2018

EVALUATION OF *TRICHODERMA* SPP. AS BIOCONTROL AGENTS FOR SOYBEAN DISEASES

Jonathan Vance Lacey
University of Kentucky, jonlacey@utk.edu
Digital Object Identifier: https://doi.org/10.13023/etd.2018.414

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Jonathan Vance Lacey, Student

Dr. Carl A. Bradley, Major Professor

Dr. Rick Bennett, Director of Graduate Studies

EVALUATION OF *TRICHODERMA* SPP. AS BIOCONTROL AGENTS FOR SOYBEAN DISEASES

THESIS

A thesis submitted in partial fulfillment

of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By

Jonathan Vance Lacey

Lexington, Kentucky

Director: Dr. Carl Bradley, Professor of Plant Pathology

Lexington, Kentucky

2018

ABSTRACT OF THESIS

EVALUATION OF *TRICHODERMA* SPP. AS BIOCONTROL AGENTS FOR SOYBEAN DISEASES

Fungi in the genus *Trichoderma* have been characterized as biocontrol agents of plant pathogens since the 1930s. The use of biologicals for disease management has increased in recent years, typically marketed as a safer alternative to chemical applications. However, biologicals often lack consistent control across varying environmental conditions. To overcome the loss in efficacy due to environmental conditions, biologicals can be combined with common fungicide seed-treatments to provide improved control. Additionally, the presence of a biological organism could slow the development of a pathogen population. Greenhouse trials were conducted to determine the baseline root colonization of three *Trichoderma* spp. used in conjunction with five commonly used seed treatments. In field trials, a stand-alone treatment of the Trichoderma isolates was assessed for management of Rhizoctonia root rot (caused by *Rhizoctonia solani*) and frogeye leaf spot (caused by *Cercospora sojina*). The greenhouse trial provided evidence that isolates of *T. virens* and *T. hamatum* can colonize the roots of plants in which seeds were treated with metalaxyl + prothioconazole + penflufen or metalaxyl + prothioconazole + penflufen + fluopyram. Surprisingly, in the Rhizoctonia root rot trials, the soybean seedlings treated with *Trichoderma* spp. had significantly reduced stand compared to the *R. solani* inoculated control. For the frogeye leaf spot trial, an application of *T. virens* conidial suspensions as a foliar treatment significantly ($P \leq 0.10$) reduced frogeye leaf spot severity of soybean compared to a non-treated control. Future research is warranted to better understand the potential efficacy in additional environments and the mechanism(s) of action used by the *Trichoderma* isolates evaluated in these e xperiments.

KEYWORDS: *Trichoderma* spp., endochitinase, seed treatment fungicide, qPCR, *Rhizoctonia solani, Cercospora sojina*

Jonathan Vance Lacey

November 6th, 2018

EVALUATION OF *TRICHODERMA* SPP. AS BIOCONTROL AGENTS FOR SOYBEAN DISEASES

By

Jonathan Vance Lacey

Carl A. Bradley

_____

Director of Thesis

Rick Bennett

_____

Director of Graduate Studies

November 6th, 2018

_____

Date

DEDICATION

Ivy Renee Triplett

A flower bloomed, but quickly wilt

May your angelic wings remove our guilt

ACKNOWLEDGMENTS

I owe my deepest gratitude to my supervisor Extension Professor Dr. Carl A. Bradley. Without his continuous optimism concerning the work, enthusiasm, encouragement, and support the study would have not been completed. The world needs more professors who exemplify the drive, passion, and determination of Dr. Carl A. Bradley.

I also want to express my warmest gratitude to my co-adviser Dr. Aardra Kachroo, who was always there to remind me science I will always have much to learn as a researcher.

Additionally, I'd like to thank the technicians, graduate students, and interns of Dr. Bradley's and Dr. Kachroo's lab. Without the guidance and support of these kind-hearted individuals, obtaining a graduate degree would have been more challenging.

Also, one of the most integral aspects of my academic journey was the support of my parents and family members.

Finally, I'd like to thank the North Central Soybean Research Program for supporting this research. It provided a valuable experience for me to develop as an individual, academic, and researcher.

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CHAPTER 1

Literature Review

Fungi in the genus *Trichoderma* are ubiquitous soil-borne organisms that exist across the world. *Trichoderma* spp. can colonize plants and develop intimate relationships that can benefit both organisms. As plant symbionts, *Trichoderma* spp. retain the ability to penetrate plant tissues without causing disease. When *Trichoderma* spp. colonize host tissue, the organisms can reduce the presence of plant pathogenic fungi by direct competition for resources, release of antibiotic compounds, or the induction of plant defenses (Harman, 2011). To communicate with a symbiotic host, *Trichoderma* spp. can release an array of volatile organic compounds (VOCs) that drastically alter gene expression in the plant (Contreras-Cornejo et al., 2016). The release of VOCs from *Trichoderma* spp. can improve plant development and elicit defense responses of the host (Contreras-Cornejo et al., 2016; Vinale et al., 2009). The unique characteristics of *Trichoderma* spp. have intrigued plant pathologist for many decades, and researchers are only beginning to harness the potential of these organisms.

Trichoderma spp. were first characterized as biocontrol agents in the 1930s (Weindling, 1932; Weindling, 1934). Initially, *Trichoderma* spp. ability to control disease was attributed to antibiosis and mycoparasitism (Chet, 1987; Elad et al., 1982; Weindling, 1934). By employing these mechanisms of action, *Trichoderma* spp. can release antibiotic compounds that inhibit the growth of competitors which help to out compete nearby microorganisms for resources. Compounds that are related to mycoparasitism and antibiosis include: 6-pentyl-α-pyrone, viridin, trichothecenes

(trichodermin and harzianum A), gliotoxin, peptaibols, harzianic acid, and siderophores (Anitha and Murugesan, 2005; Avent et al., 1992; Brian and McGowan, 1945; Jaworski et al., 1999; Malmierca et al., 2015; Vinale et al., 2009).

More recent research has indicated that most of the biocontrol activity of *Trichoderma* spp. is mediated from the induction of systemic resistance pathways (Howell, 2006; Shoresh et al., 2010). Several molecules have been isolated and identified from *Trichoderma* spp. that elicit host defense responses; harzianolide, 6-pentyl-α-pyrone, peptaibols, trichokonins, and harzianic acid (Avent et al., 1992; Claydon et al., 1987; Jaworski et al., 1999; Vinale et al., 2009; Xiao- Yan et al., 2006). Interestingly, many predicted gene clusters that regulate metabolite expression in *Trichoderma* are considered 'silent' (Hertweck, 2009). To overcome the 'silent' clusters, researchers could over-express transcription factors and potentially offer insight into the regulatory mechanisms of these unexplored genetic resources (Brakhage and Schroeckh, 2011; Strauss and Reyes-Dominguez, 2011).

Another beneficial aspect of *Trichoderma* is their ability to act as auto-regulators (Harman, 2006). *Trichoderma* metabolites acting as auto-regulators that have been identified and characterized include: 1-octen-3-ol, 3-octanone, emodin, and pachybasin (Nemcovic, 2008; Lin et al., 2012). The presence of 1-octen-3-ol will inhibit germination and colony growth of some *Trichoderma* spp. but will increase the conidiation response in the *T. atroviride* species (Nemcovic et al., 2008). In the context of mycoparasitism, the presence of pachybasin and emodin metabolites directly regulates the physiological response of coiling in *Trichoderma* spp. (Lin et al., 2012).

Trichoderma spp. as a form of crop protection has been successful for the management of various pathogens in a multitude of crops. Typically, *Trichoderma* spp. are applied as either a seed-treatment or as a conidia suspension which is applied in-furrow. However, *Trichoderma* spp. can be applied to the foliar portions of plants to manage diseases, induce plant defenses, and stimulate plant growth (Harman, 2011). In rice productionresearchers were able to successfully manage Fusarium head blight caused by *Fusarium graminearum.* Furthermore, the presence of the antagonist *Trichoderma* spp. significantly reduced deoxynivalenol contamination in the diseased kernels (Matarese et al., 2012). The use of *Trichoderma* spp. in tomato, wheat, and soybean has significantly reduced diseases caused by the pathogens *Sclerotinum rolfsii*, *Rhizoctonia solani*, *Pyrenophora tritici-repentis*, *Mycosphaerella graminicola*, *Fusarium oxysporum* f. sp. *adzuki* and *Pythium arrhenomanes* (Elad et al., 1980; Perello et al., 2006; John et al., 2010).

By combining isolates of *Trichoderma* spp. with a chemical fungicide, researchers could develop a seed treatment that offers two-tiers of protection from plant disease. Initial protection to the sensitive root tissues would be provided by the fungicide component of the seed treatment. As the fungicide efficacy begins to fade, the presence of *Trichoderma* spp. could compensate for the loss of protection by stimulating plant defenses. Currently, researchers outside of the United States have assessed *Trichoderma* isolates for their tolerance to fungicides. Initial reports of researchers combining *Trichoderma* spp. with seed-treatment chemistry are positive and could be a viable option for disease management (McLean, 2001; Madhusudan et

al., 2010; Pandya et al., 2011). Unfortunately, utilizing *Trichoderma* spp. as a form of crop protection has been slow to develop due to the lack of consistency in control when compared to synthetic fungicides (Mukherjee and Kenerley, 2010).

To overcome the lack of consistency in control, research efforts could focus on implementing *Trichoderma* spp. that are antagonist to pathogenic fungi, yet tolerant to fungicides. This thesis research was conducted to help contribute to the existing research on *Trichoderma* spp. used in row-crop systems within the United States. The first portion of research assessed the ability of various *Trichoderma* spp. to colonize roots of fungicide treated soybean. The second aspect of research determined the potential use of *Trichoderma* spp. for the management of Rhizoctonia root rot of soybean (*Glycine max*), caused by *Rhizoctonia solani*. The third component of research tested *Trichoderma* spp. potential use for the management of frogeye leaf spot of soybean, caused by *Cercospora sojina*.

CHAPTER 2

Evaluation of *Trichoderma* spp. Ability to Colonize Soybean Roots from Seeds Treated with Fungicide

Abstract

Fungi in the genus *Trichoderma* have been characterized as biocontrol agents of plant pathogens since the 1930s. Their mechanisms of control include mycoparasitism, antibiosis, competition for resources or space, and the induction of host defenses. The unique characteristics of *Trichoderma* spp. are appealing to plant pathologists and can be easily implemented in existing disease management programs, as either a stand-alone treatment or in conjunction with a fungicide seed treatment. Two fungal strains, *Trichoderma hamatum* and *Trichoderma virens* were tested to determine the efficacy of colonizing fungicide treated soybean. After 28 days of growth, qPCR was performed to determine the colonization rate of the *Trichoderma* spp. This research has provided evidence for *Trichoderma* spp. ability to colonize fungicide treated soybean. Future research is warranted to better understand the potential efficacy of *T. virens, T. hamatum,* Evergol Energy, and Evergol Energy + ILeVo combinations. Additional research is necessary to further understand the potential efficacy in additional environments and the mechanism(s) of action used by the *Trichoderma* isolates evaluated in these experiments.

Introduction

Fungi of the genus *Trichoderma* were characterized as biocontrol agents of plant diseases as early as 1930 (Wiendling, 1932). *Trichoderma* spp. are soil-borne filamentous saprophytes that can grow on plants, animals, and many other substrates (Atanasova et al., 2013; Holzlechner et al., 2016). To colonize such diverse habitats, *Trichoderma* spp. have developed pathways that produce unique secondary metabolite capable of producing an array of bioactive molecules (Atanasova et al., 2013). By harnessing the diverse chemical profiles of *Trichoderma* spp., researchers can develop naturally derived products that benefit agriculture, pharmaceutical, and industrial applications (Atanasova et al., 2013).

Initially, the ability of *Trichoderma* spp. to control disease was attributed to antibiosis and mycoparasitism (Weindling, 1934; Elad et al., 1982; Chet, 1987). Later in the 1990s, *Trichoderma* research identified additional effects on plant growth promotion and induced resistance to plant stress, but these were characterized as minor secondary benefits (Harman et al., 2004). Interestingly, recent research has indicated that most of the biocontrol activity provided by *Trichoderma* spp. is derived from the induction of systemic resistance pathways (Howell, 2006; Shoresh et al., 2010). To stimulate plant responses, *Trichoderma* spp. can release small metabolites or volatile organic compounds that easily diffuse across cellular membranes (Harman et al., 2004).

Currently, several molecules have been isolated and identified from *Trichoderma* spp. that elicit host defense responses including; harzianolide, 6-pentyl-α-pyrone, peptaibols, trichokonins, and harzianic acid (Claydon et al., 1987; Avent et

al., 1992; Jaworski et al., 1999; Xiao-Yan et al., 2006; Vinale et al., 2009). Similar to other fungi, the expression of secondary- metabolite-related genes in *Trichoderma* spp. can be influenced by interactions with other micro-organisms, fluctuations in pH, or changes in light. (Antanasova et al., 2013; Bazafkan et al., 2015; Fekete et al., 2014; Malmierca et al., 2015; Mukherjee and Kenerley, 2010; Trushina et al., 2013). Understanding the environmental factors that influence the production of metabolites can help researchers to develop bio-fungicides that are resilient and effective in any weather conditions.

Recently, interest in utilizing the secondary-metabolites produced by *Trichoderma* spp. to manage field crop diseases has increased (Moya et al., 2018; Vinale et al., 2009). The unique chemistry of *Trichoderma* spp. offers novel modes of action for disease control and can trigger plant immune responses for an entire growing season (Crutcher et al., 2013; Mukherjee and Kenerley, 2010; Mukherjee et al., 2012; Renio et al., 2008). Additionally, some *Trichoderma* spp. are tolerant to many commonly used fungicides; such as thiabendazole (Chaparro et al., 2011). Tolerance to fungicides and an ability to elicit plant defense responses are two vital components necessary for combining with a pre-existing synthetic active ingredient or formula.

Previously conducted research that evaluated in-vitro sensitivity of *Trichoderma* spp. to common fungicide active ingredients used in soybean seed treatments showed that some isolates were not greatly inhibited by prothioconazole, fludioxonil, and metalaxyl (Lacey et al., 2017). Due to the cost of novel synthetic fungicide research, plant protection products of the future will inevitably contain a

biological organism. Researchers can expedite the discovery process for novel technology by determining if the *Trichoderma* isolates selected are resistant to fungicides, can provide long-term protection throughout the season.

To date, most of the registered *Trichoderma* products are used in horticulture production systems. Research regarding *Trichoderma* spp. use in row-crop systems is limited. Specifically, research determining Trichoderma spp. ability to colonize fungicide treated soybean has only been conducted outside of the United States. To test *Trichoderma* spp. ability to colonize fungicide-treated soybean, trials were conducted with three isolates: *T. hamatum*, *T. virens*, and *Bionectria ochroleuca*. The ability of isolates of *Trichoderma* spp. to colonize the seed was compared to a treatment that had *Trichoderma* spp. without a fungicide component. All comparisons were made to a treatment that had neither a fungicide seed component nor the addition of fungi. *Trichoderma* spp. were obtained from soybean roots in fields across multiple locations in Illinois (Fakhoury et al., unpublished).

Materials and Methods

Greenhouse Preparation and Experiment Design Greenhouse trials were conducted at the University of Kentucky (Lexington, KY) at three different time periods during 2017 and 2018. The trials were set up as randomized complete block designs. Environmental conditions of the greenhouse were: 25-35°C air temperature, 65-95% relative humidity, and photosynthetic active radiation rating of 150-250 $m^{-2}s^{-1}$. Greenhouse trials contained 19 treatments replicated 5 times (95 total experimental units).

Five different fungicide combinations applied to soybean seeds and a non-

treated control were included in the trial. Fungicide combinations included 1) prothioconazole + penflufen + metalaxyl (EverGol Energy; Bayer CropScience, Research Triangle Park, NC); 2) prothioconazole + penflufen + metalaxyl + fluopyram (Evergol Energy + ILeVo; Bayer CropScience); 3) fludioxonil + metalaxyl + sedaxane (Apron Maxx + Vibrance; Syngenta Crop Protection, Greensboro, NC); 4) fludioxonil + metalaxyl + sedaxane + thiabenazole (ApronMaxx + Vibrance + Mertect; Syngenta Crop Protection); 5) pyraclostrobin + metalaxyl (Acceleron; Monsanto, St. Louis, MO).

Biological treatments consisted of three organisms: 1) *Bionectria ochroleuca*, 2) *Trichoderma hamatum*, 2) *Trichoderma virens.*Soil was prepared by mixing one-part of peat moss/vermiculite/perlite/limestone/wetting agent (Pro-mix BX Mycorrhizae; Pro-Mix, Ontario, Canada) with two parts of sand. After thorough mixing, the soil was steam pasteurized at a temperature of 65°C for a total of four hours. Polyurethane pots (Conetainer; Greenhouse Megastore, Danville, IL) with a diameter of 3.8 cm and a length of 21 cm were filled immediately after removing soil from the steamer. Planting holes with a diameter of 1 cm were made in each pot at a depth of 2.5 cm. After filling with soil, each pot was placed into a tray measuring 61 cm long by 30.5 cm wide and 17.2 cm tall.

Inoculum Preparation. Fungal isolates were cultured in laboratory conditions and grown on potato dextrose agar. Cultures were grown for two weeks in a growth room at a constant temperature of 23°C, 90% relative humidity, and under continuous soft-white fluorescent lighting (TL 841 32J/s, Phillips, Andover, MA). After two weeks of growth, conidial suspensions of each isolate were prepared by washing conidia off

plates using 15 ml of sterilized water and a bent-glass rod. Contents of the petri plate were passed through a 200-micron sieve, and sterile water was added to a volume of 500 ml. Conidia were counted using a hemocytometer (Bright- Line Hemacytometer; Hausser Scientific, Horsham, PA) and 40X objective on a compound light microscope (Zeiss Axioskop; ZEISS International, Oberkochen, Germany). The conidial suspensions were brought to a final volume of 2 L at a concentration of 1 X 10^9 conidia/ml (Harman, 2011). The conidial suspensions were applied in-furrow to each individual pot containing one soybean by means of a 10 ml glass hand pipette.

Biological Control Assessment. An indirect characterization was performed to assess the fungal ability to stimulate plant growth. To determine plant growth benefits, plant height was recorded using a ruler at 7, 14, 21, and 28 days. Plant height was measured from the soil-line to the shoot-tip. An additional qualitative measurement was conducted at 7 days to determine if the combination of fungicide and biocontrol agent exhibited systemic phytotoxic effects to the cotyledon leaf. The phytotoxicity of the cotyledons were determined as a percent relative to the affected area on the leaf. After 28 days of growth, plant roots were carefully removed from each pot. Soil was removed from each root by washing with tap water. Root length was recorded for each experimental unit and was determined by measuring from the root tip to the soil-line on the stem. After all parameters were measured, root samples were processed.

Root Sample Processing. Samples were ground by hand using a 500 ml mortar and pestle (Coors; Coorstek, Golden, CO) with liquid nitrogen added throughout the process to prevent the sample from thawing. A small subsample (100 mg) of the

homogenized tissue was weighed using a balance. Tissue samples were processed for DNA following the extraction protocol (DNAeasy Plant Kit 250 samples; Qiagen, Germantown, MD). Aliquots of dsDNA were normalized at 260/280 nm using a spectrophotometer (Biotek Synergy HT; Biotek, Winooski, VT) and brought to a final concentration of 1ng/ul. Samples were stored at -20°C until processed for qPCR.

Primers. Relative DNA quantification was determined by comparing the presence of a fungal endochitinase gene to the stable housekeeping gene, actin. The primer sequences for the endochitinase gene were: forward 5'-GGTCCACCAAYTTCCCTTCT - 3'; reverse 5'- CATCRAGCTGAGATCGGACT-3' (Fungal Endochitinase 42; Integrated DNA Technologies, Newark, NJ). The actin gene primer sequences used were: forward 5' – GAGCTATGAATTGCCTGATGG – 3'; reverse 5' – CGTITCATGAATTCCAGTAGC – 3'(Soybean Actin; Integrated DNA Technologies, Newark, NJ)

DNA quantification. A 10 ul reaction was prepared for each sample and contained: 5 ul of SYBR green master mix (SYBR Green; Bio-Rad, Hercules, CA), 2 ul of DNA, 0.8 ul of a 0.5 uM forward primer, 0.8 ul of a 0.5 uM reverse primer, and 1.4 ul of autoclaved milliQ water. Each sample was placed into a 96-well plate (MicroAmp Fast Optical 96-Well Reaction Plate 0.1 ml, Applied Biosystems, Foster City, CA) for qPCR analysis (Applied Biosystems 7900 HT, Applied Biosystems, Foster City, CA).

Plant Growth Promotion Data Analysis. Data collected for the effect of *Trichoderma* spp. on plant vigor were analyzed using the general linear model procedure (PROC GLM) in SAS (Version 9.4; SAS Institute Inc., Cary, NC). Means were compared using Fisher's protected least significant difference test ($\alpha = 0.10$).

qPCR Data Analysis. Data from qPCR analysis in the form of raw-cycle threshold values was normalized using the Delta-Delta-Ct method (Livak and Schmidtten, 2001) and Delta-Ct values were statistically analyzed by performing a Student's t-test in Excel (Excel 2016; Microsoft Corporation, Redmond, WA).

Results

Effect of Trichoderma isolates on phytotoxicity and plant vigor. To determine if adding *Trichoderma* spp. effected plant growth, height was measured and phytotoxicity was visually estimated (Tables 2.1 and 2.2). Phytotoxic effects from the fungicides in combination with the fungi were observed on cotyledon leaves at 14 days. Compared to the untreated control, phytotoxic effects were observed on cotyledon leaves of all treatments that contained ILeVo (Table 2.1). In Greenhouse Trial Two, treatments that resulted in phytotoxicity significantly greater than the untreated check were Evergol Energy + *T. virens* and all treatments that contained ILeVo (Table 2.3)

With plant height as a measured determinant of plant vigor, no treatments significantly increased plant height over the untreated check in either Greenhouse Trial One or Two (Tables– 2.4). However, in Greenhouse Trial Three, four treatments resulted into plant height greater than the untreated check, which were ApronMaxx + Vibrance + *T. virens*, ApronMaxx + Vibrance + Mertect + *T. virens*, ApronMaxx + Vibrance + Mertect + *T. hamatum*, and Acceleron + *B. ochroleuca* (Tables 2.5 and 2.6). However, some treatments did significantly reduce plant height compared to the untreated check (Tables 2.1-2.6). Treatments that consistently significantly reduced plant height compared to the untreated check at the end of the

experiment (28 days after planting) in all three trials were Evergol Energy + ILeVo + *T. hamatum* and Evergol Energy + ILeVo + *B. ochroleuca* (Tables 2.1 – 2.6). For the root length, the Evergol + ILeVo + *B. ochroleuca* was only treatment that was significantly higher than the untreated control (Table 2.5).

Ability of *Trichoderma* spp. to colonize the root of fungicide treated soybean. To determine Trichoderma spp. ability to colonize roots from fungicide treated seed, qPCR was used. An endochitnase primer was selected to detect for the presence of *Trichoderma* spp. and a housekeeping gene, actin was used as the control. (Figs. 2.1 and 2.3). In experiment 1, significant ($P \leq 0.05$) treatment effects were observed for the ability of fungi to colonize fungicide treated seed. Treatments: *Trichoderma hamatum*, *Bionectria ochroleuca*, Evergol Energy + *T. hamatum*, Evergol Energy + *T. virens*, Evergol Energy + *B. ochroleuca* Evergol Energy + ILeVo + *T. hamatum*, ApronMaxx + Vibrance + *T. virens*, ApronMaxx + Vibrance + Mertect + *T. virens*, Acceleron + *T. virens,* and Acceleron + *B. ochroleuca* were all significantly greater than the untreated control. Treatments that were significantly less than the untreated control were: *T. virens*, Evergol Energy + ILeVo + *T. virens,* Evergol Energy + ILeVo + *B.ochroleuca*, ApronMaxx + Vibrance + *T. hamatum*, ApronMaxx + Vibrance + *B. ochroleuca,* ApronMaxx + Vibrance + Mertect + *T. hamatum*, ApronMaxx + Vibrance + Mertect + *B. ochroleuca,* and Acceleron + *T. hamatum* (Figs. 2.4, 2.8, and 2.12).

For experiment 2, significant ($P \leq 0.05$) treatment effects were observed for the ability of fungi to colonize fungicide treated seed. Only one treatment was significantly greater than the untreated control: Evergol Energy + ILeVo + *T. hamatum*. Treatments that were significantly less than the untreated control were: *T. hamatum*, Evergol

Energy + *T. hamatum*, ApronMaxx + Vibrance + *T. hamatum*, ApronMaxx + Vibrance + *T. virens*, ApronMaxx + Vibrance + Mertect+ *T. virens*, and Acceleron + *T. hamatum* (Figs. 2.5, 2.9, and 2.13).

In experiment 3, significant ($P \leq 0.05$) treatment effects were observed for the ability of fungi to colonize fungicide treated seed. Treatments that were significantly greater than the untreated control were: *T. hamatum*, *T. virens*, Evergol Energy + *T. virens,* Evergol Energy + ILeVo + *T. virens,* ApronMaxx + Vibrance + *T. virens*,, ApronMaxx + Vibrance + Mertect + *T. virens,* and Acceleron + *T. virens*.

There were no treatments that were significantly less than the control for this experiment (Figs. 2.6, 2.10, and 2.14). In each greenhouse trial, the ability of *Trichoderma* spp. to colonize roots from the fungicide- treated seed varied with the organism tested and the type of fungicide applied to the seed. The fungicide treatments with the highest rates of colonization for *T. hamatum* were Evergol Energy and Evergol Energy + ILeVo (Figs. 2.4, 2.5, and 2.6). The highest rate of colonization for *T. virens* was the Evergol Energy treated seed (Figs. 2.8 and 2.10). For the *B. ochroleuca* isolate, the highest rate of colonization occurred in the Acceleron treatment (Fig. 2.7).

Discussion

Several factors can impact *Trichoderma* spp. ability to colonize roots from a fungicide treated seed including: cultivar compatibility, moisture levels, or fungicide tolerance (Harman, 2011; Mayo et al., 2015). The enhanced ability of *Trichoderma* spp. to colonize Evergol Energy and Evergol Energy + ILeVo suggests that a compound in the Evergol Energy seed treatment could be eliciting a positive response

from the fungi.

In the first greenhouse trial, the rates of colonization recorded for the *B. ochroleuca* treatments were the result of an experimental design error. Initially, the greenhouse assay was set up using a complete andomized design. However, after the data from the first greenhouse trial were analyzed, it suggested there was possible contamination occurring from the nearby pots of *Trichoderma* spp. The contamination was prevented in the second and third greenhouse trials by redesigning the experiment as a randomized complete block. When the experimental design was changed to a randomized complete block design there were no treatments of *B. ochroleuca* that were significantly different when compared to the untreated control samples (Figs. 2.12, 2.13, and 2.15).

The most important aspect in developing a qPCR-based assay for testing the colonization rate of fungi is the primer design step. Initially an internal control spacer (ITS) primer was used, instead of the endochitinase gene. However, the ITS gene was unable to differentiate between the *Trichoderma* spp. and the negative control *B. ochroleuca*, therefore, the endochitinase primer was selected as an alternative (Fig 2.2). Another issue in the experiment was that more than one control gene should have been used in the assay.

The actin primer had a variability of 6-7°C when examined by dissociation curve analysis, this high degree of variability suggests that either the actin gene expression levels were extremely variable between samples or the primer for the housekeeping gene lacked the proper specificity to be used as a control. The differences in gene expression levels could have derived from the amount of time each sample took to

process; cleaning soil from roots and weighing sample. Another possibility is that when the control primer was designed it was not rigorously tested for proper specificity. If this hypothesis were true, non-specific binding could be occurring within the samples. However, the end-point polymerase chain reaction suggests otherwise.

Aside from the primer specificity issues, the greenhouse trials exhibited similar data trends for most of the treatments (Fig 2.4-2.15). Unfortunately, in the first and second greenhouse trials there was a high degree of variability in the pure isolate treatments of *T. virens* and *T. hamatum*, respectively. Since *Trichoderma* spp. will only colonize specific portions of the root, the variability could have been the result of improper homogenization of the tissue before processing the sample for DNA. Nonetheless, the data suggests that the *T. virens* isolate would be compatible with all the fungicides tested, specifically those containing Evergol Energy. Meanwhile, the *T. hamatum* isolate would best be used in conjunction with Evergol Energy.

This study has provided evidence that *Trichoderma* spp. are able to colonize the root of fungicide treated seed. Future research could focus on developing a commercialized product based on the combination(s) of *T. virens, T. hamatum,* Evergol Energy, and Evergol Energy + ILeVo. Further characterization of the *T. virens* and *T. hamatum* isolates is necessary to determine if they are effective at controlling soil-borne pathogens. If the bio-control isolates are effective at controlling soil-borne pathogens, then researchers could begin testing viable formulations for the seed treatment.

Table 2.1 *Greenhouse experiment 1* (Treatments 1-10): Effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. Negative effects of the fungicide and biological agent were determined by observing burning to the cotyledon leaves, characterized as phytotoxicity. Potential plant growth promotion effects were determined by plant height recorded at 7, 14, 21, and 28 days. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

	Treatment	Height (cm) 7 Days	Phytotoxicity (1-100%) 14 Days	Height (cm) 14 Days	Height (cm) 21 Days	Height (cm) 28 Days
1	Untreated Check	15.7	0	28.4	42.1	54.8
2	*T. virens*	11.6	1.7	29.6	45.1	52.1
3	*T. hamatum*	15.2	0	19.7	45.7	55.0
4	*B. ochroleuca*	14.8	0	30.3	44.9	54.6
5	Evergol Energy + *T. virens*	9.1	0	23.5	42.3	54.0
6	Evergol Energy + *T. hamatum*	9.1	0	25.2	39.8	47.0
7	Evergol Energy + *B. ochroleuca*	8.5	0	25.0	40.4	49.1
8	Evergol Energy + ILevo *T. virens*	6.4	25	25.8	35.8	43.6
9	Evergol Energy + ILevo *T. hamatum*	9.3	20	26.0	38.1	45.1
10	Evergol Energy + ILevo *B. ochroleuca*	7.0	23.3	28.6	39.6	45.9
	P > F	**0.0002**	**0.0001**	**0.1696**	**0.0102**	**0.0006**
	LSD 0.10 [z]	**4.4**	**7.6**	**NS**[y]	**7.1**	**8.2**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

Table 2.2 *Greenhouse experiment 1* (Treatments 11-19): Effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. Negative effects of the fungicide and biological agent were determined by observing burning to the cotyledon leaves, characterized as phytotoxicity. Potential plant growth promotion effects were determined by plant height recorded at 7, 14, 21, and 28 days. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

	Treatment	Height (cm) 7 Days	Phytotoxicity (1-100%) 14 Days	Height (cm) 14 Days	Height (cm) 21 Days	Height (cm) 28 Days
11	ApronMaxx , Vibrance + *T. virens*	9.3	6.7	28.2	43.4	54.2
12	ApronMaxx , Vibrance + *T. hamatum*	4.7	6.7	19.3	38.5	50.2
13	ApronMaxx , Vibrance + *B. ochroleuca*	9.7	5	26.0	41.9	51.4
14	ApronMaxx , Vibrance , Mertect + *T. virens*	13.1	5	22.6	41.9	52.5
15	ApronMaxx , Vibrance , Mertect + *T. hamatum*	12.7	2.5	29.4	43.4	54.0
16	ApronMaxx , Vibrance , Mertect + *B. ochroleuca*	12.3	5	27.9	45.7	58.0
17	Acceleron + *T. virens*	14.0	1	27.3	44.9	49.7
18	Acceleron + *T. hamatum*	14.4	0	30.7	47.0	54.6
19	Acceleron + *B. ochroleuca*	8.7	0	25.4	38.9	48.9
	P > F	**0.0002**	**0.0001**	**0.1696**	**0.0102**	**0.0006**
	LSD 0.10 [z]	**4.3**	**7.6**	**NS**[y]	**7.1**	**8.2**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

Table 2.3 *Greenhouse experiment 2* (Treatments 1-10): Effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. Negative effects of the fungicide and biological agent were determined by observing burning to the cotyledon leaves, characterized as phytotoxicity. Potential plant growth promotion effects were determined by plant height recorded at 7, 14, 21, and 28 days. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

	Treatment	Height (cm) 7 Days	Phytotoxicity (1-100%) 14 Days	Height (cm) 14 Days	Height (cm) 21 Days	Height (cm) 28 Days
1	Untreated Check	5.6	0	14.9	17.0	21.3
2	*T. virens*	6.0	0	13.5	14.9	17.7
3	*T. hamatum*	5.5	4	13.0	14.4	16.9
4	*B. ochroleuca*	5.7	0	15.7	17.9	22.7
5	Evergol Energy + *T. virens*	4.4	28	14.1	15.4	17.3
6	Evergol Energy + *T. hamatum*	3.9	0	10.9	12.8	16.0
7	Evergol Energy + *B. ochroleuca*	4.3	4	12.6	14.7	18.5
8	Evergol Energy + ILevo *T. virens*	3.0	28	10.0	11.7	15.5
9	Evergol Energy + ILevo *T. hamatum*	4.1	9	11.0	12.7	16.0
10	Evergol Energy + ILevo *B. ochroleuca*	2.3	24	10.2	13.1	16.0
	P > F	**0.0049**	**<.0001**	**0.0227**	**0.0042**	**0.0015**
	LSD 0.10 [z]	**1.5**	**6.8**	**3.5**	**3.7**	**4.6**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

Table 2.4 *Greenhouse experiment 2* (Treatments 11-19): Effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. Negative effects of the fungicide and biological agent were determined by observing burning to the cotyledon leaves, characterized as phytotoxicity. Potential plant growth promotion effects were determined by plant height recorded at 7, 14, 21, and 28 days. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

	Treatment	Height (cm) 7 Days	Phytotoxicity (1-100%) 14 Days	Height (cm) 14 Days	Height (cm) 21 Days	Height (cm) 28 Days
11	ApronMaxx , Vibrance + *T. virens*	5.7	2	17.0	19.6	24.5
12	ApronMaxx , Vibrance + *T. hamatum*	3.9	4	11.9	13.2	16.6
13	ApronMaxx , Vibrance + *B. ochroleuca*	4.7	1	11.4	12.8	16.1
14	ApronMaxx , Vibrance , Mertect + *T. virens*	5.7	2	15.9	18.0	22.9
15	ApronMaxx , Vibrance , Mertect + *T. hamatum*	5.8	3	15.4	17.8	23.2
16	ApronMaxx , Vibrance , Mertect + *B. ochroleuca*	3.8	4	14.4	15.6	19.7
17	Acceleron + *T. virens*	5.8	0	16.0	19.3	24.9
18	Acceleron + *T. hamatum*	5.3	4	16.3	18.0	23.1
19	Acceleron + *B. ochroleuca*	4.4	0	10.8	11.6	16.6
	P > F	**0.0049**	**<.0001**	**0.0227**	**0.0042**	**0.0015**
	LSD 0.10 [z]	**1.5**	**6.9**	**3.6**	**3.7**	**4.6**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

Table 2.5 *Greenhouse experiment 3* (Treatments 1-10): Effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. Negative effects of the fungicide and biological agent were determined by observing burning to the cotyledon leaves, characterized as phytotoxicity. Potential plant growth promotion effects were determined by plant height recorded at 7, 14, 21, and 28 days. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

	Treatment	Height (cm) 7 Days	Phytotoxicity (1-100%) 14 Days	Height (cm) 14 Days	Height (cm) 21 Days	Height (cm) 28 Days
1	Untreated Check	5.3	0	10.2	14.9	17.1
2	*T. virens*	5.0	0	11.2	14.7	17.4
3	*T. hamatum*	5.5	0	10.3	14.5	15.2
4	*B. ochroleuca*	5.6	0	10.9	15.1	16.0
5	Evergol Energy + *T. virens*	3.6	0	11.3	14.7	17.0
6	Evergol Energy + *T. hamatum*	3.8	0	8.3	12.8	15.5
7	Evergol Energy + *B. ochroleuca*	3.8	0	9.3	15.9	17.9
8	Evergol Energy + ILevo *T. virens*	2.5	22	9.0	13.3	15.5
9	Evergol Energy + ILevo *T. hamatum*	2.9	21	8.4	12.3	14.0
10	Evergol Energy + ILevo *B. ochroleuca*	2.4	25	6.7	11.6	14.9
	P > F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	LSD 0.10 [z]	**1.2**	**5.1**	**2.2**	**2.2**	**2.2**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

Table 2.6 *Greenhouse experiment 3* (Treatments 11-19): Effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. Negative effects of the fungicide and biological agent were determined by observing burning to the cotyledon leaves, characterized as phytotoxicity. Potential plant growth promotion effects were determined by plant height recorded at 7, 14, 21, and 28 days. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

	Treatment	Height (cm) 7 Days	Phytotoxicity (1-100%) 14 Days	Height (cm) 14 Days	Height (cm) 21 Days	Height (cm) 28 Days
11	ApronMaxx , Vibrance + *T. virens*	6.9	10	10.9	19.6	21.8
12	ApronMaxx , Vibrance + *T. hamatum*	5.2	7	14.6	15.1	17.5
13	ApronMaxx , Vibrance + *B. ochroleuca*	5.0	2	11.8	17.0	19.2
14	ApronMaxx , Vibrance , Mertect + *T. virens*	6.1	7	13.3	18.3	20.8
15	ApronMaxx , Vibrance , Mertect + *T. hamatum*	5.0	3	12.2	17.0	20.6
16	ApronMaxx , Vibrance , Mertect + *B. ochroleuca*	4.6	3	10.5	15.0	18.0
17	Acceleron + *T. virens*	6.2	0	11.9	16.3	18.9
18	Acceleron + *T. hamatum*	4.7	6	11.0	16.8	20.1
19	Acceleron + *B. ochroleuca*	4.4	0	9.1	14.0	17.5
	P > F	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	LSD 0.10 [z]	**1.2**	**5.1**	**2.2**	**2.2**	**2.2**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$)

Figure 2.1 Conventional PCR for *in-planta* fungal cultures: *T. hamatum*, *T. virens*, and *B. ochroleuca*. Endochitinase primer was used and the expected amplicon size was 186 base pairs. *B. ochroleuca* was used as the negative control.

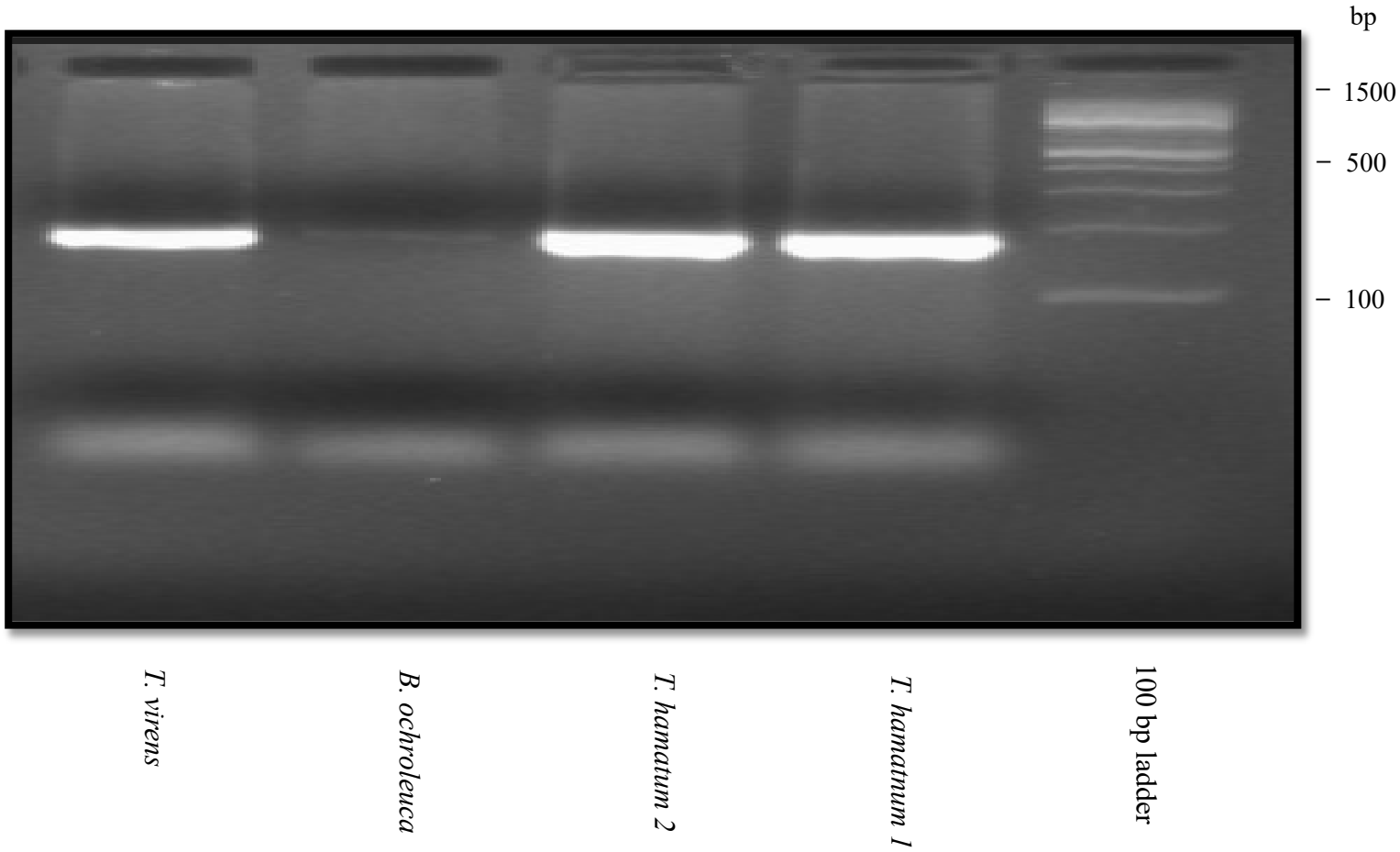


Figure 2.2 Conventional PCR for *in-planta* samples: *T. hamatum*, *T. virens*, and *B. ochroleuca*. The internal transcribed spacer primer that was used had an expected amplicon size of 613 base pairs.

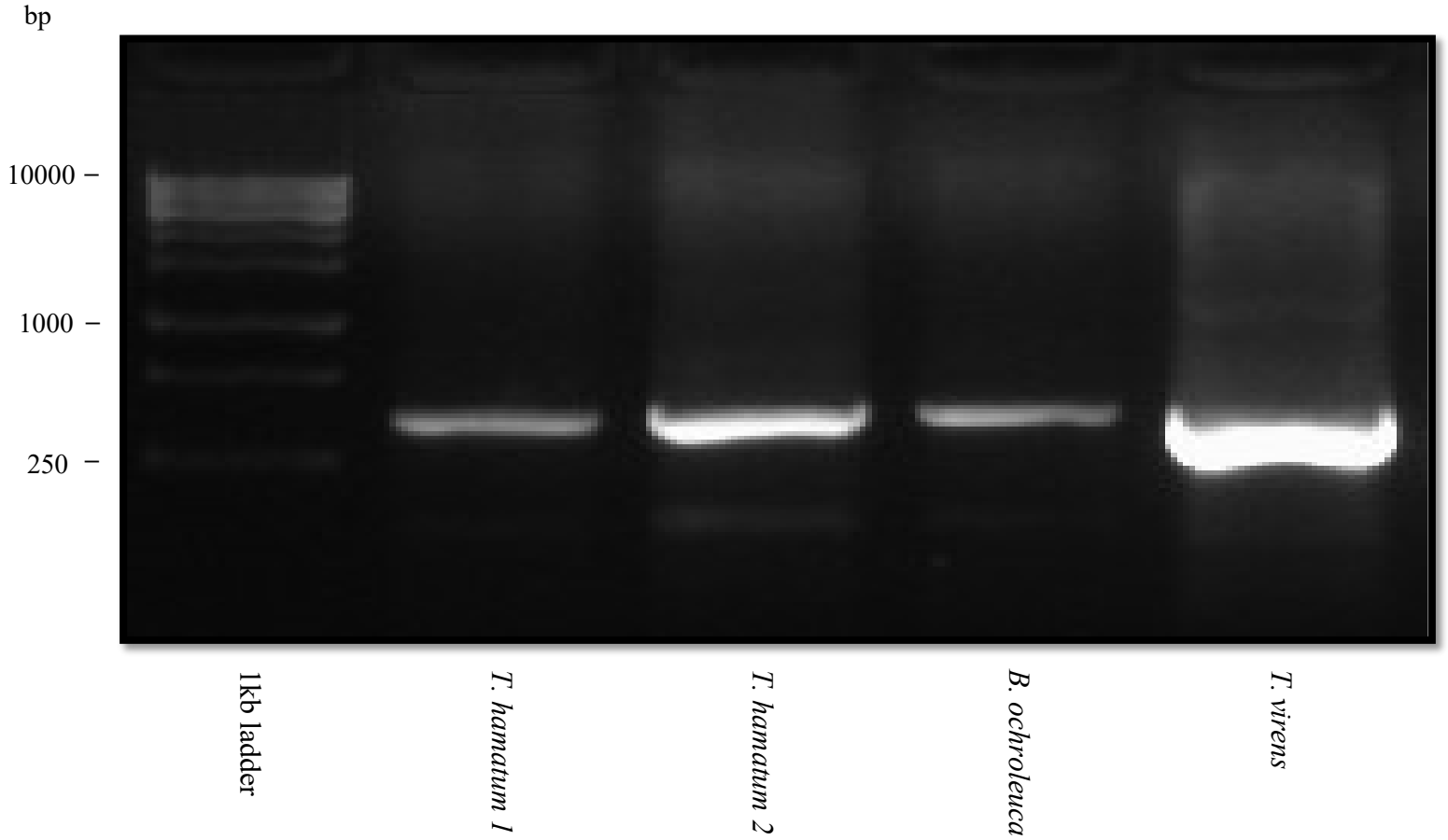


Figure 2.3 Conventional PCR for *in-planta* samples: *T. hamatum*, *T. virens*, and *B. ochroleuca*. The actin primer that was used had an expected amplicon size of 150 base pairs. *B. ochroleuca* was used as the negative control.

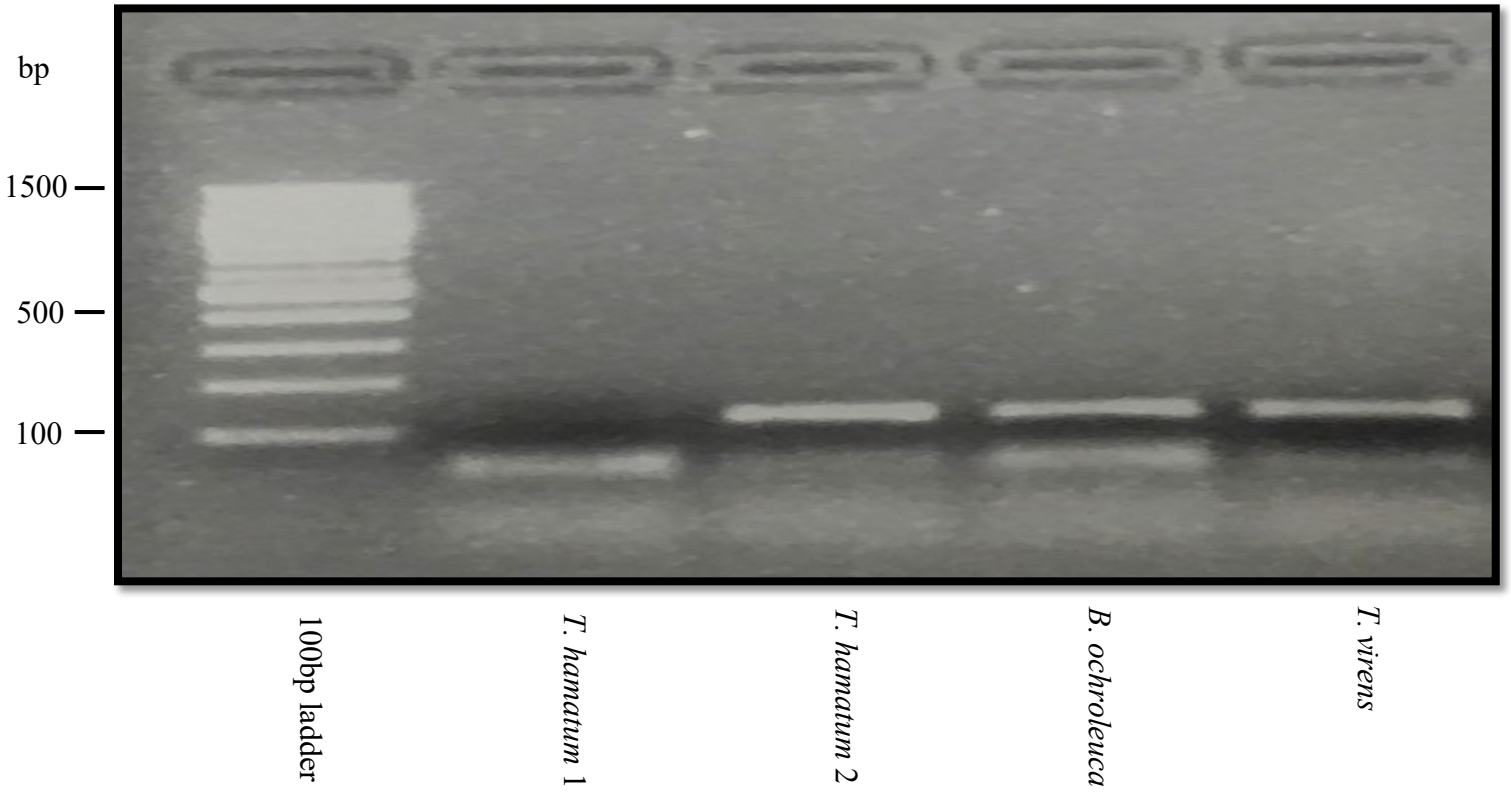


Figure 2.4 Dissociation analysis performed from experiment 3 – *T. virens*. The amplification plots on the left is for the housekeeping gene actin and the plot on the right is for the target gene, endochitinase.

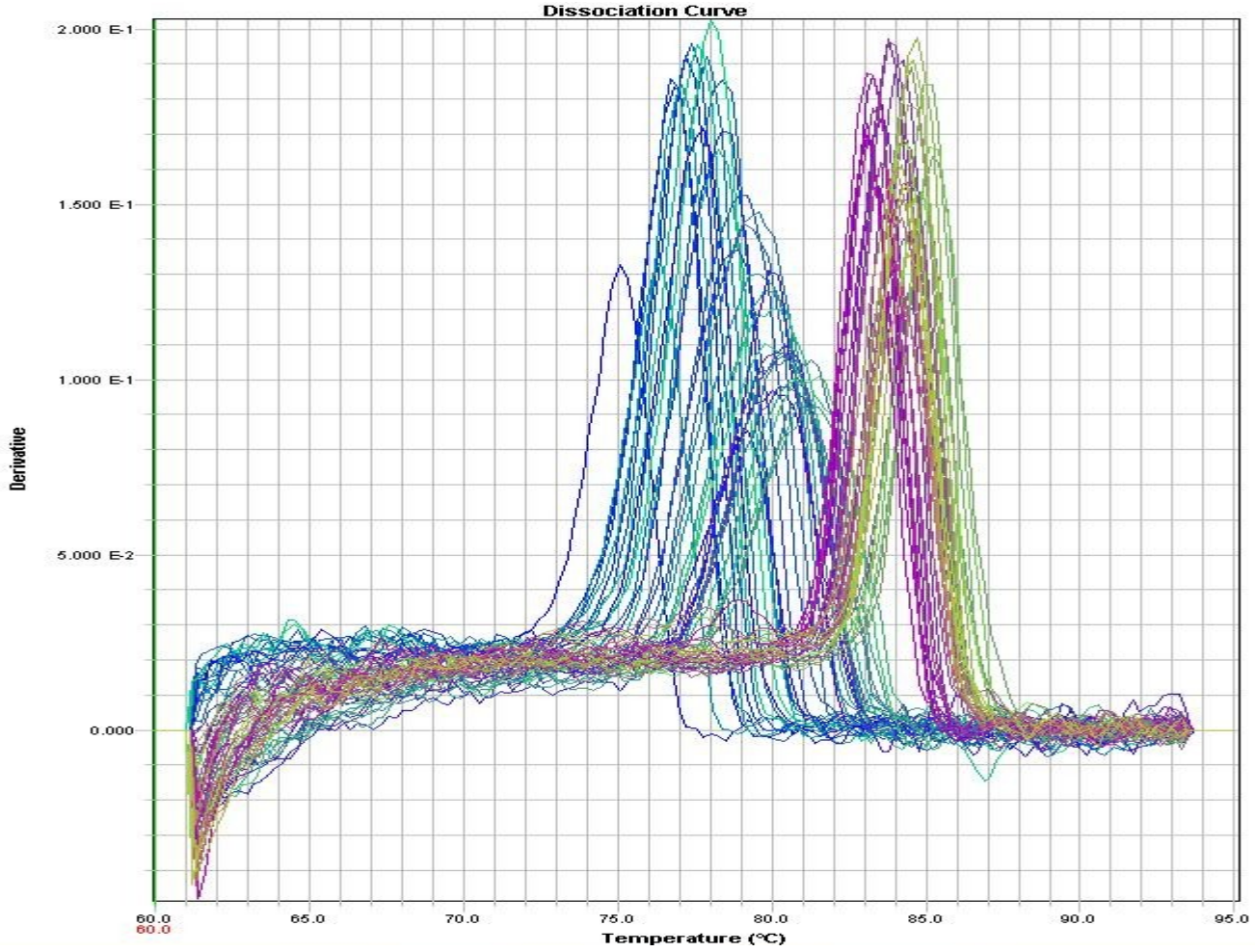


Figure 2.5 *Greenhouse experiment 1- T. hamatum*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

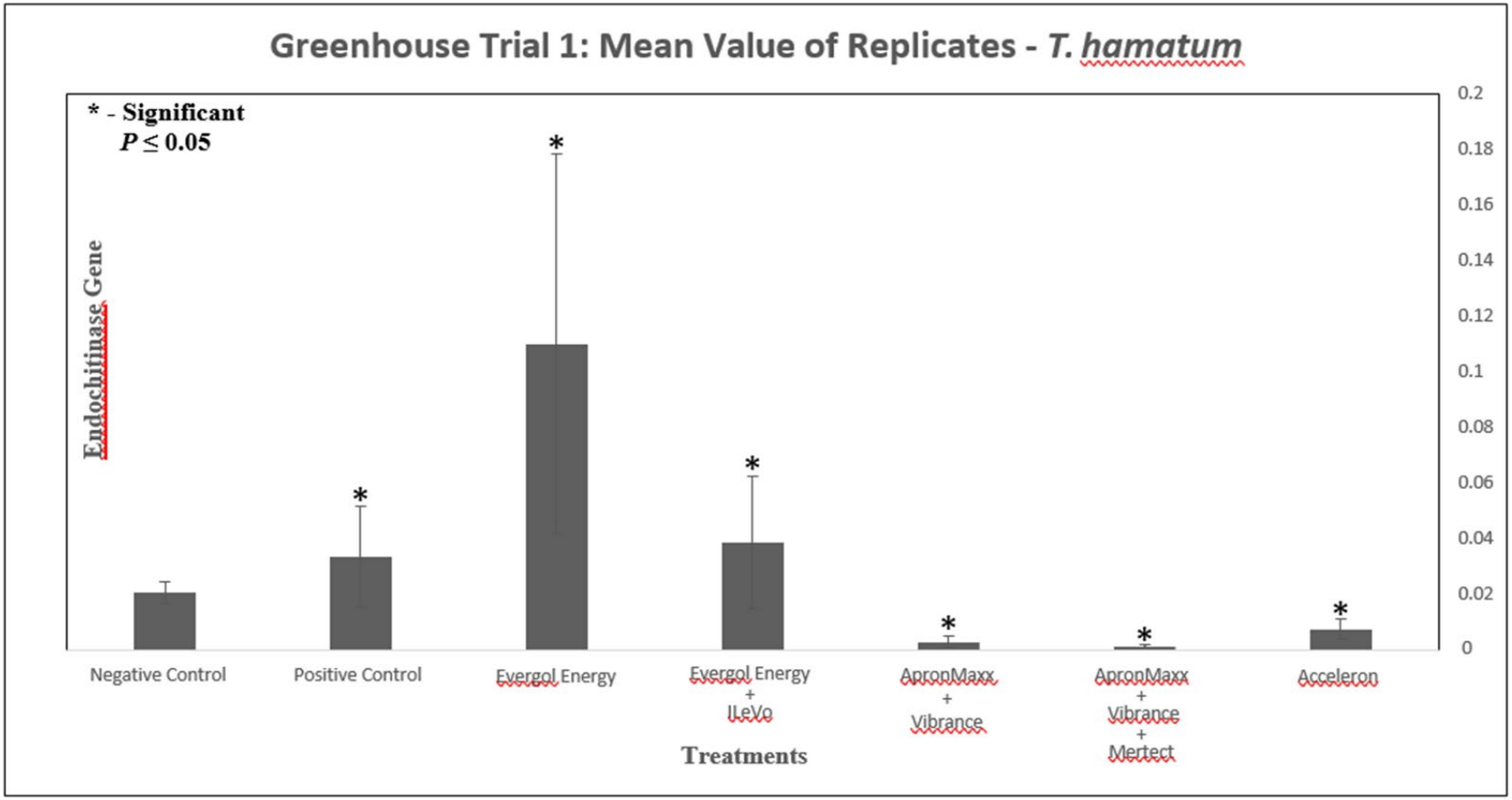


Figure 2.6 *Greenhouse experiment 2- T. hamatum*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2018.

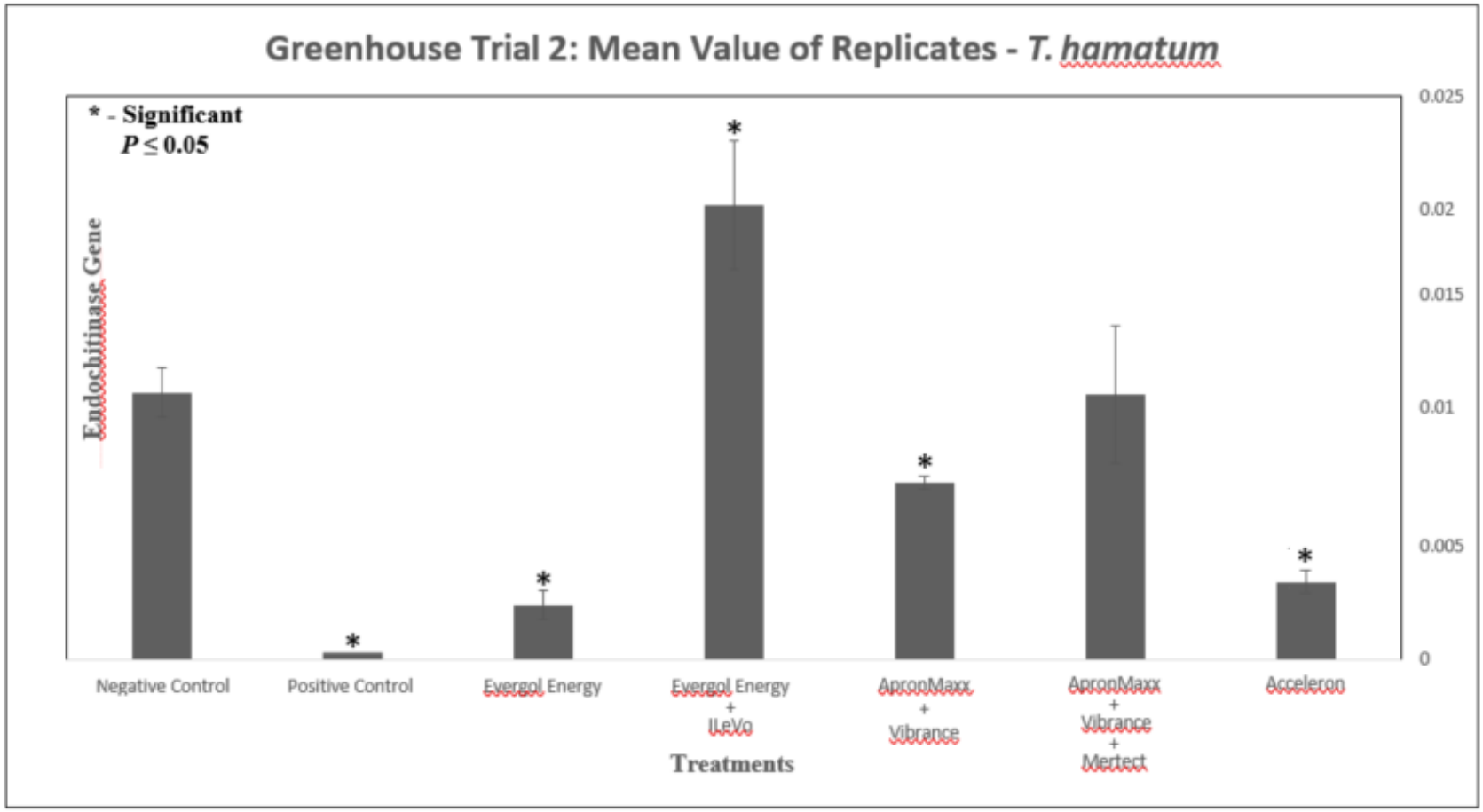


Figure 2.7 *Greenhouse experiment 3- T. hamatum*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2018.

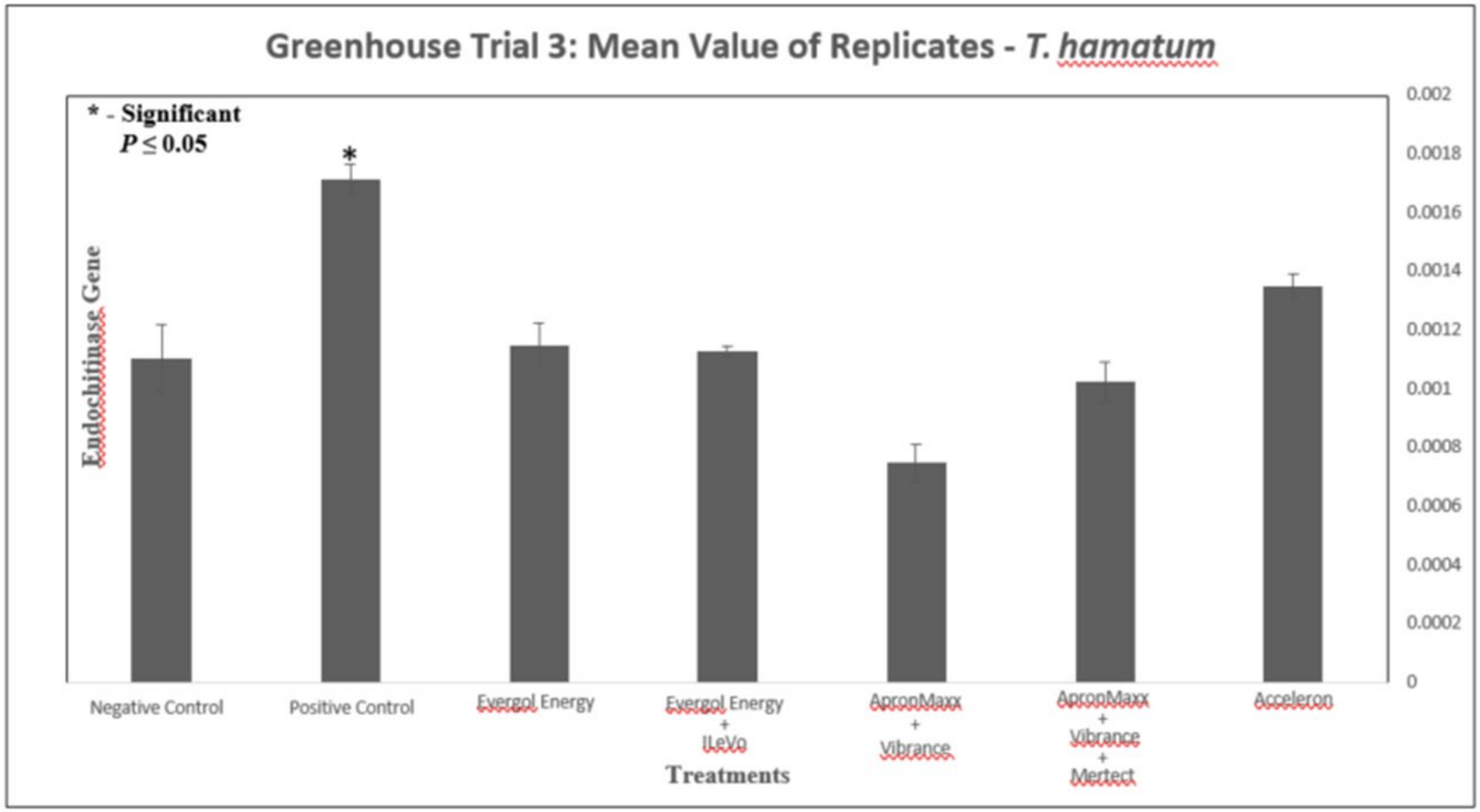


Figure 2.8 *Greenhouse experiment 1 - T. virens*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

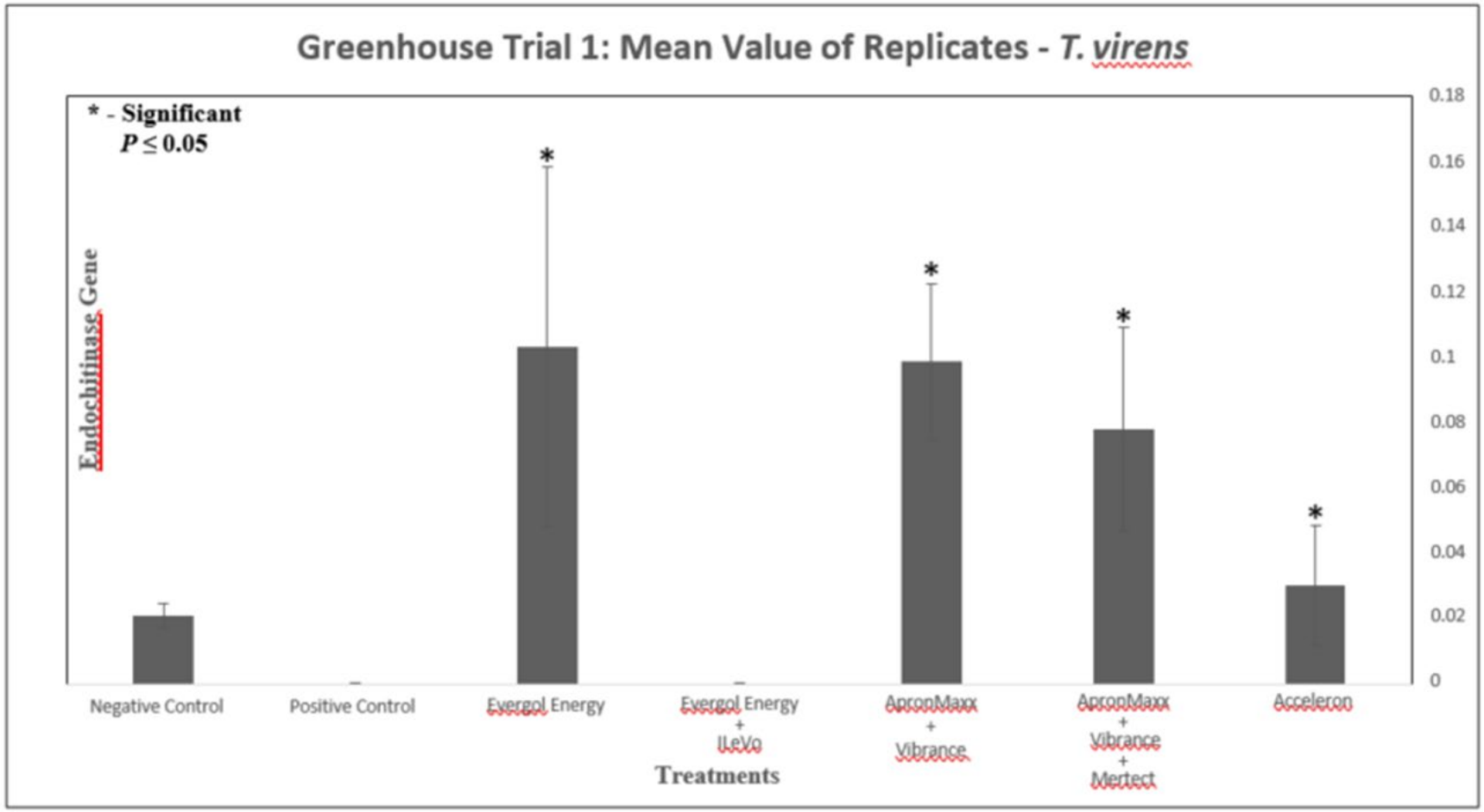


Figure 2.9 *Greenhouse experiment 2 - T. virens*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2018.

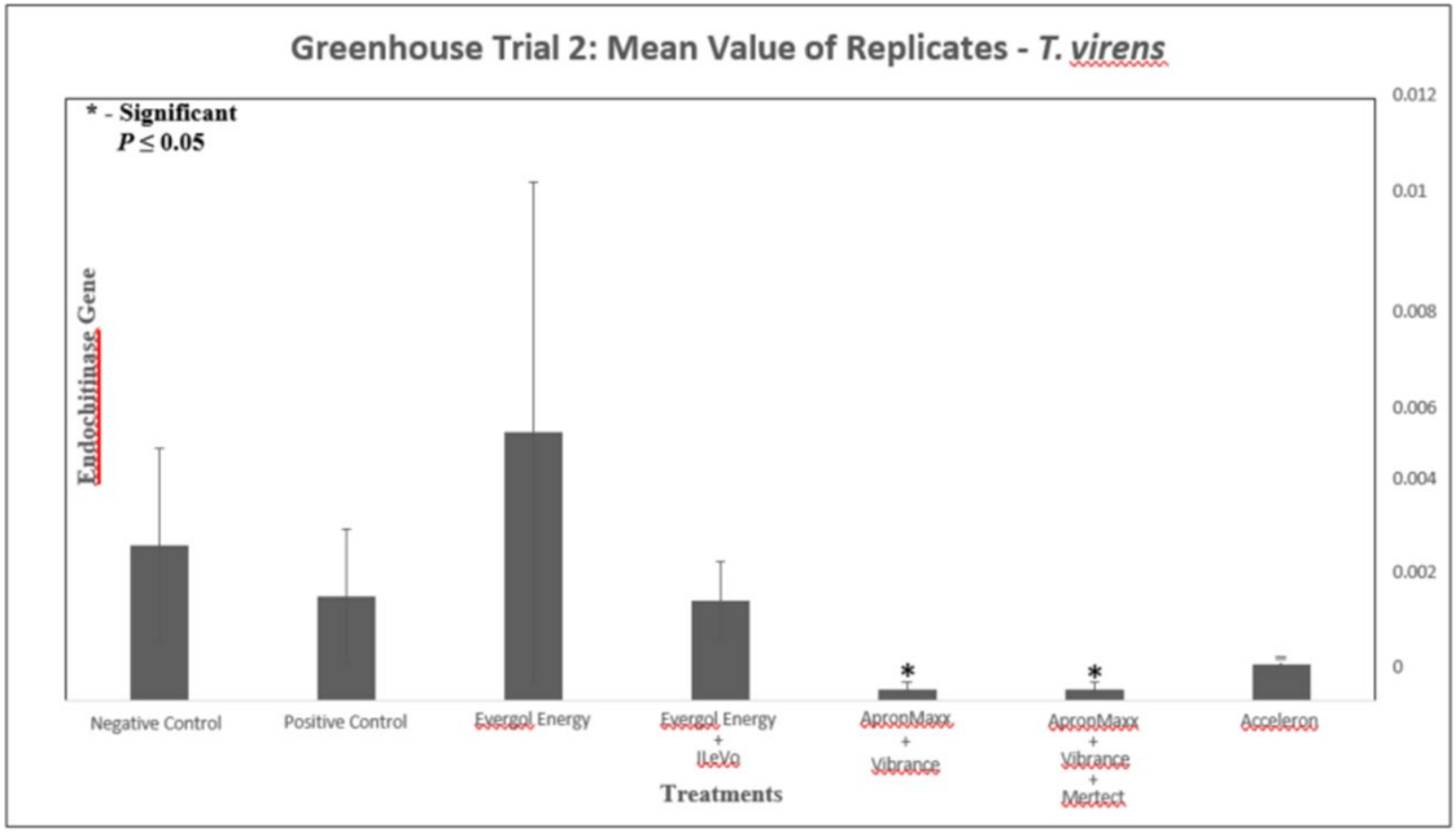


Figure 2.10 *Greenhouse experiment 3 - T. virens*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2018

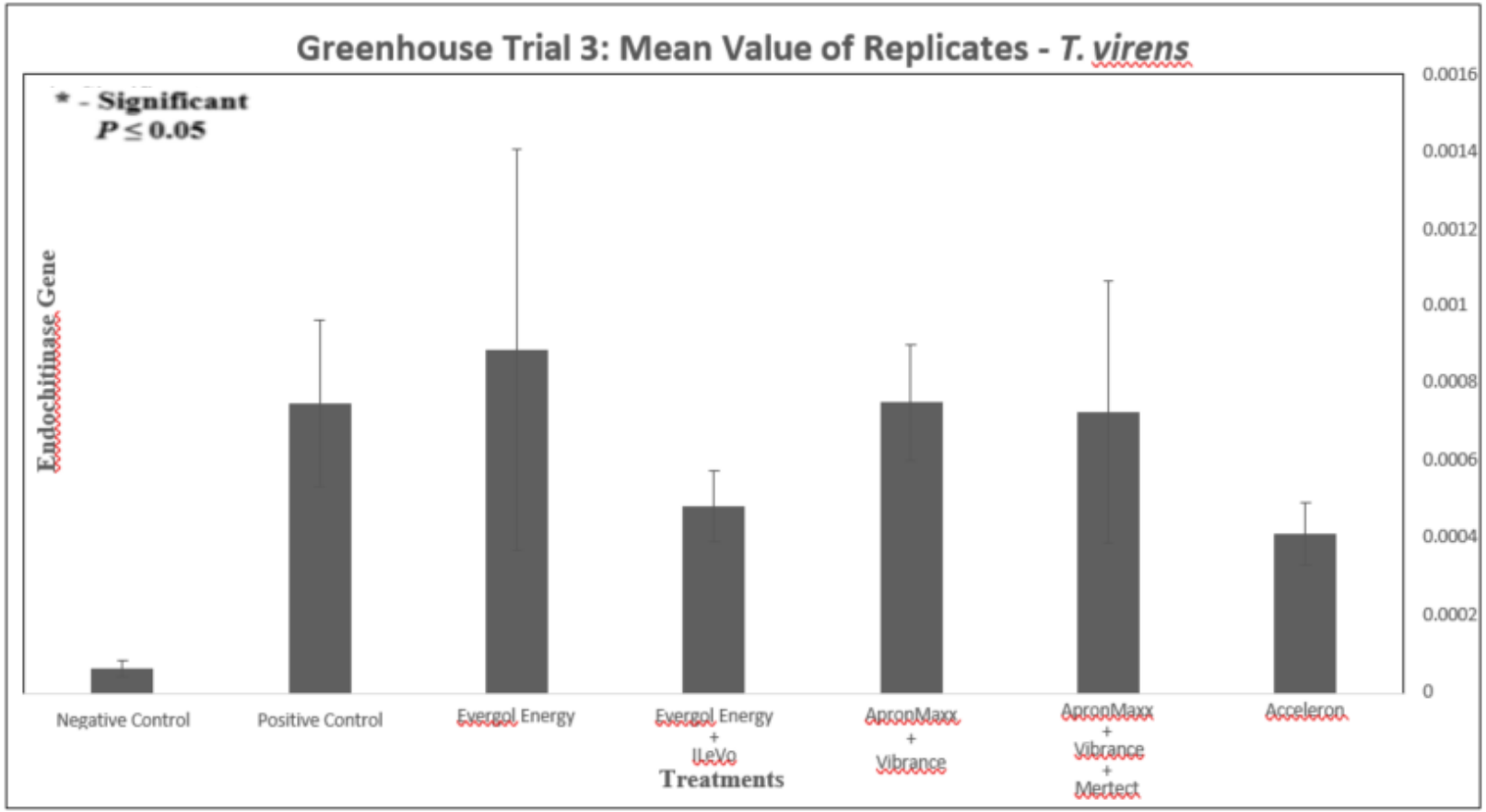


Figure 2.11 *Greenhouse experiment 1 - B. ochroleuca*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2017.

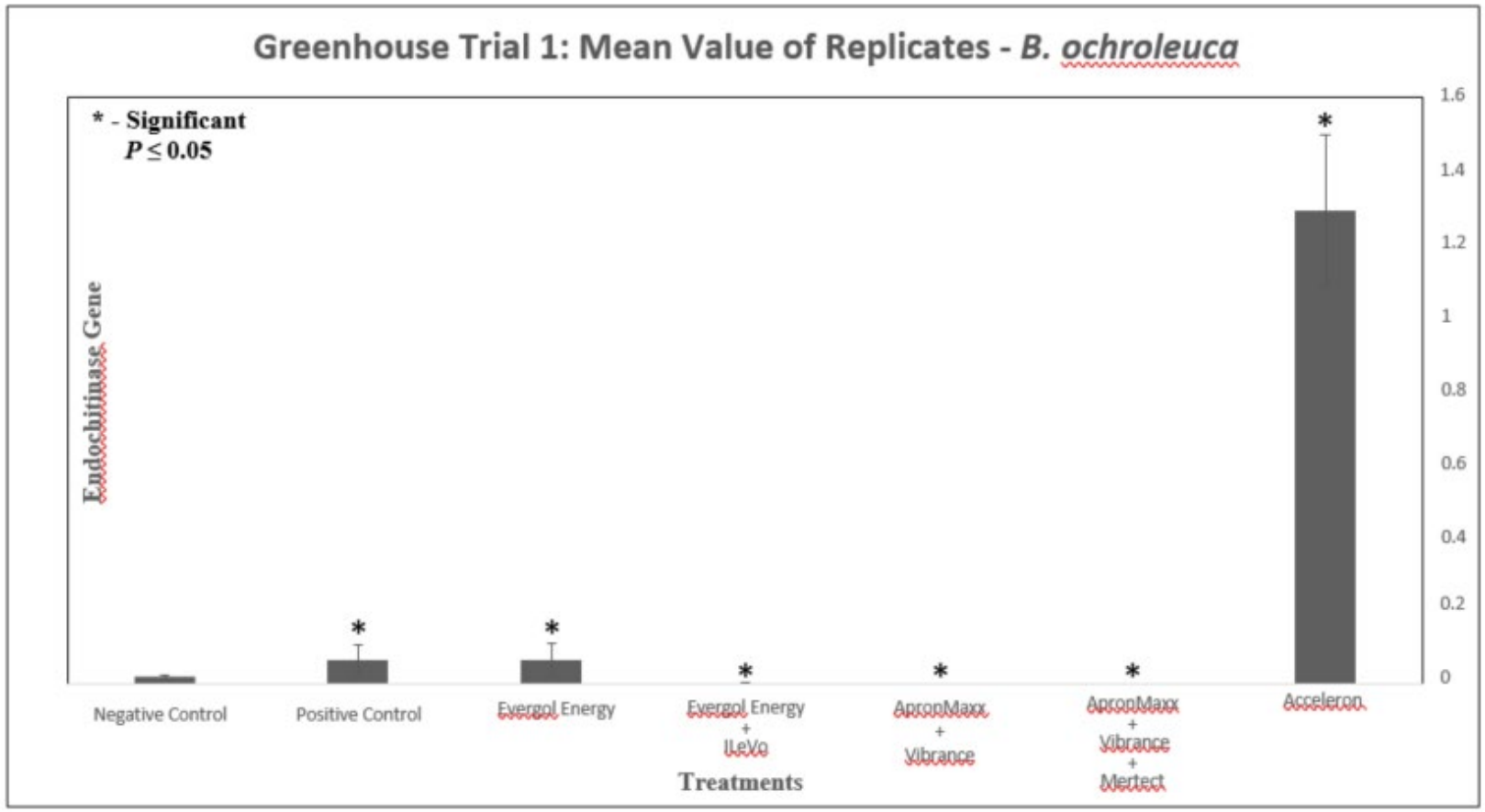


Figure 2.12 *Greenhouse experiment 2 - B. ochroleuca*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2018.

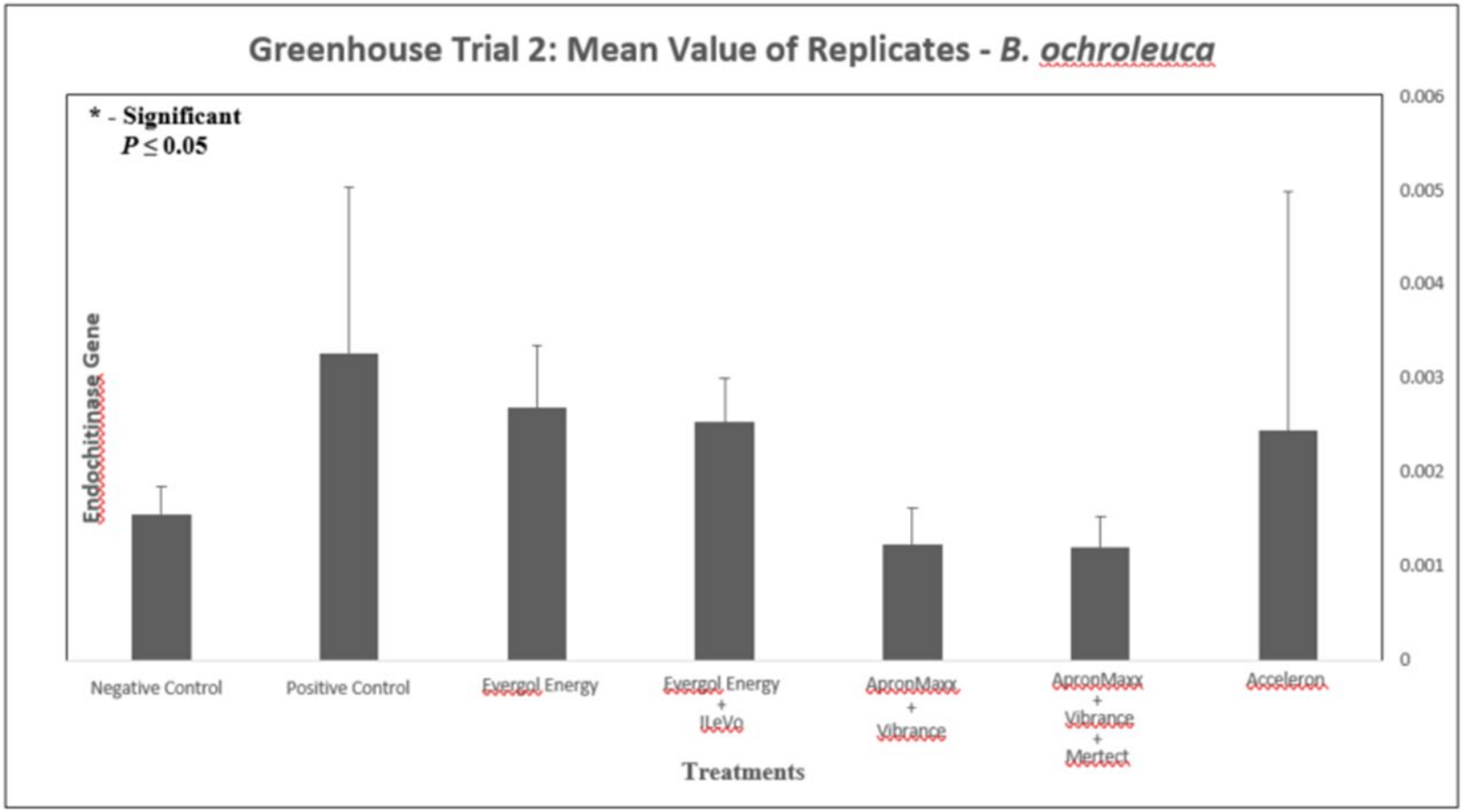
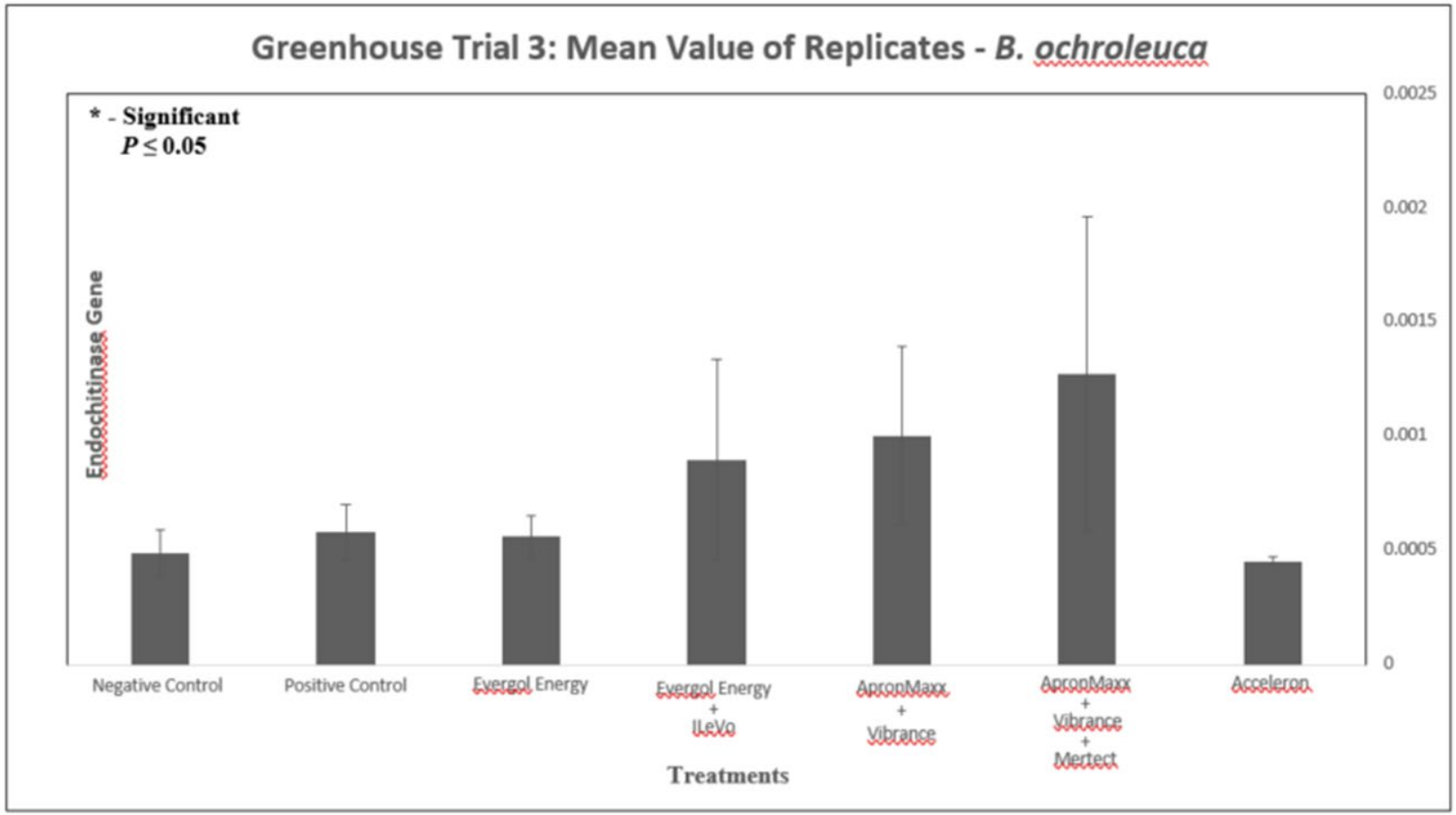


Figure 2.13 *Greenhouse experiment 3 - B. ochroleuca*: Data from qPCR analysis, effect of fungicide seed component on *Trichoderma* spp. ability to colonize roots when applied to soybean seeds in-furrow. The greenhouse experiment was performed at the University of Kentucky Agricultural Greenhouse, Lexington, KY in 2018.



CHAPTER 3

Evaluation of *Trichoderma* spp. as Potential Biocontrol Agents for the Management of Soybean Seedling Disease Caused by *Rhizoctonia solani*

Abstract

Trichoderma is a well-characterized fungal genus consisting of soil-borne ascomycetes, which are found in almost every geographical niche of the world. The fungal organisms are especially intriguing to plant pathologists due to their unique chemistry and the possibility of utilizing the molecules as novel modes of action for managing disease. This research evaluated different isolates of *Trichoderma* spp. for biocontrol of Rhizoctonia root rot (caused by *Rhizoctonia solani*) of soybean in field trials. The results of this research indicated that conidial suspensions of *T. harzianum*, *T. hamatum,* or *T. virens* applied in-furrow resulted in lower plant emergence when compared to the untreated check. Additionally, the application of *Trichoderma* spp. isolates did not result in any measurable plant growth effects. The results of this research did not show any positive effects for the control of Rhizoctonia root rot or soybean plant growth promotion by the *Trichoderma* isolates tested. However, additional research may be warranted to investigate the effect of the *Trichoderma* spp. on additional soybean cultivars and in different environmental conditions.

Introduction

Rhizoctonia solani Kühn (syn. *Thanetephorus cucumeris* (Frank) Donk.) can cause a seedling disease and root rot of soybean (*Glycine max* (L.) Merrill). The most common preemergence symptom caused by *R. solani* is seed and seedling rot, while post-emergence symptoms of Rhizoctonia seedling blight occur before the emergence of the first trifoliolate leaf develops (Yang and Hartman, 2015). Symptoms of infected plants are sunken reddish-brown lesions that girdle the entire root and sometimes the hypocotyl (Yang and Hartman, 2015). Seedling diseases of soybean, which includes those caused by species of *Rhizoctonia*, *Pythium*, *Fusarium*, and *Phomopsis*, caused an estimated soybean yield loss of nearly 6.6 billion kg from 2010 to 2014 in the United States and Ontario, Canada (Allen et al., 2017). In small plot field research trials in Iowa, Tachibana et al. (1971) reported soybean yield reductions caused by *R. solani* to be as great as 48%.

The best available methods for controlling *R. solani* integrate forms of chemical control, host-resistance, rotation with non-host crops, and tilling the soil to reduce pathogen inoculum levels (Sharon et al., 1992). Management of Rhizoctonia seedling disease of soybean can be achieved by using fungicide seed treatments from different chemistry classes, which include the quinone outside inhibitors (QoI), succinate dehydrogenase inhibitors (SDHI), demethylation inhibitors (DMI), and phenylpyrroles (PP) (Mueller et al., 2013). Unfortunately, several active ingredients effective against *R. solani* rely on a single mode of action, causing increased risk for selection of less-sensitive or resistant isolates (Mueller et al., 2013). To date, only *R. solani* AG- 1 populations have been reported to exhibit resistance to the QoI fungicide

class (Olaya et al., 2012).

Anastomosis groups (AG) of *R. solani* that have been characterized as causing seedling diseases of soybean in the Midwestern United States include AG-2-2, AG-4, and AG-5 (Ajayi- Oyetunde and Bradley, 2016; Liu and Sinclair, 1991; Muyolo et al., 1993; Nelson et al., 1996). Commercial soybean cultivars with complete resistance to *R. solani* are unavailable (Bradley, 2002). Nonetheless, sources linked to moderate genetic resistance have been identified in accessions subject to greenhouse and field screening (Muyolo et al., 1993; Zhao et al., 2005).

Although options are available for management of Rhizoctonia seedling disease and root rot of soybean, these practices may not provide complete control. Fungicide seed treatments may provide protection for a few weeks after planting, but do not provide season-long control (Dorrance et al., 2003). In addition, reduced sensitivity to fungicides may occur in *R. solani* over time (Ajayi-Oyetunde and Bradley, 2016; Hewitt, 1998; Mueller et al. 2013). Some soybean accessions have been identified with partial resistance to *R. solani*, but under severe disease pressure, losses still may occur (Muyolo et al., 1993; Zhao et al., 2005). Crop rotation may not be effective since *R. solani* has a wide host range and can survive in the soil for up to 4 years as sclerotia (Sumner, 1996). Additionally, there is evidence that epidemics of *R. solani* in field crops are positively correlated with inoculum density (Gilligan et al., 1996).

One option to potentially overcome chemical control issues attributed to diversity in *R. solani* populations would be to use biological control agents (BCAs) as a form of disease control. Microorganisms located in the rhizosphere of the soil can be

utilized as bioactive fungicides to limit damage caused by pathogenic organisms. One of the most extensively studied and well characterized BCAs found in the rhizosphere is the fungal genus, *Trichoderma* (Harman, 2004; Howell, 2003).

The success of using *Trichoderma* as a bio-fungicide is well-documented throughout literature and in the form of commercialized products, with over 60% of registered bio-active products containing a species of *Trichoderma* (Abbas et al., 2017). The ability of *Trichoderma* spp. to control various pathogens has been well-characterized in several plant families, including *Fabaceae* (Kobori et al., 2015; Larkin, 2016; Mayo et al., 2015). Strains of *Trichoderma* used as a BCA can control pathogens through various mechanisms including: 1) direct competition for space or nutrients required for pathogen to survive, 2) mycoparasitism through the production of cell wall degrading enzymes, 3) secretion of non-volatile and volatile antimicrobial compounds that directly inhibit the growth of pathogenic fungi, 4) plant growth promotion by the presence of hormones indole-3-acetic acid or gibberellic acid, and 5) stimulation of plant defense genes, either jasmonic acid or salicylic acid associated pathways (Harman, 2011; Howell, 2003; Kubicek et al., 1998).

Biological control agents are a promising field of research, but there are major limitations to consider when developing an applied biological fungicide for management of disease. When using any microbe-based fungicide, the applicator is responsible for understanding the underlying biology relative to the organism and must be aware of specific environmental factors impacting growth and persistence (Verma et al., 2007; Woo et al., 2006). For researchers developing formulations, intensive knowledge of fungal anatomy is important for developing an effective fungicide.

Trichoderma spp. can produce three types of anatomical structures: mycelia, conidia, and chlamydospores (Verma et al., 2007). Depending on the intended application, researchers can improve the effectiveness of a bio-fungicide by selecting the appropriate fungal structure with the highest probability for survival. (Harman, 2011; Woo et al., 2006).

In previous studies, *Trichoderma* has been shown to be a potential option for the control of root pathogens in *Fabaceae* (Mayo et al., 2015; Valenciano et al., 2006). However, there is a lack of research assessing the ability of *Trichoderma* spp. for controlling root pathogens in a field setting. To test the ability of *Trichoderma* spp. to manage Rhizoctonia seedling blight of soybean, trials were conducted with three isolates: *T. virens*, *T. hamatum* 1, and *T. hamatum* 2. The efficacy of the biocontrol agents for managing *R. solani* was compared with an untreated check and an inoculated untreated check. Isolates of *Trichoderma* spp. were obtained from soybean roots in fields across multiple locations in Illinois (Fakhoury et al., unpublished). In a preliminary study, an *in vitro* fungicide sensitivity analysis was performed prior to field testing, and isolates exhibiting the lowest sensitivity to sedaxane and metalaxyl were selected for field screening (Lacey et al., 2017).

Materials and Methods

Field Preparation and Experimental Design. Field trials were conducted on two different fields (Catlett Tract and Luttrell Tract) at the University of Kentucky Research and Education Center (UKREC) near Princeton, KY in 2017. The Catlett Tract had been cropped to soybean the previous year, and the Luttrell Tract had been continuously managed as tall fescue (*Festuca arundinacea* Schreb.) for several years.

To screen for the efficacy of BCAs in controlling disease, a 'hill-plot' design was utilized. The entire 'hill plot' area had a width of 5 m and a length of by 10 m. Row spacing for each individual plot within this area was 76.2 cm. Each plot was dug using a post-hole digger with a diameter of 15.9 cm. The inoculum was added at a depth of 5.1 cm and covered with a small amount of soil. A total of 10 seeds were placed as pairs within each plot and planted at a depth of 3.8 cm.

The Catlett Tract location was planted on 7 July 2017 and the Luttrell Tract location was planted on 10 July 2017. Prior to planting, glyphosate herbicide (Cornerstone Plus; Winfield Solutions, LLC, St. Paul, MN) was applied at a rate of 1.7 kg a.e./ha to control actively-growing vegetation. In addition, S-metolachlor herbicide (Dual II Magnum, Syngenta Crop Protection, Greensboro, NC) was applied at a rate of 1.9 kg a.i./ha prior to planting for residual weed control, and glyphosate was applied as a post-emergence herbicide at a rate of 1.1 kg a.e./ha during the V6 growth stage (Fehr et al., 1971). The soybean cultivar 'Armor 4744' (Armor Seed LLC., Jonesboro, AR) was planted.

Plots were set up in a randomized complete block design and contained a total of 5 treatments, replicated 6 times. Research tract dimensions were 10 m long and 3 m wide with individual plots spaced 76.2 cm apart. Treatments consisted of a non-treated control, a non-treated control inoculated with *Rhizoctonia solani* AG 2-2, and inoculated plots treated with three isolates of *Trichoderma* spp.: *T. hamatum* 1, *T. hamatum* 2, and *T. virens*.

Inoculum Preparation. The *R. solani* inoculum was prepared by placing a 5 mm diameter mycelial plug on potato dextrose agar. Cultures were grown for two weeks at

temperatures ranging from 20-25°C, relative humidity ranging from 80-90%, and under soft-white fluorescent lighting (TL 841 32J/s, Phillips, Andover, Massachusetts) set to a 12-hour photoperiod. The second step of *R. solani* inoculum production was to prepare a sterilized substrate for growth.

For this study, grain sorghum (*Sorghum bicolor* (L.) Moench) seeds were selected as the substrate for *R. solani* growth. Grain sorghum seeds were soaked in tap water for 24 hours, drained, and 1.4 kg was added to a transparent autoclavable bag. The grain sorghum seeds were then autoclaved for 1-hour each on two consecutive days. In sterile conditions, a two-week old culture of *R. solani* was added to each autoclave bag. Once visible mycelial growth was observed, the bags were shaken daily for two weeks to ensure thorough colonization of the grain sorghum seeds. After the grain sorghum seeds were well-colonized, the inoculum was placed into an industrial dryer. After drying, the inoculum was stored in paper bags at 4°C until used.

The production of the *Trichoderma* inoculum began by placing a 5 mm diameter mycelial plug cultured from each isolate on potato dextrose agar. Cultured plates were grown for two weeks in a growth room at a constant temperature of 23°C, relative humidity at 90% and under continuous soft-white fluorescent lighting (TL 841 32J/s, Phillips, Andover, MS). After two weeks of growth, conidial suspensions of each isolate were prepared by washing conidia using 15 ml sterilized water and a sterilized bent-glass rod. Contents of the Petri plate were passed through a 200 µm sieve, and sterile water was added to bring the total volume to 500 ml.

Conidial concentrations were enumerated using a hemocytometer (Bright-Line

Hemocytometer, Hausser Scientific, Horsham, PA) on the 40X objective on a compound light microscope (Zeiss Axioskop, ZEISS International, Oberkochen, Germany). Conidial suspensions were brought to a final concentration of 1 X 10^9 conidia/ml and applied to soybean plots in-furrow at a rate of 100 ml per plot.

Weather. Weather conditions were recorded for one week prior to the application and one week after the application. Additional in-depth hourly forecasts were recorded on the day of application. Weather data from Princeton, KY was compiled using the University of Kentucky's climate data website, wwwagwx.ca.uky.edu/cgi-bin/ky_clim_data_www.pl. (Tables 3.1-3.4)

Biological control assessment. Initial assessments for determining Rhizoctonia seedling disease suppression were evaluated for each plot by recording soybean plant stand at 7, 14, 21, and 28 days after planting. Plant stands for each plot were calculated as: [(number of seeds per plot/number of plants emerged per plot) X 100]. Vigor ratings were recorded at 14 and 28 days after planting using a hand-held device that measures the normalized difference vegetation index (NDVI) of an area. (GreenSeeker, Trimble, Sunnyvale, CA). After 28 days of growth, a 1 m diameter circle was measured from the center of each plot and roots were carefully dug to keep entire plants intact. Soil was washed from the roots with water using a garden hose. Fresh weight values for above and below ground biomass were recorded using a top-loading balance (VWR- 10204-992, Avantor, Phillipsburg, NJ). Above ground weight values were denoted as any plant tissue above the soil line. Whereas, below ground weight values were recorded as any plant tissue collected below the soil line. After recording fresh weight values, the plant tissue was placed into an industrial dryer for

two weeks before determining dry weight values.

Statistical Analysis. Data were analyzed using the general linear model procedure (PROC GLM) in SAS (Version 9.4; SAS Institute Inc., Cary, NC). Means were compared using Fisher's protected least significant difference test ($\alpha = 0.10$). Treatments and location were considered a fixed effect, while replication was considered a random effect.

Results

Field Trial conducted at the Catlett tract – University of Kentucky Research and Education Center in Princeton, KY. At the Catlett tract, significant ($P \leq 0.10$) treatment effects were observed for plant stand evaluations at 14, 21, and 28 days after planting, for vigor at 14 and 28 days after planting, and for root length (Tables 2.5 and 2.6). Since the inoculated untreated check is the most logical comparison to the *Trichoderma spp.* treatments, only *Trichoderma* spp. treatments that were significantly different than the inoculated untreated check will be discussed here. Significantly decreased stands, compared to the inoculated untreated check, were observed for *T. hamatum* 1 – treated plots at 14, 21, and 28 days after planting, and for *T. hamatum* 2 – treated plots at 14 days after planting. Inoculation with *R. solani* did not appear to affect any of the measured variables at this location since the inoculated untreated check did not significantly differ from the non-inoculated untreated check.

Field Trial conducted at the Luttrell tract – University of Kentucky Research and Education Center in Princeton, KY. At the Luttrell tract, significant ($P \leq 0.10$) treatment effects were observed for stand counts collected at 7, 14, 21, and 28 days after planting, and for vigor at 14 and 28 days after planting (Table 7 and Table 8). Since the

inoculated untreated check is the most logical comparison to the *Trichoderma spp*. treatments, only treatments that were significantly different than the inoculated untreated check will be discussed here. Significantly decreased stands, compared to the inoculated untreated check, were observed for *T. hamatum* 1 treated plots at 14 and 21 days after planting, and for *T. hamatum* 2 treated plots at 14 days after planting. Inoculation with *R. solani* did appear to influence stand at 7, 14, 21, and 28 days after planting, and on vigor at 14 and 28 days after planting, as the inoculated untreated check had significantly lower stands and vigor than the non-inoculated untreated check for these variables.

Discussion

Several factors can impact the persistence of bio-fungicides in an environment: volatilization, plant uptake, biotic degradation, abiotic degradation, solubility-based movement of water, and desorption to plant or soil surfaces (Mayo et al., 2015; Valenciano et al., 2006). In both trials, one environmental factor could have led to the decreased efficacy in disease control: biotic degradation. The lack of significant differences between the untreated control and the inoculated control at the Catlett Tract could be attributed to the rhizosphere community present within the soil. Prior years of production at the Catlett Tract were managed as continuous soybean, and the Luttrell Tract had been managed as tall fescue for several years. The lack of difference observed between the untreated control and inoculated untreated control at the Catlett Tract could have been caused by a well-developed rhizosphere community existing in the soil from previous soybean production years. In contrast to this assumption, the difference observed between the two control treatments at the Luttrell Tract could be attributed to a rhizosphere community that was developed specific to the previous

cropping system of tall fescue.

Notably, the performance of the *Trichoderma* treatments at both locations was extremely poor, especially at the Luttrell Tract. Soybean plots inoculated with two *Trichoderma* spp. isolates at the Catlett Tract had stand counts which were significantly lower when compared with the inoculated control, *T. hamatum* 1 at 14, 21, and 28 days and *T. hamatum* 2 at 14 days. The lower stand counts observed for the soybean plots inoculated with these isolates could be caused by the specific type of isolate used. Depending on the plant and cultivar, it is possible that some strains of *Trichoderma* spp. could exhibit pathogenic capabilities or stimulate the virulence of an existing pathogen. Prior research performed by Aly et al. (2000) observed *Trichoderma* spp. strains causing a reduction on stand counts in cotton when applied as a soil-amendment for controlling *Pythium*. An additional study in cotton observed *Trichoderma* spp. isolates stimulating the pathogenicity of *Macrophomina phaseolina* (Omar, 2005). For this study, it is possible that the presence of the *Trichoderma* could be stimulating the activity of *R. solani* and resulting in the reduced plant stand.

One of the most important aspects to consider for developing a successful BCA is the formulation. Typically, commercial forms of *Trichoderma* spp. are marketed as either conidial suspensions or substrate-based products. Surprisingly, Woo et al. (2014) discovered that only 6.2% of *Trichoderma* spp. commercialized products are applied as a substrate-based soil- amendment. For this study, a lack of substrate could be the limiting factor attributing to the reduced efficacy in controlling disease and subsequent loss of stand. Without the necessary substrate for growth, it is possible that the fungi simply utilized the seed as a carbon source for vegetative and reproductive growth.

Bio-fungicide products with various formulations are emerging as a viable form of disease management around the world. This study has provided supporting evidence that the type of isolate, plant variety, or formulation used for a BCA are important aspects that can impact the efficacy of the product. Future research using these isolates could test whether a solid substrate such as oats, rice, or sorghum would minimize the stand reductions observed in the BCA treatments. Additional research could determine if isolates of *Trichoderma* are pathogenic to certain cultivars of soybean. As the market-share of bio-fungicides increases, an important aspect of development must include thoroughly characterizing any deleterious effects caused by a specific type of formulation on different plant cultivars.

Table 3.1 Weather observations for a two-week period, one week prior to the of application of the *Trichoderma* bio-active fungicides and one week following the treatments. The highlighted region in red denotes the day of application for the fungicides. Treatments were applied on 7st, July 2017 at the Catlett Tract located on the University of Kentucky Research and Education Center, Princeton, KY. Climate data was obtained from the University of Kentucky weather database (www.agwx.ca.uky.edu/cgi-bin/ky_clim_data_www.pl).

July 2017	Temperature (°C)		Dew Point (°C)		Humidity (%)		Rain Total (cm)	Weather Events	Soil Temperature (Grass)	
	High	Low	High	Low	High	Low			Low	High
1	30.0	20.6	23.9	20.6	100	63	1.9	Rain	24.5	25.0
2	31.1	21.1	26.1	20.6	100	53	0	Fog	22.8	25.6
3	30.0	21.7	25.0	21.7	100	65	0	Fog + Rain	23.4	25.0
4	29.5	22.2	23.4	21.7	100	63	0.5	Rain	23.4	25.6
5	27.8	22.2	23.9	22.2	100	77	0.0	Rain	23.9	25.0
6	29.5	22.2	24.5	22.2	100	71	0.3	Rain	23.3	24.5
7	31.7	21.1	25.0	20.6	100	59	0.5	Fog + Rain	23.3	25.6
8	31.1	22.2	23.9	16.7	100	42	1.5	Rain	25.0	25.6
9	32.3	19.5	22.8	18.9	100	52	0.0	-	25.6	26.1
10	32.3	22.2	22.8	21.1	93	52	0.0	-	25.6	26.1
11	32.3	22.8	24.5	21.7	92	56	0.0	-	26.1	26.1
12	33.4	22.2	25.0	21.1	92	49	0.0	-	23.3	25.6
13	33.9	24.5	25.6	20.6	93	54	0.0	-	26.1	26.1
14	31.7	22.2	25.0	21.1	95	61	0.0	-	24.5	25.6

Table 3.2 Weather observations on the day of application for *Trichoderma* based bio-active fungicide. The highlighted region in red denotes the time when the application of bio-fungicides occurred. Treatments were applied on 7th, July 2017 at the Catlett Tract located on the University of Kentucky Research and Education Center, Princeton, KY. Climate data was obtained from the University of Kentucky weather database (www.agwx.ca.uky.edu/cgi-bin/ky_clim_data_www.pl).

Time (CDT)	Temperature (°C)	Dew Point (°C)	Humidity (%)	Rain (cm)
9:15 AM	24.8	24.9	100	N/A
9:35 AM	26.0	25.0	94	N/A
9:55 AM	27.0	23.9	83	N/A
10:15 AM	29.0	23.9	74	N/A
10:35 AM	29.4	23.9	72	N/A
10:55 AM	29.9	23.8	70	N/A
11:15 AM	30.2	23.8	68	N/A
11:35 AM	30.5	23.3	65	N/A
11:55 PM	30.8	23.3	65	N/A
12:15 PM	30.9	23.4	64	N/A
12:35 PM	31.2	23.5	64	N/A
12:55 PM	31.4	23.5	63	N/A
1:15 PM	31.7	23.5	62	N/A
1:35 PM	31.9	23.5	61	N/A
1:55 PM	31.7	23.0	60	N/A
2:15 PM	31.9	23.1	59	N/A

Table 3.3 Weather observations on the day of application for *Trichoderma* based bio-active fungicide. The highlighted region in red denotes the time when the application of fungicides occurred. Treatments were applied on 10th, July 2017 at the Luttrell Tract located on the University of Kentucky Research and Education Center, Princeton, KY. Climate data was obtained from the University of Kentucky weather database (www.agwx.ca.uky.edu/cgi-bin/ky_clim_data_www.pl).

July 2017	Temperature (°C)		Dew Point (°C)		Humidity (%)		Rain Total (cm)	Weather Events	Soil Temperature (Grass)	
	High	Low	High	Low	High	Low			Low	High
4	22.2	29.5	21.7	23.3	63	100	0.5	Rain	23.3	25.6
5	22.2	27.8	22.2	23.9	77	100	0.0	Rain	23.9	25.0
6	22.2	29.5	22.2	24.5	71	100	0.3	Rain	23.3	24.5
7	21.1	31.7	20.6	25.0	59	100	0.5	Fog	23.3	25.6
8	22.2	31.1	16.7	23.9	42	100	1.5	Rain	25.0	25.6
9	19.5	32.3	18.9	22.8	52	100	0.00	-	25.6	26.1
10	22.2	32.3	21.1	22.8	52	93	0.00	-	25.6	26.1
11	22.8	32.3	21.7	24.5	56	92	0.00	-	26.1	26.1
12	22.2	33.4	21.1	25.0	49	92	0.00	-	23.3	25.6
13	24.5	33.9	20.6	25.6	54	93	0.00	-	26.1	26.1
14	22.2	31.7	21.1	25.0	61	95	0.00	-	24.5	25.6
15	21.1	31.1	19.5	24.5	52	96	0.00	-	23.9	26.1
16	17.8	31.1	18.3	21.7	47	100	0.00	-	23.3	25.6
17	19.4	32.8	19.4	22.8	50	100	0.00	-	23.3	25.6

Table 3.4 Weather observations on the day of application for *Trichoderma* based bio-active fungicide. The highlighted region in red denotes the time when the application of fungicides occurred. Treatments were applied on 10th, July 2017 at the Luttrell Tract located on the University of Kentucky Research and Education Center, Princeton, KY. Climate data was obtained from the University of Kentucky weather database (www.agwx.ca.uky.edu/cgi-bin/ky_clim_data_www.pl).

Time (CDT)	Temperature (°C)	Dew Point (°C)	Humidity (%)	Rain (cm)
9:15AM	28.6	22.1	68	N/A
9:35AM	29	22.5	68	N/A
9:55AM	29.5	22.5	66	N/A
10:15AM	29.9	22.6	65	N/A
10:35AM	30.2	22.4	63	N/A
10:55AM	30.6	22.3	62	N/A
11:15AM	30.6	22.2	61	N/A
11:35AM	30.6	22	60	N/A
11:55AM	31	22.3	60	N/A
12:15 PM	31	21.3	56	N/A
12:35 PM	31.5	21.4	55	N/A
12:55 PM	31.7	22.1	57	N/A
1:15 PM	31.8	22.3	57	N/A
1:35 PM	31.8	22.5	56	N/A
1:55 PM	31.8	22.6	56	N/A
2:15 PM	31.9	21.8	55	N/A

Table 3.5 Effect of *Trichoderma* spp. applied to soybean seeds in-furrow for controlling *Rhizoctonia solani*. Efficacy of BCA's were determined by percent stand and vigor at the Catlett Tract of the University of Kentucky Research and Education Center, Princeton, KY in 2017.

Treatment	*R. solani* Inoculated	Stand (%) Day 7	Stand (%) Day 14	Stand (%) Day 21	Stand (%) Day 28	Vigor (%) Day 28
Non-inoculated Untreated Check	No	7.2	7.8	7.8	7.8	45
Untreated Check	Yes	7.5	8.0	7.7	7.7	43
T. hamatum 1	Yes	5.2	5.4	5.2	5.2	37
T. hamatum 2	Yes	6.1	6.4	6.3	6.3	43
T. virens	Yes	6.5	6.8	6.8	6.8	44
P > F		**0.1127**	**0.0624**	**0.0909**	**0.0909**	**0.7195**
LSD 0.10 [z]		**NS** [y]	**1.6**	**1.6**	**1.7**	**NS** [y]

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

Table 3.6 Effect of *Trichoderma* spp. applied to soybean seeds in-furrow for controlling *Rhizoctonia solani*. Potential plant health benefits of the BCA's were determined plant height, root length, and plant biomass at the Catlett Tract of the University of Kentucky Research and Education Center, Princeton, KY in 2017.

Treatment	*R. solani* Inoculated	Top Height (cm)	Top Weight Dry (g)	Root Length (cm)	Root Weight Dry (g)
Non-inoculated Untreated Check	No	14.0	8.6	8.1	1.5
Untreated Check	Yes	14.2	7.9	7.0	1.1
T. hamatum 1	Yes	13.0	7.3	7.8	1.3
T. hamatum 2	Yes	14.0	9.5	6.8	1.5
T. virens	Yes	14.2	8.3	7.4	1.3
P > F		**0.1323**	**0.8436**	**0.0478**	**0.6599**
LSD 0.10 [z]		**NS** [y]	**NS** [y]	**1.3**	**NS** [y]

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

Table 3.7 Effect of *Trichoderma* spp. applied to soybean seeds in-furrow for controlling *Rhizoctonia solani*. Efficacy of BCA's were determined by percent stand counts and vigor at the Luttrell Tract of the University of Kentucky Research and Education Center, Princeton, KY in 2017.

Treatment	*R. solani* Inoculated	Stand (%) Day 7	Stand (%) Day 14	Stand (%) Day 21	Stand (%) Day 28	Vigor (%) Day 28
Non-inoculated Untreated Check	No	5.8	7.1	7	7	34
Untreated Check	Yes	2.1	3.5	3.1	3.1	18
T. hamatum 1	Yes	0.5	0.6	0.6	0.6	7
T. hamatum 2	Yes	0.8	1	1	1.6	13
T. virens	Yes	2	2.8	2.8	2.8	12
P > F		**0.0125**	**0.0045**	**0.0051**	**0.0349**	**0.0807**
LSD 0.10 [z]		**2.2**	**2.2**	**2.2**	**2.6**	**0.1**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

Table 3.8 Effect of *Trichoderma* spp. applied to soybean seeds in-furrow for controlling *Rhizoctonia solani*. Potential plant health benefits of the BCA's were determined plant height, root length, and plant biomass at the Luttrell Tract of the University of Kentucky Research and Education Center, Princeton, KY in 2017.

Treatment	*R. solani* Inoculated	Top Height (cm)	Top Weight Dry (g)	Root Length (cm)	Root Weight Dry (g)
Non-inoculated Untreated Check	No	27.5	5.9	17.8	0.9
Untreated Check	Yes	25.3	6.8	16.4	1.2
T. hamatum 1	Yes	25.7	8.5	19.1	1.4
T. hamatum 2	Yes	37.1	8.8	19.7	1.5
T. virens	Yes	45.0	8.3	21.0	1.4
P > F		**0.3834**	**0.5441**	**0.8274**	**0.6615**
LSD 0.10 [z]		**NS** [y]	**NS** [y]	**NS** [y]	**NS** [y]

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

CHAPTER 4

Evaluating *Trichoderma* spp. as biocontrol agents to control frogeye leaf spot in soybean

Abstract

Trichoderma is a well-characterized fungal genus consisting of soil-borne ascomycetes, which occur in almost every geographical niche of the world. *Trichoderma* spp. have been studied extensively for their use as plant disease biocontrol agents. The most important foliar disease of soybean (*Glycine max* (L.) Merr.) in western Kentucky is frogeye leaf spot (caused by *Cercospora sojina* Hara). Management of frogeye leaf spot has become more complicated due to the widespread occurrence of *C. sojina* strains resistant to quinone outside inhibitor (QoI) fungicides. The objective of this trial was to determine if *Trichoderma* spp. applied to the foliage of soybean would reduce frogeye leaf spot severity. Field trials were conducted at two fields at the University of Kentucky Research and Education Center near Princeton, KY in 2017. A foliar application of a *T. virens* conidial suspension significantly ($P \leq 0.10$) reduced frogeye leaf spot severity of soybean compared to a non-treated control in a field environment with low to moderate disease pressure, but not in a field environment with high disease pressure. At one location, the isolate increased yield when compared to a standard foliar fungicide product containing difenoconazole + azoxystrobin (Quadris Top SBX; Syngenta Crop Protection, Greensboro, NC). This research indicated that *Trichoderma* spp. may be a potential biocontrol management option for frogeye leaf spot of soybean. Future research is warranted to better understand the potential efficacy in additional environments and the mechanism(s) of action used by the *Trichoderma* isolates evaluated in these experiments.

Introduction

Frogeye leaf spot (FLS) of soybean, is a significant foliar disease causing substantial yield loss in the United States (Allen et al., 2017). The pathogen responsible, *Cercospora sojina*, was first identified in 1915 in a Japanese soybean field (Hara, 1915). Several years later in 1924, *sojina* was observed causing frogeye leaf spot on soybean in the United States (Lehman, 1928). Symptoms can appear on the leaves, stems, or pods. Lesions begin as small, light brown circular spots exhibiting a light colored to tan center with a dark brown to purple outer margin. If the affected area on the leaf surface is greater than 50%, the leaves will begin to blight and wither (Wise and Newman, 2015). Warm temperatures and high humidity promote the disease development disease within the crop canopy (Wise and Newman, 2015). Additionally, high disease severity can reduce seed oil concentration by 2% to 7% and seed protein concentration by 4% to 5% (Gaido et al., 2013).

Race testing of *C. sojina* isolates originally collected from Brazil, China, and the U.S. revealed 22, 14, and 12 races distributed in each country, respectively (Mian et al., 2008). Isolates of *C. sojina* from Brazil, China, Nigeria, and the United States have exhibited a high degree of genetic diversity (Bradley et al., 2012). The high levels of *C. sojina* diversity suggest races of the pathogen can develop quickly due to selection pressures of a specific geographic location (Kim et al., 2013). Confirmation of population shifts caused by multiple selection pressures has recently been observed in historical isolates and samples collected in Tennessee (Shrestha et al., 2017).*Cercospora sojina* is predominately a disease of warm and humid regions in

the southern U.S. Recently *C. sojina* has begun to affect northern states including Iowa, Wisconsin, Illinois, and Ohio (Cruz and Dorrance, 2009; Mengistu et al., 2002; Yang et al., 2001; Zhang and Bradley, 2014). Disease development and associated yield loss depends on the susceptibility of the soybean cultivar planted and local weather conditions, with crop losses as high as 40%. (Wise and Newman, 2015).

Frogeye leaf spot is a polycyclic disease that remains active throughout the growing season. Conidia are dispersed by wind and splashing water (Wise and Newman, 2015). Primary and secondary inoculum sources are generated on soybean residue by conidiophores, which produce conidia (Wise and Newman, 2015). *Cercospora sojina* overwinters in plant debris, surviving at least two years (Cruz and Dorrance, 2009; Mengistu et al., 2002; Yang et al., 2001; Zhang and Bradley, 2014).

Three genes have been characterized as conveying resistance to FLS: *Rcs1*, *Rcs2*, and *Rcs3*. The *Rcs3* gene has been shown to confer resistance to all known races of *C. sojina* identified in the United States (Phillips and Boerma, 1982), but due to the high level of genetic diversity in the pathogen's population, breakdown of the *Rcs3* gene is likely just a matter of time. Control of FLS can be accomplished by a combination of management practices ranging from cultural methods, use of resistant cultivars, and fungicide applications. The most effective cultural practice is crop rotation in a two-year cycle to reduce inoculum levels (Grau et al., 2004).

Current fungicide management strategies are successful in limiting FLS severity but have increased selection pressure on pathogen populations. *C. sojina* isolates resistant to the quinone outside inhibitor (QoI) fungicide class have been

observed in the U.S. (Standish et al., 2015; Zeng et al., 2015; Zhang et al., 2012). Moreover, QoI-resistant isolates of *C. sojina* appear to be more aggressive than QoI-sensitive isolates in causing symptoms on soybean leaves (Zhang and Bradley, 2017). The success of *C. sojina* in developing resistance to multiple control methods is alarming. Currently, researchers are left with one genetic source of resistance in *Rcs3* to manage FLS outbreaks (Mian et al., 2008). As soybean breeders continue to rely on the one source of resistance from *Rcs3*, increased selection pressure will occur, and the resistance derived from the *Rcs3* gene will inevitably break down.

The initial fungicide of the QoI group was isolated from a wood-rotting fungus, *Strobilurus tenacellus* (Anke et al., 1977). Since the initial discovery, several synthetic analogs have been derived from the natural formulation (Balba, 2007). Hence, all QoI fungicides share a common biochemical mode of action that interferes with energy production in fungi. Specifically, the QoIs block electron transfer at the site of quinol oxidation in the cytochrome bc1 complex preventing adenine tri-phosphate formation (Vincelli, 2012).

Due to the single site of action for all QoIs, the group are considered high-risk fungicides, and repeated use will select for resistant isolates in fungal pathogen populations. Field resistance to QoIs has been documented in several pathogens and develops from a single point-mutation in the cytochrome b gene (Fernández-Ortuño et al., 2008). As diversity in pathogen populations increases with time, QoIs fungicide efficacy is expected to continually diminish. One strategy to slow the development of fungicide resistance in a pathogen population is to use biological control agents. These organisms could be combined with fungicides to provide a cheap secondary mode of

action and potentially delay resistance development in pathogen populations. An additional benefit provided by biological control agents could be enhanced disease protection lasting throughout the growing season and plant growth promotion.

Current research efforts must begin to focus on preservation technologies rather than prior utilitarian approaches to preserve the current tools used for managing disease. Recently, increased interest in harnessing the unique chemistry produced by biologicals has developed. Biological controls are an attractive option as a management tool due to reduced time requirements and lower development costs associated with research (Harman, 2011).

An extensively studied and well characterized biological control agent (BCA) is the fungal genus, *Trichoderma*. The ability of *Trichoderma spp.* to control plant diseases has been reported in multiple studies (Jeerapong et al., 2015; Mukherjee et al., 2013; Zeilinger et al., 2016). *Trichoderma spp.* can control plant disease by mycoparasitism, direct competition for resources, induction of plant defenses, or the production of suppressive secondary metabolites (Jeerapong et al., 2015).

Despite the possibilities of BCAs, there are factors to consider when developing effective formulations to control a pathogen. Isolates must either chemically communicate with the plant or directly penetrate the plant tissue for colonization. The viability of an isolate in several different environments must be well characterized before commercialization. Most biological control efficacy experiments are conducted in controlled environments. Unfortunately, the ability of a BCA to control a pathogen in a field setting does not always correlate to the

initial assay (Sarma et al., 2018). Additionally, secondary benefits provided by the organism should also be characterized. Potential benefits of *Trichoderma spp.* include inducing secondary plant defenses, increasing tolerance for abiotic stress, improving uptake efficiency for nutrients and water, and higher photosynthetic efficiency rates (Harman et al., 2011; Vargas et al., 2009).

Trichoderma spp. have been reported to be effective in the management of other foliar diseases of plants caused by species of *Cercospora*, such as those caused by *C. beticola* and *C. nicotiana* (Galletti et al., 2008; Maketon et al., 2008). However, there is little research assessing the ability of *Trichoderma spp*. to manage foliar pathogens in soybean production systems.Research using *Trichoderma spp.* to manage FLS severity could reveal an effective method for disease management. The *Trichoderma* spp. could be applied either as a stand-alone treatment or in combination with a synthetic fungicide. To test *Trichoderma spp*. ability to limit disease, trials were conducted with three isolates: *T. virens*, *T. hamatum* 1, and *T. hamatum* 2.

Materials and Methods

Field Preparation and Experimental Design. Field trials were conducted in two different fields at the University of Kentucky Research and Education Center (UKREC) in Princeton, KY, 2017. The Catlett Tract had been cropped to soybean the previous year; and the Luttrell Tract had been continuously managed as tall fescue (*Festuca arundinacea* Schreb.) for several years. A Kincaid Voltra research planter (Kincaid Equipment Manufacturing, Haven, KS) was used to plant the trials directly into non-tilled soil. The soybean cultivar 'Armor 4744' (Armor Seed LLC., Jonesboro, AR), which is susceptible to frogeye leaf spot, was planted. Each plot was 6.1 m long

and 4 rows wide with 76.2 cm row spacing.

The Catlett Tract location was planted on 18 May 2017 and the Luttrell Tract location was planted on 18 June 2017. Prior to planting, glyphosate herbicide (Cornerstone Plus; Winfield Solutions, LLC, St. Paul, MN) was applied at a rate of 1.7 kg a.e./ha to control actively-growing vegetation. In addition, S-metolachlor herbicide (Dual II Magnum, Syngenta Crop Protection, Greensboro, NC) was applied at a rate of 1.9 kg a.i./ha prior to planting for residual weed control, and glyphosate was applied as a post- emergence herbicide at a rate of 1.1 kg a.e./ha during the V6 growth stage (Fehr et al., 1971).

Treatments consisted of a non-treated control, a standard fungicide treatment of azoxystrobin + difenoconazole (Quadris Top SBX; Syngenta Crop Protection, Greensboro, NC), and two different isolates of *Trichoderma hamatum (T. hamatum* 1 *and T. hamatum* 2), or one isolate of *Trichoderma virens*. Treatments were applied to the two middle rows of each plot with a backpack sprayer calibrated to deliver 187 liters/ha at 276 kPa with a CO_2-pressurized hand boom. Azoxystrobin + difenoconazole was applied at a rate of 0.12 + 0.12 kg a.i./ha. Each treatment was replicated four times in a randomized complete block design.

***Trichoderma* Inoculum Preparation.** Isolates of the *Trichoderma spp.* were cultured in a laboratory on potato dextrose agar. Cultures were grown for two weeks in a growth room at a constant temperature of 23°C, 90% relative humidity, and under continuous soft-white fluorescent lighting (TL 841 32J/s, Phillips, Andover, MS). After two weeks of growth, conidial suspensions of each isolate were prepared by washing conidia off plates with 15 ml of sterilized water and a bent-glass rod. Contents of the petri plate were passed through a 200-μm sieve and sterile water was

added to a volume of 500 ml. Conidia were counted using a hemacytometer (Bright-Line Hemacytometer, Hausser Scientific, Horsham, PA) and 40X objective on a compound light microscope (Zeiss Axioskop, ZEISS International, Oberkochen, Germany).

Conidia suspensions were brought to a final concentration of $1X10^9$ conidia/ml and applied to soybean plots using a CO_2-pressurized hand boom with a rate of 187 L/ ha at 276 kPa. All treatments were applied when soybean plants were at the R3 growth stage. Catlett location treatments were applied on 29 July 2017 and the treatments at the Luttrell location were applied on 21 August 2017.

Weather. Weather conditions were recorded for one week prior to the application and one week after the application. Additional in-depth hourly forecasts were recorded on the day of application. Weather data from Princeton, KY was compiled using the University of Kentucky's climate data website, wwwagwx.ca.uky.edu/cgi-bin/ky_clim_data_www.pl. (Tables 4.1-4.4)

Biological control assessment. Frogeye leaf spot severity was evaluated for each plot by estimating the percent leaf area affected by FLS in the upper third of the soybean canopy (Price et al., 2016) at three different growth stages (R5, R6, and R8). These severity values were used to calculate an area under the disease progress curve (AUDPC) value for each plot (Van der Plank, 1963). The two middle rows of each plot were harvested with a small plot research combine (Wintersteiger Delta; Wintersteiger Inc., Salt Lake City, UT) to calculate seed weight and moisture on-the-fly (HarvestMaster Classic GrainGage; Juniper Systems, Logan, UT). Harvested seed weights were standardized to 13% moisture and used to calculate soybean yields in kg/ ha.

Statistical Analysis. Data were analyzed with the general linear model procedure (PROC GLM) in SAS (Version 9.4; SAS Institute Inc., Cary, NC). Means were compared using Fisher's protected least significant difference test ($\alpha = 0.10$). Treatments were considered a fixed effect, while replication and location were considered random.

Results

Catlett tract. Significant ($P \leq 0.10$) effects of treatment were observed for FLS severity AUDPC and for soybean yield (Table 5). Treatments did not have a significant effect on seed moisture. The azoxystrobin + difenoconazole treatment resulted in an AUDPC value that was significantly less than the non-treated control. Only the *T. virens* treatment resulted in a significantly greater soybean yield than the non-treated control. All other treatments resulted into yields that were not significantly different from each other or the non-treated control.

Luttrell tract. Significant ($P \leq 0.10$) effects of treatment were observed for FLS severity AUDPC, and for soybean yield (Table 6). Treatments did not have a significant effect on seed moisture. All treatments resulted in AUDPC values that were significantly less than the non- treated control, with azoxystrobin + difenoconazole having the lowest AUDPC value. All treatments resulted in soybean yields that were significantly greater than the non-treated control.

Discussion

At the Catlett Tract, the foliar application of *T. virens* had the highest yield. The Quadris Top SBX treatment had a significantly lower AUDPC values compared to other treatments. Quadris Top SBX's ability to control disease, but lack of yield increase

observed for this location could be the result of pathogen pressure exceeding 50% at the time of application during the experiment.

Nieto-Jacobo et al. (2017) characterized increased shoot growth in *Arabidopsis thaliana*, to the presence of various *Trichoderma spp*. The yield increase from the *T. virens* application could be attributed to plant growth promoting hormones produced by the fungus. Multiple studies show pathogenic or symbiotic fungi producing hormones that act as positive or negative regulators in plant development (De Vleesschauwer et al., 2013; Peleg and Blumwald, 2011; Pozo et al., 2015; Robert-Seilaniantz et al., 2011). Among these regulators are indole-3-acetic acid (IAA), indole-3-ethanol (IET), indole-3-acetaldehyde (IALD), and indole-3-carboxaldehyde (ICALD) secretions from the fungus, which could influence overall plant health (Zeilinger et al., 2016).

The AUDPC measurements at the Luttrell site were significantly lower in all treatments when compared to the non-treated control of the experiment. The results suggest isolates of *Trichoderma spp.* can provide some control of FLS in a field setting, depending on disease severity at the location. Increases in yield appeared to be associated with lower AUDPC levels. The effectiveness of all treatments could be the result of lower disease pressure at time of application and throughout the experiment. Additionally, applying *Trichoderma* at an earlier growth stage of R1 could provide more time for the beneficial fungus to colonize the plant and stimulate plant defenses.

This study provided evidence for using *Trichoderma spp.* to manage FLS in soybean. Future research could combine conidia from various *Trichoderma* isolates and determine if there are additive effects as a mixed treatment. Another possibility for product development would be to combine an active ingredient fungicide component with conidia. As a pre-mix addition to a fungicide, biologicals could provide multiple modes of action for disease control and potentially provide additional plant health benefits, including improved vigor and higher yields. Before combining biologicals with an active ingredient chemistry, the sensitivity of the organism must be assessed thoroughly. Depending on the type of isolate or the active ingredient there can varying degrees of tolerance. (Chaparro et al., 2011; Galletti et al. 2008)

Future research could include further characterization of peptaibols produced by the *T. virens* isolate, which could help provide a natural derivative for producing a synthetic analog. Because volatile metabolites are the initiation phase in the complex interactions between filamentous fungi and their environment, it is important to further understand the underlying mechanisms of these molecules. In doing so, researchers could develop novel forms of plant protection designed to be applied as a foliar treatment. Bio-fungicide applications applied to foliar portions of the plant could protect crops by priming plant defense genes and providing a form of secondary protection throughout the growing season.

Table 4.1 Weather observations for a two-week period, one week prior to the of application of the *Trichoderma* bio-active fungicides and one week following the treatment. The highlighted region in red denotes the day of application for the fungicides. Treatments were applied on 29 July 2017 at the Catlett Tract, University of Kentucky Research and Education Center, Princeton, KY.

July	Temperature (°C)		Dew Point (°C)		Humidity (%)		Wind Speed Average	Rain Total
2017	High	Low	High	Low	High	Low	(kph)	(cm)
23	33	23	25	21	94	50	11.3	0.0
24	33	21	25	22	100	55	4.8	2.2
25	32	22	25	22	100	64	4.8	0.0
26	34	23	27	22	96	59	3.2	0.0
27	31	23	26	23	99	72	4.8	0.5
28	30	23	26	21	100	72	9.7	0.1
29	27	18	20	16	96	51	9.7	0.0
30	28	16	19	15	99	51	4.8	0.0
31	31	16	22	17	100	44	1.6	0.0
1	31	21	22	20	100	53	1.6	0.0
2	29	20	23	18	100	63	3.2	0.6
3	32	19	22	19	98	49	4.8	0.0
4	26	17	21	13	92	44	8.1	0.0
5	27	13	17	13	100	43	3.2	0.0

Table 4.2 Weather observations on the day of application for *Trichoderma* based bio-active fungicide. The highlighted region in red denotes the time when the application of fungicides occurred. Treatments were applied on 29 July 2017 at the Catlett Tract, the University of Kentucky Research and Education Center, Princeton, KY.

Time (CST)	Temperature (°C)	Dew Point (°C)	Humidity (%)	Wind Direction	Wind Speed (kph)	Rain (cm)	Cloud Conditions
8:35 AM	22	19	81	NE	8.1	N/A	Clear
8:55 AM	22	18	79	NNE	6.9	N/A	Clear
9:15 AM	23	18	76	NE	8.1	N/A	Clear
9:35 AM	23	19	76	NNE	11.5	N/A	Clear
9:55 AM	24	18	72	NNE	9.2	N/A	Clear
10:15 AM	24	18	72	NE	8.1	N/A	Clear
10:35 AM	25	18	68	NNE	9.2	N/A	Clear
10:55 AM	25	19	68	NNE	11.5	N/A	Clear
11:15 AM	25	19	65	NE	11.5	N/A	Scattered Clouds
11:35 AM	25	18	65	ENE	12.7	N/A	Scattered Clouds
11:55 AM	26	18	64	NE	9.2	N/A	Mostly Cloudy
12:15 PM	26	18	63	NE	11.5	N/A	Scattered Clouds
12:35 PM	26	19	64	NNE	13.8	N/A	Mostly Cloudy
12:55 PM	26	19	0.7	NNE	10.4	N/A	Scattered Clouds
1:15 PM	25	14	0.5	NNE	16.7	N/A	Scattered Clouds
1:35 PM	26	14	0.5	NE	14.8	N/A	Scattered Clouds

Table 4.3 Weather observations for a two-week period, one week prior to the of application of the *Trichoderma* bio-active fungicides and one week following the treatments. The highlighted region in red denotes the day of application for the fungicides. Treatments were applied on 21 August 2017 at the Luttrell Tract, University of Kentucky Research and Education Center, Princeton, KY.

August 2017	Temperature (°C)		Dew Point (°C)		Humidity (%)		Wind Speed Average (kph)	Rain Total (cm)
	Low	High	Low	High	Low	High		
15	22	31	22	24	59	100	4.8	0.0
16	22	32	22	26	67	100	4.8	0.2
17	24	31	23	26	69	96	12.9	1.5
18	21	30	20	23	55	100	8.0	0.0
19	21	32	21	24	57	96	6.4	0.6
20	20	34	20	24	43	100	3.2	0.0
21	19	34	20	23	48	100	3.2	0.0
22	22	32	22	25	59	100	9.7	0.0
23	17	27	16	21	54	100	4.8	0.0
24	13	27	13	18	47	100	3.2	0.0
25	14	27	14	18	48	100	3.2	0.0
26	13	27	13	17	44	100	4.8	0.0
27	13	30	13	18	39	100	1.6	0.0
28	18	29	18	21	60	98	4.8	0.0

Table 4.4 Weather observations on the day of application for *Trichoderma* based bio-active fungicide. The highlighted region in red denotes the time when the application of fungicides occurred. Treatments were applied on 21 August 2017 at the Luttrell Tract, University of Kentucky Research and Education Center, Princeton, KY.

Time (CST)	Temperature (°C)	Dew Point (°C)	Humidity (%)	Wind Direction	Wind Speed (kph)	Rain (cm)	Cloud Conditions
8:35 AM	26	22	80	Calm	-	N/A	Clear
8:55AM	27	23	77	Calm	-	N/A	Clear
9:15AM	28	23	75	Calm	-	N/A	Clear
9:15AM	29	20	59	SW	5.6	N/A	Clear
9:35AM	29	21	62	Calm	-	N/A	Clear
10:15AM	31	22	59	SSW	6.9	N/A	Clear
10:35AM	32	22	58	South	3.5	N/A	Clear
10:55AM	32	23	57	South	3.5	N/A	Clear
11:15AM	33	22	53	SW	6.9	N/A	Clear
11:35AM	33	22	51	SW	3.5	N/A	Clear
11:55AM	33	21	48	SSW	4.6	N/A	Clear
12:15PM	34	22	49	Calm	-	N/A	Clear
12:35PM	34	21	49	SSW	6.9	N/A	Clear
12:55PM	33	22	52	South	5.8	N/A	Clear
1:15PM	32	21	53	South	3.5	N/A	Clear
1:35PM	30	22	61	Calm	-	N/A	Clear

Table 4.5 Effect of difenoconazole + azoxystrobin fungicide (Quadris Top SBX; Syngenta Crop Protection, Greensboro, NC) and *Trichoderma spp*. applied to soybean at the R3 growth stage on frogeye leaf spot severity area in terms of disease progress curve (AUDPC) values, seed moisture at harvest, and soybean yield at the Catlett Tract, University of Kentucky Research and Education Center, Princeton, KY in 2017.

Treatment	Frogeye leaf spot severity (AUDPC)	Yield (kg/ha)
Non-treated control	115	1,927
Difenoconazole + azoxystrobin	91	1,983
T. hamatum 1	115	1,790
T. hamatum 2	107	2,069
T. virens	105	2,529
P > F	**0.0087**	**0.0478**
LSD 0.10 [z]	**11**	**414**

[z] Fisher's protected least significant difference test value (α = 0.10).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

Table 4.6 Effect of difenoconazole + azoxystrobin fungicide (Quadris Top SBX; Syngenta Crop Protection, Greensboro, NC) and *Trichoderma spp*. applied to soybean at the R3 growth stage on frogeye leaf spot severity area in terms of disease progress curve (AUDPC) values, seed moisture at harvest, and soybean yield at the Luttrell Tract, University of Kentucky Research and Education Center, Princeton, KY in 2017.

Treatment	Frogeye leaf spot severity (AUDPC)	Yield (kg/ha)
Non-treated control	42	3,033
Difenoconazole + azoxystrobin	23	4,014
T. hamatum 1	37	3,805
T. hamatum 2	36	3,690
T. virens	32	3,689
P > F	**0.0011**	**0.0777**
LSD 0.10 [z]	**6**	**558**

[z] Fisher's protected least significant difference test value ($\alpha = 0.10$).

[y] No significant differences because of an *F*-test that was not significant ($P \geq 0.10$).

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VITA

Jonathan Vance Lacey

Education:

Southern Illinois University, Carbondale
Bachelor of Science – Plant Biology, 2016