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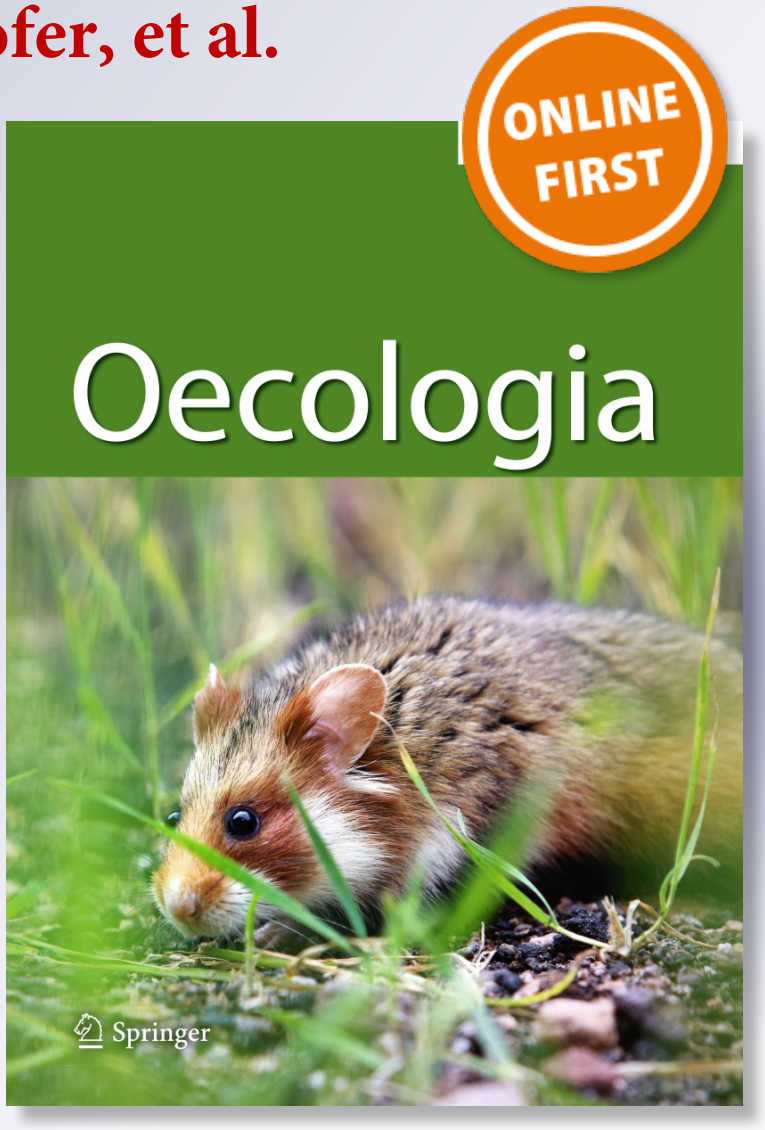

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SPECIAL TOPIC: FROM PLANTS TO HERBIVORES

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

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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 chemical analysis and gene expression measurements, we found that *E*βC production and the expression of the *E*βC synthase gene (*tps*23) were enhanced in *Pseudomonas protegens* CHA0-colonized roots after 72 h of *D. balteata* feeding. Undamaged roots colonized by *Pseudomonas* spp. showed no measurable increase in *E*βC production, but a slight increase in *tps*23 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 than on non-colonized control

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roots. The observed enhanced production of *E*βC upon *Pseudomonas protegens* CHA0 colonization may enhance the roots' attractiveness to entomopathogenic nematodes, but this remains to be tested.

Keywords Root-colonizing bacteria · *Diabrotica balteata* · (*E*)-β-caryophyllene · Terpene synthase · Maize

Introduction

Insect-damaged roots emit volatile compounds that 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 *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 further herbivore damage (Rasmann et al. 2005; Degenhardt et al. 2009). Similar root signals have been found for several other plants (Boff et al. 2001; Ali et al. 2011), but it is poorly understood how other soil organisms affect their production or may respond to them.

Besides root herbivores, numerous other organisms that live in the rhizosphere form associations with plants. 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 benefit plants by promoting plant growth, suppressing pathogens and/or inducing 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 been suggested that natural isolates of *Pseudomonas fluorescens* and *P. chlororaphis* (α-Proteobacteria: Pseudomonaceae) have a high potential to be applied as plant protection products. Since they are adapted to live on plant roots, show environmental persistence and are competitive root colonizers, they may be ideal not only to enhance plant growth, but also to control insects pests (Lugtenberg and Kamilova 2009; Kupferschmied et al. 2013). The current study is 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 volatile organic compounds have been limited to aboveground volatiles (Ballhorn et al. 2013; Pineda et al. 2013; Pangesti et al. 2015a) and the reported effects are greatly contrasting, depending on the insect herbivore attacking the plants. 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.

It remains unknown how root-associated bacteria affect the induction of belowground volatiles in response to root herbivory. This prompted this study in which we investigated these effects in maize roots damaged by larvae of another generalist, 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 maize (Chittenden 1912; Saba 1970; 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 is, therefore, a good model to test the possible effects of root-colonizing bacteria on *E*βC emissions.

We used a chemical and a molecular approach to evaluate the effects of maize root colonization by the bacterium *P. protegens* CHA0 and *P. chlororaphis* PCL1391 on the production of *E*βC. Roots were inoculated (or not) by one of the bacteria and infested or not by *D. balteata* larvae; we then collected and analyzed volatiles produced by the roots and we measured the expression of the maize terpene synthase gene *tps*23, which is responsible for *E*βC production (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 that is very similar to the insect toxin Mcf1 of the entomopathogen *Photorhabdus luminescens* (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 bacterium (Ruffner et al. 2013). It is unknown how these root-associated bacteria affect root feeding insect larvae. We,

therefore, also studied the effect of *Pseudomonas* spp. on the performance and mortality of *D. balteata* larvae.

We tested if colonization by *P. protegens* CHA0 or *P. chlororaphis* PCL1391: (1) induces a change in the production of *E*βC after *D. balteata* attack in maize roots, (2) changes the expression of the gene *tps*23, (3) affects root growth in maize plants, and (4) affects the weight gain 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 suggest the possibility of applying bacteria in combination with EPNs for the effective control of diabroticine beetle larvae in maize and other crops.

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 (v/v) was used to grow the plants. The substrate was autoclaved twice at 120 °C for 120 min. Plastic pots (11 cm, height × 4 cm, diam.) were autoclaved once at 120 °C for 120 min before 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 min, 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 °C, 8:16 h dark:light photoperiod) in summer or in a phytotron (30 ± 2 °C, 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 freshly germinated maize roots (var. Delprim). Larvae were used to infest 11 days old maize plants (after a period of 6 days of roots colonization by bacteria), by burying them (at 1–1.5 cm) 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 h in 9 cm diam. Petri dishes at 30 °C. 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 °C and 190 rpm for 16 h. Bacterial cultures were then centrifuged at 6846×*g* for 10 min to separate bacterial cells from the liquid culture media. Resulting bacterial cell pellets were diluted again in sterile distilled water. Standard bacteria concentrations, expreses as colony forming units (CFU) (1×10^6 CFU mL^{-1}) were obtained, calibrating the inoculum with a spectrophotometer at an optical density of 0.2 A at 600 nm.

At the shoot emergence stage (4–5 days after sowing), 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 distilled water. Preliminary experiments were performed before, measuring production of *E*βC after 72 h of insect feeding in the maize inbred line F268, 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 by hand 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 min 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 °C and after 24 h the numbers of 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 h 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 h of insect infestation.

Rather than trapping volatiles from the roots, we used a destructive method that quantifies the amounts of volatiles

that the roots produce. Based on previous studies (Rasmann et al. 2005; Hiltpold et al. 2011), we know that this procedure is more precise and that the measured production correlates well with emissions. Roots were harvested and washed gently with tap water 6 and 72 h 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 described by Rasmann et al. (2005): 500 mg of ground root material was transferred to 10 mL glass vials sealed with a Teflon-coated septum and stored at − 80 °C 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 °C. The compounds adsorbed onto the fiber were analyzed with an Agilent 7890a Series GC system coupled to a mass-selective detector (Agilent 5975c, transfer line 280 °C, source 230 °C, quadrupole 150 °C, ionization potential 70 eV) (Palo Alto CA, USA). The fiber was inserted into the injector port (250 °C), 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 of 0.9 mL min^{-1} was used as a carrier gas. After fiber insertion, the column temperature was maintained at 50 °C for 3 min, then increased to 180 °C at 5 °C min^{-1}, before a final ramp at 8 °C min^{-1} to reach 250 °C (hold 3 min). Chromatogram processing was 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-*tps*23 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., Thermo-Scientific, Wilmington, DE). cDNA was synthetized using Sunscript RT RNAse H + (Bioline, Germany). Real-time qPCR was performed in 100-well gene disc reaction plates (Biolabo, Scientific Instruments, Switzerland) in the Corbett Research real-time qPCR using Zm-*tps*23 specific primers (F: GTGGGCCTCTACCTATCCA, R: CTGTGGTGGTGC CGTATTT) and Zm-*actin* specific primers (F: CAGTGG TCGAACAACGGGTA, R: GGTAAGGTCACGACCAGC AA) 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 *tps*23 or for *actin* detection) both forward and reverse (0.05 µM) and SYBR Green (Bioline, Germany), and 1 µL of DNA template. The negative control contained free RNAase water instead of DNA template, to verify there is no contamination in the reactions. A qPCR analysis was carried out using the following thermal cycling conditions: a hold at 95 °C for 10 min and 40 cycles, at 95 °C for 10 s, and at 60 °C for 45 s. Relative expressions of the genes *tps*23 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, 48, and 72 h 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 with square root transformation. Transformed data from each time-point was analyzed separately with a one-way ANOVA model. Data of the relative expression of the terpene synthase gene were subject of the same transformation and analyzed with one-way ANOVA. Tukey method (5%) was used to compare Least Square Means of treatments in both cases and a *t* test was used to compare differences between time-points. Root-biomass data were analyzed with one-way ANOVA. Percentages of larvae weight gain were analyzed with two-way ANOVA, with treatment and time of feeding as variables. Mortality data were subjected to arcsin transformation and analyzed with two-way ANOVA, with treatment and time of feeding as variables. 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 not different between bacterial treatments ($F_{3,12} = 1.43$, $P = 0.35$) (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

Table 1 Quantification of root colonization by *P. protegens* CHA0 and *P. chloraphis* PCL1391 in the presence or absence of *D. balteata* ($n = 4$)

Treatment	CFU millions/g of root (mean ± SEM)	
P. protegens CHA0 + *D. balteata*	57 ± 2	a
P. chloraphis PCL + *D. balteata*	130 ± 70	a
P. protegens CHA0	240 ± 170	a
P. chloraphis PCL	35 ± 6.5	a
Control healthy	0	

Root colonization was analyzed with one-way ANOVA. Same letters indicate not significant differences between treatments

CFU colony forming units

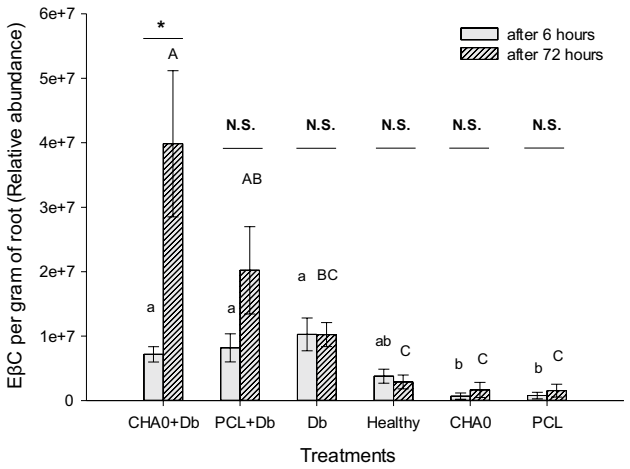

Fig. 1 Relative abundance of *E*βC (mean ± SE) released by maize roots *var.* Delprim after different treatments: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0 + Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL + Db), non-inoculated plants infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), ($n = 9$). Abundance of *E*βC was analyzed separately for each time-point with one-way ANOVA. Differences between means of the treatments were compared with Tukey tests ($P < 0.05$) and differences between time-points with a *t* test. Lower case letters indicate significant differences between treatments after 6 h of feeding. Capital letters indicate significant differences between treatments after 72 h of feeding. Stars indicate significant differences between time-points. *$P < 0.05$. *NS* no significance

post-attack) (Supplementary Fig. 1a and b). 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 h ($F_{5,42} = 9.12$, $P < 0.001$) and 72 h ($F_{5,42} = 10.8$, $P < 0.001$) of insect feeding (Fig. 1). After 6 h, there was no difference between *D. balteata*-damaged roots colonized by any of the bacteria species ($F_{5,42} = 9.12$, $P = 0.32$ and $F_{5,42} = 9.12$, $P = 0.38$) and non-colonized roots attacked by the insects. Yet, there was a difference in *E*βC quantities between non-colonized roots attacked by the insects and undamaged roots colonized by either of the bacterial species ($F_{5,42} = 9.12$, $P < 0.001$) (Fig. 1).

Seventy-two hours after *D. balteata* attack, roots colonized by *P. protegens* CHA0 produced significantly larger amounts of *E*βC ($F_{5,42} = 10.8$, $P = 0.001$) than non-colonized roots attacked by *D. balteata*, whereas roots colonized by *P. chlororaphis* PCL1391 produced similar ($F_{5,42} = 10.8$, $P = 0.22$) amounts of *E*βC as non-colonized roots attacked by the insects. Non-colonized roots attacked by *D. balteata* produced slightly higher amounts of *E*βC than undamaged roots colonized by either bacterium (Fig. 1). We found a significant higher production of *E*βC ($t = -2.86$, $P = 0.02$) after 72 h than after 6 h of insect damage in roots colonized by *P. protegens* CHA0. For the other five treatments, there were no differences between the two time points (Fig. 1).

Expression of the terpene synthase gene *tps*23 after *Diabrotica balteata* damage in maize roots colonized by *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391

The treatments also affected the expression of *tps*23 (after 6 h: $F_{5,32} = 4.41$, $P = 0.003$; after 72 h: $F_{5,23} = 18.32$, $P < 0.001$). After 6 h of insect feeding, the expression of the gene *tps*23 in roots colonized either by *P. protegens* CHA0 or *P. chlororaphis* PCL1391 and attacked by *D. balteata* was not different from non-colonized roots attacked by the insect ($F_{5,32} = 4.41$, $P = 0.83$ and $F_{5,32} = 4.41$, $P = 0.75$) (Fig. 2). We also found a significant higher expression of the gene ($F_{5,32} = 4.41$, $P = 0.001$) in non-colonized roots attacked by the insect as compared to healthy control roots (Fig. 2).

After 72 h of *D. balteata* attack, gene expression in insect-damaged roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 was significantly higher ($F_{5,23} = 18.32$, $P < 0.001$ and $F_{5,23} = 18.32$, $P = 0.001$) than in insect-damaged non-colonized roots (Fig. 2). The expression in the latter roots was not different from the expression in undamaged roots colonized by either one of the bacteria species ($F_{5,23} = 18.32$, $P = 0.99$ and $F_{5,23} = 18.32$, $P = 0.33$). Similarly to what we found for the release of *E*βC (Fig. 1), *tps*23 expression was significantly higher ($t = -3.94$, $P = 0.02$) after 72 h of insect attack than after 6 h in insect-damaged roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 (Fig. 2). In all of the other four treatments, gene expression was not statistically different between the two time-points.

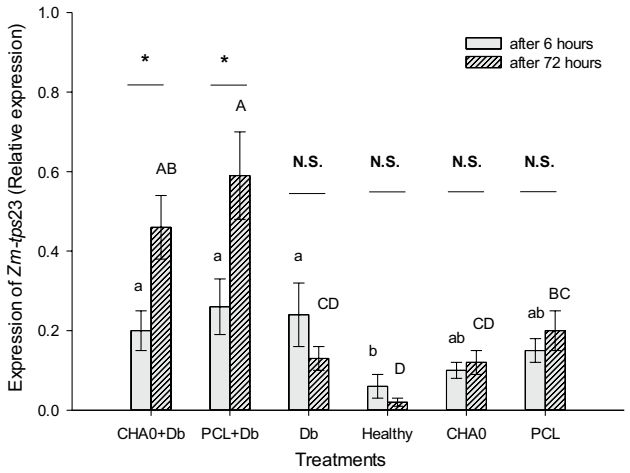

Fig. 2 Relative expression (calculated in relation to actin relative expression) of the terpene synthase gene Zm-*tps23* (mean ± SE) in maize roots *var.* Delprim after treatments: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0 + Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL + Db), non-inoculated plants infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), (n = 9). Expression of the *tps*23 gene was analyzed separately for each time-point with one-way ANOVA. Differences between means of the treatments were compared with Tukey tests ($P < 0.05$) and differences between time-points with a t test. Lower case letters indicate significant differences between treatments after 6 h of feeding. Capital letters indicate significant differences between treatments after 72 h of feeding. Stars indicate significant differences between time-points. *$P < 0.05$. *NS* no significance

Root colonization does not change root biomass

We did not find an effect of any of the treatments on root fresh weight ($F_{5,72} = 1.95$, $P = 0.09$), measured after the 72 h 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* PCL1391 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

Although no effect of treatment was found for larval weight gain ($F_{2,72} = 1.72$, $P = 0.18$), there was a trend towards increased weight gain when larvae were feeding on *P. protegens* CHA0 colonized roots than when feeding on *P. chlororaphis* PCL1391-colonized roots (Fig. 3b), which correlates with differences in root biomass (Fig. 3a). We found an overall increase in weight over time ($F_{2,72} = 8.59$, $P < 0.001$), but no significant interaction between time and treatment ($F_{4,72} = 0.72$, $P = 0.57$). In a preliminary experiment with maize plants inbred F268, we found a

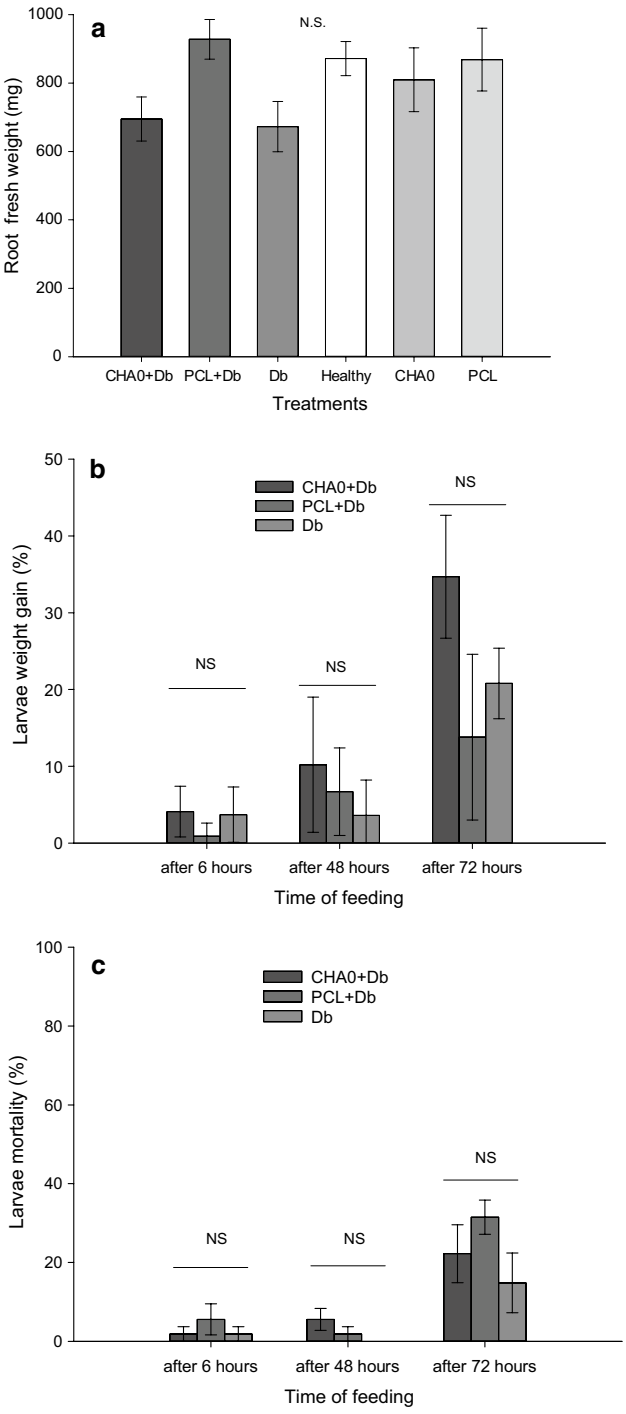

Fig. 3 **a** Root fresh weight (mean ± SE) of 14-days-old maize plants *var.* Delprim: inoculated with *P. protegens* CHA0 and infested with *D. balteata* (CHA0 + Db), inoculated with *P. chlororaphis* PCL1391 and infested with *D. balteata* (PCL + Db), non-inoculated plants infested with *D. balteata* (Db), control healthy plants, inoculated with *P. protegens* CHA0 (CHA0), and inoculated with *P. chlororaphis* PCL1391 (PCL), (n = 12). Root weight was analyzed with one-way ANOVA. **b** Weight gain (percentage, mean ± SE) of *D. balteata* larvae after 6, 48, and 72 h of feeding on maize roots *var*. Delprim with different treatments, (n = 9). Weight gain of larvae was analyzed with two-way ANOVA. **c** Percentage of mortality of *D. balteata* larvae after 6, 48 and 72 h of feeding on roots with different treatments, (n = 9). Mortality of larvae was analyzed with two-way ANOVA. Different letters show significant differences between treatments. *NS* no significance

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. 2). In this experiment, we detected a significant effect of time ($F_{2,95} = 7.09$, $P = 0.001$), but no obvious effect of the treatment ($F_{2,95} = 0.8$, $P = 0.44$), nor an interaction between time and treatment ($F_{4,95} = 0.23$, $P = 0.92$).

For the time-course 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 ($F_{2,72} = 2.03$, $P = 0.13$), nor an interaction between time and treatment ($F_{4,72} = 0.98$, $P = 0.41$) (Fig. 3c).

Discussion

We found quantitative but no qualitative differences in the volatiles produced by roots that were subjected to the different treatments. It should be emphasized that for practical reason we did not measure the actual release from the roots, but that we chose to measure root volatile content, which is a very good proxy for what the roots release (Hiltpold et al. 2011). Colonization by *P. protegens* CHA0 significantly enhanced the production of the sesquiterpene in maize after 72 h of *D. balteata* feeding (Fig. 1). Moreover, undamaged maize roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391 bacteria produced only minor quantities of the root volatile *E*βC (Fig. 1 and Supplementary Fig. 1a and b). To our knowledge, ours is the first study that evaluates how root-associated bacteria affect the production 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, but without insect damage. They found also 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. For example, *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) found 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. 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.

These differences may 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). 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.

Our findings on *E*βC emissions correlate nicely with the results for the expression of the terpene synthase gene *tps*23. In roots colonized by *P. protegens* CHA0 and *P. chlororaphis* PCL1391, the expression was enhanced after 72 h 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 *tps*23 in undamaged roots colonized by *P. chlororaphis* PCL1391 than in control healthy roots at the second time-point (72 h). This is again different from Pangesti et al. (2015a), who reported a negative effect of *P. fluorescens* colonization on the expression of the terpene synthase genes *tps*03 and *tps*04 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 *lox*2 (Pineda et al. 2012) and JA-responsive genes (van Oosten et al. 2008) and results in stronger JA-signaling after insect attack (Pangesti et al. 2015b). We also know that the gene Zm-*tps*23 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 min, resulting in an exponentially increasing production of *E*βC over 48 h 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 might be mediated by increased JA-signaling (Erb 2009; Hiltpold et al. 2011).

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* PCL1391 on the enhanced production of the root sesquiterne *E*βC and/or expression of the gene Zm-*tps*23 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 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). The latter authors 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.

In the study by Pineda et al. (2013), different volatiles were also produced in high quantities in plants colonized by *P. fluorescens* even without insect damage. It is also pertinent to note the possible role of additional volatiles and/or non-volatile secondary metabolites (Walker et al. 2012) that are produced upon bacteria root-colonization by maize plants or by bacteria themselves (D'Alessandro et al. 2014), all of which may affect the interactions with soil organisms. In the context of possible application, non-target effects on soils organisms need also to be evaluated (Ali et al. 2013). Indeed, numerous studies have shown that microbes can greatly affect the release of volatiles in plants (Ballhorn et al. 2013; Pineda et al. 2013; Pangesti et al. 2015a; Fontana et al. 2009).

We did not observe 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* PCL1391 (Fig. 3a). The relatively poor performance of the larvae on PLC-colonized plants may have contributed to this trend (Fig. 3b). Indeed, *D. balteata* larvae feeding on maize roots colonized by *P. protegens* CHA0 tended to gain relatively more weight than larvae feeding on roots colonized by *P. chlororaphis* PCL1391 after 72 h of feeding. Possibly, the increased production of *E*βC in roots colonized by *P. protegens* CHA0 stimulated feeding and/or benefitted *D. balteata* weight gain. This has previously 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 sharp contrast, in a different study, larvae of the generalist *D. speciosa* larvae gained less weight on and were 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 by feeding 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). Thus, it would be worthwhile to determine if PCL1391-colonization has an effect on these responses.

There were no significant differences in mortality among 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 higher larval mortality on *Pseudomonas*-colonized roots imply that the application of the bacteria in combination with EPNs may be an effective strategy for the control of root herbivores in maize crop. The compatibility of soil bacteria with a EPN 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. 2017). How the application of such combinations plays out against *Diabrotica* pests under realistic field condition remains to be determined.

Conclusions

Colonization of maize roots by *P. protegens* CHA0 was found to enhance the production of *E*βC after 72 h of feeding by *D. balteata* larvae. Consistent with this enhanced production of the EPN attractant, we found a higher expression of the terpene synthase gene Zm-*tps*23 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. 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. The application of beneficial pseudomonads bacteria and EPNs shows good promise (Imperiali et al. 2017), yet it remains to be tested if the observed effects of root-colonization by bacteria on *E*βC indeed enhances EPN attraction towards insect-damaged maize roots.

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Author contribution statement XCM, HG, TCJT and RC-H conceived the experiments, XCM and RC-H analyzed the data and wrote the first drafts of the paper, NI and GR provide technical assistance for microbiology techniques and GC–MS analysis, respectively. CK, MM and TCJT revised and edited the text.

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