggested to be one of the signaling molecules that induce resistant responses in soybean plants (Sukegawa et al., 2018). Riedlmeier et al. (2017) reported that a mixture of α-pinene and β-pinene induced resistant responses in *Arabidopsis*.

Detailed studies on the relationship between the effects of the volatile exposure to rice seedlings and the increased production of rice grains, especially focusing on the mechanism involved in the long-lasting effects, are needed. The effects of the timing of the exposure (i.e., which growth stages are most effective) should also be considered. Further, for achieving an effective exposure-related increase of rice grain production, it is worthwhile to evaluate whether specific weed groups (specific families or genera) are needed for the exposure, or if any weeds in the areas surrounding rice paddy fields can be used for the exposure. Thus, our findings suggest that exposure of volatiles from mechanically damaged weeds to rice seedlings has the potential to increase rice production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KS designed and conducted the field experiments. RO conducted the field experiments and the chemical analyses. MU conducted the statistical analyses. KS, RO, MU, and JT analyzed the data and wrote the manuscript. All authors gave final approval of the manuscript for publication.

FUNDING

This study was in part supported by grants for the scientific research (A) (grant number 18H03952) from the Ministry of Education, Culture, Sports, Science and Technology, and in part

by the Research Institute for Food and Agriculture of Ryukoku University (grant number FA1906).

ACKNOWLEDGMENTS

We would like to thank Asato Fukui who kindly allowed us to use his rice paddy fields for experiments, and Yoshitaka Nakashima, Kaoru Tsuji, Eizi Yano for their comments on the data. We would also like to thank Kikumi Katami and Ayako Masaoka for helping to collect data. This study was partly supported by Center for Ecological Research, Kyoto University, a Joint Usage/Research Center.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.692924/full#supplementary-material

REFERENCES

Barton, K. E., and Koricheva, J. (2010). The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. *Am. Nat.* 175, 481–493. doi: 10.1086/650722

Bate, N. J., and Rothstein, S. J. (1998). C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. *Plant J.* 16, 561–569. doi: 10.1046/j.1365-313x.1998.00324.x

Engelberth, J., Alborn, H. T., Schmelz, E. A., and Tumlinson, J. H. (2004). Airborne signals prime plants against insect herbivore attack. *Proc. Natl. Acad. Sci. U. S. A.* 101, 1781–1785. doi: 10.1073/pnas.0308037100

Farag, M. A., Fokar, M., Abd, H., Zhang, H., Allen, R. D., and Pareì, P. W. (2005). (Z)-3-Hexenol induces defense genes and downstream metabolites in maize. *Planta* 220, 900–909. doi: 10.1007/s00425-004-1404-5

Farmer, E. E., and Ryan, C. A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl Acad. Sci. U. S. A.* 87, 7713–7716. doi: 10.1073/pnas.87.19.7713

Gomi, K., Yamasaki, Y., Yamamoto, H., and Akimitsu, K. (2003). Characterization of a hydroperoxide lyase gene and effect of C6-volatiles on expression of genes of the oxylipin metabolism in Citrus. *J. Plant Physiol.* 160, 1219–1231. doi: 10.1078/0176-1617-01177

Horiuchi, J., Muroi, A., Takabayashi, J., and Nishioka, T. (2007). Exposing Arabidopsis seedlings to borneol and bornyl acetate affects root growth: specificity due to the chemical and optical structures of the compounds. *J. Plant Interact.* 2, 101–104. doi: 10.1080/17429140701575624

Karban, K., Shiojiri, K., Huntzinger, M., and Mccall, A. C. (2006). Damage-induced resistance in sagebrush: volatiles are key to intra- and interplant communication. *Ecology* 87, 922–930. doi: 10.1890/0012-9658(2006)87[922:drisva]2.0.co;2

Karban, R., Maron, J., Felton, G. W., Ervin, G., and Eichenseer, H. (2003). Herbivore damage to sagebrush induces resistance in wild tobacco: evidence for eavesdropping between plants. *Oikos* 100, 325–332. doi: 10.1034/j.1600-0706.2003.12075.x

Karban, R., Yang, L. H., and Edwards, K. F. (2014). Volatile communication between plants that affects herbivory: a meta-analysis. *Ecol. Lett.* 17, 44–52. doi: 10.1111/ele.12205

Kikuta, Y., Ueda, H., Nakayama, K., Katsuda, Y., Ozawa, R., Takabayashi, J., et al. (2011). Specific regulation of pyrethrin biosynthesis in Chrysanthemum cinerariaefolium by a blend of volatiles emitted from artificially damaged conspecific plants. *Plant Cell Physiol.* 52, 588–596. doi: 10.1093/pcp/pcr017

Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2005). Volatile C6-aldehydes and allo-ocimene activate defense genes and induce resistance against Botrytis cinerea in Arabidopsis thaliana. *Plant Cell Physiol.* 46, 1093–1102. doi: 10.1093/pcp/pci122

Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2006). Components of C6-aldehyde-induced resistance in Arabidopsis thaliana against a necrotrophic fungal pathogen, Botrysis cinerea. *Plant Sci.* 170, 715–723. doi: 10.1016/j.plantsci.2005.11.002

Mirabella, R., Rauwerda, H., Struys, E. A., Jakobs, C., Triantaphylides, C., Haring, M. A., et al. (2008). The Arabidopsis her1 mutant implicates GABA in E-2-hexenal responsiveness. *Plant J.* 53, 197–213. doi: 10.1111/j.1365-313X.2007.03323.x

Oudejans, A. M. C., and Bruin, J. (1995). Does spider-mite damage induce information transfer between plants of different species? *Med. Fac. Landbouww. Univ. Gent.* 59, 733–739.

Paschold, A., Halitschke, R., and Baldwin, I. T. (2006). Using 'mute' plants to translate volatile signals. *Plant J.* 45, 275–291. doi: 10.1111/j.1365-313X.2005.02623.x

Riedlmeier, M., Ghirardo, A., Wenig, M., Knappe, C., Kerstin, K., Georgii, E., et al. (2017). Monoterpenes support systemic acquired resistance within and between plants. *Plant Cell* 29, 1440–1459. doi: 10.1105/tpc.16.00898

SAS Institute (2018). *JMP version 14.2.0*. Cary, NC: SAS Institute Inc.

Scala, A., Mirabella, R., Goedhart, J., de Vries, M., Haring, M. A., and Schuurink, R. C. (2017). Forward genetic screens identify a role for the mitochondrial HER2 in E-2-hexenal responsiveness. *Plant Mol. Biol.* 95, 399–409. doi: 10.1007/s11103-017-0659-8

Shiojiri, K., Ozawa, R., Yamashita, K., Uefune, M., Matsui, K., Tsukamoto, C., et al. (2017). Weeding volatiles reduce leaf and seed damage to field-grown soybeans and increase seed isoflavones. *Sci. Rep.* 7:41508. doi: 10.1038/srep41508

Shiojiri, K., Ozawa, R., Yamashita, K., Uefune, M., Matsui, K., Tsukamoto, C., et al. (2020). Exposure to artificially damaged goldenrod volatiles increases saponins in seeds of field-grown soybean plants. *Phytochem. Lett.* 36, 7–10. doi: 10.1016/j.phytol.2020.01.014

Sukegawa, S., Shiojiri, K., Higami, T., Suzuki, S., and Arimura, G. (2018). Pest management using mit volatiles to elicit resistance in soy: mechanism and application potential. *Plant J.* 96, 910–920. doi: 10.1111/tpj.14077

Takabayashi, J., and Shiojiri, K. (2019). Multifunctionality of herbivory-induced plant volatiles in chemical communication in tritrophic interactions. *Curr. Opin. Insect Sci.* 32, 110–117.

Yamauchi, Y., Kunishima, M., Mizutani, M., and Sugimoto, Y. (2015). Reactive short-chain leaf volatiles act as powerful inducers of abiotic stress-related gene expression. *Sci. Rep.* 5:8030. doi: 10.1038/srep08030

Yamauchi, Y., Matsuda, A., Matuura, N., Mizutani, M., and Sugimoto, Y. (2018). Transcriptome analysis of Arabidopsis thaliana treated with green leaf volatiles: possible role of green leaf volatiles as self-made damage-associated molecular patterns. *J. Pestic. Sci.* 43, 207–213. doi: 10.1584/jpestics.D18-020

Yoneya, K., and Takabayashi, J. (2016). Plant–plant communication mediated by airborne signals: ecological and plant physiological perspectives. *Plant Biotech.* 31, 409–416. doi: 10.5511/plantbiotechnology.14.0827a

Conflict of Interest: 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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 July 2021
doi: 10.3389/fpls.2021.695867

Comparing Exogenous Methods to Induce Plant-Resistance Against a Bark-Feeding Insect

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Exogenous application of the plant hormone methyl jasmonate (MeJA) can trigger induced plant defenses against herbivores, and has been shown to provide protection against insect herbivory in conifer seedlings. Other methods, such as mechanical damage to seedlings, can also induce plant defenses, yet few have been compared to MeJA and most studies lack subsequent herbivory feeding tests. We conducted two lab experiments to: (1) compare the efficacy of MeJA to mechanical damage treatments that could also induce seedling resistance, (2) examine if subsequent insect damage differs depending on the time since induction treatments occurred, and (3) assess if these induction methods affect plant growth. We compared Scots pine (*Pinus sylvestris*) seedlings sprayed with MeJA (10 or 15 mM) to seedlings subjected to four different mechanical bark damage treatments (two different bark wound sizes, needle-piercing damage, root damage) and previous pine weevil (*Hylobius abietis*) damage as a reference treatment. The seedlings were exposed to pine weevils 12 or 32 days after treatments (early and late exposure, hereafter), and resistance was measured as the amount of damage received by plants. At early exposure, seedlings treated with needle-piercing damage received significantly more subsequent pine weevil feeding damage than those treated with MeJA. Seedlings treated with MeJA and needle-piercing damage received 84% less and 250% more pine weevil feeding, respectively, relative to control seedlings. The other treatments did not differ statistically from control or MeJA in terms of subsequent pine weevil damage. For the late exposure group, plants in all induction treatments tended to receive less pine weevil feeding (yet this was not statistically significant) compared to control seedlings. On the other hand, MeJA significantly slowed down seedling growth relative to control and all other induction treatments. Overall, the mechanical damage treatments appeared to have no or variable effects on seedling resistance. One of the treatments, needle-piercing damage, actually increased pine weevil feeding at early exposure. These results therefore suggest that mechanical damage shows little potential as a plant protection measure to reduce feeding by a bark-chewing insect.

Keywords: simulated herbivory, root damage, methyl jasmonate, forest regeneration, true insect herbivory, wounding

OPEN ACCESS

Edited by:
Toby J. A. Bruce,
Keele University, United Kingdom

Reviewed by:
Maria L. Pappas,
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *15 April 2021*
Accepted: *28 June 2021*
Published: *20 July 2021*

Citation:
Chen Y, Puentes A, Björkman C, Brosset A and Bylund H (2021) Comparing Exogenous Methods to Induce Plant-Resistance Against a Bark-Feeding Insect. Front. Plant Sci. 12:695867. doi: 10.3389/fpls.2021.695867

INTRODUCTION

Induced plant defenses can be triggered experimentally by exogenous application of methyl jasmonate (MeJA), a hormone naturally present in plants. MeJA is a methyl ester of jasmonic acid (JA), which is involved in one of three signaling pathways mediating stress responses in plants. These pathways are (1) the octadecanoid pathway, which relies on JA, (2) the shikimate pathway which mainly involves salicylic acid (SA), and (3) the ethylene pathway, which relies on ethylene molecules (Dicke and van Poecke, 2002; Kant et al., 2015). The octadecanoid pathway is most important for defense responses following insect damage (McConn et al., 1997; Kahl et al., 2000). In particular, MeJA has been shown to be involved in several plant processes such as root growth, damage signaling, and promoting plant defenses against chewing herbivores and necrotrophic pathogens (Creelman and Mullet, 1995; Thaler et al., 2012). Given its role in defense induction, exogenous MeJA application is increasingly being proposed as a plant protection strategy against various insect pests and pathogens (Moreira et al., 2012b; Zas et al., 2014; Yu et al., 2019). Inducing defenses with MeJA prior to exposure to pests has been shown to reduce levels of damage, negatively affect herbivores and increase the likelihood of plant survival. These effects have been found to occur not only in crops such as rice and soybean (Chen et al., 2018; Senthil-Nathan, 2019), but also in conifer seedlings (Zas et al., 2014; Jiang et al., 2016). Thus, it has great potential to become a practical tool within pest management and a sustainable alternative to insecticides in conifer plant protection.

Before MeJA can be promoted as a practical plant protection measure, it is necessary to consider how the use of MeJA compares to other methods of plant defense induction. Other methods to trigger plant induced defenses include previous insect feeding and mechanical damage (Mattiacci et al., 1994); but little is known on whether these responses are comparable to those induced by MeJA (Moreira et al., 2012b). Simulated herbivory, mainly by mechanical wounding and true insect herbivory, has been shown to cause defense-related responses in several plants, e.g., *Nicotiana sylvestris*, *Pinus resinosa*, and *Arabidopsis thaliana* (Baldwin, 1988; Mattiacci et al., 1994; Lombardero et al., 2006; Herde et al., 2013), and could potentially be used as a method of induction. Moreira et al. (2012b) showed that exogenous application of MeJA, mechanical stem wounding and real herbivory by the pine weevil *Hylobius abietis*, resulted in chemical defensive responses that were quantitatively similar in Maritime pine (*Pinus pinaster*) seedlings. Likewise, insect herbivory caused chemical and anatomical changes related to increased defense in Sitka spruce (*Picea sitchensis*) (Miller et al., 2005). In some plants, these defensive responses do not only happen in the damaged area, but also in undamaged parts (Wu and Baldwin, 2009). A few other studies have investigated the role of root damage on aboveground induced defenses. For example, in a study with oilseed rape (*Brassica napus*), belowground insect herbivory or mechanical damage to roots increased the proportion of indole glucosinolates in the leaves (Griffiths et al., 1994). Indole glucosinolates are defensive compounds that accumulate after damage in e.g., *A. thaliana* and other *Brassicacea* (Agerbirk et al., 2008). In general, less is known about the defense induction effects following belowground simulated herbivory (Erb et al., 2012).

Depending on the type and strength of the damage stimulus, plants can also be primed, so that once attack happens defense responses can occur more quickly and stronger (Wilkinson et al., 2019). Thus, it has been suggested that mechanical damage and previous insect herbivory can also serve as methods of defense induction to increase protection against insect pests. Regardless of the defense induction method used, most studies have focused on the extent of defensive chemical responses following induction (Miller et al., 2005; Moreira et al., 2012a), but very few have actually examined subsequent herbivory to corroborate that the induction method indeed provides efficient protection. To determine whether a method is suitable for plant protection against insect pests, a herbivore damage test following induction treatment is necessary.

Aside from its protective effects, induction methods that enhance plant resistance (such as MeJA) can result in a negative effect on growth. This trade-off can occur because plants have limited resources to be allocated among defense, growth, development and reproduction. Thus, when a plant invests more resources in defense, it is expected that less resources are available for other purposes (Herms and Mattson, 1992). Some studies have shown that application of MeJA/JA results in fewer fruits and seeds in tomato plants, and growth reductions in young conifers (Redman et al., 2001; Gould et al., 2009; Sampedro et al., 2011). In the case of tomatoes, however, fruits from JA-treated plants were larger than those from control plants (Redman et al., 2001). Thus, even if MeJA can result in a trade-off, a loss in growth or reproduction could be compensated by other benefits such as larger fruits or increased survival in the case of conifer seedlings (Redman et al., 2001; Zas et al., 2014). The effects of other induction methods on growth are less known, thus it would be of interest to investigate how such effects compare to those of MeJA.

Here, we examined and compared the efficacy of MeJA to other plant-resistance inducing methods, i.e., various kinds of mechanical damage and true insect herbivory, in providing plant protection against a bark-chewing insect. As a model system, we used the pine weevil-conifer seedling system as different studies have shown that application of MeJA enhances resistance of conifer seedlings against this herbivore (Zas et al., 2014; Fedderwitz et al., 2016; López-Goldar et al., 2020). Moreover, a study in *Pinus pinaster* examined chemical responses following mechanical stem damage, true insect herbivory and MeJA treatment, and the results showed that these induction methods all increased chemical responses to an equivalent magnitude (Moreira et al., 2012a). It would be interesting to test whether those observed changes in defensive chemistry eventually result in less insect feeding. The pine weevil, *H. abietis* (Coleoptera: Curculionidae), is an important pest of planted conifer seedlings at regeneration sites where forest stands are clear-cut. Adult pine weevils are attracted to these sites, because they use conifer stumps as breeding substrate. If seedlings are

planted during the first 3 years after clear-cutting, the parental generation and their adult offspring will feed on the stem bark of these seedlings. The feeding can cause seedling deformations, reduced growth, and high seedling mortality (Leather et al., 1999). Given increasing restrictions on the use of insecticides due to environmental and human health issues (Lalík et al., 2020), there is timely incentive to explore methods of plant protection based on plants' intrinsic defenses and how they compare to each other.

Another factor that could be essential, yet rarely addressed in other studies, is the time interval from induction stimulus to exposure of plants to the insect pest. Various time intervals between MeJA treatment and exposure to the insect have been used in the pine weevil-conifer seedling system, with less pine weevil damage being observed a week, 1 month or even longer after MeJA treatment (Heijari et al., 2005; Sampedro et al., 2010; Fedderwitz et al., 2016; Chen et al., 2020). Several studies examining defensive chemical changes in conifer seedlings showed increased concentration of terpenes or resin 2 weeks, 4 weeks, or up to 1 month after MeJA treatment (Martin et al., 2002; Miller et al., 2005; Zas et al., 2014). Thus, time is also an important factor to consider when examining induced resistance responses after using different induction methods. For example, in a study with Loblolly pine (*Pinus taeda*), decreased resin flow was observed 1 day after wounding treatment but resin levels were higher than normal 30 days after mechanical treatment (Knebel et al., 2008). Thus, examining resistance at various time periods after induction stimulus could provide more comprehensive insight into how and when to apply these stimuli to achieve the greatest effect on plant resistance.

The purpose of the study was to investigate and compare how MeJA and other potential mechanical defense induction methods affect subsequent damage to conifer seedlings by the pine weevil. Resistance to pine weevil damage was used as a measure of the extent of induced resistance, with plants that were less damaged being considered to have experienced a greater induction following treatment. Additionally, we investigated if these effects depend on the time between induction stimulus (i.e., damage treatment) and exposure to weevils, and how the different treatments affect the growth of Scots pine seedlings. We chose two time intervals (12 and 32 days after stimulus) based on a pilot study and our previous studies on MeJA (e.g., Fedderwitz et al., 2016; Chen et al., 2020). Mechanical bark damage treatments (rectangular scars of different sizes inflicted on the stem, stem needle-piercing bark damage, and root damage) were chosen based on the types of damage that seedlings may encounter naturally, but exclude any chemical or microbial stimuli from the insect feeding. True insect herbivory was also included as a reference treatment. We intended to answer the following questions:

(1) How does bark mechanical damage and previous herbivory treatments influence the levels of pine weevil damage to seedlings, relative to treatment with MeJA?
(2) Do the effects of these treatments on pine weevil damage to seedlings differ depending on the time since induction occurred? More specifically, 12 and 32 days after treatment?
(3) How is seedling growth affected following these non-MeJA treatments relative to when MeJA is used?

MATERIALS AND METHODS

Insect Material

To examine the induced resistance of seedlings following different treatments, we conducted two experiments where we subsequently exposed treated and control seedlings to pine weevils. The experiments were conducted in 2017 and 2020. The pine weevils used in these experiments were collected on May 27, 2017 and May 31, 2020, respectively, at the same sawmill (Balungstrands Sågverk AB, Enviken, Sweden) during their yearly migration. Before experiments, the weevils were kept in wooden rearing boxes in constant darkness at a room temperature of 10°C. Stems and branches from young Scots pine trees, and water tubes were placed inside each box; food and water were replaced once every month.

One week before the experiment, the pine weevils were brought for acclimatization from the cool dark room to the lab, where feeding tests were conducted (light-dark cycle 16 L/8 D, room temperature). The weevils were placed in plastic buckets with ventilated lids, and supplied with water and Scots pine branches. Female pine weevils were selected and placed individually in a Petri dish with a small Scots pine branch piece for 24 h. Those that fed on the branch during this period were selected and placed all together in a bucket, supplied only with water, in order to starve for 48 h before each feeding test.

Experiment 1: Plant Induction Treatments and Subsequent Feeding Tests at One Time Point

In order to test differences in resistance against pine weevils using different potential defense induction methods, six treatments (and undamaged controls) were applied to plants in order to trigger the induced defense of Scots pine seedlings. Since our regular nursery did not have enough seedlings, two provenances of Scots pine seedlings, known as Hade (plant height: 6–8 cm, Stora Enso Plantor AB, Sör Amsberg, Sweden) and Gotthardsberg (plant height: 7.8–13.5 cm, Stora Enso Plantor AB, Sjögränd, Sweden), were obtained from two nurseries instead. On July 17, 2017, seedlings were planted in round plastic pots (diameter: 14 cm) with commercial standard gardening soil (S-Jord, Hasselfors garden, Sweden) and kept in a greenhouse (light-dark cycle 16L/8D, temperature 20/16°C) for 1 month until the different treatments were applied. These two provenances were sown approximately at the same time of the year, and plants were 1-year-old when they were used in the experiment. However, Gotthardsberg has its origin further south in Sweden relative to Hade, and was larger in size when they were delivered to the lab.

After inflicting different potential induction treatments, area debarked by pine weevils in a feeding test was used as an inverse measure of induced resistance (seedlings receiving less damage

were those considered to exhibit higher induced resistance). The following treatments were inflicted on plants 12 days before exposing them to pine weevils:

(1) Control (C): These seedlings (n = 34 for each provenance) received no damage at all.
(2) Methyl jasmonate treatment (MeJA): Seedlings (n = 34 for each provenance) were sprayed with MeJA. The concentration of MeJA (10 mM) was created by mixing MeJA (Sigma-Aldrich 95%, Ref. No. 392707) with a carrier solution of 2.5% (v:v) ethanol. Spraying was conducted once with a plastic bottle equipped with a spraying nozzle (Free-Syringe PC 1.5 liter, Jape Products AB, Hässleholm, Sweden), in a laboratory fume hood. The spraying bottle was pumped to reach the inner air pressure limit (2.5 bar) and shaken vigorously to mix the MeJA and carrier solution before each spraying occasion. Seedlings were placed in a row and the spraying nozzle was aimed horizontally at about 40 cm from the seedlings. The spraying bottle was moved manually along the seedling row, and the pots were turned 180° to spray the other side of the plants. Each seedling got approximately one second of spraying on each side, and all aboveground parts were moistened with the solution.
(3) Previous weevil feeding (WF): Seedlings (n = 34 for each provenance) were wounded by pine weevil feeding. One pine weevil was allowed to feed restrictively using a small custom-made cage with a transparent plastic tube (diameter: 10 mm; 25 mm long; and the top of the tube was sealed with holed plastic foil). A small opening was carved with a scalpel on each cage, to allow the pine weevil to feed on the area of the stem where the cage was attached. The cage and the pine weevil were removed when the insect had fed on about 50% of the circumference, with a vertical length of about 0.5 cm. The average scar area inflicted by the pine weevil (± standard error) was 32.2 ± 3.1 mm^2.
(4) Piercing-needle damage to the stem bark (P): Seedlings (n = 34 for each provenance) were needle-pierced with a handmade tool consisting of a row of five insect pins (No. 00, diameter 0.3 mm). The five pins were fixed on a 1 cm long straight line, with a 2∼4 mm gap between pins on an eraser. With the tool, five vertical holes could be created simultaneously in the stem bark. The depth of each hole reached the xylem of the stem. Fifty holes were created by ten repeated piercings right below where the lowest needles grow, and these were evenly spread out around the stem circumference. The piercing damage area was ∼14 mm^2 (area of each hole ($0.3^2 \times 3.14$) × 50 holes). This treatment imitates sap-sucking insect damage.
(5) Root bark damage (RD): Seedlings (n = 34 for each provenance) were wounded with a scalpel on the root bark. A rectangular scar was created on the main root bark right below the soil surface. The width of the scar was about 50% of the main root circumference, and the length was 0.5 cm. All phloem tissue was removed and the xylem was exposed within the scar. This treatment imitates damage by root-bark feeding insects such as *Hylastes* sp. beetles.
(6) Small stem window (WinS): A rectangular scar was inflicted on the stem of each individual seedling (n = 17 for each provenance) with a scalpel, and was located about 1 cm above the soil or right below where the lowest needles grow. The width of the scar was about 50% of the stem circumference and the vertical length was 0.5 cm. All phloem tissue was removed, and the xylem was exposed within the scar. The average scar area (± standard error) inflicted was 16.9 ± 4.2 mm^2. This treatment imitates pine weevil damage.
(7) Large stem window (WinL): A rectangular scar was inflicted on the stem of each individual seedling (n = 17 for each provenance) as described for the WinS treatment above, but the vertical length of the scar was 1 cm. The average scar area (± standard error) inflicted was 23.3 ± 4.2 mm^2. We originally intended to include only one treatment with one stem scar/window, but we considered that the wound may be too small to trigger induced resistance, and thus inflicted a larger scar on half of the seedlings (thus n = 17 for each stem window treatment per provenance).

Twelve days after the treatments, 288 seedlings in total were exposed to pine weevils in feeding tests. Each treatment included 48 seedlings (24 for each provenance), except treatment WinL and WinS which each included 24 seedlings (12 for each provenance). The remaining seedlings in each group were monitored for their height and diameter growth without exposure to pine weevils (see description below). Each seedling was exposed to one female pine weevil for 48 h. Potted seedlings and the corresponding pine weevil were enclosed by a plastic transparent cylinder with mesh net at the top to allow air flow, but prevent insects from escaping. After the feeding test, the number of feeding scars was recorded for each seedling, and the length and width of each feeding scar were measured using millimeter paper. Areas of all scars were added together to obtain the total stem area debarked per seedling. The number of girdled (when an entire ring of stem bark around the circumference is removed) seedlings were recorded as well. Due to limited lab space, the plant treatments and corresponding feeding tests were replicated in time and thus conducted in four consecutive rounds (two rounds per week). Each round consisted of 72 seedlings. Pine weevil individuals were not reused after each test.

A total of 120 seedlings were used to compare plant growth among treatments. For each treatment, 20 seedlings (10 for each provenance) were kept in a greenhouse for growth measurements [except treatment WinL and WinS which each included 10 seedlings (5 for each provenance)]. The settings in the greenhouse were 16 h light/8 h dark, and day/night temperature was 20/16°C. The aboveground height and basal diameter of seedlings was measured once a week for three consecutive weeks from August 15 to September 4, 2017. The first measurements of height and diameter were conducted 1 day before the different treatments were applied. The average height (± standard error) and diameter (± standard error) of the provenance Hade (height: 14.82 ± 0.24 cm; diameter: 2.18 ± 0.04 mm) was significantly

lower than that of Gotthardsberg (height: 21.92 ± 0.47 cm; diameter: 2.46 ± 0.04 mm).

Experiment 2: Plant Induction Treatments and Subsequent Feeding Tests at Two Time Points

To further investigate if Scots pine resistance to weevils differed depending on the time since induction treatments were applied, we conducted a follow-up experiment. In this experiment, we examined five induction methods (in addition to non-damaged controls) and evaluated their effect on seedling resistance to pine weevil damage at two time points after treatment, 12 and 32 days. We included needle-piercing (P) of the stem bark and stem window damage (treatments P and WinS in experiment 1, respectively; average scar area Win S ± standard error: 17.8 ± 3.3 mm^2), previous pine weevil feeding (WF in experiment 1; average scar area ± standard error: 29.0 ± 3.7 mm^2), MeJA treatment (two levels: 10 mM and 15 mM) and an undamaged control group (C). The greenhouse settings and experimental set-up were the same as for experiment 1, except that only seedlings of the Hade provenance (average height: 13.21 ± 0.13 cm, diameter: 2.36 ± 0.02 mm; Stora Enso Plantor AB, Sör Amsberg, Sweden) were used. On July 16, 2020 (roughly a month earlier in the season relative to when experiment 1 was conducted), damage treatments were inflicted after seedlings had grown for 28 days in the greenhouse (planted on June 18, 2020). Seedlings were kept in this same greenhouse until it was time to expose them to pine weevils and evaluate induced resistance. The pine weevil exposure feeding tests were conducted 12 days after damage treatments (July 29, 2020, referred to as early exposure hereafter) for 5 of the treatments (8 seedlings × 5 treatments: C, 10 mM MeJA, 15 mM MeJA, WinS and Piercing), and 32 days after damage treatments (August 17, 2020, referred to as late exposure hereafter) for all six treatments (12 seedlings × 6 treatments: C, WF, 10 mM MeJA, 15 mM MeJA, WinS, and Piercing). Due to logistical challenges of restricting the amount of previous pine weevil damage (WF treatment) on seedlings and the limited number of seedlings available, we included this treatment only at late exposure. Each seedling was exposed to a starved female pine weevil for 48 h in the lab (light-dark cycle 16L/8D, room temperature), and damage inflicted was measured as described in experiment 1. Seedling height and basal diameter were measured once a week since they were planted. The room temperature during the feeding test was not recorded. However, data from the Swedish Meteorological and Hydrological Institute showed a different average air temperature for Uppsala, Sweden of 16.2 and 21.2°C, respectively, for feeding tests that happened in the early and late exposure groups.

Statistical Analyses

All statistical analyses were conducted in R software version 3.6.3 (R Core team, 2020) using R studio 1.2.5042 (RStudio Team, 2020), and graphs were generated using the *ggplot2* package (Wickham, 2016). Model fit was checked by visualizing residuals vs. predicted values using the *plot* command in R, and we found that models fitted well.

Experiment 1: Plant Induction Treatments and Subsequent Feeding Tests at One Time Point

We examined the effect of all treatments on area debarked by pine weevils, by fitting a linear mixed model (*lmer* command from *lme4* package, Bates et al., 2015). The model included treatment (C, MeJA, P, RD, WF, WinL, and WinS), plant provenance (Gotthardsberg, Hade), and their interaction, as fixed explanatory variables, round (n = 4, due to limited laboratory space the experiment was replicated in time with a total of four consecutive rounds) as a random variable, and seedling height (measured before pine weevil exposure) as a continuous covariate. A generalized linear mixed-effects model (*glmer* command from *lme4* package) was used to analyze the effect of treatment on number of scars (family = Poisson) and girdling (family = binomial) including the same explanatory variables as for area debarked. To analyze the effect of treatment on seedling height and diameter increment, a linear model (*lm* command from the base R *stats* package, R Core team, 2020) was used. Explanatory variables included treatment, provenance, the interaction of treatment and provenance, and seedling initial height (from the beginning of the growth observation period) as a covariate. After model fitting, significance of main effects and interactions was tested with analysis of deviance using the ANOVA command from the *car* package (Fox and Weisberg, 2019). When main effects were significant ($P < 0.05$), treatment means were compared using a Dunnett's test from the *contrast* command in the *emmeans* package (Lenth, 2020) and using treatment C and MeJA as reference levels.

Experiment 2: Plant Defense Induction Treatments and Subsequent Feeding Tests at Two Time Points

We examined the effects of treatment and the timing of exposure to pine weevils on seedling resistance by fitting several models. A general least square model (*gls* command from the *nlme* package, Pinheiro et al., 2020), which allows for heterogeneous error variance, was fitted with area debarked and the number of feeding scars as response variables. The explanatory variables in the models were treatment (C, MeJA 10 mM and 15 mM, P, WinS), timing of exposure (12 or 32 days) and their interaction as fixed explanatory variables, and plant height as a continuous covariate. The variance function *varIdent()* in the *weights* = argument was used to specify heterogeneous error variance for the two fixed variables.

As the number of treatments was different for the two time points (early and late exposure), we also examined the effect of treatment on seedling resistance separately for each time point. To examine differences in pine weevil area debarked and number of feeding scars at early exposure, we fitted a general least square model (*gls* command from the *nlme* package, Pinheiro et al., 2020) for each variable separately. The model included treatment (C, MeJA 10 mM and 15 mM, WinS, and P) and seedling height as explanatory fixed variables, and the variance function *varIdent()* in the *weights* = argument was used to specify heterogeneous

error variance for treatments. After model fitting, treatment estimated means were calculated by using *emmeans* command in the *emmeans* package (Lenth, 2020).

To examine differences in area debarked by pine weevils at late exposure, a linear model (*lm* command from the default *stats* package, R Core team, 2020) was fitted with area debarked being square-root transformed. The model included treatment (C, MeJA 10 mM and 15 mM, P, WF, WinS) and seedling height (measured before pine weevil exposure) as explanatory variables. A negative binomial generalized linear model (*glm.nb* command from the *MASS* package, Venables and Ripley, 2002) was used when analyzing the effect of treatment on number of scars, and this included the same explanatory variables as for area debarked.

The effect of treatment on seedling height increment and basal diameter increment (increment = last measurement–the first measurement for each individual) were analyzed with a linear model (*lm* command from the default *stats* package, R Core team, 2020). In this model, the explanatory variables were treatment (C, MeJA 10 mM and 15 mM, P, WF, WinS) and plant initial height as a covariate.

After model fitting, significance of main effects and interactions were tested with analysis of deviance using the ANOVA command from the *car* package (Fox and Weisberg, 2019). When main effects were significant ($P < 0.05$), differences among treatment levels were examined using *emmeans* command in the *emmeans* package (Lenth, 2020). If main effects were not significant, estimated means were still obtained using the *emmeans* command and used for plotting figures.

RESULTS

Experiment 1: Plant Induction Treatments and Subsequent Feeding Tests at One Time Point

Area debarked by pine weevils did not differ among Scots pine seedlings exposed to different induction treatments (**Table 1** and **Figure 1A**), and these effects were consistent for the two seedling provenances examined (non-significant treatment × provenance interaction, **Table 1** and **Supplementary Figures 1A,B**). Likewise, the number of pine weevil feeding scars was not significantly affected by induction treatments (**Table 1** and **Figure 1B**). However, a significant interaction between treatment and provenance with respect to the number of feeding scars was found, with Hade receiving overall fewer scars (**Supplementary Figures 1C,D**). Moreover, Hade showed a significantly higher girdling rate than Gotthardsberg (21% vs. 14% of seedlings were girdled, respectively) in the feeding test (**Table 1** and **Supplementary Figure 2**). Seedlings in the piercing treatment were 22% more debarked in area than control seedlings, and significantly more debarked than seedlings with MeJA treatment (Dunnett's test, df = 248, t-ratio = 2.68, $P = 0.040$). All seedlings receiving previous pine weevil feeding (WF), and mechanical stem windows (WinL and WinS) were fed upon by pine weevils in the feeding tests, while a few seedlings from other treatments remained undamaged (**Supplementary Figure 2**). Moreover, previous pine weevil feeding (WF) and small stem window (WinS) resulted in similar levels of debarked area and number of scars compared to seedlings in the MeJA treatment (**Figures 1A,B**). In addition, seedlings in the MeJA, WF, and WinS treatments experienced reductions in area debarked of 18, 12, and 22%, respectively, compared to controls. Among the three treatments for which seedlings experienced slightly less debarked area compared to controls, seedlings with previous pine weevil feeding (WF) were more girdled than those in the MeJA and WinS treatments (girdling rate 37, 13, and 13% respectively) (**Supplementary Figure 2**).

In contrast to results from feeding tests, the growth of Scots pine seedlings varied significantly among induction treatments (**Table 2**). Multiple comparisons indicated that MeJA treated seedlings had a significantly lower height growth than seedlings in all other treatments (**Figures 2A,C**). The non-significant interaction of provenance and treatment indicated that height growth patterns were similar for the two provenances across treatments (**Supplementary Figures 3A,B** and **4**). Diameter growth was also significantly affected by the different defense induction treatments (**Table 2**), and it differed for the two provenances (**Supplementary Figures 3C,D**). Overall, seedlings treated with MeJA, and those that received root damage (RD), previous weevil feeding (WF), and small stem window (WinS)

TABLE 1 | Results of analysis of deviance (df: degrees of freedom; χ^2: Chi-square value; LR χ^2: likelihood ratio Chi-square value; *P*: *P* value) from several models examining the effect of treatment on subsequent pine weevil damage in experiment 1.

	Debarked area			Number of feeding scars			Girdling rate		
Source of variance	**df**	**χ^2**	***P***	**df**	**χ^2**	***P***	**df**	**LR χ^2**	***P***
Treatment	6	11.32	0.08	6	4.75	0.58	6	6.40	0.38
Provenance	1	6.38	**0.01**	1	26.28	**<0.01**	1	5.30	**0.02**
Height	1	2.23	0.15	1	0.41	0.52	1	0.10	0.75
Treatment × Provenance	6 × 1	8.23	0.22	6 × 1	20.04	**<0.01**	6 × 1	5.92	0.43

More specifically, these models examined the effect of different plant defense induction treatments [Large stem window damage (WinL), small stem window damage (WinS), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), 10 mM MeJA (MeJA), and undamaged seedlings as controls (C)] on levels of damage (area debarked, mm^2), number of feeding scars, and girdling rate by pine weevils (H. abietis) in Scots pine (P. sylvestris) seedlings for experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment).

The models included the fixed variables: treatment, provenance (Hade, Gotthardsberg), their interaction, and seedling height (cm, measured a day before feeding test) as a continuous covariate.

Significant effects ($P < 0.05$) are in bold.

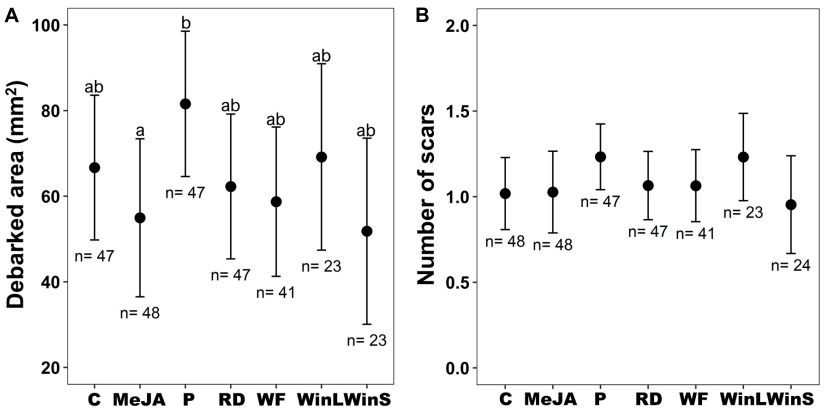

FIGURE 1 | Pine weevil damage to Scots pine (*P. sylvestris*) seedlings in experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment) after receiving different induction treatments [Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)]. **(A)** Estimated mean debarked area (±95% confidence intervals), and **(B)** Estimated mean number of feeding scars (±95% confidence intervals) for each treatment. Sample sizes (*n*) used in the statistical analyses are also shown. Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates.

did not differ in diameter growth. Yet, seedlings receiving needle-piercing damage (P) or a large stem window (WinL) grew significantly more in diameter than MeJA treated seedlings (**Figures 2B,D**).

Experiment 2: Plant Induction Treatments and Subsequent Feeding Tests at Two Time Points

Pine weevil damage to Scots pine seedlings differed among treatments, and between the two time points at which seedlings were subsequently exposed to pine weevils. Overall, seedlings were significantly more damaged at late exposure, than at early exposure (**Table 3**). At early exposure, seedlings in the needle-piercing treatment (P) received significantly more feeding damage (both in terms of debarked area and feeding scars) than seedlings in the MeJA treatment (15 mM), and it was the only group receiving 250% more damage by pine weevils relative to control seedlings (**Table 3** and **Figures 3A,B**). Seedlings in the MeJA treatments experienced a non-statistically significant reduction in area debarked (46% less for 10 mM, and 84% less for 15 mM) compared to control seedlings; while, seedlings in the WinS treatment received similar damage to controls (**Figure 3A** and **Supplementary Figure 5A**). Although not statistically significant, seedlings in the piercing and WinS treatments received 110 and 87% more feeding scars, respectively, than controls; while MeJA treated (10 mM MeJA and 15 mM MeJA) seedlings received 38 and 85% less scars, respectively, than controls (**Figure 3B** and **Supplementary Figure 5B**). In addition, many plants were not damaged at all at the early exposure, especially for seedlings that were treated with 15 mM MeJA, which had only one seedling damaged by pine weevil, while seedlings with needle-piercing had only one seedling undamaged. More than half of the seedlings treated with 10 mM and control seedlings were undamaged (**Supplementary Figure 6A**).

At late exposure, area debarked and number of feeding scars did not differ significantly among treatments (**Table 3** and **Figures 3A,B**). Seedlings in the control group, nonetheless, tended to receive the most pine weevil damage in terms of debarked area. Seedlings in the 10 mM MeJA, 15 mM MeJA, P, WF, and WinS treatments had 27, 32, 12, 17, and 23% less damage, respectively, than control seedlings (**Figure 3A** and **Supplementary Figure 5A**). The number of feeding scars was quite similar among treatments, and only seedlings with the previous pine weevil feeding (WF) and 15 mM MeJA treatments showed a 24 and 4% reduction, respectively, compared to controls. On the other hand, seedlings in the 10 mM MeJA, P, and WinS treatments received 15, 14, and 15% more scars than controls (**Figure 3B** and **Supplementary Figure 5B**). Across all treatment, the number of undamaged seedlings was lower, compared to those in the early exposure group (**Supplementary Figure 6**).

Similar to experiment 1, we found that different induction treatments significantly affected plant height and diameter growth (**Table 2**). Only seedlings treated with 15 mM MeJA grew significantly less (42% lower) in height than control seedlings. Seedlings treated with 10 mM MeJA and 15 mM MeJA grew significantly less (37 and 49%, respectively) in height than seedlings induced by previous pine weevil feeding (WF) (**Figure 4A** and **Supplementary Figure 7A**). For diameter growth, only seedlings treated with 15 mM MeJA grew significantly less (60%) than control seedlings. Seedlings receiving needle-piercing (P), previous pine weevil feeding (WF), and small window damage (WinS) treatments grew slightly more in diameter (21, 10, and 15% more, respectively) than control seedlings. Seedlings in these three treatments grew significantly more than seedlings treated with MeJA (10 and 15 mM) (**Figure 4B** and **Supplementary Figure 7B**). We also noted that wounds created by the different induction treatments had healed completely, or healed to at least half the original damaged area, by the time of late exposure (**Supplementary Figures 8A–C**).

TABLE 2 | Results of analysis of variance (ANOVA) (df: degrees of freedom; *F*: *F*-value; *P*: *P*-value) from several linear models examining the effect of treatments on plant growth in experiments 1 and 2.

		Height increment			Diameter increment		
	Source of variance	**df**	***F***	***P***	**df**	***F***	***P***
Experiment 1	Provenance	1	18.86	**<0.01**	1	0.28	0.61
	Treatment	6	15.36	**<0.01**	6	3.02	**<0.01**
	Initial height	1	5.46	**0.02**	1	14.33	**<0.01**
	Treatment × Provenance	6	1.72	0.10	6	3.28	**<0.01**
	Residuals	102			101		
Experiment 2	Treatment	5	4.63	**<0.01**	5	9.18	**<0.01**
	Initial height	1	0.22	0.64			
	Initial diameter				1	0.036	0.85
	Residuals	63			65		

More specifically, these models examined the effect of different plant defense induction treatments [Large stem window damage (WinL), small stem window damage (WinS), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), 10 mM MeJA (MeJA), and undamaged seedlings as controls (C)] for experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment), and [Small stem window damage (WinS), needle-piercing damage to the stem bark (P), previous weevil feeding damage (WF), 10 mM MeJA, 15 mM MeJA, and undamaged seedlings as controls (C)] for experiment 2 (Insect feeding tests were conducted at two time points, 12 and 32 post-treatment) on growth (height increment, cm, and diameter increment, mm) in Scots pine (P. sylvestris) seedlings.

The models included as explanatory variables: treatment, provenance (only included in experiment 1, Hade and Gotthardsberg), their interaction, and initial seedling height (cm) or initial seedling diameter (mm) (both were measured on the day of planting) as a continuous covariate.

Significant effects ($P < 0.05$) are in bold.

DISCUSSION

Our study showed that simulated bark damage as treatments for potential plant defense induction can affect levels of pine weevil damage to Scots pine seedlings. However, these effects varied depending on the type of damage inflicted and when plants were exposed to the insects after induction stimulus occurred. We found that none of the mechanical induction methods increased seedling resistance to a greater extent than MeJA, and that a shorter time between the induction stimulus and exposure to pine weevils resulted in lower damage levels. One type of stem damage, needle-piercing, even increased subsequent feeding by pine weevils to very high levels relative to all other treatments. In terms of growth, only MeJA negatively affected seedling height growth and diameter relative to the control group, in line with previous studies. All in all, our results indicate that the previous damage treatments evaluated in this study do not provide enhanced seedling resistance to bark-feeding insect damage. We discuss our findings below.

Even though studies on other conifer plants have shown that both mechanical wounding and insect herbivory can trigger induced defensive responses (Miller et al., 2005; Moreira et al., 2012a), our bark damage treatments did not result in significantly greater seedling resistance. One explanation could be that even if defensive chemistry is enhanced or altered, these changes are not enough to sufficiently deter pine weevil feeding. Previous studies have not exposed mechanically damaged plants to subsequent insect feeding, and have assumed that increased defensive chemistry responses will result in less feeding (i.e., greater resistance). Our results show that this assumption may not always be true. Exposure to the pest after induction stimulus is essential if these methods are being evaluated for use within plant protection. Our study directly examined the extent of protection provided by previous mechanical damage, and we find that it is not sufficient against damage by a bark-chewing insect. Another factor that could also be important is the extent of damage or tissue loss. A recent study on tobacco (*Nicotiana tabacum*) plants showed that the amount of leaf tissue loss is important for the level of defense induction (Lin and Felton, 2020). The authors found higher levels of trypsin protease inhibitors (which result in anti-nutritive effects and reduced insect herbivore growth) in plants subjected to whole leaf removal relative to partial leaf damage (Lin and Felton, 2020). On the other hand, a study with 1-year old Scots pine seedlings found that moderate and severe mechanical stem damage resulted in similar negative effects on plant morphology and physiology (Bansal et al., 2013). Seedlings received either one (moderate damage) or two (severe damage) window-like stem bark scars (inflicted with a scalpel), and each scar was about 10 mm in length and covering 1/3 of the stem circumference (Bansal et al., 2013). These scars are similar and even slightly greater in total area to our WinS and WinL treatments, for which we inflicted scars of 5 or 10 mm in length, respectively, across 1/2 of the stem circumference. The authors found that both treatments significantly reduced photosynthesis, needle mass and needle area relative to undamaged controls (Bansal et al., 2013). In our study, seedlings in the WinS treatment received similar pine weevil damage to those in the MeJA treatment, and even received less damage (albeit non-statistically significant) than those with larger window wounds (experiment 1, WinS and WinL, **Figure 1A**). Our results and those of Bansal et al. (2013) suggest that greater tissue loss or damage may instead be detrimental for the plants, and would not necessarily result in greater enhanced resistance to subsequent insect feeding. However, evaluation of a broader range of stem damage levels may be needed to conclusively elucidate if the extent of tissue loss plays a role.

In addition to stem bark damage, root herbivory has also been shown to trigger subsequent defensive responses in aboveground plant tissues, e.g., in cotton *Gossypium herbaceum* (Bezemer et al., 2004) and tobacco *N. tabacum* (Kaplan et al., 2008b). After belowground damage occurs, it has been observed that a reduction in herbivore growth rate, body size and food consumption of aboveground herbivores can occur (Bezemer et al., 2003; Soler et al., 2005; Van Dam et al., 2005). Thus, root herbivory has the potential to decrease overall plant damage levels of aboveground herbivores. However, there are also cases where it has not resulted in increased resistance aboveground. The magnitude of defensive responses in aboveground tissue may not be large or effective enough to decrease herbivore damage, as in the case of cotton *G. herbaceum* (Bezemer et al., 2004). Moreover, some aboveground herbivores may even benefit from belowground herbivory and inflict more damage

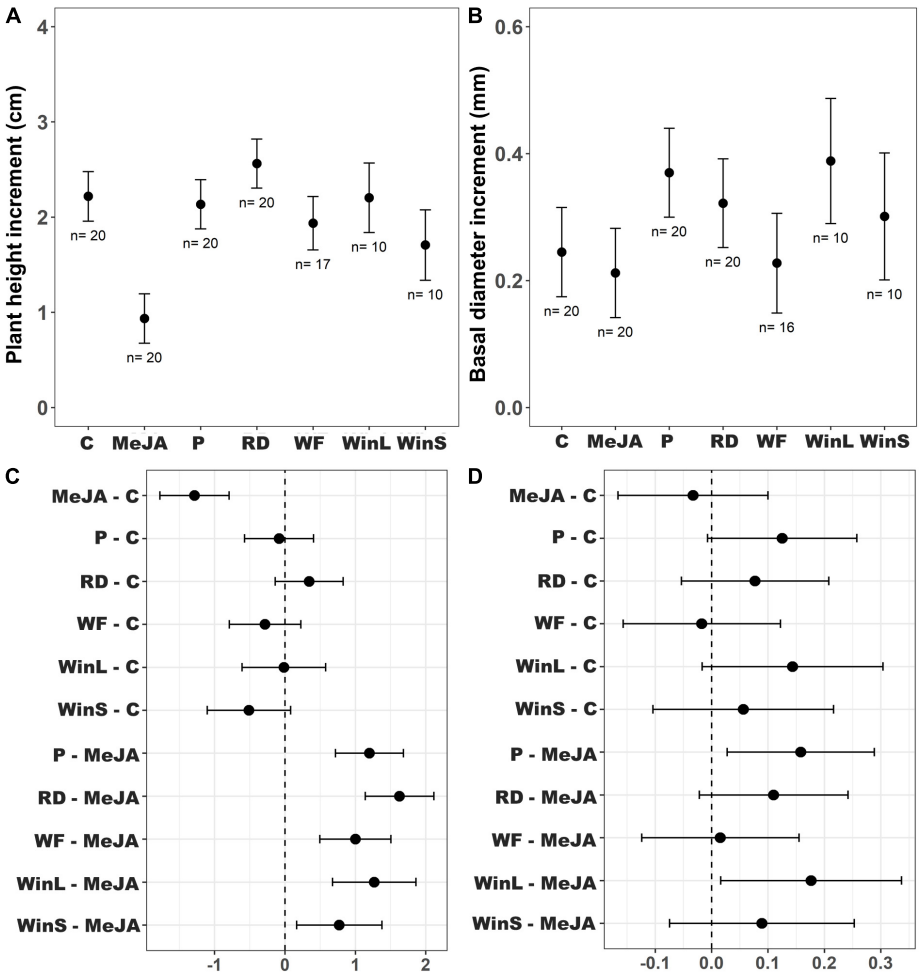

FIGURE 2 | Growth increment of Scots pine (*P. sylvestris*) seedlings and treatment mean comparisons using Dunnett's test among different plant defense induction treatments [Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)] in experiment 1. Insect feeding tests were conducted at one time point, 12 days post-treatment. The growth of seedlings was followed for 21 days post-treatment. Sample sizes (*n*) used in the statistical analyses are also shown. **(A)** Estimated mean plant height increment (cm ± 95% confidence intervals), **(B)** estimated mean basal diameter increment (mm ± 95% confidence intervals), **(C)** differences between treatments in estimated mean height increments (cm ± 95% confidence intervals), and **(D)** differences between treatments in estimated mean diameter increments (mm ± 95% confidence intervals). If an interval does not include zero, the difference between estimated means is considered to be statistically significant.

(Soler et al., 2007; Kaplan et al., 2008a). In our study, we found that root-damaged seedlings tended to grow more (15% more in seedling height increment and 33% more in basal diameter increment) and received 7% less weevil damage (debarked area) compared to control seedlings, but this difference was not statistically significant. This result suggests that the extent of root tissue loss may not have been large enough to trigger aboveground defensive responses that affected the pine weevils.

In contrast to all other induction treatments, seedlings in the needle-piercing treatment received much greater damage levels (as extreme as 250% more damage) than controls. Pine weevils are known to be attracted to the odors or compounds emitted by recently damaged seedlings (Nordlander, 1991). Given the multiple wounds that the needle piercing treatment inflicted on the stem, it could be possible that it stimulated their feeding. Even though two other treatments also inflicted large wounds (WinS and WinL), increased damage levels to the extent of those receiving piercing damage, were not observed for seedlings in these treatments. This indicates that patterns of damage are also relevant and can differentially influence pine weevil feeding behavior. However, we noted in experiment 2 that needle-piercing wounds had healed by the late exposure and at this time point, seedlings received somewhat less pine weevil damage than controls. This suggests that the cues emitted by freshly damaged seedlings could stimulate feeding, but decrease with time.

Although none of the previous damage treatments enhanced seedling resistance to a greater extent than MeJA, treatment with MeJA was also not as significantly effective as reported in previous studies. Only seedlings treated with a higher MeJA concentration (15 mM) were significantly less damaged

TABLE 3 | Results of analysis of deviance (df: degrees of freedom; χ^2: Chi-square value; LR χ^2: likelihood ratio Chi-square value; *P*: *P*-value) from several models examining the effect of treatment on subsequent pine weevil damage in experiment 2.

	Source of variance	Debarked area			Number of feeding scars		
		df	F/χ^2	*P*	df	LR χ^2	*P*
Both time points	Time point	1	82.36	**<0.01**	1	88.83	**<0.01**
	Treatment	4	13.05	**0.01**	4	22.07	**<0.01**
	Height	1	0.36	0.55	1	1.13	0.29
	Time point × Treatment	4	2.88	0.72	4	1.24	0.87
12 days after induction treatment	Treatment	4	12.53	**0.01**	4	18.85	**<0.01**
	Height	1	0.24	0.62		0.23	0.63
32 days after induction treatment	Treatment	5	0.29	0.92	5	6.67	0.25
	Height	1	1.41	0.24	1	8.06	**<0.01**

More specifically, these models examined the effect of different plant defense induction treatments and time point of exposure to pine weevils since induction, on area debarked (mm^2) and number of scars in experiment 2 (Insect feeding tests were conducted at two time points, 12 and 32 days post-treatment).
Models included treatments [Small stem window damage (WinS), needle-piercing damage to the stem bark (P), previous weevil feeding damage (WF), 10 mM MeJA, 15 mM MeJA and undamaged seedlings as controls (C); previous weevil feeding damage (WF) was not included at 12 days after induction], time point (12 or 32 days after induction), their interaction and plant height as a continuous covariate.
Significant effects (P < 0.05) are in bold.

compared to seedlings in the piercing-needle treatment after early exposure. It could be that the dose (the net amount and frequency of MeJA treatment) we used could partly explain our results. The effect of MeJA treatment on pine weevil damage has been shown to be dose dependent (Moreira et al., 2009; Zas et al., 2014). In one of our previous experiments, a higher dose of MeJA (three consecutive sprayings of 10 mM MeJA) resulted in greater Norway spruce resistance to pine weevil damage relative to plants receiving a lower dose (one spraying of 10 mM MeJA) (Chen et al., 2020). The low dose of MeJA in our previous study on Norway spruce was the same as the low dose used in this study on Scots pine seedlings, and the amount of debarked area received by these two conifer species were similar in both studies. Other studies have also used higher doses and concentrations, which have resulted in greater resistance to pine weevil damage. For example, MeJA concentrations of 100, 40, and 22 mM were used on Maritime pine (*P. pinaster*) (Moreira et al., 2009; Moreira et al., 2012a,b), 50 mM MeJA on Norway spruce (Fedderwitz et al., 2016), and 25 mM MeJA on Maritime pine, Monterrey pine (*Pinus radiata*), Scots pine and Norway spruce (Zas et al., 2014). Therefore, it appears that the MeJA dose in this study was not enough to significantly reduce pine weevil damage. Moreover, it seems that induced resistance can be better achieved by several sprayings of MeJA at lower concentrations instead of one application with a higher concentration. Concentrations higher than 10 mM can be detrimental to seedlings, and result in treatment-related damage (e.g., loss of needles, needle browning in Norway spruce; Fedderwitz et al., 2019), and we indeed observed some needle-browning in seedlings treated with 15 mM MeJA (**Supplementary Figure 8D**). Our results are thus, an important contribution to development of methods for optimum use MeJA as a seedling protection tool. Finding the MeJA treatment concentration and frequency that provides effective resistance, minimizes phytotoxicity and is compatible with nursery needs

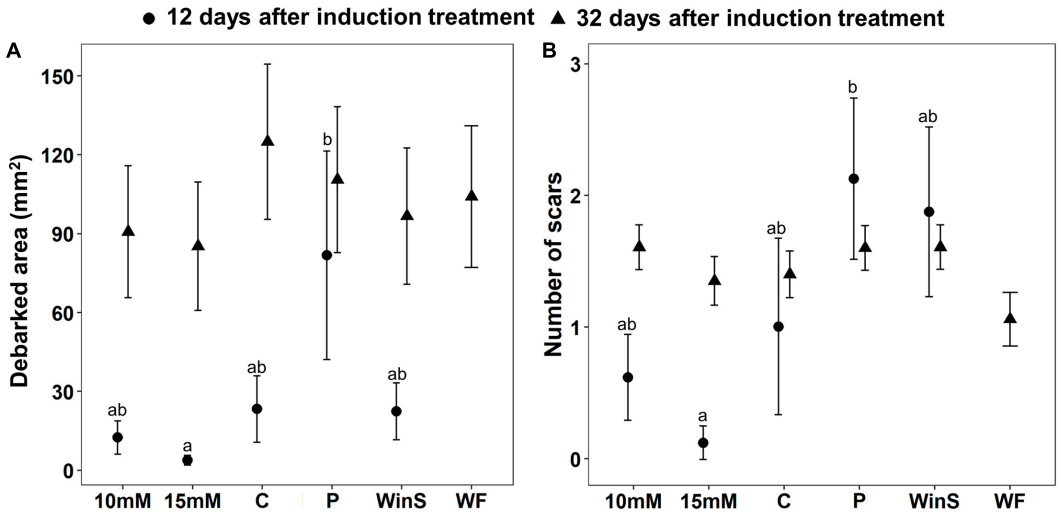

FIGURE 3 | Pine weevil damage to Scots pine (*P. sylvestris*) seedlings after receiving different induction treatments [10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF, not included in the weevil exposure test conducted 12 days post-treatment)] in experiment 2. Plants that were exposed to pine weevils 12 days after induction treatments are referred to as early exposure, and those that were exposed 32 days after, are referred to as late exposure in the main text. **(A)** Estimated mean debarked area (mm^2 ± standard error), and **(B)** Estimated mean number of feeding scars (±standard error). Sample size (*n*) is equal to 8 for all other treatments, except that the Control group (C) is equal to 7 for the early exposure, and *n* = 12 for all treatments for the late exposure. Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates.

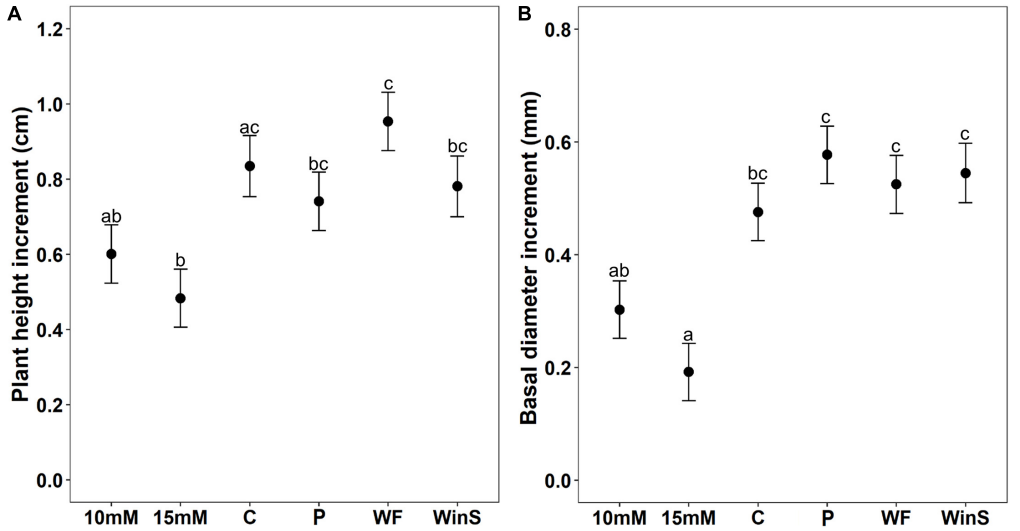

FIGURE 4 | Growth increment of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments [10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF)] in experiment 2 (n = 12 per treatment, insect feeding tests were conducted at two time points, 12 and 32 days post-treatment). The growth of seedlings was followed for 32 days post-treatment. **(A)** Estimated mean height increment (cm ± standard error), and **(B)** Estimated mean diameter growth (mm ± standard error). Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates.

and practices is essential for MeJA implementation (Fedderwitz et al., 2019, Chen et al., 2020).

Timing since induction stimulus is also an essential factor in development of plant protection strategies aimed at increasing seedling resistance prior to pest exposure. We found that the effect of previous damage and MeJA on triggering seedling resistance to pine weevils differed depending on the time since treatment. Overall, we found that plants exposed early to pine weevils (12 days after treatment) received less damage relative to those in the late exposure group (32 days after treatment). These results could indicate that treatment effects were short-lasting and tended to lose their efficacy with time. As discussed in previous paragraphs, an explanation could be that the extent of tissue loss/damage could play a role and that MeJA doses used were not enough to induce effective resistance. We observed that seedlings in the 10 mM and 15 mM MeJA groups, were often not eaten by weevils at all (**Supplementary Figure 6**) or received considerably less pine weevil feeding damage at 12 days relative to 32 days after MeJA application. Seedlings in other induction treatments also showed a similar tendency, with less damage at 12 days relative to 32 days but not as pronounced as for those in the MeJA group. Thus, if a peak in induced resistance occurs, this peak is likely closer to 12 days rather than 32 days after treatment.

Another potential cause for the different damage levels at these two time points could be that pine weevil feeding behavior differed. The average air temperature in Uppsala, Sweden at the time when the late exposure occurred (average air temperature: 21.2°C) was 5°C higher than during the early exposure (average air temperature: 16.2°C), according to data from Swedish Meteorological and Hydrological Institute (station 97510 Uppsala Aut, 59°50′50″N, 17°37′55″E, SMHI, 2020). The weevils were acclimatized in the lab for a week before the feeding test, thus, weevils in the late exposure group might have been affected by the warmer room temperature compared to those in the early exposure group. Pine weevils have been shown to consume almost four times more bark of Scots pine twigs at 20°C compared to 15°C (Leather et al., 1994). The behavior of pine weevils may thus, have been affected by a higher room temperature and resulted in increased feeding at the late exposure time point. All in all, from a plant protection perspective, our results on the timing of induction suggest that it may be better if the treatment stimulus occurs closer rather than further from pest exposure. However, additional studies where temperature is controlled for, and different levels of tissue loss and other time points since treatment are included, would help to tease apart their effect on seedling resistance.

We were also interested in examining any potential cost of induction treatments with respect to plant growth. As documented in other studies on MeJA-induced plant defense (Heijari et al., 2005; Vivas et al., 2012), we observed a significant growth reduction in seedlings receiving MeJA treatment compared to the control seedlings. Such a trade-off between growth and defense has indeed been found for seedlings of several coniferous species, e.g., Maritime pine (Moreira et al., 2012b), Monterrey pine (Gould et al., 2008), and Norway spruce (Chen et al., 2020). Also, in line with other studies, we found that growth was even more reduced for plants receiving the higher concentration of MeJA (15 mM). This suggests that resources were diverted away from growth and presumably invested in defense, yet it only resulted in a slight reduction in area debarked relative to seedlings in the control group.

Some of the non-MeJA treatments also exhibited different relationships between growth and resistance. For example, seedlings in the piercing treatment (P) and large window (WinL)

treatments received relatively higher subsequent pine weevil damage compared to all other treatments in experiment 1. Yet, there was a slight reduction in height growth and even a tendency to grow more in diameter compared to the control group. This is in line with another study that Scots pine seedlings with stem bark damage had significantly more radial growth compared to undamaged controls (Bansal et al., 2013). Moreover, seedlings with root damage showed a tendency to grow more in height, compared to control seedlings. A study on field corn *Zea mays* showed that plant dry weight was greater for plants damaged by the western corn rootworm (*Diabrotica virgifera*) relative to those not experiencing any root damage (Godfrey et al., 1993). It is also possible that the non-MeJA treatments do not induce but instead "prime" seedling defenses, which is much less costly compared to fully inducing defenses (Wilkinson et al., 2019). We observed, for example, a slight reduction in debarked area for seedlings in other non-MeJA induction methods after the late exposure, but no growth reduction compared to controls. This is in contrast to plants receiving MeJA treatments, especially at the high concentration, which exhibited distinct growth reductions, and only a reduction of 20–30% in area debarked compared to control seedlings. However, we are not able to discern from our study which of these mechanisms was involved.

CONCLUSION

Our study showed that bark damage induction treatments and a low dose of MeJA did not effectively increase the resistance of Scots pine seedlings. Induction methods that include needle-piercing stem wounding can even be detrimental for seedlings, as we found that this type of damage resulted in even more damage by pine weevils relative to all other treatments. Apart from MeJA treatments, none of the damage treatments had negative effects on seedling growth in terms of height and diameter. All in all, our results suggest that mechanical damage may not be sufficient to trigger induced resistance responses that provide adequate seedling protection. Thus, these methods of induction would not be suitable for larger scale implementation to protect conifer seedlings. Instead, improving the use of MeJA and finding optimal concentrations that enhance resistance but minimize negative effects, remains as a promising alternative. Nonetheless, further studies varying the degree of tissue loss as well as the time period between induction treatment and insect exposure, would be of interest. In addition, studies that examine the levels of chemical defense in seedlings following the treatments and subsequent exposure to insect feeding in both lab and the field, are needed to enhance our knowledge on the mechanisms of induced defense in conifer seedlings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

AUTHOR CONTRIBUTIONS

AP, CB, and HB conceived and designed the experiment. AB carried out the pilot experiment as part of her master's thesis. AP, CB, HB, and YC re-evaluated and improved the experimental design. YC conducted the experiment, carried out the statistical analyses, and wrote the draft of the manuscript with input from AP, CB, and HB. All authors contributed to subsequent revisions of the manuscript and agreed to publish the final version of the manuscript.

FUNDING

The study was supported by FORMAS grant number 942-2016-37 and the Swedish Research Council project 2018-05389.

ACKNOWLEDGMENTS

We thank Claudia von Brömssen and especially Harimurti Buntaran for help with statistical analyses and software coding. Thanks to Sunil Abeyasekera for help in the lab and greenhouse, and Balungstrands Sågverk AB sawmill (Enviken, Sweden) for access to their premises to collect migrating pine weevils.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.695867/full#supplementary-material

REFERENCES

Agerbirk, N., De Vos, M., Kim, J. H., and Jander, G. (2008). Indole glucosinolate breakdown and its biological effects. *Phytochem. Rev.* 8, 101–120. doi: 10.1007/s11101-008-9098-0

Baldwin, I. T. (1988). The alkaloidal responses of wild tobacco to real and simulated herbivory. *Oecologia* 77, 378–381. doi: 10.1007/BF00378046

Bansal, S., Hallsby, G., Lofvenius, M. O., and Nilsson, M. C. (2013). Synergistic, additive and antagonistic impacts of drought and herbivory on *Pinus sylvestris*: leaf, tissue and whole-plant responses and recovery. *Tree Physiol.* 33, 451–463. doi: 10.1093/treephys/tpt019

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Bezemer, T. M., Wagenaar, I. R., van Dam, N. M., van der Putten, W. H., and Wackers, F. L. (2004). Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.* 30, 53–67. doi: 10.1023/b:joec.0000013182.50662.2a

Bezemer, T. M., Wagenaar, R., Van Dam, N. M., and Wäckers, F. L. (2003). Interactions between above- and belowground insect herbivores as mediated by the plant defense system. *Oikos* 101, 555–562. doi: 10.1034/j.1600-0706.2003.12424.x

Chen, X., Richter, A. R., Stout, M. J., and Davis, J. A. (2018). Effects of induced plant resistance on soybean looper (Lepidoptera: Noctuidae) in soybean. *Arthropod Plant Interact.* 12, 543–551. doi: 10.1007/s11829-018-9601-5

Chen, Y., Bylund, H., Björkman, C., Fedderwitz, F., and Puentes, A. (2020). Seasonal timing and recurrence of methyl jasmonate treatment influence pine weevil damage to Norway spruce seedlings. *New Forests* 52, 431–448. doi: 10.1007/s11056-020-09803-4

Creelman, R. A., and Mullet, J. E. (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4114–4119. doi: 10.1073/pnas.92.10.4114

Dicke, M., and van Poecke, R. (2002). "Signalling in plant-insect interactions: signal transduction in direct and indirect plant defence," in *Plant Signal Transduction*, eds D. Scheel and C. Wasternack (Oxford University Press), 289–316.

Erb, M., Glauser, G., and Robert, C. A. (2012). Induced immunity against belowground insect herbivores- activation of defenses in the absence of a jasmonate burst. *J. Chem. Ecol.* 38, 629–640. doi: 10.1007/s10886-012-0107-9

Fedderwitz, F., Björklund, N., Anngren, R., Lindström, A., and Nordlander, G. (2019). Can methyl jasmonate treatment of conifer seedlings be used as a tool to stop height growth in nursery forest trees? *New Forests* 51, 379–394. doi: 10.1007/s11056-019-09737-6

Fedderwitz, F., Nordlander, G., Ninkovic, V., and Björklund, N. (2016). Effects of jasmonate-induced resistance in conifer plants on the feeding behaviour of a bark-chewing insect, *Hylobius abietis*. *J. Pest Sci.* 89, 97–105. doi: 10.1007/s10340-015-0684-9

Fox, J., and Weisberg, S. (2019). *An R Companion to Applied Regression*, 3rd Edn. Thousand Oaks CA: Sage.

Godfrey, L. D., Meinke, L. J., and Wright, R. J. (1993). Vegetative and reproductive biomass accumulation in field corn: response to root injury by western corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 86, 1557–1573. doi: 10.1093/jee/86.5.1557

Gould, N., Reglinski, T., Northcott, G. L., Spiers, M., and Taylor, J. T. (2009). Physiological and biochemical responses in *Pinus radiata* seedlings associated with methyl jasmonate-induced resistance to *Diplodia pinea*. *Physiol. Mol. Plant Pathol.* 74, 121–128. doi: 10.1016/j.pmpp.2009.10.002

Gould, N., Reglinski, T., Spiers, M., and Taylor, J. T. (2008). Physiological trade-offs associated with methyl jasmonate - induced resistance in *Pinus radiata*. *Can. J. Forest Res.* 38, 677–684. doi: 10.1139/x07-193

Griffiths, D. W., Birch, A. N. E., and Macfarlane-Smith, W. H. (1994). Induced changes in the indole glucosinolate content of oilseed and forage rape (*Brassica napus*) plants in response to either turnip root fly (*Delia floralis*) larval feeding or artificial root damage. *J. Sci. Food Agric.* 65, 171–178. doi: 10.1002/jsfa.2740650208

Heijari, J., Nerg, A. M., Kainulainen, P., Viiri, H., Vuorinen, M., and Holopainen, J. K. (2005). Application of methyl jasmonate reduces growth but increases chemical defence and resistance against *Hylobius abietis* in Scots pine seedlings. *Entomol. Exp. Appl.* 115, 117–124. doi: 10.1111/j.1570-7458.2005.00263.x

Herde, M., Koo, A. J. K., and Howe, G. A. (2013). "Elicitation of jasmonate-mediated defense responses by mechanical wounding and insect herbivory," in *Jasmonate Signaling: Methods and Protocols*, eds A. Goossens and L. Pauwels (Totowa, NJ: Humana Press), 51–61. doi: 10.1007/978-1-62703-414-2_5

Herms, D. A., and Mattson, W. J. (1992). The dilemma of plants: to grow or defend. *Q. Rev. Biol.* 67, 283–335. doi: 10.1086/417659

Jiang, D., Wang, J., Jiang, H., Zhang, W., Meng, Z., and Yan, S. (2016). Effects of locally inducing *Larix olgensis* using exogenous methyl jasmonate on the growth and development of *Lymantria dispar*. *J. Beijing Forest. Univ.* 28, 67–71.

Kahl, J., Siemens, D. H., Aerts, R. J., Gäbler, R., Kühnemann, F., Preston, C. A., et al. (2000). Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta* 210, 336–342. doi: 10.1007/pl00008142

Kant, M. R., Jonckheere, W., Knegt, B., Lemos, F., Liu, J., Schimmel, B. C., et al. (2015). Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities. *Ann. Bot.* 115, 1015–1051. doi: 10.1093/aob/mcv054

Kaplan, I., Halitschke, R., Kessler, A., Rehill, B. J., Sardanelli, S., and Denno, R. F. (2008a). Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecol. Lett.* 11, 841–851. doi: 10.1111/j.1461-0248.2008.01200.x

Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S., and Denno, R. F. (2008b). Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology* 89, 392–406. doi: 10.1890/07-0471.1

Knebel, L., Robison, D. J., Wentworth, T. R., and Klepzig, K. D. (2008). Resin flow responses to fertilization, wounding and fungal inoculation in loblolly pine (*Pinus taeda*) in North Carolina. *Tree Physiol.* 28, 847–853. doi: 10.1093/treephys/28.6.847

Lalík, M., Galko, J., Nikolov, C., Rell, S., Kunca, A., Modlinger, R., et al. (2020). Non-pesticide alternatives for reducing feeding damage caused by the large pine weevil (*Hylobius abietis* L.). *Ann. Appl. Biol.* 177, 132–142. doi: 10.1111/aab.12594

Leather, S. R., Ahmed, S. I., and Hogan, L. (1994). Adult feeding preferences of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae). *Eur. J. Entomol.* 91, 385–385.

Leather, S. R., Day, K. R., and Salisbury, A. N. (1999). The biology and ecology of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? *Bull. Entomol. Res.* 89, 3–16. doi: 10.1017/S0007485399000024

Lenth, R. (2020). *emmeans: Estimated Marginal Means, aka Least-Squares Means [Online]. R Package Version 1.4.6*. Available Online at: https://CRAN.R-project.org/package=emmeans (accessed June 01, 2021).

Lin, P., and Felton, G. W. (2020). Oral cues are not enough: induction of defensive proteins in *Nicotiana tabacum* upon feeding by caterpillars. *Planta* 251:89. doi: 10.1007/s00425-020-03385-3

Lombardero, M. J., Ayres, M. P., and Ayres, B. D. (2006). Effects of fire and mechanical wounding on *Pinus resinosa* resin defenses, beetle attacks, and pathogens. *Forest Ecol. Manag.* 225, 349–358. doi: 10.1016/j.foreco.2006.01.010

López-Goldar, X., Lundborg, L., Borg-Karlson, A. K., Zas, R., and Sampedro, L. (2020). Resin acids as inducible chemical defences of pine seedlings against chewing insects. *PLoS One* 15:e0232692. doi: 10.1371/journal.pone.0232692

Martin, D., Tholl, D., Gershenzon, J., and Bohlmann, J. (2002). Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiol.* 129, 1003–1018 doi: 10.1104/pp.011001

Mattiacci, L., Dicke, M., and Posthumus, M. A. (1994).). Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding of *Pieris brassicae* larvae: role of mechanical damage and herbivore elicitor. *J. Chem. Ecol.* 20, 2229–2247. doi: 10.1007/BF02033199

McConn, M., Creelman, R. A., Bell, E., Mullet, J. E., and Browse, J. (1997). Jasmonate is essential for insect defense in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5473–5477. doi: 10.1073/pnas.94.10.5473

Miller, B., Madilao, L. L., Ralph, S., and Bohlmann, J. (2005). Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiol.* 137, 369–382. doi: 10.1104/pp.104.050187

Moreira, X., Sampedro, L., and Zas, R. (2009). Defensive responses of *Pinus pinaster* seedlings to exogenous application of methyl jasmonate: concentration effect and systemic response. *Environ. Exp. Botany* 67, 94–100. doi: 10.1016/j.envexpbot.2009.05.015

Moreira, X., Zas, R., and Sampedro, L. (2012a). Quantitative comparison of chemical, biological and mechanical induction of secondary compounds in *Pinus pinaster* seedlings. *Trees* 26, 677–683. doi: 10.1007/s00468-011-0602-6

Moreira, X., Zas, R., and Sampedro, L. (2012b). Genetic variation and phenotypic plasticity of nutrient re-allocation and increased fine root production as putative tolerance mechanisms inducible by methyl jasmonate in pine trees. *J. Ecol.* 100, 810–820. doi: 10.1111/j.1365-2745.2011.01938.x

Nordlander, G. (1991). Host finding in the pine weevil *Hylobius abietis*: effects of conifer volatiles and added limonene. *Entomol. Exp. Appl.* 59, 229–237. doi: 10.1111/j.1570-7458.1991.tb01507.x

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and R CoreTeam (2020). *nlme: Linear and Nonlinear Mixed Effects Models*. Available online at: https://CRAN.R-project.org/package=nlme (accessed Febuary 04, 2021).

R Core team (2020). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. Available online at: https://www.R-project.org/.

Redman, A. M., Cipollini, D. F., and Schultz, J. C. (2001). Fitness costs of jasmonic acid-induced defense in tomato, *Lycopersicon esculentum*. *Oecologia* 126, 380–385. doi: 10.1007/s004420000522

RStudio Team. (2020). *RStudio: Integrated Development Environment for R [Online]*. Boston, MA: RStudio, Inc.

Sampedro, L., Moreira, X., and Zas, R. (2010). Resistance and response of *Pinus pinaster* seedlings to *Hylobius abietis* after induction with methyl jasmonate. *Plant Ecol.* 212, 397–401. doi: 10.1007/s11258-010-9830-x

Sampedro, L., Moreira, X., and Zas, R. (2011). Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. *J. Ecol.* 99, 818–827. doi: 10.1111/j.1365-2745.2011.01814.x

Senthil-Nathan, S. (2019). Effect of methyl jasmonate (MeJA)-induced defenses in rice against the rice leaffolder *Cnaphalocrocis medinalis* (Guenèe) (Lepidoptera: Pyralidae). *Pest Manag. Sci.* 75, 460–465. doi: 10.1002/ps.5139

SMHI (2020). *Meteorological Observations:Air Temperature Hourly Value* [Online]. Swedish Meteorological and Hydrological Institute. Available online at: https://www.smhi.se/data/meteorologi/ladda-ner-meteorologiska-observationer#param=airtemperatureInstant,stations=all,stationid=97510 (accessed November, 2020).

Soler, R., Bezemer, T. M., Van Der Putten, W. H., Vet, L. E., and Harvey, J. A. (2005). Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *J. Anim. Ecol.* 74, 1121–1130. doi: 10.1111/j.1365-2656.2005.01006.x

Soler, R., Harvey, J. A., Kamp, A. F. D., Vet, L. E. M., Van der Putten, W. H., Van Dam, N. M., et al. (2007). Root herbivores influence the behaviour of an aboveground parasitoid through changes in plant-volatile signals. *Oikos* 116, 367–376. doi: 10.1111/j.0030-1299.2007.15501.x

Thaler, J. S., Humphrey, P. T., and Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci.* 17, 260–270. doi: 10.1016/j.tplants.2012.02.010

Van Dam, N. M., Raaijmakers, C. E., and Van Der Putten, W. H. (2005). Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl.* 115, 161–170. doi: 10.1111/j.1570-7458.2005.00241

Venables, W. N., and Ripley, B. D. (2002). *Modern Applied Statistics with S*. New York, NY: Springer.

Vivas, M., Martín, J. A., Gil, L., and Solla, A. (2012). Evaluating methyl jasmonate for induction of resistance against *Fusarium oxysporum*, *Fusarium circinatum* and *Ophiostoma novo-ulmi*. *J. Agric. Exten. Rural Dev.* 4, 259–260. doi: 10.5897/jaerd12.064

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag.

Wilkinson, S. W., Magerøy, M. H., Sánchez, A. L., Smith, L. M., Furci, L., Cotton, T. E. A., et al. (2019). Surviving in a hostile world: plant strategies to resist pests and diseases. *Ann. Rev. Phytopathol.* 57, 505–529. doi: 10.1146/annurev-phyto-082718-095959

Wu, J., and Baldwin, I. T. (2009). Herbivory-induced signalling in plants: perception and action. *Plant Cell Environ.* 32, 1161–1174. doi: 10.1111/j.1365-3040.2009.01943.x

Yu, X., Zhang, W., Zhang, Y., Zhang, X., Lang, D., and Zhang, X. (2019). The roles of methyl jasmonate to stress in plants. *Funct. Plant Biol.* 46:197. doi: 10.1071/fp18106

Zas, R., Björklund, N., Nordlander, G., Cendán, C., Hellqvist, C., and Sampedro, L. (2014). Exploiting jasmonate-induced responses for field protection of conifer seedlings against a major forest pest, *Hylobius abietis*. *Forest Ecol. Manag.* 313, 212–223. doi: 10.1016/j.foreco.2013.11.014

Conflict of Interest: 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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ORIGINAL RESEARCH
published: 26 July 2021
doi: 10.3389/fpls.2021.691292

Nitrogen Supply Alters Rice Defense Against the Striped Stem Borer *Chilo suppressalis*

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Plant nutrition status is closely associated with plant defense against insect herbivores. However, the way nitrogen supply regulates rice anti-herbivore is not clear. This study investigated the effects of low (LN, 0.3 mM) and high (HN, 3 mM) nitrate levels on rice resistance against the striped stem borer *Chilo suppressalis* (SSB), one of the major destructive rice pests. Seven-day-old rice seedlings were cultured with different nitrate levels for 30 days and then inoculated with third instars of SSB. LN significantly enhanced rice anti-herbivore defense and lowered the total nitrogen content in the plants, but increased the content of free amino acids after SSB infestation. Additionally, LN significantly increased the accumulation of phenolic acids and flavonoids, especially lignin, resulting in enhanced constitutive defense in SSB-infested plants. SSB feeding led to a rapid accumulation of secondary metabolites. HN application led to the accumulation of metabolites derived from cinnamic acid, *p*-coumaric acid, *p*-coumaric CoA, feruloyl CoA, and apigenin, while LN led to the accumulation of metabolites derived from 3-dehydroquinic acid, phenylalanine, acetyl CoA, and aspartic acid. Collectively, our finding suggests that nitrogen deficiency enhances rice anti-herbivore defense *via* constitutive defense by the accumulation of phenolic acids and flavonoids.

Keywords: constitutive defense, induced defense, jasmonic acid, lignin, metabolome, nitrate, rice, striped stem borer

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Edited by:
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *22 April 2021*
Accepted: *24 June 2021*
Published: *26 July 2021*

Citation:
Zheng Y, Zhang X, Liu X, Qin N, Xu K, Zeng R, Liu J and Song Y (2021) Nitrogen Supply Alters Rice Defense Against the Striped Stem Borer Chilo suppressalis. Front. Plant Sci. 12:691292. doi: 10.3389/fpls.2021.691292

INTRODUCTION

In response to herbivore attacks, plants have evolved a wide spectrum of strategies to defend themselves against herbivores, such as constitutive defense and induced defense (Tiffin, 2000). Constitutive defense is always expressed, whereas induced defense is activated only after plants are attacked by herbivores (Kempel et al., 2011). Many evolutionary models of induced defense treat it as being derived from constitutive defense, the presumed ancestral state (Thaler and Karban, 1997). Trade-offs between constitutive defense and induced defense with and among species are likely to be beneficial to plants (Morris et al., 2006; Zhang et al., 2008). The constitutive defense and induced defense are both influenced by environmental factors and closely associated with plant physiological characteristics, nutritional status, and the accumulation of secondary metabolites.

Plants produce a tremendous number of secondary metabolites to defend against herbivores (Mumm and Hilker, 2006). Herbivory by *Helicoverpa zea* induced great changes in precursor amino acids in the shikimate pathway in tomato (Steinbrenner et al., 2011). Shikimate-derived amino acids and simple phenylpropanoids are precursors for many secondary metabolites (Herrmann and Weaver, 1999). In these compounds, flavonoids and phenolics are known to be effective defensive compounds against herbivores (Sampedro et al., 2011). Toxins such as flavonoids are considered primarily effective against generalist pests (Diaz Napal et al., 2010). Putative phenolic acid derivatives were also identified as important metabolites produced by plants during antibacterial defense mechanisms (Luzzatto et al., 2007).

Lignin is one of the most important phenolic acids, providing plants with physical and chemical defense mechanisms against herbivores (Liu Q. et al., 2018). Lignin serves as an important barrier that protects plants against herbivory. In rice, treatment with an insect-specific toxin peptide LqhIT2 enhanced the lignin content, leading to enhanced resistance to leafroller (Tianpei et al., 2015). In the pre-ingestion phase, host plants can limit food supplies to insects *via* physical barriers such as the cell wall fortification. Lignin and other phenolics can strengthen cell walls against digestion (Brodeur-Campbell et al., 2006; Schroeder et al., 2006). In addition, increased lignin deposition might have additional negative effects on insect fitness because phenoloxidase enzymes are involved in lignin polymerization as well as in the generation of toxic by-products such as reactive oxygen species, quinones, and peroxides (Felton et al., 1989; Stevenson et al., 1993).

Plants grown with limited resources may produce more phenolic compounds but show slow growth (Wilkens et al., 1996). Nitrogen is an essential macronutrient and a major limiting factor of plant growth and development. Besides, nitrogen can also impact the ability of plants to cope with biotic stress (Ballini et al., 2013). For example, high nitrogen fertilization enhances *Botrytis cinerea* in strawberry (Daugaard et al., 2003), while it reduces susceptibility to this fungus in tomato (Vega et al., 2015). Additionally, the form of nitrogen available can also determine the effect of nitrogen supply on plant response to biotic stress. For instance, ammonium supply reduces the resistance to *Pseudomonas syringae*, while nitrate supply enhances plant resistance to this bacterium (Mur et al., 2017).

Nitrogen content and form play a vital role in defensive primary and secondary metabolism. It can influence defense *via* amino acid metabolism and hormone production. Nitrogen may have negative effects on physical defenses and the production of phytoalexins, but positive effects on defense-related enzymes and proteins to affect local and systemic defense mechanisms (Sun Y. et al., 2020). In most of the rice-growing areas, the increasing populations of major insect pests of rice are closely related to the long-term excessive application of nitrogen fertilizers (Lu et al., 2007). Increased numbers of both adult and immature whiteflies occurred with increasing amounts of applied nitrogen (Bi et al., 2001). Increased nitrogen availability in a tomato leads to a decreased allocation to defenses and increased preference of two-spotted spider mite (*Tetranychus urticae*) females (Hoffland et al., 2000). However, the molecular mechanism and the alteration of metabolism during the interaction between plants and insect herbivores under different nitrogen supply are still unclear. Rice (*Oryza sativa* L.) is an important food crop. The striped stem borer (SSB), *Chilo suppressalis* (Walker), is one of the most economically important and destructive rice pests, which is widely distributed in rice-production countries, leading to huge rice yield losses, particularly in China (Sun et al., 2018). This study aimed to examine how nitrogen supply level affects anti-herbivore defense against SSB and metabolome responses to insect herbivory in rice plants.

MATERIALS AND METHODS

Plant Cultivation

Rice (*Oryza sativa* L. cv. Ishikari-shiroge) seeds were surface-sterilized with 10% (v/v) H_2O_2 for 15 min and rinsed with distilled water three times. The sterilized seeds were pre-imbibed in distilled water for 1 day in darkness and then transferred to seedling tray for 7 days. After germination, the seedlings were cultured with modified Kimura B nutrient solution containing two concentrations of KNO_3 (Li et al., 2016). For LN treatment, the nitrogen concentration in nutrient solution was 0.3 mM KNO_3. For HN treatment, 3 mM KNO_3 was added in Kimura B nutrient solution. Given the important role of potassium in plant defense and growth, the same concentration of potassium was added in LN group to replenish potassium. The plants were cultured in a growth chamber with a day: night temperature regime of 28°C (14 h): 22°C (10 h) and a light intensity of 30,000 lux for 30 days.

Striped Stem Borer Treatment

The original eggs of SSB were provided by the State Key Laboratory for Biology of Plant Diseases and Insect Pests, Chinese Academy of Agricultural Sciences. All cultures were kept under the conditions of 27 ± 1°C, a photoperiod of 16:8 (L:D) h, and 70–80% RH, except for adult mating and oviposition at 85–90% RH (Han et al., 2012). Plants cultured for 30 days were infested with third instars of SSB for biochemical analysis or bioassays. The moment the larva started to chew a hole was defined as time point zero for time course experiments. The stems around 3 cm of the entry hole were harvested at different time points after SSB attack (Hu et al., 2018). For the determination of feeding preference of SSB, each group contained 120 plants, and each plant was inoculated with one third-instar larva of SSB. The feeding was counted as the larva chewed a hole on the stem. The number of feeding SSB was recorded every 30 min in the first 4 h (Tong et al., 2012).

Measurement of Leaf Chlorophyll and Plant Total Nitrogen

The relative chlorophyll content of rice leaves was measured by a chlorophyll meter SPAD-plus 502 (Konica Minolta Camera Co., Ltd., Japan) according to the method previously described by Sun et al. (2019). For the determination of total nitrogen, the plant

samples were oven-dried for at least 24 h at 65°C and weighed; then the material was ground. The total nitrogen content of rice seedlings (mg N per g dry weight) was analyzed using a Foss Kjeltec 8400 analyzer (Kjeltec Analyzer Unit, Foss Tecator AB, Hoganas, Sweden) (Liu J. et al., 2018).

Determination of Free Amino Acids and Soluble Sugars

Total free amino acids (FAAs) were determined using the ninhydrin colorimetric method (Rosen, 1957). Briefly, the total FAAs from rice seedlings were extracted in ethanol/NaAc buffer (pH 5.4) and then measured using a colorimetric assay at 570 nm. The FAA content was calculated on the basis of a calibration curve by 1-Leu. The contents of soluble sugar in rice seedling were determined according to the methods described in the study by Dubois et al. (1951). Briefly, approximately 100 mg of the oven-dried sample was ground and then extracted with water at 95°C for 10 min. The solution was then centrifuged at 8,000 *g* at 25°C for 10 min. The resulting supernatants were combined and analyzed using the anthrone–sulfuric acid method (Kuang et al., 2017).

Plant Hormone Analysis

Rice seedlings were cultured with different concentrations of nitrates for 30 days. Stems were harvested at 0, 3, 9 or 24 h after SSB infestation. Samples were immediately immersed in liquid nitrogen and stored at −80°C. For each time interval, five plants were sampled. JA, JA-Ile, SA, and abscisic acid (ABA) were extracted for LC-MS analysis using labeled internal standards as described in the study by Pan et al. (2010).

Determination of Peroxidase and Polyphenol Oxidase Activities

Two defense-related enzymes, namely, determination of peroxidase (POD) (EC 1.11.1.7) and Polyphenol Oxidase (PPO) (EC1.10.3.1), were analyzed. Leaf samples (0.1 g fresh weight) were ground in liquid nitrogen and homogenized in 0.05 M phosphate buffer (pH 7.2 for POD, pH 7.8 for PPO) containing 1% (w/v) polyvinylpyrrolidone (PVP). The supernatant after 12,000 g centrifugation for 15 min at 4°C was used for enzyme assays. Activities of POD and PPO were spectrophotometrically determined according to the previous study (Song et al., 2014). For POD and PPO, the change in absorbance at 470/525 nm by 0.005/0.01 was an enzyme activity unit per minute per gram of tissue in each mL of the reaction system. Three independent biological replicates for each treatment were used for enzyme assays.

Determination of Lignin Content and Phloroglucinol Staining

Quantitative determination of lignin was measured by the method of Fukushima and Hatfield (2004). Acetyl bromide was analyzed according to the study by Foster et al. (2010). Lignin in the stem was stained as described in the previous study using the Wiesner reagent test (Shan et al., 2008). Stem near the feeding site (2 cm length) was used for staining. Each stem was cut into slices horizontally of 101 μm thickness and then stained in 1% (w/v) phloroglucinol liquor for 5 min. Slices were then mounted on slides and a few drop of HCl (18%, v/v) were added. The extent of staining was examined by a Leica microscope.

Determination the Activity of 4-Coumarate: CoA Ligase and Cinnamyl Alcohol Dehydrogenase

The activity of 4-Coumarate: CoA Ligase (4CL) was examined by the increase in A_{333} with *p*-coumarate as substrate. The reaction mixture contained crude enzyme, 0.2 mM *p*-coumarate, 0.8 mM ATP, 7.5 mM magnesium chloride anhydrous, and 38 μM CoA in 100 mM TES (pH 7.6). Protein concentrations were determined by the approach of Bradford (1976). 4CL activity was expressed as 0.01 ΔOD333/mg protein (Knobloch and Hahlbrock, 1977; Shan et al., 2008).

For the determination of CAD activity, the crude enzyme was incubated with 2 mM NADP and 1 mM *trans*-cinnamic acid at 37°C for 30 min and then terminated by adding 1 M HCl. One unit of CAD was defined as the amount of enzyme that caused a change of 0.01 in absorbance per milligram protein at 37°C (Liu et al., 2014).

Quantitative Real-Time PCR Analysis

Differential expression of selected genes was verified by quantitative real-time PCR (qRT-PCR) (Liu et al., 2016). Total RNA from the treated stem and leaves was extracted with Eastep™ Total RNA Extraction Kit (Progema). First-strand cDNA was synthesized from 1 μg of RNA using GoScript™ Reverse Transcription system (Promega). qPCR amplification was carried out using *OsActin* as endogenous control. SYBR Green probes for each gene were used. The primers are listed in **Supplementary Tables 1, 2**. Real-time PCRs proceeded with 5 μl of the 2 × UltraSYBR Mixture, 0.2 μl (0.2 μM) of each specific primer, and 1 μl of cDNA, and the final volume was adjusted to 10 μl with RNase-free water. The thermal cycle reaction condition was as follows: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60–64°C for 1 min. The specificity of amplicons was verified by the melting curve analysis. Three independent biological replicates for each treatment were used for qRT-PCR analyses.

Metabolome Analysis

Rice seedlings were treated with different nitrate levels for 30 days and then infected with SSB for 3 days. The feeding stem (3 cm from feeding sites) was collected and then stored at −80°C. For LC-MS analysis, frozen stem was ground in liquid N_2 and lyophilized overnight. A mixture of methanol and H_2O (70:30, 1.2 mL) was added to 100 mg of lyophilized tissue, shaken per 2–3 h overnight, and centrifuged for 10 min at 12,000 *g*. Aliquots of supernatant were transferred to clean tubes; the clear supernatant was filtered into glass LC autosampler vials using a PVDF filter and stored at −20°C until analyzed. Samples were analyzed by UPLC-MS/MS (ultra-performance liquid chromatography–tandem mass spectrometry). Extraction for UPLC-MS analysis was adapted from the study by Fraga et al. (2010).

RNA-Seq

Plants cultured for 30 days were infested with third instars of SSB for 24 h and then sampled for total RNA extraction with mirVana miRNA isolation kit (Ambion). The required fragments were sequenced using Illumina HiSeq 2500 instrument, with default quality parameters, at Oebiotech (Shanghai, China). The reference genome was obtained according to the previous study (Kim et al., 2015). Both portals were included in this study to allow a complete analysis of the genome. NGS QC Toolkit was used for quality control of the raw reads and then aligned to the reference genome (Patel and Jain, 2012). The software R package of DESeq was employed to capture the differentially expressed genes (DEGs) (Anders and Huber, 2012). All unigenes were annotated with gene ontology (GO) and KEGG pathway analysis.

Data Analysis

SPSS 22.0 (SPSS, Chicago, IL, United States) was used for statistical analysis. Data were assessed by three-way ANOVA, two-way ANOVA, one-way ANOVA, and independent-sample *t*-test, using Tukey's test for differences between means.

RESULTS

Low Nitrate Supply Enhances Rice Anti-herbivore Defense

To investigate the effects of nitrogen levels on rice defense against herbivore, rice seedlings were cultured with modified Kimura B nutrient solution containing different concentration of KNO_3 (0.3 mM for LN and 3 mM for HN) for 30 days and then infested with third instars of SSB for 3 days. Long LN treatment decreased seedling height and aboveground dry weight, while it increased the root length and underground dry weight, compared with HN treatment (**Supplementary Figure 1**). Additionally, 55.54 and 153.8% increases in SSB mass gain were found in the larvae fed on plants cultured with LN and HN, respectively (**Figure 1A** and **Supplementary Figure 2**). It seems that LN could enhance rice defense against SSB infestation. The feeding preference of SSB larvae was significantly different between rice plants cultured under LN and HN supply 1.5 h after larval inoculation. SSB preferred to HN-cultured plants (**Figure 1B**). Moreover, the plants cultured with LN exhibited an enhanced activity of PPO and POD compared with those with HN (**Figures 1C,D**). Collectively, the nitrate supply could regulate seedling anti-herbivore defense, and LN enhanced plant defense compared with that in HN treatment.

Nitrate Supply Alters Rice Primary Metabolism and Chlorophyll Metabolism Under SSB Infestation

To investigate the effect of nitrate supply on metabolism, total nitrogen content, total FAA content, and soluble sugar content were determined. To be consistent with the treated nitrogen concentration, the plants cultured with HN exhibited higher total nitrogen content LN regardless of SSB inoculation or not (**Figure 2A**). In the first 24 h of infestation, free amino acid content was decreased. Intriguingly, the content of free amino acid in plants cultured with LN was higher than that with HN independent of SSB inoculation (**Figure 2B**). However, no significant difference in soluble sugar was found between HN and LN treatments under SSB infestation (**Figure 2C**). Moreover, chlorophyll content was decreased after SSB inoculation, and chlorophyll content in the plants cultured with HN was significantly higher than that in LN (**Figure 2D**). qPCR analysis showed that the expression of gene (*NYC1*, *PAO*) involved in chlorophyll degradation was upregulated under SSB infestation, and the effect was more obvious in LN than that in HN (**Supplementary Figures 3A,C** and **Supplementary Table 1**). The results show that nitrate supply levels alter primary metabolism and chlorophyll metabolism in SSB-infested rice plants.

Nitrate Supply Alters the Primary and Secondary Metabolism, as Well as Constitutive Defense

Metabolome analysis was conducted to investigate the effect of nitrate supply on rice cell metabolism. The quality control for metabolome was shown as PCA (**Supplementary Figure 4**). KEGG pathway enrichment analysis was used to study the signaling pathways of all detectable metabolites (Kanehisa et al., 2008). It was shown that differentially accumulated compounds (DACs) were significantly enriched in the biosynthesis of secondary metabolites (**Figure 3A**). Further analysis showed that low nitrate significantly increased the production of phenolic acids, flavonoids, saccharides, and alcohols (others), while high nitrate significantly increased the accumulation of amino acids and their derivatives, alkaloids and terpenoids (**Figure 3B**). Each metabolite group of the phenolic acids, flavonoids, saccharides, and alcohols is shown in **Figures 3C–E**, respectively.

To further evaluate the effects of the transcriptome change on the metabolome, RNA-Seq was taken. The RNA-Seq metrics for quality control was shown as PCA, correlation analysis of differential genes (**Supplementary Figure 5**). Additionally, the transcriptomes were also validated through quantitative PCR (qPCR) analysis of selected genes over the treatment, including β-actin as an endogenous control (**Supplementary Table 2**). The results of RNA-Seq were consistent with those of qPCR. The overview of differential genes under nitrogen treatment is shown in **Supplementary Figure 5C** ($P < 0.05$, fold change > 2, FPKM ≥ 5), 1,217 DEGs were found in data between LN and HN treatments, while 638 DEGs were between LNSBB and HNSSB treatments. About 164 DEGs were co-expressed between LN and HN, and LNSSB and HNSSB (**Supplementary Figure 5D**).

The DEGs and DACs were incorporated in the KEGG pathway (**Figure 4A**). Additionally, the effects of transcriptome change on the secondary metabolites were also evaluated (**Figure 4B**). The great changes occurred in cell wall, sugar, and lignin pathway in LN. Genome-wide connection network between phenylpropanoid metabolism-associated genes and metabolites was analyzed in **Figure 4C**. Caffeic acid and coniferyl alcohol are two important intermediate metabolites of lignin biosynthesis (Zhao and Dixon, 2011). Four of six genes associated with caffeic acid metabolism and all three genes associated with coniferyl

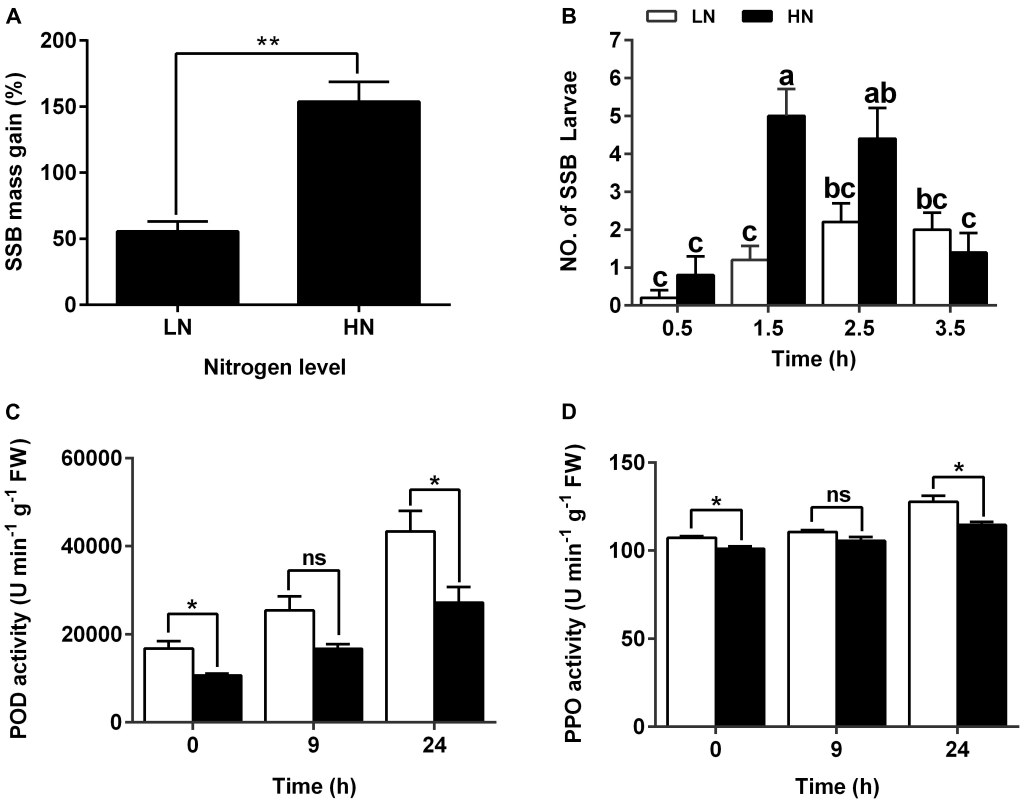

FIGURE 1 | Larval performance of the striped stem borer (SSB) and defensive enzyme activities in rice plants cultivated with different concentrations (0.3 and 3 mM) of nitrate. **(A)** Mass gain of SSB larvae fed on rice plants cultured with different concentrations of nitrogen. Seven-day-old seedlings were transplanted to nutrient solution containing 0.3 mM KNO_3 (LN) or 3 mM KNO_3 (HN) and cultured for 30 days. In low nitrogen treatment, potassium chloride was used to replenish potassium. Larvae at the third-instar stage were used for bioassays. The individual larvae were measured 3 days after inoculation, and the mean percentage of mass gain was calculated. Values are mean ± SE (n = 20). Asterisks (**) indicate Student's t-test significance at $P < 0.01$ versus the indicated samples. **(B)** Feeding preference of SSB on rice plants cultured under nitrogen supply. The number of SSB larvae fed on rice plants was recorded at 0.5, 1.5, 2.5, and 3.5 h after SSB inoculation. Each data is the mean ± SE of five replicates. Different letters indicate statistically significant differences between treatments (Tukey's multiple range test: $P < 0.05$). Enzyme activity of POD **(C)** and PPO **(D)** in the stems of rice plants cultivated with different concentrations (0.3 and 3 mM) of nitrate and inoculated with the striped stem borer (SSB). Values are mean ± SE of three replicates. Asterisks (*) indicate Student's t-test significance at $P < 0.05$ versus the indicated samples, and ns means no significant difference.

alcohol metabolism were upregulated under low nitrate regime (**Figures 4D,E**). The activity of 4CL and CAD, which are involved in the lignin biosynthesis, were upregulated under low nitrate supply (**Figures 4G,H**), resulting in the accumulation of lignin (**Figure 4F**). These results indicated that low nitrate supply might regulate rice defense against insect herbivory through lignin deposition.

Metabolism Alteration Under SSB Infestation Induced by Nitrate Supply

For the combination of nitrogen and SSB treatment, the metabolites in rice plants were divided into 12 classes according to the tendency of changes (**Supplementary Figure 6**). KEGG pathway-enrichment analysis was also used to determine the involved signaling pathways of the DACs under SSB infestation. Most of DACs were significantly enriched in the biosynthesis of secondary metabolites and metabolic pathways under LN (**Figure 5A**) and HN (**Figure 5B**) supply. Moreover, the first two enriched pathways were phenylalanine metabolism and phenylpropanoid biosynthesis in HNSSB compared with LNSSB (**Figure 5C**).

To examine the effects of nitrogen supply on primary and secondary metabolism in induced defense, the DACs were further divided into three classes (**Figure 5D**): (1) The metabolites were upregulated after SSB infestation both in LNSSB and in HNSSB group and the metabolites in LNSSB group were less than those in HNSSB group (red marked in **Figure 5D**); (2) the metabolites were upregulated after SSB infestation both in LNSSB and in HNSSB group and the metabolites in LNSSB group were more than those in HNSSB group (light green marked in **Figure 5D**); (3) the metabolites were downregulated after SSB infestation both in LNSSB and in HNSSB group but the metabolites in LNSSB group were more than those in HNSSB group (dark green marked in **Figure 5D**).

The source of red marked compounds were approximately divided into five classes: cinnamic acid, *p*-coumaric acid, *p*-coumaric CoA, feruloyl CoA and apigenin. The light green

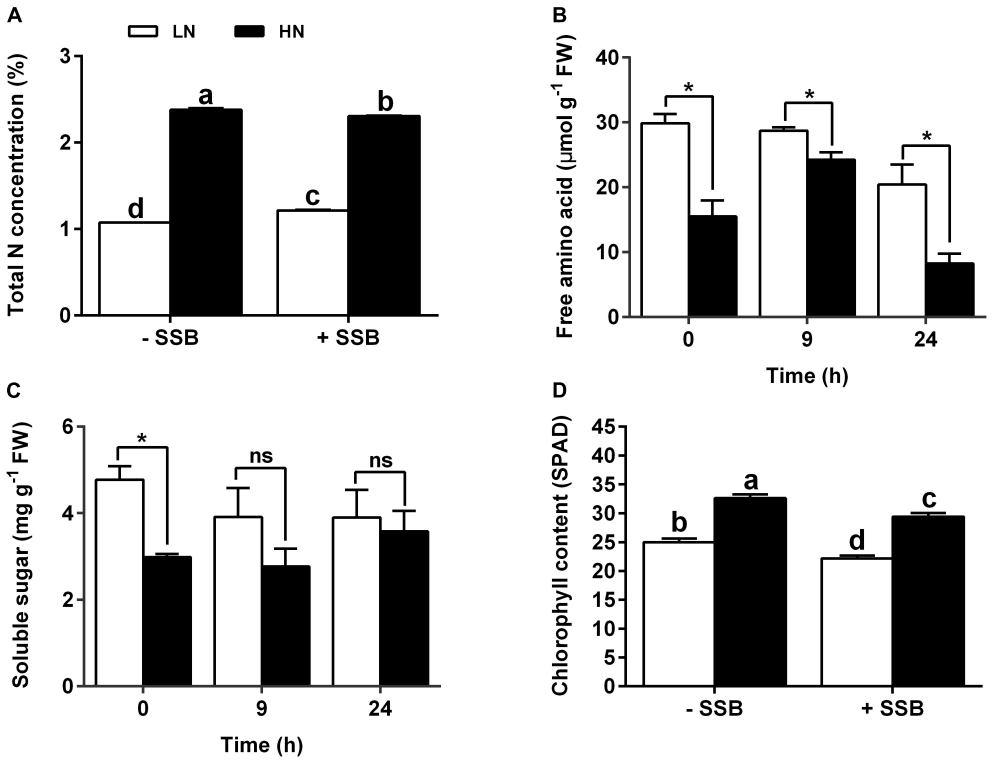

FIGURE 2 | Changes in nitrogen contents, primary metabolite, and chlorophyll metabolism in rice plants cultivated with different concentrations (0.3 and 3 mM) of nitrate and inoculated with the striped stem borer (SSB). Seven-day-old seedlings were transplanted to nutrient solution containing 0.3 mM (LN) or 3 mM KNO_3 (HN) and cultured for 30 days. **(A)** Total nitrogen content was determined at 48 h after SSB inoculation. The contents of soluble sugar content **(B)** and free amino acid content **(C)** were determined at 0, 9, and 24 h after SSB inoculation. **(D)** Total chlorophyll content was determined at 24 h after SSB inoculation. Data are the mean ± SE of three replicates **(A–C)** and 12 replicates **(D)**. Different letters indicate statistically significant differences between treatments (Tukey's multiple range test: $P < 0.05$). Asterisks (*) indicate Student's *t*-test significance at $P < 0.05$ versus the indicated samples, and ns means no significant difference.

marked compounds were screened by Venn diagram analysis (**Figure 6A**). And they were almost derived from four compounds: 3-dehydroquinic acid, phenylalanine, acetyl CoA, and aspartic acid. Each content of the compounds is shown in **Figure 6B**. The dark green marked compounds were derived from about three compounds: sinapyl CoA, apigenin, and luteolin. These compounds were screened (**Figure 6C**), and the content of the compounds are shown in heatmap (**Figure 6D**).

Additionally, Venn diagram analysis showed that six metabolites were induced by both nitrate and SSB, and the content of induced metabolites in LNSSB group was more than that in HNSSB group (**Figure 7A**). These metabolites were 3-*O*-*p*-coumaroylquinic acid *O*-glucoside, 5-*O*-*p*-coumaroylquinic acid *O*-glucoside, D-xylonic acid, D-(-)-arabinose, D-sedoheptulose 7-phosphate, and glucarate *O*-phosphoric acid (**Figure 7B**).

Alteration of Lignin Content Under Nitrate and SSB Treatment

Before SSB inoculation, the lignin content in LN-cultured plants was higher than that in HN-cultured plants (**Figure 4F**). The vast majority of compounds involved in lignin biosynthesis were upregulated by SSB feeding (**Supplementary Figure 7A**). Interestingly, after SSB infestation, lignin accumulation was higher (**Supplementary Figure 7B**) and faster in HN-cultured plants (**Supplementary Figure 7C**).

Effect of Nitrate Supply on Phytohormone Level Under SSB Infestation

To determine the effects of nitrogen levels on phytohormones possibly involved in plant defense against herbivores, LC-MS analysis was performed to quantify phytohormone levels of JA, JA-Ile, SA, and ABA in rice plants cultivated with different concentrations (0.3 and 3 mM) of nitrate and inoculated with the SSB. Phytohormone standard curve is shown in **Supplementary Figure 8**. Higher contents of JA and JA-Ile were found in LN-cultured plants before SSB inoculation. However, no significant difference in JA and JA-Ile contents was detected between LN- and HN-cultured plants 3 h after SSB inoculation (**Figures 8A,B**). However, constantly higher content of SA was found in LN-cultured plants either before or after SSB inoculation (**Figure 8C**). The changes in ABA contents were similar to those in JA and JA-Ile (**Figure 8D**). To further determine the role of JA signaling in rice defense against SSB infestation

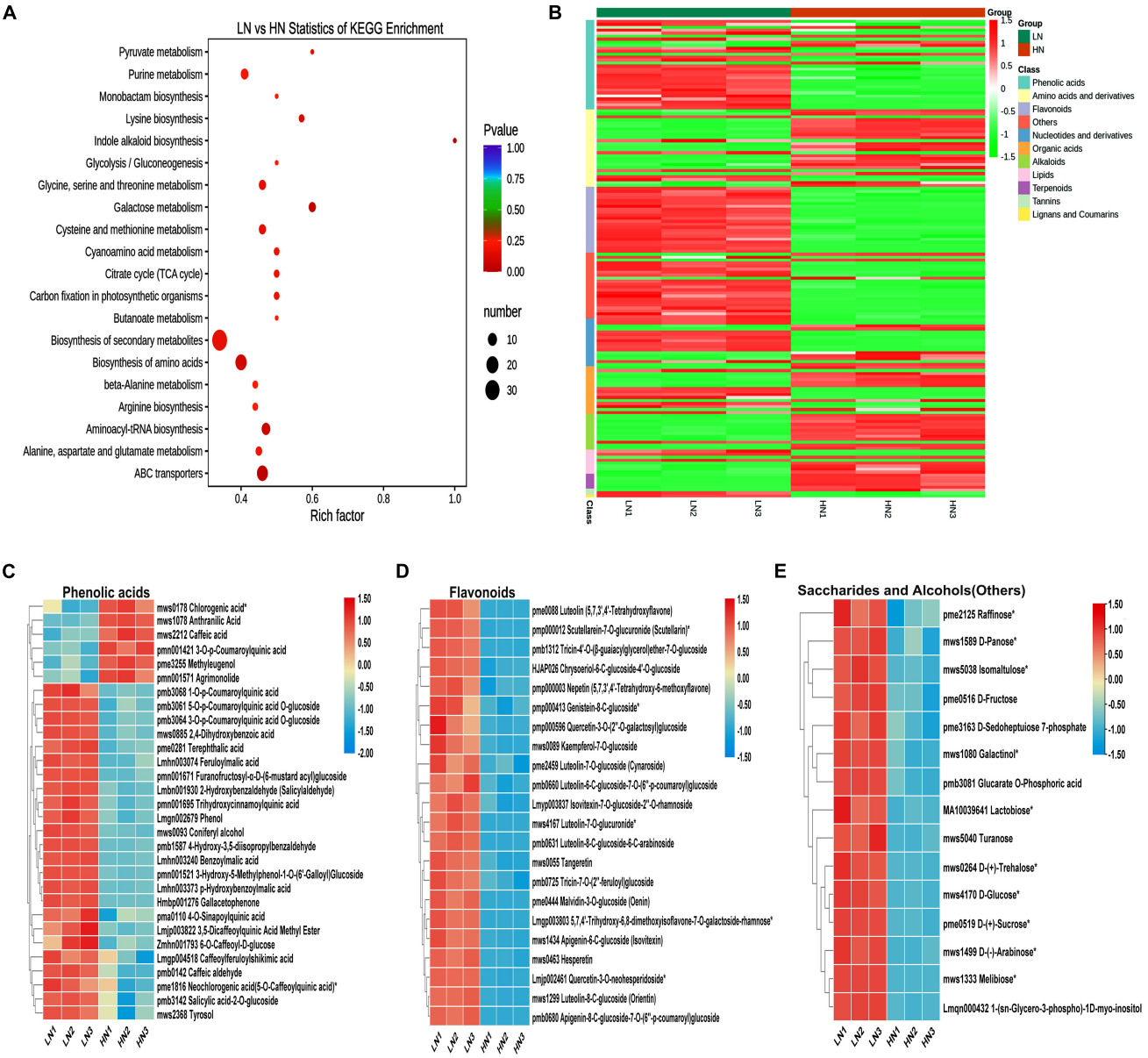

FIGURE 3 | Metabolome profiling of the stems of rice plants cultivated with different concentrations (0.3 and 3 mM) of nitrate and inoculated with the striped stem borer (SSB). Seven-day-old seedlings were transplanted to nutrient solution containing 0.3 mM (LN) or 3 mM KNO_3 (HN) and cultured for 30 days for sampling for metabolome analysis. **(A)** KEGG enrichment analysis of difference in metabolites between LN and HN treatments. Heatmap analysis of differentially accumulated metabolites **(B)**, phenolic acids **(C)**, flavonoids **(D)**, and saccharides and alcohols **(E)** between LN and HN treatments. In order to facilitate comparison, the relative content of metabolites was normalized.

under different regimes of nitrogen, two rice RNAi lines *aos* RNAi and *coil* RNAi of the JA signaling pathway were used. The two transgenic lines were obtained by silencing the expression of allene oxide synthase (*OsAOS*; active in JA biosynthesis) and CORONATINE INSENSITIVE1 (*OsCOI1*; active in JA perception) genes in rice plants *via* RNAi (Ye et al., 2012). Silencing either *OsAOS* or *OsCOI1* enhanced rice susceptibility to SSB infestation. Increased nitrogen supply decreased rice resistance to SSB regardless of the genotypes (**Supplementary Figure 9**), suggesting independence of JA signaling in nitrogen-mediated anti-herbivore defense in rice plants.

DISCUSSION

Nutrient status plays a key role in plant defense against insect herbivores. Fertilization management may serve as an important approach to manage insect pests in sustainable agriculture. In this study, low nitrogen treatment for 4 weeks significantly

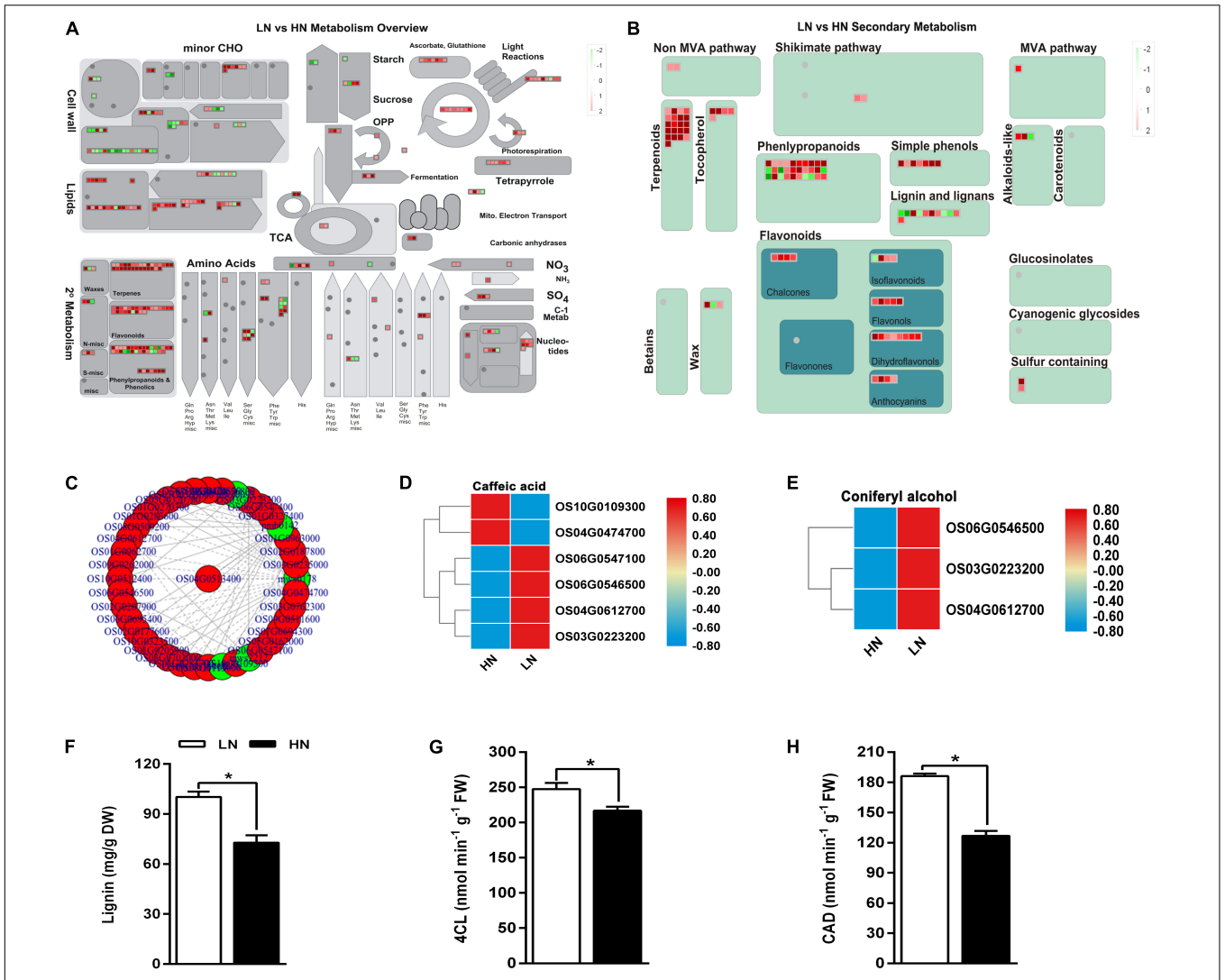

FIGURE 4 | Joint analysis of transcriptome and metabolome of the stems of rice plants cultivated with different concentrations (0.3 and 3 mM) of nitrate and inoculated with the striped stem borer (SSB). Seven-day-old seedlings were transplanted to nutrient solution containing 0.3 mM (LN) or 3 mM KNO_3 (HN) and cultured for 30 days for sampling. The overview of differential metabolites **(A)** and differential secondary metabolites **(B)**. Red means HN upregulated, while green means HN downregulated. **(C)** Joint analysis of the correlation network associated with phenylpropanoids pathway. Relative expression levels of genes involved in the biosynthesis of caffeic acid **(D)** and coniferyl alcohol **(E)**. Quantitative evaluation of lignin content **(F)** and activity of 4CL **(G)** and CAD **(H)**. Asterisks (*) indicate Student's *t*-test significance at $P < 0.05$. To facilitate comparison, normalize gene expression levels. 4CL, 4-Coumarate: CoA ligase, CAD, Cinnamyl alcohol dehydrogenase.

enhanced rice defense against SSB (**Figure 1**), one of the most destructive rice pests. Nitrogen deficiency changed both primary and secondary metabolism of rice, especially phenylpropanoid metabolism (**Figures 2–4**), leading to differential metabolic bypass in rice defense against SSB herbivory.

Trade-Off Between Growth and Defense Induced by Plant Nutrition

In plants, trade-offs exist between growth and resistance to herbivory because secondary metabolism and physical defenses divert resources from plant growth (Herms and Mattson, 1992; Huot et al., 2014; Züst and Agrawal, 2017). Immune-triggered diminished growth is a strategy to avoid starvation of essential metabolic intermediates, which is generally consistent with the acclimatory response hypothesis, i.e., diminished growth may optimize the temporal and spatial expression of defense compounds without compromising other critical roles in central metabolism (Guo et al., 2018).

Previous studies have revealed that nutrient availability could regulate plant trade-offs between growth and defense. Increased nitrogen availability decreased plant resistance to the three herbivores in cranberry, regardless of genotypes (de Lange et al., 2019). Nitrogen fertilizer affects ecological fitness of herbivores, such as selection to host plants, survival, growth, development, fecundity, and population dynamics (Lu et al., 2007). Nutrient

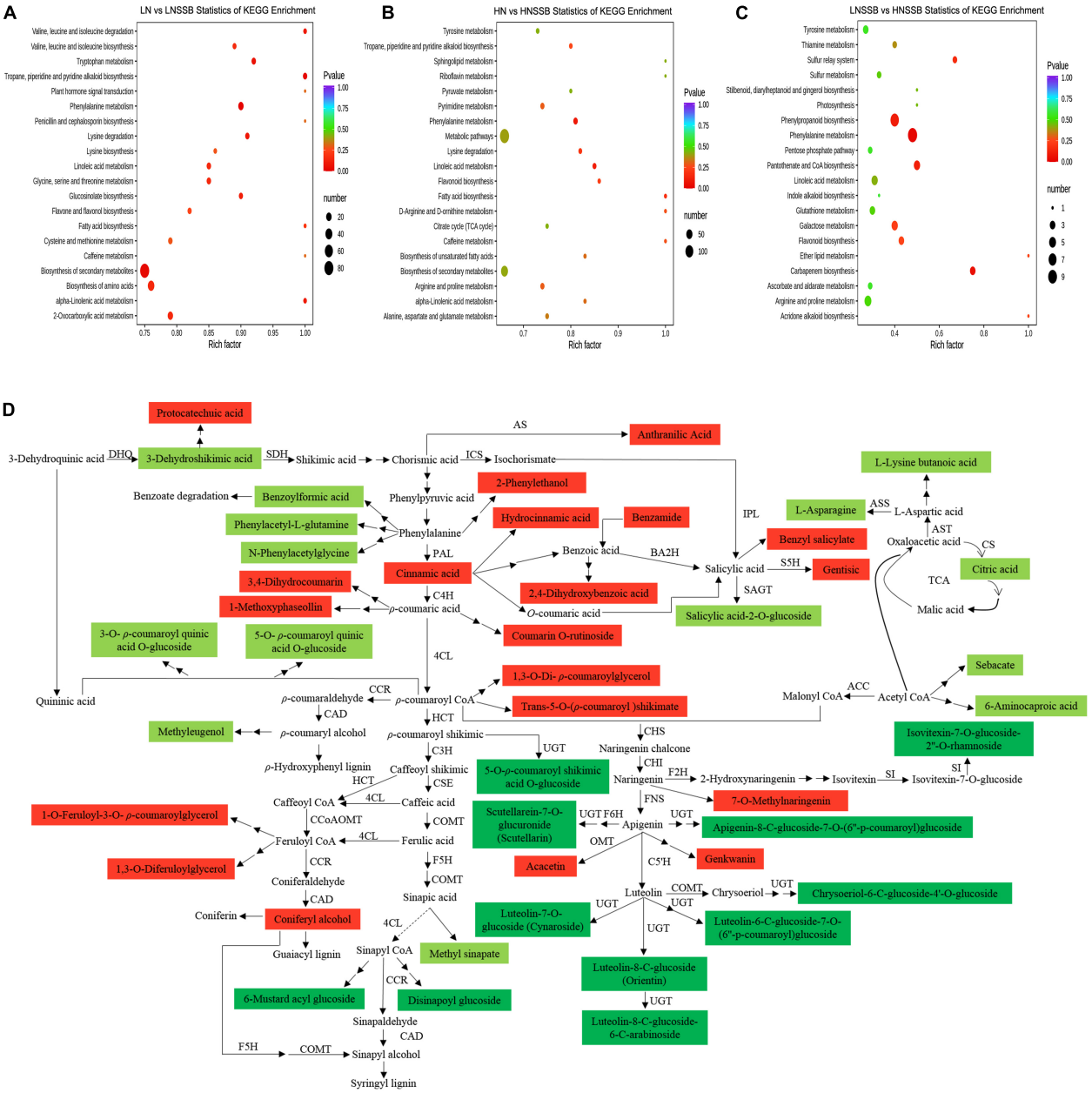

FIGURE 5 | Overview of metabolism changes in the stems of rice plants cultivated with different concentrations of nitrate and inoculated with the striped stem borer (SSB). Seven-day-old seedlings were transplanted to nutrient solution containing 0.3 mM (LN) or 3 mM KNO_3 (HN) and cultured for 30 days for sampling. KEGG enrichment analysis of differential metabolites in rice between LN and LNSSB treatments **(A)**, HN and HNSSB treatments **(B)**, and LNSSB and HNSSB treatments **(C)**. **(D)** Alteration of metabolites involved in shikimic acid and TCA pathways under nitrogen and SSB treatment. Red means that the metabolites were upregulated after SSB infestation both in LN and in HN treatment and the metabolites in LNSSB group were less than those in HNSSB group. Light green means that the metabolites were upregulated after SSB infestation both in LN and in HN treatment and the metabolites in LNSSB group were more than those in HNSSB group. Dark green means that the metabolites were downregulated after SSB infestation both in LN and in HN treatment but the metabolites in LNSSB group were more than those in HNSSB group. LNSSB, 0.3 mM KNO_3 cultured for 30 days, then infected by SSB for 3 days; HNSSB, 3 mM KNO_3 cultured for 30 days, then infected by SSB for 3 days. Single solid arrow means the established biosynthesis steps, two solid arrows means the involvement of multiple enzymatic reactions, while broken arrows represent the unestablished biosynthesis steps. DHQ, 3-dehydroquinate dehydratase; SDH, shikimate dehydrogenase; AS, anthranilate synthase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid 2-hydroxylase; SAGT, salicylic acid glucosyltransferase; S5H, salicylate 5-hydroxylase; AST, aspartate aminotransferase; ASS, asparagine synthase; CS, citrate synthase; ACC, acetyl-CoA carboxylase; PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CCR, cinnamoyl-CoA reductase; HCT, hydroxycinnamoyl-CoA: Shikimate/quinate hydroxycinnamoyl-transferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; F5H, ferulate 5-hydroxylase; CSE, caffeoyl shikimate esterase; COMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; UGT, UDP-glycosyltransferase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavonoid synthase; F6H, flavanone-6-hydroxylase; OMT, *O*-methyltransferase; C5′H, dihydroflavonol-5′-hydroxylase; F2H, flavanone-2-hydroxylase; SI, isovitexin beta-glucosyltransferase.

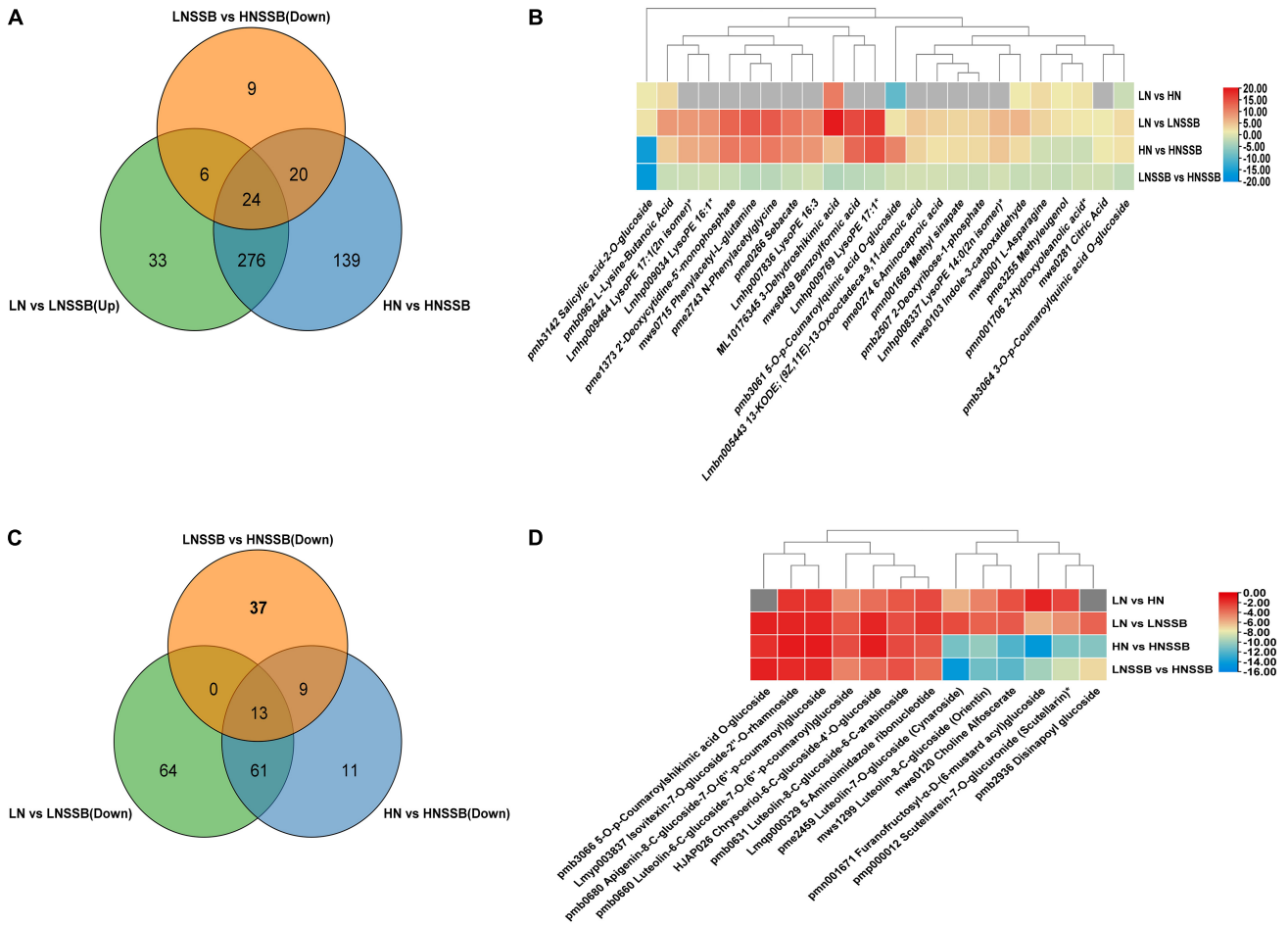

FIGURE 6 | Metabolites involved in rice responses to nitrogen deficiency and insect herbivory by the striped stem borer (SSB). LN, HN, and LNSSB are the control in the group LN vs. LNSSB, HN vs. HNSSB, and LNSSB vs. HNSSB, respectively. **(A)** Venn diagram analysis of differential metabolites under treatment from induced defense, which was upregulated after SSB infestation both in LN and in HN treatment, and the content of that in LNSSB group is more than that in HNSSB group. **(B)** Heatmap analysis of differential metabolites shared under treatment from induced defense (24). **(C)** Venn diagram analysis of differential metabolites under treatment from anti-defense, which was downregulated after SSB infestation both in LN and in HN treatment, and the content of that in LNSSB group is more than that in HNSSB group. **(D)** Heatmap analysis of differential metabolites shared under treatment from anti-defense (13). Gray square indicates no significant differences between metabolites. LN, 0.3 mM KNO_3 cultured for 30 days; HN, 3 mM KNO_3 cultured for 30 days; LNSSB, 0.3 mM KNO_3 treatment for 30 days, then infected by SSB for 3 days; HNSSB, 3 mM KNO_3 treatment for 30 days, then infected by SSB for 3 days.

availability can influence plant resistance to herbivores in various ways, such as by altering plant quality as a food source or by changing levels of secondary metabolites (Bryant et al., 1987; Altieri and Nicholls, 2003). Increasing nutrient availability may alter plant carbon allocation to chemical defensive compounds (Guo et al., 2018). Meanwhile, it also modify carbon allocation to structural defensive compounds to achieve their physical defense strategies in response to nutrient enrichment (Scalbert, 1991; Close and McArthur, 2002).

Metabolites Adjustment Induced by Nitrate in Rice Anti-herbivore Defense

Metabolome change is a strategy used by plants to cope with changes in the external environment. However, there are few related reports on priming mechanisms at the metabolome level.

Under SSB infestation, the content of chlorophyll was decreased independent of nitrogen supply (**Figure 2D**), which might be resulted in the reduced photosynthetic activity. Therefore, plants could face increased energetic and carbon demands to support inducible defenses. In comparison with rice with replete nitrate, nitrogen deficiency induced the accumulation of saccharides in the stem before SSB infection (**Figure 3E**). Once rice was infected by SSB, nitrogen deficiency promotes the degradation of saccharides; it is just the opposite for HN treatment. Therefore, rice cultured with nitrate deficiency could provide more energy from local catabolism of saccharides. Besides, the accumulation of pentoses (such as melibiose) in nitrate-deficient rice stem was also linked to the production of phenolic molecules (Gardiner, 1966).

In response to stress environments, such as drought, plants produce more phenolic compounds to serve as enzymatic

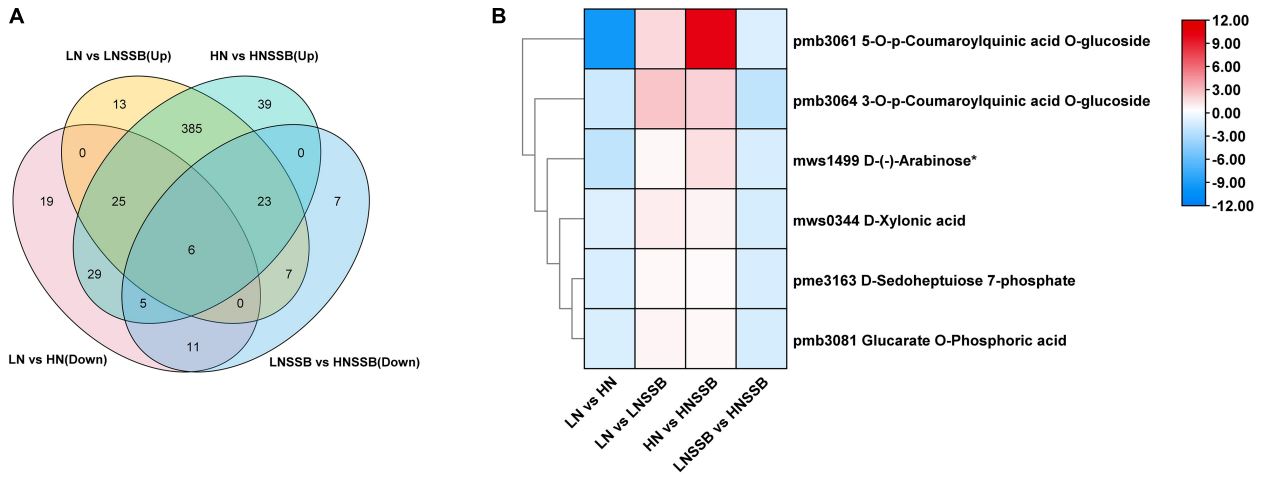

FIGURE 7 | Priming metabolites possibly involved in defense under low nitrogen and insect herbivory by the striped stem borer (SSB). LN, LN, HN, and LNSSB are the control in the group LN vs. HN, LN vs. LNSSB, HN vs. HNSSB, and LNSSB vs. HNSSB, respectively. **(A)** Venn diagram analysis of differential metabolites under treatment, which was the content of that in LN group more than that in HN group before SSB infestation, and the content of that in LNSSB group is more than that in HNSSB group after SSB infestation. **(B)** Heatmap analysis of differential metabolites shared under treatment (six). LN, 0.3 mM KNO3 cultured for 30 days; HN, 3 mM KNO3 cultured for 30 days; LNSSB, 0.3 mM KNO3 cultured for 30 days, then infected by SSB for 3 days; HNSSB, 3 mM KNO3 cultured for 30 days, then infected by SSB for 3 days.

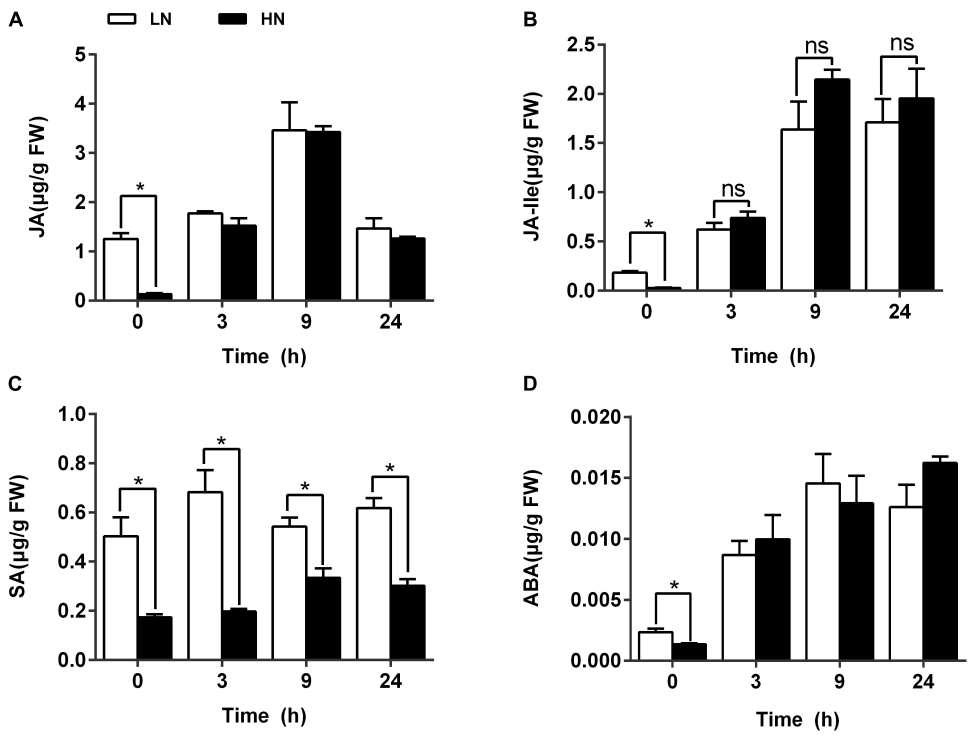

FIGURE 8 | Levels of phytohormones in rice plants cultivated with different concentrations (0.3 and 3 mM) of nitrate and inoculated with the striped stem borer (SSB). Seven-day-old seedlings were transplanted to nutrient solution containing 0.3 mM KNO_3 or 3 mM KNO_3 for 30 days. The contents of JA **(A)**, JA-Ile **(B)**, SA **(C)**, and ABA **(D)** were determined by LC-MS at 0, 3, 9, and 24 h after SSB inoculation under nitrogen supply. Each bar is the mean ± SE of three replicates. Asterisks (*) indicate Student's *t*-test significant at $P < 0.05$ versus the indicated samples, and ns means no significant difference.

antioxidants to scavenge excess ROS (Hatier and Gould, 2008; Agati and Tattini, 2010). Additionally, phenolic acid is also a leading indicator of grain resistance or susceptibility to insects (Classen et al., 1990). Previous studies have been revealed that nitrogen fertilization decreased the levels of phenolic compounds in corn (Ren et al., 2013) and tomato (Stout et al., 1998), as well

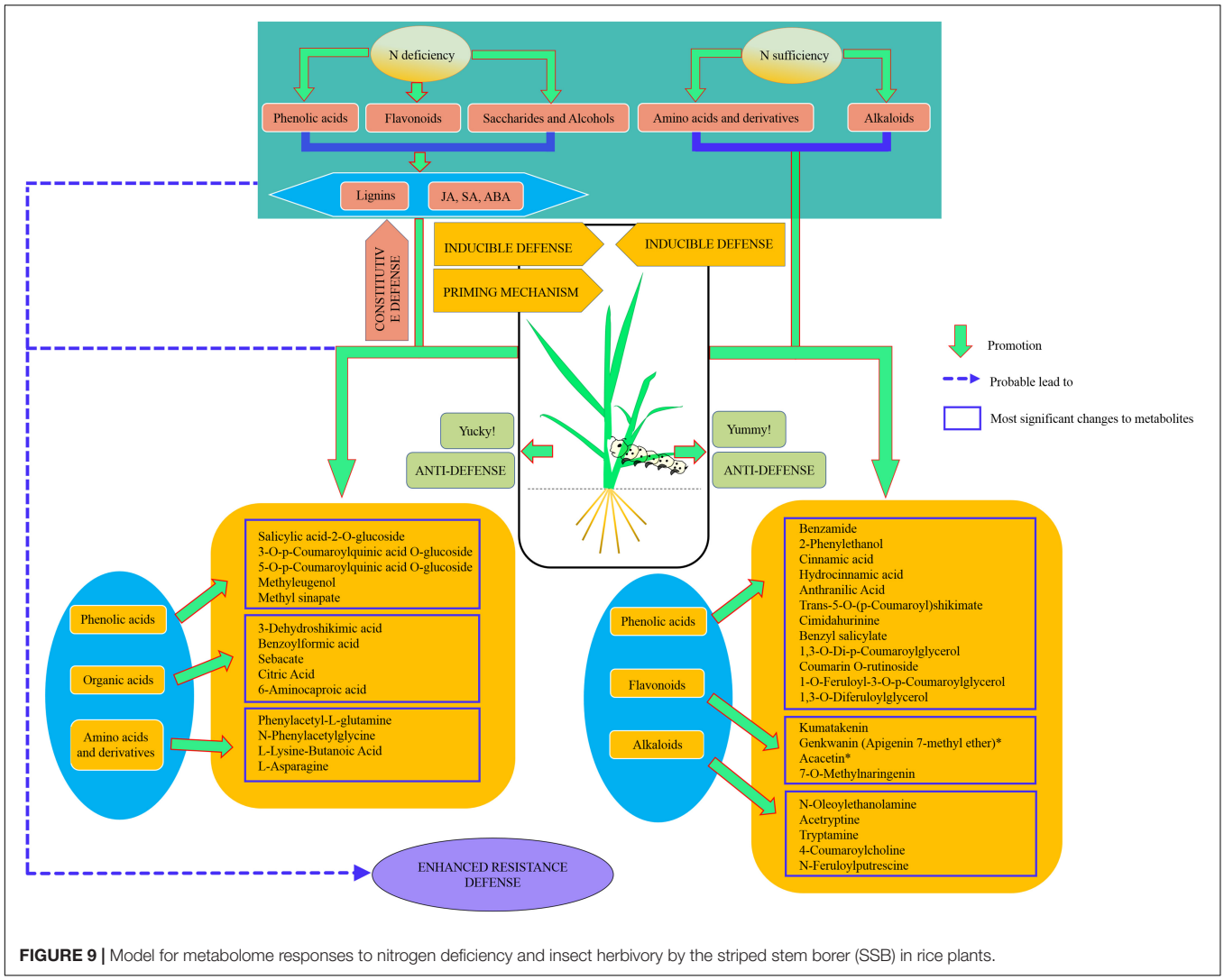

FIGURE 9 | Model for metabolome responses to nitrogen deficiency and insect herbivory by the striped stem borer (SSB) in rice plants.

as the levels of other defensive compounds in tomato (Hoffland et al., 2000; Larbat et al., 2016), peach (*Prunus persica*) (Sauge et al., 2010), and cotton (Chen et al., 2008). In this study, we also found that nitrate deficiency induced the accumulation of phenolic acids and flavonoids (**Figures 3C,D**), which might be resulted in the increase of constitutive defense.

The former study has been revealed that nitrogen deficiency leads to a marked shift from the nitrogen-containing alkaloid nicotine to carbon-rich phenylpropanoids (Fritz et al., 2006). The analysis of metabolome under nitrogen supply was also shown that the phenylpropanoid pathway was significantly different between HN group and LN group under SSB infestation (**Figure 5C**). The phenylpropanoid pathway is essential in plants, providing precursors for numerous secondary metabolites, including monolignols, flavonoids, and coumarins (Fraser and Chapple, 2011). The stimulation of phenylpropanoid metabolism is triggered by changes of nitrogen, which is mediated by the induction of a set of enzymes in the early steps of the phenylpropanoid biosynthetic pathway (such as 4CL). The differential nitrogen supply also leads to the flux of carbon into phenylpropanoids metabolism in different route under SSB infestation (**Figure 5D**).

Nitrogen deficiency promoted the accumulation of specific organic acids, phenolic acids, and saccharides before SSB infestation (**Figures 3B,C,E**). Organic acids are a unique group of metabolites, which are intermediate metabolites of critical metabolic pathways, such as the Krebs cycle, carbohydrate metabolism, ketone body metabolism, fatty acid β-oxidation, neurotransmitters turnover, and protein metabolism (Tsoukalas et al., 2017). Saccharides are omnipresent and important components for general metabolism. Recent studies show that sugars can act as critical signaling molecules in regulation of cellular metabolism in response to biotic and abiotic stress (Sheen et al., 1999; Smeekens and Hellmann, 2014). Interestingly, organic acids (such as D-xylonic acid), phenolic acids (such as 3-*O*-*p*-coumaroylquinic acid *O*-glucoside and 5-*O*-*p*-coumaroylquinic acid *O*-glucoside), and saccharides [such as D-(-)-arabinose, D-sedoheptulose 7-phosphate, and glucarate *O*-phosphoric acid] might have a similar priming mechanism effect under SSB infestation (**Figure 7**). Priming is operative

through a complex network of signaling pathways. The advantage of priming is that it offers the plant an enhanced protection without the costs of constitutively expressing their defense genes.

The Differential Regulation of Lignin Under Nitrogen Supply in Constitutive and Inducible Defense

Lignin is important for terrestrial plants by providing a structural support for the upward growth of plants and enabling the long-distance water transportation (Humphreys and Chapple, 2002). However, increased lignin accumulation is also harmful for plant growth. In plants, there are two major steps to produce lignin: monolignol biosynthesis and monolignol polymerization *via* free radical coupling. Previous study has been revealed that the levels of nitrate supplied in solution influenced the lignin production (Fritz et al., 2006; Comadira et al., 2015). In our system, we also found that low nitrate levels resulted in an increase in lignin in rice stem (**Figure 4F**). High content of lignin could make plants less palatable to herbivores, which can decrease litter decomposability and the rates of nutrient cycling concomitantly (Ostrander and Coors, 1997; Hideki and Takayuki, 2011). Additionally, secondary cell walls consisted of lignin also play a key role as a passive barrier in the defense against SSB infestation. Therefore, the higher accumulation of lignin in nitrate-deficient rice could promote the constitutive defense.

It is worth noting that the content of lignin in nitrate-replete rice was more than that in nitrate-deficient rice under SSB infestation for 3 days (**Supplementary Figure 7**). Plants cells possess various types of sensors at the plasma membrane (and possibly in the cell wall) that can probe mechanical deformations or changes in cell wall structure or composition by a rapid growth inhibition coupled with the production of ROS, ACC, and jasmonate (Wolf et al., 2012). The more injured cell wall in nitrate-replete rice might induce more deposition of lignin to reinforce their cell walls.

Phytohormone in Low-Nitrogen-Induced Priming Against SSB Infestation

Jasmonic acid is one of the most important hormones involved in the response of plants to herbivory-induced wounding, controlling the majority of insect-regulated genes in Arabidopsis leaves (Acosta and Farmer, 2010). The level of plant nutrition was closely related to the ability of rice anti-herbivore defense at least partially by regulating phytohormone signal. Previous study had revealed that Pi deficiency induced JA pathway and triggered increased resistance to *Spodoptera littoralis* in Arabidopsis, tomato, and *Nicotiana benthamiana* (Khan et al., 2016). Here, it was revealed that JA content was higher in LN group than that in HN group before SSB infection (**Figure 8A**). Previous study revealed that JA signal could regulate the production of volatile compounds, resulting in the difference in insect selectivity (Paré and Tumlinson, 1999), which was consistent with our results that the number of feeding SSB in LN group was more than that in HN group in the initial feeding time analyzed by feeding preference (**Figure 1B**). However, whether the regulation of JA signal on insect selectivity was affected by nitrate supply should be investigated in the future.

Additionally, we also found that there was no significant difference between LN and HN group after 3-h infection with SSB (**Figure 8A**). And the knockdown of JA signal did not significantly change the feeding of SSB under nitrate supply (**Supplementary Figure 9**). It seems that nitrate deficiency is not the same as Pi deficiency in rice defense against chewing herbivore infestation. Previous study has been revealed the complex signaling networks arising from cell wall alterations and leading to the upregulation of JA biosynthesis (Mielke and Gasperini, 2019). MeJA treatment prevents isoxaben-induced lignification in Arabidopsis in a concentration-dependent manner (Denness et al., 2011). Meanwhile, cell wall-degrading enzymes and cell wall fragments play a major role as triggers of the JA pathway (Ellis et al., 2002; Bömer et al., 2018). The accumulation of lignin in LN group might inhibit the initialization of JA signal in the process of rice defense against SSB infestation. Additionally, it was also found that SA levels in LN group were always higher than those in HN group in 1-day infestation by SSB (**Figure 8C**). Generally, JA signal was antagonized by SA signal in rice defense against chewing herbivore infestation, which also might lead to the similar levels of JA content in LN and HN groups after 3-h inoculation by SSB.

Besides, other phytohormones might also be involved in rice defense against SSB infestation under nitrogen supply. Previous study has revealed that LN could induce the accumulation of auxin in plant (Krouk, 2016; Sun X. et al., 2020). And herbivory-induced auxin promotes the production of anthocyanins and phenolamides in *Nicotiana attenuate* (Machado et al., 2016). Additionally, recent research has also revealed that auxin and ABA signals have a synergistic effect in plant response to drought stress. It seems that auxin could also play a positive role in stress response (Zhang et al., 2012; Wang et al., 2019). Auxin, ethylene (ET), and ABA are stress-related phytohormones that are induced upon herbivory and are well-established modulators of plant resistance to herbivores. Unfortunately, ABA content in LN group was no significantly different from that in HN group under SSB infestation (**Figure 8D**). However, previous study has revealed that auxin could promote the transduction of ET signal (Fei et al., 2017; Yue et al., 2020). Therefore, the relationship between auxin and ET signals in rice defense against SSB herbivory should be investigated in the future.

Strategy Adjustment of Nitrogen Application-Cost Less but Defense More

The expression of fitness costs depends on environmental conditions such as nutrient availability. Slow-growing plant species, which typically evolved in resource-limited environments, are less able to replace the lost tissue than fast-growing plant species from more competitive environments and should therefore invest in constitutive rather than in induced defense (Kempel et al., 2011). In this study, we found that nitrate deficiency promotes the accumulation of specific organic acids, phenolic acids, saccharides, and lignin, which might be involved in priming of rice defense against SSB infestation. Generally,

the benefits of priming outweigh its costs when stress occurs. Therefore, priming is a fine economic solution to the trade-off dilemma between plant defense protection and costs involved in enhancing defense responses (Conrath et al., 2006). The large amount of energy invested in lignin and its precursors has the potential to compensate the costly expenditure of defense, which consequently would mitigate the trade-off between growth and defense.

In conclusion, our results showed that nitrogen deficiency enhanced rice resistance to SSB. Nitrogen deficiency and sufficiency motivated the accumulation of different metabolites outlined in **Figure 9**. Nitrogen deficiency promoted the accumulation of phenolic acids, flavonoids, saccharides, and alcohols and, in particular, promoted the accumulation of lignin, while nitrogen sufficiency promoted the accumulation of amino acids and derivatives, as well as N-contained alkaloids. Upon insect herbivory, nitrogen deficiency may tend to initiate plant constitutive defense by the accumulation of phenolic acids and flavonoids.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in SRA database, accession number (PRJNA742516).

AUTHOR CONTRIBUTIONS

RZ, JL, and YS conceived and designed the experiments. YZ performed the experiments and analyzed the data. XZ, XL, NQ, and KX analyzed the data. YZ, RZ, and JL wrote and revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (31971833 and U2005208), China Postdoctoral Science Foundation (2019M652233 and 2020M671919), Natural Science Foundation of Fujian Province (2019J01375 and 2020J02030), Fujian Agriculture and Forestry University (Xjq201617), One Hundred Talents Program of Fujian Province of China (2014), and Fujian Distinguished Youth Program (2017J06010).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.691292/full#supplementary-material

REFERENCES

Acosta, I. F., and Farmer, E. E. (2010). Jasmonates. *Arabidopsis Book* 8:e0129. doi: 10.1199/tab.0129

Agati, G., and Tattini, M. (2010). Multiple functional roles of flavonoids in photoprotection. *New Phytol.* 186, 786–793. doi: 10.1111/j.1469-8137.2010.03269.x

Altieri, M. A., and Nicholls, C. I. (2003). Soil fertility management and insect pests: harmonizing soil and plant health in agroecosystems. *Soil Till. Res.* 72, 203–211. doi: 10.1016/S0167-1987(03)00089-8

Anders, S., and Huber, W. (2012). *Differential Expression of RNA-Seq Data at the Gene Level-the DESeq Package*. Available online at: http://www.genomatix.de/online_help/help_regionminer/DESeq_1.10.1.pdf (Accessed June 23, 2016)

Ballini, E., Nguyen, T. T., and Morel, J. B. (2013). Diversity and genetics of nitrogen-induced susceptibility to the blast fungus in rice and wheat. *Rice* 6:32. doi: 10.1186/1939-8433-6-32

Bi, J. L., Ballmer, G. R., Hendrix, D. L., Henneberry, T. J., and Toscano, N. C. (2001). Effect of cotton nitrogen fertilization on *Bemisia argentifolii* populations and honeydew production. *Entomol. Exp. Appl.* 99, 25–36. doi: 10.1023/A:1018959606812

Bömer, M., O'Brien, J. A., Pérez-Salamó, I., Krasauskas, J., Finch, P., Briones, A., et al. (2018). COI1-dependent jasmonate signalling affects growth, metabolite production and cell wall protein composition in Arabidopsis. *Ann. Bot.* 122, 1117–1129. doi: 10.1093/aob/mcy109

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Brodeur-Campbell, S. E., Vucetich, J. A., Richter, D. L., Waite, T. A., Rosemier, J. N., and Tsai, C. J. (2006). Insect herbivory on low-lignin transgenic aspen. *Environ. Entomol.* 35, 1696–1701. doi: 10.1093/ee/35.6.1696

Bryant, J. P., Clausen, T. P., Reichardt, P. B., McCarthy, M. C., and Werner, R. A. (1987). Effect of nitrogen fertilization upon the secondary chemistry and nutritional value of quaking aspen (*Populus tremuloides* Michx.) leaves for the large aspen tortrix (*Choristoneura conflictana* (Walker)). *Oecologia* 73, 513–517. doi: 10.1007/BF00379408

Chen, Y., Ruberson, J. R., and Olson, D. M. (2008). Nitrogen fertilization rate affects feeding, larval performance, and oviposition preference of the beet armyworm, *Spodoptera exigua*, on cotton. *Entomol. Exp. Appl.* 126, 244–255. doi: 10.1111/j.1570-7458.2007.00662.x

Classen, D., Arnason, J. T., Serratos, J. A., Lambert, J. D. H., Nozzolillo, C., and Philogene, B. J. R. (1990). Correlation of phenolic acid content of maize to resistance to *Sitophilus zeamais*, the maize weevil, in CIMMYT'S collections. *J. Chem. Ecol.* 16, 301–315. doi: 10.1007/BF01021766

Close, D. C., and McArthur, C. (2002). Rethinking the role of many plant phenolics-protection from photodamage not herbivores? *Oikos* 99, 166–172.

Comadira, G., Rasool, B., Karpinska, B., Morris, J., Verrall, S. R., Hedley, P. E., et al. (2015). Nitrogen deficiency in barley (*Hordeum vulgare*) seedlings induces molecular and metabolic adjustments that trigger aphid resistance. *J. Biol. Chem.* 66, 3639–3655. doi: 10.1093/jxb/erv276

Conrath, U., Beckers, G. J., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., et al. (2006). Priming: getting ready for battle. *Mol. Plant. Microbe Interact.* 19, 1062–1071. doi: 10.1094/MPMI-19-1062

Daugaard, H., Sorensen, L., and Loschenkohl, B. (2003). Effect of plant spacing, nitrogen fertilisation, post-harvest defoliation and finger harrowing in the control of *Botrytis cinerea* Pers. in strawberry. *Eur. J. Hortic. Sci.* 68, 77–82.

de Lange, E. S., Kyryczenko-Roth, V., Johnson-Cicalese, J., Davenport, J., Vorsa, N., and Rodriguez-Saona, C. (2019). Increased nutrient availability decreases insect resistance in cranberry. *Agr. Forest Entomol.* 21, 326–335. doi: 10.1111/afe.12335

Denness, L., McKenna, J. F., Segonzac, C., Wormit, A., Madhou, P., Bennett, M., et al. (2011). Cell wall damage-induced lignin biosynthesis is regulated by a reactive oxygen species-and jasmonic acid-dependent process in Arabidopsis. *Plant Physiol.* 156, 1364–1374. doi: 10.1104/pp.111.175737

Diaz Napal, G. N., Defagó, M. T., Valladares, G. R., and Palacios, S. M. (2010). Response of *Epilachna paenulata* to two flavonoids, pinocembrin and quercetin, in a comparative study. *J. Chem. Ecol.* 36, 898–904. doi: 10.1007/s10886-010-9823-1

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. A. J. N. (1951). A colorimetric method for the determination of sugars. *Nature* 168, 167–167. doi: 10.1038/168167a0

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J. G. (2002). The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. *Plant Cell* 14, 1557–1566. doi: 10.1105/tpc.002022

Fei, Q., Wei, S., Zhou, Z., Gao, H., and Li, X. (2017). Adaptation of root growth to increased ambient temperature requires auxin and ethylene coordination in Arabidopsis. *Plant Cell Rep.* 36, 1507–1518. doi: 10.1007/s00299-017-2171-7

Felton, G. W., Donato, K., Delvecchio, R. J., and Duffey, S. S. (1989). Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. *J. Chem. Ecol.* 15, 2667–2694. doi: 10.1007/BF01014725

Foster, C. E., Martin, T. M., and Pauly, M. (2010). Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part i: lignin. *J. Vis. Exp.* 37:e1745. doi: 10.3791/1745

Fraga, C. G., Clowers, B. H., Moore, R. J., and Zink, E. M. (2010). Signature-discovery approach for sample matching of a nerve-agent precursor using liquid chromatography-mass spectrometry, XCMS, and chemometrics. *Anal. Chem.* 82, 4165–4173. doi: 10.1021/ac1003568

Fraser, C. M., and Chapple, C. (2011). The Phenylpropanoid Pathway in Arabidopsis. *Arabidopsis Book* 9:e0152. doi: 10.1199/tab.0152

Fritz, C., Palacios-Rojas, N., Feil, R., and Stitt, M. (2006). Regulation of secondary metabolism by the carbon-nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. *Plant J.* 46, 533–548. doi: 10.1111/j.1365-313X.2006.02715.x

Fukushima, R. S., and Hatfield, R. D. (2004). Comparison of the acetyl bromide spectrophotometric method with other analytical lignin methods for determining lignin concentration in forage samples. *J. Agr. Food Chem.* 52, 3713–3720. doi: 10.1021/jf035497l

Gardiner, D. (1966). The pyrolysis of some hexoses and derived di-, tri-, and poly-saccharides. *J. Chem. Soc. C.* 1473–1476. doi: 10.1039/J39660001473

Guo, Q., Major, I. T., and Howe, G. A. (2018). Resolution of growth-defense conflict: mechanistic insights from jasmonate signaling. *Curr. Opin. Plant Biol.* 44, 72–81. doi: 10.1016/j.pbi.2018.02.009

Han, L., Li, S., Liu, P., Peng, Y., and Hou, M. (2012). New artificial diet for continuous rearing for *Chilo suppressalis* (Lepidoptera: Crambidae). *Ann. Entomol. Soc. Am.* 105, 253–258. doi: 10.1603/AN10170

Hatier, J. H. B., and Gould, K. S. (2008). "Anthocyanin function in vegetative organs," in *Anthocyanins*, eds C. Winefield, K. Davies, and K. Gould (New York, NY: Springer), 1–19.

Herms, D. A., and Mattson, W. J. (1992). The dilemma of plants: to grow or defend. *Quart. Rev. Biol.* 67, 283–335. doi: 10.1086/417659

Herrmann, K. M., and Weaver, L. M. (1999). The shikimate pathway. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 50, 473–503. doi: 10.1146/annurev.arplant.50.1.473

Hideki, K., and Takayuki, O. (2011). Ingestion and excretion of nitrogen by larvae of a cabbage armyworm: the effects of fertilizer application. *Agr. Forest Entomol.* 13, 143–148. doi: 10.1111/j.1461-9563.2010.00502.x

Hoffland, E., Dicke, M., Van Tintelen, W., Dijkman, H., and Van Beusichem, M. L. (2000). Nitrogen availability and defense of tomato against two-spotted spider mite. *J. Chem. Ecol.* 26, 2697–2711. doi: 10.1023/A:1026477423988

Hu, L., Ye, M., Kuai, P., Ye, M., Erb, M., and Lou, Y. (2018). OsLRR-RLK1, an early responsive leucine-rich repeat receptorlike kinase, initiates rice defense responses against a chewing herbivore. *New Phytol.* 219, 1097–1111. doi: 10.1111/nph.15247

Humphreys, J. M., and Chapple, C. (2002). Rewriting the lignin roadmap. *Curr. Opin. Plant Biol.* 5, 224–229. doi: 10.1016/S1369-5266(02)00257-1

Huot, B., Yao, J., Montgomery, B. L., and He, S. Y. (2014). Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Mol. Plant* 7, 1267–1287. doi: 10.1093/mp/ssu049

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., et al. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36, 480–484. doi: 10.1093/nar/gkm882

Kempel, A., Schädler, M., Chrobock, T., Fischer, M., and van Kleunen, M. (2011). Tradeoffs associated with constitutive and induced plant resistance against herbivory. *Proc. Natl Acad. Sci. U.S.A.* 108, 5685–5689. doi: 10.1073/pnas.1016508108

Khan, G. A., Vogiatzaki, E., Glauser, G., and Poirier, Y. (2016). Phosphate deficiency induces the jasmonate pathway and enhances resistance to insect herbivory. *Plant Physiol.* 171, 632–644. doi: 10.1104/pp.16.00278

Kim, D., Landmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12, 357–360. doi: 10.1038/nmeth.3317

Knobloch, K. H., and Hahlbrock, K. (1977). 4-Coumarate: CoA ligase from cell suspension cultures of *Petroselinum hortense* Hoffm: partial purification, substrate specificity, and further properties. *Arch. Biochem. Biophys.* 184, 237–248. doi: 10.1016/0003-9861(77)90347-2

Krouk, G. (2016). Hormones and nitrate: a two-way connection. *Plant Mol. Biol.* 91, 599–606. doi: 10.1007/s11103-016-0463-x

Kuang, Y., Xu, Y., Zhang, L., Hou, E., and Shen, W. (2017). Dominant trees in a subtropical forest respond to drought mainly via adjusting tissue soluble sugar and proline content. *Front. Plant Sci.* 8:802. doi: 10.3389/fpls.2017.00802

Larbat, R., Adamowicz, S., Robin, C., Han, P., Desneux, N., and Le Bot, J. (2016). Interrelated responses of tomato plants and the leaf miner *Tuta absoluta* to nitrogen supply. *Plant Biol.* 18, 495–504. doi: 10.1111/plb.12425

Li, H., Hu, B., Wang, W., Zhang, Z., Liang, Y., Gao, X., et al. (2016). Identification of microRNAs in rice root in response to nitrate and ammonium. *J. Genet. Genomics* 43, 651–661. doi: 10.1016/j.jgg.2015.12.002

Liu, J., Ji, Y. B., Zhou, J., and Xing, D. (2016). Phosphatidylinositol 3-kinase promotes v-atpase activation and vacuolar acidification and delays methyl jasmonate-induced leaf senescence. *Plant Physiol.* 170, 1714–1731. doi: 10.1104/pp.15.00744

Liu, J., Yang, Z., Dang, P., Zhu, H., Gao, Y., Ha, V. N., et al. (2018). Response of soil microbial community dynamics to *Robinia pseudoacacia* L. afforestation in the loess plateau: a chronosequence approach. *Plant Soil* 423, 327–338. doi: 10.1007/s11104-017-3516-2

Liu, Q., Luo, L., and Zheng, L. (2018). Lignins: biosynthesis and biological functions in plants. *Int. J. Mol. Sci.* 19:335. doi: 10.3390/ijms19020335

Liu, Y., Ge, Y., Bi, Y., Li, C., Deng, H., Hu, L., et al. (2014). Effect of postharvest acibenzolar-S-methyl dipping on phenylpropanoid pathway metabolism in muskmelon (*Cucumis melo* L.) fruits. *Sci. Hortic.* 168, 113–119. doi: 10.1016/j.scienta.2014.01.030

Lu, Z., Yu, X., Heong, K., and Hu, C. (2007). Effect of nitrogen fertilizer on herbivores and its stimulation to major insect pests in rice. *Rice Sci.* 14, 56–66. doi: 10.1016/S1672-6308(07)60009-2

Luzzatto, T., Golan, A., Yishay, M., Bilkis, I., Ben-Ari, J., and Yedidia, I. (2007). Priming of antimicrobial phenolics during induced resistance response towards *Pectobacterium carotovorum* in the ornamental monocot calla lily. *J. Agric. Food Chem.* 55, 10315–10322. doi: 10.1021/jf072037+

Machado, R. A., Robert, C. A., Arce, C. C., Ferrieri, A. P., Xu, S., Jimenez-Aleman, G. H., et al. (2016). Auxin is rapidly induced by herbivore attack and regulates a subset of systemic, jasmonate-dependent defenses. *Plant Physiol.* 172, 521–532. doi: 10.1104/pp.16.00940

Mielke, S., and Gasperini, D. (2019). Interplay between plant cell walls and jasmonate production. *Plant Cell Physiol.* 60, 2629–2637. doi: 10.1093/pcp/pcz119

Morris, W. F., Traw, M. B., and Bergelson, J. (2006). On testing for a tradeoff between constitutive and induced resistance. *Oikos* 112, 102–110. doi: 10.1111/j.0030-1299.2006.14253.x

Mumm, R., and Hilker, M. (2006). Direct and indirect chemical defence of pine against folivorous insects. *Trends Plant Sci.* 11, 351–358. doi: 10.1016/j.tplants.2006.05.007

Mur, L. A. J., Simpson, C., Kumari, A., Gupta, A. K., and Gupta, K. J. (2017). Moving nitrogen to the centre of plant defence against pathogens. *Ann. Bot.* 119, 703–709. doi: 10.1093/aob/mcw179

Ostrander, B. M., and Coors, J. G. (1997). Relationship between plant composition and European corn borer resistance in three maize populations. *Crop Sci.* 37, 1741–1745. doi: 10.2135/cropsci1997.0011183X003700060011x

Pan, X., Welti, R., and Wang, X. (2010). Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. *Nat. Protoc.* 5, 986–992. doi: 10.1038/nprot.2010.37

Paré, P. W., and Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121, 325–332. doi: 10.1104/pp.121.2.325

Patel, R. K., and Jain, M. (2012). NGS QC toolkit: a toolkit for quality control of next generation sequencing data. *PLoS One* 7:e30619. doi: 10.1371/journal.pone.0030619

Ren, L. L., Hardy, G., Liu, Z. D., Wei, W., and Dai, H. G. (2013). Corn defense responses to nitrogen availability and subsequent performance and feeding

preferences of beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 106, 1240–1249. doi: 10.1603/ec12091

Rosen, H. (1957). A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.* 67, 10–15. doi: 10.1016/0003-9861(57)90241-2

Sampedro, L., Moreira, X., and Zas, R. (2011). Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. *J. Ecol.* 99, 818–827. doi: 10.1111/j.1365-2745.2011.01814.x

Sauge, M. H., Grechi, I., and Poëssel, J. L. (2010). Nitrogen fertilization effects on *Myzus persicae* aphid dynamics on peach: vegetative growth allocation or chemical defence. *Entomol. Exp. Appl.* 136, 123–133. doi: 10.1111/j.1570-7458.2010.01008.x

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry* 30, 3875–3883. doi: 10.1016/0031-9422(91)83426-L

Schroeder, F. C., del Campo, M. L., Grant, J. B., Weibel, D. B., Smedley, S. R., Bolton, K. L., et al. (2006). Pinoresinol: a lignol of plant origin serving for defense in a caterpillar. *Proc. Natl Acad. Sci. U.S.A.* 103, 15497–15501. doi: 10.1073/pnas.0605921103

Shan, L. L., Li, X., Wang, P., Cai, C., Zhang, B., Sun, C. D., et al. (2008). Characterization of cDNAs associated with lignification and their expression profiles in loquat fruit with different lignin accumulation. *Planta* 227, 1243–1254. doi: 10.1007/s00425-008-0696-2

Sheen, J., Zhou, L., and Jang, J. C. (1999). Sugars as signaling molecules. *Curr. Opin. Plant Biol.* 2, 410–418. doi: 10.1016/s1369-5266(99)00014-x

Smeekens, S., and Hellmann, H. A. (2014). Sugar sensing and signaling in plants. *Front. Plant Sci.* 5:113. doi: 10.3389/fpls.2014.00113

Song, Y. Y., Ye, M., Li, C., He, X., Zhu-Salzman, K., Wang, R. L., et al. (2014). Hijacking common mycorrhizal networks for herbivore-induced defence signal transfer between tomato plants. *Sci. Rep.* 4:3915. doi: 10.1038/srep03915

Steinbrenner, A. D., Gómez, S., Osorio, S., Fernie, A. R., and Orians, C. M. (2011). Herbivore-induced changes in tomato (*Solanum lycopersicum*) primary metabolism: a whole plant perspective. *J. Chem. Ecol.* 37, 1294–1303. doi: 10.1007/s10886-011-0042-1

Stevenson, P. C., Anderson, J. C., Blaney, W. M., and Simmonds, M. S. J. (1993). Developmental inhibition of *Spodoptera litura* (Fab.) larvae by a novel caffeoylquinic acid form the wild groundnut, *Arachis paraguariensis* (Chod et Hassl.). *J. Chem. Ecol.* 19, 2917–2933. doi: 10.1007/BF00980592

Stout, M. J., Brovont, R. A., and Duffey, S. S. (1998). Effect of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato. *J. Chem. Ecol.* 24, 945–963. doi: 10.1023/A:1022350100718

Sun, L., Xu, H., Hao, H., An, S., Lu, C., Wu, R., et al. (2019). Effects of bensulfuron-methyl residue on photosynthesis and chlorophyll fluorescence in leaves of cucumber seedlings. *PLoS One* 14:e0215486. doi: 10.1371/journal.pone.0215486

Sun, X., Chen, H., Wang, P., Chen, F., Yuan, L., and Mi, G. (2020). Low nitrogen induces root elongation via auxin-induced acid growth and auxin-regulated target of rapamycin (TOR) pathway in maize. *J. Plant Physiol.* 254:153281. doi: 10.1016/j.jplph.2020.153281

Sun, Y., Wang, M., Mur, L. A. J., Shen, Q., and Guo, S. (2020). Unravelling the roles of nitrogen nutrition in plant disease defences. *Int. J. Mol. Sci.* 21:572. doi: 10.3390/ijms21020572

Sun, Y., Xu, L., Chen, Q., Qin, W., Huang, S., Jiang, Y., et al. (2018). Chlorantraniliprole resistance and its biochemical and new molecular target mechanisms in laboratory and field strains of *Chilo suppressalis* (Walker). *Pest Manag. Sci.* 74, 1416–1426. doi: 10.1002/ps.4824

Thaler, J. S., and Karban, R. (1997). A phylogenetic reconstruction of constitutive and induced resistance in *Gossypium*. *Am. Nat.* 149, 1139–1146. doi: 10.1086/286042

Tianpei, X., Li, D., Qiu, P., Luo, J., Zhu, Y., and Li, S. (2015). Scorpion peptide LqhIT2 activates phenylpropanoid pathways via jasmonate to increase rice resistance to rice leafrollers. *Plant Sci.* 230, 1–11. doi: 10.1016/j.plantsci.2014.10.005

Tiffin, P. (2000). Mechanisms of tolerance to herbivore damage: what do we know? *Evol. Ecol.* 14, 523–536. doi: 10.1023/A:1010881317261

Tong, X., Qi, J., Zhu, X., Mao, B., Zeng, L., Wang, B., et al. (2012). The rice hydroperoxide lyase OsHPL3 functions in defense responses by modulating the oxylipin pathway. *Plant J.* 71, 763–775. doi: 10.1111/j.1365-313X.2012.05027.x

Tsoukalas, D., Alegakis, A., Fragkiadaki, P., Papakonstantinou, E., Nikitovic, D., Karataraki, A., et al. (2017). Application of metabolomics: focus on the quantification of organic acids in healthy adults. *Int. J. Mol. Med.* 40, 112–120. doi: 10.3892/ijmm.2017.2983

Vega, A., Canessa, P., Hoppe, G., Retamal, I., Moyano, T. C., Canales, J., et al. (2015). Transcriptome analysis reveals regulatory networks underlying differential susceptibility to *Botrytis cinerea* in response to nitrogen availability in *Solanum lycopersicum*. *Front Plant Sci.* 6:911. doi: 10.3389/fpls.2015.00911

Wang, B., Liu, C., Zhang, D., He, C., Zhang, J., and Li, Z. (2019). Effects of maize organ-specific drought stress response on yields from transcriptome analysis. *BMC Plant Biol.* 19:335. doi: 10.1186/s12870-019-1941-5

Wilkens, R. T., Spoerke, J. M., and Stamp, N. E. (1996). Differential responses of growth and two soluble phenolics of tomato to resource availability. *Ecology* 77, 247–258. doi: 10.2307/2265674

Wolf, S., Hématy, K., and Höfte, H. (2012). Growth control and cell wall signaling in plants. *Annu. Rev. Plant Biol.* 63, 381–407. doi: 10.1146/annurev-arplant-042811-105449

Ye, M., Luo, S. M., Xie, J. F., Li, Y. F., Xu, T., Liu, Y., et al. (2012). Silencing COI1 in rice increases susceptibility to chewing insects and impairs inducible defense. *PLoS One* 7:e36214. doi: 10.1371/journal.pone.0036214

Yue, P., Lu, Q., Liu, Z., Lv, T., Li, X., Bu, H., et al. (2020). Auxin-activated MdARF5 induces the expression of ethylene biosynthetic genes to initiate apple fruit ripening. *New Phytol.* 226, 1781–1795. doi: 10.1111/nph.16500

Zhang, P. J., Shu, J. P., Fu, C. X., Zhou, Y., Hu, Y., Zalucki, M. P., et al. (2008). Trade-offs between constitutive and induced resistance in wild crucifers shown by a natural, but not an artificial, elicitor. *Oecologia* 157, 83–92. doi: 10.1007/s00442-008-1060-8

Zhang, Q., Li, J., Zhang, W., Yan, S., Wang, R., Zhao, J., et al. (2012). The putative auxin efflux carrier OsPIN3t is involved in the drought stress response and drought tolerance. *Plant J.* 72, 805–816. doi: 10.1111/j.1365-313X.2012.05121.x

Zhao, Q., and Dixon, R. A. (2011). Transcriptional networks for lignin biosynthesis: more complex than we thought? *Trends Plant Sci.* 16, 227–233. doi: 10.1016/j.tplants.2010.12.005

Züst, T., and Agrawal, A. A. (2017). Trade-offs between plant growth and defense against insect herbivory: an emerging mechanistic synthesis. *Annu. Rev. Plant Biol.* 68, 513–534. doi: 10.1146/annurev-arplant-042916-040856

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 26 July 2021
doi: 10.3389/fpls.2021.660460

Endophytic Colonization by the Entomopathogenic Fungus *Beauveria Bassiana* Affects Plant Volatile Emissions in the Presence or Absence of Chewing and Sap-Sucking Insects

OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Plant Metabolism and Chemodiversity, a section of the journal Frontiers in Plant Science

Received: *29 January 2021*
Accepted: *11 June 2021*
Published: *26 July 2021*

Citation:
González-Mas N, Gutiérrez-Sánchez F, Sánchez-Ortiz A, Grandi L, Turlings TCJ, Manuel Muñoz-Redondo J, Moreno-Rojas JM and Quesada-Moraga E (2021) Endophytic Colonization by the Entomopathogenic Fungus Beauveria Bassiana Affects Plant Volatile Emissions in the Presence or Absence of Chewing and Sap-Sucking Insects. Front. Plant Sci. 12:660460. doi: 10.3389/fpls.2021.660460

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Entomopathogenic fungi are gaining acceptance in Integrated Pest Management (IPM) systems as effective and environmental safety biological control agents to protect a great variety of crops against pest insects. Many of these insect-pathogenic fungi can establish themselves as endophytes and thereby may induce the plant immune system. The activation of plant defenses by the fungal endophytic colonization can have a direct impact on herbivores and plant pathogens. An integral component of many plant defense responses is also the release of volatile organic compounds, which may serve as an indirect defense by attracting the natural enemies of herbivores. Here we investigated the effect of endophytic colonization by the entomopathogenic fungus *Beauveria bassiana* on the volatile emission by melon and cotton plants, either unharmed or after being damaged by sap-sucking aphids or leaf chewing caterpillars. We found that when the plants are colonized by *B. bassiana* they emit a different blend of volatile compounds compared to uncolonized control plants. Some of the emitted compounds have been reported previously to be released in response to herbivory and have been implicated in natural enemy attraction. Several of the compounds are also known to have antimicrobial properties. Therefore, endophytic colonization by *B. bassiana* might help to not only direct control insect pests but also increase the resistance of plants against agronomically important pests and phytopathogens.

Keywords: endophyte, volatile organic compounds, plant-mediated interaction, *Spodoptera littoralis*, *Spodoptera frugiperda*, *Aphis gossypii*, cotton, melon

INTRODUCTION

Entomopathogenic fungi represent a large group of fungal species whose special feature is that they naturally infect and control insect populations (Lovett and St. Leger, 2017). They are gaining acceptance in Integrated Pest Management (IPM) systems, where they can serve as effective and environmental safety biological control agents of a great variety of crop pests (Zimmermann, 2007; Lacey and Shapiro-Ilan, 2008; Lacey et al., 2015; Quesada-Moraga, 2020). Besides their main habitats, soil and insect cadavers, they have recently been found to establish interesting plant associations (Meyling and Eilenberg, 2007; Vega et al., 2009; Quesada-Moraga, 2020). They can be found in the phylloplane, rhizosphere and as endophytes (Meyling and Eilenberg, 2006; Vega et al., 2008; Hu and Bidochka, 2019; Quesada-Moraga, 2020). These recent discoveries have been exploited in biological control, protecting systemically the plants from chewing and sap-sucking insects (Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017; Jaber and Ownley, 2018; Vega, 2018). Moreover, these endophytic colonizations often have the additional benefit of protecting plants against several phytopathogens and promoting plant growth (Jaber and Ownley, 2018; Vega, 2018; Dara, 2019). This is explained by the fact that endophytes induce the plant immune system and activate plant defenses, as has been confirmed by different physiological plant changes observed in several studies (Gualandi et al., 2014; Shrivastava et al., 2015; Rondot and Reineke, 2019). An integral component of many plant defense responses is the production and release of volatile organic compounds (VOCs) in response to insect attack (Dicke and Baldwin, 2010; Heil, 2014; Turlings and Erb, 2018), but also pathogen infections (Ton et al., 2007; Howe and Jander, 2008; Sobhy et al., 2017; Hijri et al., 2018). In fact, certain endophytic fungi have been reported to emit VOCs that promote growth and influence the defense responses of their host plants (Strobel et al., 2011; Li et al., 2014; Shikano et al., 2017).

Several studies have demonstrated the plant-mediated effects of endophytic colonization by entomopathogenic fungi on insects and their natural enemies (Akutse et al., 2014; Gathage et al., 2016; Sword et al., 2017; Jaber and Araj, 2018; González-Mas et al., 2019a,b), but little is known about the identity and importance of volatiles that are directly emitted by the fungus or result from the endophytic colonization (González-Mas et al., 2019b; Moloinyane and Nchu, 2019; Fingu-Mabola et al., 2020). A recent study looked at the volatiles emitted by leaves from melon plants that had been inoculated with different strains of the entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill. or *Metarhizum brunneum* (Petch) (Ascomycota: Hypocreales) (González-Mas et al., 2019b). When these plants were subsequently infested by aphids, they showed qualitative and quantitative differences depending on the entomopathogenic fungus that had colonized the leaves, which in all cases were different from control plants (González-Mas et al., 2019b). Whether the spectrum of volatile compounds changes when plants are endophytically colonized by entomopathogenic fungi is as yet unknown. Likewise, it remains unknown if these differences in the volatile profile of endophytically colonized plants is also observed when entomopathogenic fungi colonized other plant families or if the kind of emitted volatile changes when they are infested by insects with chewing feeding behavior. If so, this could have important consequences for the plants' interactions with their biotic environment.

In the current study, we addressed the effect of the endophytic colonization by the entomopathogenic fungus *B. bassiana* on the volatile emission at different time points after colonization of melon and cotton plants, and how these emissions further changed after the plants were damaged by sap-sucking aphids and leaf chewing caterpillars. We used a non-targeted approach based on metabolomic techniques and multivariate statistical analyses to reveal the potential volatile markers emitted after the endophytic colonization.

MATERIALS AND METHODS

Biological Material: Plants, Insect Populations, and Fungal Strain

Melon seeds (*Cucumis melo* L. var. Galia) were surface-sterilized in 2% sodium hypochlorite (Sigma-Aldrich, MO, USA) for 2 min, rinsed twice with sterile Mili-Q water and dried in a laminar-flow hood under sterile conditions. The soil substrate (Floragard, Germany) was also sterilized twice in an autoclave for 20 min at 121°C with a 24-h interval between each sterilization process. Surface-sterilized seeds were germinated in 400 ml pots containing a mixture of equal parts of vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and the sterilized soil substrate. Germinated seeds were maintained in an environmental chamber under controlled conditions: 25 ± 2°C and a 16-h light: 8-h dark regime. A nutritional complex of 20:20:20 (N:P:K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., PE, USA) was added to the irrigation water at a rate of 1 g l^{-1} three times a week. Wild cotton seeds (*Gossypium hirsutum* L.) were collected in 2016 from a wild population in Puerto Escondido (Oaxaca, Mexico). Most research on direct defenses in cotton comes from studies with domesticated varieties, and this could be the cause of the different volatiles found in this study. It has been observed that cotton emissions are time dependent (Loughrin et al., 1994) starting with constitutive and then moving to also *de novo* inducible volatiles and DMNT and TMTT belong to the second category. These seeds were scratched with sandpaper to improve germination and pregerminated on cotton wool moistened with sterile water. After that, germinated seeds were transferred to 200 ml pots using sterilized soil substrate (COOP, Switzerland) and maintained in an environmental chamber set at 23°C and a 16:8 h light:dark regime. Plants were fertirrigated three times a week.

Aphid colonies were obtained from a virus-free laboratory population of *Aphis gossypii* Glover (Homoptera: Aphididae) provided by the Institute of Agricultural Sciences (ICA) CSIC (Madrid, Spain) for use in the assays. The aphids were maintained in rearing cages on melon plants (*Cucumis melo* L. var. Galia) for several generations in an environmental growth chamber under controlled conditions: 25 ± 2°C, 16:8 h light:dark regime, and 70% RH. For each experiment, newly emerged apterous adult females (24–72 h after last molt) were collected from the

rearing cages using a camel-hair brush and used immediately in experiments. The caterpillars *Spodoptera littoralis* (Boisduval) and *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) were reared as described by Maag et al. (2014). Briefly, *S. littoralis* larvae were reared from eggs provided by Syngenta (Stein, Switzerland). The eggs were kept in an incubator (25°C and LD 16: 8 h) and, after emergence, larvae were placed on a wheat germ-based artificial diet at room temperature. Larvae of *S. frugiperda* were obtained from a colony established at the University of Neuchâtel. They were reared on a chickpea flour-based artificial diet in plastic boxes under artificial light conditions and at ambient temperature. For the experiments, second-instar larvae were collected and placed on the cotton plants to induce the production of volatiles. Although differences exist in the quantities of plant volatiles that they may induce, both species induce a volatile blend of similar composition. Also, application of regurgitant of both species to wounded plants, as a means of artificial induction of defenses, results in the emission of similar blends of volatiles (De Lange et al., 2020).

Beauveria bassiana strain EABb 01/33-Su was used in all the bioassays. The endophytic ability of this strain when inoculated onto melon and cotton plants has been demonstrated previously, as has its ability to cause mortality in chewing and sap-sucking insects when endophytic (Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017; González-Mas et al., 2019b). EABb 01/33-Su was originally isolated from soil from El Bosque (Cádiz) and deposited at the University of Córdoba Entomopathogenic Fungi Collection, Córdoba, Spain and at the Spanish Collection of Culture Types (CECT), University of Valencia, with accession n°. CECT 21149. Nucleotide sequences for the ITS and mtDNA intergenic regions of EABb 01/33-Su can be found in the GenBank database (EF115310 and FJ972969 for the ITS region; FJ973025 for intergenic region nad3-atp9; and FJ972914 for the intergenic region atp6-rns). For all bioassays, the EABb 01/33-Su strain was grown on potato dextrose agar (PDA) in Petri dishes for 15 days at 25°C in darkness. A cellophane film was placed on the agar prior to inoculation to prevent nutrients transferring to the conidial suspension at the time of harvest. Conidial suspensions were prepared by scraping conidia from the dishes into an aqueous sterile solution of 0.01% Tween 80. The resulting conidial suspension was filtered through several layers of sterile cheesecloth to remove mycelia, and sonicated for 5 min to homogenize the inoculum. Conidial concentrations were determined using a haemocytometer and appropriate dilutions made in 0.01% Tween 80 to achieve a concentration of 10^8 conidia ml^{-1} for experiments. Prior to experimentation conidial viability was determined on liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium and only suspensions with >97.0% germination after 24 h were used.

Inoculation of Plants With Entomopathogenic Fungi and Verification of Endophytic Colonization

For each experiment, replicate groups of five-leaf-stage melon and cotton plants were treated. Two leaves per plant were sprayed with 2 ml of the fungal suspension with an aerograph 27085 (piston compressor of 23 l min^{-1}, 15–50 PSI and a 0.3 mm nozzle diameter, China). During application the remaining leaves were protected from the spray with a transparent plastic sheet and remained uninoculated. After inoculation, all plants were covered by another plastic sheet to promote fungal growth for 24 h. Control plants were treated in the same way but only sprayed with sterile water with 0.01% Tween 80. Before the plants were transferred to the vessels to analyze their emitted volatile profile, the sprayed leaves and the soil were covered with aluminum foil to avoid the emission of volatiles from the fungal inoculum from the surface of the leaf and from the soil. This cover also avoided the insect access to the sprayed leaves.

To confirm endophytic colonization, samples of leaves from fungal sprayed and control plants were collected when each experiment had finished to avoid damaging the plant and triggering plant defenses, which may have confounded the results. Inoculated and uninoculated leaves were sampled from each replicate plant, surface-sterilized with 1% NaOCl for 2 min, rinsed twice in sterile distilled water, and dried on sterile filter paper. Sections of ∼2 cm^2 were cut with a sterile scalpel from each leaf and plated out independently in Petri dishes containing selective culture medium to determine the percentage colonized endophytically; the medium contained: 20 g of Agar Sabouraud Glucose Chloramphenicol (Cultimed Panreac, Spain), 500 mg l^{-1} streptomycin sulfate (Sigma-Aldrich Chemie, China), 500 mg l^{-1} ampicillin (Intron biotechnology, China) and 500 mg l^{-1} dodine 65 WP (Barcelona, Spain). We also plated out the last rinse water from each leaf separately to confirm the effectiveness of the surface-sterilization procedure. All plates were incubated at 25°C in darkness until fungal growth was observed.

Analysis of Volatile Compounds From Caterpillar-Infested, Endophytically-Colonized Plants

Wild cotton plants were grown and treated as described above. Volatiles emitted by control and treated plants were trapped at three different time points: (T0) Immediately after the treatment with the fungal suspension or the control solution, when the fungal colonization had not yet started; (T1) 48 h after the treatment, when the endophytic colonization was established in the plants treated with the fungal suspension; (T2) 72 h after the treatment and 24 h after the plants were damaged by caterpillars. The leaf damage treatment to induce plants to emit plant volatiles consisted in exposing each cotton plant to 10 s-instar larvae of *S. frugiperda* or *S. littoralis* were placed on each plant. Plants were infested 48 h after fungal treatment, once the collection of volatiles at T1 time was performed and the evening before the day collecting at T2 time.

These insects have been considered representative for assessing plant-induced responses to herbivory by chewing insects as they have been shown to induce chemical defenses in both wild and cultivated cotton (Chappuis and Egger, 2016).

Plants were placed in hermetic vessels and a Hayesep-Q adsorbent filter trap (25 mg, 80–100 mesh; Sigma, Switzerland) was attached to the horizontal port at the top of each odor source vessel. Volatile collection was performed during 1 h as adapted

from Turlings et al. (1998). Purified air entered the bottles at a rate of 1 L min^{-1} inflow, and air carrying the volatiles was pulled through each trap at a rate of 0.9 L min^{-1} outflow. Subsequently, filters were eluted with 100 μL dichloromethane (Super solvent; Merck, Dietikon, Switzerland), the elute was then spiked with 10 μL internal standards solution (n-octane and nonyl-acetate, [20 ng/μL] each). Samples were analyzed on a gas chromatograph (GC Agilent 6890N) coupled to a mass spectrometer detector (MSD Agilent 5973). A 2 μL aliquot of each sample was injected in pulsed splitless mode onto an Agilent HP-5MS column (30 m length × 250 μm diameter and 0.25 μm film thickness). After injection, temperature was maintained at 40°C for 3 min, increased to 100°C at a rate of 8°C per min and subsequently to 200°C at a rate of 5°C per min followed by a post run of 3 min at 250°C. Helium was used as carrier gas and kept at constant flow of 1.1 ml min^{-1}. Volatile compounds were identified at different levels of confidence on the basis of the proposed minimum criteria defined by the metabolomics standard initiative (Goodacre et al., 2007). Metabolites were definitive annotated (level 1) by comparing the MS spectra and linear retention index (LRI) against available standards purchased from Sigma-Aldrich-Merck [heptane, (*E*)-2-hexenal, (Z)-tridec-3-ene, acetic acid; hex-4-en-1-ol, ethyl heptanoate, nonanal, decanal, 2-ethylhexan-1-ol, benzaldehyde, 3,7-dimethylocta-1,6-dien-3-ol (linalool)]. Putative (or tentative) identifications (levels 2 and 3) were considered by comparing the MS spectra and LRI against existing databases. Metabolites for which MS spectra and LRI were not available in bibliography were labeled as "unknown" (level 4). Volatile compounds increases were expressed as fold-change.

There were six replicate plants for each treatment and each plant was measured at the abovementioned time points. The experiment was repeated three times using six new replicate plants, caterpillars and fungal inoculum each time, for a total of 18 plants two of these repetitions were performed using *S. frugiperda* larvae while the last one was performed with *S. littoralis*.

Analysis of Volatile Compounds From Aphid-Infested, Endophytically-Colonized Plants

Melon plants were grown and treated as described above. In this assay, volatiles emitted by control and treated plants were also trapped at three different time points: (T0) Immediately after the treatment with the fungal suspension or the control solution, when the fungal colonization had not yet started; (T1) 48 h after the treatment, when the endophytic colonization had established in the fungal treated plants; (T2) 7 days after the treatment and 6 days after the plants were damaged by aphids, a last sampling was realized 21 days after the treatment and 20 days after the plants were damaged by the aphids (T3). Each melon plant was infested with ten adult *A. gossypii* aphids. Plants were infested 48 h after fungal treatment, once the collection of volatiles at T1 time was performed. These insects have been considered representative for assessing plant-induced responses to herbivory by sap-sucking insects due to they have been shown to induce chemical defenses (Hegde et al., 2012).

Plants were transferred in a vessel covered by a plastic bag and placed in a bath heated to 30°C and allowed to equilibrate for 10 min. Volatile compounds were collected during 50 min using a solid-phase microextraction (SPME) fiber (50/30 μm DVB/CAR/PDMS Stableflex 23Ga, Autosampler, SUPELCO, Bellefonte, PA, USA) that was introduced into the top of the plastic bag. Once the volatiles were collected, they were analyzed using a High-Resolution Gas Chromatograph with Triple Quadrupole systems Mass Spectrometry (HR-GC-TQ MS) (Bruker model Scion 456-GC-TQ MS system; Bruker, Massachusetts, USA). Method parameters were set based on previous works (Sánchez-Ortiz et al., 2013; González-Mas et al., 2019b). Desorption of volatile compounds was done directly into the GC injector at 250°C for 5 min. A Supelcowax 10 capillary column (30 m × 0.25 mm, 0.25 μm, Sigma-Aldrich Co. LLC) was used with the following parameters: Helium as the carrier gas; injector was set at 250°C; column held for 5 min at 40°C and then the temperature programed to increase at a rate of 4°C min^{-1} until it reached 200°C.

The MS method was as follows: EI mode (70 eV), ion source and transfer line temperatures were all fixed at 250°C. Mass spectra were obtained in full scan mode in the 30–250 mass-to-charge ratio range at a scanning speed of 7 scan/s. Chromatograms and spectra were recorded and processed using the Bruker MS Workstation version 8.2 (Bruker, Massachusetts, USA). Volatile compounds were identified at different levels of confidence on the basis of the proposed minimum criteria defined by the metabolomics standard initiative (Goodacre et al., 2007). Metabolites were identified as describe above for cotton plants.

There were five replicate plants for each treatment and each plant was measured at the abovementioned time points. The experiment was repeated twice using five new plants, aphids and fungal inoculum each time, for a total of 10 plants.

GC-MS Pre-processing

Raw chromatograms were converted into international ANDI file format (.cdf) by means of the open source software OpenChrom. Deconvolution of the data was carried out using PARAllel FACtor analysis2 (PARAFAC2), since a minimal number of hyperparameter have to be set and it allows to handle complex chromatograms displaying coelutions, low signal-to-noise (S/N) ratios and shifts in retention times (Amigo et al., 2008). Chromatograms were divided in subintervals and independent models were performed to reduce the complexity. A total of 8 components were considered for each model, and the optimum number was optimized on the basis of the core consistency, comparison of the resolved mass spectral against raw profiles, retention times, distribution of residuals and model fit (%). Afterwards, all the deconvoluted peaks were manually inspected. The PARAFAC2 mass resolved mass spectrum was compared against the NIST (version 2.0, NIST, USA) for putative identification as described in the previous section. The freely available software PARADISe v.3.88 was used for this deconvolution procedure.

Statistical Analysis

Data on volatile compounds were analyzed using Kolmogorov–Smirnov and Levene's tests to evaluate parametricity of the data (normality and homogeneity of variance), and variables failing one of these linear model assumptions were Box-Cox transformed. Then, analysis of variance (ANOVA) was applied at a significance level of 0.05. The Tukey's HSD test was used for pairwise comparisons. Mixed models were analyzed with proc GLIMMIX-SAS 9.3; Pearson Chi-square, Kaplan Meier and linear models were analyzed with SPSS 24.0. Due to the nature of this study, we may expect a high intrinsic variability between plant replicates. Therefore, we first evaluated the variability in the repetitions to decide whether to pool them or evaluate it by separate to avoid misleading results. On this basis, the data from melon plants were pooled, since statistically significant repetition-related differences were not observed. However, the data from cotton plants were more heterogeneous across the three repetitions and we decided to separately assess each experiment.

Multivariate data analysis was performed to unravel the structure of the GC-MS data. Principal Component analysis (PCA) was built to look for trends and variability patterns. Subsequently, the most relevant metabolites (potential markers) were selected by means of an iterative procedure based on Variable Importance in Projection (VIP) scores from Partial Least Squares Discriminant Analysis (PLS-DA) and described in Muñoz-Redondo et al. (2019). Afterwards, the most discriminative metabolites were used to fit PLS-DA models double cross-validated using k-fold resampling method in the outer loop and leave-one-out in the inner loop (Szymańska et al., 2012). The PLS-DA models were validated by means of a permutation test based on balanced error rate (BER), number of misclassified (NMC) and area under the operating receiver curve (AUROC). We used $N = 1{,}000$ since it was large enough to sample the tails of the distribution and to attain a p-value up to 0.001.

When there was no statistically significant difference in the results from the repetitions on which each experiment was done the data were pooled, analyzed together, and presented as a single table. In addition, when no statistically significant difference in the results from the different sampling timepoints at the volatiles were measured, the data were pooled as well.

RESULTS

Endophytic Colonization of Melon and Cotton Plants

Microbiological techniques confirmed endophytic fungal colonization by the entomopathogenic fungus *B. bassiana* in 100% of the leaves that had received fungal sprays directly, but in only 20–40% of the unsprayed leaves (33.33 ± 17.64% for cotton plants and 56.67 ± 0.00% for melon plants) from plants that also had sprayed leaves. Individual plants were considered as positive for endophytic colonization based on detection of fungus within any of the leaves sampled on that plant. On the contrary, *B. bassiana* colonization was not observed in any of the samples from the control plants.

Effect of Endophytic Colonization on Volatile Compounds Emitted From Caterpillar-Infested Cotton Plants

A total of 101 volatile compounds were tentatively identified (**Supplementary Table 1**) including plant-derived compounds, herbivore-induced volatiles and fungal-derived compounds. To assess the impact of the endophytic colonization on volatile compounds emitted from caterpillar plants before and after infestation, two PLS-DA models were independently fitted to differentiate (i) control plants (cot-cnt) from treated plants (cot+endo) and (ii) infested control plants (cot+cat) from endophytic colonized infested plants (cot+endo+cat). An iterative procedure based on partial least squares discriminant analysis (PLS-DA) was used to select for the most relevant metabolites, which were further used to construct a final PLS-DA model shown in **Figure 1**. Satisfactory classification performances were achieved for both models optimized for four components with a BER of 0.18 ± 0.05 (i) and 0.16 ± 0.04 (ii), as it is shown in **Supplementary Table 2**. The main variation sources in the samples were explained by the first two principal components. In model (i) the first two principal components explained around the 68% of the total variance of samples. In component 1, the volatile metabolites 62 (unknown), 53 (unknown), 43 (unknown), 63 (2,7,10-trimethyldodecane), 21 (4-methyloctane), 67 (2,6-dimethyldecane), 22 [(E)-hex-2-en-1-ol], 73 (isothiocyanatocyclohexane), and 5 (3-chloro-3-methylpentane) were selected as potential markers for the endophytic colonization before the infestation due an overall increase in their concentrations (**Figure 1**). The different starting levels and the high variability of the volatiles emitted by caterpillar plants in the three experimental assays did not allow to clearly establish this behavior using the ANOVA statistic (**Supplementary Table 1**). Therefore, these results support the use of multivariate beside the univariate statistical techniques to reveal trends in complex datasets. Other relevant volatiles selected on component 2 were the metabolites 93 [2,2,6-trimethyl-9,10-dioxatricyclo(6.2.2.01,6)dodecan-3-ol], 44 (5-ethyl-2,2,3-trimethylheptane), and 90 [(3-hydroxy-2,4,4-trimethylpentyl)2-methylpropanoate], displaying higher levels in treated plants, and metabolite 36 [2,2-dimethyl-3-methylidenebicyclo(2.2.1)heptane], which was found to be increased in control plants. The lower explained variance on component 2 (12% compared to the 56% of component 1), suggests slighter changes on these compounds due to the effect of the endophytic colonization before the infestation. Meanwhile, the impact of the endophytic colonization after infestation of caterpillar plants was addressed in model (ii). The first two principal components explained around the 66% of the total variance found in samples, which made it possible to separate cot+cat from cot+endo+cat plants. An overall increase of the selected markers, which were quite different to the former approach, was again observed in treated plants. Only the volatile metabolite 90

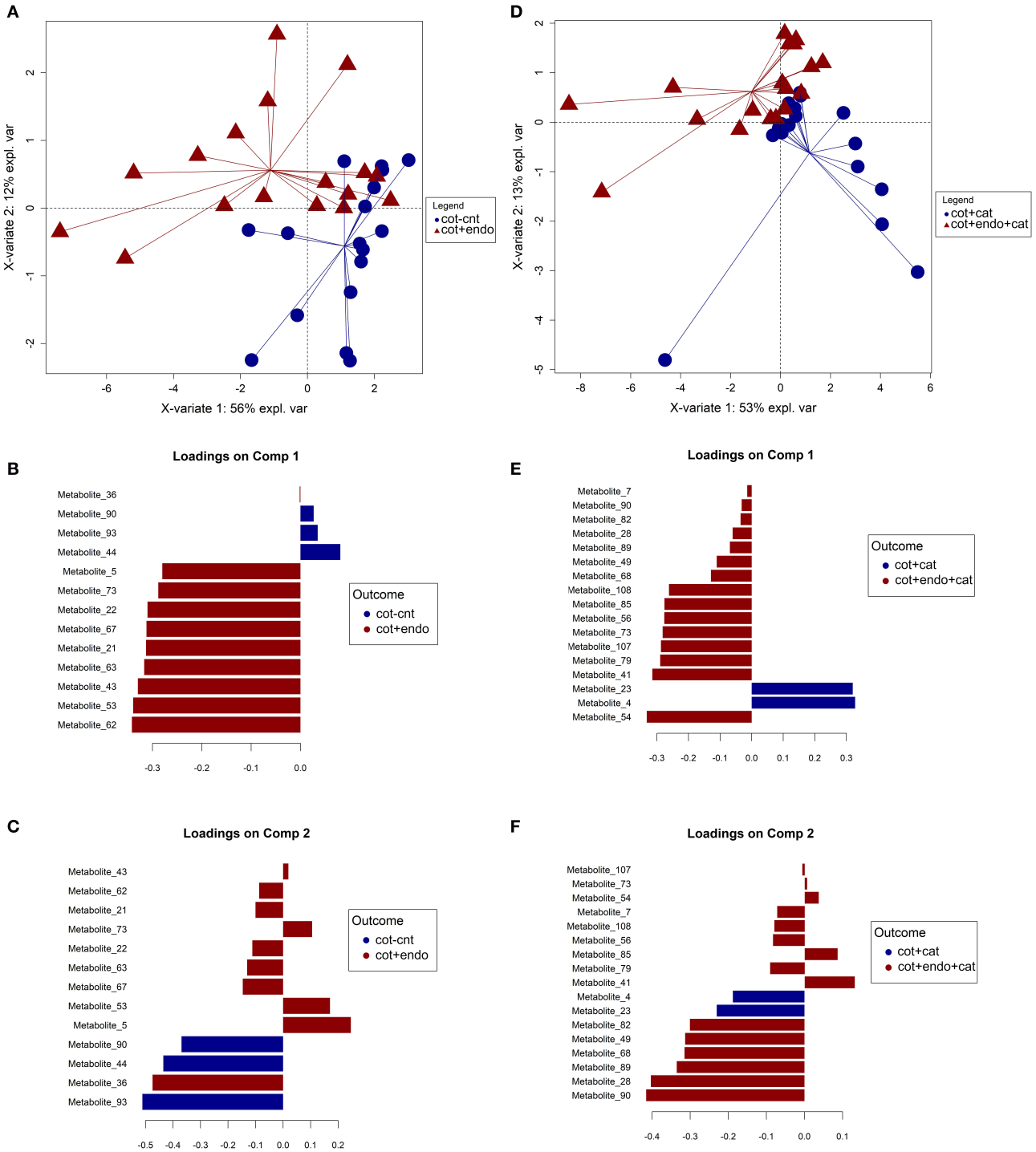

FIGURE 1 | Graphical outputs of the two Partial Least Squares Discriminant Analyses (PLS-DA) performed on volatile compounds emitted from endophytically colonized and control cotton plants before **(A–C)** and after **(D–F)** caterpillar infestation. **(A)** Scores plot for the component 1 and 2 (X-variate 1 and X variate 2); **(B)** loading contribution barplot on component 1; **(C)** loading contribution barplot on component 2. **(D)** Scores plot for the component 1 and 2 (X-variate 1 and X variate 2); **(E)** loading contribution barplot on component 1; **(F)** loading contribution barplot on component 2. Color indicates the class for which the compound has a maximal mean value. Bar length represents the multivariate regression coefficient with either a positive or negative sign for that particular feature on each component, i.e., the importance of each variable in the model. Cot-cnt: control at T0+T1, cot+cat: control and caterpillar-infested at T2, cot+endo: treated at T0+T1, cot+endo+cat: treated and caterpillar-infested at T2.

was selected in both comparisons, but displaying an opposite trend, as it can be seen in component 2 of both models (**Figure 1**). This compound diminished its concentration in cot+endo plants before infestation but increased in the same samples after the infestation. The most important volatiles selected on component 1 were the metabolites 54 [(5R)-1-methyl-5-prop-1-en-2-ylcyclohexene), 41 (benzaldehyde), 79 (3-[(E)-dodec-2-enyl]oxolane-2,5-dione), 107 (diethyl benzene-1,2-dicarboxylate), 73 (isothiocyanatocyclohexane), 56 (unknown)], 85 (cyclohex-3-en-1-ylbenzene) and 108 [(2Z,13E)-octadeca-2,13-dien-1-ol] with overall increased in TT-P plants and metabolites 4 (3-chloro-3-methylpentane), 23 (1,1,2-trimethyl-3-(2-methylpropyl)cyclopropane] and with overall lower levels in these samples. In the same line than model (i), the wide variance explained by component 1 highlights their main contribution to discriminate both labeled groups. Meanwhile, markers selected on component 2 corresponded to metabolites 90 [(3-hydroxy-2,4,4-trimethylpentyl) 2-methylpropanoate], 28 (heptanal), 89 (2-Methylpropyl 3-hydroxy-2,2,4-trimethylpentanoate), 68 (2-ethenyl-1,1-dimethyl-3-methylidenecyclohexane), 49 (octanal), and 82 (nonyl acetate), which were found in higher overall concentrations in cot+endo+cat samples.

In addition, they have been identified several compounds usually found in cotton plants. For example, (Z)-hex-3-en-1-ol (metabolite 18), 2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (metabolite 33) known as "α-pinene," 1H-indole (metabolite 78), (1R,4E,9S)-4,11,11-Trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene (metabolite 95), and (1E,4E,8E)-2,6,6,9-Tetramethylcycloundeca-1,4,8-triene (metabolite 97), known as "caryophyllene" and "α-caryophyllene or humulene" have been found by Sobhy et al. (2014) in *S. littoralis* damaged plants. 1H-indole (metabolite 78) has been showed to increase in infested plants, and endophytically colonized caterpillar infested plants emitted in highest quantities. Caryophyllene (metabolite 95) was found in higher contents in treated cotton plants than in the control, dramatically reducing the emission in endophytically colonized caterpillar infested plants. Finally, α-caryophyllene (metabolite 97) and decanal were found in general in less quantity in caterpillar-infested plants than in non-infested plants taking into account all the treatments. However, if non-infested are compared, endophytically colonized plants emitted in higher quantities than in the control ones.

It is noteworthy that the samples showed considerable differences among the three experimental assays. The endophytic colonization in the first and third assays impacted much stronger on several compounds, especially for metabolites 16 [(E)-hex-2-enal], 17 [(NZ)-N-(2-methylbutylidene)hydroxylamine], 18 [(Z)-hex-3-en-1-ol], 57 (3,7,7-trimethylbicyclo[4.1.0]hept-3-ene), 68 (2-ethenyl-1,1-dimethyl-3-methylidenecyclohexane), 78 (1H-indole), 87 (methyl anthranilate), 105 (isocaryophillene) and 107 (diethyl benzene-1,2-dicarboxylate) with increases ranging from 5 to more than 5000-fold the initial levels of the control plants (**Supplementary Table 1**). The contrary or soft variations of these compounds observed in the second experimental assay could have been prevented these metabolites to be selected as markers for the endophytic colonization during the variable reduction procedure. These differences could be due to in the assays they were used three different batches of plants grown in different time periods. In addition, despite no differences were found in other studies when the volatiles released by the two species of caterpillars were compared, it could have had an effect in this work due to the presence of the entomopathogenic fungal endophyte.

Effect of Endophytic Colonization on Volatile Compounds Emitted From Aphid-Infested Melon Plants

Just as for the cotton plants, to assess the impact of fungal colonization on the volatile emission of melon plants aphid-infested and non-infested, a wide range of volatile compounds in terms of its chemical nature were tentatively identified. In a similar fashion as the caterpillar-infested plants, the final models were fitted with the most relevant metabolites (**Figure 2**). Four clusters of samples were considered, corresponding to control (mel-cnt), control-infested (mel+aph), treated (mel+endo) and treated-infested (mel+endo+aph) and a model with a satisfactory balanced error rate (BER) of 0.14 ± 0.07 was obtained (**Supplementary Table 4**). Disclosing by groups, it was observed a slightly higher prediction accuracy for mel+endo+aph samples with a low mean error class of 0.12, due to wrong assignments to mel+aph samples during the cross-validation. While the worst prediction abilities were attained for the samples mel+aph (0.24 of mean class error) mainly driven by mislabeling as mel+endo+aph plants (**Supplementary Table 4**). The model was optimized for four components, which accounted for a high 87% of the total variability found in the plant samples. This high percentage of variability explained in the model supports the robustness of this study, taken into account the inherent variability in the volatile emission of different plants and the potential experimental errors. The subspace spanned by the first three principal components explained the most interesting information and revealed well-defined clusters of the target classes (**Figure 2**). A clear trend toward more positive values of the X-variate 1 (component 1) due to the infestation of the plants was observed, i.e., from mel-cnt to mel+aph and from mel+endo to mel+endo+aph. This trend was partially explained by an increase on levels of the metabolites 41 (2-butyloctan-1-ol), 33 (ethyl heptanoate), 37 (3-methyltridecane), 36 (hexadecan-4-yl 2,2,2-trifluoroacetate) and 1 (heptane) in mel+aph and mel+endo+aph. Variations on these compounds are expressed as foldchange in relation to the mel-cnt samples in the ANOVA of **Supplementary Table 3**. A strong increase for up to 15-fold was recorded for levels of the metabolite 41 after infestation of control plants. A similar response was observed in plants treated with the entomopathogenic fungus *B. bassiana* but even before to infestation. The metabolites 33 (ethyl heptanoate) and 36 (hexadecan-4-yl 2,2,2-trifluoroacetate) displayed the same behavior with a softer response, reaching no more than 2-fold the initial levels of the control plants. Aphid-infested plants gave rise to significant increases in Metabolite 37 (3-methyltridecane) which an average of three times over control plants. The

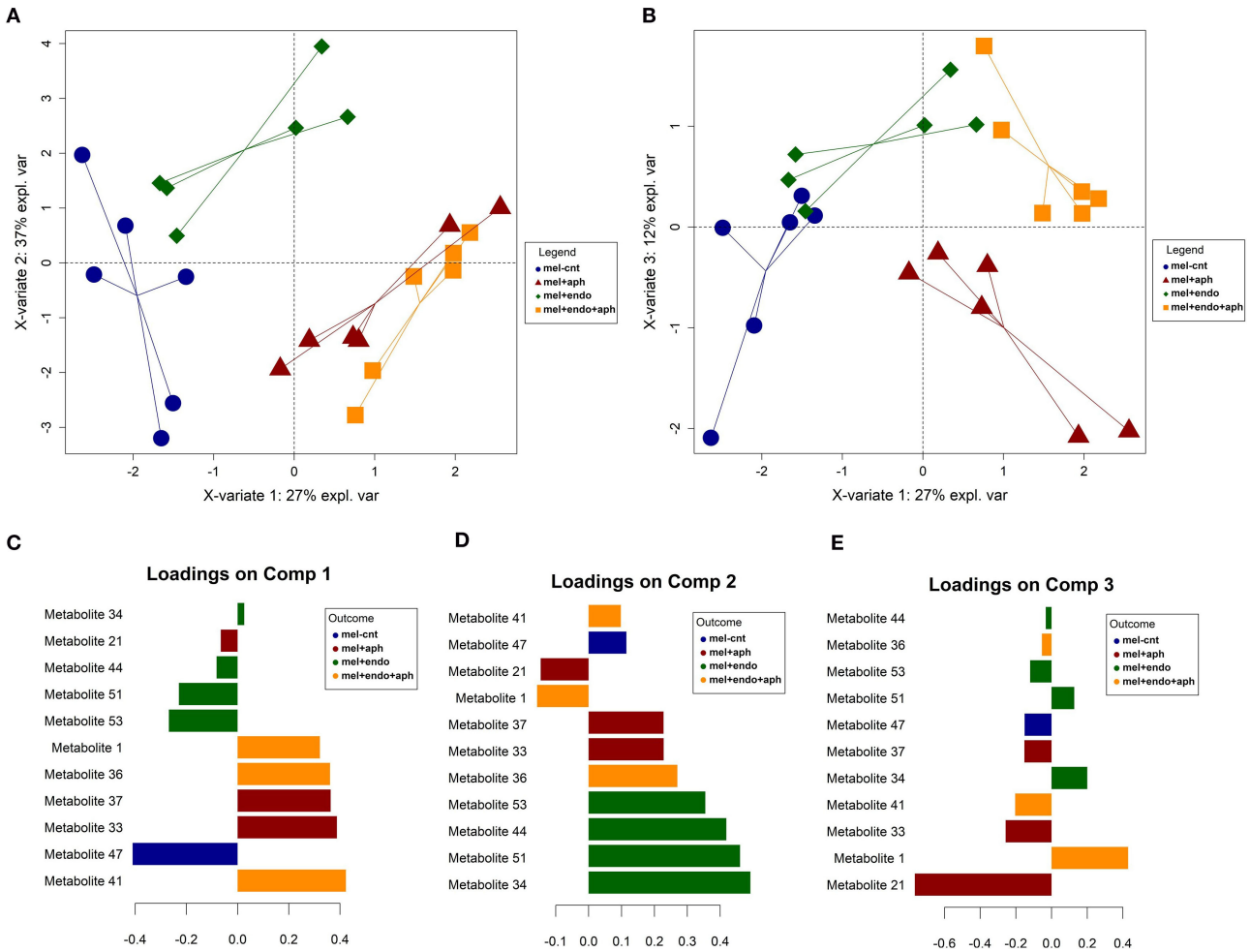

FIGURE 2 | Graphical outputs of the Partial Least Squares Discriminant Analysis (PLS-DA) performed on volatile compounds emitted from endophytically colonized and control melon plants before and after aphid infestation **(A)** Scores plot for the component 1 and 2 (X-variate 1 and X variate 2); **(B)** Scores plot for the component 1 and 3 (X-variate 1 and X variate 3); **(C)** loading contribution barplot on component 1; **(D)** loading contribution barplot on component 2; **(E)** loading contribution barplot on component 3. Color indicates the class for which the compound has a maximal mean value. Bar length represents the multivariate regression coefficient with either a positive or negative sign for that feature on each component, i.e., the importance of each variable in the model. Mel-cnt: control at T0+T1, mel+aph: control and aphid-infested at T2+T3, mel+endo:treated at T0+T1, mel+endo+aph: treated and aphid-infested at T2+T3.

behavior of metabolite 1 (heptane) was quite interesting, since its only significant variation along the experiment was observed in mel+endo-t3, growing up to 12-fold in this point (**Supplementary Table 3**; of note the high standard deviation due to a soft response in one plant assay). In addition, the trend noted on X-variate 1 was also driven by a strong decrease, ranging from 90 to 94%, on metabolite 47 (2-ethylhexan-1-ol) contents, which was particularly acute for the plants treated with *B. bassiana* (**Supplementary Table 3**). Component 2 revealed a different behavior between control and treated plants related to the infestation. While the controls did not show a clear variation on the direction of component 2, the plants treated with *B. bassiana* moved toward more negative values after infestation. This behavior was mainly explained by a decrease on certain metabolites. Specially, for metabolites 44 (unknown), 51 (oxolan-2-one) and 53 (phenyl carbamate), these decreases were of similar magnitude, between 30 and 50% in mel+endo+aph-t3 plants. The response of metabolite 51 in control and treated plants was similar, although the changes were softer in the first case. The rest of addressed metabolites displayed a reduction in its emission before aphids contact (mel-cnt and mel+endo) and a change of positive trends after aphid infested (mel+aph and mel+endo+aph) (**Supplementary Table 3**). Finally, component 3 explained new hidden information in previous components, which could be just related to the treatment effect of *B. bassiana* endophytic colonization, since both mel-cnt and mel+aph on one side and mel+endo and mel+endo+aph on the other side were between, but not within, separated. The metabolite 21 (2-ethyldecan-1-ol) was the main responsible explaining this behavior, since it was found to be decreased in plants treated with *B. bassiana* for around the 50% in mel+endo+aph-t3 compared to mel+aph-t3.

DISCUSSION

It is well-documented that microbes can significantly affect and contribute to the release of specific volatiles by plants (Pineda et al., 2010; Kanchiswamy et al., 2015), but to the best of our knowledge, ours is the first study to measure the volatile emissions from plants that have been colonized by an entomopathogenic fungus. We measure the emissions at different time points after fungal inoculation and determined how they changed after the plants were damaged by sap-sucking and chewing herbivores.

It has been proposed that the effectiveness of microbial control agents can be enhanced by manipulating or enhancing the release of VOCs, as this may boost the plants' repellence to insect herbivores and/or the attraction of other important biological control agents, such as entomophagous insects (Sword et al., 2017; Brilli et al., 2019; González-Mas et al., 2019a; Moloinyane and Nchu, 2019). In fact, several studies on endophytic entomopathogenic fungi have demonstrated that they can enhance insect plant resistance, but also a surprising increase in the plants' attractiveness to generalist predators (Vega, 2018; González-Mas et al., 2019a). A possibility is that the blend of volatiles emitted by endophytcally colonized plants could be involved in this attractiveness of plants for insects, though further research is needed to confirm this. A previous study on volatiles from processed leaves from melon plants found several changes in the blend of volatile emissions after a plant is colonized by entomopathogenic fungi and subsequently infested by aphids, in comparison to uncolonized aphid-infested plants (González-Mas et al., 2019b). The present study was motivated by the question whether what was observed using processed melon leaves is also true for intact plants. We studied this here for cotton, as well as melon plants. Results seem to indicate a regulatory effect specific on the pathway of biosynthesis of volatile compounds emitted by plants.

In general, in the assay with wild cotton plants considerably larger amounts of volatile compounds were detected as compared to melon plants, but it should be noted that different extraction methods were used. The wild cotton plants constitutively emitted various monoterpenes and sesquiterpenes such as myrcene, β-caryophellene and α-humulene, while the volatile blend of melon plants contained short chain aldehydes, alcohols, acids, acetates and their derivatives, as previously reported for this cucurbitaceous crop (Hou et al., 1997). Besides that, it was confirmed in the present research that colonization by the entomopathogenic fungus *B. bassiana* resulted in the emission of a different blend of volatile compounds as compared to control plants. In previous works it has been demonstrated that endophytic microorganisms and related plant strengtheners can strongly affect the herbivore-induced volatile blends and has been shown to render the plants considerably more attractive to natural enemies (Rostás and Turlings, 2008; D'Alessandro et al., 2014; Sobhy et al., 2018). One possible explanation for the changes in the volatile blends is that defense responses are triggered in fungally challenged plants, which involve volatile releases (Rivas-Franco et al., 2020). This has been observed for plants that were treated with methyl jasmonate or cis-jasmone, increasing the production of defense compounds and simultaneously changing the emission of VOCs (Brunissen et al., 2010; Sobhy et al., 2017).

Regarding cotton, several of the compounds that were found to be released in larger amounts after colonization by *B. bassiana* EABb 01/33-Su strain are known to attract natural enemies, which could explain the enhanced attractiveness of colonized plants to them observed in previous studies (González-Mas et al., 2019a), whereas it will need further research to determine if the volatiles released by endophytically colonized plants are in fact involved in attractiveness of plants for insects. Indeed, the aromatic compound benzaldehyde (metabolite 41) was identified here as a potential marker for caterpillar-infested *B. bassiana* endophytically colonized wild cotton plants (**Figure 1**; **Supplementary Table 1**) and is known to cause a strong response in herbivore natural enemies (Han and Chen, 2002). Similarly, we found 4-methyl-octane (metabolite 21), (E)-hex-2-en-1-ol (metabolite 22), and α-caryophyllene (metabolite 97) to be released by *B. bassiana* colonized wild cotton plants (**Figure 1**; **Supplementary Table 1**), which have been previously identified as potential attractants to natural enemies (Dickens, 1989, 1999; Han and Chen, 2002; Reddy and Guerrero, 2004). We also found that decanal (metabolite 71) and caryophyllene (metabolite 95) were released in larger quantities by endophytically colonized cotton plants than by control plants, whereas this effect of the fungal colonization was neutralized by caterpillar infestation (**Supplementary Table 1**). These two compounds are often emitted after herbivore damage and they have been reported as plant synomones that attract natural enemies (Gouinguen et al., 2001; Degen et al., 2004; Rasmann et al., 2005; Zhang et al., 2020). Furthermore, decanal (metabolite 71) and caryophyllene (metabolite 95), together with α-caryophyllene (metabolite 97) have also been reported as key plant compounds contributing to the attraction of natural enemies by cotton plants damaged by insect pests (Morawo and Fadamiro, 2016), whereas the role of these compounds in our multitrophic system remains unknown and will need a deeper study.

The emission of 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (= α-pinene) (metabolite 33) and 1H-indole (metabolite 78) have also previously been shown to increase upon caterpillar infestation of cotton plants (Sobhy et al., 2014); here we show that indole emission is higher in fungally challenged caterpillar infested plants (**Supplementary Table 1**). Indole does not have the above-mentioned synomone-like effect, and is considered a general insect repellent (Han and Chen, 2002; Schröder et al., 2015).

Regarding melon, we provide first evidence that 2-ethylhexyl nonyl sulfite (metabolite 14) can serve as a biomarker for endophytically colonized melon plants that are simultaneously infested by the cotton aphid. This compound has never previously been reported to be emitted by plants or fungi. Similar organic esters of sulfurous acids, more particularly mixed sulfite diesters of aliphatic or aromatic monohydroxy compounds and glycol ethers, are known as useful insecticides, particularly for mite control (Covey et al., 1969). A next step of the present research will explore whether or not

the emission of this kind of compounds by *B. bassiana* endophytically colonized and pest-infested plants could have an insecticidal effect on pests together with the possible role of 6-Methyl-octadecane (metabolite 50), which was more emitted by endophytically colonized melon plants, as a herbivore-induced plant volatile, that my serve as herbivore repellent and natural enemy attractant (Turlings and Ton, 2006; Nisha and Kennedy, 2017).

Some other compounds that were found to be enhanced by *B. bassiana* endophytic colonization have previously been reported to play different roles mediating tritrophic interactions insect-plants-natural enemies. For instance, benzaldehyde is an ancestral and common compound emitted by plants and insects that has been reported also to act as a sex pheromone, aggregation pheromone, alarm pheromone, and is used as defense secretion in several insect taxa (Schiestl, 2010). Another compound found in caterpillar-infested and endophytically colonized wild cotton plants that has been reported as insect sex pheromone is (2Z,13E)-octadeca-2,13-dien-1-ol (metabolite 108) (Tonini et al., 1986; Islam et al., 2007; Yang et al., 2009; Ubaid et al., 2016). We found 1-iodo-2-methylundecane (metabolite 34), as dominant compounds in VOC blends of colonized melon plants that were not also infested by aphids, it has previously been identified as a floral volatile with potential impact on insect visitation (Wang et al., 2014).

We also identified several volatile compounds with known antifungal and antimicrobial activity. For instance, benzahaldehyde (metabolite 41) and (2Z,13E)-octadeca-2,13-dien-1-ol (metabolite 108), which were found mainly for wild cotton plants that were caterpillar-infested, as well as colonized by the fungus, are known to have antimicrobial activity in other systems (Schulz et al., 1995; Wilson et al., 1999; Teles et al., 2006; Wang et al., 2006; Li et al., 2008; Shao et al., 2009; Lin et al., 2012; Sun et al., 2014; Ullah et al., 2015; Zhou et al., 2016; de Amorim et al., 2019). For example, benzahaldehyde has a negative impact on conidial germination, fungal growth speed, and conidial production of the entomopathogenic fungus *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (Lin et al., 2017). Moreover, the compounds 1-iodo-2-methylundecane (metabolite 34) and oxolan-2-one (metabolite 51), which we found in higher quantities in colonized melon plants, have been proposed to play a functional role in the chemical defense against microbial invasion (Cazar et al., 2005; Manilal and Idhayadhulla, 2014; Leena et al., 2016; Li et al., 2019). Hexadecan-4-yl 2,2,2-trifluoroacetate (metabolite 36) found in endophytically colonized melon plants with aphids, has been linked to antimicrobial activity against several bacterial and fungal species (Sarada et al., 2011; Managamuri et al., 2017; Mannaa and Kim, 2018). Finally, 6-methyl-octadecane (metabolite 50), identified in colonized melon plants, my provide resistance against phytopathogenic nematodes (Seenivasan, 2018). Both 1-iodo-2-methylundecane (metabolite 34) and 6-methyl-octadecane (metabolite 50) are bioactive compounds emitted by the entomopathogenic fungus *Paecilomyces lilacinus* (Thom) Samson (Ansari, 2019).

The enhanced production of the above compounds could be a possible explanation of the observed resistance against several plant phytopathogens when the plants are colonized by entomopathogenic fungi (Jaber and Ownley, 2018). It implies that endophytic entomopathogenic fungi produce or cause the production of secondary metabolites with inhibitory activity against mycelial growth, conidial germination, and germ-tube elongation of phytopathogenic fungi, as well as against plant virus replication and accumulation (Ownley et al., 2008, 2010; Jaber and Ownley, 2018).

In our experimental system, compounds previously reported to posse allelopathic effects on several weed species have been detected, whereas their specific origin and function in our multitrophic systems remains unraveled. This is the case for 2,7,10-trimethyldodecane (metabolite 63) that has been found to be related colonized wild cotton plants and melon plants infested with aphids (Li et al., 2016). In contrast, 6-methyl-octadecane (metabolite 50), found in colonized melon plants, has been related to the induction of seed germination (Vanitharani and Pandian, 2012; Vanitharani, 2014).

Some of the compounds found in the caterpillar-infested and endophytically colonized cotton plants had not previously been identified from plants, insects or microorganisms. This is the case for 1,1,2-trimethyl-3-(2-methylpropyl)-cyclopropane (metabolite 23) and which displayed higher overall concentrations in caterpillar-infested control plants compared to the caterpillar-infested colonized plants. Their effect on plant-insect interactions and plant-pathogen interactions should be investigated in future research. Indeed, it would also be interesting to see what effects the inducible volatile emissions may have on neighboring plants. Enhanced plant resistance mediated by VOCs will have further implications for crop protection (Brilli et al., 2019).

In summary, the present study confirms that the endophytic colonization of melon and wild cotton plants by the entomopathogenic fungus *B. bassiana* causes significant changes in the blend of volatile compounds emitted by the plants, also when they are infested by chewing or sap-sucking insects. These findings indicate an even greater potential of the use of entomopathogenic fungi as an ecologically safe strategy of biological pest control, as the enhanced volatile emissions may for example boost natural enemy attraction in agroecosystems, though further research is needed to address such a hypothesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

EQ-M, TT, and NG-M conceived and designed the study. LG, FG-S, NG-M, and AS-O did the experiments. JM, JM-R, and AS-O analyzed the data. EQ-M, NG-M, AS-O, JM, JM-R, and

TT wrote the manuscript. All authors read and approved the manuscript before submission.

FUNDING

Funding was provided by the Spanish Ministry of Science and Innovation *via* Grant PID2019-103844RB-I00 Broading the use of endophytic and rhizosphere competent entomopathogenic fungi for pest control in strategic Mediterranean crops (BENEFUNGIP).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.660460/full#supplementary-material

REFERENCES

Akutse, K. S., Fiaboe, K. K. M., Van Den Berg, J., Ekesi, S., and Maniania, N. K. (2014). Effects of endophyte colonization of *Vicia faba* (Fabaceae) plants on the life-history of leafminer parasitoids *Phaedrotoma scabriventris* (Hymenoptera: Braconidae) and *Diglyphus isaea* (Hymenoptera: Eulophidae). *PLoS ONE* 9, 1–11. doi: 10.1371/journal.pone.0109965

Amigo, J. M., Skov, T., Bro, R., Coello, J., and Maspoch, S. (2008). Solving GC–MS problems with PARAFAC2. *TrAC Trends Anal. Chem.* 27, 714–725. doi: 10.1016/j.trac.2008.05.011

Ansari, R. U. (2019). GC-MS screening of bioactive compounds from extracts of phylloplane fungi of *Markhamia lutea* (Benth.) *K. schum*. *Int. J. Bot. Stud.* 4, 52–60.

Brilli, F., Loreto, F., and Baccelli, I. (2019). Exploiting plant volatile organic compounds (VOCS) in agriculture to improve sustainable defense strategies and productivity of crops. *Front. Plant Sci.* 10, 1–8. doi: 10.3389/fpls.2019.00264

Brunissen, L., Vincent, C., Le Roux, V., and Giordanengo, P. (2010). Effects of systemic potato response to wounding and jasmonate on the aphid *Macrosiphum euphorbiae* (Sternorryncha: Aphididae). *J. Appl. Entomol.* 134, 562–571. doi: 10.1111/j.1439-0418.2009.01493.x

Cazar, M. E., Schmeda-Hirschmann, G., and Astudillo, L. (2005). Antimicrobial butyrolactone I derivatives from the Ecuadorian soil fungus *Aspergillus terreus* Thorn. var terreus. *World J. Microbiol. Biotechnol.* 21, 1067–1075. doi: 10.1007/s11274-004-8150-5

Chappuis, L., and Egger, A. (2016). *Investigation of the Factors Influencing the Chemical Defenses of Gossypium Species*. Neuchâtel: University of Neuchâtel.

Covey, R. A., Smith, A. E., and Hubbard, W. L. (1969). Method of protecting plants with sulfurous acid organic esters. *United States Pat. Off.* 3:859.

D'Alessandro, M., Erb, M., Ton, J., Brandenburg, A., Karlen, D., Zopfi, J., et al. (2014). Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic interactions. *Plant Cell Environ.* 37, 813–826. doi: 10.1111/pce.12220

Dara, S. K. (2019). Non-entomopathogenic roles of entomopathogenic fungi in promoting plant health and growth. *Insects* 10:277. doi: 10.3390/insects10090277

de Amorim, M. R., Hilário, F., Dos Santos, F. M. J., Batista, J. M. J., Bauab, T. M., Araújo, A. R., et al. (2019). New benzaldehyde and benzopyran compounds from the endophytic fungus *Paraphaeosphaeria* sp. F03 and their antimicrobial and cytotoxic activities. *Planta Med.* 85, 957–964. doi: 10.1055/a-0853-7793

De Lange, E. S., Laplanche, D., Guo, H., Xu, W., Vlimant, M., Erb, M., et al. (2020). Spodoptera frugiperda caterpillars suppress herbivore-induced volatile emissions in maize. *J. Chem. Ecol.* 46, 344–360. doi: 10.1007/s10886-020-01153-x

Degen, T., Dillmann, C., Marion-Poll, F., and Turlings, T. C. J. (2004). High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiol.* 135, 1928–1938. doi: 10.1104/pp.104.039891

Dicke, M., and Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the "cry for help." *Trends Plant Sci.* 15, 167–175. doi: 10.1016/j.tplants.2009.12.002

Dickens, J. C. (1989). Green leaf volatiles enhance aggregation pheromone of boll weevil, *Anthonomus grandis*. *Entomol. Exp. Appl.* 52, 191–203. doi: 10.1111/j.1570-7458.1989.tb01268.x

Dickens, J. C. (1999). Predator-prey interactions: olfactory adaptations of generalist and specialist predators. *Agric. For. Entomol.* 1, 47–54. doi: 10.1046/j.1461-9563.1999.00007.x

Fingu-Mabola, J. C., Martin, C., Bawin, T., Verheggen, F. J., and Francis, F. (2020). Does the infectious status of aphids influence their preference towards healthy, virus-infected and endophytically colonized plants? *Insects* 11:435. doi: 10.3390/insects11070435

Garrido-Jurado, I., Resquín-Romero, G., Amarilla, S. P., Ríos-Moreno, A., Carrasco, L., and Quesada-Moraga, E. (2017). Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). *J. Pest Sci.* 90, 319–330. doi: 10.1007/s10340-016-0767-2

Gathage, J. W., Lagat, Z. O., Fiaboe, K. K. M., Akutse, K. S., Ekesi, S., and Maniania, N. K. (2016). Prospects of fungal endophytes in the control of Liriomyza leafminer flies in common bean *Phaseolus vulgaris* under field conditions. *BioControl* 61, 741–753. doi: 10.1007/s10526-016-9761-0

González-Mas, N., Cuenca-Medina, M., Gutiérrez-Sánchez, F., and Quesada-Moraga, E. (2019a). Bottom-up effects of endophytic *Beauveria bassiana* on multitrophic interactions between the cotton aphid, *Aphis gossypii*, and its natural enemies in melon. *J. Pest Sci.* 92, 1271–1281. doi: 10.1007/s10340-019-01098-5

González-Mas, N., Sánchez-Ortiz, A., Valverde-García, P., and Quesada-Moraga, E. (2019b). Effects of endophytic entomopathogenic ascomycetes on the life-history traits of *Aphis gossypii* Glover. *Insects* 10:165. doi: 10.3390/insects10060165

Goodacre, R., Broadhurst, D., Smilde, A. K., Kristal, B. S., Baker, J. D., Beger, R., et al. (2007). Proposed minimum reporting standards for data analysis in metabolomics. *Metabolomics* 3, 231–241. doi: 10.1007/s11306-007-0081-3

Gouinguen,é, S., Degen, T., and Turlings, T. C. J. (2001). Variability in herbivore-induced odour emissions among maize cultivars and their wild ancestors (teosinte). *Chemoecology* 11, 9–16. doi: 10.1007/PL00001832

Gualandi, R. J., Augé, R. M., Kopsell, D. A., Ownley, B. H., Chen, F., Toler, H. D., et al. (2014). Fungal mutualists enhance growth and phytochemical content in *Echinacea purpurea*. *Symbiosis* 63, 111–121. doi: 10.1007/s13199-014-0293-z

Han, B., and Chen, Z. (2002). Behavioral and electrophysiological responses of natural enemies to synomones from tea shoots and kairomones from tea aphids, *Toxoptera aurantii*. *J. Chem. Ecol.* 28, 2203–2219. doi: 10.1023/A:1021045231501

Hegde, M., Oliveira, J. N., Da Costa, J. G., Loza-Reyes, E., Bleicher, E., Santana, A. E. G., et al. (2012). Aphid antixenosis in cotton is activated by the natural plant defence elicitor cis-jasmone. *Phytochemistry* 78, 81–88. doi: 10.1016/j.phytochem.2012.03.004

Heil, M. (2014). Herbivore-induced plant volatiles: targets, perception and unanswered questions. *New Phytol.* 204, 297–306. doi: 10.1111/nph.12977

Hijri, M., Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., Pozo, M. J. M. J., Toju, H., et al. (2018). Production of biomolecules of interest to the anxiolytic herbal medicine industry in yellow passionfruit leaves (*Passiflora edulis f. flavicarpa*) promoted by mycorrhizal inoculation. *J. Sci. Food Agric.* 10, 1–12. doi: 10.1002/jsfa.9598

Hou, Z. Y., Chen, X., Zhang, Y., Guo, B. Q., and Yan, F. S. (1997). EAG and orientation tests on the parasitoid *Lysiphlebia japonica* (Hym., Aphidiidae) to volatile chemicals extracted from host plants of cotton aphid *Aphis gossypii* (Hom., Aphidae). *J. Appl. Entomol.* 121, 495–500. doi: 10.1111/j.1439-0418.1997.tb01439.x

Howe, G. A., and Jander, G. (2008). Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* 59, 41–66. doi: 10.1146/annurev.arplant.59.032607.092825

Hu, S., and Bidochka, M. J. (2019). Root colonization by endophytic insect-pathogenic fungi. *J. Appl. Microbiol.* 2019, 1–12. doi: 10.1111/jam.14503

Islam, M. A., Yamamoto, M., Sugie, M., Naka, H., Tabata, J., Arita, Y., et al. (2007). Synthesis and characterization of 2,13- and 3,13-octadecadienals for the identification of the sex pheromone secreted by a clearwing moth. *J. Chem. Ecol.* 33, 1763–1773. doi: 10.1007/s10886-007-9334-x

Jaber, L. R., and Araj, S. E. (2018). Interactions among endophytic fungal entomopathogens (Ascomycota: Hypocreales), the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae), and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae). *Biol. Control* 116, 53–61. doi: 10.1016/j.biocontrol.2017.04.005

Jaber, L. R., and Ownley, B. H. (2018). Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? *Biol. Control* 116, 36–45. doi: 10.1016/j.biocontrol.2017.01.018

Kanchiswamy, C. N., Malnoy, M., and Maffei, M. E. (2015). Bioprospecting bacterial and fungal volatiles for sustainable agriculture. *Trends Plant Sci.* 20, 206–211. doi: 10.1016/j.tplants.2015.01.004

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015). Insect pathogens as biological control agents: back to the future. *J. Invertebr. Pathol.* 132, 1–41. doi: 10.1016/j.jip.2015.07.009

Lacey, L. A., and Shapiro-Ilan, D. I. (2008). Microbial control of insect pests in temperate orchard systems: potential for incorporation into IPM. *Annu. Rev. Entomol.* 53, 121–144. doi: 10.1146/annurev.ento.53.103106.093419

Leena, P., Hukuman, N. H. Z., and Jisha, M. (2016). *In vitro* antimicrobial efficacy and GC-MS analysis of bioactive components from *Lepidagathis keralensis* (Acanthaceae). *World J. Pharm. Res.* 5, 937–948. doi: 10.20959/wjpr201612-7472

Li, D. L., Li, X. M., Li, T. G., Dang, H. Y., Proksch, P., and Wan, B. G. (2008). Benzaldehyde derivatives from *Eurotium rubrum*, an endophytic fungus derived from the mangrove plant *Hibiscus tiliaceus*. *Chem. Pharm. Bull.* 56, 1282–1285. doi: 10.1248/cpb.56.1282

Li, J., He, S. Y., and Qin, X. D. (2016). Allelopathic potential and volatile compounds of *Manihot esculenta* Crantz against weeds. *Allelopath. J.* 37, 195–206.

Li, T., Blande, J. D., Gundel, P. E., Helander, M., and Saikkonen, K. (2014). Epichloë endophytes alter inducible indirect defences in host grasses. *PLoS ONE* 9:e101331. doi: 10.1371/journal.pone.0101331

Li, Y., Wei, W., Wang, R. L., Liu, F., Wang, Y. K., Li, R., et al. (2019). Colletolides A and B, two new γ-butyrolactone derivatives from the endophytic fungus *Colletotrichum gloeosporioides*. *Phytochem. Lett.* 33, 90–93. doi: 10.1016/j.phytol.2019.08.004

Lin, Q., Li, M., Zhou, R., and Liu, Y. (2012). Chemical composition and anti-bacterial activity of essential oil from *Cedrela sinensis* (A. Juss.) Roem. seed. *African J. Biotechnol.* 11, 1789–1795. doi: 10.5897/AJB11.2947

Lin, Y., Qasim, M., Hussain, M., Akutse, K. S., Avery, P. B., Dash, C. K., et al. (2017). The herbivore-induced plant volatiles methyl salicylate and menthol positively affect growth and pathogenicity of entomopathogenic fungi. *Sci. Rep.* 7:40494. doi: 10.1038/srep40494

Loughrin, J., Manukian, A., Heath, R., Turlings, T. C. J., Tumlinson, J. H. (1994). Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11836–11840. doi: 10.1073/pnas.91.25.11836

Lovett, B., and St. Leger, R. J. (2017). The insect pathogens. *Microbiol. Spectr.* 5, 923–943. doi: 10.1128/microbiolspec.FUNK-0001-2016

Maag, D., Dalvit, C., Thevenet, D., Köhler, A., Wouters, F. C., Vassão, D. G., et al. (2014). 3-β-d-Glucopyranosyl-6-methoxy-2-benzoxazolinone (MBOA-N-Glc) is an insect detoxification product of maize 1,4-benzoxazin-3-ones. *Phytochemistry* 102, 97–105. doi: 10.1016/j.phytochem.2014.03.018

Managamuri, U., Vijayalakshmi, M., Ganduri, V. S. R. K., Rajulapati, S. B., Bonigala, B., Kalyani, B. S., et al. (2017). Isolation, identification, optimization, and metabolite profiling of Streptomyces sparsus VSM-30. *3 Biotech* 7:217. doi: 10.1007/s13205-017-0835-1

Manilal, A., and Idhayadhulla, A. (2014). Potential *in vitro* antimicrobial efficacy of *Holigarna arnottiana* (Hook F). *Asian Pac. J. Trop. Biomed.* 4, 25–29. doi: 10.1016/S2221-1691(14)60203-3

Mannaa, M., and Kim, K. D. (2018). Biocontrol activity of volatile-producing *Bacillus megaterium* and *Pseudomonas protegens* against *Aspergillus* and *Penicillium* spp. predominant in stored rice grains: study II. *Mycobiology* 46, 52–63. doi: 10.1080/12298093.2018.1454015

Meyling, N. V., and Eilenberg, J. (2006). Isolation and characterisation of *Beauveria bassiana* isolates from phylloplanes of hedgerow vegetation. *Mycol. Res.* 110, 188–195. doi: 10.1016/j.mycres.2005.09.008

Meyling, N. V., and Eilenberg, J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biol. Control* 43, 145–155. doi: 10.1016/j.biocontrol.2007.07.007

Moloinyane, S., and Nchu, F. (2019). The effects of endophytic *Beauveria bassiana* inoculation on infestation level of *Planococcus ficus*, growth and volatile constituents of potted greenhouse grapevine (*Vitis vinifera* L.). *Toxins* 11:72. doi: 10.3390/toxins11020072

Morawo, T., and Fadamiro, H. (2016). Identification of key plant-associated volatiles emitted by *Heliothis virescens* larvae that attract the parasitoid, *Microplitis croceipes*: implications for parasitoid perception of odor blends. *J. Chem. Ecol.* 42, 1112–1121. doi: 10.1007/s10886-016-0779-7

Muñoz-Redondo, J. M., Ruiz-Moreno, M. J., Puertas, B., Cantos-Villar, E., and Moreno-Rojas, J. M. (2019). Multivariate optimization of headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry for the analysis of terpenoids in sparkling wines. *Talanta* 208:120483. doi: 10.1016/j.talanta.2019.120483

Nisha, R., and Kennedy, J. S. (2017). Life cycle of papaya mealybug *Paracoccus marginatus* Williams and Granara de Willink on different host plants vis-à-vis divergent natural selection. *J. Entomol. Zool. Stud.* 5, 91–102.

Ownley, B. H., Griffin, M. R., Klingeman, W. E., Gwinn, K. D., Moulton, J. K., and Pereira, R. M. (2008). *Beauveria bassiana*: endophytic colonization and plant disease control. *J. Invertebr. Pathol.* 98, 267–270. doi: 10.1016/j.jip.2008.01.010

Ownley, B. H., Gwinn, K. D., and Vega, F. E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl* 55, 113–128. doi: 10.1007/s10526-009-9241-x

Pineda, A., Zheng, S. J., van Loon, J. J. A., Pieterse, C. M. J., and Dicke, M. (2010). Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends Plant Sci.* 15, 507–514. doi: 10.1016/j.tplants.2010.05.007

Quesada-Moraga, E. (2020). Entomopathogenic fungi as endophytes: their broader contribution to IPM and crop production. *Biocontrol Sci. Technol.* 30, 864–877. doi: 10.1080/09583157.2020.1771279

Rasmann, S., Köllner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., et al. (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434, 732–737. doi: 10.1038/nature03451

Reddy, G. V. P., and Guerrero, A. (2004). Interactions of insect pheromones and plant semiochemicals. *Trends Plant Sci.* 9, 253–261. doi: 10.1016/j.tplants.2004.03.009

Resquín-Romero, G., Garrido-Jurado, I., Delso, C., Ríos-Moreno, A., and Quesada-Moraga, E. (2016). Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. *J. Invertebr. Pathol.* 136, 23–31. doi: 10.1016/j.jip.2016.03.003

Rivas-Franco, F., Hampton, J. G., Narciso, J., Rostás, M., Wessman, P., Saville, D. J., et al. (2020). Effects of a maize root pest and fungal pathogen on entomopathogenic fungal rhizosphere colonization, endophytism and induction of plant hormones. *Biol. Control* 150:104347. doi: 10.1016/j.biocontrol.2020.104347

Rondot, Y., and Reineke, A. (2019). Endophytic *Beauveria bassiana* activates expression of defence genes in grapevine and prevents infections by grapevine downy mildew *Plasmopara viticola*. *Plant Pathol.* 68, 1719–1731. doi: 10.1111/ppa.13089

Rostás, M., and Turlings, T. C. J. (2008). Induction of systemic acquired resistance in *Zea mays* also enhances the plant's attractiveness to parasitoids. *Biol. Control* 46, 178–186. doi: 10.1016/j.biocontrol.2008.04.012

Sánchez-Ortiz, A., Pérez, A. G., and Sanz, C. (2013). Synthesis of aroma compounds of virgin olive oil: significance of the cleavage of polyunsaturated fatty acid hydroperoxides during the oil extraction process. *Food Res. Int.* 54, 1972–1978. doi: 10.1016/j.foodres.2013.03.045

Sarada, K., Jothibai Margret, R., and Mohan, V. R. (2011). GC–MS determination of bioactive components of *Naringi crenulata* (Roxb) Nicolson. *Int. J. ChemTech Res.* 3, 1548–1555.

Schiestl, F. P. (2010). The evolution of floral scent and insect chemical communication. *Ecol. Lett.* 13, 643–656. doi: 10.1111/j.1461-0248.2010.01451.x

Schröder, M. L., Glinwood, R., Webster, B., Ignell, R., and Krüger, K. (2015). Olfactory responses of *Rhopalosiphum padi* to three maize, potato, and wheat

cultivars and the selection of prospective crop border plants. *Entomol. Exp. Appl.* 157, 241–253. doi: 10.1111/eea.12359

Schulz, B., Sucker, J., Aust, H. J., Krohn, K., Ludewig, K., Jones, P. G., et al. (1995). Biologically active secondary metabolites of endophytic *Pezicula* species. *Mycol. Res.* 99, 1007–1015. doi: 10.1016/S0953-7562(09)80766-1

Seenivasan, N. (2018). Phytochemical profiling of burrowing nematode (*Radopholus similis*) resistant and susceptible banana (*Musa* spp.) genotypes for detection of marker compounds. *Fruits* 73, 48–59. doi: 10.17660/th2018/73.1.6

Shao, C., Wang, C., Wei, M., Jia, Z., She, Z., and Lin, Y. (2009). Two new benzaldehyde derivatives from mangrove endophytic fungus (No. ZZF 32). *Chem. Nat. Compd.* 45, 779–781. doi: 10.1007/s10600-010-9509-5

Shikano, I., Rosa, C., Tan, C. W., and Felton, G. W. (2017). Tritrophic interactions: microbe-mediated plant effects on insect herbivores. *Annu. Rev. Phytopathol.* 55, 313–331. doi: 10.1146/annurev-phyto-080516-035319

Shrivastava, G., Ownley, B. H., Aug,é, R. M., Toler, H., Dee, M., Vu, A., et al. (2015). Colonization by arbuscular mycorrhizal and endophytic fungi enhanced terpene production in tomato plants and their defense against a herbivorous insect. *Symbiosis* 65, 65–74. doi: 10.1007/s13199-015-0319-1

Sobhy, I. S., Bruce, T. J. A., and Turlings, T. C. J. (2018). Priming of cowpea volatile emissions with defense inducers enhances the plant's attractiveness to parasitoids when attacked by caterpillars. *Pest Manag. Sci.* 74, 966–977. doi: 10.1002/ps.4796

Sobhy, I. S., Erb, M., Lou, Y., and Turlings, T. C. J. (2014). The prospect of applying chemical elicitors and plant strengtheners to enhance the biological control of crop pests. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20120283. doi: 10.1098/rstb.2012.0283

Sobhy, I. S., Woodcock, C. M., Powers, S. J., Caulfield, J. C., Pickett, J. A., and Birkett, M. A. (2017). *cis*-jasmone elicits aphid-induced stress signalling in potatoes. *J. Chem. Ecol.* 43, 39–52. doi: 10.1007/s10886-016-0805-9

Strobel, G., Singh, S. K., Riyaz-Ul-Hassan, S., Mitchell, A. M., Geary, B., and Sears, J. (2011). An endophytic/pathogenic Phoma sp. from creosote bush producing biologically active volatile compounds having fuel potential. *FEMS Microbiol. Lett.* 320, 87–94. doi: 10.1111/j.1574-6968.2011.02297.x

Sun, J. F., Lin, X., Zhou, X. F., Wan, J., Zhang, T., Yang, B., et al. (2014). Pestalols A-E, new alkenyl phenol and benzaldehyde derivatives from endophytic fungus *Pestalotiopsis* sp. AcBC2 isolated from the Chinese mangrove plant Aegiceras corniculatum. *J. Antibiot.* 67, 451–457. doi: 10.1038/ja.2014.24

Sword, G. A., Tessnow, A., and Ek-Ramos, M. J. (2017). Endophytic fungi alter sucking bug responses to cotton reproductive structures. *Insect Sci.* 24, 1003–1014. doi: 10.1111/1744-7917.12461

Szymańska, E., Saccenti, E., Smilde, A. K., and Westerhuis, J. A. (2012). Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics* 8, 3–16. doi: 10.1007/s11306-011-0330-3

Teles, H. L., Sordi, R., Silva, G. H., Castro-Gamboa, I., da Silva Bolzani, V., Pfenning, L. H., et al. (2006). Aromatic compounds produced by *Periconia atropurpurea*, an endophytic fungus associated with Xylopia aromatica. *Phytochemistry* 67, 2686–2690. doi: 10.1016/j.phytochem.2006.09.005

Ton, J., D'Alessandro, M., Jourdie, V., Jakab, G., Karlen, D., Held, M., et al. (2007). Priming by airborne signals boosts direct and indirect resistance in maize. *Plant J.* 49, 16–26. doi: 10.1111/j.1365-313X.2006.02935.x

Tonini, C., Cassani, G., Massardo, P., Guglielmetti, G., and Castellari, P. L. (1986). Study of female sex pheromone of leopard moth, *Zeuzera pyrina* L. isolation and identification of three components. *J. Chem. Ecol.* 12, 1545–1558. doi: 10.1007/BF01012371

Turlings, T. C. J., and Erb, M. (2018). Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. *Annu. Rev. Entomol.* 63, 433–452. doi: 10.1146/annurev-ento-020117-043507

Turlings, T. C. J., Lengwiler, U. B., Bernasconi, M. L., and Wechsler, D. (1998). Timing of induced volatile emissions in maize seedlings. *Planta* 207, 146–152. doi: 10.1007/s004250050466

Turlings, T. C. J., and Ton, J. (2006). Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. *Curr. Opin. Plant Biol.* 9, 421–427. doi: 10.1016/j.pbi.2006.05.010

Ubaid, J. M., Kadhim, M. J., and Hameed, I. H. (2016). Study of bioactive methanolic extract of *Camponotus fellah* using gas chromatography–mass spectrum. *Int. J. Toxicol. Pharmacol. Res.* 8, 434–439.

Ullah, I., Khan, A. L., Ali, L., Khan, A. R., Waqas, M., Hussain, J., et al. (2015). Benzaldehyde as an insecticidal, antimicrobial, and antioxidant compound produced by *Photorhabdus temperata* M1021. *J. Microbiol.* 53, 127–133. doi: 10.1007/s12275-015-4632-4

Vanitharani, J. (2014). Sustainable management of forest through ecosystem services of bats. *Scrut. Int. Res. J. Biol. Environ. Sci.* 1, 7–24.

Vanitharani, J., and Pandian, M. (2012). "A Probe into chemical signaling in fruit bats for quick forest restoration in foot hill reserves of southern western ghats," in *Recent Advances in Biodiversity of India*, eds. C. Raghunathan, C. Sivaperu, and K. Venkataraman (Kolkata: Zoological Survey of India), 457–470.

Vega, F. E. (2018). The use of fungal entomopathogens as endophytes in biological control: a review. *Mycologia* 110, 4–30. doi: 10.1080/00275514.2017.1418578

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., et al. (2009). Fungal entomopathogens: new insights on their ecology. *Fungal Ecol.* 2, 149–159. doi: 10.1016/j.funeco.2009.05.001

Vega, F. E., Posada, F., Catherine Aime, M., Pava-Ripoll, M., Infante, F., and Rehner, S. A. (2008). Entomopathogenic fungal endophytes. *Biol. Control* 46, 72–82. doi: 10.1016/j.biocontrol.2008.01.008

Wang, R., Xu, S., Liu, X., Zhang, Y., Wang, J., and Zhang, Z. (2014). Thermogenesis, flowering and the association with variation in floral odour attractants in *Magnolia sprengeri* (Magnoliaceae). *PLoS ONE* 9:e99356. doi: 10.1371/journal.pone.0099356

Wang, S., Li, X. M., Teuscher, F., Li, D. L., Diesel, A., Ebel, R., et al. (2006). Chaetopyranin, a benzaldehyde derivative, and other related metabolites from *Chaetomium globosum*, an endophytic fungus derived from the marine red alga *Polysiphonia urceolata*. *J. Nat. Prod.* 69, 1622–1625. doi: 10.1021/np060248n

Wilson, C. L., Solar, J. M., El Ghaouth, A., and Fravel, D. R. (1999). Benzaldehyde as a soil fumigant, and an apparatus for rapid fumigant evaluation. *HortScience* 34, 681–685. doi: 10.21273/HORTSCI.34.4.681

Yang, C. Y., Kim, J., Kang, T. J., and Jeon, H. Y. (2009). Identification and field bioassays of the sex pheromone of *Synanthedon haitangvora*. *J. Chem. Ecol.* 35, 1197–1201. doi: 10.1007/s10886-009-9694-5

Zhang, L., Lu, G., Huang, X., Guo, H., Su, X., Han, L., et al. (2020). Overexpression of the caryophyllene synthase gene GhTPS1 in cotton negatively affects multiple pests while attracting parasitoids. *Pest Manag. Sci.* 76, 1722–1730. doi: 10.1002/ps.5695

Zhou, J. Y., Li, X., Zheng, J. Y., and Dai, C. C. (2016). Volatiles released by endophytic *Pseudomonas fluorescens* promoting the growth and volatile oil accumulation in *Atractylodes lancea*. *Plant Physiol. Biochem.* 101, 132–140. doi: 10.1016/j.plaphy.2016.01.026

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Sci. Technol.* 17, 553–596. doi: 10.1080/09583150701309006

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 August 2021
doi: 10.3389/fpls.2021.709858

Caterpillar-Induced Volatile Emissions in Cotton: The Relative Importance of Damage and Insect-Derived Factors

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In response to herbivore attack, plants release large amounts of volatiles that can serve as attractants for the natural enemies of the attacking herbivores. Such responses are typically triggered by damage- and insect-associated factors. Cotton plants are somewhat peculiar because they release specific blends of volatiles in two waves in response to caterpillar attack. They first emit constitutively stored volatile compounds, and after about 24 h a second wave that includes various *de novo* synthesized compounds. The relative importance of damage-associated and insect associated-factors in this induction of cotton volatile emissions is not yet fully clear. We evaluated how cotton plants respond to mechanical damage and to the application of the oral secretion from the generalist lepidopteran pest *Spodoptera exigua*, by measuring the local and systemic emissions of volatile compounds from their leaves. Our results confirm that cotton plants respond to damage-associated molecular patterns (DAMPs) as well as to herbivore-associated molecular patterns (HAMPs) present in the caterpillars' oral secretion. Interestingly, a stronger response was observed for cotton plants that were treated with oral secretion from cotton-fed caterpillars than those fed on maize. We tested the possibility that volicitin, a common fatty acid-derived elicitor in caterpillar regurgitant plays a role in this difference. Volicitin and volicitin-like compounds were detected in equal amounts in the oral secretion of *S. exigua* fed on either cotton or maize leaves. We conclude that other elicitors must be involved. The identification of these eliciting cues is expected to contribute to the development of novel strategies to enhance the resistance of cotton plants to insect pests.

Keywords: cotton, *Spodoptera* spp., plant indirect defenses, volatile emissions, herbivore-associated molecular patterns, damage-associated molecular patterns

OPEN ACCESS

Edited by:
Toby J. A. Bruce,
Keele University, United Kingdom

Reviewed by:
Wang Jie,
Fujian Agriculture and Forestry University, China
Luis Sampedro,
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *14 May 2021*
Accepted: *28 June 2021*
Published: *03 August 2021*

Citation:
Arce CM, Besomi G, Glauser G and Turlings TCJ (2021) Caterpillar-Induced Volatile Emissions in Cotton: The Relative Importance of Damage and Insect-Derived Factors. Front. Plant Sci. 12:709858. doi: 10.3389/fpls.2021.709858

INTRODUCTION

During the millions of years of interaction with herbivorous insects, plants have evolved a multitude of defense strategies. These include constitutive defenses as well as induced defenses, which are produced only upon herbivore attack (Karban and Baldwin, 1997; Farmer, 2014; Erb and Reymond, 2019). Among the inducible defenses are so-called "herbivore-induced plant

volatiles" (HIPVs), that can serve as foraging cues for natural enemies of herbivores (Dicke et al., 1988; Turlings et al., 1990; Paré and Tumlinson, 1999; Turlings and Wäckers, 2004; Dicke and Baldwin, 2010; Heil and Land, 2014; Aljbory and Chen, 2018; Turlings and Erb, 2018).

Plants are able to distinguish between herbivory and mere mechanical damage. This ability allows plants to avoid wasting valuable resources for defenses in situations where they are not needed. Damage itself is a key factor by which plants recognize that they are under attack (Heil, 2009), but alone it is not sufficient to trigger full plant defense responses (Fürstenberg-Hägg et al., 2013; Acevedo et al., 2015; Schmelz, 2015). This is also the case for HIPVs (Turlings et al., 1990; Dicke, 2016; Turlings and Erb, 2018). While feeding, herbivores introduce molecules from their oral secretion into plant tissue, which can lead to the release of endogenous signal molecules from disrupted cells (Acevedo et al., 2015; Duran-Flores and Heil, 2016; Erb and Reymond, 2019). Hence, plants rely on the recognition of non-self elicitors and damaged-self associated molecules to launch appropriate defense responses to an attack. These insect-derived elicitors are known as herbivore-associated molecular patterns (HAMPs), whereas plant-derived inducers are known as damage-associated molecular patterns (DAMPs). HAMPs can be found in the oral secretions and oviposition fluids of insects (Felton and Tumlinson, 2008; Wu and Baldwin, 2009). After perceiving the attacker, the plant activates downstream signaling mechanisms, resulting in activation of the immune system. To date, various elicitors in insect oral secretions have been shown to play a role in the recognition of herbivore attackers by plants (Turlings et al., 1993, 2000; Mattiacci et al., 1995; Alborn et al., 1997, 2007; Schmelz et al., 2006; Louis et al., 2013; Acevedo et al., 2015; Basu et al., 2018). Specific examples of caterpillar-produced HAMPs include β-glucosidase, found in the oral secretion of *Pieris brassicae* fed on cabbage, which triggers the release of volatiles that attract the parasitic wasp *Cotesia glomerata* (Mattiacci et al., 1994, 1995), volicitin, which is a fatty acid-derived elicitor, first isolated from *Spodoptera exigua* caterpillars fed on maize plants (Alborn et al., 1997; Turlings et al., 2000), and inceptin, a peptide found in the oral secretions of *Spodoptera* caterpillars after they ingest chloroplast-containing plant tissue (Schmelz et al., 2006, 2009).

Damage-associated molecular patterns, the second category of signal molecules, are the basis for the mechanism of plant self-recognition (Green and Ryan, 1972; Heil, 2009; Duran-Flores and Heil, 2016). They are endogenous plant-derived indicators of injury, which are released from damaged cells into the extracellular space (Lotze et al., 2007; Heil and Land, 2014; Choi and Klessig, 2016; Gust et al., 2017). The signals generated upon herbivory allow injured cells to communicate their damage status to other cells, thereby activating downstream signaling defense mechanisms (Henry et al., 2012). DAMPs comprise molecules such as cell wall components, fragmented DNA, ATP, and peptides (Quintana-Rodriguez et al., 2018; Hou et al., 2019). A well-studied DAMP is systemin, a polypeptide formed in tomato leaves and perceived by the plant as a primary signal for systemic defense responses (Pearce et al., 1991; Pearce and Ryan, 2003). Such peptides are released by damaged cells and trigger an immune response in the plant (Huffaker et al., 2006, 2013; Yamaguchi et al., 2006). Several studies have demonstrated the effects of DAMPs by applying plant extracts to several species of plants, which resulted in enhanced plant resistance (Mattiacci et al., 1995; Devaiah et al., 2009; Quintana-Rodriguez et al., 2018). In recent years, it has been suggested that the combined recognition of DAMPs and HAMPs, rather than single molecules, provides plants with specific information regarding the nature of an ongoing attack (Huffaker and Ryan, 2007; Duran-Flores and Heil, 2016).

Cotton (*Gossypium hirsutum*) is a plant of great economic importance. It is known to have direct defenses such as gossypol, a terpenoid with insecticidal properties, and indirect defenses such as extra-floral nectar and HIPVs (Loughrin et al., 1994; McCall et al., 1994; McAuslane et al., 1997; Röse and Tumlinson, 2004; Rose et al., 2006). Despite these defenses, cotton plants are subject to attack by rich and complex groups of arthropod herbivores, and are known to be one of the "dirtiest" crops in the world because of the large quantities of pesticides used against these pests (Naranjo et al., 2008; Hagenbucher et al., 2013). The moth *S. exigua* is a widely distributed polyphagous pest of numerous cultivated crops, including cotton plants (Eveleens et al., 1973; Greenberg et al., 2001; Zheng et al., 2011). Previous studies have shown that cotton releases constitutively stored volatiles immediately following attack by *S. exigua* caterpillars, whereas several other volatiles are *de novo* synthesized and only emitted after more than 24 h of feeding damage (Loughrin et al., 1994; McCall et al., 1994). These truly inducible volatiles can be systemically released, and are also released from undamaged parts of the plant (Paré and Tumlinson, 1998; Röse and Tumlinson, 2005). Paré and Tumlinson (1997) found that cotton plants treated with *S. exigua* oral secretion released higher amounts of volatiles than plants that had only been mechanically damage.

Only a handful of elicitors have been identified so far, yet considering the vast number of herbivorous insect species and the plants they feed on, more can be expected to be involved in mediating plant-insect interactions (Bonaventure et al., 2011). No specific elicitor has been identified from insect oral secretions that trigger defense responses in cotton leaves. From previous studies (described above), we surmise that it is likely that cotton is able to perceive elicitor-like molecules from the caterpillars that feed on them, but is unclear to what extent DAMPs and HAMPs jointly contribute to the responses in cotton plants. We tested this by measuring local and systemic HIPV emissions in cotton plants after different treatments with the regurgitant (R) of *S. exigua* caterpillars fed on different plants. We also measured the amount of the known elicitor volicitin and volicitin-like compounds in the caterpillars regurgitant. The results indicate that the inducible responses in cotton are driven by HAMPs as well as DAMPs, and that the oral secretion of cotton-fed caterpillars is particularly active.

MATERIALS AND METHODS

Plants

Cultivated cotton seeds (*G. hirsutum* L., var. STAM 59A) were obtained from CIRAD (La recherche agronomique pour le développement, Montpellier, France). Seeds were soaked in tap water and covered with aluminum foil for 24 h at 24°C. Subsequently, they were kept in plastic boxes with a 4-cm layer of moist vermiculite until germination. Around 4 days after germination, the seedlings were transplanted to individual plastic pots filled with plant substrate soil (Profi Substrat soil, Einheitserde, Germany). The plants were grown under greenhouse conditions (L16:D8, T = 30°C ± 5, and R.H. = 60–80%) and watered every 2 days. All the plants used in the experiments had either two or four fully developed leaves. Maize seeds (*Zea mays* L., var. Delprim) were purchased from DSP Delley seeds and plants genetics Ltd., (Delley, Switzerland) and they were germinated in individual plastic pots filled with substrate soil (Profi Substrat soil, Einheitserde, Germany) and kept under greenhouse conditions (as above). The plants used in the experiments were 10 days old.

Insects

Spodoptera exigua (Hübner; Lepidoptera; Noctuidae) eggs were purchased from Entocare (Wageningen, Netherlands). The caterpillars were reared on wheat-germ based artificial diet (Frontier Scientific Services, Newark, United States) under laboratory conditions (25 ± 2°C, 60% relative humidity, 16:8 h L/D). Regurgitant (R) was collected according to Turlings et al. (1993). Briefly, third to fourth instar caterpillars were gently squeezed close to the head, which forced them to regurgitate (about 10 µl per caterpillar), which was collected through 25 µl capillaries inserted into a 3-ml vial and attached to a vacuum pump (low pressure). The caterpillars were previously fed on either cotton or maize leaves for 24 h. The regurgitant samples were stored at −80°C until further use.

Local and Systemic Induction of Cotton Plants by Simulating Herbivory

In order to test whether cotton plants respond specifically to HAMPs present in the regurgitant of *S. exigua* fed on cotton plants, we performed a series of experiments to measure local and systemic volatile emissions over time. First, we carried out an experiment with cotton plants that had two developed leaves and were induced by simulating caterpillar damage and applying regurgitant to the wounds (**Figures 1A,B**). In brief, we collected emitted volatiles from cotton plants (n = 4–5) that were either mechanically damaged (MD), were MD and with application of regurgitant from *S. exigua* fed on cotton plants (MD + CR) and were mechanically damaged and with application of regurgitant from *S. exigua* fed on maize plants (MD + MR). Control plants were not damaged. Mechanical damage was inflicted by scratching one leaf (~2 cm^2) with a pair of serrated forceps. Three sites on each side of the leaf were damaged in this way, and 10 µl of regurgitant (the approximate equivalent to the volume collected per caterpillar) was immediately applied directly onto the wounded sites (Turlings et al., 1990, 1998; Erb et al., 2015; De Lange et al., 2020). The collection volatiles were performed 2 h after the first induction and repeated 24 and 48 h later. The induction treatments were repeated 2 h before each collection timing alternating the leaf damaged (De Lange et al., 2020).

To evaluate the importance of HAMPs in the systemic response of HIPVs in cotton plants, we collected emitted volatiles from plants induced with *S. exigua* regurgitant. Plants with four developed leaves were used and induced the same way as described above (mechanical damage and regurgitant application). However, this time the two lower leaves (1st and 2nd leaves) were treated, and after the final induction were removed, and the volatiles were collected only from the untreated 3rd and 4th leaves (**Figures 1C–E**). Cotton plants (n = 6) were induced twice a day (morning and evening) for 2 consecutive days. The plants were induced by MD, mechanical damage with regurgitant from *S. exigua* fed on cotton plants (MD + CR), and mechanical damaged with regurgitant from *S. exigua* fed on maize plants (MD + MR). Control plants were not damaged, but the two lower leaves were also removed. On the 3rd day, after 2 days of being induced by simulated herbivory, the plants received the last induction treatment at 8 am. The damaged leaves (1st and 2nd leaves) were removed 2 h later at 10 am, and the collection of systemic HIPVs was immediately performed.

Lastly, we used detached cotton and maize plants to incubate them in a regurgitant solution (**Supplementary Figure 1**), as the plants have to uptake the regurgitant to be induced. We could, therefore, evaluate the effect of the regurgitant itself. The incubation was performed according to Turlings et al. (2000). The plants were subjected to the following treatments: cotton and maize plants incubated in water, cotton plants incubated in regurgitant from *S. exigua* fed on cotton plants (CR), cotton plants incubated in regurgitant from *S. exigua* fed on maize plants (MR), and maize plants incubated in regurgitant from *S. exigua* fed on maize (MR). Based on the previous study, maize plants are known to respond to this form of induction, and (Turlings et al., 2000); that is why were used as a positive control. Plants were cut close to the soil and immediately placed in a 1.5-ml Eppendorf tube with 500 µl of 10% regurgitant solution (50 µl of filtered regurgitant in 450 µl of Milli-Q water). To avoid the regurgitant oxidation, the tubes were covered with aluminum foil. In order to prevent bacterial growth, the regurgitant was filtered (Turlings et al., 1993). To this end, the regurgitant samples were centrifuged for 20 min at 12,000 rpm and the supernatant was collected and filtered (13 mm Syringe filter, PTFE hydrophilic, 0.22 µm, BGB, Switzerland). The time course of the volatile collection was the same as described above (2, 24, and 48 h after induction). For this, we used three different batches of plants. The plants were incubated for 2 h before volatile collection (morning period). After the end of the volatile collection, plants were kept for the next 2 h in the regurgitant solution. In total, the plants were incubated in the regurgitant solution for 6 h. After that, plants were removed from the regurgitant solution and 0.5 cm of the stem was cut off. Plants were kept overnight in falcon tubes containing 10 ml of Milli-Q water. This procedure

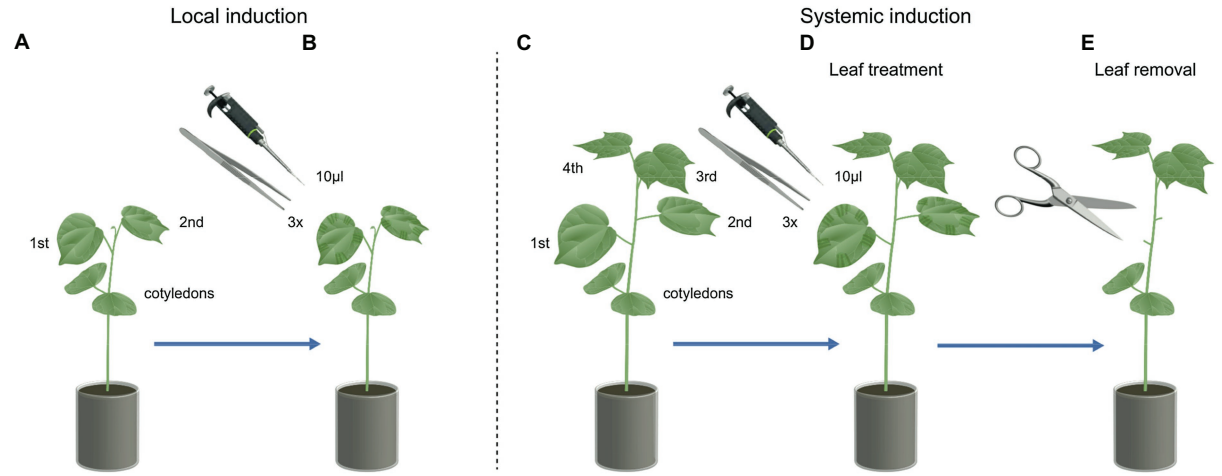

FIGURE 1 | Illustration of simulated herbivory on cotton plants. Local induction: **(A)** Two-leaves stage cotton plants used to measure local herbivore-induced plant volatile (HIPV) emissions. **(B)** The first and second leaf were wounded three times with a serrated forceps and immediately afterward 10 µl of regurgitant were applied on the wounded sites, the HIPVs were collected over time. Systemic induction: **(C)** Four-leaves stage cotton plants used to measure systemic HIPV emissions. **(D)** The first and second leaf were wounded three times with a serrated forceps and immediately afterward 10 µl of regurgitant were applied on the wounded sites. **(E)** To measure systemically induced volatiles emissions (from the third and fourth leaf), the treated first and second leaves were removed right before the volatile collection.

was performed to keep the plants hydrated during the entire experiment, which lasted for 48 h. The next day the same incubation treatment was carried out: incubation in regurgitant solution for 2 h prior to volatile collection, and then followed by the incubation in water overnight.

Collection of Volatiles

Plants were carefully placed in one-port glass bottles to collect the volatiles as described by Turlings et al. (1993, 2000, 2004). The volatiles were collected using trapping filters containing 25 mg of 80/100 mesh Hayesep-Q adsorbent (Ohio Valley Specialist Company, Marietta, United States) for 2 h between 11 am and 1 pm. This period of the day is when cotton plants emit the highest amounts of volatiles (Rose et al., 1996). After each collection, the filters were eluted with 150 µl of dichloromethane (Honeywell, Riedel-de Haën, DE), and 10 µl of internal standard was added (*n*-octane and *n*-nonyl acetate, 20 ng/µl each; Turlings et al., 2000). The samples were stored at −80°C until further analyses.

Analysis of Volatiles

The volatile samples were analyzed on a gas chromatograph (Agilent 7890B) coupled with a mass spectrometer (Agilent 5977B GC/MSD) on TIC mode. About 2 µl of sample were injected in pulsed splitless mode onto an Agilent HP-5MS column (30 m length x 0.25 mm diameter and 0.25 µm film thickness). The temperature program was as follows: kept at 40°C for 3 min, increased to 100°C at a rate of 8°C min^{-1} and subsequently at 5°C min^{-1} to 200°C, followed by a post run period of 3 min at 250°C. Helium was used as a carrier gas and kept at constant flow of 1.1 ml min^{-1}. The identification and quantification of compounds were performed using comparisons to the mass spectra of commercial standards and NIST 17 library spectra.

Volicitin Extraction and Analysis

In order to check whether *S. exigua* fed on cotton plants produce elicitors, we measured relative amounts of volicitin and volicitin-like compounds content in regurgitant of *S. exigua* caterpillars fed on either cotton or maize plants. FACs, specially volicitin *N*-(17-hydroxylinolenoyl)-L-glutamine were chosen based on previous results from Alborn et al. (2000) and Turlings et al. (2000), where it was shown that *S. exigua* fed on maize plants produce this elicitor, which is responsible for inducing HIPV emission in maize plants. To this end, the regurgitant was collected as previously described, each sample (n = 5) corresponded to 10 µl of regurgitant collected from two caterpillars. For the extraction, 1 ml of MeOH:H_2O 50% (50:50) was added to each sample, which was then vortexed and centrifuged at 10,000 rpm and at 4°C for 15 min. The supernatant was collected and used for volicitin analysis.

The analysis of volicitin was performed by UHPLC MS instrument (Waters) made up with using ultra-high performance liquid chromatography quadrupole-time-of-flight mass spectrometry. Specifically, an Acquity UPLC (Waters) coupled to a Synapt G2 high-resolution mass spectrometer. QTOF was employed. The column used for separation was Acquitting UPLC BEH C18 1.7 µm, 2.1 × 50 mm (Waters). The temperature was maintained at 25°C. Two eluants were used: water and 0.05% of formic acid (eluant A) and acetonitrile and 0.05% of formic acid (eluant B). A linear gradient from 10 to 100% B in 7 min was applied. The injection volume was 2.5 µl. The mass spectrometer source was operated in electrospray negative ionization and data were acquired in data-independent acquisition

(DIA) mode (so-called MSe which alternates between low and high collision energies). Exact mass measurements (<2 ppm) were ensured by infusing a 500 ng/ml solution of leucine-enkephalin at 15 µl/min through the Lockspray probe. For data acquisition and processing, we used the software Masslynx v.4.1 (Waters). Volicitin and related molecules were identified based on the determination of the most probable molecular formula as well as fragmentation pattern (typical fragment at *m/z* 145.0615 corresponding to a glutamine moiety). Peaks corresponding to volicitin were volicitins were automatically integrated using TargetLynx XS with a 0.1 min chromatographic window centered on the retention time of each compound and a 0.02 Da mass window centered on the $(M\text{-}H)^-$ ion.

Statistical Analysis

Statistical tests were carried out in R (v. 4.0.0; Team, 2001) using Analysis of Deviance (ANODEV; a maximum likelihood equivalent of ANOVA), followed by residual analysis to verify suitability of distributions of the tested models. Generalized Linear Models (GLM) with a Gaussian distribution were used to verify the differences in volatile emissions and volicitin. Least Squares Means (*LSMeans*) were used to compare significant differences among treatments. An orthogonal partial least squares discriminant analysis (OPLS-DA) and hierarchical clustering heatmap were carried using MetaboAnalyst 5.0 (Wiklund et al., 2008; Chong et al., 2019) to check for differences among treatments on local and systemic HIPVs profiles after 48 h.

RESULTS

Cotton Plants Induced Local and Systemic HIPV After Simulated Herbivory

To evaluate the effect of HAMPs and DAMPs on the induction of plant volatile emissions, we measured the volatiles emitted by cotton plants treated with the regurgitant of *S. exigua* fed on either cotton or maize plants. Overall, plants treated with *S. exigua* regurgitants emitted more volatiles than the mechanically damaged plants and that untreated plants (**Figure 2**). We found differential effects depending on whether the regurgitant originated from maize-fed or from cotton-fed caterpillars. These effects were larger after 48 h of elicitation (**Figures 2A,C**). After 24 h, the plants responded similarly to the application of regurgitant of both cotton-fed and maize-fed caterpillars (**Figure 2C**). Interestingly, only after 48 h the emission rate of HIPVs in plants treated with the regurgitant of maize-fed caterpillars did not differ from the volatiles emitted by mechanically damaged plants (**Figures 2A,C**). On the other hand, plants treated with the regurgitant of cotton-fed caterpillars had a more pronounced response after 48 h (**Figures 2A,C**). During the first 24 h, a total of seven compounds were detected (**Supplementary Tables 1, 2**). Furthermore, (*E*)-4,8–dimethyl–1,3,7-nonatriene (DMNT), (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), and indole were emitted only after 48 h of elicitation (**Figure 2B**; **Supplementary Tables 1–3**). The most abundant compounds emitted from plants treated with the regurgitant of cotton-fed caterpillars were 4-hexen-1-ol, acetate; α-pinene; β-myrcene; β-ocimene; DMNT, and Indole (**Figures 2A,B**). Most of these compounds were emitted at higher concentrations by plants treated with the regurgitant of cotton-fed *S. exigua* than by plants treated with the regurgitant of maize-fed caterpillars.

The HIPVs emitted by systemic leaves of cotton plants treated with the regurgitant of either cotton-fed or maize-fed *S. exigua* caterpillars were collected after 48 h. The emission rate of volatiles showed the same patterns as the patterns observed for locally induced plants (**Figures 3A,C**). In general, plants treated with the regurgitant of cotton-fed caterpillar emitted more volatiles than the plants treated with the regurgitant of maize-fed caterpillars. After 48 h of elicitation, we detected eight different volatiles (**Supplementary Table 4**). Three of them were significantly higher in plants treated with the regurgitant of cotton-fed caterpillars (**Figures 3A,B**). The monoterpene β-myrcene and the sesquiterpene (*E*)-β-farnesene were emitted mostly by plants treated with the regurgitant of cotton-fed caterpillars, whereas the monoterpene α-pinene were emitted by plants treated with both types of regurgitant, but in higher amounts by plants treated with the regurgitant of cotton-fed caterpillars (**Figures 3A,B**). Multivariate analyses, OPLS-DA and hierarchical clustering heatmaps, show clear differences in the volatile emission patters after the different plant elicitation treatments of local induction (**Figures 4A,B**) and systemic induction (**Figures 4C,D**).

To evaluate the effect of HAMPs and minimizing the effect of mechanical damage on the induction of volatile emissions, we measured volatiles in plants that were incubated in a solution of caterpillar regurgitant using the method developed by Turlings et al. (2000). Unexpectedly, cotton plants incubated in a regurgitant solution did not show a pronounced induction of volatile emission (**Figure 5A**; **Supplementary Figure 2**). Nevertheless, the plants emitted three compounds independently of the treatment (α-pinene, β-myrcene, and caryophyllene) plus DMNT, which was emitted only by plants incubated in regurgitant of cotton-fed caterpillars (**Supplementary Table 5**). Maize plants were used as a positive control and showed the expected pattern of responses, especially after 24 h of incubation (**Figure 5B**; **Supplementary Table 6**; **Supplementary Figure 3**). The amounts of regurgitant solution uptaken by the plants did not differ, showing that this is not the reason why cotton plants did not respond to the treatments (**Figure 5C**).

Volicitin Is Present in the Regurgitant of *Spodoptera exigua* Fed on Cotton Plants

The chemical analysis of *S. exigua* regurgitant fed on either cotton or maize plants revealed the presence of six different fatty acid amino acids conjugates (FACs): 18:3-OH-glutamine (volicitin), 18:2-OH-glutamine, 16:1-OH-glutamine, 18:01-OH-glutamine, 18:03-glutamine, and 18:2-glutamine. Interestingly, all the six FACs found in the regurgitant showed similar levels regardless of the plant food source that the insect fed on (**Figure 6**).

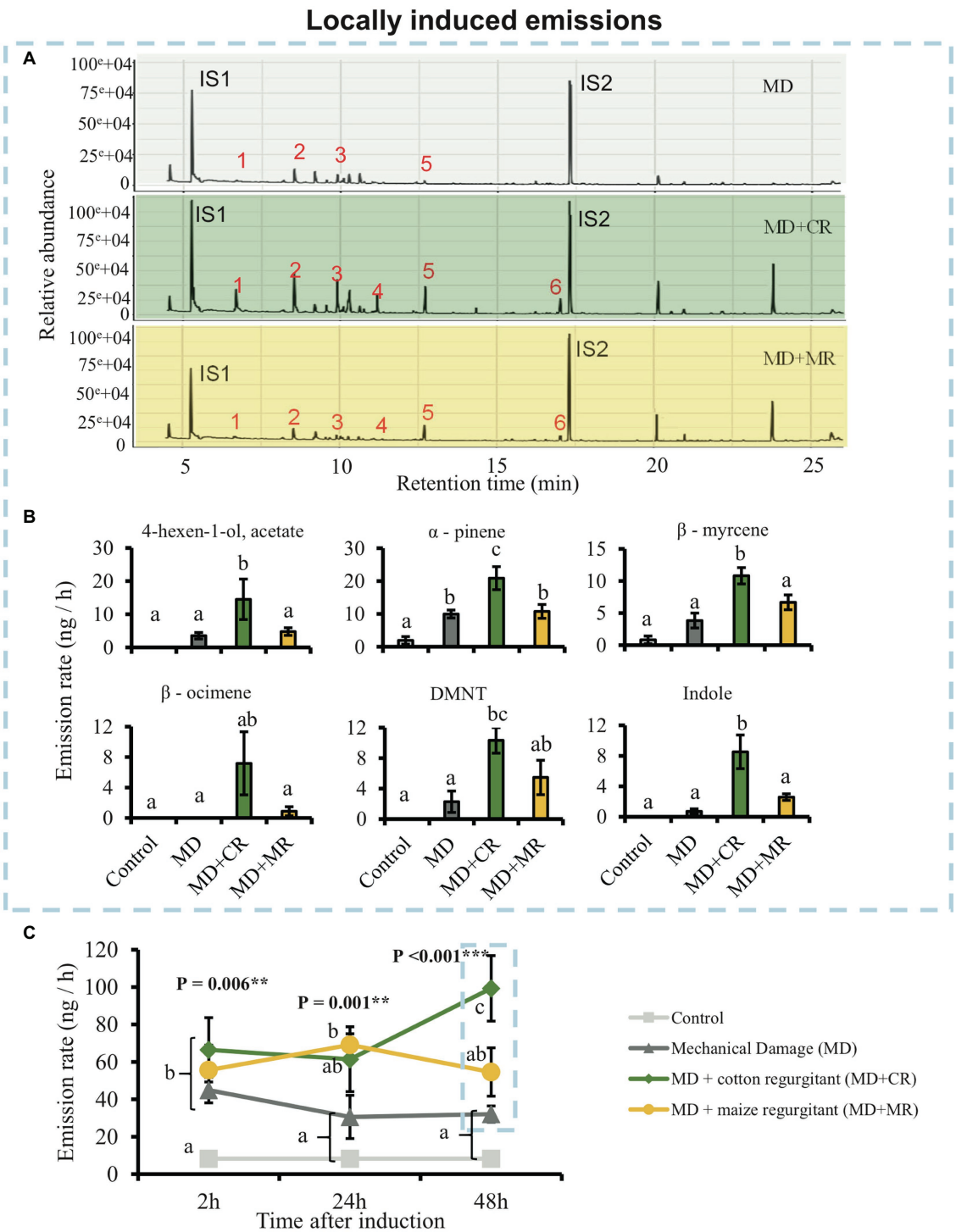

FIGURE 2 | Cotton plants locally emit different amount of volatiles in response to elicitation by different caterpillar regurgitants. Plants were subjected to the following treatments: undamaged (Control), mechanically damaged (MD), mechanically damaged and application of regurgitant of cotton-fed *Spodoptera exigua* (MD + CR), and mechanically damaged and application of regurgitant of maize-fed *S. exigua* (MD + MR; n = 4–5). **(A)** Typical GC-MS chromatograms of HIPV from cotton plants 48 h after elicitation. The identities of the compounds are 1: 4-hexen-1-ol, acetate; 2: α-pinene; 3: β-myrcene; 4: β-ocimene; 5: DMNT; 6: Indole; and IS1 and IS2: internal standards (20 ng/μl), n-octane and nonyl acetate, respectively. **(B)** Average (±SE) of the most representative compounds emitted by cotton plants 48 h after elicitation. Different letters indicate significant differences between treatments ($p < 0.05$). **(C)** Average (±SE) of total amount of volatiles emitted by treated cotton plants 2, 24, and 48 h after elicitation. Different letters indicate significant differences between treatments within each time point. p values are given for treatments [generalized linear model (GLM; family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). $^{**}p < 0.01$, $^{***}p < 0.001$.

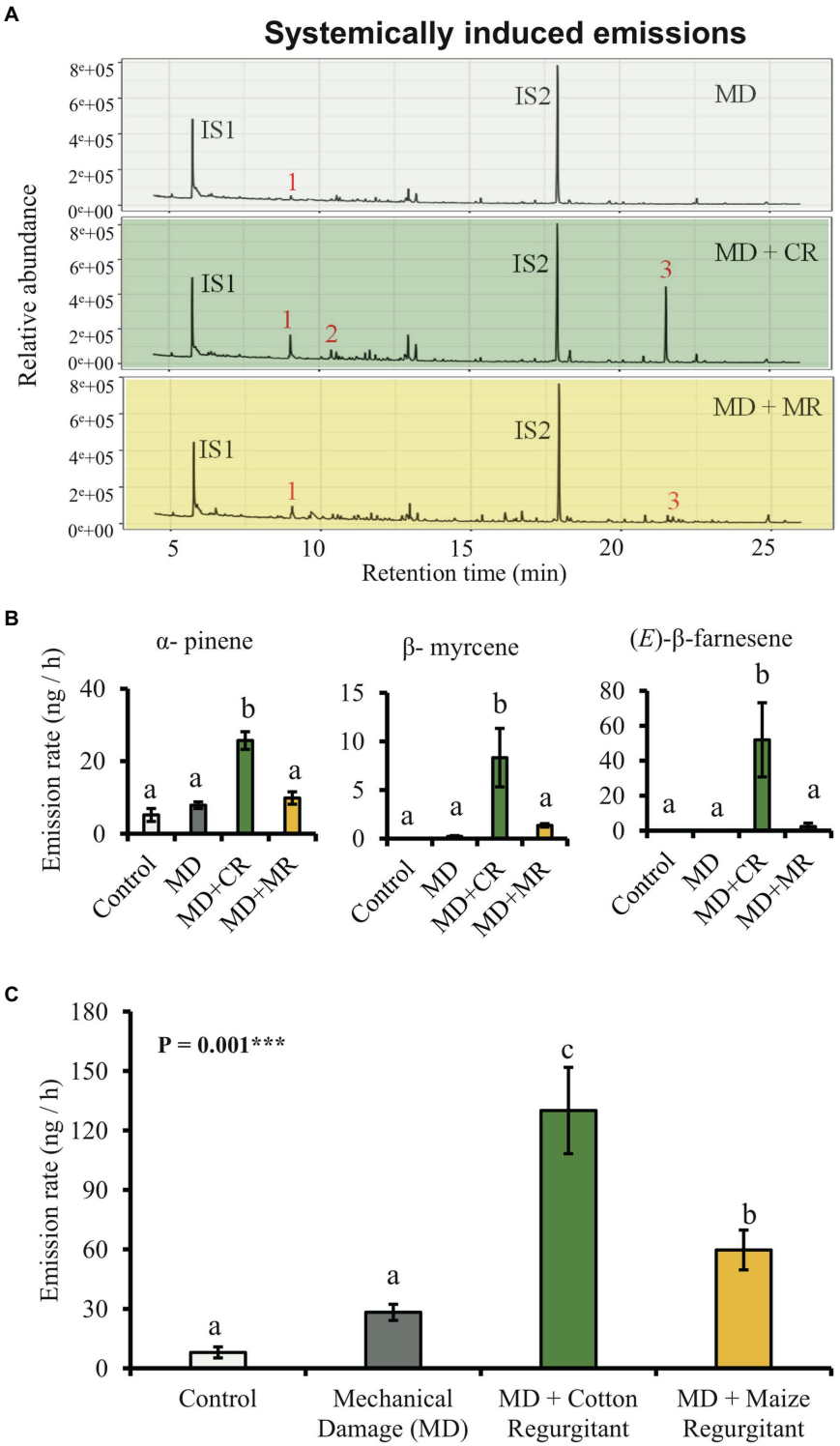

FIGURE 3 | Cotton plants systemically emit different amount of volatiles in response to elicitation by different caterpillar regurgitants. Plants were subjected to the following treatments: undamaged (Control), mechanically damaged (MD), and application of regurgitant of cotton-fed *S. exigua* (MD + CR), and mechanically damaged and application of regurgitant of maize-fed *S. exigua* (MD + MR; $n = 6$). **(A)** Typical GC-MS chromatograms of HIPV from cotton plants 48 h after elicitation. The identities of the compounds are: 1: α-pinene, 2: β-myrcene, 3: (*E*)-β–farnesene, IS1 and IS2: internal standards (20 ng/μl), and *n*-octane and nonyl acetate, respectively. **(B)** Average (±SE) of the most representative compounds emitted by cotton plants 48 h after elicitation. **(C)** Average (±SE) of total amount of volatiles emitted by systemically treated cotton plants 48 h after elicitation. Different letters indicate significant differences among treatments ($p < 0.05$). *p* values are given for treatments [GLM (family, Gaussian)] followed by pairwise comparisons of LSMeans. ***$p < 0.001$ and *$p < 0.05$.

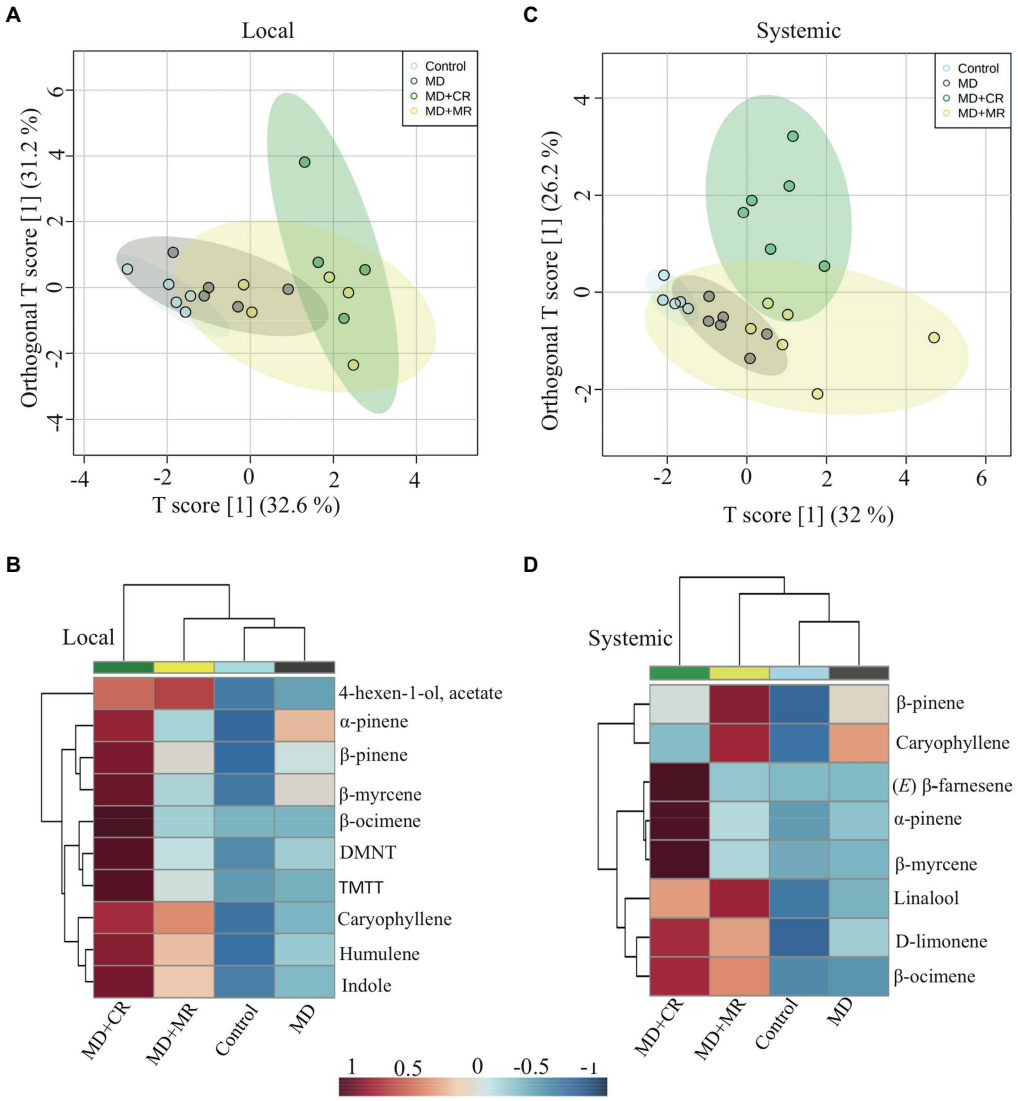

FIGURE 4 | Differences in local and systemically emitted volatiles by cotton plants in response to elicitation by different caterpillar regurgitant. Plants were subjected to the following treatments: undamaged (Control), mechanically damaged (MD), and application of regurgitant of cotton-fed *S. exigua* (MD + CR), and mechanically damaged and application of regurgitant of maize-fed *S. exigua* (MD + MR; n = 4–6). The HIPVs were collected after 48 h of elicitation. **(A)** Results of a orthogonal partial least squares discriminant analysis (OPLS-DA) and **(B)** hierarchical clustering heatmaps of the local emission of volatiles by cotton plant treated with different regurgitants. **(C)** Results of a discriminant analysis (OPLS-DA) and **(D)** hierarchical clustering heatmaps of the systemically emitted volatiles by cotton after treatment with different regurgitants.

DISCUSSION

The identification of both DAMPs and HAMPs, and the evaluation of their relative contribution to triggering plant defenses are essential to understand the factors that regulate plant-herbivore interactions. It is known that wounding itself and/or elicitors present in the regurgitant of the herbivores can trigger the emission of plant volatiles (Mithöfer et al., 2005; Heil and Karban, 2010; Turlings and Erb, 2018). The specificity of the response depends on the recognition of these molecules by the plant and the responses may be different for different plant species. Our study shows that volicitin, a potent HAMP, is present in the regurgitant of both cotton-fed and maize-fed *S. exigua* caterpillars, but only the regurgitant from cotton-fed caterpillars elicit a clear induction of volatiles in cotton plants. Therefore, it is unlikely that volicitin or any other FAC play a role in the induction, but that other, yet unidentified, DAMPs are involved in the regulation of HIPV responses in cotton plants.

Our study is in good agreement with previous studies that show that mechanical damage and the application of *S. exigua* regurgitant on cotton leaves induces higher rates of overall responses and systemic volatile emissions than only mechanical damage (McCall et al., 1994; Paré and Tumlinson, 1997;

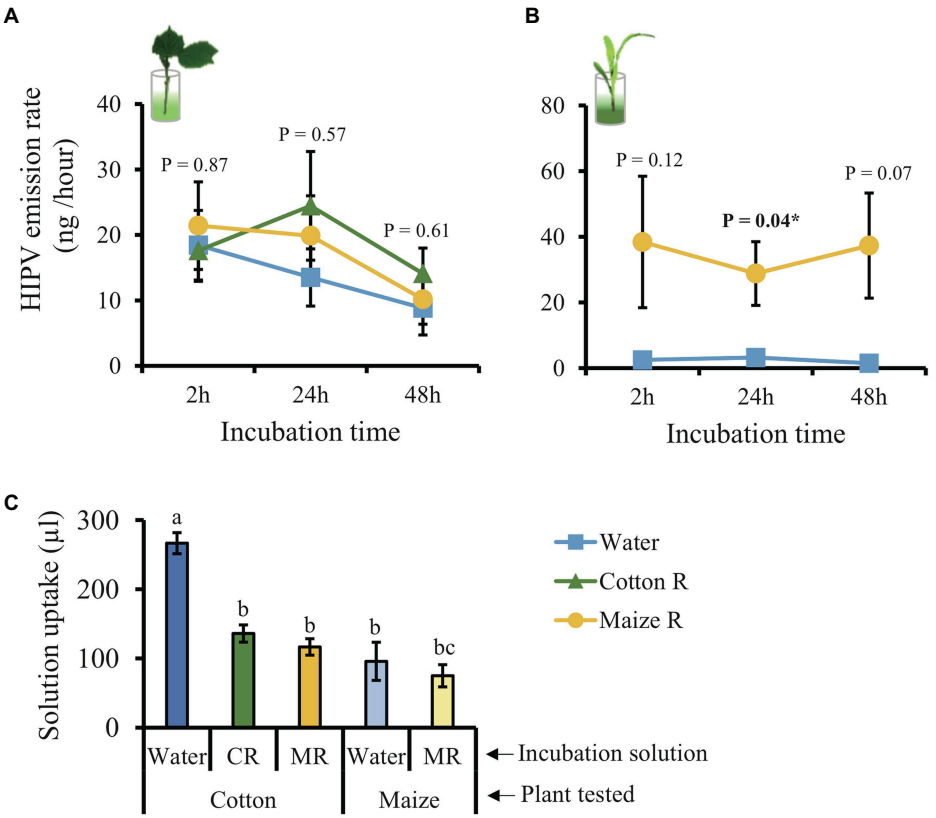

FIGURE 5 | Incubation in caterpillar regurgitant does not induce volatile emissions in cotton plants. **(A)** Total emission of volatiles released by cotton plants incubated in water (blue line), regurgitant of cotton–fed *S. exigua* (green line) and regurgitant of maize–fed *S. exigua* (yellow line) after 2, 24, and 48 h (n = 4–6). **(B)** Total emission of volatiles released by maize plants (n = 4) incubated in water (blue line) and regurgitant from maize–fed *S. exigua* (yellow line) after 2, 24, and 48 h. **(C)** Mean quantity of solution taken up by either cotton or maize plants over 48 h. Lines and bars represent the average (±SE). Different letters indicate significant differences among treatments ($p < 0.05$). p values are given for treatments [GLM (family, Gaussian)] followed by pairwise comparisons of *LSMeans*. ***$p < 0.001$ and *$p < 0.05$.

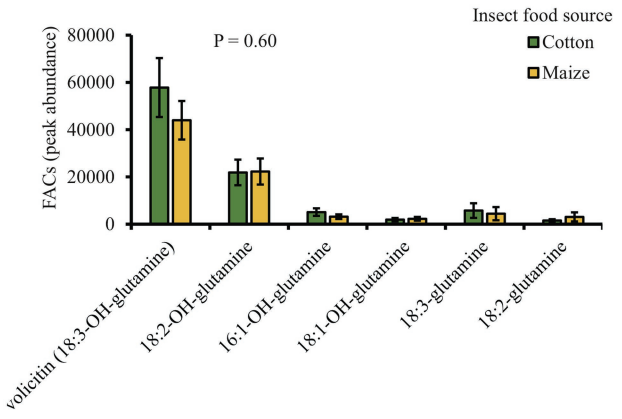

FIGURE 6 | Volicitin and volicitin-like compounds are present in the regurgitant of *S. exigua* caterpillars fed on cotton and maize leaves (n = 5). Bars represent average (±SE). p values are given for treatment comparisons [GLM (family, Gaussian)], followed by pairwise comparisons of LSMeans.

Röse and Tumlinson, 2005). Our finding that the response elicited by regurgitant of cotton-fed *S. exigua* is stronger than the response elicited by the regurgitant of maize-fed *S. exigua* suggests the involvement of specific cotton-derived DAMPs. The self-recognition by cotton plants when treated with regurgitant of *S. exigua* fed on cotton leaves might be responsible for the observed induction of HIPVs emission (Huffaker et al., 2006; Yamaguchi et al., 2006; Heil and Land, 2014; Duran-Flores and Heil, 2016). This can only be confirmed when, the chemical nature of the responsible molecules are known. Few molecules have been identified as DAMPs, e.g., extracellular ATP, extracellular DNA, and extracellular sucrose. These molecules may also include cell-wall fragments (e.g., oligosaccharides, oligogalacturonides; Bonaventure et al., 2011; Duran-Flores and Heil, 2014). Turlings et al. (1993) found that incubating maize plants in a maize leaf juice solution induced a weak, but significant emission of volatiles, which did not occur when the plants were incubated only with water. Yet, incubating the plants in caterpillar regurgitant was far more potent in inducing volatile emissions (Turlings et al., 1993), implying that HAMPs play a more important role than DAMPs in the maize-caterpillar interaction. Mattiacci et al. (1994, 1995) found the same for the volatile responses of cabbage plants to the regurgitant of *Pieris* caterpillars. For bean plants, the application of leaf homogenate is sufficient to induce the production of reactive oxygen species and extra floral nectar (Duran-Flores and Heil, 2014), but they are also responsive to a HAMP, a

peptide which has been named inceptin (Schmelz et al., 2006, 2009). Some authors have argued that wounding and the resulting exposure to DAMPs is enough to trigger the emission of HIPVs (Mithöfer et al., 2005; Heil and Karban, 2010), but the relative contribution of DAMPs remains unclear. Most likely, DAMP and HAMP molecules are used in combination by plants to identify the nature of the organisms that initiate an interaction with them reviewed in Dicke (2016).

It is evident that chemical cues in herbivore oral secretions play key roles in eliciting plants defense, and the outcome of the response can vary among herbivores species (Acevedo et al., 2015; Schmelz, 2015; Turlings and Erb, 2018). Several elicitors have been identified, e.g., volicitin, caeliferins, inceptins, and β-glucosidase (Mattiacci et al., 1995; Alborn et al., 1997; Schmelz et al., 2006). FACs like volicitin are oral secretion components of many lepidopteran, but also present in other insect's order such as Orthoptera and Diptera (Yoshinaga et al., 2007, 2010). They are sufficient to elicit herbivory-specific responses in several plant species including maize and the wild-type tobacco (Turlings et al., 1993; Alborn et al., 1997; Halitschke et al., 2001). Our results showed that volicitin levels in the regurgitant of both cotton- and maize-fed *S. exigua* are the same. The fact that the regurgitant of cotton-fed caterpillars induced stronger HIPVs emissions than the regurgitant of maize-fed caterpillars, strongly suggests that other types of elicitors are involved. Indeed, it has been suggested that insect oral secretion may contain more than a single elicitor with eliciting activity and that these can act synergically or independently to regulate plant defense responses (Spiteller et al., 2001; Acevedo et al., 2017; Basu et al., 2018; Jones et al., 2019). FACs are expected to act specifically and, therefore, different plant species recognize them by different mechanisms, or they are active only in certain plants where they induce a *de novo* production of JA e.g., tobacco, eggplant, maize, and soybean (Halitschke et al., 2001; von Dahl et al., 2007; Wu et al., 2007; Schmelz et al., 2009). Spiteller et al. (2001) found that synthetic volicitin does not induced volatiles in lima bean and cotton, but the FAC *N*-linolenoylglutamine does induced the biosynthesis of volatiles in lima bean (Koch et al., 1999). Also, FACs do not affect JA production in *Arabidopsis* (Schmelz et al., 2009). The species-specific responses are likely due to plant-specific receptors (pattern recognition *receptor*, PRRs). One such receptor was recently identified inceptin (Steinbrenner et al., 2020), confirming that they play a key role in elicitor perception (Truitt et al., 2004; Santamaria et al., 2018; Malik et al., 2020). To date, this research area still lacks enough knowledge to fully characterize specific HAMPs-PRRs interactions.

The overall blend of HIPV emitted by cotton plants treated with *S. exigua* regurgitant was composed mainly by monoterpenes and sesquiterpenes (**Figures 2, 4**). These results are in a good agreement with those found by McAuslane et al. (1997); Röse and Tumlinson (2005) and Loughrin et al. (1995). The main constitutive terpenoids emitted by treated plants were: α-pinene, β-pinene, myrcene, and caryophyllene. They are known to be stored in the glands located near the surface of cotton leaves and be immediately released when the glands are ruptured (Elzen et al., 1985; Rose et al., 1996). Previous studies have shown that in addition to these constitutive volatiles, attack by *S. exigua* caterpillars, also results in *de novo* biosynthesis of several other volatiles that are released with considerable delay (Loughrin et al., 1994; McCall et al., 1994). In accordance, 48 h after treatment, we observed an additional release of mainly non-cyclic terpenoids, which included, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (*E*)-β-farnesene. These truly inducible volatiles are also systemically released from non-attacked leaves of cotton plants (Paré and Tumlinson, 1998; Röse and Tumlinson, 2005). The same pattern of emission was found in our study, but we did not detect the presence of DMNT, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), and indole in the systemic response. Indeed, Rose et al. (1996, 2006) detected the systemic release of these compounds only after 3 or 4 days. We collected the HIPVs after 48 h damage, which might have been too early to find these compounds in the systemic leaves in our experiment. Somewhat surprisingly, incubating cotton plants in *S. exigua* regurgitant solution did not result in any induction of HIPV. This is in sharp contrast to maize plants that respond strongly to the incubation in regurgitant solution of *S. exigua* with the emission of typical maize HIPVs (Turlings et al., 1993), as we confirm here (**Figure 5**). Importantly, the fact that the cotton plants treated with regurgitant from cotton-fed caterpillars emitted larger quantities of volatiles than plants treated with regurgitant from maize-fed caterpillars, is in line with the notion that DAMPs are also involved in the response (Duran-Flores and Heil, 2016). Testing additional types of regurgitant and conducting similar experiments with other plant species may shed more light on the importance of self-damage recognition. It appears that this will vary for different plant species, as, for instance, maize plants respond weaker to cotton-derived regurgitant than to maize-derived regurgitant but stronger to soybean-derived regurgitant (Turlings et al., 1993).

We draw two primary conclusions from our work investigating the factors involved in caterpillar induced cotton volatiles. Firstly, mechanical damage alone is not sufficient to induce a full response and elicitors present in caterpillar oral secretions enhance the response. Secondly, the difference in emissions from plants treated with cotton- and maize-derived secretions may imply that unidentified cotton DAMPs, help the plants to recognize self-damage. Cotton plants have been shown to particularly amenable to priming with inducible volatiles; they can become considerably resistant to insect attack if they have been exposed to volatiles from attacked plants (Renou et al., 2011; Llandres et al., 2018). Therefore, further unraveling the respective roles of HAMPs and DAMPs present in the oral secretion of caterpillars fed in inducing cotton leaf volatiles may provide crucial information that can help to improve cotton defenses against important pests in an agricultural context.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

TT and CA designed the study. CA and GB collected data and analyzed and interpreted the data. GG developed the method to analyze volicitin. CA and GA wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Swiss National Science Foundation Project number 185319.

ACKNOWLEDGMENTS

We would like to thank Thomas Degen for the creation of **Figure 1**. We are grateful to Ricardo Machado, Mary Clancy, and Thomas Degen for the critical evaluation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.709858/full#supplementary-material

REFERENCES

Acevedo, F. E., Peiffer, M., Tan, C. W., Stanley, B. A., Stanley, A., Wang, J., et al. (2017). Fall armyworm-associated gut bacteria modulate plant defense responses. *Mol. Plant-Microbe Interact.* 30, 127–137. doi: 10.1094/MPMI-11-16-0240-R

Acevedo, F. E., Rivera-Vega, L. J., Chung, S. H., Ray, S., and Felton, G. W. (2015). Cues from chewing insects—the intersection of DAMPs, HAMPs, MAMPs and effectors. *Curr. Opin. Plant Biol.* 26, 80–86. doi: 10.1016/j.pbi.2015.05.029

Alborn, H. T., Hansen, T. V., Jones, T. H., Bennett, D. C., Tumlinson, J. H., Schmelz, E. A., et al. (2007). Disulfooxy fatty acids from the American bird grasshopper *Schistocerca americana*, elicitors of plant volatiles. *Proc. Natl. Acad. Sci. U. S. A.* 104, 12976–12981. doi: 10.1073/pnas.0705947104

Alborn, H. T., Jones, T. H., Stenhagen, G. S., and Tumlinson, J. H. (2000). Identification and synthesis of volicitin and related components from beet armyworm oral secretions. *J. Chem. Ecol.* 26, 203–220. doi: 10.1023/A:1005401814122

Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhagen, G., Loughrin, J. H., and Tumlinson, J. H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276, 945–949. doi: 10.1126/science.276.5314.945

Aljbory, Z., and Chen, M. S. (2018). Indirect plant defense against insect herbivores: a review. *Insect Sci.* 25, 2–23. doi: 10.1111/1744-7917.12436

Basu, S., Varsani, S., and Louis, J. (2018). Altering plant defenses: herbivore-associated molecular patterns and effector arsenal of chewing herbivores. *Mol. Plant Microbe Interact.* 31, 13–21. doi: 10.1094/MPMI-07-17-0183-FI

Bonaventure, G., VanDoorn, A., and Baldwin, I. T. (2011). Herbivore-associated elicitors: FAC signaling and metabolism. *Trends Plant Sci.* 16, 294–299. doi: 10.1016/j.tplants.2011.01.006

Choi, H. W., and Klessig, D. F. (2016). DAMPs, MAMPs, and NAMPs in plant innate immunity. *BMC Plant Biol.* 16:232. doi: 10.1186/s12870-016-0921-2

Chong, J., Wishart, D. S., and Xia, J. (2019). Using metaboanalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr. Protoc. Bioinformatics* 68:e86. doi: 10.1002/cpbi.86

De Lange, E. S., Laplanche, D., Guo, H., Xu, W., Vlimant, M., Erb, M., et al. (2020). *Spodoptera frugiperda* caterpillars suppress herbivore-induced volatile emissions in maize. *J. Chem. Ecol.* 46, 344–360. doi: 10.1007/s10886-020-01153-x

Devaiah, S. P., Mahadevappa, G. H., and Shetty, H. S. (2009). Induction of systemic resistance in pearl millet (*Pennisetum glaucum*) against downy mildew (*Sclerospora graminicola*) by *Datura metel* extract. *Crop Prot.* 28, 783–791. doi: 10.1016/j.cropro.2009.04.009

Dicke, M. (2016). Plant phenotypic plasticity in the phytobiome: a volatile issue. *Curr. Opin. Plant Biol.* 32, 17–23. doi: 10.1016/j.pbi.2016.05.004

Dicke, M., and Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends Plant Sci.* 15, 167–175. doi: 10.1016/j.tplants.2009.12.002

Dicke, M., Sabelis, M. W., and de Jong, M. (1988). Analysis of prey preference in phytoseiid mites by using an olfactometer, predation models and electrophoresis. *Exp. Appl. Acarol.* 5, 225–241. doi: 10.1007/BF02366096

Duran-Flores, D., and Heil, M. (2014). Damaged-self recognition in common bean (*Phaseolus vulgaris*) shows taxonomic specificity and triggers signaling via reactive oxygen species (ROS). *Front. Plant Sci.* 5:585. doi: 10.3389/fpls.2014.00585

Duran-Flores, D., and Heil, M. (2016). Sources of specificity in plant damaged-self recognition. *Curr. Opin. Plant Biol.* 32, 77–87. doi: 10.1016/j.pbi.2016.06.019

Elzen, G. W., Williams, H. J., Bell, A. A., Stipanovic, R. D., and Vinson, S. B. (1985). Quantification of volatile terpenes of glanded and glandless *Gossypium hirsutum* L. cultivars and lines by gas chromatography. *J. Agric. Food Chem.* 33, 1079–1082. doi: 10.1021/jf00066a015

Erb, M., and Reymond, P. (2019). Molecular interactions between plants and insect herbivores. *Annu. Rev. Plant Biol.* 70, 527–557. doi: 10.1146/annurev-arplant-050718-095910

Erb, M., Veyrat, N., Robert, C. A., Xu, H., Frey, M., Ton, J., et al. (2015). Indole is an essential herbivore-induced volatile priming signal in maize. *Nat. Commun.* 6:6273. doi: 10.1038/ncomms7273

Eveleens, K. G., Van Den Bosch, R., and Ehler, L. E. (1973). Secondary outbreak induction of beet armyworm by experimental insecticide applications in cotton in California. *Environ. Entomol.* 2, 497–504. doi: 10.1093/ee/2.4.497

Farmer, E. E. (2014). *Leaf Defence*. Oxford, UK: Oxford Univ. Press.

Felton, G. W., and Tumlinson, J. H. (2008). Plant–insect dialogs: complex interactions at the plant–insect interface. *Curr. Opin. Plant Biol.* 11, 457–463. doi: 10.1016/j.pbi.2008.07.001

Fürstenberg-Hägg, J., Zagrobelny, M., and Bak, S. (2013). Plant defense against insect herbivores. *Int. J. Mol. Sci.* 14, 10242–10297. doi: 10.3390/ijms140510242

Green, T. R., and Ryan, C. A. (1972). Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* 175, 776–777. doi: 10.1126/science.175.4023.776

Greenberg, S., Sappington, T., Legaspi, B., Liu, T.-X., and Setamou, M. (2001). Feeding and life history of *Spodoptera exigua* (Lepidoptera: Noctuidae) on different host plants. *Ann. Entomol. Soc. Am.* 94, 566–575. doi: 10.1603/0013-8746(2001)094[0566:FALHOS]2.0.CO;2

Gust, A. A., Pruitt, R., and Nürnberger, T. (2017). Sensing danger: key to activating plant immunity. *Trends Plant Sci.* 22, 779–791. doi: 10.1016/j.tplants.2017.07.005

Hagenbucher, S., Olson, D. M., Ruberson, J. R., Wäckers, F. L., and Romeis, J. (2013). Resistance mechanisms against arthropod herbivores in cotton and their interactions with natural enemies. *Crit. Rev. Plant Sci.* 32, 458–482. doi: 10.1080/07352689.2013.809293

Halitschke, R., Schittko, U., Pohnert, G., Boland, W., and Baldwin, I. T. (2001). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiol.* 125, 711–717. doi: 10.1104/pp.125.2.711

Heil, M. (2009). Damaged-self recognition in plant herbivore defence. *Trends Plant Sci.* 14, 356–363. doi: 10.1016/j.tplants.2009.04.002

Heil, M., and Karban, R. (2010). Explaining evolution of plant communication by airborne signals. *Trends Ecol. Evol.* 25, 137–144. doi: 10.1016/j.tree.2009.09.010

Heil, M., and Land, W. G. (2014). Danger signals–damaged-self recognition across the tree of life. *Front. Plant Sci.* 5:578. doi: 10.3389/fpls.2014.00578

Henry, G., Thonart, P., and Ongena, M. (2012). PAMPs, MAMPs, DAMPs and others: an update on the diversity of plant immunity elicitors. *BASE* 16, 257–268.

Hou, S., Liu, Z., Shen, H., and Wu, D. (2019). Damage-associated molecular pattern-triggered immunity in plants. *Front. Plant Sci.* 10:646. doi: 10.3389/fpls.2019.00646

Huffaker, A., Pearce, G., and Ryan, C. A. (2006). An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proc. Natl. Acad. Sci. U. S. A.* 103, 10098–10103. doi: 10.1073/pnas.0603727103

Huffaker, A., Pearce, G., Veyrat, N., Erb, M., Turlings, T. C. J., Sartor, R., et al. (2013). Plant elicitor peptides are conserved signals regulating direct and indirect antiherbivore defense. *Proc. Natl. Acad. Sci. U. S. A.* 110, 5707–5712. doi: 10.1073/pnas.1214668110

Huffaker, A., and Ryan, C. A. (2007). Endogenous peptide defense signals in *Arabidopsis* differentially amplify signaling for the innate immune response. *Proc. Natl. Acad. Sci. U. S. A.* 104, 10732–10736. doi: 10.1073/pnas.0703343104

Jones, A. G., Mason, C. J., Felton, G. W., and Hoover, K. (2019). Host plant and population source drive diversity of microbial gut communities in two polyphagous insects. *Sci. Rep.* 9:2792. doi: 10.1038/s41598-019-39163-9

Karban, R., and Baldwin, I. T. (1997). *Induced Responses to Herbivory*. Chicago: University of Chicago Press.

Koch, T., Krumm, T., Jung, V., Engelberth, J., and Boland, W. (1999). Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiol.* 121, 153–162. doi: 10.1104/pp.121.1.153

Llandres, A. L., Almohamad, R., Brévault, T., Renou, A., Téréta, I., Jean, J., et al. (2018). Plant training for induced defense against insect pests: a promising tool for integrated pest management in cotton. *Pest Manag. Sci.* 74, 2004–2012. doi: 10.1002/ps.5039

Lotze, M. T., Zeh, H. J., Rubartelli, A., Sparvero, L. J., Amoscato, A. A., Washburn, N. R., et al. (2007). The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol. Rev.* 220, 60–81. doi: 10.1111/j.1600-065X.2007.00579.x

Loughrin, J. H., Manukian, A., Heath, R. R., and Tumlinson, J. H. (1995). Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *J. Chem. Ecol.* 21, 1217–1227. doi: 10.1007/BF02228321

Loughrin, J. H., Manukian, A., Heath, R. R., Turlings, T. C., and Tumlinson, J. H. (1994). Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plant. *Proc. Natl. Acad. Sci.* 91, 11836–11840. doi: 10.1073/pnas.91.25.11836

Louis, J., Peiffer, M., Ray, S., Luthe, D. S., and Felton, G. W. (2013). Host-specific salivary elicitor(s) of European corn borer induce defenses in tomato and maize. *New Phytol.* 199, 66–73. doi: 10.1111/nph.12308

Malik, N. A. A., Kumar, I. S., and Nadarajah, K. (2020). Elicitor and receptor molecules: orchestrators of plant defense and immunity. *Int. J. Mol. Sci.* 21:963. doi: 10.3390/ijms21030963

Mattiacci, L., Dicke, M., and Posthumus, M. A. (1994). Induction of parasitoid attracting synomone in *Brussels sprouts* plants by feeding of *Pieris brassicae* larvae: role of mechanical damage and herbivore elicitor. *J. Chem. Ecol.* 20, 2229–2247. doi: 10.1007/BF02033199

Mattiacci, L., Dicke, M., and Posthumus, M. A. (1995). Beta-glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. U. S. A.* 92, 2036–2040. doi: 10.1073/pnas.92.6.2036

McAuslane, H. J., Alborn, H. T., and Toth, J. P. (1997). Systemic induction of terpenoid aldehydes in cotton pigment glands by feeding of larval *Spodoptera exigua*. *J. Chem. Ecol.* 23, 2861–2879. doi: 10.1023/A:1022575313325

McCall, P. J., Turlings, T. C. J., Loughrin, J., Proveaux, A. T., and Tumlinson, J. H. (1994). Herbivore-induced volatile emissions from cotton (*Gossypium hirsutum* L.) seedlings. *J. Chem. Ecol.* 20, 3039–3050. doi: 10.1007/BF02033709

Mithöfer, A., Wanner, G., and Boland, W. (2005). Effects of feeding Spodoptera littoralis on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* 137, 1160–1168. doi: 10.1104/pp.104.054460

Naranjo, S. E., Ruberson, J. R., Sharma, H. C., Wilson, L., and Wu, K. (2008). "The present and future role of insect-resistant genetically modified cotton in IPM," in *Integration of Insect-Resistant Genetically Modified Crops Within IPM Programs*. eds. J. Romeis, A. M. Shelton and G. G. Kennedy. (Dordrecht: Springer), 159–194.

Paré, P. W., and Tumlinson, J. H. (1997). De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol.* 114, 1161–1167. doi: 10.1104/pp.114.4.1161

Paré, P. W., and Tumlinson, J. H. (1998). Cotton volatiles synthesized and released distal to the site of insect damage. *Phytochemistry* 47, 521–526. doi: 10.1016/S0031-9422(97)00442-1

Paré, P. W., and Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121, 325–332. doi: 10.1104/pp.121.2.325

Pearce, G., and Ryan, C. A. (2003). Systemic signaling in tomato plants for defense against herbivores: isolation and characterization of three novel defense-signaling glycopeptide hormones coded in a single precursor gene. *J. Biol. Chem.* 278, 30044–30050. doi: 10.1074/jbc.M304159200

Pearce, G., Strydom, D., Johnson, S., and Ryan, C. A. (1991). A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* 253, 895–897. doi: 10.1126/science.253.5022.895

Quintana-Rodriguez, E., Duran-Flores, D., Heil, M., and Camacho-Coronel, X. (2018). Damage-associated molecular patterns (DAMPs) as future plant vaccines that protect crops from pests. *Sci. Hortic.* 237, 207–220. doi: 10.1016/j.scienta.2018.03.026

Renou, A., Téréta, I., and Togola, M. (2011). Manual topping decreases bollworm infestations in cotton cultivation in Mali. *Crop Prot.* 30, 1370–1375. doi: 10.1016/j.cropro.2011.05.020

Rose, U. S. R., Lewis, J., and Tumlinson, J. H. (2006). Extrafloral nectar from cotton (*Gossypium hirsutum*) as a food source for parasitic wasps. *Funct. Ecol.* 20, 67–74. doi: 10.1111/j.1365-2435.2006.01071.x

Rose, U. S., Manukian, A., Heath, R. R., and Tumlinson, J. H. (1996). Volatile semiochemicals released from undamaged cotton leaves (a systemic response of living plants to caterpillar damage). *Plant Physiol.* 111, 487–495. doi: 10.1104/pp.111.2.487

Röse, U. S., and Tumlinson, J. H. (2004). Volatiles released from cotton plants in response to *Helicoverpa zea* feeding damage on cotton flower buds. *Planta* 218, 824–832. doi: 10.1007/s00425-003-1162-9

Röse, U. S., and Tumlinson, J. H. (2005). Systemic induction of volatile release in cotton: how specific is the signal to herbivory? *Planta* 222, 327–335. doi: 10.1007/s00425-005-1528-2

Santamaria, M. E., Arnaiz, A., Gonzalez-Melendi, P., Martinez, M., and Diaz, I. (2018). Plant perception and short-term responses to phytophagous insects and mites. *Int. J. Mol. Sci.* 19:1356. doi: 10.3390/ijms19051356

Schmelz, E. A. (2015). Impacts of insect oral secretions on defoliation-induced plant defense. *Curr. Opin. Insect Sci.* 9, 7–15. doi: 10.1016/j.cois.2015.04.002

Schmelz, E. A., Carroll, M. J., LeClere, S., Phipps, S. M., Meredith, J., Chourey, P. S., et al. (2006). Fragments of ATP synthase mediate plant perception of insect attack. *Proc. Natl. Acad. Sci. U. S. A.* 103, 8894–8899. doi: 10.1073/pnas.0602328103

Schmelz, E. A., Engelberth, J., Alborn, H. T., Tumlinson, J. H., and Teal, P. E. A. (2009). Phytohormone-based activity mapping of insect herbivore-produced elicitors. *Proc. Natl. Acad. Sci. U. S. A.* 106, 653–657. doi: 10.1073/pnas.0811861106

Spiteller, D., Pohnert, G., and Boland, W. (2001). Absolute configuration of volicitin, an elicitor of plant volatile biosynthesis from lepidopteran larvae. *Tetrahedron Lett.* 42, 1483–1485. doi: 10.1016/S0040-4039(00)02290-5

Steinbrenner, A. D., Muñoz-Amatriaín, M., Chaparro, A. F., Aguilar-Venegas, J. M., Lo, S., Okuda, S., et al. (2020). A receptor-like protein mediates plant immune responses to herbivore-associated molecular patterns. *Proc. Natl. Acad. Sci. U. S. A.* 117, 31510–31518. doi: 10.1073/pnas.2018415117

Team, R. C. (2001). "R installation and administration". R Foundation for Statistical Computing, Vienna, Austria, 2015a.

Truitt, C. L., Wei, H. X., and Pare, P. W. (2004). A plasma membrane protein from *Zea mays* binds with the herbivore elicitor volicitin. *Plant Cell* 16, 523–532. doi: 10.1105/tpc.017723

Turlings, T. C. J., Alborn, H. T., Loughrin, J. H., and Tumlinson, J. H. (2000). Volicitin, an elicitor of maize volatiles in oral secretion of *Spodoptera exigua*: isolation and bioactivity. *J. Chem. Ecol.* 26, 189–202. doi: 10.1023/A:1005449730052

Turlings, T. C. J., Davison, A. C., and TamÒ, C. (2004). A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. *Physiol. Entomol.* 29, 45–55. doi: 10.1111/j.1365-3032.2004.0362.x

Turlings, T. C. J., and Erb, M. (2018). Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. *Annu. Rev. Entomol.* 63, 433–452. doi: 10.1146/annurev-ento-020117-043507

Turlings, T. C. J., Lengwiler, U. B., Bernasconi, M. L., and Wechsler, D. (1998). Timing of induced volatile emissions in maize seedlings. *Planta* 207, 146–152. doi: 10.1007/s004250050466

Turlings, T. C. J., McCall, P. J., Alborn, H. T., and Tumlinson, J. H. (1993). An elicitor in caterpillar oral secretions that induces corn seedlings to emit chemical signals attractive to parasitic wasps. *J. Chem. Ecol.* 19, 411–425. doi: 10.1007/BF00994314

Turlings, T. C. J., Tumlinson, J. H., and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250, 1251–1253. doi: 10.1126/science.250.4985.1251

Turlings, T. C. J., and Wäckers, F. (2004). "Recruitment of predators and parasitoids by herbivore-injured plants," in *Advances in Insect Chemical Ecology*. eds. R. T. Cardé and J. G. Millar (Cambridge: Cambridge University Press), 21–71.

von Dahl, C. C., Winz, R. A., Halitschke, R., Kühnemann, F., Gase, K., and Baldwin, I. T. (2007). Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*. *Plant J.* 51, 293–307. doi: 10.1111/j.1365-313X.2007.03142.x

Wiklund, S., Johansson, E., Sjöström, L., Mellerowicz, E. J., Edlund, U., Shockcor, J. P., et al. (2008). Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. *Anal. Chem.* 80, 115–122. doi: 10.1021/ac0713510

Wu, J., and Baldwin, I. T. (2009). Herbivory-induced signalling in plants: perception and action. *Plant Cell Environ.* 32, 1161–1174. doi: 10.1111/j.1365-3040.2009.01943.x

Wu, J., Hettenhausen, C., Meldau, S., and Baldwin, I. T. (2007). Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. *Plant Cell* 19, 1096–1122. doi: 10.1105/tpc.106.049353

Yamaguchi, Y., Pearce, G., and Ryan, C. A. (2006). The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in *Arabidopsis*, is functional in transgenic tobacco cells. *Proc. Natl. Acad. Sci. U. S. A.* 103, 10104–10109. doi: 10.1073/pnas.0603729103

Yoshinaga, N., Aboshi, T., Ishikawa, C., Fukui, M., Shimoda, M., Nishida, R., et al. (2007). Fatty acid amides, previously identified in caterpillars, found in the cricket *Teleogryllus taiwanemma* and fruit fly *Drosophila melanogaster* larvae. *J. Chem. Ecol.* 33, 1376–1381. doi: 10.1007/s10886-007-9321-2

Yoshinaga, N., Alborn, H. T., Nakanishi, T., Suckling, D. M., Nishida, R., Tumlinson, J. H., et al. (2010). Fatty acid-amino acid conjugates diversification in lepidopteran caterpillars. *J. Chem. Ecol.* 36, 319–325. doi: 10.1007/s10886-010-9764-8

Zheng, X. L., Cong, X. P., Wang, X. P., and Lei, C. L. (2011). Pupation behaviour, depth, and site of *Spodoptera exigua*. *Bull. Insectol.* 64, 209–214.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 August 2021
doi: 10.3389/fpls.2021.700242

Application of Plant Defense Elicitors Fails to Enhance Herbivore Resistance or Mitigate Phytoplasma Infection in Cranberries

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Synthetic elicitors of the salicylic acid (SA) and jasmonic acid (JA) plant defense pathways can be used to increase crop protection against herbivores and pathogens. In this study, we tested the hypothesis that elicitors of plant defenses interact with pathogen infection to influence crop resistance against vector and nonvector herbivores. To do so, we employed a trophic system comprising of cranberries (*Vaccinium macrocarpon*), the phytoplasma that causes false blossom disease, and two herbivores—the blunt-nosed leafhopper (*Limotettix vaccinii*), the vector of false blossom disease, and the nonvector gypsy moth (*Lymantria dispar*). We tested four commercial elicitors, including three that activate mainly SA-related plant defenses (Actigard, LifeGard, and Regalia) and one activator of JA-related defenses (Blush). A greenhouse experiment in which phytoplasma-infected and uninfected plants received repeated exposure to elicitors revealed that both phytoplasma infection and elicitor treatment individually improved *L. vaccinii* and *L. dispar* mass compared to uninfected, untreated controls; however, SA-based elicitor treatments reduced *L. vaccinii* mass on infected plants. Regalia also improved *L. vaccinii* survival. Phytoplasma infection reduced plant size and mass, increased levels of nitrogen (N) and SA, and lowered carbon/nitrogen (C/N) ratios compared to uninfected plants, irrespective of elicitor treatment. Although none of our elicitor treatments influenced transcript levels of a phytoplasma-specific marker gene, all of them increased N and reduced C/N levels; the three SA activators also reduced JA levels. Taken together, our findings reveal positive effects of both phytoplasma infection and elicitor treatment on the performance of *L. vaccinii* and *L. dispar* in cranberries, likely *via* enhancement of plant nutrition and changes in phytohormone profiles, specifically increases in SA levels and corresponding decreases in levels of JA. Thus, we found no evidence that the tested elicitors of plant defenses increase resistance to insect herbivores or reduce disease incidence in cranberries.

Keywords: false blossom disease, blunt-nosed leafhoppers, gypsy moth, carbon/nitrogen ratios, phytohormones, plant-phytoplasma-herbivore interactions

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *25 April 2021*
Accepted: *19 July 2021*
Published: *12 August 2021*

Citation:
Rodriguez-Saona C, Polashock JJ, Kyryczenko-Roth V, Holdcraft R, Jimenez-Gonzalez G, De Moraes CM and Mescher MC (2021) Application of Plant Defense Elicitors Fails to Enhance Herbivore Resistance or Mitigate Phytoplasma Infection in Cranberries. Front. Plant Sci. 12:700242. doi: 10.3389/fpls.2021.700242

INTRODUCTION

Plants respond to biotic antagonists *via* immune responses that are modulated by phytohormone signaling (Verhage et al., 2010; Pieterse et al., 2012). In particular, the phytohormones salicylic acid (SA) and jasmonic acid (JA) play a critical role in a plant's response to attack by pathogens and herbivores (Howe and Jander, 2008; Robert-Seilaniantz et al., 2011). The SA pathway is mostly associated with resistance against biotrophic pathogens (Thomma et al., 1998) as well as defenses against piercing/sucking insects (War et al., 2012), whereas the JA pathway is often induced after herbivore feeding, particularly those with chewing mouthparts, and against necrotrophic pathogens (Ballaré, 2011). Plants that have been domesticated for high yield may, however, have weakened immune defenses compared to their wild ancestors (Lindig-Cisneros et al., 2002; Rodriguez-Saona et al., 2011; Szczepaniec et al., 2013; Chen et al., 2015; Gaillard et al., 2018). To boost the defenses of domesticated plants, synthetic elicitors can be used to enhance the activation of the SA and JA defensive pathways and thus protect plants against both pathogens and insect herbivores (Stout et al., 2002; Holopainen et al., 2009; Pickett et al., 2014; Sobhy et al., 2014; Bektas and Eulgem, 2015).

Four synthetic elicitors of plant defenses commercially available at present include Regalia®, LifeGard® WG Biological Plant Activator, Actigard® 50WG Plant Activator, and Blush® 2X. Regalia, LifeGard, and Actigard are biofungicides that induce plant resistance to several fungal and bacterial diseases, likely through activation of the SA defense pathway and production of pathogenesis-related proteins, indicators of systemic acquired resistance (Cole, 1999; Ziadi et al., 2001; Bargabus et al., 2002; Darolt et al., 2020; Margaritopoulou et al., 2020). Conversely, the active ingredient in Blush is a synthetically produced jasmonate that acts as an analog of JA in plants and increases plant resistance against herbivores (Mandour et al., 2013; Uefune et al., 2014; Sobhy et al., 2015) and pathogens (Yoshida et al., 2010). Despite considerable evidence that such elicitors can provide protection against pathogens and herbivores, there is also reason to suspect that exogenous application of these elicitors could lead to increased plant susceptibility to one or more attackers due to potential cross talk between the SA and JA pathways (Inbar et al., 1998; Koornneef and Pieterse, 2008; Thaler et al., 2012).

Such cross talk between defense pathways might have particular relevance in the case of vector-borne plant pathogens, such as phytoplasmas. Phytoplasmas are cell-wall-less plant pathogenic bacteria belonging to the class Mollicutes (Phylum Tenericutes), which are transmitted by phloem-feeding insects (Hemiptera), including leafhoppers (Ciccadellidae), planthoppers (Fulgoroidea), and psyllids (Psyllidae; Weintraub and Beanland, 2005; Hogenhout et al., 2008; Gross et al., 2021). In cranberries (*Vaccinium macrocarpon* Aiton, Ericaceae), false blossom disease is caused by *Candidatus* phytoplasma sp. subgroup 16SrIII-Y (Lee et al., 2014; Polashock et al., 2017). False blossom disease symptoms include the formation of a witches' broom that causes several branches to appear at the internode and a malformation of flowers (Dobroscky, 1931). Blunt-nosed leafhoppers, *Limotettix vaccinii* Van Duzee (Hemiptera: Cicadellidae), are the only known vectors of this disease in cranberries (Beckwith and Hutton, 1929; Dobroscky, 1931; Chen, 1971; De Lange and Rodriguez-Saona, 2015; Polashock et al., 2017). A recent study by Pradit et al. (2020) found that *L. vaccinii* adults grew larger when developing on phytoplasma-infected cranberry plants relative to uninfected controls, but that females nevertheless laid more eggs on uninfected plants. Another study (Pradit et al., 2019a) showed that three nonvector herbivores of cranberries, spotted fireworm (*Choristoneura parallela* Robinson), Sparganothis fruitworm (*Sparganothis sulfureana* Clemens; both Lepidoptera: Tortricidae), and gypsy moth (*Lymantria dispar* L.; Lepidoptera: Erebidae), also grew larger and damaged more leaves on phytoplasma-infected than on uninfected cranberries. The latter results were found to correlate with an increase in nitrogen (N) levels and reduced levels of defensive proanthocyanidins (a class of polyphenolic compounds important in plant defense against pathogens and herbivores; Fisk, 1980; Bernays, 1981; Treutter, 2006) in infected cranberry plants (Pradit et al., 2019a).

These previous findings (Pradit et al., 2019a, 2020) suggest that the phytoplasma causing false blossom may manipulate cranberry plants by increasing their nutritional quality and reducing defenses, likely for its own benefit but also benefitting vector and nonvector herbivores (at least in the short term). Here, we asked whether this host-plant manipulation by the pathogen affects the efficacy of synthetic elicitors against insect herbivores. We hypothesized that the effects of these elicitors on vector and nonvector herbivore performance differ between uninfected and infected cranberry plants. To test this hypothesis, we examined how the four elicitors of plant defenses described above (i.e., Regalia, LifeGard, Actigard, and Blush) interact with phytoplasma infection itself to influence the performance (survival and growth) of the false blossom vector *L. vaccinii*, as well as the nonvector herbivore *L. dispar* (gypsy moth). In addition, we measured effects on several plant traits, including size, mass, and nutrient (i.e., N) and phytohormone (i.e., JA and SA) levels, that may provide insight into possible mechanisms.

MATERIALS AND METHODS

Plant Material

Phytoplasma-infected and uninfected cranberries, *V. macrocarpon* (cv. Crimson Queen), were collected in the fall 2018 from a commercial cranberry farm located in Chatsworth (New Jersey, United States) and placed in a coldframe at ~10°C until propagation in February 2019. For propagation, stem cuttings (~7.5 cm) from uninfected and infected plants were transferred to individual 4 × 4-cm cells in 96-cell plug trays and placed in a greenhouse [23 ± 2°C, 70 ± 10% relative humidity (RH), and 14:10 light:dark (L:D)] under high-pressure sodium lights for rooting. After root development, cuttings were transplanted to single pots (10 × 10 cm^2). Plants were grown in the greenhouse until use in experiments in a 50:50 v/v peat:sand mix, were fertilized once a month (from March until June) with

Jack's Professional Water Soluble 20-20-20 N-P-K General Purpose fertilizer (J.R. Peters Inc., Allentown, Pennsylvania, United States) at a rate of 12.3 g per 7 L of water, and were watered weekly as needed. Each of these plants (from individual cuttings) was considered a replicate, and the numbers of plants used for each experiment are provided below.

Prior to experiments, 10 random plants (five infected and five uninfected) were tested using a nested PCR assay (Lee et al., 2014) to confirm phytoplasma infection only in infected plants. Only infected plants tested positive for the presence of phytoplasma. Furthermore, only the phytoplasma-infected plants showed symptoms associated with false blossom disease (e.g., short and straight uprights). All plants were at the vegetative stage when used in experiments.

Insects

We used the following two herbivore species: the blunt-nosed leafhopper *L. vaccinii*, a vector of the phytoplasma that causes false blossom, and the nonvector herbivore *L. dispar* (gypsy moth). Both herbivores are pests of cranberries in the northeastern United States (Averill and Sylvia, 1998), with immatures that feed on cranberries in the spring during new growth development. Therefore, we synchronized crop phenology with the time when immatures of these herbivores are active. *Limotettix vaccinii* nymphs, mostly second instars, were collected at the end of May 2019 *via* sweep net sampling of commercial cranberry beds (Chatsworth, New Jersey), showing no signs of false blossom disease. To check whether these field-collected nymphs had the phytoplasma, we tested 20 randomly selected individuals for phytoplasma infection using a nested PCR assay (Lee et al., 2014) and found that most (70%) of them were free of phytoplasma. *Limotettix vaccinii* nymphs were used in the experiments on the same date of collection.

Egg masses of *L. dispar* were sourced from the USDA APHIS Otis Laboratory (Buzzards Bay, Massachusetts, United States) in early June 2019 and placed in an environmental chamber at 25 ± 1°C, 65% RH, and 14:10 L:D. Neonates were used for all experiments.

Experimental Design and Treatments

An experiment was conducted to test two infection levels (i.e., phytoplasma-infected and uninfected plants) and five treatments: four commercial elicitors of plant defenses and a distilled-water control, in a 2 × 5 factorial design. The plant elicitors tested and their application rates are shown in **Table 1**. Application rates were based on the maximum recommended label rate and did not to exceed the maximum amount allowed per ha per season. All applications were mixed with water to reach 467 L/ha of spray. Plants in pots were treated three times (once a month) with one of the four elicitors or were treated with distilled water only (control). Elicitors were applied on April 15, May 13, and June 10, 2019 (**Figure 1**), using 236-ml spray bottles (Goody Products, Inc., Atlanta, Georgia, United States; ~0.5 ml per pot), which were held ~20 cm above the cranberry canopy; different bottles were used for each treatment. The treatments were initiated as soon as new growth started and continued for 3 months to maximize treatment effects while avoiding phytotoxicity. No phytotoxicity symptoms were detected.

Plant Growth

To quantify growth differences of phytoplasma-infected and uninfected plants across treatments, the size of 10 randomly selected plants from each infection/treatment combination (n = 2 infection levels × 5 treatments × 10 replicates = 100 plants) was determined by measuring the length (in cm) of all vines per plant on June 19, 2019 (**Figure 1**). The length of all the vines was averaged to obtain one value for each plant. Fresh and dry plant mass (in *g*) were also measured on July 09 and July 19, 2019, respectively (**Figure 1**). For this, all vines from the same plants used to assess plant size were cut aboveground and weighed on a Mettler Toledo AE 50 analytical balance (Mettler Toledo, Columbus, Ohio, United States). To attain the dry mass, after determining the fresh mass, plants were oven-dried at 70°C for at least 48 h and reweighed.

Performance Assays

Blunt-Nosed Leafhopper (*Limotettix vaccinii*)

To investigate the survival and growth of the vector *L. vaccinii* on phytoplasma-infected and uninfected cranberry plants treated with the elicitors, a portion (12-cm length) of plants (n = 2 infection levels × 5 treatments × 10 replicates = 100 plants) containing 2–3 vines was enclosed in a plastic dialysis tube cage (2.5-cm diameter × 18-cm length), with the ends of each cage closed by cylindrical sponges (2.5-cm diameter × 2.5-cm length) to prevent the leafhoppers from escaping. Each cage was considered a replicate, and a total of 10 cages were set up for each infection/treatment combination. Four *L. vaccinii* nymphs (second instars) of similar size were placed in each tube cage on May 28, 2019 (n = 40 nymphs per infection/treatment combination; total of 400 nymphs used for entire experiment). This experiment was conducted in a greenhouse under 23 ± 2°C, 70 ± 10% RH, and 15:9 L:D conditions for 20 days. After 20 days (on June 17, 2019, 1 week after the last elicitor application; **Figure 1**), *L. vaccinii* mortality was recorded, the number of live nymphs and adults was counted, and their body mass was measured using a high precision balance (Sartorius BP211D; Göttingen, Germany). These growth and developmental parameters were used as proxy for *L. vaccinii* performance on cranberry plants since a previous study showed that they were influenced by phytoplasma infection (Pradit et al., 2020). The ratio of adults to nymphs was calculated as a measure of stage development, with a higher proportion of adults indicating faster development (reflected in an older population stage structure). We also checked to determine whether leafhoppers acquired the phytoplasma after feeding on infected plants by randomly collecting three leafhoppers from each elicitor treatment of infected plants (n = 15) and conducting a nested PCR assay (Lee et al., 2014); we found that most (93%) of the leafhoppers were infected by the end of the experiment.

TABLE 1 | List of product trade names, active ingredients, rates/concentrations, and manufacturers of the elicitors of plant defenses used in this study.

Product trade name	Active ingredient	Rate[1]	Concentration	Manufacturer
Regalia®	Giant knotweed (*Reynoutria sachalinensis*)	4.75 L/ha	1.0%	Marrone Bio Innovations, Inc., Davis, California, United States
LifeGard® WG Biological Plant Activator	Bacterium *Bacillus mycoides* isolate J	160 g/ha	0.07%	Certis USA L.L.C., Columbia, Maryland, United States
Actigard® 50WG Plant Activator	Acibenzolar-S-methyl (BTH)	53.25 g/ha	0.012%	Syngenta, Basel, Switzerland
Blush® 2X PGR	Prohydrojasmon (propyl-3-oxo-2-pentylcyclo-pentylacetate)	11.5 L/ha	0.99%	Fine Americas, Inc., Walnut Creek, California, United States

[1]*Based on maximum label rates allowed per ha per season.*

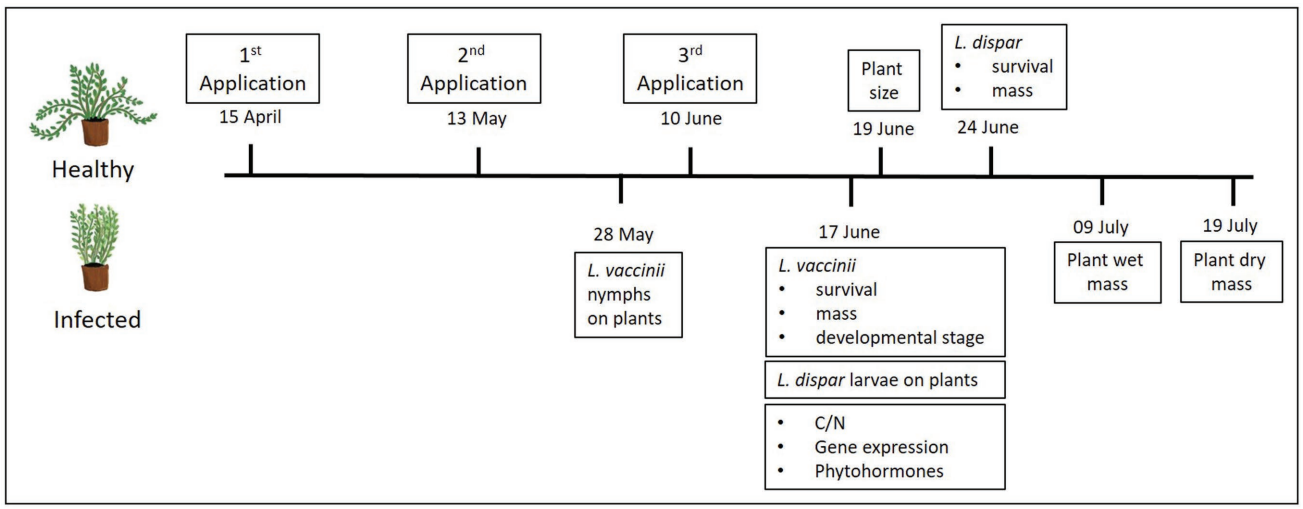

FIGURE 1 | Timeline showing the dates of elicitor applications, insect performance assays, and plant measurements and tissue sampling.

Gypsy Moth (*Lymantria dispar*)

To assess the larval performance of the nonvector *L. dispar* on phytoplasma-infected and uninfected cranberry leaves treated with the elicitors, we conducted a separate experiment in the same greenhouse described above. One hundred plants (two infection levels × 5 treatments × 10 replicates) were individually covered with 6-L (50 × 61 × 19 cm) Super-Aire fiber plant sleeves (A-ROO Company, Strongsville, Ohio, United States). Each plant then received three *L. dispar* neonates (n = 30 neonates per infection/treatment combination; total of 300 neonates used for entire experiment). Each plant was considered a replicate, and each infection/treatment combination was replicated 10 times. The experiment was set up on June 17, 2019 (a week after the last elicitor application), and larval mortality and mass (using a Sartorius BP211D balance) were assessed after 7 days (on June 24, 2019; **Figure 1**); a previous study by Pradit et al. (2019a) showed that phytoplasma infection can positively affect larval mortality and mass of nonvector insects, including *L. dispar*, in cranberries.

Plant Water and Nutrient Analysis

Data from fresh and dry mass were used to calculate water content (g) by subtracting dry plant mass from fresh mass. To determine the interactive effects of phytoplasma infection and elicitor treatments on plant carbon (C) and N, all vines were taken from three randomly selected plants from each infection/treatment combination on June 17, 2019 (**Figure 1**; n = 2 infection levels × 5 treatments × 3 replicates = 30 plants). Vines from each plant were kept in separate paper bags and allowed to dry. The dried samples (1.5 g) were weighed using a Mettler Toledo AE 50 analytical balance and then analyzed for total C and N by combustion with an Elementar Vario Max N/C analyzer (Pella, 1990; Horneck and Miller, 1998) by the Penn State University Agricultural Analytical Service Laboratory.[1] Percent N and C/N ratios are reported based on plant sample dry mass. C and N were used as proxy for changes in host nutritional quality since a previous study showed positive effects of phytoplasma infection on N levels in cranberries (Pradit et al., 2019a).

Gene Expression Analysis

To test whether the four synthetic elicitors of plant defenses reduce expression of a phytoplasma-specific marker gene, leaves

[1]http://agsci.psu.edu/aasl

from vines were taken from three randomly selected elicitor-treated and untreated plants on June 17, 2019 (**Figure 1**; n = 5 treatments × 3 replicates = 15 plants) and stored at −80°C until used for real-time quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from the leaves immediately after harvest *via* previously described methods (Rodriguez-Saona et al., 2013; De Lange et al., 2019; Pradit et al., 2019b). The total RNA extract was treated with Optizyme DNase I (Fisher Scientific, Hampton, New Hampshire, United States) to remove any residual DNA, suspended in 50 µl RNAse-free water, and quantified using a ND-1000 NanoDrop spectrophotometer. The quality of the RNA was assessed by gel electrophoresis; then, cDNA was synthesized using 100 ng of RNA per reaction and the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) according to the manufacturer's (Invitrogen) protocol.

The target gene ("UnkPhyto") was selected based on previous transcriptome data and RT-qPCR data showing that this gene is stable and expressed only in cranberry leaves infected by phytoplasma (Pradit et al., 2019b). The primer used for the target gene is listed in **Table 2**. The expression of actin was used as the endogenous control (Rodriguez-Saona et al., 2013; De Lange et al., 2019; Pradit et al., 2019b). The primers of the target gene and endogenous control were designed using PrimerQuest (Integrated DNA Technologies Inc., Skokie, Illinois, United States).

Real-time quantitative PCR reactions were set up using the Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, California, United States) according to the manufacturer's directions and run on a QuantStudio 5 RT-qPCR System (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 58°C for 1 min, with the melting curve set at 95°C for 15 s, 60°C for 1 min, and 95°C for 1 s. We used 500 ng of cDNA per reaction. There were three biological replicates (individual plants) of each sample, and three technical replicates were run for each biological replicate. The technical replicates for each biological replicate were averaged. The expression level of the target gene relative to the endogenous control gene was calculated and compared among elicitor-treated and untreated phytoplasma-infected plants with the $^{\Delta\Delta}$CT method using the QuantStudio Design & Analysis Software version 1.4.3 (Applied Biosystems).

Phytohormone Analysis

We assessed the interactive effects of phytoplasma infection and elicitor treatment on JA and SA, two key defensive phytohormones (Schmelz et al., 2003). All vines from four plants of each phytoplasma/treatment combination were collected on June 17, 2019 (**Figure 1**; n = 2 infection levels × 5 treatments × 4 replicates = 40 plants) and stored at −20°C before analysis. Phytohormone levels in the leaves were analyzed by liquid chromatography-mass spectrometry (LC-MS) at the Department of Environmental Systems Science at ETH Zürich (Zürich, Switzerland), as described in Pradit et al. (2020). Freeze-dried samples (10–20 mg) were placed in a 2-ml round-bottom Eppendorf tube and frozen in liquid nitrogen. Frozen samples were ground to powder using the Genogrinder and a 100-µl extraction solution (80:20 isopropanol:methanol) supplemented with isotopically labeled standards of the phytohormones (4 ng/µl). The samples were vortexed and then sonicated in a water bath for 30 min. After sonication, samples were centrifuged at 10,000 rpm at 4°C for 15 min, and the supernatant was transferred to a 2-ml Eppendorf tube. The remaining sample was extracted *via* the same process mentioned above two more times, using 40 µl of the extraction solution each time. The combined supernatant (approximately 180 µl) was centrifuged at 13,000 rpm for 30 min to remove any solids. After centrifugation, the supernatant was transferred to a vial for LC-MS analysis. The amount of phytohormone was calculated using the Masshunter Quantitative analysis software (Agilent, Santa Clara, California, United States).

For LC-MS analysis, separation was done with ultra-high-performance liquid chromatographs equipped with a Zorbax SB-C18 column (1.8 µm, 2.1 × 100 mm; Agilent). The analysis of phytohormones was done with Quadrupole Time-of-Flight LC-MS (Agilent) with positive and negative ion modes. In the positive ion mode, water +0.1% formic acid was used as solvent A and acetonitrile +0.1% formic acid was used as solvent B. In the negative ion mode, water +5 mm ammonium formate was used as solvent A and acetonitrile as solvent B. The elution gradient in the positive ion mode was 99.5% A/0.5% B at 1 min, 97.0% A/3.0% B at 5 min, and 1.0% A/99.0% B at 15 min and 17 min, whereas the elution gradient in the negative ion mode was 99.5% A/0.5% B at 1 min, 97.0% A/3.0% B at 5 min, 1.0% A/99.0% B at 15 min, and 0.2% A/99.8% B at 17 min. The flow rate was 0.6 ml/min, and the injection volume was 5 µl. The column temperature was 50°C. The diode-array detector detected UV wavelengths between 190 and 640 nm.

Statistical Analysis

Data analyses for all experiments were performed using Minitab version 18 (Minitab Inc., State College, Pennsylvania, United States).

TABLE 2 | Target and endogenous control genes used for real-time qPCR and primer sequences indicating the presence of a phytoplasma causing cranberry false blossom disease in cranberry leaves.

Target gene	Real-time F primer	Real-time R primer
Phytoplasma gene[1]	GCAAGAACGCTTTCTGAACTAAA	CCTGCCTTATGAGGATACAACTC
Actin[2]	TTCACCACCACGGCTGAAC	AGCCACGTATGCAAGCTTTTC

[1]*Unknown "UnkPhyto" gene based on transcriptome data and RT-qPCR data from Pradit et al. (2019b).*
[2]*Endogenous control gene.*

We tested for the main effects of elicitor treatment, phytoplasma infection, and the elicitor × phytoplasma interaction on herbivore performance traits (*L. vaccinii* and *L. dispar* mass and survival) by using two-way ANOVA; a significant level of $\alpha = 0.05$ was applied. Because each plant was considered a replicate, the mass and survival of *L. vaccinii* and *L. dispar* were each averaged to obtain a single (mean) value per plant before statistical analysis. The same statistical analysis was performed for all plant traits, i.e., size, mass, water and N content, C/N level, and phytohormone (JA and SA) levels. Before performing the ANOVA, data were checked for normality by using the Anderson-Darling test and for homogeneity of variances by using the Levene's tests. Differences in $^{\Delta\Delta}$CT among elicitor treatments and untreated control were analyzed using one-way ANOVA. Nonnormal data were Ln-transformed to achieve normality. Percent data were arcsine square-root-transformed before analysis. After significant ANOVAs were attained, multiple comparisons were performed using Tukey's honestly significant difference tests for means separation.

RESULTS

Plant Growth

Compared to uninfected plants, phytoplasma-infected cranberry plants were 34% lighter (mean mass ± *SE*: infected plants = 1.58 ± 0.11 g; uninfected plants = 2.38 ± 0.15 g; **Table 3**; **Figure 2A**) and 32% smaller (mean size ± *SE*: infected plants = 9.09 ± 0.29 cm; uninfected plants = 13.35 ± 0.47 cm; **Table 3**; **Figure 2B**). There was, however, no effect of elicitor treatment on plant mass or size nor an infection × treatment interaction (**Table 3**; **Figure 2**).

Herbivore Performance

Blunt-Nosed Leafhopper (*Limotettix vaccinii*)

Limotettix vaccinii mass was not affected by phytoplasma infection (**Table 3**) and was only marginally affected by elicitor treatment (**Table 3**); however, the infection and elicitor treatment interaction significantly affected *L. vaccinii* mass (**Table 3**; **Figure 3A**). All elicitors increased *L. vaccinii* mass on healthy plants. Phytoplasma infection also increased leafhopper mass by > 3-fold on untreated control plants, but this positive effect was attenuated in the elicitor-treated plants, indicating possible trade-offs in the plant's response to infection and the elicitors. These differences in mass across treatments likely reflect differences in the proportion of *L. vaccinii* adults present. Phytoplasma infection did not influence the proportion of *L. vaccinii* adults (**Table 3**), but this proportion was significantly affected by elicitor treatment (**Table 3**) and by the infection × treatment interaction (**Table 3**; **Figure 3B**). Phytoplasma infection increased the proportion of adults by 3.4-fold in untreated control plants, indicative of faster development, but not in the Regalia-, LifeGard-, and Actigard-treated plants.

Limotettix vaccinii mortality was 30% lower on phytoplasma-infected plants than on uninfected plants (mean % mortality ± *SE*: infected plants = 23 ± 0.04%; uninfected plants = 33 ± 0.03%; **Table 3**; **Figure 3C**). Elicitor treatment also affected *L. vaccinii* mortality (**Table 3**), with Regalia increasing survival by ~28% compared to the control (**Figure 3C**); however, there was no infection × treatment interaction (**Table 3**).

Gypsy Moth (*Lymantria dispar*)

Phytoplasma infection increased *L. dispar* larval mass by 2-fold (mean mass ± *SE*: infected plants = 6.58 ± 0.4 mg; uninfected plants = 3.44 ± 0.27 *g*; **Table 3**; **Figure 4A**). Elicitor treatment also positively affected *L. dispar* larval mass (**Table 3**), and this effect was not influenced by the interaction between elicitor and infection (**Table 3**; **Figure 4A**). Compared to larvae on untreated control plants, both Regalia and Actigard treatments increased larval mass.

There was no effect of infection, elicitor treatment, or their interaction on *L. dispar* mortality (**Table 3**). About 40% of larvae died across all treatments (**Figure 4B**).

Plant Water and Nutrient Content

Phytoplasma infection, elicitor treatment, and the interaction between them affected water content (**Table 3**). Actigard treatment decreased water content compared to untreated control plants, but this effect was ameliorated in phytoplasma-infected plants (**Figure 5A**). Phytoplasma infection increased water content in LifeGard-treated plants (**Figure 5A**).

Phytoplasma infection increased N concentrations in plants by 10% (mean % *n* ± *SE*: infected plants = 1.033 ± 0.03%; uninfected plants = 0.939 ± 0.04%; **Table 3**; **Figure 5B**). Similarly, elicitor treatment increased N concentrations in plants by ~30% compared to the untreated controls (**Table 3**), and this was independent of phytoplasma infection, i.e., no infection × treatment interaction (**Table 3**; **Figure 5B**). As a result, phytoplasma infection decreased C/N ratios in plants (mean C/N ratio ± *SE*: infected plants = 48.79 ± 1.58; uninfected plants = 54.35 ± 2.48; **Table 3**; **Figure 5C**). Elicitor treatment also decreased C/N levels in plants compared to the untreated controls (**Table 3**), independently of phytoplasma infection (**Table 3**; **Figure 5C**).

Gene Expression

Elicitor treatment had no significant effects on the expression levels of our target gene (UnkPhyto) in phytoplasma-infected cranberry plants (mean $^{\Delta\Delta}$CT ± *SE*: Actigard = 0.13 ± 0.04; LifeGard = 0.34 ± 0.23; Regalia = 0.68 ± 0.21; Blush = 0.42 ± 0.23; Control = 0.42 ± 0.27; $F_{4,10} = 0.88$, $p = 0.511$), indicating that elicitors of plant defenses did not reduce the expression of this phytoplasma-specific gene.

Phytohormone Levels

Phytoplasma infection increased the SA concentrations in plants by ~4 times (**Table 3**; **Figure 6B**) but had no effect on JA levels (**Table 3**; **Figure 6A**). In contrast, elicitor treatment affected JA levels (**Table 3**), but this effect was influenced by phytoplasma infection (significant elicitor × hytoplasma interaction; **Table 3**; **Figure 6A**). Regalia, LifeGard, and Actigard reduced concentrations of JA compared to the Blush and control

TABLE 3 | Results of two-factor ANOVA on the effects of phytoplasma infection and chemical elicitors on plant traits and insect performance.

Variables		Source	*F*	*df*[1]	Value of *p*[2]
Plant growth	Mass	Infection	16.13	1, 110	**<0.001**
		Elicitor	1.1	4, 110	0.361
		Infection × Elicitor	0.3	4, 110	0.875
	Size	Infection	65.19	1, 110	**<0.001**
		Elicitor	0.7	4, 110	0.594
		Infection × Elicitor	0.69	4, 110	0.598
Limotettix vaccinii	Mass	Infection	0.09	1, 86	0.768
		Elicitor	2.24	4, 86	0.071
		Infection × Elicitor	14.67	4, 86	**<0.001**
	Adult:nymph	Infection	0.35	1, 86	0.555
		Elicitor	2.82	4, 86	**0.03**
		Infection × Elicitor	5.08	4, 86	**<0.001**
	Mortality	Infection	4.28	1, 90	**0.042**
		Elicitor	3.09	4, 90	**0.02**
		Infection × Elicitor	0.42	4, 90	0.797
Lymantria dispar	Mass	Infection	40.29	1, 88	**<0.001**
		Elicitor	3.07	4, 88	**0.02**
		Infection × Elicitor	2.18	4, 88	0.078
	Mortality	Infection	1.39	1, 100	0.241
		Elicitor	1.31	4, 100	0.27
		Infection × Elicitor	1.2	4, 100	0.317
Water content		Infection	5.68	1, 107	**0.019**
		Elicitor	5.42	4, 107	**0.001**
		Infection × Elicitor	2.96	4, 107	**0.023**
Nutritional content	Nitrogen	Infection	7.56	1, 20	**0.012**
		Elicitor	8.66	4, 20	**<0.001**
		Infection × Elicitor	1.42	4, 20	0.263
	Carbon:nitrogen	Infection	9.06	1, 20	**0.007**
		Elicitor	10.62	4, 20	**<0.001**
		Infection × Elicitor	1.69	4, 20	0.192
Phytohormones	Salicylic acid	Infection	41.68	1, 30	**<0.001**
		Elicitor	2.56	4, 30	0.059
		Infection × Elicitor	0.55	4, 30	0.703
	Jasmonic acid	Infection	2.13	1, 25	0.157
		Elicitor	225.57	4, 25	**<0.001**
		Infection × Elicitor	2.9	4, 25	**0.042**

[1]*Numerator, denominator (error).*
[2]*Numbers in bold indicate statistically significance (α = 0.05).*

treatments and were not affected by infection; however, infection did increase JA levels in the Blush treatment. Somewhat surprisingly, elicitor treatment had no effect on SA (**Table 3**) concentrations, and there was no elicitor × phytoplasma interaction on SA (**Table 3**; **Figures 6B**).

DISCUSSION

Limited options are currently available for the control of false blossom disease in cranberries, and the only method shown to effectively reduce disease incidence so far targets the disease vector *L. vaccinii*. Early breeding efforts aimed at enhancing plant resistance to false blossom assessed varietal resistance based on susceptibility to *L. vaccinii* feeding; in recent decades, however, the focus of breeding efforts has shifted toward increased fruit yield without regard to resistance (Vorsa and Zalapa, 2020). These changes in breeding priorities came about due, in part, to the development, during the 1940–1950s, of broad-spectrum insecticides (e.g., organophosphates and carbamates) that were effective at controlling *L. vaccinii* populations. However, recent restrictions on the use of these insecticides, including the EPA Food Quality Protection Act (US Environmental Protection Agency, 1996), have prompted the search for alternative management practices. In this study, we attempted to manipulate cranberry resistance against *L. vaccinii*, through the use of elicitors of plant defenses to inhibit the phytoplasma that causes false blossom disease, as an alternative to chemical control.

Our results revealed that phytoplasma infection causes significant stunting of vines, so that infected plants were significantly smaller and lighter than uninfected plants, as has been reported previously (e.g., Chen, 1971). Infection by phytoplasma also made cranberries more susceptible to insect herbivores. Previous studies have reported mixed effects of phytoplasma infection in plants on insect vectors, including negative effects on survival (e.g., Bressan et al., 2005a, 2005b; D'Amelio et al., 2008; Mayer et al., 2011) and reproduction

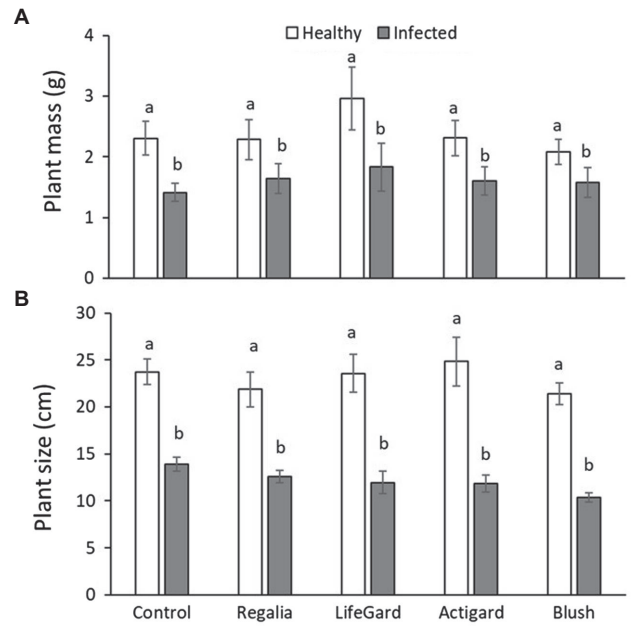

FIGURE 2 | Effects of phytoplasma infection and elicitor treatments on fresh mass **(A)** and size **(B)** of cranberry plants. Data are means ± *SE*. Different letters above bars indicate that means among elicitor treatments differ for uninfected (healthy) and infected plants [Tukey's honestly significant difference (HSD); tests, $p \leq 0.05$]. Control plants did not receive any elicitor (i.e., treated with distilled water only). $n = 10$ replicates per infection/treatment combination.

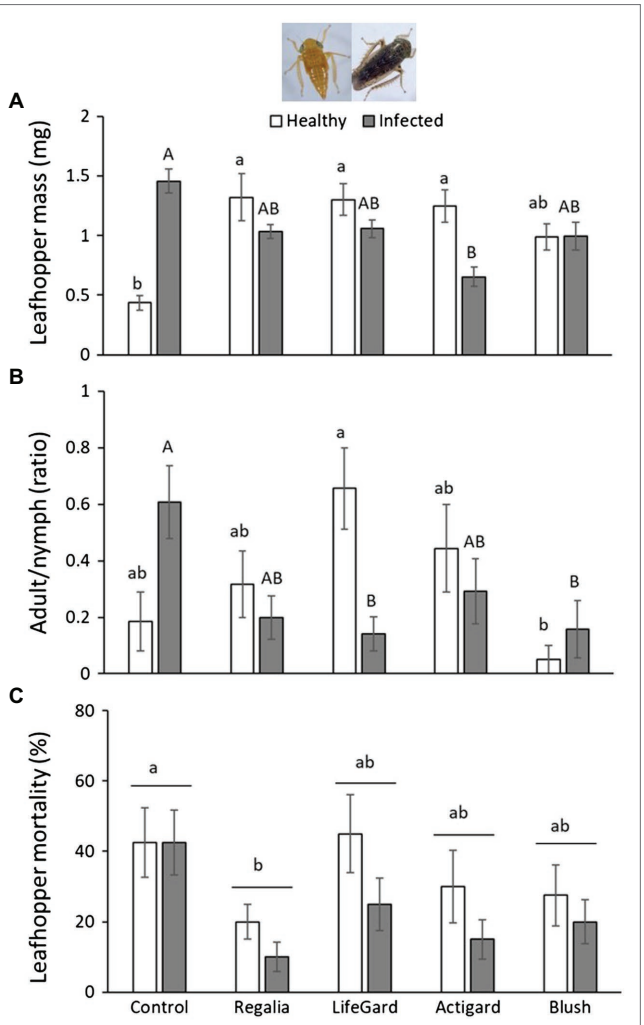

FIGURE 3 | Effects of phytoplasma infection and elicitor treatments on *Limotettix vaccinii* mass **(A)**, ratio of adults vs. nymphs **(B)**, and percent mortality **(C)**. Data are means ± *SE*. Different lowercase letters above bars indicate that means among elicitor treatments differ for uninfected (healthy) plants, and different uppercase letters show treatment differences for infected plants (Tukey's HSD tests, $p \leq 0.05$). Control plants did not receive any elicitor (i.e., treated with distilled water only). $n = 10$ replicates per infection/treatment combination.

(e.g., Bressan et al., 2005a, 2005b; Mayer et al., 2011; Pradit et al., 2020); positive effects on survival (e.g., Purcell, 1988; Beanland et al., 2000; Ebbert and Nault, 2001; Kingdom and Hogenhout, 2007), growth (e.g., Pradit et al., 2020), and development (e.g., Pradit et al., 2020); or no effects (e.g., D'Amelio et al., 2008; Kaul et al., 2009). Our study agrees with findings by Pradit et al. (2019a, 2020) that phytoplasma infection in cranberries increases the masses of the vector *L. vaccinii* and the nonvector *L. dispar*. Clearly, infection by phytoplasma in cranberries enhances the performance of both vector and nonvector herbivores, at least in the short term (i.e., immature growth).

The observed improvement of herbivore performance on infected plants was associated with an increase in N levels, a critical nutrient for insect survival, growth, and development (Mattson, 1980). Correspondingly, infected cranberry plants had lower C/N ratios, an important parameter during insect food selection. Pradit et al. (2019a) reported similar increases in N concentration in phytoplasma-infected cranberries, along with lower concentrations of defensive polyphenolics, i.e., proanthocyanidins. In contrast, Raiesi and Golmohammadi (2020) reported higher phenolics and lower N concentrations in Mexican lime [*Citrus aurantifolia* (Christm.) Swingle] infected by "*Candidatus Phytoplasma aurantifolia*," suggesting that the effects of phytoplasmas on plant nutritional and biochemical composition depend on the specific crop-phytoplasma interaction. In cranberries, the current results together with previous findings by Pradit et al. (2019a, 2020) indicate that phytoplasma infection increases nutrient levels while weakening plant defenses, likely benefitting not only the pathogen (e.g., Mitchell et al., 2003) but also vector and nonvector herbivores. This suppression of the plant defenses could result from the observed increase in SA levels in phytoplasma-infected plants. Previous studies examining the effects of phytoplasma infection on phytohormones have produced mixed results (Dermastia, 2019), with some showing activation of the SA pathway (Ahmad et al., 2013; Prezelj et al., 2016), while others have shown activation of the JA pathway (Musetti et al., 2013; Luge et al., 2014; Paolacci et al., 2017). Contrary to this study, however, previous studies in cranberries found no effect of phytoplasma infection on SA concentrations (Pradit et al., 2020) or on the expression

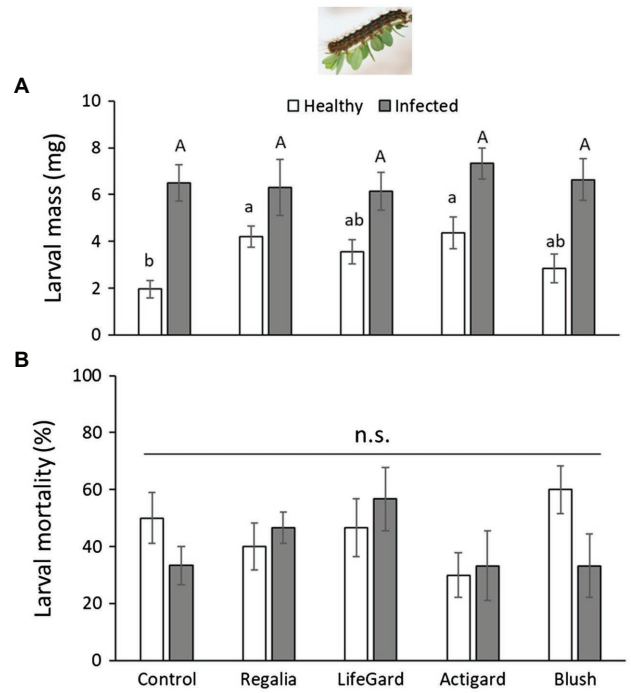

FIGURE 4 | Effects of phytoplasma infection and elicitor treatments on *Lymantria dispar* mass **(A)** and percent mortality **(B)**. Data are means ± *SE*. Different lowercase letters above bars indicate that means among elicitor treatments differ for uninfected (healthy) plants, and different uppercase letters show treatment differences for infected plants (Tukey's HSD tests, $p \leq 0.05$); n.s. = not significantly different (Tukey's HSD tests, $p > 0.05$). Control plants did not receive any elicitor (i.e., treated with distilled water only). $n = 10$ replicates per infection/treatment combination.

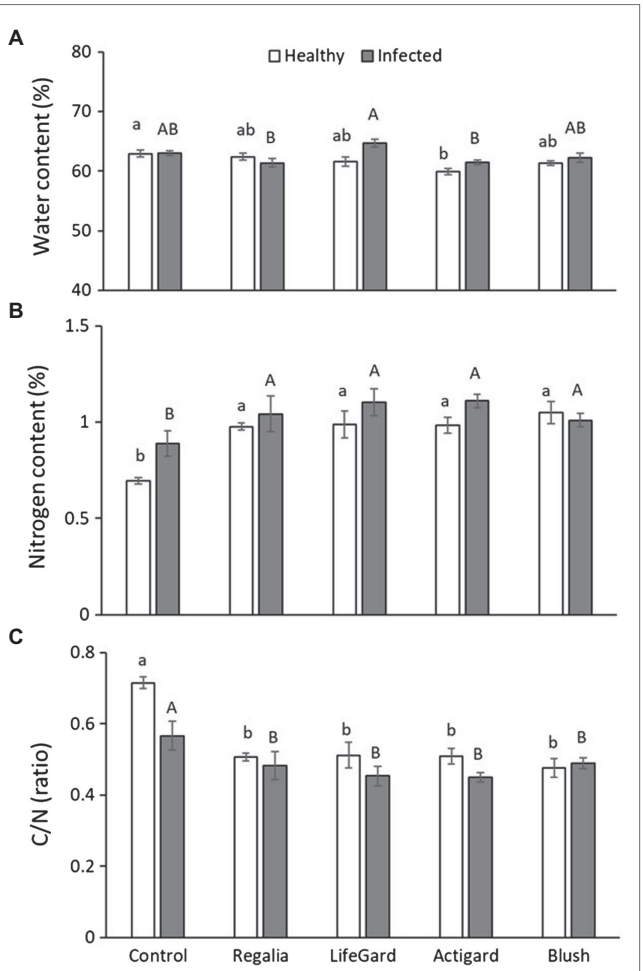

FIGURE 5 | Effects of phytoplasma infection and elicitor treatments on water **(A)** and nitrogen **(B)** content and ratio of carbon vs. nitrogen (C/N) **(C)** in cranberry plants. Data are means ± *SE*. Different lowercase letters above bars indicate that means among elicitor treatments differ for uninfected (healthy) plants, and different uppercase letters show treatment differences for infected plants (Tukey's HSD tests, $p \leq 0.05$). Control plants did not receive any elicitor (i.e., treated with distilled water only). $n = 10$ (water content) and three (nitrogen and C/N) replicates per infection/treatment combination.

of SA-related genes (Pradit et al., 2019b). Although the mechanisms underlying the variable effects of infection observed across study systems remain unclear, it is known that phytohormone levels can change based on plant age and growing conditions (Paolacci et al., 2017). Future studies should therefore examine the influence of abiotic and biotic factors on phytohormone levels in phytoplasma-infected cranberries. We can also not discard the possibility that other phytohormones besides SA and JA, such as ethylene and abscisic acid (which were not measured here), might play a role in interactions among cranberries, phytoplasma, and insect herbivores (Dermastia, 2019). It is worth noting that phytoplasma infection not only exclusively affects the plant nutritional and biochemical composition, but also induces morphological and physiological changes in their respective host plants (Gallinger et al., 2021); thus, other factors not measured here could have also contributed to the observed improved herbivore performance.

The current results reveal that treating cranberries with the salicylate-based elicitors (Regalia, LifeGard, and Actigard) improved leafhopper (*L. vaccinii*) and gypsy moth caterpillar (*L. dispar*) performance, a possible indication of negative cross talk between the SA and JA signaling pathways (Felton and Korth, 2000; Koornneef and Pieterse, 2008). These findings agree with the previous studies showing increased susceptibility of tomato (*Solanum lycopersicum* L.) plants to larvae of the beet armyworm *Spodoptera exigua* (Hübner) and of cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merr.] to larvae of the fall armyworm *Spodoptera frugiperda* (J. E. Smith) when treated exogenously with acibenzolar-S-methyl (BTH), the active ingredient in Actigard (Thaler et al., 1999; Gordy et al., 2015). However, they contradict other reports indicating that Actigard provides protection against leafminers, *Liriomyza* spp. (Inbar et al., 1998) and the Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval; Sobhy et al., 2015) in tomatoes as well as the wheat stem sawfly *Cephus cinctus* Norton in wheat (*Triticum aestivum* L.; Shrestha et al., 2018). Acibenzolar-S-methyl also reduced green peach aphid, *Myzus persicae* (Sulzer), fecundity in tomatoes (Boughton et al., 2006). Furthermore, both

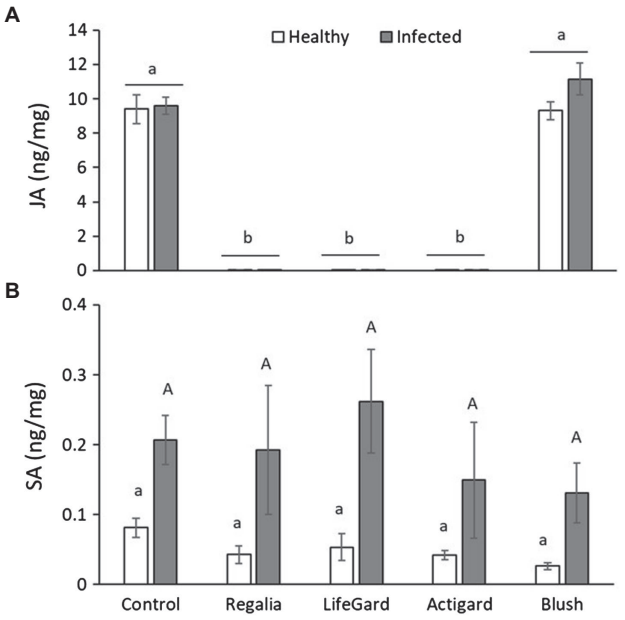

FIGURE 6 | Effects of phytoplasma infection and elicitor treatments on levels of the phytohormones jasmonic acid (JA) **(A)** and salicylic acid (SA) **(B)** in cranberry plants. Data are means ± *SE*. Different lowercase letters above bars indicate that means among elicitor treatments differ for uninfected (healthy) plants, and different uppercase letters show treatment differences for infected plants (Tukey's HSD tests, $p \leq 0.05$). Control plants did not receive any elicitor (i.e., treated with distilled water only). $n = 4$ replicates per infection/treatment combination.

Actigard and Regalia were found to adversely affect the performance of the soybean looper *Chrysodeixis includens* (Walker) in soybean (Chen et al., 2018). In overview, these studies suggest that the effects of SA-based elicitors on herbivores are difficult to predict and likely depend on the specific plant-herbivore interaction (Stout et al., 2002; Gordy et al., 2015). In the current study, these SA-based elicitors attenuated some of the positive effects of phytoplasma infection on *L. vaccinii*, but not to a level that would justify their use as a control tactic. SA mimics have previously been shown to increase the expression of pathogenesis-related protein genes and to reduce the incidence of several bacterial and fungal diseases (e.g., Inbar et al., 1998; Thaler et al., 1999; Obradovic et al., 2005; Thakur and Sohal, 2013). In our study, however, the application of synthetic SA mimics did not appear to reduce the incidence of false blossom disease in cranberries based on the expression levels of a phytoplasma-specific marker gene. Furthermore, in a previous study, Actigard failed to reduce early canker disease when sprayed on foliage throughout the season in citrus [*Citrus paradisi* Macfad. × *Poncitrus trofoliata* (L.) Raf.] orchards (Graham and Leite, 2004). In the current study, elicitor treatment also did not alleviate false blossom symptoms, as both treated and untreated infected cranberry plants had similar stunted growth at the end of our experiment. However, it should also be noted that we applied the elicitors as a potential curative treatment to plants already infected by the phytoplasma, and thus, it is unknown whether elicitor applications to healthy plants might prevent them from acquiring the disease.

To explore whether the effects of elicitor treatment on herbivore performance were mediated by chemical changes in cranberries, we measured the nutritional (N and C/N) and phytohormone (SA and JA) levels in plants. Our results suggest that the improved performance of herbivores, particularly on plants treated with the SA-based elicitors, might be attributable, at least in part, to increased N concentration, lower C/N ratios, lower JA concentrations, or some combination of these factors. The finding that elicitors increased N concentration was unexpected, but could reflect higher N assimilation by treated plants because of increased metabolic (i.e., enzymatic) activity—whether this plant N is readily available to insect herbivores is unknown. Elicitor treatment had no negative effects on plant growth, suggesting the absence of physiological costs associated with defense activation, but elicitor treatment did interact with phytoplasma infection to affect water content, *via* mechanisms that remain unclear. Elicitor treatment also did not cause any detectable phytotoxicity (CRS, personal observation) in cranberries. A surprising result of the current study was that the application of SA-based elicitors did not result in an increase in SA concentrations in either infected or uninfected plants. However, it is plausible that the elicitors tested in this study do not directly trigger SA production but instead act on downstream components of the SA pathway. Indeed, JA levels were attenuated by application of the SA mimics Regalia, LifeGard, and Actigard, another indicator of potential trade-offs between these two defensive pathways together with improved herbivore performance in cranberries (Thaler et al., 1999; Felton and Korth, 2000; Koornneef and Pieterse, 2008). It is also possible that the production of other defensive hormones not measured here was induced by the elicitors (Bektas and Eulgem, 2015).

In summary, the results of the current study indicate that the exogenous application of synthetic defense elicitors had little effect on tripartite interactions between cranberries, a phytoplasma that causes false blossom disease, and insect herbivores (including both a vector and nonvector). In fact, phytoplasma infection and elicitor treatment acted independently to influence *L. dispar* performance, as well as plant traits, such as N, C/N, and SA levels. Only for *L. vaccinii* did elicitors interact with phytoplasma to affect growth and development. Furthermore, contrary to our initial expectations, none of the elicitors of the SA and JA defensive pathways tested here neither increased the plant resistance against the vector blunt-nosed leafhopper (*L. vaccinii*) or the nonvector gypsy moth (*L. dispar*) nor reduced the degree of phytoplasma infection in cranberry plants. On the contrary, treatment with these elicitors made cranberry plants more susceptible to both herbivores. While the mechanisms underlying this increased susceptibility are not entirely clear, both phytoplasma infection and elicitor treatment increased N levels and reduced C/N ratios, while infection also increased SA levels in cranberry plants, which might be expected to suppress JA defenses that are thought to play a more important role in resistance to herbivory.

Taken together, these findings show that the tested elicitors of plant defenses failed to increase resistance to insect herbivores or reduce disease incidence in cranberries; consequently, their use for this purpose is not recommended. Because under field conditions there are cranberry beds with plants already infected by phytoplasma, in this study, we attempted to use elicitors of plant defenses to prevent further infection of young, new growing tissues in infected plants (i.e., curative control); future studies are needed to determine whether these elicitors can be used to "vaccinate" cranberries by protecting uninfected plants from acquiring this disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CR-S, JP, MM, and CM conceived and designed the experiments. VK-R, RH, JP, and GJ-G carried out the experiments and collected the data. CR-S conducted the statistical analyses and wrote the first draft of the manuscript. JP, MM, and CM contributed to interpreting the results. All authors edited the manuscript and gave final approval for publication.

FUNDING

This work was supported financially by the Hatch projects no. NJ08140 and NJ08252 to CR-S and by the New Jersey Blueberry and Cranberry Research Council, Inc., Cape Cod Cranberry Growers Association, and Ocean Spray Cranberries, Inc. to CR-S.

ACKNOWLEDGMENTS

We dedicate this paper to the memory of Giovanna Jimenez-Gonzalez. We thank Vanessa Garzón and Timothy Schwanitz for assistance with plant propagation and greenhouse experiments; Kristy Adams for assistance with molecular analysis; James W. Sims for assistance with phytohormone analysis; and Elvira de Lange, Brittany Dodson, and three reviewers for helpful comments on an earlier draft of the manuscript.

REFERENCES

Ahmad, J. N., Renaudin, J., and Eveillard, S. (2013). Expression of defence genes in stolbur phytoplasma infected tomatoes, and effect of defence stimulators on disease development. *Eur. J. Plant Pathol.* 139, 39–51. doi: 10.1007/s10658-013-0361-x

Averill, A. L., and Sylvia, M. M. (1998). *Cranberry Insects of the Northeast—A Guide to Identification, Biology and Management*. Amherst, MA, USA: University of Massachusetts Extension.

Ballaré, C. L. (2011). Jasmonate-induced defenses: a tale of intelligence, collaborators and rascals. *Trends Plant Sci.* 16, 249–257. doi: 10.1016/j.tplants.2010.12.001

Bargabus, R. L., Zidack, N. K., Sherwood, J. E., and Jacobsen, B. J. (2002). Characterisation of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. *Physiol. Mol. Plant Pathol.* 61, 289–298. doi: 10.1006/pmpp.2003.0443

Beanland, L., Hoy, C. W., Miller, S. A., and Nault, L. R. (2000). Influence of aster yellows phytoplasma on the fitness of aster leafhopper (Homoptera: Cicadellidae). *Ann. Entomol. Soc. Am.* 93, 271–276. doi: 10.1603/0013-8746(2000)093[0271:IOAYPO]2.0.CO;2

Beckwith, C. S., and Hutton, S. B. (1929). Cranberry false blossom and the blunt-nosed leafhopper. *New Jersey Agr. Exp. St. Bull.* 491, 1–16.

Bektas, Y., and Eulgem, T. (2015). Synthetic plant defense elicitors. *Front. Plant Sci.* 5:804. doi: 10.3389/fpls.2014.00804

Bernays, E. A. (1981). Plant tannins and insect herbivores: an appraisal. *Ecol. Entomol.* 6, 353–360. doi: 10.1111/j.1365-2311.1981.tb00625.x

Boughton, A. J., Hoover, K., and Felton, G. W. (2006). Impact of chemical elicitor applications on greenhouse tomato plants and population growth of the green peach aphid. *Entomol. Exp. Appl.* 120, 175–188. doi: 10.1111/j.1570-7458.2006.00443.x

Bressan, A., Clair, D., Sémétey, O., and Boudon-Padieu, É. (2005b). Effect of two strains of Flavescence dorée phytoplasma on the survival and fecundity of the experimental leafhopper vector *Euscelidius variegatus* Kirschbaum. *J. Invert. Pathol.* 89, 144–149. doi: 10.1016/j.jip.2005.03.001

Bressan, A., Girolami, V., and Boudon-Padieu, E. (2005a). Reduced fitness of the leafhopper vector *Scaphoideus titanus* exposed to Flavescence dorée phytoplasma. *Entomol. Exp. Appl.* 115, 283–290. doi: 10.1111/j.1570-7458.2005.00240.x

Chen, T. A. (1971). Mycoplasmalike organisms in sieve tube elements of plants infected with blueberry stunt and cranberry false blossom. *Phytopathology* 61, 233–236. doi: 10.1094/Phyto-61-233

Chen, Y. H., Gols, R., and Benrey, B. (2015). Crop domestication and its impact on naturally selected trophic interactions. *Annu. Rev. Entomol.* 60, 35–58. doi: 10.1146/annurev-ento-010814-020601

Chen, X., Richter, A. R., Stout, M. J., and Davis, J. A. (2018). Effects of induced plant resistance on soybean looper (Lepidoptera: Noctuidae) in soybean. *Arthropod Plant Interact.* 12, 543–551. doi: 10.1007/s11829-018-9601-5

Cole, D. L. (1999). The efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance, against bacterial and fungal diseases of tobacco. *Crop Prot.* 18, 267–273. doi: 10.1016/S0261-2194(99)00026-5

Darolt, J. C., Fassini, C. G., Wulff, N. A., and Di Piero, R. M. (2020). Gene expression of salicylic acid and jasmonic acid pathways and photosynthesis parameters of sweet orange trees in response to acibenzolar-S-methyl. *Trop. Plant Pathol.* 45, 691–700. doi: 10.1007/s40858-020-00373-6

De Lange, E. S., and Rodriguez-Saona, C. (2015). *Blunt-Nosed Leafhopper: A Vector of Cranberry False Blossom Disease (Factsheet 1248)*. New Brunswick, New Jersey, USA: Rutgers Cooperative Extension.

De Lange, E. S., Salamanca, J., Polashock, J., and Rodriguez-Saona, C. (2019). Genotypic variation and phenotypic plasticity in gene expression and emissions of herbivore-induced volatiles, and their potential tritrophic implications, in cranberries. *J. Chem. Ecol.* 45, 298–312. doi: 10.1007/s10886-018-1043-0

Dermastia, M. (2019). Plant hormones in phytoplasma infected plants. *Front. Plant Sci.* 10:477. doi: 10.3389/fpls.2019.00477

Dobroscky, I. D. (1931). Studies on cranberry false blossom disease and its insect vector. *Contribs. Boyce Thompson Inst.* 3, 59–83.

D'Amelio, R., Palermo, S., Marzachì, C., and Bosco, D. (2008). Influence of chrysanthemum yellows phytoplasma on the fitness of two of its leafhopper vectors, *macrosteles quadripunctulatus* and *Euscelidius variegatus*. *Bull. Insectol.* 61, 349–354.

Ebbert, M. A., and Nault, L. R. (2001). Survival in *Dalbulus* leafhopper vectors improves after exposure to maize stunting pathogens. *Entomol. Exp. Appl.* 100, 311–324. doi: 10.1046/j.1570-7458.2001.00878.x

Felton, G. W., and Korth, K. L. (2000). Trade-offs between pathogen and herbivore resistance. *Curr. Opin. Plant Biol.* 3, 309–314. doi: 10.1016/S1369-5266(00)00086-8

Fisk, J. (1980). Effects of HCN, phenolic acids and related compounds in *Sorghum bicolor* on the feeding behaviour of the planthopper *Perregrinus maidis*. *Entomol. Exp. Appl.* 27, 211–222. doi: 10.1111/j.1570-7458.1980.tb02968.x

Gaillard, M. D. P., Glauser, G., Robert, C. A. M., and Turlings, T. C. J. (2018). Fine-tuning the 'plant domestication-reduced defense' hypothesis: specialist vs generalist herbivores. *New Phytol.* 217, 355–366. doi: 10.1111/nph.14757

Gallinger, J., Zikeli, K., Zimmermann, M. R., Görg, L. M., Mithöfer, A., Reichelt, M., et al. (2021). Specialized 16SrX phytoplasmas induce diverse morphological and physiological changes in their respective fruit crops. *PLoS Pathog.* 17:e1009459. doi: 10.1371/journal.ppat.1009459

Gordy, J. W., Leonard, B. R., Blouin, D., Davis, J. A., and Stout, M. J. (2015). Comparative effectiveness of potential elicitors of plant resistance against *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) in four crop plants. *PLoS One* 10:e0136689. doi: 10.1371/journal.pone.0136689

Graham, J. H., and Leite, R. P. Jr. (2004). Lack of control of citrus canker by induced systemic resistance compounds. *Plant Dis.* 88, 745–750. doi: 10.1094/PDIS.2004.88.7.745

Gross, J., Gallinger, J., and Görg, L. M. (2021). Interactions between phloem-restricted bacterial plant pathogens, their vector insects, host plants, and natural enemies, mediated by primary and secondary plant metabolites. *Entomol. Gen.* (in press).

Hogenhout, S. A., Oshima, K., Ammar, E.-D., Kakizawa, S., Kingdom, H. N., and Namba, S. (2008). Phytoplasmas: bacteria that manipulate plants and insects. *Mol. Plant Pathol.* 9, 403–423. doi: 10.1111/j.1364-3703.2008.00472.x

Holopainen, J. K., Heijari, J., Nerg, A.-M., Vuorinen, M., and Kainulainen, P. (2009). Potential for the use of exogenous chemical elicitors in disease and insect pest management of conifer seedling production. *Open Sci. J.* 2, 17–24. doi: 10.2174/1874398600902010017

Horneck, D. A., and Miller, R. O. (1998). "Determination of total nitrogen in plant tissue," in *Handbook of Reference Methods for Plant Analysis*. ed. Y. Kalra (New York: CRC Press), 75–83.

Howe, G. A., and Jander, G. (2008). Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* 59, 41–66. doi: 10.1146/annurev.arplant.59.032607.092825

Inbar, M., Doostdar, H., Sonoda, R. M., Leibee, G. L., and Mayer, R. T. (1998). Elicitors of plant defensive systems reduce insect densities and disease incidence. *J. Chem. Ecol.* 24, 135–149. doi: 10.1023/A:1022397130895

Kaul, C., Seitz, A., Maixner, M., and Johannesen, J. (2009). Infection of bois-noir tuf-type-I stolbur phytoplasma in *Hyalesthes obsoletus* (Hemiptera: Cixiidae) larvae and influence on larval size. *J. Appl. Entomol.* 133, 596–601. doi: 10.1111/j.1439-0418.2009.01406.x

Kingdom, H. N., and Hogenhout, S. A. (2007). Aster yellows phytoplasma witches' broom (AY-WB; '*Candidatus* Phytoplasma asteris') increases survival rates of *macrosteles quadrilineatus* and *Dalbulus maidis* on various plant species. *Bull. Insectol.* 60, 225–226.

Koornneef, A., and Pieterse, C. M. J. (2008). Cross talk in defense signaling. *Plant Physiol.* 146, 839–844. doi: 10.1104/pp.107.112029

Lee, I.-M., Polashock, J., Bottner-Parker, K. D., Bagadia, P. G., Rodriguez-Saona, C., Zhao, Y., et al. (2014). New subgroup 16SrIII-Y phytoplasmas associated with false-blossom diseased cranberry (*Vaccinium macrocarpon*) plants and with known and potential insect vectors in New Jersey. *Eur. J. Plant Pathol.* 139, 399–406. doi: 10.1007/s10658-014-0396-7

Lindig-Cisneros, R., Dirzo, R., and Espinosa-García, F. (2002). Effects of domestication and agronomic selection on phytoalexin antifungal defense in *Phaseolus* beans. *Ecol. Res.* 17, 315–321. doi: 10.1046/j.1440-1703.2002.00491.x

Luge, T., Kube, M., Freiwald, A., Meierhofer, D., Seemüller, E., and Sauer, S. (2014). Transcriptomics assisted proteomic analysis of *Nicotiana occidentalis* infected by *Candidatus* Phytoplasma Mali strain AT. *Proteomics* 14, 1882–1889. doi: 10.1002/pmic.201300551

Mandour, N. S., Kainoh, Y., Ozawa, R., Uefune, M., and Takabayashi, J. (2013). Effects of prohydrojasmon-treated corn plants on attractiveness to parasitoids and the performance of their hosts. *J. Appl. Entomol.* 137, 104–112. doi: 10.1111/j.1439-0418.2012.01721.x

Margaritopoulou, T., Toufexi, E., Kizis, D., Balayiannis, G., Anagnostopoulos, C., Theocharis, A., et al. (2020). *Reynoutria sachalinensis* extract elicits SA-dependent defense responses in courgette genotypes against powdery mildew caused by *Podosphaera xanthii*. *Sci. Rep.* 10:3354. doi: 10.1038/s41598-020-60148-6

Mattson, W. J. (1980). Herbivory in relation to plant nitrogen content. *Ann. Rev. Ecol.* 11, 119–161. doi: 10.1146/annurev.es.11.110180.001003

Mayer, C. J., Vilcinskas, A., and Gross, J. (2011). Chemically mediated multitrophic interactions in a plant-insect vector-phytoplasma system compared with a partially nonvector species. *Agric. For. Entomol.* 13, 25–35. doi: 10.1111/j.1461-9563.2010.00495.x

Mitchell, C. E., Reich, P. B., Tilman, D., and Groth, J. V. (2003). Effects of elevated CO_2, nitrogen deposition, and decreased species diversity on foliar fungal plant disease. *Glob. Chang. Biol.* 9, 438–451. doi: 10.1046/j.1365-2486.2003.00602.x

Musetti, R., Farhan, K., De Marco, F., Polizzotto, R., Paolacci, A., Ciaffi, M., et al. (2013). Differentially-regulated defence genes in *Malus domestica*. *Eur. J. Plant Pathol.* 136, 13–19. doi: 10.1007/s10658-012-0147-6

Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., et al. (2005). Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Dis.* 89, 712–716. doi: 10.1094/PD-89-0712

Paolacci, A. R., Catarcione, G., Ederli, L., Zadra, C., Pasqualini, S., Badiani, M., et al. (2017). Jasmonate-mediated defence responses, unlike salicylate-mediated responses, are involved in the recovery of grapevine from bois noir disease. *BMC Plant Biol.* 18:39. doi: 10.1186/s12870-017-1069-4

Pella, E. (1990). Elemental organic analysis. Part 1. *Am. Lab.* 22, 116–125.

Pickett, J. A., Aradottír, G. I., Birkett, M. A., Bruce, T. J. A., Hooper, A. M., Midega, C. A. O., et al. (2014). Delivering sustainable crop protection systems via the seed: exploiting natural constitutive and inducible defence pathways. *Phil. Trans. R. Soc. B.* 369:20120281. doi: 10.1098/rstb.2012.0281

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Polashock, J. J., Caruso, F. L., Averill, A. L., and Schilder, A. C. (2017). *Compendium of Blueberry, Cranberry, and Lingonberry Diseases and Pests. 2nd Edn*. St. Paul, Minnesota, USA: The American Phytopathological Society.

Pradit, N., Mescher, M. C., De Moraes, C., and Rodriguez-Saona, C. (2020). Phytoplasma infection of cranberry affects development and oviposition, but not host-plant selection, of the insect vector *Limotettix vaccinii*. *J. Chem. Ecol.* 46, 722–734. doi: 10.1007/s10886-019-01137-6

Pradit, N., Mescher, M. C., Wang, Y., Vorsa, N., and Rodriguez-Saona, C. (2019a). Phytoplasma infection of cranberries benefits non-vector phytophagous insects. *Front. Ecol. Evol.* 7:181. doi: 10.3389/fevo.2019.00181

Pradit, N., Rodriguez-Saona, C., Kawash, J., and Polashock, J. (2019b). Phytoplasma infection influences gene expression in American cranberry. *Front. Ecol. Evol.* 7:178. doi: 10.3389/fevo.2019.00178

Prezelj, N., Covington, E., Roitsch, T., Gruden, K., Fragner, L., Weckwerth, W., et al. (2016). Metabolic consequences of infection of grapevine (*Vitis vinifera* L.) cv. "Modra frankinja" with Flavescence dorée phytoplasma. *Front. Plant Sci.* 7:711. doi: 10.3389/fpls.2016.00711

Purcell, A. H. (1988). Increased survival of *Dalbulus maidis*, a specialist on maize, on non-host plants infected with mollicute plant pathogens. *Entomol. Exp. Appl.* 46, 187–196. doi: 10.1111/j.1570-7458.1988.tb01110.x

Raiesi, T., and Golmohammadi, M. (2020). Changes in nutrient concentrations and biochemical characteristics of Mexican lime (*Citrus aurantifolia*) infected by phytoplasma. *J. Gen. Plant Pathol.* 86, 486–493. doi: 10.1007/s10327-020-00944-0

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Rodriguez-Saona, C. R., Polashock, J., and Malo, E. A. (2013). Jasmonate-mediated induced volatiles in the American cranberry, *Vaccinium macrocarpon*: from gene expression to organismal interactions. *Front. Plant Sci.* 4:115. doi: 10.3389/fpls.2013.00115

Rodriguez-Saona, C., Vorsa, N., Singh, A. P., Johnson-Cicalese, J., Szendrei, Z., Mescher, M. C., et al. (2011). Tracing the history of plant traits under domestication in cranberries: potential consequences on anti-herbivore defences. *J. Exp. Bot.* 62, 2633–2644. doi: 10.1093/jxb/erq466

Schmelz, E. A., Alborn, H. T., Engelberth, J., and Tumlinson, J. H. (2003). Nitrogen deficiency increases volicitin-induced volatile emission, jasmonic acid accumulation, and ethylene sensitivity in maize. *Plant Physiol.* 133, 295–306. doi: 10.1104/pp.103.024174

Shrestha, G., Briar, S. S., and Reddy, G. V. P. (2018). Plant defense elicitors: plant fitness versus wheat stem sawfly. *PeerJ* 6:e5892. doi: 10.7717/peerj.5892

Sobhy, I. S., Erb, M., Lou, Y., and Turlings, T. C. J. (2014). The prospect of applying chemical elicitors and plant strengtheners to enhance the biological control of crop pests. *Phil. Trans. R. Soc.* 369:20120283. doi: 10.1098/rstb.2012.0283

Sobhy, I. S., Mandour, N. S., and Sarhan, A. A. (2015). Tomato treatment with chemical inducers reduces the performance of *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Appl. Entomol. Zool.* 50, 175–182. doi: 10.1007/s13355-014-0319-2

Stout, M. J., Zehnder, G. W., and Baur, M. E. (2002). Potential for the use of elicitors of plant resistance in arthropod management programs. *Arch. Insect Biochem. Physiol.* 51, 222–235. doi: 10.1002/arch.10066

Szczepaniec, A., Widney, S. E., Bernal, J. S., and Eubanks, M. D. (2013). Higher expression of induced defenses in teosintes (*Zea* spp.) is correlated with greater resistance to fall armyworm, *Spodoptera frugiperda*. *Entomol. Exp. Appl.* 146, 242–251. doi: 10.1111/eea.12014

Thakur, M., and Sohal, B. S. (2013). Role of elicitors in inducing resistance in plants against pathogen infection: a review. *ISRN Biochem.* 2013:762412. doi: 10.1155/2013/762412

Thaler, J. S., Fidantsef, A. L., Duffey, S. S., and Bostock, R. M. (1999). Trade-offs in plant defense against pathogens and herbivores: a field demonstration of chemical elicitors of induced resistance. *J. Chem. Ecol.* 25, 1597–1609. doi: 10.1023/A:1020840900595

Thaler, J. S., Humphrey, P. T., and Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci.* 17, 260–270. doi: 10.1016/j.tplants.2012.02.010

Thomma, B. P. H., Eggermont, K., Penninckx, I. A. M. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A., et al. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. U. S. A.* 95, 15107–15111. doi: 10.1073/pnas.95.25.15107

Treutter, D. (2006). Significance of flavonoids in plant resistance: a review. *Environ. Chem. Lett.* 4, 147–157. doi: 10.1007/s10311-006-0068-8

U.S. Environmental Protection Agency. (1996). Summary of the Food Quality Protection Act. Public Law 104–170. Available at: https://www.epa.gov/laws-regulations/summary-food-quality-protection-act (Accessed July 28, 2021).

Uefune, M., Ozawa, R., and Takabayashi, J. (2014). Prohydrojasmon treatment of lima bean plants reduces the performance of two-spotted spider mites and induces volatiles. *J. Plant Interact.* 9, 69–73. doi: 10.1080/17429145.2012.763146

Verhage, A., van Wees, S. C. M., and Pieterse, C. M. J. (2010). Plant immunity: it's the hormones talking, but what do they say? *Plant Physiol.* 154, 536–540. doi: 10.1104/pp.110.161570

Vorsa, N., and Zalapa, J. (2020). "Domestication, genetics, and genomics of the American cranberry," in *Plant Breeding Reviews. 1st Edn. Vol. 43*. ed. I. Goldman (Hoboken, New Jersey, USA: John Wiley & Sons, Inc.), 279–315.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., et al. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signal. Behav.* 7, 1306–1320. doi: 10.4161/psb.21663

Weintraub, P. G., and Beanland, L. (2005). Insect vectors of phytoplasmas. *Annu. Rev. Entomol.* 51, 91–111. doi: 10.1146/annurev.ento.51.110104.151039

Yoshida, K., Ogino, A., Yamada, K., and Sonoda, R. (2010). Induction of disease resistance in tea (*Camellia sinensis* L.) by plant activators. *JARQ* 44, 391–398. doi: 10.6090/jarq.44.391

Ziadi, S., Poupard, P., Brisset, M.-N., Paulin, J.-P., and Simoneau, P. (2001). Characterization in apple leaves of two subclasses of PR-10 transcripts inducible by acibenzolar-S-methyl, a functional analogue of salicylic acid. *Physiol. Mol. Plant Pathol.* 59, 33–43. doi: 10.1006/pmpp.2001.0343

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 August 2021
doi: 10.3389/fpls.2021.695701

Effects of Prohydrojasmon on the Number of Infesting Herbivores and Biomass of Field-Grown Japanese Radish Plants

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Prohydrojasmon (PDJ), an analog of jasmonic acid (JA), was found to induce direct and indirect defenses against herbivores in non-infested plants. To test whether PDJ can be used for pest control in crop production, we conducted experiments in pesticide-free Japanese radish fields from October 4 to December 12 in 2015. Twenty-four Japanese radish plants in three plots were treated with a 100 times-diluted commercial formulation (5%) of PDJ (treated plants), and 24 plants in three different plots were treated with water (control plants) until November 29 every week. Throughout the observation period, the number of aphids, leaf-mining fly larvae, vegetable weevils, and thrips was significantly lower on the treated plants than on the control plants. In contrast, the number of lepidopteran larvae was not significantly different between the treated and control plants throughout the study period. Parasitized aphids (mummies) were also observed in both plots. Poisson regression analyses showed that a significantly higher number of mummies was recorded on the treated plants as compared to that on the control plants when the number of aphids increased. This suggested that PDJ application to Japanese radish plants attracted more parasitoid wasps on the treated plants than on the control plants. We also identified eight terpenoids and methyl salicylate as the PDJ-induced plant volatiles in the headspace of the treated plants. Some of these volatiles might be responsible for attracting aphid-parasitoid wasps in the field. However, for other insect pests, we did not find any natural enemies. Interestingly, the genes of the JA and salicylic acid signaling pathways were differentially upregulated in the treated plants. We also observed that the PDJ treatments induced the expression of the genes related to glucosinolate biosynthesis and the subsequent isothiocyanate formation. Additionally, the weights of both the aboveground and belowground parts of the treated plants were significantly lower than those of the respective parts of the control plants. These results indicated that the treatment of Japanese radish plants with a 100 times-diluted commercial formulation of PDJ induced their direct and indirect defenses against several insect pest species to reduce their numbers, and negatively affected their biomass.

Keywords: prohydrojasmon, *Raphanus sativus* L. var. *hortensis* Backer, aphids, parasitoids, thrips, vegetable weevils, leaf-mining fly larvae, lepidopteran larvae

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *15 April 2021*
Accepted: *12 July 2021*
Published: *12 August 2021*

Citation:
Yoshida K, Uefune M, Ozawa R, Abe H, Okemoto Y, Yoneya K and Takabayashi J (2021) Effects of Prohydrojasmon on the Number of Infesting Herbivores and Biomass of Field-Grown Japanese Radish Plants. Front. Plant Sci. 12:695701. doi: 10.3389/fpls.2021.695701

INTRODUCTION

In response to herbivory, plants become more resistant either directly (by decreasing their palatability to herbivores) or indirectly (by increasing the effectiveness of carnivorous natural enemies of herbivores). Jasmonic acid (JA), a plant hormone, is involved in the induction of direct defenses against herbivores in infested plants (Smith et al., 2009), and is also involved in the production of herbivory-induced plant volatiles, which attract the natural enemies of the infesting herbivores (Takabayashi and Dicke, 1996; Arimura et al., 2009; McCormick et al., 2012; Takabayashi and Shiojiri, 2019). Exogenous application of JA to non-infested plants can induce direct defenses, making them less suitable resources for herbivores (Farmer and Ryan, 1992; Thaler et al., 1996; Heijari et al., 2005; Cooper and Rieske, 2008). Furthermore, JA-treated non-infested plants become more attractive to predators and parasitoids than untreated plants (indirect defense induction) (Hopke et al., 1994; Dicke et al., 1999; Gols et al., 1999; Thaler, 1999; Ozawa et al., 2000, 2004, 2008; Lou et al., 2005).

Prohydrojasmon (PDJ) [propyl(1*RS*,2*RS*)-(3-oxo-2-pentylcyclopentyl) acetate], an analog of JA, was first registered as a plant growth regulator, particularly for coloring apples and grapes (Koshiyama et al., 2006). Subsequently, similar to JA, PDJ was found to induce direct and indirect defenses against herbivores in non-infested plants (Mandour et al., 2013; Uefune et al., 2014; Sobhy et al., 2015; Matsuura et al., 2020). A report on PDJ treatment of non-infested maize plants suggests a reduction in the weights and survival rates of the common armyworm, *Mythimna separata* (Walker), larvae and their increased attraction to *Cotesia kariyai* Watanabe, a parasitoid wasp of the herbivore (Mandour et al., 2013). Furthermore, Uefune et al. (2014) reported that two-spotted spider mites (*Tetranychus urticae* Koch) laid significantly fewer eggs on PDJ-treated leaf disks than on control leaf disks. They also showed that PDJ treatment of non-infested lima bean plants induced the production of volatiles, including (*E*)-β-ocimene and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), which attract *Phytoseiulus persimilis* Athias-Henriot, a predatory mite (Dicke et al., 1990). Moreover, under greenhouse conditions, PDJ treatment of tomato plants negatively affects the damage caused by thrips (Matsuura et al., 2020). These laboratory and greenhouse studies indicate that PDJ treatment of crops increases their direct and indirect defenses against several insect pests under open field conditions.

Therefore, in the present study, we investigated whether the treatment of field-grown Japanese radish plants (*Raphanus sativus* L. var. *hortensis* Backer) with commercially formulated PDJ could reduce the number of insect pests throughout the cultivation season and affect the biomass of PDJ-treated Japanese radish plants after harvesting. We also analyzed the volatiles emitted from, and the genes expressed in the treated and control plants. The possible use of PDJ for pest management was discussed.

MATERIALS AND METHODS

Prohydrojasmon

We used a 100 times-diluted commercial formulation with 5% PDJ (Jasmonate-Ekizai®, Meiji Seika Pharma, Tokyo, Japan) for the experiments. This concentration was determined based on the results of our previous studies, wherein the induction of direct and indirect defense responses was detected at this concentration in corn (Mandour et al., 2013) and lima bean plants (Uefune et al., 2014). As a plant growth regulator and for controlling thrips, 1000- to 2,000-fold dilution and 500-fold dilution are suggested by the company, respectively.

Experiments in a Common Garden

We used an open common garden (ca. 3 m × 6 m) located in Gifu City, Gifu Prefecture, Japan for the field experiments in 2015. The common garden was divided into 12 plots (140 cm × 60 cm). We sowed three seeds in each spot (8 spots in two lines per plot: **Supplementary Figure 1**) on September 27. Seedlings were recognized a week after seeding (that is, October 4), and then the seedlings in each spot were reduced to one (i.e., eight seedling pre plot). Three each of treatment (PDJ-treated) and control (water-treated) plots were randomly chosen using the "RAND" function in Microsoft Excel (**Supplementary Figure 1**). The observation was performed on October 4, 11, 18, and 25; November 1, 15, 22, and 29; December 6, 13. On October 4, 11, 18, and 25; November 1, 11, 22, and 29, we sprayed the PDJ solution. The 24 Japanese radish plants in the three treatment plots were sprayed with 3 mL of the 100 times-diluted commercial formulation (5%) of PDJ (treated plants), and the 24 plants in the control plots were sprayed with water (control plants) using a hand sprayer. In December, we did not spray PDJ (the land owner's request). The plants were harvested on December 20. The weather conditions in the experimental period are shown in **Supplementary Table 1**.

We counted the number of arthropods on the plants, including insect pests and their carnivorous natural enemies, in each plot from October 4 to December 13. The insect pests observed in the plots were aphids, thrips, vegetable weevils (*Listroderes obliquus* Klug), leaf-mining fly larvae, and lepidopteran larvae. The major lepidopteran species were the cabbage butterfly (*Pieris rapae* L.) that is a crucifer specialist, and few species of the subfamilies Hadeninae and Plusiinae those are not crucifer specialists. We also observed aphids parasitized by wasps (mummies). However, the species of aphids, aphid parasitoid wasps, leaf- mining flies, and thrips were not identified.

During the first five observation weeks, we observed herbivorous insects on all eight plants in each plot. We randomly chose six and four plants per plot for the sixth-to-eighth and the ninth weeks, respectively, to observe the herbivorous insects.

After the harvest on December 20, we separated the aboveground and belowground parts of all the harvested plants from the treated and control plots, and weighed the parts to assess the effects of PDJ treatment on the growth of the plants.

Analyses of Headspace Volatiles of Japanese Radish Plants

To test whether PDJ treatment resulted in the induction of volatiles in Japanese radish plants, we cultivated the plants and collected the headspace volatiles in the laboratory. Between 24 and 27 days after seeding, the PDJ- and water-sprayed plants were placed in a growth chamber at 25 ± 1°C, with an RH of 60 ± 22%, and a 16L:8D light regimen for 24 h. Headspace volatiles from 25- to 28-day-old PDJ-treated and control Japanese radish plants were collected using a Twister (a 10-mm long magnetic stir bar coated with a 0.5-mm thick film of polydimethylsiloxane) (Gerstel Twister®, Gerstel GmbH and Co., KG, Germany). A potted plant and 0.1 μg of *n*-tridecane infiltrated into a piece of filter paper (1 cm^2) were placed in a 2 L glass flask as an internal standard. Two stir bars were then placed inside the glass flask using magnetic rods for 2 h, and volatile collection was replicated five times.

Headspace volatiles collected using the stir bars were analyzed by gas chromatography-mass spectrometry (GC-MS; GC: Agilent 6890; MS: Agilent 5973) with an HP-5MS capillary column (Agilent Technologies Inc., United States) equipped with a thermo-desorption system, cooled injection, and cold trap (Gerstel GmbH and Co., KG, Germany). The GC oven temperature was programmed to increase from 40°C (9 min-hold) to 280°C at 10°C/min. The compounds were tentatively identified using the Wiley database and their mass-spectra and retention times were then verified with those of authentic compounds, except for 4-methylthio-3-butenyl isothiocyanate, α-copaene, germacrene D, and caryophyllene oxide. (*Z*)-3-Hexen-1-yl acetate and (*E*)-β-caryophyllene were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Myrcene, menthol, and (*E,E*)-α-farnesene were purchased from FUJIFILM Wako Chemical Co., Ltd. (Osaka, Japan); α-Humulene was purchased from Sigma-Aldrich LLC. (St. Louis, MO, United States). DMNT was synthesized in our laboratory. The ion intensity of each peak was normalized by dividing the ion intensity of each peak by that of the internal standard (*n*-tridecane) and plant weights (g). The normalized data were referred to as "relative peak areas."

qRT-PCR Analyses

To test whether PDJ-treatment resulted in the induction of defense-related genes in Japanese radish plants, we cultivated the plants and conducted qRT-PCR analyses in the laboratory. Total RNA was extracted from Japanese radish leaves (200 mg FW) using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), and the DNA was degraded using an AccuRT Genomic DNA Removal kit (Applied Biological Materials, Richmond, BC, Canada). cDNA was then synthesized from the total RNA (4 μg) using the Thermoscript RT-PCR kit (Invitrogen, Carlsbad, CA, United States) with oligo$(dT)_{20}$ as a primer, according to the manufacturer's instructions. Subsequently, qRT-PCR was performed using Power SYBR Green Master mix (Thermo Fisher Scientific, Waltham, MA, United States) on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, United States). The primers used are shown in the **Supplementary Table 2**.

Statistical Analyses

We analyzed the effects of the PDJ treatment, observation week, and their interaction on the number of aphids, mummies, thrips, and vegetable weevils and the leaf-mining fly larvae, *P. rapae*, and species of the subfamilies Hadeninae and Plusiinae on the radish plants. For the analyses, we used generalized linear mixed models (GLMMs) with Poisson distribution using the "glmer" function in the "lme4" package version 1.1-21 (Bates et al., 2015) in R version 3.3.5 (R Core Team, 2019). The identification of plants was a random effect in all the models, and the significant values from the GLMMs were calculated by the type II Wald chi-square tests using the "Anova" function in the "car" package version 3.0.2 (Fox and Weisberg, 2011) in R.

When the interaction significantly affected the number of insects, we analyzed the effect of treatment on the number in each observation week using a GLMM with Poisson distribution using the "glmer" function in the "lme4" package in R. Additionally, the observation weeks with no target insect in any of the plots were excluded from the analysis. The plots were included as random effects in all the models, and the significant values from the GLMMs were calculated by the likelihood ratio tests using the "anova" function in R. In the event of convergence errors, the models were fitted using the "bobyqa" optimizer in R.

The effect of the treatment on the weights of the aboveground and belowground parts of the plants was analyzed using a GLMM with Gaussian distribution using the "lmer" function in the "lme4" package in R. The plots were included as random effects in all the models, and the significant values from the GLMMs were calculated using the likelihood ratio tests and the "anova" function in R.

The emitted volatiles and the expression of genes in the radish plants were analyzed using *t*-tests in JMP version 14.2.0 (SAS Institute, 2018). The data were Box-Cox transformed in JMP before the analyses. When a dataset had 0 values, 1 was add to all values in the dataset before Box-Cox transformation.

To clarify whether the structure of insect community and the blend of volatile compounds differed between treated plants and control plants in October, November and December, we conducted principal coordinates analysis (PCoA) and a permutational multivariate analysis of variance (PERMANOVA; number of permutations performed = 9999) based on the Bray-Curtis dissimilarities of the number of insects or the relative peak area of volatile compounds of the Japanese radish plants. PCoA and PERMANOVA were calculated using the functions "capscale" and "adonis" in the "vegan" package (Oksanen et al., 2020) in R. Before PERMANOVA was conducted, we used the "betadisper" function of PERMDISP to evaluate whether the dispersion of the insect community structure and volatile compound composition of treated plants differed significantly from those of control plants. Only when no significant difference in the dispersion is observed does a significant difference determined by the PERMANOVA indicate a difference in the insect community structure and the volatile compound composition. We also identified major insects and

volatile compounds that are responsible for the differences between treatments via SIMPER analysis using the function "simper." Before PCoA, PERMANOVA, and PERMDISP were conducted, the number of aphids and mummies was square root transformed. The data on the radish plants with no insects were removed from the analysis using PCoA and PERMANOVA. For graphics, we used the "ggplot2" package (Wickham, 2016).

RESULTS

Herbivorous Arthropods Found on Japanese Radish Plants

Aphids were observed on both the treated and control plants from October 18 to December 13, with a gradual increase in their number during the observation period (**Figure 1A**). The effects of treatment, observation week, and their interaction (treatment × week) on the number of aphids were significant (treatment: $P = 0.0002$, week: $P < 0.0001$, and treatment × week: $P < 0.0001$) (**Figure 1A**). Therefore, we compared the number of aphids in the treatment and control plots on each observation week using GLMM. Except for October 18 and November 1, the incidence of aphids in the treatment plots was significantly lower than that in the control plots.

Mummies (aphids parasitized by wasps) were also observed on both the treated and control plants from November 15 to December 13, with a gradual increase in their number (**Figure 1B**). The effects of the treatment and the observation week were significant (treatment: $P < 0.0001$ and week: $P < 0.0001$), while the effect of their interaction was not significant ($P = 0.45$) **(Figure 1B)**. These results indicated that the number of wasps was always lower in the treatment plots than in the control plots.

We then analyzed the relationship between the number of aphids and that of mummies in the treatment and control plots using Poisson regression analyses (**Figure 1C**). The effects of the treatment, number of aphids, and the interaction between treatment and the number of aphids on the number of mummies were significantly different (treatment: $P < 0.0001$, number of aphids: $P < 0.0001$, and treatment × number of aphids: $P < 0.0001$).

Thrips, leaf-mining fly larvae, and vegetable weevils gradually increased on both the treated and control plants from October 18 (October 25 for vegetable weevils) to December 13 (**Figures 2A–C**). The effects of the treatment ($P = 0.0029$ for thrips, $P < 0.0001$ for leaf-mining fly larvae, and $P = 0.0064$ for vegetable weevil) and the observation week ($P < 0.0001$ for thrips, $P < 0.0001$ for leaf-mining fly larvae, and $P < 0.0001$ for vegetable weevil) on their number were significant, while that of their interaction was not ($P = 0.11$, $P = 0.86$, and $P = 0.22$ for thrips, leaf-mining fly larvae, and vegetable weevil, respectively), indicating that their incidences were always significantly lower in the treatment plots than those in the control plots (**Figures 2A–C**).

The incidences of the numbers of Lepidopteran larvae of the subfamilies Hadeninae and Plusiinae were quite low (**Supplementary Figure 2**). The larvae of the cabbage butterfly and species of the subfamily Hadeninae were observed in both the treatment and control plots from October 18 to December 13 (**Figure 2D** and **Supplementary Figure 2A**). The effects of treatment ($P = 0.098$ for cabbage butterfly larvae and $P = 0.63$ for Hadeninae larvae) and the interaction between the treatment and the observation week ($P = 0.60$ for cabbage butterfly larvae and $P = 0.87$ for Hadeninae larvae) on the number of larvae were not significant, while that of the observation week was significant ($P < 0.0001$ for the larvae of both the cabbage butterfly and Hadeninae species), indicating that their numbers were not significantly different between the treatment plots and the control plots, but fluctuated significantly during the observation period.

Larvae of the subfamily Plusiinae were observed in the treatment and the control plots from October 4 to December 13 (**Supplementary Figure 2B**). The number of larvae did not differ significantly between the treatment, the observation week, and the treatment × week interaction (treatment: $P = 0.95$, week: $P = 0.16$, and treatment × week: $P = 0.26$), indicating that the number of larvae was not significantly different between the treatment and control plots without significant fluctuations in their numbers during the observation period.

To detect compositional differences, we applied a PCoA to the insect communities on the plants treated with water and those treated with PDJ. The first two axes (MDS1 and MDS2) explained the difference (29.5% and 24.8% in October, 33.7% and 13.4% in November, and 42.4% and 12.5% in December) in composition of the insect community between plants. We found that in November and December they were significantly different (PERMDISP: November, $P = 0.72$; December, $P = 0.27$, PERMANOVA: November, $P = 0.0001$; December, $P = 0.0001$, **Figures 3B,C**) but in October they were not (PERMDISP: $P = 0.61$, PERMANOVA: $P = 0.12$, **Figure 3A**). The insect species that significantly contributed to the difference between the two groups were aphids (40.1%, $P = 0.0002$), vegetable weevils (10.8%, $P = 0.0002$), leaf mining flies (9.8%, $P = 0.0002$), and thrips (8.1%, $P = 0.0002$) in November and aphids (52.7%, $P = 0.0001$), vegetable weevils (15.9%, $P = 0.0009$), mummies (8.3%, $P = 0.0016$), and cabbage butterflies (4.2%, $P = 0.028$) in December.

Biomass of the Harvested Japanese Radish Plants

The weights of both the aboveground and belowground parts of the radish plants treated with PDJ were significantly lower than those of the control plants (aboveground parts: $P = 0.0032$; belowground parts: $P = 0.027$) (**Figure 4**).

Analysis of the Headspace Volatiles of Japanese Radish Plants

Thirteen and 12 compounds were detected in the headspaces of the treated and control plants, respectively (**Table 1**). To detect compositional differences, we applied a PCoA. The first two axes (MDS1 and MDS2) explained 90.3% and 5.3% of the difference in volatile composition between the plants. The blend of volatiles from the control and treated plants were grouped in different areas with significant difference

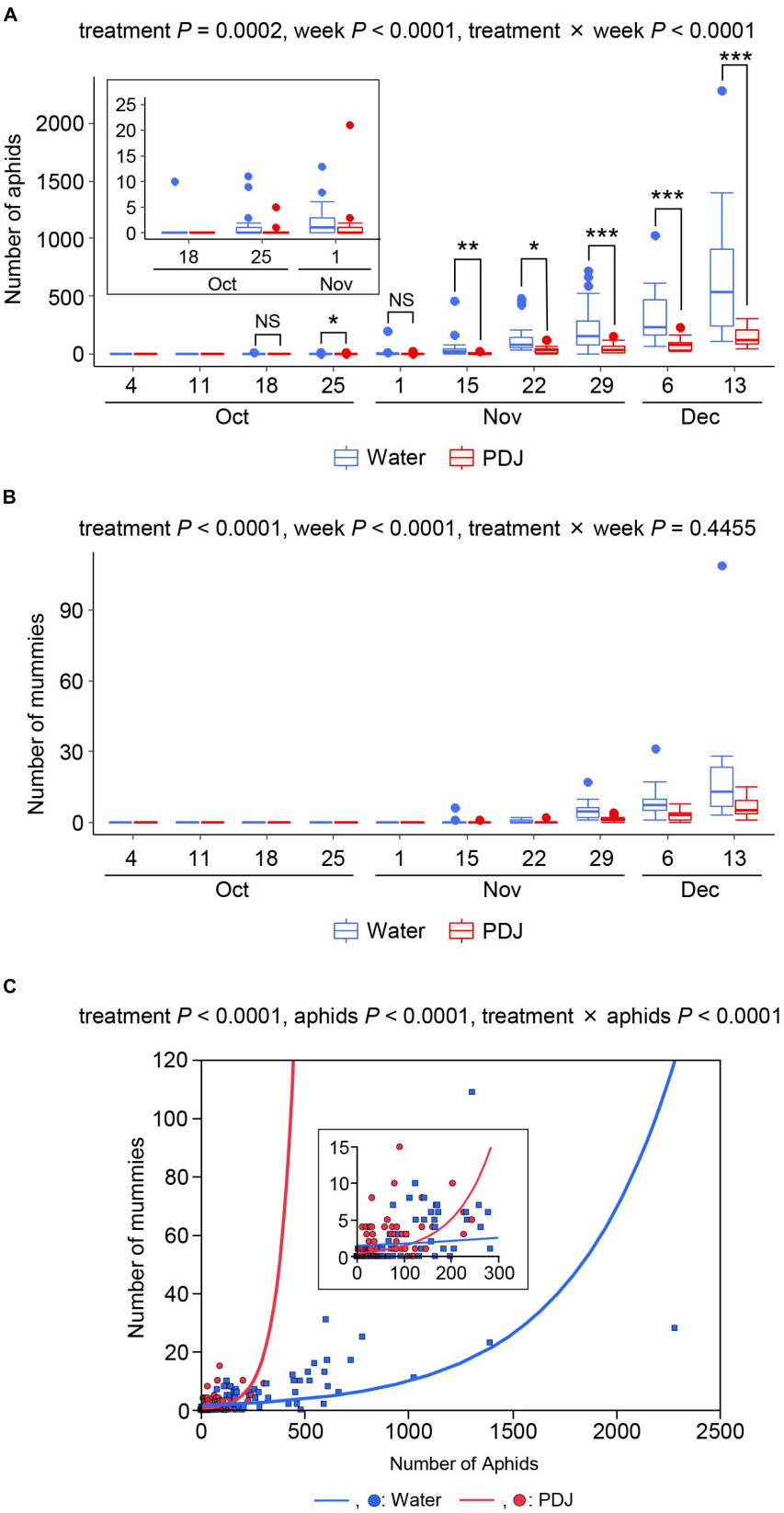

FIGURE 1 | Occurrences of aphids **(A)** and mummies **(B)** on Japanese radish plants in the field during the observation period. **(C)** Poisson regression analysis of the relationship between the numbers of mummies and that of the aphids. NS: $0.05 < P$, $^{*}0.01 < P < 0.05$, $^{**}0.001 < P < 0.01$, and $^{***}P < 0.001$ (GLMM).

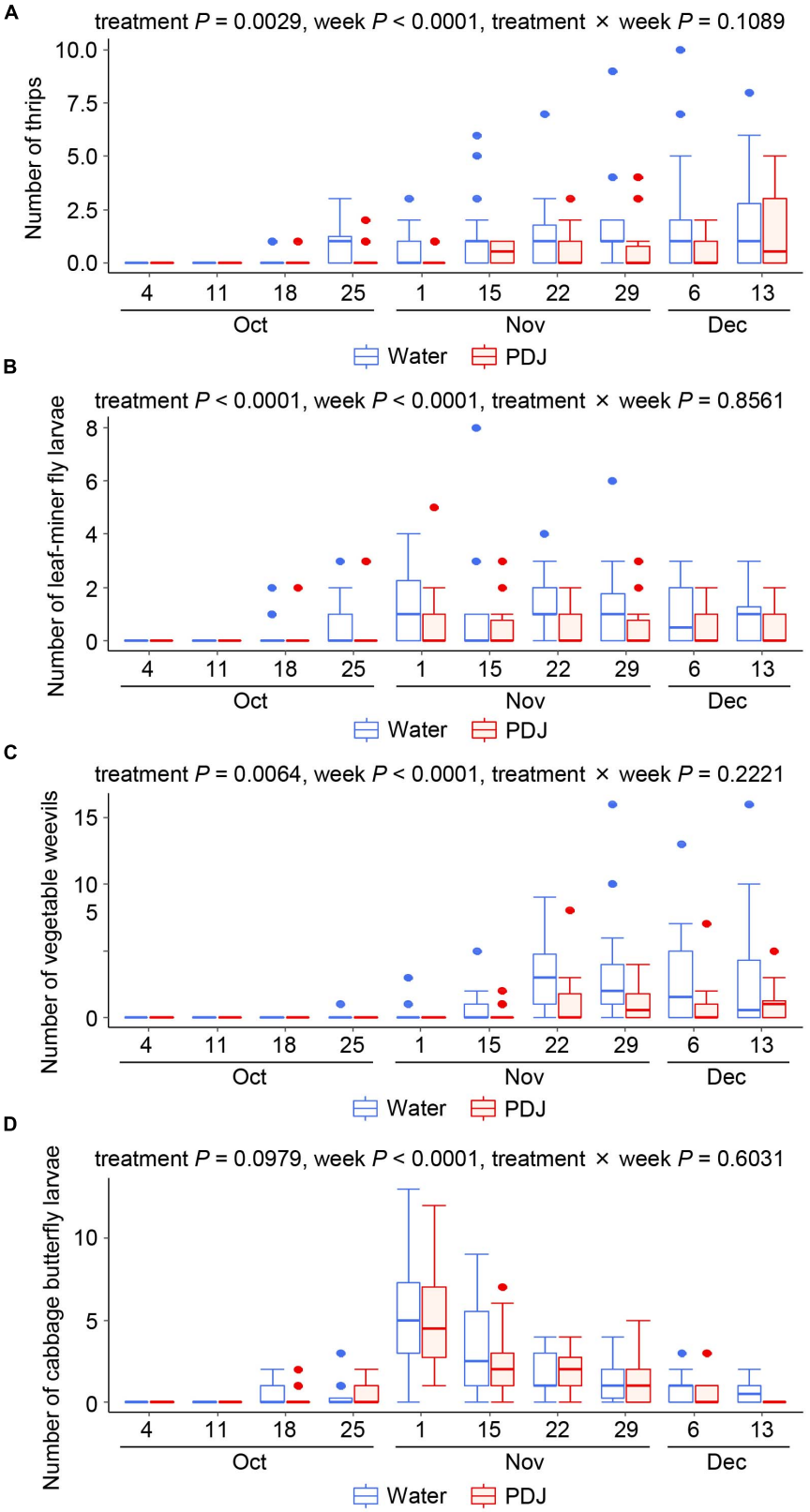

FIGURE 2 | Occurrences of thrips **(A)**, leaf-mining fly larvae **(B)**, vegetable weevils **(C)**, and cabbage butterfly larvae **(D)** on Japanese radish plants in the field during the observation period (GLMM).

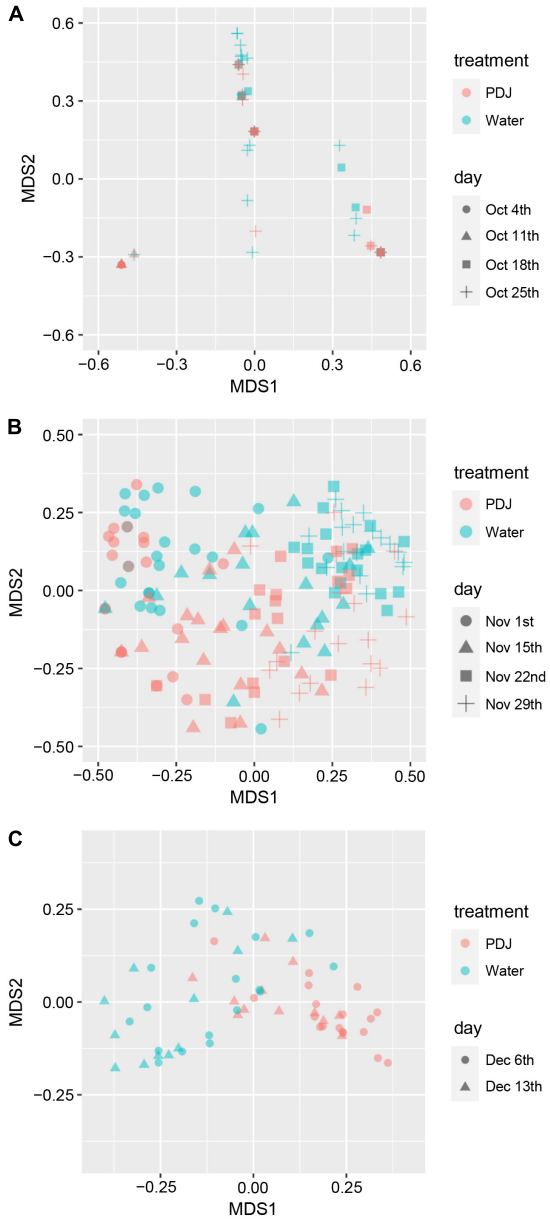

FIGURE 3 | Multidimensional scaling plot (for the first two axes) using a principal coordinates analysis (PCoA) of a Bray–Curtis distance matrix on the data of insect community composition on plants treated with water and those treated with prohydrojasmon (PDJ) in October **(A)**, November **(B)**, and December **(C)**.

(PERMDISP: $P = 0.1$, PERMANOVA: $P = 0.01$, **Supplementary Figure 3**). The compounds that significantly contributed to the difference between the two groups were (*E*)-β-caryophyllene (72.5%, $P = 0.01$), DMNT (17.3%, $P = 0.0091$), (*E,E*)-α-farnesene (3.3%, $P = 0.0091$), α-humulene (3.0%, $P = 0.0091$), caryophyllene oxide (1.1%, $P = 0.0091$), α-copaene (0.8%, $P = 0.0091$), methyl salicylate (0.4%, $P = 0.022$), and germacrene D (0.2%, $P = 0.047$). The amounts of these compounds detected in the headspace of the treated plants were significantly higher than those of the control plants ($P < 0.001$, **Table 1**). The amounts of (*Z*)-3-hexen-1-yl acetate, 4-methylthio-3-butenyl isothiocyanate, menthol, and an unknown terpenoid were not significantly different between the PDJ-treated and control plants (**Table 1**).

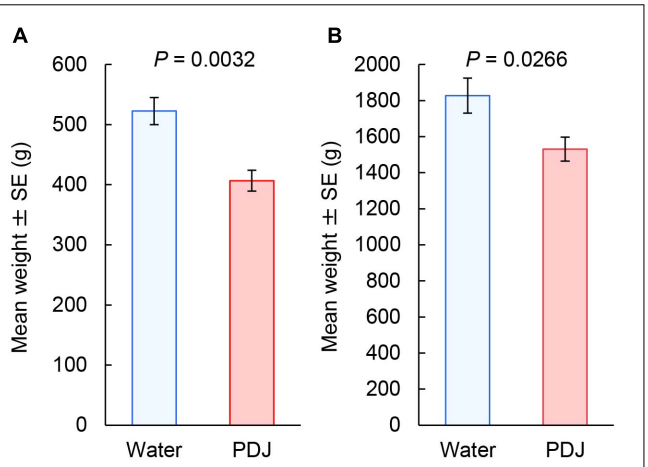

FIGURE 4 | Weights (g) of both the aboveground **(A)** and belowground **(B)** parts of Japanese radish plants treated with PDJ and water (GLMM).

qRT-PCR Analyses

We studied the effects of PDJ treatment on the expression levels of *RsLOX*, and *RsMYC2*, which are regulated by the JA signaling pathway, and *RsPAL*, *RsPR1*, *RsPR2, and RsPR3* which are regulated by the salicylic acid (SA) signaling pathway. The expression of *RsLOX*, *RsMYC2*, *RsPR1* and *RsPR3* was significantly upregulated and that of *RsPR2* was marginally significantly upregulated when the plants had been treated

TABLE 1 | The volatiles emitted from PDJ-treated Japanese radish plants and those emitted from water-treated conspecific plants.

	Relative peak area ($\times 10^{-5}$)	
Compounds	**PDJ**	**Water control**
(*Z*)-3-Hexen-1-yl acetate	144.7 ± 36.2	133.8 ± 38.0
Methyl salicylate	108.4 ± 19.1	9.1 ± 2.3 ***
4-Methylthio-3-butenyl isothiocyanate	45.2 ± 40.2	34.0 ± 19.9
Myrcene	41.9 ± 2.2	14.0 ± 2.1 ***
(*E*)-4,8-dimethyl-1,3,7-nonatriene	4387.0 ± 897.0	15.9 ± 2.5 ***
Menthol	107.9 ± 5.9	95.4 ± 4.6
α-Copaene	203.8 ± 9.4	10.5 ± 1.9 ***
Germacrene D	52.4 ± 3.2	6.1 ± 1.3 ***
(*E*)-β-Caryophyllene	17777.2 ± 1158.9	118.8 ± 24.9 ***
α-Humulene	731.6 ± 60.5	0.0 ± 0.0 ***
(*E,E*)-α-Farnesene	789.7 ± 67.1	5.4 ± 1.0 ***
Caryophyllene oxide	272.8 ± 25.0	12.2 ± 3.4 ***
Unknown terpenoid	219.7 ± 32.9	370.5 ± 116.7

Data are represented by mean ± standard error.
Asterisks indicate that there is a significant difference in quantity between treatments.
****P < 0.001 (t-test).*

with PDJ (*RsLOX*: $P < 0.0001$; *RsMYC2*: $P < 0.0001$; *RsPR1*: $P = 0.0043$; *RsPR2*: $P = 0.084$; *RsPR3*: $P = 0.0005$). In contrast, the expression level of *RsPAL* was not significantly different after PDJ treatment ($P = 0.69$) (**Figure 5**).

We also studied the effects of PDJ treatment on the expression levels of *Rsmyb28* and *Rsmyb29*, the orthologs of *Atmyb28* and *Atmyb29* which regulate aliphatic glucosinolate biosynthesis, *Rscyp79b2* and *Rscyp79b3*, orthologs of *Atcyp79b2* and *Atcyp79b3* which regulate indole glucosinolate biosynthesis, and *RsTGG1* and *RsTGG2*, orthologs of myrosinase genes, *AtTGG1* and *AtTGG2*. The expression of *Rscyp79b2*, *Rscyp79b3*, *RsTGG1a,* and *RsTGG1b* was significantly upregulated when the plants had been treated with PDJ (*Rscyp79b2*: $P < 0.0001$; *Rscyp79b3*: $P < 0.0001$; *RsTGG1a*: $P = 0.038$ and *RsTGG1b*: $P < 0.0001$). In contrast, the expression levels of *Rsmyb28* and *Rsmyb29* were not significantly different after PDJ treatment (*Rsmyb28*: $P = 0.29$ and *Rsmyb29*: $P = 0.13$) (**Figure 5**).

DISCUSSION

Previous laboratory and greenhouse studies have shown that crops treated with PDJ become more resistant against herbivores directly or indirectly (see section "Introduction") (Mandour et al., 2013; Uefune et al., 2014; Sobhy et al., 2015; Matsuura et al., 2020). These studies indicated that PDJ has the potential to increase the defense responses in non-infested plants of different classes (monocotyledons and dicotyledons) against herbivores with different feeding guilds (chewing pests: common armyworms; sucking pests: two-spotted spider mites). In the present study, we showed that PDJ treatment was effective in reducing the number of aphids, leaf-mining fly larvae, vegetable weevils, and thrips, without affecting the number of lepidopteran larvae, including those of *P. rapae* and few species of the subfamilies Hadeninae and Plusiinae, under open common garden conditions. Furthermore, PCoA analyses revealed that the community composition on the treated plants was significantly different from that on control plants in November and December, but not in October. In both November and December, aphids were the most important factor that explained the difference in the community composition. The other herbivores that influenced the composition were different between these 2 months: leaf miner fly larvae and thrips in November, and vegetable weevils, mummies, and cabbage butterflies in December.

The number of parasitized aphids (mummies) was significantly lower on the treated plants than on the control plants, which could be explained by the reduction in the number of host aphids. Poisson regression analyses revealed that significantly higher number of mummies were recorded on the treated plants when the number of aphids increased, suggesting that the treated plants attracted a higher number of aphid parasitoids than the control plants. PCoA analyses showed that the composition of volatiles from the treated plants and that from the control plants was significantly different, because the treatment also resulted in the production of PDJ-induced volatile compounds: one monoterpene (myrcene), six sesquiterpenes, one homoterpen (DMNT), and methyl salicylate. Some PDJ-induced volatiles from the treated plants probably attracted the parasitoid wasps (induced indirect defense). Among the induced volatiles, (*E*)-β-caryophyllene has been reported to attract *Aphidius ervi* Haliday, a parasitoid of several aphid species, including Brassica aphids (Sasso et al., 2007). However, *A. ervi* did not occur in the experimental area. Additionally, corn plants treated with PDJ have been reported to attract parasitic wasps (see section "Introduction") (Mandour et al., 2013). The PDJ-induced compounds found in this study were not recorded in the headspace of Brussels sprouts sprayed with 1 mM JA solution except (*Z*)-3-hexen-1-yl acetate, a common green leaf volatile compound (Bruinsma et al., 2009). van Dam et al. (2010) identified biologically relevant compounds involved in the attraction of parasitoid wasps, *Cotesia glomerata*, in the headspace of feral *Brassica oleracea* plants that are treated with JA and host herbivory. They identified DMNT and six monoterpenes, the latter of which were not induced in this study, except myrcene. Qiu et al. (2012) reported that JA treatment to shoots of feral *B. oleracea* resulted in increased attraction of *Cotesia glomerata*, a parasitoid of cabbage white butterfly (*Pieris rapae*) larvae.

We did not detect the natural enemies of the leaf-mining fly larvae, vegetable weevils, thrips, and lepidopteran larvae (*P. rapae*, Hadeninae spp. and Plusiinae spp.). Thus, we could not evaluate the possible effects of PDJ on the indirect defenses against these herbivores.

JA and SA signaling pathways are antagonistic to each other (Yang et al., 2019). Under laboratory conditions, spraying PDJ on Japanese radish plants resulted in the induction of *LOX* and *MYC2* (regulated by the JA signaling pathways). Interestingly, we also observed an inconsistent induction of genes regulated by the SA signaling pathways; *PR1, PR2, and PR3* were induced, while *PAL* was not. In Arabidopsis, only the JA signaling pathway was induced in response to PDJ treatment (H. Abe, unpublished data). Clarifying this inconsistency, and the molecular mechanisms involved in the difference between Japanese radish plants and Arabidopsis are interesting. Papadopoulou et al. (2018) showed that *PR1* in *Brassica rapa* shoots was a unique marker gene for the SA pathway, while *B. rapa* roots *PR1* was induced not only by SA treatment, but also by the treatment of ethephon that did not affect SA levels. Thus, the widely used marker genes may not show specific responsiveness to single hormone applications in Japanese radish plants.

Induction of JA and SA signaling pathways results in the induction of direct defense responses in plants (Smith et al., 2009). Here, spraying the 100 times-diluted commercial formulation (5%) of PDJ on Japanese radish plants differentially activates their JA and SA signaling pathways. The induction of these pathways would in part explain the reduction of insect pests either directly or indirectly, except the lepidopteran larvae. In this study, as Japanese radish plants were cultivated for commercial purpose, we were not allowed to sample leaves to analyze JA and/or SA-inducible secondary compounds that might confer direct defense against aphids, thrips, vegetable weevils, and leaf-mining fly larvae during the experiments.

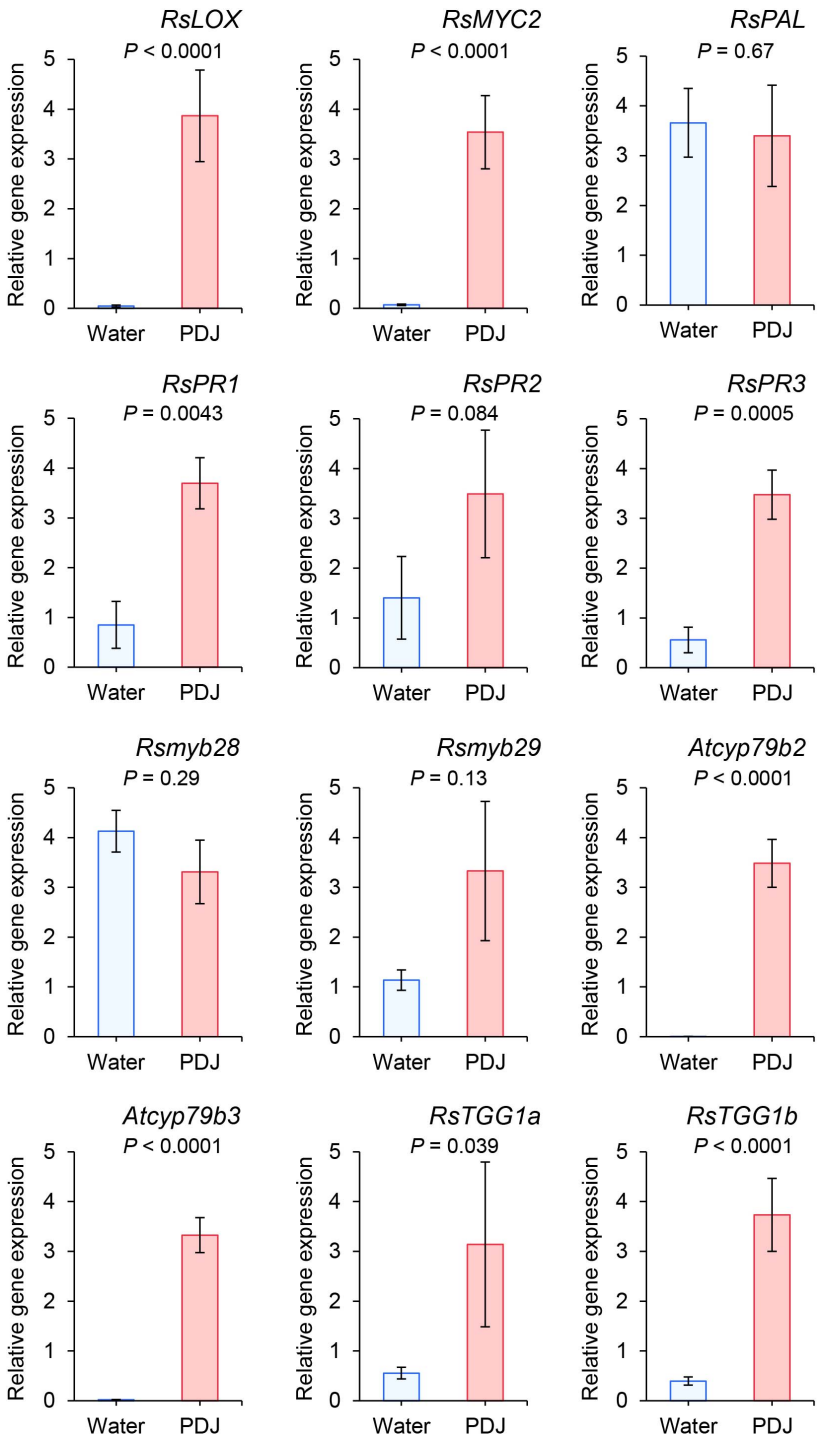

FIGURE 5 | Expression levels of *RsLOX* and *RsMYC2*, which are regulated by the jasmonic acid signaling pathways, *RsPAL*, *RsPR1*, *RsPR2*, and *RsPR3*, which are regulated by the salicylic acid signaling pathways; *Rsmyb28* and *Rsmyb29*, orthologs of *Atmyb28* and *Atmyb29* which regulated aliphatic glucosinolate biosynthesis, *Rscyp79b2* and *Rscyp79b3*, orthologs of *Atcyp79b2* and *Atcyp79b3* which regulate indole glucosinolate biosynthesis; and *RsTGG1* and *RsTGG2*, orthologs of myrosinase genes, *AtTGG1* and *AtTGG2*. The number of replications for all genes was five, except for *RsPR1* (n = 4) for PDJ-treated Japanese radish plants (t-test).

We also observed the effects of the PDJ treatments on the expression of the genes related to glucosinolate biosynthesis. The increase of *Rscyp79b2*, *Rscyp79b3*, *RsTGG1a*, and *RsTGG1b* expression in the treated plants indicated the induction of indole glucosinolate biosynthesis and the subsequent isothiocyanate formation. Whether these inductions affected the community

composition on the treated plants remained unanswered. Both aliphatic and indole glucosinolates make plants more susceptible to attack by specialist herbivores, *P. rapae* and *Plutella xylostella* (Müller et al., 2010). Kim et al. (2008) reported that the post-ingestive breakdown of indole glucosinolates provides a defense against herbivores such as aphids.

Induced defenses are suggested to have evolved to save costs in the absence of herbivores (Agrawal et al., 1999). This mean, trade-offs between induced defense and plant growth are expected. In agricultural crops, these trade-offs may not be found, due to the addition of nutrients to the system and alleviation of competition (e.g., Siemens et al., 2002; Backmann et al., 2019). In contrast, we found that both the weights of the aboveground and belowground parts of the treated Japanese radish plants were significantly lower than those of the control plants, even though the PDJ-treated plants suffered less herbivory by aphids, leaf-mining fly larvae, and thrips than the control plants. This indicates that also in agricultural setting the costs of induction may outweigh the benefits. Alternatively, the direct effects of PDJ might explain the results. The concentration of PDJ used in this study was 5–20 times higher than that of what farmers used. This may have triggered a stronger response in PDJ- treated Japanese radish plants than those grown under normal conditions, thus leading to growth inhibition. Matsuura et al. (2020) reported that spraying PDJ on greenhouse-grown tomato slightly inhibited their initial growth but the fruit yield and quality were not affected. Azis et al. (2020) reported that no significant suppressive effect of PDJ was observed in the aerial parts of komatsuna plants (*Brassica rapa* var. periviridis) and eggplant (*Solanum melongena* L.), but a significant inhibitory effect was found in the roots of both the plant species when treated with PDJ at certain concentrations. Furthermore, in komatsuna, a lower concentration of PDJ resulted in an increase in root weight (Azis et al., 2020).

In conclusion, this study revealed a novel function of PDJ in pest control under open common garden conditions. Spraying the 100 times-diluted commercial formulation (5%) of PDJ every week reduced the incidences of aphids, leaf-mining fly larvae, vegetable weevils, and thrips; however, the incidences of lepidopteran larvae (*P. rapae* Hadeninae spp. and Plusiinae spp.) were not affected by the treatment. Further, the weights of the aboveground and belowground parts of treated plants were significantly lower than those of the control plants. These positive and negative effects might depend on the dose of PDJ as reported by Azis et al. (2020). Therefore, further investigations on the effects of treating Japanese radish plants, as well as other crops (see section "Introduction"), with different concentrations of the formulated PDJ on their growth and defenses against herbivores are required.

DATA AVAILABILITY STATEMENT

The datasets for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

KeY, MU, HA, RO, and YO designed and conducted the experiments and analyzed the data. KiY and JT analyzed the data. JT, MU, and HA wrote the manuscript. All authors have approved the final manuscript for publication.

FUNDING

This study was supported by grants for the scientific research (A) (Grant Number 18H03952) from the Ministry of Education, Culture, Sports, Science and Technology.

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.com) for English language editing. This study was partly supported by Center for Ecological Research, Kyoto University, a Joint Usage/Research Center.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.695701/full#supplementary-material

REFERENCES

Agrawal, A. A., Strauss, A. Y., and Stout, M. J. (1999). Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* 53, 1093–1104. doi: 10.1111/j.1558-5646.1999.tb04524.x

Arimura, G., Matsui, K., and Takabayashi, J. (2009). Chemical and molecular ecology of herbivore-induced plant volatiles: proximate factors and their ultimate functions. *Plant Cell Physiol.* 50, 911–923. doi: 10.1093/pcp/pcp030

Azis, H. R., Takahashi, S., Koshiyama, M., Fujisawa, H., and Isoda, H. (2020). Effect of prohydrojasmon on the growth of eggplant and komatsuna. *Plants* 9:1368. doi: 10.3390/plants9101368

Backmann, P., Grimm, V., Jetschke, G., Lin, Y., Vos, M., Baldwin, I. T., et al. (2019). Delayed chemical defense: timely expulsion of herbivores can reduce competition with neighboring plants. *Am. Nat.* 193, 125–139. doi: 10.1086/700577

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Bruinsma, M., Posthumus, M. A., Mumm, R., Mueller, M. J., van Loon, J. J. A., and Dicke, M. (2009). Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *J Exp. Bot.* 60, 2575–2587. doi: 10.1093/jxb/erp101

Cooper, W. R., and Rieske, L. K. (2008). Differential responses in American (*Castanea dentata* Marshall) and Chinese (*C. mollissima* Blume) chestnut (Falales: Fagaceae) to foliar application of jasmonic acid. *Chemoecology* 18, 121–127. doi: 10.1007/s00049-008-0399-y

Dicke, M., Gols, R., Ludeking, D., and Posthumus, M. A. (1999). Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25, 1907–1922. doi: 10.1023/A:1020942102181

Dicke, M., Vanbeek, T. A., Posthumus, M. A., Bendom, N., Vanbokhoven, H., and Degroot, A. E. (1990). Isolation and identification of volatile kairomone that affects acarine predator-prey interactions: involvement of host plant in its production. *J. Chem. Ecol.* 16, 381–396. doi: 10.1007/BF01021772

Farmer, E. E., and Ryan, C. A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4, 129–134. doi: 10.1105/tpc.4.2.129

Fox, J., and Weisberg, S. (2011). *An R Companion to Applied Regression*, 2nd Edn. Thousand Oaks, CA: Sage.

Gols, R., Posthumus, M. A., and Dicke, M. (1999). Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis*. *Entomol. Exp. Appl.* 93, 77–86. doi: 10.1046/j.1570-7458.1999.00564.x

Heijari, J., Nerg, A. M., Kainulainenm, P., Viiri, H., Vuorinen, M., and Holopainen, J. K. (2005). Application of methyl jasmonate reduces growth but increases chemical defence and resistance against *Hylobius abietis* in Scots pine seedlings. *Entomol. Exp. Appl* 115, 117–124. doi: 10.1111/j.1570-7458.2005.00263.x

Hopke, J., Donath, J., Blechert, S., and Boland, W. (1994). Herbivore induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by β-glucosidase and jasmonic acid. *FEBS Lett.* 352, 146–150. doi: 10.1016/0014-5793(94)00948-1

Kim, J. H., Lee, B. W., Schroeder, F. C., and Jander, G. (2008). Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid). *Plant J.* 54, 1015–1026. doi: 10.1111/j.1365-313X.2008.03476.x

Koshiyama, M., Seto, H., Kamuro, Y., and Kateora, M. (2006). A jasmonic acid analog, PDJ, comes into practical use as a plant growth regulator (in Japanese). *Plant Growth Regul.* 38, 35–47.

Lou, Y. G., Du, M. H., Turlings, T. C. J., Cheng, J. A., and Shang, W. F. (2005). Exogenous application of jasmonic acid induces volatile emissions in rice and enhances parasitism of *Nilaparvata lugens* eggs by the parasitoid *Anagrus nilaparvatae*. *J. Chem. Ecol.* 31, 1985–2002. doi: 10.1007/s10886-005-6072-9

Mandour, N. S., Kainoh, Y., Ozawa, R., Uefune, M., and Takabayashi, J. (2013). Effects of prohydrojasmon-treated corn plants on attractiveness to parasitoids and the performance of their hosts. *J. Appl. Entomol.* 137, 104–112. doi: 10.1111/j.1439-0418.2012.01721.x

Matsuura, S., Ohya, T., Sakurai, T., Mitomi, M., and Abe, H. (2020). Suppressive effect of prohydrojasmon on western flower thrips *Frankliniella occidentalis* (Pergande) (*Thysanoptera*: Thripidae) on greenhouse tomato plants. *Int. J. Pest Manag.* doi: 10.1080/09670874.2020.1817620

McCormick, A. C., Unsicker, S. B., and Gershenzon, J. (2012). The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. *Trends Plant Sci.* 17, 303–310. doi: 10.1016/j.tplants.2012.03.012

Müller, R., de Vos, M., Sun, J. Y., Sønderby, I. E., Halkier, B. A., Wittstock, U., et al. (2010). Differential effects of indole and aliphatic glucosinolates on Lepidopteran herbivores. *J. Chem. Ecol.* 36, 905–913. doi: 10.1007/s10886-010-9825-z

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2020). *vegan: Community Ecology Package. R package version 2.5-7*. Available online at: https://CRAN.R-project.org/package=vegan (accessed November 11, 2020).

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T., and Nishioka, T. (2000). Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant Cell Physiol.* 41, 391–398. doi: 10.1093/pcp/41.4.391

Ozawa, R., Shiojiri, K., Sabelis, M. W., Arimura, G., Nishioka, T., and Takabayashi, J. (2004). Corn plants treated with jasmonic acid attract more specialist parasitoids, thereby increasing parasitization of the common armyworm. *J. Chem. Ecol.* 30, 1797–1808. doi: 10.1023/B:JOEC.0000042402.04012.c7

Ozawa, R., Shiojiri, K., Sabelis, M. W., and Takabayashi, J. (2008). Maize plants sprayed with either jasmonic acid or its precursor, methyl linolenate, attract armyworm parasitoids, but the composition of attractants differs. *Entomol. Exp. Appl.* 129, 189–199. doi: 10.1111/j.1570-7458.2008.00767.x

Papadopoulou, G. V., Maedicke, A., Grosser, K., van Dam, N. M., and Martínez-Medina, A. (2018). Defence signalling marker gene responses to hormonal elicitation differ between roots and shoots. *AoB Plants* 10:ly031. doi: 10.1093/aobpla/ply031

Qiu, B.-L., van Dam, N. M., Harvey, J. A., and Vet, L. E. M. (2012). Root and shoot jasmonic acid induction differently affects the foraging behavior of *Cotesia glomerata* under semi-field conditions. *BioControl* 57, 387–395. doi: 10.1007/s10526-011-9410-6

R Core Team (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

SAS Institute (2018). *JMP version 14.2.0*. Cary, NC: SAS Institute, Inc.

Sasso, R., Iodice, L., Digilio, M. C., Carretta, A., Ariati, L., and Guerrieri, E. (2007). Host-locating response by the aphid parasitoid *Aphidius ervi* to tomato plant volatiles. *J. Plant Interact.* 2, 175–183. doi: 10.1080/17429140701591951

Siemens, D. H., Garner, S. H., Mitchell-Olds, T., and Callaway, R. M. (2002). Cost of defense in the context of plant competition: Brassica rapa may grow and defend. *Ecology* 83, 505–517. doi: 10.2307/2680031

Smith, J. L., De Moraes, C. M., and Mescher, M. C. (2009). Jasmonate- and salicylate-mediated plant defense responses to insect herbivores, pathogens and parasitic plants. *Pest Manag. Sci.* 65, 497–503. doi: 10.1002/ps.1714

Sobhy, I. S., Mandour, N. S., and Sarhan, A. A. (2015). Tomato treatment with chemical inducers reduces the performance of *Spodoptera littoralis* (*Lepidoptera*: Noctuidae). *Appl. Entomol. Zool.* 50, 175–182. doi: 10.1007/s13355-014-0319-2

Takabayashi, J., and Dicke, M. (1996). Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci.* 1, 109–113. doi: 10.1016/S1360-1385(96)90004-7

Takabayashi, J., and Shiojiri, K. (2019). Multifunctionality of herbivory-induced plant volatiles in chemical communication in tritrophic interactions. *Curr. Opin. Insect Sci.* 32, 110–117. doi: 10.1016/j.cois.2019.01.003

Thaler, J. S. (1999). Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399, 686–688. doi: 10.1038/21420

Thaler, J. S., Stout, M. J., Karban, R., and Duffey, S. S. (1996). Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* 22, 1767–1781. doi: 10.1007/BF02028503

Uefune, M., Ozawar, R., and Takabayashi, J. (2014). Prohydrojasmon treatment of lima bean plants reduces the performance of two-spotted spider mites and induces volatiles. *J. Plant Interact.* 9, 69–73. doi: 10.1080/17429145.2012.763146

van Dam, N. M., Qiu, B.-L., Hordijk, C. A., Vet, L. E. M., and Jansen, J. J. (2010). Identification of biologically relevant compounds in aboveground and belowground induced volatile blends. *J. Chem. Ecol.* 36, 1006–1016. doi: 10.1007/s10886-010-9844-9

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag.

Yang, J., Duan, G., Li, C., Liu, L., Han, G., Zhang, Y., et al. (2019). The crosstalks between jasmonic acid and other plant hormone signaling highlight the involvement of jasmonic acid as a core component in plant response to biotic and abiotic stresses. *Front. Plant Sci.* 10:1349. doi: 10.3389/fpls.2019.01349

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 August 2021
doi: 10.3389/fpls.2021.710268

Effects of Methyl Salicylate on Host Plant Acceptance and Feeding by the Aphid *Rhopalosiphum padi*

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Methyl salicylate (MeSA) is a volatile shown to act as an inducer of plant defense against pathogens and certain herbivores, particularly aphids. It has been shown to have potential for aphid pest management, but knowledge on its mode of action is lacking, particularly induced plant-mediated effects. This study investigated the effects of exposing plants to MeSA on the host searching, host acceptance and feeding behavior of the bird cherry-oat aphid *Rhopalosiphum padi*. Barley plants were exposed to volatile MeSA for 24 h, after which biological effects were tested immediately after the exposure (Day 0), and then 1, 3 and 5 days after the end of the exposure. Aphid settling on MeSA-exposed plants was significantly reduced on days 0, 1 and 3, but not on day 5. In olfactometer tests, aphids preferred the odor of unexposed plants on days 1 and 3, but not on day 0 or 5. Analysis of volatiles from exposed and unexposed plants showed higher levels of MeSA from exposed plants, most likely absorbed and re-released from plant surfaces, but also specific changes in other plant volatiles on days 0, 1 and 3. High doses of MeSA did not affect aphid orientation in an olfactometer, but lower doses were repellent. Analysis of aphid feeding by Electronic penetration graph (EPG) showed that MeSA exposure resulted in resistance factors in barley plants, including surface factors and induced systemic factors in other tissues including the phloem. The results support the potential of MeSA as a potential tool for management of aphid pests.

Keywords: plant defense, plant resistance, herbivores, plant volatiles, VOCs, olfactory response, semiochemicals, methly salicylic acid

OPEN ACCESS

Edited by:
Islam S. Sobhy,
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Reviewed by:
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Consiglio Nazionale delle Ricerche (CNR), Italy

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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *15 May 2021*
Accepted: *14 July 2021*
Published: *13 August 2021*

Citation:
Ninkovic V, Glinwood R, Ünlü AG, Ganji S and Unelius CR (2021) Effects of Methyl Salicylate on Host Plant Acceptance and Feeding by the Aphid Rhopalosiphum padi. Front. Plant Sci. 12:710268. doi: 10.3389/fpls.2021.710268

INTRODUCTION

Volatile semiochemicals are increasingly being considered as promising components of integrated management strategies against insect pests (Smart et al., 2014). Semiochemicals may include compounds that directly repel pests, attract natural enemies, or they may be "elicitors" that induce defensive pathways that confer resistance in the host plant (Maffei et al., 2012). Certain semiochemicals may potentially have more than one of these modes of action. The salicylic acid biochemical pathway in plants, mainly activated in response to the attack of sap feeders, is generally considered to provide defense against biotrophic pathogens, but it also appears to function against certain herbivorous arthropods, particularly those with a piercing/sucking feeding mode (Aerts et al., 2021). Methyl salicylate (MeSA) is a volatile compound associated with the salicylic acid

pathway in plants. MeSA has been shown to act as a mobile signal for systemic acquired resistance (SAR) by being converted to salicylic acid (Park et al., 2007) and is known to promote the expression of defense related genes in response to herbivores and pathogens (Li et al., 2002).

MeSA may have different modes of action that can be exploited for pest management (James, 2003; Ninkovic et al., 2003; Byers et al., 2021). It is attractive to a range of natural enemies of arthropod pests (Mallinger et al., 2011; Orre Gordon et al., 2013) and possibly birds (Rubene et al., 2019), and has shown repellency against aphids (Glinwood and Pettersson, 2000; Prinsloo et al., 2007; Digilio et al., 2012). MeSA is often reported as a plant volatile that is induced by insect feeding, and may play a role in defense signaling within (Heil and Ton, 2008) or between plants (Shulaev et al., 1997). Thus, the compound can act as a defense-elicitor (Heil and Ton, 2008), providing an additional mode of action against pests.

MeSA may have particular potential against piercing/sucking pests such as aphids. In fact, the capacity of MeSA to suppress aphid populations in crops has been demonstrated in a number of studies (Pettersson et al., 1994; Ninkovic et al., 2003; Prinsloo et al., 2007; Xu et al., 2018). Outside of the attraction of natural enemies, the mode of action of the compound is not always clear, particularly regarding the relative importance of direct and plant-mediated effects. This is particularly true for the bird cherry-oat aphid, *Rhopalosiphum padi* L. which has been successfully managed by releasing MeSA in cereal fields (Pettersson et al., 1994; Ninkovic et al., 2003). *R. padi* alternates between a winter host, *Prunus padus* L., and summer hosts including grasses and cereals. There is evidence that MeSA is involved in this host alternation process, and may act as an aphid feeding-induced cue mediating migration from winter host (Glinwood and Pettersson, 2000; Pickett et al., 2017). Moreover, previous studies have shown that the exposure of cereal plants to MeSA can reduce *R. padi* host acceptance (Ninkovic et al., 2003; Glinwood et al., 2007), suggesting that MeSA could affect this aphid both directly as an ecological cue in the lifecycle, and indirectly by eliciting unfavorable changes in the host plant.

Aphids find, select and colonize host plants through a step-wise process (Pettersson et al., 2007). Initially volatile chemical and/or visual cues mediate attraction. After landing on the plant cues from the plant surface may be involved in the decision to begin feeding. The feeding process is highly developed; the stylet probes the plant tissues and navigates mostly between cells eventually piercing the phloem sieve elements to commence feeding. Protective effects against aphids induced by exposing plants to MeSA could therefore be expressed at several different stages or in different plant tissues. Thus, although MeSA has been shown to suppress *R. padi* populations in crops, it is still unclear how plant-mediated effects, including disruption of feeding, may contribute to this.

R. padi is a major pest of cereals in many temperate regions including Sweden (Wiktelius et al., 1990), with increasingly limited options for insecticide treatment. The application of MeSA could be promising in the development of more sustainable approaches to control *R. padi* and other aphid species. Still, the direct effects on plants and plant-mediated effects against aphids need further attention. The aim of this study is therefore to investigate the effect of exposing barley plants to volatile MeSA on the behavior of *R. padi* related to host finding, host acceptance and feeding. We exposed barley plants to volatile methyl salicylate for 24 h, then investigated the dynamics of *R. padi* responses to MeSA exposed plants over a number of days including olfactory responses, host acceptance and feeding behavior. We also analyzed MeSA-induced changes in the volatile profiles of exposed plants.

MATERIALS AND METHODS

Aphids

Bird Cherry-oat aphid, *R. padi*, was reared on oat plants (*Avena sativa* cv. Belinda) in multi-clonal cultures in a greenhouse at 20 ± 2°C with a L16:D8 light cycle. Apterous viviparous aphids of larval instars 2–4 used in the all experiments were collected from cultures immediately prior to bioassays.

Plants

Barley plants, *Hordeum vulgare L.* (cv. Scandium) were grown in plastic pots (8 × 8 × 6 cm) with soil (Hasselfors Garden Special, Sweden) with one plant/pot (apart from volatile collections where 10 plants/pot were used). Plants were grown in a greenhouse chamber at 20±2°C with 16D:8L light cycle with supplementary lighting from HQIE lamps.

Chemicals

Commercially available authentic standards and other chemicals were obtained from (Sigma-Aldrich, Sweden). Methyl salicylate triple-deuterated on the methoxy group (D-MeSA), was synthesized by hydrolysis of ethyl salicylate to get salicylic acid. The second step was an acid-catalyzed (HCl) esterification with deuterated methanol (See **Supplementary Material** for spectroscopic data).

Plant Exposure to Methyl Salicylate

Barley plants were exposed to volatile MeSA inside a twin-chamber exposure cage system (Ninkovic et al., 2002). The system consisted of a series of clear Perspex cages divided in to two chambers (each chamber 10 × 10 × 40 cm) connected by an opening (Ø 7 cm) in the middle wall. Each Perspex cage was connected to a vacuum tank pump from which the air was removed by a fan and vented outside the greenhouse. Airflow through the cages was 1.3 l/min. MeSA (98%, CAS no. 119-36-8) was purchased from Sigma-Aldrich, Sweden. 10 μl MeSA was applied to a 9 cm filter in a Petri dish placed in the front chamber, and pots with plants were placed in the rear chamber. The mean aerial concentration of MeSA in the exposure cages using this method was measured in a previous study as: first 4 h 300 ng/l (1.9 nM) (peak 625 ng/l, 4.5 nM), following 8 h 50 ng/l (0.3 nM) (Glinwood et al., 2007).

Control plants were treated in the same way except that no MeSA was applied to the filter papers. For each experiment, barely plants at the two-leaf stage (11 days after sowing) were used. Only one plant per pot was grown to avoid pseudo replication and unplanned differences in the cage caused by

treatment. Individual pots were watered using an automated drop system (DGT Volmatic) without additional fertilizer. The two-chamber cages were kept in a greenhouse chamber at 20±2°C with 16D:8L light cycle with supplementary lighting from HQIE lamps.

The filter papers with and without MeSA were removed from the inducing chamber after 24 h. After filter papers were removed, plants were used immediately in experiments (Day 0), or kept for 1 day (Day 1), 3 days (Day 3) or 5 days (Day 5) in the responding chambers.

Aphid Plant Acceptance

The effect of plant exposure to MeSA on aphid plant acceptance was assessed by means of a no-choice settling test (Ninkovic et al., 2002). A 50-ml polystyrene tube was placed over the second leaf of plants at the two-leaf stage. The upper end of the tube was covered with a net and the lower end with a plastic sponge plug with a slit for the leaf. The tube was supported with a stick to avoid mechanical damage to the plant. Ten aphids were placed in the tube and the number of aphids settled (not walking) on the leaf was recorded after 2 h, since this is sufficient time for aphids to settle and reach the phloem with their stylets (Prado and Tjallingii, 1997). The tests were performed on a bench in a separate greenhouse chamber under the same conditions as the MeSA exposures. Twenty plants per treatment were tested.

Analysis of Aphid Probing Behavior Using Electronic Penetration Graphs

The effect of plant exposure to MeSA on aphid probing and feeding in plant tissues was monitored using the DC-EPG technique (Tjallingii, 1986, 1988). Four plants treated with MeSA and four control plants were placed in a Faraday cage where aphid probing and feeding behavior was recorded simultaneously during 8 h for each experiment. Each pot with one plant was placed in a Petri dish. To prevent interaction between treated and control plants via volatiles, the Faraday cage was divided into two chambers each with independent airflow and ventilation. The placement of treated and control plants was alternated between chambers and between tests.

Aphids were collected using a soft brush and then held in place by a vacuum device to apply a small drop of conductive water-based silver glue on the dorsum to which a thin gold wire (20 μm) electrode about 2 cm long was attached. The other end of this electrode was attached to a 3 cm long copper wire connected to a channel (a first stage amplifier with 1 GΩ input resistance) (Tjallingii, 1988). Each wired insect was placed on the abaxial side of the second leaf of a barely plant, about 1 h after collection from the aphid culture. A second electrode (2 mm in diameter and 10 cm long copper rod) was inserted into the soil of the potted plant and connected to Giga-8 (plant voltage output of EPG device) (Wageningen University, The Netherland). The EPG device was connected to A/D converter at 100 Hz (DATAQ Instruments, USA) and it was in turn connected to a computer where waveform signals of eight plants, divided between two Faraday cage chambers, were recorded using PROBE 3.0 software.

EPG signals were analyzed using STYLET A software (epgsystems. eu). Identification of the waveform patterns was made according to Tjallingii (1990). Aphid probing behavior was divided into five main phases: the non-probing phase (np), the probing phase (C) representing aphid stylet penetration in plant tissues which was further divided into the pathway phases (epidermis first stylet contact waveform A, intercellular sheath salivation waveform B; stylet movements waveform C, and an intracellular stylet puncture waveform pd "potential drop"), the phloem phase (E) divided in "salivation activity" (El) and sap ingestion (E2), stylet penetration difficulties (F) and xylem phase (G) active sucking of water from xylem elements. A number of variables (parameters) most relevant for aphid resistance, comparable to those used by Marchetti et al. (2009), were derived from the EPG signals. EPG variables were expressed in mean numbers, total durations of waveform periods and durations of waveform periods before or after certain events per treatment.

Collection and Identification of Plant Volatiles

To investigate the effect of plant exposure to MeSA on barley volatile production, volatiles were collected and identified as described below. Pots containing 10 barley plants were exposed to methyl salicylate as described above. Control plants were kept inside two-chamber cages without exposure to the chemical. Volatiles from barley plants were collected by air-entrainment (Glinwood et al., 2011). The whole pot with 10 plants was placed inside a polyester (PET) cooking bag (Stewart-Jones and Poppy, 2006) (60 × 55 cm, Toppits, Melitta, Sweden). A glass liner containing 50 mg of the molecular adsorbent Tenax TA (Atas GL Intl., Veldhoven, Netherlands) was inserted through a small hole cut in one corner of the bag. A positive pressure push–pull system was used, with charcoal-filtered air pushed in through a Teflon tube inserted through a small hole in the bottom of the bag, at 600 ml/min and pulled out over the adsorbent at 400 ml/min. Bags were baked in an oven at 140°C for 2 h immediately prior to the entrainment. Charcoal filters and Tenax tubes were baked at 180 and 220°C respectively under a flow of nitrogen for 16 h. At time points Day 0, Day 1 and Day 3 (after the end of the 24 h exposure to methyl salicylate), pots of plants were removed from exposure cages and volatile collection was started immediately for a total duration of 24 h. For each treatment and time point, six separate pots of plants were entrained. Three collections were made from plastic pots containing only soil and only volatiles appearing in the plant samples but not in the soil controls were quantified.

Volatiles were analyzed by gas chromatography-mass spectrometry (GC/MS) on an Agilent 7890N (Agilent Technologies) GC coupled to an Agilent 5975C mass selective detector (electron impact 70 eV). The GC was equipped with an HP-1 column (100% dimethyl polysiloxane, 50 m, 0.32 mm i.d. and 0.52 μm film thickness, J&W Scientific, USA), and fitted with an Optic 3 thermal desorption system (Atas GL Intl., Veldhoven, Netherlands). The liner containing the Tenax with absorbed volatiles was placed directly into the injector and volatiles were thermally desorbed starting at 30°C/0.5 min, and rising at 30°C/s to 250°C. The GC temperature program

was 30°C/4 min, 5°C/min to 150°C/0.1 min, 10°C/min to 250°C/15 min, using helium as carrier with a flow rate of 1.3 ml/min. Volatile compounds were identified by comparison against a commercially available library (NIST 08) and by comparison of mass spectra and retention indices with commercially available authentic standards (Sigma-Aldrich, Sweden). Amounts of compounds in the samples were quantified based on the cumulative abundances for three ions selected with the criteria that they were typical and abundant for the target compound. These were then compared with response curves constructed using commercially available chemical standards (Sigma-Aldrich, Sweden) to estimate the amount present in the sample.

Estimation of Absorption/Re-release Compared With *De Novo* Production of MeSA in Exposed Plants

The aim was to estimate the relative contribution to the total MeSA measured in the headspace of exposed plants by absorption/re-release from the leaves compared with *de novo* emission by the plant. Plants were exposed to deuterated-MeSA (D-MeSA) (see **Supplementary Material** - Synthesis of Deuterated MeSA), using the same method described above.

As described above, D-MeSA was removed from the exposure cage, and pots containing 10 barley plants were used for volatile collection (24 h) immediately (Day 0) or 1 or 3 days after removal of D-MeSA. Volatiles were collected according to the method described above, except that glass collection tubes contained the adsorbent Porapak Q (50 mg, mesh 50/80, Supelco, Bellefonte, PA, USA). These tubes were prepared by rinsing with dichloromethane and baking for 4 h at 140 °C under nitrogen flow. After the volatile collection, collected volatiles were eluted from the absorbent tubes using 500 μl dichloromethane then concentrated to 50 μl under a low flow of nitrogen.

A 2 μL aliquot of the extracted sample was injected into an Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a cold-on-column injector and fitted with an HP-1 column (30 m, 0.25 mm i.d., and 0.25 μm film thickness; J &W Scientific, Folsom, CA, USA) coupled to an Agilent 5975C mass selective detector (electron impact 70 eV, 230 °C). The GC program was set to start at 30 °C for 1 min, and set to rise 20 °C/min to 250 °C. The carrier gas was helium with a flow rate of 1 ml/min. The mass selective detector was programed in selected ion monitoring (SIM) mode, with the quantification ions m/z =155 for D-MeSA and $m/z = 152$ for MeSA and a confirmation ion $m/z = 92$. Quantifications were made using the abundances of quantification ions in the collected samples compared with those in an injected authentic standard of D-MeSA or MeSA (10 ng).

Olfactometer Bioassays

The effect of plant exposure to MeSA on aphid responses to plant volatiles was tested using a two way airflow olfactometer (Vucetic et al., 2014). *Three* separate experiments were carried out:

(i) *aphid olfactory response to odor from exposed and unexposed plants.* Plants used as odor sources were kept in two-chamber cages, as described above. A two-chamber cage with a plant previously exposed to MeSA was directly connected to one arm of the olfactometer, and a two-chamber cage with an unexposed plant was connected to the opposite arm.

(ii) *aphid olfactory response to synthetic odor blends*. Using chemical standards, odor blend mixtures were constructed based on the occurrence and proportions of compounds identified by GC/MS in the plant headspace for MeSA-exposed and unexposed plants at Day 1 (1 day after removal of MeSA from the exposure chambers). For each treatment, five blends were constructed over a range of different concentrations: 100×, 10×, 1×, 1/10 and 1/100 the concentration in the headspace collections. Synthetic blends (10 μl of a solution in hexane) of MeSA-exposed and unexposed plants were dosed onto small filter paper squares placed in a tube connected to the arm of the olfactometer. Chemicals were obtained from commercial suppliers: (Z)-3-hexen-1ol (98 % purity, Sigma Aldrich Inc., St. Louis, MO, USA), 6-methyl-5-hepten-one (99 % purity, Sigma Aldrich), myrcene (90 % purity, Fluka, Buchs, Switzerland), (Z)-3-hexen-1-yl acetate (99 % purity, Sigma Aldrich), linalool (97 % purity, mixture of (S) and (R) isomers, Sigma Aldrich), methyl salicylate (98 % purity, Sigma Aldrich), longifolene (99 % purity, ABCR GmbH & Co., Karlsruhe, Germany).

(iii) *aphid olfactory response to individual chemicals differing significantly between profiles of MeSA-exposed and unexposed plants*. The procedure was as for (ii) above using the same concentration range of substances, with hexane lone as control.

Airflow in the olfactometer was 180 ml/min, measured with a flowmeter at the arm inlets. A single aphid was introduced into the olfactometer and, after an adaptation period of 10 min, the aphid's position in the arena was recorded every 3 min over a 30-min period. The accumulated number of visits in the arm zones (excluding the central neutral zone) was regarded as one replicate. Each test was repeated 20 times (aphids) for each treatment. Each individual aphid was used only once. If an aphid was inactive in the olfactometer (i.e. observed to be stationary in the same position for three consecutive observations) it was removed and the bioassay restarted with a fresh aphid. To account for any positional bias the position of treatments in the olfactometer was switched between the left and right arms in each separate olfactometer.

Statistical Analysis

Statistical differences in aphid settling between treated and control plants were analyzed using t-tests for independent samples. Data for all variables were subjected to tests for homogeneity of variances. As the EPG data were not normally distributed, paired comparison of means of MeSA treatments with controls was done by nonparametric Mann–Whitney *U*-test. Plant volatile data followed the assumptions of normality and were analyzed by one-way analysis of variance (ANOVA) following SQRT transformation to reduce heteroscedasticity when necessary. Aphid olfactory response data were analyzed by Wilcoxon matched pairs tests. All statistical tests were performed with the Statistica software (TIBCO Software Inc., 2018).

RESULTS

Aphid Settling on MeSA-Exposed and Unexposed Plants

Immediately after a 24 h exposure to MeSA (Day 0), aphid settling was significantly reduced on exposed barley plants in comparison to unexposed plants ($p = 0.037$). Aphid settling was also significantly reduced on exposed plants at Day 1 ($p = 0.0004$) and Day 3 ($p = 0.01$) after removing MeSA, before returning to the same level as unexposed plants at Day 5 after removal of MeSA ($p = 0.104$) (**Figure 1**).

Aphid Olfactory Response to Odor From MeSA-Exposed and Unexposed Plants

Aphids were observed significantly less often in the olfactometer arm containing odor of barley plants previously exposed to MeSA than in the arm with odor of unexposed plants at Day 1 (Wilcoxon test, $Z = 2.79$, $p = 0.006$) and Day 3 (Wilcoxon test, $Z = 2.35$, $p = 0.018$). These significant reductions in aphid preference to treated plants were not observed at Day 0 (Wilcoxon test, $Z = 1.36$, $p = 0.173$) or Day 5 (Wilcoxon test, $Z = 1.63$, $p = 0.103$) (**Figure 2**).

Volatile Profiles of MeSA-Exposed and Unexposed Plants

Significantly more methyl salicylate was detected in the headspace of MeSA exposed plants than of unexposed plants (**Figure 3**) at Day 0 (Tukey test, $p = 0.0002$), Day 1 (Tukey test, $p = 0.0002$) and Day 3 h (Tukey test, $p = 0.013$) after the end of exposure.

Exposure to methyl salicylate resulted in significant quantitative and qualitative changes in the occurrence of certain volatiles in barley headspace. At Day 0 (**Figure 4A**), exposed plants released significantly less of the green leaf alcohol (*Z*)-3-hexen-1-ol than unexposed plants (ANOVA, $F_{1,10} = 9.04$, $p = 0.013$). At Day 1 (**Figure 4B**), exposed plants released significantly less (Z)-3-hexen-1ol than unexposed plants (ANOVA, $F_{1,10} = 15.17$, $p = 0.002$) and significantly more of the terpenoids myrcene (ANOVA, $F_{1,10} = 9.33$, $p = 0.12$) and linalool (ANOVA, $F_{1,10} = 13.8$, $p = 0.004$). At Day 3 (**Figure 4C**), exposed plants released the sesquiterpene (*E*)-β-caryophyllene, which was not detected from unexposed plants (ANOVA, $F_{1,10} = 20.18$, $p = 0.001$).

Estimation of Absorption/Re-release Compared With *De Novo* Production of MeSA in Exposed Plants

The relative abundances in the headspace of deuterated MeSA (characterized by ion $m/z = 155$) and undeuterated MeSA ($m/z = 152$) showed that a very high proportion of the methyl salicylate quantified in the headspace of exposed plants in the experiments above was most likely absorbed and re-released from the plant (98.7% at Day 0, 96.2% at Day 1, 87.7% at Day 3) (**Supplementary Table 1**). The amount of deuterated MeSA recorded was initially high at Day 0 and decreased on Days 1 and 3 in a similar pattern to that seen with the earlier experiment above. A higher amount of undeuterated MeSA in the headspace of exposed compared to unexposed plants would indicate induced *de novo* production, but this was significantly higher only on Day 1 ($p \leq 0.01$ Mann-Whitney U Test).

Aphid Olfactory Response to Synthetic Odor Blends

Aphids did not show any significant response to the synthetic blend of MeSA-exposed barley compared with the synthetic blend of unexposed barley (**Figure 5**). Aphids did discriminate between the synthetic blends of MeSA-exposed and unexposed barley plants when MeSA was excluded from the synthetic blends (**Figure 6**). Aphids made significantly fewer visits to the olfactometer arm containing the synthetic blend of MeSA-exposed barley than to the arm containing the blend of unexposed barley when solutions with concentrations of 10 ng/μl (Wilcoxon test: $Z = 2.897$, $p = 0.004$, $n = 15$), 1 ng/μl (Wilcoxon test: $Z = 2.225$, $p = 0.026$, $n = 17$) and 0.01 ng/μl (Wilcoxon test: $Z = 3.416$, $p = 0.0006$, $n = 20$) were used as odor sources (**Figure 7**).

Aphid Olfactory Response to Individual Compounds That Differed Significantly Between Volatile Profiles of MeSA-Exposed and Unexposed Plants

Aphids made significantly fewer visits to the olfactometer arm containing MeSA than to the arm containing hexane control, but only at the two lowest concentrations tested, 0.03 ng/μl (Wilcoxon test: $Z = 2.44$, $p = 0.015$, $n = 17$) and 0.003 ng/μl (Wilcoxon test: $Z = 3.07$, $p = 0.002$, $n = 22$) (**Figure 7**). Aphids did not respond to (*Z*)-3-hexen-1-ol or linalool (**Supplementary Figures 1, 2**).

Probing Behavior of Aphids on MeSA-Exposed and Unexposed Plants

Table 1 gives a summary of effects on aphid feeding, the full data are presented in **Supplementary Table 2**. Immediately after 24 h exposure to MeSA (Day 0), the phloem feeding time in MeSA-exposed plants was significantly shorter than in unexposed plants ($p = 0.02$) and the number of feeding periods longer than 10 and 60 mins were significantly fewer in MeSA-exposed compared to unexposed plants ($p = 0.01$; $p = 0.001$) (**Supplementary Table 2A**). The duration of pathway (C), which represents intercellular stylet penetration from epidermis to phloem, was not significantly different in exposed plants ($p = 0.44$). There was no significant difference in the number of short probes (< 3 min) ($p = 0.63$) or the number of short probes before the 1st phloem phase ($p = 0.72$). There was no significant difference in salivation period (E1) between MeSA-exposed and unexposed plants ($p = 0.44$).

Twenty four hours after removal of MeSA (Day 1) the significantly longer salivation period (E1) ($p = 0.03$) and the higher number of E1 ($p = 0.02$) and duration of all E1 fractions ($p = 0.046$) in MeSA-exposed plants (**Supplementary Table 2B**). Mean duration of the feeding period (E2) was shorter in

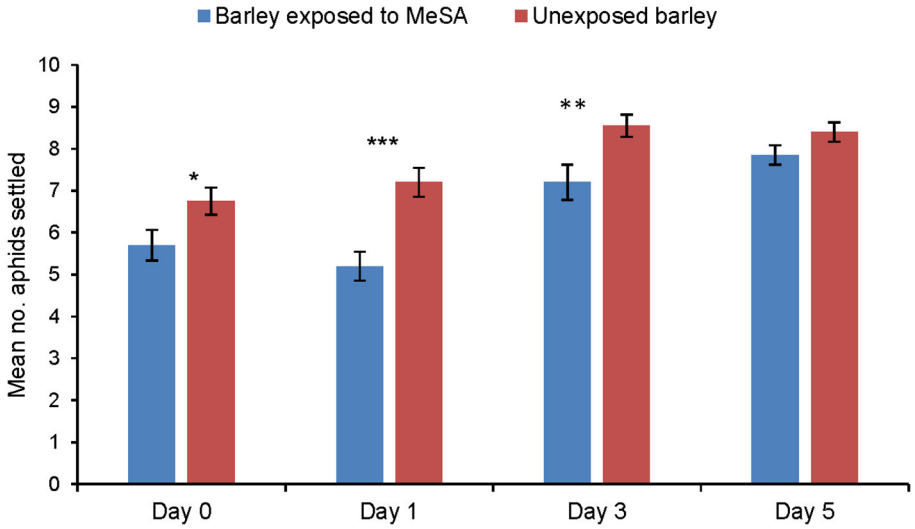

FIGURE 1 | Settling (mean number of aphids settled ± SE) of *R. padi* on unexposed barley plants or on plants exposed to MeSA (directly after removal of MeSA exposure = Day 0 and at time points 1, 3 and 5 days after removal of MeSA exposure). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ after t-tests.

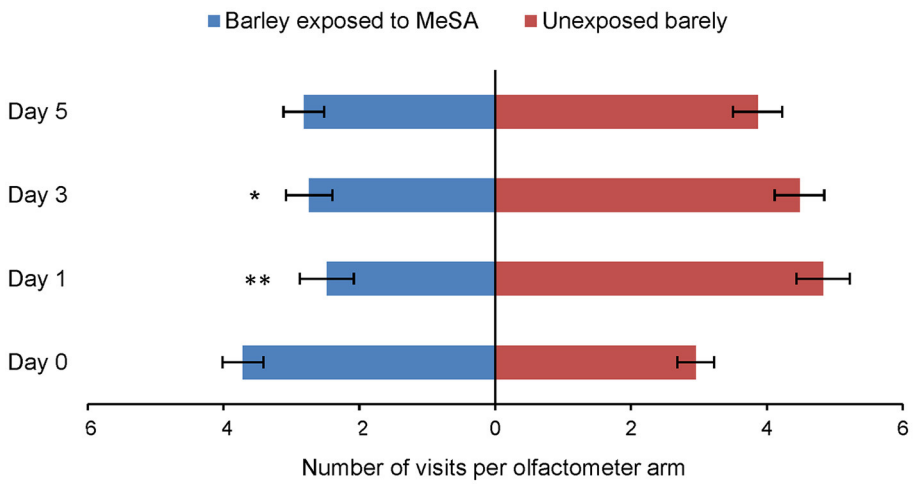

FIGURE 2 | Olfactory response of *R. padi* (mean visits in olfactometer arm ± SE) to odor of unexposed barley plants or to plants exposed to MeSA (directly after removal of MeSA exposure = Day 0 and at time points 1, 3 and 5 days after removal of MeSA exposure). *$p \leq 0.05$; **$p \leq 0.01$ after Wilcoxon matched pairs tests.

MeSA-exposed plants, but not significantly. Duration of probing did not differ between exposed and unexposed plants ($p = 0.57$) but in exposed plants significantly longer time was spent in the path period (about 40%) ($p = 0.014$) and less time in the feeding period (E2) (about 29%) compared to unexposed plants (26 and 50% respectively). Number of probes before the 1st feeding attempt (1st E1) was significantly higher in MeSA-exposed plants ($p = 0.042$). The duration of the xylem feeding period was significantly longer and the number of xylem feeding periods (G) was significantly higher in MeSA-exposed plants ($p = 0.034$; $p = 0.008$ respectively).

Three days after removal of MeSA (Day 3) the duration of non-probing period was significantly longer ($p = 0.024$) while probing period was shorter ($p = 0.023$) in MeSA-exposed plants than unexposed (**Supplementary Table 2C**). The effect was mainly due to a significantly shorter duration of feeding period (E2) in exposed plants ($p = 0.03$). The number of long sustained feeding periods (> 10 and 60 mins) was significantly higher in unexposed plants ($p = 0.037$; $p = 0.048$). A further indication of resistance to feeding in MeSA-exposed plants is the longer time between the 1st E1 and 1st E12 (sustained feeding) period ($p = 0.013$). This means that aphids took a longer time to establish a successful feeding period in exposed plants.

Five days after removal of MeSA (Day 5), the EPG results show no significant effects of plant exposure to MeSA on aphid probing (**Supplementary Table 2D**).

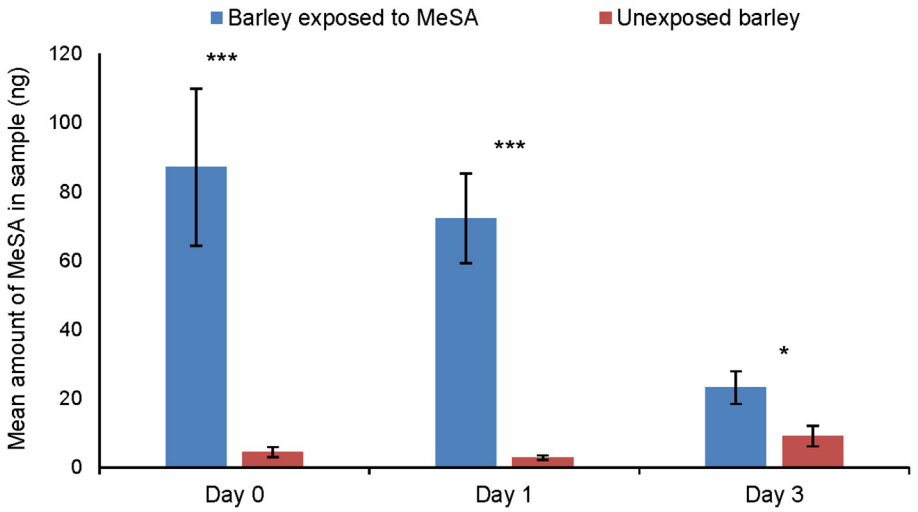

FIGURE 3 | Amount of MeSA (mean ng ± SE) quantified in the headspace of unexposed barley plants or on plants exposed to MeSA (directly after removal of MeSA exposure = Day 0 and at time points 1 and 3 days after removal of MeSA exposure). *$p \leq 0.05$; ***$p \leq 0.001$, after Tukey test.

DISCUSSION

This study shows that exposing plants to volatile MeSA can impact the step-wise process of *R. padi* host plant selection by affecting aphid behavior at more than one step. The findings support the potential of MeSA as a compound that can protect crop plants against this aphid pest by interfering with the host selection process by different modes of action.

MeSA has been shown to act as a mobile signal for systemic acquired resistance (SAR) via reconversion to salicylic acid (SA) in the plant (Park et al., 2007). SA triggers defenses and plays a critical role in plant immunity to phloem-feeding insects, including aphids (Kaloshian and Walling, 2005; Goggin, 2007; Smith and Boyko, 2007; Spoel and Dong, 2012; van Dam et al., 2018). Exogenous application of the SA analog benzothiadiazole (BTH) induces plant defenses and has been shown to disrupt aphid colonization and feeding behavior in wheat (Cao et al., 2014) and population growth rates on susceptible and resistant tomato cultivars (Li et al., 2002; Cooper et al., 2004). In the current study we show that exposure of barley to the volatile signal MeSA negatively affects aphid host selection. We also report for the first time MeSA-induced disruption of different stages of the aphid feeding process as revealed by EPG. SA analogs tend to be less phytotoxic than SA itself (Durrant and Dong, 2004; Tripathi et al., 2019), and application of BTH reduced foliar thickness and caused necrotic lesions on sprayed tomato leaves (Boughton et al., 2006). The plants exposed to MeSA in the current study did not show any visible changes during the experiment period, but further investigation of effects on plant development and yield are needed.

The first step in host selection by aphids involves orientation to color and volatile cues from the host plant (Pettersson et al., 2007). In the olfactometer, aphids avoided the odor of MeSA-exposed plants but only 1 and 3 days after termination of the 24 h exposure to MeSA; there was no significant aphid response immediately after termination of exposure (Day 0) or 5 days after termination. Exposure to MeSA did cause significant changes in the volatile profile of barley. This suggests that MeSA can induce changes in the biochemistry of exposed plants. Compounds that were induced or upregulated by MeSA-exposure were mainly terpenoids, which are known to be involved in plant defensive responses (Mumm et al., 2008). However, it is unclear whether these induced changes in barley volatile profiles affected aphid behavior; an artificial volatile blend designed to replicate that of MeSA-exposed plants at Day 1 was less attractive to aphids than the blend of unexposed plants, but only when the blend lacked the high proportion of MeSA itself that was recorded in the headspace. Further, several of the volatiles that were altered in MeSA-exposed plants did not affect aphid orientation in the olfactometer when tested individually. Plant volatiles may have different effects on aphid behavior when encountered alone or together with other compounds in blends (Bruce and Pickett, 2011). For practical reasons, our study used wingless aphid morphs for the experiments, whereas it can be argued in nature that the initial steps in host location and selection are carried out by winged morphs.

Aphids were repelled by MeSA itself in the olfactometer, but only when it was introduced at the lower concentrations in a dose-response experiment. Thus the olfactory responses to MeSA-exposed plants observed at Days 1 and 3 could be due to a concentration-dependent response to MeSA in the headspace. Repellency of MeSA has been reported for *R. padi,* the Russian wheat aphid *Diuraphis noxia* (Glinwood and Pettersson, 2000; Prinsloo et al., 2007) and black bean aphid, *Aphis fabae*

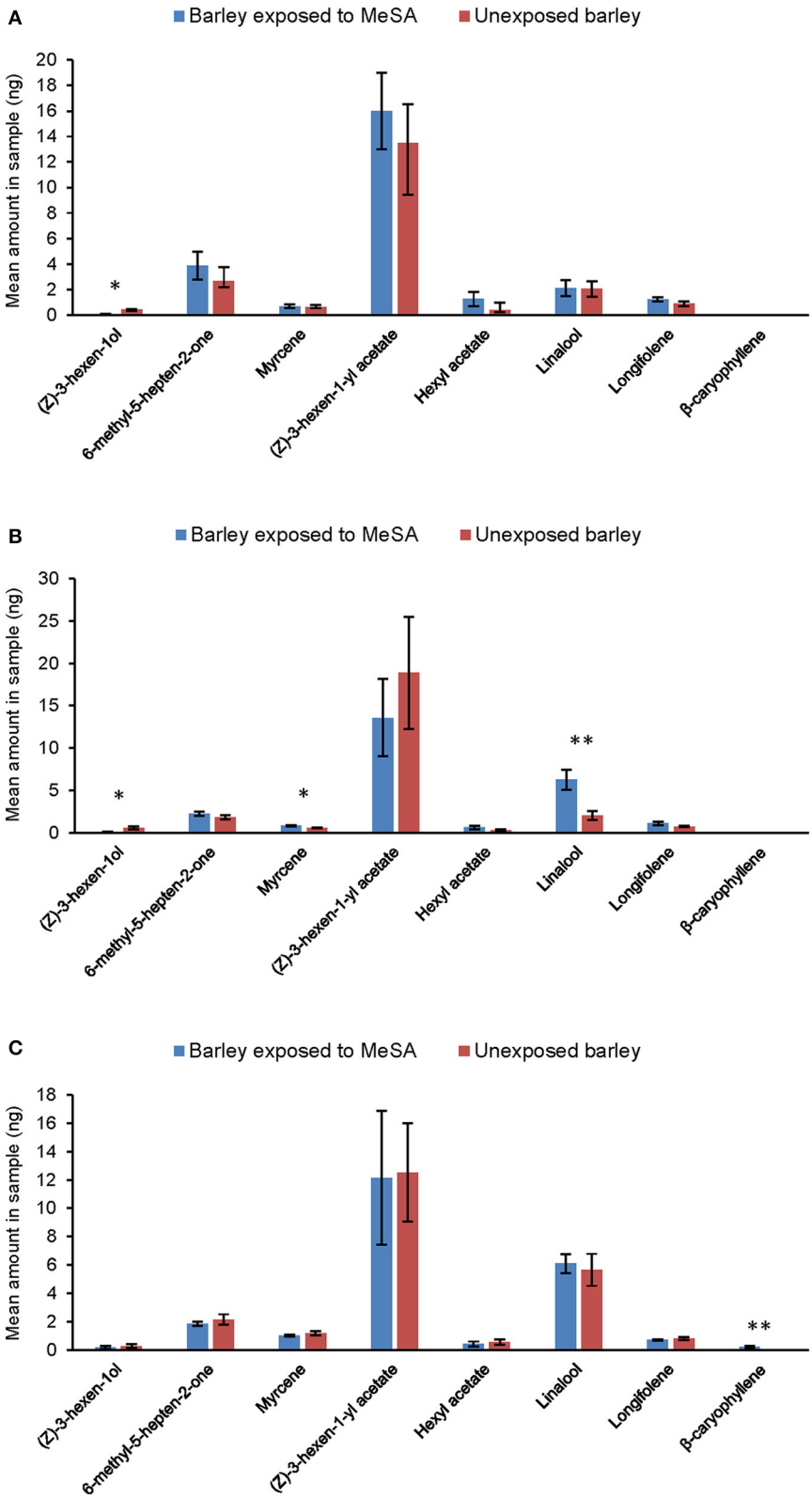

FIGURE 4 | Amounts of volatile compounds (mean ng ± SE) quantified in the headspace of unexposed barley plants and plants exposed to MeSA [directly after removal of MeSA exposure (Day 0) **(A)** and at time points 1 **(B)** and 3 days **(C)** after removal]. *$p \leq 0.05$; **$p \leq 0.01$, after ANOVA. MeSA was analyzed in the headspace but, due to large differences in amounts, it is shown in a separate figure (**Figure 3**).

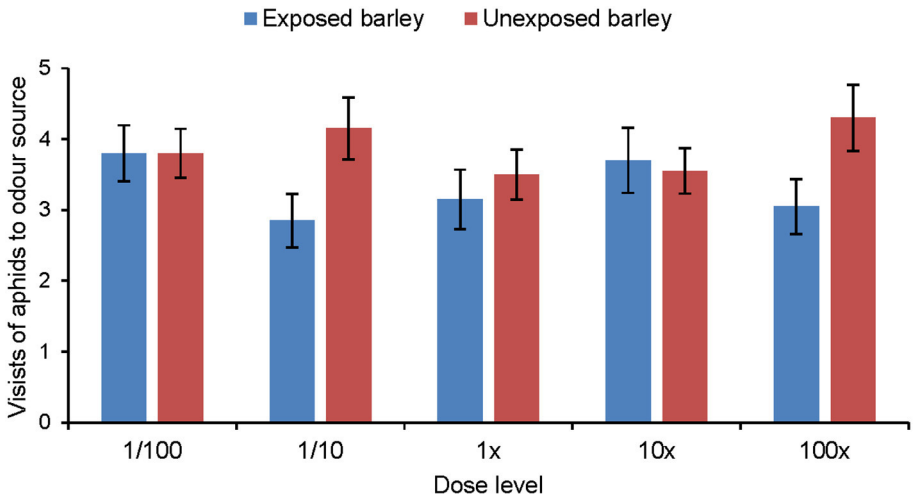

FIGURE 5 | Olfactory response of *R. padi* (mean visits in olfactometer arm ± SE) to different concentrations of a synthetic volatile blend resembling the headspace of unexposed barley plants or plants exposed to methyl salicylate. (Wilcoxon matched pairs tests did not show significant differences).

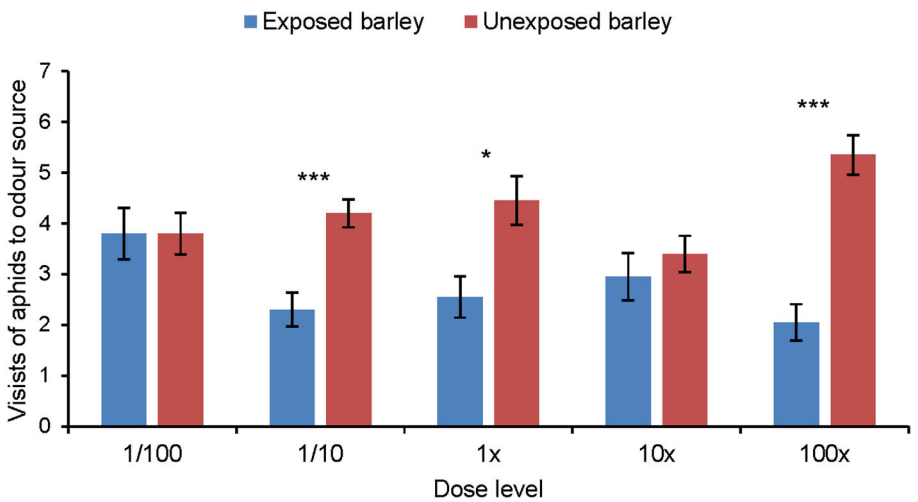

FIGURE 6 | Olfactory response of *R. padi* (mean visits in olfactometer arm ± SE) to different concentrations of a synthetic volatile blend resembling the headspace of unexposed barley plants or plants exposed to methyl salicylate, but with MeSA not included in the blend. *$p \leq 0.05$; ***$p \leq 0.001$ after Wilcoxon matched pairs tests.

(Hardie et al., 1994).The current results suggest that the *R. padi* olfactory response to MeSA is dynamic and concentration-dependent, possibly representing an adaptation to biologically-relevant levels. Interestingly, the olfactory response of *R. padi* to MeSA has also been shown to vary dynamically within migratory aphid individuals depending on life stage (Glinwood and Pettersson, 2000). It is probable that the olfactory response of *R. padi*, a host-alternating species, to MeSA has evolved in relation to the compound's role in plant defense and the aphid's lifecycle including dispersal of apterous aphids on the summer host plant (Glinwood and Pettersson, 2000; Pickett et al., 2017).

By exposing plants to deuterated MeSA, we attempted to determine whether the high levels of MeSA in the headspace of exposed plants were most likely absorbed and re-released from the plant or produced *de novo*. While the results show some *de novo* production, they suggest that the majority of MeSA from exposed plants that was available to aphids as an olfactory cue in our experiments was absorbed then re-released from the leaves/stem. It is still unknown whether uninfested plants can adsorb and re-release MeSA produced by infested neighbors, thus gaining protection without the metabolic costs of producing the compound. The absorption and re-release of volatiles from plant tissues has been shown to affect arthropods and pathogens in several systems, and this mechanism could potentially contribute protective effects to crops in pest management (Himanen et al., 2010; Mofikoya et al., 2019; Camacho-Coronel et al., 2020).

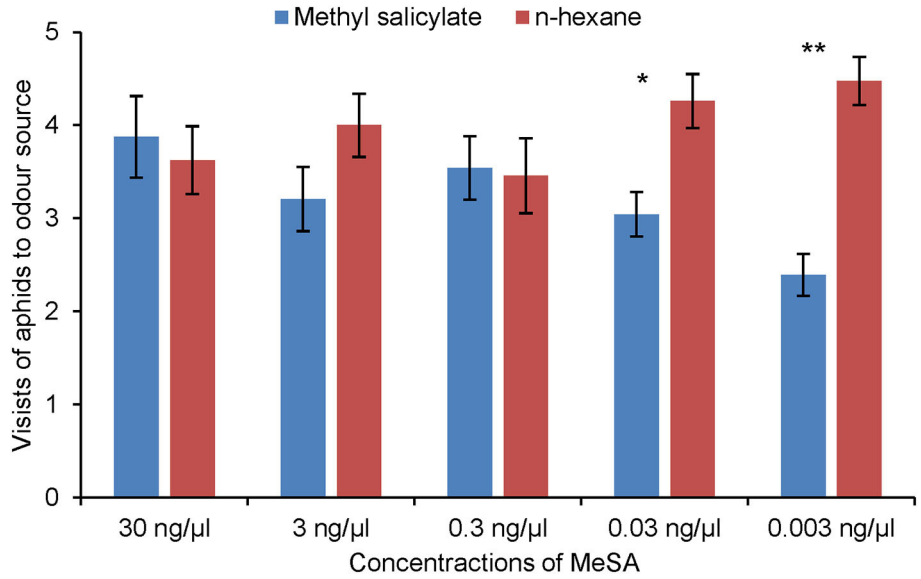

FIGURE 7 | Olfactory response of *R. padi* (mean visits in olfactometer arm ± SE) to different concentrations of MeSA compared with control (hexane). *$p \leq 0.05$; **$p \leq 0.01$ after Wilcoxon matched pairs tests.

Ideally, the dynamics between re-release and *de novo* production after MeSA exposure should be studied over a longer period of plant development and in a field situation.

When aphids have contacted the host plant, they make an assessment of host suitability before settling and initiating the probing and feeding process (Pettersson et al., 2007). The settling bioassay showed that after exposure to MeSA, aphid settling was significantly reduced on exposed plants immediately following the termination of the exposure period (Day 0). A significant reduction in settling on exposed plants was also found at two subsequent time points after termination of exposure Day 1 and Day 3, but on Day 5 there was no significant difference between settling on exposed and unexposed plants. This reduction in settling could be partially explained by a response to MeSA absorbed on the leaves, and the EPG data do suggest responses to plant surface factors at Day 3. However, the settling data do not correlate fully with the aphid's olfactory responses, suggesting that plant exposure to MeSA also induced systemic resistance factors within the plant. These were investigated by the Electrical penetration graph (EPG) study of aphid probing and feeding.

EPG has been widely used to monitor aphid stylet activities on plants and to identify plant tissues where resistance factors against aphids are expressed (Tjallingii, 2006). The EPG data in the current study suggest that exposure to MeSA results in a leaf surface resistance factor, indicated by a contact effect on the initial probing behavior (a longer time from the start of the EPG recording until the first probe) of *R. padi* on exposed compared to unexposed plants at Day 3. A prolonged period before the first probe reflects the effect of repellent or deterrent surface factors (Alvarez et al., 2006). However, this effect was not statistically significant at Day 0 and Day 1, which may indicate slow induction of systemic resistance factors.

Probing and non-probing time, an indication of the suitability of the plant for feeding, was not significantly different at Day 0 suggesting the absence of induced resistance factors on plant surface and in the epidermis and mesophyll. Aphids spent less time in the phloem phase (E12) and had a shorter first sustained feeding period (E12) in exposed compared to unexposed plants. Shorter feeding times and fewer long feeding periods in exposed plants suggest that a relative short 24 h exposure to MeSA can induce changes in barley phloem sap making the plant less suitable for aphids.

After Day 1, there was no significant difference in probing time and sustained phloem feeding time, but there was a significantly longer xylem feeding time in exposed plants. It appears that resistance factors are located in both mesophyll and phloem sieve elements. Resistance in the mesophyll is suggested by the significantly longer all path period (C) and significantly higher number of probes before the first feeding attempt (1st E1) in exposed plants. Resistance in the phloem is suggested by significantly longer salivation (E1) period and lower mean feeding time (E2) in exposed plants. Cao et al. (2014) suggested that the increased salivation period is due to the fact that SA primes phloem clogging processes. Tjallingii (2006) found the salivation period (single E1 and all E1 fractions) was considerably increased in frequency and duration in resistant cultivars of melon and potato, suggesting aphids had difficulties to initiate phloem sap ingestion. Garzo et al. (2002) suggested that a prolonged E1 salivation and reduced E2 indicate a reduced ability to suppress the phloem wound response in resistant cultivars. The consequence of this is that aphids spend more time searching for a suitable feeding site through the mesophyll, and more time in combating resistance factors in the sieve elements.

Resistance factors affecting *R. padi* outside of the phloem have been reported in wild barley (a possible role of hydroxamic acids)

TABLE 1 | Summary of effects of exposure of barley to MeSA on feeding behaviors of *R. padi* on barley plants investigated using electronic penetration graph (EPG) directly after removal of MeSA exposure and at time points 1 and 3 days after removal.

EPG variables	24 h after exposure	Days after exposure		
		1 day	3 days	5 days
Plant surface				
1. Time to 1st probe	–	–	–	–
2. Duration of all non-probing	–	–	++(longer)	–
Epidermis and mesophyll				
3. Total duration of all probes	–	–	++(shorter)	–
4. Number of probing before 1st E1	–	++(higher)	–	–
5. Total duration of all path (C)	–	++(longer)	–	–
6. Number of all path (C)	–	++(higher)	–	–
Xylem				
6. Duration of all G	–	++(longer)	–	–
7. Number of all G	–	++(higher)	–	–
Phloem				
8. Duration of 1st E12 period	++(shorter)	–	–	–
8. Duration of all E12 period	++(shorter)	–	++(shorter)	–
10. Duration of all E1 fractions	–	++(longer)	–	–
11. Duration between 1st E1 and 1st E12	–		++(longer)	–
12. Duration between 1st E1 and 1st E12 w/o non–probing	–	–	++(longer)	–
13. Duration all single E1	–	++(longer)	–	–
14. Duration of 1st E2 fractions	++(shorter)	–	–	–
15. Duration of all E2 fractions	++(shorter)	–	++(shorter)	–
16. Duration of all E	++(shorter)	–	++(shorter)	–
17. Number of all single E1	–	++(higher)	–	–
18. Number of E2 fraction more than 10 min	++(lower)	–	++(lower)	–
19. Number of E2 fraction more than 60 min	++(lower)	–	++(lower)	–
20. Duration of all E1	–	++(longer)	–	–
21. Number of all E12 periods	–	–	++(lower)	–

Effects noted in the table with ++ indicate statistical significant differences between exposed and unexposed plants (see Results and ***Supplementary Table 2****).*

(Niemeyer, 1990). A high gramine content has been detected in barley epidermis and mesophyll parenchyma cells, but not in phloem vessels, causing *R. padi* to take longer to reach the phloem in seedlings with high gramine levels (Zúñiga et al., 1988). There is a clear correlation between the presence of a resistance factor (hydroxamic acids) in the mesophyll and the time cereal aphids including *R. padi* take to reach the phloem. The aphids spent more time searching for a suitable phloem vessel, with increasing frequency of probes and periods of ingestion from xylem (Givovich and Niemeyer, 1995). In a study on the aphid *Sitobion fragariae* on wheat, Ramírez and Niemeyer (1999) concluded that a high concentration of hydroxamic acids is associated with a delay in the time to start salivation in the sieve elements and an increase in the process of salivation itself, suggesting that these compounds may act both in the epidermis/mesophyll and in the phloem.

Three days after treatment (Day 3) *R. padi* allocated significantly less time in probing and more to non-probing on MeSA-exposed plants, suggesting systemic induction of resistance factors. Further evidence for this is the shorter duration of phloem phase (E12) and feeding period (E2) in exposed plants. Long feeding periods (longer than 10 and 60 mins), indicating stable, sustained feeding, were also significantly reduced in exposed plants suggesting a resistance factor in the phloem.

The EPG data show that exposure of barley to MeSA can induce resistance factors against aphids that negatively affect probing even 3 days after exposure has ended. However, 5 days after exposure ended there were no significant effects on the first steps in aphid host selection.

The potential of MeSA to contribute to sustainable plant protection has been demonstrated for a number of different pests and crops, and via different mechanisms including attraction of natural enemies (James, 2003; Sasso et al., 2007, 2009; Mallinger et al., 2011; Orre Gordon et al., 2013; Rubene et al., 2019; Byers et al., 2021). Several studies have shown that releasing

volatile MeSA in cereal crops can reduce aphid populations (Pettersson et al., 1994; Ninkovic et al., 2003; Prinsloo et al., 2007; Xu et al., 2018). The current study confirms this potential against aphid pests, and suggests that MeSA may disrupt aphid host selection at several stages, including olfactory orientation, settling and feeding. The EPG data suggest that exposing plants to MeSA can induce systemic resistance factors that interfere with aphid feeding. Our results support previous hypotheses that MeSA may play a multi-functional role in plant protection against aphids, contributing both with a repellent olfactory effect and an induced plant resistance effect (Pettersson et al., 1994; Ninkovic et al., 2003; Sasso et al., 2007; Digilio et al., 2012). The disruption of aphid host selection induced by MeSA disappeared 5 days after the exposure had been terminated. This suggests that to take advantage of induced plant resistance, a continuous release of MeSA in the crop during the establishment of aphid populations would be preferred over a short-lived application. In fact, the studies reporting reduction of aphid populations in crops used slow-release formulations of MeSA (Pettersson et al., 1994; Ninkovic et al., 2003; Prinsloo et al., 2007; Xu et al., 2018). Multiple modes of action could be an advantage in a pest management scenario since it may reduce the risks of aphids developing genotypes that overcome one of the modes, and offer protection against one of the modes being disrupted, for example by abiotic factors such as extreme weather.

In conclusion this study has revealed new insights into the mechanisms by which methyl salicylate can disrupt aphid host selection and supports its potential as a tool for sustainable management of aphid pests in cereals. A key question for its success will be the development of environmentally and economically sustainable application technologies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

VN designed the study. VN and AGÜ conducted the experiments. RG, SG, and CU conducted chemical analysis. VN analyzed the data. VN and RG wrote the manuscript. All authors read, contributed to revisions, and approved the manuscript.

FUNDING

We greatly appreciate the financial support provided by the following funding agency Formas—the Swedish Research Council for Sustainable Development (Grant No. 2019-00727).

ACKNOWLEDGMENTS

We thank Annhild Andersson and Elham Ahmed for their assistance with rearing insects and plants. We thank Dr. Sate Al Abassi for his assistance with the EPG technique. We also thank Dr. Dimitrije Markovic for constructive discussions. We would also like to thank reviewers for their feedback on the previous version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.710268/full#supplementary-material

REFERENCES

Aerts, N., Pereira Mendes, M., and Van Wees, S. C. M. (2021). Multiple levels of crosstalk in hormone networks regulating plant defense. *Plant J.* 105, 489–504. doi: 10.1111/tpj.15124

Alvarez, A. E., Tjallingii, W. F., Garzo, E., Vleeshouwers, V., Dicke, M., and Vosman, B. (2006). Location of resistance factors in the leaves of potato and wild tuber-bearing Solanum species to the aphid *Myzus persicae*. *Entomol. Exp. Appl.* 121, 145–157. doi: 10.1111/j.1570-8703.2006.00464.x

Boughton, A. J., Hoover, K., and Felton, G. W. (2006). Impact of chemical elicitor applications on greenhouse tomato plants and population growth of the green peach aphid, *Myzus persicae*. *Entomol. Exp. Appl.* 120, 175–188. doi: 10.1111/j.1570-7458.2006.00443.x

Bruce, T. J. A., and Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects—finding the right mix. *Phytochemistry* 72, 1605–1611. doi: 10.1016/j.phytochem.2011.04.011

Byers, J. A., Maoz, Y., Cohen, B., Golani, M., Fefer, D., and Levi-Zada, A. (2021). Protecting avocado trees from ambrosia beetles by repellents and mass trapping (push–pull): experiments and simulations. *J. Pest Sci.* 94, 991–1002. doi: 10.1007/s10340-020-01310-x

Camacho-Coronel, X., Molina-Torres, J., and Heil, M. (2020). Sequestration of exogenous volatiles by plant cuticular waxes as a mechanism of passive associational resistance: a proof of concept. *Front. Plant Sci.* 11:121. doi: 10.3389/fpls.2020.00121

Cao, H. H., Wang, S. H., and Liu, T. X. (2014). Jasmonate- and salicylate-induced defenses in wheat affect host preference and probing behavior but not performance of the grain aphid, *Sitobion avenae*. *Insect Sci.* 21, 47–55. doi: 10.1111/1744-7917.12023

Cooper, W. C., Jia, L., and Goggin, F. L. (2004). Acquired and r-gene-mediated resistance against the potato aphid in tomato. *J. Chem. Ecol.* 30, 2527–2542. doi: 10.1007/s10886-004-7948-9

Digilio, M. C., Cascone, P., Iodice, L., and Guerrieri, E. (2012). Interactions between tomato volatile organic compounds and aphid behaviour. *J. Plant Interact.* 7, 322–325. doi: 10.1080/17429145.2012.727104

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

Garzo, E., Soria, C., Gomez-Guillamon, M. L., and Fereres, A. (2002). Feeding behavior of *Aphis gossypii* on resistant accessions of different melon genotypes (*Cucumis melo*). *Phytoparasitica* 30, 129–140. doi: 10.1007/BF02979695

Givovich, A., and Niemeyer, H. M. (1995). Comparison of the effect of hydroxamic acids from wheat on five species of cereal aphids. *Entomol. Exp. Appl.* 74, 115–119. doi: 10.1111/j.1570-7458.1995.tb01882.x

Glinwood, Gradin, T., Karpinska, B., Ahmed, E., Jonsson, L. M. V., and Ninkovic, V. (2007). Aphid acceptance of barley exposed to volatile phytochemicals differs between plants exposed in daylight and darkness. *Plant Signal. Behav.* 2, 321–326. doi: 10.4161/psb.2.5.4494

Glinwood, R., Ahmed, E., Qvarfordt, E., and Ninkovic, V. (2011). Olfactory learning of plant genotypes by a polyphagous insect predator. *Oecologia* 166, 637–647. doi: 10.1007/s00442-010-1892-x

Glinwood, R. T., and Pettersson, J. (2000). Change in response of Rhopalosiphum padi spring migrants to the repellent winter host component methyl salicylate. *Entomol. Exp. Appl.* 94, 325–330. doi: 10.1046/j.1570-7458.2000.00634.x

Goggin, F. L. (2007). Plant–aphid interactions: molecular and ecological perspectives. *Curr. Opin. Plant Biol.* 10, 399–408. doi: 10.1016/j.pbi.2007.06.004

Hardie, J., Isaacs, R., Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. (1994). Methyl salicylate and (–)-(1 R,5 S)-myrtenal are plant-derived repellents for black bean aphid, *Aphis fabae* Scop. (Homoptera: Aphididae). *J. Chem. Ecol.* 20, 2847–2855. doi: 10.1007/BF02098393

Heil, M., and Ton, J. (2008). Long-distance signalling in plant defence. *Trends Plant Sci.* 13, 264–272. doi: 10.1016/j.tplants.2008.03.005

Himanen, S. J., Blande, J. D., Klemola, T., Pulkkinen, J., Heijari, J., and Holopainen, J. K. (2010). Birch (*Betula* spp.) leaves adsorb and re-release volatiles specific to neighbouring plants—a mechanism for associational herbivore resistance? *New Phytol.* 186, 722–732. doi: 10.1111/j.1469-8137.2010.03220.x

James, D. G. (2003). Synthetic herbivore-induced plant volatiles as field attractants for beneficial insects. *Environ. Entomol.* 32, 977–982. doi: 10.1603/0046-225X-32.5.977

Kaloshian, I., and Walling, L. L. (2005). Hemipterans as plant pathogens. *Annu. Rev. Phytopathol.* 43, 491–521. doi: 10.1146/annurev.phyto.43.040204.135944

Li, X., Schuler, M. A., and Berenbaum, M. R. (2002). Jasmonate and salicylate induce expression of herbivore cytochrome P450 genes. *Nature* 419, 712–715. doi: 10.1038/nature01003

Maffei, M. E., Arimura, G. I., and Mithöfer, A. (2012). Natural elicitors, effectors and modulators of plant responses. *Nat. Prod. Rep.* 29, 1288–1303. doi: 10.1039/c2np20053h

Mallinger, R. E., Hogg, D. B., and Gratton, C. (2011). Methyl salicylate attracts natural enemies and reduces populations of soybean aphids (Hemiptera: Aphididae) in soybean agroecosystems. *J. Econ. Entomol.* 104, 115–124. doi: 10.1603/EC10253

Marchetti, E., Civolani, S., Leis, M., Chicca, M., Tjallingii, W. F., Pasqualini, E., et al. (2009). Tissue location of resistance in apple to the rosy apple aphid established by electrical penetration graphs. *Bull. Insectol.* 62, 203–208.

Mofikoya, A. O., Bui, T. N. T., Kivimäenpää, M., Holopainen, J. K., Himanen, S. J., and Blande, J. D. (2019). Foliar behaviour of biogenic semi-volatiles: potential applications in sustainable pest management. *Arthropod. Plant. Interact.* 13, 193–212. doi: 10.1007/s11829-019-09676-1

Mumm, R., Posthumus, M. A., and Dicke, M. (2008). Significance of terpenoids in induced indirect plant defence against herbivorous arthropods. *Plant Cell Environ.* 31, 575–585. doi: 10.1111/j.1365-3040.2008.01783.x

Niemeyer, H. M. (1990). "Secondary plant chemicals in aphid host interactions," in *Proc. Int. Symp.* Aphid—*Plant Interactions, Populations to Molecules,* eds D. C. Peters, J. A. Webster, and C. S. Chlouber (Oklahoma, OK: Stillwater), 101–111.

Ninkovic, V., Ahmed, E., Glinwood, R., and Pettersson, J. (2003). Effects of two types of semiochemical on population development of the bird cherry oat aphid *Rhopalosiphum padi* in a barley crop. *Agric. For. Entomol.* 5, 27–34. doi: 10.1046/j.1461-9563.2003.00159.x

Ninkovic, V., Olsson, U., and Pettersson, J. (2002). Mixing barley cultivars affects aphid host plant acceptance in field experiments. *Entomol. Exp. Appl.* 102, 177–182. doi: 10.1046/j.1570-7458.2002.00937.x

Orre Gordon, G. U. S., Wratten, S. D., Jonsson, M., Simpson, M., and Hale, R. (2013). "Attract and reward": combining a herbivore-induced plant volatile with floral resource supplementation—multi-trophic level effects. *Biol. Control* 64, 106–115. doi: 10.1016/j.biocontrol.2012.10.003

Park, S. W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Pettersson, J., Pickett, J. A., Pye, B. J., Quiroz, A., Smart, L. E., Wadhams, L. J., et al. (1994). Winter host component reduces colonization by bird-cherry-oat aphid, *Rhopalosiphum padi* (L.) (homoptera, aphididae), and other aphids in cereal fields. *J. Chem. Ecol.* 20, 2565–2574. doi: 10.1007/BF02036192

Pettersson, J., Tjallingii, W. F., and Hardie, J. (2007). "Host-plant selection and feeding," in *Aphids as Crop Pests*, eds E. H. F. Van and R. Harrington (Wallingford, OX: CABI), 173–195. doi: 10.1079/9780851998190.0087

Pickett, J. A., Bruce, T. J. A., and Glinwood, R. T. (2017). "Chemical ecology," in *Aphids as Crop Pests*, eds E. H. F. Van and R. Harrington (Wallingford, OX: CABI), 148–172. doi: 10.1079/9781780647098.0148

Prado, E., and Tjallingii, W. F. (1997). Effects of previous plant infestation on sieve element acceptance by two aphids. *Entomol. Exp. Appl.* 82, 189–200. doi: 10.1046/j.1570-7458.1997.00130.x

Prinsloo, G., Ninkovic, V., van der Linde, T. C., van der Westhuizen, J., Pettersson, J., and Glinwood, R. (2007). Test of semiochemicals and a resistant wheat variety for Russian wheat aphid management in South Africa. *J. Appl. Entomol.* 131, 637–644. doi: 10.1111/j.1439-0418.2007.01213.x

Ramírez, C. C., and Niemeyer, H. M. (1999). "Salivation into sieve elements in relation to plant chemistry: the case of the aphid *Sitobion fragariae* and the wheat, *Triticum aestivum*," in *Entomologia Experimentalis et Applicata* (Netherlands: Springer), 111–114. doi: 10.1046/j.1570-7458.1999.00472.x

Rubene, D., Leidefors, M., Ninkovic, V., Eggers, S., and Low, M. (2019). Disentangling olfactory and visual information used by field foraging birds. *Ecol. Evol.* 9, 545–552. doi: 10.1002/ece3.4773

Sasso, R., Iodice, L., Digilio, M. C., Carretta, A., Ariati, L., and Guerrieri, E. (2007). Host-locating response by the aphid parasitoid *Aphidius ervi* to tomato plant volatiles. *J. Plant Interact.* 2, 175–183. doi: 10.1080/17429140701591951

Sasso, R., Iodice, L., Woodcock, C. M., Pickett, J. A., and Guerrieri, E. (2009). Electrophysiological and behavioural responses of *Aphidius ervi* (Hymenoptera: Braconidae) to tomato plant volatiles. *Chemoecology* 19, 195–201. doi: 10.1007/s00049-009-0023-9

Shulaev, V., Silverman, P., and Raskin, I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385, 718–721. doi: 10.1038/385718a0

Smart, L. E., Aradottir, G. I., and Bruce, T. J. A. (2014). "Role of semiochemicals in integrated pest management," in *Integrated Pest Management: Current Concepts and Ecological Perspective* (Amsterdam: Elsevier), 93–109. doi: 10.1016/B978-0-12-398529-3.00007-5

Smith, C. M., and Boyko, E. V. (2007). The molecular bases of plant resistance and defense responses to aphid feeding: current status. *Entomol. Exp. Appl.* 122, 1–16. doi: 10.1111/j.1570-7458.2006.00503.x

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Stewart-Jones, A., and Poppy, G. M. (2006). Comparison of glass vessels and plastic bags for enclosing living plant parts for headspace analysis. *J. Chem. Ecol.* 32, 845–864. doi: 10.1007/s,10886-006-9039-6

TIBCO Software Inc (2018). *Statistica (data analyse software system) version 13.* Available online at: http://tibco.com

Tjallingii, W. F. (1986). Wire effects on aphids during electrical recording of stylet penetration. *Entomol. Exp. Appl.* 40, 89–98. doi: 10.1111/j.1570-7458.1986.tb02159.x

Tjallingii, W. F. (1988). "Electrical recording of stylet penetration activities," in *Aphids, Their Biology, Natural Enemies and Control*, eds A. K. Minks, and P. Harrewijn (Amsterdam: Elsevier Science Publishers), 95–108.

Tjallingii, W. F. (1990). "Continuous recording of stylet penetration activities by aphids," in *Aphid–Plant Genotype Interactions*, eds R. K. Campbell, and R. D. Eikenbary (Amsterdam: Elsevier), 89–99.

Tjallingii, W. F. (2006). Salivary secretions by aphids interacting with proteins of phloem wound responses. *J. Exp. Bot.* 57, 739–745. doi: 10.1093/jxb/erj088

Tripathi, D., Raikhy, G., and Kumar, D. (2019). Chemical elicitors of systemic acquired resistance—salicylic acid and its functional analogs. *Curr. Plant Biol.* 17, 48–59. doi: 10.1016/j.cpb.2019.03.002

van Dam, N. M., Wondafrash, M., Mathur, V., and Tytgat, T. O. G. (2018). Differences in hormonal signaling triggered by two root-feeding nematode species result in contrasting effects on aphid population growth. *Front. Ecol. Evol.* 6:88. doi: 10.3389/fevo.2018.00088

Vucetic, A., Dahlin, I., Petrovic-Obradovic, O., Glinwood, R., Webster, B., and Ninkovic, V. (2014). Volatile interaction between undamaged plants affects tritrophic interactions through changed plant volatile emission. *Plant Signal. Behav.* 9:e29517. doi: 10.4161/psb.29517

Wiktelius, S., Weibull, J., and Pettersson, J. (1990). "Aphid host plant ecology: the bird cherry–oat aphid as a model," in *Aphid–Plant Genotype Interactions*, eds. R. K. Campbell and R. D. Eikenbary (Amsterdam: Elsevier), 21–36.

Xu, Q., Hatt, S., Lopes, T., Zhang, Y., Bodson, B., Chen, J., et al. (2018). A push–pull strategy to control aphids combines intercropping with semiochemical releases. *J. Pest Sci.* 91, 93–103. doi: 10.1007/s10340-017-0888-2

Zúñiga, G. E., Varanda, E. M., and Corcuera, L. J. (1988). Effect of gramine on the feeding behavior of the aphids *Schizaphis graminum* and *Rhopalosiphum padi*. *Entomol. Exp. Appl.* 47, 161–165. doi: 10.1111/j.1570-7458.1988.tb01131.x

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 August 2021
doi: 10.3389/fpls.2021.691768

Seed Treatment With Jasmonic Acid and Methyl Jasmonate Induces Resistance to Insects but Reduces Plant Growth and Yield in Rice, *Oryza sativa*

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *07 April 2021*
Accepted: *19 July 2021*
Published: *16 August 2021*

Citation:
Bhavanam S and Stout M (2021) Seed Treatment With Jasmonic Acid and Methyl Jasmonate Induces Resistance to Insects but Reduces Plant Growth and Yield in Rice, Oryza sativa. Front. Plant Sci. 12:691768. doi: 10.3389/fpls.2021.691768

When applied exogenously to plants, jasmonates [i.e., jasmonic acid (JA) and methyl jasmonate (MeJA)] increase plant resistance against herbivores, and their use in pest management has been suggested. For integration into pest management programs, the benefits of the resistance induced by jasmonates must outweigh the costs of jasmonates on plant growth and yield. A previous field study in rice found that seed treatment with MeJA reduced densities of the rice water weevil, *Lissorhoptrus oryzophilus,* but also reduced plant growth. Yields from MeJA plots were similar to yields from control plots. Because this study was conducted under field conditions with natural levels of pest populations, it was unclear whether effects on growth and yield were due to direct effects of MeJA treatment on the plant or due to lower reductions in rice water weevil densities. Therefore, the present study was designed to characterize the effects of JA and MeJA seed treatment on rice plant growth and yield in a pest-free environment under greenhouse conditions. Seed treatment with 2.5 mM JA and 2.5 mM MeJA enhanced resistance in rice plants to rice water weevils when plants were exposed to weevils 30 days after planting. Seed treatment with MeJA reduced seedling emergence and plant height at 4 and 14 days after planting, respectively, compared to JA and control treatments. However, numbers of tillers per plant at 45 days after planting and days to heading were unaffected by jasmonate seed treatment. Of four yield components (panicles per plant, filled grains per panicle, percent unfilled grains, and filled grain mass) that were measured, only filled grain mass was reduced by seed treatment. Plants grown from MeJA-treated seeds showed 31% lower grain masses compared to plants grown from control-treated seeds. Thus, the effects of seed treatment with MeJA on plant growth were stronger immediately post-treatment and subsided over time, such that plant growth mostly recovered 6 weeks after treatment. At maturity, MeJA may reduce one but not all components of yield. Despite similar effects on rice water weevil resistance, the negative effects of JA seed treatment on plant growth and yield were smaller compared to MeJA seed treatment.

Keywords: plant elicitors, jasmonates, induced resistance, trade-offs, rice water weevil

INTRODUCTION

Jasmonic acid (JA), its methylated derivative methyl jasmonate (MeJA) and its conjugate with isoleucine (JA-Ile), collectively referred to as jasmonates, are phytohormones that regulate several physiological and developmental processes in plants (Ali and Baek, 2020). Jasmonates play a critical role in plant resistance against insect pests, pathogens, and abiotic stresses (Ali and Baek, 2020). Feeding by chewing insects, necrotrophic pathogens, and certain types of abiotic stresses activate the JA signaling pathway (Raza et al., 2020), which stimulates direct defense in plants through formation of morphological structures, such as trichomes, or biochemical responses, such as production of plant secondary metabolites and resistance-related enzymes, all of which can interfere with preference and performance of insect pests (Walling, 2000). Moreover, jasmonate-induced defenses include changes in the qualitative and quantitative composition of plant volatile compounds that can directly affect herbivores and attract insect natural enemies; the latter can lead to increased parasitization and predation rates of herbivores, thereby providing indirect resistance to plants against herbivores (Lou et al., 2005; Okada et al., 2015).

Over the years, increased understanding of defense signaling pathways and induced resistance in plants has led to the development of natural or synthetic elicitors that can mimic responses to natural herbivory (Karban and Kuc, 1999). Exogenous application of JA, like feeding by chewing insects, rapidly increases the levels of endogenous JA, which in turn triggers the expression of defense-related genes (Pauwels et al., 2009; Benevenuto et al., 2019). Exogenous MeJA also elicits JA-related responses after MeJA is demethylated to JA in plants (Wu et al., 2008). Exogenous application of JA and MeJA through foliar sprays or soil drenching enhances resistance against a broad spectrum of insects in a wide range of agricultural crops under greenhouse and field conditions (Rodriguez-Saona et al., 2001; Choh et al., 2004; Hamm et al., 2010; Nabity et al., 2013; Gordy et al., 2015; Haas et al., 2018; Nouri-Ganbalani et al., 2018). Recently, studies have attempted to use application of JA or MeJA to seeds before germination to induce resistance in plants. Seed treatments with plant elicitors have mostly been investigated in tomato, *Solanum lycopersicum* L., with a few additional studies on cabbage, *Brassica oleracea* and rice, *Oryza sativa* L. These studies provide evidence that seed treatment with JA or MeJA can increase resistance against herbivores in plants (Worrall et al., 2012; Paudel et al., 2014; Strapasson et al., 2014; Haas et al., 2018; Kraus and Stout, 2019). However, variations in JA-induced responses existed among cultivars within a plant species as well as among plant species as seeds of different varieties were not equally receptive to seed treatment with plant elicitors (Smart et al., 2013; Mouden et al., 2020). Moreover, unlike applications of plant elicitors by foliar spray or soil drench, which are usually made at or near the time of pest infestation, seed treatments are made before seeds are planted, and hence, the duration of induction of defenses by seed treatments is a critical consideration for the efficacy of seed treatments (Worrall et al., 2012; Haas et al., 2018; Kraus and Stout, 2019).

The utility of JA and MeJA as elicitors in insect pest management programs is potentially limited because, while exogenous jasmonates may induce resistance to pests, they may incur a cost to the plant by reducing plant growth and yield. These costs may occur through trade-offs in resource allocation; that is, plants have limited resources that can be invested either in growth or defense and increased allocation of resources to one trait limits the resources available for the other (Jimenez-Aleman et al., 2017; Guo et al., 2018). Thus, diversion of resources away from growth and to defense may have an impact on plant vegetative and reproductive growth. Another possible mechanism for reduced growth and yields in jasmonate-treated plants involves hormonal cross talk between JA signaling and other plant hormones (Hou et al., 2013). The costs of induced resistance to plants may be manifested in the form of delays in seed germination and post-germination seedling development, as seen in many cereal crops (Zwar and Hooley, 1986; Norastehnia et al., 2007; Lahuta et al., 2018) but not in cowpea, *Vigna unguiculata* (L.) Walp and soybean, *Glycine max* (L.) Merr (Muttucumaru et al., 2013). In wild mustard, *Brassica kaber* D.C., exogenous application of JA increased the activities of trypsin inhibitor (TI) and peroxidase (POD) and the levels of glucosinolates but also increased the time to first flower (Cipollini and Sipe, 2001). However, JA and MeJA application may not always incur costs to growth because of increased allocation to defense (Van Dam et al., 2004). A few studies have shown that, after initial reductions in plant growth and development, applications of jasmonates have no effect (Worrall et al., 2012) or a positive effect (Barbosa et al., 2008; Feng et al., 2012) on plant growth and yield. To integrate the use of plant elicitors in pest management programs, further studies are needed to characterize the effects of JA and MeJA application on plant growth and yield parameters under pest-free conditions, which may facilitate the development of strategies to mitigate negative effects of plant elicitors on plant growth and reproduction.

The rice water weevil, *Lissorhoptrus oryzophilus* Kuschel, is the most important insect pest of rice in the United States (Villegas et al., 2021). Adults feed on leaves of young rice plants leaving longitudinal scars on the leaf blades (Stout et al., 2002; Zou et al., 2004). Adult oviposition largely commences after flooding of rice fields (Stout et al., 2002). Eggs are laid inside the tissues of submerged leaf sheaths (Grigarick and Beards, 1965; Bowling, 1972). Upon egg hatching, first instars mine the tissue of leaf sheaths for a period of time before moving to the root system and feed on roots to complete larval and pupal development (Bowling, 1972). Pruning of roots by larvae negatively affects plant height, numbers of tillers and panicles, and ultimately, rice yields (Zou et al., 2004). The yield losses caused by rice water weevil infestation can reach up to 30% in untreated plots in southern United States (Villegas et al., 2021). Among different management practices, management of rice water weevil by chemical insecticides is the most commonly used tactic by growers (Aghaee and Godfrey, 2014), probably because of lack of effective alternative management tactics (Vyavhare et al., 2016). The use of plant elicitors to enhance

resistance in plants to rice water weevil may reduce pest damage early in the season and thereby reduce the frequent application of insecticides, delay development of insect resistance to insecticides, and reduce harmful effects on non-target organisms.

In a previous study, Kraus and Stout (2019) showed that, under field conditions, MeJA-treated rice seeds enhanced resistance to the rice water weevil in rice plants, leading to reductions in population densities of rice water weevils on rice roots. The resistance induced by the MeJA seed treatment came, however, with a cost to plant growth. Seedling emergence was delayed, plant biomass was reduced, and time from emergence to heading increased in plants treated with MeJA plots relative to control plots. However, yields, measured in terms of per-panicle grain mass, were similar in plants grown from MeJA-treated seeds and plants from untreated seeds. In addition, yields in MeJA-treated plants were lower than yields from plants treated with insecticide to protect them from rice water weevils. These yield results were observed despite the fact that plants grown from MeJA-treated seeds harbored fewer numbers of rice water weevil relative to controls, raising the question of whether yields were directly impacted by MeJA treatment. The experimental design of the study was not sufficient to tease apart whether reductions in yield in MeJA-treated plants were due to elicitor treatment or lower reductions in RWW densities in the MeJA treatment compared to the insecticide treatment. Therefore, the present study was designed to characterize the effects of JA and MeJA seed treatment on plant vegetative and reproductive traits in pest-free conditions in a greenhouse, while simultaneously confirming the JA- and MeJA-induced resistance against rice water weevils.

MATERIALS AND METHODS

Seed Treatment

Seeds of the rice cultivar "Cheniere," obtained from the Louisiana State University (LSU) Agricultural Center Rice Research Station, Crowley, Louisiana, were used in all experiments. For seed treatments, 2.5 mM solutions of both JA (J0004-2200N-KF, Sigma-Aldrich, St. Louis, Missouri) and MeJA (Lot# MKCF2439, Sigma-Aldrich, St. Louis, Missouri) were prepared. The concentration of jasmonates used in this study was based on the results of Kraus and Stout (2019). Solutions were prepared by dispersing 75 µl of JA or 82 µl MeJA in 150 ml distilled water that contained 0.1% v/v (150 µl) of Tween-20 (Sigma-Aldrich, St. Louis, Missouri) in a 250 ml Erlenmeyer flask. The control solution included 0.1% v/v Tween-20 in the same volume of distilled water. The solutions in the flasks were mixed thoroughly on a magnetic stirrer before adding 50 g of rice seeds to the solutions. The flasks containing the seeds and treatment solutions were covered with aluminum foil, then placed on an orbital shaker, and gently shaken for 24 h at room temperature. Following the soaking, rice seeds were washed three times in distilled water, dried with paper towels, and used immediately in experiments.

Greenhouse Experiment

A greenhouse experiment was conducted to characterize the effects of seed treatment on plant growth and yield while also verifying the induction of resistance to rice water weevil by seed treatments. The experiment was conducted in a greenhouse facility located near the campus of LSU, Baton Rouge, Louisiana. Plastic pots (1.9 L) that were filled with soil mix consisting of two parts topsoil, one part sand, and one part peat moss were used as the growth medium. Fifteen pots were prepared for each treatment (control, 2.5 mM JA, and 2.5 mM MeJA seed treatments) and, in each pot, five seeds were placed in the soil mix. Each pot was considered an experimental unit. Out of 15 pots prepared for each treatment, five pots were used to verify induction of resistance by the seed treatments and the remaining pots were used to characterize the effects of seed treatments on rice growth and yield. On the 14th day after planting, plants were thinned to one per pot and fertilized with approximately 2 g of 13-13-13 controlled release fertilizer (Carl Pool Products, Gladewater, Texas). Greenhouse temperature was maintained between 25 and 30°C. All plants were grown under ambient light and watered as needed.

Five pots of each treatment were used to verify the induction of resistance to rice water weevils by seed treatments as previously reported by Kraus and Stout (2019). This experiment was initiated 30 days after seed treatment. By this time, plants possessed one tiller. Rice water weevil adults were collected from research plots at LSU AgCenter H. Rouse Caffey Rice Research Station, Crowley, LA, on the day of experiment. Adults were kept in a Mason jar and provided with rice leaves and water. Five cylindrical cages, each consisting of a frame made of chicken wire (46 cm diameter × 61 cm height) covered with fine mesh, were placed on a wooden basin that was lined with a plastic pond liner to allow flooding of basins. A single pot from each of the three treatments was randomly selected and placed inside each cage; thus, each cage contained one pot of each of the three treatments. The wooden basin was filled with water up to a height of 25 cm. Nine adult rice water weevils (6 females and 3 males) were introduced into each cage, and the cage was covered with mesh. A total of five cages (replicates) were established in this manner. Adults were allowed to feed, mate, and oviposit inside the cages for 5 days. On day 5, the three pots inside a cage were removed and surviving adults found on the plants were killed. The plants were carefully removed from the soil, and roots were rinsed to remove the soil. Each plant was placed in a test tube that was filled with water and labelled appropriately. The test tubes with rice plants were brought to the lab and held in an environmentally-controlled room (25°C). The following day, each plant was vigorously shaken in the water in the test tube and the water was poured into a Petri dish. The numbers of first instar rice water weevils found in each dish were counted. Plants were returned to test tubes and tubes refilled with water. This procedure was repeated every day until no additional larvae were found in test tubes. The numbers of larvae emerging in each test tube were summed, and the sum was used for analyses.

To characterize the effects of seed treatment with JA and MeJA on rice plant growth and yield, several agronomically important plant traits were measured at appropriate time points during plant development from the 10 replicates of each treatment, as follows:

i. Seedling emergence: All pots of each treatment were observed for seedling emergence (visible appearance of coleoptile above the soil) on the fourth day after planting. For each pot, the number of emerged seedlings was counted and percent emergence was calculated using the formula,

$$\text{Percent emergence} = \frac{\text{Number of emerged seedlings}}{\text{Number of seeds placed in a pot}} \times 100$$

ii. Plant height: Plant height was measured on the 14th day after planting and before thinning. For all emerged seedlings in a pot, plant height from the top of the soil surface to the tip of the uppermost leaf was measured to the nearest 0.1 cm. Mean plant height per pot was calculated and used for analysis.

iii. Tillers: Data on numbers of tillers per plant were recorded on the 45th day after planting.

iv. Days to heading: Beginning on the 55th day after planting, plants were observed daily for heading (panicle exertion from the boot). For each plant, the date of first heading was noted and the number of days from planting to heading was calculated.

v. Yield components: At harvest (removal of grain-bearing panicles from the plant), several components of plant yield were recorded. The number of panicles on each plant was counted. Then, all panicles on a plant were harvested and each panicle was placed in a separate labelled coin envelope. Panicles were oven-dried at 65°C for 7 days. After drying, individual panicles were hand-threshed. For each panicle, filled grains were separated from unfilled grains and the number of filled and unfilled grains was recorded. The same procedure was followed for all panicles of a plant. The number of filled grains and unfilled grains of a plant was totaled. Filled grains per panicle were calculated as below:

$$\text{Filled grains per panicle} = \frac{\text{Total number of filled grains of a plant}}{\text{Number of panicles of a plant}}$$

Percent unfilled grains were calculated as below:

$$\text{Percentage unfilled grains} = \frac{\text{Total numbers of unfilled grains of a plant}}{\text{Total numbers of grains (unfilled + filled) of a plant}} \times 100$$

Mass of filled grains per plant was also measured.

Laboratory Study

A separate study was conducted to assess in more detail the effects of JA and MeJA seed treatments on rice seed germination. Seeds were subjected to the three treatments described above. From each treatment, 20 seeds were randomly selected and placed in 4 rows of 5 seeds each in a square Petri dish (9 × 9 cm) that was lined with double-layered moistened germination paper (Anchor Paper Company, St. Paul, Minnesota). All seeds were oriented in the same direction. Seeds were covered with a VWR-light duty tissue wiper (VWR International LLC, Pennsylvania), and lids were placed on the Petri dishes. Nine Petri dishes for the control treatment (0 mM) and three Petri dishes for each of the other two treatments (2.5 mM MeJA and 2.5 mM JA) were prepared. Each Petri dish was treated as a replicate, and three replicates of each treatment were placed in separate plastic boxes (36 cm length × 20 cm width × 12.5 cm height). In addition, three Petri dishes of the control treatment were placed in each of the boxes with the dishes of the MeJA and JA treatments. Each plastic box was closed with a lid. To maintain conditions of high humidity, a wet paper towel was placed at the bottom of each plastic box before Petri dishes were placed on boxes. All the plastic boxes were placed in an incubator kept at 27°C in dark. On the fourth day after seed placement, Petri dishes were removed from the incubator and data on seed germination and lengths of root and shoot were collected. For each Petri dish, germination percentage was calculated by dividing total number of germinated seeds (seeds that possessed a radicle at least 1 mm in length) by total number of seeds placed and multiplied by 100. In each Petri dish, germinated seeds were separated, and for each seed, length of root and shoot were measured with a ruler to the nearest mm. The means per Petri dish were calculated.

Statistical Analysis

To determine whether seed treatment increased plant resistance to rice water weevil, a generalized linear mixed model with Poisson distribution and log link was performed. In the model, the seed treatment was included as a fixed effect and cage as a random effect. Data on seedling emergence, plant height, number of tillers per plant, days to heading, panicle densities per plant, filled grains per panicle, percentage unfilled grains, and mass of filled grains per plant were analyzed using one-way MANOVA followed by univariate one-way ANOVA and multiple pairwise comparisons for each variable. MANOVA was used to test for overall differences among treatments because there were several dependent variables that may have been correlated, and using separate ANOVAs would have increased the risk of Type I errors. However, for one of the dependent variables (mass of filled grains), the assumption of homogeneity of variances was violated even after transformation. Although MANOVA is typically robust enough that unequal variances can be ignored, a non-parametric Welch test, which can be used in cases in which variances are unequal but distributions are normal, was also performed on filled grain mass. The dependent variable, seedling emergence, was not normal even after transformation, and hence, a Kruskal-Wallis test was conducted followed by *post-hoc* multiple comparison of means using Dunn's test.

For the laboratory study, there were no differences in germination between controls placed in separate boxes and control dishes placed in boxes with MeJA- and JA-treated seeds, and hence, all the data from controls were pooled. Data on germination percentage, root length, and shoot length were

analyzed using ANOVA followed by Tukey's HSD multiple mean comparison test. All data were checked for normality and homogeneity in variances using Shapiro-Wilk's test and Levene's test, respectively. Data analyses were performed in RStudio version 1.4 (RStudio Team, 2021). All graphs were prepared in RStudio using packages "cowplot" (Wilke, 2020), "ggplot2" (Wickham, 2016), and "ggpur" (Kassambara, 2020).

RESULTS

Greenhouse Experiment

The numbers of first instar rice water weevils emerging from plants grown from seeds treated with MeJA ($z = -2.94$, $p = 0.009$) or JA ($z = -3.14$, $p = 0.005$) were lower than the numbers of first instars that emerged from plants grown from untreated seeds (**Figure 1**), verifying that seed treatment with MeJA- and JA-induced resistance to rice water weevil in rice. Seed treatment with MeJA and JA reduced emergence of rice water weevil larvae by 25 and 26%, respectively, compared to the control.

The results of the MANOVA revealed a significant effect of treatment on plant growth parameters ($F_{16, 42} = 2.26$, $p = 0.02$). Seed treatment with MeJA reduced the emergence of seedlings (measured at 4 days of planting) by 48% relative to control ($\chi^2 = 14.34$, $df = 2$, $p = 0.0008$; **Figure 2A**). The effects of seed treatment with MeJA were also detected on plant height at 14 days of planting ($F_{2, 27} = 5.17$, $p = 0.01$; **Figure 2B**). Plants grown from seeds treated with MeJA were approximately 22% shorter, on average, compared to those grown from control seeds. Mean numbers of tillers produced per plant at 45 days after planting were not affected by seed treatment with MeJA or JA ($F_{2, 27} = 0.25$, $p = 0.78$; **Figure 2C**). Plants grown from MeJA- and JA- treated seeds took similar amounts of time to reach heading as plants grown from untreated seeds ($F_{2, 27} = 0.77$, $p = 0.47$; **Figure 2D**). Significant effects of seed treatment with MeJA and JA were observed on one but not all yield-related traits. Total numbers of panicles on plants grown from MeJA- and JA-treated seeds did not differ significantly from numbers on control plants ($F_{2, 27} = 0.41$, $p = 0.66$; **Figure 3A**). There were no significant differences among treatments in numbers of filled grains per panicle ($F_{2, 27} = 3.06$, $p = 0.06$; **Figure 3B**) and percent unfilled grains ($F_{2, 27} = 1.32$, $p = 0.28$; **Figure 3C**). However, the percentages of unfilled grains were 32% higher in the MeJA treatment and 18% higher in the JA treatment relative to control. The numbers of filled grains per panicle were reduced in plants developed from seeds treated with MeJA and JA by 37 and 21%, respectively. Although the differences in numbers of filled grains per panicle and percentage of unfilled grains were not statistically significant, the combination of reductions in these two parameters resulted in a significant reduction in mass of filled grains per plant in the MeJA-treated plants compared to JA seed-treated plants and control plants ($F_{2, 27} = 3.64$, $p = 0.04$; **Figure 3D**). The mass of filled grains in JA-treated plants was intermediate and did not differ significantly from masses of filled grains in control- or MeJA-treated plants.

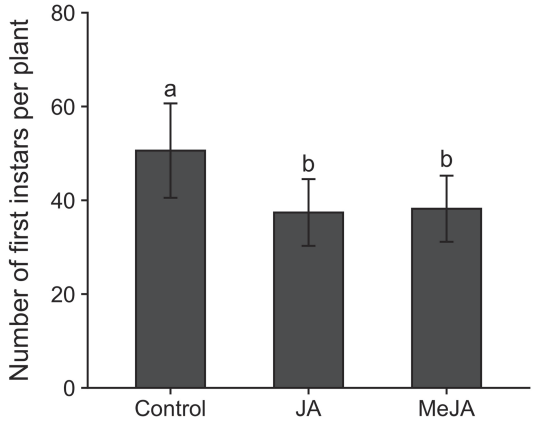

FIGURE 1 | Mean (± SE) numbers of first instar rice water weevils, *Lissorhoptrus oryzophilus,* that emerged from plants grown from untreated seeds or from seeds treated with either jasmonic acid (JA; 2.5 mM) or methyl jasmonate (MeJA; 2.5 mM). Bars accompanied by different lowercase letters represent means that differed significantly ($p < 0.05$).

Laboratory Study

Treatment of rice seeds with 2.5 mM JA and 2.5 mM MeJA significantly reduced percent seed germination (**Table 1**) at 4 days after placing seeds on germination paper. Post-germination seed development (growth of roots and shoots) was significantly reduced by seed treatment with JA and MeJA (**Table 1**). The reductions in lengths of root and shoot were significantly greater in the MeJA than in the JA seed treatment.

DISCUSSION

The utility of elicitor seed treatment for a given crop will depend on the balance of the positive effects of elicitors (reductions in yield-reducing pest populations) and the negative effects of elicitors (reductions in yield resulting from activation of defense-related pathways). This study was undertaken to determine the possible effects of jasmonate seed treatments on the growth and yield of rice plants in an environment lacking a major pest, the rice water weevil, while also investigating whether seed treatment with MeJA and JA enhances resistance in rice against rice water weevil. As reported earlier by Kraus and Stout (2019), the application of jasmonates to rice seeds enhanced the resistance of rice plants to rice water weevils when exposed to weevils 30 days after seed treatment, when plants were producing their first tiller. Seed treatment reduced the numbers of first instar weevils emerging from plants by about 25% relative to controls, a level of induction similar to that found by Kraus and Stout (2019) in both the greenhouse and the field. Kraus and Stout (2019) also reported that MeJA-induced resistance declined over time, with resistance stronger when plants were infested at 15 days after seed treatment than at 30 days after seed treatment (as in this study). Paudel et al. (2014) reported

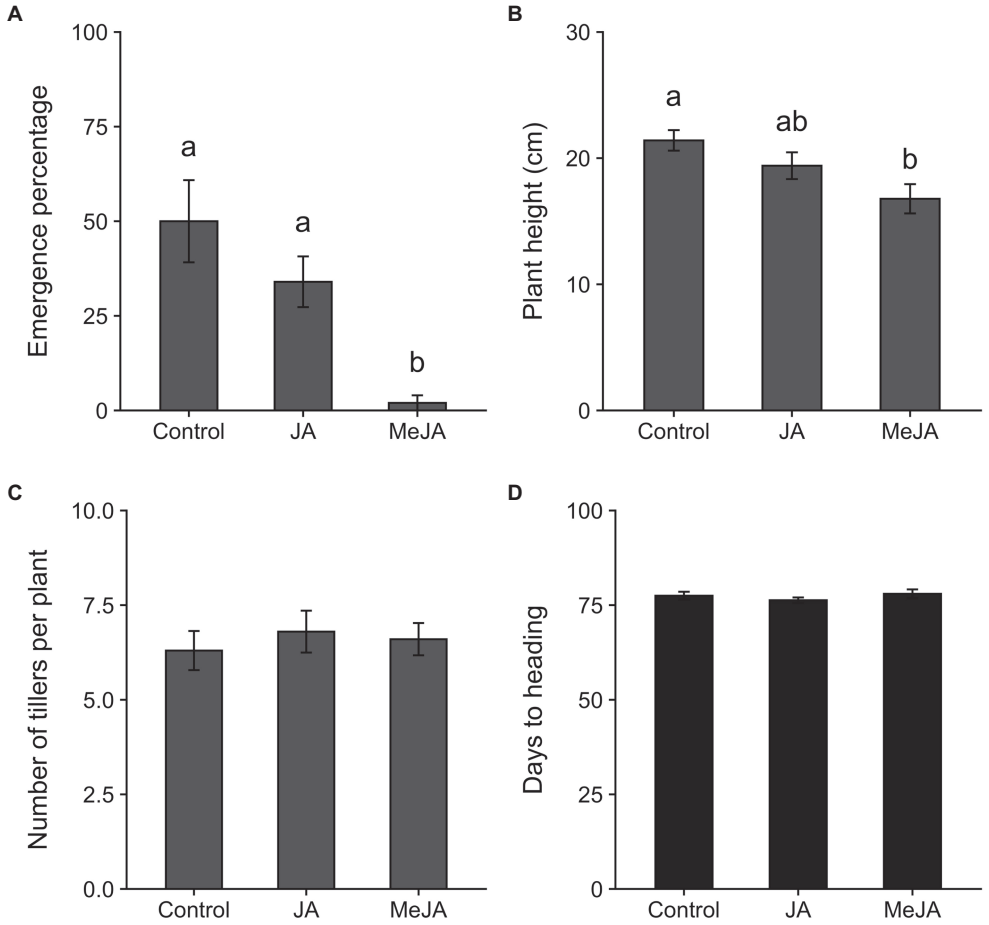

FIGURE 2 | Effects of seed treatment with 2.5 mM jasmonic acid (JA), 2.5 mM methyl jasmonate (MeJA) and control (0 mM) on seedling emergence at 4 days after planting **(A)**; plant height at 14 days after planting **(B)**; numbers of tillers per plant at 45 days after planting **(C)**; and days to heading **(D)**. Bars represent means ± SE. Within each graph, different lowercase letters above the bars indicate significant difference among treatments ($p < 0.05$).

that increased resistance by MeJA treatment in tomato plants decreased over time, but plants developed from MeJA-treated seeds were resistant to tomato fruitworm, *Helicoverpa zea* Boddie, for as long as 75 days after seed treatment.

The mechanisms of induced resistance were not investigated in this study, but treatment of seeds with JA and MeJA may have increased the activities of resistance-related enzymes or the production of secondary metabolites, such as phenolics, that in turn may have interfered with the ovipositional preference of female rice water weevils or the survival and emergence of first instar weevils from plants, or both. Elevated activities of plant defense enzymes after application of JA and MeJA have been reported in other studies. For example, Paudel et al. (2014) reported higher levels of polyphenol oxidase (PPO) in tomato plants treated as seeds with MeJA. In larch, *Larix olgensis* Henry, treatment with JA or MeJA reduced the survival and pupal weight of the gypsy moth, *Lymantria dispar* L., and these increases in resistance were associated with increased levels of plant defense proteins, including PPO, phenylalanine ammonia lyase, TI, and chymotrypsin inhibitor (Jiang and Yan, 2018). Similarly, in rice, changes in total plant phenolics and elevated activities of POD and PPO in response to feeding by the fall armyworm, *Spodoptera exigua* Hubner, have been observed (S.B., unpublished data). Further studies will be needed to determine the full range of resistance-related traits stimulated by jasmonate seed treatments in rice.

This study provided clear evidence that the induction of resistance to rice water weevils by jasmonate seed treatment was accompanied by reductions in plant growth in the absence of pests. These effects on plant growth were particularly severe immediately following treatment of seeds. In the greenhouse experiment, only 2% of seedlings had emerged from soil in the MeJA treatment at 4 days after planting, whereas 33% and 50% of seeds had emerged in the JA and control treatments, respectively. In the laboratory study, >80% of seeds had germinated in both the JA and MeJA treatments at 4 days, but the germination percentage was still lower in these treatments relative to control (**Table 1**). In addition, lengths of plant roots (radicles) and shoots (coleoptiles) were reduced by 50–70% by JA and MeJA treatments, with reductions in lengths greater in the MeJA treatment than in the JA treatment. Taken together, the results of both the laboratory and greenhouse experiments

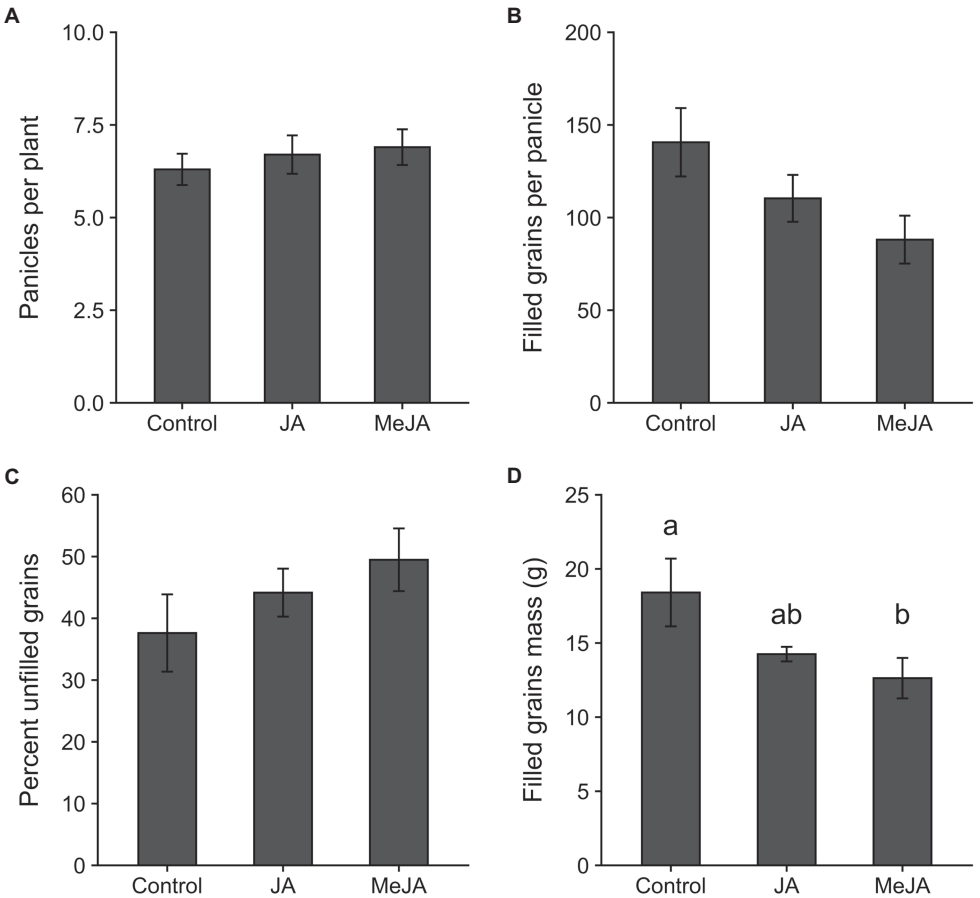

FIGURE 3 | Effects of seed treatment with 2.5 mM jasmonic acid (JA), 2.5 mM methyl jasmonate (MeJA) and control (0 mM) on panicle densities per plant **(A)**; number of filled grains per panicle **(B)**; percent unfilled grains **(C)**; and filled grains mass of each plant **(D)**. Bars represent mean ± SE. Within each graph, different lowercase letters above the bars indicate significant difference among treatments ($p < 0.05$).

TABLE 1 | Effects of seed treatment with 2.5 mM jasmonic acid (JA) and 2.5 mM methyl jasmonate (MeJA) on seed germination and post-germination seed growth in rice, *Oryza sativa* seeds.

Treatment	Germination percentage	Root length (cm)	Shoot length (cm)
Control	96.11 ± 1.11^a	5.36 ± 0.11^a	1.74 ± 0.05^a
2.5 mm JA	86.67 ± 3.33^b	2.40 ± 0.15^b	0.87 ± 0.03^b
2.5 mm MeJA	83.33 ± 3.33^b	1.43 ± 0.17^c	0.50 ± 0.00^c
F	12.45	218.3	122.2
df	2.12	2.12	2.12
p	0.00	<0.0001	<0.0001

The data presented in the table are means ± SE. In each column, different lowercase letter indicates means differ significantly among treatments ($p < 0.05$).

indicate that JA and MeJA at the tested concentration inhibit or delay germination but do not ultimately reduce seed germination. Similarly, delays in seed germination and seedling emergence and decreased root and shoot growth in various agricultural crops due to application of MeJA or JA have been reported in several studies (Norastehnia et al., 2007; Paudel et al., 2014).

Although the effects of jasmonate seed treatment on the germination and early growth of rice plants were quite severe, the effects on growth appeared to erode over time. At 14 days after planting, plants in the MeJA treatment were significantly shorter than controls, but the same was not true of plants in the JA treatment. Reductions in plant heights may have been caused by delays in seedling emergence rather the costs of MeJA application *per se*. At 45 days after planting, the numbers of tillers per plant were unaffected by seed treatment with JA or MeJA. Similarly, days until exertion of the first panicle (heading) did not differ among treatments. Diminished effects of exogenous JA and MeJA over time have been observed in a few other crops. In corn, Feng et al. (2012) reported that JA application to above- and below-ground portions of plants resulted in reductions in root length, root surface area, and root biomass but increased the levels of the plant defense compounds, DIMBOA, and phenolics, 2 weeks after application. However, the effects on plant growth were not detected 4 weeks after application. Moreover, 4 weeks after treatment, shoot and root biomasses of JA-treated plants were greater relative to untreated plants indicating no apparent defense allocation costs. Similarly, treatment of tomato seeds with JA reduced plant

heights but had no long-term effects on plant growth (Worrall et al., 2012). Our results indicate that JA and MeJA seed treatment in rice had a strong inhibitory effect on plant growth immediately after treatment but that the effects of JA and MeJA diminish over time, such that growth in 6-week-old plants had fully recovered.

The most critical potential impacts of jasmonate treatments on plants are those involving negative effects on crop yield. In the current study, four components of yield – panicles per plant, total number of filled grains per panicle, percent unfilled grains, and mass of filled grains per plant – were measured. The first three yield components were not significantly affected by seed treatment with jasmonates, although the numbers of filled grains per panicle trended lower in jasmonate treatments, while percent unfilled grains trended higher in treated plants. In contrast, masses of filled grains per plant, which is a composite of the other three yield components, were reduced (p = 0.04) in the MeJA treatment relative to JA and control treatments when MANOVA followed by pairwise comparisons was performed. The results of the non-parametric Welch test showed no differences in masses of filled grains among JA, MeJA, and control treatments. Thus, it appears that decreases in numbers of filled grains per panicle, increases in percent unfilled grains, and perhaps other effects (e.g., average grain weights) combined to result in marginal decreases in overall yields in plants treated with MeJA as seeds, with yields intermediate (but not significantly different from the control treatment) in JA-treated plants.

Constitutive expression of jasmonate-induced defenses is costly to plants. For example, Cipollini (2007) showed that overexpression of *At*JMT (jasmonic acid carboxyl methyltransferase – an enzyme that methylates JA to MeJA) in *Arabidopsis thaliana* (L.) Heynh resulted in development of stunted plants and reduced seed germination and yields measured as total seed mass. Likewise, Kim et al. (2009), in rice, showed that overexpression of JMT, which increased the levels of MeJA in young panicles, had a drastic effect on rice yield due to decreased numbers of spikelet per panicle and lower filling rates that ultimately resulted in lower grain masses. The above studies attributed decreases in plant yield/fitness to the overproduction of MeJA. In contrast to constitutive resistance, induced resistance, in which resistance-related traits are expressed only when plants are subjected to biotic and abiotic stresses, allows plants to use their limited resources for growth and reproduction when defenses against stresses are not needed. Dietrich et al. (2005) reported that given the time and resources, *A. thaliana* plants treated with the chemical elicitor BION [benzo (1,2,3) thiadiazole-carbothioic acid S-methylester, a mimic of salicylic acid and elicitor of induced resistance against biotrophic pathogens] were able to recover from a single application of chemical elicitor without losses in yield.

Other studies that have investigated the effects of application of MeJA and JA on plant yields have found contrasting results. In tomato, repeated applications of JA at 15 day intervals until harvest resulted in a delay in time to fruit set and reduced the number of fruits per plant, fruit weight, and number of seeds per plant (Redman et al., 2001). Likewise, Agrawal et al. (1999) reported that JA-induced responses reduced the plant fitness in the absence of herbivory in terms of time to first flower formation and number of pollen per flower but not in seed number and seed weight. However, Thaler (1999) reported that, regardless of the presence of herbivory, foliar sprays of JA reduced the number of flowers produced in treated plants relative to untreated plants but had no effect on fruit weight and number of fruits produced per plant. In tomato, seed treatment with 3 mM JA did not reduce the fruit dry weights (Worrall et al., 2012). These differences in JA/MeJA effects of plant fitness and yield are probably due to the differences in concentrations of elicitors, application methods (foliar and seed treatment), time and frequency of elicitor application, plant stage, days to senescence, and levels of herbivory.

The inhibitory effects on seed germination, plant height, and yields were stronger in MeJA-treated plants compared to the JA-treated plants. One possible reason for these differences is that MeJA more effectively reached its target sites in the seed. This may have been the case because the different polarities of the two compounds allowed MeJA to more easily penetrate into rice seeds. Alternatively, because MeJA is a volatile compound and has a lower vapor pressure than JA, it may have penetrated faster and moved more quickly through plant tissues (Farmer and Ryan, 1990; Seo et al., 2001) and more quickly elicited plant defense genes compared to JA (Jiang and Yan, 2018). However, it is important to note that these different effects on growth and yield occurred despite the fact that JA- and MeJA-induced resistance to rice water weevil to roughly equivalent extents, which suggests that different jasmonate elicitors may have differing effects on plant growth, yield, and defense. Qi et al. (2016) found that mutant *os*JMT1 rice plants that overexpress the jasmonic acid carboxyl methyltransferase gene had high levels of MeJA and low levels of JA and JA-Ile. The *os*JMT1 rice lines were resistant to brown planthopper, *Nilaparvatha lugens* Stal nymphs, but adult females were more attracted to *os*JMT1 rice lines compared to wild types, perhaps because of low levels of JA. However, in *os*JMT1 rice lines, plant height and yields were reduced relative to the wild type. The authors suggested that MeJA might have a greater role in plant growth and development than in plant defense, while JA and JA-Ile might be more involved in defense against pathogens and insects.

Explanations for reduced growth and yields in jasmonate-treated plants generally fall into two categories. On the one hand, the applications of jasmonates can result in increased allocation of resources to resistance-related pathways, processes, and metabolites at the expense of growth and reproduction (Walters and Heil, 2007; Guo et al., 2018). On the other hand, negative effects of JA and MeJA treatment on growth and development could result from hormonal cross talk between hormones and hormonal pathways, such as those involving JA and gibberellic acid (GA). Upregulation of the JA signaling pathway has been shown to downregulate the GA pathway (Hou et al., 2013). Downregulation of GA, for example, might have impacted rice germination and seedling growth in two ways in the current study. First, GA regulates the growth rate of plant tissues by affecting cell proliferation and expansion (Achard et al., 2009; Ubeda-Tomás et al., 2009). Second, in

cereal crops, upon imbibition of water, GA present in the seeds induces the expression of an α-amylase gene and other hydrolytic enzymes and proteases involved in germination (Sugimoto et al., 1998). During germination, α-amylase plays a critical role in degradation of the insoluble starch granules to soluble sugars and mobilization of energy reserves to the plant tissues (Palmiano and Juliano, 1972), which are then utilized for growth and elongation of embryonic roots and shoots (Nomura et al., 1969; Jones and Jacobsen, 1991). Treatment of seeds with MeJA decreased activity of α-amylase in germinating seeds that led to a delay in seed germination and reduction in seedling root elongation in corn, *Zea mays* L. (Norastehnia et al., 2007). Similarly, Lahuta et al. (2018) reported that in triticale (*Triticosecale* Witmmack), reduced seed germination, lower fresh and dry weight of embryo and fewer root hairs on embryonic roots were associated with decreased starch degradation by α-amylase in the presence of MeJA.

The results of this current study help clarify results from the recently published field study (Kraus and Stout, 2019) on induction of resistance to rice water weevils by MeJA seed treatment. In this prior study, treatment of rice seeds by MeJA resulted in an approximately 30% reduction in densities of rice water weevil larvae on the roots of rice plants, but reductions in weevil densities by MeJA treatment were not as large as those obtained by treating plants multiple times with a pyrethroid. Reductions or delays in seedling emergence and plant growth resulting from MeJA seed treatment were similar to those observed in the current study. Yields (panicle masses) did not differ between untreated and MeJA-treated plants, but yields were higher from insecticide-treated plots than from MeJA-treated plots. However, it was unclear whether the lower yields from MeJA-treated plots were the result of higher densities of yield-reducing weevil larvae in MeJA-treated plots relative to insecticide-treated plots, or from direct effects of MeJA on plant yield. The results of the current study, which demonstrates reductions in yield in MeJA-treated plants in the absence of weevils, suggest that the lower yields in MeJA-treated plots than in insecticide-treated plots in Kraus and Stout (2019) may have resulted partly from the direct effects of MeJA on plant yield. Further work will be needed to define the conditions under which the benefits of reduced rice water weevil densities in MeJA-treated plants outweigh the costs associated with the treatment. However, the marginal reductions in yield, including the non-significant effect of JA on yield, and the temporary nature of reductions in growth caused by jasmonate treatment in this study suggest that use of elicitors could be integrated into management programs for pests in rice under certain circumstances.

Plant elicitors applied to seeds may be advantageous as they are simple to implement and are not accompanied by the same deleterious effects on non-target organisms and the environment as is the use of insecticides. Further research on the positive effects of elicitor seed treatments on plant defense under field conditions is warranted, including possible activation of indirect defenses and defense priming mechanisms by elicitors. However, cost-effective methods for applying elicitors to seeds will need to be developed. In addition, cultural practices (e.g., seeding rates) may need to be adjusted to compensate for the negative effects of elicitors on plant growth. Finally, as noted above, further research on different crops under field conditions will be needed to further define those situations in which the benefits accruing from reduced densities of pests outweigh reductions in yield resulting from activation of plant defenses, which are likely to be species-specific.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MS conceived the study. SB and MS designed the study and reviewed and edited the manuscript. SB conducted the study, collected and analyzed the data, and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by the Louisiana Agricultural Experiment Station and USDA-NIFA Hatch Project # 94288. This article was approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript No2021-234-36576.

ACKNOWLEDGMENTS

We would like to thank Dr. Marc Cohn at the LSU Department of Plant Pathology and Crop Physiology for his advice and Tyler for his assistance with seed counting.

REFERENCES

Achard, P., Gusti, A., Cheminant, S., Alioua, M., Dhondt, S., Coppens, F., et al. (2009). Gibberellin signaling controls cell proliferation rate in *Arabidopsis*. *Curr. Biol.* 19, 1188–1193. doi: 10.1016/j.cub.2009.05.059

Aghaee, M.-A., and Godfrey, L. D. (2014). A century of rice water weevil (Coleoptera: Curculionidae): a history of research and management with an emphasis on the United States. *J. Integr. Pest Manag.* 5, D1–D14. doi: 10.1603/IPM14011

Agrawal, A. A., Strauss, S. Y., and Stout, M. J. (1999). Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* 53, 1093–1104. doi: 10.1111/j.1558-5646.1999.tb04524.x

Ali, M., and Baek, K.-H. (2020). Jasmonic acid signaling pathway in response to abiotic stresses in plants. *Int. J. Mol. Sci.* 21:621. doi: 10.3390/ijms21020621

Barbosa, M. A. G., Laranjeira, D., and Coelho, R. S. B. (2008). Physiological cost of induced resistance in cotton plants at different nitrogen levels. *Summa Phytopathol.* 34, 338–341. doi: 10.1590/S0100-54052008000400007

Benevenuto, R. F., Seldal, T., Hegland, S. J., Rodriguez-Saona, C., Kawash, J., and Polashock, J. (2019). Transcriptional profiling of methyl jasmonate-induced defense responses in bilberry (*Vaccinium myrtillus* L.). *BMC Plant Biol.* 19:70. doi: 10.1186/s12870-019-1650-0

Bowling, C. (1972). Note on the biology of rice water weevil, *Lissorhoptrus oryzophilus*. *Ann. Entomol. Soc. Am.* 65, 990–991. doi: 10.1093/aesa/65.4.990

Choh, Y., Ozawa, R., and Takabayashi, J. (2004). Effects of exogenous jasmonic acid and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), a functional analogue of salicylic acid, on the egg production of a herbivorous mite *Tetranychus urticae* (Acari: Tetranychidae). *Appl. Entomol. Zool.* 39, 311–314. doi: 10.1303/aez.2004.311

Cipollini, D. (2007). Consequences of the overproduction of methyl jasmonate on seed production, tolerance to defoliation and competitive effect and response of *Arabidopsis thaliana*. *New Phytol.* 173, 146–153. doi: 10.1111/j.1469-8137.2006.01882.x

Cipollini, D. F., and Sipe, M. L. (2001). Jasmonic acid treatment and mammalian herbivory differentially affect chemical defenses and growth of wild mustard (*Brassica kaber*). *Chemoecology* 11, 137–143. doi: 10.1007/s00049-001-8319-4

Dietrich, R., Ploss, K., and Heil, M. (2005). Growth responses and fitness costs after induction of pathogen resistance depend on environmental conditions. *Plant Cell Environ.* 28, 211–222. doi: 10.1111/j.1365-3040.2004.01265.x

Farmer, E. E., and Ryan, C. A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase-inhibitors in plant-leaves. *Proc. Natl. Acad. Sci. U. S. A.* 87, 7713–7716. doi: 10.1073/pnas.87.19.7713

Feng, Y. J., Wang, J. W., Luo, S. M., Fan, H. Z., and Jin, Q. (2012). Costs of jasmonic acid induced defense in aboveground and belowground parts of corn (*Zea mays* L.). *J. Chem. Ecol.* 38, 984–991. doi: 10.1007/s10886-012-0155-1

Gordy, J. W., Leonard, B. R., Blouin, D., Davis, J. A., and Stout, M. J. (2015). Comparative effectiveness of potential elicitors of plant resistance against *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) in four crop plants. *PLoS One* 10:e0136689. doi: 10.1371/journal.pone.0136689

Grigarick, A., and Beards, G. (1965). Ovipositional habits of the rice water weevil in California as related to a greenhouse evaluation of seed treatments. *J. Econ. Entomol.* 58, 1053–1056. doi: 10.1093/jee/58.6.1053

Guo, Q., Major, I. T., and Howe, G. A. (2018). Resolution of growth–defense conflict: mechanistic insights from jasmonate signaling. *Curr. Opin. Plant Biol.* 44, 72–81. doi: 10.1016/j.pbi.2018.02.009

Haas, J., Lozano, E. R., Haida, K. S., Mazaro, S. M., De Souza Vismara, E., and Poppy, G. M. (2018). Getting ready for battle: do cabbage seeds treated with jasmonic acid and chitosan affect chewing and sap-feeding insects? *Entomol. Exp. Appl.* 166, 412–419. doi: 10.1111/eea.12678

Hamm, J. C., Stout, M. J., and Riggio, R. M. (2010). Herbivore- and elicitor-induced resistance in rice to the rice water weevil (*Lissorhoptrus oryzophilus* Kuschel) in the laboratory and field. *J. Chem. Ecol.* 36, 192–199. doi: 10.1007/s10886-010-9751-0

Hou, X. L., Ding, L. H., and Yu, H. (2013). Crosstalk between GA and JA signaling mediates plant growth and defense. *Plant Cell Rep.* 32, 1067–1074. doi: 10.1007/s00299-013-1423-4

Jiang, D., and Yan, S. C. (2018). MeJA is more effective than JA in inducing defense responses in *Larix olgensis*. *Arthropod Plant Interact.* 12, 49–56. doi: 10.1007/s11829-017-9551-3

Jimenez-Aleman, G. H., Machado, R. A., Baldwin, I. T., and Boland, W. (2017). JA-Ile-macrolactones uncouple growth and defense in wild tobacco. *Org. Biomol. Chem.* 15, 3391–3395. doi: 10.1039/C7OB00249A

Jones, R. L., and Jacobsen, J. V. (1991). "Regulation of synthesis and transport of secreted proteins in cereal aleurone," in *International Review of Cytology*. eds. K. W. Jeon and M. Friedlander (San Diego, CA: Academic Press), 49–88.

Karban, R., and Kuc, J. (1999). "Induced resistance against pathogens and herbivores: an overview," in *Induced Plant Defenses Against Pathogens and Herbivores*. eds. A. A. Agrawal, S. Tuzun and E. Bent (St. Paul, MN: APS Press), 1–15.

Kassambara, A. (2020). ggpubr: 'ggplot2' based publication ready plots. R package Version 0.4.0.

Kim, E. H., Kim, Y. S., Park, S.-H., Koo, Y. J., Do Choi, Y., Chung, Y.- Y., et al. (2009). Methyl jasmonate reduces grain yield by mediating stress signals to alter spikelet development in rice. *Plant Physiol.* 149, 1751–1760. doi: 10.1104/pp.108.134684

Kraus, E. C., and Stout, M. J. (2019). Seed treatment using methyl jasmonate induces resistance to rice water weevil but reduces plant growth in rice. *PLoS One* 14:e0222800. doi: 10.1371/journal.pone.0222800

Lahuta, L. B., Zalewski, K., Głowacka, K., Nitkiewicz, B., and Amarowicz, R. (2018). Effect of methyl jasmonate on carbohydrate composition, α-amylase activity and growth of triticale (*Triticosecale* Witmmack) seedlings. *J. Agr. Sci. Tech.* 19, 1127–1137.

Lou, Y. G., Du, M. H., Turlings, T. C. J., Cheng, J. A., and Shan, W. F. (2005). Exogenous application of jasmonic acid induces volatile emissions in rice and enhances parasitism of *Nilaparvata lugens* eggs by the Parasitoid Anagrus nilaparvatae. *J. Chem. Ecol.* 31, 1985–2002. doi: 10.1007/s10886-005-6072-9

Mouden, S., Kappers, I. F., Klinkhamer, P. G., and Leiss, K. A. (2020). Cultivar variation in tomato seed coat permeability is an important determinant of Jasmonic acid elicited defenses against western flower thrips. *Front. Plant Sci.* 11:576505. doi: 10.3389/fpls.2020.576505

Muttucumaru, N., Powers, S. J., Gaur, H. S., Kurup, S., and Curtis, R. H. (2013). Differential defence response due to jasmonate seed treatment in cowpea and tomato against root-knot and potato cyst nematodes. *Nematology* 15, 15–21. doi: 10.1163/156854112X641754

Nabity, P. D., Zavala, J. A., and Delucia, E. H. (2013). Herbivore induction of jasmonic acid and chemical defences reduce photosynthesis in *Nicotiana attenuata*. *J. Exp. Bot.* 64, 685–694. doi: 10.1093/jxb/ers364

Nomura, T., Kono, Y., and Akazawa, T. (1969). Enzymic mechanism of starch breakdown in germinating rice seeds II. Scutellum as the site of sucrose synthesis. *Plant Physiol.* 44, 765–769. doi: 10.1104/pp.44.5.765

Norastehnia, A., Sajedi, R., and Nojavan-Asghari, M. (2007). Inhibitory effects of methyl jasmonate on seed germination in maize (*Zea mays*): effect on α-amylase activity and ethylene production. *Gen. Appl. Plant Physiol.* 33, 13–23.

Nouri-Ganbalani, G., Borzoui, E., Shahnavazi, M., and Nouri, A. (2018). Induction of resistance against *Plutella xylostella* (L.) (Lep.: Plutellidae) by jasmonic acid and mealy cabbage aphid feeding in *Brassica napus* L. *Front. Physiol.* 9:859. doi: 10.3389/fphys.2018.00859

Okada, K., Abe, H., and Arimura, G. (2015). Jasmonates induce both defense responses and communication in monocotyledonous and dicotyledonous plants. *Plant Cell Physiol.* 56, 16–27. doi: 10.1093/pcp/pcu158

Palmiano, E. P., and Juliano, B. O. (1972). Biochemical changes in the rice grain during germination. *Plant Physiol.* 49:751. doi: 10.1104/pp.49.5.751

Paudel, S., Rajotte, E. G., and Felton, G. W. (2014). Benefits and costs of tomato seed treatment with plant defense elicitors for insect resistance. *Arthropod Plant Interact.* 8, 539–545. doi: 10.1007/s11829-014-9335-y

Pauwels, L., Inzé, D., and Goossens, A. (2009). Jasmonate-inducible gene: what does it mean? *Trends Plant Sci.* 14, 87–91. doi: 10.1016/j.tplants.2008.11.005

Qi, J., Li, J., Han, X., Li, R., Wu, J., Yu, H., et al. (2016). Jasmonic acid carboxyl methyltransferase regulates development and herbivory-induced defense response in rice. *J. Integr. Plant Biol.* 58, 564–576. doi: 10.1111/jipb.12436

Raza, A., Charagh, S., Zahid, Z., Mubarik, M. S., Javed, R., Siddiqui, M. H., et al. (2020). Jasmonic acid: a key frontier in conferring abiotic stress tolerance in plants. *Plant Cell Rep.*, 1–29. doi: 10.1007/s00299-020-02614-z [Epub ahead of print]

Redman, A. M., Cipollini, D. F., and Schultz, J. C. (2001). Fitness costs of jasmonic acid-induced defense in tomato, *Lycopersicon esculentum*. *Oecologia* 126, 380–385. doi: 10.1007/s004420000522

Rodriguez-Saona, C., Crafts-Brandner, S. J., Paré, P. W., and Henneberry, T. J. (2001). Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* 27, 679–695. doi: 10.1023/A:1010393700918

RStudio Team (2021). RStudio: Integrated Development Environment for *R*. PBC, Boston, MA. Available at: http://www.rstudio.com/ (Accessed June 25, 2021).

Seo, H. S., Song, J. T., Cheong, J.-J., Lee, Y.- H., Lee, Y.- W., Hwang, I., et al. (2001). Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc. Natl. Acad. Sci. U. S. A.* 98, 4788–4793. doi: 10.1073/pnas.081557298

Smart, L. E., Martin, J. L., Limpalaër, M., Bruce, T. J., and Pickett, J. A. (2013). Responses of herbivore and predatory mites to tomato plants exposed to jasmonic acid seed treatment. *J. Chem. Ecol.* 39, 1297–1300. doi: 10.1007/s10886-013-0345-5

Stout, M. J., Rita Riggio, M., Zou, L., and Roberts, R. (2002). Flooding influences ovipositional and feeding behavior of the rice water weevil (Coleoptera: Curculionidae). *J. Econ. Entomol.* 95, 715–721. doi: 10.1603/0022-0493-95.4.715

Strapasson, P., Pinto-Zevallos, D. M., Paudel, S., Rajotte, E. G., Felton, G. W., and Zarbin, P. H. (2014). Enhancing plant resistance at the seed stage: low concentrations of methyl jasmonate reduce the performance of the leaf miner *Tuta absoluta* but do not alter the behavior of its predator *Chrysoperla externa*. *J. Chem. Ecol.* 40, 1090–1098. doi: 10.1007/s10886-014-0503-4

Sugimoto, N., Takeda, G., Nagato, Y., and Yamaguchi, J. (1998). Temporal and spatial expression of the α-amylase gene during seed germination in rice and barley. *Plant Cell Physiol.* 39, 323–333. doi: 10.1093/oxfordjournals.pcp.a029373

Thaler, J. S. (1999). Induced resistance in agricultural crops: effects of jasmonic acid on herbivory and yield in tomato plants. *Environ. Entomol.* 28, 30–37. doi: 10.1093/ee/28.1.30

Ubeda-Tomás, S., Federici, F., Casimiro, I., Beemster, G. T., Bhalerao, R., Swarup, R., et al. (2009). Gibberellin signaling in the endodermis controls *Arabidopsis* root meristem size. *Curr. Biol.* 19, 1194–1199. doi: 10.1016/j.cub.2009.06.023

Van Dam, N. M., Witjes, L., and Svatoš, A. (2004). Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. *New Phytol.* 161, 801–810. doi: 10.1111/j.1469-8137.2004.00984.x

Villegas, J. M., Wilson, B. E., Way, M. O., Gore, J., and Stout, M. J. (2021). Tolerance to rice water weevil, *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae), infestations among hybrid and inbred rice cultivars in the southern US. *Crop Prot.* 139:105368. doi: 10.1016/j.cropro.2020.105368

Vyavhare, S. S., Gealy, D. R., Way, M. O., Tabien, R. E., and Pearson, R. A. (2016). Evaluation of host-plant resistance of selected rice genotypes to the rice water weevil (Coleoptera: Curculionidae). *Environ. Entomol.* 45, 1439–1444. doi: 10.1093/ee/nvw120

Walling, L. L. (2000). The myriad plant responses to herbivores. *J. Plant Growth Regul.* 19, 195–216. doi: 10.1007/s003440000026

Walters, D., and Heil, M. (2007). Costs and trade-offs associated with induced resistance. *Physiol. Mol. Plant Pathol.* 71, 3–17. doi: 10.1016/j.pmpp.2007.09.008

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Wilke, C. O. (2020). Cowplot: streamlined plot theme and plot annotations for 'ggplot2'. R package Version 1.1.0.

Worrall, D., Holroyd, G. H., Moore, J. P., Glowacz, M., Croft, P., Taylor, J. E., et al. (2012). Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. *New Phytol.* 193, 770–778. doi: 10.1111/j.1469-8137.2011.03987.x

Wu, J., Wang, L., and Baldwin, I. T. (2008). Methyl jasmonate-elicited herbivore resistance: does MeJA function as a signal without being hydrolyzed to JA? *Planta* 227, 1161–1168. doi: 10.1007/s00425-008-0690-8

Zou, L., Stout, M. J., and Dunand, R. T. (2004). The effects of feeding by the rice water weevil, *Lissorhoptrus oryzophilus* Kuschel, on the growth and yield components of rice, *Oryza sativa*. *Agric. For. Entomol.* 6, 47–54. doi: 10.1111/j.1461-9555.2004.00203.x

Zwar, J. A., and Hooley, R. (1986). Hormonal regulation of α-amylase gene transcription in wild oat (*Avena fatua* L.) aleurone protoplasts. *Plant Physiol.* 80, 459–463. doi: 10.1104/pp.80.2.459

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 01 October 2021
doi: 10.3389/fpls.2021.711896

Effects of *cis*-Jasmone Treatment of Brassicas on Interactions With *Myzus persicae* Aphids and Their Parasitoid *Diaeretiella rapae*

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There is a need to develop new ways of protecting plants against aphid attack. Here, we investigated the effect of a plant defence activator, *cis*-jasmone (CJ), in a range of cultivars of *Brassica napus*, *Brassica rapa* and *Brassica oleracea*. Plants were sprayed with *cis*-jasmone or blank formulation and then tested with peach potato aphids (*Myzus persicae* Sulzer) (Hemiptera: Aphididae) and their parasitoid *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Braconidae). CJ treated plants had significantly lower aphid settlement than control plants in a settlement bioassay. Conversely, in a foraging bioassay, *D. rapae* parasitoids spent a significantly longer time foraging on CJ treated plants. Our results reveal that CJ treatment makes plants less attractive to and less suitable for *M. persicae* but more attractive to *D. rapae* in a range of brassica cultivars. It is likely that these effects are due to changes in volatile emission indicating activation of defence and presence of conspecific competitors to aphids but presence of prey to parasitoids. Increases in volatile emission were found in CJ induced plants but varied with genotype. Among the synthetic volatile compounds that were induced in the headspace of CJ treated brassica cultivars, methyl isothiocyanate, methyl salicylate and *cis*-jasmone were most repellent to aphids. These results build on earlier studies in *Arabidopsis* and show that tritrophic interactions are influenced by CJ in a wide range of brassica germplasm. The implication is that CJ is a promising treatment that could be used in brassica crops as part of an integrated pest management system.

Keywords: induced defence, aphid, tritrophic interactions, biological control, crop protection

OPEN ACCESS

Edited by:
Víctor Flors,
University of Jaume I, Spain

Reviewed by:
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University of the Free State, South Africa
Paloma Sanchez-Bel,
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *19 May 2021*
Accepted: *23 August 2021*
Published: *01 October 2021*

Citation:
Ali J, Covaci AD, Roberts JM, Sobhy IS, Kirk WDJ and Bruce TJA (2021) Effects of cis-Jasmone Treatment of Brassicas on Interactions With Myzus persicae Aphids and Their Parasitoid Diaeretiella rapae. Front. Plant Sci. 12:711896. doi: 10.3389/fpls.2021.711896

INTRODUCTION

cis-Jasmone (CJ) is a benign plant defence activator shown to have considerable promise in enhancing plant defence against hemipteran insect pests in *Arabidopsis thaliana* (Bruce et al., 2008) as well as crops such as wheat (Bruce et al., 2003a,b), maize (Oluwafemi et al., 2013), cotton (Hegde et al., 2012), sweet pepper (Dewhirst et al., 2012) and potato (Sobhy et al., 2017, 2020). CJ was first tested and reported as a plant defence activator by Birkett et al. (2000) after the compound was found to be highly electrophysiologically active with aphids. It was hypothesised to have a role in plant defence due to structural similarities with the plant hormone jasmonic acid and experimental

evidence confirmed this hypothesis. Genes activated by CJ are, however, different from the ones activated by jasmonic acid or methyl jasmonate (Matthes et al., 2010).

Basic studies done in *A. thaliana* demonstrated that CJ makes plants less attractive to aphids but more attractive to their parasitoid natural enemies (Bruce et al., 2008; Matthes et al., 2010). We hypothesised that similar effects would occur in brassica crops, but data on this were not previously available and there was a knowledge gap relating to this. Therefore, the current study was designed to test the hypotheses that CJ treatment could (1) reduce aphid performance and colonisation and (2) increase parasitoid foraging in a range of cultivars of *Brassica napus*, *B. rapa* and *B. oleracea*. Our study focused on *Myzus persicae* Sulzer (Hemiptera: Aphididae), commonly known as peach potato aphid, because it is the main aphid pest of brassica crops (van Emden et al., 1969). *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Braconidae) was chosen as the parasitoid species because a survey conducted by ADAS revealed it was the most common parasitoid of *M. persicae* in the UK (Jude Bennison, *pers. comm.*).

Our study is timely because there is an urgent need for new approaches to crop protection in brassicas as *Myzus persicae* aphids have evolved resistance to many insecticides (Bass et al., 2014) and the neonicotinoid restriction in Europe has further reduced conventional control options (Dewar, 2017).

MATERIALS AND METHODS

Insects

Myzus persicae aphids (clone O) were reared on pak choi (*Brassica rapa* subsp. *chinensis* cv. Hanakan) in BugDorm cages (46 × 46 × 46 cm; NHBS Ltd, Devon, UK) under controlled environmental conditions (24°C, 38 % RH, 16:8 photoperiod) in the Centre of Applied Entomology and Parasitology (CAEP) insectary at Keele University (UK). *Diaeretiella rapae* parasitoid wasps were reared on surplus *M. persicae* infested pak choi plants in BugDorms (46 × 46 × 46 cm) under controlled conditions (20°C, 40% RH, 16:8 photoperiod) in a growth chamber (MLR-352-PE; Panasonic, The Netherlands). Both cultures were originally obtained from Harper Adams University, UK.

Plants

Six brassica cultivars, representing a wide range of germplasm, from three species were grown for use in experiments: *Brassica napus* cultivars Samurai, Wesway, English Giant and Turnip Rutabaga 57, *Brassica oleracea* cv. Warwick L9 (Chinese kale) and *Brassica rapa* subsp. *chinensis* cv. Hanakan (pak choi) were obtained from Warwick University, UK. The effect of CJ treatment has not been investigated previously in these cultivars. All plants were individually grown in 7.5 cm pots filled with John Innes No. 2 compost (Westland Horticulture Limited, Tyrone, UK) under controlled environment conditions (20°C, 37 % RH, 16:8 photoperiod) in a growth chamber (MLR-352-PE; Panasonic). Plants at BBCH growth stage 14 (i.e., five true leaves) were used for all experiments (Meier, 1997).

Chemicals and *cis*-Jasmone Treatment

Chemical standards tested individually in olfactometer bioassays were *cis*-3-hexenyl acetate (≥98%), methyl salicylate (≥99%), *cis*-jasmone (≥97%), methyl isothiocyanate (97%), benzyl nitrile (98%), limonene (96%), nonanal (≥98%), *trans*-β-farnesene (90 %), and β-elemene (≥98%). Concentrations of 100 ng/μl of each synthetic volatile eluted in hexane (≥95%) were prepared. All standards including hexane were purchased from Sigma Aldrich (Gillingham, UK).

Plants were sprayed with an aqueous emulsion of *cis*-jasmone (CJ) (Sigma Aldrich, Buchs, Switzerland) as described in Bruce et al. (2003a). A stock CJ emulsion was formulated by mixing 25 μl of CJ with 100 μl of Tween 80 (Sigma Aldrich) in 100 ml of deionized water, while a blank formulation to act as a control was formulated by mixing 100 μl of Tween 80 in 100 ml of deionised water. Spray treatment was carried out using an Oshide spray bottle (100 ml; Zhengzhou Xinrui Tongda Metal and Material Co., Ltd. Henan Sheng, China) by applying three trigger pulls of spray formulation (250 μl) to each plant at a distance of 30 cm. Sprayed plants were left for 24 h and then used for experiments. Control plants and CJ treated plants were placed in different compartments to avoid any plant-plant interaction.

Aphid Performance Clip-Cage Bioassay

Performance of *M. persicae* was assessed on the different brassica cultivars. There were two separate series of experiments with different plants: the first series recorded observations after 48 h and the second recorded observations after 96 h. Fresh plants and aphids were used in each replicate observation in each experiment. In each replicate, 10 adult alate *M. persicae* were placed in a clip cage (2.5 cm diameter, Bioquip Products Inc., USA), which was attached to the lower surface of plant leaves (Sobhy et al., 2020). Two clip cages were placed on each plant. Ten replicates (control and CJ treated) were performed for each cultivar. To assess the survival and fecundity of aphids, plants were left undisturbed in a controlled environment room (25°C, 37% RH, 16:8 photoperiod). Plants were assessed after 48 h (series 1) or 96 h (series 2). For assessment, leaves containing the cages were cut and cages were removed without losing any aphids. Numbers of live adults and newly larviposited nymphs were recorded.

Aphid Settlement Bioassay

In this choice test bioassay, CJ treated and control plants were placed in a BugDorm insect cage (60 × 60 × 60 cm; NHBS Ltd, Devon, UK) and were kept in a controlled environment room (25°C, 37% RH, 16:8 photoperiod). Each BugDorm contained four plants (two treated and two control) at alternate positions. A vial containing 50 alate *M. persicae* was positioned in the centre of the cage, opened and then the cage was left for 24 h. Counts of settled aphids were recorded 24 h after release. Ten replicates were done for each cultivar. The position of treatments was alternated between replicates.

Parasitoid Foraging Bioassay

To test CJ effect on parasitoids, the foraging behaviour of *D. rapae* females was monitored. Noldus Observer 4.1 software was used

to record the behavioural observations. In an open-fronted cage, a single female parasitoid was released from a vial onto a plant leaf and then its foraging behaviour was monitored by direct observation. Time spent walking, still, and cleaning was recorded, as well as total time spent before the parasitoid left the plant. An observation was terminated when a parasitoid flew away from the plant, which was considered as leaving the foraging "patch." Ten replicates of CJ treated and control plants were done for each cultivar. Treated and control replicates were observed alternately. All experiments were done between 9:00 a.m. and 2:00 p.m.

Parasitism Bioassay

Effect of CJ on the parasitism rate of *D. rapae* was assessed in BugDorm (60 × 60 × 60 cm) cages as described above. Bioassays were repeated 10 times on different experimental days. Each plant was infested with 50 adult *M. persicae* that were released 2 h prior to the release of *D. rapae*. Eight female parasitoids were released into the cage following the experimental procedure of Sun et al. (2020). After 24 h, parasitoid females were collected from the cages and plants were kept contained in bread bags while aphid mummies developed. Experiments were conducted in a controlled environment room (20°C, 37% RH, 16:8 photoperiod). After 15 days, the number of mummified aphids on the treated and control plants was recorded.

Volatile Collection

Plant volatiles were collected following a procedure adapted from Agelopoulos et al. (1999) in which whole plants were contained inside oven bags (35 × 43 cm; Bacofoil, UK). The bag was partially sealed, so that only volatiles produced by the plants were collected. Prior to entrainments, bags were baked in an oven (Heraeus, Thermo Electron corporation, Mark Biosciences, UK) at 120°C overnight. Porapak Q philtres (0.05 g, 60/80 mesh; Supelco, Bellefonte, PA, USA) were washed with diethyl ether and then conditioned before use. Plants were enclosed in bags individually. Each bag was open at the bottom and closed at the top. An outlet hole was made in the upper part of the bag to connect the Porapak Q filter, whereas the bag bottom was closed by attaching a rubber band around the pot. Charcoal filtered air was pumped into bags at 600 ml min^{-1} and sampled air was pulled out at 400 ml min^{-1} through a Porapak Q filter in which the plant volatiles were trapped. To avoid entry of unfiltered air, positive pressure was maintained using differing air flows rates. Connections were made with 1.6 mm (i.d.) polytetrafluoroethylene (PTFE) tubing (Alltech Associates Inc., Lancashire, UK) with Swagelok brass ferrules and fittings (North London Valve Co., London, UK) and sealed with PTFE tape (Gibbs & Dandy Ltd., Luton, UK). Volatile collection was done for a 48 h period, after which the Porapak filters were eluted with 500 μl of diethyl ether into sample vials (Supelco, 2 ml, PTFE/silicone) and stored at −20°C in a freezer (Lec Medical, UK) for use in olfactometer bioassays and chemical analysis.

Olfactometer Bioassay

The behavioural responses of alate *M. persicae* to brassica volatiles were investigated using a Perspex 4-arm olfactometer in a controlled environment room (24°C, 30% RH). The central area at the top of the olfactometer contained a hole into which a single aphid was introduced, and which was connected to a low-pressure air pump to remove air at a rate of 200 ml min^{-1}. PTFE tape was used to ensure airtight seals between the olfactometer and the Teflon tubing. Holes connected to odour source tubes were covered with a layer of muslin to prevent aphid escape during the bioassays. The olfactometer arena was split into five areas; four areas by each arm (one treatment arm vs. three control arms or two treatment and two control arms) and a central area (Webster et al., 2010). Adult aphids were collected from rearing cages in a separate insectary room and transferred to the olfactometer laboratory for acclimatisation and starved for 2 h before each trial. Individual aphids were added using a 000 paintbrush. Each aphid was exposed to a test sample for 12 min, and after every 3 min the position of the olfactometer was rotated clockwise by 90° to eliminate directional bias. Time spent in each arm was recorded using a software program (OLFA, F. Nazzi, Udine, Italy). Ten replicates were done for each comparison. For each experiment, filter paper (Whatman No. 1, Buckinghamshire, UK) strips (cut to 5 × 20 mm) were treated with an aliquot (10 μl) of the volatile sample treatment or solvent control, applied using a micropipette (Drummond "microcaps"; Drummond Scientific Co., USA), and allowed to evaporate for 30 s before placing in odour source tubes. If an aphid remained motionless for the first 2 min of a replicate it was recorded as unresponsive and excluded from analysis. All bioassays were performed between 10:00 a.m. and 1:00 p.m.

Headspace Samples of Plant Naturally Emitted Plant Volatile Blends

Three series of experiments were conducted with the natural volatile blends collected from plants: (series 1) volatiles from control plants (treated with blank formulation of water + tween) vs. solvent control (diethyl ether), (series 2) volatiles from CJ treated plants (CJ + tween + water) vs. solvent control (diethyl ether), and (series 3) a choice test with volatiles from control plants vs. CJ treated plants vs. solvent (diethyl ether). For the first two series experiments, one arm was assigned to the collected volatiles from plants whereas three control arms were treated similarly with the same volume of solvent (diethyl ether used for eluting the plant volatiles). In the third series experiment, the two treatments being compared were assigned to one arm each whereas the other two opposite arms were assigned to solvent control (diethyl ether).

Individual Synthetic Volatile Compounds

The behavioural responses of alate *M. persicae* to nine synthetic chemical compounds were investigated as described above. These volatiles were induced in the headspace of CJ treated brassica cultivars (see below). Each synthetic chemical compound was tested at a concentration of [100 ng/μl in hexane]. In these experiments, similar to series 1 and 2 described above, one arm was assigned to one synthetic volatile compound whereas three control arms were treated similarly with the same volume of solvent.

Volatile Analysis

Analyses were carried out on a 7820A GC coupled to a 5977B single quad mass selective detector (Agilent Technologies, Cheadle, UK). The GC was fitted with a non-polar HP5-MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness) coated with (5%-phenyl)-methylpolysiloxane (Agilent Technologies) and used hydrogen carrier gas at a constant flow rate of 1.2 ml/min. Automated injections of 1 μl were made using a G4513A autosampler (Agilent Technologies) in splitless mode (285°C), with oven temperature programmed from 35°C for 5 min then at 10°C/min to 285°C. Compounds were identified according to their mass spectrum, linear retention index relative to retention times of *n*-alkanes, and co-chromatography with authentic compounds.

Statistical Analysis

Aphid Clip Cage Bioassay

Differences in the mean number of live aphids on control and CJ treated plants was compared for each brassica cultivar at two time-points (48 and 96 h) using generalised linear models (GLM) fitted with Poisson probability distributions. Differences in the mean number of aphid nymphs larviposited onto control and CJ treated plants were compared for each brassica cultivar at two time-points (48 and 96 h) using GLMs fitted with quasi-Poisson probability distributions to account for overdispersion. Plant treatment (i.e., control vs. CJ treated) was a fixed factor.

Aphid Settlement Bioassay

Differences in the mean number of aphids settling on control and CJ treated plants were compared for each brassica cultivar using GLMs with Poisson or quasi-Poisson probability distributions depending on dispersion. Plant treatment (i.e., control vs. CJ treated) was a fixed factor.

Parasitoid Foraging Bioassay

The total time spent by parasitoid wasps foraging on control and CJ treated plants was first analysed for each brassica cultivar using Shapiro-Wilk tests to determine whether the underlying data were Gaussian. As data for this bioassay was non-Gaussian, the response variable (i.e., time) was square root transformed and re-analysed using Shapiro-Wilk tests to confirm that transformed data were Gaussian. After transformation, differences in mean total parasitoid foraging time between control and CJ treated plants was evaluated for each brassica cultivar using two-sample *t*-tests.

Parasitism Bioassay

Differences in the mean number of mummified aphids on control and CJ treated plants were compared for each brassica cultivar using GLMs with Poisson or quasi-Poisson probability distributions depending on dispersion. Plant treatment (i.e., control vs. CJ treated) was a fixed factor.

Volatile Profiling

To visualise the overall differences in volatile profiles emitted from the six studied brassica cultivars, a principal component analysis (PCA) was performed using the concentrations of the detected volatiles as dependent variables. Loading and score plots were derived after mean-centering and log transformation of volatile data. Average linkage hierarchical clustering based on Ward clustering algorithm of the Euclidean distance measure for the differentially emitted VOCs was used to construct a heatmap displaying the concentrations of different volatiles. Visualisation, together with hierarchical clustering of VOC data, was done using the MetaboAnalyst online tool suite (Chong et al., 2018). Subsequently, univariate analyses of variances were performed to investigate whether the concentrations of individual volatile compounds differed with and without CJ treatment using SigmaPlot 12.3 (Systat Software Inc., USA).

Olfactometer Bioassays

The behavioural response of *M. persicae* was tested in two ways. For experiments with one treated arm vs. three solvent control treatments, data were analysed by a paired *t*-test. In this analysis, the time spent by aphids in treated and solvent arms of the four-arm olfactometer were compared. In experiments where the response in two treatment arms vs. two arms of solvent control was compared, data were first converted into proportions then log-ratio transformed before analysis by one-way analysis of variance and Holm-Sidak mean separation (Mwando et al., 2018). Data were examined for a Gaussian distribution using the Shapiro-Wilk test prior to analysis.

All statistical analyses were carried out using R (v 4.0.3) (R Core Development Team, 2021).

RESULTS

Aphid Clip Cage Bioassay

After 48 h there was no significant reduction in adult *M. persicae* survival on five brassica cultivars treated with CJ in clip cage experiments (**Figure 1A**). However, treating the Chinese kale cultivar with CJ reduced survival two-fold (generalised linear model with Poisson distribution: $X^2 = 12.50$; *d.f.* = 1,38; $P = 0.04$). There was no significant reduction in adult *M. persicae* survival on four brassica cultivars treated with CJ in clip cage experiments after 96 h (**Figure 1B**). Both the Chinese kale (generalised linear model with Poisson distribution: $X^2 = 40.35$; *d.f.* = 1,38; $P < 0.001$) and Samurai (generalised linear model with Poisson distribution: $X^2 = 12.50$; *d.f.* = 1,38; $P = 0.04$) cultivars treated with CJ showed significant increases in *M. persicae* mortality compared to their control (blank formulation) treated counterparts.

There was a significant reduction in nymph production on CJ treated plants across both time points (**Figures 2A,B**). Mean larviposition on CJ treated plants of all brassica cultivars was significantly reduced after 48 hours (**Figure 2A**), decreasing by 46% from 26.15 on control treated plants to 14.03 on CJ treated plants. Larviposition was reduced most on Chinese kale (GLM with quasi-Poisson distribution: $F = 76.57$; *d.f.* = 1,38; $P < 0.001$; 82 % reduction in larviposition) and the least on Samurai (GLM with quasi-Poisson distribution: $F = 33.03$; *d.f.* = 1,38; $P < 0.001$; 21% reduction in larviposition). Similarly, mean larviposition on CJ treated plants of all brassica cultivars was also significantly reduced after 96 h (**Figure 2B**), decreasing by 41% from 55.80 on control plants to 32.65 on CJ treated plants. Larviposition

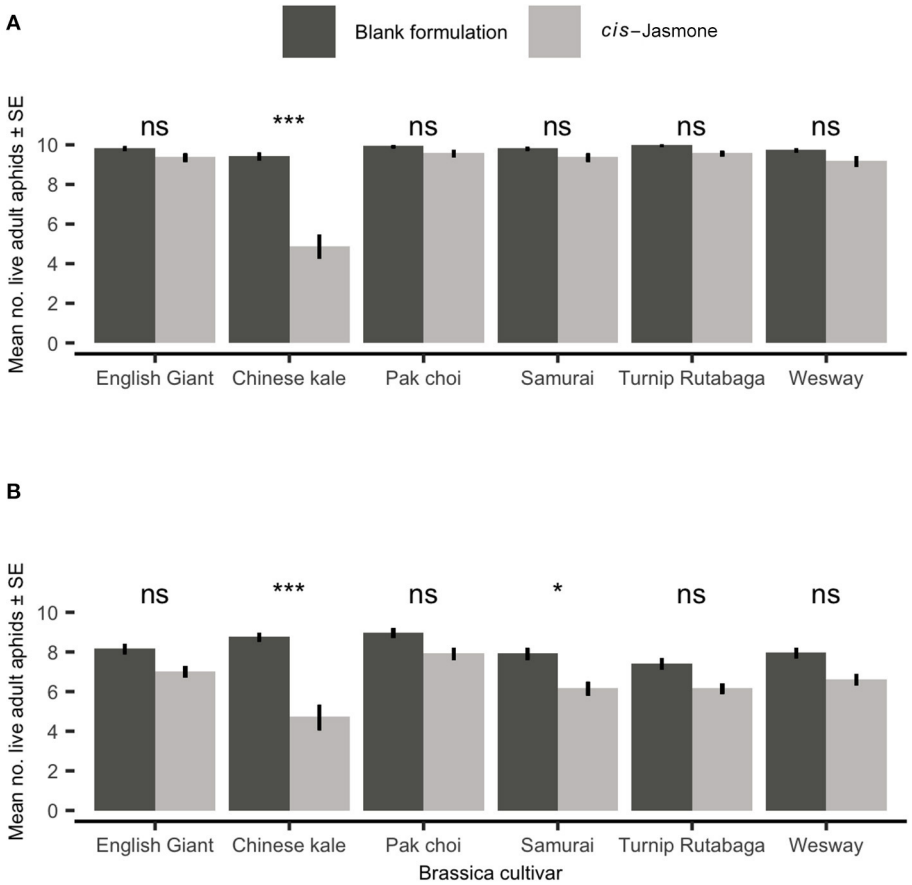

FIGURE 1 | Adult *Myzus persicae* survival (Mean ± SE) out of the original 10 individuals after **(A)** 48 h and **(B)** 96 h in clip cages on six brassica cultivars ($n = 10$) treated with *cis*-jasmone or blank formulation (control). Brassica cultivars capped with "ns" do not show a significant difference between control and *cis*-jasmone treatment while asterisks denote differing levels of statistical significance: * < 0.05 and *** < 0.001 (generalised linear models with Poisson probability distribution).

was reduced most on Chinese kale (GLM with quasi-Poisson distribution: $F = 18.31$; *d.f.* = 1,38; $P < 0.001$; 55% reduction in larviposition) and the least on Turnip Rutabaga (GLM with quasi-Poisson distribution: $F = 9.43$; *d.f.* = 1,38; $P = 0.004$; 24% reduction in larviposition).

Aphid Settlement Bioassay

In settlement bioassays, where aphids were offered a choice between CJ treated and control plants, a clear and statistically significant reduction in aphid settlement was observed on CJ treated plants (**Figure 3**). This effect was consistent across all brassica cultivars tested. The preference for control over CJ treated plants was strongest for the *B. napus* cultivar Wesway, with a mean of only 7.05 aphids settling on CJ treated plants compared to 16.2 aphids settling on control treated plants (GLM with quasi-Poisson distribution: $F = 32.39$; *d.f.* = 1,38; $P < 0.001$; 30.3% of aphid settlement occurring on CJ treated plants). Similarly, aphid settlement was significantly reduced in pak choi (GLM with quasi-Poisson distribution: $F = 98.81$; *d.f.* = 1,38; $P < 0.001$), English Giant (GLM with Poisson distribution: $X^2 = 86.63$; *d.f.* = 1,38; $P < 0.001$), Samurai (generalised linear model with Poisson distribution: $X^2 = 70.4$: *d.f.* = 1,38; $P < 0.001$), Turnip Rutabaga (GLM with Poisson distribution: $X^2 = 41.12$; *d.f.* = 1,38; $P < 0.001$) and Chinese kale (GLM with Poisson distribution: $X^2 = 86.57$; *d.f.* = 1,38; $P < 0.001$). Pooling data across all cultivars tested, mean aphid settlement on CJ treated plants was 1.86 times lower than on control plants.

Parasitoid Foraging Bioassay

In a foraging bioassay, parasitoid wasps spent substantially longer on CJ treated plants than on control plants (**Figure 4**). There was a 5.1× increase in the mean time spent on CJ treated pak choi plants (two-sample *t*-test: Welch's $t = 3.59$; *d.f.* = 10.21; $P = 0.004$), a 4.6× increase on Turnip Rutabaga (two-sample *t*-test: Welch's $t = 3.67$; *d.f.* = 17.26; $P = 0.001$), a 4.5× increase on Wesway (two-sample *t*-test: Welch's $t = 3.89$; *d.f.* = 15.48; $P = 0.001$), a 3.9× increase on Samurai (two-sample *t*-test: Welch's $t = 2.84$; *d.f.* = 14.10; $P = 0.013$), a 2.8× increase on English Giant (two-sample *t*-test: Welch's $t = 2.22$; *d.f.* = 17.39; $P = 0.04$) and no significant increase on Chinese kale (two-sample *t*-test: Welch's $t = 0.65$; *d.f.* = 16.96; $P = 0.525$).

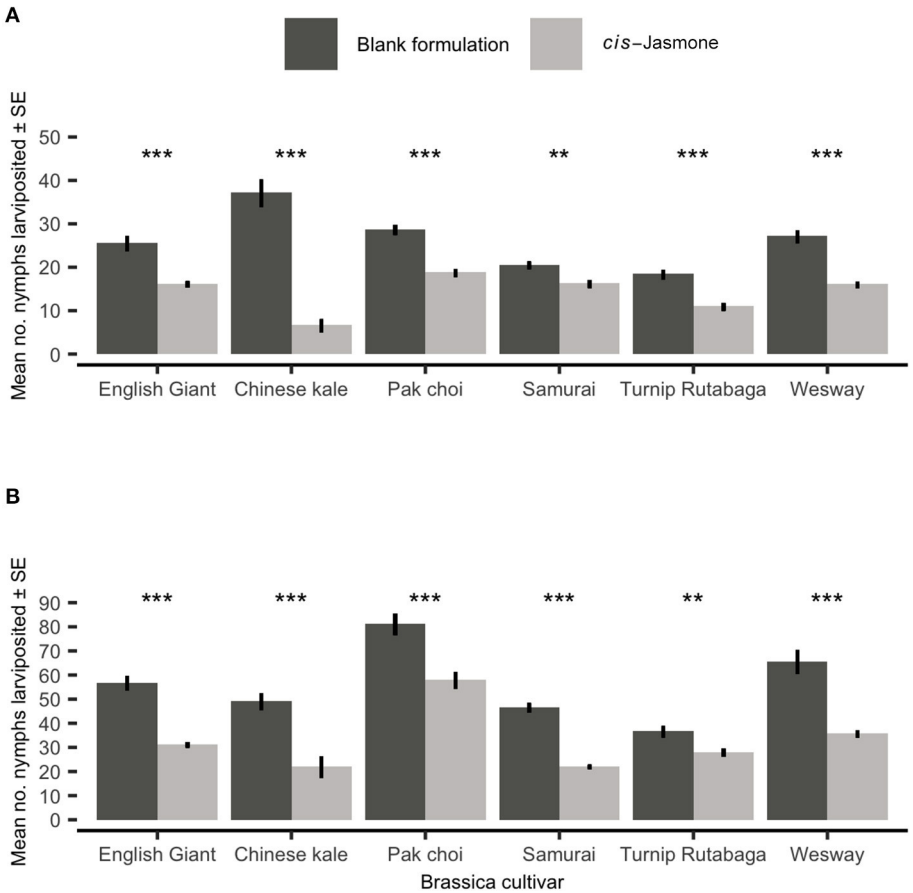

FIGURE 2 | *Myzus persicae* larviposition (Mean ± SE) after **(A)** 48 h and **(B)** 96 h in clip cages on six brassica cultivars ($n = 10$) treated with *cis*-jasmone or blank formulation (control). Asterisks denote differing levels of statistical significance: ** < 0.01 and *** < 0.001 (generalised linear models with quasi-Poisson probability distribution).

Parasitism Bioassay

Parasitism rates were significantly increased by CJ application in three brassica cultivars, though all six cultivars treated with CJ showed some level of increase (**Figure 5**). The largest increase in parasitism rates was observed on Samurai (generalised linear model with quasi-Poisson distribution: $F = 11.74$; $d.f. = 1,38$; $P = 0.001$; 121 % increase), though pak choi had the greatest total number of mummified aphids. Low levels of parasitism were observed on Chinese kale irrespective of plant treatment (**Figure 5**).

Olfactometer Bioassay

Natural Volatile Blends

When presented with a choice between a solvent control (diethyl ether; DEE) and volatiles collected from plants treated with a blank formulation, adult aphids showed no significant preference for either odour source in five of the tested cultivars (**Figure 6A**). Aphids did, however, spend significantly less time in the treated zone for Wesway (paired *t*-test: $t = 4.69$; $d.f. = 9$; $P = 0.001$; **Figure 6A**). However, when plants were CJ treated and aphids were presented with a choice of volatiles from a treated plant vs. solvent control (DEE), aphids spent significantly less time in the treated zone of the olfactometer with volatiles from CJ treated plants for 5 out of the 6 cultivars tested (**Figure 6B**). Turnip Rutabaga was the only cultivar in which volatiles from CJ-treated plants were not repellent. CJ-treated Chinese kale and Samurai were the most repellent. When also allowed to choose between volatiles from CJ treated plants and untreated control plants (**Figure 6C**), aphids spent significantly longer in the olfactometer zone with volatiles from blank formulation control plants for pak choi and the Samurai and Chinese kale cultivars.

Individual Synthetic Volatile Compounds

When presented with a choice between a solvent control (diethyl ether) and one of the eight individual synthetic compounds, adult aphids either showed no significant preference for either odour source or avoided the olfactometer arms containing a synthetic chemical (**Figure 7**). Aphids spent significantly less time in the olfactometer arms containing CJ (paired *t*-test: $t = 2.62$; $d.f. = 9$; $P = 0.03$), methyl isothiocyanate (paired *t*-test: $t = 7.86$; $d.f. = 9$; $P < 0.001$) or methyl salicylate (paired *t*-test: $t = 2.59$; $d.f. = 9$; $P = 0.03$).

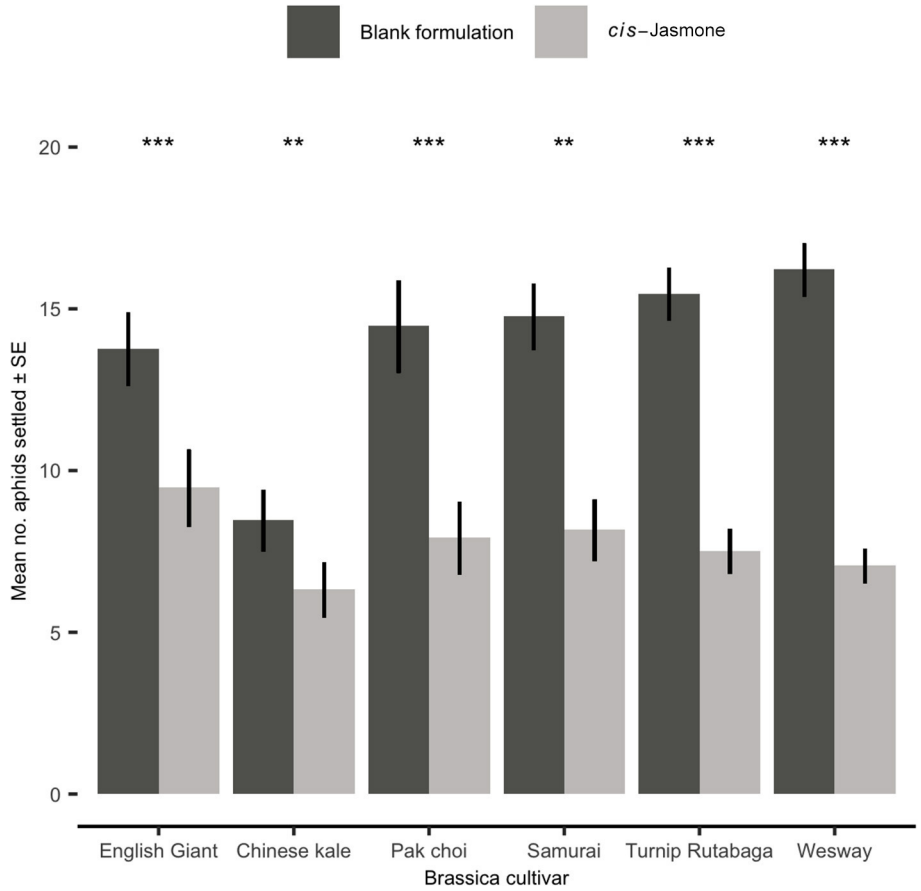

FIGURE 3 | Settlement of *Myzus persicae* (Mean ± SE) after 24 h on blank formulation (control) and *cis*-jasmone treated plants in a series of choice test bioassays (50 aphids released in each replicate; $n = 10$). Asterisks denote differing levels of statistical significance: ** < 0.01 and *** < 0.001 (generalised linear models with either Poisson or quasi-Poisson probability distributions).

Volatile Analysis

GC-MS analysis of headspace collections from the six studied brassica cultivars revealed 25 detectable VOCs in 8 functional classes (alcohols, aldehydes, aliphatic hydrocarbons, benzenoids, esters, ketones, N-containing compounds and terpenes). Volatile emission was found to increase with CJ treatment in all brassica cultivars except Chinese kale. There were both quantitative and qualitative changes in the volatile profile of CJ treated plants compared to control plants. Quantitative changes in total volatile emission are shown in **Figure 8**. Volatile emission was increased ∼14-fold in Wesway, 5-fold in pak choi, 4-fold in Samurai and Turnip Rutabaga, and 2-fold in English Giant but there was no change in Chinese kale. **Table 1** shows the compounds emitted. In English Giant, nonanal, (*E*)-3-tetradecene, dihydrojasmone, CJ, methyl isothiocyanate and benzyl nitrile were the main compounds induced. In Chinese kale *de novo* production of methyl isothiocyanate and benzyl nitrile was the main difference observed with CJ treatment and there was reduced emission of other compounds such as (*E*)-3-tetradecene. In pak choi there was a large increase in green leafy volatile (*Z*)-3-hexenyl acetate and 2-ethyl-1-hexanol production together with CJ, limonene, and citronellol with CJ treatment. In Samurai, induced emission of CJ itself was the main change, together with a smaller increase in emission of (*Z*)-3-hexenyl acetate and 2-ethyl-1-hexanol. In turnip rutabaga, the biggest changes were increases in (*E,E*)-α-farnesene, 2-ethyl-1-hexanol and p-cymen-7-ol with also notable induction of methyl salicylate (MeSA) and CJ. Finally, in Wesway there were marked increases in emission of 9 volatiles including CJ, 2-ethyl-1-hexanol, (*Z*)-3-hexenyl acetate and p-cymen-7-ol. **Figure 9** provides a PCA analysis showing qualitative differences where the first two principal components accounted for 42.3% of the total variation in the volatile data. A clear separation based on the first principal component (PC1) is visible between CJ treated Samurai, Turnip Rutabaga, and Wesway plants, whereas another separation but based on the second principal component (PC2) is obvious for the volatile profiles of CJ treated English Giant, pak choi and Samurai plants. In descending order, the greatest loadings of PC1, were for β-elemene (0.218), *cis*-jasmone (0.213), and (*E,E*)-α-farnesene (0.170), whereas the major loadings of PC2 were for *cis*-jasmone (0.495), citronellol (0.382), and dihydrojasmone (0.257). These VOCs shown to contribute to PC1 and PC2 may impact the behaviour of *M.*

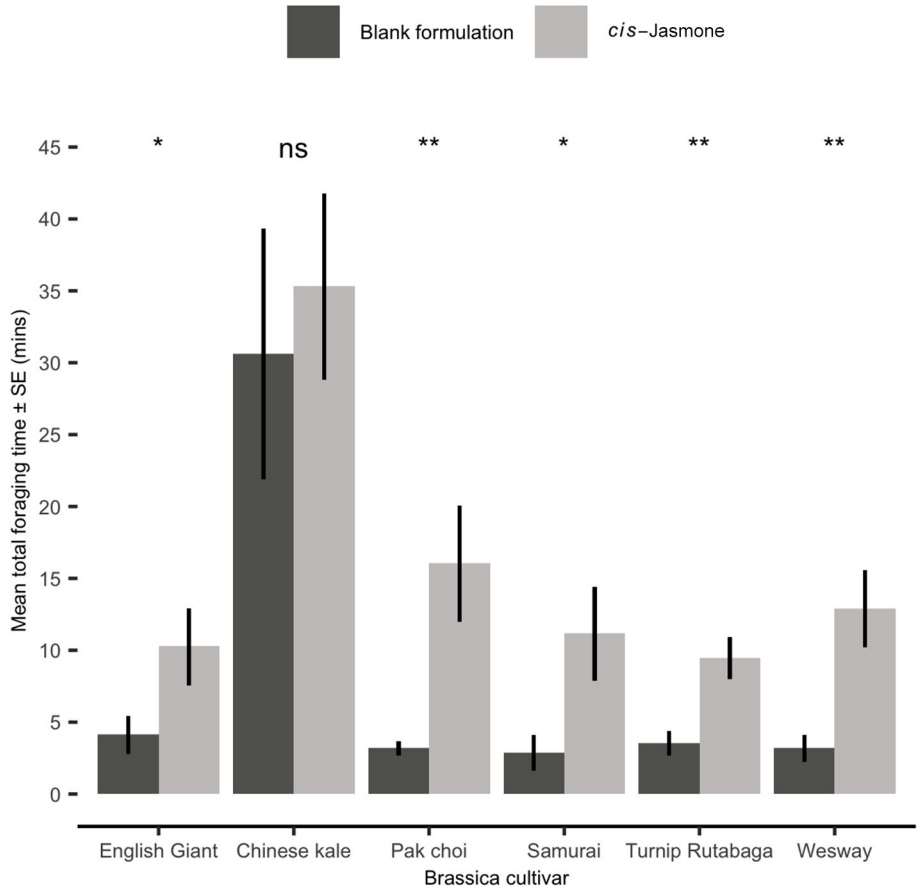

FIGURE 4 | Mean total time spent foraging (Mean ± SE) by *Diaeretiella rapae* on blank formulation (control) and *cis*-jasmone treated plants ($n = 10$). Brassica cultivars capped with "ns" do not show a significant difference between control and CJ treatment while asterisks denote differing levels of statistical significance: * < 0.05 and ** < 0.01 (two-sample *t*-tests).

persicae and *D. rapae*. A heatmap showed differential magnitude of volatile emission from the six studied brassica cultivars with and without CJ treatment (**Supplementary Figure 1**).

DISCUSSION

Taken together, our data show that CJ treatment of a diverse sample of crop brassica cultivars can, as hypothesised, make the plants less suitable and less attractive for *M. persicae* aphids but more attractive to their key parasitoid, *D. rapae*. Aphid survival and nymph production were reduced on CJ treated plants and aphids avoided CJ treated plants. Conversely, *D. rapae* spent longer foraging on CJ treated plants. However, this effect was brassica cultivar dependent. This is not surprising because any induction of plant defence triggered by a chemical elicitor depends on the existence of an inducible defence trait(s) (Bruce, 2014). Evidence can be seen for differential response between brassica cultivars from the volatile analysis with the biggest changes in volatile emission in Wesway and no significant change in Chinese kale. Despite this, CJ-induced Chinese kale was the best-performing brassica in terms of reduced aphid survival on plants.

Interestingly, there appeared to be a difference between cultivars in direct and indirect defence: Chinese kale (*Brassica oleracea* cv. "Warwick L9") was the only cultivar where adult aphid survival was substantially and significantly reduced by CJ treatment indicating that direct defence against the herbivore had increased. In contrast, CJ treatment of Chinese kale had no effect on parasitoid foraging or parasitism levels indicating that there was no effect on indirect defence via tritrophic interactions with the aphid parasitoid. In all other cultivars parasitoid foraging time was significantly increased by CJ treatment and in three of them (pak choi, Turnip Rutabaga and Wesway) a significant increase in number of aphid mummies was found.

With aphids confined in clip cages in no-choice experiments, adult survival was reduced by CJ treatment in Chinese kale at both 48 and 96 h time intervals with approximately half as many aphids surviving on the treated plants. There was also a significant reduction in adult survival with CJ treatment in Turnip Rutabaga at 96 h, but the effect size was not as large as in Chinese kale. Aphid reproduction was affected more than adult

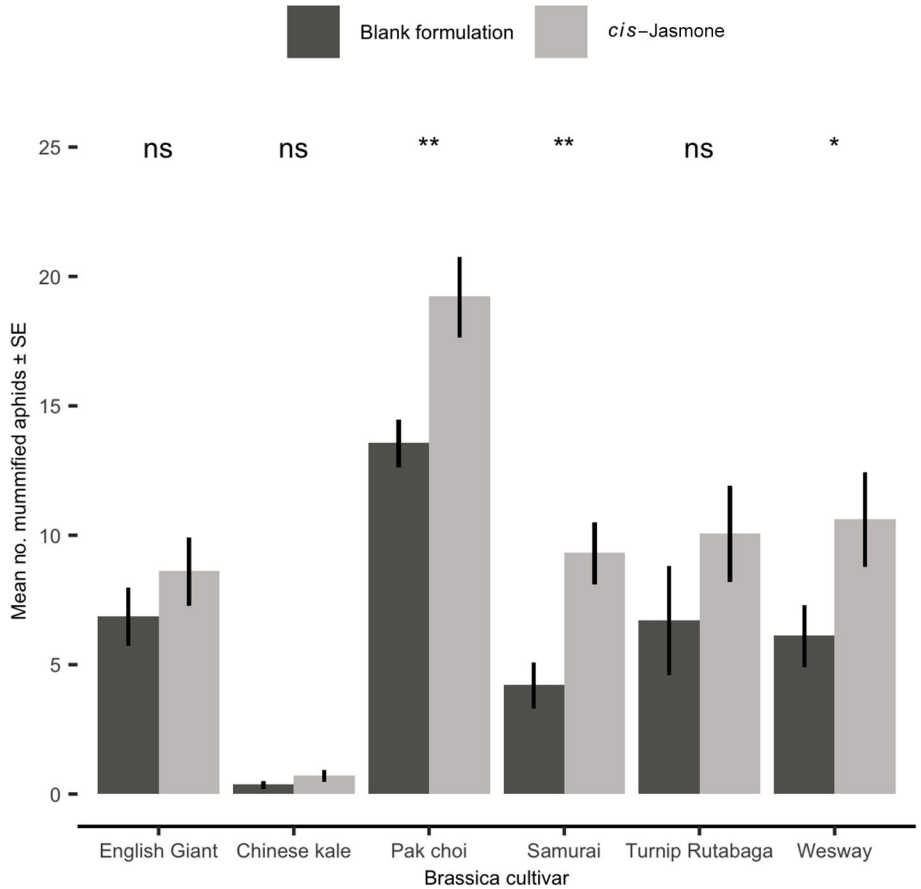

FIGURE 5 | Mean number (± SE) of mummified *Myzus persicae* on blank formulation (control) and *cis*-jasmone treated plants 15 days after exposure to *Diaeretiella rapae* for 2 h ($n = 10$). Brassica cultivars capped with "ns" do not show a significant difference between control and CJ treatment while asterisks denote differing levels of statistical significance: * < 0.05 and ** < 0.01 (generalised linear models with either Poisson or quasi-Poisson probability distributions).

survival because a significant reduction in nymph larviposition was found with CJ treatment in all six cultivars tested, which was also the case for other crop plants treated with CJ (Dewhirst et al., 2012; Sobhy et al., 2020). A reduction in larviposition would slow down aphid population growth rates and this would be valuable in pest management because the high reproduction rate of aphids is one of the main reasons why they are such formidable pests (Leather et al., 2017).

Settlement bioassays revealed that aphid colonisation of CJ treated plants was significantly lower in all six cultivars tested. This included Chinese kale although the effect size was lower with this cultivar. Reduced aphid settlement would further slow down aphid infestations because initial populations settling on plants would also be lower with CJ treatment. Olfactometer bioassays revealed that volatiles from CJ treated plants were repellent to aphids in most cultivars. Surprisingly, no aphid repellence was observed with aphids exposed to volatiles from CJ-treated Turnip Rutabaga although volatile analysis showed a 4-fold increase in volatile emission with CJ treatment. This suggests that the chemical composition or quality matters more than the absolute quantity for aphid repellence. Also, a repellent effect was found with Chinese kale although total volatile emission was not increased by CJ-treatment. This again suggests the type of volatile emitted matters. Methyl isothiocyanate and benzyl nitrile were not detected in control Chinese kale volatiles but were found in volatiles from CJ-treated plants and it is possible these compounds could have caused the observed repellent effect but further studies would be needed to confirm this. *cis*-Jasmone itself was found in the volatiles of control English Giant and Turnip Rutabaga with increased emission after CJ treatment. It was not found in the other cultivars until after CJ treatment and the largest amounts were found in Wesway and Samurai. As CJ is highly volatile, was found in some control plants and varied considerably between varieties, we interpret this as being production of CJ by the plants themselves. The precursor dihydrojasmone was also found in English Giant, pak choi and Samurai.

A foraging bioassay was used to investigate how long parasitoids spent on treated plants after being released on them. This bioassay was used because retention of parasitoids after arrival is more important in determining parasitism levels than initial arrival rates (Budenberg et al., 1992).

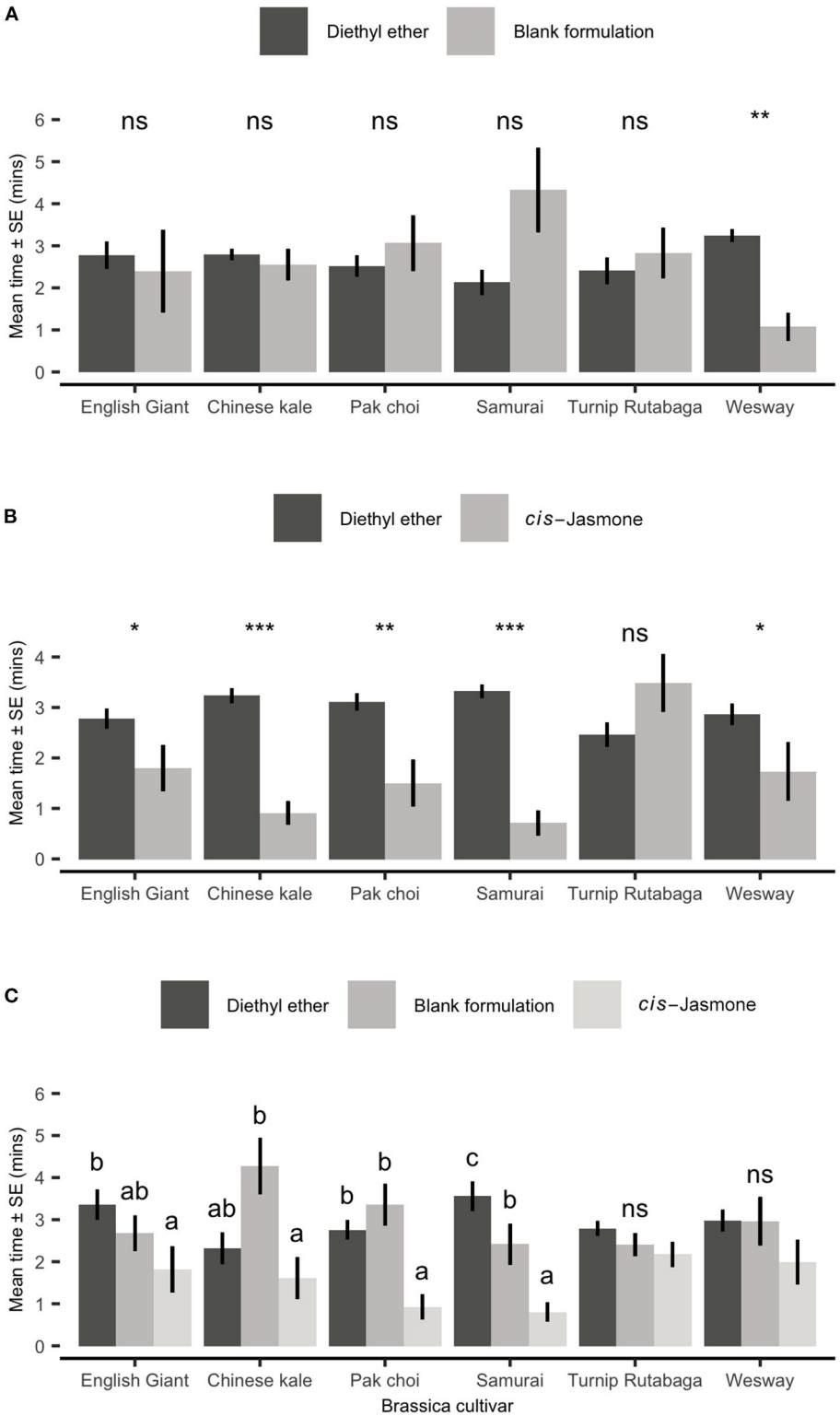

FIGURE 6 | Behavioural responses of *Myzus persicae* females to volatiles from six brassica cultivars in an olfactometer bioassay. Individual aphids were given 12 min to make a choice between **(A)** one arm of blank formulation treated plants (BF—tween 80 and water) vs. three solvent diethyl ether (DEE) arms, **(B)** one arm of *cis*-jasmone treated plants (CJ) vs. three solvent control arms (DEE) and **(C)** two different treatment arms (BF, blank formulation treated plants and CJ, *cis*-jasmone treated plants) vs. two solvent arms (DEE). The shown values are mean time spent in arm ± SE ($n = 10$). For **(A,B)**, brassica cultivars capped with "ns" do not show a significant difference between plant treatment while asterisks denote differing levels of statistical significance: * < 0.05, ** < 0.01 and *** < 0.001 (paired *t*-test). For **(C)**, different letters above bars indicate statistically significant differences between treatments ($P < 0.05$), based on Holm-Sidak method.

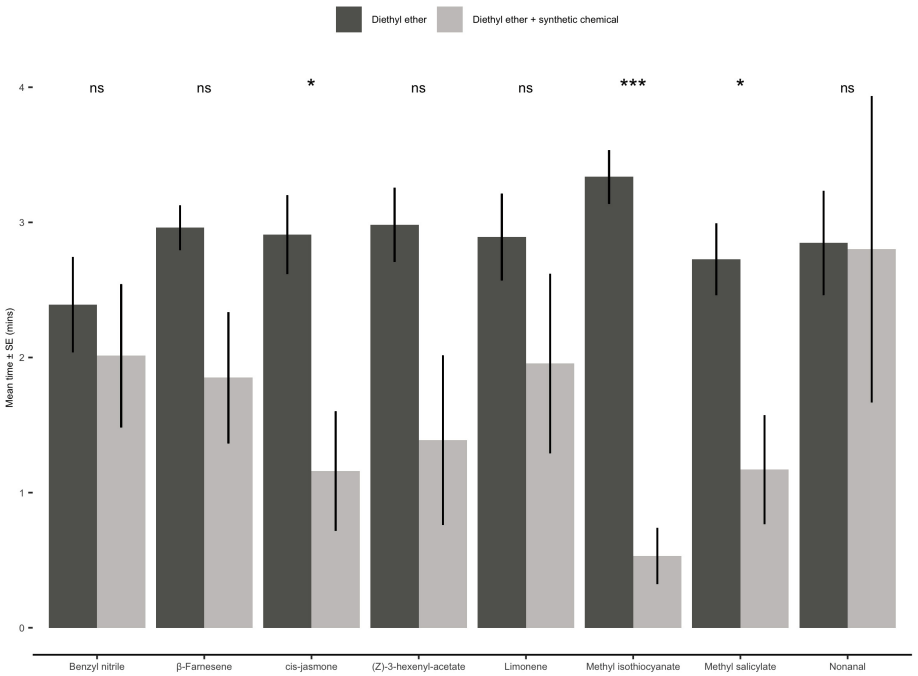

FIGURE 7 | Behavioural responses of *Myzus persicae* females to eight synthetic chemical compounds. Individual aphids were given 12 min to make a choice between one arm containing a synthetic chemical compound vs. three solvent diethyl ether arms. The shown values are mean time spent in arm ± SE ($n = 10$). Synthetic chemical compounds capped with "ns" do not show a significant difference while asterisks denote differing levels of statistical significance: * < 0.05 and *** < 0.001 (paired *t*-test).

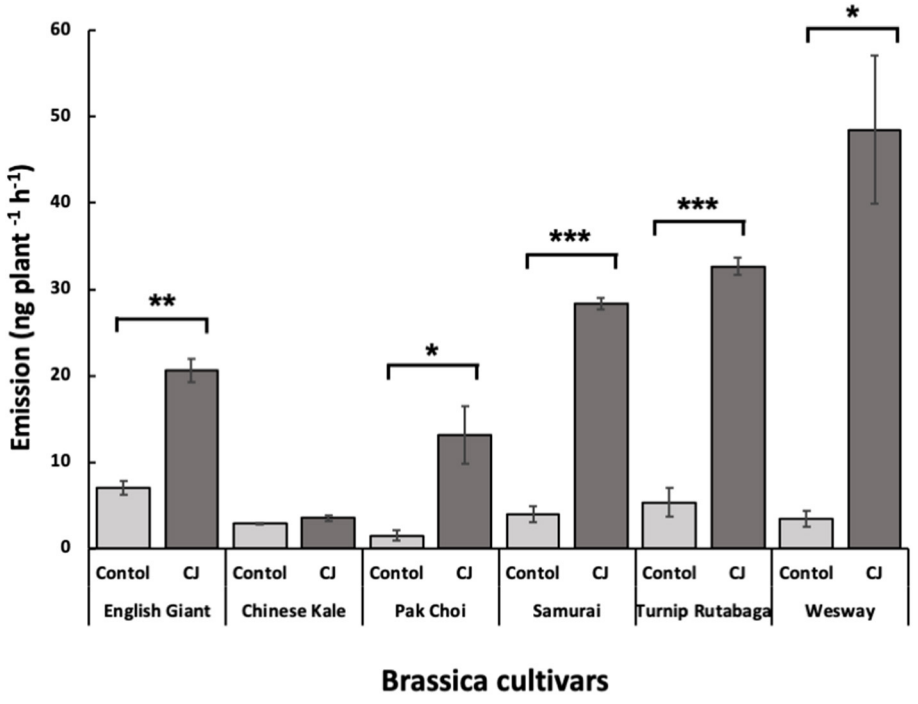

FIGURE 8 | Total amount (mean nanograms plant^{-1} h^{-1} ± SE) of identified volatile organic compounds (VOCs) emitted from the six brassica cultivars with and without CJ-treatment. Asterisks indicate statistically significant differences: *** < 0.001 and * < 0.05 (paired *t*-test).

TABLE 1 | Emission (in ng; mean ± SE; $n = 3$) of volatiles released by *cis*-jasmone treated (CJ) and non-treated (Control) plants [a].

Plant volatile	KI	English giant		Chinese kale		Pak choi		Samurai		Turnip rutabaga		Wesway	
		Control	CJ	Control	CJ	Control	CJ	Control	CJ	Control	CJ	Control	CJ
Alcohols													
2-ethyl-1-hexanol	1030	0.6 ± 0.1	0.7 ± 0.1	0.61 ± 0.1	0.27 ± 0.01	**0.34 ± 0.02**	**2.17 ± 0.3**	**0.51 ± 0.06**	**3.35 ± 0.4**	**0.97 ± 0.5**	**3.61 ± 0.5**	**0.36 ± 0.2**	**5.92 ± 0.9**
1-octanol	1071	ND	ND	ND	ND	ND	ND	**ND**	**0.31 ± 0.08**	**ND**	**0.37 ± 0.1**	**ND**	**0.67 ± 0.2**
2-butyl-1-octanol	1277	0.02 ± 0.01	0.6 ± 0.2	0.16 ± 0.01	0.20 ± 0.03	**ND**	**0.59 ± 0.2**	0.06 ± 0.04	0.41 ± 0.2	**0.03 ± 0.02**	**0.52 ± 0.1**	0.05 ± 0.02	1.41 ± 0.5
Aldehydes													
Nonanal	1104	**0.7 ± 0.2**	**3.4 ± 0.4**	ND	ND	ND	0.06 ± 0.04	**0.07 ± 0.06**	**1.15 ± 0.2**	**0.43 ± 0.2**	**1.61 ± 0.3**	**0.08 ± 0.06**	**2.62 ± 0.6**
Decanal	1206	**0.03 ± 0.01**	**0.17 ± 0.01**	ND	ND	ND	0.17 ± 0.06	0.05 ± 0.04	0.34 ± 0.1	0.22 ± 0.1	0.41 ± 0.02	0.04 ± 0.03	0.97 ± 0.2
Aliphatic hydrocarbons													
Dodecane	1200	0.06 ± 0.05	0.13 ± 0.03	ND	0.02 ± 0.01	0.03 ± 0.01	0.07 ± 0.05	0.04 ± 0.03	0.17 ± 0.09	0.05 ± 0.04	0.18 ± 0.07	**ND**	**0.62 ± 0.06**
(E)-3-tetradecene	1385	0.4 ± 0.2	1.5 ± 0.2	0.43 ± 0.06	0.24 ± 0.12	1.16 ± 0.1	1.34 ± 0.6	0.18 ± 0.07	0.92 ± 0.6	0.13 ± 0.1	0.51 ± 0.2	**ND**	**3.48 ± 0.9**
Benzenoids													
MeSA#	1192	ND	ND	ND	ND	ND	ND	ND	ND	**0.05 ± 0.04**	1.57 ± 0.4	ND	2.5 ± 0.6
Benzothiazole	1229	ND	ND	ND	ND	ND	0.19 ± 0.1	ND	0.77 ± 0.3	**ND**	**0.63 ± 0.1**	ND	0.39 ± 0.3
Esters													
cis-3-hexenyl acetate	1005	2.3 ± 0.3	2.9 ± 0.5	ND	ND	0.43 ± 0.3	2.81 ± 1.2	**0.91 ± 0.1**	**2.32 ± 0.2**	**1.05 ± 0.2**	**2.11 ± 0.09**	**1.28 ± 0.4**	**5.3 ± 0.8**
2-ethylhexyl acetate	1129	0.17 ± 0.01	0.22 ± 0.01	ND	ND	0.08 ± 0.03	ND	0.14 ± 0.07	0.19 ± 0.08	0.05 ± 0.03	0.19 ± 0.01	ND	ND
Ketones													
Dihydrojasmone	1369	0.10 ± 0.04	1.09 ± 0.6	ND	ND	**ND**	**0.27 ± 0.03**	**ND**	**0.63 ± 0.07**	ND	ND	ND	ND
cis-Jasmone	1394	**0.12 ± 0.06**	**1.53 ± 0.3**	**ND**	**0.11 ± 0.02**	**ND**	**1.76 ± 0.5**	**ND**	**10.9 ± 2.7**	**0.16 ± 0.13**	**1.6 ± 0.4**	**ND**	**11.7 ± 2.9**
N-containing compounds													
Methyl isothiocyanate	992	**0.08 ± 0.06**	**0.5 ± 0.1**	ND	0.10 ± 0.08	0.05 ± 0.04	0.09 ± 0.07	**0.09 ± 0.07**	**0.42 ± 0.01**	ND	ND	ND	0.23 ± 0.2
Benzyl nitrile	1144	**ND**	**1.7 ± 0.2**	**ND**	**0.31 ± 0.07**	0.04 ± 0.03	0.73 ± 0.24	ND	ND	**ND**	**1.26 ± 0.3**	ND	0.95 ± 0.8
Terpenes													
β-phellandrene	1031	ND	ND	0.18 ± 0.1	0.53 ± 0.09	ND	ND	ND	ND	ND	ND	ND	ND
D-limonene	1030	0.2 ± 0.1	0.5 ± 0.1	0.39 ± 0.06	0.55 ± 0.06	ND	1.97 ± 1.1	0.61 ± 0.4	0.56 ± 0.2	0.41 ± 0.2	1.27 ± 0.4	ND	2.17 ± 1.1
Eucalyptol	1032	ND	0.3 ± 0.1	0.75 ± 0.03	0.51 ± 0.05	ND	ND	0.07 ± 0.05	ND	ND	ND	ND	ND
Citronellol	1229	0.15 ± 0.09	0.22 ± 0.1	0.14 ± 0.1	0.31 ± 0.1	**ND**	**1.03 ± 0.2**	0.11 ± 0.08	ND	ND	ND	ND	ND
DMNT#	1116	ND	ND	ND	ND	ND	ND	ND	ND	**ND**	**0.79 ± 0.2**	ND	ND
p-cymen-7-ol	1289	0.35 ± 0.2	0.54 ± 0.08	0.15 ± 0.01	0.16 ± 0.01	**0.24 ± 0.12**	**0.85 ± 0.02**	0.32 ± 0.08	0.67 ± 0.3	0.32 ± 0.1	3.31 ± 0.5	0.23 ± 0.1	4.67 ± 1.2
α-cedrene	1411	ND	ND	ND	ND	0.06 ± 0.02	0.24 ± 0.05	ND	ND	ND	ND	ND	ND
β-elemene	1391	**1.41 ± 0.1**	**2.47 ± 0.1**	0.08 ± 0.03	0.19 ± 0.04	ND	ND	**0.75 ± 0.1**	**2.79 ± 0.4**	**0.47 ± 0.3**	**7.7 ± 1.7**	**1.23 ± 0.4**	**4.75 ± 0.5**
β-curcumene	1514	0.18 ± 0.02	0.38 ± 0.01	ND	ND	**ND**	**0.31 ± 0.07**	ND	ND	**ND**	**0.36 ± 0.1**	**ND**	**0.38 ± 0.03**
(*E, E*)-α-farnesene	1508	**0.05 ± 0.04**	**0.69 ± 0.1**	ND	ND	0.12 ± 0.1	0.31 ± 0.06	**0.03 ± 0.02**	**1.22 ± 0.03**	**0.68 ± 0.1**	**4.58 ± 0.7**	**0.18 ± 0.1**	**0.66 ± 0.06**

[a] *Plants were treated 24 h before the start of VOCs air entrainment. Under each chemical class, VOCs are ordered in accordance with their increasing retention time in a gas chromatograph and Kovats index. Bold values indicate significant differences between treatments (t-test; $P < 0.05$). # [DMNT: (E)-4, 8-dimethyl-1, 3, 7-nonatriene; MeSA: Methyl salicylate; ND: Not Detected]. VOCs were tentatively identified based on spectra, Kovats retention index and NIST 17 library matches. KI: Kovats index determined on the intermediately non polar HP5-MS column (https://webbook.nist.gov/; http://www.pherobase.com/).*

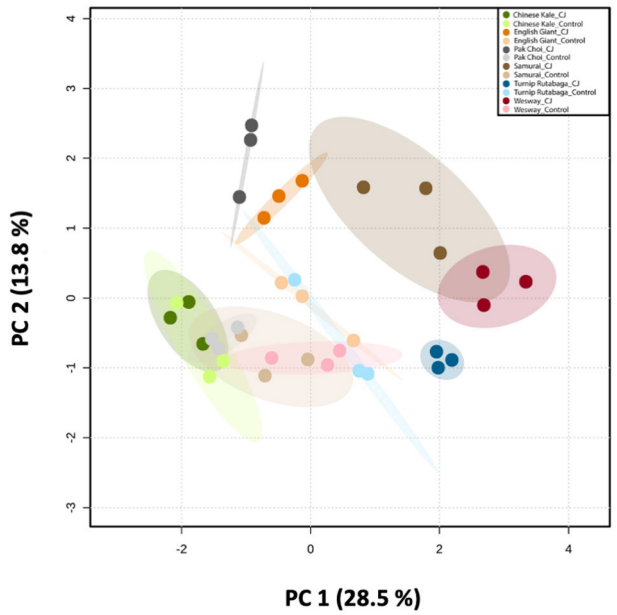

FIGURE 9 | Principal Component Analysis (PCA) of the volatile compounds emitted by six brassica cultivars (i.e., Chinese kale, English Giant, pak choi, Samurai, Wesway, and Turnip Rutabaga) ($n = 3$). Score plot visualises the ordination of collected samples according to the first two PCs based on the quantitative values of emitted VOCs from different brassica cultivars with and without CJ-treatment, with the percentage of explained variation in parentheses. The ellipses show 95% confidence regions.

Significant increases in parasitoid foraging time were found in five out of the six cultivars tested. Earlier studies in *Arabidopsis* (Bruce et al., 2008) had shown that parasitoids spent approximately twice as long foraging on CJ treated plants compared to control plants. For several of the crop brassica species tested here the size of the effect was considerably bigger, for example, on pak choi and Wesway parasitoids spent 5 and 4.5 times longer on CJ treated plants than on control plants. The cultivars in which there was the biggest effect on parasitoid foraging were also the ones where the biggest increases in volatile emission were observed. This suggests that parasitoid foraging could be influenced by CJ-induced volatile emission although further experiments would be required to confirm this. A parasitism bioassay showed that numbers of aphid mummies found was significantly increased with CJ treatment in the cultivars pak choi, Turnip Rutabaga, and Wesway. CJ treatment had no effect on parasitism in Chinese kale but it is interesting to note that although parasitism on Chinese kale was low parasitoids spent an extended time foraging even on untreated Chinese kale. The most likely explanation for this is that CJ-treatment not only affected aphid survival with the direct defence effect discussed above but also made the aphids less suitable as hosts for the parasitoid.

Volatile analysis revealed both quantitative and qualitative changes in the volatile profile of CJ treated plants which is clearly illustrated by heatmap analysis. With the exception of Chinese kale, the tested brassica cultivars exhibited higher volatile emission, reached more than 4-fold in some cultivars, of key volatile compounds such as CJ, methyl isothiocyanate, benzyl nitrile, (*Z*)-3-hexenyl acetate, 2-ethyl-1-hexanol, nonanal, β-elemene, (*E,E*)-α-farnesene, and MeSA. Concordant with this, treating other crop plants with CJ induced the emission of several VOCs, including MeSA (Hegde et al., 2012; Sobhy et al., 2017, 2020), CJ (Sobhy et al., 2017, 2020), (*E*)-β-farnesene (Delaney et al., 2013; Oluwafemi et al., 2013; Sobhy et al., 2017, 2020) and (*Z*)-3-hexenyl acetate (Hegde et al., 2012; Delaney et al., 2013). These induced volatiles have been shown previously to mediate brassica/aphid/parasitoid interactions (Bruce et al., 2008; Pope et al., 2008; Verheggen et al., 2013). Thus, one possible interpretation for the notable reduction in aphid settlement and survival observed on CJ treated plants could be attributed to the higher emission of aphid defence-related herbivore-induced plant volatiles (HIPVs) following CJ application. Aphids may therefore perceive such elevated volatile emissions from CJ-treated plants as signals of a greater risk of competition from conspecifics or as a greater risk of predation from natural enemies (Bruce and Pickett, 2011). Supporting the first assumption, a reduced settlement (Cascone et al., 2015; Markovic et al., 2019), fecundity (Digilio et al., 2012; Maurya et al., 2020), and feeding behaviour (Kang et al., 2018) of several aphid species were reported when reared on plants exposed to HIPVs. The latter scenario is further supported by our parasitoid foraging and parasitism rate bioassays where parasitoid females spent substantially longer time on CJ treated plants and higher numbers of mummified aphids were recorded on CJ treated plants. Indeed, studies have previously shown that CJ treatment induce the plant indirect defence increasing their attractiveness to parasitic wasps (Bruce et al., 2008; Dewhirst et al., 2012). When individually testing the synthetics of these elevated volatiles following CJ treatment, methyl isothiocyanate, MeSA and CJ were most repellent to aphids. Indeed, these VOCs are key semiochemicals in aphid trophic interactions and are directly associated with aphid repellency and/or attraction of aphid natural enemies (Bruce et al., 2003a, 2008; Zhu and Park, 2005; Mallinger et al., 2011; Lin et al., 2016).

While there was variation between cultivars, the current results are promising because effects on aphid performance and settlement or parasitoid foraging were found in all cultivars. In five of the six cultivars tested, both aphid settlement was reduced and parasitoid foraging increased. This simultaneous "slow down" of aphid growth combined with a "speed up" of parasitoid activity could be very valuable in pest management because it could perhaps mean that natural enemies are better able to keep pace with pest population growth. Combined effects on aphids and parasitoids would be useful in an integrated pest management framework (Stenberg, 2017). The next step in development of CJ as a treatment for use against aphids in brassicas is to conduct field experiments and we have this planned for future research. We found an increase in volatile emission from CJ treated plants and this could perhaps explain why aphid settlement was reduced and parasitoid foraging increased. It would be

interesting to look at the genetics underpinning these effects in future research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JA, JR, IS, WK, and TB conceived the ideas and designed the experiments. JA, AC, and JR performed the experiments and collected the data. JA, JR, and IS analyzed the data. JA, JR, IS, and TB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

FUNDING

The research was funded by BBSRC project BB/R021708/1. JA was supported by the Government of India.

ACKNOWLEDGMENTS

We thank Dr. Graham Teakle and Gill Prince of Warwick University (UK) for providing seeds and Dr. Tom Pope of Harper Adams University (UK) for providing the initial aphid and parasitoid cultures.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.711896/full#supplementary-material

REFERENCES

Agelopoulos, N. G., Hooper, A. M., Maniar, S. P., Pickett, J. A., and Wadhams, L. J. (1999). A novel approach for isolation of volatile chemicals released by individual leaves of a plant *in situ*. *J. Chem. Ecol.* 25, 1411–1425. doi: 10.1023/A:1020939112234

Bass, C., Puinean, A. M., Zimmer, C. T., Denholm, I., Field, L. M., Foster, S. P., et al. (2014). The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*. *Insect Biochem. Mol. Biol.* 51, 41–51. doi: 10.1016/j.ibmb.2014.05.003

Birkett, M. A., Campbell, C. A., Chamberlain, K., Guerrieri, E., Hick, A. J., Martin, J. L., et al. (2000). New roles for *cis*-jasmone as an insect semiochemical and in plant defense. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9329–9334. doi: 10.1073/pnas.160241697

Bruce, T. J. A. (2014). Variation in plant responsiveness to defense elicitors caused by genotype and environment. *Front. Plant Sci.* 5:349. doi: 10.3389/fpls.2014.00349

Bruce, T. J. A., Martin, J. L., Pickett, J. A., Pye, B. J., Smart, L. E., and Wadhams, L. J. (2003a). *cis*-Jasmone treatment induces resistance in wheat plants against the grain aphid, *Sitobion avenae* (Fabricius) (Homoptera: Aphididae). *Pest Manag. Sci.* 59, 1031–1036. doi: 10.1002/ps.730

Bruce, T. J. A., Matthes, M. C., Chamberlain, K., Woodcock, C. M., Mohib, A., Webster, B., et al. (2008). *cis*-Jasmone induces *Arabidopsis* genes that affect the chemical ecology of multitrophic interactions with aphids and their parasitoids. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4553–4558. doi: 10.1073/pnas.0710305105

Bruce, T. J. A., and Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects-finding the right mix. *Phytochemistry* 72, 1605–1611. doi: 10.1016/j.phytochem.2011.04.011

Bruce, T. J. A., Pickett, J. A., and Smart, L. (2003b). *cis*-Jasmone switches on plant defence against insects. *Pestic. Outlook* 14:96. doi: 10.1039/b305499n

Budenberg, W. J., Powell, W., and Clark, S. J. (1992). The influence of aphids and honeydew on the leaving rate of searching aphid parasitoids from wheat plants. *Entomol. Exp. Appl.* 63, 259–264. doi: 10.1111/j.1570-7458.1992.tb01582.x

Cascone, P., Iodice, L., Maffei, M. E., Bossi, S., Arimura, G., and Guerrieri, E. (2015). Tobacco overexpressing β-ocimene induces direct and indirect responses against aphids in receiver tomato plants. *J. Plant Physiol.* 173, 28–32. doi: 10.1016/j.jplph.2014.08.011

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., et al. (2018). MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* 46, W486–W494. doi: 10.1093/nar/gky310

Delaney, K. J., Wawrzyniak, M., Lemańczyk, G., Wrzesińska, D., and Piesik, D. (2013). Synthetic *cis*-jasmone exposure induces wheat and barley volatiles that repel the pest cereal leaf beetle, *Oulema melanopus* L. *J. Chem. Ecol.* 39, 620–629. doi: 10.1007/s10886-013-0281-4

Dewar, A. M. (2017). The adverse impact of the neonicotinoid seed treatment ban on crop protection in oilseed rape in the United Kingdom. *Pest Manag. Sci.* 73, 1305–1309. doi: 10.1002/ps.4511

Dewhirst, S. Y., Birkett, M. A., Loza-Reyes, E., Martin, J. L., Pye, B. J., Smart, L. E., et al. (2012). Activation of defence in sweet pepper, *Capsicum annum*, by *cis*-jasmone, and its impact on aphid and aphid parasitoid behaviour. *Pest Manag. Sci.* 68, 1419–1429. doi: 10.1002/ps.3326

Digilio, M. C., Cascone, P., Iodice, L., and Guerrieri, E. (2012). Interactions between tomato volatile organic compounds and aphid behaviour. *J. Plant Interact.* 7, 322–325. doi: 10.1080/17429145.2012.727104

Hegde, M., Oliveira, J. N., da Costa, J. G., Loza-Reyes, E., Bleicher, E., Santana, A. E. G., et al. (2012). Aphid antixenosis in cotton is activated by the natural plant defence elicitor *cis*-jasmone. *Phytochemistry* 78, 81–88. doi: 10.1016/j.phytochem.2012.03.004

Kang, Z.-W., Liu, F.-H., Zhang, Z.-F., Tian, H.-G., and Liu, T.-X. (2018). Volatile β-ocimene can regulate developmental performance of peach aphid *Myzus persicae* through activation of defense responses in Chinese cabbage *Brassica pekinensis*. *Front. Plant Sci.* 9:708. doi: 10.3389/fpls.2018.00708

Leather, S. R., Awmack, C. S., and Garratt, M. P. D. (2017). "Growth and development," in *Aphids as Crop Pests*, eds H. F. van Emden, and R. Harrington (Oxfordshire: CAB International), 98–113. doi: 10.1079/9781780647098.0098

Lin, Y., Hussain, M., Avery, P. B., and Qasim, M. (2016). Volatiles from plants induced by multiple aphid attacks promote conidial performance of *Lecanicillium lecanii*. *PLoS ONE* 11:e0151844. doi: 10.1371/journal.pone.0151844

Mallinger, R. E., Hogg, D. B., and Gratton, C. (2011). Methyl salicylate attracts natural enemies and reduces populations of soybean aphids (Hemiptera: Aphididae) in soybean agroecosystems. *J. Econ. Entomol.* 104, 115–124. doi: 10.1603/EC10253

Markovic, D., Colzi, I., Taiti, C., Ray, S., Scalone, R., Ali, J. G., et al. (2019). Airborne signals synchronize the defenses of neighboring plants in response to touch. *J. Exp. Bot.* 70, 691–700. doi: 10.1093/jxb/ery375

Matthes, M. C., Bruce, T. J. A., Ton, J., Verrier, P. J., Pickett, J. A., and Napier, J. A. (2010). The transcriptome of cis-jasmone-induced resistance in *Arabidopsis thaliana* and its role in indirect defence. *Planta* 232, 1163–1180. doi: 10.1007/s00425-010-1244-4

Maurya, A. K., Patel, R. C., and Frost, C. J. (2020). Acute toxicity of the plant volatile indole depends on herbivore specialization. *J. Pest Sci.* 93, 1107–1117. doi: 10.1007/s10340-020-01218-6

Meier, U. (1997). *Growth Stages of Mono- and Dicotyledonous Plants : BBCH-Monograph*. Berlin; Boston, MA: Blackwell Wissenschafts-Verlag.

Mwando, N. L., Tamiru, A., Nyasani, J. O., Obonyo, M. A. O., Caulfield, J. C., Bruce, T. J. A., et al. (2018). Maize chlorotic mottle virus induces changes in host plant volatiles that attract vector thrips species. *J. Chem. Ecol.* 44, 681–689. doi: 10.1007/s10886-018-0973-x

Oluwafemi, S., Dewhirst, S. Y., Veyrat, N., Powers, S., Bruce, T. J. A., Caulfield, J. C., et al. (2013). Priming of production in maize of volatile organic defence compounds by the natural plant activator *cis*-jasmone. *PLoS ONE* 8:e62299. doi: 10.1371/journal.pone.0062299

Pope, T. W., Kissen, R., Grant, M., Pickett, J. A., Rossiter, J. T., and Powell, G. (2008). Comparative innate responses of the aphid parasitoid *Diaeretiella rapae* to alkenyl glucosinolate derived isothiocyanates, nitriles, and epithionitriles. *J. Chem. Ecol.* 34, 1302–1310. doi: 10.1007/s10886-008-9531-2

R Core Development Team (2021). "R: a language and environment for statistical computing.," in *R Foundation for Statistical Computing*, eds R. S. Cowles, C. R. Soana, R. Holdcraft, and G. M. Loeb (Vienna). Available online at: http://www.r-project.org/

Sobhy, I. S., Caulfield, J. C., Pickett, J. A., and Birkett, M. A. (2020). Sensing the danger signals: *cis*-Jasmone reduces aphid performance on potato and modulates the magnitude of released volatiles. *Front. Ecol. Evol.* 7:499. doi: 10.3389/fevo.2019.00499

Sobhy, I. S., Woodcock, C. M., Powers, S. J., Caulfield, J. C., Pickett, J. A., and Birkett, M. A. (2017). *cis*-Jasmone elicits aphid-induced stress signalling in potatoes. *J. Chem. Ecol.* 43, 39–42. doi: 10.1007/s10886-016-0805-9

Stenberg, J. A. (2017). A conceptual framework for integrated pest management. *Trends Plant Sci.* 22, 759–769. doi: 10.1016/j.tplants.2017.06.010

Sun, Y. L., Dong, J. F., Huang, L. Q., and Wang, C. Z. (2020). The cotton bollworm endoparasitoid *Campoletis chlorideae* is attracted by *cis*-jasmone or *cis*-3-hexenyl acetate but not by their mixtures. *Arthropod Plant Interact.* 14, 169–179. doi: 10.1007/s11829-019-09738-4

van Emden, H. F., Eastop, V. F., Hughes, R. D., and Way, M. J. (1969). The ecology of *Myzus persicae*. *Annu. Rev. Entomol.* 14, 197–270. doi: 10.1146/annurev.en.14.010169.001213

Verheggen, F. J., Haubruge, E., De Moraes, C. M., and Mescher, M. C. (2013). Aphid responses to volatile cues from turnip plants (*Brassica rapa*) infested with phloem-feeding and chewing herbivores. *Arthropod. Plant. Interact.* 7, 567–577. doi: 10.1007/s11829-013-9272-1

Webster, B., Bruce, T., Pickett, J., and Hardie, J. (2010). Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. *Anim. Behav.* 79, 451–457. doi: 10.1016/j.anbehav.2009.11.028

Zhu, J., and Park, K. (2005). Methyl salicylate, a soybean aphid-induced plant volatile attractive to the predator *Coccinella septempunctata*. *J. Chem. Ecol.* 31, 1733–1746. doi: 10.1007/s10886-005-5923-8

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 October 2021
doi: 10.3389/fpls.2021.698882

Convergence of *Bar* and *Cry1Ac* Mutant Genes in Soybean Confers Synergistic Resistance to Herbicide and *Lepidopteran* Insects

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OPEN ACCESS

Edited by:
Islam S. Sobhy,
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Reviewed by:
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Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *22 April 2021*
Accepted: *13 September 2021*
Published: *14 October 2021*

Citation:
Nguyen TD, La VH, Nguyen VD, Bui TT, Nguyen TT, Je YH, Chung YS and Ngo XB (2021) Convergence of Bar and Cry1Ac Mutant Genes in Soybean Confers Synergistic Resistance to Herbicide and Lepidopteran Insects. Front. Plant Sci. 12:698882. doi: 10.3389/fpls.2021.698882

Soybean is a globally important crop species, which is subject to pressure by insects and weeds causing severe substantially reduce yield and quality. Despite the success of transgenic soybean in terms of *Bacillus thuringiensis* (*Bt*) and herbicide tolerance, unforeseen mitigated performances have still been inspected due to climate changes that favor the emergence of insect resistance. Therefore, there is a need to develop a biotech soybean with elaborated gene stacking to improve insect and herbicide tolerance in the field. In this study, new gene stacking soybean events, such as bialaphos resistance (*bar*) and pesticidal crystal protein (*cry*)*1Ac* mutant 2 (M#2), are being developed in Vietnamese soybean under field condition. Five transgenic plants were extensively studied in the herbicide effects, gene expression patterns, and insect mortality across generations. The increase in the expression of the *bar* gene by 100% in the leaves of putative transgenic plants was a determinant of herbicide tolerance. In an insect bioassay, the *cry1Ac*-M#2 protein tested yielded higher than expected larval mortality (86%), reflecting larval weight gain and weight of leaf consumed were less in the T1 generation. Similarly, in the field tests, the expression of *cry1Ac*-M#2 in the transgenic soybean lines was relatively stable from T0 to T3 generations that corresponded to a large reduction in the rate of leaves and pods damage caused by *Lamprosema indicata* and *Helicoverpa armigera*. The transgenic lines converged two genes, producing a soybean phenotype that was resistant to herbicide and lepidopteran insects. Furthermore, the expression of *cry1Ac*-M#2 was dominant in the T1 generation leading to the exhibit of better phenotypic traits. These results underscored the great potential of combining *bar* and *cry1Ac* mutation genes in transgenic soybean as pursuant of ensuring resistance to herbicide and lepidopteran insects.

Keywords: *Cry1Ac* mutation 2, herbicide resistance, *Lepidoptera* insects, soybean transformation, VX93

INTRODUCTION

Soybean is one of the most important oilseed crops and its growth in many regions of the world is subject to pressure by insects, which is the main factor affecting the yield and quality. Insect pests cause 20–30% biomass losses in soybean (*Glycine max* L.) worldwide (Bengyella et al., 2018). In the Lepidoptera order, *Helicoverpa armigera* Hubner (*H. armigera*) is the main insect pest of several crops such as cotton, chickpea, and soybean (Homrich et al., 2012; Martins-Salles et al., 2017). The application of chemical insecticides is a common measure to control insect pests in soybean fields. However, this practice has raised many problems in terms of the environment and human health. Genetically modified (GM) crops could be an alternative to minimize the application of chemical pesticides. The GM crops express an insecticidal gene to control major lepidopteran insects. This approach not only provides an effective alternative tool for insect management but also addresses other issues such as environmental protection and economic advantages to farmers. In 2017, soybean varieties harboring insecticidal traits stacked with herbicide tolerance were grown in ∼95.9 million ha worldwide with an economic value of up to USD 17.6 billion (ISAAA, 2017).

The insecticidal crystal protein (ICPs) imparts resistance against lepidopteran insects. Synergistic activities among different ICPs to augment insect pest resistance have hitherto been reported (Xue et al., 2014). Due to high specificity for exclusive receptors on the membrane of the insect gut epithelial cells (Carroll et al., 1997; Badran et al., 2016), ICPs are shown to be harmless to non-target insects and are compatible with integrated pest management (IPM) systems (Naranjo, 2011). Thus, the expression of ICPs in commercialized *Bacillus thuringiensis* (*Bt*) soybean is a highly important agronomic trait. However, *Bt* soybean varieties have not yet been fully commercialized. Genetic engineering is an important technique to develop *Bt* crops with the pesticidal crystal protein (*cry*) gene (Homrich et al., 2012; Bengyella et al., 2018). The *cry1Ac* gene is one of the *cry* genes isolated from the bacterium *Bt*, and this gene codes for an insecticidal crystal protein. There are several successful reports of genetic transformation into soybean (Yu et al., 2013), cotton (Wei et al., 2003), and sugarcane (Gao et al., 2016). Evidence from laboratory bioassay (Stewart et al., 1996) and field experiments (Walker et al., 2000) shows that transgenic soybean lines with high expression levels of the *cry1Ac* gene significantly increase larval mortality. However, *Bt* transgenic plants often show variable resistance responses against insect pests because of lower *cry* genes expression (Martins-Salles et al., 2017). Thus, there is a need to develop transgenic lines with elaborated gene stacking to limit the emergence of insect resistance. Furthermore, recombinant fusion proteins and domain swapping can be leveraged to protection against insects (Nachimuthu and Kumar, 2004; Martins-Salles et al., 2017). Hence, the challenge is to develop insect-resistant lines by increasing and stabilizing the level of the *cry1Ac* gene in target tissues or use a fusion protein to confer complete protection against insects.

In this study, our goal was to improve the lepidopteran insect resistance in a Vietnamese soybean, by introducing a newly released high expression of *cry1Ac* mutant (M#2). We recommended herein that bialaphos resistance (*bar*) and *cry1Ac*-M#2 genes transferred to soybean offer an enhanced herbicide and insect resistance. The elevated resistance was identified by screening and analyzing plant phenotypes, *cry1Ac* mutant transcriptional level, Southern blotting, and larval insect responses in laboratory bioassays.

MATERIALS AND METHODS

Materials

The plasmids pOB, pENTRTM/D-TOPO, pCambia 3301, and pB2GW7 were sourced from the Invitrogen (Thermo Fisher Scientific, MA USA 02451, www.thermofisher.com). *Escherichia coli* (*E. coli*) strain DH5α and *Agrobacterium tumefacines* (*A. tumefacienes*) strain EHA105 were provided by Prof. Young Soo Chung, Dong-A University, Busan, South Korea. The *cry1Ac* mutants in pOB-Mutant-*cry1Ac*-M#2 (**Supplementary Figures 1–3**) were developed by a collaboration between the Department of Biotechnology and Food Technology, Thai Nguyen University of Agriculture and Forestry, Vietnam, and Dong-A University and Seoul University, South Korea. The soybean cultivar VX93 used for transformation was provided by the Genetic Institute and Maize Research Institute, Ha Noi, Vietnam.

Vector Construction

For the construction of binary vector, pOB-Mutant-*cry1Ac* was originally derived from pIM-Mod-*cry1Ac* with Xba I and Bgl II restriction enzymes for *cry1Ac* mutation region (821 bp). The *cry1Ac* mutant gene was constructed by integrating it into the pOB vector. The pOB vector harboring the *cry1Ac* mutant gene, AcNPV $3'$-$5'$ homologous regions, and AcNPV Polh promoter finally constructed pOB-Mut-*cry1Ac* (8,085 bp) (**Supplementary Figure 2**). Cassettes containing the *bar* and *cry1Ac*-M#2 genes, 35S promoter, and 35S terminator (T35S) were digested from the pOB-Mutant-*cry1Ac* and pENTR^{TM}/D-TOPO vector using restriction enzymes, *Bam*HI and *Eco*RI (Invitrogen, USA) (**Figure 7A**, **Supplementary Figure 3**). The expression vector pB2GW7 was digested by *Bam*HI and *Eco*RI and linked to the cassette with T4-DNA ligase containing the *cry1Ac*-M#2 (**Supplementary Figure 4**). The mutation of *cry1Ac*-M#2 was confirmed by sequencing and comparing it with the *cry1Ac* gene available in GenBank (https://www.ncbi.nlm.nih.gov/genbank) (**Supplementary Figure 5; Tables 1, 2**).

Genetic Transformation and Screening

Genetic transformation of soybean used the "half seed" method described in the study by Olhoft et al. (2003), with minor modifications. Briefly, mature soybean seeds were sterilized with chlorine gas prepared by mixing 3.5 ml of 12 N HCl and 100 ml bleach (5.25% sodium hypochlorite) for 24 h and the sterilized seeds were thoroughly rinsed with sterilized water. From a seed, one cotyledonary node was removed and cut apart of the hypocotyl. The epicotyl was subsequently removed and

both axillary bud and cotyledonary node were wounded by scratching with a blade. Then, 50 explants were inoculated in the 50 ml co-cultivation (CC) *A. tumefaciens* suspension (OD = 0.8). Afterward, it was rapidly sonicated for 30 s and vacuumed for 30 s. After 30 min, 10 explants were placed on a solid CC medium of Acetosyringone (0.2 mM) and inoculated at 25°C for 5 days under 16 h light period per day. After this time, excess *A. tumefaciens* were removed from the explants by liquid shoot induction medium (SIM) prepared by adding cefotaxime (250 mg L^{-1}) and ticarcillin (250 mg L^{-1}). Explants were then transferred into solidified SIM1 containing phosphinothricin (PPT) (10 mg L^{-1}) and grown at 25°C under fluorescence lighting 200 μmol photons m^{-2} s^{-1} in 16/8 h light/dark cycles. After 14 days, the newly developed shoots were sub-cultured to fresh SIM2 containing 5 mg L^{-1} PPT for the selection of transformed shoots, and this culture and selection process were continued up to 28 days in shoot elongation medium (SEM) containing PPT 5 mg L^{-1}. When the elongated shoot length was over 4 cm, it was transferred to a rooting medium (RM) supplied with indol-3-butyric acid (IBA) at the rate of 0.5 mg L^{-1} for new root induction. After 14 days, T0 plants were grown in the greenhouse until maturity under natural light and 80% relative humidity at 28 ± 2°C.

Screening of Transgenic Plants Using Herbicide Biochemical Test

Screening the glufosinate-resistant transgenic plants by leaf painting method was carried out as described in the study of by Paz et al. (2004). Briefly, plants with four trifoliate and R1 (early fruiting stage) were screened for putative transformants that expressed the bar gene. The upper surface of a leaf was painted with PPT along with the midrib using a cotton bud (Q-tip). The concentration of active ingredient PPT 0.3, 0.5, 0.7, and 1 mg ml^{-1} was tested. The plant was scored based on the tolerance of leave tissue at 3–5 days after painting.

Under field test, the transgenic and control (non-transgenic) plants were grown in a field experiment at the Thai Nguyen University of Agriculture and Forestry, Thai Nguyen, Vietnam (22°03′ N, 106°14′ E). The randomized completed block design with four blocks of 300 m^2 (15 × 20 m) each was used to grow plants. Seeds were planted in eight random subsections of each block with two transgenic lines separated by the lines of the control plants. The plants were grown under natural temperature, light, and humidity conditions during the season. Furthermore, 30-day-old plants were preliminarily screened using PPT 0.5 mg ml^{-1} and sprayed with a chemical herbicide (Basta 0.3%, Bayer, Germany) based on a preliminary test referring to the previous study (Paz et al., 2004). After 5 days, the occurrence of yellow leaves and plant death were evaluated. The survival of plants with maintaining green leaves exhibited herbicide tolerance. The following agronomic traits were also measured: plant height, pods per plant, seed number per plant, and thousand-seed weight, and yield.

Insect Bioassay

An insect bioassay was conducted with *Lamprosema indicata* (F.) larvae based on the previous method of using an artificial diet (Gupta et al., 2004; Singh et al., 2016). Four-day-old *L. indicata* larvae were used for assessing the feeding behavior on leaf tissue of the selected transgenic lines. The young and tender soybean leaflets were clipped from three transgenic lines and non-transgenic plants (control) at 40 days old. One complete leaf was placed on a petri dish on a moistened filter paper. For the insect feeding bioassay, five four-day-old larvae were carefully released on the soybean leaflet in each petri dish to assess the sensitivity of *L. indicata* to the protein encoded by the transferred *cry1Ac* mutant in the leaf tissue. The lids were perforated with a paper pin to ensure air circulation in a room set at 27 ± 1°C, with a relative humidity of 65 ± 5% and a 16-h light/8-h dark cycle. The percentage mortality was calculated from 72 h on daily basis (Abedi et al., 2014). After 3 days, the remaining leaves were evaluated to determine the amount of leaf consumed (g) by the neonates. Similarly, the larval weight gain (g), the percent mortality, and the weight of the leaf consumed on the 3 days were recorded.

Confirmation of *cry1Ac* Gene Mutant in Transgenic Plants by RT-PCR

Total RNA was isolated from 0.1 g young leaf of PPT- and basta-resistance plants by using customized plant RNAiso Plus Kit (Takara, Takara Bio Inc, Japan, https://www.takara-bio.com/). The cDNA was synthesized using the GoScript Reverse Transcription System (Promega, USA, https://worldwide.promega.com). The presence of the *bar* and *cry1Ac* genes in the putative transgenic soybean were confirmed by RT-PCR using specific primer sequences (**Supplementary Table 3**). The thermal amplification was carried out using 10X SYBR® Premix Ex TaqTM buffer (including Taq DNA polymerase 5U, Takara, Takara Bio Inc, Japan, https://www.takara-bio.com/), 2.5 mM dNTP mix, 10 pM primers, in 20 μl volume. The RT-PCR reaction condition was as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s, with a final extension at 72°C for 2 min, and finally samples were maintained at 4°C. Gene expression levels were quantified *via* real-time PCR by using detection on a Bio-Rad system (Bio-Rad, USA, https://www.bio-rad.com). Products were resolved on 1% agarose gel stained with ethidium bromide in 1X TAE buffer and images were captured by Gel Documentation system (BioRad Gel Doc XR, US). The qPCR reaction was performed in triplicate for each of the three independent samples. Quantification of the relative transcript levels used the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Southern Blot Analysis

Total DNA was extracted from 0.5 g fresh leaves using the CTAB method (Saghai-Maroof et al., 1984) and the DNA was purified using a genomic DNA purification kit (Thermo Fisher Scientific, USA). Southern blot analysis was undertaken following the method of Wang (2005). To produce unique fragments for T-DNA insertion from the pB2GW7 vector, 15 μl total DNA was digested with restriction enzymes *Bam*HI and *Eco*RI. The products were separated on a 1.5% (w/v) agarose gel and blotted onto Amersham Hybond^{TM}–N^+ membrane (GE Healthcare

products, Sigma-Aldrich, USA) according to the instructions of the manufacturer. The membrane was prehybridized at 65°C for a minimum of 2 h in 100 ml of 10X SSC solution, pH = 7 (50 M NaCl, 25 mM Na_3Citrat, 1% SDS) and 100 ml of hybridization solution 20X SSPE, pH = 7.7 [mM NaCl 100, 20 mM NaH_2PO_4, 10 mM EDTA, 2% (w/v) SDS]. The probes for *bar*- and *cry1Ac*-M#2 genes-labeled dUTP-11-biotin were inoculated with the hybridization membranes at 65°C. After 4 h, the hybridization membrane was washed with 10X AP solution, pH = 7.5 (40 mM Tris HCl, 25 mM NaCl, 10 mM $MgCl_2$) and inoculated with 10 μl of streptavidin alkaline phosphatase (Promega MADISON, WI, USA) in a Blocking solution (Thermo Fisher Scientific, USA) at 37°C for 30 min, then washed again using 10X AP solution. The expected band was detected by inoculation with 200 μl NBT/BCIP solution and the reaction was terminated by 0.5 M EDTA.

ELISA Analysis of cry1Ac-M#2 Protein in Transgenic Lines

The cry1Ac-M#2 protein content in putative transgenic soybean progenies was measured by using a quantitative ELISA Kit (Envirologix, USA) based on a double-antibody sandwich enzyme-linked immunosorbent assay. Total soluble protein was isolated from young leaf tissue using bicarbonate buffer [15 mM sodium carbonate, 35 mM sodium bicarbonate, 0.1% TritonX 100, 0.05% Tween 20, 1% polyvinylpyrrolidone (PVP), and 1 mM phenylmethanesulfonylfluoride (PMSF)]. The ELISA was performed according to the instructions of the manufacturer (QuliPlate^{TM} Kit for cry1Ab/cry1Ac, Envirologix, Cat. No AP003 CRBS). *Cry1Ac*-M#2 specific primary antibody and second antibody were incubated with soluble protein in each well of ELISA plate. The absorbance of the samples was measured at 405 nm wavelength in ELISA reader (Synergy H1 Hybrid Reader, Biotek, Korea). The quantification of the *cry1Ac*-M#2 protein was based on absorbance values of these protein test samples onto the standard curve of purified cry1Ac-M#2 protein-extracted from *E. coli* DH5α.

Statistical Analysis

A randomized complete block design was used with five replicates for field trial and sampling date. Moreover, ANOVA was applied to all data. Duncan's multiple range test was employed to compare the means of separate treatments. All statistical tests were performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA 2002–2003, https://www.sas.com/), and the differences at $P < 0.05$ were considered significant.

RESULTS

Production of Transgenic Lines Expressing the *bar* and *cry1Ac*-M#2 Genes

In this study, we validated the ability to receive *bar* and *cry1Ac*-M#2 genes of a commercial Vietnamese soybean variety VX93. *Cry1Ac*-M#2-mediated *A. tumefaciens* using genetic transformation was attempted to VX93. *Cry1Ac*-M#2 gene-optimized for transformation was constructed with nucleotide sequences that were encoded by T-DNA fragment (**Figure 7A**). In this process, the construct-harbored *bar*- and *cry1Ac*-M#2 were transferred to cotyledonary explants (**Figure 1A**). A total of 4,740 explants were used for transformation, of which 214 new shoots were developed on the selection medium (**Figures 1D,E**), with a transformation efficiency of 4.51% (**Table 1**). Eight putative transgenic plants of T0 generation were grown in the soil (**Figures 1F,G**) and tested for their PPT resistance ability. Out of eight, five plants showed survival in the different concentrations of PPT range from 0.3 to 1 mg ml^{-1}. The leaves of the resistant plants maintained green color after 3 days of PPT painting (**Figure 2A**). The frequency of green leaf in the putatively transformed lines was 62.5% higher than that in the control lines (**Figures 2B,C**; **Table 2**).

Herbicide Tolerance Revealed the Efficiency Transformation From T1 to T3 Generations

In the field trials, the plants of T1 to T3 generations were tested PPT and Basta, positive and exhibited stable green leaves, whereas all the non-transgenic plants exhibited yellow leaves followed by leaf death after 7 days of treatment (**Figures 2D,E**). Similarly, T1 generation-putative transgenic plants exhibited the highest survival with 100% plant resistance which gradually decreased in T2 and T3 generations (**Table 3**).

These results are consistent with the RT-PCR and validated that the transgenic plants induced herbicide tolerance. *Bar* gene was remarkably expressed in T0 (**Figure 3A**), accompanied by relative gene expression. A much higher *bar* gene expression was randomly observed in the T1 generation (**Figure 3E**). However, the intensity of this gene expression was largely decreased in T2 and T3 generations (**Figures 3E,F**). On the other hand, locus integration in genomic DNA digested with *Sac*I enzyme was detected in southern blot analysis. As expected, the *cry1Ac*-M#2 transgenic lines resistance to PPT was positive for hybridization with the probe *bar* and *cry1Ac* mutant 2 genes (**Figure 4A**). Notably, a single band (0.5 kb) was detected in all of the transgenic events corresponding to homozygous in T2 and T3 generations, respectively (**Figures 4B,C**).

Expression of *cry1Ac*-M#2 Reduced the Negative Effects of Insect

After 72 h, the detached leaf bioassay showed that the weight of leaf consumed was lower in T1-transgenic lines than in T2 and T3 generations (**Figures 5A–E**). Similarly, larval weight gain was the lowest in the *Bt*-soybean transgenic line of T1 generation, i.e., 38.2% fewer larvae weight gain compared with the non-transgenic plants ($P < 0.001$). Consequently, the leaf weight consumed of T1 plants was less than that of T2 and T3 generations (**Figure 5E**). In contrast, the survival of leaf roller (*L. indicata*) larvae was significantly different when fed the leaves from transgenic lines of T1 to T3 generations. Indeed, the percentage of larval mortality was significantly increased in transgenic plants of T1 generation with a rate of 86.5% compared with non-transgenic plants ($P < 0.001$), whereas it was slightly decreased in T2 and T3 generations ($P < 0.05$) (**Figure 5G**).

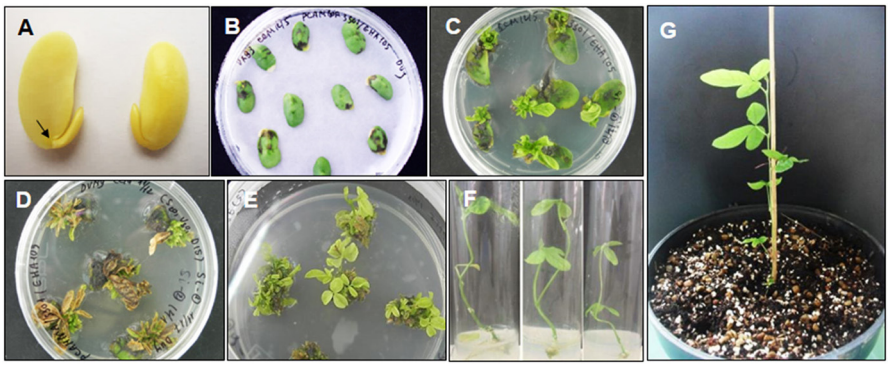

FIGURE 1 | VX93 soybean transformation bearded *bar* and *cry1Ac*-M#2 genes. **(A)** Half seed inoculated Agrobacterium tumefaciens on CCM medium with Whatman filter paper 3MM, **(B)** Shoots induction on SIM media, **(C)** The first selection shoots transgenic in SEM medium with phosphinothricin (PPT) 10 mg L^{-1}, **(D)** The second selected shoot-resistance PPT 10 mg L^{-1} in SEM medium, **(E)** Root induction in RM medium, **(F, G)** transgenic plants in soil. Values are represented as mean ± SE ($n = 3$).

TABLE 1 | The transformation efficiency of *cry1Ac*-M#2-mediated *Agrobacterium tumefaciens* transferred to VX93 soybean cultivar.

Soybean cultivar	No of explants	Percent shoot induced (%)	Shoots survival in phosphinothricin selection medium (shoot)	Percentage selection (%)	Transgenic line grown in greenhouse
VX93-cry1A(c)-M#2	1,871	79.00	46	3.11	3
	1,126	95.20	82	7.65	2
	1,743	87.89	86	5.61	3
	4,740	86.08	214	4.51	8

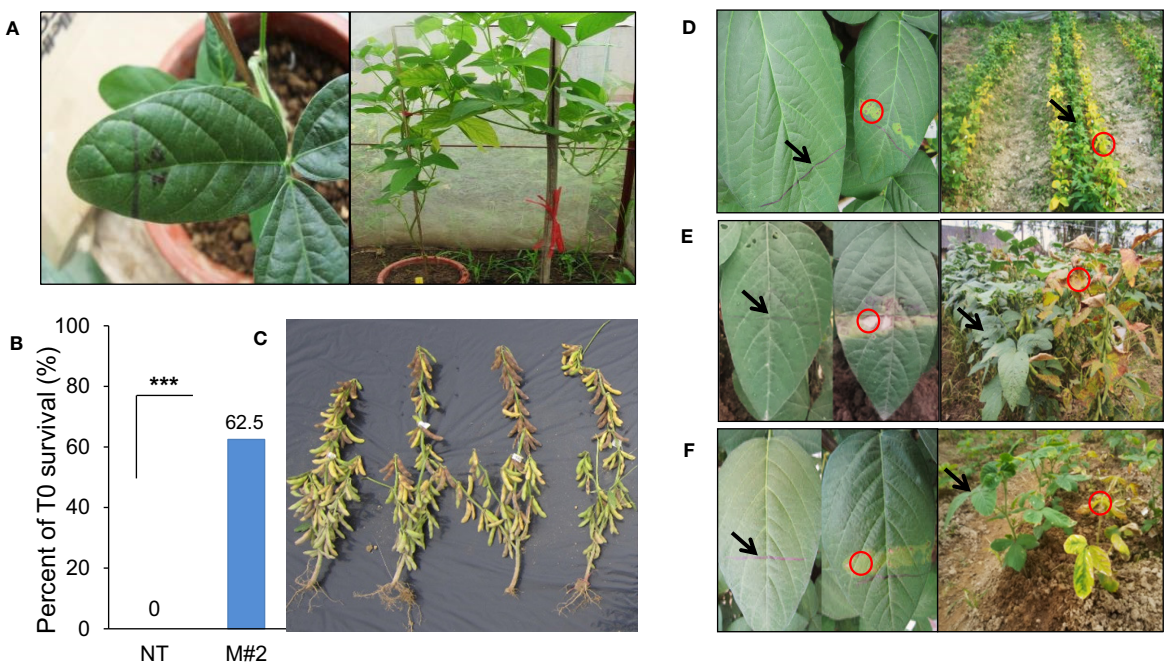

FIGURE 2 | Evaluation of herbicide tolerance from T0 to T3 generation. Three days visualized the morphology of leaf response to PPT.5% following line marker and sprayed basta 0.3% for VX93 transgenic plants in the field after 5 days. **(A)** T0 generation assessed PPT.5 mg mL^{-1}. **(B)** T0 survival. **(C)** T0 plants. **(D–F)** T1 to T3 generations tested herbicide tolerance by PPT 0.5 mg mL^{-1} and basta 0.3% in the field, respectively. Arrows indicate the PPT/basta resistant leaves of transgenic plants and circles are the PPT/basta sensitive leaves of control plants. ***$P < 0.001$.

TABLE 2 | Evaluation of the transformation efficiency of VX93-*cry1Ac-M#2* in T0 herbicide-resistance gene (*bar*) by testing phosphinothricin (PPT).

T0 regeneration	No.	Transgenic lines phenotype response to PPT (mg mL^{-1})			
		0.3	0.5	0.7	1.0
VX93-cry1A(c)-M#2	1	–	–	–	–
	2	–	–	–	–
	3	+	+	+	+
	4	–	–	–	–
	5	+	+	+	+
	6	+	+	+	+
	7	+	+	+	+
	8	+	+	+	+
VX93 Non-transgenic	NT	–	–	–	–

Leaf number 4 was used to test different concentrations of PPT. After 3 days, the obtained yellow leaf is negative (–), while others keeping stable blue color leaf is positive (+).

TABLE 3 | Evaluation of the potential ability of VX93 transgenic lines to herbicide resistance (Basta 0.3%) in T1 to T3 generations under field condition.

Progenies	No. plant resistant to basta 0.3%	No. plant non-resistant to basta 0.3%	Percent of plant resistance (%)
T1			
Transgenic	84***	0	100.0***
Non-transgenic	0	88	0.0
T2			
Transgenic	873***	54	94.2***
Non-transgenic	0	927	0.0
T3			
Transgenic	456***	121	79.0***
Non-transgenic	0	436	0.0

*Asterisks indicate significant differences between the non-transgenic (control) and transgenic plants; ***$P < 0.001$.*

Similar to the pattern of the cry1Ac-M#2 protein content was detected in T1 to T3 generations (**Figure 5H**).

Cry1Ac-M#2 Induced Insect Resistance Under Field Conditions

Under field conditions, the rate of insect resistance observed in T1 to T3 generations was significantly different between transgenic and non-transgenic lines. Generally, *L. indicata* and *H. armigera* were affected to a greater extent in the T2 and T3 generations compared with the T1 generation (**Table 4**). Transgenic plants were more resistant to *L. indicata*, considering the significantly lower rate of leaf damage ($P < 0.05$) and less than four-fold of the rate of plant damage in T2 ($P < 0.001$) compared with non-transgenic lines. However, no significant difference between transgenic and non-transgenic lines was observed on the rate of plants damaged by *H. armigera* insect in the T1 generation, but it was less in T2 and T3 generations.

These results were confirmed by the presence of *cry1Ac* mutant expression in *Bt*-soybean lines. The *cry1Ac* expression levels of *Bt*-soybean were significantly different compared with the non-transgenic plants. The expression pattern of *cry1Ac*-M#2 in five transgenic plants of T1 and T2 generations was similar (**Figures 6A,B**). However, the result in Southern blot digested by *Bam*HI and *Eco*RI enzymes (**Figure 7A**) revealed that four transgenic lines had an expected band of 0.5 kb (**Figures 7B,C**). Subsequently, the *cry1Ac* mutant expression levels were less in the transgenic line of T2 and T3 generations, whereas *cry1Ac*-M#2-4 was not detected by RT-PCR (**Figures 6C–F**) and Southern blot (**Figures 7C,D**) in the T2 transgenic line, similar to one plant in the T3 generation.

Agronomic Performance of Soybean Transgenic Lines Under Field Conditions

Agronomic traits such as plant height, primary branches, pods per plant, seed per plant, and seed weight of the T1 to T3 transgenic plant were compared with the non-transgenic plants grown in the field (**Table 5**). Generally, the average height of transgenic plants was greater than that of non-transgenic plants. Indeed, the majority of transgenic plants in the T1 generation exhibited superior plant growth compared with the non-transgenic line, T2, and T3 generations during the experiment period ($P < 0.05$). There was no difference in the primary branches measured in T1 to T3 generations. The yield of mature fruit and numbers of seeds per plant were greater in the T1 and T2 generations. Similarly, seed weight per plant of transgenic plants in the T1 generation was significantly higher than that of the non-transgenic plants ($P < 0.05$).

DISCUSSION

Transgenic soybean containing multiple *Bt* genes are conferred with resistance to important insect pests (Romeis et al., 2019). A transgenic lineage of soybean expressing the *cry1Ac* gene has enhanced resistance to *Lepidoptera* insects (Walker et al., 2000; Yu et al., 2013), and further research developed with respect to maintaining insect resistance (Badran et al., 2016; Singh et al., 2016; Romeis et al., 2019). A study showed that *Bt*-soybean with *cry1Ac* expression provided good protection against *H. armigera*, however, limited resistance efficiency was found in transgenic soybean (Yu et al., 2013). Therefore, determining the thresholds of regulatory expression at which the *cry1Ac* gene switches from hypersensitive responses to insect resistance and survival in transgenic soybean would provide valuable insights into insect resistance. Accordingly, one of the aims of the present study was to test the effect of the *cry1Ac* mutants that would accelerate the resistance of the soybean against *Lepidoptera* insects, because this gene produces *cry* protein toxicity for this insect (Romeis et al., 2006). Therefore, this present study assessed preferentially relative transcriptional expression, insect mortality levels, and inheritance of *cry1Ac* mutant.

It has been well documented that the combination of different traits or genes in genetically modified plants has been a trend. It

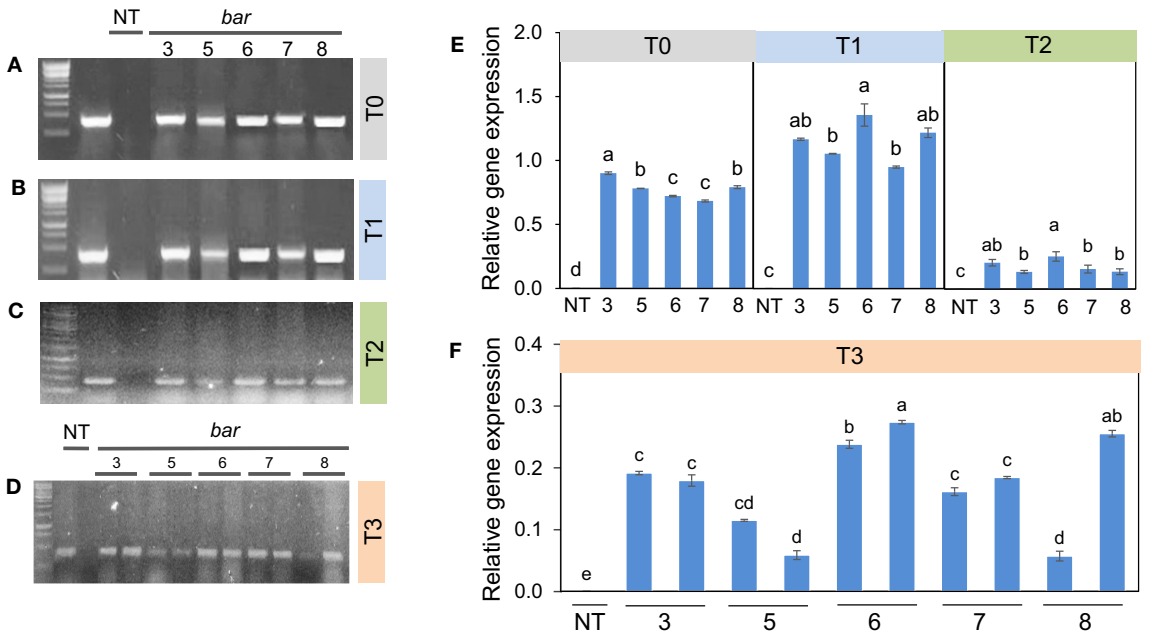

FIGURE 3 | *Bar* gene expression in transgenic plants from T0 to T3 generations. Among these 3, 5, 6, 7, 8 are transgenic lines and non-transgenic plants (NT). *Bar* gene expression of five plants **(A–C)**, left to the right border, in which lane 1 (marker 1kb), lane 2 (plasmid contained pB2WG7 vector), lane 3 negative plant (VX93 non-transgenic, NT), lanes 4 to 8 T0 and T2 generation, respectively. **(D)** left to right border, it showed 10 plants in the T3 generation (from lanes 4 to 13), therein lane 1 is maker 1 kb, lane 2 is plasmid contained pB2WG7 vector, lane 3 is a negative plant (VX93 non-transgenic), and lanes 4 to 13 are transgenic plants. Relative gene expression of T0 to T2 **(E)**, and T3 **(F)** for each plant consistently.

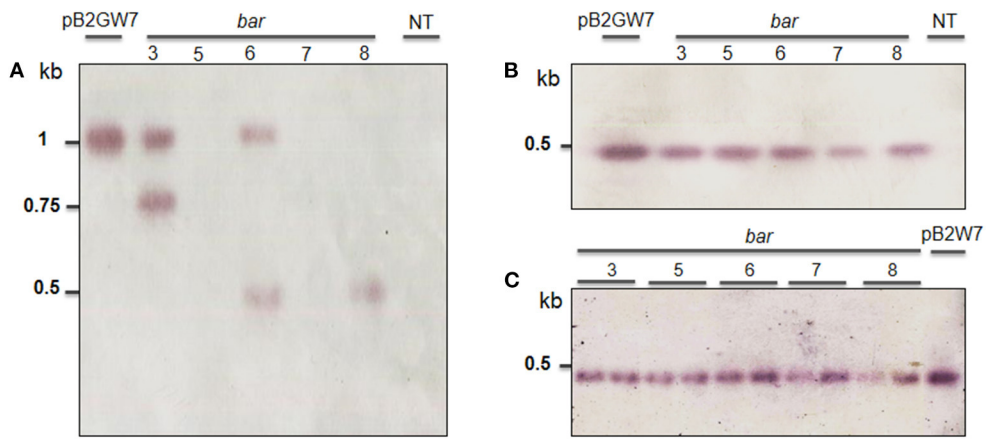

FIGURE 4 | Southern blot analysis of T1 to T3 transgenic plants obtained from *Agrobacterium*-mediated transformation of VX93 cultivar. The DNA soybean transgenic plant was cut by *Sac*I enzyme. The process used the probe-*bar* gene expression in NBT-BCIP. The figures **(A, B)** indicated T1 and T2 generation (left to right), lane 1 is pB2GW7 vector, lane 2 to 6 (five plants), lane 7 is a non-transgenic plant (NT). **(C)** T3 generation (left to right), lanes 1–10 (ten plants), and lane 11 is pB2GW7 vector. Plants were chosen randomly for southern blot analysis.

is advantageous to provide desirable characteristics in genetically modified plants, e.g., stacking multiple herbicides and insect resistance in soybean. Among these, the *bar* gene is a highly efficient selectable marker in plant genetic transformation and attribute to plant resistance to herbicides (Gordon-Kamm et al., 1990; Abdeen and Miki, 2009; Yun et al., 2009), which is detoxifying by the phosphinothricin N-acetyltransferase enzyme (Lutz et al., 2001; Yun et al., 2009; Huang et al., 2014). It has been widely used in many plant species, including soybean, due to its advantage in screening putative transformants (Kita et al., 2009). Indeed, in this study, the *cry1Ac* mutant 2 transformation efficiency was released on *bar* gene expression, because the *bar* gene in the T-DNA segment was harbored with *cry1Ac*-M#2 (**Figure 7A**), accompanied to shoot induction

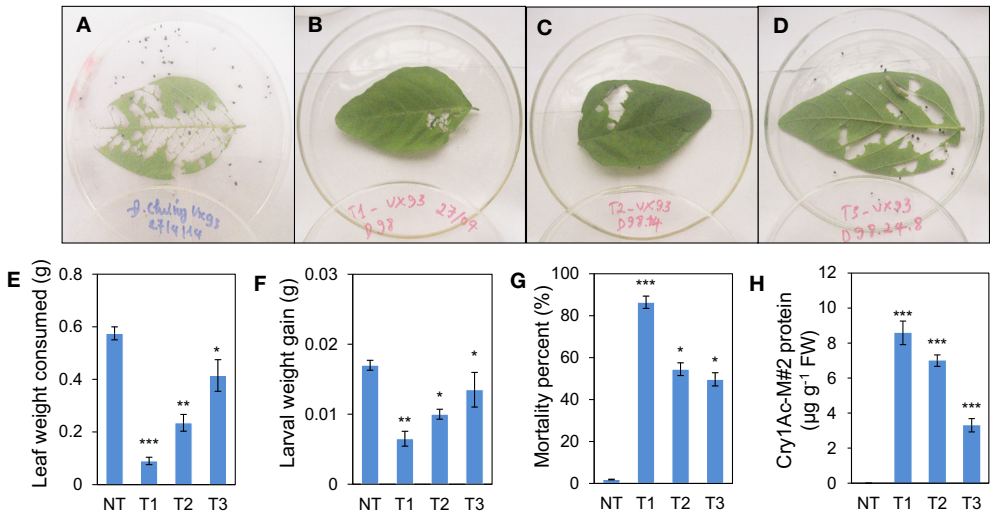

FIGURE 5 | Insect feeding bioassay of transgenic lines No. 98. **(A)** Detached leaf assay in VX93 non-transgenic **(A)** and transgenic line (VX93-M#2) from T1 to T3 generations **(B–D)**, respectively. **(E)** average leaf consumed by neonates in all transgenic lines T0 to T3 compared with non-transgenic VX93 (control). **(F)** Lower average larval weight gain. **(G)** Significant higher percent mortality of neonates in transgenic VX93 line (T1 to T3 generations) compared to VX93 non-transgenic (NT). **(H)** Cry1Ac-M#2 protein content detected by Elisa analyzing. Values are represented as mean ± SE ($n = 3$). Asterisks indicate significant differences between the control and pathogen-stressed plants; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

TABLE 4 | Main insect effect of VX93 transgenic lines from T1 to T3 generations under field condition.

Progenies	*Lamprosema indicata*		*Helicoverpa armigera*	
	Rate of plant damage (%)	Rate of leaves damage (%)	Rate of plant damage (%)	Rate of pod damage (%)
T1				
Transgenic	0.00*	4.12*	1.19	3.25*
Non-transgenic	2.10	5.38	2.38	5.80
T2				
Transgenic	2.00***	5.30*	2.40**	2.10
Non-transgenic	8.00	7.05	8.33	3.03
T3				
Transgenic	9.20*	4.40*	6.20*	3.00*
Non-transgenic	13.33	6.85	8.20	5.61

Asterisks indicate significant differences between the non-transgenic (control) and transgenic plants; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

survival of 4.51% (**Table 1**; **Figure 1**). Moreover, in the field test, VX93 transgenic exhibited much greater Basta resistance compared with the non-transgenic line (**Table 3**). Putative transformants surviving in tissue culture or field trial tests could be screened by treatment with PPT or Basta herbicide. This procedure allowed rapid identification of positive transgenic plants because the leaves in tissue cultures of transgenic plants are green (**Figure 1E**), while non-transgenic leaves turned yellow (**Figure 1D**), these symptoms were recorded similarly the following spraying with Basta 0.3% (**Figure 3**). The non-transgenic-induced hypersensitive response was accompanied by yellow leaves and the death of leaves (**Figures 3D–F**). Severe yellow leaves in the non-transgenic plants reflected no *bar* gene expression. According to VX93, putative transgenic plants from T0 to T3 generations induced PPT resistance-mediated *bar* gene activity (**Figures 2**, **3**), which in turn increased PPT-resistance levels (**Table 2**). Expression of the *bar* phenotype was not consistent from one generation to the next in only five lines. In the T1 plants 5 and 7, no *bar* expression was detected in leaf tissue by Southern blot (**Figure 4A**). However, *bar* expression was segregated in the leaf of T2 and T3 plants (**Figures 4B,C**). Such change in *bar* expression between generations has been reported in soybean Bert (Olhoft et al., 2003) and Jack (Reddy et al., 2003). It is possible that unstable *bar* expression was a result of silencing or elimination between the generations or difference in *bar* expression in the particular tissue analyzed. According to this, several studies have reported that soybean transformation efficiency tends to be low, because of transgene silencing or transgene loss, in which silencing of transgene expression in the progeny plants was reported in 10% of transgenic lines (Vain

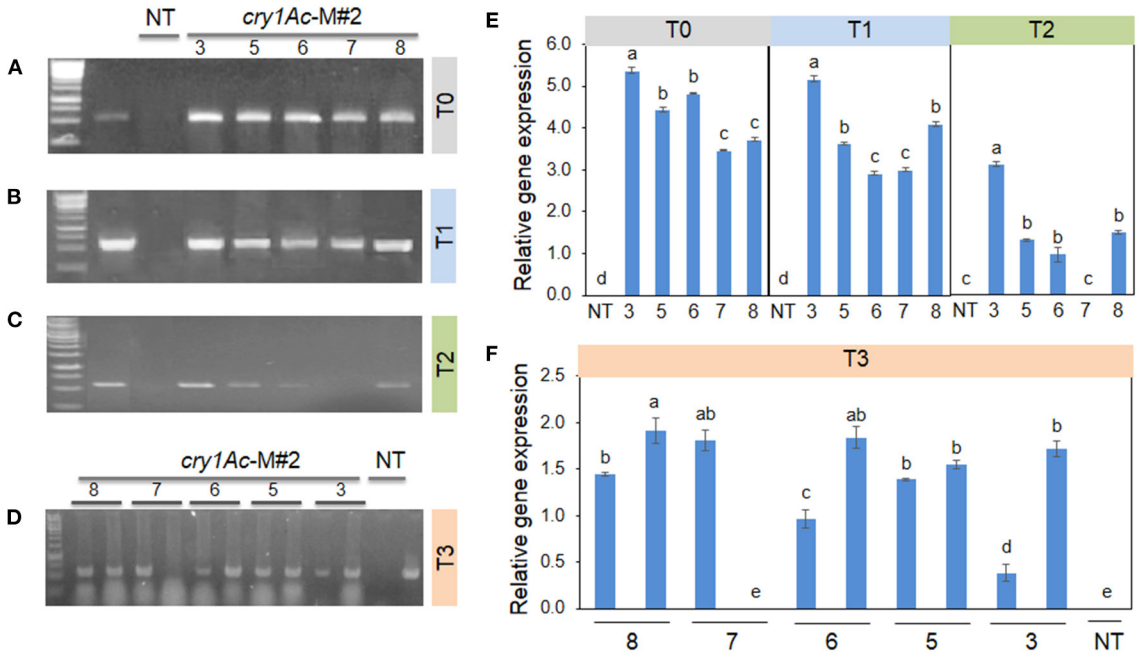

FIGURE 6 | *Cry1Ac* mutant (M#2) expression in transgenic plants from T0 to T3 generations. Among these 3, 5, 6, 7, 8 are transgenic lines and non-transgenic plants (NT). *Cry1Ac* mutant (M#2) gene expression of five plants **(A–C)** left to the right border, in which lane 1 (marker 1kb), lane 2 (plasmid contained pB2WG7 vector), lane 3 negative plant (VX93 non-transgenic, NT), lanes 4 to 8 T0 and T2 generation for cry1Ac mutant (M#2), respectively. **(D)** showed 10 plants in the T3 generation (from lanes 2 to 11), therein lane 1 is maker 1 kb, lane 12 is a negative plant (VX93 non-transgenic), and lane 13 is plasmid contained pB2WG7 vector). Relative gene expression of T0 to T2 **(E)**, and T3 **(F)**.

et al., 2002; Olhoft et al., 2003). Testing the *bar* gene silencing was due to transcriptional or post-transcriptional level. RT-PCR showed that the *bar* gene was expressed in five transgenic lines during T1 to T3 progenies (**Figures 3B,E**). This result suggested that *bar* transgene silencing in these five transgenic lines may not be due to post-transcriptional gene silencing, similar to what was previously reported (Reddy et al., 2003; Zhang et al., 2006; De Bolle et al., 2007), and *cry1Ac* transgene silencing (Gao et al., 2016). Similarly, the study of Zhenyu et al. (2019) reviewed that the overexpression of the *Bt* gene at earlier stages of transgenic cotton plants resulted in gene regulation at the post-transcription level and caused the gene silencing consequently. Moreover, the increased *bar* gene coincided with the green leaf mediated stable *bar* gene inheritance during T0 to T3 progenies (**Figure 3**), which significantly reduced leaf toxicity and leaf death (**Figure 2**), thereby alleviating the negative symptoms-induced by PPT or Basta treatments. Therefore, a significant expression of the *bar* gene was observed in leaves of VX93 transgenic plants, possibly activating regulatory mechanism resistance to PPT or Basta herbicide. Thus, stable inheritance of the *bar* transgene is important to obtain commercially useful soybean transgenic lines.

Several reviews have documented herbicide and insect resistance in transgenic crops that are important agronomic traits (Lutz et al., 2001; Singh et al., 2016; Martins-Salles et al., 2017; Romeis et al., 2019). Useful genes can be introduced to crops without leading to interference with normal plant metabolism (Block et al., 1995; Gao et al., 2016; Gupta et al., 2020). Thus, the simultaneous expression of the *bar* and *cry1Ac* genes has been postulated to be a key to the resistance capabilities of soybean (Kita et al., 2009). Numerous studies have indicated that *cry* genes encoded for *Bt* protein can reduce the effects of insects (Bravo et al., 2011; Lu et al., 2012). A previous study described *cry* gene expression in soybean and observed insecticidal activity, e.g., soybean Jack-*Bt* expressed *cry1Ac* gene exhibited five times less defoliation (Walker et al., 2000), and provided good protection against corn earworm (Yu et al., 2013). Most studies of *Bt* transgenic soybean with a *cry* protein coded by *cry1Ac* gene (Yu et al., 2013), thereby providing a possible *Bt* soybean-mediated *cry1Ac* expression option to regulate *Lepidoptera* resistance. It is well known that *cry* genes produce endotoxins specific to some major insects of important crops (Gatehouse, 2008; Yu et al., 2013). Many *cry* genes have been characterized and tested against insects (Bengyella et al., 2018). However, from the first testing of *Bt* crops to the present, the development of resistance to *cry* toxins in insects has remained a major concern (Tabashnik et al., 2008; Romeis et al., 2019). The much subscribed strategy for delaying resistance development is "high dosage/refuge" (Bates et al., 2005; Gryspeirt and Gregoire, 2012). The success of this strategy depends on using a refuge zone containing non-*Bt* plants susceptible to the insect and *Bt* plants expressing a high concentration of cry toxins. Among these, high dosage *cry* toxins released on insecticidal and closely related to *cry1Ac* gene expression levels

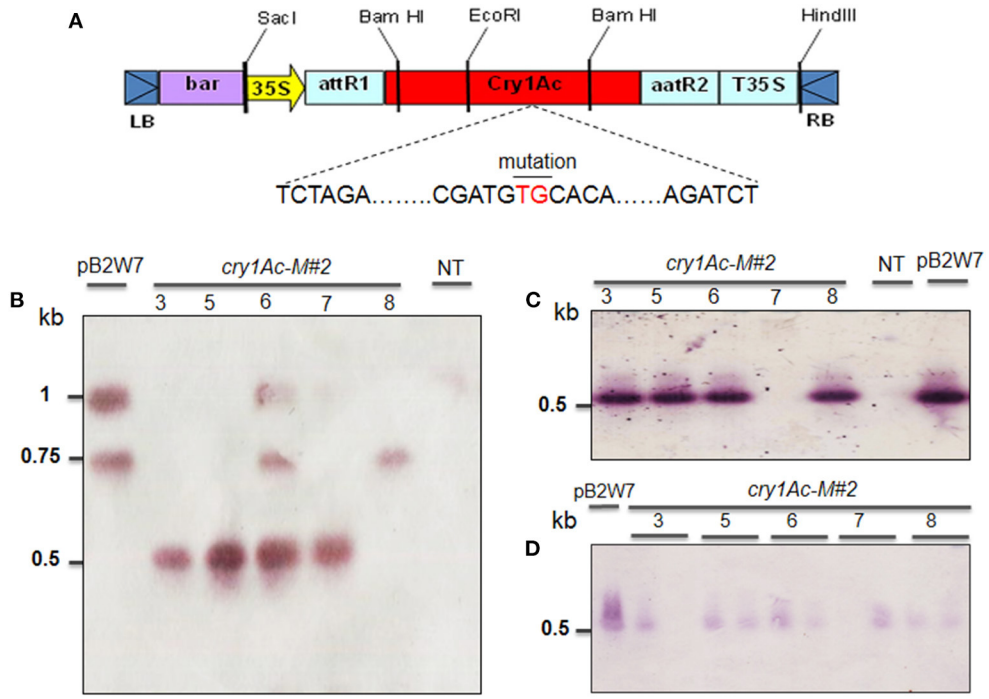

FIGURE 7 | Southern blot analysis of transgenic plant in T1 to T3 generations obtained from *Agrobacterium*-mediated transformation of VX93 cultivar. The DNA soybean transgenic plant cut by cut by *Bam*HI and *Eco*RI enzymes. The process used the probe-bar gene expression in NBT-BCIP. **(A)** T-DNA structure harbored *bar* and *cry1Ac-M#2* genes (TG nucleotides mutation, red color indicator). **(B)** T1 generation (left to right), lane 1 is pB2GW7 vector, lane 2 to 6 (five plants), lane 7 is a non-transgenic plant (NT). **(C)** T2 generation (left to right), lane 1 to 5 (five plants), lane 6 is a non-transgenic plant (NT), and lane 7 is the pB2GW7 vector. **(D)** T3 generation (left to right), lanes 2–11 (10 plants), and lane 1 is pB2GW7 vector. Plants were chosen randomly for southern blot analysis.

TABLE 5 | Growth and development of VX93 transgenic lines from T1 to T3 generations under field condition.

Progenies	Plant height (cm)	Sub-branches	Mature fruit per plant	Seeds per plant	Seeds weight per plant (g)
T1					
Transgenic	100.8*	4.0	59.7	118.6	19.3*
Non-transgenic	81.0	4.0	57.4	116.0	16.1
T2					
Transgenic	80.1	3.5	59.9	131.6	23.1
Non-transgenic	78.0	3.8	57.8	126.0	22.3
T3					
Transgenic	87.8	4.2	58.0	119.4	21.4
Non-transgenic	86.0	3.3	60.7	121.0	20.5

*Asterisks indicate significant differences between the non-transgenic (control) and transgenic plants; *P < 0.05.*

(Gao et al., 2016, Singh et al., 2016). Thus, it is important to determine the copy number of transgenes in transgenic plants, because the copy number can affect genetic stability and expression level. Thus, we developed transgenic events with *cry1Ac*-M#2, both containing a nucleotide-modified truncated *cry1Ac* gene (**Supplementary Figures 1–3**). Furthermore, the expression of *cry1Ac*-M#2 in VX93 transgenic soybean was remarkably increased in the T0 and T1 generations but slightly decreased in the T2 and T3 generations (**Figure 6**). Meanwhile, quantification of the *bar* and *cry* gene expression level between Southern blotting and RT-PCR in T2 and T3 suggested a generation-dependent pattern (**Figures 3, 6**), as shown in the significant herbicide- and insect resistance (**Tables 3**, **4**). Among the many networks involved in insect resistance-dependent *Bt* toxin gene expression in a temporal and spatial variation, overexpression of the *cry1Ac* gene at the post-transcription level leading to consequent gene silencing has also been found (Adamczyk Jr et al., 2009). According to the study of Walker et al. (2000), a transgenic Jack-Bt showed greater resistance than untransformed Jack to the natural infestation of lesser cornstalk borer. It should be noted that *cry1Ac*-M#2 expression levels and the copy number of *cry1Ac* gene in T2 and T3 generations

(**Figures 6C,D** and **7C, D**) were not detected in RT-PCR and Southern blot, which could result in co-suppression due to multi-copy integration; thus, leading to transgene silencing. Transgenic silencing has hitherto been reported in soybean (Olhoft et al., 2003) and sugarcane (Zhou et al., 2018). The results of several other studies have reported the *Bt*-soybean against insects in regulating *cry1Ac* response, e.g., *cry1Ac*-activated *cry* protein toxin (Walker et al., 2000; Jamil et al., 2021), involvement of insecticidal activity-induced insect resistance (Gao et al., 2016), and *cry1Ac* gene-induced *Lepidoptera* resistance (Yu et al., 2013; Jamil et al., 2021). The insect bioassay revealed a significant increase in the larval mortality rate and larval weight gain of *L. indicata* (**Figures 5F,G**), but reduced leaf weight consumed (**Figure 5E**) when compared with that of the non-transgenic plants, as well as coincided with the expression of *cry1Ac*-M#2 gene (**Figure 6**). Indeed, the highest expression of *cry1Ac*-M#2 in leaves of transgenic soybean occurred in T0 and T1 generations, thereby alleviating the rate of damage and negative symptoms induced by insects (**Table 4**; **Figures 6A–D**). It is worth noting that there was a remarkable difference in the insect symptoms of *cy1Ac*-M#2, e.g., leaf weight consumed and larval weight gain, even though plants in both *cry1Ac* mutant expression exhibited a significant inheritance in T0 to T3 generations. These results demand further discussion of *cry1Ac* mutant regulatory insecticidal activity involved in the integrative process of insect resistance, and this should emphasize the most distinct differences in the mortality of insects and *cry1Ac*-M#2 expression in leaves (**Figures 6A–F**). The variable insect mortality level was mostly in agreement with the *cry* protein-dependent intensity of *cry1Ac* mutant expression levels. In addition, as far as we know, this study provided the first report on the high expression of *cry1Ac*-M#2 increased *Lepidoptera* resistance level. Given that the *cry1Ac* mutant triggers insecticidal activity, specifically induced transgenic soybean defense signaling, it is reasonable to conclude that the *Bt*-VX93 soybean has mediated the overproduction of *cry1Ac* mutant, thereby functioning as crucial regulatory insect resistance.

In the *Bt*-soybean, high-level expression of the *cry1Ac* gene in soybean leaves is important to obtain insect resistance (Yu et al., 2013). In the present study, *cry1Ac*-M#2 expression was highest in VX93 transgenic leaves, but not expressed in the leaves of non-transgenic plants (**Figures 5A,B**). A significant *Bt* soybean against *Lepidoptera* for *cry1Ac* mutant gene-transduction insecticidal activity was evaluated in insect bioassay (**Figures 5**, **7**). Increasing evidence demonstrates that *cry1Ac* expression is the first plant-produced insecticidal protein and that *cry1Ac* is the master activator of *Lepidoptera* resistance (Walker et al., 2000; Yu et al., 2013; Martins-Salles et al., 2017). In the field evaluation, there was substantively less *Lepidoptera* insect damage on plants, leaves, and pods in the transgenic VX93 compared with non-transgenic plants (**Table 4**). The insect resistance level and response to insect feeding were observed in the insect bioassay (**Figures 5A–D**), which was consistent with the pattern of the *cry1Ac* mutant gene (**Figures 5C,D** and **6**). The *cry1Ac* specifically responds to *Lepidoptera* insect, e.g., *L. indicata* and *H. armigera* (McPherson and MacRae, 2009; Tabashnik et al., 2009; Yu et al., 2013; Martins-Salles et al., 2017), thereby providing a venue for soybean transgenic induced insect resistance (**Figure 5**; **Table 4**) in high *cry1Ac*-M#2 expression during T1 to T3 generations. These results were higher than that of previous studies in sugarcane (Weng et al., 2006) and soybean (Yu et al., 2013) which were used with normal *cry1Ac*. According to this study, larval mortality was dependent on the highest *cry1Ac*-M#2 expression levels in *Bt*-soybean leaves (**Figure 5**). In the feeding leaf test, the results indicated that larval mortality exceeded 86% in the T1 generation (**Figure 5G**), which is higher than *cry1Ac* un-mutation in *Bt*-soybean MON87701 (Yu et al., 2013). Indeed, the study of Yu et al. (2013) reported larval mortality around 76% when fed with transgenic soybean leaves. These results demonstrated that *cry1Ac*-M#2 has been proven more effective than *cry1Ac* non-mutation and this gene is essential for insect control. However, the expression of *cry1Ac*-M#2 in transgenic soybean was declined consistently during the growing period, which confirmed *cry1Ac*-M#2 protein level found in the leaf of transgenic lines from T1 to T3 generations at vegetative stages (before anthesis) (**Figures 5G**, **6**). In accordance with this, leaf weight consumed and larval weight gain significantly increased in T2 and T3 generations (**Figures 5E,F**), accompanied to the efficiency against *L. indicata* in artificially infested active larval mortality (**Figure 5G**) and *cry1Ac*-M#2 protein lower (**Figure 5H**). It supported that a high degree of resistance against *Lepidoptera* insects dependent *cry1Ac*-M#2 levels. On the other hand, the study of Weng et al. (2006) reported a modified *cry1Ac* gene in sugarcane ROC16 and YT79-177 had comprised 62% of the transgenic plant which were resistant to stem borer damage in both greenhouse and field trials. The resistance of *Bt* crops to target insects is generally correlated with the levels of insecticidal protein (Walker et al., 2000; Gao et al., 2016; Singh et al., 2016). In the present study, the highest expression of *cry1Ac*-M#2 in transgenic leaves was detected in the vegetative stage (before anthesis), as well as the reduced rate of *L. indicata* and *H. armingera* insects damage (**Table 4**). Thus, transgenic soybean lines revealed efficacy against these insects feeding in the field. Significant reductions in the larval populations of *L. indicata* and *H. armingera* were observed in transgenic soybean lines expressing *cry1Ac*-M#2 compared with the non-transgenic plants at the field. Similarly, the study of Yu et al. (2013) reported that *Bt*-soybean expressing *cry1Ac* targeted *H. armingera* before anthesis. Compared with the non-transgenic, transgenic soybeans were more efficient to larval mortality in the T1 generation, despite this symptom decreased in T2 and T3 generations. This is in agreement with earlier reports of *Bt*-soybean expressing *cry1Ac* caused high first-instar mortality in *H. armingera* (Yu et al., 2013).

Given that agronomic traits depend on the *cry1Ac* genes, responsive-related genes expression has been reported. The previous study evaluated that the majority yield was not affected by *cry1Ac* expression in transgenic soybean (Homrich et al., 2008) or found that there were no unintended changes in the seed composition of transgenic chickpea-expressed a truncated-*cry1Ac* gene (Gupta et al., 2020). In the present study, agronomic traits in the transgenic plants were similar to the non-transgenic plants (**Table 5**). However, in the T1 generation, the transgenic

plants exhibited variable plant height and seed weight per plant ($P < 0.05$) compared with the non-transgenic plants, but these traits were not different in the T2 and T3 generations. The plant height was higher in *Bt* transgenic than in the non-transgenic plants, which was consistent with the *cry1Ac*-M#2 expression with the greatest effect in the T1 generation (**Table 5**), thereby providing a possible *cry1Ac* mutant-mediated option to regulated plant growth and development reactions, e.g., leafing speed, branches forming, and burning effective (Chen et al., 2019a). Increasing evidence demonstrates that *cry8*-like gene expressing in *Jinong28* soybean enhances plant growth and yield (Qin et al., 2019). Moreover, there is a synergistic and significant interaction between insect resistance and plant growth for the improvement of seed weight (**Tables 3–5**), which may potentially interact with carbon and nitrogen metabolism (Coviella et al., 2002; Rochester, 2006) and integrative cellular hormone (GA_3) regulation processes that promote *Bt* cotton yield (Chen et al., 2019b). However, the mechanisms by which *cry1Ac* mutant-elicited nitrogen metabolism improves plant growth remains unclear and requires further investigation.

In summary, the results of both the present study and previous reports (Zhang et al., 2006; Yu et al., 2013) provided evidence on *Bt*-soybean-mediated *bar*- and *cry1Ac*-M#2 transcriptional response, which may promote herbicidal and insecticidal activity. *Bt*-soybean-mediated modulations were characterized by (1) herbicide tolerance stably inherited in T1 to T3 progeny, (2) negative insect-induced symptoms were largely alleviated in *Bt* soybean, and (3) synergistic interactions occurred between insect resistance, plant growth, and seed yield in *Bt*-soybean.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TDN, VHL, YSC, and XBN designed the experiment, interpreted the data, and wrote the manuscript. TDN, VHL, VDN, and TTB carried out the chemical analysis. TTN carried out the field experiments. YHJ made vector construction. All authors reviewed and approved the submitted version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.698882/full#supplementary-material

REFERENCES

Abdeen, A., and Miki, B. (2009). The pleiotropic effects of the *bar* gene and glufosinate on the *Arabidopsis* transcriptome. *Plant Biotechnol. J.* 7, 266–282. doi: 10.1111/j.1467-7652.2008.00398.x

Abedi, Z., Saber, M., Vojaudi, S., Mahdavi, V., and Parsaeyan, E. (2014). Acute, sublethal, and combination effects of azadirachtin and *Bacillus thuringiensis* on the cotton bollworm, *Helicoverpa armigera*. *J. Insect Sci.* 14:30. doi: 10.1673/031.014.30

Adamczyk Jr, J. J., Perera, O., and Meredith, W. R. (2009). Production of mRNA from *cry1Ac* transgene differ among Bollgard® lines which correlates to the level of subsequent protein. *Trans. Res.* 18, 143–149. doi: 10.1007/s11248-008-9198-z

Badran, A. H., Gunzov, V. M., Huai, Q., Kemp, M. M., Vishwanath, P., Kain, W., et al. (2016). Continuous evolution of *Bacillus thuringiensis* toxins overcomes insect resistance. *Nature* 533, 58–63. doi: 10.1038/nature17938

Bates, S. L., Zhao, J. Z., Roush, R. T., and Shelton, A. M. (2005). Insect resistance management in GM crops: past, present and future. *Nat. Biotechnol.* 23, 57–62. doi: 10.1038/nbt1056

Bengyella, L., Yekwa, E. L., Iftikhar, S., Nawaz, K., Jose, R. C., Fonmboh, D. J., et al. (2018). Global challenges faced by engineered *Bacillus thuringiensis Cry* genes in soybean (*Glycine max* L.) in the twenty-first century. *Biotech* 8:464. doi: 10.1007/s13205-018-1484-8

Block, M. D., Sonville, A. D., and Debrouwer, D. (1995). The selection mechanism of phosphinothricin is influenced by the metabolic status of the tissue. *Planta* 197, 619–626. doi: 10.1007/BF00191569

Bravo, A., Likitvivatanavong, S., Gill, S. S., and Soberon, M. (2011). *Bacillus thuringiensis*: a story of a successful bioinsecticide. *Insect Biochem. Mol. Biol.* 41, 423–431. doi: 10.1016/j.ibmb.2011.02.006

Carroll, J., Convents, D., VanDamme, J., Boets, A., VanRie, J., and Ellar, D. J. (1997). Intramolecular proteolytic cleavage of *Bacillus thuringiensis* cry3A delta-endotoxin may facilitate its coleopteran toxicity. *J. Invertebr. Pathol.* 70, 41–49. doi: 10.1006/jipa.1997.4656

Chen, Y., Li, Y., Chen, Y., Abidallha, E. H. M. A., Hu, D., Li, Y., et al. (2019a). Planting density and leaf-square regulation affected square size and number contributing to altered insecticidal protein content in *Bt* cotton. *Field Crops Res.* 205, 14–22. doi: 10.1016/j.fcr.2017.02.004

Chen, Y., Li, Y., Zhou, M., Cai, Z., Tambel, L. I. M., Zhang, X., et al. (2019b). Nitrogen deficit decreases seed *cry1Ac* endotoxin expression in *Bt* transgenic cotton. *Plant Physiol. Biochem.* 141, 114–121. doi: 10.1016/j.plaphy.2019.05.017

Coviella, C. E., Stipanovic, R. D., and Trumble, J. T. (2002). Plant allocation to defensive compounds: interactions between elevated CO_2 and nitrogen in transgenic cotton plants. *J. Exp. Bot.* 53, 323–331. doi: 10.1093/jexbot/53.367.323

De Bolle, M. F. C., Utave, K. M. J., Goderis, I. J. W. M., Wouters, P. F. J., Jacobs, A., Delauré, S. L., et al. (2007). The influence of matrix attachment regions on transgene expression in *Arabidopsis thaliana* wild type and gene silencing mutants. *Plant Mol. Biol.* 63, 533–543. doi: 10.1007/s11103-006-9107-x

Gao, S., Yang, Y., Wang, C., Guo, J., Zhou, D., Wu, Q., et al. (2016). Transgenic sugarcane with a *cry1Ac* gene exhibited better phenotypic traits and enhanced resistance against sugarcane borer. *PLoS ONE* 11:e0153929. doi: 10.1371/journal.pone.0153929

Gatehouse, J. A. (2008). Biotechnological prospects for engineering insect-resistant plants. *Plant Physiol.* 146, 881–887. doi: 10.1104/pp.107.111096

Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., et al. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* 2, 603–618. doi: 10.2307/3869124

Gryspeirt, A., and Gregoire, J. C. (2012). Effectiveness of the high dose/refuge strategy for managing pest resistance to *Bacillus thuringiensis* (Bt) plants expressing one or two toxins. *Toxins* 4, 810–835. doi: 10.3390/toxins4100810

Gupta, G. P., Birah, A., and Rani, S. (2004). Development of artificial diet for mass rearing of American bollworm, Helicoverpa armigera. *Indian J. Agric. Sci.* 74, 548–551. doi: 10.1093/jis/14.1.93

Gupta, R., Baruah, A. M., Acharjee, S., and Sarmah, B. K. (2020). Compositional analysis of transgenic Bt-chickpea resistant to *Helicoverpa amigera*. *GM Crops Food* 11, 262–274. doi: 10.1080/21645698.2020.1782147

Homrich, M. S., Passaglia, L. M. P., Pereira, J. F., Bertagnolli, P. F., Salvadori, J. R., Nicolau, M., et al. (2008). Agronomic performance, chromosomal stability and resistance to velvetbean caterpillar of transgenic soybean expressing *cry1Ac* gene. *Pesq. Agropec. Bras.* 43, 801–807. doi: 10.1590/S0100-204X2008000700003

Homrich, M. S., Wiebke-Strohm, B., Weber, R. L. M., and Bodanese-Zanetitini, M. H. (2012). Soybean genetic transformation: a valuable tool for the functional study of genes and the production of agronomically improved plants. *Genet. Mol. Biol.* 35, 998–1010. doi: 10.1590/S1415-47572012000600015

Huang, W. K., Peng, H., Wang, G. F., Cui, J. K., Zhu, L. F., Long, H. B., et al. (2014). Assessment of gene flow from glyphosate-resistant transgenic soybean to conventional soybean in China. *Acta Physiol. Plant* 36, 1637–1647. doi: 10.1007/s11738-014-1539-3

ISAAA. (2017). *Global status of commercialized biotech/GM crops in 2017: Biotech crop adoption surges as economic benefits accumulate in 22 years.* Available online at: https:/www.isaaa.org/resources/publications/briefs/53/download/isaa-brief-53-2017

Jamil, S., Shahzad, R., Rahman, S. U., Iqbal, M. Z., Yaseen, M., Ahmad, S., et al. (2021). The level of *cry1Ac* endotoxin and its efficacy against *H.* armigera in Bt cotton at large scale in Pakista*n. GM Crops Food* 12, 1–17. doi: 10.1080/21645698.2020.1799644

Kita, Y., Hanafy, M. S., Deguchi, M., Hasegawa, H., Terakawa, R., Kitamura, K., et al. (2009). Generation and characterization of herbicide-resistant soybean plant expressing novel phosphinothricin N-acetyltransferase genes. *Breed. Sci.* 59, 245–251. doi: 10.1270/jsbbs.59.245

Livak, J. K., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lu, Y., Wu, K., Jiang, Y., Guo, Y., and Desneux, N. (2012). Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. *Nature* 487, 362–365. doi: 10.1038/nature11153

Lutz, K. A., Knapp, J. E., and Maliga, P. (2001). Expression of *bar* in the plastid genome confers herbicide tolerance. *Plant Physiol.* 125, 1585–1590. doi: 10.1104/pp.125.4.1585

Martins-Salles, S., Machado, V., Massochin-Pinto, L., and Fiuza, L. M. (2017). Genetically modified soybean expressing insecticidal protein (*cry1Ac*): management risk and perspectives. *FACETS* 2, 496–512. doi: 10.1139/facets-2017-0006

McPherson, R. M., and MacRae, T. C. (2009). Evaluation of transgenic soybean exhibiting high expression of a synthetic *Bacillus thuringiensis cry1A* transgene for suppressing *Lepidopteran* population densities and crop injury. *J. Econ. Entomol.* 102, 1640–1648. doi: 10.1603/029.102.0431

Nachimuthu, S., and Kumar, P. A. (2004). Protein engineering of delta-endotoxins of *Bacillus thuringiensis*. *Elect. J. Biotechnol*. 7, 178–188. doi: 10.2225/vol7-issue2-fulltext-3

Naranjo, S. E. (2011). Impacts of Bt transgenic cotton on integrated pest management. *J. Agric. Food Chem*. 59, 5842–5851. doi: 10.1021/jf102939c

Olhoft, P. M., Flagel, L. E., Donovan, C. M., and Somers, D. A. (2003). Efficient soybean tranfomation using hygromycin B selection in the cotyledonary - node method. *Planta* 216, 723–735. doi: 10.1007/s00425-002-0922-2

Paz, M. M., Shou, H., Guo, Z., Zhang, Z., Banerjee, A. K., and Wang, K. (2004). Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant. *Euphytica* 136, 167–179. doi: 10.1023/B:EUPH.0000030670.36730.a4

Qin, D., Liu, X. Y., Miceli, C., Zhang, Q., and Wang, P. W. (2019). Soybean plants expressing the *Bacillus thuringiensis* cry8-like gene show resistance to *Holotrichia parallela*. *BMC Biotechnol.* 19:66. doi: 10.1186/s12896-019-0563-1

Reddy, M. S. S., Dinkins, R. D., and Collins, G. B. (2003). Gene silencing in transgenic soybean plants transformed *via* particle bombardment. *Plant Cell Rep*. 21, 676–683. doi: 10.1007/s00299-002-0567-4

Rochester, I. J. (2006). Effect of genotype, edaphic, environmental conditions, and agronomic practices on *cry1Ac* protein expression in transgenic cotton. *J. Cott. Sci.* 10, 252–262. doi: 10.3389/fpls.2017.02107

Romeis, J., Meissle, M., and Bigler, F. (2006). Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nat. Biotechnol.* 24, 63–71. doi: 10.1038/nbt1180

Romeis, J., Naranjo, S. E., Meissle, M., and Shelton, A. M. (2019). Genetically engineered crops help support conservation biological control. *Biol. Control* 130, 136–154. doi: 10.1016/j.biocontrol.2018.10.001

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. USA* 81, 8014–8019. doi: 10.1073/pnas.81.24.8014

Singh, A. K., Paritosh, K., Kant, U., Burma, P. K., and Pental, D. (2016). High expression of *cry1Ac* protein in cotton (*Gossypium hirsutum*) by combining independent transgenic events that target the protein to cytoplasm and plastids. *PLoS ONE* 11:e0158603. doi: 10.1371/journal.pone.0158603

Stewart, J. R., Adang, M. J., All, N. H., Boerma, H. R., Cardineau, G., Tucker, D., et al. (1996). Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis* cryIAc gene. *Plant Physiol.* 112, 121–129. doi: 10.1104/pp.112.1.121

Tabashnik, B. E., Gassmann, A. J., Crowder, D. W., and Carriere, Y. (2008). Insect resistance to Bt crops: evidence versus theory. *Nat. Biotechnol.* 26, 199–202. doi: 10.1038/nbt1382

Tabashnik, B. E., Van Rensburg, J. B., and Carriere, Y. (2009). Field-evolved insect resistance to Bt crops: definition, theory, and data. *J. Econ. Entomol.* 102, 2011–2025. doi: 10.1603/029.102.0601

Vain, P., James, A., Worland, B., and Snape, W. (2002). Transgene behavior across two generations in a large random population of transgenic rice plants produced by particle bombardment. *Theor. Appl. Genet*. 105, 878–889. doi: 10.1007/s00122-002-1039-5

Walker, D. R., All, J. N., McPherson, R. M., Boerma, H. R., and Parrott, W. A. (2000). Field evaluation of soybean engineered with a synthetic *cry1Ac* transgene for resistance to corn earworm, soybean looper, velvetbean caterpillar (Lepidoptera: Noctuidae), and lesser cornstalk borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 93, 613–622. doi: 10.1603/0022-0493-93.3.613

Wang, C. Y. (2005). *Southern blotting protocol.* Sugden lab: 1–3.

Wei, H. R., Wang, M. L., Moore, P. H., and Albert, H. H. (2003). Comparative expression analysis of two sugarcane polyubiquitin promoters and flanking sequences in transgenic plants. *J. Plant Phys.* 160, 1241–1251. doi: 10.1078/0176-1617-01086

Weng, L. X., Deng, H. H., Xu, J. L., Li, Q., Wang, L. H., Jiang, Z. D., et al. (2006). Regeneration of sugarcane elite breeding lines and engineering of strong stem borer resistance. *Pest. Manage Sci.* 62, 178–187. doi: 10.1002/ps.1144

Xue, C., Wang, B. C., Yu, Z., and Sun, M. (2014). Structural insights into *Bacillus thuringiensis cry*, *cyt* and parasporin toxins. *Toxins* 6, 2732–2770. doi: 10.3390/toxins6092732

Yu, H., Li, Y., Li, X., Romeis, J., and Wu, K. (2013). Expression of *cry1Ac* in transgenic Bt soybean lines and their efficiency in controlling lepidopteran pests. *Pest Manag. Sci.* 69, 1326–1333. doi: 10.1002/ps.3508

Yun, C. S., Hasegawa, H., Nanamiya, H., Terakawa, T., and Tozawa, Y. (2009). Novel Bacterial N acetyltransferase gene for herbicide detoxification in land plants and selection maker in plant transformation. *Biosci. Biotechnol. Biochem.* 73, 1000–1006, doi: 10.1271/bbb.80777

Zhang, Y., Yang, B. Y., and Chen, S. Y. (2006). Inheritance analysis of herbicide-resistant transgenic soybean lines. *Act. Gen. Sinic.* 33, 1105–1111. doi: 10.1016/S0379-4172(06)60148-0

Zhenyu, L., Abidallha, E. H. M. A., Huimin, W., Mingyuan, Z., Xiang, Z., Yuan, C., et al. (2019). Bt insecticidal efficacy variation and agronomic regulation in Bt cotton. *J. Cott. Res*. 2:23. doi: 10.1186/s42397-019-0042-1

Zhou, D., Liu, X., Gao, S., Guo, J., Su, Y., Ling, H., et al. (2018). Foreign *cry1Ac* gene integration and endogenous borer stress-related genes synergistically improve insect resistance in sugarcane. *BMC Plant Biol.* 18:342. doi: 10.1186/s12870-018-1536-6

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