Effect of Rhizobacteria on Induction of Volatile Organic Compounds and Consequences for Corn Herbivores and Tritrophic Interactions

by

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Abstract

Herbivorous insects depend on plants for survival. When herbivores feed on a plant the immobile plant responds directly by mobilizing it defenses, or indirectly by attracting natural enemies of the herbivore. Although plant defense is mostly constitutive, induced defense can provide an advantage to the plant. Insect oral secretions, plant volatiles and microbes, such as rhizobacteria, are common elicitors of induced systemic defense in plants. Rhizobacteria are plant activators with broad applicability that includes promotion of soil health, growth of plant and health. However, little information is available on how rhizobacteria mediate crop plant-insect and tritrophic interactions. This study used *Zea mays* (corn), its important herbivores (*Ostrinia nubilalis* and *Diabotrica virgifera virgifera*) and *D. v. virgifera* natural enemy, as model systems to examine the effects rhizobacteria have on the induction of plant defense. The goal of this dissertation is to understand how rhizobacteria and plant-derived elicitor mediate plant-insect and tritrophic interactions.

In chapter II, I investigated the role of rhizobacteria in mediation of oviposition of *O. nubilalis* in choice bioassays. Also, headspace volatile organic compounds (VOCs) from plants were analyzed by gas chromatography-mass spectrometry (GC-MS). *Ostrinia nubilalis* laid significantly fewer eggs on bacilli-treated plants compared to untreated plants. When a pair of bacilli-treated versus untreated plants were presented in two-choice oviposition experiments, significantly higher numbers of eggs were laid on untreated plants compared to bacilli-treated plants. Results showed that bacilli-treated plants emitted fewer VOCs than untreated plants, which in part, explain the relatively fewer numbers of eggs on bacilli-treated plants. These

results indicate that selected bacilli treatments can alter corn plant volatiles with important ramifications for plant-insect interactions.

In chapter III, I tested the hypothesis that application of bacilli as seed treatments to corn affects host-selection and feeding behavior of second instar larvae of *D. v. virgifera.* Corn, *Bacillus pumilus* strain INR-7 and *D. v. virgifera* second instar were used as a model system in horizontal olfactometer assays. Treatments for this study were *Bacillus pumilus* strain INR-7, two bacilli mixtures (Blend-8 or Blend-9) or untreated (control). Each bacteria treatment (plant) was compared with the untreated group (plant) in feeding preference studies. The result showed that *D. v. virgifera* larvae preferred untreated plants (76%) compared to plants treated with the *B. pumilus* strain INR-7 (24%). A follow up no-choice feeding test showed that *D. v. virgifera* larvae fed INR-7 treated plants weighed significantly less than larvae fed untreated plants or plants treated with bacilli blends. Overall, the results demonstrate that *B. pumilus* INR-7 can enhance resistance of corn against damage by *D. v. virgifera* larvae.

Following the result of chapter III where *B. pumilus* strain INR-7 showed promise as biological control agent against *D. v. virgifera* larvae, I conducted a study with natural enemies of *D. v. virgifera* larvae in chapter IV. The preference of *Heterorhabditis bacteriophora* to *D. v. virgifera*-infested corn roots was investigated using belowground four-choice olfactometer. Treatments for this study were *B. pumilus* strain INR-7, heat-killed INR-7 (HK), untreated or sand control. Intact plants were presented in four-choice olfactometer in the presence or absence of *D. v. virgifera* larvae in growth chambers. Furthermore, volatile root extract was sampled from plants with similar treatments above and effects were evaluated using four-choice olfactometer in a laboratory setting. The results showed that significantly higher numbers of *H. bacteriophora* choose *B. pumilus* strain INR-7 inoculated intact corn roots compared to untreated

plants or sand control both in the presence or absence of *D. v. virgifera* larvae. A further test of corn root VOC extract showed that a higher number of *H. bacteriophora* was recovered from the arm containing VOC from INR-7 treated plants without infestation but the number was not significantly different from other treatments. Also, *H. bacteriophora* recovered from the arm containing VOC from INR-7 treated plants that were infested with *D. v. virgifera* larvae was not statistically different from other treatments, suggesting that other factors than volatile cues may have affected *H. bacteriophora* choice to bacillus-treated plants. These studies have increased our understanding of the role of rhzobacteria in mediation of host plant indirect resistance.

In chapter V, I studied the molecular mechanisms of *cis*-Jasmone (CJ) in mediating changes in terpenoid genes and emission of VOCs. The role of VOC in oviposition behavior of insects was also tested. First, I quantified VOC and the transcript levels of key genes that encode VOC biosynthesis in CJ-treated plants with *Spodoptera exigua* caterpillar infestation (CJI), untreated plants with *S. exigua* caterpillar infestation (UI), CJ-treated plants without *S. exigua* caterpillar infestation (CJ), and untreated plants without *S. exigua* caterpillar infestation (U). In addition, oviposition preference of *S. exigua* was compared between CJI and UI, and between CJ and U. The result of GC-MS analyses showed qualitative and quantitative differences in CJI compared to UI, CJ or U. The result also showed that the transcript levels of certain terpene synthase genes involved in the biosynthesis of many VOC were higher in CJI plants. Consequently, *S. exigua* laid fewer numbers of eggs on CJI than UI. Moreover, in an *in vitro* oviposition choice test using filter paper, *S. exigua* laid significantly fewer eggs on filter papers containing VOC from CJI compared to UI. These results indicate that CJ treatment followed by caterpillar infestation can prime tomato plant defense with potential ramifications for insect oviposition.

In chapter VI, I provided a general conclusion that summed up the entire project. Opportunities for future research were identified.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Host Plant Resistance

Resistant crop variety is a sustainable means of controlling pests in agroecosystem. Resistant plants can alter the relationship between herbivores and their host plants with negative effects on herbivores while susceptible plants support pest abundance or are less likely to suppress herbivore activities (Duffey and Stout 1996). Host plant resistance can manifest in several forms namely: antibiosis, antixenosis or tolerance. Immobile plants utilize these mechanisms, especially non-preference mechanism to manipulate insect behavior by repelling herbivorous insects and/or luring “friends” that either feed on the herbivores or oviposit in them (Paré and Tumlinson 1999; Peñaflor et al. 2011; Tonelli et al. 2016). One main disadvantage of using resistant plant is the long period it takes to develop resistant plant variety. This disadvantage can be mitigated by using rhizobacteria to induce plant resistance. Beneficial rhizobacteria are ecological and environmentally friendly, inexpensive biological agent that promote plant growth as well as induce systemic resistance against biotic plant stressors.

1.2 Rhizobacteria-mediated Interactions

Rhizobacteria such as plant growth promoting rhizobacteria (PGPR) are microorganisms with main characteristics of elicitation of vigorous plant growth. The most well studied species include *Pseudomonas* and *Bacillus* (Raj et al. 2003; Kloepper et al. 2004a; Kloepper et al. 2004b; Shaharoona et al. 2006; Shaharoona et al. 2008; Huang et al. 2017). Arbuscular mycorrhizae also have growth promotion ability and its effect on plants are well characterized (Gange et al. 2005; Adesemoye et al. 2010). Certain *Bacillus* spp. (e.g., *B. pumilus* T4 and *B. amyloliquefaciens* IN937a) and the arbuscular mycorrhizae fungi (AMF), *Glomus interadices* have shown potential for large scale use in nutrient management in agriculture (Adesemoye et al. 2009). Increased levels of nitrogen in tissues of plants that previously received microbial inoculums such as AMF (Gange et al. 2005; Adesemoye et al. 2010) and *Bacillus* spp. (Adesemoye et al. 2010) have been reported. These increases of some nutrients in plant tissues suggest that beneficial microorganisms increase nutrient content of plants.

In addition to growth promotion, rhizobacteria mediate systemic resistance of plants against several plant diseases and phytophagous insects. This defensive characteristic of rhizobacteria suggests they are a potential tool to mitigate or reduce chemical pesticides in our environment. Bhattacharyya and Jha (2012) and Kloepper and Schroth (1981) reported suppression of soil pathogens by beneficial rhizobacteria that enhanced the performance of treated plants due to reduction of antagonistic pathogens effects. Several studies have reported that rhizobacteria induce systemic resistance against foliar plant diseases (Jetiyanon and Kloepper 2002; Kloepper et al. 2004b; Ryu et al. 2005; Van der Ent et al. 2009; Liu et al. 2016) and herbivorous insects (Zehnder et al. 1997; Van Oosten et al. 2008; Pineda et al. 2010; Santos et al. 2014; Zebelo et al. 2016; Coy et al. 2017). Also, rhizobacteria regulate plant defense by

inducing blends of complex plant volatiles that affect plant-insect communities: plant, plant-herbivores, natural enemies of herbivores in diverse ways (Schausberger et al. 2012; Pineda et al. 2013; Pangesti et al. 2015; Zebelo et al. 2016). Induction of non-volatile secondary metabolites and increased expression of defense enzymes are some suggested mechanisms by which rhizobacteria mediate plant defense but it is not known how rhizobacteria interact with above and belowground crop pests and the mechanisms involved. This knowledge might contribute to the incorporation of rhizobacteria in integrated pest management strategies and reduce reliance on chemical pesticides.

1.2.1 Rhizobacteria Interactions with Herbivores

Rhizobacteria-induced plant defenses 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 *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 bacilli 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 *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.) plants with *Pseudomonas fluorescens* (Migula) WCS417r had a negative effect on *S. exigua*, a generalist chewing herbivore but did not

affect the specialist herbivore, *Pieris rapae* (Linnaeus). Pangesti et al. (2015a) 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*, but had no effect on the crucifer aphid *Brevicoryne brassicae*. Similarly, Shavit et al. (2013) found that *B. 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 and Gange 2016). These studies suggest that the effects of rhizobacteria treatment on insect pests can vary in relation to the feeding guild of insects. Overall, rhizobacteria seem to negatively affect development of generalist insects, irrespective of the feeding guild.

1.2.2 Rhizobacteria Interactions with Natural Enemies

Rhizosphere-associated microbes alter chemical profiles of volatile organic compounds and influence the searching behavior of parasitoids and predators. While several plant volatiles may be passively emitted (constitutive), others are induced by insect-feeding injuries, elicitors or rhizosphere microbes (De Moraes et al. 2001; Guerrieri et al. 2004; Fontana et al. 2011; Oluwafemi et al. 2013; Kloepper et al. 2013; Zebelo et al. 2014) that prime distal parts of plants, making them ready to quickly respond to herbivory or by recruiting natural enemies of the attacking herbivores (indirect defense) (Girling et al., 2011; Erb et al., 2010). Previous studies showed that rhizobacteria mediate production of plants volatiles that can have consequences for

plant herbivores and their natural enemies (Guerrieri et al. 2004; Schausberger et al. 2012; Battaglia et al. 2013; Pangesti et al. 2015b). Pineda et al. (2013) showed that rhizobacterium *P. fluorescens* WCS417r with aphid infestation on *A. thaliana* Col-0 resulted in reduction in attraction of parasitoid, *Diaeretiella rapae,* to lay eggs in the aphid (*Myzus pericae*) and subsequent reduction in the performance of the parasitoid as determined by number of mummies. In contrast, Pangesti et al. (2015b) reported that treatment with rhizobacterium *P. fluorescens* WCS417r led to increased attraction of the parasitoid *Microlitis mediator* to caterpillar-infested plants. Interestingly, these studies on tritrophic interactions have been demonstrated for aboveground systems. There is a need to test whether root colonization by *Bacillus* species affect tritrophic interactions belowground.

1.3 *cis*-Jasmone as an Elicitor of Plant Defense

Induction of host plant defense by plant-derived semiochemicals is showing some promise in sustainable management of economically important pests. Prominent in this category is the natural plant elicitor, *cis*-Jasmone (CJ). CJ is a component of volatile organic compounds (VOCs) first identified in herbivore damaged cotton plants (Paré and Tumlinson 1999). CJ can prime or induce plant defenses in receiver plants through increased or primed production of plant metabolites (Pope et al. 1997; Bruce et al. 2003; Moraes et al. 2008; 2009; Matthes et al. 2010; Dewhirst et al. 2012; Delaney et al. 2013; Vieira et al. 2013). Birkett et al. (2000) showed that *Triticum aestivum* (L.), winter wheat plants pretreated with CJ repelled *Nasonovia ribisnigri* (Mosley), damson-hop aphid. Simultaneously, the CJ-pretreated wheat plants were attractive to natural enemies of aphids, *Coccinella septempunctata* (Linnaeus), seven-spot ladybird and *Aphidius ervi* (Haliday), aphid parasitoid. Bruce et al. (2003) reported that CJ treatment altered the composition of volatiles emitted by *A. thaliana*. This change in composition repelled the

generalist aphid, *Myzus persicae*, in an olfactometer bioassay but attracted *A. ervi*, a parasitoid of *M. persicae*.

In a related study, Oluwafemi et al. (2013) reported that CJ treatment by itself or CJ treatment without insect infestation did not induce VOC emission in maize or affect the response of the leafhopper, *Cicadulina storeyi* (China) in olfactometer bioassays. However, pre-treatment of maize plants with CJ followed by herbivore infestation increased the emission of defensive VOCs and repelled *C. storeyi* in the olfactometer. Other reported effects of CJ include an antixenosis effect against aphid feeding on CJ-treated cotton plants (Hegde et al. 2012), attraction and enhancement of stink bug egg parasitoids to induced signals from soybean (Moraes et al. 2009; Vieira et al. 2013), preference for the aphid parasitoid *A. ervi* to sweet pepper (Dewhirst et al. 2012), and arrestment effect on *A. ervi* foraging on CJ-treated *A. thaliana* (Matthes et al. 2010). Recently, Egger and Koschier (2014) reported that feeding damage by *Frankliniella occidentalis* (Pergande) was significantly reduced when CJ was applied on *Phaseolus vulgaris* (L.). These studies demonstrate the effect of CJ on xylem and phloem feeders and their natural enemies, but little is known about CJ priming effects on oviposition preference of lepidopteran pests. Oviposition preference is an important fitness index for insect species whose offspring survival is dependent on the mother's choice of suitable egg laying site (Gripenberg et al. 2010).

1.4 Model Systems

1.4.1 *Bacillus* spp.-corn Model

The ability of plants to maintain a symbiotic relationship with soil microorganisms such as rhizobacteria has potential for improving plant and soil health. Some rhizobacteria 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). Aside from the fact that these strains induce growth promotion, the bacilli form dormant spores, a characteristic that helps the strains survive inclement environments. They are also used as research model. This study uses selected strains that have been used in a biological control lab at the Department of Entomology and Plant Pathology, Auburn University to promote growth of corn (Calvo et al. 2013) and to induce emission of VOCs in cotton (Kloepper et al. 2013). The strains studied include *B. pumilus* strain INR-7, bacilli 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.*) stain AP-217, and *B. velezensis* strain AP-218; and bacilli 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). The interaction between these strains and hybrid non-GMO corn (Jacobsen 4704) was also investigated.

1.4.2 Herbivore Model Organisms for Rhizobacteria-mediated Interactions

This study uses the aboveground generalist herbivore *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) and the belowground specialist herbivore *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae) as model organisms. *Ostrinia nubilalis* is a polyphagous insect feeding on multiple plant hosts including corn in the United States. The larval stages feed on leaf whorls, borrow into maize stalks, and can cause significant yield loss by damaging growing seedlings and ears (Godfrey et al. 1991; Bohn et al. 1999). *Ostrinia nubilalis* is selected for the rhizobacteria-corn-herbivore interactions because it is a generalist pest and its interaction with corn is well documented. Also, there is evidence that some rhizobacteria strains negatively affect development of generalist insects, including chewing insects (Van Oosten et al. 2008,

Pangesti et al. 2015a, Zebelo et al. 2016) and phloem feeders (Valenzuela-Soto et al. 2010). Furthermore, *O. nubilalis* females exploit plant volatiles (Solé et al. 2010; Leppik & Frérot 2012; Molnár et al. 2015) and 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). Thus, improving plant and soil health by rhizobacteria application may potentially deter *O. nubilalis* from laying eggs on treated-plants.

Diabrotica virgifera virgifera is an important specialist insect pest of *Zea mays* (L.) in North America and Europe. Costs of control and yield loss are estimated at $1 billion per year in the United States (Metcalf 1986). Adult *D. v. virgifera* usually deposits eggs in the soil near corn plants. Larvae from overwintering sites exploit semiochemicals from corn roots (Bjostad and Hibbard 1992; Robert et al. 2012a; Erb et al. 2010) and feeding stimulants (Bernklau and Bjostad 2008; Robert et al. 2012b) to orient toward corn plants where they actively feed on roots, causing extensive root damage. *Diabrotica v. virgifera* beetles feed on leaves and silk of corn, but the larval stages are the most economically important life stages because injury from feeding on roots eventually causes the plant to lodge, thereby reducing yield of the crop (Urías-López and Meinke 2001). Much of the research on corn-belowground pest interactions has been done with neonate and second instar larvae life stages (Bernklau and Bjostad 2008; Robert et al. 2012a; Hiltpold and Hibbard 2016).

1.4.3 Tomato-*Spodoptera exigua* Model for CJ

Tomato-*S. exigua* system is a good model to investigate the role of elicitors in secondary metabolite-mediated plant-insect interactions since gene expression and volatile profiles of tomato plants damaged by chewing insects such as *S. exigua* are well documented (e.g., Zebelo et al. 2014). Furthermore, the larval stage of *S. exigua* feeds on multiple hosts including tomato

and has assumed a significant status as an agricultural pest in the Southeastern United States of America (Lange and Bronson 1981; Taylor and Riley 2008).

1.4.4 Natural Enemy Model

Infective juveniles of *Heterorhabditis bacteriophora* (Poinar) is the main parasitic model organism tested in this study. *Heterorhabditis bacteriophora* and *Heterorhabditis megidis* (Poinar et al.) have been extensively studied for their attraction to *D. v. virgifera* damaged corn root volatiles (Rasmann et al. 2005; Hiltpold et al. 2009; Hiltpold et al. 2011; Turlings et al. 2012).

1.5 Justification of the Study

The United States exports over one-third of the worlds' corn. Although corn production has increased over the years, price per bushel has also risen significantly hitting a record high of $8.40 in 2012 (http://www.macrotrends.net/2532/corn-prices-historical-chart-data) but has gradually dropped to $3.50 per bushel in 2016. Increased production cost due to bad weather, world demand for products from corn and expenditure on crop protection are some leading factors. *Diabrotica v. virgifera* and *O. nubilalis* are destructive pests that affect root and foliage of sweet and grain corns. These pests have evolved strategies to avoid toxic effects of insecticides that were once effective in managing them. For example, economic loss associated with *D. v. virgifera* infestations amount to about 1 billion dollars per year (Metcalf 1986). Corn farmers across the Corn Belt of the United States had relied on the transgenic corn with the Cry resistance gene since its first release in 1996 to control *D. v. virgifera* but report shows *D. v. virgifera* has developed resistance (Gassmann et al. 2011).

Growing concerns about insecticide resistance and negative environmental impacts of use of chemicals necessitate a search for a more sustainable pest control alternative. A biological control alternative to chemicals such as use of rhizobacteria is a promising and effective control measure. Some rhizobacteria application presents several advantages: (1) plant grows healthier because they utilize nutrients more efficiently (Adesemoye et al. 2009); (2) plants are protected against pests and disease stressors via induced systemic resistance (ISR) and priming of defense mechanisms (Murphy et al. 2000; Zehnder et al. 2001; Kloepper et al. 2004b). Not much has been reported on how combined bacilli strains affect plant-insect interactions compared to the effect of single bacteria (Zehnder et al., 1997; Pineda et al., 2013). Also, it is believed that unraveling how VOC induced by CJ and rhizobacteria-treated plants interact with most agriculturally important crops, especially cereals, might contribute to the incorporation of rhizobacteria in integrated pest management strategies and reduce over-reliance on chemical pesticides.

1.5.1 Dissertation Goal and Objectives

The goal of this dissertation is to understand how rhizobacteria and plant-derived elicitor mediate plant-insect and tritrophic interactions. The main objective is to characterize mechanisms of rhizobacteria and CJ-induced host plant resistance to develop sustainable management tactics against aboveground and subterranean herbivores of corn and tomato. Specific objectives are:

1. Investigate the effect and mechanism of rhizobacteria-mediated host plant preference by *O. nubilalis*
2. Test the effects of rhizobacteria on feeding and development of *D. v. virgifera* in corn

3. Evaluate the effects of rhizobacteria on attraction of a natural enemy of *D. v. virgifera*
4. Investigate molecular mechanisms of *cis*-Jasmone-mediated tomato VOC

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CHAPTER 2

SEED INOCULATION WITH BENEFICIAL RHIZOBACTERIA AFFECTS EUROPEAN CORN BORER (LEPIDOPTERA: PYRALIDAE) OVIPOSITION ON CORN

2.1 Introduction

Plants maintain symbiotic relationships with soil dwelling microorganisms such as plant growth-promoting rhizobacteria (PGPR). Rhizobacteria 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 rhizobacteria 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 and Schroth 1981; Bhattacharyya and Jha 2012) and induction of host plant resistance against foliar plant diseases (Jetiyanon and 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 rhizobacteria 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 rhizobacteria strain *Bacillus pumilus* INR-7 reduced numbers of cucumber beetles (*Diabrotica undecimpunctata hawardi* 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 *S. exigua* larvae and pupae. Similarly, reduced development of whitefly (*Bemisia tabaci* on tomato plants treated with *Bacillus subtilis* was reported by Valenzuela-Soto *et al*. (2010). Van Oosten *et al*. (2008) reported that inoculation of *Arabidopsis thaliana* (L.) plants with *Pseudomonas fluorescens* WCS417r had a negative effect on a generalist chewing herbivore, *S. exigua* but did not affect the specialist herbivore, *Pieris rapae*. Pangesti et al. (2015) also found that a generalist caterpillar, *Mamestra brassicae* 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*, but had no effect on the crucifer aphid *Brevicoryne brassicae*. 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 and Gange 2016). These studies suggest that the effects of rhizobacteria treatment on insect pests can vary in relation to the feeding guild of insects. Overall, rhizobacteria 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 rhizobacteria 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 and Schroth 1981; Bhattacharyya and 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 Mexican bean beetle, *Epilachna varivestis* to rhizobia-treated plants. In contrast, Jallow et al. (2008) reported that root fungal endophyte, *Acremonium strictum* reduced emission of volatiles by tomato plants which rendered plants more attractive to *Helicoverpa armigera* oviposition. These studies suggest that altered production of volatiles could be a mechanism by which beneficial microbes mediate plant-insect interactions.

In this study, I used European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) as insect model to investigate effect of bacilli treatment on insect oviposition. *Ostrinia*

nubilalis is a polyphagous insect feeding on multiple plant hosts including corn in the United States. The larval stages feed on leaf whorls, borrow into corn 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 bacilli-treated plants. *Ostrinia nubilalis* females can exploit plant volatiles and have been used as research model (Solé et al. 2010; Leppik and 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 fewer number of eggs laid on plants raised on organic soils is a result of abundance of beneficial microorganisms prevalent in such system.

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

2.2 Materials and Methods

2.2.1 Rhizobacteria and Seeds. Bacilli strains were selected from a rhizobacteria collection in the Department of Entomology and Plant Pathology, Auburn University based on

their capacity to promote growth of corn (Calvo et al. 2013) and to induce emission of VOCs in cotton (Kloepper et al. 2013). Bacilli strains tested include *B. pumilus* strain INR-7, bacilli 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 bacilli 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^{o}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 strains was combined into a 50-ml sterile tube. This concentration was used for inoculating corn seeds at the time of planting.

Hybrid non-GMO corn 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, Washington). Seeds were then inoculated with 1 ml of PGPR spore suspensions (-log 7.0 CFU/ml/seed). One milliliter of water without bacteria 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 ± 1°C and 60 ± 5% RH using daylight fluorescent tubes (270 μmol m^{-l} s^{-s}) with a light phase of 16 h. Twelve-day-old plants were used for collection of plant VOCs.

2.2.2 Insect. *Ostrinia nubilalis* pupae were purchased from Benzon Research, PA, USA. The pupae were kept in separate cages (25 cm × 15 cm) at 25 ± 2^{o}C, 75 ± 5% RH and

14:10 h (L: D) photoperiod. The cages were monitored daily for adults that will 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 hours before they were paired in a single cage to encourage mating. At the end of 96 hours, females were separated from males and were used for oviposition preference tests.

2.2.3 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 × 38 × 76 cm high (no-choice) and 115 × 115 × 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 ± 2^{o}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 bacilli in a confined environment. Cages (38 × 38 × 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 hours and left for two hours 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 there was a total of seven replicates tested over seven consecutive days.

Dual-choice oviposition bioassay: The aim of this test was to determine whether *O. nubilalis* female can distinguish between bacilli-treated and untreated plants. In this test, bacilli 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, bacilli-treated and untreated control were spaced 80 cm apart within each cage at the opposite diagonals of the cage (115 × 115 × 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 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 bacilli. 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 (13 replicates), and each group of four plants was considered a replicate.

2.2.4 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 corn plants (n=4). An individual 12-day-old plant was placed in a 5-liter headspace volatile collection

chamber (ARS, Inc., FL). 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 ± 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) 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) coupled with 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, Gaithersburg, Maryland (NIST) 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, Missouri).

2.2.5 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. Corn leaves (one-centimeter-long) were then transferred into individual Petri dish. One 2nd 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.

2.2.6 Data Analyses. All analyses were performed in SAS 9.4 (SAS Institute USA). Oviposition data (number of eggs laid on plants) was over-dispersed 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 and 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 modelled as fixed effect with blocks (replicates) modelled 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 bacilli treatments and those of control plants.

2.3 Results

2.3.1 Effect of Bacilli 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; Figure 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 bacilli-treated plant was paired with untreated plant, significantly fewer eggs were laid by *O. nubilalis* on bacilli-treated plants than on untreated plants (Figure 1B). Block (days) effect was observed for the comparison between bacilli 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; Figure 1C).

2.3.2 Effect of Bacilli Treatment on VOC Emission. The GC-MS analyses showed both qualitative and quantitative differences in the chemical profiles of corn plants treated with bacilli than untreated plants (Table 4). A total of six compounds were detected from all the treatments. Fewer compounds were detected in plants treated with bacilli 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 plants treated with Blend-9 than in plants treated with INR-7 or Blend-8 ($F_{3, 11}$ = 6.28; P < 0.01) but the amount in untreated plants compared to INR-7 and Blend-8 treated plants. (*E*)-5- Methyl-2-methylene-2-hexen-1-ol was emitted in significantly higher amounts in untreated plants than in any of the bacilli treatments ($F_{3, 11}$ = 991.02; P < 0.0001).

Principal component analysis (PCA) was conducted to further visualize qualitative differences among treatments. PC 1 accounted for 54.6% of the total variance, while PC 2 accounted for 22.3% of the total variance (Figure 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) (Figure 2A). The cluster analysis of the VOCs from different treatments showed that PGPR treatments were like one another than untreated control (Figure 2B).

2.3.3 Effect of Bacilli 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 bacilli 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 bacilli-treated plants on the last day of the experiment compared to untreated plants but the difference was not statistically significant (Figure 3B).

2.4 Discussion

In this study, *Ostrinia nubilalis* females differentiated between bacilli-treated and untreated corn plants, and laid significantly fewer eggs on bacilli-treated plants. However,

conspecific larval weight was not affected by bacilli. These differential effects suggest that PGPR may affect conspecific adult and larval stages of insect herbivores differently. Negative effect (weight loss) of rhizobacteria 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 rhizobacteria on adult Lepidoptera species remain relatively unexplored. Analysis of headspace volatiles showed that bacilli treatment induced both qualitative and quantitative differences in the VOC profiles of corn plants, with fewer compounds detected in bacilli-treated plants. These results support our central hypothesis that treatment of seeds with rhizobacteria 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 corn. Past studies showed that some root colonizing bacteria can suppress harmful soil pathogens (Kloepper and Schroth 1981; Bhattacharyya and Jha 2012) which was believed to provide a healthy environment for plants to grow. In this study, we recorded significantly fewer eggs on bacilli-treated plants compared to untreated plants (Figure 1), suggesting a potential role of rhizobacteria in integrated management of *O. nubilalis* in corn 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 corn (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 rhizobacteria and untreated plants seems to explain the fewer numbers of eggs deposited by *O. nubilalis* on plants. 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 bacilli-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* 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 and Kang et al. 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 bacilli-treated plants may have implications for plant-insect interactions. Plant VOCs are stress-induced (insect feeding) (Turlings et al. 1998; Bruce et al. 2005) but induction by abiotic stressors has also been reported (Gouinguene´ and Turlings,

2002). The question arises why bacilli-treated plants in this current study produced less VOCs or are less "smelly"? Reduction in VOC production by bacilli-treated corn 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 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 rhizobacteria mediate plant-insect interactions but effects of volatile may be plant species-specific. We suggest that addition of specific rhizobacteria 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 bacilli-treated plants had similar weights as those fed untreated plants (Figure 3), suggesting no effect of bacilli treatment on larval development. This result deviates from previous studies that reported negative effects of rhizobacteria 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 corn (Bethenod et al. 2005; Leppik and 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 corn but has evolved a wide dietary breadth to be able to switch host to survive changing environments.

This study showed that the tested bacilli strains mediate oviposition behavior of *O. nubilalis* on corn. The recorded qualitative and quantitative differences in VOC profiles of bacilli-treated plants versus untreated plants, particularly the fewer VOCs in bacilli-treated plants may explain the non-preference of bacilli-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 bacilli-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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Table 1 Effects of treatment of corn seeds with bacilli (INR-7, Blend-8, and Blend-9) or untreated in no-choice oviposition by *O. nubilalis* on plants.

Parameter	DF	Estimate	Se	Wald Chi-Square	Pr > 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, $P \leq 0.05$)

Table 2 Effects of treatment of corn seeds with bacilli (INR-7, Blend-8, and Blend-9) or untreated in dual-choice oviposition by *O. nubilalis* on plants.

Parameter	Estimate	Se	Z	Pr > |Z|	Parameter	Estimate	Se	Z	Pr > |Z|	Parameter	Estimate	Se	Z	Pr > |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							

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

Table 3 Effects of treatment of corn seeds with bacilli (INR-7, Blend-8, and Blend-9) or untreated in four-choice oviposition by *O. nubilalis* on plants.

Parameter	Estimate	Se	Z	Pr > |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 equations (GEE) was adopted to compare bacilli treatment effects with untreated control (Z; $P \leq 0.0001$).

Table 4 Headspace volatile organic compounds (VOCs) (ng g^{-1} FW) emitted by plants treated with bacilli (INR-7, Blend-8, and Blend-9) and untreated plants. Means (± SE) within the same row having different letters are significantly different (Tukey-Kramer HSD test, $P < 0.05$).

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 (mins).

Figure legend

Figure 1. Number of eggs oviposited by *Ostrinia nubilalis* when offered corn plants treated with single *Bacillus* strain (INR-7), mixture of bacilli 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. * indicates significant difference among treatments (Wald χ^2; $P \leq 0.05$); (b) Number of eggs laid overnight (12 h) by *O. nubilalis* when offered a dual-choice between plants treated with bacilli (i.e., INR-7, Blend-8, and Blend-9) and untreated plants. **** indicates significant difference (Z; $P \leq 0.0001$); (c) Figure shows number of eggs laid overnight (12 h) by *O. nubilalis* in four-choice tests comprising plants treated with bacilli (INR-7, Blend-8 and Blend-9) or untreated plants ($P \leq 0.0001$).

Figure 2. (A) Dendrogram showing similarity in composition of VOC from bacilli-treated plants. (B) Principal component analysis (PCA) score plot of VOCs of bacilli-treated plants or untreated plants.

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

Figure 1

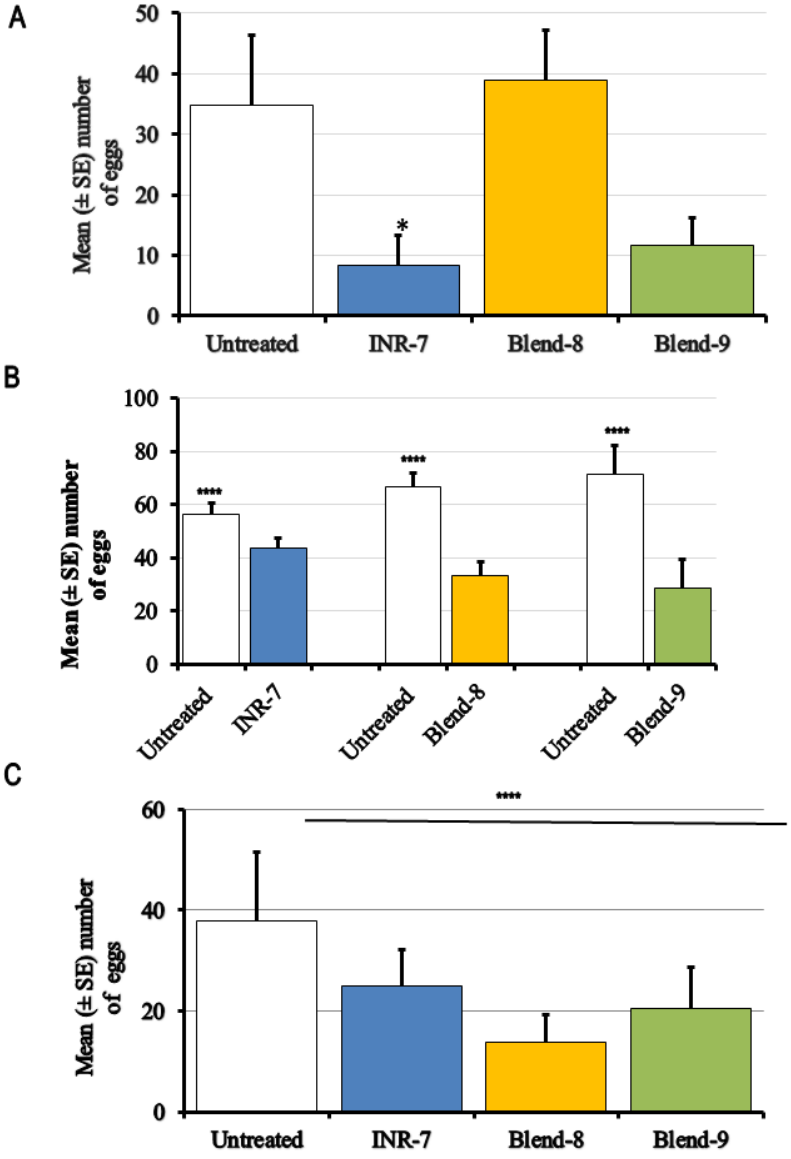

A
Mean (± SE) number of eggs
50
40
30
20
10
0
*
Untreated
INR-7
Blend-8
Blend-9
B
Mean (± SE) number of eggs
100
80
60
40
20
0
****
****
****
Untreated
INR-7
Untreated
Blend-8
Untreated
Blend-9
C
Mean (± SE) number of eggs
60
40
20
0
****
Untreated
INR-7
Blend-8
Blend-9

Figure 2

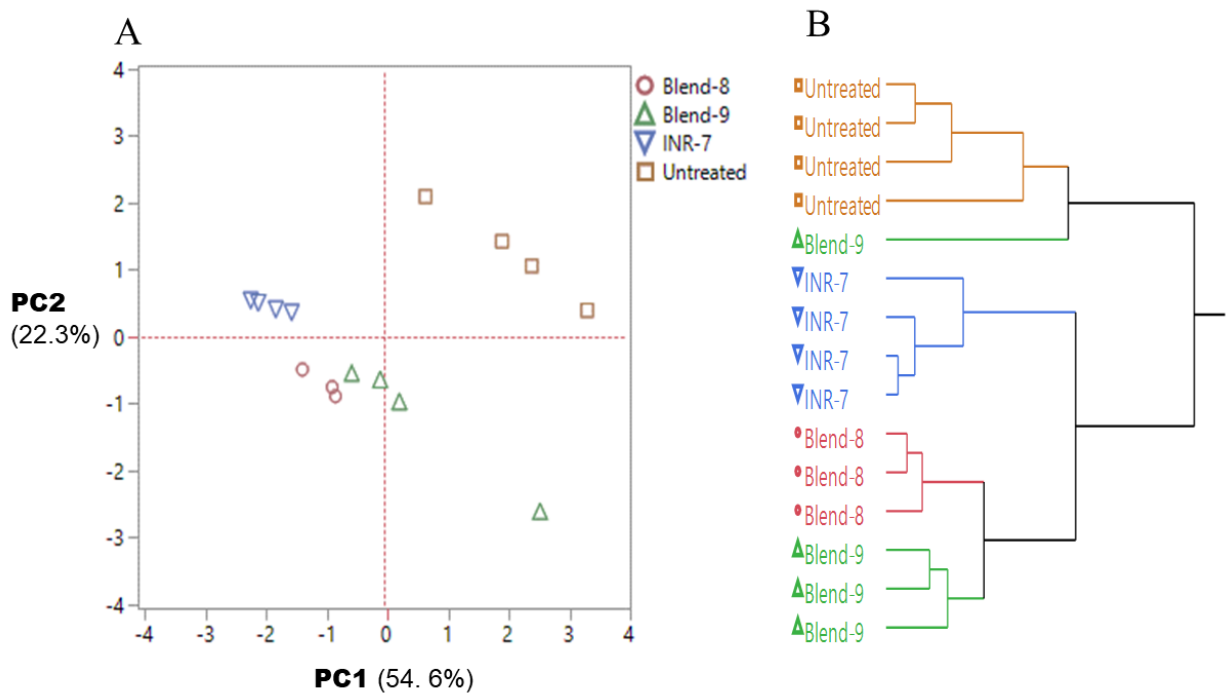
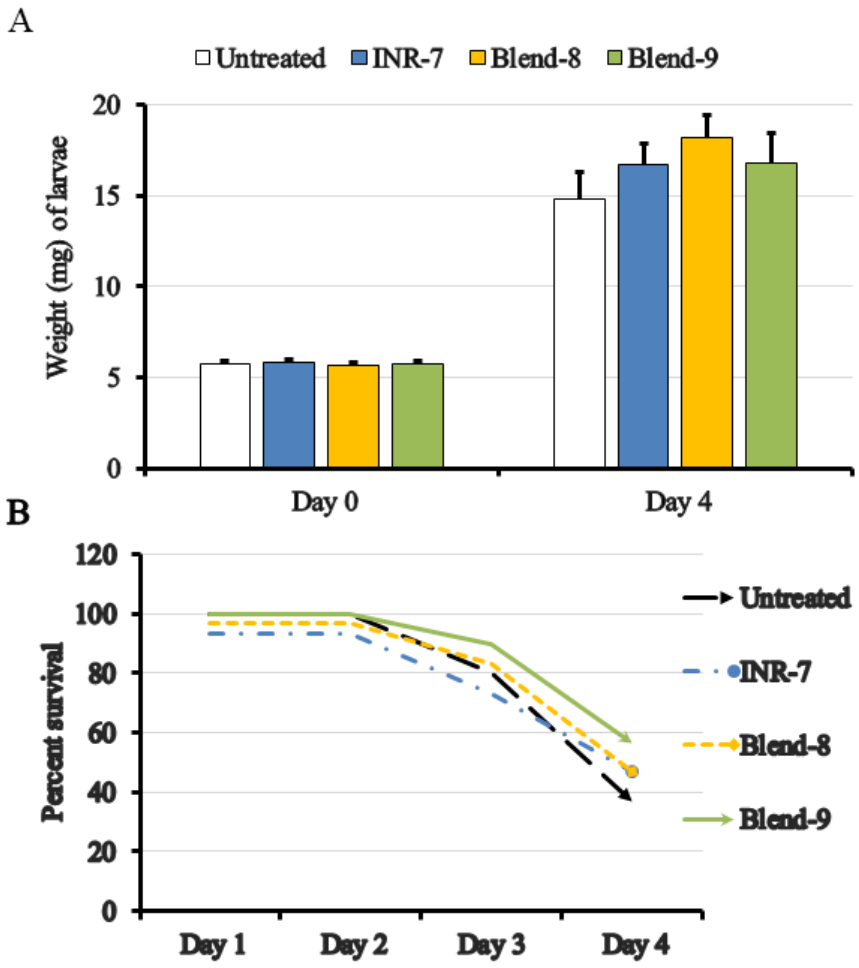

A
B
Blend-8
Blend-9
INR-7
Untreated
PC2
(22.3%)
PC1 (54. 6%)
Untreated
Untreated
Untreated
Untreated
Blend-9
INR-7
INR-7
INR-7
INR-7
Blend-8
Blend-8
Blend-8
Blend-9
Blend-9
Blend-9

Figure 3


A
Untreated
INR-7
Blend-8
Blend-9
Weight (mg) of larvae
20
15
10
5
0
Day 0
Day 4
B
Percent survival
120
100
80
60
40
20
0
Day 1
Day 2
Day 3
Day 4
Untreated
INR-7
Blend-8
Blend-9

CHAPTER 3

SEED TREATMENT OF CORN WITH *BACILLUS PUMILUS* STRAIN INR-7 AFFECTS HOST LOCATION AND FEEDING BY WESTERN CORN ROOTWORM, *DIABROTICA VIRGIFERA VIRGIFERA*

3.1 Introduction

The structural and spatial differences in terrestrial plants ensure that plants function effectively in their environment. Plant roots interact with soil and rhizosphere microorganisms, including plant growth-promoting rhizobacteria (PGPR). PGPR elicit plant growth, and some also suppress soil pathogens (Kloepper and Schroth 1981; Bhattacharyya and Jha 2012). Some PGPR strains also induce host plant resistance against foliar plant diseases and herbivorous insects (Zehnder et al. 1997; Jetiyanon and Kloepper 2002; Kloepper et al. 2004a; Ryu et al. 2007; Van der Ent et al. 2009; Noumavo et al. 2013; Pineda et al. 2012; Liu et al. 2016).

Studies have shown that the efficacy of rhizobacteria as biological control agents against foliar pathogens is enhanced by mixing individual strains (Raupach and Kloepper 1998; Jetiyanon and Kloepper 2002; Kloepper et al. 2004b; Kloepper et al. 2007; Liu et al. 2016). Similarly, several reports demonstrated that PGPR can affect phylloplane-feeding insects. Saravanakumar et al. (2008) showed that treatment of rice plants with a combination of three *Pseudomonas fluorescens* strains (Pf1, TDK1, and PY15) led to higher activity of polyphenol

oxidase (PPO) and lipoxygenase (LOX) compared to plants treated with individual strains, chemical or untreated controls. This increase in PPO and LOX correlated with malformation of adult leaf folder, *Cnaphalocrocis medinalis* in rice plant (Saravanakumar et al. 2008). Kloepper et al. (2013) reported that cotton plants treated with bacilli blends (e.g., Blend-9) emitted higher amounts of plant volatiles following *Heliothis virescens* larvae infestation. A recent study by Gadhave and Gange (2016) showed that both individual strains of the tested *Bacillus* spp. (i.e., *Bacillus cereus, Bacillus subtilis,* and *Bacillus amyloliquefaciens*) and their mixtures suppressed growth and development of *Brevicoryne brassicae*. In a similar study, Gange et al. (2005) showed that arbuscular mycorrhiza fungi (AMF) increased nutrient content of *Leucanthemum vulgare,* as well as enhanced the infestation levels of seed-feeding insects. Despite the demonstrated roles of rhizobacteria in eliciting plant growth and defense against foliar insects, it is still not known if *Bacillus* species negatively affects soil-dwelling herbivores. Previous study showed that inoculation of soil with AMF reduced larval growth of *Otiorhynchus sulcatus* on *Taraxacum officinale* (Gange et al. 1994). Similarly, root colonization of *Asclepias* species affected the survivorship of larvae of *Bradysia* species (Vannette and Rasmann 2012).

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is an important specialist insect pest of *Zea mays* in North America and Europe. Costs of control and yield loss are estimated at $1 billion per year in the United States (Metcalf 1986). Adult *D. v. virgifera* usually deposits eggs in the soil near maize plants. Larvae from overwintering sites exploit semiochemicals from maize roots (Bjostad and Hibbard 1992; Robert et al. 2012a; Erb et al. 2013) and feeding stimulants (Bernklau and Bjostad 2008; Robert et al. 2012b) to orient toward maize plants where they actively feed on roots, causing extensive root damage. Adult WCR feed on leaves and silk of maize, but the larval stages are the most

economically important life stages because injury from feeding on roots eventually causes the plant to lodge, thereby reducing yield of the crop (Urías-López and Meinke 2001). Much of the research on maize-belowground pest interactions has been done with both neonate and second instar larvae life stages (Bernklau and Bjostad 2008; Robert et al. 2012a; Hiltpold and Hibbard 2016). However, little is known about how beneficial rhizobacteria affect the foraging behavior of WCR second instar larvae, the target of parasitic nematodes. Such investigation is important for developing alternative management strategy such as biological control for the pest.

Control of WCR is difficult under the current corn production system in the US and Europe. Commonly-used tactics to manage WCR include behavioral modification (adults) (Chandler 2003), chemical insecticides (Meinke et al. 1998), and application of *Bacillus thuringiensis* (*Bt*) (Moellenbeck et al. 2001; Vaughn et al. 2005). However, these approaches are not sustainable because WCR has evolved several resistance mechanisms including avoidance to crop rotation (adults) (Gray et al. 2009), and rapid detoxification of several insecticides (Parimi et al. 2006) and *Bt* CRY protein (Meihls et al. 2008; Gassmann et al. 2011). Currently, there are no known alternative control tactics for managing WCR, and all commercially available maize cultivars are susceptible to feeding injury by the larvae.

This study tested the effects of selected *Bacillus* species single strain and mixtures on host location behavior and feeding of *D. v. virgifera*. The objectives of the study were to: i) test the capacity of selected bacilli treatments to promote growth of maize plants; and ii) test if inoculation of maize with the same bacilli affects host preference and feeding of *D. v. virgifera* larvae. The biological control effect of *B. pumilus* strain INR-7 against foliar pathogens and insects is well established (Zehnder et al. 1997; Raupach and Kloepper 1998; Domenech et al. 2006). Some bacilli mixtures (Blend-8, and Blend-9) were recently demonstrated to have a

negative effect on *Spodoptera exigua* in cotton (Zebelo et al. 2016). In addition, seven of the individual strains that constitute Blends 8 and 9, including AP-136, AP-188, AP-209, AP-217, AP-218, AP-219, and AP-295 were reported to induce systemic resistance against black rot disease in Chinese cabbage, caused by the bacterial pathogen *Xanthomonas campestris* (Liu et al. 2016). We hypothesized that bacilli applied as seed treatments to maize affects host-selection and feeding behavior of second instar larvae of *D. v. virgifera.*

3.2 Materials and Methods

3.2.1 Preparation of Bacilli Strains and Insect. The following bacilli treatments were tested: 1) *B. pumilus* strain INR-7, 2) mixed bacilli strain Blend-8, 3) mixed bacilli strain Blend-9, and 4) untreated (control). Blend-8 contained bacilli strains: *B. velezensis* strain AP-188, *B. mojavensis* strain AP-209, *Fictibacillus solisalsi* stain AP-217, and *B. velezensis* strain AP-218. Blend-9 contained bacilli strains: *B. velezensis* strain AP-136, *B. velezensis* strain AP-188, *B. velezensis* strain AP-219, and *B. velezensis* strain AP-295. To prepare the inoculum, bacteria from cold storage (-80^{o}C) were plated on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI, USA) and incubated at 28°C for two days (Zhang et al. 2010; Liu et al. 2016). A Loop-full bacterium was transferred into 20 ml of sterilized water and then vortexed for one minute. Two ml of each strain were combined into sterile 50 ml tubes, and the bacterial suspension was adjusted to a final concentration of 10^7 CFU/ml by adding sterilized water. This concentration was then used to inoculate maize seeds during planting. Western corn rootworm second instars (non-diapause strain) used in this study were purchased from French Agricultural Research, Inc., Lamberton, Minnesota. The larvae were shipped overnight by air in 10 cm diameter Petri dish lined with a moist pad containing 4-5-day-old corn root (Pioneer 9917). The corn roots provided

by the supplier during shipping were used to feed the larvae until the larvae were used for behavioral assays. Larvae were maintained at room temperature in the Petri dish approximately 24h from the time they were received before using them for bioassays.

3.2.2 Greenhouse Growth Promotion. Hybrid non-GMO maize seeds (Jacobsen 4704) were planted 2 cm deep in 9-cm diameter plastic pots (one seed per pot) filled with a 1:1 mixture (v/v) of sand and Sunshine mix (SunGro Horticulture, Agawam, MA). After placing single seeds in the potted soil, a one-time application of 1.0 ml of bacilli spore suspensions (10^7 CFU/ml/seed) was pipetted over each seed which was then covered with soil. Control seeds received 1.0 ml/seed of distilled water. Pots were watered every other day as needed. Twenty-five ml of water-soluble NPK fertilizer 20-10-20 (Buddies Plant Food, Ballinger, TX, USA) were applied once, on the fifth day after planting (DAP) to both treated and untreated plants. All plants were maintained at 27 ± 2°C and 60 ± 5% RH with a light and dark phase of 14:10 h in a greenhouse at the Plant Science Research Center, Auburn University. Potted plants were arranged in a completely randomized design. After 12 days of planting, plants were harvested, and plant roots were washed to remove soil. Plant biomass (shoot and root dry weights) was measured for eight replicates per treatment. Before drying, fresh roots of the 12-d plant were analyzed for eight replicates with WinRhizo root scanner (Regent Instruments Inc., Quebec, Canada) to determine root architecture including root length (cm), root volume (cm^3), root surface area (cm^2), and root diameter (0.00-0.05 mm). Scanned root tissues were then placed in paper bags, and dried at 70 °C for five days, and weighed.

3.2.3 Feeding Bioassays

3.2.3.1 *Diabrotica v. virgifera* Larval Feeding Preference. The same bacilli treatments described above were used in this experiment. Previous studies showed that the use of soilless

bioassays does not affect WCR behavior (Robert et al 2012). An initial preference test in which WCR larva presented with two choices (i.e., olfactometer arm with 5-day old maize plant and another arm without maize plant (blank)) was used to determine whether WCR larvae can detect maize plants. Here, plants with bacilli treatments (INR-7, Blend-8, and Blend-9) and untreated plants, and untreated versus untreated plants were presented in two-choice tests to second instar WCR in a Petri dish olfactometer without soil. The olfactometer consists of a center Petri dish (diameter 14 cm) with orifice that serves as point for releasing individual larva. Two connecting tubes (7.0 cm each) modified from 25 ml Falcon tubes (VWR, Radnor, PA USA) were each joined to other two Petri dishes on the opposite sides (diameter 14 cm) that served as container for the plants (Figure 1). The complete olfactometer setup was 56 cm in length. The distance from the center dish (the point where the WCR larva was transferred) to the odor source (plant) on both arms was 11 cm. A 5-d old plant was carefully removed from the pot, shook gently to reduce soil that are attached to roots, and placed inside the soilless Petri dishes on both side arms, and a Petri dish lid (100 × 15 mm) was placed over it and secured with a binder (Fig. 1). The setup was then transferred to an open plastic bowl and covered with black cloth to exclude light. For each olfactometer experiment, an individual second instar larva of *D. v. virgifera* was gently transferred with forceps into the center dish to choose between bacilli-treated and control plants. New sets of plants and larvae were used each time. After 12 h (8:00 AM -8:00 PM), the setup was dismantled to determine the position of each larva and to recover the larva. This experiment was replicated 28 times.

3.2.3.2 No-choice Larvae Feeding. The treatments included: i) *B. pumilus* strain INR-7, ii) mixed bacilli strains Blend-8, iii) mixed bacilli strain Blend-9, and iv) untreated (control). Plants were treated as described in the growth promotion assay section above. Plates (709 ml,

Anyware™ plastic, Arrow Plastic MFG. CO, ELK GROVE, IL USA) were first filled to about 1 cm deep with sand and two pre-weighed second instar larvae were then transferred. One pot containing a 12 days-old plant was then placed on top of the sand-infested with WCR larvae (Figure 2). The openings below the pot allowed the WCR larvae to move into the pot to feed on roots. The larvae were given six days to feed undisturbed after which they were recovered, and their weights determined. The two larvae were weighed together before transferring them to the sand contained in the plastic plate. This initial weight of larvae was then subtracted from the weight of recovered larvae to determine weight gain. There were 18 replicates per treatment.

3.2.4 Statistical Analyses. Growth promotion and no-choice feeding data were first checked for normality of distribution by normal probability plot (Q-Q plot) and skewness of data while Levene test was conducted to check for equality of variance. All data that met analysis of variance (ANOVA) assumptions were then analyzed using one-way ANOVA followed by Tukey-Kramer HSD multiple comparison test ($P < 0.05$). Larval preference to plants on both arms of the olfactometer was modelled as a binary response (non-responders were excluded before data analyses) using Logistic Regression Analysis. Likelihood Ratio test was conducted to choose the most suitable model as described (Morawo and Fadamiro 2014). All data analyses were done using SAS 9.4 (SAS Institute USA) with 0.05 level of significance.

3.3 Results

3.3.1 Greenhouse Growth Promotion. One of the bacilli mixtures significantly increased maize plant biomass and root architecture including root length, root volume, root surface area, and root diameter compared to untreated control plants (Figure 3; Table 1). For shoot dry weight (SDW), Blend-9 treated plants weighed significantly more than untreated plants ($F_{3, 28} = 3.55$; $P = 0.03$). However, there were no significant differences among the treatments in root dry weight (RDW) (Table 1). Plants treated with blends-8 and 9 had significantly longer roots than those treated with *B. pumilus* strain INR-7 or untreated plants ($F_{3, 28} = 6.04$; $P = 0.003$). Root volume of plants treated with Blend-9 were significantly greater than the untreated control ($F_{3, 28} = 3.24$; $P = 0.04$). For root diameter, plants treated with Blend-9 were statistically bigger compared to untreated plants ($F_{3, 28} = 4.94$; $P = 0.007$) but all bacilli-treated plants had similar root diameter size. Root surface area was significantly bigger for plants treated with Blend-9 compared to *B. pumilus* strain INR-7 and untreated plants ($F_{3, 28} = 5.31$; $P = 0.005$).

3.3.2 Feeding Bioassays.

3.3.2.1 *Diabrotica v. virgifera* Larval Feeding Preference. In a two-choice olfactometer study conducted to compare WCR preference between untreated control maize plants versus bacilli-treated plant or blank (no plant), WCR larvae preferred the arm with maize plants compared to the blank arm (without plant) (X^2 (1, $N = 18$) = 1639, $P = 0.0001$) but no significant difference was observed when WCR larvae were presented a pair of untreated plants X^2 (1, $N = 19$) = 0.1050, $P = 0.7459$ (Figure 4). When WCR larvae were presented a choice between plants treated with the *B. pumilus* strain INR-7 and untreated plants, a significantly higher number chose untreated plants (76%) compared to plants treated with INR-7 (24%) X^2 (1, $N = 21$) =

10.31, $P = 0.0013$ (Figure 4). However, a higher number of WCR larvae chose plants treated with bacilli-blends (Blend-8 and Blend-9) compared to untreated plants but the difference was not significant (Figure 4).

3.3.2.2 No-choice Larval Feeding. Plants treated with *B. pumilus* strain INR-7 negatively affected weight gain of WCR larvae. Western corn rootworm larvae gained significantly less weight on plants treated with INR-7 compared to those fed untreated or Blend-8 and Blend-9 plants ($F_{3, 69} = 5.11$; $P = 0.003$) (Figure 5A). The weights of larvae that were fed Blend-8 or Blend-9 treated plants did not differ significantly from those fed untreated plants. Furthermore, the results of experiments conducted to test the effect of individual strains showed that weights of larvae fed with plants treated with mixed bacilli-strains (Blend-8 or Blend-9) did not differ significantly from those fed plants treated with individual strains or untreated control plants ((Figures. 5A and C).

3.4 Discussion

The results of this study showed that *B. pumilus* strain INR-7 negatively affected the host-seeking behavior and development of WCR larvae. However, the same result was not recorded for the bacilli mixtures (Blend-8 and Blend-9). Although application of Blend-9 led to increased shoot dry weight and root architecture of maize plants compared to untreated plants, WCR larvae were slightly attracted to it and the weight gain by larvae was significantly higher on plants treated with the Blend-9. Studies on the effects of beneficial rhizobacteria treatment on plant-insect interactions have focused mainly on foliar-feeding (aboveground) insects (Zehnder et al. 1997; Saravanakumar et al. 2008; Pineda et al. 2012; Pangesti et al. 2015; Zebelo et al. 2016; Gadhave and Gange 2016), with very little attention paid to belowground herbivorous

insects. The few studies that investigated belowground microbial interactions with insects focused on AMF (Gange et al. 1994; Vannette and Rasmann 2012). Our results demonstrate that plants treated with the *B. pumilus* strain INR-7 were less attractive to WCR larvae than untreated (Figure 4).

Elicitation of vigorous plant growth is a main characteristic of some rhizobacteria in addition to their biological control potential. Several studies have shown that with some rhizobacteria strains, early plant growth is often related to induced systemic resistance and improved marketable yields (Domenech et al. 2006; Ban et al. 2011; Liu et al. 2016). Our results showed that plants treated with Blend-9 significantly increased several plant growth indices including shoot biomass, root length, root volume, root surface area, and root diameter of maize plants compared to untreated plants (Table 1). Blend-9 treated plants corroborated past studies that some mixtures of beneficial microorganisms elicit a greater magnitude of plant growth than single strains (Requena et al. 1997; Sandheep et al. 2013; Parra-Cota et al. 2014; Yadav and Verma 2014; Liu et al. 2016).

Host location behavior and feeding results in our study showed that *B. pumilus* strain INR-7, was less preferred by WCR larvae (Figure 4). Also, WCR larvae fed *B. pumilus* strain INR-7 treated plants weighed significantly less than control and plants treated with bacilli blends (Fig. 5). Past studies reported that *P. fluorescens* WCS417r did not affect the foliar specialist herbivorous insects, *Pieris rapae* (Van Oosten et al. 2008) or *Mamestra brassicae* (Pangesti et al. 2015), and at least one study reported that *P. fluorescens* WCS417r induced susceptibility of Arabidopsis to the generalist sucking insect, *Myzus persicae* (Pineda et al. 2012). Shavit et al. (2013) showed that *Bamisia tabaci* nymphs developed faster and survived more after they fed on tomato plants that were pre-inoculated with *P. fluorescens* WCS417r. Our results are partly in

agreement with reports of previous studies that showed that *B. pumilus* strain INR-7 induced systemic resistance against foliar pathogens and insects (Zehnder et al. 1997; Zhang et al. 2010; Liu et al. 2016). Our data also corroborates a recent finding that the single beneficial rhizobacteria strain *Azospirillum* affected host selection by root-feeding *Diabrotica speciose* (Santos et al. 2014). Whether the effect of bacilli blends (e.g., Blend-9 and Blend-8) in our study is specific to *D. v. virgifera* in maize is not clear. In no-choice feeding test in which weight of WCR larvae were compared among plants treated with the individual strains that constitute Blends-8 and 9, larval weight did not differ significantly from the individual strains (Fig. 5b and c), suggesting a lack of antagonism among tested bacilli strains. A recent study has shown that the behavior of neonate and second instar larvae of WCR are different in relation to use of volatile in locating maize roots (Hiltpold and Hibbard 2016). Future research conducted under similar conditions reported in this paper will be important to show whether these results on second instar larvae can be replicated with neonates.

The quality of nutrient source can affect herbivore development (Bukovinszky et al. 2008), and PGPR have been shown to enhance nitrogen uptake by plants (Adesemoye et al. 2010). Gange et al. (2005) reported higher nitrogen contents by PGPR-treated plants. In this present study, we found that plants treated with the mixture of bacilli strains (e.g., Blend-9) had higher biomass compared to control plants (Table 1), which seem to support the increased weight of WCR larvae (Figure 5). However, the results of this study differ from a previous report by our group and that of other laboratories that mixing beneficial microorganism enhances their efficacies against plant-feeding insects. For example, Zebelo et al. (2016) reported that mixtures of bacilli strains (Blend-8 and Blend-9) induced cotton resistance and reduced growth and development of *S. exigua* via increased level of gossypol. Furthermore, a mixture of *P. fluorescens* strains (Pf1,

TDK1, and PY15) increased the activities of PPOLOX that were shown to correlate with malformation of adult leaf folder, *C. medinalis* in rice plant (Saravanakumar et al. 2008). These studies in the preceding lines suggest that rhizobacteria treatment negatively affected foliar feeding insects. Further studies are needed to investigate the role of bacilli-mediated maize metabolites (primary and secondary) on host-seeking behavior and feeding by WCR larvae.

The results presented here show that *B. pumilus* strain INR-7 enhanced resistance of maize plants to WCR 2nd instar larvae. Selected mixtures of bacilli strains reported in this study did not have negative effect on host-selection or feeding behavior of WCR larvae. Plant scientists and entomologists should take into consideration how application of mixtures of biological control agents, such as *Bacillus* species, may affect targeted insect pests.

3.5 Acknowledgements

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Table 1

Effects of *Bacillus* species on maize biomass and root architecture

	Plant Biomass		Root Architecture			
Treatment	SDW (g)	RDW (g)	Root length (cm)	Root volume (cm^3)	Root Surface area (cm^2)	Root diameter (mm)
Blend-8	0.22 ± 0.01ab	0.06 ± 0.00a	522.26 ± 35.58a	2.20 ± 0.14ab	119.9 ± 7.17ab	299.40 ± 25.92ab
Blend-9	0.24 ± 0.01a	0.07 ± 0.00a	647.78 ± 35.58a	2.46 ± 0.14a	141.07 ± 7.17a	386.17 ± 25.92a
INR-7	0.21 ±0.01ab	0.06 ± 0.00a	498.33 ± 35.58b	1.96 ± 0.14ab	110.49 ± 7.17b	287.57 ± 25.92ab
Untreated	0.19 ± 0.01b	0.05 ± 0.00a	440.19 ± 35.58b	1.92 ± 0.14b	102.93 ± 7.17b	249.98 ± 25.92b

Means ± SE within the same column having different letters are significantly different ($P < 0.05$), and SDW and RDW refer to shoot and root dry weight, respectively.

Figure Legend

Figure 1 Behavioral assay set up for host-plant preference of western corn rootworm larvae. A) shows a horizontal olfactometer with one arm containing a maize plant and the other arm without plant (blank), B) shows a horizontal olfactometer with one arm containing a bacilli-treated plant and the other arm containing an untreated plant.

Figure 2 No-choice assay of western corn rootworm larvae feeding on undisturbed roots of 12-day-old maize plants.

Figure 3 Image showing root morphology of Bacillus-treated plant in comparison with untreated plant.

Figure 4 Number and percentage of western corn rootworm (WCR) larvae that chose between bacilli-treated (INR7, Blend-8 or Blend-9 versus untreated maize plants in two-choice olfactometer bioassays. Figure shows a comparison between untreated plant versus bacilli-treated or blank (no plant). Chi-square goodness of fit test ($P < 0.05$).

Figure 5 Mean (± SE) weight gain of WCR larvae in no-choice feeding test. A) Shows effects of the *B. pumilus* strain INR-7, bacilli mixtures (Blend-8 and Blend-9) or untreated plants on performance of *Diabrotica virgifera virgifera* larvae in no-choice feeding test. Bars with different letters are significantly different Tukey-Kramer HSD (ANOVA, $P <0.05$). B) shows

effects of Blend-8 and individual strains on performance of *D. v. virgifera* larvae in no-choice feeding test, C) shows effects of Blend-9 and individual strains on performance of *D. v. virgifera* larvae in no-choice feeding test (ANOVA, $P > 0.05$).

Figure 1

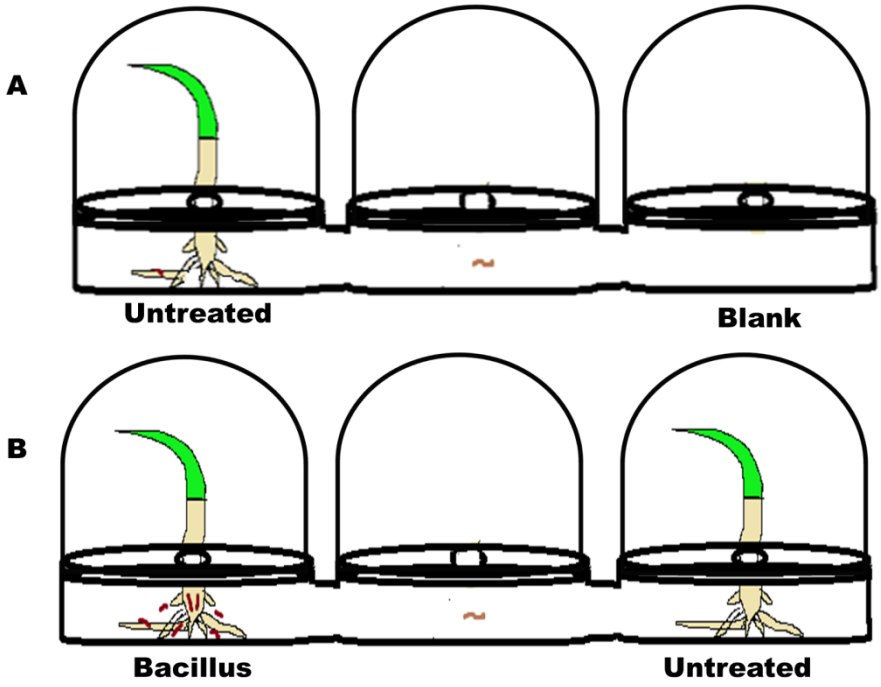
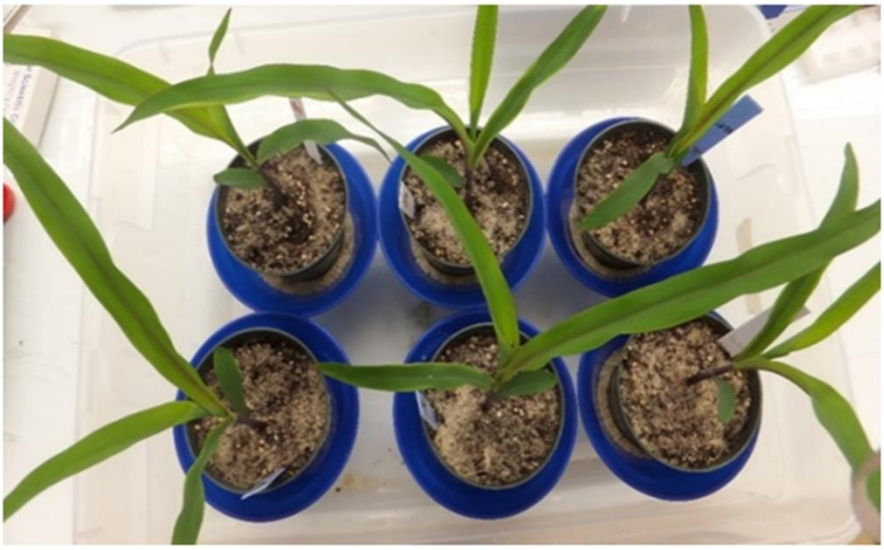


Figure 2



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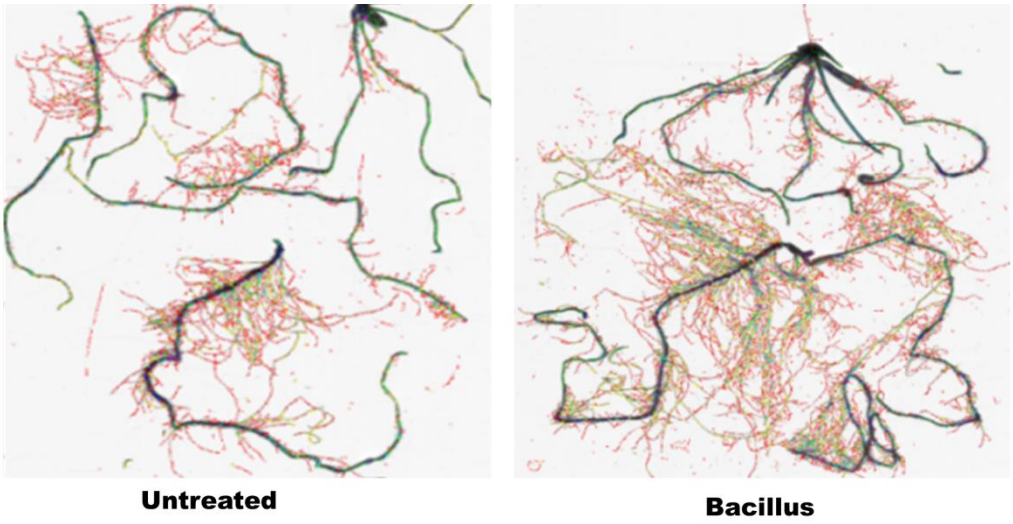


Figure 4

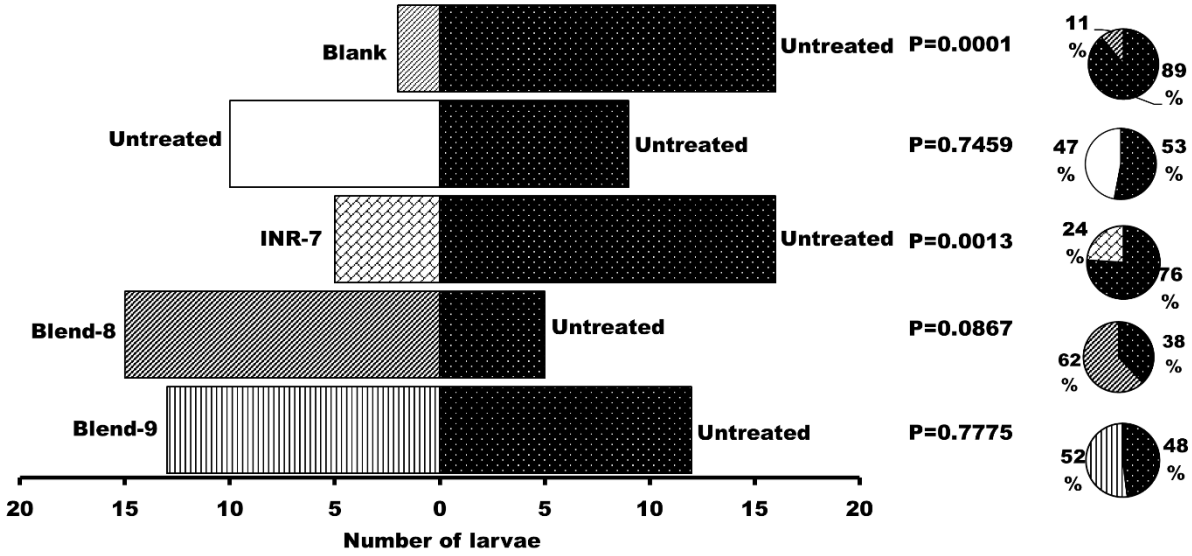
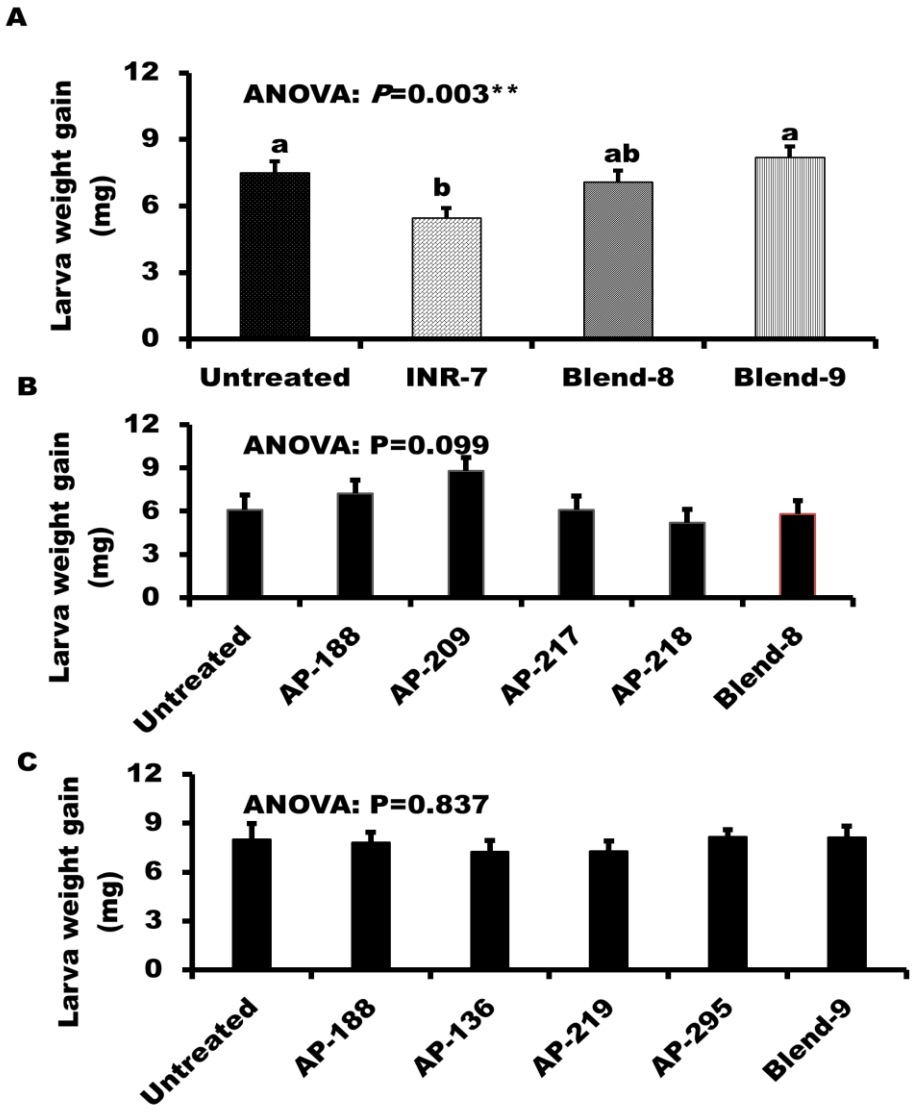

Blank
Untreated
P=0.0001
Untreated
Untreated
P=0.7459
INR-7
Untreated
P=0.0013
Blend-8
Untreated
P=0.0867
Blend-9
Untreated
P=0.7775
20
15
10
5
0
5
10
15
20
Number of larvae
11 %
89 %
47 %
53 %
24 %
76 %
62 %
38 %
52 %
48 %

Figure 5


A
ANOVA: P=0.003**
Larva weight gain (mg)
12
9
6
3
0
a
b
ab
a
Untreated
INR-7
Blend-8
Blend-9
B
ANOVA: P=0.099
Larva weight gain (mg)
Untreated
AP-188
AP-209
AP-217
AP-218
Blend-8
C
ANOVA: P=0.837
Larva weight gain (mg)
Untreated
AP-188
AP-136
AP-219
AP-295
Blend-9

CHAPTER 4

EFFECTS OF RHIZOBACTERIA ON INDUCTION OF CORN VOLATILES AND CONSEQUENCES FOR ENTOMOPATHOGENIC NEMATODES

4.1 Introduction

Immobile plants protect themselves against herbivory through direct and indirect defenses. As a direct defense, plants produce toxic compounds that negatively affect the biology of plant-eating insects (Duffey and Stout 1996). On the other hand, herbivore-attacked plants release volatile organic compounds (VOCs) that are exploited by natural enemies to locate their host either for food or oviposition (Wei et al. 2007). Although, VOC-mediated tritrophic interactions are well studied for aboveground plant systems (Guerrieri et al. 2004; Turlings et al. 2012; Pangesti et al. 2015), studies with belowground system are still in their infancy. Initial studies identified corn root herbivore-induced volatile sesquiterpene that attracts entomopathogenic nematodes (EPN) to herbivore-damaged but this compound is only found in wild relatives of corn (Köllner et al. 2008). Breeding effort aimed at restoring the terpene has begun (Köllner et al. 2004) but breeding takes time and other ethical issues may delay the use of developed lines. Hence, there is need to explore sustainable method that can be used to induce tritrophic interactions belowground via VOC in corn.

Rhizosphere-associated micro-organisms can regulate plant physiology and response to

the environment. Rhizobacteria can increase the rate of plant growth (Adesemoye et al. 2008; Adesemoye et al. 2010) and induce systemic resistance against a plethora of plant diseases (Raupach and Kloepper 1998; Jetiyanon and Kloepper 2002; Kloepper et al. 2004; Domenech et al. 2006; Thilagavathi et al. 2007; Ryu et al. 2007; Liu et al. 2016) and plant-eating insects (Pineda et al. 2010; Pangesti et al. 2013). Several aboveground studies have shown that rhizobacteria mediate production of plant volatiles with consequences for plant herbivores and their natural enemies (Guerrieri et al. 2004; Schausberger et al. 2012; Battaglia et al. 2013; Pangesti et al. 2015). For example, colonization of *Arabidopsis thaliana* Col-0 roots by *Pseudomonas fluorescens* WCS417r was found to suppress the emission of (*E*)-α-bergamotene and methyl salicylate, which subsequently enhanced attraction of *Mamestra brassicaae* to treated plants. An earlier study by Pineda et al. (2013) demonstrated that application of the same non-pathogenic rhizobacteria (*P. fluorescens* WCS417r) interfered with the composition of volatiles emitted by *A. thaliana* plants and that these volatiles were less attractive to the aphid parasitoid, *Diaeretiella rapae*. However, it remains to be tested whether root colonization by *Bacillus* species affects tritrophic interactions belowground.

Plant volatile terpenes are essential indirect defense cues. These VOCs signal availability of oviposition host or food resource to parasitoids and predators aboveground (Paré and Tumlinson 1999; Peñaflor et al. 2011). Belowground, beneficial nematodes exploit VOCs to locate insect hosts feeding on plant roots for oviposition (Rasmann et al. 2005; Kollner et al. 2008; Hiltpold et al. 2010; Ali et al. 2010; Errera et al. 2012; Turlings et al. 2012; Laznik and Trdan 2016; Tonelli et al. 2016). Although some of these plant VOCs are emitted constitutively, the rate of emission may depend on many factors including plant genetic background, herbivory and other environmental factors. Mass spectrometry analyses of maize landrace and inbred lines

showed both qualitative and quantitative difference in VOC emission by the lines (Degen et al. 2004; Köllner et al. 2004; Köllner et al. 2008; Fantaye et al. 2015; Tamiru et al. 2017). Herbivore damage has been shown to significantly increase the rate of emission of VOCs by plants (Dicke et al. 2009). Other abiotic factors that affect the quantity or quality of VOCs emitted by plants include temperature and sunlight (Turlings and Gouinguené 2007), and chemical elicitors (Xu et al. 1994; Birkett et al. 2000; Disi et al. 2017). Rhizosphere-associated organisms have also emerged as important players in mediating plant physiology and plant-insect interactions (Pineda et al. 2012; Santos et al. 2014; Disi et al. 2017).

Zea mays-*D. v. virgifera* LeConte (Coleoptera: Chrysomelidae)- *Heterorhabditis bacteriophora* has emerged as an important model system to study tritrophic interactions belowground, but there is limited information on rhizobacteria mediation of tritrophic interactions belowground. The larva of *D. v. virgifera* is a specialist insect pest of corn in North America and Europe. Although *D. v. virgifera* beetle is known to feed on leaves and silk of corn, the larval stage is by far the most economically important life stage because injury from feeding on roots causes the plant to lodge, thereby reducing yield (Urías-lópez et al. 2001). Costs of control and yield loss are estimated at $1 billion per year in the United States (Metcalf et al. 1982). Moreover, WCR is resistant to currently available control tactics and there is no known effective management option available to control the pest (Meinke et al. 1998; Moellenbeck et al. 2001; Chandler 2003; Vaughn et al. 2005). (*E*)-β-caryophyllene (*E*βC), a sesquiterpene released by *D. v. virgifera* larval-damaged corn is known to attract foraging infective juveniles of *H. bacteriophora* and *Heterorhabditis megidis* (Rasmann et al. 2005; Hiltpold et al. 2010; Hiltpold et al. 2011; Turlings et al. 2012) but this compound is lacking in corn lines in the United States (Köllner et al. 2008). Building on previous literature reports on the ability of root colonizing

rhizobacteria to induce production of VOCs belowground (Santos et al. 2014), we tested the hypothesis that inoculation of corn seeds with root colonizing *Bacillus* bacteria will affect attraction of *H. bacteriophora* to *D. v. virgifera*-damaged corn roots.

4.2 Materials and Methods

4.2.1 Bacteria, Plant, Insect, and Entomopathogenic Nematode. The bacterial strain, *B. pumilus* (INR-7) used in this study was from freezer stock from the biological control lab at the Department of Entomology and Plant Pathology, Auburn University, Alabama. Bacterial culture was prepared as previously described in chapter III. Briefly, the bacteria from cold storage (-80^{o}C) were plated on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI, USA) and incubated at 28°C for two days. Loop-full bacteria were transferred into 20 ml of sterilized water and then vortexed for one minute. Four ml of bacteria was pipetted into sterile 50 ml tubes, and the bacterial suspension was adjusted to a final concentration of 10^7 CFU/ml by adding sterilized water. This concentration was then used to inoculate corn seeds during planting. From this concentration, heat-killed INR-7 (HK) was prepared as one of the negative controls. Briefly, spore suspension of INR-7 was divided into two parts. One part was autoclaved at 121^{o}C for 30 mins while the second part was kept in 4^{o}C fridge. The HK suspension was kept at room temperature to cool. Prior to planting, the HK and the non-autoclaved bacteria were tested for viability by plating them on TSA media and incubated at 28^{o}C for 48 h (Figure 1).

Hybrid non-GMO maize seeds (Jacobsen 4704) were sterilized by washing in 70% ethyl alcohol and rinsed three times in distilled water and then planted in the arms of olfactometer (one seed per arm) filled with a 1:1 mixture (v/v) of sand and Sunshine mix (SunGro Horticulture, Agawam, MA). After placing single seeds in the different arms of the olfactometer, a one-time

application of 1.0 ml each of bacillus spore suspensions (10^7 CFU/ml/seed) and HK were made over seeds and then covered with soil. Control seeds received 1.0 ml/seed of distilled water. The 4^{th} arm was filled with sand but no seed was planted in it. Pots (arms containing planted seeds) were watered every other day as needed. Twenty-five ml of water-soluble NPK fertilizer 20-10-20 (Buddies Plant Food, Ballinger, TX, USA) were applied once on the fifth day after planting (DAP) to both treated and untreated plants. All plants were maintained at 27 ± 2°C and 60 ± 5% RH with a light and dark phase of 14:10 h in a growth chamber. Twelve (12) days-old plants were used for bioassays.

Western corn rootworm second instars (non-diapause strain), *Diabrotica v. virgifera* were purchased from French Agricultural Research, Inc., Lamberton, Minnesota. The larvae were shipped overnight by air in 10 cm diameter Petri dish lined with a moist pad containing 4-5-day-old corn root (Pioneer 9917). The corn roots provided by the supplier during shipping were used to feed the larvae until the larvae were used for behavioral assays the next day. Larvae were maintained at room temperature in the Petri dish approximately 24 h from the time they were received before using them for bioassays.

Entomopathogenic nematodes, *H. bacteriophora* were purchased from Arbico Organics, Tucson, Arizona, USA. Upon arrival, nematodes were immediately removed from the shipping box and transferred to 4 ^oC fridge where they were stored until used. Nematodes were placed in distilled water and shook gently to dissolve. Number of nematodes were normalized by repeatedly pipetting 500 ml (n= 3) from the mixture to establish the number to be applied.

4.2.2 Bacterial Recovery from Root and Plant Growth. The goal of this experiment was to test whether the bacterial strain can be recovered several days after application on seeds. In this greenhouse/laboratory study, INR7 rif^R strain was collected from the biological control

lab at the Department of Entomology and Plant Pathology, Auburn University, Alabama. The INR7 rif^R strain was streaked onto TSA plate and incubated at 28 ˚C for 24 hours. One colony of bacterial strain was transferred into 35 ml TSB in a sterile 50 ml centrifuge tube and kept in a shaking incubator at 28 ˚C with 180 rpm for 48 hours. After 24 hours' incubation, Rifampicin (Sigma-Aldrich, Product code 101594249, USA) antibiotic at 50 µg/ml concentration was added to 50 ml centrifuge tube and covered with aluminum foil to avoid direct light. At the end of 48 hours, INR-7 rif^R strain was centrifuged at 3600 x g for 10 minutes to obtain pellets and washed three times with sterile water. INR-7 rif^R strain vegetative cells CFU were adjusted to 10^{-7} CFU/ml and 1 ml inoculated directly on the corn seed surface. Similar amount of water was used to inoculate control seeds. Ten days after planting, both INR-7 rif^R and control plants were each divided into two groups. A group was returned to growth chamber in the laboratory and infested with six *D. v. virgifera* second instar while the other was left uninfested. Two days later, both INR-7 rif^R and control plants with and without infestation were harvested by gently removing soils from the roots. Roots with attached rhizosphere soils were serially diluted and incubated on TSArif plates at 28 ˚C for 48 hours to count INR-7 rif^R strain population in each plate. Whole dry weight was also determined for 11 replicates.

4.2.3 Response of Nematodes to Corn Roots Treated with INR-7. In this study, plants treated with INR-7, heat-killed INR-7, untreated control, and sand only were presented in four-choice olfactometer to *H. bacteriophora* in the presence or absence of *D. v. virgifera* larval infestation. The olfactometer consisted of a center PVC connector with orifice that serves as point for releasing the *H. bacteriophora*. This central PVC joint was used as connector to the olfactometer arms (cups) that served as container for plants (Figure 2). The distance from the center PVC (the point where the *H. bacteriophora* juveniles were transferred) to the odor source

(plant) arms was 11 cm. The olfactometer connectors and the central PVC were filled with 10% moist sand as previously described (Hiltpold et al. 2011) three days before the *H. bacteriophora* was transferred with pipette. The setup was then transferred to an open plastic bowl and kept in the growth chamber. For each olfactometer experiment, plants were grown in the olfactometer arms for at least 10 days prior to start of experiments.

Experiment 1: Plants with the following treatments: INR-7 inoculated plant infested with six *D. v. virgifera* larvae, heat-killed INR-7 inoculated plant infested with six *D. v. virgifera* larvae, non-inoculated plant infested with six *D. v. virgifera* larvae, sand (without plant) infested with six *D. v. virgifera* larvae were presented as a four choice to *H. bacteriophora* infective juveniles. After 72 h of larval infestation, 500 ml of newly emerged infective juvenile of *H. bacteriophora* (~2000) was pipetted at the center of the olfactometer containing plants growing in different arms. The setup was left for an additional 24 h and then disassembled to recover nematodes. *Heterorhabditis bacteriophora* were recovered from the different arms using Sieving and Sucrose Centrifugation methods as described by Xiang et al. (2017). Soil sample from the olfactometer arm was mixed in a plastic bowl. The supernatant was poured through 75/25 µm stacked mesh sieve. The remaining silt and *H. bacteriophora* on the lower sieve were washed into a centrifuge tube. Tubes were filled with sugar solution at room temperature. Samples were then centrifuged at 1700 rpm for 1 minute. The tubes were left to sit for about 3 minutes. Suspended nematodes in sugar supernatant were gently decanted into mesh sieve as described above. The *H. bacteriophora* in the sieve were gently rinsed off using a fine spray water bottle and transferred to labelled plastic cups. Nematodes recovered were counted under a compound microscope (Nikon TS100) at 4X by counting those found in the diagonal squares of the Petri dish. Relative numbers of nematodes recovered were calculated based on the formula: (Number

of nematodes recovered/6) ×77.25. The experiment was replicated four times with two time repeats.

Experiment 2: In this test, *H. bacteriophora* infective juveniles were given a choice among INR-7 inoculated plant; heat-killed INR-7 inoculated plant, non-inoculated plant and sand. A pipette was used to release infective juvenile of *H. bacteriophora* (~2000) at the center of the olfactometer. The setup was left for an additional 24h and then disassembled to recover *H. bacteriophora*. The experimental setup here is similar to the setup in experiment 1 but the only difference was the absence of *D. v. virgifera* larval infestation. This experiment was replicated four times with two time repeats.

4.2.4 Volatile organic compound collection

Volatile organic compounds (VOCs) were collected from 12-day-old plants with treatments as described above using the undisturbed root volatile collection technique described by Ali et al. (2010) with modifications. Plants were grown in a 15-cm tall pot made from PVC pipe. An opening was made at the base of the pot approximately 5 cm from the ground base. Through this opening, a Teflon tube connected with 50 mg Super-Q (Alltech Associates, Deerfield, IL) adsorbent trap was linked to a vacuum pump (ARS, Gainsville, FL, USA) with air flow rate of 0.8 ml/min to trap root volatiles. At the end of 24 h, the Super-Q trap was eluted with 300 µl of methylene chloride into 2.0 ml vials and stored at -20 ^{o}C.

4.2.5 Response of Entomopathogenic Nematodes to Corn Root Extracts Induced by INR-7.

The goal of this experiment is to confirm that entomopathogenic nematode responds to volatile extracts from bacilli-treated plant. This experiment was designed similar to the ones where intact plant roots treated with INR-7, heat-killed INR-7, untreated control, and sand only were presented in a four-choice olfactometer to *H. bacteriophora*. Here, filter paper was

permeated with 10 µl of VOC extract from corn roots and then placed in sand-filled glass olfactometer (Figure 3). Briefly, the four-choice olfactometer has a center chamber with orifice (0.64 cm wide) for releasing infective juveniles of *H. bacteriophora*. This central chamber was joined with connecting arms (each 2.54 cm long) to the air source. The complete olfactometer setup was 12 cm long including the central chamber. The olfactometer arms and the central chamber were filled with 10% moist sand five minutes prior to start of experiment. The setup was then transferred to a circular glass stand to secure the setup before the olfactometer arms were connected to purified air source. *Heterorhabditis bacteriophora* count was documented as above for seven replicates.

Experiment 1: Prior to using corn root extracts, we tested whether *H. bacteriophora* will show attraction to (*E*)-β-caryophyllene (*EβC*), a plant sesquiterpene attractive to *H. bacteriophora* and *H. megidis* (Rasmann et al. 2005; Kollner et al. 2008; Rasmann and Turlings 2008). Commercially available *EβC,* purity 95% (Sigma® Chemical Co., St. Louis, Missouri) was dissolved in GC grade methylene chloride solvent and 10 µl was applied on filter paper. Similar amount of methylene chloride was applied as control on another arm while the remaining two arms were left without *EβC* or solvent. A 500 ml of infective juvenile (~2000) was pipetted into the central chamber of the olfactometer through the orifice. The setup was left for 12h and then disassembled to recover *H. bacteriophora* using the method described above.

Experiment 2: This experiment is similar to the experiment with *EβC*. VOCs were sampled from INR-7 inoculated plant; heat-killed INR-7 inoculated plant, non-inoculated (control) plant and sand (no plant) in the presence or absence of *D. v. virgifera* larvae damage. A 500 ml of infective juvenile (~2000) of *H. bacteriophora* was pipetted into the central chamber of the olfactometer through the orifice. The setup was left for 12h and then disassembled to

recover nematodes. *Heterorhabditis bacteriophora* were recovered from the different arms using methods described above. The experiment was replicated seven times with each of the VOCs from plants in the presence or absence of *D. v. virgifera* larval infestation.

4.2.6 Statistical Analyses

Data were mostly over dispersed. To control for this over dispersion, negative binomial regression distribution with log link modelling was used to analyze all data. Where dispersion is found to persist after performing negative binomial, zero inflated negative binomial distribution model was implemented. Treatment was modeled as fixed effect with time as random effect for EPN response to bacilli-treated plant roots. For response of nematodes to VOC extract, treatment was modelled as fixed effect while days of the data collection was modeled as random effect. For bacteria colony recovered from 12-day-old plants, nematode counts were modeled as fixed effects. All data analyses were performed in SAS 9.4 at $P < 0.05$ level of significance.

4.3 Results

4.3.1 Bacterial Recovery from Root and Plant Growth. Bacteria were recoverable 12 days after inoculating seeds under greenhouse and laboratory conditions. The total CFU recovered from *B. pumilus* (INR-7)-treated plants were significantly higher both in the absence or presence of *D. v. virgifera* larvae than the number recovered from untreated plants (Table 1; Figure 4A). Furthermore, the result showed that total CFU recovered from INR-7-treated plants decreased with *D. v. virgifera* larval infestation. No significant difference in plant dry weight was recorded for INR-7 treated plant with and without larval infestation compared to untreated plants (Figure 4B).

4.3.2 Response of Nematodes to Corn Roots Treated with INR-7. The result showed that *B. pumilus* strain INR-7 inoculation of corn can mediate attraction of *H. bacteriophora* under controlled environment. In a four choice olfactometer, a significantly higher number of *H. bacteriophora* were recovered from the arm containing INR-7 treated plants in the presence of *D. v. virgifera* larval infestation than other treatments including heat-killed INR-7, untreated plants and sand (Table 2; Figure 5A). A similar trend was recorded for INR-7 treated plants without insect infestation (Figure 5B). When plants without larval infestation were presented in choice experiment, higher numbers of *H. bacteriophora* chose the arm containing INR-7 treated plants than other treatments. The relative number of *H. bacteriophora* attracted to treatment INR-7 were significantly higher for plants without larval infestation compared to heat-killed INR-7, untreated or sand.

4.3.3 Response of Nematode to Corn Root Extracts Induced by INR-7. Initial result with (*E*)-β-caryophyllene shows that *H. bacteriophora* was weakly attracted to *EβC* than to the solvent (methylene chloride) but the difference was not significant (Figure 6A). In general, there was a fewer number of *H. bacteriophora* recovered from sand column. *Heterorhabditis bacteriophora* response to VOC extract from larval infested plants did not differ for all treatments (Figure 6B). Similar result was recorded for VOC extract from plants without *D. v. virgifera* larval infestation (Figure 6C).

4.4 Discussion

This study demonstrates that a higher number of infective juvenile of the obligate parasitic nematode, *H. bacteriophora* chose *B. pumilus* strain INR-7 inoculated corn root in the presence or absence of *D. v. virgifera* larval damage. *Heterorhabditis bacteriophora* were

weakly attracted to VOC extracts from undamaged INR-7 inoculated corn root. Interestingly, bacteria were recoverable 12 days after inoculating seeds under greenhouse and laboratory conditions with significantly higher number of CFU found in INR-7 treated plants both in the absence or presence of WCR larval infestation. These results support our hypothesis that inoculation of corn seeds with root colonizing *Bacillus* species will affect attraction of *H. bacteriophora* to *D. v. virgifera*-damaged corn roots. Past studies showed that entomopathogenic nematodes in the family Rhabditidae preferentially orient towards *D. v. virgifera* larval-damaged corn root (Rasmann et al. 2005; Hiltpold et al. 2010; Hiltpold et al. 2011; Turlings et al. 2012). However, to the best of our knowledge, this is the first study to report that corn root colonization by *Bacillus* species mediate tritrophic interactions belowground especially the preference of beneficial nematodes.

One characteristic that sets some gram-positive *Bacillus* species apart from others is the ability to form spores under unfavorable conditions and to bounce back their populations when the environment becomes favorable (Nicholson 2002). My data showed that *B. pumilus* (INR-7) can colonize corn as bacteria were recovered after several days of inoculation under greenhouse conditions (Figure 4). The data also showed that *D. v. virgifera* larval feeding did not affect total numbers of CFU recovered from *B. pumilus* (INR-7)-treated plants which were significantly higher in both WCR larvae infested or uninfested plants. This result addresses the question often asked by plant biologists whether beneficial rhizobacteria can be recovered from plant roots after several days of inoculation. It also suggests that *Bacillus* species can be used in integrated management of agricultural pest as they seem to persist longer in the soil.

In this study, I found that higher numbers of *H. bacteriophora* chose INR-7 inoculated corn root (Figure 5), suggesting the potential for integrating rhizobacteria in the management of

D. v. virgifera in corn production. *Heterorhhabditis bacteriophora* was consistently attracted to both *D. v. virgifera* larval damaged and undamaged INR-7 inoculated corn roots. This data corroborates other reports on effects of mycorrhiza and rhizobacterium (e.g., *P. fluorescens* WCS417r) treatment on plant-insect and tritrophic interactions aboveground (Schausberger et al. 2012; Pineda et al. 2013; Pangesti et al. 2015). Pangesti et al. (2015) showed that rhizobacterial colonization of *A. thaliana* roots increased attraction of the parasitoid *Microplitis mediator* to *M. brassicae* caterpillar infested plants. Similarly, mycorrhizal fungus, *Glomus mosseae* treated bean plants *Phaseolus vulgaris* attacked by spider mites, *Tetranychus urticae* were attractive to the spider mites' predatory mite, *Phytoseiulus persimilis* (Schausberger et al. 2012). In contrast, Pineda et al. (2013) reported that treatment of *A. thaliana* with *P. fluorescens* WCS417r reduced attraction of the parasitoid *Diaeretiella rapae* to *Myzus persicae* aphid feeding-induced volatiles. To the best of our knowledge, this is the first report on bacillus mediation of tritrophic interactions involving a very destructive belowground pest of corn and a natural enemy of the pest, *H. bacteriophora*.

Attraction of *H. bacteriophora* to corn root VOC extracts from plants without *D. v. virgifera* larval infestation followed similar pattern as the result of experiment with intact plant root but the number of *H. bacteriophora* was not significantly difference (Figure 6). This result seems to suggest that INR-7 induced VOC play a little or no role in the attraction of *H. bacteriophora*. Hiltpold et al. (2010) showed that volatile from *D. v. virgifera* larval damaged corn roots significantly attracted *H. bacteriophora* than volatile from undamaged corn roots. In chapter II, I demonstrated that altered production of volatiles could be a common mechanism by which rhizobacteria mediate plant-insect interactions in aboveground system. Whether a similar effect is the case for belowground tritrophic interactions is yet to be investigated. Future study is

needed to understand the role of bacilli-mediated root exudates in *H. bacteriophora* interaction with hosts.

The results of this study suggest that *Bacillus* species colonization of corn roots affects the behavior of entomopathogenic nematodes. However, the study showed a minimal effect of bacilli-induced VOC, at least in this study system. Other sensory stimuli, such as non-volatile contact cues (e.g., exudates) also contribute to entomopathogenic nematodes choice of intact plant (Nicholson 2002; Hiltpold et al. 2015). There is need to include bacilli-induced root exudates in future behavioral studies to test the effect of identified compounds.

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Table 1 Population of *Bacillus pumillus* INR-7 recovered from 12-day-old plants.

Parameter	DF	Estimate	SE	Wald 95% Confidence Limits		Wald χ^2	Pr > χ^2
Intercept	1	11.2157	0.2126	10.7991	11.6323	2784.24	<.0001
Untreated infested	1	0.33	0.3129	-0.2832	0.9433	1.11	0.2915
INR-7	1	6.5122	0.2719	5.9793	7.0451	573.64	<.0001
INR-7 infested	1	5.9456	0.2719	5.4127	6.4785	478.16	<.0001
Untreated	0	0	0	0	0	.	.
Dispersion	1	0.3162	0.072	0.2024	0.494		

Maximum likelihood parameter estimates (zero inflated negative binomial distribution) was adopted to compare treatments with untreated control (Wald χ^2, $P \leq 0.0001$)

Table 2 Effect of *Bacillus pumillus* INR-7 on attraction of entomopathogenic nematodes to 12-day-old plants.

Parameter	DF	Estimate	Standard	Wald 95% Confidence Limits		Wald χ^2	Pr > χ^2
Intercept	1	2.959	0.9427	1.1113	4.8067	9.85	0.0017
HK infested	1	0.9906	1.24	-1.4396	3.4209	0.64	0.4243
INR-7 infested	1	3.0751	1.2127	0.6983	5.4518	6.43	0.0112
Sand infested	1	0.4011	1.1713	-1.8946	2.6967	0.12	0.732
Untreated infested	0	0	0	0	0	.	.
Time	1	0	0.5962	-1.1686	1.1686	0	1
Time*HK infested	1	-0.7016	0.814	-2.2971	0.8938	0.74	0.3887
Time*INR-7 infested	1	-1.2816	0.7913	-2.8325	0.2694	2.62	0.1053
Time*Sand infested	1	-0.4024	0.7559	-1.8839	1.079	0.28	0.5945
Time*Untreated infested	0	0	0	0	0	.	.
Dispersion	1	0.3025	0.0984	0.1599	0.5723		

Maximum likelihood parameter estimates (zero inflated negative binomial distribution) adopted to compare treatments with untreated control (Wald χ^2, $P \leq 0.05$)

Table 3 Effect of *Bacillus pumillus* INR-7 on attraction of entomopathogenic nematodes to 12 day-old plants.

Parameter	DF	Estimate	SE	Wald 95% Confidence Limits		Wald χ^2	Pr > χ^2
Intercept	1	4.4035	0.6265	3.1756	5.6314	49.41	<.0001
HK	1	3.4722	0.8783	1.7508	5.1936	15.63	<.0001
INR-7	1	2.0184	0.824	0.4033	3.6334	6	0.0143
Sand	1	0.2518	0.8391	-1.3928	1.8965	0.09	0.7641
Untreated	0	0	0	0	0	.	.
Time	1	-0.6443	0.3846	-1.3981	0.1095	2.81	0.0939
Time*HK	1	-2.0194	0.5973	-3.1902	-0.8486	11.43	0.0007
Time*INR-7	1	-0.5655	0.5143	-1.5735	0.4424	1.21	0.2714
Time*Sand	1	-0.203	0.5263	-1.2346	0.8286	0.15	0.6997
Time*uUntreated	0	0	0	0	0	.	.
Dispersion	1	0.2212	0.0627	0.1269	0.3856		

Maximum likelihood parameter estimates (zero inflated negative binomial distribution) adopted to compare treatments with untreated control (Wald χ^2, $P \leq 0.0001$)

Table 4 Attraction of entomopathogenic nematodes to (*E*)-β-caryophyllene).

Parameter	DF	Estimate	Standard	Wald 95% Confidence Limits		Wald χ^2	Pr > χ^2
Intercept	1	-3.7748	14.5082	-32.2103	24.6606	0.07	0.7947
(*E*)-β-caryophyllene	1	7.2699	14.51	-21.1692	35.7091	0.25	0.6164
Sand	1	6.3298	14.5121	-22.1133	34.7729	0.19	0.6627
methylenechloride	0	0	0	0	0	.	.
Days	1	-0.2464	3.2655	-6.6467	6.1539	0.01	0.9398
Dispersion	0	0.0002	0	0.0002	0.0002		

Maximum likelihood parameter estimates (zero inflated negative binomial distribution) adopted to compare (*E*)-β-caryophyllene with untreated control (Wald χ^2, P >0.05)

Table 5 Attraction of entomopathogenic nematodes to VOC extract from *Bacillus pumillus* INR-7 treated plants infested with second instar *Diabrotica virgifera virgifera*.

Parameter	DF	Estimate	Standard	Wald 95% Confidence Limits		Wald χ^2	Pr > χ^2
Intercept	1	2.319	0.333	1.6663	2.9717	48.49	<.0001
HK infested VOC	1	0.3186	0.2325	-0.1371	0.7743	1.88	0.1706
INR-7 infested VOC	1	0.2317	0.2383	-0.2354	0.6988	0.95	0.3308
Sand infested VOC	1	0.3191	0.2708	-0.2116	0.8499	1.39	0.2386
Untreated	0	0	0	0	0	.	.
Days	1	0.0492	0.0603	-0.069	0.1675	0.67	0.4145
Dispersion	1	0.0232	0.0324	0.0015	0.3579		

Maximum likelihood parameter estimates (zero inflated negative binomial distribution) adopted to compare treatment with untreated control (Wald χ^2, $P > 0.05$)

Table 6 Attraction of entomopathogenic nematodes to VOC extract from *Bacillus pumillus* INR-7 treated plants without infestation.

Parameter	DF	Estimate	Standard	Wald 95% Confidence Limits		Wald χ^2	Pr > χ^2
Intercept	1	3.6415	2.7572	-1.7625	9.0456	1.74	0.1866
HK VOC	1	-0.2438	3.8115	-7.7143	7.2267	0	0.949
INR-7 VOC	1	2.1599	4.4578	-6.5773	10.8971	0.23	0.628
Sand VOC	1	-2.3906	3.4012	-9.0568	4.2756	0.49	0.4821
Untreated VOC	0	0	0	0	0	.	.
Days	1	-0.7087	0.6799	-2.0413	0.624	1.09	0.2973
Dispersion	1	4.3125	1.6416	2.0451	9.0939		

Maximum likelihood parameter estimates (negative binomial distribution) adopted to compare treatment with untreated control (Wald χ^2, $P > 0.05$)

Figure Legend

Figure 1 Image showing *Bacillus pumilus* strain INR-7 and Heat-killed INR-7 (HK) after 48h growth on TSB media

Figure 2 Image of belowground sand olfactometer

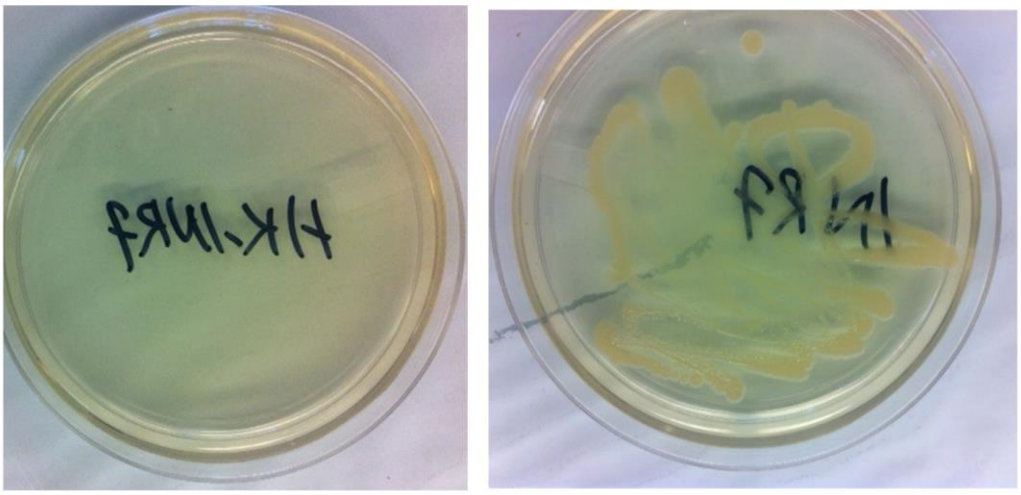
Figure 3 Image of glass olfactometer for testing the role of volatiles belowground

Figure 4 Numbers of *Bacillus pumillus* strain INR-7 recovered from corn root and plant dry weight. A) Shows mean (±SE) of CFU/ml recovered from root of 12-day-old corn, *** indicates significant difference (Wald χ^2; $P \leq 0.05$); and B) shows mean (±SE) of plant dry weight (g) of *B. pumillus* treated plants (ANOVA, $P > 0.05$).

Figure 5 Mean relative numbers of entomopathogenic nematodes choosing different treatments. A) Shows mean (±SE) of numbers nematodes attracted to *Bacillus pumillus* strain INR-7 treated plants that were infested with second instar *Diabrotica virgifera virgifera* larvae, ** indicates significant difference (Wald χ^2; $P \leq 0.05$); B) shows mean (±SE) of numbers nematodes attracted to *B. pumillus* strain INR-7 treated plants without infestation, *** indicates significant difference (Wald χ^2; $P \leq 0.05$).

Figure 6 Mean relative numbers of entomopathogenic nematodes choosing arms with volatile organic compounds (VOCs) from different treatments. A) Shows mean (±SE) of numbers nematodes attracted to filter paper with (*E*)-β-caryophyllene (*E*βC); B) shows mean (±SE) of numbers nematodes attracted to VOCs from *Bacillus pumillus* strain INR-7 treated plants that were infested with second instar *Diabrotica virgifera virgifera* larvae; C) shows mean (±SE) of numbers nematodes attracted to VOCs from *B. pumillus* strain INR-7 treated plants without infestation. NS indicates no significant difference (Wald χ^2; $P > 0.05$).

Figure 1



HK-INR-7 *Bacillus pumilus* INR-7

Figure 2

Figure 3

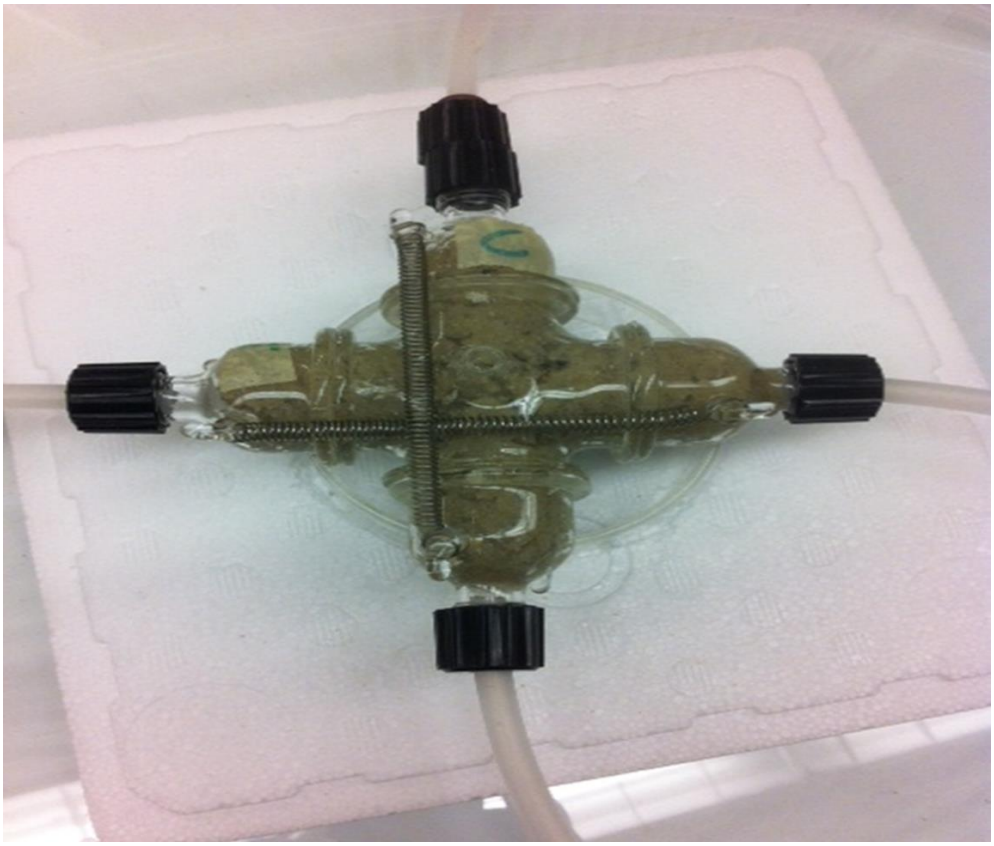


Figure 4

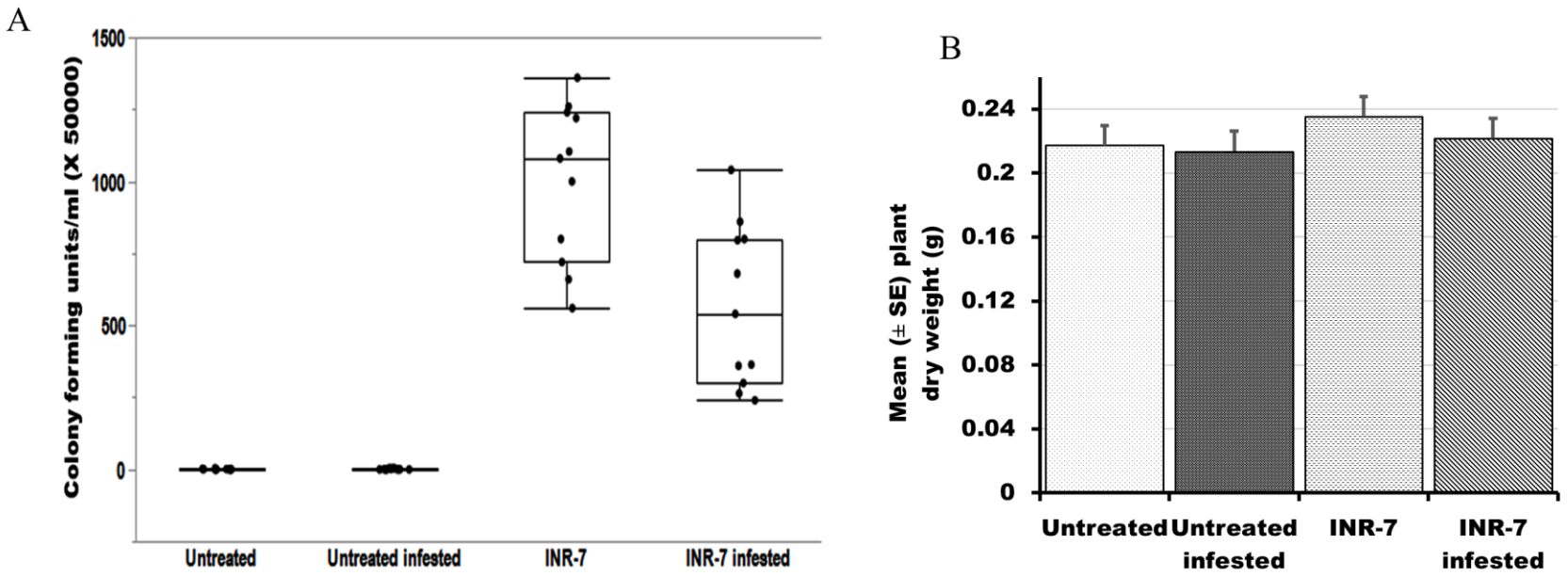
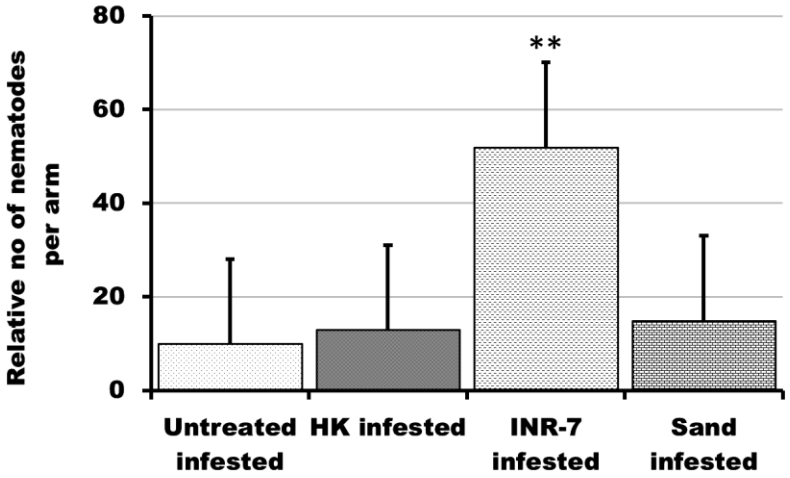

A
Colony forming units/ml (X 50000)
1500
1000
500
0
Untreated
Untreated infested
INR-7
INR-7 infested
B
Mean (± SE) plant dry weight (g)
0.24
0.2
0.16
0.12
0.08
0.04
0
Untreated
Untreated infested
INR-7
INR-7 infested

Figure 5


Relative no of nematodes per arm
80
60
40
20
0
**
Untreated infested
HK infested
INR-7 infested
Sand infested

B

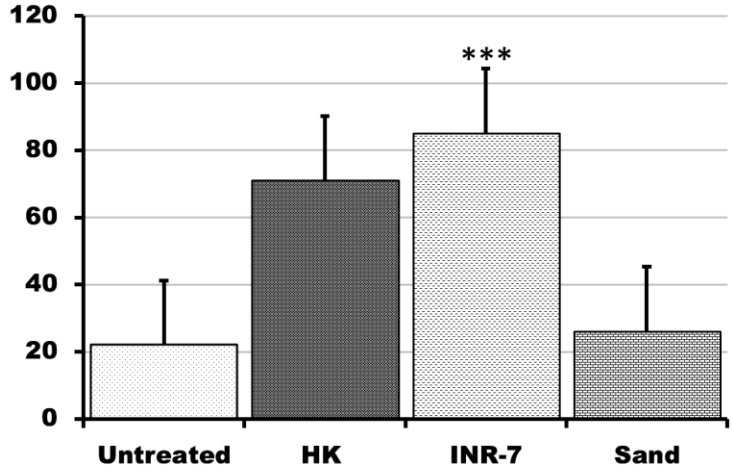
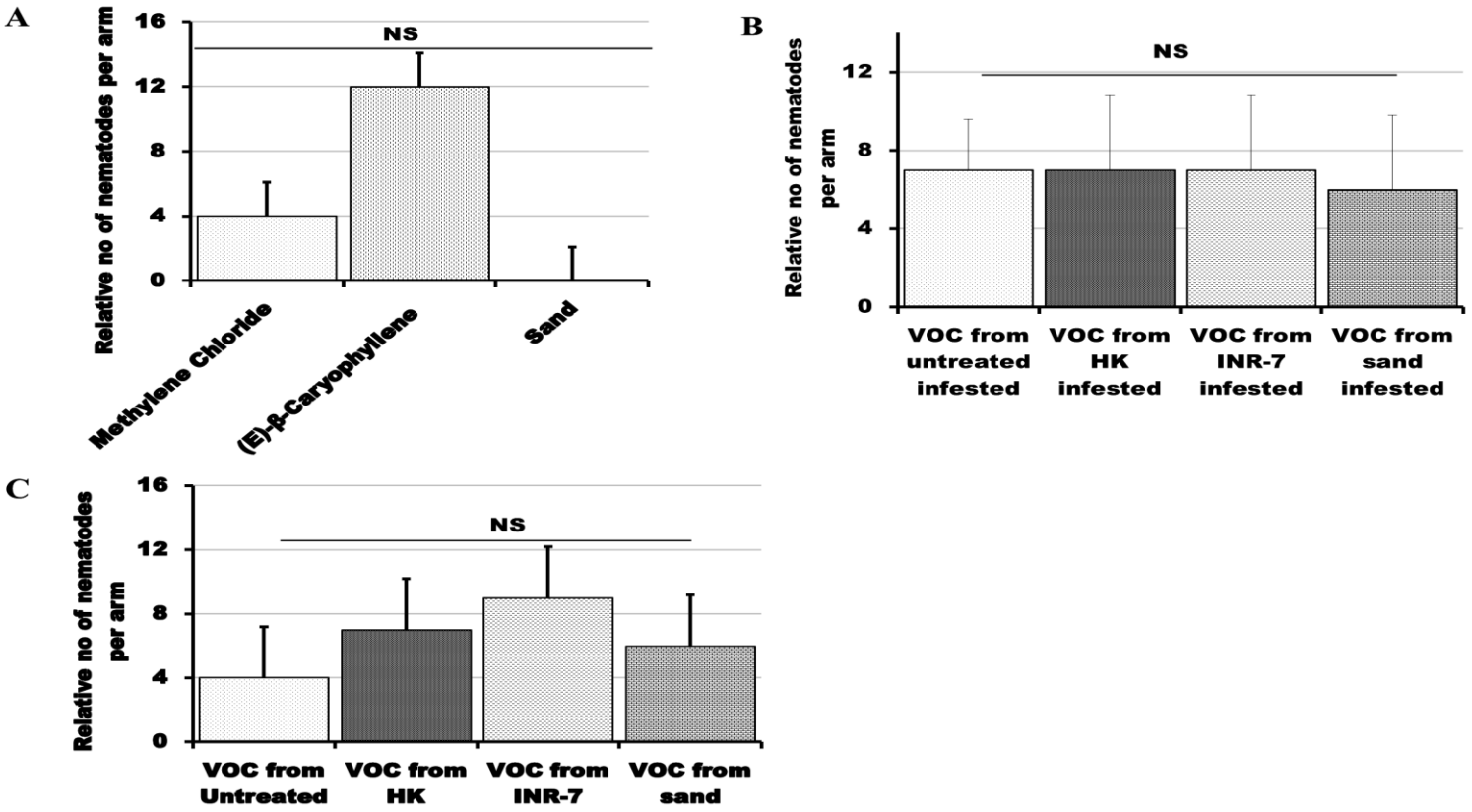

120
100
80
60
40
20
0
***
Untreated
HK
INR-7
Sand

Figure 6


A
Relative no of nematodes per arm
16
12
8
4
0
NS
Methylene Chloride
(E)-β-Caryophyllene
Sand
B
Relative no of nematodes per arm
12
8
4
0
NS
VOC from untreated infested
VOC from HK infested
VOC from INR-7 infested
VOC from sand infested
C
Relative no of nematodes per arm
16
12
8
4
0
NS
VOC from Untreated
VOC from HK
VOC from INR-7
VOC from sand

CHAPTER 5

cis-JASMONE PRIMES DEFENSE PATHWAYS IN TOMATO VIA EMISSION OF VOLATILE ORGANIC COMPOUNDS AND REGULATION OF GENES WITH CONSEQUENCES FOR *SPODOPTERA EXIGUA* OVIPOSITION

5.1 Introduction

Upon insect herbivory, plants emit volatile organic compounds (VOCs) that mediate plant-insect interactions. These VOCs can be exploited by some herbivorous insects for host finding (Bruce et al. 2005; von Mérey et al. 2013; Ajayi et al. 2015) or by their natural enemies as host location cues (Du et al. 1998; Bruce and Pickett 2011). Furthermore, plant VOCs may act as airborne information signaling molecules that boost direct and indirect defenses in remote parts of the same plants or neighboring plants (Kost and Heil 2006; Heil and Ton 2008; Peng et al. 2011). Plants perceive airborne chemicals and can be induced into a unique physiological state called "priming" (Conrath et al. 2006). Defense priming occurs when a plant is conditioned for the superactivation of defenses against environmental challenges without or only slightly affecting plant fitness (van Hulten et al. 2006; Hedge et al. 2012; Martinez-Medina et al. 2016), and can be induced by several compounds such as herbivore-induced plant volatiles (HIPVs) (Paschold et al. 2006), jasmonic acid (JA) (Menzel et al. 2014), salicylic acid (SA) (Conrath et

al. 2002), and *cis*-Jasmone (CJ) (Birkett et al. 2000; Bruce et al. 2003a; Ton et al. 2007; Bruce et al. 2008; Moraes et al. 2009; Oluwafemi et al. 2013).

The plant hormone jasmonic acid (JA) is a ubiquitous signal for tissue injury and for the subsequent activation of defense responses to many insect herbivores (Howe and Jander 2008). JA is involved in a wide range of defense-related processes, including the synthesis of secondary metabolites (e.g., volatile and non-volatile compounds) (Pauwels et al. 2009; Menzel et al. 2014). The role of JA or its volatile derivative methyl jasmonate (MeJA) in defense signaling pathway has been investigated in many plant species (Farmer and Ryan 1990; Xu et al. 1994; Gols et al. 2003). For example, effects of MeJA has been shown to vary in plants including production of fewer quantity of volatiles released by JA-treated *Vicia faba* plants (Birkett et al. 2000), and direct and indirect defense against spider mites (Gols et al. 2003). Koch et al. (1997) postulated that inactivation of JA to the volatile phase (i.e., CJ) relaxes stressed plants but a recent report has shown that the biosynthesis of CJ follows a different route from other oxylipin pathway in *Arabidopsis thaliana* (Matthes et al. 2010).

cis-Jasmone is a component of VOCs first identified in herbivore damaged cotton plants (Paré and Tumlinson 1999). CJ can prime or induce plant defenses in receiver plants through increased or primed production of plant metabolites (Pope et al. 1997; Bruce et al. 2003b; Moraes et al. 2008; 2009, Matthes et al. 2010; Dewhirst et al. 2012; Delaney et al. 2013; Vieira et al. 2013). Birkett et al. (2000) showed that *Triticum aestivum* (winter wheat plants) pretreated with CJ repelled *Nasonovia ribisnigri* (damson-hop aphid). Simultaneously, the CJ-pretreated wheat plants were attractive to natural enemies of aphids, *Coccinella septempunctata* (seven-spot ladybird) and *Aphidius ervi* (aphid parasitoid). Bruce et al. (2003) reported that CJ treatment altered the composition of volatiles emitted by *A. thaliana*. This change in composition repelled

the generalist aphid, *Myzus persicae*, in an olfactometer bioassay but attracted *A. ervi*, a parasitoid of *M. persicae*, thereby providing the plant with double protection.

In a recent related study, Oluwafemi et al. (2013) reported that CJ by itself or CJ treatment without insect infestation did not induce VOC emission in maize or affect the response of the leafhopper, *Cicadulina storeyi* in olfactometer bioassays. However, pre-treatment of maize plants with CJ followed by herbivore infestation increased the emission of defensive sesqueterpenes (especially those designated as herbivore repellents), and repelled *C. storeyi* in the olfactometer. Other reported effects of CJ include an antixenosis effect against aphid feeding on CJ-treated cotton plants (Hegde et al. 2012), attraction and enhancement of stink bug egg parasitoids to induced signals from soybean (Moraes et al. 2009; Vieira et al. 2013), preference for the aphid parasitoid *A. ervi* to sweet pepper (Dewhirst et al. 2012), and arrestment effect on *A. ervi* foraging on CJ-treated *A. thaliana* (Matthes et al. 2010). Recently, Egger and Koschier (2014) reported that feeding damage by *Frankliniella occidentalis* larvae was significantly reduced when CJ was applied on *Phaseolus vulgaris*. These studies demonstrate the effect of CJ on xylem and phloem feeders and their natural enemies, but little is known about CJ priming effects on oviposition preference of lepidopteran pests. Oviposition preference is an important fitness index for insect species whose offspring survival is dependent on the mother's choice of suitable egg laying site (Gripenberg et al. 2010).

Tomato-*S. exigua* system is a good model to investigate the role of elicitors in secondary metabolite-mediated plant-insect interactions since gene expression and volatile profiles of tomato plants damaged by chewing insects such as *S. exigua* are well documented (e.g., Zebelo et al. 2014). In addition, the larval stage of *S. exigua* feeds on multiple hosts including tomato

and has assumed a significant status as an agricultural pest in the Southeastern United States of America (Lange and Bronson 1981; Taylor and Riley 2008).

In this study, we hypothesized that CJ pre-treatment of tomato will prime expression of terpene biosynthesis related genes and lead to enhanced emission of VOCs, which consequently will affect the oviposition behavior of beet armyworm, *S. exigua*. To test this hypothesis, we first quantified and compared plant VOCs and transcript levels of genes regulating their production in tomato plants treated with CJ or not in the presence or absence of *S. exigua* caterpillar. The oviposition preference of *S. exigua* female moths was also compared between the above treatments.

5.2 Materials and Methods

5.2.1 Plants. Tomato seeds (*Solanum lycopersicon cv. Rutgers*) were surface sterilized with 10% bleach and 70% alcohol, rinsed a minimum of seven times in distilled water (to obtain sterile seeds free of unwanted microorganisms), and pre-germinated on moist sterilized paper towel (Proctor and Gamble, USA) for five days. The germinating seedlings were then transplanted to 8-cm- diameter plastic pots filled with sterilized Sunshine potting mix (SunGro Horticulture, Washington). Scotts® peat lite special fertilizer (Scotts-Sierra Horticultural Product Company, Marysville, Ohio), 20-10-20 water soluble NPK fertilizer mixture with micronutrients was applied one week after transplant (one-time application). Twenty-five milliliter of water was applied once every two days, depending on water needs of plants. The application amount was then increased to 50 ml when the plants were three weeks old. Plants were maintained at 24 ± 1°C, 60 ± 5 % RH 16:8 h (L: D) photoperiod. Five weeks old non-flowering potted tomato plants were used for the experiments.

5.2.2 Insects. S*podoptera exigua* eggs and pupae were purchased from Benzon Research (Carlisle, PA) and used to start laboratory colonies at Auburn University (Auburn, AL). Caterpillars were reared on laboratory-prepared pinto bean artificial diet (Shorey and Hale 1965) and maintained at 25 ± 1°C, 75 ± 5% RH and 14:10 h (L: D) photoperiod. Following emergence, adults were supplied with 10% sugar water and maintained at 25 ± 1°C, 75 ± 5% RH and 14:10 h (L: D) photoperiod (same environmental conditions for the caterpillars). Twenty-four hours after emergence, female and male moths were paired in a plastic container to encourage mating. Females aged 48 h were used for oviposition experiments.

5.2.3 *cis*-Jasmone Treatment. *cis*-Jasmone (94 % purity, Alfa Aesar, UK) was dissolved in 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer pH 6 aqueous solutions with 0.1 % non-ionic surfactant Tween-20 (Amresco, OH, USA). Using hydraulic nozzle, 753.6 µl of CJ solution which is equivalent to the 50 g/ha in 200 liters/ha used by Bruce et al. (2008) was applied to 5-week-old plants and a similar amount of water (753.6 µl) was applied to control plants. After allowing the plants to dry up, each plant was enclosed for 24 h in separate free air circulation jars to prevent interaction between treatments. Past studies have demonstrated that neither MES buffer plus Tween-20 (Zebelo et al. 2014) nor water alone (van Hulten et al. 2006) induces plant defense signaling in tomato and *Arabidopsis*, respectively. The plant treatments compared were: 1) CJ-treated plants with *S. exigua* caterpillar infestation (CJI), 2) untreated plants with *S. exigua* caterpillar infestation (UI), 3) CJ-treated plants without *S. exigua* caterpillar infestation (CJ), 4) untreated plants without *S. exigua* caterpillar infestation (U), 5) CJ-treated mechanically injured (CJMI), and 6) untreated mechanically injured (UMI). After 24 h has elapsed (i.e. post CJ and water treatments), both CJ- and U (control) plants were then exposed to caterpillar infestation and mechanical damage. Twenty 2nd instar *S. exigua* caterpillars were placed on individual plants

for a period of 6 h. The caterpillars were then removed at the end of 6 h and the plants were immediately thereafter used for VOC collection and oviposition bioassays. The same plant materials from which the VOCs were collected were used for gene expression analysis.

5.2.4 Headspace VOC Collection and Analysis. Collection of headspace plant VOCs was done as described by Ngumbi et al. (2009) with minor modifications. Headspace VOCs were collected from six biological replicates of the following treatments: 1) CJI, 2) UI, 3) CJ, 4) U, 5) CJMI, and 6) UMI. Plants used for headspace volatile collection were five weeks old. The pot with the potting soil was wrapped with aluminum foil to minimize contamination. The pot was then placed in an air-tight 5 L volatile collection chamber and a purified (activated charcoal) air stream (350 ml min^{-1}) was passed over the plant at room temperature for 24 h under an artificial light source generating 50 μmol m^{-2} s^{-1} and 16:8h light-night photoperiod. Headspace VOCs were trapped using a trap containing 50 mg of Super-Q (Alltech Associates, Deerfield, IL) and eluted with 300 μl of methylene chloride. The resulting extracts were stored in a freezer (at -20 °C) until use. One microliter of each headspace VOC extract was analyzed by gas chromatography (Agilent Technologies, mod. 7890A) coupled with a mass spectrometry (Agilent technologies, mod. 5975C) as described (Zebelo et al. 2014). Compounds were identified by comparing mass spectra and retention times with the mass spectra of search software v2.0 using the National Institute of Standards and Technology 98 library (NIST, Gaithersburg, Maryland). To quantify the compounds that were identified in the samples, external calibration curves were made with standard solutions of commercially available synthetic compounds, purity 95-99% (Sigma® Chemical Co., St. Louis, Missouri) of representative compounds (cis-3-hexenal, α-pinene, and (*Z*)-β-caryophyllene (Zebelo et al. 2014).

5.2.5 Quantification of Transcript Levels of Defense Related Genes in Tomato Plants

Plants.To determine the transcript levels of defense related genes, tomato leaves were collected from the same plants (CJI, UI, CJ, U, CJMI, and UMI) used in the headspace VOC collection experiment and immediately frozen in liquid nitrogen. Frozen samples were ground to fine powder in liquid nitrogen with mortar and pestle and total RNA was isolated from 100 mg of each leaf tissue using Spectrum™ plant total RNA kit (Sigma Aldrich USA), according to the manufacturer's instructions. RNA concentration and purity was determined using a NanoDrop™ Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, USA), and the integrity of RNA was also assessed by 1% agarose gel electrophoresis and ethidium bromide staining. The presence of contaminant DNA in the RNA samples was verified by PCR using specific primers of a known gene and gel electrophoresis analysis. Since the analysis did not show fragments of genomic DNA in all the samples tested, first strand cDNA was then synthesized from 200 ng RNA using a Goscrpit™ Reverse Transcription System Kit (Promega, USA) at the manufacturer's specifications.

The transcript levels of lipoxygenase (*LOX2*), allene oxide synthase (*AOS*) and four terpene synthase (*TPS*) genes (*TPS5*, *TPS7*, *TPS12*, and *TPS27*) known to be involved in tomato defense were measured by quantitative RT-PCR (Table 1). *TPS5* and *TPS7* genes are involved in biosynthesis of certain monoterpenes while *TPS12* and *TPS27* genes are involved in sesquiterpene biosynthesis pathway (Table 1). PCR was carried out on an ABI 7500 Real Time PCP System (Life Technologies, Carlsbad, CA, USA) with a 96-well rotor. The amplification reactions were performed with 25 µl of mixture consisting of 12.5 µl of PerfeCTA® SYBR® Green Fastmix®ROX qPCR Master Mix (Quanta Biosciences, Inc, USA), 0.5 µl of cDNA and 100 nM primers (Integrated DNA Technologies, Coralville, IA, US). Relative RNA levels were

calibrated and normalized with a reference housekeeping gene Ubiquitin carboxyl-terminal hydrolase 6 (*UBP6)*. PCR conditions were determined by comparing threshold values in a dilution series of the RT product, followed by non-template control for each primer pair. Calculation of relative expression levels of genes was done using the method developed by Pfaffl (2001). A suitable melt curve analysis was also performed for three biological samples and three technical replicates.

5.2.6 Oviposition Preference Test with Tomato Plants. Several experiments were designed to test the oviposition preference of female *S. exigua*. Choice experiments included CJI versus UI and CJ versus U. We also compared oviposition choice between CJMI and UMI. All experiments were carried out in the greenhouse by presenting the pair of treatments to females in a black cloth cage (76 × 80 × 115 cm tall). The two plants were separated 70 cm apart in the cage. Previous reports have showed that a minimum distance of 50 cm could eliminate interference effect of volatiles from emitter plant on neighboring receiver plants (Heil and Adame-Álvarez 2010; Kautz et al. 2014). Both pairs of plants had the same number of leaves and there were no noticeable height differences. Pots were wrapped in aluminum foil to cover the soil surface so that only the stem and leaves of the plant were exposed. The cage was placed on a greenhouse bench and left overnight. Eight mated *S. exigua* females were placed in the center of the cage (18:00 to 6:00) and to oviposit overnight. The number of eggs on each plant was recorded, and the test was replicated eight times and new sets of plants and insects were used for each replication.

5.2.7 Oviposition Preference Test with Filter Papers. In order to confirm the role of VOCs released from the plant treatments in mediating oviposition behavior of *S. exigua*, two oviposition experiments were carried out using filter papers permeated with headspace VOCs

collected from the plant treatments. Experiment 1 compared VOCs from CJI versus UI, while experiment 2 compared VOCs from CJ versus U. The main difference between this experiment and the experiment with plants (see above) was that filter papers permeated with VOCs collected from the treatments mentioned above were tested in these experiments instead of presenting whole plants to moths for choice of oviposition host. The experiment was carried out in cages in the laboratory at 25 ± 1 ^{o}C and 70 ± 5% RH. Two 5.5 cm diameter filter papers (Whatman #1, Maryland) representing a pair of treatments were permeated with 30 µl of headspace VOCs from the test plant treatments and presented to moths in a fine mesh plastic container (15 × 6 × 15 cm) in the laboratory. The filter papers were placed at diagonal ends of the container in the laboratory. Headspace VOCs were collected as previously described. Eight mated female *S. exigua* were placed in the center of each cage and left overnight to oviposit on the filter paper treatments. The numbers of eggs on each filter paper were recorded, and the test was replicated five times using new sets of filter papers and insects.

5.2.8 Plant Fitness. Fitness cost of CJ treatment was investigated with five weeks old plants. Treatments for this experiment included: CJI, UI, CJ, U, CJMI, and UMI. Fifteenth day post treatment, the numbers of flowers were recorded for five replicates per treatment and presented as average number of flowers on individual plants.

5.2.9 Statistical Analyses. Data on the emission of VOCs and relative gene expression values were analyzed and compared using a one-way ANOVA followed by Tukey-Kramer HSD at $P < 0.05$. The VOCs data was analyzed also with multivariate statistics (i.e., principal component analysis (PCA)) to better visualize which compounds contributed most to the separation between our samples. Relative gene expression data were first log10 [X+1] transformed prior to analysis.

Previous report showed that *S. exigua* females prefer to lay eggs on cage wall than on plants (Greenberg et al. 2002). Also, our preliminary data showed that *S. exigua* laid more eggs on cages compared to the filter paper treated with methylene chloride solvent and filter paper without solvent choices. Based on this observation, the numbers of eggs laid only on plants were considered as the key measure of oviposition choice by moths. Data from the oviposition experiments did not follow a normal distribution even after appropriate data transformation was done, so, they were analyzed using the non-parametric Wilcoxon rank-sum test at $P<0.05$. All analyses were performed in JMP software (JMP® 12, SAS Institute USA).

5.3 Results

5.3.1 *cis*-Jasmone Affects the Emission of Headspace VOCs in Tomato Plants. A total of 18 compounds were identified from the headspace of CJ-treated plants with *S. exigua* caterpillar infestation (CJI) compared to the 17 compounds detected in the headspace of untreated plants with *S. exigua* caterpillar infestation (UI), nine compounds detected in untreated mechanically injured plants (UMI), nine compounds detected in CJ-treated mechanically injured plants (CJMI), eight compounds detected in CJ-treated plants without caterpillar infestation (CJ) and nine compounds detected in untreated plants without caterpillar infestation) (U) (Table 2). The CJI and UI treatments had 17 compounds in common. The only qualitative difference was the detection of τ-terpinene in CJI, which was not detected in UI. Six of the 17 compounds common to CJI and UI treatments were significantly elevated in CJI including one green leaf volatile (i.e., *cis*-3-hexenal), four monoterpenes (i.e., β–cymene, τ-terpinene, m-cymene and α-phellandrene), and one sesquiterpene (germacerene-C). In contrast, certain monoterpenes (i.e., α-terpinene, *cis*-ocimene) and sesquiterpenes (i.e., (Z)-β caryophyllene and α-humulene) were

emitted in significantly higher amounts in UI compared to CJI (Table 2). The data also showed qualitative differences between CJ and U, and between CJMI and UMI treatments (Table 2). Three of the VOCs (α-pinene, linalyl acetate and τ-terpinene) were detected only in U while two (α-terpinolene and α-humulene) were detected only in CJ plants. One compound, (+)-4-carene was significantly higher in CJMI than the in the other treatments. The data also showed qualitative differences between CJMI and UMI: α-terpinolene was detected in CJMI but not in UMI, whereas α-humulene was detected in UMI but not in CJMI.

The score plot of PCA 1 and 2 showed clear demarcation in the VOC profile between samples from different treatments. The score plot also showed that the VOC profile of CJI and UI plants are clearly different from each other and from the other treatments (i.e. U, CJ, UMI, and CJMI). Volatile profiles of U, CJ, UMI, and CJMI nearly overlapped (Fig. 1). PCA 1 accounted for 55.4% of the total variance, while PCA 2 accounted for 18.2% of the total variance (Figure 1).

5.3.2 *cis*-Jasmone affects Transcript of Defense Genes in Tomato Plants.

Quantitative RT-PCR analysis revealed significant differences among the treatments in the transcript levels of defense-related genes including genes encoding several terpene synthase genes (Figure 2). Two key genes involved in the biosynthesis of jasmonic acid (JA) and green leaf volatiles, *LOX2* ($F_{5, 12}$ = 310.52; P = 0.0001) and *AOS* ($F_{5, 12}$ = 15.69; P = 0.0001), were significantly up-regulated in CJI compared to UI, CJ, U, CJMI, and UMI (Figure 2A). Interestingly, CJ treatment without caterpillar infestation did not up-regulate the expression of *LOX2* (Figure 2A). Similarly, the transcript levels of the genes involved in synthesis of certain monoterpenes including *TPS5* ($F_{5, 12}$ = 18.30; P = 0.0001) and *TPS7* ($F_{5, 12}$ = 13502; P = 0.0001) were significantly higher in CJI compared to other treatments (Figure. 2B). In contrast, the

transcript level of *TPS12* was significantly higher in CJMI ($F_{5, 12}$ = 130.32; P = 0.0001) while *TPS27* was significantly higher in UI and UMI compared to other treatment plants ($F_{5, 12}$ = 19.71; P = 0.0005) (Figure 2C). These results are consistent with the VOC emission data which showed elevated emission of a green leaf volatile (*cis*-3-hexenal) and certain monoterpenes (i.e., β–cymene and α-phellandrene) in CJI plants.

5.3.3 *cis*-Jasmone Affects the Oviposition Behavior of *S. exigua*. *S. exigua* laid numerically fewer eggs on CJI plants than on UI plants. Approximately two and a half times more eggs were deposited on UI plants (407 eggs) than on CJI plants (174 eggs) but this was not significant (Z = 0.90, P = 0.37), possibly due to the high variation among the replicates (Figure. 3A). Similar results were observed in the filter paper substrate choice test in which *S. exigua* laid significantly fewer eggs on filter papers permeated with VOC collected from CJI plants than on filter papers permeated with VOC collected from UI (Z = 2.1, P = 0.04) (Figure 3B). However, in the experiment in which CJ plant was paired with U plant, *S. exigua* laid more eggs on CJ plant although the difference was not significant (Z = -023, P = 0.79) (Figure 4A). Similarly, no significant difference was recorded on the filter paper substrate choice test CJ and U plant treatments (Figure 4B). A comparison between mechanically injured plants showed that *S. exigua* laid more eggs on CJMI than UMI plants, although the difference was not significant (Figure 5).

5.3.4 Tomato Flowering is Not Affected by CJ Treatment. Plant fitness cost was determined by counting numbers of flowers produced by CJI, UI, CJ, CJMI, and UMI. There was no significant difference in the numbers of flowers produced by all treatment plants although CJI plants produced more flowers ($F_{5, 23}$ = 0.67; P = 0.65) (Figure 6).

5.4 Discussion

Our results showed that pre-treatment of tomato plants with *cis*-jasmone (CJ) followed by caterpillar infestation caused quantitative and qualitative changes in the emission of VOCs that influenced the oviposition preference of *S. exigua*. The results of the oviposition preference tests showed that *S. exigua* females could distinguish between CJ-treated plants with *S. exigua* caterpillar infestation (CJI) and untreated plants with *S. exigua* caterpillar infestation (UI) and also their headspace odors and laid significantly fewer eggs on CJI plants. The results recorded in the oviposition tests are consistent with the VOC and molecular data, indicating that many defense-related genes were up-regulated in CJI plants. Together, our results demonstrate that CJ can prime herbivore induced VOC emission in plants with important ramifications for oviposition by herbivorous insect.

The results of the headspace VOC analysis showed increased emission of six VOCs, including green leaf volatiles (i.e., *cis*-3-hexenal), monoterpenes (i.e., *β*–cymene, *τ*-terpinene, m-cymene and *α*-phellandrene), and one sesquiterpene (germacerene-C) in CJI plants, suggesting that CJ can manipulate tomato plant physiology and induce changes in the production or emission of herbivore induced plant VOCs. Several plant VOCs including (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), (*E*)- *β*-ocimene were induced by pre-treatment with CJ (Birkett et al. 2000; Hegde et al. 2012). In this study, CJ-treated plant without caterpillar infestation did not emit any of the common green leafy volatiles which supports our gene expression result indicating that CJ may suppress the activity of LOX genes, an important gene family involved in plant defense pathway (Matthes et al. 2010). These results

suggest that CJ treatment in combination with caterpillar infestation enhanced emission of plant VOCs.

The biosynthesis of most plant VOCs is mediated by terpene synthase genes and the results of the gene expression studies suggest that their activity can be enhanced by priming tomato plants with CJ combined with caterpillar infestation. Specifically, our data showed that many defense-related genes were up-regulated in CJI plants compared to UI plants. These include *LOX2* and *AOS,* key genes involved in the biosynthesis of JA and green leaf volatiles (GLVs) (Park et al. 2007; Zebelo et al. 2014), and *TPS5 and TPS7***,** which encode monoterpene synthases that catalyze the formation of volatiles (Falara et al. 2011). These results suggest that treatment of plants with CJ and subsequent caterpillar feeding can prime tomato plants to increase levels of genes encoding terpene synthases in tomato, as reported by Oluwafemi et al. (2013). Menzel et al. (2014) also reported a similar result in which treatment of lima bean with JA and simultaneous or sequential herbivory by *Tetranychus urticae* increased the transcript levels of the gene involved in the biosynthesis of (*E*)-*β*-ocimene. Our data also showed that *TPS12* was highly expressed in CJ and CJMI plants and minimally expressed in UI and CJI plants but this high expression did not correlate with metabolite products. Falara et al. (2011) reported that the expression of *TPS12* gene correlated with the biosynthesis of *β*-caryophyllene and *α*-humulene in tomato trichromes. Similarly, Zebelo et al. (2014) showed that the emission of *β* -caryophyllene and *α*-humulene correlated with increase in transcript level of *TPS12* genes in tomato plants injured by *S. exigua* caterpillars. Furthermore, *TPS27* encodes the biosynthesis of sesquiterpene in tomato (Falara et al. 2011). However, in Arabidopsis, *TPS27* is involved in the biosynthesis of 1,8-cineole and other minor monoterpene products including myrcene, *α*-pinene, *β*-pinene, sabinene, and limonene produced from roots of the plant (Roos et al. 2015).

Priming sometimes can have fitness cost in plants (see Martinez-Medina et al. 2016 for recent review) but our data showed that priming with CJ did not result in a fitness cost in tomato. Interestingly, CJ-treated plants with caterpillar infestation produced more number of flowers compared to untreated plants with *S. exigua* caterpillar infestation (UI) or CJ-treated plants without caterpillar infestation (CJ), although the difference was not significant. Whether the rate of defense response in terms of release of secondary metabolites that mediate direct or indirect defense is faster in CJ-primed plants than in untreated plants was not investigated in this study. Hegde et al. (2012) reportd that subsequent attack by herbivores on CJ-primed cotton plants led to faster production of DMNT and TMTT than water-treated controls. Together, our results suggest that the combined effect of CJ treatment and insect herbivory likely regulates defense pathways that are under the control of JA, even though both compounds have different signaling roles (Matthes et al. 2010). As shown in this study, CJ treatment of plants without caterpillar infestation did not increase the expression level of *LOX2*, an important gene involved in biosynthesis of JA. JA plays a critical role in the regulation of plant defense against herbivores. Whether this suppression of *LOX2* gene is responsible for the higher numbers of eggs recorded on CJ plants is not clear.

cis-Jasmone has been reported as an inducer of plant defense against many insect species (Pickett et al. 2007; Moraes et al. 2008; Bruce et al. 2008), but studies on the role of CJ in mediating oviposition behavior of insects are limited. Our results agree with those reported by Oluwafemi et al. (2013), which showed that pre-treatment of maize plants with CJ followed by herbivore infestation increased the emission of defensive sesquiterpenes and repelled the leafhopper, *C. storeyi*. In the present study, *S. exigua* females deposited numerically fewer eggs on CJI plants than on UI plants. Moreover, *S. exigua* laid significantly fewer eggs on filter papers

permeated with VOC collected from CJI plants than on filter papers permeated with VOC collected from UI plants. Although, plant volatiles mediate oviposition behavior in many insect species (De Moraes et al. 2001; Reddy et al. 2004; Proffit et al. 2011), some insects also rely on tactile and visual cues for selecting host plants either for reproductive functions or for food. Our data showed that *S. exigua* females laid more eggs on cage walls (95%) than on filter papers treated with or without solvent (5%). In field cage experiments, Greenberg et al. (2002) demonstrated that *S. exigua* prefers to lay eggs on walls of cages than on plants. This behavior may not be surprising given that oviposition behavior is a heritable trait. Rearing insects continuously in artificial environment can affect their innate choice for oviposition host (Damodaram et al. 2014).

These results demonstrate that CJ treatment followed by caterpillar infestation can prime tomato plant to defend itself through enhanced production of defensive secondary metabolites which may confer resistance against herbivore oviposition. Farmer and Ryan (1990) reported that exogenous application of methyl jasmonate (MeJA), a common plant secondary compound, induced the synthesis of defensive proteinase inhibitor proteins in treated tomato plants. Although our aim was not to investigate the role of nonvolatile cues in this study, leaf surface metabolites have been shown to play a role in the acceptance of plants as host for oviposition by several lepidopteran insects including *Cydia pomonella* (Lombarkia and Derridj 2008) and *Polygonia c-album* (Moz et al. 2012). There is the likelihood that the reduced oviposition recorded on CJI plants in this study was due to a combined effect of production of VOCs and other nonvolatile cues but this aspect will be the subject of future studies. In addition to conferring protection to treated plants, CJ treatment can also prime neighboring plants for defense since CJ is produced naturally by herbivore infested plants (Loughrin et al. 1994;

Oluwafemi et al. 2013). Our findings may have potential applications in pest management by using CJ treatments plus minimal caterpillar infestation to repel herbivores or attract natural enemies of herbivores. Further studies are needed to investigate the tritrophic effects of CJ priming of tomato plants and to explore the potential use of CJ to manage tomato pests in the field.

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Table 1 Primers used for RT-qPCR

Defense Genes	Primer pairs	Primer sequences (5'-3')	GenBank (AN)	References
LOX2	Forward	TGCAACACGCACCATTTATT	U37840	Heitz et al. 1997
	Reverse	GTGACAACACGTTTGGATCG		
AOS	Forward	GGGTGAAATCCTATTCGGGT	AF230371	Howe et al., 2000
	Reverse	CGCACTGTTTATTCCCCACT		
TPS5	Forward	CTATTTCCACCACAAGGCGT	AY840091	Bernays and Chapman, 2000
	Reverse	TTCATCATGTGATCCCTCCA		
TPS7	Forward	CAAGGAGTATGTTAATGTCAGG	JN412082	Bernays and Chapman, 2000
	Reverse	GCTTCATATAAGTTCAATATTCC		
TPS12	Forward	GCCCAATGGTTAAACAATGATAATC	JN412092	Bernays and Chapman, 2000
	Reverse	ATATAACGTGTTTATCACGCGTGTG		
TPS27	Forward	ATGGGCAGAATTTTGCAAAGCACTG	JN412084	Bernays and Chapman, 2000
	Reverse	CCATCTCCAGAGAGGTACATGACA		

Lipoxygenase (*LOX2*), Allene oxide synthase (*AOS*), Tomato monoterpene synthase 5 (*TPS5*), Tomato terpene synthase 7 (*TPS7*), Tomato terpene synthase 12 (*TPS12*), and Tomato terpene synthase 27 (*TPS27*). Accession Number = AN.

Table 2 Quantitative analysis of emission of volatile organic compounds (VOCs)

Compounds	U	CJ	UMI	CJMI	UI	CJI
Green leafy volatiles						
cis-3-hexenal	0	0	0	0	1.96±0.39b	3.69±0.39a
4-hexen-1-ol	0	0	0	0	2.26±0.53ab	3.31±0.53a
Monoterpenes						
α-pinene	2.24±6.05bc	0	10.45±7.41bc	19.98±7.41abc	41.84±6.05a	27.69±6.05ab
β-pinene	9.12±31.57b	11.87±31.57b	0	0	227.43±31.57a	211.03±31.57a
β-cymene	8.57±18.94b	16.40±18.94b	2.05±23.20b	4.392±23.20b	8.53±18.94b	198.65±18.94a
α-terpinene	201.67±296.92b	330.05±296.92b	6.09±363.65b	13.39±363.65b	2522.55±296.92a	1373.15±296.92ab
α-phellandrene	29.63±225.25b	41.02±225.25b	22.43±275.87b	48.60±275.87b	457.95±225.25b	2385.23±225.25a
τ-terpinene	8.626±16.71	0	0	0	0	182.56±16.71
Sabinene	4.86±1115.20b	1046.82±1115.20b	494.49±1365.80b	1028.02±1365.80b	8330.42±1115.20a	8164.05±1115.20a
(+)-4-carene	0	0	126.77±35.96ab	216.40±35.96a	15.73±29.36b	5.14±29.36b
cis-ocimene	0	0	0	0	56.23±6.09a	31.69±6.09a
α-terpinolene	0	3.32±0.80ab	0	3.70±0.98ab	4.95±0.80a	5.26±0.80a
m-cymene	0	0	0	0	2.60±4.64b	50.46±4.64a
Ester(s)						
linalyl acetate	31.93±11.17b	0	0	0	97.55±11.17a	46.89±11.17b
Sesquiterpenes						
δ-elemene	0	0	13.85±9.96ab	8.73±9.96ab	45.34±8.13a	46.14±8.13a
(Z)-β-caryophyllene	1.39±31.98c	25.56±31.98bc	16.99±39.16bc	29.51±39.16bc	282.86±31.98a	144.89±31.98ab
α-humulene	0	1.3038±4.86c	4.55±5.96bc	0	40.58±4.86a	22.40±4.86ab
Germacrene-C	0	0	0	0	3.78±8.04b	89.60±8.04a

Table shows VOCs (μg g−1 fw) emitted by CJ-treated plants with *S. exigua* caterpillar infestation (CJI), untreated plants with *S. exigua* caterpillar infestation (UI), CJ-treated mechanically injured plants (CJMI), untreated mechanically injured plants (UMI), CJ-treated plants without *S. exigua* caterpillar infestation (CJ), and untreated plants without *S. exigua* caterpillar infestation (U). Data was collected from four to six plants (i. e. 4-6 biological replicates) per treatment. Means (±SE) within the same row having different letters are significantly different ($P < 0.05$).

Figure Legend

Figure 1 Score plot of principal component analysis (PCA) of volatile organic compounds identified from the headspace of CJ-treated plants with *S. exigua* caterpillar infestation (CJI), untreated plants with *S. exigua* caterpillar infestation (UI), CJ-treated plants without *S. exigua* caterpillar infestation (CJ), untreated plants without *S. exigua* caterpillar infestation (U), CJ-treated mechanically injured (CJMI), and untreated mechanically injured (UMI). n = 4-6 replicates per treatment.

Figure 2 Relative expressions of genes involved in the biosynthesis of green leaf volatiles (A), monoterpenes (B), and sesquiterpenes (C) in CJ-treated plants with *S. exigua* caterpillar infestation (CJI), untreated plants with *S. exigua* caterpillar infestation (UI), CJ-treated plants without *S. exigua* caterpillar infestation (CJ), untreated plants without *S. exigua* caterpillar infestation (U), CJ-treated mechanically injured (CJMI), and untreated mechanically injured (UMI). Different letters indicate difference ($P < 0.05$, Tukey-Kramer HSD). n = 3 replicates.

Figure 3 Number of eggs oviposited by *S. exigua* when offered a choice between CJ-treated plants with caterpillar infestation (CJI) versus untreated plants with caterpillar infestation (UI) (A) and numbers of eggs on filter paper permeated with headspace VOCs from CJ-treated plants with caterpillar infestation (CJI) versus filter paper permeated with headspace VOCs from untreated plants with caterpillar infestation (UI) (B). Fewer eggs were laid on CJI plants than UI plants. Significant difference was indicated by * (Wilcoxon rank sum test, $P < 0.05$).

Figure 4 Number of eggs oviposited by *S. exigua* when offered a choice between CJ-treated plants without *S. exigua* caterpillar infestation (CJ) versus untreated plants without *S. exigua* caterpillar infestation (U) (A) and number of eggs oviposited by *S. exigua* when offered a choice between filter paper permeated with headspace VOCs from CJ-treated plants without *S. exigua* caterpillar infestation (CJ) versus filter paper permeated with headspace VOCs from untreated plants without *S. exigua* caterpillar infestation (U) (B). The difference was not statistically significant (Wilcoxon rank sum test, $P > 0.05$).

Figure 5 Number of eggs oviposited by *S. exigua* when offered a choice between CJ-treated mechanically injured (CJMI) and untreated mechanically injured (UMI). The difference was not statistically significant (Wilcoxon rank sum test, $P > 0.05$).

Figure 6 Fitness of tomato plants induced by CJ priming in the presence or absence of *S. exigua* caterpillar infestation, and mechanical injury. CJ-treated and untreated five-week-old plants were infested with third instar caterpillar (10 per plant) for 48 hours. Number of flowers produced was recorded 15-day post infestation in CJ-treated plants with *S. exigua* caterpillar infestation (CJI), untreated plants with *S. exigua* caterpillar infestation (UI), CJ-treated plants without *S. exigua* caterpillar infestation (CJ), untreated plants without *S. exigua* caterpillar infestation (U), CJ-treated mechanically injured (CJMI), and untreated mechanically injured (UMI). The difference was not statistically significant (ANOVA, $P > 0.05$). n = 5 replicates.

Figure 1

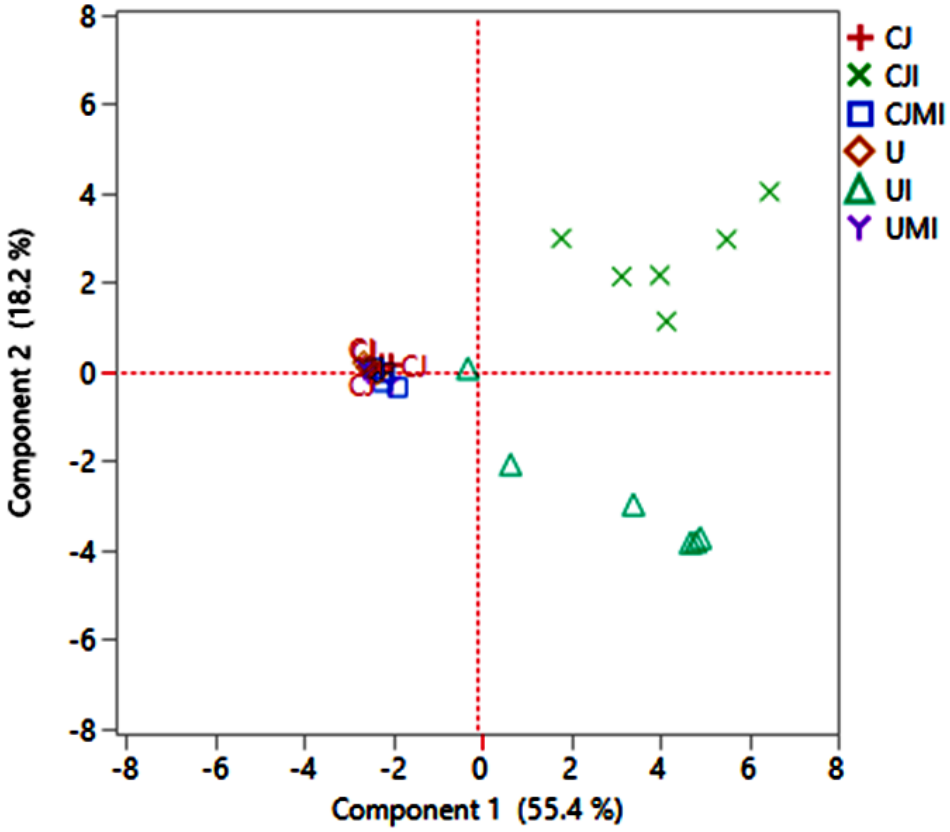

CJ
CJI
CJMI
U
UI
UMI
Component 2 (18.2 %)
Component 1 (55.4 %)
8
6
4
2
0
-2
-4
-6
-8

Figure 2

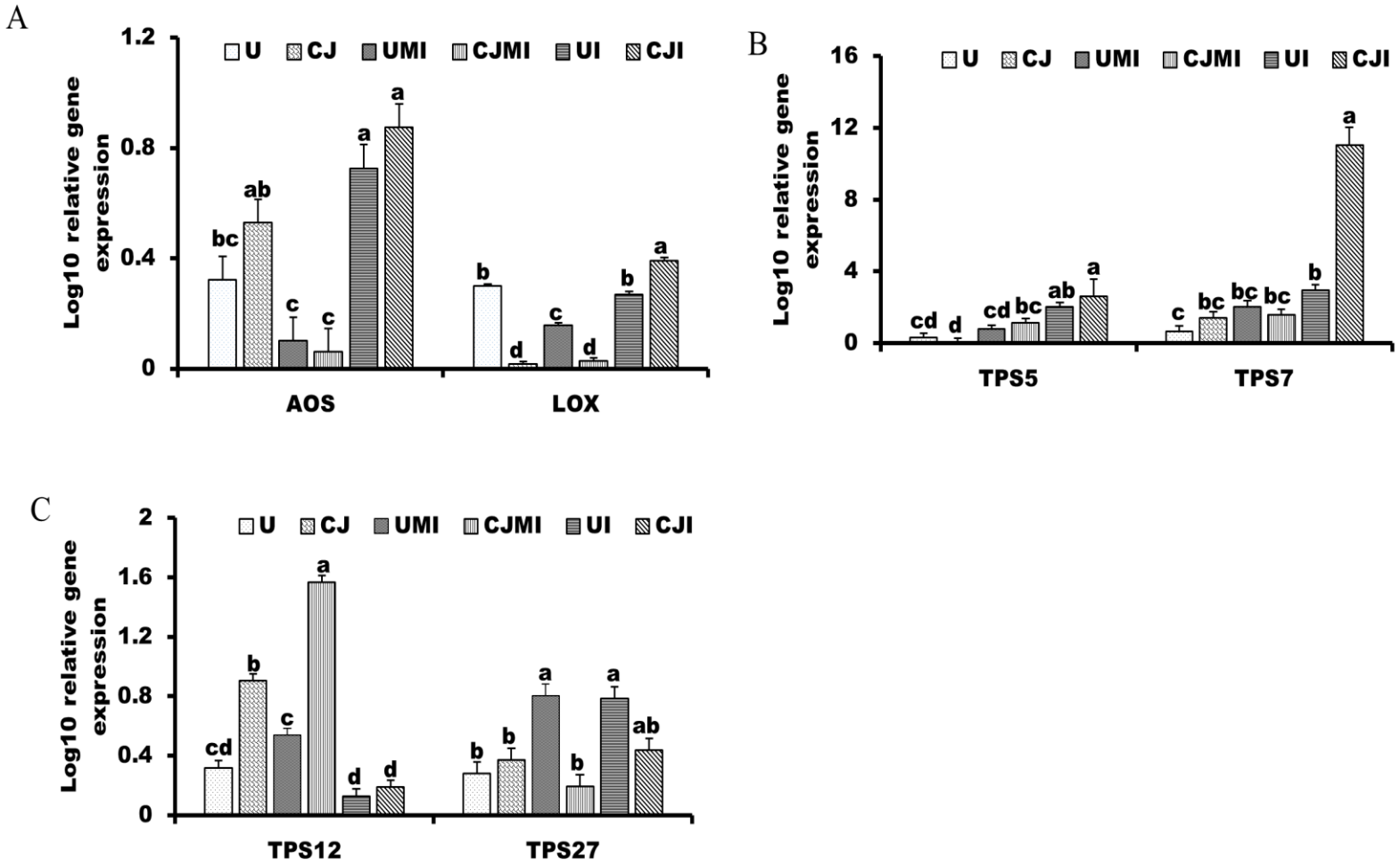

A
Log10 relative gene expression
U
CJ
UMI
CJMI
UI
CJI
AOS
LOX
B
TPS5
TPS7
C
TPS12
TPS27

Figure 3

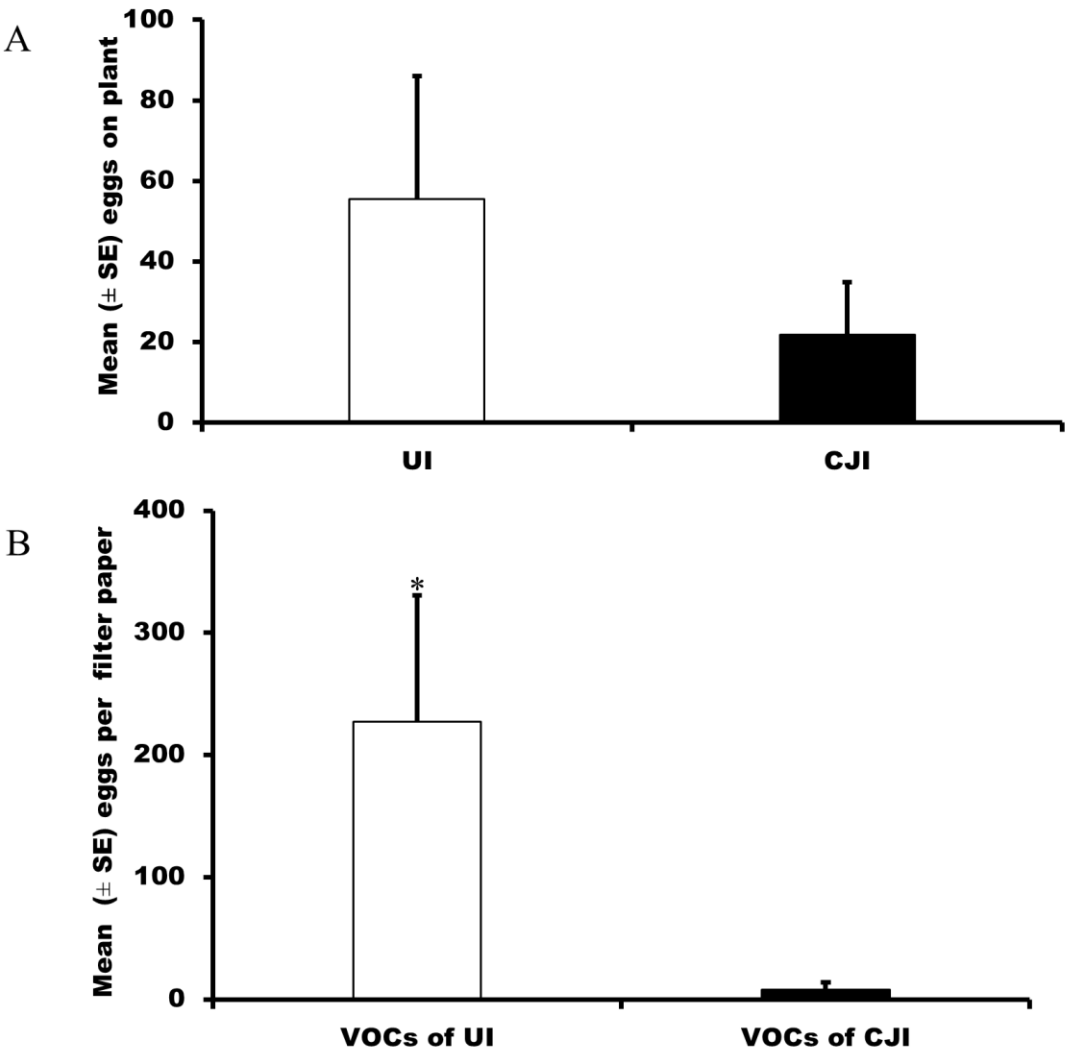

A
Mean (± SE) eggs on plant
100
80
60
40
20
0
UI
CJI
B
Mean (± SE) eggs per filter paper
400
300
200
100
0
*
VOCs of UI
VOCs of CJI

Figure 4

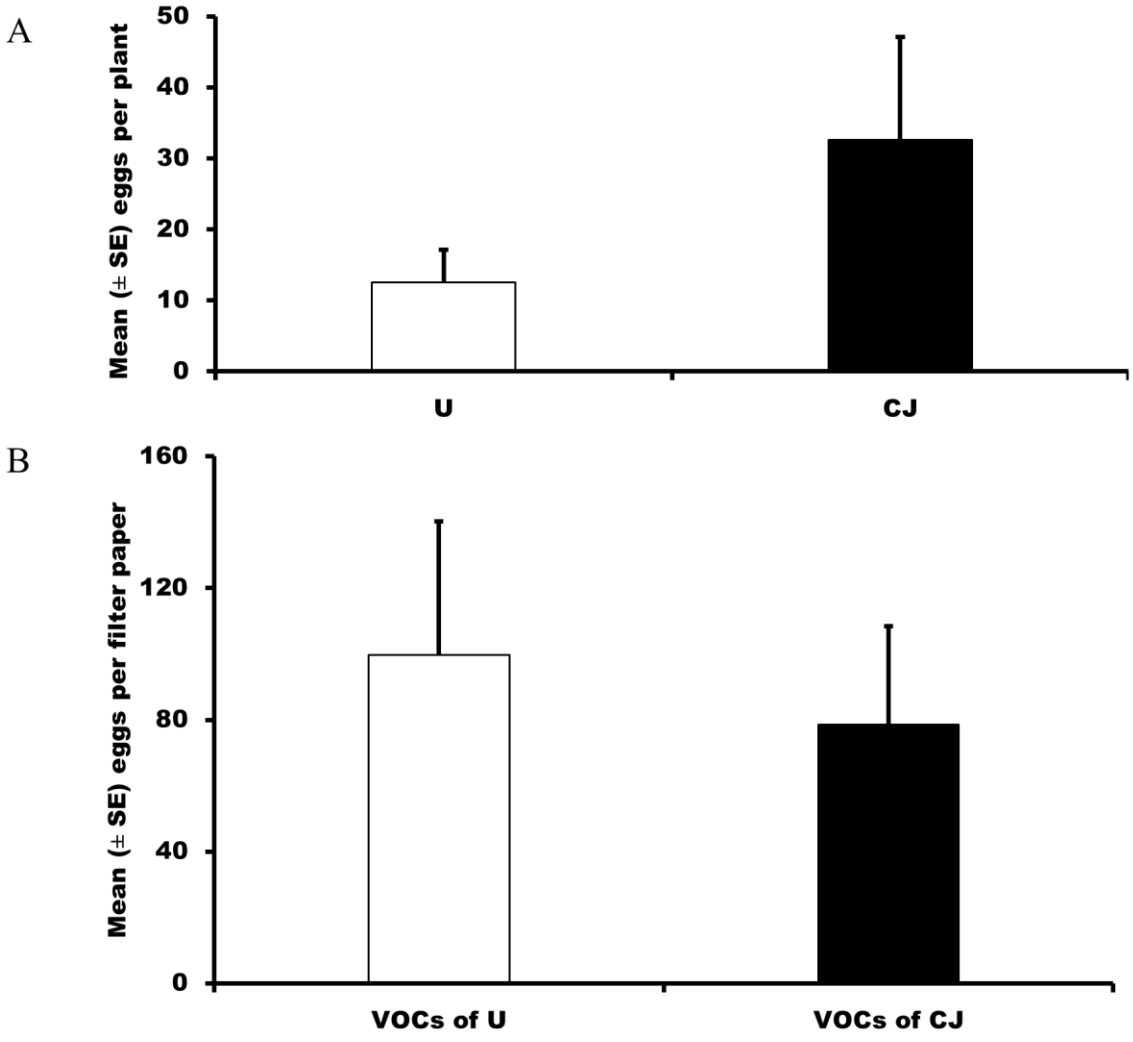

A
Mean (± SE) eggs per plant
50
40
30
20
10
0
U
CJ
B
Mean (± SE) eggs per filter paper
160
120
80
40
0
VOCs of U
VOCs of CJ

Figure 5

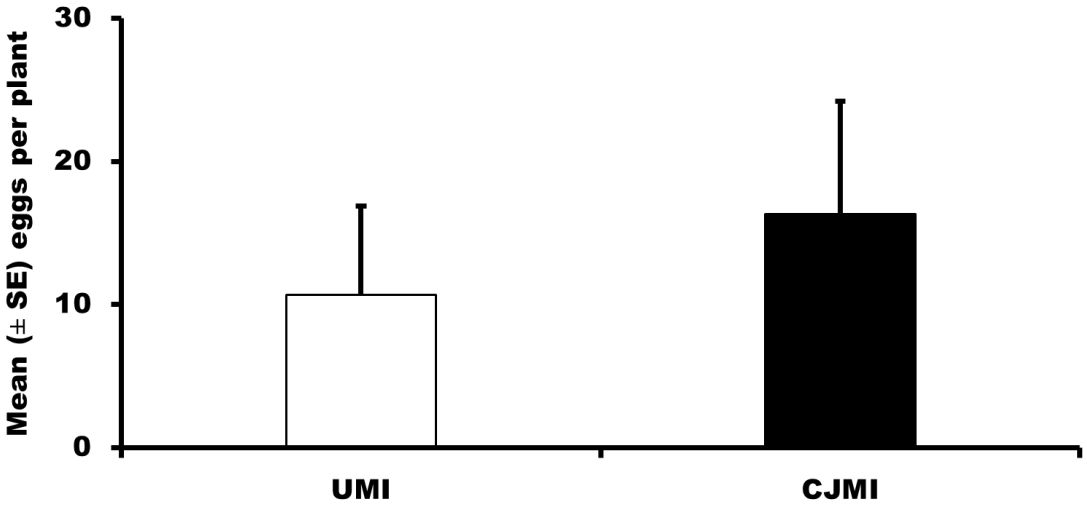
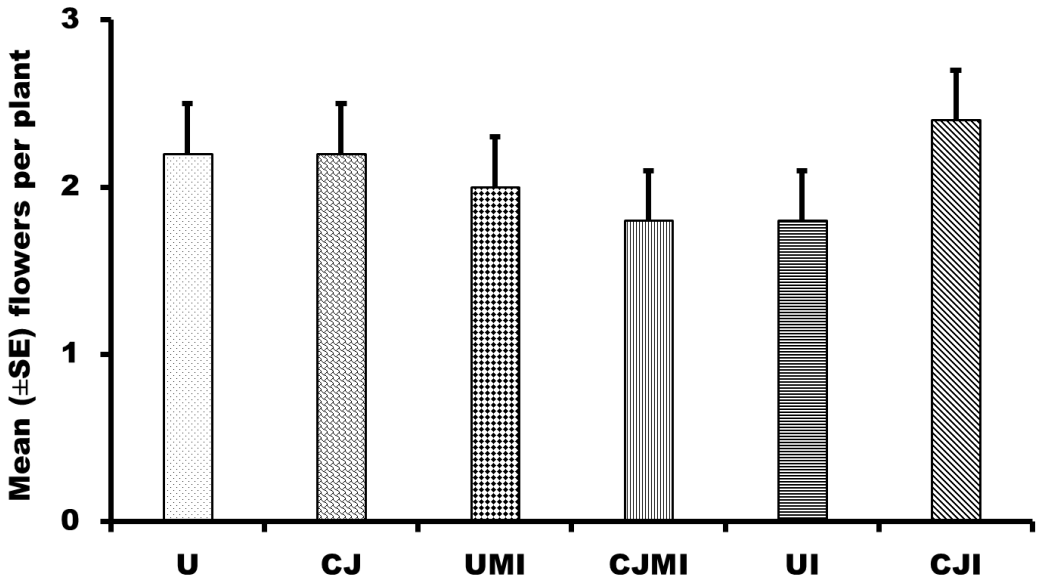


Figure 6



CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

6.1 Summary and Future Research

The first part of this dissertation investigated the co-evolutionary relationship between rhizobacteria and corn. The second aspect sought to understand the molecular mechanisms of *cis*-Jasmone (CJ)-mediated plant defense against lepidopteran herbivores via emission of volatile organic compounds (VOCs). Together, the application of spore-forming bacilli as seed treatments mediated host plant resistance against two herbivorous insect pests of corn: *Ostrinia nubilalis* (an aboveground generalist herbivore) and *Diabrotica virgifera virgifera* (a belowground specialist root herbivore). Results from the CJ study showed priming as key element in CJ induction of tomato plant defense against *Spodoptera exigua*.

The first study on rhizobacteria-mediated plant interactions investigated effects of spore forming bacilli on oviposition behavior of *O. nubilalis* moths as well as quantified VOCs in bacilli-treated corn. The results show that *O. nubilalis* moths preferred to lay eggs on untreated plants suggesting that the bacilli-treated plant was less attractive to moths. GC-MS analysis showed that bacilli-treated plants emitted fewer volatile organic compounds than untreated plants. This alteration in composition of VOC seems to explain the fewer number of eggs on bacilli-treated plants and could be a possible mechanism of rhizobacteria-mediated host plant resistance to herbivores. This study corroborates previous studies (Phelan et al. 1995; Phelan et al. 1996) that reported that improvement of soil quality by addition of compost affects plant health as well as deter *O. nubilalis* from laying eggs on plants. Colonization of lima bean by nitrogen-fixing rhizobia has been reported to alter the composition and quantity of plant volatiles that were

shown to deter Mexican bean beetle (*Epilachna varivestis*) from rhizobia-colonized plants (Ballhorn et al. 2013). Although bacilli treatment affected oviposition behavior of *O. nubilalis*, the data showed there was no effect on conspecific larval feeding on corn leaves, suggesting a possible involvement of other chemical, contact or morphological mechanisms. Further studies should be conducted to determine how individual strains differ from mixed bacilli strains in induction of plant secondary metabolites, using a metabolomic approach. Also, insect community ecology should be investigated to tease out the effect of environment on the performance of rhizobacteria in nature.

The second study of the dissertation shifted emphasis to belowground herbivores. Here, the effect of bacilli application on feeding preference and development of *D. v. virgifera* larvae was evaluated. The results showed that only the single *B. pumilus* strain INR-7 negatively affected feeding preference and development of the belowground specialist root feeder, suggesting that *Bacillus* strains could have varying effects on herbivores. Based on this finding, further feeding experiments with the individual strains that constitute blends 8 and 9 were conducted. The result showed that weight of *D. v. virgifera* larva fed plants treated with individual strains did not differ from those fed plant treated with blends of the bacilli, confirming that individual strains in the formulated bacilli were not antagonistic to one another. Furthermore, Blend-9 treated plant had increased growth in terms of all growth indices measured including shoot dry weight and root architecture. Thus, conducting a similar study over a longer time may reveal whether Blend-9 bacilli treatment can induce tolerance. Future mechanistic studies using gnotobiotic crop systems to discover novel genes and metabolic pathways involved in bacilli-mediated crop plant-insect interactions should be conducted. Analyses of the total transcriptome, metabolome, and proteome in the presence and absence of herbivores will be a

good place to start the inquiry. The data from transcriptome, metabolome, and proteome study can be confirmed in transgenic plants and compared with similar data from field studies.

Given that only *B. pumilus* strain INR-7 showed positive results against *D. v. virgifera* larvae from the second study, the strain was adopted to investigate rhizobacteria mediated belowground plant-insect tritrophic interactions. *Heterorhabditis bacteriophora*, a natural enemy of *D. v. virgifera* larvae was attracted to *B. pumilus* strain INR-7 treated intact plants with or without infestation with *D. v. virgifera* larvae. On the other hand, *H. bacteriophora* did not show attraction to VOC extracts from either corn roots infested or uninfested. In fact, the data showed that very few *H. bacteriophora* responded to VOC extracts than the bacilli-treated intact plants. Cautiously, the conclusion is that bacilli-mediated plant VOCs did not play a role in the response of *H. bacteriophora*. Root cap exudates at low concentrations have been reported to increase the activity of *H. bacteriophora* against *Galleria mellonella* larvae (Hiltpold et al. 2015). However, it is not known whether specific *Bacillus* spp. mediate composition of root exudates or organic acids. Studies should be conducted to test the hypothesis whether *Bacillus* spp. alter composition or abundance of root exudates /or organic acids and the consequences on behavior and activities of infective juveniles of *H. bacteriophora*.

Having established the effect of rhizobacteria on mediation of host plant resistance, using corn-*D. v. virgifera*-*H. bacteriophora* as model system, the study digressed and focused on natural plant elicitors. This last part of the dissertation investigated mechanisms of CJ-mediated plant defense against lepidopteran herbivores. The gene expression and VOCs data indicated that priming is the key element in CJ induction of tomato plant defense against *S. exigua*. CJ treatment without caterpillar feeding played minimal role in mediating VOCs and oviposition by *S. exigua.* Evidence of CJ's role in plant defense against phloem-sucking insects is well

documented but this study is the first report of the effect of CJ on behavioral preference of moths. The main conclusion from this study, as it relates to the test insect is that CJ alone, in the absence of biological modulators like caterpillars may induce plant susceptibility, but this scenario is not likely to occur in the field as plant-eating insects are abundant in nature. Investigation of CJ-mediated feeding guild interactions should provide insight on potential application in agriculture.

This dissertation showed that alteration of corn VOCs by spore-forming *Bacillus* spp. is a mechanism by which bacilli mediate host plant resistance. *O. nubilalis* females preferred untreated plant as an oviposition host than bacilli-treated plant. *Bacillus pumilus* strain INR-7 influenced belowground corn-insect and tritrophic interactions, a finding which is new and presents the potential for integration in management of aboveground and belowground plant herbivores. Also, mechanisms of plant-derived elicitors indicated priming as a major element of defense against leaf chewing insects. In summary, this gap-filling study illustrates the potential of rhizobacteria and natural plant products as insect pest control tools in agriculture.

6.2 References

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Phelan PL, Mason JF, Stinner BR (1995) Soil-fertility management and host preference by European corn borer, *Ostrinia nubilalis* (Hübner), on Zea mays L.: a comparison of organic and conventional chemical farming. Agric Ecosyst Environ 56:1-8.

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