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Abstract:	When larvae of rootworms feed on maize roots they induce the emission of the sesquiterpene (E)-β-caryophyllene (EβC). EβC is attractive to entomopathogenic nematodes, which parasitize and rapidly kill the larvae, thereby protecting the roots from further damage. Certain root-colonizing bacteria of the genus Pseudomonas also benefit plants by promoting growth, suppressing pathogens or inducing systemic resistance (ISR), and some strains also have insecticidal activity. It remains unknown how these bacteria influence the emissions of root volatiles. In this study, we evaluated how colonization by the growth-promoting and insecticidal bacteria Pseudomonas protegens CHA0 and Pseudomonas chlororaphis PCL1391 affects the production of EβC upon feeding by larvae of the banded cucumber beetle, Diabrotica balteata Le Conte (Coleoptera: Chrysomelidae). Using a combination of chemical analysis and

	gene expression measurements, we found that EβC emission and the expression of the EβC synthase gene (TPS23) was enhanced in Pseudomonas-colonized roots after 72 hours of D. balteata feeding. Undamaged roots colonized by Pseudomonas spp. showed no measurable increase in EβC production, but a slight increase in TPS23 expression. Pseudomonas colonization did not affect root biomass, but larvae that fed on roots colonized by P. protegens CHA0 tended to gain more weight than larvae that fed on roots colonized by P. chlororaphis PCL1391. Larvae mortality on Pseudomonas spp. colonized roots was slightly, but not significantly higher. The observed enhanced production of EβC upon Pseudomonas spp. colonization may enhance the protective role of entomopathogenic nematodes and other soil beneficial organisms.

Root-colonizing bacteria enhance the levels of (*E*)-β-caryophyllene produced by maize roots in response to rootworm feeding

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XCM, HG, TCJT and RC-H conceived the experiments, XCM and RC-H analyzed the data and wrote the paper, NI and GR provide technical assistance for microbiology techniques and GC-MS analyses and, respectively. CK, MM and TCJT edited the text and approve the paper for publication.

Abstract

When larvae of rootworms feed on maize roots they induce the emission of the sesquiterpene (*E*)-β-caryophyllene (*E*βC). *E*βC is attractive to entomopathogenic nematodes, which parasitize and rapidly kill the larvae, thereby protecting the roots from further damage. Certain root-colonizing bacteria of the genus *Pseudomonas* also benefit plants by promoting growth, suppressing pathogens or inducing systemic resistance (ISR), and some strains also have insecticidal activity. It remains unknown how these bacteria influence the emissions of root volatiles. In this study, we evaluated how colonization by the growth-promoting and insecticidal bacteria *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391 affects the production of *E*βC upon feeding by larvae of the banded cucumber beetle, *Diabrotica balteata* Le Conte (Coleoptera: Chrysomelidae). Using a combination of chemical analysis and gene expression measurements, we found that *E*βC emission and the expression of the *E*βC synthase gene (TPS23) was enhanced in *Pseudomonas*-colonized roots after 72 hours of *D. balteata* feeding. Undamaged roots colonized by *Pseudomonas* spp. showed no measurable increase in *E*βC production, but a slight increase in TPS23 expression. *Pseudomonas* colonization did not affect root biomass, but larvae that fed on roots colonized by *P. protegens* CHA0 tended to gain more weight than larvae that fed on roots colonized by *P. chlororaphis* PCL1391. Larvae mortality on *Pseudomonas* spp. colonized roots was slightly, but not significantly higher. The observed enhanced production of *E*βC upon *Pseudomonas* spp. colonization may enhance the protective role of entomopathogenic nematodes and other soil beneficial organisms.

Key words: Root-colonizing bacteria, *Diabrotica balteata*, (*E*)-β-caryophyllene, terpene synthase, maize

Introduction

During the past decade it has been found that insect-damaged roots emit volatile compounds that may serve as attractants for the natural enemies of the damaging insects (Rasmann et al. 2005; Ali et al. 2010; Tonelli et al. 2016). The first such attractant was identified for maize roots, which respond to feeding by larvae of the beetle *Diabrotica virgifera virgifera* Le Conte (Coleoptera: Chrysomelidae) with the release of the sesquiterpene (*E*)-β-caryophyllene (*E*βC). This herbivore-induced volatile (HIPV) attracts entomopathogenic nematodes (EPNs) and, thereby, helps to protect maize roots against herbivore damage (Rasmann et al. 2005; Degenhardt et al. 2009). Although similar root-produced EPN attractants have been identified for several other plants (Boff et al. 2001; Ali et al. 2011), it is still poorly understood how other soil organisms affect the production or may respond to these signals.

Besides root herbivores, numerous other organisms that live in the rhizosphere may form associations with a plant. Their effects may be beneficial (e.g. mycorrhizal fungi, N-fixing bacteria) or detrimental (e.g. pathogenic fungi or bacteria) to plant performance (Brussaard 1998; Rasmann and Turlings, 2016). There is increasing interest in some strains of root-associated bacteria of the genus *Pseudomonas* that have plant-beneficial properties. They can promote plant growth, suppress pathogens and/or induce systemic plant defenses (Kupferschmied et al. 2013; Lugtenberg and Kamilova 2009; Van Oosten et al. 2008). Recent studies have also revealed that specific *Pseudomonas* strains possess insecticidal activity against several insect herbivore species (Ruffner et al. 2013). It has become increasingly evident that natural isolates of *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* (γ-Proteobacteria: Pseudomonaceae) have a high potential to be applied as plant protection products against various insect pests (Kupferschmied et al. 2013). Since many strains of the *P.*

fluorescens group are adapted to live on plant roots, show environmental persistence and are competitive and strong root colonizers, they may be ideal not only to enhance plant growth, but also to control insects pests (Lugtenberg & Kamilova 2009; Kupferschmied et al. 2013). The current study is part of an interdisciplinary effort to explore potential synergies in applying combinations of plant beneficial soil organisms (http://www.nrp68.ch/en).

Studies measuring the effects of root-associated bacteria on volatiles organic compounds have been largely limited to aboveground volatiles (Ballhorn et al. 2013; Pineda et al., 2013; Pangesti et al., 2015a) and the reported effects are greatly contrasting. Pineda et al. (2013) and Pangesti et al. (2015a) both used the bacterium *P. fluorescens* WCS417r to colonize *Arabidopsis thaliana* roots, but they employed aboveground herbivores of different feeding guilds to induce the leaves. It was found that *Myzus persicae* (Homoptera: Aphididae), a phloem feeder, induced increased levels of volatiles in colonized plants (Pineda et al., 2013), whereas colonized-plants that were damaged by leaf chewing caterpillars of *Mamestra brassicae* (Lepidoptera: Noctuidae) had reduced levels of HIPVs (Pangesti et al., 2015a). These differences can be explained by the different hormonal pathways that are activated by different plant antagonists. Chewing insects and necrotrophic pathogens typically induced the jasmonic acid pathway, whereas phloem-feeding insects and biotrophic pathogens usually upregulate the salicylic acid pathway (Zarate et al. 2006; Thaler et al., 2012; Jacobs et al. 2011; Pieterse et al. 2012). Thus, crosstalk between the two pathways may result in their mutual suppression (Zhang et al., 2009; Thaler et al., 2012). This is also a possible explanation for the results found by Ballhorn et al. (2013), who compared volatile emissions by rhizobia-colonized lime bean plants after experimental induction with jasmonic acid. Colonized plants produced higher amounts of shikimic acid-derived compounds than non-colonized plants, whereas the emission of compounds produced via the octadecanoid, mevalonate and non-mevalonate pathways was reduced.

We are aware of only one study that looked at the effects of root-colonizing bacteria on root-produced HIPVs. Santos et al. (2014) found that maize root colonization by *Azospirillum brasilense* (α-Proteobacteria: Rhodospirillaceae) produced higher amounts of *E*βC compared to non-colonized maize roots, in this case without insect damage. They further found that larvae of the generalist root feeder *Diabrotica speciosa* (Coleoptera: Chrysomelidae) oriented preferentially towards non-inoculated maize roots *versus* inoculated roots and gained less weight when feeding on inoculated roots. Interestingly, larvae of the maize specialist *D. virgifera virgifera*, which were initially studied in the context of inducible *E*βC (Rasmann et al., 2005), are attracted to *E*βC and perform better on already infested root systems (Robert et al., 2012a).

It remains unknown how root-associated bacteria affect the induction of belowground volatiles in response to root herbivory. This prompted the current study in which we studied these effects in maize roots damaged by larvae of another generalist *Diabrotica* beetle, the banded cucumber beetle *Diabrotica balteata* Le Conte (Coleoptera: Chrysomelidae). *D. balteata* larvae induce lesser amounts of *E*βC in maize roots than *D. virgifera* larvae, but this still results in some attraction of EPN (Rasmann and Turlings 2008). *D. balteata* is an important agricultural pest in Central and North America (Capinera 2011), attacking a broad spectrum of crops, including cucumber, squash, beet, bean, soybean, pea, sweet potato, okra, maize, lettuce, onion, and various cabbages (Saba, 1970; Chittenden, 1992; Capinera, 2011). It may damage all parts of a plant, but the most serious injury caused by *D. balteata* is to the roots (Capinera 2011). Enhancing *E*βC emissions in maize roots damaged by *D. balteata* might render EPN more effective in finding and killing the larvae of this important generalist root pest.

This pest is therefore a good model to test the possible effects of plant-beneficial root colonizing bacteria on *E*βC emissions.

In the present study, we used a chemical as well as a molecular approach to evaluate the effects of maize root colonization by the bacteria *P. chlororaphis* PCL1391 and *P. protegens* CHA0 on the emission of (*E*)-β-caryophyllene. Roots were inoculated (or not) by one of the bacteria and infested or not by *D. balteata* larvae. We then collected and analyzed the volatiles emissions from the roots and we measured the expression of the maize *E*βC synthase gene (TPS23) (Köllner et al. 2008).

The species *P. protegens* CHA0 is a root-associated bacterium that not only produces antifungal metabolites, but also an insecticidal protein. This protein is very similar to the potent insect toxin Mcf1 of the entomopathogen *Photorhabdus luminescens* (γ-Proteobacteria: Enterorbacteriaceae) (Péchy-Tarr et al., 2008). *P. protegens* CHA0 causes insect toxicity in experimental infections of aboveground feeding insect larvae (Péchy-Tarr et al., 2008) and also in feeding assays with artificial diets or leaves treated with the bacteria (Ruffner et al., 2013). It is unknown how these root-associated bacteria affect root feeding insect larvae. We therefore also studied the effect of the bacteria on the performance and mortality of *D. balteata* larvae.

Hence, we studied if colonization by *P. protegens* CHA0 or *P. chlororaphis* PCL1391: i) induces a change in the production of *E*βC after *D. balteata* attack in maize roots, ii) changes the expression of the maize *E*βC synthase gene TPS23, iii) affects root growth in maize plants, and iv) affects the performance and mortality of *D. balteata* larvae. We discuss our results in terms of the physiological changes that may occur in plants upon *Pseudomonas* colonization and how these changes may influence HIPVs. We further address the possibility of applying the bacteria in combination with EPNs for the effective control of diabroticine beetle larvae in maize and other cropping systems.

Materials and methods

Soil, plants and insect larvae

A substrate containing potting soil (Terreau semis Capito, Landi-Switzerland, pH = 5.8-6.8) and white sand (Migros, Switzerland) in proportion 1:1 was used to grow the plants. The substrate was autoclaved twice at 120 ºC for 120 min. Plastic pots (11 cm, height x 4 cm, diameter) were autoclaved once at 120 ºC for 120 min before each sowing.

Maize seeds (var. Delprim and var. F268) were surface sterilized by washing them with ethanol 70% for 2 min and sodium hypochlorite 3% for 2 minutes and rinsing them with sterile water. Plants were watered with 20 mL of sterile distilled water every 2-3 days. Plants were grown either in a greenhouse (30±5 ºC, 8:16 h dark:light photoperiod) in summer or in a phytotron (30±2 ºC, 8:16 h dark:light photoperiod, 300 µmol m^{-2} s^{-1}, CLF Plant Climatics, Germany) in winter.

Second instar larvae of *D. balteata* were reared from eggs provided by Syngenta (Stein, Switzerland) and they were fed with maize germinate. Larvae were used to infest 11 days old maize plants (after a period of 6 days of roots colonization by bacteria), by burying them in small holes in the soil. Each plant was infested with six *D. balteata* larvae.

Bacteria cultures and inoculation

The bacteria *P. protegens* CHA0 and *P. chlororaphis* PCL1391 (Department of Fundamental Microbiology, University of Lausanne) were cultured in LB agar (Miller, Sigma-Aldrich) supplemented with 100 µg/mL of rifampicin (≥ 97% powder, Sigma-Aldrich) for 48 hours in 9 cm diam. Petri dishes at 30 ºC. Bacteria were scratched from the plates under sterile conditions and transferred to 100 mL of sterile rifampicin supplemented-LB broth. Both species were cultivated independently in an orbital agitator (IKA-KS 4000) at 30 ºC and 190 rpm for 16 hours. Bacterial cultures were then centrifuged at 6846 x g for 10 minutes to separate bacterial cells from the liquid culture media. Resulting bacterial cell pellets were diluted again in sterile distilled water. Standard bacteria concentrations (1×10^6 CFU ml^{-1}) were obtained, calibrating the inoculum with a spectrophotometer at an optical density of 0.2A at 600 nm.

After 4-5 days of sowing, at the shoot emergence stage, plants were selected for the application of different treatments: a) inoculated with *P. protegens* CHA0, and infested with *D. balteata* (CHA0+Db), b) inoculated with *P. chlororaphis* PCL1391, and infested with *D. balteata* (PCL+Db), c) not inoculated with bacteria, infested with *D. balteata* (Db), d) control healthy plants (Healthy), e) only inoculated with *P. protegens* CHA0 (CHA0), and f) only inoculated with *P. chlororaphis* PCL1391 (PCL). Plants treated with root-colonizing bacteria were inoculated with 20 mL of *P. protegens* CHA0 or *P. chlororaphis* PCL1391 inoculum prepared as described above. Plants infested only with *D. balteata* and control-healthy were watered with 20 mL of sterile water. Preliminary experiments were performed before, measuring production of *E*βC after 72 hours of insect feeding, with six replicates per treatment (n = 6). Nine replicates (n = 9) per treatment were done in a final time-course experiment. Plants of different treatments were kept separated in different plastic trays to avoid cross-contamination and kept either in a greenhouse or a phytotron for 6 days during the root colonization period.

Colonization of maize roots with *P. protegens* CHA0 or *P. chlororaphis* PCL1391 was verified for a subset of plants of the same batch used for the volatiles and gene expression analysis. For this, roots of inoculated plants were harvested and the soil was gently removed and roots were weighed. Then the roots were suspended in flasks with 40 mL of sterile water and the flasks were shaken vigorously for 10 minutes to wash off the bacteria from the roots. Serial dilutions of the washed roots were prepared and plated on rifampicin-LB agar Petri dishes. Plates were incubated at 30 ºC and after 24 h the numbers of colony-forming units (CFU) were counted and CFU per gram of root calculated.

Volatile extraction and analyses

In preliminary experiments, we analyzed volatiles produced by the whole root system after 72 hours of *D. balteata* infestation, whereas in the final time-course experiment, we standardized the amount of ground root sample per vial for volatile analysis. We quantified the amount of *E*βC produced by roots of maize plants var. Delprim after 6 and 72 hours of insect infestation.

Roots were harvested and washed gently with tap water 6 and 72 hours after insect infestation and immediately frozen in liquid nitrogen for grinding. Roots were ground in a frozen mortar with liquid nitrogen. Root volatiles were extracted following the standard procedure by Rasmann (2005): 500 mg of ground root material were weighed and transferred to 10-mL glass vials sealed with a Teflon-coated septum and stored at -80 ºC for analysis. A 100 µm polydimethylsiloxane SPME fiber (Supelco, Sigma-Aldrich Chemie SA, Buchs, Switzerland) was inserted through the septum and exposed in the headspace for 60 min at 40 ºC. The compounds adsorbed onto the fiber were analyzed with an Agilent 7890a Series GC system coupled to mass-selective detector (Agilent 5975c, transfer line 280 ºC, source 230 ºC, quadrupole 150 ºC, ionization potential 70 eV) (Palo Alto CA, USA). The fiber was inserted into the injector port (250 ºC), desorbed and the volatile compounds were separated on a non-polar column (HP1-MS; 30 m, 0.25 mm internal diameter, 0.25 mm film thickness; J & W Scientific, Agilent Technologies SA, Basel, Switzerland). Helium at a constant flow mode 0f 0.9 mL min^{-1} (127.9 kPa) was used as a carrier gas. After fiber insertion, the column temperature was maintained at 50 ºC for 3 min, then increased to 180 ºC at 5 ºC min^{-1}, before a final ramp at 8 ºC min^{-1} to reach 250 ºC (hold 3 min). Chromatograms processing were carried out with ChemStation software (Agilent Technologies SA, Basel, Switzerland). Relative abundance of the root volatiles was calculated by integrating peaks and values were corrected for sample weight to calculate relative abundance of the volatile per gram of root.

cDNA synthesis and gene expression analysis

Approximately 60 mg of ground root material was used for the analysis of Zm-TPS23 gene expression. RNA from roots was extracted using the Isolate II RNA Plant Kit (Bioline, Germany), and RNA concentration was determined using a Nanodrop (Control Program ND-1000 v.3.3.0., ThermoScientific, Wilmington, DE). cDNA was synthetized using Sunscript RT RNAse H+ (Bioline, Germany). Real-time qPCR was performed in 100-well gene discs reaction plates (Biolabo, Scientific Instruments, Switzerland) in the Corbett Research real-time qPCR using Zm-TPS23 specific primers (F: GTGGGCCTCTACCTATCCA, R: CTGTGGTGGTGCCGTATTT) and Zm-actin specific primers (F: CAGTGGTCGAACAACGGGTA, R: GGTAAGGTCACGACCAGCAA) as a reference gene (Köllner et al. 2008). The qPCR mix was adjusted to a final volume of 10 µL, using RNA-free water, specific primers (either for TPS23 or for actin detection) both forward and reverse (0.05 µM) and SYBR Green (Bioline, Germany) and 1 µL of DNA template. Negative control contained free RNAase water instead of DNA template, to verify there is not contamination in the reactions. A qPCR analysis was carried out using the following thermal cycling conditions: a hold at 95 ºC for 10 min and 40 cycles, at 95 ºC for 10 s and at 60 ºC for 45 s. Relative expressions of the genes TPS23 and actin for different treatments were obtained using the correction method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

Assessment of larvae weight gain and mortality

For this evaluation, we used the same set of plants that we used for volatile extraction in the time-course experiment. We weighed *D. balteata* larvae (Mettler Toledo MX5 microbalance) before placing them on the plants and we recorded weight gain of the larvae after 6 hours, 48 hours and 72 hours of feeding. We also recorded the number of dead larvae per treated plant.

Statistical analysis

Relative abundance of volatiles per gram of root values (*E*βC) were normalized prior statistical analysis by log transformation. We employed a Linear mixed-effects model, each time-point was analyzed separately. Relative expression of terpene synthase gene data was analyzed with a Generalized linear model with a quasi-Poisson distribution. Tukey method was used to compare Least square means in both cases and T-test was used to compare differences between time-points. Root growth data was analyzed with One-way ANOVA. Larvae weight gain data were analyzed with Two-Way ANOVA. Mortality data were arcsin transformed and analyzed with Two-way ANOVA, differences between means were obtained with the Tukey method in all cases. All data were analyzed using R 3.3.2. (2016). Data is presented as mean ± SEM of untransformed values.

Results

Maize root colonization by Pseudomonas spp. and production of (E)-β-caryophyllene after *Diabrotica balteata* damage

The root colonization by *Pseudomonas* spp. was similar for all bacterial treatments (ANOVA, $F_{3,4} = 1.4$, $P > 0.1$) (Table 1). Our preliminary experiments, in which we analyzed the roots from two maize genotypes (var. Delprim and inbred line F268), showed a trend of higher production of *E*βC in response to *D. balteata* feeding on *Pseudomonas*-colonized roots as compared to non-colonized roots (72 h post-attack) (Supplementary Fig.1). However, variability within the treatments was high and no significant differences were detected.

The subsequent experiments showed that the production of *E*βC in maize roots was affected by treatment after 6 hours ($F_{5,40} = 9.12$, $P < 0.001$) and 72 hours ($F_{5,7} = 10.9$, $P < 0.01$) of insect feeding (Fig. 1; Supplementary Table 1). After 6 hours, non-inoculated roots attacked by the insects produced significantly larger amounts of *E*βC than control healthy roots ($P < 0.01$). There was a marginal difference in *E*βC quantities between insect-damaged roots colonized by any of the bacteria species and control healthy roots. However, there was no difference between insect-damaged roots colonized by any of the bacteria species and non-colonized roots attacked by the insect ($P > 0.1$) (Fig.1).

Seventy two hours after *D. balteata* attack, roots colonized by *P. protegens* CHA0 produced significantly larger amounts of *E*βC (P < 0.05) than non-colonized roots attacked by the insects whereas roots colonized by *P. chlororaphis* PCL produced similar (P > 0.1) amounts of *E*βC than non-colonized roots attacked by *D. balteata*. Control healthy roots produced the same amounts of *E*βC (P > 0.1) as undamaged roots colonized by either bacterium (Fig. 1). We found a significant higher production of *E*βC (P < 0.05) after 72 hours than after 6 hours of insect damaged in roots colonized by *P. protegens* CHA0. For the other treatments, there were no differences between the two time points, neither for insect-damaged plants colonized by *P. chlororaphis* PCL1391 ($P > 0.1$).

Expression of the terpene synthase-TPS23 after *Diabrotica balteata* damage in maize roots colonized by Pseudomonas protegens CHA0 and Pseudomonas chlororaphis PCL1391

The treatments also affected the expression of TPS23 (after 6 hours: $F_{5,37} = 3.27$, $P < 0.05$; after 72 hours: $F_{5,28} = 18.32$, $P < 0.001$). After 6 hours of insect feeding, the expression of the gene was significantly higher in roots colonized by *P. chlororaphis* PCL1391 and attacked by *D. balteata* ($P < 0.05$), and in non-colonized roots attacked by the insect ($P < 0.05$), as compared to healthy control roots (Fig. 2; Supplementary Table 2).

After 72 hours of *D. balteata* attack, gene expression in insect-damaged roots colonized by *P. protegens* CHA0 ($P < 0.01$) and *P. chlororaphis* PCL1391 ($P < 0.05$) was significantly higher than in insect-damaged non-colonized roots. The expression in the latter roots was not different from the expression in control healthy roots ($P = 0.1$), nor from the expression in undamaged roots colonized by either one of the bacteria species ($P > 0.1$) (Fig. 2; Supplementary Table 2). As found for the release of *E*βC (Fig. 1), TPS23 expression was significantly higher ($P < 0.01$) after 72 hours of insect attack than after 6 hours in insect-damaged roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL. In all of the other treatments, the expression was not statistically different ($P > 0.01$) between the two time-points.

Root colonization does not change roots biomass

We did not find an effect of any of the treatments on root fresh weight ($P = 0.09$), measured after the 72 hours of *D. balteata* feeding (Fig. 3A). However, there was a trend that biomass of insect-damaged roots was higher for plants colonized by *P. chlororaphis* PCL as compared to the insect-damaged roots grown in presence of *P. protegens* CHA0 or in absence of bacterial inoculants.

Effects of bacterial colonization on the weight gain and mortality of *Diabrotica balteata* larvae

Overall, there was no effect of the treatments on larval weight gain ($F_{2,72} = 1.72$, $P = 0.18$), but there was a trend of better weight gain when larvae were feeding on *P. protegens* CHA0 colonized roots than when feeding on *P. chlororaphis* PCL-colonized roots (Fig. 3b), and this correlates with differences in root biomass (Fig. 3a and Supplementary Fig. 2). We measured an overall increase in weight over time ($F_{2,72} =$

8.59, $P < 0.001$) (Fig. 3b), but no significant interaction between time and treatment ($F_{4,72} = 0.72$, $P = 0.57$). Within each treatment, weight over time varied only significant for larvae that had fed on roots colonized by *P. protegens* CHA0.

In a preliminary experiment with maize plants var. F268, we found a similar pattern of weight gain for *D. balteata* feeding on roots colonized by *P. protegens* CHA0, *P. chlororaphis* PCL1391 and non-colonized roots (Supplementary Fig. 3). In this experiment, we detected a significant effect of time ($F_{4,123} = 10.85$, $P < 0.01$), but no obvious effect of the treatment ($F_{2,123} = 1.11$, $P > 0.1$), nor an interaction between time and treatment ($F_{5,123} = 0.26$, $P > 0.1$).

For the main experiment, we also found an effect of time on the mortality of *D. balteata* larvae ($F_{2,72} = 21.76$, $P < 0.001$), but no effect of the treatment ($F_{2,72} = 2.03$, $P > 0.1$), nor an interaction between time and treatment ($F_{4,72} = 0.98$, $P > 0.1$) (Fig. 3C).

Discussion

We found quantitative but no qualitative differences in the volatile profiles for the different treatments. Maize roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 bacteria without insect infestation produced only minor quantities of the root volatile *E*βC (Fig.1 and Supplementary Fig.1.), but colonization by *P. protegens* CHA0 significantly enhanced the production of the sesquiterpene in maize after 72 hours of *D. balteata* feeding. To our knowledge, ours is the first study that evaluates how root-associated bacteria affect the emissions of a belowground HIPV upon root herbivory. Yet, Santos et al. (2014), using the same maize variety (Delprim), showed that the plant-beneficial bacterium *Azospirillum brasilense* affects *E*βC emissions in plants without insect damage. They found that colonized roots released more *E*βC and repelled larvae of *Diabrotica speciosa*.

Other studies on how root-associated bacteria affect volatile emissions have focused on volatiles released from aboveground plant parts, and show contrasting results. Root colonization by pseudomonads can decrease (Pangesti et al. 2015a) or increase (Pineda et al., 2013) aboveground HIPVs. *Arabidopsis thaliana* plants colonized by *Pseudomonas fluorescens* WCS417r and subsequently attacked by *Mamestra brassicae* caterpillars, produced lower amounts of methyl salicylate, lilial and the terpene (*E*)-α-bergamotene in comparison with non-colonized plants infested with caterpillars (Pangesti et al. 2015a). In contrast, Pineda et al. (2013) showed with the same plant-bacteria system, but using the aphid *Myzus persicae* as herbivore, that the aphid-induced production of eight leaf volatiles (2-nonenal, isovaleric acid, dimethyl sulfoxide, 2-cyclopente-1-one, (*R*)-verbenone, (*E*)-2-heptanal, 1-pentanol and 5,5 dimethyl-2(5H)-furanone) was enhanced in soil bacteria-colonized plants compared with non-colonized plants. Some other volatiles were produced in high quantities in plants colonized by *P. fluorescens* even without insect damage in the same study. Hence, effects of root colonizing bacteria on inducible volatiles appear to vary strongly, depending on the plants species, root-associated bacteria and on the insect herbivores.

Our findings on *E*βC emissions correlate nicely with the results for the expression of the terpene synthase gene Zm-TPS23. In roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391, the expression was enhanced after 72 hours of *D. balteata* infestation in comparison with non-colonized roots attacked by the insect (Fig. 2). Interestingly, we also found a higher expression of the gene TPS23 in undamaged roots colonized by *P. chlororaphis* PCL1391 than in control healthy roots at the second time-point (after 72 hours). This is again different from Pangesti et al. (2015a), who reported a negative effect of *P. fluorescens* colonization on the expression of the terpene synthases TPS03 and TPS04 in *Arabidopsis* upon insect leaf herbivory. These contrasting results confirm, as mentioned above, that the effects of root-associated bacteria on volatile emissions may vary depending on the system under study.

Inducible plant defenses, including volatile emissions, are mediated by wound-induced jasmonic acid (JA), which is derived from the lipoxygenase (*LOX*) pathway (Turner et al. 2002; Schmelz et al. 2003; Maffei et al. 2011; Dudareva et al, 2013). Previous studies found that *Pseudomonas* colonization of *A. thaliana* plants promotes the expression of the gene LOX2 (Pineda et al. 2012) and JA-responsive genes (Oosten et al. 2008), and results in stronger JA-signaling (Pangesti et al., 2015b) after insect attack. We also know that the gene Zm-TPS23 is locally and systemically induced in maize roots in response to feeding by *D. virgifera*. This appears to be triggered by local induction of jasmonic acid (JA) and its isoleucine conjugate (JA-Ile) after 30 minutes, resulting in an exponentially increasing production of *E*βC over 48 hours of feeding (Erb 2009; Hiltpold et al. 2011). Taking all together, we can hypothesize that belowground enhanced production of *E*βC in maize roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 might be mediated by increased JA-signaling.

Pangesti et al. (2015b) point out that differences in soil composition may explain some of the variable outcomes of plant-mediated effects of root-associated microbes on volatile signals and insect performance. It remains to be investigated if the effects of *P. protegens* CHA0 and *P. chlororaphis* PCL-1391 on the enhanced production of the root sesquiterne *E*βC are consistent in different types of soils. We previously showed the importance of studying the dynamics of *E*βC production and diffusion under different soil conditions (Chiriboga M. et al. 2017).

It has also been proposed that the effect of root-associated microbes on insect herbivores is different for specialist and generalist herbivores and for insects with different modes of feeding. Pineda et al. (2010) expect a negative effect on generalist chewing insects and mesophyll feeders, and positive or neutral on specialist chewing insects and phloem feeders. The effects on herbivore performance are directly related to the activation of defensive responses in the plant, including the production of HIPVs. It is pertinent to investigate what additional volatiles are produced upon root-colonization by bacteria, also by the bacteria themselves (D'Alessandro et al., 2014), and how these affect the interactions with other soil organisms.

We did not measure a clear effect of any treatment on root biomass (Fig. 3A), but there was a trend of lower biomass for insect-damaged roots that were colonized by *P. protegens* CHA0 compared to insect-damaged roots colonized by *P. chlororaphis* PCL (Fig. 3A). The poorer performance of the larvae on PLC-colonized plants may have contributed to this trend (Fig. 3B and Supplementary Fig 2.). Indeed, *D. balteata* larvae feeding on maize roots colonized by *P. protegens* CHA0 tended to gain more weight than larvae feeding in roots colonized by *P. chlororaphis* PCL1391 after 72 hours of feeding. Possibly, the increased emissions of *E*βC in roots colonized by *P. protegens* CHA0 stimulated feeding and/or benefitted *D. balteata* weight gain. This has been shown for larvae of the maize specialist *D. virgifera*, which are attracted to *E*βC (Robert et al. 2012a) and perform better on already infested roots (Robert et al. 2012b). In contrast, larvae of the generalist *D. speciosa* larvae gained less weight on and are less attracted to roots that produce increased amounts of *E*βC (Santos et al. 2014).

It is further possible that the differences in weight gain on roots with different treatments were due to differences in nutritional quality and/or biomass of the roots. Mutualistic microorganisms are known to influence plant tolerance to herbivory

(Strauss and Agrawal 1999). *Diabrotica* feeding also triggers tolerance responses, including regrowth of roots and resource reallocation in maize (Erb, 2009) and it would be worthwhile to determine if PCL1391-colonization has an effect on these responses.

There were no significant differences in mortality among different treatments (Fig. 3C), but there was a trend for higher mortality in larvae feeding 72 h on *P. chlororaphis* PCL-treated plants. If we had let the larvae feed longer this might have resulted in clearer effects, as pathogenicity of *Pseudomonas* bacteria can be rather a long process that involves several steps: bacteria ingestion, release of the toxin, toxin binding, breaking of the gut wall and insect death (Kupferschmied et al. 2013, Keel 2016). The observed enhanced signaling ability and possible higher larval mortality on Pseudomonas-colonized roots imply that the application of the bacteria in combination with EPNs might be a highly effective strategy for the control of root herbivores in maize production. This compatibility was confirmed in a field study, in which two species of *Pseudomonas* in combination with the EPN *Heterorhabitis bacteriophora* were found to be best in enhancing wheat plant performance (Imperiali et al., under review). How the application of such combinations plays out against *Diabrotica* pest under realistic field condition remains to be determined.

Conclusions

Colonization of maize roots by *P. protegens* CHA0 was found to enhance the emission of *E*βC after 72 h of feeding by *D. balteata* larvae. Consistent with this enhanced emission of the EPN attractant, we found a higher expression of the terpene synthase gene Zm-TPS23 after 72 h of insect infestation in colonized roots. The gene expression data revealed a positive effect of both *Pseudomonas* strains. Undamaged roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 also had a slightly enhanced expression of the terpene synthase gene. The mechanisms that are involved in this enhanced production of *E*βC are still unclear. The same is true for the observed differences in larval growth and mortality on roots of the different treatments. Yet, it is evident from this study that the application of beneficial *Pseudomonad* bacteria and EPN is compatible and may be a highly complementary strategy for the control of soil pests and to enhance crop performance.

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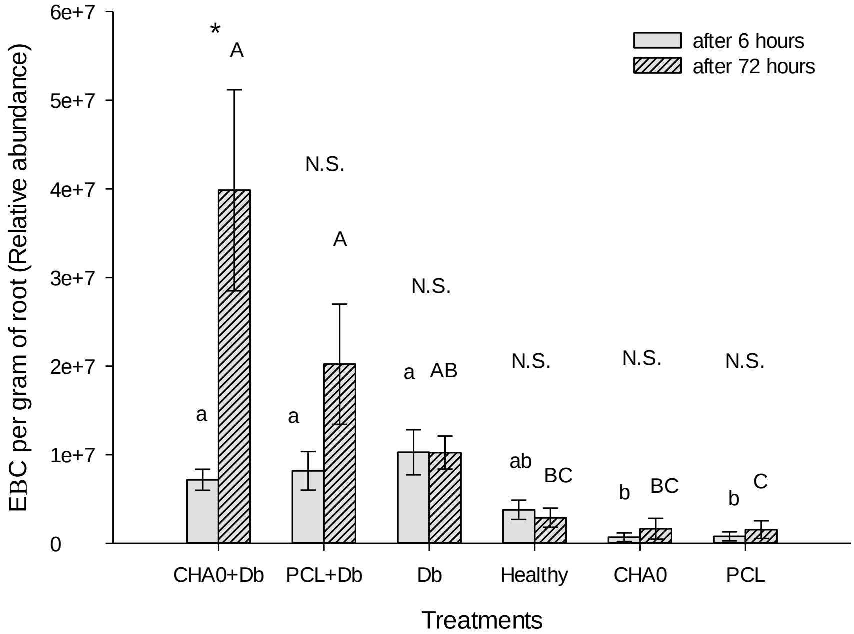
Figure Legends

Fig. 1 Relative abundance of *E*βC (mean ± SE) released by maize roots *var.* Delprim after different treatments: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0+Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D.balteata* (PCL+Db), infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), (N=9). Lower case letters indicate significant differences between treatments after 6 hours of feeding. Capital letters indicate significant differences between treatments after 72 hours of feeding. Stars indicate significant differences between times. N.S. indicate not significant differences between times.

Fig. 2 Relative expression (calculated in relation to actin relative expression) of the terpene synthase gene *Zm*-TPS23 (mean ± SE) in maize roots *var.* Delprim after treatments: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0+Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL+Db), infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), (N=9). Lower case letters indicate significant differences between treatments after 6 hours of feeding. Capital letters indicate significant differences between treatments after 72 hours of feeding. Stars indicate significant differences between times. N.S. indicate not significant differences between times.

Fig. 3a Root fresh weight (mean ± SE) of 14-days-old maize plants *var.* Delprim: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0+Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL+Db), infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), (N=12) **b** Weight gain (percentage, mean ± SE) of *D. balteata* larvae after 6 hours, 48 hours and 72 hours of feeding on maize roots *var*. Delprim with different treatments, (N=9) **c** Percentage of mortality of *D. balteata* larvae after 6 hours, 48 hours and 72 hours of feeding on roots with different treatments, (N=9). Different letters show significant differences between treatments. N.S. not significant differences.

Figure 1



6e+7
5e+7
4e+7
3e+7
2e+7
1e+7
0
EBC per gram of root (Relative abundance)
after 6 hours
after 72 hours
*
A
N.S.
A
N.S.
a
AB
N.S.
N.S.
N.S.
N.S.
a
a
ab
BC
b
BC
b
C
CHA0+Db
PCL+Db
Db
Healthy
CHA0
PCL
Treatments

Figure 2

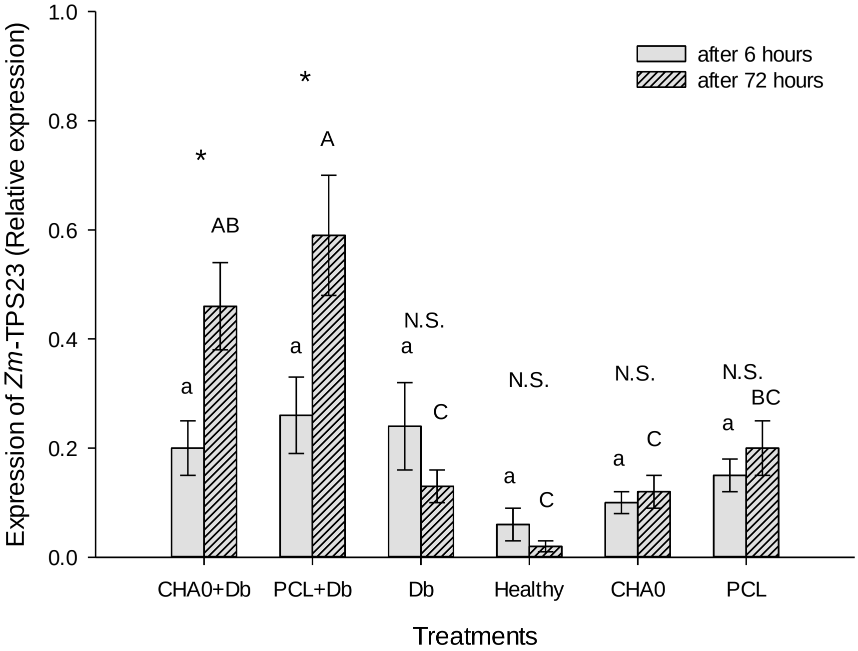

1.0
0.8
0.6
0.4
0.2
0.0
Expression of Zm-TPS23 (Relative expression)
after 6 hours
after 72 hours
*
*
A
AB
N.S.
a
a
a
a
C
N.S.
N.S.
N.S.
BC
a
C
a
C
CHA0+Db
PCL+Db
Db
Healthy
CHA0
PCL
Treatments

Figure 3

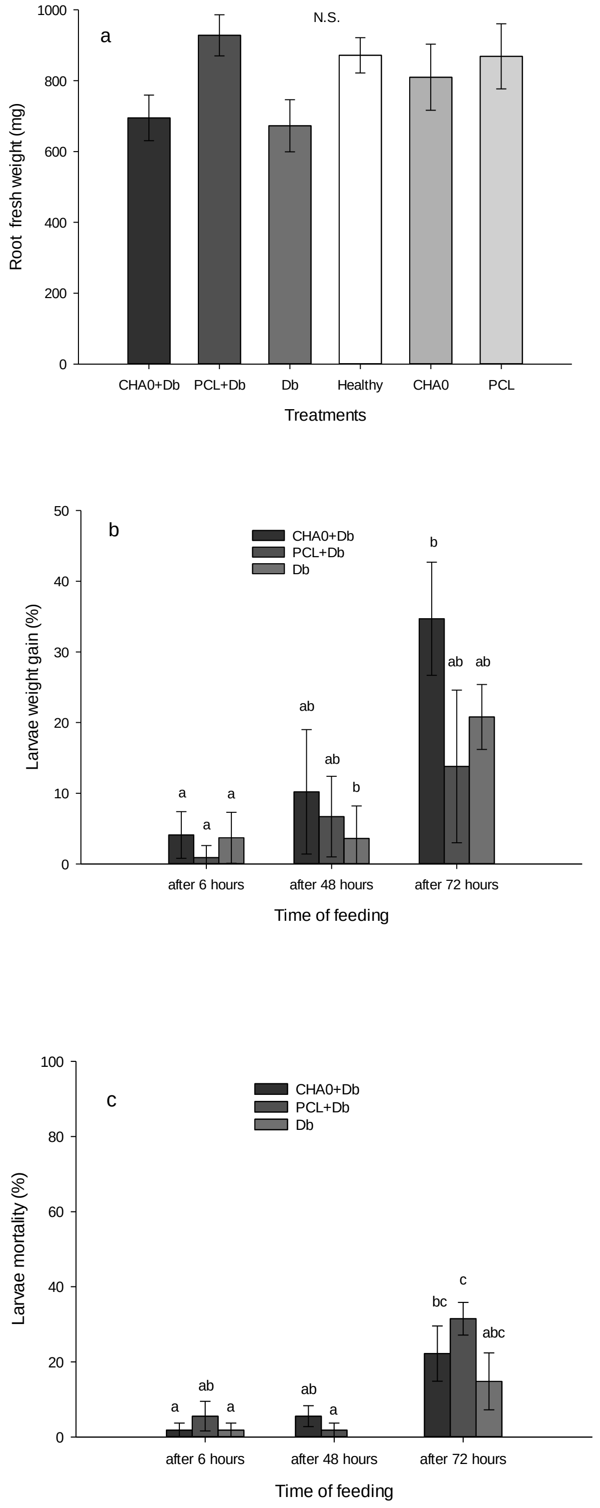

a
N.S.
Root fresh weight (mg)
0
200
400
600
800
1000
CHA0+Db
PCL+Db
Db
Healthy
CHA0
PCL
Treatments
b
Larvae weight gain (%)
CHA0+Db
PCL+Db
Db
0
10
20
30
40
50
a
a
a
ab
ab
b
b
ab
ab
after 6 hours
after 48 hours
after 72 hours
Time of feeding
c
Larvae mortality (%)
CHA0+Db
PCL+Db
Db
0
20
40
60
80
100
a
ab
a
ab
a
bc
c
abc
after 6 hours
after 48 hours
after 72 hours
Time of feeding

Table 1. Quantification of root colonization by *P. protegens* CHA0 and *P. chloraphis* PCL1391 in different treatments

Treatment	**C.F.U. / g of root (±SEM)**
P. protegens* CHA0 + *D.balteata	$5.7 \times 10^{7} \pm 0.20$ a
P. chloraphis* PCL + *D.balteata	$1.3 \times 10^{8} \pm 0.07$ a
***P. protegens* CHA0**	$2.4 \times 10^{8} \pm 1.70$ a
***P. chloraphis* PCL**	$3.5 \times 10^{7} \pm 0.65$ a
Control healthy	0