Effects of Plant Growth-Promoting Rhizobacteria (PGPR) Treatment of Cotton on the Oviposition Behavior of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae)

by

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Abstract

Treating crops with a group of naturally-occurring root bacteria, termed plant growth-promoting rhizobacteria (PGPR), has been shown to increase plant growth and enhance plant health in different ways. Although much is known about the effects of PGPR treatment on plant growth and disease resistance, very few studies have explored how the use of PGPR may affect plant-insect interactions. This study was carried out to investigate the effect of PGPR treatment of cotton plants on the host location and acceptance behavior of a generalist herbivorous insect, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (beet armyworm). Specifically, the goal of this study was to investigate the effects of PGPR treatment of cotton plants on the oviposition behavior of *S. exigua* with experimental design considerations for the presence of headspace volatile organic compounds which may affect this behavior.

In chapter II, choice and no-choice oviposition studies were used to evaluate the influence of treating cotton plants with PGPR on the oviposition behavior of *S. exigua*. In choice and no-choice bioassays, the oviposition behavior and preference of females was compared among four treatments: i) untreated cotton plants (control), cotton plants treated with ii) *Bacillus pumilis* strain INR-7 (a single PGPR strain), iii) Blend 8 (a blend of 4 PGPR strains), and iv) Blend 9 (a blend of 4 PGPR strains). In choice oviposition bioassays, females preferred to oviposit on untreated cotton plants (control) compared to any of the three PGPR treatments. In particular, Blend 9-treated plants had significantly fewer eggs compared to untreated plants. In no-choice bioassays, however, similar numbers of eggs were deposited on untreated plants and

PGPR-treated plants. These results show that *S. exigua* females are capable of distinguishing between the PGPR-treated and untreated plants, as well as between some of the PGPR-treated plants. More notably the results showed that PGPR treatments influence the oviposition behavior of *S. exigua* and that some PGPR treatments may be able to increase plant health by reducing oviposition by lepidopteran pest species.

In chapter III, three different designs of no-choice oviposition studies used to evaluate the oviposition behavior of a *S. exigua* were compared and discussed. Females were allowed to oviposit overnight on one of four treatments: i) untreated cotton plants (control), cotton plants treated with ii) *Bacillus pumilis* strain INR-7 (a single PGPR strain), iii) Blend 8 (a blend of 4 PGPR strains), and iv) Blend 9 (a blend of 4 PGPR strains). In two of the designs, all four of these treatments were evaluated, but distances separating individual cages were increased in the second design because it was suspected that there was contamination between treatments from volatile organic compounds (VOCs) released by the plants. Reductions in P-values with increased distances between individual cages correspond with the hypothesis that VOCs associated with the headspace of different treatments studied are capable of diffusing through cloth and affecting the oviposition behavior of *S. exigua*. This hypothesis was further supported by a third design evaluating only two of the treatments (the untreated control and Blend 9), which reduced the numbers of treatments whose VOC profiles may be design contaminants. A comparison of the results reveals the potential for PGPR treatment of plants to either stimulate or reduce egg-laying behavior of Lepidoptera under short-range, no-choice conditions.

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Table of Contents

List of Tables

List of Figures

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1   Insects and plant hosts

Many studies of plant-insect interactions have focused on understanding the factors and

mechanisms affecting selection of host plants by herbivorous insects. In the area of crop

protection, this information can be especially valuable. In 2009, the United States cotton industry

spent more than $57 million to treat cotton fields with insecticides at planting, over $36 million

to do so throughout the season, more than $34 million to scout and monitor for insects, and

incurred an estimated loss of $502 million due to insects, among which bollworms, armyworms,

and budworms all ranked in the top five (Williams, 2010). Despite current pest control measures,

including the use of transgenic crops and chemical insecticide applications, lepidopteran pests

continue to be significant agricultural pests requiring new management tactics as resistant

populations develop (Temple et al., 2009). Identification of factors affecting host-plant selection

by herbivorous insects allows agricultural systems to be managed in ways that support plant

health and decrease pest populations without the use of harmful chemical pesticides.

Although the exact mechanisms mediating insect host-plant choice are still not fully

understood feeding preferences of herbivorous insect pests are often correlated with host-plant

characteristics such as nitrogen and free amino acid content (Showler, 2001 and Chen et al.,

2008). These correlations are useful when they relate physiological states of the plant, which can

often be managed by growers, to the susceptibility of the plant to damage by insect pests.

Understanding factors affecting host-plant selection by herbivorous insect pests provides the

1

opportunity to protect plants from insect damage before it occurs and can be used to develop integrated pest management tactics. In large monoculture systems, understanding the factors that influence host-plant oviposition preferences of gravid females is particularly useful when the most damaging stage of the pest is the larval stage. For many of the generalist herbivore species, including Lepidoptera, this is the case.

1.2 *Spodoptera exigua* and host-plant preference studies

In several experiments, beet armyworms preferred to lay eggs on plants which were also preferred for feeding by third-instar larvae. For example, Showler (2001) reported that *S. exigua* preferred to oviposit and feed on pigweed instead of cotton. Analysis of the leaves revealed higher free amino acid levels in pigweed plants, and these levels were proposed to have an effect on the preference of the herbivore (Showler, 2001). Likewise, in cotton, high nitrogen levels have been positively correlated with shorter development times, oviposition preference, feeding preference, and subsequently proposed as a factor affecting both oviposition and feeding choice behavior (Chen et al., 2008). However, this is not always the case. In a study evaluating the effects of drought stress on cotton, *S. exigua* preferred to oviposit on drought stressed plants, but the larvae showed no preference (Showler and Moran, 2003). Similarly, in a study evaluating two plant species, feeding preference and oviposition preference did not correlate with host plant fitness of *S. exigua* larvae (Berdegué et al., 1998). Information from these studies are helpful in identifying potential factors affecting the ecology of these plant-insect interactions, but larval and adult host preferences cannot always be used to predict one another.

Many studies attempt to draw conclusions about the host-plant oviposition choice behavior of Lepidoptera based on correlations with host-plant fitness, but the results from various parameters can be confusing. For example, in the study evaluating the effects of drought

2

stress on cotton, larval weights were higher on the drought stressed cotton (the preferred oviposition substrate), but survival of these larvae was also lower on drought stressed cotton (Showler and Moran, 2003). Such correlations are most useful for understanding the impact of agricultural practices on pest populations.

Determining the ecology of these correlations can be difficult and inappropriate as all of the mechanisms involved in plant responses to insects have not been fully explored. Recently, it was shown that oviposition by *Spodoptera frugiperda* caused maize plants to release different volatile compounds known to affect insect behavior in as few as six hours (Peñaflor et al., 2011). Several hypotheses for variation in correlations between parameters for host-plant fitness and oviposition preference involve tritrophic interactions and propose that the plant-herbivore interaction may be strongly influenced by the behavior of natural enemies. Studies which draw conclusions about the evolution of these preferences based on the survival interests of different insects may be misleading. For example, it is well documented that herbivorous insects and their natural enemies alike are able to utilize plant volatiles to locate their respective hosts (Dicke and Van Loon, 2000). However, how these volatiles and their blends affect insect behavior and relate to plant physiology are not yet fully understood and plants which have adapted effective direct defense mechanisms to reduce pest fitness on the plant will not necessarily be identified through investigating herbivore preference alone.

With so many factors potentially playing a role, it can be difficult to fully understand the mechanisms and predict consequences of oviposition preferences of herbivorous insects. Oviposition choice studies may not be able to predict which plant(s) will provide the highest fitness for an herbivore insect pest, but they do provide information about an important step in

plant defense. For these reasons, it is best to use behavioral bioassays with whole plant material to evaluate one of the first steps in plant defense from insects: oviposition.

1.3   Oviposition behavior of Lepidoptera

Over the past few decades, technological advances in chemistry have allowed researchers to isolate, identify, and assay the biochemicals involved in plant-insect interactions with very interesting outcomes. While the tools used to evaluate the ecology have improved, the questions and behaviors surrounding the ecology persist. Several known aspects of lepidopteran behavior are important to consider as researchers strive to understand the effects of chemicals and other treatments of plants. Renwick and Chew (1994) provide a wonderful overview of some of these behaviors, and as per this review, it is helpful to divide lepidopteran oviposition behavior into the following steps: searching, orientation, encounter, landing, contact evaluation, and acceptance or rejection. Depending on the limits of an oviposition study (no-choice, choice, distance, wind tunnel), these mechanisms must often be assayed in isolation of one another while considering the whole process in the discussion. Results from these studies are helpful for formulating hypotheses and determining directions of future experiments but should not be used to draw conclusions about other steps. For example, attraction to volatile stimuli preferences observed in a wind tunnel should not be used to assume oviposition substrate acceptance preferences, and vice versa.

There are several reasons as to why the behavioral preferences of a Lepidoptera may not remain consistent throughout all steps of oviposition. Regardless of the variable evolutionary ecologies determining behavioral responses of Lepidoptera to cues from plants, insects, and other aspects of their surroundings, it is known that these insects have sensory receptors involved in oviposition on their antennae, tarsi, proboscis, and ovipositors (Renwick and Chew, 1994).

4

There are also studies which indicate that the oviposition preferences of nocturnally active Lepidoptera (such as *S. exigua*) may be more dependent on tactile surface and chemical (both volatile and surface) cues (Renwick and Chew, 1994). From antennae to tarsi, female Lepidoptera are equipped with the receptors needed to acquire data from many sources and the preferences which result from the processing of this information may never be fully understood. However, understanding that there are different steps involved in oviposition behavior is powerful knowledge for those hoping to exploit the mechanisms.

In monoculture producers may benefit most from attracting (for trapping) or repelling Lepidoptera, which would only require study of the searching and orientation steps. Previously, although the exact chemistry and physiology remain unknown, the volatiles from a resistant cultivar of soybean were shown to repel a Noctuid pest (Khan et al., 1987). Many studies attempt to draw correlations between multiple aspects of the oviposition behavior of Lepidoptera and subsequent ecology of plant-insect interactions of the offspring. While this information can be useful, it is always important to consider the goal of research and to remember that understanding all of the mechanisms involved may not always be possible or necessary.

1.4 PGPR: current uses and potential applications

The negative environmental impacts, increased resistance, suppression of natural enemy populations, and negative health effects of chemical pesticides have prompted studies to identify and explore alternative pest control methods. One such approach involves treating crops with plant growth-promoting rhizobacteria (PGPR). PGPR can help plants to tolerate abiotic stresses through the induction of induced stress tolerance (IST), which includes increased tolerance to drought or high salinity soils and increased uptake of phosphate or nitrate (Yang et al., 2008). Although the exact mechanisms have not always been identified, PGPR have also been shown to

provide resistance against many plant pathogens including nematodes, viruses, insect-transmitted diseases, fungi, and bacteria (Kloepper et al., 1999 and Zehnder et al., 2001). Currently, PGPR are sold commercially for seed formulations, seedling root dip, soil application, and drip irrigation. PGPR strains of the genus *Bacillus* are particularly common because they form spores which can be stored and shipped under a range of conditions that are commonly encountered by commercial products. In addition to improving the health of plants by providing resistance, PGPR treatments can be beneficial as supplements, replacements, or additions to agricultural management practices. For example, PGPR treatment of tomato plants allowed for similar growth rates of plants receiving lower fertilization rates (Adesemoye et al., 2009). These results may have consequences on pest populations, considering the increased oviposition and development of pests like *S. exigua* on plants with high nitrogen fertilizer rates (Chen et al., 2008). Integrating PGPR in agriculture management practices has many benefits that have already been established, but there are still potential benefits that have not been fully explored.

Although the effects of PGPR treatment on plant growth and disease resistance have been studied extensively, few studies have explored their effects on plant-insect interactions. Field studies with PGPR-treated cucumber plants revealed PGPR-related resistance to cucurbit wilt but the exact mechanism of resistance remained unknown until a later study (Tuzun and Kloepper, 1995). Eventually, it was revealed that the PGPR-treated cucumber plants produced less cucurbitacin which reduced feeding by cucumber beetles known to transmit the disease (Zehnder et al., 1997). Evidence that PGPR play an important role in plant-insect interactions was also supported in later studies. While evaluating a PGPR strain of *Bacillus subtilis* known to increase plant tolerance to salinity stress, investigators noted lower whitefly populations on PGPR-treated tomato plants grown under greenhouse conditions (Hanafi et al., 2007). The mechanism of this

resistance was not identified but a later study showed that treatment of tomato plants with PGPR reduced adult whitefly emergence, and even restored whitefly population suppression normally achieved by jasmonic acid (JA)-pathway plant responses (Valenzuela-Soto et al., 2010). Although the factors responsible for this resistance are not fully understood, the effects observed further supported the theory that these bacteria may be important for resistance to insect pests, as well as plant diseases.

1.5   Model system

This study uses a plant-pest model which includes the crop, cotton (*Gossypium hirsutum*), known to be attacked by several economically important species of arthropod pests including the generalist herbivore, beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). *Spodoptera exigua* is considered a secondary pest and it has been shown that early insecticide applications in cotton systems are correlated with an increase in the numbers of *S. exigua* larvae present later in the season as well as increased cost and pesticide applications over the whole growing season (Gross and Rosenheim, 2011). As a generalist lepidopteran pest, several potential factors affecting host-plant oviposition preferences of *S. exigua* have been previously studied (Berdegué et al., 1998, Showler, 2001, Cardoza et al., 2003, Showler and Moran, 2003, Chen et al., 2008). Armyworms like *S. exigua* are among the top five arthropod pests causing loss in cotton despite the current use of management tactics (Williams, 2010). The three PGPR treatments evaluated for defense from these armyworms have already been screened for effectiveness in promoting plant growth and improving plant health. The single strain treatment tested, INR-7, has previously been studied and discussed in literature (Zehnder et al., 1997 and 2001). All three treatments have been shown to increase plant growth and cause changes in the profiles of headspace volatile organic compounds released from cotton plants (Ngumbi, 2011).

1.6   Justification of the study

Many studies of plant-insect interactions have focused on understanding the factors and mechanisms affecting selection of host plants by herbivorous insects. In the area of crop protection, this information can be especially valuable. In 2009, the United States cotton industry incurred an estimated loss of $502 million due to insects, among which bollworms, armyworms, and budworms all ranked in the top five (Williams, 2010). Despite current pest control measures, including the use of Bt transgenic crops and chemical insecticide applications, lepidopteran pests continue to be significant agricultural pests requiring new management tactics as resistant populations develop (Temple et al., 2009). Due to negative environmental impacts, increased resistance, and negative health effects of chemicals used in such pesticides, alternative pest control methods have been explored. One such approach involves treating crops with plant growth-promoting rhizobacteria (PGPR) which have been shown to increase plant growth and to provide resistance against many plant pathogens including nematodes, viruses, fungi, and bacteria (Kloepper et al., 1999).

The effects of PGPR treatment on plant growth and resistance to several diseases have been studied, but few studies have explored their effects on plant-insect interactions. Field studies with PGPR-treated cucumber plants revealed PGPR-related resistance to cucurbit wilt which was later attributed to the reduced feeding by cucumber beetles on the PGPR-treated plants (Zehnder et al., 1997). More recent studies have provided some evidence that PGPR treatment of plants may provide some resistance to whitefly (Hanafi et al., 2007 and Valenzuela-Soto et al., 2010). Although the factors responsible for this resistance are not fully understood, the effects observed further supported the theory that these bacteria may be important for resistance to insect pests, in addition to plant diseases.

Because large monoculture systems contain high densities of a single crop type, it would be beneficial for crop protection to reduce oviposition of lepidopteran insect pests whose larvae are the most damaging life stage. It is well documented that herbivorous insects and their natural enemies alike are able to utilize plant volatiles in order to help them locate their respective hosts (Dicke and Van Loon, 2000), and treatment of cotton plants with PGPR can change the profile of headspace volatiles (Ngumbi, 2011). Considering the established effects of headspace volatiles on the oviposition behavior of herbivorous insects and the various benefits of PGPR treatment of plants, it is possible that treating plants with PGPR may reduce the oviposition of insect pests. If they do provide plants with resistance from insect pests, PGPR could be applied in new ways and could be integrated into current pest management programs. PGPR spores are easily stored, can be applied through sprayers, are appropriate for use by organic growers, and are added easily to seed coat formulations. PGPR are already being used in agriculture and understanding the effects they may be having on pest populations, whether positive or negative, is important.

1.7   Thesis goal and outline

The goal of this research is to study the role that PGPR may be playing in plant-insect interactions. No-choice and choice bioassays were used to investigate the effects of PGPR treatment of cotton plants on the oviposition behavior of a lepidopteran pest. Specifically, the goal of this study was to investigate the effect of PGPR treatment of cotton plants on the host location and acceptance behavior of a generalist herbivorous insect, *S. exigua,* with experimental design considerations for the presence of headspace volatile organic compounds which may affect this behavior.In chapter II, choice and no-choice oviposition studies were used to evaluate the influence of treating cotton plants with PGPR on the oviposition behavior of *S. exigua*. In choice and no-choice bioassays, the oviposition behavior and preference of females was

compared among four treatments: i) untreated cotton plants (control), cotton plants treated with

ii) *Bacillus pumilis* strain INR-7 (a single PGPR strain), iii) Blend 8 (a blend of 4 PGPR strains),

and iv) Blend 9 (a blend of 4 PGPR strains). In choice oviposition bioassays, females preferred

to oviposit on untreated cotton plants (control) compared to any of the three PGPR treatments. In

particular, Blend 9-treated plants had significantly fewer eggs compared to untreated plants. In

no-choice bioassays, however, similar numbers of eggs were deposited on untreated plants and

PGPR-treated plants. These results show that *S. exigua* females are capable of distinguishing

between the PGPR-treated and untreated plants, as well as between some of the PGPR-treated

plants. More notably, the results showed that PGPR treatments influence the oviposition

behavior of *S. exigua* and that some PGPR treatments may be able to increase plant health by

reducing oviposition by lepidopteran pest species.

In chapter III, three different designs of no-choice oviposition studies used to evaluate the

oviposition behavior of *S. exigua* were compared and discussed. Females were allowed to

oviposit overnight on one of four treatments: i) untreated cotton plants (control), cotton plants

treated with ii) *Bacillus pumilis* strain INR-7 (a single PGPR strain), iii) Blend 8 (a blend of 4

PGPR strains), and iv) Blend 9 (a blend of 4 PGPR strains). In two of the designs, all four of

these treatments were evaluated, but distances separating individual cages were increased in the

second design because it was suspected that there was contamination between treatments from

volatile organic compounds (VOCs) released by the plants. Reductions in P-values with

increased distances between individual cages correspond with the hypothesis that VOCs

associated with the headspace of different treatments studied are capable of diffusing through

cloth and affecting the oviposition behavior of *S. exigua*. This hypothesis was further supported

by a third design evaluating only two of the treatments (the untreated control and Blend 9),

which reduced the numbers of treatments whose VOC profiles may be design contaminants. A comparison of the results reveals the potential for PGPR treatment of plants to either stimulate or reduce egg-laying behavior of Lepidoptera under short-range, no-choice conditions. When designing future studies to evaluate the effects of microorganisms on plant-inset interactions, investigators should consider the possible influences of volatile compounds on insect behavior.

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CHAPTER 2

EFFECTS OF PGPR TREATMENT OF COTTON PLANTS ON THE OVIPOSITION

BEHAVIOR OF THE BEET ARMYWORM, SPODOPTERA EXIGUA (HÜBNER)

(LEPIDOPTERA: NOCTUIDAE)

2.1   Introduction

In 2009, the United States cotton industry incurred an estimated loss of $502 million due

to insects, among which bollworms, armyworms, and budworms all ranked in the top five

(Williams, 2010). Despite current pest control measures, including the use of Bt transgenic crops

and chemical insecticide applications, lepidopteran pests continue to be significant agricultural

pests requiring new management tactics as resistant populations develop (Temple et al., 2009).

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is one of these

major pests. In addition to cotton, it has over 90 known species of host plants including other

economically important crops such as tomato and corn (Pearson, 1982). The current reliance on

insecticide treatments for insect control in agriculture is of particular concern in systems where

S. exigua may be present because early insecticide applications in cotton systems are correlated

with an increase in the numbers of armyworms present later in the season as well as increased

pesticide applications over the whole growing season (Gross and Rosenheim, 2011). Because of

these secondary pest outbreak concerns, potential reductions of natural enemy populations,

negative environmental impacts, increased insect resistance, and negative health effects of

insecticides, many alternative pest control methods are being explored. One such approach

involves treating crops with a class of naturally-occurring root bacteria, termed plant growth-

promoting rhizobacteria (PGPR), which have been shown to increase plant growth and enhance plant health in a variety of ways (Kloepper and Schroth, 1978).

The effects of PGPR treatment on plant growth and disease resistance have been studied extensively. However, only a few studies have explored their effects on plant-insect interactions. Field studies with PGPR-treated cucumber plants revealed PGPR-related resistance to cucurbit wilt, but the exact mechanism of resistance remained unknown until a later study (Tuzun and Kloepper, 1995). Eventually, it was revealed that the PGPR-treated cucumber plants produced less cucurbitacin, which reduced feeding by cucumber beetles known to transmit the disease (Zehnder et al., 1997). A few other studies have supported the idea that PGPR play an important role in plant-insect interactions. While evaluating a PGPR strain of *Bacillus subtilis* known to increase plant tolerance to salinity stress, investigators noted lower whitefly populations on PGPR-treated tomato plants grown under greenhouse conditions (Hanafi et al., 2007). Later, another group reported that treatment of tomato plants with PGPR reduced adult whitefly emergence and even restored whitefly population suppression normally achieved by jasmnic acid (JA)-pathway plant responses (Valenzuela-Soto et al., 2010). Although the exact mechanisms responsible for the observed resistance to whitefly were not identified, the effects observed supported the theory that these bacteria may be important for resistance to insect pests, as well as plant diseases.

In this study, we evaluated the treatment of cotton plants with PGPR on beet armyworm behavior in order to explore the possibility that PGPR may be playing a role in the interactions between lepidopteran pests and their host plants. Feeding preference and larval development studies carried out with *S. exigua* provide useful information about which types of plants might be at higher risk for damage when larvae are present, but cannot be used to predict oviposition

preference by adult females (Berdegué et al., 1998). Because oviposition precedes egg hatch and larval feeding, it is important to consider this initial step of host finding behavior. The hypothesis that *S. exigua* oviposition behavior would be affected by PGPR treatments was tested using oviposition choice and no-choice experiments.

2.2   Materials and Methods

2.2.1   PGPR treatments. As shown in Table 1, a total of eight strains of *Bacillus spp.* (all from Auburn University) were used to develop the three PGPR treatments studied: i) *Bacillus pumilis* strain INR-7 (AP 18), ii) Blend 8, containing four strains of *Bacilli* (AP 188, 209, 217 218), and iii) Blend 9, containing four strains of *Bacilli* (AP 136, 188, 219, 295).

2.2.2   Plants. Commercial variety (*Gossypium hirsutum*) Max-9 cotton seeds (All-Tex Seed, Inc.) were grown individually in round plastic pots (9 cm high, 11 cm diameter) filled with a top soil/vermiculite/peat moss mixture. Plants were grown in a greenhouse facility at Auburn University (Auburn, AL) at 27 °C ± 10, 14:10 h (L/D) photoperiod and 50 ± 10% relative humidity. At planting, six pots of each treatment were placed inside a single $60 \times 60 \times 60$ cm thrips-proof insect tent (Megaview Science Co., Ltd.) to prevent insect damage without the use of insecticides. After two weeks, these six pots were thinned to four. Tents remained closed during watering and fertilizing and were rotated in the greenhouse every three days to account for positional differences. Plants were fertilized once weekly with a 250 ppm solution of Peters Professional 20–10–20 Peat-Lite Special® (The Scotts Co., Marysville, OH).

PGPR treatments were applied at seeding (1ml/seed) as aqueous spore suspensions (1×10^{7} spores/ml). Weekly, PGPR-treated plants received 1 ml additional treatments as an

aqueous bacterial suspension (1×10^9cfu/ml). Plants used were 3-5weeks old from day of planting. Each plant was only used once for any experiment.

2.2.3 *Spodoptera exigua.* *Spodoptera exigua* eggs purchased from Benzon Research (Carlisle, PA) were used to start laboratory colonies at Auburn University (Auburn, AL). Larvae were fed a laboratory-prepared pinto bean diet (Shorey and Hale, 1965) at 25 ± 1^oC, $75 \pm 5\%$ relative humidity, and 14:10-h (L/D) photoperiod. Males and females were separated as pupae. Emerged males were labeled by clearing scales from the thorax with a paintbrush before painting with a drop of white Tulip® Dimensional Fabric Paint (iLoveToCreate, Fresno, CA). Newly emerged (<1-day old) adults were allowed to mate for ~ 48h and provided with paper towels for egg-laying**.** Adults were given water and 10% sucrose in water solution on cotton wicks supported by 25 ml Erlenmeyer flasks**.** Mated females used for experiments were 3 to 4 days old.

2.2.4 **Oviposition choice study.** Thirty mated female *S. exigua* were released between 6:00 and 6:30pm CDT from the center of a black cloth cage ($115 \times 115 \times 76$ cm tall). The cage was placed on a greenhouse table in a darkened, interior, windowless room with a temperature range of 24 ± 2 °C. Four cotton plants were placed inside the cage with one plant in each corner (Figure 1). The cage contained one cotton plant each of the following four treatments: Untreated, INR-7, Blend 9, or Blend 8. All plants had the same number of leaf pairs and height differences were ≤4.0 cm. On each side of the square, plant stems were placed 80 cm apart and pots were wrapped in aluminum foil which covered the soil surface so that only the stem and leaves of the cotton plant were exposed. Covering the pot and soil also prevented *S. exigua* females from making direct contact with chemical compounds on these surfaces and reduced their exposure to volatile compounds emanating from the soil. Females were allowed to oviposit for 12 h before

the number of eggs and egg clutches were recorded for each plant. Eight replicates were carried out with two full rotations of plants that had been randomized twice.

      2.2.5   **Statistical analysis of oviposition choice data.** For each treatment and replicate, the number of eggs per plant was recorded and converted to a ratio as follows: (eggs laid per treatment plant)/(total number of eggs laid on all four treatment plants). The data followed a Poisson distribution and could not be transformed to fit assumptions of normality. Non-parametric analysis (Kruskal-Wallis) was performed. Following the detection of a significant difference by Kruskal-Wallis testing methods ($P < 0.05$), Mann-Whitney U-tests were used for pairwise comparisons (Musser et al., 2002 and Costa et al., 2000).

      2.2.6   **No-choice oviposition studies.** In two studies, treatments were presented to *S. exigua* in separate cages, under the same conditions, to determine if females would accept each treatment for oviposition when presented with no other choice. In the first no-choice study, four treatments were compared. The second no-choice study tested only two treatments (untreated versus Blend 9). In each study, eight mated female *S. exigua* were released between 6:00 and 6:30pm CDT in a black cloth cage ($38 \times 38 \times 76$ cm tall) containing one cotton plant. Pots were wrapped in aluminum foil which covered the soil surface so that only the stem and leaves of the cotton plant were exposed. Covering the pot and soil also prevented *S. exigua* females from making direct contact with chemical compounds on these surfaces and reduced their exposure to volatile compounds emanating from the soil. The individual cages were placed on a greenhouse table in a darkened, interior, windowless room with a temperature range of 24 ± 2 °C. For the first no-choice study (evaluating four treatments), four cages were arranged in a square design with a distance of 240 cm between plant stems (Figure 2). Twelve replicates were carried out with full rotations of three randomized treatment positions. For the second no-choice study

19

evaluating only two treatments, cages containing untreated plants were placed on one table, and cages containing Blend 9 treatments were placed on the opposite table. On each table, four cages of a single treatment were spaced with 80 cm between stems. Between the two tables (each for a separate treatment), stems were spaced with a minimum distance of 240 cm. Treatments' positions were rotated for a total of 8 replicates.

2.2.7   Statistical analysis of no-choice oviposition data. The data for the no-choice study evaluating four treatments was not normally distributed. Thus, a square root transformation was performed prior to analysis. The transformed data were analyzed by analysis of variance (ANOVA) followed by the Tukey-Kramer HSD multiple comparison test ($P < 0.05$; JMP Version 8.0.2, SAS Institute, 2008) to establish significant differences between treatments. The data for the no-choice study evaluating only two treatments (untreated versus Blend 9) were not normally distributed. A square root transformation was performed prior to comparison of the two treatments using the Student's t-test ($P < 0.05$; JMP Version 8.0.2, SAS Institute, 2008).

2.3   Results

2.3.1   Oviposition choice study. In the oviposition choice study, the percentage of eggs laid on each treatment relative to the total number of eggs laid on all treatments was calculated for each treatment and used as a measure of oviposition preference of *S. exigua.* Significantly more eggs were laid on untreated cotton plants compared to those treated with PGPR Blend 9 or INR-7 ($F = 6.7015$, d.f. = 3, 27, $P = 0.0016$; Figure 4). Although not significant, more eggs were also laid on untreated cotton plants compared to those treated with PGPR Blend 8 (Figure 4).

2.3.2   No-choice oviposition studies. In the first no-choice study in which each of the four treatments was presented separately to *S. exigua*, a significantly greater number of eggs were laid on PGPR treatment INR-7 compared to PGPR treatment Blend 9 ($F = 3.8470$, d.f. = 3,

44, P = 0.0157; Figure 5). However, no significant differences were recorded between untreated plants and any of the PGPR treatments. In the second no-choice study designed to compare only untreated plants versus Blend 9-treated plants, significantly more eggs were laid on untreated cotton plants as compared to Blend 9-treated plants (P = 0.0165; Figure 6).

2.4 Discussion

The results showed that *S. exigua* (a generalist lepidopteran species) can differentiate between PGPR-treated cotton plants versus untreated plants and that PGPR treatment can reduce oviposition by females. Given a choice, *S. exigua* preferred to lay eggs on untreated cotton plants than plants treated with PGPR Blend 9 or PGPR strain INR-7. Interestingly, oviposition on plants treated with PGPR Blend 8 was not significantly lower than on untreated plants, suggesting that not all PGPR treatments have an effect on oviposition choice or acceptance by *S. exigua*. The observed variation in the effects of PGPR treatments on oviposition behavior stresses the importance of investigating additional strains and blends of PGPR. Historically, studies investigating the role of PGPR have used a single strain of bacteria to draw conclusions about the mechanisms and effects of PGPR in plant systems. Our data showed that treating plants with different PGPR treatments could have variable effects on insect behavior. Therefore, it is important to consider PGPR treatments individually and not to draw conclusions about all PGPR based on limited model systems.

Although the exact mechanisms responsible for the observed reduction in oviposition by *S. exigua* on some of the PGPR- treated plants have not been determined, the no-choice oviposition studies demonstrated that *S. exigua* will lay eggs on both PGPR-treated plants and untreated plants if presented no choice. Because the results of the second no-choice study revealed a significant difference in the number of eggs laid on untreated plants versus Blend 9-

21

treated plants (Figure 6), but the results of the first no-choice study did not (Figure 5), it was

thought that the statistical differences between the results of these two no-choice studies may

have been due to differences in cage placement and presence of additional treatment plants in the

same room. In the first design, one plant of each of the four treatments was present, although

each was individually caged in black cloth at a minimum distance of 240 cm. In the second

design only two treatments (untreated and Blend 9) were in the room, and the treatments were

spaced 240 cm apart. The statistical differences between the two no-choice studies demonstrate

the importance of reporting materials, treatment types, numbers of each plant (with treatment

type also specified), and all distances between plants used in oviposition studies. More

importantly, because plants were individually caged with black cloth, these results provide

strong evidence for the role of volatile organic compounds (VOCs) in stimulating oviposition by

S. exigua.

It is possible that VOCs may have influenced the outcomes of these two no-choice

studies. A recent study by our group showed that treating cotton plants with PGPR resulted in

differences in headspace profiles of volatile organic compounds (VOCs) (Ngumbi, 2011).

Considering the established roles of VOCs in the oviposition behavior of herbivorous insects,

and the data presented in this study, it is likely that these changes in VOCs, induced by PGPR

treatment, are an important factor in reducing oviposition by *S. exigua*. Neither the distance over

which these compounds diffuse overnight nor the distance at which they may be perceived and

subsequently affect *S. exigua* behavior has been identified. Factors such as leaf area, air flow,

temperature, and compound type affect the movement of VOCs, and until they are better

understood, it is paramount that studies report the specifics of their designs for these oviposition

studies. Because significant differences in egg number were obtained in one design but not in

another, there is evidence that the use of cloth is not sufficient to eliminate the behavioral effects of VOCs from several PGPR-treated cotton plants.

Many studies investigate the role of volatile compounds in the attraction of gravid females, but in order to evaluate treatments with potential for use in monoculture, it is important to identify effects of treatments on oviposition behavior (including stimulation) under short-range conditions. Final acceptance or rejection of an oviposition substrate by Lepidoptera involves processing many sensory cues, which means that orientation to a plant may be influenced by preferences for nectar sources or other plant characteristics that do not relate to oviposition preference (Renwick and Chew, 1994). Therefore, in order to identify effects of PGPR treatment of plants, which may improve crop protection from insects, both choice and no-choice studies with whole plant material should be used.

Differences between headspace VOCs among the treatments may be related to physiological and/or morphological differences in the treated plants that are important to ovipositing females. Although the exact mechanisms mediating insect host-plant choice are still not fully understood, feeding preferences of herbivorous insect pests are often correlated with host-plant characteristics such as nitrogen and free amino acid content (Showler, 2001 and Chen et al., 2008). These correlations are useful when they relate physiological states of the plant, which can often be managed by growers, to the susceptibility of the plant to damage by insect pests. However, it should be noted that gravid females may not need to detect these plant characteristics directly to make decisions about host-plant selection as long as VOCs or other cues provide information which they perceive to be reliable. Understanding the effects of VOCs in stimulating or reducing oviposition by Noctuids is especially important in agriculture because

many lepidopteran species lay eggs at night when visual cues may be difficult to perceive at a distance.

Studies have shown that beet armyworms preferred to lay eggs on plants which are also preferred for feeding by third-instar larvae (Berdegué et al., 1998 and Showler, 2001). For example, *S. exigua* preferred to oviposit and feed on pigweed over cotton. Analysis of the leaves revealed higher free amino acid levels in pigweed plants, and these levels were proposed to have an effect on the preference of the herbivore (Showler, 2001). Likewise, in cotton, high nitrogen levels have been positively correlated to shorter development times, oviposition preference, feeding preference, and subsequently proposed as a factor affecting both oviposition and feeding choice behavior (Chen et al., 2008). However, in other studies of *S. exigua* host-plant preferences, larval choice and gravid female oviposition do not correlate with the host plants on which larval fitness was highest (Berdegué et al., 1998). With information about the mechanisms and factors involved in insect selection of host plants being currently limited, it is important to consider the many possible influences on insect behavior, in addition to those being evaluated by a single experiment. For these reasons, making assumptions about the type of information perceived by herbivores via VOCs should be avoided at this time.

As responses of plants resulting from treatment with PGPR have not been fully explored, it is not appropriate to attribute the reduction in egg-laying to differences in VOCs alone. Instead, it will be important to continue to investigate the effects of these PGPR treatments on changes of the plants and document correlations between these changes and subsequent changes in insect behavior. Regardless of the mechanisms responsible for the observed reduction in oviposition following treatment with PGPR, it is a newly identified means by which some PGPR may provide plant defense.

In the future, identification of the mechanisms of the observed reduction in oviposition on PGPR-treated plants could improve the efficiency of screening for plants with resistance to damage by herbivore pests and provide information about behavioral mechanisms used by insects to select host plants. Field studies should evaluate the persistence of the bacteria in the soil at levels that can protect crops via significant effects on insect pest behavior. Identification of factors, including VOCs, which may influence oviposition behavior, provides tools which may be exploited and manipulated in order to enhance crop protection. Because these beneficial bacteria occur naturally, they are not only likely candidates for use in insect defense in agriculture, but their role in the ecology of plant-insect interactions should continue to be explored. Further study of the effects of treating plants with PGPR on insect behavior has the potential to provide valuable information about plant-insect interactions and to improve current crop protection measures without the negative effects of harmful chemicals.

2.5   Acknowledgements

2.6   References cited

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Table 1. Table of bacterial strains used in preparation of each of the three PGPR treatments studied.

PGPR Preparation	Identification
Blend 8	*Bacillus amyloliquefaciens* strain AP-188 *Bacillus mojavensis* strain AP-209 *Bacillus solisalsi* strain AP-217 *Bacillus amyloliquefaciens* strain AP-218
Blend 9	*Bacillus amyloliquefaciens* strain AP-136 *Bacillus amyloliquefaciens* strain AP-188 *Bacillus amyloliquefaciens* strain AP-219 *Bacillus amyloliquefaciens* strain AP-295
INR-7	*Bacillus pumilus* strain AP-18

Figure Legend

Figure 1. Design for the oviposition choice study. One plant of each treatment type was placed in a square design as shown.

Figure 2. Design for a single replicate of the oviposition no-choice study. Four treatments were placed in a square design as shown.

Figure 3. Design of a no-choice oviposition study with two treatments separated by 240 cm. Plants were arranged as shown. Four replicates were carried out each night.

Figure 4. Effect of various PGPR on oviposition behavior of *S. exigua* (choice). For each replicate, the number of eggs per plant of each treatment type was recorded and converted to a percentage of eggs laid as follows: (eggs laid per treatment plant)/(total number of eggs laid on all four treatment plants). Means with different letters are significantly different ($P < 0.05$, Kruskal-Wallis, Mann-Whitney U-tests, n = 8).

Figure 5. Effect of various PGPR treatments on oviposition behavior of *S. exigua* (no-choice). For each replicate, the number of eggs per plant of each treatment type was recorded. Means

28

with different letters are significantly different (P < 0.05, ANOVA, Tukey-Kramer HSD multiple

comparison test, n = 12).

Figure 6. Effect of PGPR treatment Blend 9 on oviposition behavior of *S. exigua* (no-choice).

For each replicate, the number of eggs per plant of each treatment type was recorded. Means

with different letters are significantly different (P < 0.05, Mann-Whitney U-test, n = 8).
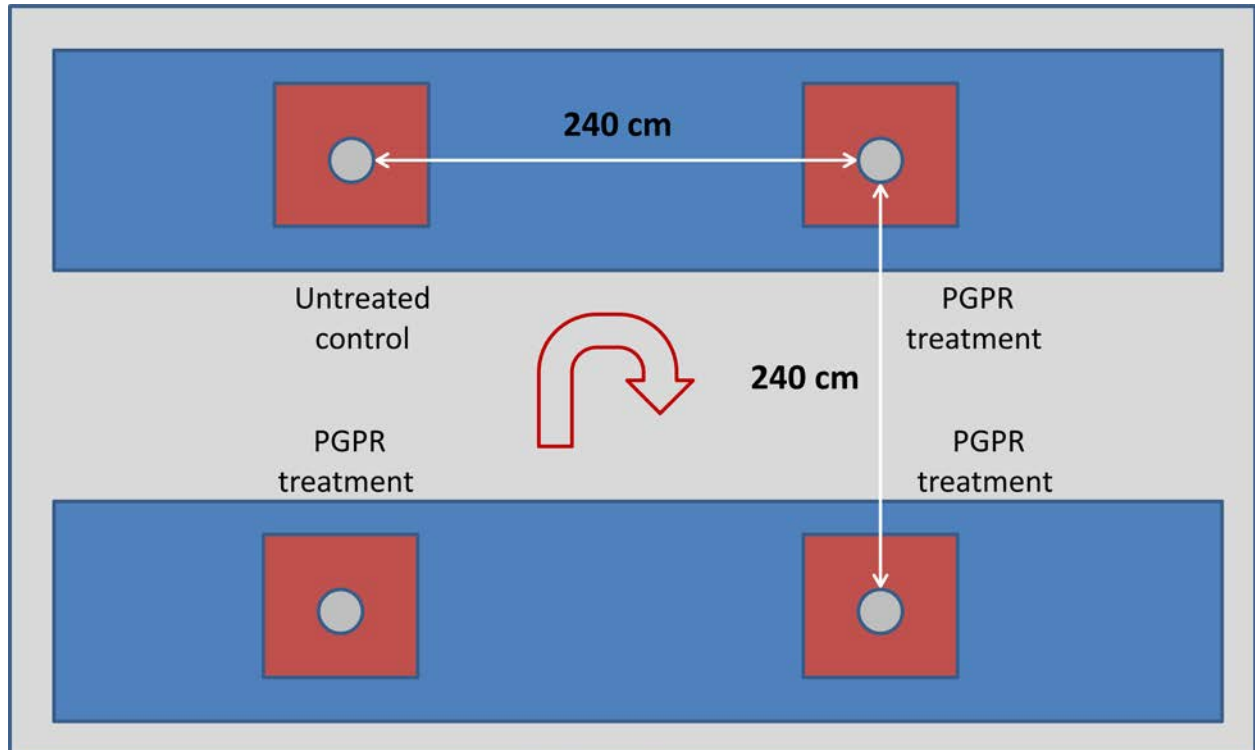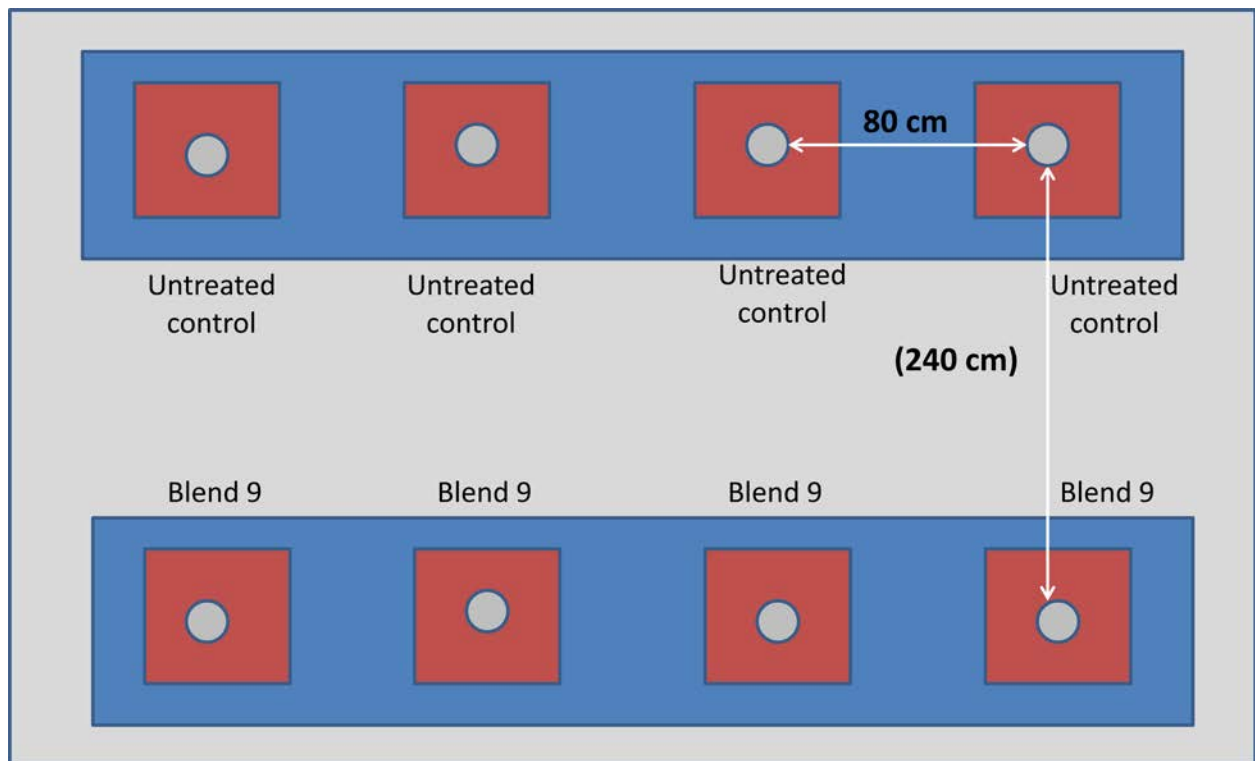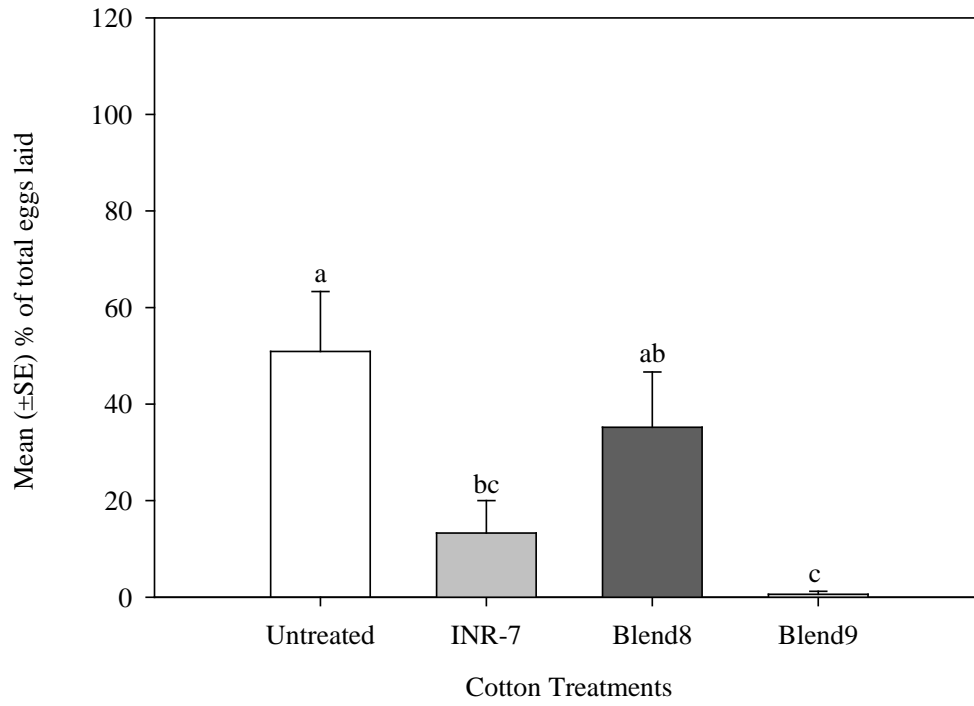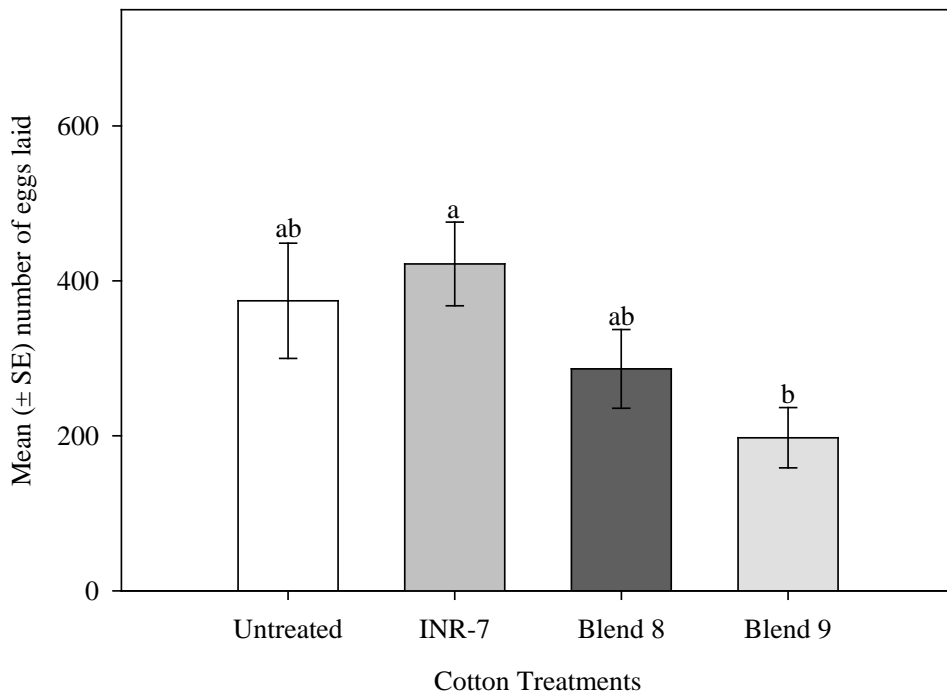
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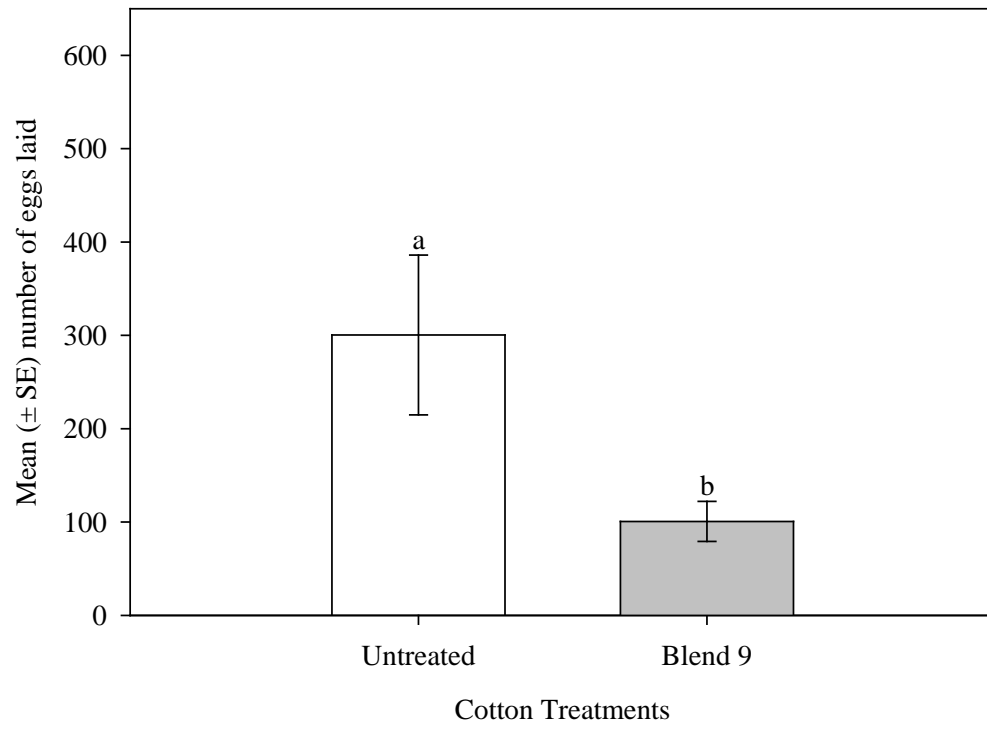
CHAPTER 3

CONSIDERATIONS FOR DESIGNING EXPERIMENTS TO EVALUATE

OVIPOSITION BEHAVIOR OF NOCTURNAL LEPIDOPTERA

3.1   Introduction

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a pest of

several important agricultural crops. In addition to cotton, it has over 90 known species of host

plants including other important crops such as tomato and corn (Pearson, 1982). As a generalist

lepidopteran pest, several potential factors affecting host-plant oviposition preferences of *S.*

exigua have been previously studied (Berdegué et al., 1998, Showler, 2001, Cardoza et al., 2003,

Showler and Moran, 2003, Chen et al., 2008). In the area of crop protection, this information can

be especially valuable. Armyworms like *S. exigua* are among the top five arthropod pests causing

loss in cotton (Williams, 2010). Despite current pest control measures, including the use of Bt

transgenic crops and chemical insecticide applications, lepidopteran pests continue to be

significant agricultural pests requiring new management tactics as resistant populations develop

(Temple et al., 2009). Considering the large host-plant range of the damaging pest, *S. exigua*, and

the need to find alternative pest control measures in agriculture, I evaluated in Chapter 2 the

possibility that treating cotton plants with growth-promoting rhizobacteria (PGPR) may have

applications for crop protection against insects like the beet armyworm.

Although no effects of PGPR treatment of plants on insect oviposition behavior had

previously been identified, I showed in Chapter 2 that the beet armyworm is able to distinguish

between PGPR-treated and untreated cotton plants, and that some PGPR treatments significantly

reduce egg-laying by adult females. For the evaluation of the effects of PGPR treatment of cotton plants on the oviposition behavior of *S. exigua*, both choice and no-choice oviposition studies were utilized. Oviposition choice studies are helpful for identifying potential factors affecting the ecology of plant-insect interactions. Choice studies do not exclude any of the steps involved in lepidopteran oviposition so the resulting egg numbers are a measure of the effects of a treatment on the combination of the following behaviors: searching, orientation, encounter, landing, contact evaluation, and acceptance or rejection (Renwick and Chew, 1994). For this reason, no-choice oviposition studies can also be very important for understanding plant-insect interactions. No-choice studies are more representative of popular monoculture conditions which contain high densities of a single plant type. Searching behavior of adult females may not play a large role under these conditions, and the newlyhatched larvae may not be able to disperse over distances required to reach and feed on non-crop host plants. The no-choice studies were used to determine the effects of PGPR treatment of cotton plants on short-range, no-choice oviposition behavior of *S. exigua*, which would be the most relevant oviposition behavior for crop protection.

In chapter 2, two different no-choice study designs were reported. These two designs were chosen after an evaluation of three different designs based on treatments present and spacing of those treatments. Published designs for such experiments are variable, and effects of several factors on the short-range oviposition behavior of Lepidoptera are not fully understood. Although no-choice studies were designed to isolate each treatment plant, some distance-dependent factors were shown to have an effect on the statistical significance of observed trends in oviposition behavior. An evaluation of all three no-choice designs highlights the importance of reporting as many details of methodology as possible and provides new considerations for future experimental designs.

With so few factors of oviposition behavior fully understood, designs for ovipostition studies tend to be highly variable between research groups, and publications do not always fully report materials and methods. Some oviposition behavior studies even use cuttings from the plant as substrates (Allison et al., 2007). Others may leave the plant intact but restrict ovipositing females to sections of the plant (Chen et al., 2008). Because the effects of PGPR on plant physiology have not been fully explored, limiting oviposition to parts of whole plants may fail to identify influences of PGPR in the model system. The length of these studies is also variable. Commonly, oviposition studies allow female *S. exigua* to oviposit on plant material for 48 hours or more (Berdegué et al., 1998, Showler, 2001, and Chen et al., 2008). However, it has been shown in maize that oviposition by *Spodoptera frugiperda* can alter the amount of volatile organic compounds (VOCs) released by plants in as little as six hours (Peñaflor et al., 2011). Volatile organic compound release, and other responses of plants to oviposition, should be considered in such studies as it is well documented that herbivorous insects and their natural enemies alike are able to utilize plant volatiles in order to help them locate their respective hosts (Dicke and van Loon, 2000). Currently, distances at which *S. exigua* can detect such VOCs, distances at which these VOCs may diffuse under various conditions, and specific effects of these VOCs on oviposition behavior are all unknown. Possible differences in plant responses to oviposition caused by treatment of plants with various PGPR have also not previously been explored. Therefore, oviposition studies lasting several days may not be appropriate. Collecting egg-laying data after 12 and 48 hours could provide significantly different results.

Statistical differences observed between several of the designs evaluated in this chapter provide strong evidence that VOCs have an effect on oviposition behavior under no-choice conditions. Differences in VOC profiles released by cotton plants of the different treatments

35

used (Ngumbi, 2011) must be recognized as a significantly influential factor affecting the oviposition behavior of *S. exigua*. Furthermore, I propose new consideration for these VOCs in future experimental designs.

3.2 Materials and Methods

3.2.1 PGPR treatments. As shown in Table 1, a total of eight strains of *Bacillus spp.* (all from Auburn University) were used to develop the three PGPR treatments studied: i) *Bacillus pumilis* strain INR-7 (AP 18), ii) Blend 8, containing four strains of *Bacilli* (AP 188, 209, 217 218), and iii) Blend 9, containing four strains of *Bacilli* (AP 136, 188, 219, 295).

3.2.2 Plants. Commercial variety (*Gossypium hirsutum*) Max-9 cotton seeds (All-Tex Seed, Inc.) were grown individually in round plastic pots (9 cm high, 11 cm diameter) filled with a top soil/vermiculite/peat moss mixture. Plants were grown in a greenhouse facility at Auburn University (Auburn, AL) at 27 °C ± 10, 14:10 h (L/D) photoperiod and 50 ± 10% relative humidity. At planting, six pots of each treatment were placed inside a single $60 \times 60 \times 60$ cm thrips-proof insect tent (Megaview Science Co., Ltd.) to prevent insect damage without the use of insecticides. After two weeks, these six pots were thinned to four. Tents remained closed during watering and fertilizing and were rotated in the greenhouse every three days to account for positional differences. Plants were fertilized once weekly with a 250 ppm solution of Peters Professional 20–10–20 Peat-Lite Special® (The Scotts Co., Marysville, OH).

PGPR treatments were applied at seeding (1ml/seed) as aqueous spore suspensions (1×10^{7}spores/ml). Weekly, PGPR-treated plants received 1 ml additional treatments as an aqueous bacterial suspension (1×10^{9}cfu/ml). Plants used were 3-5weeks old from day of planting. Each plant was only used once for any experiment.

3.2.3 *Spodoptera exigua. Spodoptera exigua* eggs purchased from Benzon Research (Carlisle, PA) were used to start laboratory colonies at Auburn University (Auburn, AL). Larvae were fed a laboratory-prepared pinto bean diet (Shorey and Hale, 1965) at 25 ± 1°C, $75 \pm 5\%$ relative humidity, and 14:10-h (L/D) photoperiod. Males and females were separated as pupae. Emerged males were labeled by clearing scales from the thorax with a paintbrush before painting with a drop of white Tulip® Dimensional Fabric Paint (iLoveToCreate, Fresno, CA). Newly emerged (<1-day old) adults were allowed to mate for ~ 48h and provided with paper towels for egg-laying. Adults were given water and 10% sucrose in water solution on cotton wicks supported by 25 ml Erlenmeyer flasks. Mated females used for experiments were 3 to 4 days old.

3.2.4 **No-choice oviposition with four treatments separated by 80 cm.** Eight mated female *S. exigua* were released between 6:00 and 6:30pm CDT in a black cloth cage ($38 \times 38 \times$ 76 cm tall) containing a single cotton plant. Pots were wrapped in aluminum foil which covered the soil surface so that only the stem and leaves of the cotton plant were exposed. Covering the pot and soil also prevented *S. exigua* females from making direct contact with chemical compounds on these surfaces and reduced their exposure to volatile compounds emanating from the soil. Four individual cages were placed on one of two greenhouse tables (for a total of two replicates per night) in a darkened, interior, windowless room with a temperature range of 24 ± 2 °C. On each table, four individually-cage treatments were spaced with a distance of 80 cm between plant stems. Between the two tables, plant stems were at least 240 cm apart (Figure 1). Eight replicates were carried out with two full rotations of randomized treatment positions. The data were not normally distributed. Thus, a square root transformation was performed prior to analysis. The transformed data were analyzed by analysis of variance (ANOVA) followed by the

Tukey-Kramer HSD multiple comparison test (P < 0.05; JMP Version 8.0.2, SAS Institute, 2008) to establish significant differences between treatments.

3.2.5   **No-choice oviposition with four treatments separated by 240 cm.** Eight mated female *S. exigua* were released between 6:00 and 6:30pm CDT in a black cloth cage (38 × 38 × 76 cm tall) containing one cotton plant. Pots were wrapped in aluminum foil which covered the soil surface so that only the stem and leaves of the cotton plant were exposed. Covering the pot and soil also prevented *S. exigua* females from making direct contact with chemical compounds on these surfaces and reduced their exposure to volatile compounds emanating from the soil. The four individual cages (each containing a plant of one of the four treatments) were placed on a greenhouse table in a darkened, interior, windowless room with a temperature range of 24 ± 2 °C in a square design with a distance of 240 cm between treatments (Figure 2). Twelve replicates were carried out with full rotations of three randomized treatment positions. The data were not normally distributed. Thus, a square root transformation was performed prior to analysis. The transformed data was analyzed by analysis of variance (ANOVA) followed by the Tukey-Kramer HSD multiple comparison test (P < 0.05; JMP Version 8.0.2, SAS Institute, 2008) to establish significant differences between treatments.

3.2.6   **No-choice oviposition with two treatments separated by 240 cm.** Eight mated female *S. exigua* were released between 6:00 and 6:30pm CDT in a black cloth cage (38 × 38 × 76 cm tall) containing one cotton plant. Cages were placed on a greenhouse table in a darkened, interior, windowless room with a temperature range of 24 ± 2 °C. Untreated cotton plants were placed on one table, while those treated with Blend 9 were placed on the opposite table. On each table, supporting four individually caged plants of a single treatment, plants were spaced with 80cm between stems. Between the two tables, stems were spaced at a minimum of 240 cm

38

(Figure 3). Pots were wrapped in aluminum foil which covered the soil surface so that only the stem and leaves of the cotton plant were exposed. Covering the pot and soil also prevented *S. exigua* females from making direct contact with chemical compounds on these surfaces and reduced their exposure to volatile compounds emanating from the soil. Treatment positions were rotated for a total of 8 replicates. The data were not normally distributed. A square root transformation was performed prior to comparison of the two treatments using the Student's t-test ($P < 0.05$; JMP Version 8.0.2, SAS Institute, 2008).

3.3   Results

3.3.1   No-choice oviposition with four treatments separated by 80 cm. There were no significant differences in the number of eggs laid among the four treatments ($F = 0.7482$, d.f. = 3, 28, $P = 0.5325$; Figure 4)

3.3.2   No-choice oviposition with four treatments separated by 240 cm. Significant differences were recorded among treatments ($F = 3.8470$, d.f. = 3, 44, $P = 0.0157$; Figure 5), but none of the PGPR treatments had significantly fewer eggs laid on them as compared to the untreated control. However, significantly more eggs were laid on plants treated with INR-7 compared to those treated with Blend 9 ($P = 0.0134$). More eggs were laid on untreated plants compared to plants treated with PGPR Blend 9 ($P = 0.0911$), but this difference was not significant using $P < 0.05$.

3.3.3   No-choice oviposition with two treatments separated by 240 cm. Significantly more eggs were laid on untreated cotton plants as compared to Blend 9-treated plants ($P = 0.0165$; Figure 6).

3.4   Discussion

3.4.1   Discussion of statistical differences among three experimental designs.

Despite the use of black cloth for the no-choice oviposition cage material, the spacing of

cages containing different treatment types had a significant effect on treatment comparisons. At a

distance of only 80 cm between each of the four treatments (Figure 1), the P-value indicated that

there were no significant differences among treatments (P = 0.5325; Figure 4). However, when

the four treatments were spaced 240 cm apart (Figure 2), a significant difference was observed (P

= 0.0157; Figure 5). In each experiment no block effects were observed. This data suggest that

the spacing between treatments and not their positions in the room was responsible for the

observed differences. Cages were made of black cloth and closed in a darkened room. Visual

cues did not differ between the two experimental designs. The plants and moths used were also

handled similarly prior to and during each replicate. The results from the two experiments

indicate that a factor affecting the oviposition behavior of *S. exigua* was distance-dependent,

despite the use of individual cloth cages.

Volatile compounds released by one treatment plant may have passed through the cloth

of neighboring cages. In this way, the cages were not sufficient to create completely closed

systems. It is now well documented that herbivorous insects and their natural enemies are able to

utilize plant volatiles in order to help them locate their respective hosts (Dicke and van Loon,

2000) and there are differences in VOC the profiles released by cotton pants of the different

treatments used (Ngumbi, 2011). Therefore, in the design of these studies, we considered the

potential of these VOCs to contaminate separate treatments and significantly influence

oviposition behavior.

The benefits of using cloth for the cages included: the exclusion of minimal light and

reflections thereof, passage of air with similar humidity, background odors, and temperatures

through each of the cages, ease of cleaning between replicates, ability to be compacted for storage, and minimal release of compounds into the system (in comparison to materials such as plywood). Controlling for replicate-wide environmental conditions was a priority because conditions such as humidity and background odors in the room itself could not be precisely regulated. Complete isolation of each plant was never the intent but it was hoped that the black cloth would reduce the flow of air in the immediate vicinity of the plant and exclude some VOCs diffusing from a distance. Effects of VOCs from neighboring plants had been considered. However, their short-range effects in no-choice systems, diffusion properties, and the distance at which they are perceived by female *S. exigua* had not yet been determined. Whether or not the black cloth and minimal spacing of 80cm would provide reliable information about the oviposition behavior of *S. exigua* was unknown. However, distances between treatments are rarely reported in no-choice oviposition studies, and to my knowledge, there have been no previous studies reporting distances with the use of cloth cages. Although the data reported for the second no-choice design (240 cm spacing) contain egg counts from 12 replicates and the first no-choice design (80 cm spacing) contain egg counts from 8 replicates, a reduction in P-values from $P>0.50$ to $P<0.05$ can still be obtained after removing four replicates from the second design. The increase in distance between treatments, not the number of replicates, is responsible for the lower P-value. The hypothesis that VOCs from cotton plants of one treatment were present in cages containing plants of a different treatment was further supported by a third design. Because the distance at which these VOCs may be affecting oviposition behavior in the no-choice designs had not been determined, a third design was used to investigate only two treatments (Figure 3). In a post-hoc analysis of data from the second design (at 240 cm), the number of eggs laid on untreated plants was lower than on Blend 9-treated plants, but not at $P<$

0.05 (P = 0.0911; Figure 5). Considering the effects of treatment spacing observed previously, I used a third design which eliminated two of the treatments (and potential contamination by their VOCs) to evaluate differences between only untreated and Blend 9-treated plants. In this last design, only two treatments were present in the room, but the treatments were still spaced 240 cm apart. Four replicates of a single treatment type (one plant per cage) were placed 80 cm apart on the same table. The distance between the two tables allowed for the two treatments to be spaced approximately 240 cm apart. Treatments were rotated between the two tables, but a single table always had four cages of the same treatment, which maintained a distance of 240 cm between treatment types.

In this (the third) design, a significant difference was recorded. Significantly more eggs were laid on untreated plants compared to Blend 9-treated plants (P = 0.0165; Figure 6). In the first design with only 80 cm between each of the four treatments, no significant difference in egg numbers was recorded between untreated plants and Blend 9-treated plants (P = 0.6320; Figure 4). In the third design, as in the first design, the spacing of only 80 cm on each table most likely allowed for volatiles from one cage to affect the oviposition behavior of female *S. exigua* in a neighboring cage. With only a single treatment type on each table, the VOCs may have passed between cages but would have been passing between cages containing similar VOC profiles. Thus, although VOCs may have been passing between different cages, they were not having a significant effect on oviposition behavior, because the treatments in those cages were the same. The 240 cm between different treatments likely reduced the mixing of VOCs from different treatments. The reduction in P-values corresponded with increased spacing between cages containing plants of different treatments. Increased spacing could have reduced the amount of passing VOCs passing from one experimental cage into another. In light of the factors controlled

42

for in the design, volatile contamination is the most likely explanation for the variation in oviposition behavior of *S. exigua* observed between experiments with different distances separating treatments. Despite differences in standard error and other statistical significances, in all three designs, the same trends for relative numbers of eggs laid were observed. Such consistent trends support the hypothesis that the design of the study may need to be altered to reveal differences which otherwise would not be considered statistically significant at lower thresholds for type I error. Reporting spacing, materials, and conditions and discussing results within the limitations of the study prevent authors from making possible type II errors. Had the results from only the no-choice study with only 80 cm between treatments been reported, we may have concluded that treating cotton plants with PGPR had no effect on the oviposition behavior of *S. exigua* under no-choice conditions. However, after redesigning the experiment under new consideration of VOC contamination, we found that this was not the case. Treating cotton plants with PGPR can reduce oviposition by *S. exigua*, even under no-choice conditions.

3.4.2 Discussion of designs and statistical analysis of several oviposition studies.

There is no single standard design for studying the oviposition behavior of nocturnal Lepidoptera, which makes comparisons between various studies difficult. As we gain more knowledge about the many factors affecting oviposition behavior and host-plant selection, it becomes prudent to provide as many details about experimental designs as possible. Methods sections rarely list completed specifications including cage material, cage distance, treatment and growing conditions of plants, plant sizes, placement of plants inside the cages (distances, relative locations), and environment housing the cages. Full disclosure regarding materials and environmental conditions is crucial for correct interpretation of experimental results in the field of oviposition studies. Often, only some of these details are reported. The existing body of

literature lacks continuity of design, particularly in the areas of cage material, cage size, plant sections presented to females, number of plants presented to females, duration of study, and numbers of females used. Variation among these different factors is most evident in the discussion of statistics. With so many possible factors affecting the final parameters selected for data, a discussion of a few examples will stress the need for investigators to design and discuss oviposition experiments within the parameters set by the limitations of our current understanding of the factors and mechanisms involved in the control of the outcome(s).

Because the spacing of different treatment plants had such a significant effect on behavior, it is crucial that distances between treatments always be reported and given attention in oviposition studies. In designing experiments it is not always possible to control for all factors affecting the outcome, but it is important to recognize those that may still influence the data and to report these with as much detail as possible. Deciding which factors to maintain between treatments should depend most on the question(s) being investigated, and the discussion should not draw conclusions beyond the limits of the design.

In the data reported, a whole and single plant was presented to gravid females in a ratio of approximately eight females per plant, in both choice and no-choice studies. Because the data were used to discuss effects of plant treatments on oviposition choice and behavior, it is crucial to be able to determine levels of variation within treatments. There are several examples of oviposition studies examining the behavior of *S. exigua* which use more than one plant per replicate. Discussing egg count data pooled from several plants is not statistically appropriate without testing to make sure the results do not vary significantly within treatments. If those data are to be analyzed in this manner, and plant types will constitute treatments in discussions, it is more direct to design studies with replicates to measure egg numbers on single plants.

Failure to report specific details of a design and the use of multiple plants of a single treatment type per replicate can be confusing when discussing the statistics and ecology of the behavioral results. For example, in order to investigate the correlation between free amino acid content and oviposition behavior of *S. exigua*, Showler (2001) initially reported egg-laying preferences based on a choice study. The methods failed to specify cage size, material, and location. The months during which the replicates were carried out were reported, which leads me to believe that the cage was placed outside. As for specifics regarding the spatial arrangement of plants, four pots of 3 pigweed plants and four pots of 3 cotton plants were "randomly placed" inside these cages without record of distance or relative positions. Just four gravid females were allowed to oviposit for 48 hours on any of a total of 24 plants. Twelve plants, each of two treatments, were grown three per pot. For analysis of this choice experiment, egg number and number of egg clusters were reported in a table as means. As replicates were defined, each of these means represents a number of eggs or egg batches laid on any of twelve different plants (pooled data) by four different females. In the written portion of the results section, none of these means were reported. The table is referred to, but the results were given in terms of ratios. In the end, the data reported were average total egg numbers laid by four females on a total of 12 plants. Controlling for total size, height, color, different growing conditions and exposure to damaging agents can be extremely difficult when selecting plants for use, which is why it is important to be able to test for effects between plants of a single treatment.

Neither number of eggs laid per treatment plant nor number of eggs laid per female moth can be determined. If number of eggs laid per treatment plant were available, it would be more appropriate to discuss the effect of treatment on the oviposition behavior of *S. exigua*, whose population was represented by a sample size of n = 4 per replicate. However, as is similar to the

data presented here, Showler (2001) discussed some data in terms of ratios. With so many factors affecting total egg numbers laid per each female *Spodoptera*, it would be less confusing to discuss oviposition choice as a ratio of eggs laid per treatment plant. Ratios indicate a relative amount, which is most often what investigators wish to determine when analyzing oviposition choice between different treatment plants.

Within the same study, Showler (2001) did not conserve a plant to female ratio. Reportedly, to determine if volatiles were playing a role in oviposition preference, five females were released in a cage (dimensions specified) with two separate choice chambers, made of plywood, attached by plastic tubes at an unspecified distance from each other. Each chamber contained one pot of 3 plants. Two fans blew air into the choice chambers and through the tubes. Females were allowed to oviposit for 48 hours, and egg clusters laid in the choice chambers (made of plywood and screen) as well as on the plants were scored. In this assay, compared to cotton plants, 3.27 more egg clusters were laid on pigweed *plants* and 2.05 more egg clusters were laid on the *cage* housing the cotton plants. Here, the ratios were confusing because they were used to compare total egg cluster numbers on two different plant treatments in combination with the cages by which each was contained. By counting egg batch numbers on the plywood substrate of the cages and comparing these between cages containing different plant types, the author introduced a third choice for oviposition but failed to include these numbers in the total used to calculate ratios. In the discussion section, the author concluded that these numbers indicate that "cotton may be less preferred than pigweed once the moths entered the chamber." The danger in drawing this conclusion is that there were no reports of *how many moths* entered the chamber housing the cotton plants. Measuring actual egg-laying was not the most direct measure of attraction. When asking questions about attraction to the volatiles of a substrate, it

would be more accurate to discuss data relating to only the attraction phase of the behavior. Observing the number of females entering each chamber would have provided some insight into the influence of volatile attraction. However, in this case, an even number of females should have been used for each replicate.

Even when design parameters are described in detail, failure to recognize potential variability of plants can still be of concern. Pooling data from multiple treatment plants within a single replicate is not the only way to fail to account for possible differences within treatments. A study by Berdegué et al. (1998) specified the cage design, material, and arrangement of two different host plants in their experiment designed to evaluate oviposition preference of adult *Spodoptera exigua* and to examine the correlations between oviposition preference and the fitness of larvae on those plants. Citing previous results indicating that third-instar *S. exigua* preferred to feed on leaf discs and whole plants of *Chenopodium murale* rather than *Apium graveolens*, the authors hypothesized that adults would prefer to oviposit and larvae would develop at a faster rate on *Chenopodium murale* rather than *Apium graveolens*. However, the opposite trend was observed in both oviposition choice as well as development studies. Oviposition choice studies included transplanted *Chenopodium murale* and *Apium graveolens* plants. The authors also compared oviposition preference to the fitness of larvae on the two plants. Although the oviposition study was described in detail, the condition of the plants was not. The field site from which *C. murale* plants were transplanted is specified, but there is no mention of the origin of *A. graveolens* seedlings. Developmental studies used plant material grown under field conditions, which might not be comparable to the material use in the oviposition choice studies. In discussing oviposition choice preferences, egg numbers and clutch numbers were reported as a mean calculated per cage, despite the fact that there were two plants

47

in each cage. As in the study by Showler, (2001), variability across plants within a single treatment was not reported. Conclusions regarding the ecology of oviposition preference and development were made using plants grown under very different environmental conditions. Plant stress and damage conditions could have resulted in differential induction of plant defense pathways and should have been controlled for if the goal of the authors had been to investigate the ecological relationship between adult oviposition preference and larval fitness.

Growing conditions of plants should always be controlled when these plants will comprise different treatments discussed within a single study. Differences in oviposition behavior in the study by Berdegué et al. (1998) may have actually been due to the growth conditions of the plants and not the plant type. Additionally, it has been shown that plants can signal to one another, which requires consideration for separation of treatments during growth and experiments alike (Bruin and Dicke, 2001). If growth conditions were not kept consistent between oviposition and larval choice studies, it is not appropriate to correlate preferences between the two studies. These conditions may also have implications in discussions of other studies. For example, the data presented in this chapter were obtained using young cotton plants because the growth promotion and volatile compound data had been gathered using this age of plant. Under field conditions, gravid female *S. exigua* would generally not be present until the cotton plants are of an older age. Methods employed to grow these cotton plants under conditions which reduced damage from pests also limited the size of plants used. Even with the best efforts to standardize growing conditions, controlling for total size, height, color, and exposure to damaging agents can be extremely difficult when selecting plants for use, which is why it is important to be able to test for effects between plants of a single treatment.

Some methods historically considered to be standard, such as greenhouse fertilization rates, should also be reported. A study by Chen et al. (2008) investigated the effects of nitrogen fertilization rates on *S. exigua* oviposition preference by presenting a single female with one leaf from each of two different cotton treatment plants. In this study, females did not have access to the whole plant for oviposition. Instead, leaf type was controlled for by enclosing the third true leaf of each plant. The arena used was described in detail. According to the design, the wooden ring used for the perimeter resulted in a height of just 3.6cm. Not only was a small portion of the entire plant used for this oviposition choice study, but the cage design itself greatly limited the movements of the moths. In order to report findings related to the oviposition preference of *S. exigua* on various host plants or different treatments of similar plants, the range of behaviors involved in making those decisions must be considered at the experimental design level. The limitations of this study include a significantly short range for choice and exclusion of plant surfaces that may be differentially preferred for oviposition. The behavior involved during the searching period of oviposition choice is limited in this design. Discussion of correlations between larval behavior and development and adult oviposition preference may not be accurate if nitrogen fertilizer rates have effects on the searching behavior of gravid females.

The designs of oviposition behavior studies are understandably variable, depending on the limitations of plant size and available materials. However, discussions of these designs should not reach beyond the limitations of the data recorded. With so many potential factors playing a role in determining insect behavior, it is important to recognize which may be present in each design, and to report findings as precisely as possible. Studies should always be designed to answer specific questions within the breadth of the parameters measured. Drawing

conservative conclusions and having realistic discussions about treatment effects on insect

behavior will result in a most useful body of applicable literature.

3.5   Acknowledgements

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Table 1. Table of bacterial strains used in preparation of each of the three PGPR treatments studied

PGPR Preparation	Identification
Blend 8	*Bacillus amyloliquefaciens* strain AP-188 *Bacillus mojavensis* strain AP-209 *Bacillus solisalsi* strain AP-217 *Bacillus amyloliquefaciens* strain AP-218
Blend 9	*Bacillus amyloliquefaciens* strain AP-136 *Bacillus amyloliquefaciens* strain AP-188 *Bacillus amyloliquefaciens* strain AP-219 *Bacillus amyloliquefaciens* strain AP-295
INR-7	*Bacillus pumilus* strain AP-18

Figure Legend

Figure 1. Design of a no-choice oviposition study with four treatments separated by 80 cm. Plants were arranged in a design with 80cm between treatments and 240 cm between replicates. Two replicates were carried out each night

Figure 2. Design of a no-choice oviposition study with four treatments separated by 240 cm. Plants were arranged in a square design with 240cm between treatments. One replicate was carried out each night.

Figure 3. Design of a no-choice oviposition study with two treatments separated by 240 cm. Plants were arranged as shown. Four replicates were carried out each night.

Figure 4. Effect of various PGPR treatments on oviposition behavior of *S. exigua* (no-choice). Plants were arranged in a design with 80cm between treatments. For each replicate the number of

eggs per plant of each treatment type was recorded. Means with different letters are significantly different (P < 0.05, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 8).

Figure 5. Effect of various PGPR treatments on oviposition behavior of *S. exigua* (no-choice). Plants were arranged in a square design with 240 cm between treatments. For each replicate the number of eggs per plant of each treatment type was recorded. Means with different letters are significantly different (P < 0.05, ANOVA, Tukey-Kramer HSD multiple comparison test, n = 12).

Figure 6. Effect of PGPR treatment Blend 9 on oviposition behavior of *S. exigua* (no-choice). Each table supported four individually caged plants (constituting four replicates) of a single treatment. Between the two tables, treatments were spaced at a minimum of 240 cm. For each replicate the number of eggs per plant of each treatment type was recorded. Means with different letters are significantly different (P < 0.05, Student's t-test, n = 8).

Figure 1.

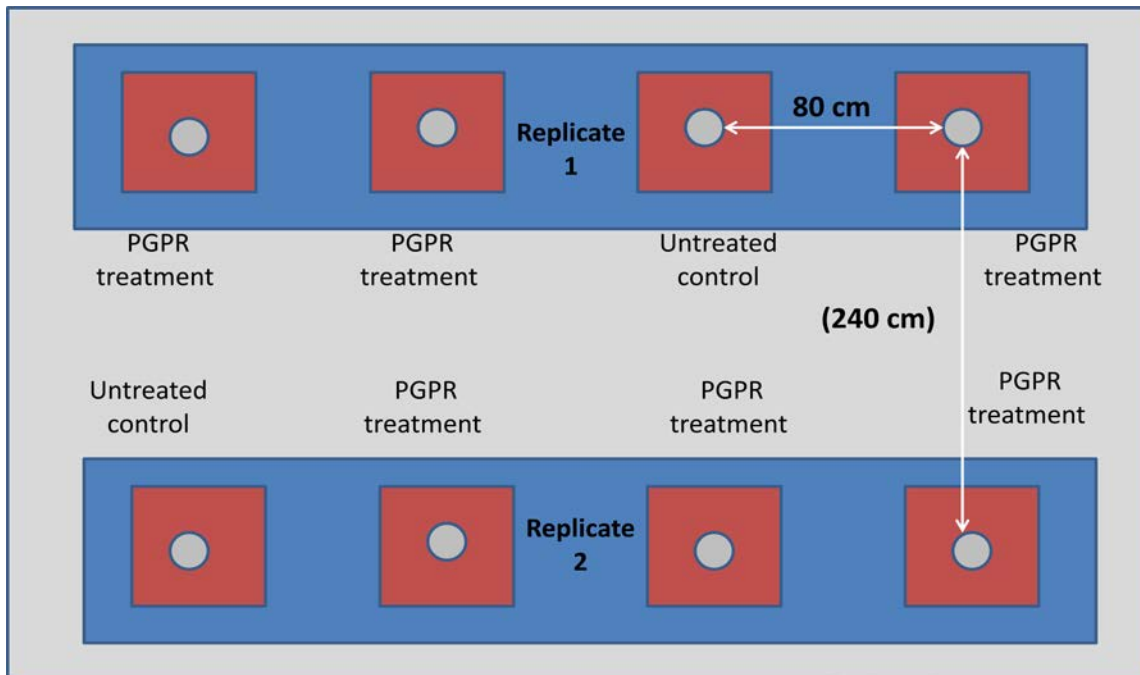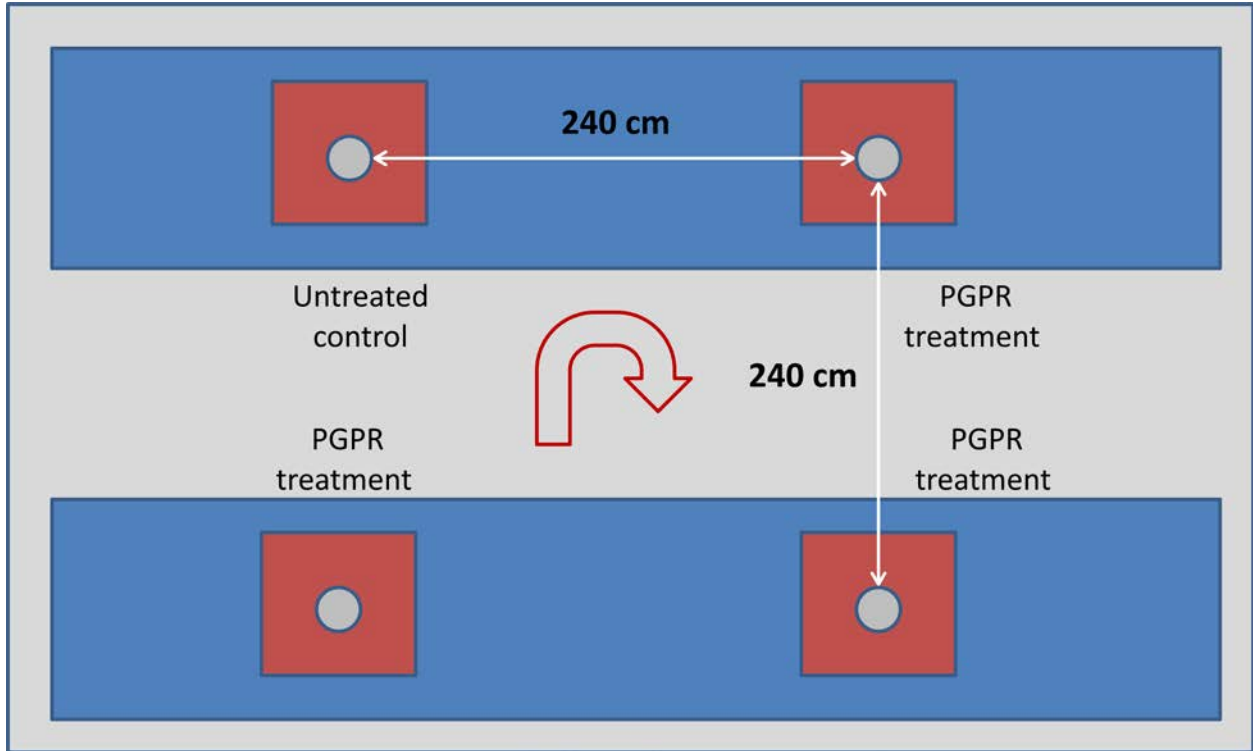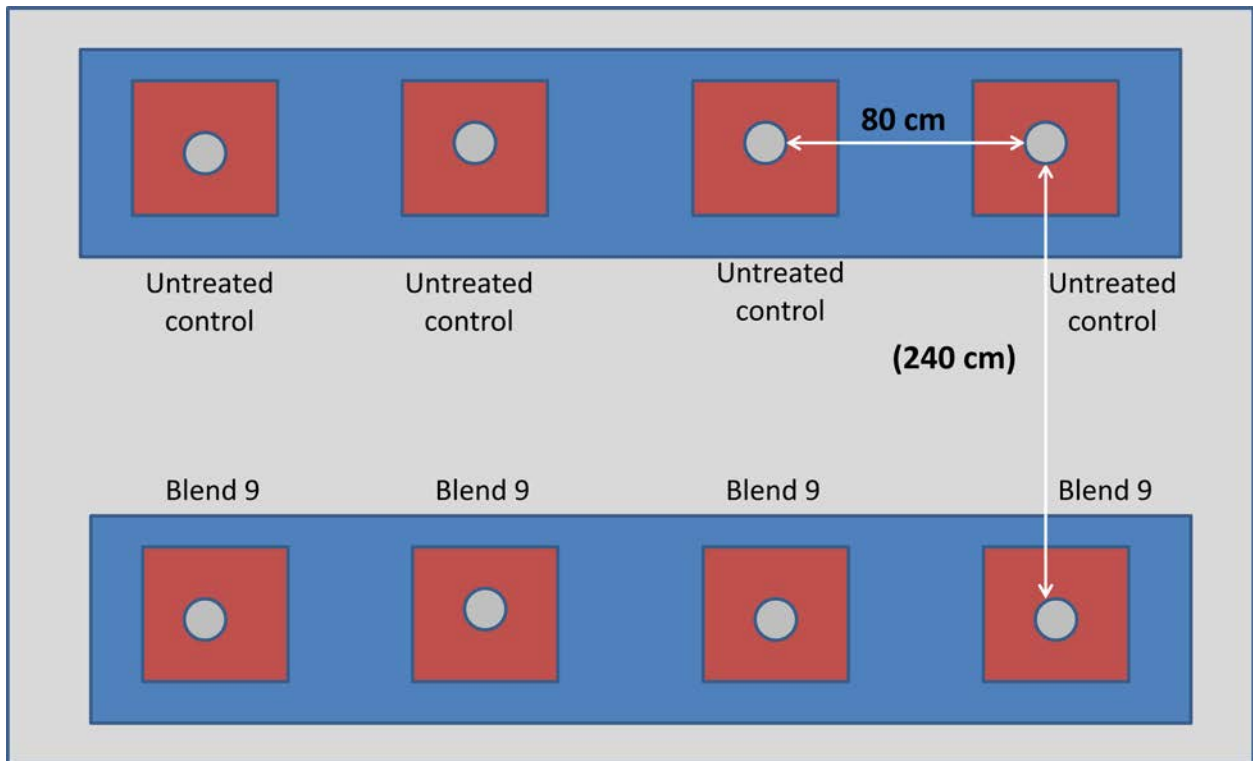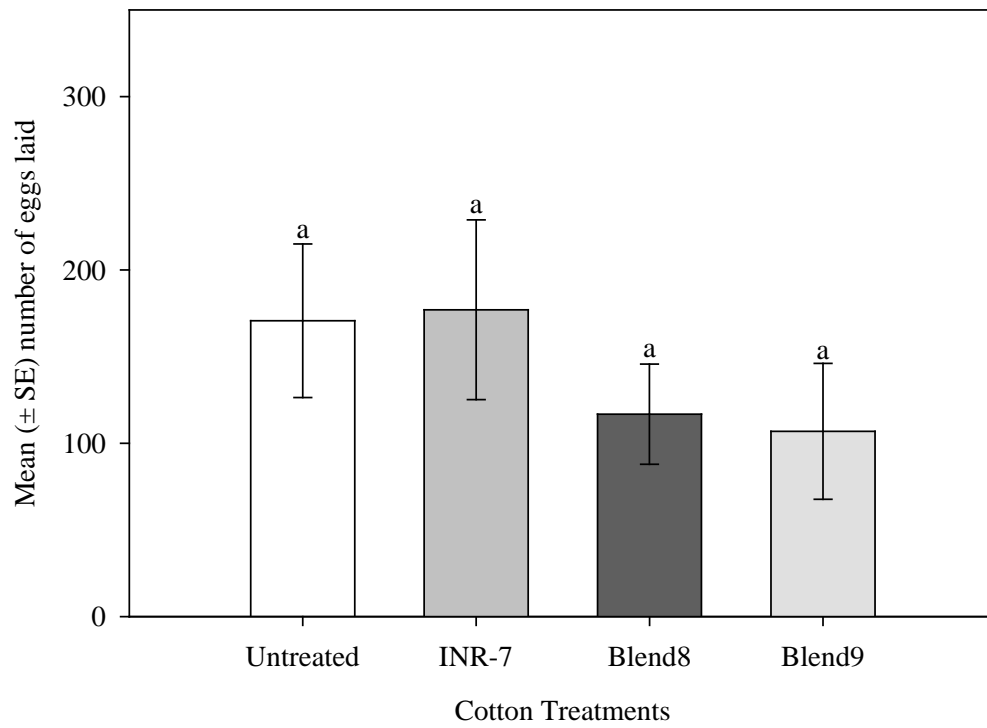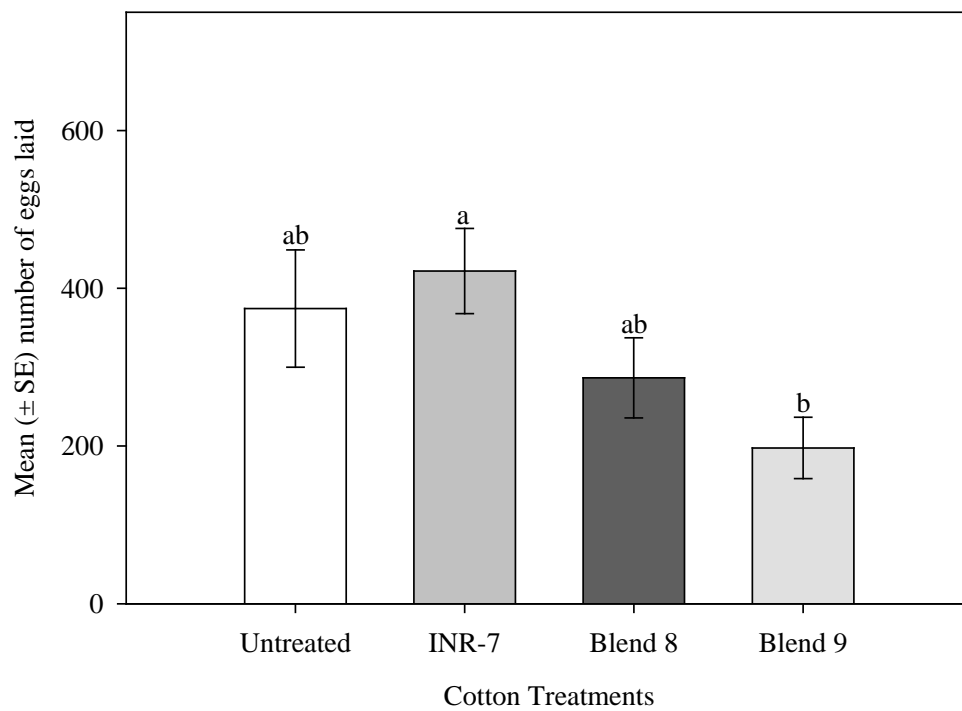**Figure 2.**

Figure 3.

Figure 4.

Figure 5.

Figure 6.