ECOLOGICAL ROLES OF TWO ENTOMOPATHOGENIC ENDOPHYTES: BEAUVERIA BASSIANA AND PURPUREOCILLIUM LILACINUM IN CULTIVATED COTTON

A Dissertation

by

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ABSTRACT

The ecological roles of two entomopathogenic endophytes: *Beauveria bassiana* and *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*), were investigated in both applied and basic studies utilizing cultivated cotton, *Gossypium hirsutum,* as a model system. Fungal endophytes are defined as microorganisms living inside plant tissues, but do not cause symptoms of disease to the host. First, we evaluated the plant performance of cotton and the negative effects, if any, on two different herbivores feeding *in planta* under greenhouse and field conditions. We first tested cotton aphid (*Aphis gossypium* Glover) in greenhouse and field experiments in 2012 and 2013 and found a reduction in aphid reproduction when feeding on plants inoculated with the target endophytes. Our research also evaluated the effects of the endophytes on both the performance of cotton and development of its chewing herbivore pest (*Helicoverpa zea*). The results showed an increase in dry biomass and number of reproductive tissues on plants treated with the endophytes, along with reduced longevity and slower developmental rates across multiple *H. zea* life history stages. One of the mechanisms suggested behind endophytic-mediated plant protection from herbivores is a systemic response in the plant induced by the presence of the fungus. Thus, the plant hormone profile of plants inoculates with target endophytes was investigated using UPLC-MS/MS analysis. Results showed a priming effect of plant defense response when the plants were inoculated with the endophytes and then challenged by an insect herbivore, *A. gossypi*. Lastly, a field experiment was conducted to investigate the role of host plant genotype and its local genetic environment in shaping fungal endophyte communities. The diversity of endophytes was different depending on date of sampling, plant genotype and its local environment. Overall, our research results provide support for the beneficial effects of

manipulating fungal endophytes in plants as part of reliable insect pest management strategies in the near future.

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CHAPTER I

INTRODUCTION

Fungal endophytes can protect plants from a wide range of stressors including insect pests (Porras-Alfaro & Bayman 2011). In this study, we refer to endophytes, as defined by Schulz (2005), as microorganisms (fungi or bacteria) found in asymptomatic plant tissues for all or part of their life cycle without causing detectable damage to the host. In particular, we focus on entomopathogenic fungal endophytes (Vega *et al.* 2009) and the ecological roles these fungi can play in agricultural systems. Entomopathogenic fungal endophytes have been isolated from a variety of plant species and tissues, and single isolates can be inoculated to establish as an endophyte across a range of phylogenetically divergent plants (Vega *et al*. 2009; Rodriguez *et al.* 2009; Gurulingappa *et al.* 2010; Porras-Alfaro & Bayman 2011). These entomopathogenic fungal endophytes are classified as non-clavicipitaceous (Rodriguez *et al*. 2009), referring to fungal endophytes that are usually horizontally transmitted. Several non-clavicipitaceous entomopathogens including *Beauveria bassiana*, *Lecanicillium lecanii, Metharizium anisoplae* and *Isaria* (*Paecilomyces*) *spp.* can have negative effects on insect pests when *in planta*, may antagonize plant pathogens, and also promote plant growth (Ownley *et al*. 2004, 2008; Vega *et al.* 2009). For example, the application of *B. bassiana* as an endophyte to tomato and cotton seedlings increased plant stand counts and height of the plants when these were infected by damping off disease caused by the fungal plant pathogen, *Rhizoctonia solani* (Ownley *et al*. 2004; Griffin *et al*. 2005; Ownley *et al*. 2008). The fungus *Paecilomyces lilacinum,* more widely known as *Paecilomyces lilacinus* (Luangsa-ard *et al*. 2011), has been mainly considered a nematophagous, egg-parasitizing fungus, specifically against the root-knot nematode, *Meloidogyne incognita,* and several other nematode species

including *Radopholus similis*, *Heterodera spp*, *Globodeera spp* (Carrion & Desgarennes 2012; Kannan 2012; Khan 2012; Sharma & Trivedi 2012). However, *P. lilacinum* can also be pathogenic to insects and occur as a endophyte in plants (Castillo-Lopez *et al.* 2014). The mode of establishment and duration of presence of endophytic fungi in plants varies among the different plant-endophyte combinations tested to date (Posada & Vega 2005; Gurunlingappa *et al*. 2010; Posada *et al*. 2007; Akello *et al*. 2008; Powell *et al*. 2009; Reddy *et al*. 2009; Brownbridge *et al*. 2012, etc). In some cases, intentionally inoculated endophytes can be retained within plants for considerable amounts of time, including *B. bassiana* found for as long as eight months in coffee (Posada *et al*. 2007) or nine months in *Pinus radiata* (Brownbridge *et al*. 2012).

Demonstrations of negative effects of endophytic entomopathogens including *B. bassiana* on herbivores in more natural whole plant feeding assays are relatively rare, but have been shown for a few species including aphids (Akello & Bikora 2012; Martinuz *et al*. 2012). Similarly, there are only a few examples of negative effects on lepidopteran species caused by endophytic colonization by *B. bassiana* using whole plant assays including *Ostrinia nubilalis* and *Helicoverpa zea* (Bing & Lewis 1991; Powell *et al*. 2009). To our knowledge, there are no reports in the literature of negative endophytic effects of *P. lilacinum* on herbivorous insects. This is not surprising since this fungus was, until recently, thought to mainly have pathogenic properties against nematodes and not insects.

The mechanisms by which herbivores can be negatively affected by clavicipitaceous obligate endophytes have been studied in a few different grass species and can vary from antixenosis and/or antibiosis mediated by constitutive production and or induction of secondary compounds produced by the plant (Clay *et al*. 1993; Clay 1996; Carriere *et al*.

1998) or secondary metabolites produced by the endophytes themselves (Gindin *et al*. 1994; Ball *et al*. 1997a,b; Bush *et al*. 1997; Wang *et al*. 1997;Jaber & Vidal 2010; Saari *et al*. 2010; Guru 2011). It is important to mention that infection rates of natural populations of grasses by these endophytes can vary depending on the genetic and environmental background the population and these factors can determine if this symbiosis goes from mutualistic to antagonistic (Saikkonen *et al*. 2006; Rasmussen *et al*. 2008; Saikkonen *et al*. 2010; Saari *et al*. 2010; Young & Wilkinson 2010). Another hypothesis for the mechanism by which endophytes can negatively affect herbivores is based on the idea that endophytes can alter the phytosterol profiles of plants and compete with insects for these compounds which are essential for insect development (Dugassa-Gobena *et al*. 1996; Raps & Vidal 1998). The literature also suggests a systemic response that can induced in the plant by the presence of some entomopathogenic endophytes including *B. bassiana* that confers resistance against plant pathogens (Ownley *et al*. 2008, 2010). Endophytes have also been suggested to increase the production of superoxides or to induce an indirect systemic defense response in the plant, thus conferring resistance to insect feeding (Raps & Vidal 1998; Schardl 2004, 2007; Tanaka 2006; Huang 2007; Hartley & Gange 2009; White Jr & Torres 2010).

Cultivated cotton accounts for 35% of total world fiber use, with the United States, China, and India producing two-thirds of the world's cotton (USDA, World Agricultural Outlook board, Sep 2014). Furthermore, the cotton industry in the United States alone accounts for more than $25 billion in products and services per year (USDA, www.ers.usda.gov). Cotton plants are hosts of an array of herbivorous insects including the cotton aphid (*Aphis gossypii* [Glover]) and the New World lepidopteran species *Helicoverpa zea*, most commonly known as the cotton bollworm or corn earworm. Cotton aphids, *A.*

gossypii, have a broad range of host plants including cultivated cotton, causing damage directly by plant feeding and indirectly through virus transmission and physical contamination of cotton by honeydew production (Godfrey *et al*. 1997). Most commonly, *A. gossypii* is considered a mid- to late-season pest in cotton. However, extensive use of insecticides such as pyrethroids can decrease its natural enemy community, thereby contributing to the establishment of the aphid as a season-long pest across cotton production areas (King *et al*. 1987; Godfrey *et al*. 2000). Chronic insecticide use for aphid control has also increased its resistance to several classes of insecticides (O'Brien *et al*. 1990; Grafton-Caldwell 1991; Kerns & Gaylor 1992). The cotton bollworm, *H. zea* has been reported to feed on over 100 plant species, including important economic crops in the United States such as corn, soybean, cotton and peanuts (Cho *et al.* 2008). Management of this insect has relied mostly on chemical control either by insecticidal sprays or by the use of genetically modified crops expressing transgenic insecticidal proteins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) (Jackson *et al.* 2007).

Considering the increasing need for alternative insect management strategies in agricultural systems, we set out to evaluate the ecological role of two entomopathogenic endophytes, namely a commercially available strain of *B. bassiana* and a strain of *P. lilacinum* isolated from cotton as part of a state-wide fungal endophyte survey conducted by our laboratory in 2011 (Ek-Ramos *et al*. 2013). The first part of my research was focused on re-inoculating these endophytes into cotton plants to test for plant growth enhancing effects and negative effects, if any, on two different herbivores, the cotton aphid and the cotton bollworm. We utilized *in planta* feeding assays with the insects fed on endophyte-inoculated plants under both greenhouse and field conditions. The third chapter of my dissertation was

focused on investigating the mechanisms behind cotton plant-endophyte interaction known to confer resistance to different stressors by quantifying the acidic plant hormones profile responses of cotton plants when inoculated with the two different entomopathogenic endophytes and challenged with an insect herbivore. And as a last research chapter we set out to investigate the role of host plant genotype and its local genetic environment in shaping fungal endophyte communities in cultivated varieties of cotton.

CHAPTER II

THE ENTOMOPATHOGENIC FUNGAL ENDOPHYTES PURPUREOCILLIUM LILACINUM (FORMERLY PAECILOMYCES LILACINUS) AND BEAUVERIA BASSIANA NEGATIVELY AFFECT COTTON APHID REPRODUCTION UNDER BOTH GREENHOUSE AND FIELD CONDITIONS[*]

Introduction

Fungal endophytes can protect plants from a wide range of stressors including insect pests (Porras-Alfaro & Bayman 2011). Entomopathogenic fungal endophytes have been isolated from a variety of different plant species and tissues, and can be inoculated to establish endophytically in a range of other plants to test for adverse effects, if any, on different insect herbivores (Vega *et al*. 2009; Gurunlingappa *et al*. 2010; Porras-Alfaro & Bayman 2011). These entomopathogenic fungal endophytes are classified as non-clavicipitaceous (Rodriguez *et al*. 2008).

A number of benefits to plants are also conferred by non-clavicipitaceous endophytes (Omacini *et al*. 2001; Jung *et al*. 2006; Hartley & Gange 2009; Jaber & Vidal 2010; Gange *et al*. 2012). As endophytes, several non-clavicipitaceous entomopathogens including *Beauveria bassiana*, *Lecanicillium lecanii, Metharizium anisoplae* can have negative effects

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[*] Reprinted with permission from Castillo-Lopez, D. Zhu-Salzman, K. Ek-Ramos, M.J. Sword, G.A. 2014. The Entomopathogenic Fungal Endophytes Purpureocillium lilacinum (Formerly Paecilomyces lilacinus) and Beauveria bassiana Negatively Affect Cotton Aphid Reproduction under Both Greenhouse and Field Conditions. *PLOS ONE*: DOI: 10.1371/journal.pone.0103891**.** Copyright 2014 PLOS ONE.

on insect pests when *in planta*, antagonize plant pathogens and promote plant growth (Vega *et al*. 2008, 2009). The activity of *B. bassiana* has received particular attention due to its negative effects on a variety of insect herbivores including the cotton aphid (Bing & Lewis 1991; Posada & Vega 2005; Posada *et al*. 2007; Akello *et al*. 2008; Powell *et al*. 2009; Gurunlingappa *et al*. 2010; Biswas *et al*. 2011).

The fungus *P. lilacinum* is more widely known as *Paecilomyces lilacinus*, having undergone a recent taxonomic revision (Luangsa-ard *et al*. 2011). To our knowledge there are no studies demonstrating *P. lilacinum* as an endophytic fungus causing negative effects on insect herbivores, but there are reports of it being an pathogenic to a number of insects including *Ceratitis capitata*, *Setora nitens*, *A. gossypii,* and *Triatoma infestans* (Marti *et al*. 2006; Fiedler & Sosnowaska 2007; Imoulan 2011; Rao *et al*. 2012; Wakil *et al*. 2012). Both *B. bassiana* and *P. lilacinum* are commercially available for use as biocontrol agents, but *P. lilacinum* is mainly considered to be a nematophagous, egg-parasitizing fungus, specifically against root-knot nematode, *Meloidogyne incognita,* and several other nematode species including *Radopholus similis*, *Heterodera spp*, *Globodeera spp* (Kannan 2010; Carrion & Desgarennes 2012; Khan 2012).

Cotton aphids, *A. gossypii,* have a broad range of host plants including cultivated cotton, causing damage directly by plant feeding and indirectly through virus transmission and physical contamination of cotton by honeydew production (Godfrey *et al*. 1997). Most commonly, *A. gossypii* is considered a mid- to late-season pest in cotton. However, extensive use of insecticides such as pyrethroids can decrease its natural enemy community, thereby contributing to the establishment of the aphid as a season-long pest across cotton production areas (King *et al*. 1987; Godfrey *et al*. 2000). Chronic insecticide use for aphid control has

also increased its resistance to several classes of insecticides (O'Brien *et al*. 1990; Grafton & Caldwell 1991; Kerns & Gaylor 1992). In this chapter we set out to investigate the effects of two endophytic entomopathogens, *B. bassiana* and *P. lilacinum*, on the cotton aphid when present endophytically in cotton. Specifically, we tested: 1) the ability of *B. bassiana* and *P. lilacinum* to establish as endophytes in cotton seedlings when inoculated at the seed stage, and 2) the effects of these endophytes on cotton aphid reproduction using *in planta* feeding trials in both greenhouse and field environments.

Materials and Methods

Plants and endophytic fungi strains: The cotton seeds used for all experiments were variety LA122 (All-Tex Seed, Inc.). The *P. lilacinum* strain was isolated from a field survey of naturally-occurring fungal endophytes in cotton (Ek-Ramos *et al*. 2013). This strain was confirmed to be *P. lilacinum* (formerly *P. lilacinus*) by diagnostic PCR and subsequent sequencing of the ribosomal ITS region using specific species primers (Atkins et al. 2004). The *B. bassiana* was cultured from a commercially obtained strain (Botanigard, BioWorks Inc, Victor, NY). Stock spore solutions of each fungus were made by adding 10 ml of sterile water to the fungi cultured on potato dextrose agar (PDA) in 10 cm diameter petri dish plates and scraping them with a sterile scalpel. The resulting mycelia and spores were then filtered through cheese cloth into a sterile beaker. A haemocytometer was used to calculate the conidia concentrations of the resulting stock solutions. Final treatment concentrations were reached by dilution using sterile water.

Cotton seed inoculation: Seeds were surfaced sterilized prior to soaking in different spore concentrations by immersion in 70% ethanol for 3 minutes with constant shaking, then 3 minutes in 2% sodium hypochlorite (NaOCl) followed by three washes in sterile water, based on Posada et al. [18]. The third wash was plated on PDA media to confirm surface sterilization efficiency. Seeds were then soaked for 24 hours in two different spore concentrations of the two fungi and sterile water was used as control. Spore concentrations for each fungus were zero (control), $1x10^6$ spores/ml (treatment 1) and $1x10^7$ spores/ml (treatment 2) based on inoculum concentrations used in previous studies of endophytic entomopathogens (Posada *et al*. 2005, 2007; Vega *et al*. 2008; Gurunlingappa *et al*. 2010; Guru *et al*. 2011). Beakers containing the seeds were placed in a dark environment chamber at 28ºC until the next day for planting. Soaked seeds were planted in individual pots (15 cm diameter) containing unsterilized Metro mix 900 soil consisting of 40-50% composted pine bark, peat moss, vermiculite, perlite and dolomitic limestone (Borlaug Institute, Texas A&M). All plants were grown in a greenhouse at ~25ºC with natural photoperiod for the duration of the experiment. Pots were placed in a complete randomized design, watered as needed, and no fertilizer was applied throughout the experiments.

Confirmation of plant colonization by endophytic fungi: We have no reason to assume that 100% of the endophyte-treated plants are always colonized by the endophytes when inoculated as seed treatments. Given this constraint, we decided to use two detection methods simultaneously, PDA culturing and diagnostic PCR analysis, to positively confirm the presence of the target endophytes in the experimental plants from the greenhouse experiments, but not for our field experiments. At the end of each greenhouse trial, all treated and control plants were harvested, and each plant was cut in half longitudinally using a sterile

scalpel. Fragments of leaves of 1cm^2, stems and roots of 1 cm length were plated on PDA media and placed in growth chamber at 28°C to check for presence of the endophytes. The other half of the plant was freeze dried and DNA was extracted utilizing the CTAB protocol [41]. Species specific oligonucleotide primers for *B. bassiana* 5'CGGCGGACTCGCCCCAGCCCG 3', 3' CCGCGTCGGGGTTCCGGTGCG 5' [39] and *P. lilacinum* 5' CTCAGTTGCCTCGGCGGGAA 3', 3' GTGCAACTCAGAGAAGAAATTCCG 5'[40] (Sigma-Aldrich, Inc St Louis, MO) were used for diagnostic PCR assays. PCR products were visualized on a 2% agarose gel to determine the presence of the inoculated fungal endophytes based on amplification of a DNA fragment of the expected size (positive control). Given the larger size of the plants utilized in our field trials and the impracticality of PDA plating and extracting genomic DNA from entire large plants, we did not test for the presence of the target endophytes in the experimental plants. Instead, we analyzed our data as treatment groups [control, *B. bassiana* (10^6), *B. bassiana* (10^7), *P. lilacinum* (10^6) and *P. lilcainum* (10^7)] with concentration effects nested within endophyte treatment and present our results as such.

Cotton aphid reproduction tests: A colony of *A. gossypii* was maintained on caged cotton plants in the same greenhouse as the experimental plants as described above. For all endophyte-aphid greenhouse trials, second instar nymphs were placed directly on to the experimental control and endophyte-treated cotton plants. Experimental and control plants with aphids were placed in individual clear plastic cages of 45 cm height and 20 cm diameter, then sealed on top with no-see-um mesh (Eastex products, NJ) to avoid aphid escape or movement between plants.

***B. bassiana* cotton aphid greenhouse experiments:** Greenhouse assays of the effects of endophytic *B. bassiana* on cotton aphid reproduction consisted of three independent tests, each utilizing slightly different protocols. The first was initiated when plants were 13 days old (1st true leaf stage) with aphids allowed to feed for seven days on 10 plants per treatment group. For the second trial, we used older plants (20 days old/third true leaf stage) and aphids were left to reproduce for a longer period of time (14 days) on 10 plants per treatment. At the end of each trial, total aphid numbers were recorded on each individual plant. The third independent test consisted of only a single reproduction trial in which ten 2nd instar aphids were placed on 15 day old plants (second true leaf stage) and left to reproduce 14 days on 15 plants per treatment group, but the cohorts of aphids on each plant were sampled twice at 7 and then again at 14 days.

***P. lilacinum* cotton aphid greenhouse experiments:** We conducted two replicate experiments testing for effects of endophytic *P. lilacinum* on cotton aphid reproduction utilizing the same reproduction test protocol for each trial. In these trials, ten 2nd instar aphids were left to reproduce on the same plants for 14 days consecutively and sampled twice at 7 and then again at 14 days. Ten 1st true leaf stage plants per treatment group were utilized for the first trial; 15 plants per treatment group were used for the second trial.

Cotton aphid field trials for both *B. bassiana* and *P. lilacinum*: During the summers of 2012 and 2013, experimental field trials were conducted at the Texas A&M University Field Station located near College Station in Burleson, Co., TX (N 30° 26’ 48” W 96° 24’ 05.12”) at an elevation of 68.8 m. We utilized a randomized block design with five seed inoculation treatments (T1: Control, T2: *B. bassiana* $1x10^6$, T3: *B. bassiana* $1x10^7$, T4: *P. lilacinum* $1x10^6$ and T5: *P. lilacinum* $1x10^7$). Surface sterilized seeds were inoculated with

the different treatments as described in our greenhouse assay protocol. Treatments were replicated six times, making a total of 30 plots in the field. Each plot was comprised of 4 rows of 16.6m length and planted with 15 seeds per meter. For the aphid reproduction experiments, we utilized the same protocol during both field seasons whereby a total of 75 cone shaped metal framed cages (0.35m of height) were randomly assigned to be placed over endophyte-inoculated and control plants (15 cages / treatment) and set up on May 17, 2012 and June 24, 2013, respectively (delayed experiment due to rain in 2013). Predators were eliminated if found prior to enclosing the caged plants with no-see-um mesh (Eastex products, NJ) to prevent aphid escapes and entrance of predators. Ten second instar aphid nymphs from the laboratory colony were placed on each plant and left to reproduce for 14 days. At the end of the experiment, cages were removed, the entire plant was bagged and brought back to laboratory for total aphid number counts. .

Fungal pathogenicity experiment: To assess pathogenicity of both the *P. lilacinum* strain recovered in our endophyte survey of cotton [39], and the commercial *B. bassiana* strain utilized in our endophyte trials, we performed a cotton aphid survival experiment as per Gurunlingappa et al. 2010 and Vega et al. 2008 with slight modification. The same spore concentrations used in our endophyte *in planta* experiment were used for this test for both endophytes (0, $1x10^6$ and $1x10^7$ spores/ml). Thirty 2nd instar aphids per treatment were dipped in spore solutions for 5 seconds, and then placed on fresh cotton leaves kept on moistened filter paper (to prevent drying out) inside 10cm diameter petri dishes sealed with parafilm (Bemis flexible packaging, Neenah, WI). Ten aphids per petri dish were placed in three replicate petri dishes per treatment. Aphids were checked daily for mortality and dead

aphids were removed, plated and incubated on PDA media to confirm emergence of the entomopathogens from aphid cadavers.

Statistical analyses: All data were tested for normality assumptions using a qqplot, Levene's homogeneity test and the Shapiro-Wilk normality test at alpha=0.05 significance level. For the first independent *B. bassiana* greenhouse experiment, ANOVA and t-tests were performed to compare aphid reproduction differences among plants after 7 days of feeding. In the second and third B. *bassiana* tests, the data were non-normal and nonparametric Kruskal-Wallis and Mann-Whitney U tests were used..For both *P. lilacinum* greenhouse trials, a repeated measures ANOVA was performed with time as a repeated factor to test for differences in aphid numbers between plants after 7 and 14 days of reproduction because aphids on the same plants were sampled sequentially. Aphid field trials for both 2012 and 2013 were analyzed using ANOVA followed by pairwise comparisons (control vs. treatment). We conducted a combined ANOVA analysis of the field data across both 2012 and 2013 to test for year, treatment, and year by treatment effects. For the cotton aphid pathogenicity experiment, a Kaplan-Meier survival analysis was performed to compare the cumulative survival of treated vs. untreated control aphids. All analyses were conducted using SPSS 22 (IBM SPSS, Armonk NY).

Results

Plant colonization by endophytic fungi: Our culturing results showed no fungal growth on the PDA plating of the third sterile water wash of either the surface sterilized seeds or plant samples, indicating the efficacy of our surface sterilization. Thus, we assume

that the fungi growing in the media from surface-sterilized plant materials were endophytes that came from within plant tissues and not epiphytes from the plant surface. Utilizing combined PDA plating and diagnostic PCR detection methods revealed 30-45% more instances of positive endophytic colonization relative to PDA plating alone. *B. bassiana* was detected in 35% and 55% of the treated plants in the first (7 day) and second (14 day) greenhouse trials, respectively. For the third *B. bassiana* trial which consisted of using the same plants for both measurements of aphid reproduction at 7 and 14 days, B. *bassiana* was detected in 53.3% of the treated plants. In the *P. lilacinum* experiments, the target endophyte was detected in 55% and 45% of plants in the first and second trials, respectively.

***B. bassiana* cotton aphid greenhouse experiments:** Our results were analyzed both as treatments (control, low and high concentration) and by confirmed positive colonization of plants by the target endophyte (colonized vs. uncolonized). In the first test, the mean number of cotton aphids per plant on *B. bassiana* treated plants was not significantly different from those on control plants after 7 days of reproduction when analyzed by treatment groups ($F=2.07$; $df=2,29$; $P=0.145$), but was significantly different when analyzed by positive colonization of the endophyte (t-test; $P=0.014$) (Fig 1a). In the second test, we observed a significant negative effect on reproduction of cotton aphids after 14 days when analyzed by treatment groups (Kruskal-Wallis=6.744; $P=0.034$) as well as by positive colonization of the endophyte (Mann Whitney $U=44$; $P=0.004$) (Fig 1b). In our third *B. bassiana* trial, there was no significant effect on the number of aphids per plant after 7 days when analyzed by treatment (Kruskal-Wallis=4.74; $P=0.093$), but there was a significant effect on aphids when analyzed by positive colonization by the endophyte (Mann-Whitney $U=60.50$; $P=<0.0001$) (Fig 1c). Similarly at the end of the 14 days in the same experiment, there were no significant

effects on the number of aphids when the data were analyzed by treatment (Kruskal Wallis=3.069; P=0.216), but a significant effect was observed when the data were analyzed by plant positive colonization by the endophyte (Mann Whitney U=58; P<0.0001) (Fig 1d).

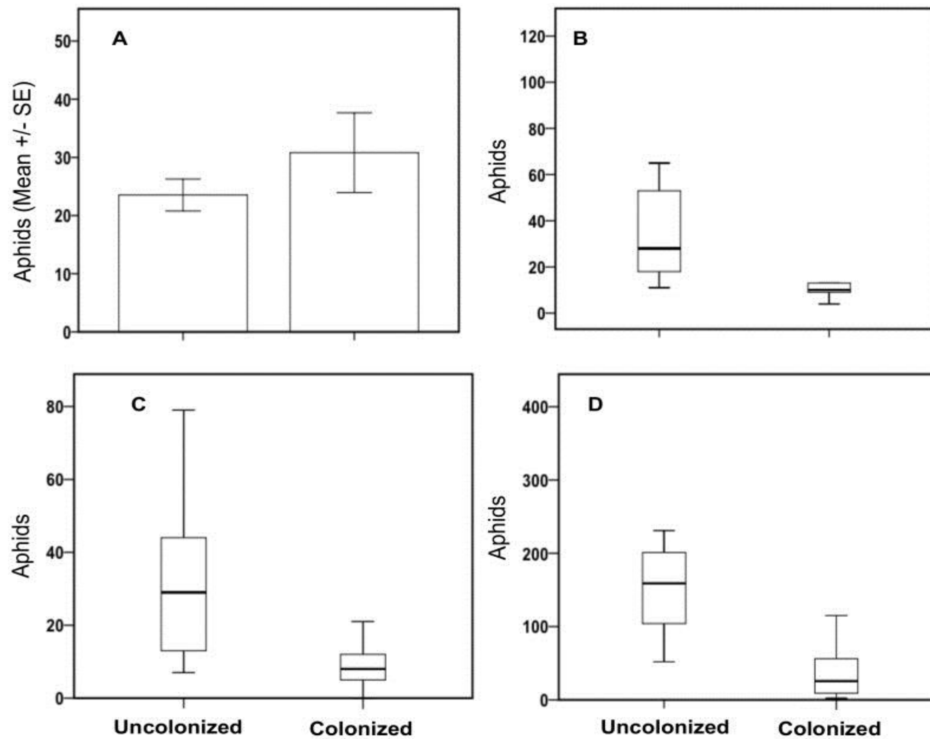

Figure 1: Effects of endophytic *B. bassiana* on cotton aphid reproduction in three independent greenhouse assays. A: aphid reproduction at 7 days first trial. B: aphid reproduction at 14 days second trial. C: aphid reproduction at 7 days second trial. D: aphid reproduction at 14 days second trial.

***P. lilacinum* cotton aphid greenhouse experiments:** As with the *B. bassiana* trials above, we present the results of analyses categorizing the data as both treatment groups and positive versus negative colonization. In the first *P. lilacinum* trial, aphid numbers varied

significantly with time (Repeated Measures ANOVA F=60.40; df=1,28; P=0.0001), but no significant endophyte treatment effect was observed when data were analyzed by plant positive colonization (F=0.026; df=1,28; P=0.873). However, when analyzed based on treatment groups, there was a significant effect of time (F=69.56; df=1,27; P<0.0001) as well as endophyte treatment (F=140.48; df=2,27; P=0.049) (Fig 2a). After increasing our sample size in the second trial, we observed a significant effect of both time (F=53.73; df=1,42;P=0.0001) and treatment when analyzed based on plant positive colonization by the endophyte (F=8.05; df=1,42; P=0.007) (Fig 2c). Although there was a significant effect of time (F=52.52; df=1,41; P<0.000) on the number of aphids when we analyzed our data by treatment groups (control, low or high concentration), the effect of endophyte treatment was not significant (F=0.546; df=241; P=0.583).

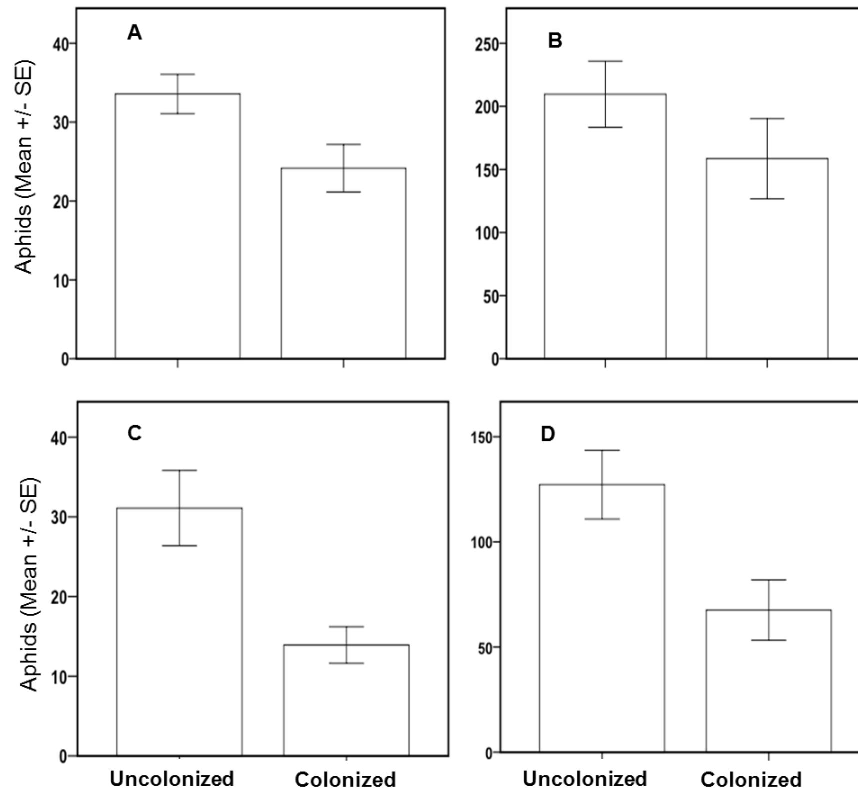

Figure 2: Effects of endophytic *P. lilacinum* on cotton aphid reproduction in two replicate greenhouse assays. A: aphid reproduction at 7 days first trial. B: aphid reproduction at 14 days first trial. C: aphid reproduction at 7 days second trial. D: aphid reproduction at 14 days second trial.

Cotton aphid field trials of both *B. bassiana* and *P. lilacinum*: In both 2012 and 2013 there was no effect of seed treatment spore concentration within each endophyte treatment (2012 Nested ANOVA, F=1.95; df= 2,77; P=0.149 and 2013 Nested ANOVA F=.935; df=2,67; P=0.398), therefore data from both concentrations were grouped for each endophyte in subsequent analyses. Across both years of the field trial, there was a significant effect of endophyte treatment (ANOVA, F=7.31; df=5,132; P=0.001) and also a significant year effect (ANOVA, F=17.43; df=5,132; P<0.0001), but no endophyte by year interaction (ANOVA, F=0.547; df=5,132; P=0.580). During the summer of 2012, there was a significant

overall effect of endophyte treatment on the number of cotton aphids per plant at the end of 14 days of reproduction (ANOVA, F=4.12; df=2,73; P=0.02). Follow-up pairwise comparisons revealed that there were significantly fewer aphids on cotton plants from *B. bassiana*-treated vs. control plots (P =0.006). The difference in aphid numbers on plants in *P. lilacinum*-treated vs. control plots exhibited a similar but non-significant reduction (P=0.085) (Fig 3a). Similarly in 2013, there was a significant overall effect of endophyte treatment on aphid reproduction at the end of 14 days (ANOVA, F=3.13; df=2,59; P=0.05). Pairwise comparisons indicated that inoculation of plants with *B. bassiana* had a significant negative effect on aphid reproduction vs. control (P=0.016), but only a non-significant trend was observed with *P. lilacinum* vs. the control (P=0.086) (Fig 3b).

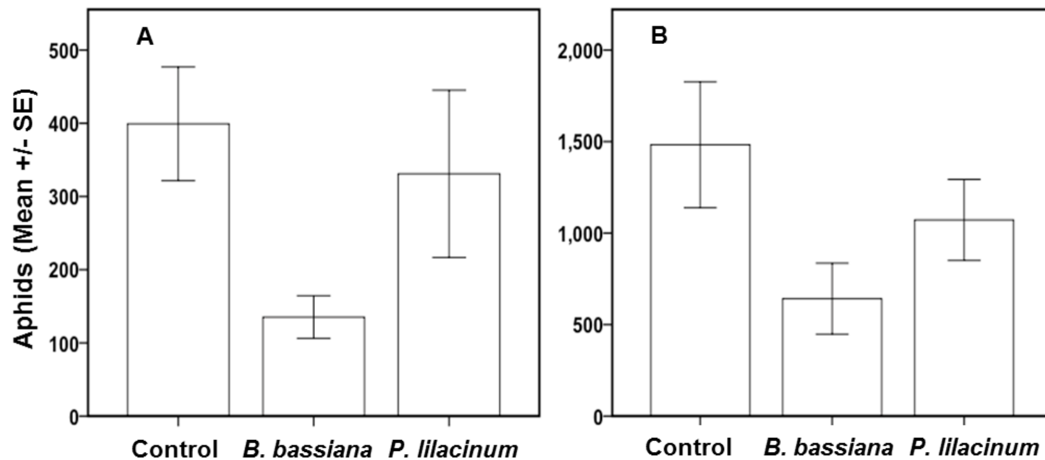

Figure 3*:* Effects of endophytic *B. bassiana* and *P. lilacinum* on cotton aphid reproduction under field conditions. A: aphid reproduction in 2012. B: aphid reproduction in 2013.

Cotton aphid survival experiment: There was no significant difference in aphid mortality between those treated with two different concentrations ($1x10^6$ or $1x10^7$) of conidia solutions of each fungus. Thus, the data from both concentrations were pooled and analyzed together for each fungus. There was a highly significant increase in mortality between aphids treated with either *P. lilacinum* (60%) or *B. bassiana* (57%) vs. the controls (10%) (Kaplan-Meier, $P<0.0001$ for both fungi).

Discussion

Our results provide the first report of the negative effects of two endophytic entomopathogenic fungi, *B. bassiana* and *P. lilacinum,* on cotton aphid reproduction when feeding on whole intact cotton plants inoculated as seed treatments. Importantly, we observed negative effects under both greenhouse and field conditions. We also provide the first evidence for an endophytic effect of *P. lilacinum* on herbivorous insect performance. After analyzing our data based on positive plant colonization by the target endophyte, we found that aphid reproduction on cotton plants positively colonized by *B. bassiana* was reduced in three independent greenhouse trials. Although the results of our first trial testing the effects of *P. lilacinum* as an endophyte on aphid reproduction revealed only a significant effect of time but not treatment, we attributed this to a small sample size for the given effect size based on the results of power analysis (Power=0.175) (Fig. 2b). After increasing the sample size in the second *P. lilacinum* trial, we observed a significant effect of both time and treatment on the reproduction of cotton aphid with lower aphid numbers on endophyte-colonized plants (Figs. 2c & 2d). Our greenhouse endophyte trial results using *A. gossypii* are

similar to those of Martinuz *et al*. 2012 in which whole squash plants were inoculated with *Fusarium oxysporum* as an endophyte via soil drench, resulting in negative effects on *A. gossypii* choice and performance. Similarly, Akello & Bikora 2012 showed that *Aphis fabae* feeding on bean plants colonized independently by strains of either *B. bassiana*, *Trichoderma asperellum* or *Gibberella moniliformis* reproduced poorly compared to those on control plants. Both Martinuz *et al*. 2012 and Akello & Bikora 2012 attribute the negative effects on aphid fitness to be due to chemical changes in the plant that were systemically induced by the presence of the endophyte, though the specific mechanism by which these fungi activated a systemic response within the plants was not investigated.

The ability of *B. bassiana* to establish as an endophyte across a range of plants has been well established [e.g., cotton, corn, bean, wheat, pumpkin, tomato (Gurulingappa *et al*. 2010); coffee (Posada *et al*. 2007); sorghum (Reddy *et al*. 2009); banana (Akello *et al*. 2008); tomato (Powell *et al*. 2009); jute (Biswas *et al.* 2011 and pine (Brownbridge *et al*. 2012). A number of plant-endophyte-insect interaction experiments, including a cotton aphid study by Gurunlingappa *et al*. 2010 have been performed using cut leaf bioassays rather than whole intact plant experiments (Raps & Vidal 1998; McGee 2002; Vicari *et al*. 2002; Wakil *et al*. 2012). Utilizing leaf cuts rather than whole intact plants can potentially cause release of allelochemicals due to direct plant damage that may have negative effects on insects that could obscure those caused by the presence of an endophyte (Price *et al*. 2011). Alternatively, cutting plants and abscising leaves may induce changes in plant chemistry that alter the interaction between the endophyte and host in ways not observed in intact plants (Price *et al*. 2011). Demonstrations of negative effects of endophytic entomopathogens including *B. bassiana* on herbivores in more natural whole plant feeding assays are relatively

rare, but have been shown for a few species including aphids (Martinuz *et al*. 2012; Akello & Sikora 2012). Similarly, there are only a few examples of negative effects on lepidopteran species caused by endophytic colonization by *B. bassiana* using whole plant assays including *Ostrinia nubilalis* and *Helicoverpa zea* (Bing & Lewis 1991; Powell *et al*. 2009).

To our knowledge, there are no reports in the literature of negative endophytic effects of *P. lilacinum* on herbivorous insects. This is not surprising since this fungus was until recently thought to mainly have pathogenic properties against nematodes and not insects. Historically, *P. lilacinum* has been considered largely as a soil-born nematode egg parasite and used as a biocontrol agent against nematode pests such as root-knot, *Meloidogyne incognita,* and reniform, *Rotylenchulus reniformis,* nematodes (Munawar *et al*. 2011; Kiewnick 2011; Chaudhary & Kaul 2012). However, recent evidence indicates that *P. lilacinum* can also be an entomopathogen (Marti *et al*. 2006; Fiedler & Sosnowaska 2007; Imoulan 2011; Rao *et al*. 2012; Wakil *et al*. 2012). Our results indicate that the *P. lilacinum* strain isolated from cotton by Ek-Ramos et al. 2013 can negatively affect insect herbivores when present as an endophyte and that it is also pathogenic to insects. Interestingly, the same strain has also been observed to parasitize root-knot nematode eggs in simple lab bioassays and negatively affect nematode reproduction when present as an endophyte in *in planta* assays (W. Zhou, J.T. Starr and G.A. Sword, unpublished results).

The mechanisms by which herbivores can be negatively affected by clavicipitaceous obligate endophytes have been studied in a few different grass species and can vary from antixenosis and/or antibiosis mediated by constitutive production and or induction of secondary compounds produced by the plant (Clay *et al*. 1993; Clay 1996; Carriere *et al*. 1998) or secondary metabolites produced by the endophytes themselves (Latan 1993; Gindin

et al. 1994; Ball et al 1997a,b; Bush *et al*. 1997; Wang *et al*. 2007; Jaber & Vidal 2010; Saari *et al*. 2010; Guru 2011). It is important to mention that infection rates of natural populations of grasses by these endophytes can vary depending on the genetic and environmental background the population and these factors can determine if this symbiosis goes from mutualistic to antagonistic (Saikkonen *et al*. 2006; Rasmussen *et al*. 2008; Saari *et al*. 2010; Saikkonen *et al*. 2010; Young & Wilkinson 2010). Another hypothesis for the mechanism by which endophytes can negatively affect herbivores is based on the idea that endophytes can alter the phytosterol profiles of plants and compete with insects for these compounds which are essential for insect development (Dugassa-Govena *et al*. 1996; Raps & Vidal 1998). The mechanisms by which entomopathogenic endophytic fungi may protect plants from insect herbivores are unknown. Although these endophytes do produce secondary metabolites (Guru 2011; Ownley *et al*. 2008), we do not know if this is the main cause for the negative effects on aphids when feeding on endophytically-colonized plants observed in our study. The literature also suggests a systemic response in the plant can induced by the presence of some entomopathogenic endophytes including *B. bassiana* that confers resistance against plant pathogens (Ownley *et al*. 2008, 2010). Whether an induced systemic response accounts for the negative effects on insects observed in our study remains to be determined. The mode of establishment and duration of presence of endophytic fungi in plants varies among the different plant-endophyte combinations tested to date (Posada & Vega 2005; Posada *et al*. 2007; Reddy *et al*. 2009; Gurunlingappa *et al*. 2010; Akello *et al*. 2008; Powell *et al*. 2009; Biswas *et al*. 2011; Brownbridge *et al*. 2012). In some cases, intentionally inoculated endophytes can be retained within plants for considerable amounts of time, including *B. bassiana* found for as long as eight months in coffee (Posada *et al*. 2007) or nine months in

Pinus radiata (Brownbridge *et al*. 2012). Our study indicates that *B. bassiana* and *P. lilacinum* were still present in cotton plants up to 34 days following inoculation as a seed treatment. This duration does not necessarily indicate that *B. bassiana* and *P. lilacinum* can only be present in cotton as endophytes for this period of time, but rather that we did not test for the presence/absence of the endophytes beyond 34 days. The average recovery success of the target endophytes used in our studies ranged from 35-55%. Though not a high colonization frequency, we were still able to detect negative effects on aphids feeding on plants colonized by the endophytes. We have not yet rigorously studied the endophytic colonization of cotton by *P. lilacinum* and *B. bassiana*, but *P. lilacinum* was primarily detected in the root tissues whereas *B. bassiana* was found mostly in the above ground tissues. Fungal endophytes are known to occur throughout an entire plant including leaves, stems, roots and reproductive parts, however, tissue specific presence in plants is not required for negative effects on target herbivores. For example, endophytic fungi inhabiting roots can negatively affect the performance and fitness of caterpillars feeding on above ground tissues (Raps & Vidal 1998; Jaber & Vidal 2010). Our results support this scenario given that *P. lilacinum* negatively affects aphids feeding on cotton leaves above ground, but is recovered more commonly from below ground root tissues.

The manipulation of endophytic fungi, many of which are completely unstudied, has the potential to protect plants from insect herbivores and other stress factors (Porras-Alfaro & Bayman 2011). We have provided novel evidence showing that the endophytic establishment in cotton of the entomopathogens *B. bassiana* and *P. lilacinum* when inoculated as seeds can adversely affect cotton aphid reproduction not only in greenhouse assays, but also under field conditions. Although we observed a significant year effect, this was due to differences in the

total aphid numbers across years (Fig. 3a&b). Importantly, there was no year by endophyte treatment interaction effect. Our field results exhibited the same pattern of negative effects of endophytes on cotton aphids across years in both 2012 and 2013. The consistency of results across years under field conditions that can vary in variety of uncontrolled environmental variables (e.g. precipitation and temperature regimes) is particularly encouraging for the potential reliability of incorporating of fungal endophyte manipulations into IPM strategies. Future directions of our work include testing these entomopathogenic endophytes against other insect and nematode herbivores along with phytohormone and transcriptomic analysis to investigate the mechanisms by which these endophytes confer protection to their plant hosts.

CHAPTER III

THE ENDOPHYTIC FUNGAL ENTOMOPATHOGENS BEAUVERIA BASSIANA AND PURPUREOCILLIUM LILACINUM ENHANCE THE GROWTH OF CULTIVATED COTTON (GOSSYPIUM HIRSUTUM) AND NEGATIVELY AFFECT SURVIVAL OF THE COTTON BOLLWORM (HELICOVERPA ZEA)

Introduction

Fungal endophytes can protect plants from a wide range of stressors including insect pests (Porras-Alfaro & Bayman 2011). In this study, we refer to endophytes as defined by Schulz (2005), as microorganisms (fungi or bacteria) found in asymptomatic plant tissues for all or part of their life cycle without causing detectable damage to the host. Here we focus on entomopathogenic fungal endophytes (Vega *et al.* 2009) and the ecological roles these fungi can play in agricultural systems. Entomopathogenic fungal endophytes have been isolated from a variety of plant species and tissues, and single isolates can be inoculated to establish as an endophyte across a range of phylogenetically divergent plants (Vega *et al*. 2009; Rodriguez *et al.* 2009; Gurulingappa *et al.* 2010; Porras-Alfaro & Bayman 2011). These entomopathogenic fungal endophytes are classified as non-clavicipitaceous (Rodriguez *et al*. 2009), referring to fungal endophytes that are usually horizontally transmitted. Several non-clavicipitaceous entomopathogens including *Beauveria bassiana*, *Lecanicillium lecanii, Metharizium anisoplae* and *Isaria* (*Paecilomyces*) *spp.* can have negative effects on insect pests when *in planta*, may antagonize plant pathogens, and also promote plant growth (Ownley *et al*. 2004, 2008; Vega *et al.* 2009). For example, the application *B. bassiana* as an endophyte to tomato and cotton seedlings increased plant stand counts and height of the

plants when infected by damping off disease caused by the fungal plant pathogen, *Rhizoctonia solani* (Ownley *et al*. 2004; Griffin *et al*. 2005; Ownley *et al*. 2008). The mechanisms by which *B. bassiana* had a positive effect on plant growth may have been due to its antagonistic activity to *R. solani* either due to direct competition or by a systemic induced resistance in the plants (Ownley *et al*. 2008). A similar study using *M. anisoplae* conidia applications to seedlings for control of wireworms increased the stand count of corn and increased the yield at the end of the field season (Kabaluk & Ericsson 2007). The mechanism underlying the increase in yield was suggested to be due to the reduction in wireworms attacking roots, thereby allowing plants to better obtain soil nutrients and water (Kabaluk & Ericsson 2007).

The Heliothinae are a subfamily of about 365 species of noctuid moths, including a number of the world's most economically important crop pests, such as the Old World bollworm (*Helicoverpa armigera*) (Fitt 1989; Mathews 1999). Among the heliothis complex in the New World, *H. zea*, most commonly known as the corn earworm or cotton bollworm, has been reported to feed on over 100 plant species, including important economic crops in the United States such as corn, soybean, cotton and peanuts (Cho *et al.* 2008). Management of this insect has relied mostly on chemical control either by insecticidal sprays or by the use of genetically modified crops expressing transgenic insecticidal proteins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) (Jackson *et al.* 2007). The endophytic activity of *B. bassiana* has received particular attention due to its negative effects on a variety of insect herbivores including the cotton bollworm (Bing & Lewis 1991; McGee *et al*. 2002; Cherry 2004; Powell *et al.* 2009; Leckie *et al.* 2014). The fungus, *Purpureocillium lilacinum,* more widely known by its former name, *Paecilomyces lilacinus* (Luangsa-ard *et al*. 2011),

has been mainly considered a nematophagous, egg-parasitizing fungus, specifically against the root-knot nematode, *Meloidogyne incognita,* and several other plant-parasitic nematode species including *Radopholus similis*, *Heterodera spp*, *Globodeera spp* (Carrion & Desgarennes 2012; Kannan 2012; Khan 2012; Sharma & Trivedi 2012). However, *P. lilacinum* can also be pathogenic to insects (Castillo-Lopez *et al.* 2014). To our knowledge, the only study to date demonstrating negative endophytic effects of *P. lilacinum* on insect herbivores is Castillo-Lopez *et al.* (2014) who showed negative effects when present as a endophyte in cotton under on reproduction of the cotton aphid, *Aphis gossypium* Glover, both greenhouse and field conditions.

Several studies using fungal endophytes in *in planta* feeding assays or utilizing fungal extracts from endophytes have tested for negative effects on lepidopteran fitness (Bing & Lewis 1991; Cherry 2004; Powell *et al*. 2009; Reddy *et al.* 2009; Jaber & Vidal 2010; Mantzoukas *et al*. 2014; Leckie *et al.* 2014). Most of these studies have evaluated the survivorship and developmental rate of lepidopteran species, and mainly through the duration of the larval stage only. In contrast, Jaber & Vidal (2010) showed negative effects on adult life history parameters (i.e., fecundity) of the lepidopteran *H. armigera* feeding on endophyte inoculated plants versus control. The same significant negative effects were also observed in the F2 generation. The effects of *B. bassiana* as an entomopathogenic endophyte on *H. zea* have not been tested in *in planta* feeding assays utilizing cultivated cotton. Similarly, there are no published studies to date testing for effects of the entomopathogenic endophyte *P. lilacinum* on any lepidopteran species. Here we, (i) examined the plant growth enhancing effects of endophytic *B. bassiana* and *P. lilacinum* in cotton when inoculated as seed treatments using two different conidial concentrations, and (ii) tested the same endophytic

entomopathogens against *H. zea* in cotton for effects on survivorship, larval weight, pupal weight, days to pupation and days to eclosion using whole plant *in planta* feeding assays.

Materials and Methods

Plants and endophytic fungi strains: The cotton seeds used for all experiments were variety LA122 (All-Tex Seed, Inc.). The *P. lilacinum* strain was isolated from a field survey of naturally-occurring fungal endophytes in cotton (Ek-Ramos *et al*. 2013). This strain was confirmed to be *P. lilacinum* (formerly *P. lilacinus*) (Luangsa-ard *et al*. 2011) by diagnostic PCR and subsequent sequencing of the ribosomal ITS region using specific species primers (Atkins *et al*. 2004). The *B. bassiana* was cultured from a commercially obtained strain (Botanigard, BioWorks Inc, Victor, NY). Stock spore solutions of each fungus were made by adding 10 ml of sterile water to the fungi cultured on potato dextrose agar (PDA) in 10 cm diameter petri dish plates and scraping them with a sterile scalpel. The resulting mycelia and spores were then filtered through cheese cloth into a sterile beaker. A haemocytometer was used to calculate the conidia concentrations of the resulting stock solutions. Final treatment concentrations were reached by dilution using sterile water.

Cotton seed inoculation: Seeds were surface sterilized by immersion in 70% ethanol for 3 minutes with constant shaking, then 3 minutes in 2% sodium hypochlorite (NaOCl), followed by three washes in sterile water, based on Posada *et al*. 2007. The third wash was plated on PDA media to confirm surface sterilization efficiency. Seeds were then soaked for 24 hours in two different conidia concentrations of the two fungi and sterile water was used as the control. Spore concentrations for each fungus were zero (control), $1x10^6$ spores/ml

(treatment 1) and $1x10^7$ spores/ml (treatment 2) based on inoculum concentrations used in previous studies of endophytic entomopathogens (Posada & Vega 2005; Posada *et al*. 2007, Vega *et al*. 2008; Gurulingappa *et al*. 2010, 2011) including one of our own using the same protocol in which positive endophytic colonization frequencies of at least 50% were conservatively estimated for both fungi using the same variety of cotton (Castillo-Lopez *et al*. 2014). Beakers containing the soaking seeds were placed in a dark environment chamber at 28ºC until the next day for planting. Soaked seeds were planted in individual pots (15 cm diameter) containing unsterilized Metro mix 900 soil consisting of 40-50% composted pine bark, peat moss, vermiculite, perlite and dolomitic limestone (Borlaug Institute, Texas A&M). All plants were grown in a greenhouse at ~25ºC with natural photoperiod for the duration of the experiment. Pots were placed in a complete randomized design, watered as needed, and not fertilized throughout the experiments.

Cotton plant performance test: A factorial design was used to evaluate performance of plants inoculated as seeds with two different *B. bassiana* concentrations ($1x10^6$ and $1x10^7$ spores/ml), two different *P. lilacinum* concentrations ($1x10^6$ and $1x10^7$ spores/ml), and plants inoculated with water only. Seeds were treated as described in the previous section. The first trial was planted on June 6, 2013 and the second on June 25, 2014. Harvesting times were July 29, 2013 for the first trial and August 20th, 2014 for the second trial. At the end of each trial, the number of nodes was recorded and the aboveground and belowground dry biomass was measured after plants were harvested and dried at 60ºC for 7 days. The number of developing flowers (referred to as squares in cotton) was counted in the second trial only. The experiment was replicated twice with 12 and 15 plants per treatment in

the first and second trials, respectively. Plants were watered as needed, and no fertilizer was applied during the experiments.

***H. zea* developmental test:** We conducted two replicate experiments to test for effects of the two entomopathogenic fungal endophytes, *B. bassiana* and *P. lilacinum*, on the survival and development of cotton bollworm (*H. zea*). Seed treatments, planting dates, and harvesting times were the same as the plant performance experiment described above. *H. zea* eggs were obtained from Benzon Research (Carlisle, PA) and hatched at 22ºC. First instar neonates were fed on control (un-inoculated) cotton until they reached the 2nd instar in order to acclimate larvae to feed on live plants versus artificial diet. One second-instar larva was placed on a single plant with N=12 replicates per each endophyte x concentration treatment group during the first trial for a total of 60 plants. For our second replicate experiment, we increased the number of plants to 15 per treatment due to the high mortality observed during our first trial. Whole cotton plants were covered with cylindrical plastic cages containing a window of non-see um-mesh material (Eastex products, NJ) to allow air flow, but prevent the escape of larvae. Larvae were checked daily for mortality. Larvae were weighed twice throughout the experiment at 5 and 10 days during both experiment trials. We also recorded longevity of insects (from larva to adult death) where adults were separated by treatment and kept in medium plastic cages with lids 30 x 50 x 25 cm (Amazon Inc, Seattle, WA) were fed honey water and mortality was recorded daily. Days to pupation, pupal weight and days to eclosion (larva to adult) from control and treated plants was also recorded during both greenhouse trials.

Statistical analyses: All data were tested for normality assumptions using a qqplot, Levene's homogeneity test and the Shapiro-Wilk normality test at alpha=0.05 significance

level. Data from the two replicate trials were pooled within each experiment (plant and insect experiments) to test for overall trial and endophyte effects using a two-way ANOVA. Nested ANOVA analyses were performed for each experiment to test for concentration effect within endophyte treatments. Results from the cotton plant performance experiment were analyzed using a MANOVA with follow up univariate and pairwise analysis for dry biomass and number of nodes. The number of squares from the second greenhouse plant performance experiment was analyzed by non-parametric Kruskal-Wallis test with pairwise comparisons between endophyte treatments. For the first and second insect performance trials, a repeated measures ANCOVA was conducted with time as a repeated factor and initial larval weight as a covariate to test for differences in larval weight at 5 and 10 days of feeding on treated plants. Kaplan-Meier curve analyses, followed by pairwise comparisons, were used to analyze patterns of (i) insect survivorship (ii) days to pupation and (iii) days to eclosion of insects. All analyses were conducted using SPSS 22 (IBM SPSS, Armonk NY).

Results

Cotton plant performance test: There were no significant effects of seed treatment spore concentration one either plant dry biomass or number of nodes. There were significant overall effects of endophyte treatment (MANOVA, $F=3.16$, $df = 4,220$, $P=0.015$) and trial (MANOVA, $F=201.37$, $df=2,109$, $P<0001$) on plant performance, but no significant interaction effect. Follow-up examinations of the underlying univariate responses revealed significant effects of both endophyte treatment ($F=3.87$, $df=2,110$, $P=0.024$) and trial (and $F=391.63$, $df=1,10$,$P<0.0001$) on dry biomass. Both treatment and trial had significant effects

on number of nodes as well ($F=3.80$, $df= 2,110$, $P=0.025$ and $F=128.93$, $df=1,10$, $P<0.0001$, respectively) (Fig. 4a,b). There were no significant interactions between treatment and trial for either variable. Pairwise comparisons showed that the control plants had significantly lower biomass than plants treated with *B. bassiana* (LSD, $P=0.006$). *P. lilacinum* treated plants were similarly larger than the controls, but the difference was only marginally significant (LSD, $P=0.062$)(Fig. 1a,b). In pairwise comparisons, control plants also had fewer nodes than both *B. bassiana* (LSD, $P=0.004$) and *P. lilacinum* (LSD, $P=0.007$) treated plants (Fig 4c,d). The number of squares was only measured in the second trial and was significantly different among treatments (Kruskal-Wallis=7.237; $df=2,68$; $P=0.027$). Median number of squares per plant was one for control plants whereas it was two per plant for both the *B. bassiana* and *P. lilacinum* treated plants (Fig 5)

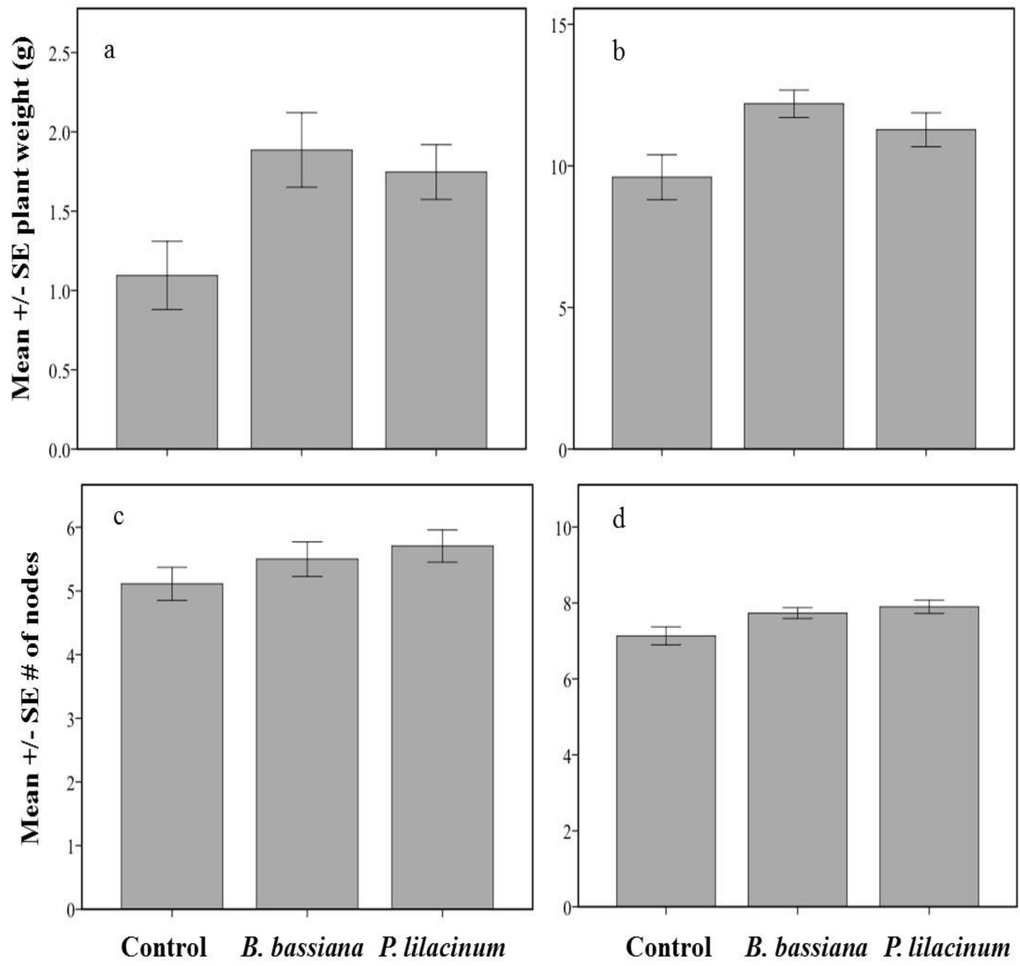

Figure 4. The effects of endophyte treatment on cotton dry biomass first and second trial (a & b) (P=0.024). Number of nodes first and second trial (c & d) P=0.025).

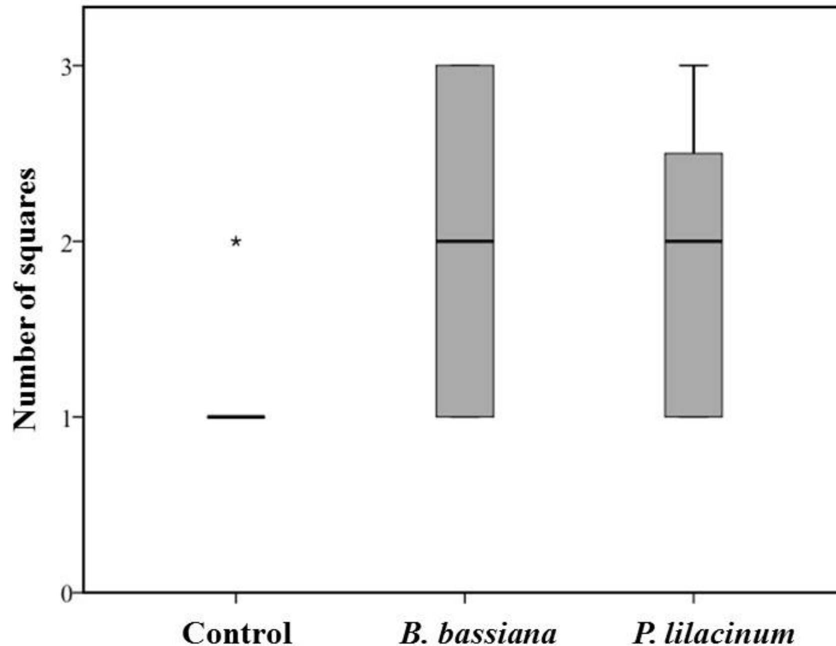

Figure 5. Box plots depicting the median and 5, 25, 75 and 95% range values of the number of developing flowers (squares) per plant in control and endophyte treated plants. The asterisk represents an outlier and was the only plant with two developing squares in the control group at the time of sampling.

***H. zea* survivorship:** No effect of endophyte concentration was observed on the number of days to death of *H. zea* individuals when data were pooled for both greenhouse trials (Nested ANOVA, F=0.2.35; df=2,131; P=0.09). Thus the data from both concentrations were pooled for subsequent analyses. There was a significant trial effect (ANOVA, F=22.86; df=1,30; P<0.000) and a significant endophyte treatment effect (ANOVA, F=3.05; df=2,130; P=0.05) on days to death, but no significant trial by endophyte treatment interaction. Analysis of Kaplan-Meier survival curves showed a significant difference in the survival of insects feeding on control plants vs. endophyte treated plants (P=0.020). Pairwise

comparisons showed control individuals lived longer on average than those on *B. bassiana* treated plants (20 days ± 2.48 vs. 14 days ± 1.34) (P=0.004). Control individuals also lived longer than those *P. lilacinum* treated plants (20 days ± 2.48 vs. 16.6 ±1.7), but the difference was only marginally significant (P=0.07) (Fig 6).

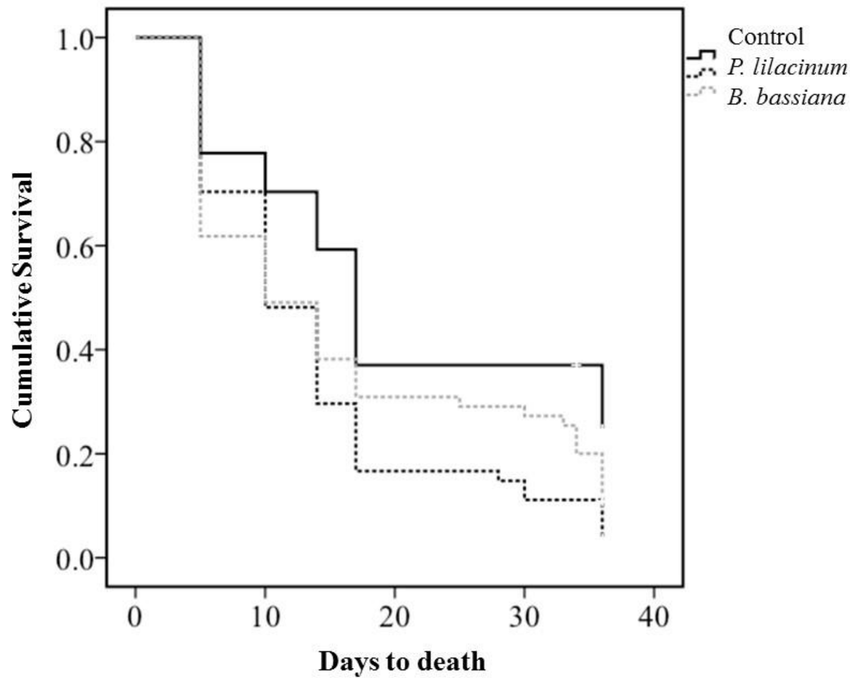

Figure 6. Kaplan-Meier survivorship curves of *H. zea* from 2nd instar to adult when fed on either control or endophyte-treated plants.

***H. zea* larval and pupal weights:** No effect of endophyte concentration was observed on the larval weights of *H. zea* individuals when data were pooled for both greenhouse trials (Nested ANOVA, F=1.89; df=2,62; P= 0.160). Thus data for both concentrations were pooled in for subsequent analysis. There was a significant trial effect (ANOVA, F=23.87; df=2,62; P<0.000), but no significant endophyte treatment effect or trial by endophyte treatment interaction effects. As expected, there was a significant effect of time on larval weight after 10 days of feeding *in planta* (Repeated Measures ANCOVA, F=42.09; df=1,62; P<0.000). There was also a strong effect of covariate (initial weight) (Repeated Measures ANCOVA, F=19.25; df=2,62; P<0.000), but no endophyte treatment effect was observed (Repeated Measures ANCOVA, F=1.12; df=2, 62; P=0.330) (Fig. 7). No effect of endophyte concentration was observed on the pupal weights of *H. zea* individuals when data were pooled for both greenhouse trials (Nested ANOVA, F=0.207; df=2,25; P= 0.815). Thus data for both concentrations were pooled in for subsequent analysis. There was a significant trial effect (ANOVA, F=14.17; df=2,25; P<0.000), but no significant endophyte treatment effect or trial by endophyte treatment interaction effects on the pupal weights (Fig. 8).

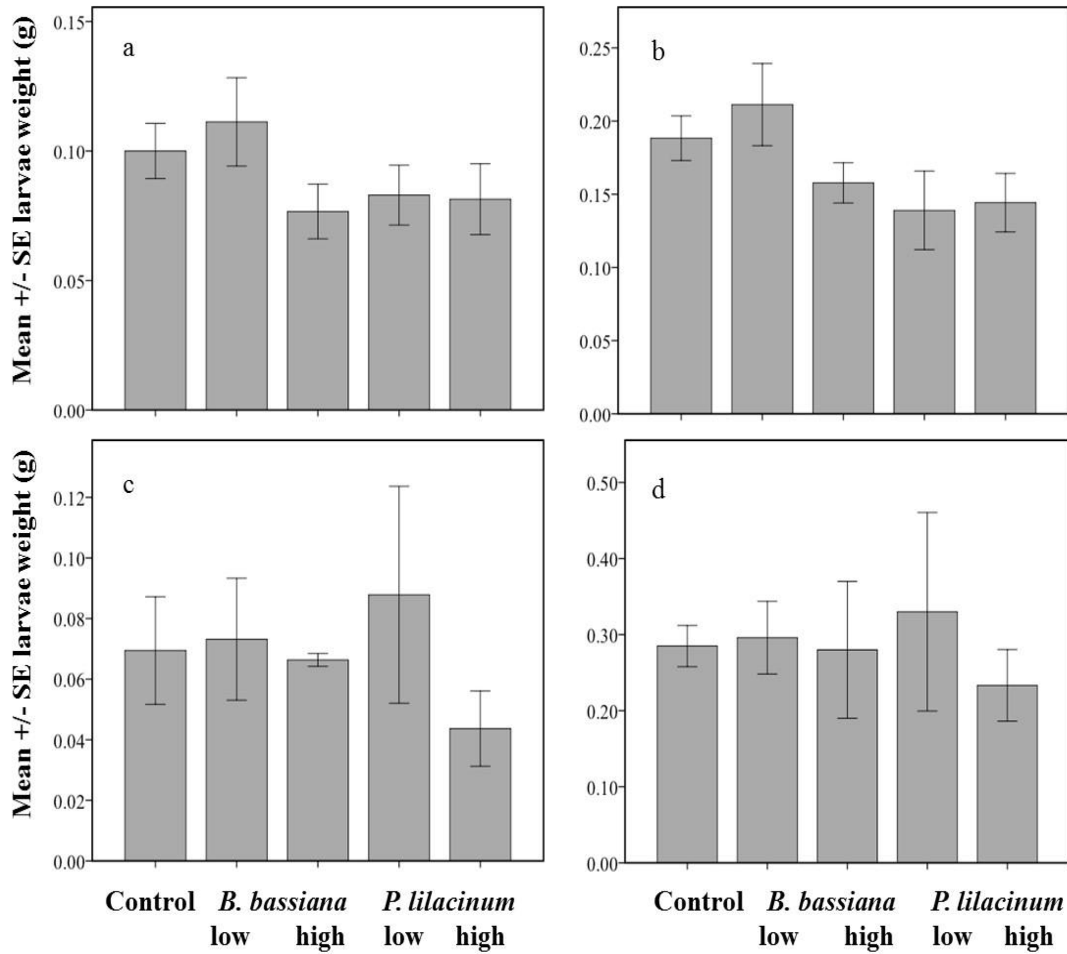

Figure 7. *H. zea* larval weights after feeding *in planta* on uninoculated control and endophyte-inoculated plants. (a & b) First trial weights after 5 and 10 days, respectively. (c & d) Second trials weights after 5 and 10 days, respectively.

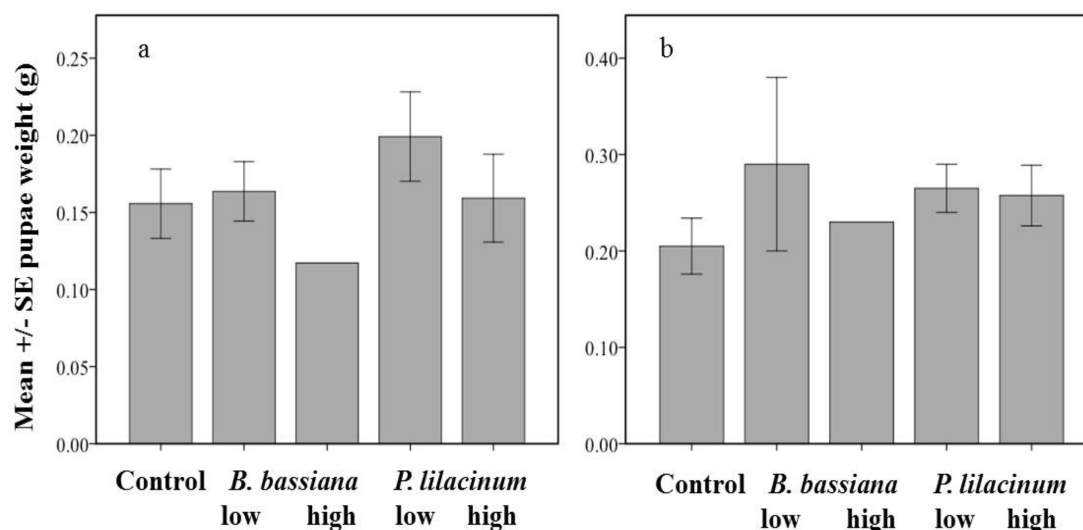

Figure 8. *H. zea* pupal weights after feeding *in planta* on uninoculated control and endophyte-inoculated plants. (a) First trial (b) second trials weights. Note that only one individual from *B. bassiana* high concentration survived as pupa. Thus no standard error could be calculated.

***H. zea* days to pupation and days to eclosion:** No effect of endophyte concentration was observed on the days to eclosion of *H. zea* individuals when data were pooled for both greenhouse trials (Nested ANOVA, F=0.949; df=2,35; P= 0.397). Thus data for both concentrations were pooled in for subsequent analysis. There was no significant effect of trial, endophyte or endophyte by trial interaction effects on days to pupation. Also, the Kaplan-Meier curve showed no significant effect of endophyte treatment on the days to pupation among *H. zea* individuals (P=0.756). For days to eclosion there was no effect of endophyte concentration when data were pooled for both greenhouse trials (Nested ANOVA, F=0.773; df=2,23; P= 0.473). Thus data for both concentrations were pooled in for subsequent analysis. There was a significant trial effect (ANOVA, F=66.05; df=2,23;

P<0.000), but no significant endophyte treatment effect or trial by endophyte treatment interaction effects on the days to eclosion of *H. zea* individuals. The Kaplan-Meier curve analysis indicated a marginally significant difference in days to eclosion among treatments (P=0.07). Pairwise comparisons showed control *H. zea* insects emerged faster as adults than those in the *B. bassiana* treatment group (Mantel-Cox, P=0.033) (28 days vs. 33 days). Individuals from the *P. lilacinum* treatment group also emerged faster as adults than those in the *B. bassiana* treatment group (Mantel-Cox, P=0.047) (29 days vs. 33 days).

Discussion

Our results provide the first report for plant growth enhancing effects in cotton in response to inoculation with the endophytes, *B. bassiana* and *P. lilacinum.* Additionally, our results indicate negative effects of the presence of these endophytic fungi on *H. zea* survivorship and marginal effects on performance-related traits when feeding on whole intact cotton plants inoculated as seed treatments. The mode of establishment and duration of presence of non-clavicipitaceous endophytic fungi in plants varies among the different plant-endophyte combinations tested to date (e.g., Posada & Vega 2005; Gurunlingappa *et al*. 2010; Posada *et al*. 2007; Akello *et al*. 2008; Powell *et al*. 2009; Reddy *et al*. 2009; Brownbridge *et al*. 2012). In some cases, intentionally inoculated endophytes can be retained within plants for considerable amounts of time, including *B. bassiana* found for as long as eight months in coffee (Posada *et al*. 2007) or nine months in *Pinus radiata* (Brownbridge *et al*. 2012). Our previous studies have indicated that *B. bassiana* and *P. lilacinum* can be detected in cotton plants for at least 34 days following inoculation as a seed treatment. This

duration does not necessarily indicate that *B. bassiana* and *P. lilacinum* can only be present in cotton as endophytes for this period of time, but rather that we have not tested for the presence/absence of the endophytes beyond 34 days. The average recovery success of the target endophytes used in this experiment ranges from 53-55% of the plants sampled (Castillo-Lopez *et al*. 2014). Due to difficulty in adequately sampling all tissues from larger plants and the possibility that surface sterilization treatments and competition with other endophytes during plating affect fungal culturing and recovery success, these colonization frequencies are likely underestimates.

Our results showed significant plant growth enhancing effects of fungal endophyte treatment on plant dry biomass, number of nodes, and number of reproductive tissues (squares) in plants treated with entomopathogenic endophytes. The literature suggests that genotype-specific interactions between plants and endophytes may either enhance, reduce, or have no effect on plant fitness (Rodriguez *et al*. 2009 Saikkonen *et al*. 2010). A few studies have shown positive effects on plant growth following the conidial application of entomopathogenic endophytes including higher stand count, root and shoot growth (Lee *et al*. 1999; Ownley *et al*. 2004, 2008; Griffin *et al*. 2005; Kabaluk & Ericsson 2007). The effects on plant growth shown in these studies were observed in experiments in which the endophytic entomopathogens were used as targeted biocontrol agents against soil pathogens or herbivores, and increased plant growth was attributed to reducing the damage associated with the stressors. However, the potential for either direct or interactive plant growth enhancement mediated by the endophytes in the absence of the stressor as observed in our study cannot be rule out. In terms of the mechanisms underlying plant growth enhancement, a few studies done mostly on endophytic bacteria-plant interactions have shown that the

effects can be due either to the fixation of nutrients from the soil, production of a growth regulating metabolite by the microbe itself or due to up-regulation of growth hormones in plants (e.g. auxins, gibberellins, cytokinins and ethylene) (Windham *et al*. 1986; Kleifeld & Chet 1992; Glick 1998; Malinowski & Belesky 2000; Tudzynski & Sharon 2002; Pierik *et al*. 2006; Berg 2009). Though we do not know the mechanism behind the plant growth enhancement observed in our study, we provide the first report of increased growth (biomass and number of nodes) and reproductive tissue development in cultivated cotton inoculated with the entomopathogenic endophytes, *B. bassiana* and *P. lilacinum,* in the absence of any experimentally imposed stressors.

Several non-mutually exclusive mechanisms have been proposed to account for negative effects of fungal endophytes on insect survival and performance. These include production of secondary metabolites, production of superoxides, change of the phytosterol profile of plants, or by inducing an indirect systemic defense response in the plant thus conferring resistance to insect feeding (Raps & Vidal 1998; Schardl 2004, 2007; Tanaka 2006; Huang 2007; Hartley & Gange 2009; White Jr & Torres 2010). A study done by Powell *et al*. (2009) showed some mortality of *H. zea* larvae due to mycosis when dead individuals were removed from *B. bassiana* inoculated tomato plants and then incubated on fungal growth media (Powell *et al*. 2009). A recent experiment conducted in our laboratory supports the systemic induced resistance hypothesis and suggests there is a priming effect of the plant defense system when colonized by *B. bassiana* and *P. lilacinum* and then challenged by herbivore feeding (D. Castillo-Lopez, E. Borrego, M. Kolomiets, J. Wulff and G.A. Sword, unpublished results) (see Chapter 4).

Rather than analyzing our survivorship data as a percentage of individuals alive at the end of our experiment, we conducted a Kaplan-Meier survival analysis where the survival rate is expressed as the survival function [S(time) = # of individuals surviving longer than (time)/total number of individuals in study] (Kaplan & Meier 1958). *H. zea* individuals feeding on plants inoculated with endophytes exhibited significantly lower survival compared to those feeding on control plants from the 2nd instar to pupation (Fig. 2)**.** Similar results were found by Jaber & Vidal (2010) who showed that *H. armigera* feeding on endophyte inoculated *V. faba* plants in the first generation of the insects had reduced survival rate but this effect was not observed in the subsequent generation. A reduction in survival was also observed for *H. zea* larvae feeding on *B. bassiana* inoculated tomato plants (Powell *et al*. 2009). Although our data from both greenhouse trials were pooled for analysis and no significant seed inoculation concentration effect was detected within the endophyte treatments in the ANOVA, by the end of our experiments, no individuals from the *B. bassiana* high conidia concentration treatment made it to the adult stage in either of our greenhouse trials. To our knowledge, no studies in the literature using *in planta* herbivore feeding assays on plants colonized by endophytes have shown a differential response due to conidial concentration. Importantly, survivorship was generally low in our experiments, even among the control group insects in both trials. We attribute this effect to the reduced vigor of lab colony insects maintained on artificial diets across generations and then forced to feed on living host plants. Nonetheless, our results clearly showed poorer survival of the insects that fed on endophyte treated plants relative to the uninoculated controls.

The significantly lower survival of the larvae feeding on endophyte inoculated plants observed in our study was not due to mycosis of insects as evidenced by a lack of fungal

growth emerging from the cadavers after being placed on wet filter paper to observe any subsequent sporulation of the target endophytes (D. Castillo-Lopez, pers. observ). In a study done by Jaber & Vidal (2010), the endophyte *Acremonium strictum* could not be re-isolated from *V. faba* leaves, thus the authors concluded that the observed negative effects on *H. armigera* larval performance were not due to direct contact between the endophyte and the insect, but rather to an endophyte-triggered indirect effect when the insects fed on endophyte inoculated plants.

The weights of larvae and pupae did not differ between endophyte treatment groups in either of our greenhouse trials. Several studies investigating the role of endophytes in protecting several different crops against lepidopteran species did not measure insect life history or performance parameters, but rather looked for reduction of plant damage caused by the insect (Bing & Lewis 1991; Cherry *et al*. 2004; Reddy *et al*. 2009; Mantzoukas *et al*. 2014). Some studies have found an effect of endophytes on gained weight of lepidopteran species including *H. armigera*, *H. punctigera* and *H. zea*, but these effects were observed by incorporating fungal broth extracts of the endophytes into artificial diets rather than evaluating effects on insect growth via *in planta* feeding assays (McGee *et al*. 2002; Powell *et al*. 2009 and Leckie et al. 2014). Two studies similar to ours that utilized *in planta* feeding assays (Jallow *et al*. 2004 and Jaber & Vidal 2010) found a significant decrease in relative growth rate of *H. armigera* larvae feeding on *A. strictum*-inoculated plants, but they did not observe a difference in pupal weights among treatments. A limitation of our study was that we did not measure consumption of plant material by larvae feeding on plants inoculated with endophytes versus control plants, which could have shed some light on possible inhibition or compensatory feeding behavior of larvae. As suggested by Moran & Hamilton

(1980), some phytophagous insects feeding on low quality plants tend to compensate through increased consumption of plant material. This was also suggested by Jaber & Vidal (2010) who observed that *H. armigera* larvae feeding on endophyte-inoculated plants consumed more plant tissues than larvae feeding on control plants, possibly explaining why they found no difference in pupal weights even though they observed lower larval weights.

Even though we did not observe any difference in days to pupation in our greenhouse trials, a study done by Leckie *et al*. (2014) using *H. zea* individuals feeding on *B. bassiana* broth extracts incorporated into artificial diet showed that larvae feeding on endophyte treatments pupated faster than control (13 vs. 15 days). In contrast, Jaber & Vidal (2010) showed that *H. armigera* feeding on endophyte treated plants in *in planta* trials exhibited slower larval and pre-pupal developmental times than individuals feeding on control plants. Differences in days to adult eclosion were only marginally significant. In our experiment using *in planta* trials, control insects reached the adult stage faster than the *B. bassiana* endophyte treatment (P=0.033), but no difference was observed with individuals that fed on *P. lilacinum* treated plants. Similar delayed emergence of adults following larval development on endophyte treated plants was observed in *H. armigera* by Jaber & Vidal (2010) and in *H. zea* by Liecke *et al*. (2014).

In conclusion, the manipulation of endophytic fungi has the potential to protect plants from insect herbivores and other stressors (Vega *et al*. 2009; Rodriguez *et al*. 2008; Hartley & Gange 2009; Porras-Alfaro & Bayman 2011). Our study demonstrated for the first time the positive effects of the endophytic entomopathogens *B. bassiana* and *P. lilacinum* on plant growth enhancement in cultivated cotton. In addition to positive effects on plant growth, we also observed negative effects of the survival and development of a key herbivorous insect

pest, *H. zea*. In conjunction with previously reported examples of successfully manipulating the presence of these endophytes in cotton under field conditions (Castillo-Lopez *et al*. 2014), our study further supports the use of these beneficial entomopathogenic fungal endophytes as a component of IPM practices to protect plants from pests and enhance plant growth.

CHAPTER IV

ENDOPHYTIC FUNGAL ENTOMOPATHOGENS ALTER PLANT DEFENSIVE HORMONE SIGNALING PATHWAYS IN CULTIVATED COTTON (*GOSSYPIUM HIRSUTUM*)

Introduction

Plants interact with a variety of organisms in their environment such as bacteria, fungi, viruses and herbivores (Van-Wees *et al*. 2008). These interactions can have negative, neutral or positive effects on the plant (Kogel *et al*. 2006). Among the interactions between plants and microorganisms are mutualisms, in which both the plant and microbe benefit from the association (Kogel *et al*. 2006). Some of these mutualistic relationships include the interactions between plants and an assortment of mycorrhizae, rhizobium bacteria and endophytes (Van der Heijden 1998; Smith & Read 2008; Porras-Alfaro & Bayman 2011).

Endophytes are defined as microorganisms (fungi or bacteria) found in asymptomatic plant tissues for all or part of their life cycle without causing detectable damage to the host (Schulz 2005). Fungal endophytes have been found to protect plants from a wide range of stressors (Porras-Alfaro & Bayman 2011, Rodriguez *et al*. 2009). Entomopathogenic fungal endophytes, classified as non-clavicipitaceous endophytes have been isolated from a variety of different plant species and tissues, and can be inoculated to establish endophytically in a range of other plants to test for negative effects, if any, against pathogens, herbivores or other stressors (Ownley *et al*. 2008, 2010; Rodriguez *et al*. 2009; Vega *et al*. 2008, 2009). Clavicipitaceous endophytes, on the other hand, have been studied more extensively. These endophytes are typically found as part of an obligate relationship in grasses in which they are

vertically transmitted across generations via seed and exhibit higher infection levels within their hosts (Schardl *et al*. 2004; Hartley & Gange 2009; Rodriguez *et al*. 2009).

The plant defense system is similar to the animal innate immune system where recognition of non-self molecules occurs (Howe & Jander 2008, Jones & Dangl 2006; Pieterse *et al*. 2009, 2012). Plant immune responses to beneficial microbes have started to receive more attention in the past decade (Van-Wees *et al*. 2008; Pieterse *et al*. 2009; 2012). It has been suggested that beneficial microbes are recognized by the plant immune system in the same way as pathogenic microorganisms, resulting in a mild systemic activation of the immune response through microbe-associated molecular patterns (MAMPs) (Van-Wees *et al*. 2008; Pieterse *et al*. 2009). Thus, active interaction with the plant immune system is thought to be fundamental for the establishment of an intimate mutualistic relationship with the plant (Pieterse *et al*. 2009; Zamioudis & Pieterse 2012). Among the most studied beneficial plant-microbe associations are those involving Nitrogen fixing bacteria, non-pathogenic *Pseudomonas* species, species of *Trichoderma* fungi, the clavicipitaceous fungal endophyte *Epichloe festucae,* and a few species of arbuscular mycorrhizae (AM) (Ryu *et al*. 2004; Meziane *et al*. 2005; Ahn *et al*. 2007; Campos-Soriano *et al*. 2010, 2011, 2012; Fernandez *et al*. 2014). To name just a few examples, *P. fluorenses* SS10 and *P. putida* WC5358 are able to activate systemic resistance in tomato (Iavicoli *et al*. 2003; Tran *et al*. 2007). The fungi *T. virens* and *T. asperellum* can induce a systemic response in maize and cucumber, respectively (Djonovic *et al*. 2007; Shoresh *et al*. 2005). In the *E. festucae* and perennial grass system, 59 pathways have been shown to be involved in maintaining the mutualistic relationship between endophyte and plant including the stress-activated MAP kinase sakA61 and the transcription factor proA (Eaton *et al*. 2010; Tanaka *et al*. 2013).

Local and systemic immune responses triggered by beneficial microbes are controlled by a complex signaling network where plant hormones play an important role by cross-communicating in an antagonistic or synergistic manner (Pieterse *et al*. 2009, 2012; Jaillais & Chory 2010; Robert-Seilaniantz *et al*. 2011a). Among the best-studied plant hormones are salicylic acid (SA), the oxylipin jasmonic acid (JA), and ethylene (ET) as primary signals in local and systemic induced defense in plants (Pieterse *et al*. 2009). It has been suggested that biotrophic pathogens generally activate plant defense responses regulated by SA, whereas pathogens with a necrotrophic lifestyle induce defenses controlled by JAs and ET (Glazebrook 2005; Van-Wees *et al*. 2008). Similar to the defense response to necrotrophs, response to herbivore and mechanical damage is generally regulated by the JA signaling pathway (Howe 2004). A long-lasting and broad-spectrum induced disease resistance is referred to as systemic acquired resistance (SAR) and this response is SA-dependent (Pieterse *et al*. 2009; 2012). In contrast to SAR, induced systemic resistance (ISR) is triggered by beneficial microbes and is generally regulated by JA- and ET-dependent signaling pathways, associated with priming the plant defense response rather than a direct activation of defense (Conrath 2006; Pozo *et al*. 2008; Van-Wees *et al*. 2008; Pieterse *et al*. 2009).

Other less studied, but nonetheless important, plant hormones include azelaic acid (AZA), 12-oxo-phytodienoic acid (OPDA), traumatic acid (TA) and cinnanimic acid (CA). AZA is a mobile molecule found to confer pathogen resistance to local and systemic tissues by priming SA accumulation and SA-associated gene expression in *Arabidopsis* (Jung *et al*. 2009). Upstream of the octadecadenoid pathway (JA-pathway) is OPDA, a molecule found to have similar signaling capabilities as JA, but also found to regulate gene expression through

electrophilic activity (Farmer *et al*. 2003; Beckers & Spoel 2006). TA, formed from linolenic acid such as JA, has a wound response thought to modify auxin levels or auxin activity (Gaspar *et al*. 1996; Noordermeer *et al*. 2001). CA is involved in the alternative pathway that has been studied in tobacco for the synthesis of salicylic acid (SA) from phenylalanine via benzoic acid (Shah 2003).

The most common phytohormone studies of plant-fungi interactions involve arbuscular mycorrhizal (AM)-plant associations. Hormones such as abscisic acid (ABA), SA, and JAs are believed to play a key role not only in the establishment, but also in the maintenance of the symbiosis (Hause *et al*. 2007; Herrera-Medina *et al*. 2007; Lopez-Raez *et al*. 2010a; Ludwig-Muller 2010). However, the results of investigations into this symbiosis have been contrasting; some reports have shown reduced SA levels upon root colonization by fungi whereas others have shown unaltered or higher SA levels (Herrera-Medina *et al*. 2003; Herrera-Medina *et al*. 2007; Khaosaad *et al*. 2007; Lopez-Raez *et al*. 2010a; Campos-Soriano & Segundo 2011; Roman *et al*. 2011). Similarly variable responses have been shown for ABA ranging from an increase, no change, or decrease in content levels (Meixner *et al*. 2005; Aroca *et al*. 2008 Lopez-Raez *et al*. 2010a; Martinez-Medina *et al*. 2011). Oxylipins such as JA's, on the other hand, have been shown to have both positive and negative effects on the plant-mycorrhizae symbioses (Hause & Schaarschmidt 2009; Lopez-Raez *et al*. 2010a; Leon-Morcillo *et al*. 2012).

Plant defense hormone studies in cultivated cotton (*Gossypium hirsutum*) have mainly focused on either pathogen, herbivore, fiber elongation or abiotic stress responses (Dowd *et al*. 2004; Lin *et al*. 2006; Shi *et al*. 2007; Yao *et al*. 2011; Zhang *et al*. 2011, etc). To our knowledge there have been no defensive phytohormone profiling studies to date in cultivated

cotton when inoculated with beneficial microbes. Recently, colonization of cotton by the entomopathogenic fungal endophytes, *Beauveria bassiana* and *Purpureocillium lilacinum,* was shown to have negative effects on cotton aphid (*Aphis gossypi*) reproduction under both greenhouse and field conditions (Castillo-Lopez *et al.* 2014). Though the mechanism underlying endophyte-mediated resistance to insects was not elucidated in the study, several hypotheses have been proposed including that such a response may be due to the induction of an indirect systemic defense response (priming effect) in the plant due to the presence of the endophyte (Tanaka 2006; Huang 2007; Hartley & Gange 2009; White Jr & Torres 2010). Given this background, we set out to empirically address the following questions: (1) Are endogenous hormonal profiles in cotton affected when endophytically colonized by *B. bassiana* and *P. purpureocillium* in the absence of aphid herbivory as a stressor? (2) Is there a priming effect when cotton is colonized by either endophyte and then challenged by aphid feeding? (3) Are cotton hormonal responses the same for both fungal endophytes?.

Materials and Methods

Plant growth and endophyte inoculation: The cotton seeds used for all experiments were variety LA122 (All-Tex Seed, Inc.). The *P. lilacinum* strain was isolated from a field survey of naturally-occurring fungal endophytes in cotton (Ek-Ramos *et al*. 2013). This strain was confirmed to be *P. lilacinum* (formerly *P. lilacinus*) (Luangsa-ard *et al*. 2011) by diagnostic polymerase chain reaction (PCR) and subsequent sequencing of the ribosomal internal transcribed spacer (ITS) region using specific species primers (Atkins *et al*. 2004). The *B. bassiana* was cultured from a commercially obtained strain (Botanigard, BioWorks Inc, Victor, NY). Seeds were surface sterilized by immersion in 70% ethanol for 3 minutes

with constant shaking, then 3 minutes in 2% sodium hypochlorite (NaOCl), followed by three washes in sterile water, based on the methods of Posada *et al*. (2007). The third water wash was plated on potato dextrose agar (PDA) media to confirm surface sterilization efficiency. Fungal endophytes were cultured on liquid Sabouraud dextrose agar (SDA) for 7-10 days to produce biomass. Fungal biomass was harvested, freeze-dried and ground to create a powder of conidia and mycelia from each fungus. The powder of each endophyte was then mixed in a methylcellulose solution (35mg/3ml of methylcellulose) (Carolina Biological Supply Company, Burlington, NY) and applied to cotton seeds. Seeds were coated with the solution from each endophyte or with methylcellulose sticker only as the control. Coated seeds were allowed to dry overnight, then planted in individual pots (15 cm diameter) containing unsterilized Metro mix 900 soil consisting of 40-50% composted pine bark, peat moss, vermiculite, perlite and dolomitic limestone (Borlaug Institute, Texas A&M). All plants were grown in a controlled environmental room at ~25ºC with a 12:12 light:dark photoperiod. Pots were placed in a completely randomized design, watered as needed, and not fertilized throughout the experiments. The first trial experiment was planted on September 5, 2014 and, the second trial was planted on October 5, 2014.

Herbivore feeding assays: A colony of cotton aphids (*Aphis gossypii* [Glover]) was maintained on caged cotton plants in the same environmental conditions as the experimental plants. Three cotton aphid adults were placed on each plant from endophyte and control treatments. Individual clear plastic cages of 45 cm height and 20 cm diameter were used to prevent aphid escape or movement between plants. For the cotton aphid feeding assays, we utilized cotton plants on the 3rd true leaf stage. Aphids were left to feed on plants for one, four, eight, 24 or 48 hours. At the end of the herbivory treatment period, aphids were

removed, plants were harvested and freeze dried in liquid nitrogen for processing as described below. Cohorts of cotton plants from both the endophyte and control treatments that were not challenged with aphid feeding were similarly processed in order to measure endogenous levels of hormones prior to herbivory. There were five biological replicates per treatment. Each biological replicate consisted of two plants pooled into the same sample making a total of 90 biological replicates per experimental trial. We repeated the experiment twice in time.

Hormone quantification in cotton by UPLC-MS/MS: Each plant was divided into leaf and root tissues, ground using a mortar and pestle, and stored at -62ºC until hormone quantification. Hormones were extracted by placing ~100 ± 10 mg of tissue from each sample into a 1.5 ml vial. Precise weights were recorded for later hormone calculations. A mixture containing 10 µl of 5 µM internal standards and 500 ul of extraction buffer [1-propanol/H2O/concentrated HCl (2:1:0.002, vol/vol/vol) was added to each sample. Samples were agitated for 30 min at 4°C, then 500µl of Dichloromethane (CH2Cl2) were added. Samples were agitated again for 30 min at 4°C, and then centrifuged at 13,000g for 5 min. in darkness. The lower layer was removed into a glass vial and the organic solvent was evaporated by drying samples for 30-40 min. Samples were re-solubilized in 150ul of MeOH, shaken for 1 min and centrifuged at 14,000g for 2 min. A supernatant of 90ul was transferred into the autosampler vial and hormones were analyzed by ultraperformance liquid chromatography, coupled to mass spectrometry (UPLC-MS/MS). Quantifications were carried out with MassLynx 4.1 software (Waters), using the internal standards as a reference for extraction recovery. Leaf and root tissue was saved in -62ºC and saved for subsequent gene expression analysis.

Statistical analyses: All data were tested for normality assumptions using a qqplot, Levene's homogeneity test and the Shapiro-Wilk normality test at alpha=0.05 significance level. For the hormone quantification results using UPLC/MS-MS data from both greenhouse trials were initially pooled but was analyzed separately by aboveground (leaves) and belowground (root) tissue. Using a one-way ANOVA, we tested for endogenous responses of each hormone to endophyte-treatment in the absence of aphid herbivory. We also used a One-Way ANOVA to test for changes in phytohormone levels overall and tested for herbivory, endophyte, and trial as main effects, along with any interactions between these independent variables. In the event of significant interactions between endophyte treatment and trial, the results were then reanalyzed separately by trial to further examine differential responses among the endophyte treatments associated with these factors. Significant endophyte by time interactions were followed up using pairwise comparisons at each time to further examine the differential responses mediated by the endophytes over time.

Results

Aboveground tissue: The endogenous levels of ABA in the absence of herbivory were not significantly affected by endophyte treatment or the interaction between endophyte and trial. However, the endogenous response was significantly different by trial ($F=77.53$; $df=1,31$; $P<0.001$) when both greenhouse trials were pooled (Fig 9, Table 1 see page 64). One-way ANOVA results showed that the overall response of ABA varied significantly with herbivory time ($F=4.05$; $df=5,142$; $P=0.002$) and by endophyte treatment ($F=4.51$; $df=2,142$; $P=0.013$) but there was no significant interaction between endophyte and herbivory time or

endophyte and trial (Fig 9, Table 1). Pairwise comparisons between endophye treatments showed that control plants had higher ABA levels than *P. lilacinum* treated plants at one hour (LSD, P=0.004).

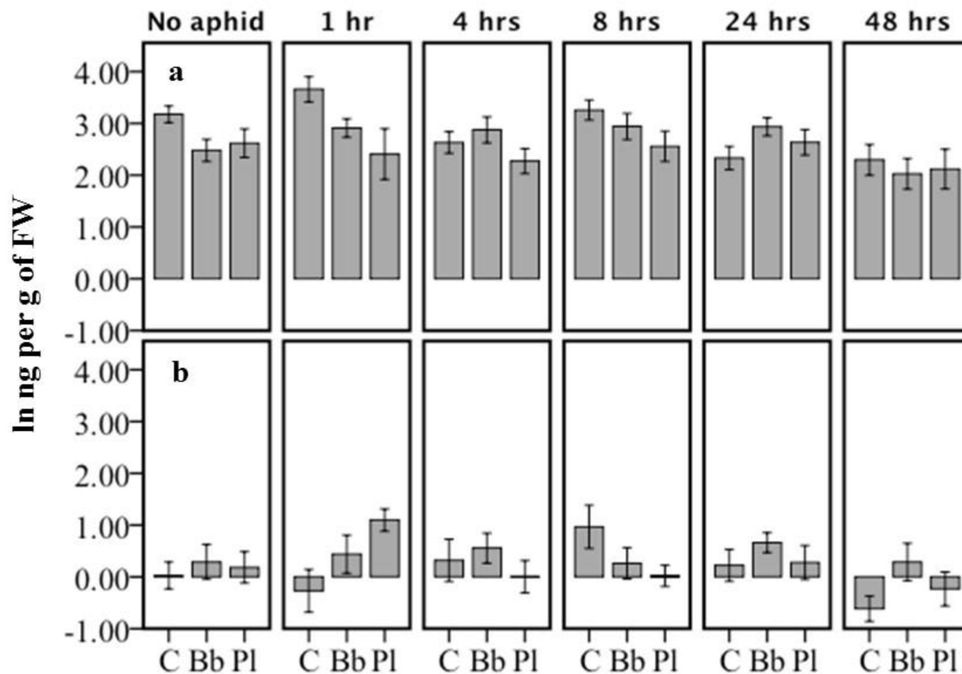

Figure 9**.** Abscisic acid (ABA) levels in endophyte-treated plants before and after aphid herbivory in a) leaves and b) root tissue. C=control, Bb=*B. bassiana* and Pl=*P. lilacinum*.

The endogenous levels of AZA were not significantly different by endophyte treatment, by trial or by the interaction between endophyte treatment and trial. One-way ANOVA results showed the overall response of AZA was significant by endophyte treatment

(F=3.02; df=2,142; P=0.05) and trial (F=76.99; df=1,142; P<0.001), with marginally significant variation across herbivory time (F=2.02; df=5,142; P=0.07). There were no significant interactions between either endophyte and trial or endophyte and herbivory time. Endophyte treatment pairwise comparisons showed control plants had lower levels of AZA than *B. bassiana* treated plants at one and four hours (LSD, P=0.010) and lower levels than *P. lilacinum* plants at 1 hour (P=0.029). Also *B. bassiana* had higher levels of AZA than *P. lilacinum* treated plants at eight hours (LSD, P=0.007) (Fig 10, Table 1).

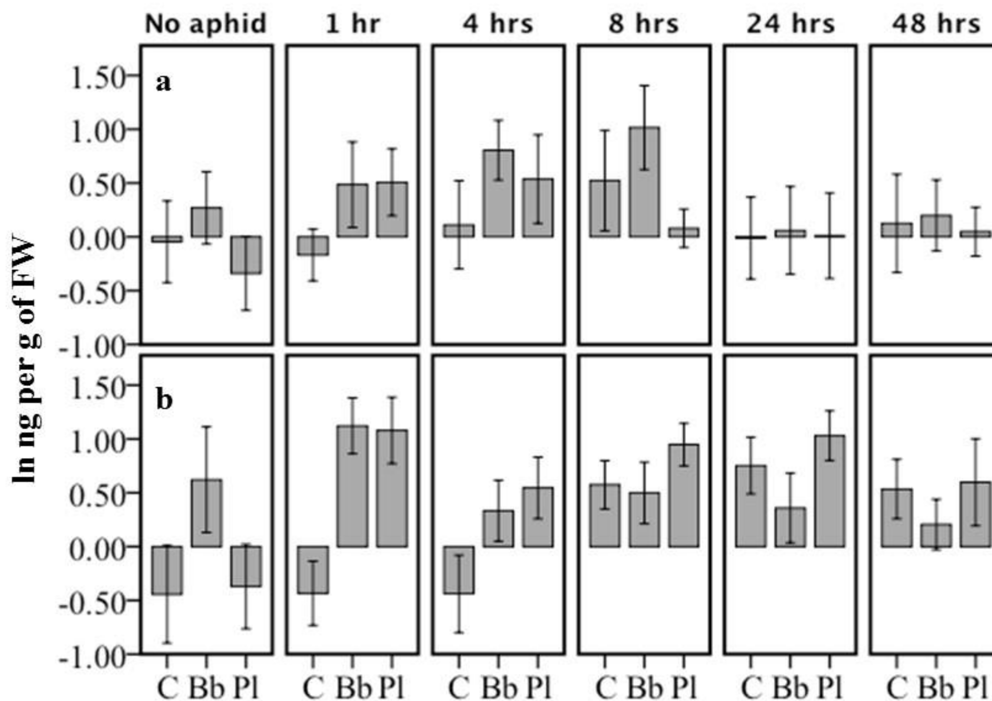

Figure 10**.** Azelaic acid (AZA) levels in endophyte-treated plants before and after aphid herbivory in a) leaves and b) root tissue. C=control, Bb=*B. bassiana* and Pl=*P. lilacinum*.

The endogenous levels of OPDA were not different among endophyte treatments. The endogenous response was significantly different between trials (F=55.93; df=1,31; P<0.001) when both greenhouse trials were pooled, but there was no significant endophyte by trial interaction. One-way ANOVA results showed the overall response of OPDA was significantly different across herbivory time (F=3.58; df=5,142; P=0.001), by endophyte treatment (F=5.22; df=2,142; P=0.028), by trial (F=305.90; df=1,142; P<0.001), but there was no interaction between endophyte and trial or endophyte and herbivory time. Endophyte treatment pairwise comparisons showed control plants had lower OPDA levels than *B. bassiana* treated plants at 1 hour (LSD, P=0.023) and lower than *P. lilacinum* plants at one hour (LSD, P=0.003). Also *B. bassiana* plants had higher OPDA levels than *P. lilacinum* plants at four hours (LSD, P=0.041) (Fig 11, Table 1).

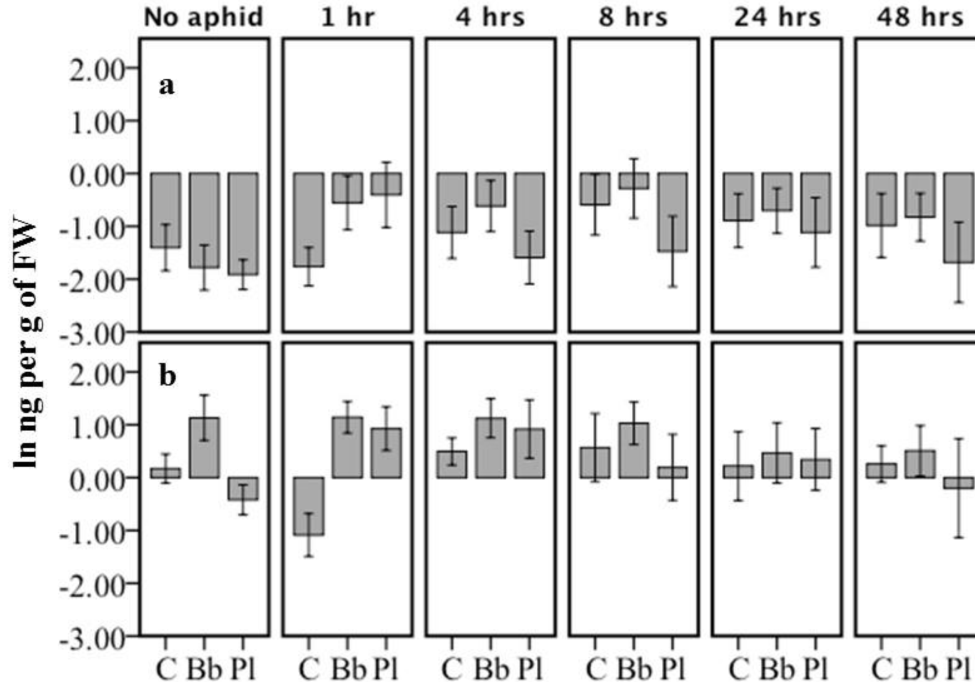

Figure 11. 12-oxo-phytodienoic acid (OPDA) levels in endophyte-treated plants before and after aphid herbivory in a) leaves and b) root tissue. C=control, Bb=*B. bassiana* and Pl=*P. lilacinum.*

The endogenous levels of JA were not significantly different among endophyte treatments, but were significant by trial (F=10.76; df=1,31; P=0.003) and by the interaction between endophyte and trial (F=3.50; df=2,31; P=0.04) when data from both greenhouse trials was pooled. One-way ANOVA results showed the overall response of JA was significantly different across herbivory time (F=4.81; df=5,141; P<0.001), by endophyte treatment (F=3.11; df=2,141; P=0.048) and by trial (F=234.81; df=1,141; P<0.001) and an interaction between endophyte and herbivory time (F=2.73; df=10,141; P=0.004), but there

were no significant interactions between endophyte and trial. Endophyte treatment pairwise comparisons showed control plants had lower levels of JA than *P. lilacinum* and *B. bassiana* plants at one hour (LSD, P=0.004, P=0.041,, respectively). Also the response of JA was higher in *B. bassiana* treated plants than *P. lilacinum* treated plants at eight hours (LSD, P=0.014) (Fig 12, Table 1).

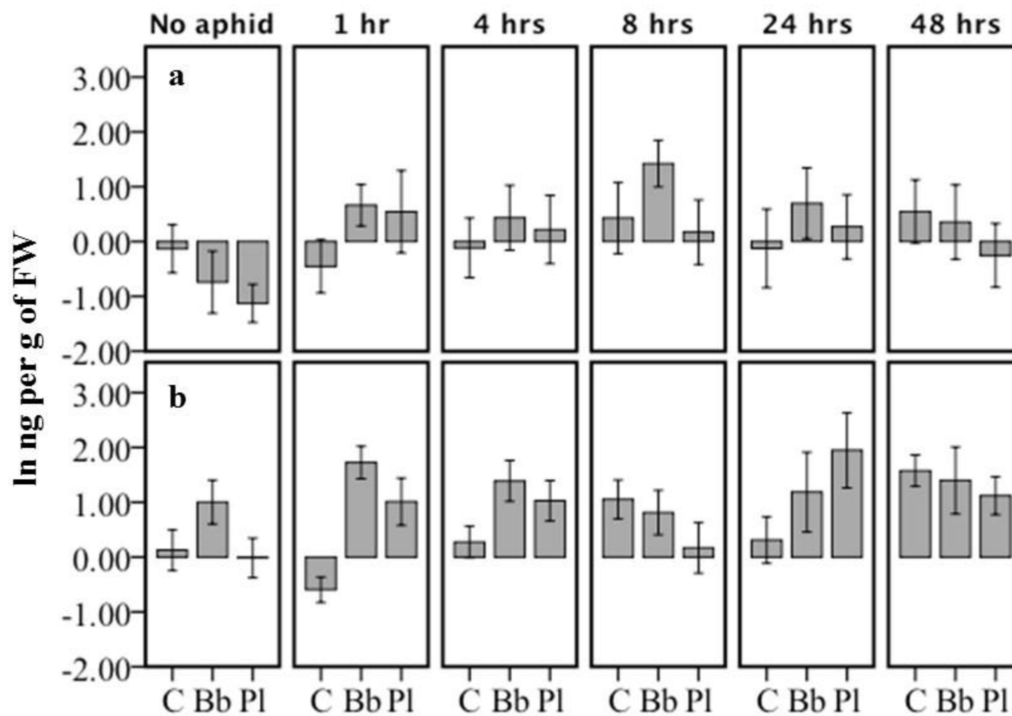

Figure 12**.** Jasmonic acid (JA-Ille) levels in endophyte-treated plants before and after aphid herbivory in a) leaves and b) root tissue. C=control, Bb=*B. bassiana* and Pl=*P. lilacinum*.

The endogenous levels of SA were significantly different among endophyte treatments ($F=4.66$; $df=2,31$; $P=0.019$), by trial ($F=386.35$; $df=1,31$; $P<0.001$), but there was no endophyte by trial interaction when data from both greenhouse was pooled. Pairwise comparisons showed both control plants and *B. bassiana* plants had lower endogenous levels of SA than *P. lilacinum* plants (2.91 ±0.90 and 2.62 ± 0.10 vs. 3.06 ± 0.10). One-way ANOVA results showed the overall response of SA was significantly different across herbivory time ($F=7.75$; $df=5,142$; $P<0.001$), by trial ($F=11.21$; $df=1,142$; $P=0.001$), and the interaction between endophyte and herbivory time was significant ($F=2.05$; $df=10,142$; $P=0.03$). Pairwise comparisons showed SA levels in control plants were higher than *B. bassiana* treated plants at four hours (LSD, $P=0.014$). There was not a significant main effect of endophyte treatment or an interaction between endophyte and trial (Fig 13, Table 1).

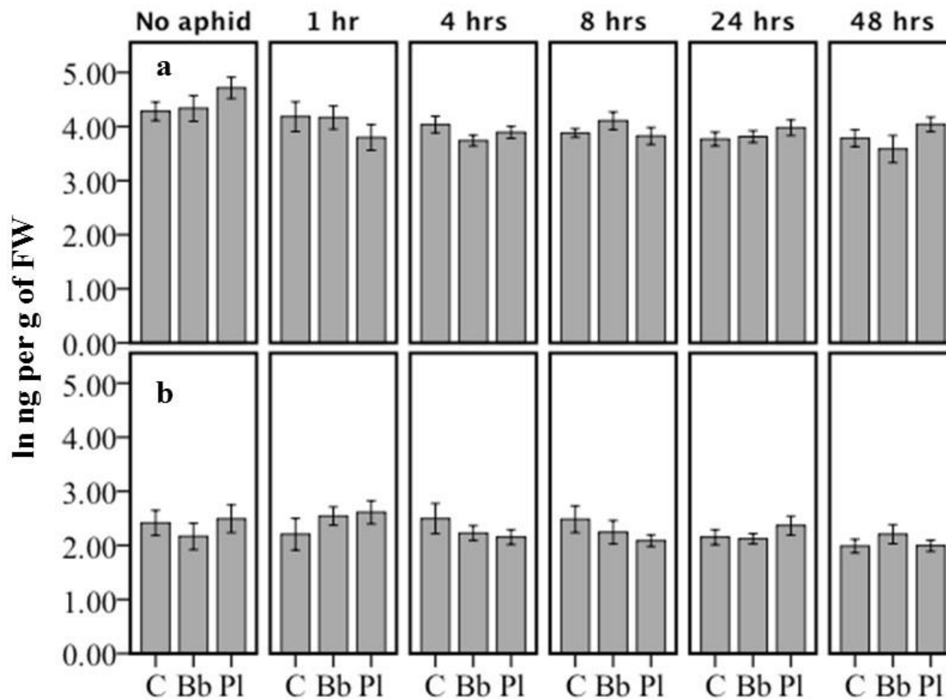

Figure 13**.** Salicylic acid (SA) levels in endophyte-treated plants before and after aphid herbivory in a) leaves and b) root tissue. C=control, Bb=*B. bassiana* and Pl=*P. lilacinum*.

The endogenous levels of TA were not significantly different by trial, or endophyte treatment, but there was a significant interaction between endophyte and trial (F=14.61; df=1,31; P<0.001). One-way ANOVA results showed the overall response of TA was not significantly different across herbivory time, by endophyte treatment or by the interaction of endophyte and trial. However, there was a significant difference by trial (F=131.71; df=1,141; P<0.001) and a marginally significant interaction between endophyte and herbivory time (F=1.76; df=10,141; P=0.072). Pairwise comparisons showed control plants

had higher levels of TA than *B. bassiana* and *P. lilacinum* treated plants at 48 hours (LSD, P<0.001, P=0.009) respectively (Fig 14, Table 1).

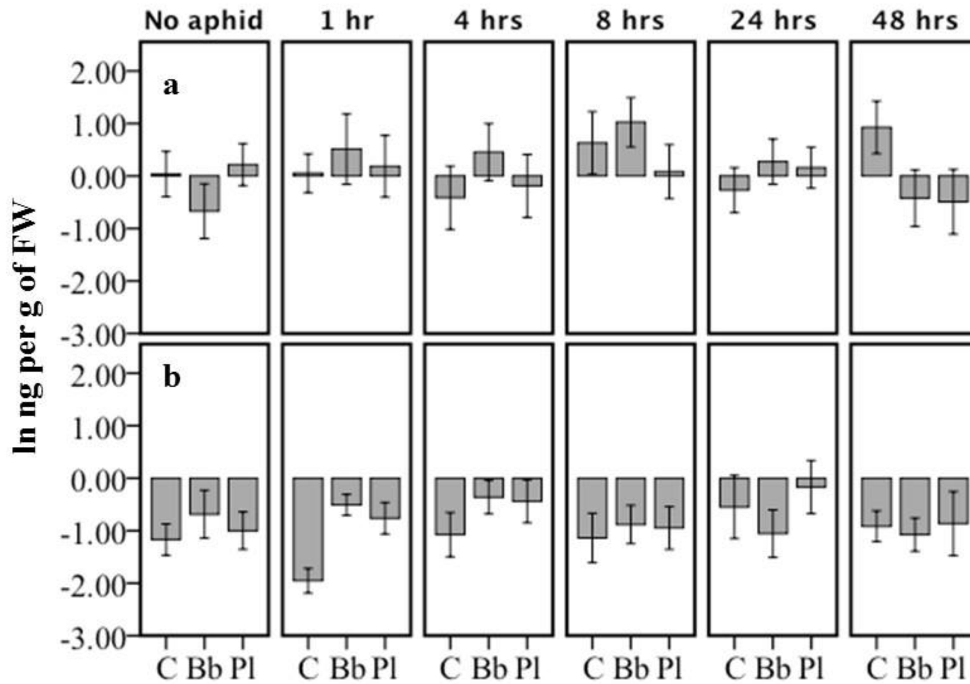

Figure 14**.** Traumatic acid (TA) levels in endophyte-treated plants before and after aphid herbivory in a) leaves and b) root tissue. C=control, Bb=*B. bassiana* and Pl=*P. lilacinum*.

The endogenous levels of CA were not different among endophyte treatment, tissue, trial or endophyte and trial interaction. One-way ANOVA results showed the overall response of CA was significantly different across herbivory time (F=7.25; df=5,108; P<0.001), by trial (F=6.03; df=1,108; P=0.016), by endophyte (F=20.01; df=2,108; P<0.001)

and by the interaction of endophyte and herbivory time ($F=7.13$; $df=10,108$; $P<0.001$). There was also a significant interaction between endophyte and trial ($F=19.86$; $df=2,108$; $P<0.001$). Thus analysis was conducted separately by trial. One-way ANOVA results from the first trial showed there was a significant effect of endopyte treatment ($F=59.98$; $df=2,72$; $P<0.001$), herbivory time ($F=8.38$; $df=5,72$; $P<0.000$) and the interaction between endophyte and herbivory time ($F=15$; $df=10,72$; $P<0.001$). Pairwise comparisons showed control plants had lower levels of CA than *P. lilacinum* plants at eight, 24 and 48 hours (LSD, $P<0.001$, $P<0.001$, $P<0.001$ respectively). Also *P. lilacinum* plants had higher levels of CA than *B. bassiana* plants at 24 and 48 hours (LSD, $P<0.001$, $P<0.001$ respectively) (Fig 15, Table 1). Second trial one-way ANOVA results showed no significant difference among endophyte treatments, herbivory time or the interaction between endophyte and herbivory time.

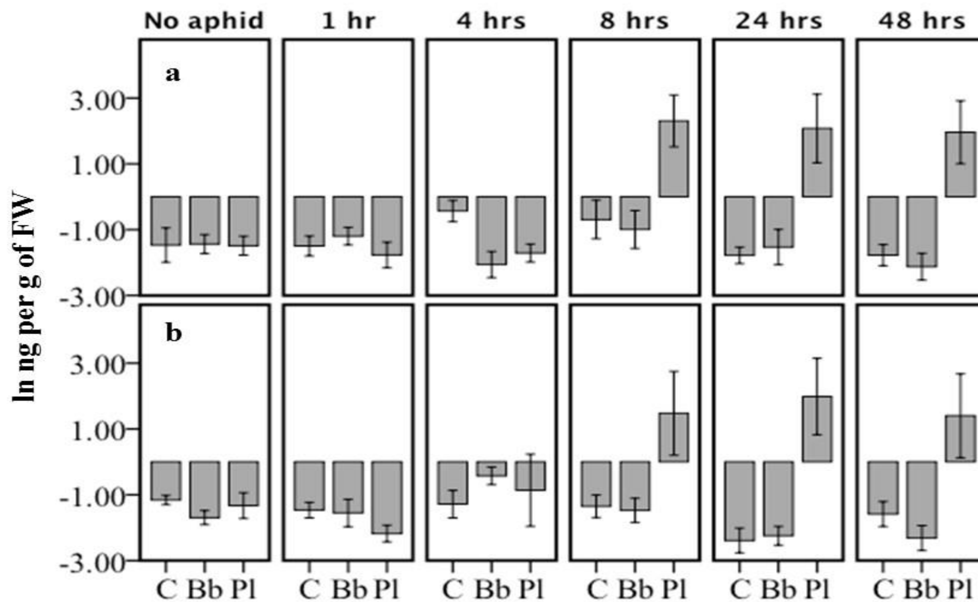

Figure 15**.** Cinnamic acid (CA) levels in endophyte-treated plants before and after aphid herbivory in a) leaves and b) root tissue. C=control, Bb=*B. bassiana* and Pl=*P. lilacinum*.

Belowground tissue: The endogenous levels of ABA in the absence of herbivory were not significantly affected by endophyte treatment. The endogenous response was significantly different by trial (F=116.11; df=1,22; P<0.001), but there was no interaction between endophyte and trial when both greenhouse trials were pooled (Fig 9, Table 1). One-way ANOVA results showed that the overall response of ABA varied significantly within herbivory time (F=2.27; df=5,139; P=0.05), by trial (F=9.57; df=2,139; P=0.002) and by the interaction of endophyte and herbivory time (F=2.59; df=10,139; P=0.007). Pairwise comparisons showed control had lower levels of ABA than *P. lilacinum* plants at 1 hour (LSD, P=0.001) and at 48 hours (LSD, P=0.07). Control plants also had higher ABA levels

than both endophyte treated plants at eight hours (LSD, P=0.001, P=0.004, respectively). There was no main effect of endophyte treatment, nor an interaction between endophye and trial (Fig 19, Table 1).

The endogenous levels of AZA were not significantly different across endophyte treatments, by trial, or by the interaction of endophyte and trial. One-way ANOVA results showed the overall response of AZA was significant across herbivory time (F=3.58; df=5,138; P=0.004), by endophyte treatment (F=5.94; df=2,138; P=0.003) and by endophyte by herbivory time (F=2.59; df=1,138; P=0.007). There was no significant effect of trial or by the interaction between endophyte and trial. Endophyte treatment pairwise comparisons showed control plants had lower levels of AZA than *B. bassiana* and *P. lilacinum* treated plants at one, and four hours (LSD, *B. bassiana*: P=0.004, P=0.026; *P. lilacinum:* P<0.001, P=0.042) (Fig 10, Table 1).

The endogenous levels of OPDA were not different among endophyte treatments, nor was there an endophyte by trial interaction. However, the endogenous response was significantly different between trials (F=15.25; df=1,22; P=0.004) when both greenhouse trials were pooled. One-way ANOVA results showed the overall response of OPDA was not significantly different across herbivory time, nor was there an endophyte by trial interaction. However, there was a significant effect of endophyte treatment (F=9.62; df=2,139; P<0.001), trial (F=168.93; df=1,139; P<0.001) and the interaction of endophyte and herbivory time (F=2.35; df=10,139; P=0.013). Endophyte treatment pairwise comparisons showed control plants had lower levels of OPDA than *B. bassiana* treated plants at one and four hours (LSD, P<0.001, P=0.024, respectively). Also, *B. bassiana* plants had higher OPDA levels than *P. lilacinum* plants at eight hours (LSD, P=0.011). Control plants had lower levels of OPDA

than *P. lilacinum* plants at one and four hours (LSD, $P=0.002$, $P=0.003$, respectively) (Fig 11, Table 1).

The endogenous levels of JA were significantly affected by endophyte treatment effect ($F=4.31$; $df=1,22$; $P=0.026$), but did not significantly vary between trials, nor was there a significant interaction between endophyte and trial. Control plants had lower endogenous levels of JA than *B. bassiana* treated plants (LSD, $P=0.041$) (0.67 ± 0.40 vs. 1 ± 0.38). Also *B. bassiana* plants had higher levels of endogenous JA than *P. lilacinum* plants (LSD, $P=0.010$) (1 ± 0.38 vs. -0.58 ± 0.40). One-way ANOVA results showed the overall response of JA was significantly different across herbivory time ($F=4.86$; $df=5,139$; $P<0.001$), by endophyte treatment ($F=12.02$; $df=2,139$; $P<0.001$), and by trial ($F=182.89$; $df=1,139$; $P<0.001$). There were also significant interactions between endophyte and herbivory time ($F=5.13$; $df=10,139$; $P<0.001$) and endophyte and trial ($F=5.26$; $df=2,139$; $P=0.006$). Thus, the analysis was conducted separately by trial. One-way ANOVA results from the first trial showed there was no significant difference among endophyte treatments or herbivory times. However, there was a significant interaction between endophyte and herbivory time ($F=2.07$; $df=10,71$; $P=0.038$) where control plants had lower levels of JA than *P. lilacinum* plants at eight and 24 hours (LSD, $P=0.028$, $P=0.06$, respectively). Second trial one-way ANOVA results showed the levels of JA were significantly different between endophyte treatments ($F=7.73$; $df=2,68$; $P=0.001$), by herbivory time ($F=10.31$; $df=5,68$; $P<0.001$) and by the interaction of endophyte and herbivory time ($F=3.2$; $df=10,68$; $P=0.002$). Endophyte treatment pairwise comparisons showed control plants had lower levels of JA than *B. bassiana* plants at one,four,, 24 and 48 hours (LSD, $P=0.002$, $P<0.001$, $P<0.001$, $P=0.017$,

respectively). Control plants also had lower levels than *P. lilacinum* plants at one, four, 24 and 48 hours (LSD, P<0.001, P=0.001, P=0.001, P=0.032, respectively) (Fig 12, Table 1). The endogenous levels of SA were not significantly different by endophyte treatment, but they were significantly different by trial (F=148.83; df=1,22; P<0.001) with no interaction between endophyte and trial. One-way ANOVA results showed the overall response of SA was significantly different across herbivory time (F=3.36; df=5,138; P=0.007), by trial (F=92.24; df=1,138; P<0.001), and marginally affected by the interaction between endophyte and herbivory time (F=1.80; df=10,138; P=0.07). Pairwise comparisons showed control plants had higher levels of SA than B.bassiana and P. lilacinum treated plants (LSD, P=0.04 and P=0.045, respectively). There was not a significant main effect of endophyte treatment or of the interaction between endophyte and trial (Fig 13, Table 1).

The endogenous levels of TA were not significantly affected by endophyte treatment or by the interaction of endophyte and trial. However, there was an overall trial effect on endogenous TA levels (F=5.11; df=1,21; P=0.03). One-way ANOVA results showed the overall response of TA was not significantly different across herbivory time. There was a significant endophyte treatment effect (F=2.99; df=1,125; P=0.05) (Fig 14, Table 1), but no significant interactions between either endophyte and trial or endophyte and herbivory time. Endophyte treatment pairwise comparisons showed control plants had lower levels of TA than both *B. bassiana* and *P. lilacinum* plants at one hour (LSD, P=0.004, P=0.05, respectively) (Fig 14, Table 1).

The endogenous levels of CA were not different among endophyte treatments, tissue, trial, nor was there an endophyte by trial interaction. One-way ANOVA results showed levels of CA were significantly different across endophyte treatments (F=16.11; df=2,107;

P<0.001) and by trial (F=48.12; df=1,107; P<0.001). There were also significant interactions between endophyte and herbivory time (F=5.73; df=10,107; P<0.001) and endophyte and trial (F=32; df=2,107; P<0.001). Thus, analyses were conducted separately by trial. One-way ANOVA results from the first trial showed the CA levels were significantly different by endophyte treatment (F=8.58; df=2,71; P<0.001), but there was no effect of herbivory time or an interaction between endophyte and herbivory time. Endophyte treatment comparisons showed control plants had significantly lower levels of CA than *P. lilacinum* plants at eight, 24 and 48 hours (LSD, P<0.001, P<0.001, P<0.001) respectively. Also, *P. lilacinum* plants had higher levels of CA than *B. bassiana* plants at eight, 24 and 48 hours (LSD, P<0.001, P<0.001, P<0.001, respectively) (Fig 15, Table 1). Second trial one-way ANOVA results showed CA levels were not significantly affected by endophyte, herbivory time or the interaction between endophyte and herbivory time.

Table 1. Summary of differences in hormone levels in leaf and root tissue of endophyte-treated plants before and after aphid herbivory. Arrows represent significant differences and the direction of response between control and treatment plants at each sampling time. Different color arrows represent statistical differences in hormone levels between the *B. bassiana* and *P. lilacinum* treatments.

		ABA		AZA		OPDA		JA	
	Tissue	*Bb*	*Pl*	*Bb*	*Pl*	*Bb*	*Pl*	*Bb*	*Pl*
1 hour	**Leaf**		↓	↑	↑	↑	↑	↑	↑
	Root		↑	↑	↑	↑	↑	↑	↑
4 hours	**Leaf**			↑					
	Root			↑	↑	↑		↑	↑
8 hours	**Leaf**							↑ (blue)	
	Root	↓	↓						
24hours	**Leaf**	↑							
	Root								↑
48 hours	**Leaf**								
	Root	↑							

Table 1. Continued.

		SA		TA		CA		JA	
	Tissue	*Bb*	*Pl*	*Bb*	*Pl*	*Bb*	*Pl*	*Bb*	*Pl*
1 hour	**Leaf**							↑	↑
	Root			↑	↑			↑	↑
4 hours	**Leaf**	↓				↓	↓		
	Root							↑	↑
8 hours	**Leaf**						↑	↑	
	Root						↑		
24hours	**Leaf**						↑		
	Root						↑		↑
48 hours	**Leaf**						↑		
	Root			↓	↓		↑		

Discussion

Our results provide the first report of differential phytohormone responses in cultivated cotton when inoculated by the entomopathogenic endophytes *B. bassiana* and *P. lilacinum* in both the absence and presence of herbivory. Entomopathogenic fungal endophytes have been isolated from a variety of plant species and tissues, and single isolates can be inoculated to establish as an endophyte across a range of phylogenetically divergent plants (Vega *et al*. 2009; Rodriguez *et al.* 2009; Gurulingappa *et al.* 2010; Porras-Alfaro & Bayman 2011). Several entomopathogens including *Beauveria bassiana*, *Lecanicillium lecanii, Metharizium anisoplae* and *Isaria* (*Paecilomyces*) *spp.* can have negative effects on insect pests when *in planta* including a recent study done in our laboratory which showed

negative effects on cotton aphid reproduction when *B. bassiana* and *P. lilacinum* were present in cotton as endophytes (Bing & Lewis 1991; Vega *et al.* 2008, 2009; Porras-Alfaro & Bayman 2011, Castillo-Lopez *et al*. 2014). Negative effects on herbivory when feeding on endophyte-inoculated plants have been extensively reported in the literature. However, the mechanisms underlying endophyte-mediated effects on plant resistance to herbivorous insects are poorly understood. Several non-mutually exclusive hypotheses have been suggested including the production of secondary metabolites, production of superoxides, change of the phytosterol profile of plants, or by inducing an indirect systemic defense response in the plant thus conferring resistance to insect feeding (Raps & Vidal 1998; Schardl 2004, 2007; Tanaka 2006; Huang 2007; Hartley & Gange 2009; White Jr & Torres 2010).

A long-lasting and broad-spectrum induced disease resistance in plants is referred to as systemic acquired resistance (SAR). SAR starts by the accumulation of endogenous SA at the site of infection then a similar response is triggered in distal plant parts activating a large set of pathogenesis-related genes (PR) to protect undamaged tissues against subsequent pathogen attack (Van Loon *et al*. 2006b; Pieterse *et al*. 2009; 2012). Our results provide evidence for changes in endogenous levels in cotton when colonized by entomopathogenic fungal endophytes. In the absence of aphid herbivory, our results illustrated different endogenous levels of SA in the leaf tissue when plants were endophytically colonized by *P. lilacinum* (Table 1). The endogenous levels of SA were higher in the *P. lilacinum* plants compared to the control and *B. bassiana* treated plants. In contrast to our observations, Navarro-Melendez & Heil (2014) showed levels of SA were significantly reduced on intact and mechanically damaged Lima bean leaves colonized by *Fusarium sp* and *Bartalinia*

pondoensis endophytes compared to uncolonized plants. Another example showed no differences in endogenous SA levels in root samples of rice when colonized by the mycorrhizal fungus *Glomus intraradices* (Campos-Soriano & Segundo 2011).

Presence of the target endophytes also affected endogenous levels of JA-Ile (receptor-active derivative of JA) in the plant roots, where *B. bassiana* treated plants had higher levels than both control and *P. lilacinum* treated plants (Table 1). Comparable to our results, Hause *et al*. (2002) found that mycorrhizal colonization of barley roots by *G. intraradices* led to elevated endogenous JA levels. Though we did not see any endogenous changes in JA-Ile levels in the leaf tissue, Navarro-Melendez & Heil (2014) showed elevated endogenous JA levels in Lima bean leaves when colonized by the endophyte *Fusarium sp,* but the suppression of endogenous JA levels in mechanically damaged leaves of *B. pondoensis* colonized plants. Similarly, Ren & Dai (2012) showed the inoculation of *Atractylodes lancea* plants with the endophyte *Gilmaniella sp.* enhanced endogenous JA levels in the plant leaf tissue. It has also been extensively documented that beneficial rhizobacteria such as *Pseudomonas sp.* (*P. fluorescences* and *P. putida*) along with the beneficial fungi *Trichoderma sp*. are known to enhance endogenous JA levels upon plant colonization (Iavicoli *et al*. 2003; Ryu *et al*. 2004; Shoresh *et al*. 2005; Anh *et al*. 2007; Djonovic *et al*. 2007; Tran *et al*. 2007). Stress-induced accumulation of JA-Ile occurs in both above and below-ground tissues and depending on the eliciting signal and tissue type, is also considered a systemic response (Campos *et al*. 2014).

Our study shows differential induction of endogenous levels of SA and JA-Ile in *P. lilacinum* and *B. bassiana* colonized plants, respectively. The SA resistance signaling defense pathway is typically (but not exclusively) effective against biotrophic pathogens (Pieterse *et*

al. 2012). The idea of an endophyte activating the SA-pathway and providing subsequent protection against pathogens has been extensively reported in the literature. To name a few, the endophyte *Pirimisfora indica* provided subsequent resistance against *Blumeria graminis* and *Fusarium culmorum* in Barley, *Verticillium dahliae* in tomato and Arabidopsis, *Fusarium Verticillioides* in corn and *Pseudocercosporella herpotrichoides* in wheat (Waller *et al*. 2005; Serfling *et al*. 2007; Kumar *et al*. 2009; Fakhro *et al*. 2010; Sun *et al*. 2014). Also, the entomopathogen *B. bassiana* has been shown to provide subsequent resistance to plant pathogens such as *Rhizoctonia solani* (Ownley *et al*. 2004, 2008), and *Pythium myriotylum* (Clark *et al*. 2006). These studies did not report the mechanism behind these interactions and we did not see any SA endogenous changes in *B. bassiana* colonized plants in our study. Rather our results indicated induction in endogenous levels of SA in cotton plants colonized by *P. lilacinum* and this observation may support the hypothesis that this endophyte to confer subsequent resistance to multiple stressors by activation of SAR.

The antagonistic and synergistic interactions between SA and JA as major defense hormones is called cross-talk (Mundy et al. 2006; Jaillais & Chory 2010). Generally trade-offs between SA-dependent resistance to biotrophs and JA-dependent defense against insect herbivores or necrotrophs have been reported, and the majority of these studies have been done in *Arabidopsis*, tomato and tobacco plants (e.g., Kunkel & Brooks 2002; Bostock 2005; Spoel 2007; Uppalapati *et al*. 2007; Leon-Reyes 2010b; Verhage *et al*. 2010; Pieterse *et al*. 2012; Van der Does *et al*. 2013; Sun *et al*. 2014). Although many reports describe an antagonistic interaction between the SA and JA pathways, neutral and synergistic interactions have also been described (Schenk *et al*. 2000; Van-Wees *et al*. 2000; Mur *et al*. 2006). However, plants are often simultaneously challenged by different stressors, thus SA-JA cross

talk may be advantageous for the plant to prioritize one pathway over the other. Importantly, timing of initiation of SA or JA signaling also depends on the sequence and type of stress encountered, all of which can affect the defense response outcome (Koornneef *et al*. 2008a, Leon-Reyes *et al*. 2010a; Pieterse *et al*. 2012).

In contrast to SAR, induced systemic resistance (ISR) is triggered by beneficial microbes and is generally regulated by JA- and ET-dependent signaling pathways, which is associated with priming the plant defense response rather than a constitutive activation of defense (Conrath *et al*. 2006; Van-Wees *et al*. 2008; Pozo *et al*. 2008; Pieterse *et al*. 1996, 2000, 2009, 2012). Primed plants display either faster, stronger, or both, activation of various cellular defense responses (Conrath *et al*. 2006; Van-Wees *et al*. 2008). Our results provide strong evidence for defensive priming effects in cotton when colonized by either *B. bassiana* or *P. lilacinum*. When challenged by aphid herbivory, plants colonized by *B. bassiana* exhibited priming effects for ABA, AZA, OPDA, and JA-Ile in the leaf tissue and for ABA, AZA, OPDA, JA-Ile and TA in the root tissue (Table 1). Similarly, when colonized by *P. lilacinum*, we observed priming effects for AZA, OPDA, and JA-Ile and CA in the leaf tissue and for AZA, OPDA, JA-Ile, TA and CA in the roots. Our results are comparable to a study done by Song et al. (2013) where the arbuscular mycorrhizal fungus *G. mosseae* was inoculated to tomato plants that were then challenged with feeding by the caterpillar *Helicoverpa armigera* at different times. Their study showed how mycorrhiza treated tomato plants upregulated JA related defense genes faster than control plants in response to herbivory.

The systemic resistance responses induced by beneficial microbes are not associated with major changes in the expression of defense genes (PR genes) because this would lead to

higher investments in resources and reduced fitness of the host plant (Heil 2002; Pieterse *et al*. 2002; Van Hulten *et al*. 2006; Conrath *et al*. 2006). For example, colonization of tomato roots by mycorrhizal fungi systemically protects the plant against the pathogen *Phytophthora parasitica* without the accumulation of PR proteins (Pozo *et al*. 1999, 2002). Also in *Arabidopsis*, ISR triggered by *Pseudomonas fluorescens* has been shown to be effective against different types of pathogens, but it is not associated with the activation of PR genes, but rather mediated by the JA and ET defense pathways (Pieterse *et al*. 1996, 2002). Several studies have argued how direct defense responses represent a high cost to a plant in terms of growth and reproduction (Simms & Fritz 1990; Baldwin 1998; Agrawal *et al*. 1999; Redman *et al*. 2001; Heil 2002). For example, it was demonstrated in *Arabidopsis thaliana* that the costs of priming were substantially lower than those of the directly induced defense responses against pathogens (Van Hulten *et al*. 2006).

Although *B. bassiana* and *P. lilacinum* both elicited changes in endogenous phytohormone levels as well as strong priming effects when present as endophytes in cotton, there were several species-specific differences in their effects. Similar patterns were observed in the phytohormone analysis done by Fernandez et al. (2014) on plant-AM symbioses in corn, tomato and soybean with two different AM fungi (*Funneliformis mosseae* and *Rhizophagus irregularis*). They found that levels of ABA and JA depended on both partner genotypes and their interactions with the AM fungi. A separate study done on Lima bean inoculated with *Bartalinia pondoensis*, *Fusarium sp*., and *Cochliobolus lunatus* as endophytes also provided evidence that changes in hormonal profiles observed in the plant depended on both the plant and the specific endophytic fungus (Navarro-Melendez & Heil 2014). Although we used only one cultivar of cotton and did not control for genetic variation

among the individual plants samples, our results clearly illustrate that different taxa of endophytic entomopathogens do not all elicit identical responses within the plant. Defense responses and downstream signaling pathways are regulated mainly by phytohormones and though these signaling networks are rather complex, the induction of plant defense depends on the identity, sequence, and intensity of the plant-symbiosis established (Conrath *et al*. 2006; Stout *et al*. 2006; Howe & Jander 2008; Thaler *et al*. 2010, 2012; Stam *et al*. 2014).

In summary, our study provides evidence for changes in defense related hormones in cultivated cotton when endophytically colonized by the fungal entomopathogens *B. bassiana* and *P. lilacinum*. Phytohormone hormonal levels were differently affected by the presence of the endophytes in both the absence and presence of herbivory. Our results generally coincide with what has been reported in the literature for other beneficial microorganisms-plant symbiosis. Our study strongly supports induced systemic defense responses in the plant as a mechanism underlying endophyte mediate-resistance to herbivory in cotton. Given that colonization of plants by fungal endopythes is pervasive and negative effects on herbivores have been shown in a variety of endophyte-plant systems, similar effects are likely to be widespread, but modulated by the specific plant-endophyte combination involved in the interaction.

CHAPTER V

INDIVIDUAL AND SPATIAL VARIATION IN PLANT GENOTYPE AFFECT FUNGAL ENDOPHYTE COMMUNITIES IN COTTON

Introduction

Studies of plant-endophyte interactions in both natural and agricultural systems often focus on investigating the benefits these microorganisms can provide to their host plants against an array of biotic and abiotic stresses (Clay 1996; Rodriguez *et al*. 2009; Vega *et al*. 2009; Farr *et al*. 1989; Boyle *et al*. 2001; Liu 2001; McGee 2002; Redman *et al*. 2002; Schulz *et al*. 2002; Vega 2008). More specifically, studies have considered several facets of the interaction including colonization frequencies, the identification and extraction of novel secondary fungal metabolites, antibiosis effects, and the enhancement of plant growth and fitness (e.g., Farr *et al*. 1989; Bing & Lewis 1992; Gindin *et al*. 1994; Raps & Vidal 1998; Omacini *et al*. 2001; Vicari *et al*. 2002; Wang *et al*. 2007; Rodriguez *et al*. 2008; Vega 2008; Gurulingappa *et al*. 2010, 2011; Kiewnick 2011; Munawar *et al*. 2011; Martinuz *et al*. 2012; Lau & Lennon 2012; Castillo Lopez *et al*. 2014). Fungi are able to express several different symbiotic lifestyles that are defined by fitness benefits to the host plant or to the symbiont (Lewis 1985; Rodriguez & Redman 2008). The range of symbiotic lifestyle from mutualism to parasitism is described as the symbiotic continuum (Carroll 1988; Johnson *et al*. 1997; Saikkonen *et al*. 1998; Schulz *et al*. 1999; Schardl & Leuchtmann 2005). Studies on host genotype versus symbiotic lifestyle expression suggest that individual isolates of some fungal species can express either mutualistic or pathogenic lifestyles depending on the host plant genotype and environmental background (Redman *et al*. 2001; Rodriguez & Redman 2008).

Thus, it is suggested that host plants are likely to have particularly strong effects on the structure of fungal endophyte communities since these microrganisms live asymptomatically for all or part of their life cycle in both above and below ground tissues of plants and rely completely on them for resources (Pan & Clay 2003, 2004; Schulz & Boyle 2005; Schulz 2005).

The genotype, species and local diversity of plants have been shown to significantly influence the community composition and structure of organisms at different trophic levels. Considering this, a few studies have shown how increased plant species diversity led to increased herbivore diversity (Southwood 1961; Murdoch *et al*. 1972; Tscharntke & Greiler 1995; Panzer & Schwartz 1998; Siemann *et al*. 1998; Wimp *et al*. 2005). Similarly, genetic variation among hybrid groups can also affect the arthropod community by presenting herbivores with new genotypes and different levels of chemical compounds than parental species (Fritz & Price 1988; Maddox & Root 1990; Boecklen & Spellenberg 1990; Fritz *et al*. 1994; Floate & Whitham 1995; Dungey *et al*. 2000). Given that genetic differences among plant species and within a hybridizing complex can affect the associated herbivore communities, it is predicted these effects may also influence the composition of other trophic levels including predators, parasites, and symbiotic microbes (Hunter & Price 1992, Johnson & Agrawal 2006, Saikkonen 2007, Prober *et al*. 2014). Similarly plant diversity is predicted to promote soil microbe diversity by increasing food resources (soil exudates and litter), physical microhabitats and environmental conditions (Prober *et al*. 2014). With respect to fungal endophytes, Saikkonen (2007) suggested that within a forest stand, the seasonal and spatial variation of infection frequencies of endophytes depend largely on the host identity,

density, surrounding vegetation and environmental conditions such as weather and moisture of the plant microclimate.

Literature examples including arbuscular mycorrhizal show how fungal symbiont communities can vary depending on various biotic or abiotic factors (Opik *et al*. 2010; Lopez-Garcia *et al*. 2014). Specifically, fungal community composition may vary depending on (i) the tissue from which they were originally isolated (Carroll 1988; Halmschlager *et al*. 1993; Fisher *et al*. 1995; Hata & Futai 1996; Gamboa & Bayman 2001, etc), (ii) host plant genotype (Todd 1988; Bailey *et al*. 2005; Pan *et al*. 2008; Yurkonis *et al*. 2012; Rajala *et al*. 2013), (iii) precipitation levels (Hawkes *et al*. 2011), and (iv) space and time within the same plant (Helander *et al*. 1994; Martinson *et al*. 2012). Only four studies to date have explicitly surveyed asymptomatic fungal endophyte communities in cultivated cotton (*Gossypium hirsutum*); one in Australia (McGee 2002), two in Brazil (Wang *et al*. 2007; Vieira *et al*. 2011) and most recently one in the United States (Ek-Ramos *et al*. 2013). There are multiple older reports dating back to the 1920's in which fungi were isolated from different tissues of cotton, but mostly with the purpose of identifying potential fungal pathogens (e.g., Crawford 1923). The more extensive analyses done by Vieira *et al*. (2011) isolated endophytes from transgenic *Bt* and conventional non-*Bt* cotton in Brazil, but did not find any difference in community diversity between the different cotton genotypes. The study done by Ek-Ramos *et al*. (2013) in which cotton tissues were surveyed at two different time points from organic and conventional agricultural practices across multiple locations in Texas found no differences in endophyte species richness or diversity among different cotton varieties, but detected differences over time and from different plant tissues sampled.

In this study, we set out to empirically test for the effects of host plant species (*G. hirsutum* vs. *G. barbadense*) and host genotype among *G. hirsutum* (varieties LA122, OL220 and 1203) on fungal endophyte community composition at both local (α-diversity) and landscape (β-diversity) scales in cotton plants. We utilized three different varieties of *G. hirsutum* and one variety of *G. barbadense* grown in three different local genetic diversity environments and addressed the following questions: (1) Are fungal endophyte communities affected by host plant genotype within a field? (2) Are fungal endophyte community diversity and composition affected by local genetic variation among neighboring conspecifics? And, (3) how do these fungal communities change over time?

Materials and Methods

Study location: The field experiment was conducted at the Texas A&M University Field Laboratory located near College Station in Burleson, Co., TX (N 30° 26' 48" W 96° 24' 05.12") at an elevation of 68.8 m. The experiment was planted on April 18, 2012.

Experimental design and cotton genotypes: Each replicate plot was comprised of 8 rows (101.60cm apart), and each row was 14.30m in length. Four different commercial cotton genotypes were used: (1) LA122, (2) OL220, (3) 1203 (*G. hirsutum*) and (4) P-203 (*G. barbadense*) (all from All-Tex Seed, Inc.). Untreated chemically-delinted black seeds were planted at a density of 2.66 seeds per 0.30m. The cotton genotypes were planted in the following three different spatial treatments to manipulate genetic diversity within the plots: (1) *Monoculture* containing same genotype in all eight rows of the plot, (2) *Quad random* with all four genotypes mixed within each row of the plot, and (3) *Quad by row* with each

row of the plot planted with a different genotype (see Fig 9). Each treatment plot was replicated five times in a randomized block design and only the inner rows of the plot were sampled to avoid edge effects.

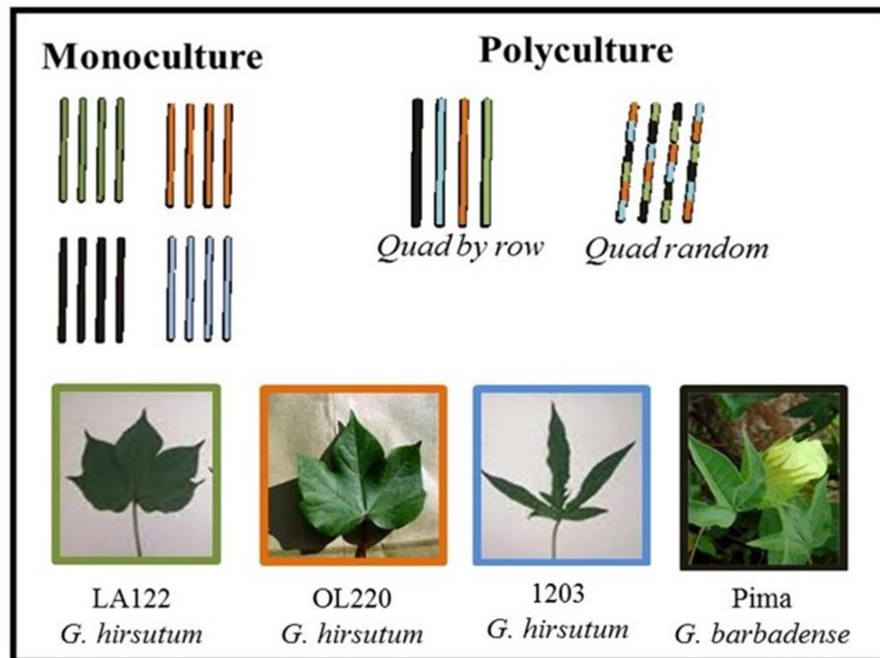

Figure 16. Field experimental design diagram illustrating the spatial arrangement of cotton genotypes in the inner four rows of in plots of each treatment.

Plant sampling: Sampling occurred twice in time during the experiment at 40 and 80 days post-planting. Plants were randomly collected from all treatment plots comprising a total of 60 plants per treatment (3 plants per row x 4 rows x 5 replicate plots). Cotton plants were collected and bagged in the field then brought back to the laboratory for endophyte culturing of surface sterilized plant tissues on sterile potato dextrose agar media (PDA). The endophyte culturing protocol was based on Posada *et al*. (2007) and consisted of immersing tissues in 70% ethanol for 3 minutes, 2% sodium hypochlorite (NaCl) for 3 minutes, followed by three washes in sterile water. The third sterile water wash was also plated on PDA media to confirm surface sterilization effectiveness. Following surface sterilization, asymptomatic leaves were cut into small fragments of approximately 1 cm^2 with a standardized leaf puncher, and placed on PDA media plates. Only one leaf punch was taken from each leaf of the plant, thus the number of fragments plated varied according to the size of the plant. Stems fragments were cut every 10cm from the bottom to the top of the plant and also varied in number depending on the size of the plant collected. Antibiotics Penicillin G (100 Units/mL) and Streptomycin (100 mg/mL) were added to the PDA media (Sigma, St Louis MO) to prevent bacterial growth. Plates were incubated in the dark at room temperature (approximately 25ºC).

Fungal endophyte isolation and identification: Fungal plates were visually screened every two days to check for any fungal growth from within tissues. Different fungal morphotypes observed on plates were subcultured on new PDA plates for later DNA extraction and PCR identification. Genomic DNA was extracted from mycelium of each different morphotype using the CTAB protocol (Doyle & Doyle 1987). We utilized fungal specific primers to amplify the ITS (Internal Transcribed Spacer) region of nuclear ribosomal

DNA with the primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS2 (5' GCT GCG TTC TTC ATC GAT GC 3') as per Ek-Ramos *et al*. (2013) (Sigma-Aldrich, Inc St Louis, MO). An expected band of 240bp was visualized on a 2% agarose gel ran at 70V. The PCR products were cleaned utilizing Invitrogen Superscript kit (Invitrogen. Grand Island, NY) and sent for sequencing to Macrogen USA Corp. (Maryland, USA). The resulting sequences were aligned as query sequences against GenBank nucleotide, UNITE and PlutoF available databases (Abarenkov *et al*. 2010a, b). Only hits with an E-value $<1E^{-10}$ were considered as matches (GenBank accession numbers are provided).

Statistical analyses: To quantify fungal endophyte species diversity at a local scale within plots (α-diversity), we calculated the Shannon-Wiener biodiversity index (H') using frequency of different fungal species isolated per environment, genotype and time of sampling (EstimateS software) (Colwell 2009; Colwell *et al*. 2012). In order to assess species richness and determine if our sampling intensity was sufficient, we generated two Hurlbert rarefaction curves for our sampling times (40 and 80 days post planting) to calculate fungal endophyte taxa accumulation curves using 1000 randomizations (R software, version 3.1). We also compared variation in identity of fungal species among treatments to assess community composition differences (β-diversity) among environments and cotton genotypes using two different similarity indices. The Jaccard's index compares fungal taxa presence or absence among samples (binary data) and the Bray-Curtis similarity index compares fungal taxa presence or absence and their relative abundances among samples (Anderson *et al*. 2011). We utilized the XLSTAT software to calculate both similarity indices, and the matrices generated from these calculations were graphed on 2D non-metric multidimensional scale (NMDS) plots. The Kruskal's stress value was used in the multidimensional scaling to

decide which grouping of the data was the most accurate (commonly acceptable value < 0.2) (Quinn & Keough 2002). Effects of environment, genotype and sampling time on species richness and community composition utilizing the Shannon-Wiener values were analyzed using Analysis of Variance (ANOVA) when data were normally distributed and non-parametric Kruskall-Wallis tests when data were not normally distributed (SPSS 20.0, IBM North America, New York, USA).

Results

Fungal endophyte isolation and identification: There was no fungal growth on the PDA plating of the third sterile water wash of the surface sterilized plant tissues, indicating the efficacy of our surface sterilization. Thus, we assume that the fungi growing in the media from surface-sterilized plant materials were endophytes that came from within plant tissues and not epiphytes from the plant surface. There were 10 different fungal taxa isolated from our study (Table 1). Even though only a small number of fungal taxa was isolated, both Hulbert rarefaction curves generated for our 40 and 80 day post-planting sampling events indicated that our sampling intensity was sufficient to adequately sample for the number of culturable taxa present in the plants collected (Fig 10a,b).

Table 2. Fungal taxa and abundances isolated during the field experiment.

Sequence accession GenBank number	Fungal taxa	Isolates
KP407570	*Verticillium lecani*	9
KP407571	*Aspergillus sp.*	269
KP407572	*Xylaria polymorpha*	30
KP407573	*Pseudogymnoascus pannorum*	44
KP407574	*Agaricus semotus*	99
KP407575	*Heterobasidion parvispora*	13
KP407576	*Pezizales sp.*	11
KP407577	*Stereum sanguinolentum*	8
KP407578	*Cladosporium sp.*	2

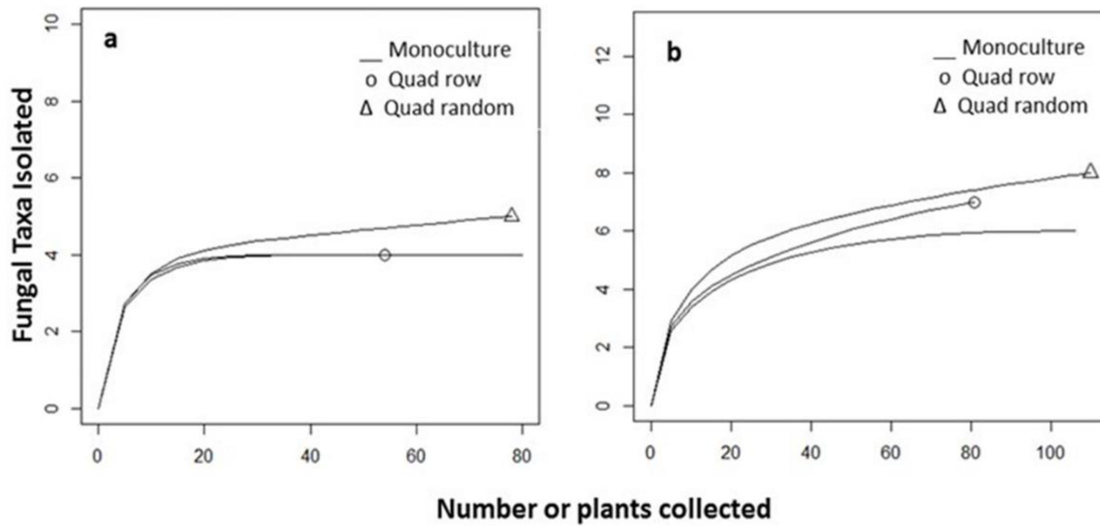

Figure 17. Rarefaction curve for both collections times (a: 40 days post planting, b: 80 days post planting).

Number of fungal taxa isolated: One-way ANOVA results on the number of total fungal taxa isolated (abundance) were not significantly different by environment (F=0.336; df=2,244; P=0.715), genotype (F=0.353; df=2,244; P=0.787), or time of sampling (F=0.363; df=1,244; P=0.547) (SPSS 20.0, IBM North America, New York, USA).

Fungal endophyte α-diversity: Results from the Shannon-Weiner biodiversity index indicated that fungal community composition was significantly different among environments at both 40 days (Kruskal-Wallis= 46.48; df=2,136; P<.0001) (Fig 11a) and 80 days post-planting (Kruskal-Wallis= 62.70; df=2,160; P<.0001) (Fig 11b). Pairwise comparisons showed that the quad-random environment (all genotypes mixed in a row/4 rows per plot) had a significantly higher Shannon-Weiner biodiversity value compared to the monoculture (P< 0.0001) and the quad by row environment (P< 0.0001) at the 40 day post-planting sampling time. During the second sampling time (80 days), the quad by row (each genotype in one row/4 rows per plot) environment had a significantly higher Shannon-Weiner biodiversity value compared to the monoculture (P< 0.0001) and the quad-random environment (P< 0.0001). Overall at both sampling times, the monoculture environment had a lower Shannon-Weiner biodiversity value than any of the mixed genotype plots. Across both sampling times (40 and 80 days post planting) we found a difference in the fungal community composition (ANOVA, F=37.23; df=1,296; P<.0001), with a significant interaction between environment and time based on the Shannon-Weiner values (ANOVA, F=6.77; df=2,296; P=0.001). When we analyzed the Shannon-Weiner index by genotype we found that fungal endophyte communities were different between the four different cotton genotypes during the first sampling event (40 days) (Kruskal-Wallis= 42.84; df=3,105; P<.0001) (Fig 12a), but not after 80 days post planting (Kruskal-Wallis= 3.17; df=3,105;

P<.0.365) (Fig 12b). Pairwise comparisons during the first sampling time showed that OL220 (*G. hirsutum*) and Pima (*G.barbadense*) genotypes had a more diverse fungal community composition compared to 1203 (*G. hirsutum*) (OL220 vs. 1203 P=0.003; Pima vs. 1203 P=< 0.0001), but were not different from the LA122 cotton genotype (*G. hirsutum*) (P=0.107; P=0.286) (Fig 12).

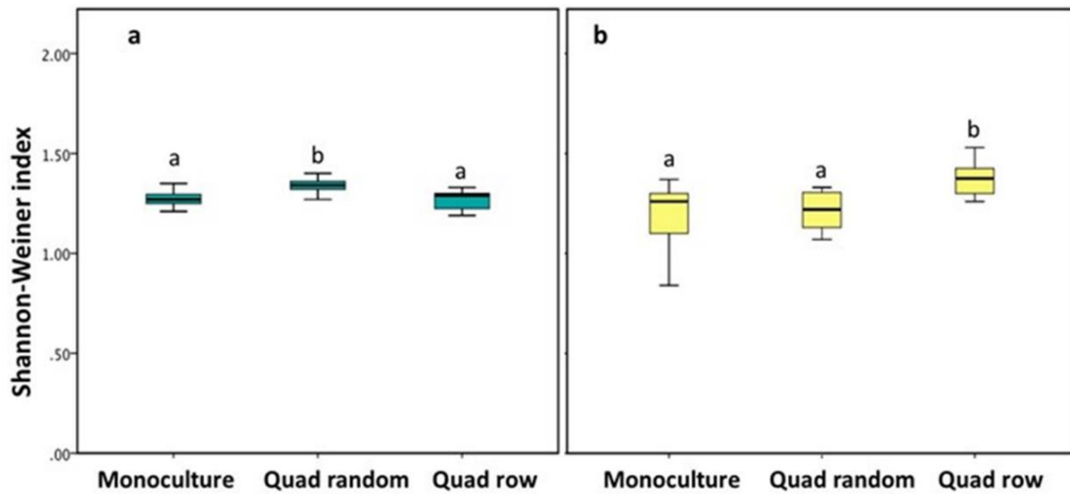

Figure 18. Shannon-Weiner diversity index by environment at: 40 days after planting and b: 80 days after planting.

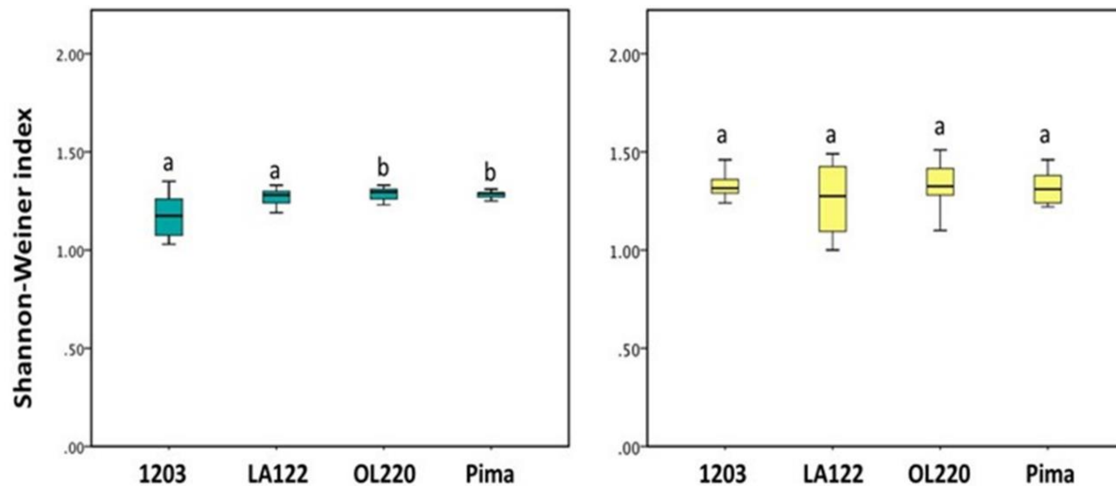

Figure 19. Shannon-Weiner diversity index by cotton genotype at a: 40 days post planting and b: 80 days post planting.

Fungal endophyte β-diversity: We utilized a two dimensional (2D) non-metric multidimensional scale plot to represent our clustering analyses results using fungal endophyte community similarity coefficients (Jaccard and Bray-Curtis). The Jaccard index results showed that fungal endophyte communities were significantly different or dissimilar both when analyzed by environment (Kruskal stress=0.193) (Fig 13) and by genotype (Kruskal stress=0.187) (Fig 13). The Bray-Curtis coefficient (raw abundance of species) indicated that fungal endophyte communities were not dissimilar from each other when analyzed by environment (Kruskal stress=1.701) (Fig 13), but were dissimilar when analyzed by genotype (Kruskal stress= 0.028) (Fig 13). Overall, the two different ecological similarity measures used did not consistently show the same observed patterns of cluster formation based on the genetic environment treatments, but did consistently differentiate communities based on the plant genotype treatments (Fig 13).

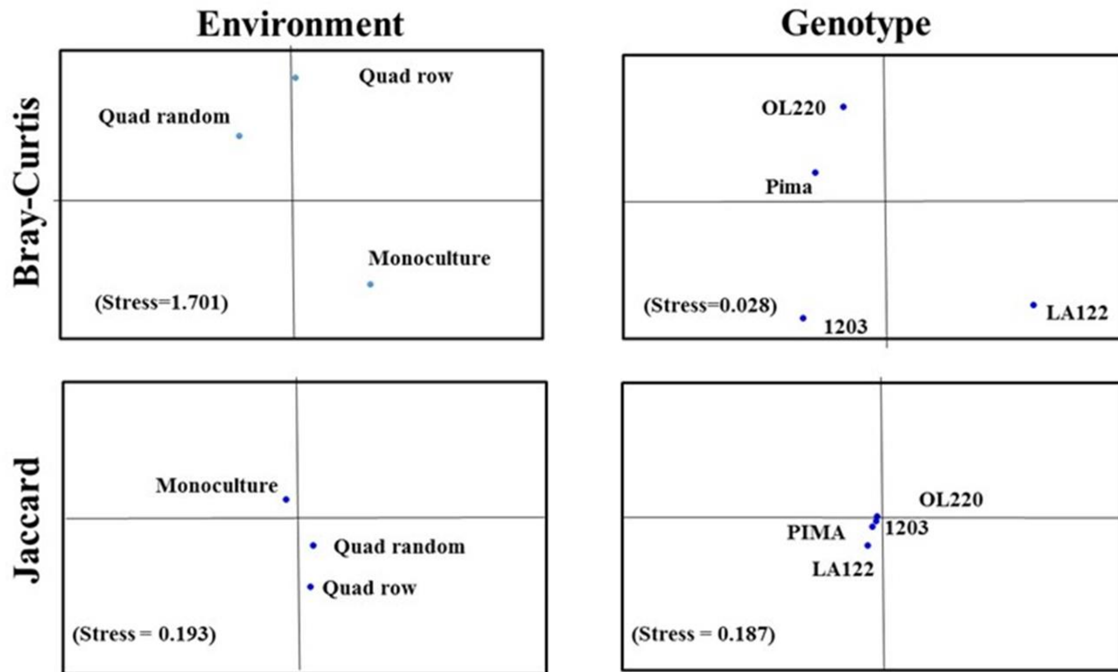

Figure 20. NMDS plots for Bray-Curtis and Jaccard similarity indices.

Discussion

In order to better understand the ecology and structure of fungal communities in cultivated cotton, our study investigated two aspects: (i) plant genotype and (ii) spatial distribution of genotypes, and how these may affect the structure and composition of fungal endophytes in an experimental field setting. Cultivated cotton accounts for 35% of total world fiber use, with the United States, China, and India producing two-thirds of the world's cotton (USDA, World Agricultural Outlook board, Sep 2014). Furthermore, the cotton

industry in the United States alone accounts for more than $25 billion in products and services per year (USDA, www.ers.usda.gov). It is well known that plants host a diversity of microbes. All wild and agricultural plant species surveyed to date harbor a diverse array of fungi, and most members of these communities are endophytes (Stone *et al*. 2000; Arnold 2007; Saikkonen 2007; Rodriguez et al. 2008). Previous studies of cultivated cotton isolated higher numbers of fungal endophytes than we did in this study (Wang *et al*. 2007; Vieira *et al*. 2011; Ek-Ramos *et al*. 2013). For example, Vieira *et al*. 2011 isolated 23 fungal taxa from both transgenic and non-transgenic cotton, whereas Ek-Ramos *et al*. 2013 isolated a total of 69 fungal taxa compared to our study where only 10 different fungal taxa were identified (Table 1). Both of the studies mentioned above utilized samples from whole plants including leaves, shoots, squares and bolls (Ek-Ramos *et al*. 2013) and leaves, stems and roots (Viera *et al*. 2011) whereas our study only utilized single cuts of leaves and stems. The reason behind selecting a single cut of both leaves and stems was because our study was part of a larger experiment from which dry-weight of plants was needed to be collected to measure plant biomass (Fiene 2014, unpublished data) and tissue was utilized for plant carbohydrate and protein analysis (Deans 2014, unpublished data) thus, limiting the amount of tissue available for endophyte isolation. Additionally, the identification of fungal endophytes was not done by microscopy, but using DNA identification only. By not using microscopy we risked underestimating fungal morphotypes that looked similar to the naked eye, but may have been different species. Regardless, the Hulbert rarefaction curve generated in our study indicated our sampling intensity was sufficient to adequately sample for the number of culturable taxa present in the plants (Fig 10).

Even though we did not see any statistical difference in the number of taxa (richness) isolated from the different agricultural settings (monoculture vs. polyculture) or genotype across both sampling events (40 and 80 days post planting) we did find a difference in the fungal community composition over time. Similarly, Ek-Ramos *et al*. (2013) found fungal endophyte community composition to be different among cotton plants sampled at different points in time during the growing season. Based on the Shannon-Weiner diversity index results our main finding was that for both sampling times (40 and 80 days post planting) the monoculture agricultural setting was less diverse than the plots with mixed cotton genotypes (Figure 11). Several studies support the correlation between higher plant diversity associated with higher arthropod, predators, omnivores and microbial community diversity (Arnold *et al.* 2003; Wimp *et al*. 2005; Johnson and Agrawal 2006; Crawford 2007; Saikkonen 2007; Broekling *et al.* 2008; Rowntree *et al.* 2011; Prober *et al.* 2014). Similar to our study, LeBlanc *et al.* (2014) investigated the fungal community of two grasses and two legume species grown in two spatial settings (monoculture vs. polyculture) and showed how fungal communities associated with the plants growing in monoculture were distinct from communities associated with the same plant hosts growing in polyculture; however, fungal communities associated with grasses and legumes were not distinct when plants were grown in polyculture (LeBlanc *et al.* 2014). This is relevant to both alpha diversity, described by the number of taxa and their abundance within communities and beta diversity, defined here as variation in community composition and measured in terms of pair-wise dissimilarity between plots (Whittaker 1972; Anderson *et al.* 2011). We also found that Shannon-Weiner diversity index was different among some cotton genotypes (Fig 12) whereas Ek-Ramos *et al*. 2013 did not find any diversity differences among the >10 cotton varieties sampled across

Texas. The clustering analysis of our data using two similarity coefficients was only consistent when our data was grouped by genotype but not by spatial setting (*Monoculture*, *Quad-row* or *Quad-random*).

Overall, both the literature and our study illustrate how fungal communities may fluctuate in plants, and these variations may be due to host traits, fungal traits or both as well as environmental factors (Saunders *et al*. 2010). For example, the interaction between fungal species in the family Clavicipitaceae and their grass hosts have been very well studied (Clay & Schardl 2002; Belesky & Bacon 2009; Saunders *et al*. 2010). Many of these fungal species provide fitness benefits to the host by increasing tolerance to environmental stressors, although it has been documented that the direction of this relationship can change with environmental conditions and plant–fungus genotype combinations (Meijer & Leuchtmann 2000; Clay & Schardl 2002).

Among the fungal species we isolated, a few are considered in the literature as beneficial endophytes to different host plants (Table 1). *Lecanicillium lecani* is known to be an endophytic entomopathogen that causes negative effects on aphids, scale and whitefly insects when *in planta* (Vega *et al*. 2008; Gurulingappa *et al.* 2010). Similarly, *Cladosporium sp*, is known to produce Befeldin, a secondary metabolite with antimicrobial activity against plant pathogens (Wang *et al*. 2007). We also isolated *Xylaria polymorpha* reported both as a saprophytic and as an endophyte that produces an array of secondary metabolites with antifungal characteristics against plant pathogens among others (Jang *et al*. 2009). These examples illustrate the potential some of these fungal taxa isolated from our study may have in future biological control experiments and practices. Although our study isolated a relatively small number of fungal taxa, our empirical manipulations of plant genotype and

local genetic environment under field conditions demonstrate that these factors can play an important role in affecting endophyte community composition. Given the benefits fungal endophytes may provide to their host plants, a more comprehensive understanding of the host traits are involved in these symbiotic relationships and how these traits interact with the local genetic diversity to influence fungal communities will provide deeper knowledge of causes and consequences of microbial mediated interactions in both natural and agricultural ecosystems.

CHAPTER IV

CONCLUSIONS

The main focus of our research was to study the ecological roles of two different entomopathogenic endophytes in cultivated cotton. Through the use of basic and applied tools we were able to make progress in the field of plant-microbe interactions regarding facultative endophytes in cotton plants. The major findings of our study included (1) the successful inoculation of *Beauveria bassiana* and *Purpureocillium lilacinum* into cotton plants via seed inoculation, (2) Negative fitness effects on two different herbivorous insects when feeding on endophyte colonized cotton plants, (3) Phytohormone profile changes in plants inoculated with target endophytes as a proposed plant-endophyte defense mechanism against herbivorous insects and (4) The composition of fungal communities in cotton are affected by both genotype and spatial variation of plants.

We provide the firt comprehensive study of the ecological roles these two entomopathogenic endophytes have in cultivated cotton and how their manipulation has the potential to protect plants from insect herbivores and potentially other stress factors. The consistency of results across years and given the need of environmentally friendly strategies into Agriculture our research shows the potential reliability of incorporating fungal endophyte manipulations into insect pest management strategies.

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