ORIGINAL ARTICLE

Seed inoculation with beneficial rhizobacteria affects European corn borer (Lepidoptera: Pyralidae) oviposition on maize plants

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Abstract

Larvae of *Ostrinia nubilalis* (Hübner) cause significant damage to maize ears and reduce market value of fresh sweet corn. Females rely on volatile cues to locate and oviposit preferentially on maize plants. In addition, oviposition behavior of females is influenced by soil management practices as they usually lay more eggs on maize plants grown on conventional soil than on organic soils that harbor rich microbial diversity. Since some plant growth-promoting rhizobacteria (PGPR) are known to mediate plant health via suppression of soil pathogens and enhanced uptake of nutrients; we hypothesized that inoculation of maize seeds with PGPR will alter emission of maize volatile and reduce the attractiveness of plants to ovipositing *O. nubilalis*. Plants treated with the single PGPR strain *Bacillus pumilus* INR-7, two PGPR mixtures (Blend-8 or Blend-9) or untreated plants were presented to *O. nubilalis* females in oviposition choice bioassays. Headspace volatile organic compounds (VOCs) from the plants were analyzed by gas chromatography–mass spectrometry (GC–MS). *Ostrinia nubilalis* laid significantly fewer eggs on PGPR-treated plants compared to untreated plants. In two-choice oviposition experiments, significantly higher numbers of eggs were laid on untreated plants compared to PGPR-treated plants. PGPR-treated plants emitted fewer VOCs than untreated plants which, in part, explains the relatively fewer eggs on PGPR-treated plants. These results indicate that selected PGPR treatments can alter maize plant volatiles with important ramifications for plant-insect interactions. The implication of this finding is discussed in the context of integrated management of soil health to improve crop resistance to biotic stressors.

Key words: *Bacillus* spp., *Ostrinia nubilalis*, plant-insect interactions, soil health, volatile organic compounds.

INTRODUCTION

Plants maintain symbiotic relationship with soil dwelling microorganisms such as plant growth-promoting rhizobacteria (PGPR). PGPR use root exudates as a source of carbon, nitrogen and other required nutrients (Bais *et al.* 2006; Yuan *et al.* 2015) and in the process, elicit increased rates of plant growth and yield (Kloepper *et al.* 2004a). Some PGPR strains also increase the rate of nutrient uptake by plants, thereby helping to remove excess chemical fertilizers from agricultural soils (Adesemoye *et al.* 2008; Adesemoye *et al.*

2010; Calvo *et al.* 2013). Other ecological services provided by PGPR include suppression of soil pathogens (Kloepper & Schroth 1981; Bhattacharyya & Jha 2012) and induction of host plant resistance against foliar plant diseases (Jetiyanon & Kloepper 2002; Kloepper *et al.* 2004b; Ryu *et al.* 2005; Van der Ent *et al.* 2009; Liu *et al.* 2016) and herbivorous insects (Pineda *et al.* 2013; Santos *et al.* 2014). PGPR application has also been shown to alter levels and composition of secondary metabolites in plants (Zehnder *et al.* 1997; Zebelo *et al.* 2016).

Treatment of plant with some PGPR strains can affect insect behavior. Reduced levels of secondary metabolites that serve as phygostimulants may negatively impact feeding by specialist insect herbivores (e.g. Zehnder *et al.* 1997), whereas an increase of secondary metabolites may negatively impact generalist herbivores (e.g. Zebelo *et al.* 2016). Zehnder *et al.* (1997) showed that inoculation

of cucumber seeds or seedlings with PGPR strain *Bacillus pumilus* (Meyer and Gottheil) INR-7 reduced numbers of cucumber beetles (*Diabrotica undecimpunctata hawardi* Barber) on leaves. This reduction in beetle population correlated with a lower level of curcubitacin, a feeding stimulant, in inoculated plants. Zebelo *et al.* (2016) demonstrated that treatment with mixtures of PGPR strains (Blend-8 and Blend-9) elevated the level of gossypol in cotton leaves, thereby reducing the development of *Spodoptera exigua* (Hübner) larvae and pupae. Similarly, reduced development of whitefly (*Bemisia tabaci* Gennadius) on tomato plants treated with *Bacillus subtilis* (Ehrenberg) was reported by Valenzuela-Soto *et al.* (2010). Van Oosten *et al.* (2008) reported that inoculation of *Arabidopsis thaliana* (L.) Heynh. plants with *Pseudomonas fluorescens* (Migula) WCS417r had a negative effect on a generalist chewing herbivore, *S. exigua,* but did not affect the specialist herbivore, *Pieris rapae* (Linnaeus). Pangesti *et al.* (2015) also found that a generalist caterpillar, *Mamestra brassicae* (Linnaeus), weighed less upon feeding on roots of *A. thaliana* treated with *P. fluorescens* WCS417r, but there was no effect on the weight of the specialist *Pieris brassicae*. In contrast, *P. fluorescens* WCS417r treatment induced systemic susceptibility to phloem feeders (Pineda *et al.* 2012). Pineda *et al.* (2012) showed that treatment of *A. thaliana* with *P. fluorescens* WCS417r positively affected weight gain and the intrinsic rate of increase of the generalist aphid *Myzus persicae* (Sulzer), but had no effect on the crucifer aphid *Brevicoryne brassicae* (Linnaeus). Similar results were reported by Shavit *et al.* (2013) who found that *Bemisia tabaci* nymphs developed faster and had higher survivorship after they fed on tomato plants that were pre-inoculated with rhizobacteria. However, treatment of Calabrese (broccoli) with *Bacillus* species suppressed the growth and development of *B. brassicae* (Gadhave & Gange 2016). These studies suggest that the effects of PGPR treatment on insect pests can vary in relation to the feeding guild of insects. Overall, PGPR seem to negatively affect development of generalist insects, irrespective of the feeding guild.

It is not clear whether improved nutrient availability and plant health as a result of PGPR application affect VOC emission. Arbuscular mycorrhiza fungi (AMF)-treated plants often grow more and appear healthier than untreated plants (Gange *et al.* 2005). Previous studies indicate that some PGPR and AMF enhance nutrient use efficiency (Adesemoye *et al.* 2008) and increase plant uptake of nitrogen from soil (Gange *et al.* 2005; Adesemoye *et al.* 2009; Adesemoye *et al.* 2010; Calvo *et al.* 2013) or via suppression of soil pathogens (Kloepper & Schroth 1981; Bhattacharyya & Jha 2012). Therefore,

it may be reasonable to allude that a less stressed plant will be better prepared to mount defense against herbivores. However, studies showed that stressed plants are more likely to release volatiles (Turlings *et al.* 1998; Degen *et al.* 2004; Bruce *et al.* 2005). Some of these volatiles play direct defense role by deterring insect herbivory (Disi *et al.* 2017). Ballhorn *et al.* (2013) demonstrated that colonization of lima bean by rhizobia altered the composition of plant volatiles thereby deterring the Mexican bean beetle *Epilachna varivestis* (Mulsant) to rhizobia-treated plants. In contrast, Jallow *et al.* (2008) reported that the root fungal endophyte *Acremonium strictum* (W. Gams) reduced emission of volatiles by tomato plants which rendered plants more attractive to *Helicoverpa armigera* (Hübner) oviposition. These studies suggest that altered production of volatiles could be a mechanism by which beneficial microbes mediate plant-insect interactions.

In this study, we used European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), as insect model to investigate effect of PGPR treatment on insect oviposition. *Ostrinia nubilalis* is a polyphagous insect feeding on multiple plant hosts, including maize, in the United States. The larval stages feed on leaf whorls, burrow into maize stalks, and can cause significant yield loss by damaging growing seedlings and ears (Godfrey *et al.* 1991; Bohn *et al.* 1999). Due to demonstrated evidence that some PGPR strains negatively affect development of generalist insects, including chewing insects (Van Oosten *et al.* 2008; Pangesti *et al.* 2015; Zebelo *et al.* 2016) and phloem feeders (Valenzuela-Soto *et al.* 2010), we hypothesized that *O. nubilalis* will be deterred from laying eggs on PGPR-treated plants. *Ostrinia nubilalis* females can exploit plant volatiles and have been used as research model (Solé *et al.* 2010; Leppik & Frérot 2012; Molnár *et al.* 2015). In addition, previous studies showed that *O. nubilalis* females respond to differences in soil management practices (soil health); often preferring to oviposit on plants grown on conventional soils than those on organic soils (Phelan *et al.* 1995; Phelan *et al.* 1996). Fewer eggs have also been reported on arbuscular mycorrhizal colonized plants (Murrell *et al.* 2015). Hence, it is possible that the smaller number of eggs laid on plants raised on organic soils is a result of abundance of beneficial microorganisms prevalent in such systems.

The objectives of the study were to: (i) investigate the effect of the single PGPR strain *B. pumilus* INR-7 and mixtures of PGPR strains (i.e. Blend-8 and Blend-9) on oviposition preference of *O. nubilalis*; (ii) test whether inoculation of maize with PGPR alters emission and composition of VOCs in maize plants; and (iii) determine

whether PGPR inoculation affects feeding and survivorship of *O. nubilalis* larvae.

MATERIALS AND METHODS

PGPR and seeds

The PGPR strains were selected from a PGPR collection in the Department of Entomology and Plant Pathology, Auburn University, based on their capacity to promote growth of maize (Calvo *et al.* 2013) and to induce emission of VOCs in cotton (Kloepper *et al.* 2013). PGPR strains tested include *B. pumilus* strain INR-7, PGPR mixture Blend-8 containing *Bacillus velezensis* (Ruiz-García *et al.*) strain AP-188, *Bacillus mojavensis* (Roberts *et al.*) strain AP-209, *Fictibacillus solisalsi* (Glaeser *et al.*) strain AP-217, and *B. velezensis* strain AP-218; and PGPR mixture Blend-9 containing *B. velezensis* strain AP-136, *B. velezensis* strain AP-188, *B. velezensis* strain AP-219, and *B. velezensis* strain AP-295. PGPR preparation was carried out as described by Zhang *et al.* (2010). Bacterial strains from cold storage ($-80°C$) were streaked on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI, USA) and incubated at 28°C for two days. Bacteria were then mixed with sterilized water to a final concentration of –log 7.0 CFU/mL. To prepare the mixtures (blends), 2.0 mL of each of the strain was combined into a 50-mL sterile tube. This concentration was used for inoculating maize seeds at the time of planting.

Hybrid non-GMO maize seeds (Jacobsen 4704) were used for the study. One seed was placed 2.0 cm deep in a plastic pot (volume 307 cm^3) filled with Sunshine potting mix (SunGro Horticulture, Agawam, MA, USA). Seeds were then inoculated with 1 mL of PGPR spore suspensions ($-$log 7.0 CFU/mL/seed). One milliliter of water without PGPR was applied to the control. Twenty-five mL of water-soluble NPK fertilizer 20–10-20 (Buddies Plant Food, Ballinger, TX, USA) was applied once on the fifth day after planting (DAP) to both treated and control plants. Water was applied as needed every other day. All plants were maintained in growth chambers at $26 \pm 1°C$ and $60 \pm 5\%$ RH using daylight fluorescent tubes (270 µmol m^{-2} s^{-1}) with a light phase of 16 h. Twelve-day-old plants were used for collection of plant VOCs.

Insect

Ostrinia nubilalis pupae were purchased from Benzon Research, Carlisle, PA, USA. The pupae were kept in separate cages (25 cm × 15 cm) at $25 \pm 2°C$, $75 \pm 5\%$ RH and 14:10 h (L:D) photoperiod. The cages were monitored daily for adults to emerge. Males and females were separated by looking at the size of their wingspan, which was about 26–35 mm for females and about 20–26 mm for males. Also, females have pale yellowish coloration while males are usually small, pale brown or grayish brown in color. When the male is at rest, the abdomen usually extends beyond the hind wing. Male and female moths were reared in separate cages containing cotton wool soaked with 10% sucrose sugar solution until 48 h before they were paired in a single cage to encourage mating. At the end of 96 h, females were separated from males and were used for oviposition preference tests.

Oviposition bioassays

The treatments for this test were: (i) plants treated with INR-7; (ii) plants treated with Blend-8; (iii) plants treated with Blend-9; and (iv) control plants (untreated). Black cloth cages measuring $38 \times 38 \times 76$ cm high (no-choice) and $115 \times 115 \times 76$ cm high (choice) were used for oviposition bioassays. All oviposition tests were carried out during scotophase (from 18:00 to 07:00) at $25 \pm 2°C$. Used plants and insects were discarded each morning after counting eggs laid on plants and cages were then washed thoroughly with tap water. New set of plants and insects were used in every replicate.

No-choice oviposition bioassay: A no-choice test was used to evaluate the response of female *O. nubilalis* to plants treated with PGPR in a confined environment. Cages ($38 \times 38 \times 76$ cm high) on bench tops were spaced 60 cm apart in black room (room without lighting). For this experiment, pots containing individual plants (single treatment) were wrapped with aluminum foil to minimize odor from the plastic pot and soil. A single plant (pot) was then placed in the center of a black cloth cage at 16:00 h and left for 2 h before female *O. nubilalis* were released. Three females were released overnight (18:00 to 07:00) in the center of the cage containing the single plant. The numbers of eggs laid by *O. nubilalis* on plants were recorded the following morning. Each potted plant in each cage represented a replicate and a total of seven replicates were tested over seven consecutive days.

Dual-choice oviposition bioassay: The aim of this test was to determine whether *O. nubilalis* female can distinguish between PGPR-treated and untreated plants. In this test, PGPR treatments (INR-7, Blend-8 and Blend-9) were compared with the control. The experimental setup and handling of plants was as described above. Two plants, PGPR-treated and untreated control were spaced 80 cm apart within each cage at the opposite diagonals of the cage ($115 \times 115 \times 76$ cm high). Three *O. nubilalis* females were released overnight (18:00 to 07:00) in the center of the cage containing the pair of plants. The numbers of eggs laid on the plants were recorded the following morning. The experiment was

conducted over a period of six to eight days and the experiment was blocked by days (i.e. a minimum of six replicates).

Four-choice oviposition bioassay: The four-choice oviposition preference test was designed to mimic a natural environment where insects are exposed to several kinds of odor sources. It was designed to test whether *O. nubilalis* females can discriminate among control and plants treated with different strains/mixtures of PGPR. The experimental setup, conditions and treatments were like the dual-choice experiments. Plants were spaced 80 cm apart within each cage. Four mated *O. nubilalis* females were released overnight (18:00 to 07:00) in the center of black cloth cage containing the four plants. The following morning, insects were recovered and the numbers of eggs laid on the plants were counted and compared. This experiment was conducted over 13 days, and each group of four plants was considered a replicate.

Volatile collection and analysis

Standard protocols developed in our laboratory (Ngumbi *et al.* 2009; Zebelo *et al.* 2014) were used to collect headspace VOCs from maize plants ($n = 4$). An individual 12-day-old plant was placed in a 5 L headspace volatile collection chamber (ARS, Inc., Gainesville, FL, USA). Volatiles were collected for 24 h from each plant by pushing purified air at a rate of 350 mL min^{-1} over the plant in the jar (chamber) at $25 \pm 2°C$. Plants were provided an artificial light source with florescent light bulbs at the rate of 270 μmol m^{-2} s^{-1} under a 16 h photoperiod. Volatile compounds were trapped using 50 mg of Super-Q (Alltech Associates, Deerfield, IL, USA) adsorbent traps and eluted with 300 μl of methylene chloride solvent. The resulting extract was stored in a freezer ($-20°C$) until use. To analyze samples, one microliter of solvent extract was injected into a gas chromatography (Agilent Technologies, mod. 7890A, Santa Clara, CA, USA) mass spectrometry (Agilent technologies, mod. 5975C) as described by Morawo and Fadamiro (2014) with slight modifications. The sample was injected at an initial temperature of 40°C, held at 40°C for 4 min, and gradually increased by 5°C/min until 230°C for a total run time of 30 min. The injector and detector temperatures were maintained at 200°C. Mass spectra were obtained using electron impact (EI, 70 eV). Identification of compounds was done per their retention times and mass spectra in comparison with the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) 98 library (Fadamiro *et al.* 2010; Morawo & Fadamiro 2014). For the quantification, external calibration curve was made with standard solution of the commercially available synthetic compound linalool, purity 95% (Sigma Chemical Co., St. Louis, MO, USA).

Larval feeding bioassay

The treatments for this test were: (i) plants treated with INR-7; (ii) plants treated with Blend-8; (iii) plants treated with Blend-9; and (iv) control plants (untreated). Leaf tissues from plants 15 DAP were used for the feeding test. The inside of a 9-cm diameter Petri dish was lined with moist paper towel. Maize leaves (1-cm-long) were then transferred into individual Petri dishes. One second instar *O. nubilalis* was weighed and transferred with forceps into the Petri dish. Leaves were replaced daily with fresh ones to reduce the potential for opportunistic organisms that may favor larval mortality. Numbers of surviving larvae were recorded daily for a period of five days and weight of surviving larvae were taken on the last day of the experiment. There were 30 replicates per treatment.

Data analyses

All analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA). Oviposition data (number of eggs laid on plants) was overdispersed and did not conform to the assumptions of normality of distribution and equality of variance. Transformation is usually applied to manage data with these characteristics but transformation is not encouraged for count data as models are available to deal with such data (O'Hara & Kotze 2010). Here, we used negative binomial regression distribution with log link to analyze number of eggs laid on plants for the no-choice test. Treatment was modeled as fixed effect with day modeled as random effect. There were no significant block effects for no-choice and there were no interaction effects among replicates. To account for overdispersion and dependency in our study, oviposition data in the dual and four-choice tests were analyzed with generalized estimating equations (GEE). Repeated subject option and the correlation structure (exchangeable) in GEE accounts for interrelatedness of observations but assumes that correlations are constant over time. Data from control cohorts were used as base reference in all analyses. No transformation was necessary for the VOC data since the data met the assumptions of normality of distribution and equality of variance. ANOVA was used to examine the treatment effects on the amounts of VOCs emitted by plants and the larval weight. Mean difference was separated by Tukey–Kramer honestly significant difference (HSD). The VOC data was also subjected to principal component analysis (PCA) to allow for accurate separation of compounds by treatments. Hierarchical

cluster analysis was used to show similarity of VOC profile of PGPR treatments and those of control plants.

RESULTS

Effect of PGPR treatment on oviposition behavior of *O. nubilalis*

No-choice oviposition tests: The oviposition results showed that *O. nubilalis* females were less likely to lay eggs on plants treated with INR-7 and Blend-9 than on untreated control plants (Table 1; Fig. 1A). The data showed that *O. nubilalis* laid more eggs on the untreated control and Blend-8.

Dual-choice oviposition tests: For the two-choice oviposition tests in which each PGPR-treated plant was paired with untreated plant, significantly fewer eggs were laid by *O. nubilalis* on PGPR-treated plants than on untreated plants (Fig. 1B). Block (days) effect was observed for the comparison between PGPR and untreated plants as indicated by GEE results for comparisons involving INR-7 vs. untreated and Blend-9 *vs.* Untreated but such effect was minimal for Blend-8 *vs.* untreated (Table 2).

Four-choice oviposition tests: The results of the four-choice oviposition tests followed the general pattern recorded for the two-choice oviposition tests. Egg counts per plant showed that *O. nubilalis* was less likely to lay eggs on Blends 8- and 9-treated plants compared to INR-7 treated and untreated plants tests. Significantly fewer eggs were recorded on Blends 8- and 9-treated plants compared to INR-7 treated and untreated plants (Table 3; Fig. 1C).

Effect of PGPR treatment on VOC emission

The GC–MS analyses showed both qualitative and quantitative differences in the chemical profiles of maize plants treated with PGPR than untreated plants (Table 4). A total of six compounds were detected from all the treatments. Fewer compounds were detected in plants treated with PGPR compared to untreated plants: five compounds were detected in untreated plants, four in plants treated with Blend-9, three in plants treated with INR-7, and two in plants treated with Blend-8. Linalool was detected only in plants treated with INR-7, while 3-Hexen-1-ol was detected only in untreated plants (Table 4). α-Copaene was emitted in significantly higher amounts in untreated plants and those treated with Blend-9 than in plants treated with INR-7 or Blend-8 ($F_{3,11} = 6.28$; $P < 0.01$). (E)-5- Methyl-2-methylene-2-hexen-1-ol was emitted in significantly higher amounts in untreated plants than in any of the PGPR treatments ($F_{3, 11} = 991.02$; $P < 0.0001$).

Principal component analysis (PCA) was conducted to further visualize qualitative differences among treatments. PC1 accounted for 54.6% of the total variance, while PC2 accounted for 22.3% of the total variance (Fig. 2A). The score plot of principal components showed that PC1 had three compounds (Methyl-2-methylene-2-hexen-1-ol, Pentanal, Cyclosativene) while PC2 had two compounds (3-Hexen-1-ol and linalool) (Fig. 2A). The cluster analysis of the VOCs from different treatments showed that PGPR treatments were like one another than untreated control (Fig. 2B).

Effect of PGPR treatment on *O. nubilalis* larval development

Larvae generally grew larger as they aged for all treatments (Fig. 3A). The weight of larvae that were fed plants treated with PGPR was not significantly different from those fed untreated plants ($F_{3, 52} = 0.82$; $P = 0.49$). However, percentages of surviving larvae were higher for PGPR-treated plants on the last day of the experiment compared to untreated plants but the difference was not statistically significant (Fig. 3B).

DISCUSSION

In this study, *O. nubilalis* females differentiated between PGPR-treated and untreated maize plants, and laid significantly fewer eggs on PGPR-treated plants. However, conspecific larval weight was not affected by PGPR. These differential effects suggest that PGPR may affect conspecific adult and larval stages of insect herbivores differently. Negative effect (weight loss) of PGPR treatment on generalist caterpillars that were fed Bacillus- (Zebelo *et al.* 2016) or Pseudomonas-treated plants (Van Oosten *et al.* 2008; Pangesti *et al.* 2015) are well documented but effects of PGPR on adult

Table 1 Negative binomial distribution estimates showing the effects of treatment of maize seeds with PGPR (INR-7, Blend-8, and Blend-9) or untreated in no-choice oviposition by *Ostrinia nubilalis* on maize plants

Parameter	DF	Estimate	Se	Wald Chi-Square	$P >$ ChiSq
Intercept	1	3.98	0.61		0.0001
INR-7	1	−1.29	0.65	42.87	0.0487
Blend-8	1	0.31	0.65	3.88	0.6337
Blend-9	1	−1.18	0.64	0.23	0.0657
Untreated	0	0	0	3.39	.
Day	1	−0.13	0.12	.	0.2631
Dispersion	1	1.35	0.42	1.25	

Maximum likelihood parameter estimates (negative binomial distribution) was adopted to compare treatments with untreated control (Wald χ^2, $P \leq 0.05$).
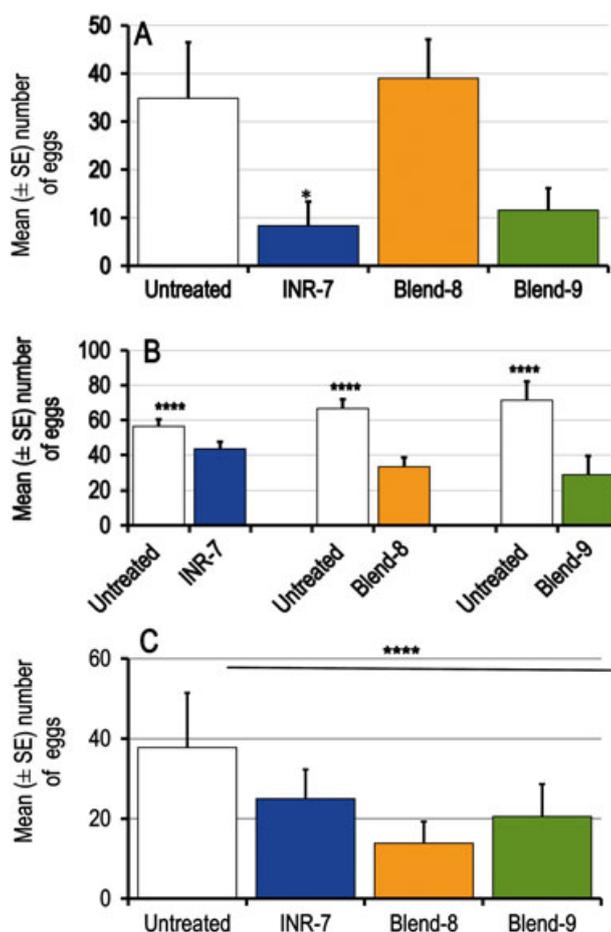
Figure 1 Number of eggs oviposited by *Ostrinia nubilalis* when offered maize plants treated with single PGPR strain (INR-7), mixture of PGPR strains (Blend-8 or Blend-9) or untreated plants. (A) Number of eggs laid overnight (12 h) in no-choice tests by *O. nubilalis* per plant; (B) number of eggs laid overnight (12 h) by *O. nubilalis* when offered a dual-choice between plants treated with PGPR (i.e. INR-7, Blend-8, and Blend-9) and untreated plants; (C) number of eggs laid overnight (12 h) by *O. nubilalis* in four-choice tests comprising plants treated with PGPR (INR-7, Blend-8 and Blend-9) and untreated plants. *indicates significant difference (Wald χ^2; $P \leq 0.05$); ****indicates significant difference (Z; $P \leq 0.0001$).

Table 2 Generalized estimating equation (GEE) (negative binomial distribution) estimates showing the effects of treatment of maize seeds with PGPR (INR-7, Blend-8, and Blend-9) or untreated in dual-choice oviposition by *Ostrinia nubilalis* on maize plants

Parameter	Estimate	Se	Z	$P > |Z|$	Parameter	Estimate	Se	Z	$P > |Z|$	Parameter	Estimate	Se	Z	$P > |Z|$
Intercept	3.25	0.06	72.13	<.0001	Intercept	2.94	0.05	103.15	<.0001	Intercept	2.39	0.23	100.68	<.0001
INR-7	−0.37	0.10	−45.54	<.0001	Blend-8	−0.70	0.01	−38.57	<.0001	Blend-9	−1.06	0.28	−22.45	<.0001
Untreated	0	0	.	.	Untreated	0	0	.	.	Untreated	0	0	.	.
Day1	0.25	0.02	2.61	0.009	Day1	0.03	0.10	0.66	0.5086	Day1	−0.40	0.26	−3.47	0.0005
Day 2	0.18	0.05	16.85	<.0001	Day 2	−0.09	0.01	−12.1	<.0001	Day 2	0.36	0.20	9.84	<.0001
Day 3	0.25	0.04	6.3	<.0001	Day 3	0.04	0.04	1.22	0.2219	Day 3	−0.19	0.11	−3.8	0.0001
Day 4	−0.82	0.02	−5.09	<.0001	Day 4	0.05	0.16	1.67	0.0945	Day 4	−0.34	0.07	−2.73	0.0064
Day 5	0.21	0.06	6.24	<.0001	Day 5	−0.00	0.03	−0.07	0.9439	Day 5	−0.90	0.17	−146.08	<.0001
Day 6	−0.34	0.01	−20.93	<.0001	Day 6	0	0.02	.	.	Day 6	0	0	.	.
Day 7	0.10	0.01	187.09	<.0001			0.00							
Day 8	0	0	.	.			0							

Generalized estimating equation (GEE) was adopted to compare PGPR treatment effects with untreated control (Z; $P \leq 0.0001$).

Table 3 Generalized estimating equation (GEE) (negative binomial distribution) estimates showing the effects of treatment of maize seeds with PGPR (INR-7, Blend-8, and Blend-9) or untreated in four-choice oviposition by *Ostrinia nubilalis* s on maize plants

Parameter	Estimate	Se	Z	$P > |Z|$
Intercept	1.84	0.37	4.93	<.0001
Blend-8	−0.55	0.14	−3.84	0.0001
Blend-9	−1.75	0.14	−12.31	<.0001
INR-7	−0.47	0.29	−1.6	0.1102
Untreated	0	0	.	.
Day1	−0.21	0.52	−0.4	0.6898
Day2	−0.02	0.31	−0.05	0.9595
Day3	0.10	0.64	0.16	0.8743
Day 4	−1.13	0.95	−1.19	0.2331
Day 5	0.64	0.66	0.97	0.3328
Day 6	−0.14	0.61	−0.22	0.8229
Day 7	−0.93	0.24	−3.83	0.0001
Day 8	−0.47	0.97	−0.48	0.6278
Day 9	0.35	0.69	0.5	0.6167
Day 10	−0.01	0.32	−0.02	0.9866
Day 11	−0.69	0.54	−1.28	0.2021
Day 12	−0.38	0.78	−0.49	0.6232
Day 13	0	0	.	.

Generalized estimating equation (GEE) was adopted to compare PGPR treatment effects with untreated control (Z; $P \leq 0.0001$).

Table 4 Headspace volatile organic compounds (VOCs) (ng g^{-1} FW) emitted by maize plants treated with PGPR (INR-7, Blend-8, and Blend-9) and untreated plants

VOCs	RT	Untreated	INR-7	Blend-8	Blend-9
(*E*)-5- Methyl-2-methylene-2-hexen-1-ol	4.9	1.0(0.0)a	0.3(0.0)b	0d	0.3(0.0)b
Pentanal	6.9	0.5(0.1)	0.4(0.1)	0.4(0.0)	0.4(0.1)
3-Hexen-1-ol	9.7	0.6(0.0)a	0b	0b	0b
Linalool	12.4	0b	0.2(0.0)a	0b	0b
Cyclosativene	19.9	1.6(0.4)	0	0.9(0.3)	1.8(0.8)
α-Copaene	20.0	0.7(0.2)ab	0b	0b	1.0(0.3)a

RT, retention time (min). Means (± SE) within the same row having different letters are significantly different (Tukey–Kramer HSD test, $P < 0.05$).

Lepidoptera species remain relatively unexplored. Analysis of headspace volatiles showed that PGPR treatment induced both qualitative and quantitative differences in the VOC profiles of maize plants, with fewer compounds detected in PGPR-treated plants. These results support our central hypothesis that treatment of seeds with PGPR will mediate host plant physiology and negatively affect the host location by herbivorous insects.

Incorporation of microbial inoculum can affect host preference of *O. nubilalis* on maize. Past studies showed that some speices of root colonizing bacteria can suppress harmful soil pathogens (Kloepper & Schroth 1981; Bhattacharyya & Jha 2012) which was believed to provide a healthy environment for plants to grow. In this study, we recorded significantly fewer eggs on PGPR-treated plants compared to untreated plants (Fig. 1), suggesting a potential role of PGPR in integrated management of *O. nubilalis* in maize production. This result corroborates a previous finding that a reduction in the amount of fertilizer in addition to arbuscular mycorrhizal deterred *O. nubilalis* from ovipositing on maize (Murrell *et al.* 2015). Host acceptance oviposition behavior of *O. nubilalis* is influenced by soil management practice (soil health) (Phelan *et al.* 1995; Phelan *et al.* 1996). Phelan *et al.* (1996) reported that *O. nubilalis* preferred to lay eggs on plants grown on conventional soil than those grown on organic soil that had higher organic matter/nutrient content that supports diverse microbial community. This result suggests that adding beneficial microorganisms to cultivated soils may offer another level of integrated pest management.

The qualitative variation in VOCs between plants treated with PGPR and untreated plants seems to explain the smaller number of eggs deposited by *O. nubilalis* on plants treated with PGPR reported in this study. Ratios of VOCs also play a major role in host plant location and recognition by insects (Cha *et al.* 2011; Molnár *et al.* 2015). Our GC–MS results showed differences in the VOC profiles of PGPR-treated and untreated plants, with methyl-2-methylene-2-hexen-1-ol significantly emitted in higher amounts by untreated plants (Table 4). In our study, a monoterpene, linalool was recorded only in plants treated with INR-7. The increased emission of linalool seems to explain the fewer eggs oviposited by *O. nubilalis* on plants treated with INR-7 but this effect contrasted with McCallum *et al.* (2011) who showed that transgenic tobacco plants expressing increased emission of (S)-linalool were attractive to *H. armigera*. Similarly, *Manduca sexta* (Linnaeus) oviposited preferentially on plants treated with different enantiomers of linalool (Reisenman *et al.* 2010). Trans/cis-3-Hexen-1-ol has been reported to attract insect herbivores (Wei & Kang 2011). In this study, 3-Hexen-1-ol (a green leaf volatile) was detected only in untreated control plants which corresponded with significantly higher numbers of eggs laid by *O. nubilalis*. GLVs are utilized by herbivorous insects to find their host plants (Bruce *et al.* 2005; Carroll *et al.* 2006; von Arx *et al.* 2011; von Mérey *et al.* 2013) and can serve as long-range host location cues by natural enemies (Girling *et al.* 2011; Peñaflor *et al.* 2011). It is not clear why plants treated with PGPR in this study did not produce detectable amounts of any GLVs.

Decreased emission of volatiles by PGPR-treated plants may have implications for plant-insect interactions. Plant
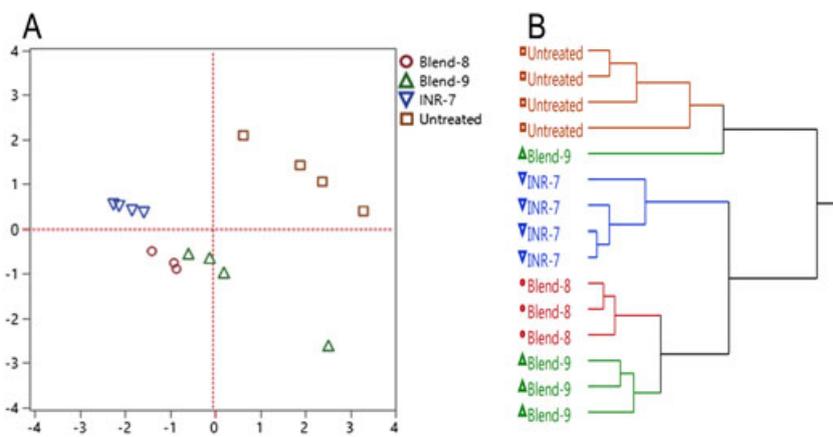
Figure 2 (A) Principal component analysis (PCA) score plot of VOCs of PGPR-treated plants or untreated plants. (B) Dendogram showing similarity in composition of VOC from PGPR-treated plants.
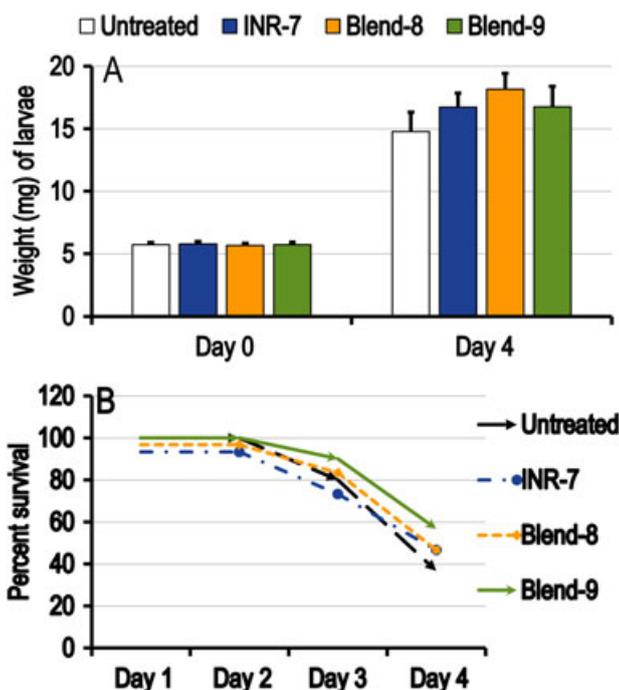

Figure 3 Feeding performance of *Ostrinia nubilalis* larvae. (A) Mean weight (± SE) of *O. nubilalis* larvae fed PGPR-treated maize plant; (B) percentage of surviving larvae after feeding on PGPR-treated versus untreated plants. (ANOVA; $P > 0.05$).

VOCs are stress-induced (insect feeding) (Turlings *et al.* 1998; Bruce *et al.* 2005) but induction by abiotic stressors has also been reported (Gouinguené & Turlings 2002). The question arises why PGPR-treated plants in this current study produced less VOCs or are less "smelly"? Reduction in VOC production by PGPR-treated maize plants in our study correlated with fewer eggs laid on plants, which contrasted with a report from a previous study. Jallow *et al.* (2008) reported that the fungal endophyte *Acremonium strictum* reduced emission of volatiles by tomato plants that rendered plants more attractive to *H. armigera* oviposition. Our result corroborates Ballhorn

et al. (2013) who demonstrated that rhizobia colonization of lima bean altered the composition and quantity of plant volatiles that deterred Mexican bean beetle to rhizobia-colonized plants. These studies, in addition to our current results, showed that altered production of volatiles could be a common mechanism by which PGPR mediate plant-insect interactions but effects of volatile may be plant species-specific. We suggest that addition of specific PGPR that improve soil health by reducing soil nitrate (Calvo *et al.* 2013) or increase nutrient availability to plants (Gange *et al.* 2005) may contribute to protection against herbivores such as ovipositing *O. nubilalis* females.

Ostrinia nubilalis larvae that were fed PGPR-treated plants had similar weights as those fed untreated plants (Fig. 3), suggesting no effect of PGPR treatment on larval development. This result deviates from previous studies that reported negative effects of PGPR on generalist caterpillars such as *S. exigua* (van Oosten *et al.* 2008; Zebelo *et al.* 2016) and *M. brassicae* caterpillars (Pangesti *et al.* 2015). Although *O. nubilalis* is a generalist, it does show strong preference for maize (Bethenod *et al.* 2005; Leppik & Frérot 2012). The feeding response of *O. nubilalis* larvae in this study is like those in previous studies where weight of specialist caterpillars, *P. rapae* (van Oosten *et al.* 2008) and *P. brassicae* (Pangesti *et al.* 2015), that were fed *A. thaliana*-treated with *P. fluorescens* WCS417r were not affected. The similarity between the feeding performance (weight gain) of *O. nubilalis* in our study and other specialist herbivores suggests that the Z strain of *O. nubilalis* used in this study may be a specialist on maize but has evolved a wide dietary breath to be able to switch host to survive changing environments.

This study showed that the tested PGPR strains mediate oviposition behavior of *O. nubilalis* on maize. The recorded qualitative and quantitative differences in VOC profiles of PGPR-treated plants versus untreated plants, particularly the fewer VOCs in PGPR-treated plants may explain the non-preference of PGPR-treated plants by *O. nubilalis*. However, other factors such as plant morphology, contact or visual cues may have also played a role in mediating the reduced oviposition on PGPR-treated plants. Leaf-surface sugars can stimulate oviposition and are used for host plant recognition and acceptance for oviposition by *O. nubilalis* (Derridj *et al.* 1986; Derridj *et al.* 1989), and this aspect should be investigated in future studies. Further studies should also be conducted to investigate the role of specific VOC compounds in the oviposition behavior of *O. nubilalis*.

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