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Impact of Soil Rhizobacteria Inoculation and Leaf-Chewing Insect Herbivory on *Mentha piperita* Leaf Secondary Metabolites

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Abstract

Secondary metabolites commonly play important physiological roles in plants and can be modified quantitatively and qualitatively by exposure to biotic and abiotic interactions. Plant growth promoting rhizobacteria (PGPR) and herbivory induce systemic resistance. In the present study, we analyzed the induction of secondary metabolites in peppermint plants in response to chewing insect herbivory on PGPR-inoculated *Mentha piperita* plants. The secondary metabolites of *M. piperita* plants were increased when plants were inoculated with PGPR and also exposed to caterpillar herbivory. It was found that the total essential oil yield in inoculated plants with insect damage was ~2.6-fold higher than in controls. The yield was similar to that of plants either damaged by insects or inoculated, indicating that there was no synergism. The same trend was observed for phenolic compounds. In contrast, VOC emissions were significantly higher in plants infested by insects, independent of whether they were inoculated. Insect damaged plants had 5.5 times higher monoterpene emissions than control plants, and ~ 2-fold higher emissions than on PGPR-inoculated plants without insects. To gain a better understanding of how herbivory on PGPR-inoculated plants can cause an increase in secondary metabolites of peppermint, we examined changes in plant defense hormones in inoculated plants after herbivory. We found that the combination of both treatments increased the endogenous jasmonic and salicylic acid levels to the same extent as in plants only inoculated or only insect-damaged. Because different interactions can alter the phytochemistry of plants such as *M. piperita*, this topic is both ecologically and economically relevant.

Keywords Secondary metabolites · Rhizobacteria · Peppermint · Herbivory · VOC · Essential oil · Total phenolic compounds

Abbreviations

ABA	abscisic acid
EO	essential oil
JA	jasmonic acid
PGPR	plant growth-promoting rhizobacteria
SA	salicylic acid
SM	secondary metabolites
TPC	total phenolic content
VOC	volatile organic compounds

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Introduction

Plants are sessile organisms which are forced to discriminate between, and respond to the different challenges present in their biotic and abiotic environment in order to allocate their resources to growth, reproduction and defenses (Yang et al. 2018). It has been postulated that the enormous phytochemical diversity in plants has resulted from interactions of increasing complexity (Raguso et al. 2011). Secondary metabolites play a variety of roles in response to changing environments. In fact, many reports have shown a variety of ecological functions for plant secondary metabolites (SM), including protection against pathogen and herbivore attack (Freeman et al. 2008). The stress response in plants comprises a range of molecular and signalling responses, initiated after perception of specific or combined biotic or abiotic stresses which may result in the induction of SM. Such induced responses play an important role in plant interactions and are considered to be a major source of allelochemicals (Saharkhiz et al. 2010).

Aromatic plants include a great diversity of plant species whose common characteristic is the production of essential

oils (EO)(Güenther 1948). One of these, peppermint (*Mentha x piperita* L), is an aromatic and medicinal plant of the Lamiaceae family. It is cultivated worldwide mainly because its EOs are used as additives in the cosmetic, food and pharmaceutical industries. Fresh or dried leaves are used as condiments or in infusions (Jullien 2007; Bakkali et al. 2008; Lubbe and and Verpoorte 2011). The chemical composition of *M. piperita* EO is very complex; the leaves contain ~3% EO which consists of more than 50 different compounds. Its principal EO components, which comprise ~60% of the total oil volume, are limonene, linalool, (−) menthone, (−) menthol and (+) pulegone (Santoro et al. 2011).

Phenolic compounds are plant secondary metabolites that can be induced under the influence of multiple biotic and abiotic stresses (Cheynier 2012). Phenolic compounds act as pigments, antioxidants and signaling agents both above- and below-ground between the plant and other organisms. They are also involved in defense, because of their antibiotic or antinutritional properties (Latanzzio 2013). Total phenolic compound production can be induced by environmental stress and exogenous elicitor treatment (Farmer et al. 2003). Peppermint leaves are rich in phenolic compounds, including caffeic acid, rosmarinic acid, eriocitrin and luteolin-7-O-glucoside (Farnad et al. 2014), and represent about 20% of the dry weight of the plant.

Plants maintain a close relationship with soil microorganisms, such as plant growth promoting rhizobacteria (PGPR), which use root exudates as a source of carbon, nitrogen and other required nutrients for their growth and reproduction (Backer et al. 2018). The microorganisms provide benefits to the host by stimulating plant growth (Pieterse et al. 2014). This resultant plant growth can be due to the PGPRs fixing atmospheric nitrogen and augmenting the uptake of nutrients (e.g. phosphorus and iron). In addition, numerous microbes are able to synthesize plant hormones such as auxins, cytokinins and gibberellins, which are key for promoting growth (Backer et al. 2018). Furthermore, PGPR have the capacity to decrease the numbers of pathogens and induce systemic resistance (ISR) (Pieterse et al. 2014).

A series of studies on PGPR strains which induce systemic protection in several plants has presented evidence that the physiological response of the plant during induction is different from classical systemic acquired resistance (SAR) (reviewed in van Loon et al. 1998). In contrast to SAR, where the signaling pathway is mediated by SA (Romeiro 2007), PR proteins are not activated in plants showing protection mediated by non-pathogenic bacteria (Pieterse et al. 1998; van Loon and van Strien 1999). This means that SA is not an intermediate in induced systemic protection induced by PGPRs. Another distinguishing feature is that systemic resistance induced by PGPR requires responsiveness to jasmonates, whereas classical SAR does not (Pieterse et al. 1998). This PGPR-mediated sensitization of the plant tissue for enhanced defense expression is called 'priming,' and is characterized by a faster or stronger activation of cellular defenses upon pathogen or insect attack, resulting in enhanced host resistance (Van der Ent et al. 2009; Martinez-Medina et al. 2016). However, some studies have reported that several PGPR increase the levels of salicylic acid (SA) after an inoculation and infection with a pathogen (Park et al. 2017). This was particularly the case in *M. piperita* inoculated with the PGPR *Bacillus amyloliquefaciens* GB03 or *Pseudomonas putida* SJ04 (Cappellari et al. 2019). Examples of wild-type PGPR that have been demonstrated to induce SA-dependent SAR are *Paenibacillus Alvei* K165 (Tjamos et al. 2005) and *P. fluorescens SS101* (Van de Mortel et al. 2012). Also, a role for SA in the induction of systemic resistance has been established for several *Trichoderma* species (Martinez-Medina et al. 2013). The systemic protection which some PGPR strains can induce on medicinal and aromatic plants is reflected in the induction of SM (Cappellari et al. 2015)

Induced defenses against insect herbivory are triggered upon feeding. They involve the direct production of defenses, such as the biosynthesis of SM, which act as toxins or feeding deterrents (Karban and Baldwin 1997). Herbivore-induced SM can also act as an indirect defense, such as the emission of VOCs that attract enemies of the herbivores (Dicke and Baldwin 2010). JA is an important signal in this response (Browse and Howe 2008), but also SA has been implicated in the induction of indirect defense against herbivory (Van Oosten et al. 2008).

In general, PGPR can induce ISR to herbivory (Pieterse et al. 2014; Beardon et al. 2014). Despite the importance of plant defense responses to PGPR inoculation, the underlying mechanisms and consequences for the production of SM are relatively understudied. Previously, we reported that inoculation with *P. putida* SJ04 or *B. amyloliquefaciens* GB03, two beneficial PGPR strains, led to increased peppermint growth, caused a systemic induction of EO synthesis and also enhanced phenolic production in peppermint leaves (Cappellari et al. 2015, 2017). Building on these results, we decided to compare secondary metabolite levels in peppermint plants inoculated with PGPR and subsequently damaged by insect leaf chewers.

Rachiplusia nu (Lepidoptera: Noctuidae) is a polyphagous noctuid pest endemic to southern South America. The larval stage of *R. nu* can cause substantial damage to crops. It particularly causes damage to soybean, but also to sunflower, maize, alfalfa, tobacco, and certain horticultural and aromatic species such as *M. piperita*. (Specht et al. 2006; Rimoldi et al. 2012). Interaction with rhizobacteria can benefit crops and in particular peppermint plants by increasing SM biosynthesis and improving plant resistance to herbivory. In addition, the increase of nutrient intake mediated by PGPR can facilitate tissue re-growth after herbivory, which helps compensate for the biomass lost due to the herbivore (Rashid and Chung

2017). However, there is no report on the effect of *R. nu* feeding on *M. piperita*. In particular, it is unknown how plants regulate SM when plants are damaged by insects after colonization by rhizobacteria. Thus, the objective of the present study was to compare secondary metabolite levels in peppermint plants in response to (i) chewing insect-herbivory, (ii) inoculation with PGPR and (iii) herbivory on PGPR-inoculated plants. In addition, we measured the levels of endogenous phytohormones in plants exposed to herbivory and PGPR to obtain a better understanding of how the combined effect of herbivory and PGPR inoculation modified SM biosynthesis in peppermint leaves.

Inducible chemical changes are of particular interest in medicinal and aromatic plants, not only in relation to defensive mechanisms, but also because the altered SM may have bioactive properties that enhance the economic value of the plant (Banchio et al. 2005). Therefore, a better understanding of the factors that modify SM quantity and quality in aromatic plants will be useful for improving the production of these natural products and in pest management strategies.

Material and Methods

Plant Material, Bacterial Inoculation, and Treatments

The two following plant growth-promoting rhizobacterial strains were evaluated: *Pseudomonas putida* SJ04, a native strain isolated from rhizosphere soil of *M. piperita* collected from a commercial crop in Córdoba, Argentina (GenBank KF312464.1), and *Bacillus amyloliquefaciens* GB03 (formerly known as *Bacillus subtilis* GB03), previously reported as a plant growth promoting bacterium (Cappellari et al. 2015). In previously studies, we have shown that GB03 or SJ04 have beneficial effects on *M. piperita*, including growth promotion, and EO yield, plant volatile organic compound (VOC) emission and total phenolic content (TPC) increase (Cappellarie et al. 2015; 2017).

Bacteria were grown on LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) for routine use and maintained with 20% glycerol at −80 °C for long-term storage. Prior to inoculation on plant roots, a single colony of each rhizobacterial species was transferred to LB liquid medium and grown in an incubator shaker at 30 °C at 120 rpm until reaching the exponential phase. The bacterial cells were collected, washed twice in 0.9% NaCl by Eppendorff centrifugation (1000 rpm, 10 min), resuspended in 0.9% NaCl, and adjusted to a final concentration of ~10^9 CFU/ mL for use as inoculum. The seedlings were inoculated with 100 μL bacterial suspension.

Young shoots from *M. piperita* plants grown in the Traslasierra Valley (Córdoba Province, Argentina) were surface-disinfected and micropropagated as described previously by Santoro et al. (2016). The seedlings obtained were transferred to plastic pots (diameter 12 cm, depth 22 cm) containing sterilized vermiculite (one per pot), with all plants receiving Hoagland's nutrient medium (20 mL/pot) once per week (Cappellari et al. 2015). Plants were grown in a growth chamber under controlled conditions of light (16/8-h light/dark cycle), temperature (22 ± 2 °C), and relative humidity (~70%). After 7 days, the seedlings were inoculated as a soil drench around the plant base stem with 100 μl bacterial suspension. Sterile water was applied to the control seedlings.

At 28 days, the plants were exposed to three 3rd instar *R. nu* larvae which were starved for 24 h. After 4 h, these larvae were removed. All experiments were standardized at 4 h, because the presence of three herbivores for 4 h was found to cause about 30% of leaf damage. The total damage was similar in all treatments.The experiments were repeated 3 times (10 pots per treatment; 1 plant per pot), and the pots were arranged randomly in the growth chamber. Plants exposed to larvae were placed well apart in a separate chamber (using exactly the same controlled conditions as described above) until VOC collection to avoid the possibility of volatile compounds influencing plants in other treatments. The treatments used were: control (non-inoculated plants), plants inoculated with *P. putida* SJ04 (SJ04), plants inoculated with *B. amyloliquefaciens* GB03 (GB03), plants infested with *R. nu* (larvae), plants inoculated with *P. putida* SJ04 and infested with *R. nu* (Larvae+S04), and plants inoculated with *B. amyloliquefaciens* GB03 and infested with *R. nu* (Larvae+GB03). Two days after infestation, plant VOC emission were sampled as detailed below. Immediately thereafter, the plants were removed from the pots and the leaves were frozen in liquid nitrogen. Sample used for phytohormone analysis were lyophilized and kept at room temperature, while samples used for total phenolic compounds (TPC) and EO yields analysis were stored at −80 °C. Several studies have revealed changes in SM after 48 h herbivory (Zebelo et al. 2016; de Bobadilla et al. 2017) At the end of the experiment plant experienced 30 days of inoculation.

Insect

The *R. nu* larvae used were provided by the AgIdea (Agricultural Innovation Applied Research-Argentina) company, which were obtained from a colony without previous insecticide exposure. These larvae were kept on a semi-synthetic diet (Greene et al. 1976) at 23–25 °C in a 70% humidified chamber, with a 16:8 h light/dark photoperiod.

Determination of Total Phenolic Compounds

The total phenolic content of the extract was determined by the Folin–Ciocalteu method (Cappellari et al. 2017). Briefly, 200 mg of frozen tissue were ground, with a mortar and pestle,

and homogenized in 5 ml distilled water, and incubated in the dark at room temperature. After 24 hs, 0.5 mL of the supernatant or gallic acid (standard phenolic compound) was mixed with Folin–Ciocalteu reagent (0.5 mL, diluted with 8 mL distilled water) and aqueous Na_2CO_3 (1 mL, 1 M). After 1 h, the level of total phenols was determined by colorimetry at a wavelength of 760 nm. The TPC were expressed in terms of μg gallic acid (a common reference compound) equivalent per g plant fresh weight (fw) using the standard curve.

Extraction of EOs

The remainder of the frozen samples was weighed and extracted in a Clevenger-like apparatus for 40 min. The volatile fraction was collected in dichloromethane, and β-pinene (1 μL in 50 μL ethanol) was added as an internal standard (β-pinene was reported not to be present in peppermint plants; Cappellari et al. 2015). *M. piperita* plants contain ~3% volatile oils, consisting of >50 different compounds. The major EO components, which comprise ~60% of total oil volume, are limonene, linalool, (−) menthone, (−) menthol, and (+) pulegone. These compounds were quantified in relation to the standard added during the distillation procedure as described above. Flame ionization detector (FID) response factors for each compound generated essentially equivalent areas (differences<5%).

Chemical analyses were performed using a Perkin-Elmer Q-700 gas chromatograph (GC) equipped with a CBP-1 capillary column (30 m × 0.25 mm, film thickness 0.25 μm) and a mass selective detector. The analytical conditions were: injector temperature 250 °C, detector temperature 270 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4 °/min; carrier gas = helium at a constant flow rate of 0.9 mL/min; source 70 eV. Oil components (limonene, linalool, (−) menthone, (−) menthol, and (+) pulegone) were identified by comparison of the diagnostic ions (NIST 2014 library) and GC retention times with those of respective authentic standard compounds purchased from Sigma-Aldrich (St. Louis, MO, USA) (Santoro et al. 2015). GC analysis was performed using a Shimadzu GC-RIA gas chromatograph fitted with a 30 m × 0.25 mm fused silica capillary column coated with Supelcowax 10 (film thickness 0.25 μm; Supelco. Inc. Bellefonte, PA, USA). The GC operating conditions were: injector and detector temperatures 250 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4 °/min; detector = FID; carrier gas = nitrogen at a constant flow rate of 0.9 mL/min.

Collection of Plant VOCs

The collection system consisted of a vacuum pump that created a constant airflow (300 mL/min) through a polyethylene terephthalate (PET) chamber (volume 1500 mL) containing one plant. This chamber was closed at one end with a cap pre-drilled to fit the collection trap. At the other end was a cap with a hole, through which the plant stem fitted. This separated the bottom of the chamber from the base of the pot. Air exited the chamber through a re-usable glass collection trap packet with 30 mg Super-Q absorbent (80–100 mesh; Alltech, Deerfield, IL, USA), which was rinsed 5 x with 10 mL dichloromethane prior to each collection to remove impurities. Headspace VOCs were collected for 2 h and eluted immediately from the absorbent traps with 200 mL dichloromethane, after which the internal standard was added (1 μL α-pinene in 50 μL ethanol) (Banchio et al. 2007). Six plants, one from each treatment, were collected at the same time (one plant per collection system). The collection was completed in two days. Collected VOCs were analyzed by gas chromatography as described above. Following VOC collection, each plant was cut and weighed, with VOCs also being collected from control (uninoculated) plants. Collections from an empty chamber showed that the background level of monoterpenes was negligible.

Phytohormone Analysis

The phytohormone analysis was based on a procedure by Schmidt et al. (2011). Approximately 0.10 g of ground lyophilized plant material was homogenized in 1 mL of methanol spiked with 40 ng of $[^2H_2]$JA, 40 ng $[^2H_4]$SA and 40 ng $[^2H_6]$ABA shaken for 60 min. Homogenates were centrifuged at 20,000 g for 20 min at 4 °C, the methanol phase was collected, and the homogenate was re-extracted with 1.0 ml methanol. The organic phases were combined, and the samples were evaporated to dryness in a vacuum concentrator at 30 °C. The dry residue was reconstituted in 0.5 ml of 70% (*v*/v) methanol/water, and analyzed by LC-MS/MS. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany), with separation being achieved on an XDB C18 column (1.8 μm, 50 mm × 4.6 mm; Agilent Technologies). The mobile phase, comprising of solvent A (0.05% formic acid) and solvent B (acetonitrile), was used in a gradient of 0–0.5 min, 5% B; 0.5–9.5 min, 0–58% B; 9.5–9.52 min, 58–100% B; 9.52–11 min, 100% B; 11–11.1 min, 5% B and 11.1–14 min, 5% B, with a flow rate of 1.1 mL min^{-1}. The column temperature was maintained at 25 °C, with an injection volume of 2 μL being used for all samples.

An API 5000 tandem mass spectrometer (AB Sciex, Darmstadt,. Germany) equipped with a turbospray ion source was operated in the negative ionization mode, and the ion spray voltage was maintained at −4500 V, with settings used of a turbo gas temperature of 700 °C, nebulizing gas at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. The data analysis was performed using Analyst

Software 1.6 Build 3773 (AB Sciex), and JA, SA and ABA were quantified according to the labeled standard compounds.

Statistical Analyses

The data were subjected to an analysis of variance (ANOVA) followed by a comparison of multiple treatment levels with controls using Fisher's post hoc LSD (least significant difference) test. In addition, we evaluated the individual effects and interaction between PGPR and herbivory using a two-way ANOVA. Differences between means were considered significant for P values <0.05. The Infostat software program, version 2008 (Group Infostat, Universidad Nacional de Córdoba, Argentina), was used for all the statistical analyses.

Results

Essential Oils

EO yield of plants damaged by *R. nu* increased 2.6 fold compared to control plants (Fig. 1), and increased by approximately 3.5 fold in comparison to controls when plants were inoculated with SJ04 or GB03 ($df = 5$, $F = 3.53$, $P = 0.01$, Table S1) (Fig. 1). This is similar to what was previously observed by Cappellari et al. (2015). The total EO content in plants inoculated and damaged by insects was similar to that in plants only damaged by insects or only inoculated, indicating no synergistic effects, which was confirmed by the lack of statistically significant interaction effects ($df = 2$, $F = 2.77$, $P = 0.07$, Table S2). Regarding the main components of the EOs, feeding of *R. nu* resulted in an increase in linalool, limonene, (−)-menthone, (−)-menthol, and (+)-pulegone (Table 1), with their content being approximately 3.5 times higher in plants damaged by insects than in control plants ($P < 0.05$, Table S1). Inoculation with rhizobacteria also led to significant variations in the major EO components (Table 1), with similar levels being recorded to those of infested plants. Although the yields for each of the major EO components were higher in plants inoculated with GB03 than in those treated with SJ04, these differences were not significantly significant ($p > 0.05$, Table S1). However, the feeding of *R. nu* on inoculated plants did not cause an additional increase in the content of the main components of the EO.

Emission of Plant VOCs

The emission of VOCs was significantly higher in plants infested by insects ($df = 5$, $F = 5.76$, $P < 0.001$, Table S1). Plants with *R. nu* only, emitted 6 times more monoterpenes than control plants (Fig. 2). Inoculated plants released ~3-fold more VOCs compared to control plants, which is similarly to our previous results (Cappellari et al. 2017). However, no statistical differences were observed between plants only infested with *R. nu* larvae and plants inoculated with PGPR, and subsequently infested with larvae ($P > 0.05$). No statistically significant interaction between herbivory and inoculation effect was found ($df = 2$, $F = 2.45$, $P = 0.64$, **Table S2**).

The emission of major VOCs was affected by herbivore damage (Table 2). The amounts of (+)-pulegone and (−)-menthone in the headspace were ~30- and 17 fold higher, respectively, for infested than for control plants. In inoculated plants, the emission of both compounds increased, but was not significantly ($P > 0.05$, Table S1) higher than in non-inoculated plants. For plants inoculated and also infested with *R. nu* larvae, the emissions were similar to those of non-inoculated plants damaged by insects.

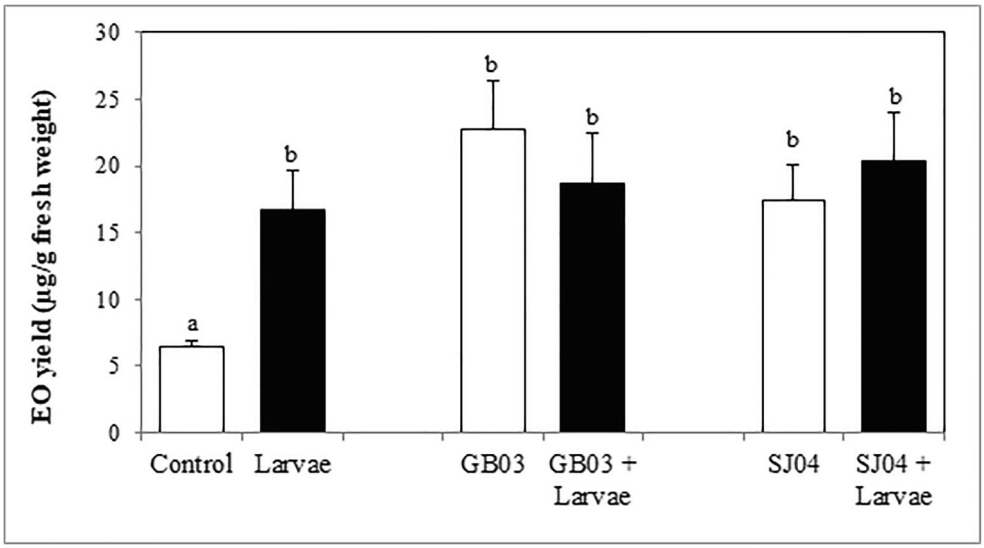

Fig. 1 EO yields in *Mentha piperita* inoculated with PGPR strains (*Bacillus amyloliquefaciens* GB03 and *Pseudomonas putida* SJ04) and/or attacked by *Rachiplusia nu*. Values are means ± standard error (SE). Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

Table 1 Concentrations of major essential oil (EO) components in *Mentha piperita* inoculated with PGPR strains and/or infested by *R. nu*. Data shown are means ± standard error (SE). Different letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$)

Treatment	Linalool (μg/g fw)	Limonene (μg/g fw)	(−)-Menthone (μg/g fw)	(−)-Menthol (μg/g fw)	(+)-Pulegone (μg/g fw)
Control	0.15 ± 0.08 *a*	0.21 ± 0.04 *a*	0.43 ± 0.26 *a*	0.33 ± 0.20 *a*	3.63 ± 1.16 *a*
Larva	0.61 ± 0.08 *bc*	0.63 ± 0.10 *b*	1.43 ± 0.29 *b*	1.12 ± 0.16 *b*	12.74 ± 1.09 *c*
GB03	0.69 ± 0.06 *bc*	0.78 ± 0.22 *b*	1.67 ± 0.29 *b*	1.24 ± 0.16 *b*	12.59 ± 1.24 *c*
GB03 + larva	0.68 ± 0.08 *bc*	0.80 ± 0.04 *b*	1.78 ± 0.29 *b*	1.15 ± 0.16 *b*	13.87 ± 1.24 *c*
SJ04	0.50 ± 0.10 *b*	0.77 ± 0.08 *b*	1.34 ± 0.22 *b*	1.08 ± 0.15 *b*	9.05 ± 0.99 *b*
SJ04 + larva	0.77 ± 0.08 *c*	0.63 ± 0.09 *b*	1.36 ± 0.35 *b*	1.37 ± 0.17 *b*	11.45 ± 1.24 *bc*

Total Phenolic Content (TPC)

The accumulation of phenolic compounds was higher in inoculated *M. piperita* plants compared to non-inoculated plants (Fig. 3), which is in line with our previous results (Cappellari et al. 2017). When plants were damaged by *R. nu* larvae, the total phenolic content increased by approximately 30% in comparison to control plants ($df = 5$, $F = 23.78$, $P < 0.001$, Table S1). When plants were inoculated and subsequently infested with *R. nu*, the TPC values were similar to individual treatments ($P > 0.05$). No statistically significant interaction between herbivory and the inoculation effect was found ($df = 2$, $F = 1.79$, $P = 0.18$, Table S2).

Endogenous Phytohormones

When endogenous phytohormones were measured 48 h after larval damage, the SA levels were approximately 4 times higher than in control plants ($df = 5$, $F = 2.82$, $P = 0.0208$, Table S1). (Fig. 4-**A**). When plants were inoculated alone (regardless of the strain), non-inoculated or inoculated and subsequently damaged by larvae, the levels of SA were similar to those observed in plants damaged by the larvae only.

The JA levels were 2.5 fold higher in damaged plants than in controls after 48 h of damage ($df = 5$, $F = 2.35$, $P = 0.0460$, Table S1). (Fig. 4-**B**). When plants were inoculated and damaged by larvae, the JA levels slightly decreased. However, this was only statistically significant for plants treated with larvae+ SJ04 compared with plants infested by the larvae alone. The JA levels in plants inoculated with SJ04 or GB03 were similar to those in herbivore damaged plants.

Finally, ABA levels were higher in insect infested plants compared to non-inoculated plants (Fig. 4-**C**), but the differences were not statistically significant ($df = 5$, $F = 2.25$, $P = 0.06$, Table S1). Overall, no statistically significant interaction between herbivory and inoculation effect was found for any of the phytohormones analyzed ($P > 0.05$, Table S2).

Discussion

In the present study, we demonstrated that the levels of SMs in *M. piperita* increased when plants were inoculated with PGPR as well as in response to caterpillar herbivory. The combination of both treatments produced the same effects on EO and TPC as each treatment individually. Thus, we concluded that

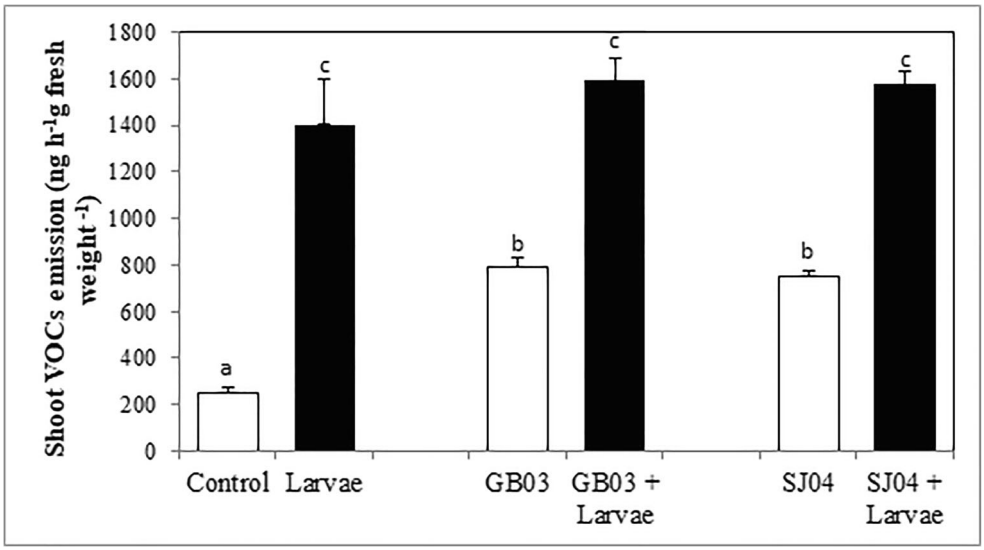

Fig. 2 Emission of shoot VOCs by *Mentha piperita* plants inoculated with PGPR strains (*Bacillus amyloliquefaciens* GB03 or *Pseudomonas putida* SJ04) and/or damaged by *Rachiplusia nu*. Values are means ± standard error (SE). Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$)

Table 2 Concentrations of (−) menthol and (+) pulegone (mean ± SE) in VOC emissions of *M. piperita* plants inoculated with PGPR strains and/or infested by *R. nu*. Means followed by the same letter within a column are not significantly different according to Fisher's LSD test ($p < 0.05$)

Treatment	(−)-Menthol (ng/ h g fw)	(+)-Pulegone (ng/h g fw)
Control	20.78 ± 2.69 *a*	18.09 ± 2.06 *a*
Larva	359.15 ± 213.58 *b*	521.15 ± 362.84 *b*
GB03	45.12 ± 8.09 *a*	46.34 ± 9.38 *a*
GB03 + larva	298.19 ± 127.96 *b*	393.20 ± 165.30 *b*
SJ04	32.84 ± 2.55 *a*	37.49 ± 8.36 *a*
SJ04 + larva	239.39 ± 41.03 *b*	469.47 ± 305.9 *b*

there was no synergism between the inoculation of PGPR strains and the damage caused by herbivory. The emission of VOCs was significantly higher in plants infested by insects, whether or not they were inoculated with PGPR. Their monoterpene emission where 6 times higher than in control plants and 2 -fold higher than in plant inoculated with PGPR only.

It is well known that the biochemistry of plant tissues changes following mechanical damage or herbivory. These phytochemical changes can increase plant resistance to subsequent herbivore attack, thereby improving the plant's defense level (Van Oosten et al. 2008; Rashid and Chung 2017). In the present study, we observed that damage produced by feeding larvae increased EO and plant VOC emission (2.5 and 5.6 fold, respectively). The induction of secondary metabolites involves JA and SA. Of these, JA has repeatedly been shown to be the most important mediator of plant–herbivore interactions, and to be responsible for VOC activation, including the synthesis of terpenes (Dicke and Baldwin 2010; Turling and Erb 2018). Many cases of cross-talk between the SA and JA defense pathway have been reported (Vos et al. 2015). This cross-talk between the hormone signaling pathways is supposed to activate the most effective defenses.

Soil is the major source of microorganisms, which are not only crucial for enhancing plant survival, growth and tolerance to abiotic stress, but also induce systemic resistance against pathogens and insects (Pieterse et al. 2014). In previous studies, we have shown that GB03 and SJ04 have beneficial effects on *M. piperita*, including growth promotion, EO yield, plant VOC emission and TPC increase (Cappellari et al. 2015, 2017). We observed similar effects in the present study. In the current investigation, when *M. piperita* plants were inoculated and infested with *R. nu,* the level of EO was similar as for infestation alone, indicating there was no synergism in the defense response. In our set-up we placed plants with caterpillars in a different growth room. This was done to avoid that herbivore-induced VOCs would trigger responses in plants without herbivores. Even though the two chambers were set to exactly the same conditions, there may be a slight chance that synergistic effects were not detected due to uncontrolled differences between the chambers.

The rise of EO and VOC emission was correlated with an increase in endogenous JA levels. JA controls the production of a number of secondary metabolites, including terpenoids, and upregulates the genes and enzymes of secondary metabolite biosynthetic pathways (Wasternack and Strnad 2017). Similar results were also observed in other aromatic plants, where the biosynthesis of monoterpenes was induced by a leaf miner and a gall insect in *Minthostachys mollis* (Valladares et al. 2002; Banchio et al. 2005). *Spodoptera littoralis* feeding

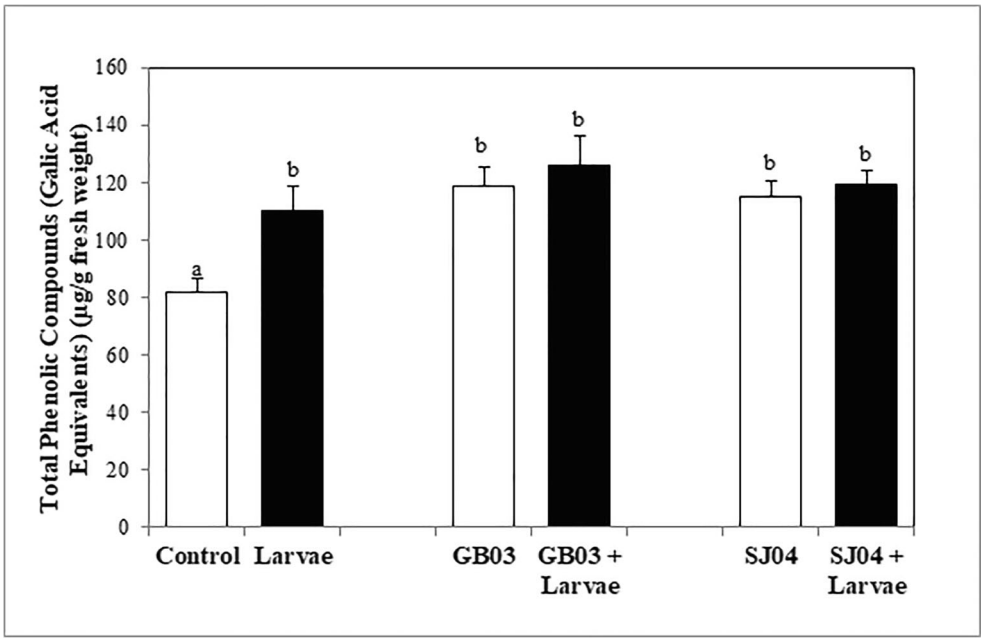

Fig. 3 Total phenolic content (TPC) in *Mentha piperita* inoculated with PGPR strains (*Bacillus amyloliquefaciens* GB03 or *Pseudomonas putida* SJ04) and/or damaged by *Rachiplusia nu*. Values are means ± standard error (SE). Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$)

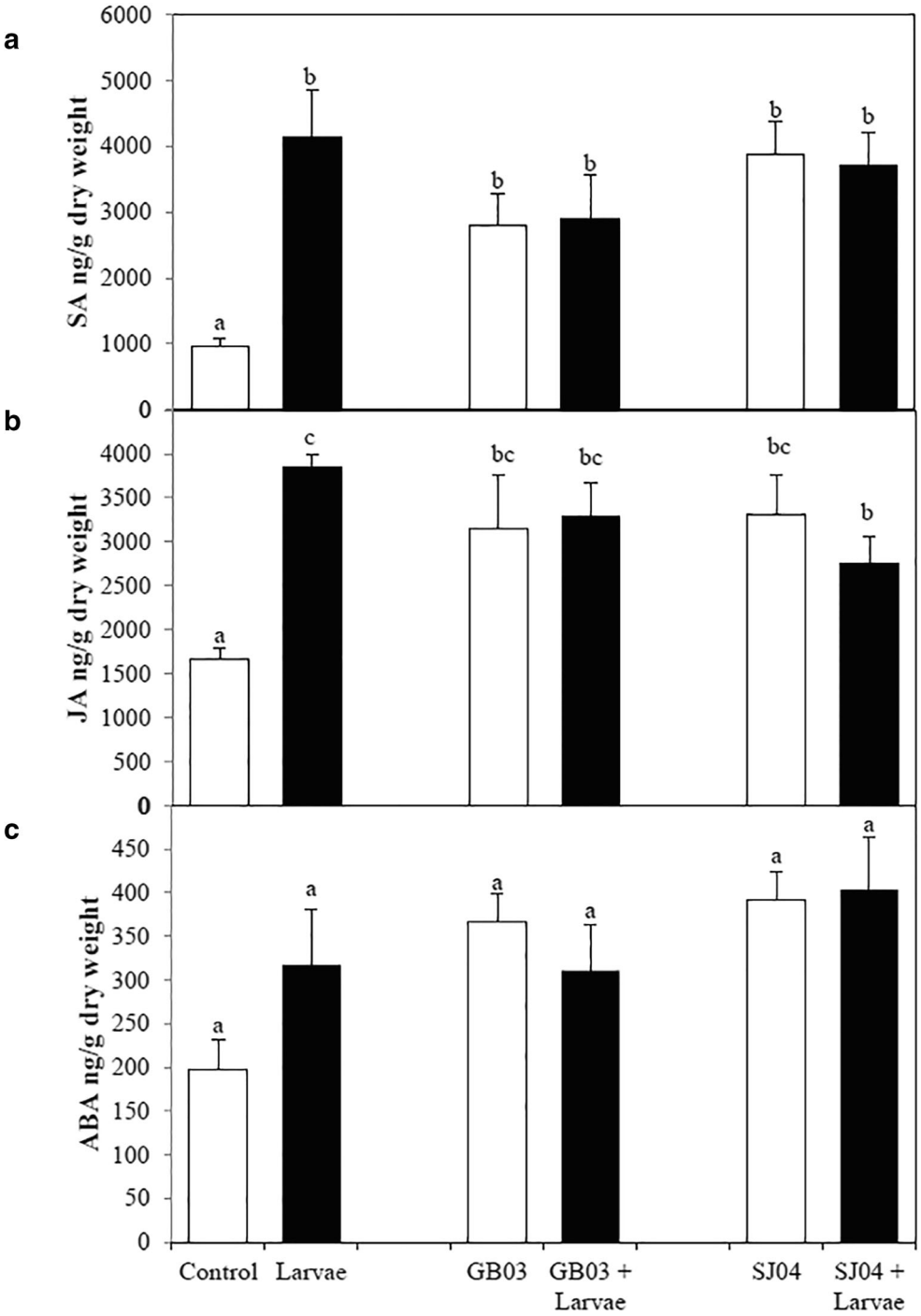

Fig. 4 Endogenous phytohormone content A) salicylic acid (SA) - B) jasmonic acid (JA) - C) abscisic acid (ABA) in *Mentha piperita* plants inoculated with PGPR strains (*Bacillus amyloliquefaciens* GB03 or *Pseudomonas putida* SJ04) and/or infested with *Rachiplusia nu.* Values are means ± standard error (SE), n = at least 10 per treatment group). Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$)

causing chemical and genetic modulation of terpenoids in *Origanum vulgare* (Agliasa and Maffei 2018). In addition, Zebelo et al. (2011) reported that *Chrysolina herbacea* activated genes for terpenoid biosynthesis when fed on *Mentha aquatica*. An increase in biosynthesis provides resistance to phytophagous organisms. The toxicity of terpenoids to herbivores has been extensively documented, including insecticidal effects of several components from the EO of *M. piperita* (Bakkali et al. 2008; Singh and Pandey 2018).

Plant VOCs can directly and indirectly protect the plant, as they are involved in plant-plant communication, resistance to abiotic stress factors, as well as in defense (Heil and Ton 2008). In response to feeding arthropods, plants emit a large variety of VOCs, as we have observed in the present study. These may directly affect herbivore physiology and behaviour due to their toxic repelling or deterring properties (Dong et al. 2016; Kalske et al. 2019). The emission of pulegone and menthol also increased due to the damage caused by *R. nu.* Plants

damaged by herbivorous insects can increase the total emission as well as the proportion of volatiles in the mixture (Bautista-Lozada et al. 2012). The increase in VOC emissions observed was not proportional to that recorded in the EOs. EO accumulation in *M. piperita* inoculated with rihozobacteria or damaged by larvae increased approximately 3 times, whereas the total VOC increased approximately 6 times. This difference probably is due to the fact that larval movement and chewing ruptured the glandular trichomes, responsible for producing and storing EOs, on the leaf surface (Duke et al. 2000). However, it is not fully understood how volatiles are released from plant cells. It has been proposed that volatiles must cross multiple cellular compartments to reach the environment. Additional biological mechanisms involved in the transport of other hydrophobic compounds may contribute to volatile emission as well. It is not known how metabolite movement occurs between various subcellular compartments, with the molecular mechanisms involved in VOC efflux remaining largely unknown (Dudareva 2013; Tissier et al. 2017).

In the present study, we observed that TPC increased significantly in response to leaf damage by *R. nu* feeding. In agreement with our results, in wild cotton plants (*Gossypium hirsutum*) the synthesis of phenolic compounds was significantly induced by leaf damage caused by *S. frugiperda* (Abdala-Roberts et al. 2019). The increase of phenolic compounds after leaf damage has been considered to be a defense against insects due to their ability to inhibit insect feeding by their toxicity (Karban and Baldwin 1997; Durak et al. 2019). Also, some phenolic compounds have been reported to affect insects negatively by acting as feeding deterrents and growth inhibitors (Zhang et al. 2017a). However, in the present study, the TPC level was similarly induced by *R. nu* feeding on inoculated and non-inoculated plants. As for the VOCs and EO, there was no synergistic effect of inoculation with PGPR strains and the damage caused by herbivory, suggesting that the effect of the herbivory prevailed. In contrast to our results, Bano and Muqarab (2016) observed no effect on the content of phenolic compounds in tomato plants inoculated with PGPR or infested with *S. litura.* They found that only plants inoculated with PGPR and infested with herbivores showed an increase in the phenolic content (Bano and Muqarab, 2016). This means that the effects of PGPR on herbivore-induced responses are plant species specific (Santoro et al. 2015).

In order to gain a better understanding of how the combined effects of herbivory and PGPR-inoculation caused increased EO, VOC emissions and TPC levels, we measured changes in the plant hormones in peppermint leaves. The endogenous JA and SA levels increased in inoculated plants compared to non-inoculated ones, regardless of the strain used, as we have previously observed (Cappellari et al. 2019). Previous work on the mechanisms of PGPR effects found that beneficial microorganisms in plant roots can improve plant health by priming the entire plant to increase its defense levels via ISR (Pieterse et al. 2014). ISR is activated by non-pathogenic bacteria in SA-independent and -dependent manners, and somewhat intersects with the JA/ET pathway. In infested plants, we observed that plant defence hormones (JA and SA) also increased to the same extent as in inoculated plants. It is well known that the JA pathway is activated in response to chewing herbivores (Turling and Erb 2018). In contrast, the SA pathway is activated when the plant is attacked by piercing-sucking insects or biotrophic pathogens (Schweiger et al. 2014). However, in the present study, we also observed an increase of SA in *M. piperita* leaves damaged the chewing herbivore *R. nu*. SA accumulation in host plants can be induced by chewing insects to suppress JA-mediated defenses (Thaler et al. 2012; Caarls et al. 2015). Oral secretion of *S.exigua* induces higher SA levels in *N. attenuata*, which suppresses JA accumulation (Diezel et al. 2009). Moreover, several insects carry viruses or microbes that trigger SA accumulation (Diezel et al. 2009; Zhang et al. 2017b). When we exposed *M. piperita* plants to the combination of both treatments, the level of SA or JA did not greatly differ from the level of individual treatments, with the JA endogenous levels being slightly higher only in insect infested plants, but this was not statistically significant.

In general, knowledge concerning secondary metabolite content in aromatic plants inoculated with PGPR after herbivory still remains limited. The promotion of plant growth induced by PGPR can affect plant-insect interactions, resulting in a benefit for the insect and/or for the plant (Pineda et al. 2010). The enhancement of plant growth increases food availability for herbivores. The improvement in the nutritional composition of the plant can affect the performance of insects of different trophic levels (herbivores, natural enemies and pollinators) (Bukovinszky et al. 2008; Shikano et al. 2017). On the other hand, by interacting with rhizobacteria the plant can benefit from increasing its tolerance or resistance to the herbivore (Pineda et al. 2010). The increase in water intake and nutrients mediated by PGPR can facilitate tissue regrowth after the attack of herbivore, which represents a compensation of the biomass or yield lost due to the herbivore (Rashid and Chung 2017). The present study highlights the importance of PGPR and chewing insects as determinants of induced SM, but also indicates the critical need for further study to demonstrate the role of rhizobacteria in the performance of insects and plants.

Conclusions

In summary, the combination of both treatments produced the same effects on EO and TPC as each treatment individually. Thus, we concluded that there was no synergism between the

inoculation of PGPR strains and the damage caused by herbivory. The emission of VOCs was significantly higher in plants infested by insects or simultaneously inoculated and infested. Because different interactions can alter the phytochemistry of plants such as *M. piperita*, this topic is both ecologically (e.g. insect-plant; plant-plant microorganism-plant interactions) and economically (e.g. essential oil production) relevant.

In nature, plants are exposed to multiple interactions, either simultaneously or sequentially. In our current investigation, these interactions were mostly studied in isolation or in combination of two interactors (PGPR inoculation and insect herbivory). When two or more interactions occur together, their effects may be additive, while in other cases the influence of one may have priority (Holopainen and Gershenzon 2010). The results presented here corroborate that PGPR inoculation and insect herbivory increase EO yield, VOC emission and TPC. However, adding a caterpillar to inoculated plants did not modify the levels of the SM compared to either treatment alone. This suggests that the response to herbivory may have had priority (Holopainen and Gershenzon 2010).

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