EVALUATION OF ATOXIGENIC FUSARIUM VERTICILLIOIDES AND BENEFICIAL FUNGAL SYMBIONTS TOWARDS IMPROVING MAIZE MYCOTOXIN RESISTANCE, PLANT GROWTH, AND PEST RESISTANCE

A Dissertation

by

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ABSTRACT

Infection of seed with two major ear rot pathogens of maize, *Fusarium verticillioides* and *Aspergillus flavus*, lead to high yield losses and detrimental health effects to humans and animals through the production of mycotoxins, fumonisin and aflatoxin, respectively. These carcinogenic compounds are some of the most agriculturally important mycotoxins, consumption of which in food and feed can lead to various cancers and stunted growth. While atoxigenic strains of *A. flavus* are used for biocontrol of the pathogenic strain, no similar biocontrol agent exists for *F. verticillioides*. I hypothesized that *F. verticillioides* may antagonize *A. flavus* and promote plant growth. Here I screened 150 putative *Fusarium* isolates collected from maize across Texas that produce a broad range of fumonisin levels from highly toxigenic to almost non-detectable levels. I confirmed that selected isolates do not produce fumonisin *in vitro* and determined that their effects on plant growth promotion are variable. Atoxigenic strains of *F. verticillioides*, 302-A6 and PAL 401, significantly inhibited aflatoxin contamination in a kernel bioassay compared to kernels only infected with *A. flavus* but were unable to definitively inhibit aflatoxin or fumonisin by *in planta* experiments.

A broad range of microbes have been studied for their ability to induce defense pathways in plants towards improved resistance to pathogens and insect herbivores, as well as for promoting plant growth and yield. Here, I show that a selection of beneficial

fungal symbionts applied in two locations over two years hold potential for reducing mycotoxin contamination by inducing maize resistance to *A. flavus* and *F. verticillioides*. In particular, *Epicoccum nigrum* TAMU32, *Pleospora herbarum* TAMU473, and *Acremonium alternatum* TAMU505 led to reductions of aflatoxin in B73 inbred and specific hybrid maize lines. Emphasizing that each fungal treatment must be rigorously tested before marketing, treatments with *E. nigrum* 89 and *P. herbarum* 473 increased aflatoxin in the already resistant Tx779 hybrid. Seed treatment with *A. alternatum* 505 before planting led to promising reductions of aflatoxin and fumonisin in College Station and Lubbock, and showed reduced occurrence of insect damage, suggesting it may improve resistance to both mycotoxins and herbivores depending on genotype and location.

DEDICATION

I dedicate this dissertation and all projects I have undertaken in my time at Texas A&M to God, my Mom and Dad, and my fiancée Shelby. They have all provided unwavering support and encouragement for which I am eternally grateful.

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Firstly, I would like to thank Mike Kolomiets for his mentorship, endless patience, support, and advice throughout my graduate program and for allowing me to pursue my passion for scientific research in agriculture. I wish to thank my committee members Tom Isakeit, Greg Sword, and Charles Kenerley for their continual support, insights, and review of my work. I also would like to thank all the members of the Kolomiets lab who have helped me along the way and provided daily encouragement and advice. Additionally, my gratitude to all the many members of the Department of Plant Pathology and Microbiology I have had the privilege to meet and work with and that have assisted me throughout my career here. I also wish to thank the many interns I have had the privilege to mentor during my program, and without whose diligent help I would not be done yet or have been able to complete so much. Finally, and most importantly, my gratitude to my Mom and Dad for always being there for me and for your love, and to family who have always believed in me. In particular, thank you to my wonderful fiancée Shelby for your incredible love, support, and devotion, and for being there with me through it all, from prelims to my defense and beyond; you mean the world to me.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors Michael V. Kolomiets, Thomas Isakeit, and Charles Kenerley of the Department of Plant Pathology and Microbiology, and Professor Gregory Sword of the Department of Entomology.

Fusarium isolates screened for atoxigenic strains seen in Chapter III were collected by Dr. Thomas Isakeit in the Department of Plant Pathology and Microbiology. Fungal symbionts used as treatments in Chapter IV were collected by Dr. Maria Julissa Ek Ramos while in the lab of Dr. Gregory Sword of the Department of Entomology. Dr. Eli Borrego and Dr. Zachary Gorman, of the Department of Plant Pathology and Microbiology, assisted with quantification and analysis of mycotoxins in both Chapters III and IV. All other work conducted for the dissertation was completed by the student independently.

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NOMENCLATURE

32 Epic.	*Epicoccum nigrum* TAMU32
89 Epic.	*Epicoccum nigrum* TAMU89
117 Chaet.	*Chaetomium globosum* TAMU117
473 Pleo.	*Pleospora herbarum* TAMU473
505 Acrem.	*Acremonium alternatum* TAMU505
534 Clado.	*Cladosporium oxysporum* 534
AFB1	Aflatoxin B1
Chaet. 554	*Chaetomium globosum* 554
FB1	Fumonisin B1
ISR	Induced systemic resistance
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Phial. 490	*Phialemonium inflatum* 490

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CHAPTER I

INTRODUCTION

Maize (*Zea mays* L.), as one of the most produced cereal crops in the world, comprises a commodity that yields a wide range of food, animal feed, and products for industrial targets. The USDA-NASS reported that in the US in 2020, 90.82 million acres of maize were planted and an estimated 14.2 billion bushels were harvested (USDA-NASS, 2021). In Texas, corn is third only to cotton and wheat, with 2.25 million acres planted in 2020 and 231 million bushels harvested with a value of approximately 1.02 billion dollars (USDA-NASS, 2020). Yield losses due to mycotoxin contamination varies widely year to year, but can be severe depending on weather conditions. In 2018, adverse wet and delayed harvest conditions in the US and Ontario, Canada led to unsafe mycotoxin contamination of an estimated 2.5 billion bushels of corn grain (Mueller et al., 2020).

Fusarium verticillioides (Sacc.) Nirenberg (syn. *F. moniliforme* Sheldon, teleomorph *G. fujikuroi* (Sawada) Wr.) is a major pathogen of maize that leads to loss of yield through diseases such as Fusarium stalk rot and Fusarium ear rot. In addition, fumonisin production by this pathogen contaminates maize and renders it unacceptable for certain uses such as cattle feed and food products, depending on contamination level. Maize plants can be infected by *F. verticillioides* either systemically through the plant from root infection, through wounds in any part of a plant, or by infection through the

silks (Leslie & Summerell, 2006). Most strains of *F. verticillioides* naturally produce fumonisin B1 (FB1), which can cause esophageal cancer, liver damage, and defects in embryo development in humans and other diseases in animals and humans (Leslie & Summerell, 2006). FB1 has been shown to cause equine leukoencephalomalacia in horses (Marasas et al., 1988) and has been linked to liver cancer in lab rats (Jaskiewicz et al., 1987). Fumonisin (B1, B2, and B3) cumulative contamination limits are set at 2 ppm for dry milled corn commodities to 30 ppm for corn and corn derived products for breeding poultry and ruminants, the highest limit being 100 ppm for poultry intended for slaughter. Other countries including the EU set the most stringent regulation of 1 ppm for maize products intended for humans (Farhadi et al., 2019).

Aspergillus flavus is another fungal pathogen of maize that infects kernels and causes Aspergillus ear rot. A significant problem occurs when the highly carcinogenic mycotoxin aflatoxin is produced (Atehnkeng *et al*., 2008), which causes liver cancer in animals and humans and suppression of the immune system and disruption of protein metabolism in lab and farm animals (Williams et al., 2004). US maize losses due to aflatoxin alone amount to an estimated $280 million annually (Nierman *et al*., 2015), and in Indonesia, Philippines, and Thailand, estimated losses are $900 million annually (Schmale III & Munkvold, 2017). Aflatoxin is of particular concern in the production of dried distiller's grains and solubles (DDGS), which is a valuable source of feed for livestock such as cattle, swine, and chicks (Bowers & Munkvold, 2014). Mycotoxins can become more concentrated during the process of DDGS production, posing a higher risk from mycotoxins in commercial uses of DDGS. One control method for *A. flavus* is

biocontrol using atoxigenic strains of *A. flavus* that are unable to produce aflatoxin (Atehnkeng *et al*., 2014). In field studies conducted in 1991, atoxigenic strains were found to exclude aflatoxin-producing strains so efficiently that aflatoxin levels were reduced by 80 to 95% (Brown *et al*., 1991). However, recent evidence that sexual recombination occurs between different strains of *A. flavus* in nature creates the concern recombination between native toxigenic and widely released commercial atoxigenic strains may occur (Horn et al., 2009). This could lead to the development of new, potentially more virulent and toxic strains of *A. flavus*, or new fungal populations that are not inhibited by atoxigenic strains leading to biocontrol methods becoming less effective. Either of these scenarios prompts the necessity for finding additional alternative methods of control.

In this study, I have identified naturally occurring atoxigenic strains of *F. verticillioides* from diverse Texas locations to test for their ability to reduce contamination of both aflatoxin and fumonisin. These results are reported in Chapter III of this dissertation. To reduce contamination with the mycotoxins, a second project tested several fungal symbionts as seed treatments for their efficacy in reducing mycotoxin contamination as well as other biotic stresses. These results are reported in Chapter IV.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS FOR CHAPTER III

1. Plant and fungal materials

F. verticillioides (teleomorph, *Gibberella fujikuroi*) strain 7600 was used as the reference strain for purposes of comparative fumonisin production. *A. flavus* (teleomorph, *Petromyces flavus*) strain NRRL3357 was used for infections of kernel bioassays and ears with developing kernels around blister stage in field trials. Fungal isolates to be screened for atoxigenic *F. verticillioides* were obtained from a collection of morphologically-selected *Fusarium* spp. isolates derived from maize research plots in College Station, Corpus Christi, Kingsville, and Palacios, TX in 2014. B73 inbred maize was obtained from seed propagated in 2014 in College Station, TX.

2. Testing *Fusarium* isolates for fumonisin production

To screen for atoxigenic isolates of *Fusarium* sp., harvested kernels from individual ears in the collection mentioned above were placed on sterilized damp paper towels and incubated for ~7 days in plastic bags to create a humidity chamber. Spores of *Fusarium* colonies from individual kernels were collected onto sterile cotton swabs and prepared for long-term storage in vials of silica at 4-7 °C. Individual isolates were cultured by inoculating plates of potato dextrose agar containing streptomycin (PDAS) with one isolate per plate and incubating at 28 °C for 3-5 days. An individual colony from each plate was transferred to a 10 mL test tube with ~3 ml of ½ PDAS on a slant

for maximum fungal growth on the surface area and incubated for 14 days. To quantify fumonisin production by each isolate, 6 mL of 1:1 acetonitrile:sterile distilled water (SDW) was added to each test tube and extracted overnight. Approximately 0.5 mL of extract was then syringe-filtered through 25 mm filters with 0.45 µm cellulose acetate membranes into amber vials. 100 µL of filtrate were then analyzed by LC-MS/MS system (Gorman et al., 2020) following methods from Dall'Asta et al. (2007).

3. Morphological and molecular identification

Putative isolates of *Fusarium* sp. found to be fumonisin non-producing by LCMS were identified by morphological and molecular identification techniques. Colonies were identified according to the nomenclature proposed by Leslie and Summerell (2006) including identification of chains of microconidia characteristic of *F. verticillioides*. Isolates identified as *F. verticillioides* were subcultured for further testing and maintained in silica and glycerol stocks. DNA of *F. verticillioides* isolates found to be atoxigenic, as described below, were extracted from isolates grown in liquid culture and extracted following the *A. nidulans* genomic DNA isolation protocol from Bok and Keller (2012). Isolates identified as *F. verticillioides* were tested with species-specific primers from Rodriguez Estrada et al. (2011) and confirmed by positive PCR identification.

4. Kernel bioassays

To conduct kernel bioassays, ears of B73 inbred maize were screened to identify seed that exhibited low infection by pathogens including *Fusarium* spp., *A. flavus*, and *A. niger*. Eight seeds per ear were surface sterilized in 0.65% sodium hypochlorite for 2 mins and rinsed in SDW two times for 1 min each. Cleaned seeds were then incubated at 20-24°C on damp autoclaved paper towels in humidity chambers. Ears with lowest infection, 0/8 to 3/8 kernels showing infection, were selected for use in kernel bioassays experiments to reduce the chance of preexisting mycotoxin contamination. Kernel bioassays were performed with slight modifications from the protocol developed at our laboratory (Christensen et al., 2012). Kernels for this bioassay were sterilized by autoclaving at 140 °C for 30 min to eliminate the possibility of internal infection of *A. flavus* or *F. verticillioides*. Kernels were wounded with a sterile scalpel to a depth of 0.5 mm at the base of the embryo. Conidial suspensions of atoxigenic *F. verticillioides* isolates, toxigenic *F. verticillioides* 7600, and *A. flavus* NRRL3357 were prepared by flooding plates with 5 mL 0.01% Tween-20 solution, scraping with a sterile spatula to liberate spores, and pipetting the suspension through at least 4 layers of cheesecloth to remove mycelium by gravity filtration. The resulting spore suspensions were diluted in Falcon tubes to 40 mL with sterile distilled water (SDW), and conidia were enumerated by hemocytometer and diluted to $1x10^6$ spores/mL. Four wound-treated kernels were placed into sterile 20 mL glass scintillation vials and weighed, then inoculated with either 200 µL of control treatment of 0.01% Tween solution or with 200 µL of spore suspension, vortexed, and separated to reduce fungal growth crossing between kernels.

Vials were then loosely capped and incubated in a humidity chamber at 28 °C with 12/12 hr light:dark. 200 µL of SDW were added to each vial daily until the experiment was terminated.

The ability of atoxigenic isolates of *F. verticillioides* to inhibit aflatoxin production was determined in a modified kernel assay. Treatment vials that were intended to be inoculated with *A. flavus* were treated with 200 µL of 0.01% Tween solution (control) or $1x10^6$ spores/mL spore suspensions of atoxigenic *F. verticillioides* isolates. After 2 days incubation, vials were treated with either control treatment of 0.01% Tween solution or $1x10^6$ spores/mL *A. flavus* spores. All kernels were incubated for an additional 7 days followed by aflatoxin extraction with 10 mL of 2:1 chloroform:methanol and mycotoxin quantification by LC-MS/MS following the protocols from Borrego (2014).

5. Growth chamber and greenhouse assays with atoxigenic fusarium and ear rot pathogens

5.A Experimental design

For growth chamber and greenhouse trials conducted to determine growth promotion and biocontrol effects of atoxigenic *F. verticillioides* isolates, ears of B73 inbred maize propagated from the field in 2014 were screened to identify seed that exhibited high germination and low infection by pathogens including *Fusarium* spp., *A. flavus*, and *A. niger*, seeds were surface sterilized following methods above, and samples of seeds with highest germination, 5/8 to 8/8, and lowest infection, 0/8 to 3/8, were

selected for use in kernel bioassays experiments. Seeds from those selected ears to be planted for growth chamber and greenhouse experiments were surface sterilized with 70% ethanol for 2 mins, SDW for 1 min, 0.65% sodium hypochlorite for 2 mins, and SDW three times for 1 min each. Clean seeds were then planted and grown, following methods from Gao et al. (2007) with modifications, in Metro Mix 360 RSi soil (Sun Gro Horticulture, Agawam, MA) that was twice-autoclaved at 140 °C for 1 hour and allowed to cool overnight between sterilizations. Seedlings were watered every 3-4 days with 1 g/L of 21-7-7 quick-release fertilizer (ICL Specialty Fertilizers, Summerville, SC) in Deepots (Stuewe and Sons, Inc., Tangent, OR) used for growth chamber experiments or in Ray Leach cone-tainers (Stuewe and Sons, Inc., Tangent, OR) for later transplanting to 5 gallon pots for greenhouse experiments.

Seedlings were prepared for inoculation at approximately V2 stage at two weeks after planting by forming two holes in the soil with sterile 1 mL pipette tips, one hole half the length of the tip and the second on the opposite side of the pot to its full length. Spore suspensions of atoxigenic *F. verticillioides* isolates at $1x10^6$ spores/mL were prepared as described above, and 5 mL aliquots of either control treatment of 0.01% Tween-20 or the spore suspensions were injected into each hole for a total of 10 ml/plant. Injection sites were then covered with soil by additional sterile pipette tips while avoiding cross-contamination.

For plants grown in Deepots, plant heights were measured from soil level to the tip of the longest leaf. Stem circumferences were measured in the middle of the thickest

internode above the first leaf, and stem diameters were measured at the thickest internode above the first leaf but across the thinner direction of the stalk to avoid including the thicker midribs of the sheaths around the stalk in the measurement. Shoots were collected when experiments were terminated and dried at 70 °C in foil bags to measure dry weight.

Plants grown for greenhouse experiments were grown in Ray Leach cone-tainers and soil-treated as described above, then transplanted to 5 gallon pots. Plant heights of mature plants at VT stage were measured from soil level to the top of each tassel. Yield was measured from ears harvested at least 1 month after pollination and dried under hot ventilated greenhouse conditions at ~38°C. Ears were shelled and whole kernel yields per cob were recorded.

5.B Inoculation with F. verticillioides 7600 and atoxigenic isolates for stalk rot evaluation

B73 plants were grown in Deepots and inoculated as described above, then transferred to 5 gallon pots under outdoor conditions in College Station to the VT developmental stage, at which maize tassels are fully visible, and 10 plants per genotype were selected and stalks inoculated following previously described methods (Thon et al., 2002; Gao et al., 2007). Briefly, the three internodes above the last node with brace roots were wounded with an 18G hypodermic needle to ¼ inch depth. Sterile cotton swabs were dipped in treatment of 0.01% Tween-20 (control) or spore suspensions ($1x10^6$ spores/mL) of *F. verticillioides* 7600 or atoxigenic *F. verticillioides* isolates, and

wrapped in place on the wound site with parafilm to create a humid chamber. Nine days after inoculation, stalks were harvested, split open, and photographed. Lesion areas were measured in mm^2 with ImageJ.

5.C F. verticillioides and A. flavus inoculations and quantification of aflatoxin and fumonisin

To inoculate ears of B73 maize for testing the biocontrol potential of atoxigenic *F. verticillioides*, spore suspensions of $1x10^6$ spores/mL of *A. flavus* were made from plate cultures as described above and maize ears were inoculated following methods from Wahl et al. (2016). Briefly, 3 mL of inoculum was injected in the silk channel 10-14 days after midsilking with a tree Arborjet and inoculation needle (SKU: 35780SO, Treestuff, Greensboro, NC, United States). After senescence, ears were harvested, shelled, and 100 kernel weights and whole kernel yields per cob were recorded. For the AFB1 analysis, all inoculated ears from each plot were ground with a Romer mill (EQMMS2010, Romer Labs), 0.1 g weighed into 20 mL glass scintillation vials and extracted with 10 mL of 2:1 chloroform:methanol for 24 hrs, and 0.5 mL of extract was filtered through 25 mm filters with 0.45 µm cellulose acetate membranes into amber vials. 100 to 200 µL aliquots of filtrate were then analyzed for aflatoxin by LC-MS/MS as described above.

6. Field trial: seed treatments and experimental design

To test the biocontrol potential of atoxigenic *F. verticillioides* isolates under field conditions, seeds of B73 were surface sterilized as described previously and coated with 2% methylcellulose and either 0.01% Tween-20 (control) or $1x10^7$ spores/mL of atoxigenic *F. verticillioides* isolates suspended in 0.01% Tween-20. The seeds were dried overnight and planted in a randomized complete block design (RCBD) at 25 seeds/plot with four reps at the Texas A&M AgriLife Center at Corpus Christi in 2016. Ears inoculations with mock or *A. flavus* spore suspension and quantification of resulting aflatoxin contamination were conducted as described above.

MATERIALS AND METHODS FOR CHAPTER IV

1. Plant and fungal materials

Maize seed selected for this project included maize hybrids, Spirit 596 (Sp596) and Spirit 799 (Sp799), from Indigo Ag, Inc., and Tx777 and Tx779 hybrids from the Texas maize breeding program of Dr. Seth Murray (Murray et al., 2019), and B73 inbred propagated in 2017 and 2018 in College Station. Beneficial fungal seed treatments were selected from fungal isolates originating from a field survey of facultative fungal endophytes of cultured cotton in Texas (Ek-Ramos et al., 2013). The fungal isolates selected from this consortium consisted of *Phialemonium inflatum* TAMU490, *Chaetomium globosum* TAMU554, *Epicoccum nigrum* TAMU32, *Epicoccum nigrum* TAMU89, *Chaetomium globosum* TAMU117, *Pleospora herbarum* TAMU473, *Acremonium alternatum* TAMU505, or *Cladosporium oxysporum* TAMU534. The

maize pathogens *Colletotrichum graminicola* strain CgM2 (syn. M.1.001) used for Anthracnose stalk rot infection assays, *A. flavus* (NRRL 3357) used for Aspergillus ear rot inoculations, and *F. verticillioides* 7600 (M3125; Fungal Genetics Stock Center, Kansas City, KS, U.S.A.) were all cultured from 60% glycerol stock stored in a -80°C freezer. *F. verticillioides* (teleomorph, *Gibberella fujikuroi*) strain 7600 was used as the reference strain for purposes of comparative fumonisin production. *A. flavus* (teleomorph, *Petromyces flavus*) strain NRRL3357 was used for infections of kernel bioassays and ears with developing kernels around blister stage in field trials.

2. Kernel bioassay

To conduct kernel bioassays, ears of B73 inbred maize were screened to identify seed that exhibited low infection by pathogens including *Fusarium* spp., *A. flavus*, and *A. niger*. Eight seeds per ear were surface sterilized in 0.65% sodium hypochlorite for 2 mins and rinsed in SDW two times for 1 min each. Cleaned seeds were then incubated at 20-24°C on damp autoclaved paper towels in humidity chambers. Ears with lowest infection, 0/8 to 3/8 kernels showing infection, were selected for use in kernel bioassays experiments to reduce the chance of preexisting mycotoxin contamination. Kernel bioassays were performed with slight modifications from the protocol developed at our laboratory (Christensen et al., 2012). Kernels for this bioassay were sterilized by autoclaving at 140 °C for 30 min to eliminate the possibility of internal infection of *A. flavus* or *F. verticillioides*. Kernels were wounded with a sterile scalpel to a depth of 0.5 mm at the base of the embryo. Conidial suspensions of atoxigenic *F. verticillioides*

isolates, toxigenic *F. verticillioides* 7600, and *A. flavus* NRRL3357 were prepared by flooding plates with 5 mL 0.01% Tween-20 solution, scraping with a sterile spatula to liberate spores, and pipetting the suspension through at least 4 layers of cheesecloth to remove mycelium by gravity filtration. The resulting spore suspensions were diluted in Falcon tubes to 40 mL with sterile distilled water (SDW), and conidia were enumerated by hemocytometer and diluted to $1x10^6$ spores/mL. Four wound-treated kernels were placed into sterile 20 mL glass scintillation vials and weighed, then inoculated with either 200 µL of control treatment of 0.01% Tween solution or with 200 µL of spore suspension, vortexed, and separated to reduce fungal growth crossing between kernels. Vials were then loosely capped and incubated in a humidity chamber at 28 °C with 12/12 hr light:dark. 200 µL of SDW were added to each vial daily until the experiment was terminated.

The ability of atoxigenic isolates of *F. verticillioides* to inhibit aflatoxin production was determined in a modified kernel assay. Treatment vials that were intended to be inoculated with *A. flavus* were treated with 200 µL of 0.01% Tween solution (control) or $1x10^6$ spores/mL spore suspensions of atoxigenic *F. verticillioides* isolates. After 2 days incubation, vials were treated with either control treatment of 0.01% Tween solution or $1x10^6$ spores/mL *A. flavus* spores. All kernels were incubated for an additional 7 days followed by aflatoxin extraction with 10 mL of 2:1 chloroform:methanol and mycotoxin quantification by LC-MS/MS following the protocols from Borrego (2014).

3. Field trials

3.A Seed treatments and field design

Seeds of each maize genotype were screened to remove seed with visual infection and in the 2016 trial in Corpus Christi at the Texas A&M AgriLife Center at Corpus Christi, seeds of B73 were treated with *Phialemonium inflatum* TAMU490, *Chaetomium globosum* TAMU554, or 2% methylcellulose (control) and planted in a randomized complete block designs (RCBD).

For the repeated field trials in College Station and Lubbock, seeds were coated at Indigo labs with treatments of proprietary chemistry (*personal communication*, 2019) and control or spores of selected fungal symbionts. In 2018, the selected fungal seed treatments in College Station consisted of control, *Epicoccum nigrum* TAMU32, *Epicoccum nigrum* TAMU89, *Chaetomium globosum* TAMU117, *Pleospora herbarum* TAMU473, and *Acremonium alternatum* TAMU505, while in Lubbock the same seed treatments were applied, excluding *E. nigrum* 89. Maize genotypes tested in 2018 for both locations were B73 inbred and the hybrids Sp596, Sp799, Tx777, and Tx779. In 2019, the seed treatments tested were modified and consisted of control, *Epicoccum nigrum* TAMU32, *Epicoccum nigrum* TAMU89, *Acremonium alternatum* TAMU505, and *Cladosporium oxysporum* TAMU534. Maize genotypes tested in 2019 for both locations were B73 inbred and the hybrids Sp596 and Sp799. Seeds were planted in field trials in an RCBD at 21-25 seeds per plot. Fields were located on the Texas A&M University Farm in College Station, TX, and on the Quaker Ave. Research Farm

operated by Texas Tech University in Lubbock, TX. Six reps were planted in College Station while three were planted in Lubbock in 2018, and in 2019 six reps were planted in both locations.

3.B F. verticillioides and A. flavus inoculations and quantification of aflatoxin and fumonisin

To inoculate ears with *A. flavus* and *F. verticillioides*, inocula were prepared by inoculating sterilized maize kernels following the methods of Wahl et al. (2017). Conidia were liberated by adding 0.01% Tween-20 and removing mycelia by filtering with at least four layers of cheesecloth. Spore suspensions of all inocula were adjusted to final spore concentrations of $1x10^6$ mL^{-1} for field inoculations and kept on ice during transportation and until inoculation. The time to midsilk was determined from a subsample of plots for each genotype as silking was reached at different times. Primary open-pollinated ears were inoculated at 10-14 days after midsilk with 3 mL of either mock treatment of 0.01% Tween or fungal inoculum of *A. flavus* or *F. verticillioides*, by silk channel-inoculation following the methods from Anderson et al. (2016). All inoculated ears from each plot were hand harvested at plant senescence at least 30 days after inoculation and dried under hot ventilated greenhouse conditions at ~38°C. Ears were shelled and 1000 kernel weights and whole kernel yields per cob were recorded.

For the AFB1 analysis, all inoculated ears from each plot were ground with a Romer mill (EQMMS2010, Romer Labs). For the initial field trial in Corpus Christi in 2016 (Fig. 12), aflatoxin was extracted from 0.1 g samples of ground meal placed in

glass scintillation vials with 10 mL of 2:1 chloroform:methanol and quantified by LC-MS/MS following the protocols from Borrego (2014). For subsequent field trials in 2018 and 2019 (Figs. 13 & 14), fifty-gram samples of ground meal were tested for aflatoxin concentration using the VICAM AflaTest® (VICAM, Milford, MA) per manufacturer's instructions. Fumonisin was quantified from 0.3 g samples of ground meal by adding 10 mL acetonitrile:sterile distilled water (SDW) and extracting overnight. ~1.0 mL of extract was syringe-filtered through 25 mm filters with 0.45 µm cellulose acetate membranes into amber vials. 100 µL of filtrate were then analyzed by the LC-MS/MS system (Gorman et al., 2020) following the methods from Dall'Asta et al. (2007).

3.C Inoculation with C. graminicola for Anthracnose stalk rot evaluation

For inoculation of stalks with *C. graminicola,* cultures were grown on potato dextrose agar plates for at least two weeks before conidia were collected following methods as previously described by Gao et al. (2007). Final spore concentrations of $1x10^6$ mL^{-1} were prepared for inoculations and kept on ice during transportation and use. Ten plants per genotype x treatment combination were selected 14 days after midsilking and inoculated following previously described methods (Thon et al., 2002; Gao et al., 2007). Briefly, the four internodes above the last node with brace roots were wounded with an 18G hypodermic needle to ¼ inch depth. Sterile cotton swabs were dipped in *C. graminicola* spores and wrapped in place on the wound site with parafilm to create a humid chamber. Nine to eleven days after inoculation, stalks were harvested, split open,

and photographed. Lesion areas were measured in cm^2 with ImageJ (Schneider et al., 2012).

3.D Phenotyping of agronomic traits

Germination was recorded by counting the number of seeds germinated in all plots and dividing by the number of seeds planted per plot. Data for all locations and years were exponentially transformed to improve normality.

Plant heights were measured by selecting three representative plants from each plot and from a three blocks per field selected randomly. Plants were measured in cm from the soil level at the base of each plant to the tip of the tallest leaf pulled taught. Heights were measured at 4 weeks after planting in 2018 and at three different time points, at 4, 6, and 8 weeks in 2019 in College Station and two time points, at 3 and 5 weeks, in Lubbock. Results were analyzed of both individual data points and averages of the three plants per plot.

The rate at which plants reached midsilking was quantified by recording the number of ears with silk and without silk in all plots in 2-3 blocks per field. For determining differences in time to midsilking the number of ears were divided by the final number of ears that were recorded as developing in each plot.

In field trials in 2019, incidence of insect damage was measured at both locations at two timepoints; in College Station at ~V4 stage 4 weeks after planting and again 10

weeks after planting at the reproductive stage as ears were silking, and in Lubbock at ~V4 stage and again two weeks later at V6-V7 stage.

4. Data analysis

Analysis of results was performed with JMP 12.0.1 (SAS Institute, Inc) statistical package was utilized after recording and sorting data in Microsoft Office Excel 2007 (Microsoft Corporation). Data were tested for normal distribution and the appropriate log-transformation was performed if needed following Ferrigo et al. (2020). Since aflatoxin results had a large variation, including some means with no aflatoxin, data were transformed using log(aflatoxin ppb +1) (Snedecor and Cochran, 1967) to stabilize the variance (Gorman et al., 1992). Germination results in proportions were exp transformed before statistical analysis. All results were analyzed by one-way ANOVA or factorial ANOVA depending on the experiment and means were separated by post hoc analysis with Dunnett's tests or Tukey's Highly Significant Difference (HSD) tests, unless otherwise specified, using the least significant difference at a 5% significance level (Weaver et al., 2017). To exam relationships between fungal symbionts treatments and plant phenotypic results, principal component analysis was conducted using JMP.

CHAPTER III

IDENTIFICATION OF NATURALLY OCCURRING ATOXIGENIC ISOLATES OF FUSARIUM VERTICILLIOIDES AND THEIR POTENTIAL AS BIOCONTROL AGENTS OF MAIZE EAR ROT PATHOGENS

INTRODUCTION

Bacon and Hinton (1996) reported that *F. verticillioides* persists in maize tissues as a symptomless endophyte that colonizes intercellular spaces without causing disease and may only become pathogenic when environmental conditions induce changes in the host plant. A report of *F. verticillioides* living in asymptomatic tissues suggests it causes disease only when environmental conditions result in a stress to the plant (Leslie et al. 1990). Fusarium may even promote growth enhancement of maize when it colonizes maize as a symptomless endophyte. Growth enhancement, albeit nonsignificant, was observed in early growth stages of maize when Fusarium was inoculated into sterilized soil (Oren et al. 2003). Yates et al. (1997) found that *F. verticillioides* seed treatment led to significantly increased stem diameter and plant weight, though not plant height, 28 days after planting. Atoxigenic strains have been identified before, such as an atoxigenic strain referred to as M-5496, discovered in Nepal while screening 10 different strains for fumonisin production (Nelson et al., 1991). This prompted us to screen local isolates in Texas for atoxigenic strains and test them for growth promotion and biocontrol potential.

A. flavus and *F. verticillioides* display an antagonistic interaction when coinfecting the same maize ear, which raises the possibility of using one fungus to control the other. When inoculated with both *F. verticillioides* and *A. flavus*, aflatoxin accumulation was much lower than in ears inoculated with *A. flavus* alone (Zummo & Scott, 1992). Lillehoj *et al.* (1982) also found lower aflatoxin in ears treated with a mixture of *A. flavus* and *F. verticillioides* compared to *A. flavus* only treatment across two locations, and aflatoxin levels in ears treated with *F. verticillioides* were lower than control levels in Georgia. This may not always be the case as Ono *et al.* (2010) found the presence of fumonisin and aflatoxin in Brazilian maize, but fumonisin was still more prevalent than aflatoxin which they interpreted as an ability of *F. verticillioides* to suppress aflatoxin in agreement with Zummo & Scott (1992).

The precedent of isolating and utilizing atoxigenic strains of *A. flavus* for biocontrol and the potential for *F. verticillioides* exhibiting antagonism towards *A. flavus* prompted us to screen a collection of *F. verticillioides* isolates from local fields for naturally occurring non-fumonisin-producing (atoxigenic) strains. The goal of this research was to identify isolates of atoxigenic *Fusarium verticillioides* and test them for their potential as plant growth enhancers and biocontrol agents of toxigenic *F. verticillioides* and *A. flavus*. I hypothesized that one or more strains of *F. verticillioides* would be non-producers of fumonisin and that one or more could significantly improve maize growth and reduce contamination by fumonisin or aflatoxin by inhibiting infection of toxigenic *F. verticillioides* or *A. flavus*, respectively.

RESULTS

Screening for atoxigenic Fusarium

A total of 149 isolates of *F. verticillioides* grown on potato dextrose agar demonstrated high variability of fumonisin B1 (FB1) production, both higher and lower than the reference *F. verticillioides* strain 7600 (Fig. 1). *F. verticillioides* 7600 was tested from three separate cultures and averaged 15.2 nM FB1 concentration. Over half the isolates (55.7%) produced more fumonisin, up to 65 nM FB1, than the reference strain *F. verticillioides* 7600. The rest produced less fumonisin than *F. verticillioides* 7600 and included potential atoxigenic candidates with FB1 accumulation levels as low as 0.011 nM FB1. Figure 1 shows the eventually selected two isolates, 302-A6 and PAL 401, labeled with green diamonds, which produced almost non-detectable levels of fumonisin comparable to controls containing no fungal colonies.

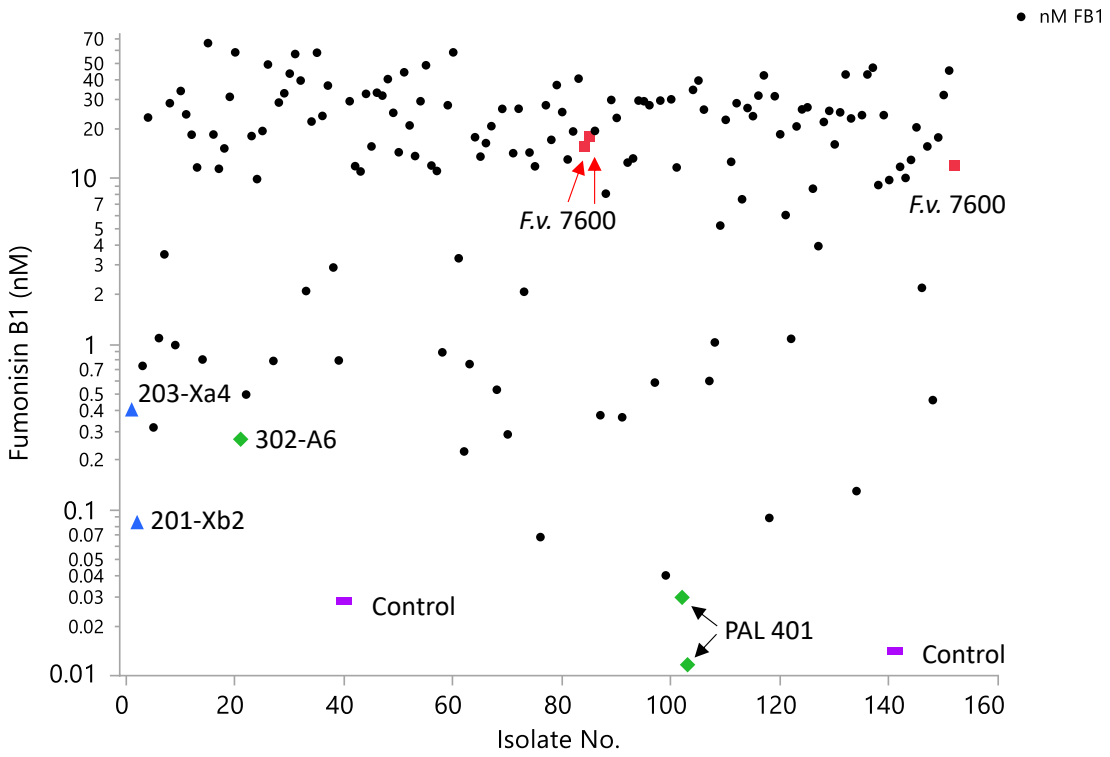

Figure 1 Fumonisin B1 production by *F. verticillioides* isolates collected from cob samples in 2014 and grown in test tubes containing slanted 3 mL of ½ PDA. Purple rectangles are controls; red squares are three replicates of the reference strain *F. verticillioides* 7600; green diamonds are isolates 302-A6 and two replicates of PAL 401, the final selections of atoxigenic isolates; blue triangles are isolates initially atoxigenic but found to produce fumonisin in kernels (Fig. 2). Black circles are all other isolates tested (n = 1 unless otherwise noted).

Morphological identification

Thirty four of the lowest FB1-producing isolates were selected and cultured on ½ PDAS plates for morphological identification. Colonies were analyzed and identified according to the nomenclature proposed by Leslie and Summerell (2006) including identification of microconidia characteristic of *F. verticillioides*. Of those 34 isolates, 29 were identified as *F. verticillioides* and five could not be identified to species level.

PCR amplification

Two isolates, 302-A6 and PAL 401 were tested with the species-specific primers FusqPCR_F/R (Table 1) and confirmed by positive PCR identification.

Table 1 List of species-specific primers for identification of *Fusarium verticillioides* by PCR.

Primer Name	Primer Sequence	Reference
FusqPCR_F	**TCGCTCTAGGCCAGATTACCA**	Rodriguez Estrada et al., 2011
FusqPCR_R	**GAACCAGGAAAGTCGATGGTG**	Rodriguez Estrada et al., 2011

Confirmation of the selected isolates inability for fumonisin production

As fumonisin production is inducible under certain conditions such as oxidative stress (Ferrigo et al., 2015) and the isolates tested above may not produce fumonisin on PDA while retaining the ability to produce fumonisin in other conditions, four of the lowest fumonisin-producing isolates, 201-Xb2, 203-Xa4, 302-A6, and PAL 401, and the reference strain *F. verticillioides* 7600, were applied to sterilized non-viable kernels of B73 inbred maize in a kernel bioassay. This was tested to confirm the inability of these isolates to produce fumonisin. ANOVA showed a significant effect among kernel treatments ($P < 0.0001$), and isolates 302-A6 and PAL 401 showed almost non-detectable levels of FB1, which were not statistically different from control-treated kernels (Dunnett's test, "control" set as control; $P = 1.000$) (Fig. 2). These two strains were considered to be atoxigenic. Interestingly, when grown on kernels, two initially

atoxigenic isolates 201-Xb2 and 203-Xa4 not only produced as much FB1 as *F. verticillioides* 7600, but in the case of isolate 201-Xb2, produced nearly significantly more FB1 (Dunnett's test, *F. verticillioides* 7600 set as control; P = 0.0570).

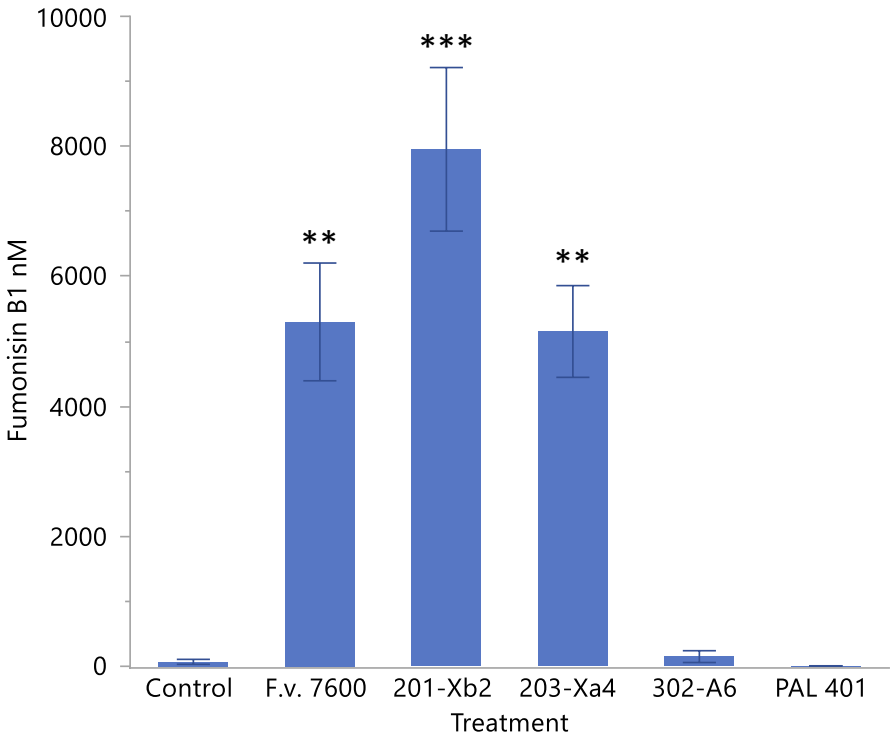

Figure 2 *F. verticillioides* 302-A6 and PAL 401 fumonisin levels were indistinguishable from control-treated kernels. Bars represent the mean fumonisin B1 in nM ± SE of four biological replicates, quantified 7 days after inoculation onto sterilized, wounded kernels. Pairwise statistical differences between treatments and the control group were compared using Dunnett's test (*$P < 0.05$; **$P < 0.01$ ***$P \leq 0.0001$).

Effects on plant growth and yield by atoxigenic *F. verticillioides* strains

To determine whether our two atoxigenic isolates of *F. verticillioides* could induce growth promotion in maize as reported for some strains by Oren et al. (2003), B73 inbred was treated with 10 mL of $1x10^6$ spores/mL of the isolates by soil injection

about two weeks after planting, and heights and stem circumferences of the plants were measured four weeks after soil injection treatment. Plant height and stem circumference were measured showed a trend of promoting plant growth, but results were not significant (ANOVA; $\alpha \leq 0.05$) (Fig. 3A&B).

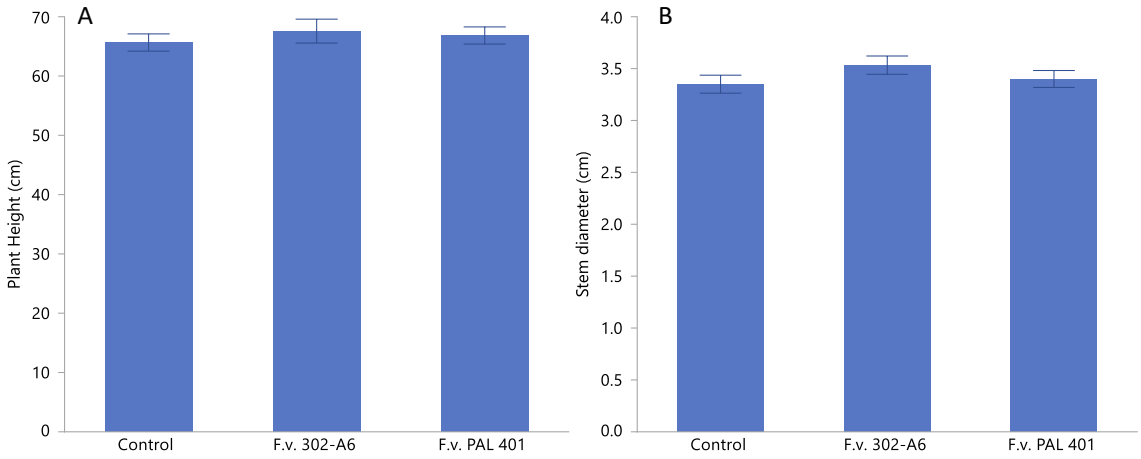

Figure 3 Plant height A) and stem diameter B) of B73 plants displayed slightly increased growth in treatments with atoxigenic *F. verticillioides* isolates. Bars represent means of plant height and stem diameter in cm ± SE of 4, 4, and 6 biological replicates, respectively (ANOVA, $\alpha < 0.05$).

Shoot dry weights in a second growth chamber trial were also measured and *F. verticillioides* 302-A6 demonstrated a trend of growth promotion, however there was no significant difference among treatments (ANOVA; $\alpha \leq 0.05$) (Fig. 4).

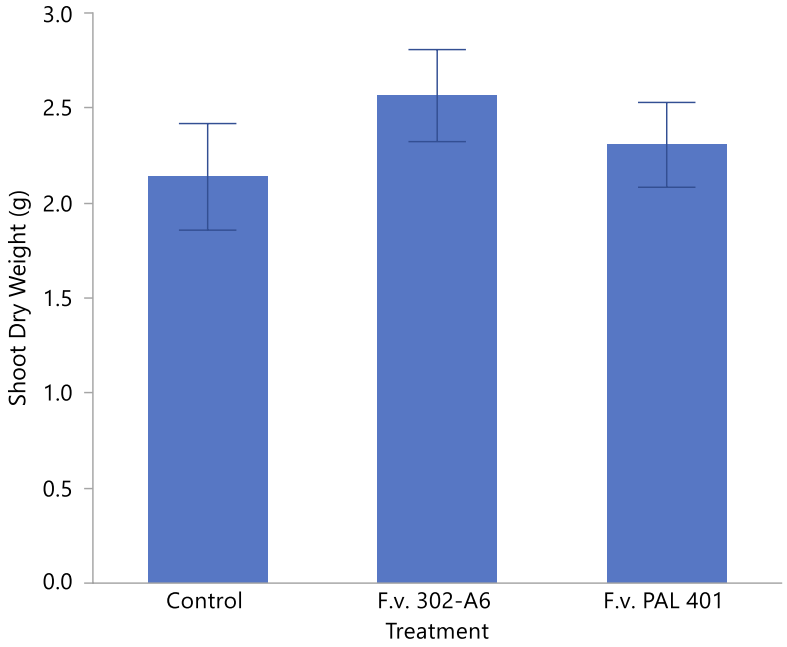

Figure 4 Shoot dry weight of B73 inbred maize displayed slightly increased growth in treatments with atoxigenic *F. verticillioides* isolates. Bars represent means of shoot dry weights in g ± SE of 4, 4, and 6 biological replicates, respectively (ANOVA, $\alpha < 0.05$).

In order to determine if atoxigenic *F. verticillioides* could promote growth in maize at later maturity stages, B73 inbred seedlings were treated with spores of both atoxigenic isolates and plants were allowed to grow to full maturity. Seed treatments had were significantly different (ANOVA; $P = 0.0005$), and *F. verticillioides* PAL 401 led to a significant increase in plant height (Dunnett's test; $P = 0.0003$) (Fig. 5A). Stem circumferences were also measured and were significantly different among treatments (ANOVA; $P = 0.0456$), and *F. verticillioides* PAL 401 led to a significant decrease in stem circumference (Dunnett's test; $P = 0.0408$) (Fig. 5B).

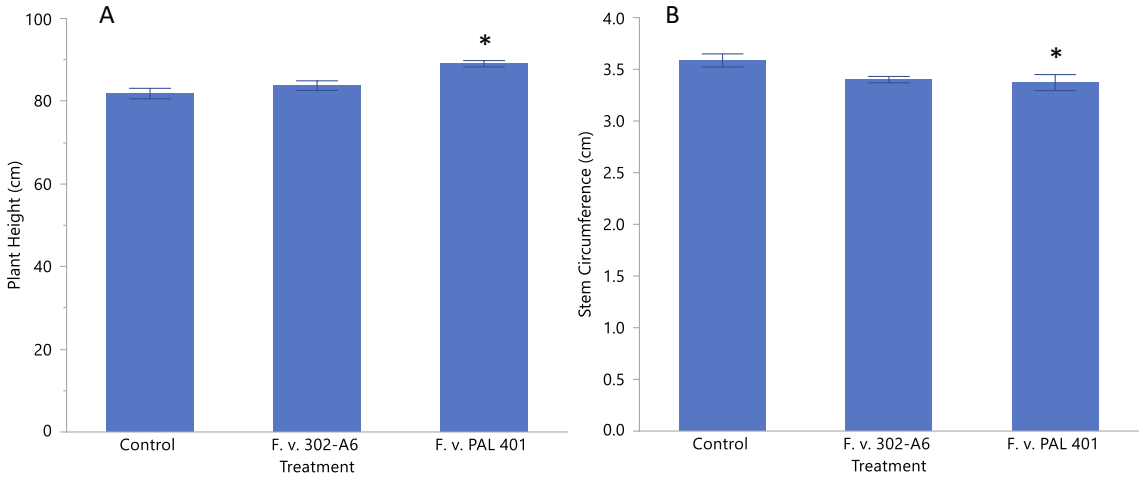

Figure 5 Plant height of B73 plants displayed increased growth with the treatment of atoxigenic *F. verticillioides* PAL 401. Bars represent means of plant heights in cm ± SE of 7 biological replicates (Dunnett's method, $\alpha < 0.05$).

To determine if the inability to produce fumonisin affected virulence of the atoxigenic isolates, the ability to cause Fusarium stalk rot was next tested. Mature B73 plants at the VT developmental stage were wound-inoculated in three internodes starting one internode above the brace roots, with *F. verticillioides* strain 7600, or isolates 302-A6 or PAL 401, and incubated for 9 days. Lesion areas reached an average of 150 mm^2 in the first internode and averaged 340 mm^2 in the second and third internodes (Fig. 6). No significant difference was found among treatments or in treatment by internode interaction (ANOVA; $\alpha \leq 0.05$), indicating that lack of fumonisin does not affect the virulence of these atoxigenic isolates.

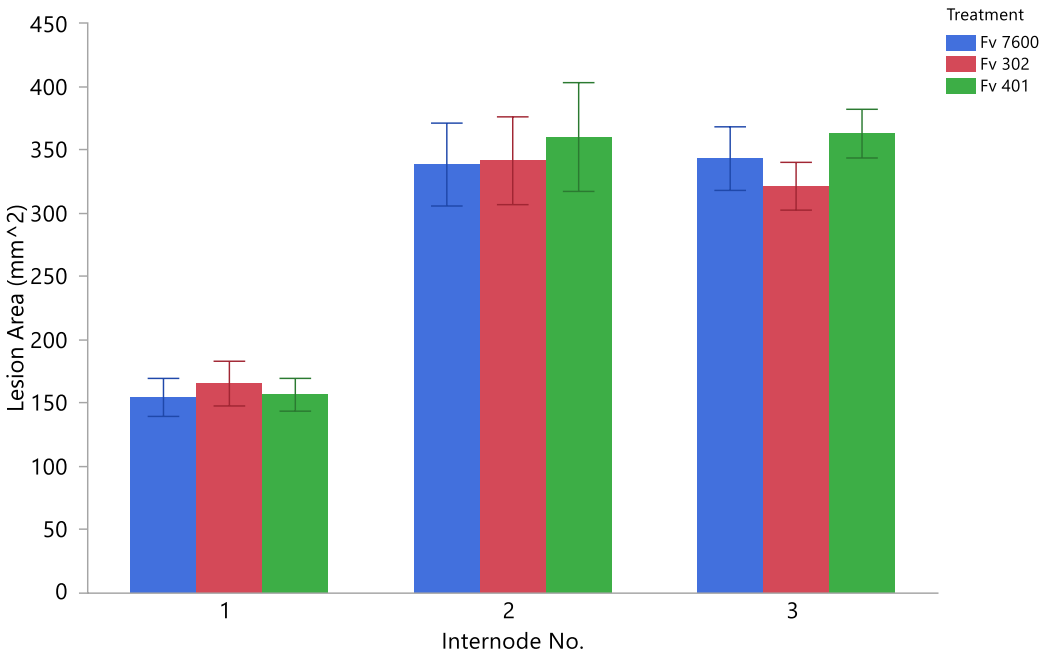

Figure 6 Lesion areas of stalk rot showed no change in the pathogenicity of atoxigenic *Fusarium verticillioides* isolates to cause Fusarium stalk rot. Bars indicate average lesion areas in mm^2 ± SE of necrotic area following stalk inoculation with *F. verticillioides* 7600, 302-A6, or PAL 401 in 9 biological replicates (ANOVA; $\alpha < 0.05$).

Potential of atoxigenic *F. verticillioides* for biocontrol of aflatoxin contamination

To determine if atoxigenic *F. verticillioides* isolates could act as biocontrol agents of *A. flavus* and aflatoxin contamination, kernels were inoculated with isolates 302-A6 and PAL 401 two days prior to inoculation with *A. flavus*. There was a significant difference in aflatoxin among treatments by ANOVA ($P < 0.0001$). AFB1 content was significantly lower in kernels treated with either isolate of *F. verticillioides* compared to kernels infected by *A. flavus* alone (Fig. 7). AFB1 content was 489 µg/g

kernel in *A. flavus*-treated kernels, whereas 302-A6-treated seed accumulated 49 µg/g kernel and treated with PAL 401 accumulated 3.7 µg/g kernel.

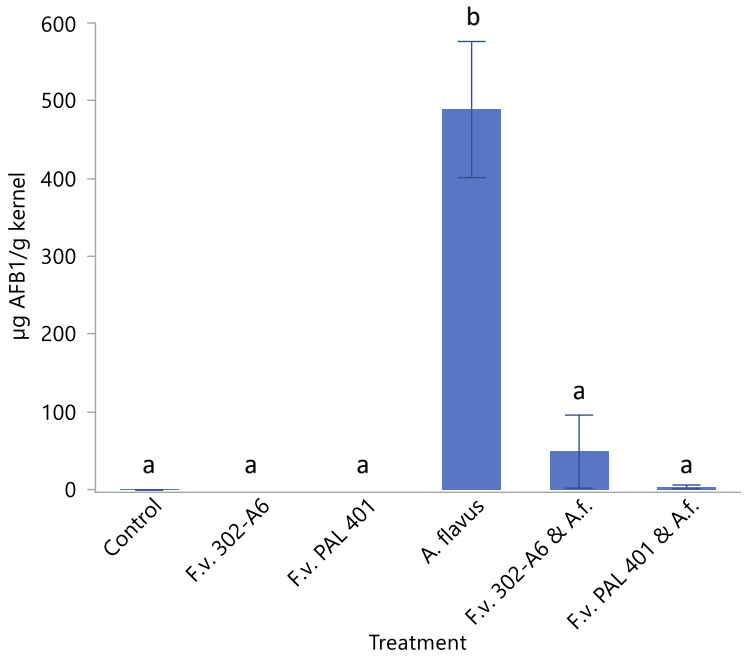

Figure 7 Aflatoxin B1 (AFB1) accumulation was inhibited in kernels pre-treated with atoxigenic *F. verticillioides* followed by inoculation with *A. flavus* two days later. Autoclaved kernels were treated with control or atoxigenic *F. verticillioides* spores and incubated for 48 hours before inoculation with *A. flavus*. Bars are mean AFB1 accumulation per g kernel in 3 biological replicates quantified 7 days after inoculation with *A. flavus*. Differences in letters indicate statistically significant differences (n=3; ± SE; Tukey's HSD; $P < 0.05$)

We next investigated whether the atoxigenic isolates could reduce aflatoxin accumulation *in planta*. Thus, B73 seedlings were treated by soil-injection with atoxigenic *F. verticillioides* isolates then transplanted to 10-gallon pots, grown to maturity, and silk channel-inoculated with *A. flavus* 10-14 days after midsilking. Low pollen production led to low yield (Fig. 8A) and likely led to the high variability in aflatoxin accumulation seen in the small ears produced. ANOVA showed an almost

significant effect among seed treatments (P = 0.0534) and Dunnett's post hoc test showed a nearly significant increase in yield by PAL 401 compared to control (P = 0.0575). Analysis of aflatoxin results showed no differences among treatments (ANOVA; P = 0.911) (Fig. 8B) and both isolates, 302-A6 and PAL 401, showed a slight, though not significant, trend of inhibiting aflatoxin contamination in treated plants.

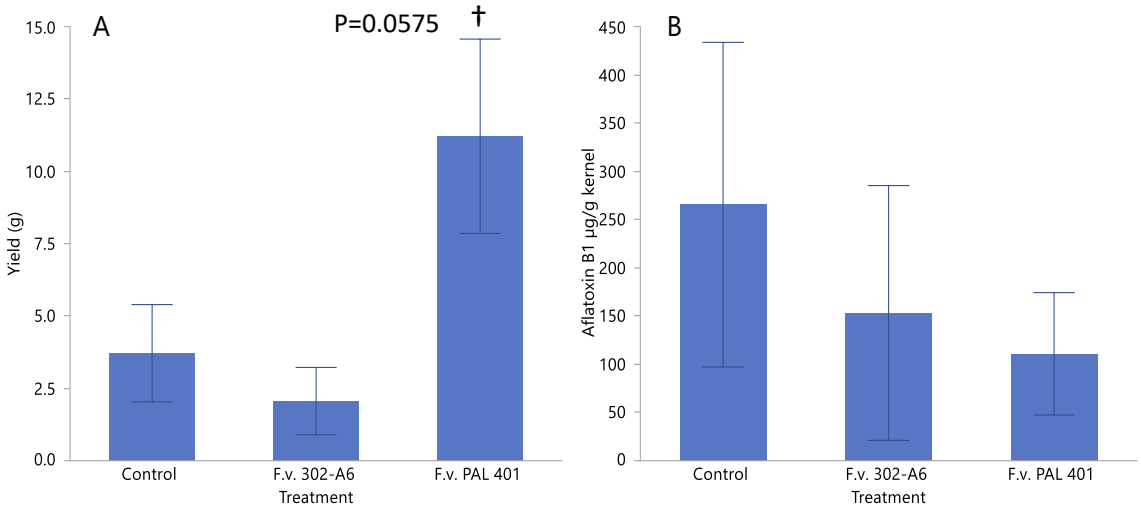

Figure 8 Atoxigenic *F. verticillioides* isolate PAL 401 displayed a trend of A) increased yield and B) reduced aflatoxin B1 accumulation in B73 maize soil injection-treated with atoxigenic *F. verticillioides* 302-A6 and PAL 401. Bars are A) yield in g per ear ± SE in 8-10 biological reps and B) mean AFB1 in µg/g ground meal ± SE in 5, 2, and 4 biological reps, respectively). Differences between means were analyzed for statistical significance by Dunnett's pairwise test of yield and of log transformed aflatoxin (* $P < 0.05$, † ≥ 0.05: indicates non-significant differences).

The results from the greenhouse trial were inconclusive, but suggested potential for the reduction of aflatoxin contamination by treatments with atoxigenic *F. verticillioides* isolates. The isolates' potential as biocontrol agents under the field

conditions was investigated by applying atoxigenic isolates as seed treatments in a field trial in Corpus Christi. Plants were grown to maturity and the highest ear of all plants were silk channel-inoculated with spores of *A. flavus* 10-14 days after midsilking. Aflatoxin levels in atoxigenic *F. verticillioides* seed-treated plots showed no significant difference compared to control-treated plants (Fig. 9).

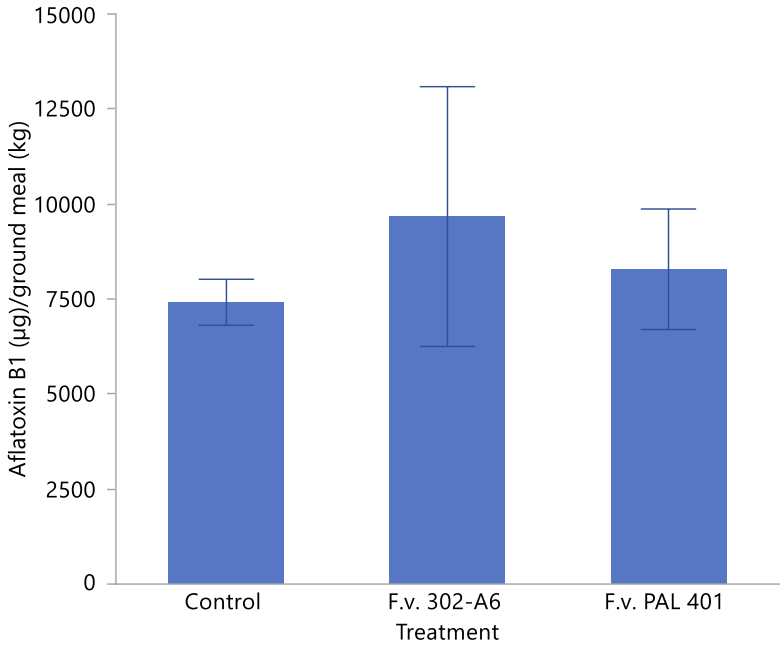

Figure 9 Aflatoxin B1 accumulation was not different in B73 maize ears seed-treated with atoxigenic *F. verticillioides* grown in Corpus Christi in 2016. Bars are mean AFB1 in µg/kg ground meal ± SE of 4 biological replicates (ANOVA, $\alpha < 0.05$).

Potential of atoxigenic *F. verticillioides* for biocontrol of fumonisin

The ability of atoxigenic *F. verticillioides* isolates to inhibit fumonisin production by toxigenic *F. verticillioides* in maize was determined by measuring fumonisin in ears silk channel-inoculated with *F. verticillioides*. Atoxigenic *F. verticillioides* treatments showed no significant changes in fumonisin B1 levels

(ANOVA, $\alpha \leq 0.05$) (Fig. 10) or in visual colonization levels, suggesting a lack of systemic colonization by these isolates or a lack of induced systemic resistance.

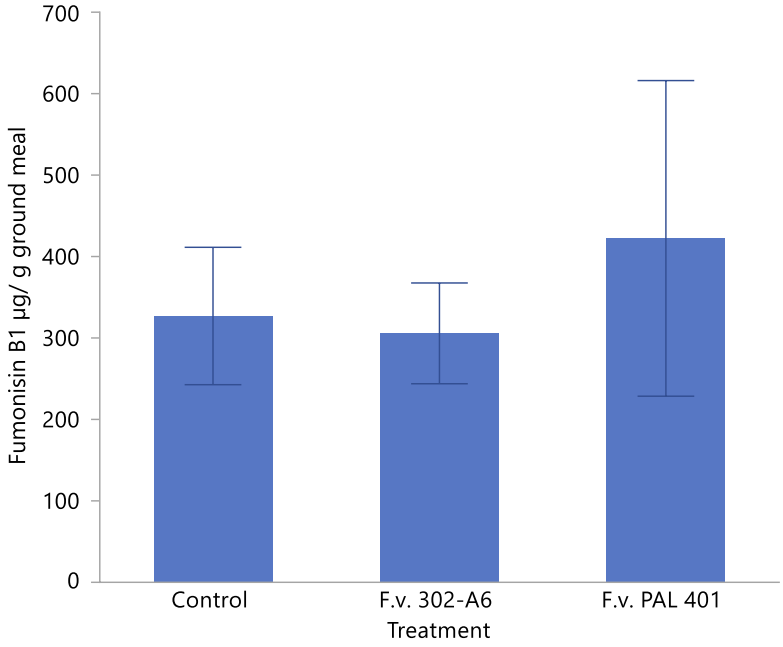

Figure 10 Fumonsin B1 accumulation was not different in B73 maize ears seed-treated with atoxigenic *F. verticillioides* grown in greenhouse. Bars are mean fumonisin B1 in µg/g ground meal ± SE of 4 biological replicates (ANOVA, $\alpha < 0.05$).

DISCUSSION

Quantification of fumonisin from *F. verticillioides* isolates grown on PDA media indicated these isolates produce a wide range in levels of fumonisin, with the majority producing levels similar to that of reference strain *F. verticillioides* 7600 (Fig. 1). Thirty four of the lowest fumonisin-producing isolates were screened morphologically and molecularly with species-specific primers. Interestingly, following the application of the lowest fumonisin-producing *F. verticillioides* isolates onto kernels in a kernel bioassay, I

found that three isolates remained atoxigenic for fumonisin production while two other strains produced as much as or more fumonisin than *F. verticillioides* 7600 (Fig. 2). This was not unexpected, as Bailly et al. (2005) demonstrated that toxigenic *F. verticillioides* produced significantly different amounts of fumonisin depending on whether they were grown on whole kernels, cracked corn, corn flour, or polenta (course corn meal), and Ferrigo et al. (2015) found fumonisin biosynthesis is inducible under oxidative stress such as the presence of H_2O_2. To our knowledge no atoxigenic strains of *F. verticillioides* have been identified before in Texas. A study by Ortiz et al. (2015) found that all 164 isolates tested showed positive for the *FUM1* gene, a polyketide synthase encoding gene that is required for fumonisin biosynthesis. Furthermore, when samples were taken from maize that tested negative for fumonisin, all ten *F. verticillioides* isolates were able to produce fumonisin levels even higher than *F. verticillioides* 7600 when inoculated into kernels (Ortiz et al., 2015).

Upon identifying the fumonisin-nonproducing *F. verticillioides* isolates 302-A6 and PAL 401, I next tested their ability to promote plant growth of maize. Isolate 302-A6 showed a trend of increasing shoot dry weight, plant height, and stem diameter, but significant differences were not seen until plant were allowed to grow to full height. Isolate PAL 401 treatment showed increased yield of maize. *F. verticillioides* has been demonstrated to produce variable results as a growth promoting treatments, inducing greater stem diameter and plant weight, though not plant height (Yates et al., 1997). Since *F. verticillioides* is also known as a stalk rot pathogen of maize depending upon the environment, atoxigenic strains were evaluated for their ability to cause disease.

Maize inoculated with the atoxigenic strains showed induction of lesions, but the lack of fumonisin made them no more virulent than *F. verticillioides* 7600. Jardine and Leslie (1999) also found that a fumonisin-nonproducing strain was just as capable of infection and causing stalk rot as fumonisin-producing strains. Stalk rot caused by *F. verticillioides* is dependent on the environmental conditions and does not appear to be considered a major threat in (Bell et al., 2003). This leads to the possibility that these atoxigenic isolates, if developed into biocontrol agents, could be used in locations such as Texas where Fusarium stalk rot rarely occurs.

Finally, atoxigenic *F. verticillioides* strains 302-A6 and PAL 401 were tested for their ability to inhibit aflatoxin production in kernels under laboratory, greenhouse, and field conditions. Both isolates showed they were able to inhibit aflatoxin accumulation by 89.9% and 99.2%, respectively, when allowed to colonize kernels for two days before inoculation with *A. flavus*. In greenhouse conditions, plants exhibited low yield due to poor seed set, leading to high variation of results. However, PAL 401 treatment showed a trend of improving yield and decreasing aflatoxin by 52%. In order to corroborate these results under field conditions, a field trial was conducted in Corpus Christi. However, the results indicated there were no differences in aflatoxin between control and ears of plants seed treated with atoxigenic Fusarium. Promising results of biocontrol effects found *in vitro* frequently do not translate to field conditions as environmental conditions play a major role in plant-microbe interactions. Ferrigo et al (2020) found that treatment with *Trichoderma harzianum* strain INAT11 greatly reduced *F. verticillioides* mycelial growth *in vitro* and disease symptoms in stalks under greenhouse conditions,

but showed no effect on fumonisin contamination under field conditions. Finally, I also applied atoxigenic *F. verticillioides* strains to maize and tested them in greenhouse conditions to see if treatment could induce resistance to toxigenic strains of *F. verticillioides* and fumonisin accumulation. *F. verticillioides* can colonize maize as a symptomless endophyte (Bacon and Hinton, 1996), opening the possibility of treating maize with these atoxigenic strains to assess whether they can exclude fumonisin by colonizing ears before toxigenic Fusarium infection initiates. Results showed no significant differences in fumonisin levels in ears. There are various reasons fumonisin levels were not different including that these strains did not induce ISR, that one or both did not colonize part or all of the plant and ears and limit infection by toxigenic strains, or that one or both did colonize the ears but was not effective at competitively excluding infection by the toxigenic *F. verticillioides*.

CHAPTER IV

MAIZE GENOTYPE AND ENVIRONMENT MEDIATE FUNGAL SYMBIONT EFFECTS ON MYCOTOXIN CONTAMINATION, PLANT GROWTH, AND PEST RESISTANCE.

INTRODUCTION

The Ascomycete fungal pathogens *Aspergillus flavus* and *Fusarium verticillioides* (Sacc.) Nirenberg [G] (synonym: *F. moniliforme* Sheld.) are two of the most problematic ear rot pathogens of maize. *A. flavus* infection results in the production of aflatoxin and *F. verticillioides* infection produces fumonisin in maize kernels, and together present a significant part of the mycotoxin risk that maize growers face in the US. These two mycotoxins can individually or together limit or exclude corn grain from use for animal or human consumption due to their potential for hepatotoxic, mutagenic, and carcinogenic effects (Bennett and Klich, 2003; Marasas et al. 1988; Marasas 1996). Many developed countries have regulations on limits for these mycotoxins in maize, including the USA which sets limits on aflatoxins (B1, B2, G1 and G2) at 20ppb for corn, peanut products, and other animal foods intended for dairy animals, and up to 300 ppb for corn and peanut products intended for finishing (i.e. feedlot) beef cattle (FDA, 2019). Fumonisin (B1, B2, and B3) cumulative contamination limits are set at 2 ppm for dry milled corn commodities to 30 ppm for corn and corn derived products for breeding poultry and ruminants, the highest limit being 100 ppm for poultry intended for

slaughter. Other countries including the EU set the most stringent regulation of 1 ppm for maize products intended for humans (Farhadi et al., 2019).

Beneficial microbes comprise a large, diverse group of microorganisms that have been shown to associate with most plant species and confer valuable traits. Endophytes are a group of microbes that can partially or fully colonize plants and live asymptomatically or confer many beneficial traits in a wide range of economically important crops. Beneficial bacterial genera such as *Pseudomonas, Serattia,* and *Bacillus*, and fungal genera such as *Trichoderma* and *Piriformospora*, have been shown to induce resistance to pathogenic microbes and herbivorous insects (Pieterse *et al*., 2014). This increased resistance, termed Induced Systemic Resistance (ISR), is a priming of the host plant by stimuli such as beneficial microbes for faster, more robust responses leading to increased resistance to pathogens, insects, or other biotic and abiotic stressors (Mauch-Mani, 2017). Beneficial fungi may also be able to influence production of defense phytohormones or their signaling pathways, as in the example of *Laccaria bicolor*, an ectomycorrhizal fungus of *Populus trichocarpa* that produces the effector MiSSP7, which interacts with host plant JA signaling repressors, blocking JA action (Plett *et al*., 2014).

Numerous studies have attempted approaches to reduce mycotoxins in maize, but the majority have investigated biocontrol effects *in vitro*, such as Samsudin and Magan (2016) who found that *Clonostachys rosea* inhibited FB1 production *in vitro* on milled maize agar by 73-100% depending on water availability of the media. Applications of

the beneficial bacteria *Bacillus cereus sensu lato* strain B25 as seed treatments led to greatly reduced fumonisin contamination in the field (Lizarraga-Sanchez et al., 2015), while treatment with *Trichoderma harzianum* strain INAT11 reduced Fusarium stalk rot but was unable to affect fumonisin levels under the field conditions (Ferrigo et al., 2020).

In an effort to identify new fungal symbionts that could serve as biocontrol agents of *A. flavus* and *F. verticillioides*, several fungal isolates that originated from a field survey of facultative fungal endophytes of cultured cotton in Texas (Ek-Ramos et al. 2013) were selected for testing. This group of potential endophytes included *Epicoccum nigrum* strains TAMU32 and TAMU89, *Chaetomium globosum* TAMU117, *Pleospora herbarum* TAMU473, *Acremonium alternatum* TAMU505, and *Cladosporium oxysporum* 534. There are several possible modes of action for these beneficial fungi to promote plant health. Some fungal symbionts may increase plant resistance to pathogens through the production of antimicrobial or fungicidal compounds or induction of ISR. *Chaetomium globosum*, a saprophytic ascomycete, produces two metabolites, 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT) and the epidithiadiketopiperazine, chaetomin, compounds which *C. globosum* uses to antagonize the "damping-off" pathogen, *Pythium ultimum* (Di Pietro *et al*., 1992). *Acremonium alternatum* was found to suppress infection by the obligate biotroph *Plasmodiophora brassicae* and subsequent clubroot symptoms in *Arabidopsis thaliana* (Jaschke *et al.*, 2010). Additionally, an isolate of *A. alternatum* was discovered on cucurbits acting as a hyperparasite of the powdery mildew fungus *Sphaerotheca*

fuliginea throughout Crete (Malathrakis, 1985). Further research found that both live and autoclave-, oven-, or UV- killed *A. alternatum* spores significantly increased the obligate pathogen *Leveillula taurica* required to reach 50% severity of leaf infection. The effect was more pronounced with non-viable spores, indicating the inhibition of the pathogen must be due to ISR rather than parasitism (Kasselaki *et al*., 2006). *Acremonium* species are also known to produce metabolites, including pyrrocidine A and B, with inhibitory activities *in vitro* against the fungal pathogen Pyricularia oryzae, but interestingly, not against *A. flavus* or *F. verticillioides* (Poling et al., 2008). The globally distributed ascomycete *Epicoccum nigrum* exhibits biocontrol effects in numerous crops including peaches, *Prunus persica* (L.) Batch, where it is currently used as a biocontrol agent for brown rot caused by the fungal pathogens, *Monilinia laxa* and *M. fructigena* (De Cal *et al*., 2009). In addition, it holds potential as a beneficial endophyte of sugarcane for its antifungal compounds that reduced *in vitro* colony growth of the sugarcane pathogens *Ceratocystis paradoxa* and *F. verticillioides* (Fávaro *et al*., 2012). *Pleospora herbarum* (anamorph *Stemphylium vesicarium*) is a little-studied ascomycetous fungus commonly found in nature as a saprophyte on plant debris such as decaying grass leaves. *P. herbarum* is largely known only as a causal agent of purple spot of asparagus, wherein airborne conidia are detected throughout the growing season but mostly following the last spear harvest, indicating this fungus is primarily a pathogen on the asparagus fern (Granke & Hausbeck, 2012). *P. herbarum* also causes brown rot of pears, but strains found on asparagus or onions are not pathogenic on pears, suggesting strains of the pathogen are host-specific (Köhl *et al*., 2009). Finally, the fungal symbiont

Cladosporium oxysporum TAMU534 was included for the 2019 field trials. *C. oxysporum* is listed as a rather common and widespread saprobic hyphomycete able to inhabit dead parts of leaves and stems of both herbaceous and woody plants, and as a pathogen able to induce leaf spots in certain plant species including citrus, pepper, and greenhouse tomato (Bensch et al., 2012; Fisher, 1967, Hammouda 1992, Lamboy & Dillard 1997).

Two fungal strains, *Beauveria bassiana*, GHA strain, commercially available as BotaniGard®, and *Phialemonium inflatum* TAMU490, isolated as an endophyte from cotton, were shown to inhibit cotton aphid reproduction under greenhouse and field conditions (Lopez *et al*., 2014), and increase plant growth while reducing larval weight of cotton bollworms (*Helicoverpa zea*) feeding on endophyte-treated cotton under greenhouse conditions (Lopez *et al*., 2014; Lopez & Sword, 2015). Seeing as these were potential inducers of plant resistance to herbivores, these two fungal symbionts were tested in initial field trials on maize (unpublished) and *Phialemonium inflatum* TAMU490 was selected for further analysis, presented here.

In year one of field trials (2018), five fungal symbionts, *Epicoccum nigrum* strains TAMU32 and TAMU89, *Chaetomium globosum* TAMU117, *Pleospora herbarum* TAMU473, and *Acremonium alternatum* TAMU505, were tested on five maize genotypes, B73 inbred line, Hybrid 1 (Spirit 596) and Hybrid 2 (Spirit 799) provided by Indigo Ag, and two hybrids from the breeding program of Dr. Seth Murray, Texas A&M University, Tx777 x PHG39 (Tx777) and LH195 x Tx779 (Tx779). These

were planted in field trials conducted in College Station and Lubbock, TX, with both trials receiving silk-channel inoculations of *A. flavus*, *F. verticillioides*, or mock and with six replicates in College Station and three in Lubbock. In year two of testing (2019), the maize lines Tx777 and Tx779, found to be resistant to mycotoxin accumulation, were excluded from testing. Additionally, *C. globosum* 117 and *P. herbarum* 473 were replaced by and *Cladosporium oxysporum* 534 for proprietary reasons by Indigo, and the number of replicates in Lubbock was increased from three to six. I hypothesized that one or more fungal symbionts would prove effective at reducing mycotoxin contamination in one or more maize genotypes. This would inform a tailored use of specific beneficial fungi to particular maize lines and growing regions for the control of ear rot and mycotoxin contamination.

RESULTS

Fungal symbiont-mediated defense against fungal pathogens of maize *in vitro* and under field conditions

Initial evaluation of aflatoxin inhibition by fungal symbionts in vitro and in planta:

P. inflatum 490 and *C. globosum* 554 both significantly inhibited aflatoxin accumulation in a kernel bioassay where seeds of susceptible B73 inbred maize were treated with beneficial fungal strains followed by *A. flavus* spores two days later. Seeds inoculated solely with *A. flavus* spores accumulated 488.7 µg aflatoxin B1/g kernel, while *P. inflatum* 490 treatment led to significant inhibition with 234.1 µg aflatoxin B1/g

kernel (P = 0.015), while *C. globosum* 554 treatment displayed an almost significant reduction with 282.7 µg aflatoxin B1/g kernel (P = 0.057) (Figure 11).

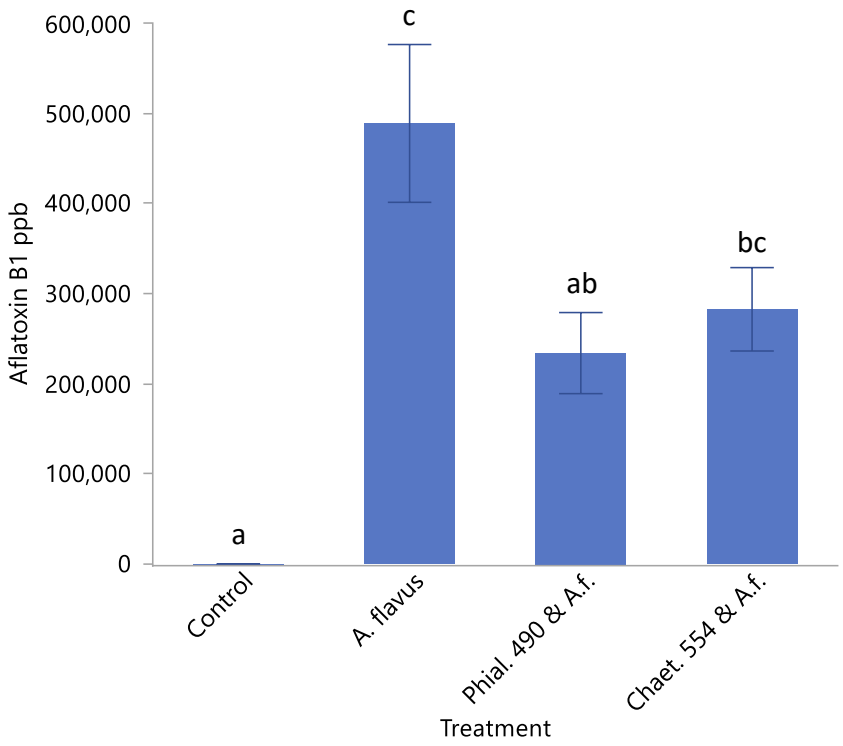

Figure 11 Aflatoxin accumulation was inhibited in kernels treated with spores of *P. inflatum* 490 and *C. globosum* 554 and incubated for 48 hours before inoculation with *A. flavus*. Bars are mean aflatoxin B1 µg/g kernel quantified 7 days after *A. flavus* inoculation. Average and significance were calculated over three biological replicates; error bars indicate ± SE. Differences in letters indicate significant differences between means determined by Tukey's HSD (P < 0.05).

Effects of fungal symbionts on aflatoxin contamination of maize under field conditions:

Since *C. globosum* 554 and *P. inflatum* 490 held potential for suppressing aflatoxin accumulation in maize kernels and were linked to systemic ISR in cotton, it was hypothesized these strains could systemically affect plant defense against *A. flavus* and result in reduced aflatoxin levels when were applied as seed treatments to maize. To test this hypothesis, I seed-treated susceptible B73 inbred maize with the fungal symbionts and conducted a field trial in Corpus Christi in summer 2016. ANOVA revealed a significant difference among seed treatments ($P = 0.0079$) and aflatoxin was reduced by 58%, from 7414 ppb aflatoxin in control plots to 3090 ppb aflatoxin in plots treated with *P. inflatum* 490, though this result was not significant according to a post hoc analysis using Dunnett's test ($P = 0.0613$) (Fig. 12). *C. globosum* 554 increased the levels of aflatoxin by 151% to 18,579 ppb, though this was also not significant by Dunnett's ($P = 0.1025$).

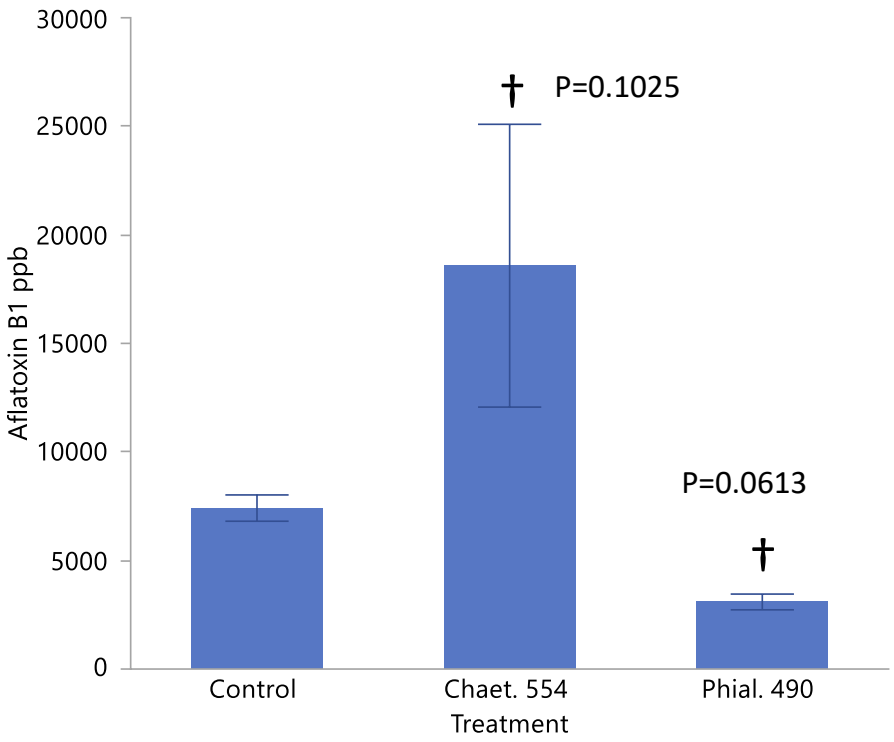

Figure 12 Aflatoxin accumulation was affected by the seed treatments *C. globosum* 554 and *P. inflatum* 490 in B73 inbred maize inoculated with *A. flavus* in Corpus Christi in 2016. Bars are mean aflatoxin B1 ppm accumulation quantified from ground meal. Average and significance were calculated over three biological replicates; error bars indicate ± SE. Differences between means were analyzed for statistical significance by Dunnett's pairwise test of log transformed aflatoxin (* $P \leq 0.05$, † > 0.05: indicates non-significant differences).

In order to evaluate fungal symbionts for their ability to reduce the risk of aflatoxin contamination in maize under the field conditions at other Texas locations, seeds of B73 inbred and Sp596, Sp799, Tx777, and Tx779 hybrid maize were treated with the fungal symbionts and planted in field trials in College Station and Lubbock in 2018 and 2019. Ears were injected 10-14 days past midsilking with either mock-inoculum or *A. flavus* spore suspensions. In 2018, resulting aflatoxin levels analyzed by

ANOVA indicated seed treatments were significantly different in B73 plots treated with *A. flavus* in College Station ($P = 0.0151$) (Fig. 13A). In College Station, Tx779 plots treated with *A. flavus* showed a significant difference between seed treatments ($P = 0.0137$) and in these plots, *E. nigrum* 89 treatment resulted in increased aflatoxin content compared to control by almost 4.3-fold ($P = 0.0316$) (Fig. 13E). In Lubbock, Tx799 plots treated with *A. flavus* displayed differences among the treatments ($P = 0.0678$), and *P. herbarum* 473 and *A. alternatum* 505 had a trend of increased aflatoxin compared to control ($P = 0.0721$; $P = 0.0809$, respectively).

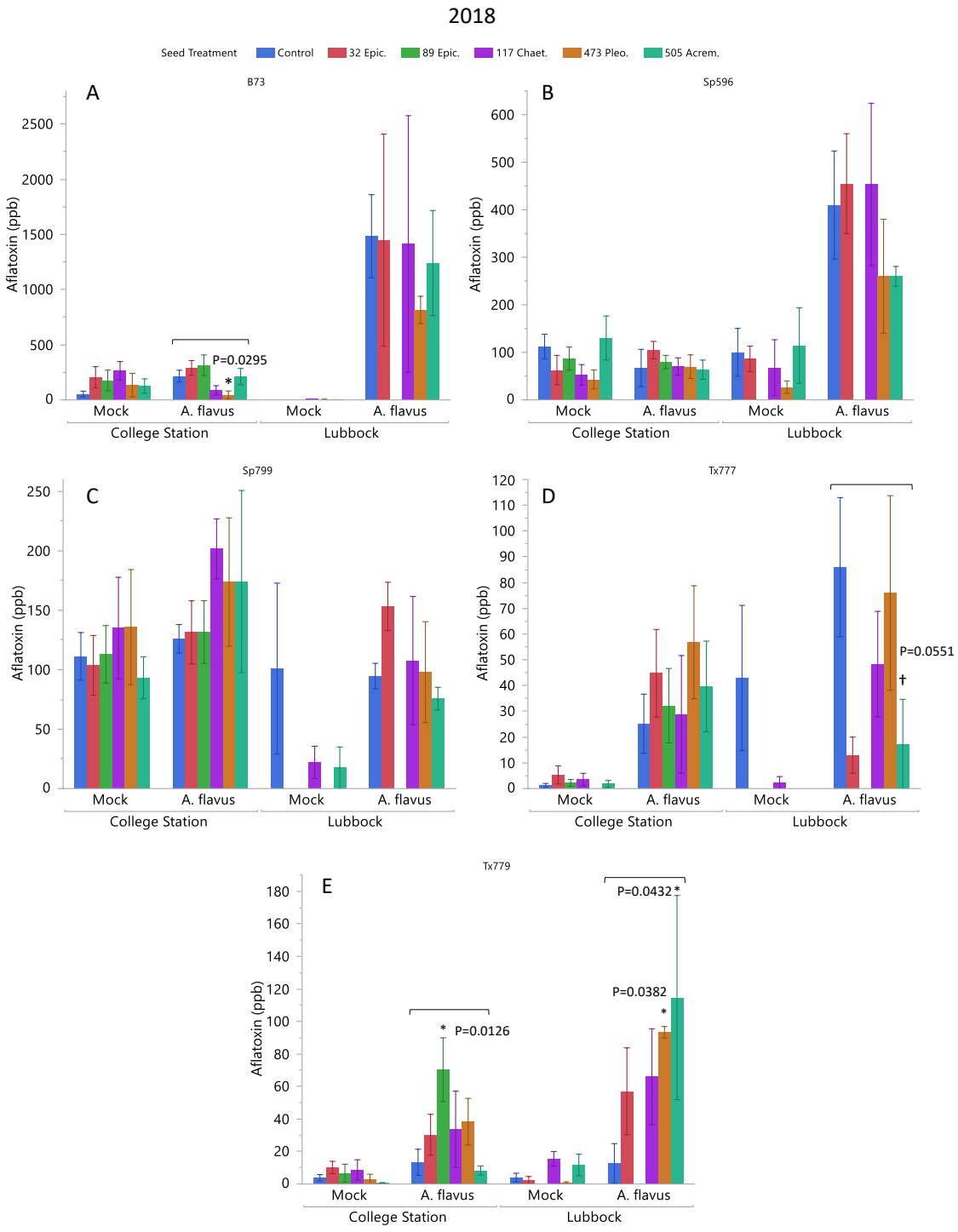

Figure 13 Aflatoxin contamination in field trials was highly variable and not significantly influenced by fungal symbionts in College Station in 2018. *E. nigrum* 89 was not tested in Lubbock. Bars represent average aflatoxin B1 ppb in maize of 6 and 3

biological replicates in College Station and Lubbock, respectively; error bars indicate ± SE. Differences between means were analyzed for statistical significance by Dunnett's pairwise test of log(aflatoxin+1) transformed data (* $P < 0.05$, † ≥ 0.05: indicates non-significant differences).

In 2019, year 2 of the field trials, maize hybrids Tx777 and Tx779, which had been determined to be resistant to mycotoxin contamination from the results above, were removed from the experimental design in favor of increasing the replicate numbers in Lubbock of the remaining genotypes. Thus, for field trials in 2019, B73 inbred and Sp596 and Sp799 hybrid maize were planted in field trials in College Station and Lubbock. Since fungal symbionts may be chosen individually as biocontrol agents and analyzed for effectiveness across multiple genotypes, seed treatments were individually compared to control treatments across genotypes to show significant results. The interaction between maize genotype and fungal seed treatment was significant (P = 0.0224) in College Station so means were separated within each genotype by Tukey's HSD (Fig. 14). *E. nigrum 89* treatments showed significantly reduced aflatoxin in B73 inbred, from 156.2 ppb in control to 17.3 ppb in the *E. nigrum* 89-treated mock-inoculated plots, a decrease of 89.1% (P = 0.0420). In addition, *A. alternatum* 505 reduced aflatoxin in mock-inoculated B73 in College Station by 76.3% though results were not significant. Conversely, although ANOVA showed no differences between seed treatments in Sp596 and Sp799 hybrid genotypes (P = 0.9780 and P = 0.0602, respectively), the treatment with the other strain of *E. nigrum*, TAMU89, resulted in an increase of 265% in *E. nigrum* 32 treated plots of Sp799 maize though not significantly (P = 0.1493).

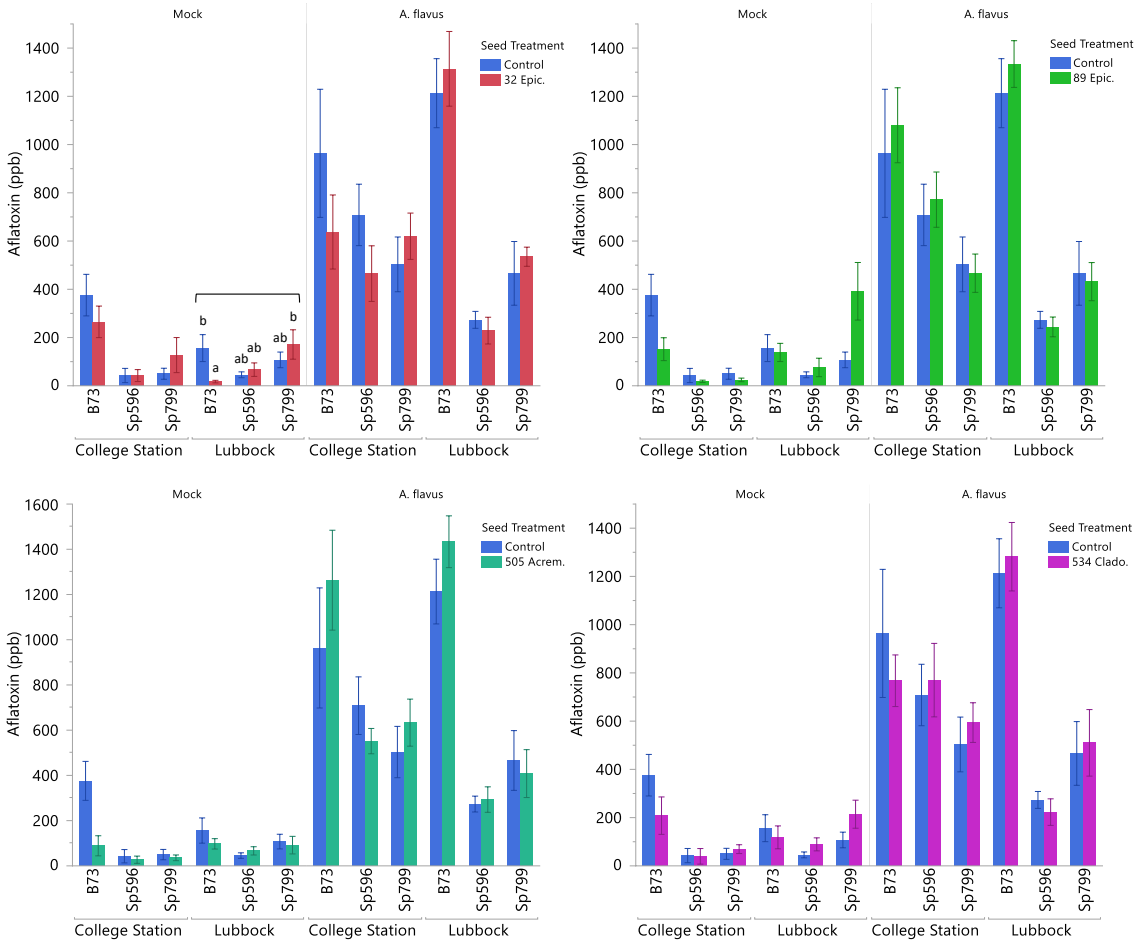

Figure 14 Aflatoxin contamination in field trials was reduced significantly by *E. nigrum* 89 in Lubbock in 2019. Bars represent average aflatoxin B1 ppb in maize of 6 biological replicates; error bars indicate ± SE. Different letters indicate significant differences between means determined by Tukey's HSD of log transformed data ($P \leq 0.05$).

Effects of fungal symbionts on fumonisin contamination of maize under field conditions:

In order to evaluate fungal symbionts for the ability to reduce the risk of fumonisin contamination in maize under the field conditions, seeds of B73 inbred and

Sp596, Sp799, Tx777, and Tx779 hybrid maize were treated with fungal symbionts and planted in field trials in College Station and Lubbock in 2018. Ears were injected 10-14 days past midsilking with either mock-inoculum or *F. verticillioides* spore suspensions. In 2018, resulting fumonisin levels analyzed by ANOVA indicated seed treatments were significantly different in Lubbock (Fig. 15). Plots of Tx779 treated with mock-inoculum were almost significantly different ($P = 0.0663$), however there were no significant differences found between seed treatments tested by Tukey's HSD ($P \leq 0.05$) within location by genotype by ear treatment groups.

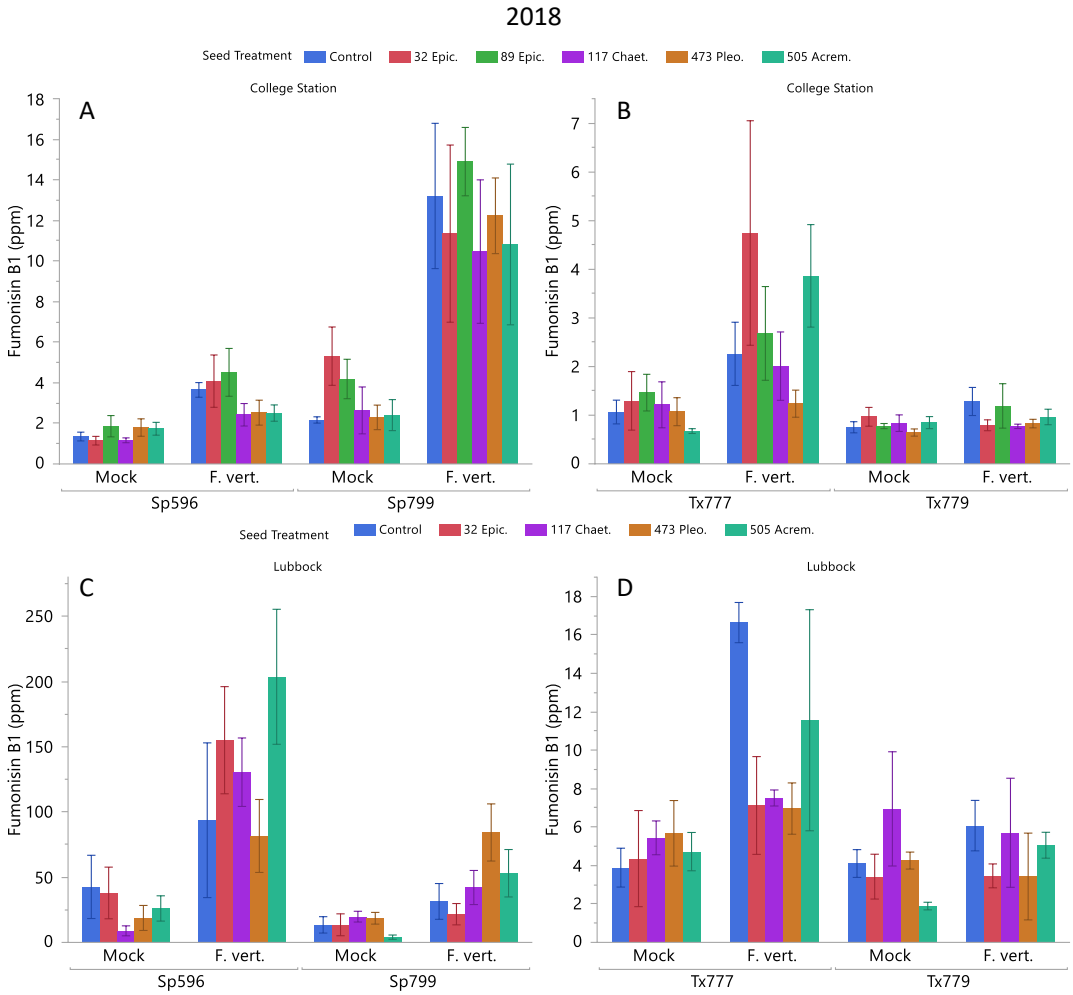

Figure 15 Fumonisin contamination in field trials was not significantly affected by fungal symbionts in College Station in 2018. *E. nigrum* 89 was not tested in Lubbock. Bars represent average fumonisin B1 concentrations in maize of 6 and 3 biological replicates in College Station and Lubbock, respectively; error bars indicate ± SE. Differences between means were analyzed for statistical significance by Dunnett's pairwise test of log(fumonisin) transformed data (* $P \leq 0.05$, † ≥ 0.05: indicates non-significant differences).

In 2019, fumonisin levels among the two locations and three genotypes were significantly different (both $P < 0.0001$) while there were no difference in seed treatments ($P = 0.1681$). Genotype x seed treatment interaction was also not significant

(P = 0.7722), however, location x seed treatment interaction was (P = 0.0494), and thus seed treatments were analyzed within locations (Fig. 16). No significant differences were seen among seed treatments when comparing means within genotype x ear treatment groups by Tukey's HSD.

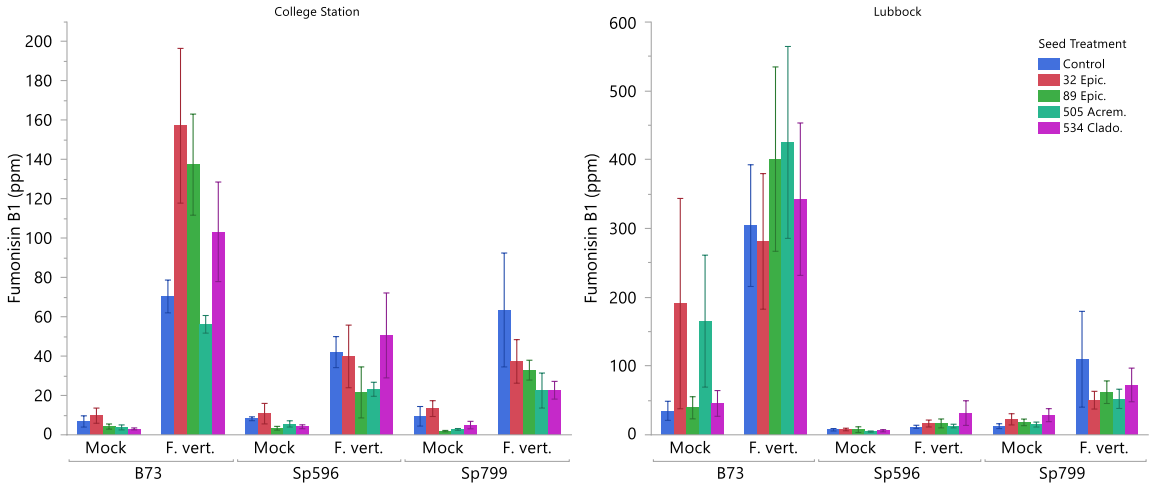

Figure 16 Fumonisin contamination in field trials was not significantly affected by fungal symbionts in College Station and Lubbock in 2019. Bars represent average fumonisin B1 ppm in maize of 6 biological replicates; error bars indicate ± SE. Differences between means were analyzed for statistical significance by Dunnett's pairwise test of log(fumonisin) transformed data (* $P < 0.05$, † ≥ 0.05: indicates non-significant differences).

When the fumonisin levels above were analyzed in separate genotypes, ANOVA showed that in Sp596 in College Station there were significant differences among seed treatments (P = 0.0327) and ear treatments ($P < 0.0001$) (Fig. 17A). However, the seed treatment by ear treatment interaction was not significant (P = 0.6620), which allowed for the analysis of seed treatment means in combined seed treatments. This analysis

showed that *E. nigrum* 89 reduced fumonisin accumulation by 56.6% (P = 0.0186). *A. alternatum* 505 treatment showed a consistent trend of reducing fumonisin in all three maize genotypes (Fig. 17B). When only compared to the control, *A. alternatum* 505 reduced fumonisin in *F. verticillioides*-inoculated ears of Sp799 by 60.5% (P = 0.0028).

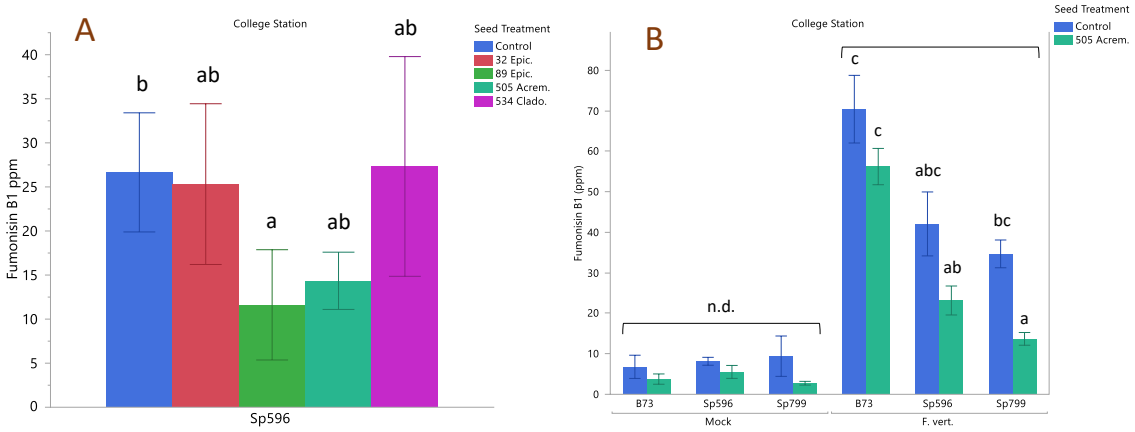

Figure 17 Fumonisin contamination in 2019 was significantly reduced A) in Sp596 hybrid by *E. nigrum* 89 in combined plots of mock and *F. verticillioides* ear treatment, and B) in Sp799 hybrid treated with *A. alternatum* 505 and ear inoculated with *F. verticillioides* in College Station. Bars represent average fumonisin B1 concentrations in maize of A) 12 and B) 6 biological replicates; error bars indicate ± SE. Means were compared by post hoc analysis with Tukey's HSD of log transformed data (P < 0.05).

Effects of fungal symbionts on Anthracnose stalk rot in maize:

To determine if one or more beneficial fungal symbionts could improve plant health through increasing disease resistance, a subset of plants within field plots in College Station only in both years of field trials were inoculated with *C. graminicola* and evaluated after 9-11 days of incubation. In 2018, ANOVA indicated genotypes, seed

treatments, and genotype x seed treatment interaction effects were all significant ($P <$ 0.0001) and thus seed treatments were compared within each genotype separately (Fig. 18). In Sp596 hybrid, *E. nigrum* 32, *C. globosum* 117, *P. herbarum* 473, and *A. alternatum* 505 all significantly increased lesion areas by 117%, 163%, 115%, and 122%, respectively. *E. nigrum* 89 also increased lesion area in Sp596 but not significantly by Dunnett's test. In contrast, *A. alternatum* 505 alone affected Sp799, where the treated plots had a 29.4% increase in lesion area. B73 inbred and Tx777 and Tx779 hybrids were not significantly affected by any of the fungal seed treatments.

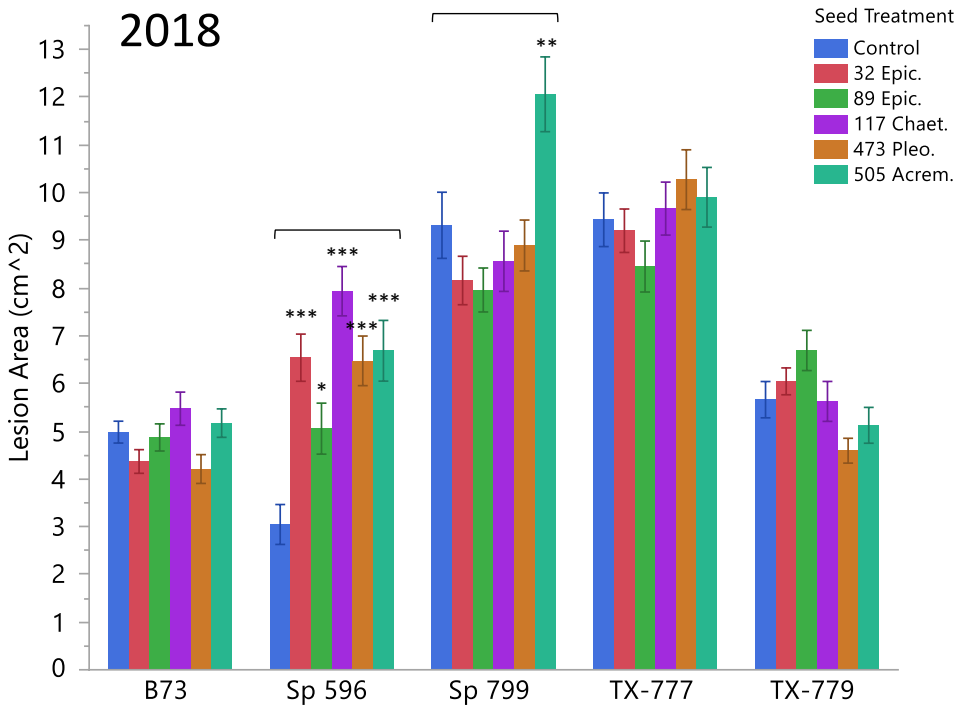

Figure 18 Lesion areas due to Anthracnose stalk rot show fungal symbionts induce susceptibility to *C. graminicola* in 2018. Bars represent average lesion areas in cm^2 calculated from four internodes per plant with 10 biological replicates each; error bars indicate ± SE. Pairwise statistical differences between treatments and the control group were compared using Dunnett's test (*$P < 0.05$; **$P < 0.01$ ***$P \leq 0.0001$).

Results from the field trial in 2019 showed significant differences between block and genotype (both $P < 0.0001$), block x genotype ($P = 0.0069$), seed treatment ($P = 0.0002$), and block x seed treatment ($P = 0.0164$) but not between genotype x seed treatment interactions ($P = 0.1629$) or rep x genotype x seed treatment ($P = 0.1048$) (Fig. 19). *E. nigrum* 89 had a trend of reduced lesion area in Sp596. *A. alternatum* 505 significantly reduced lesion areas in B73 ($P = 0.0063$) and Sp799, though not significantly by Tukey's HSD.

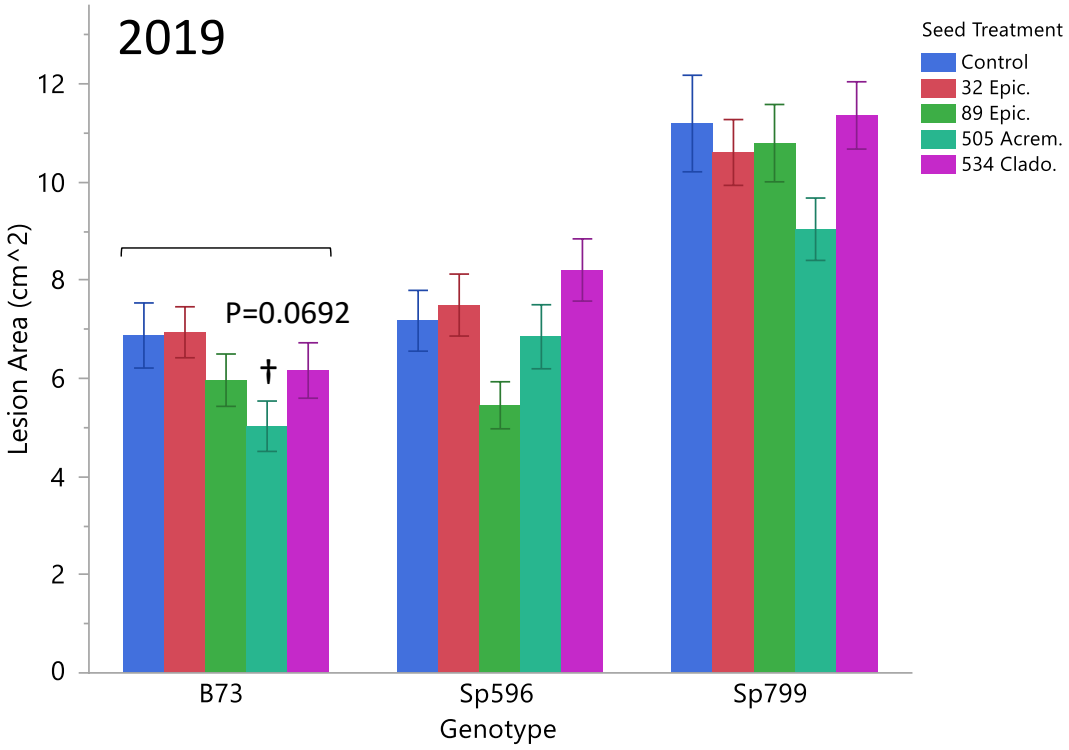

Figure 19 Reduction of lesion areas due to Anthracnose stalk rot show induction of ISR by *E. nigrum* 89 and *A. alternatum* 505 seed treatments in 2019. Bars represent average lesion areas in cm^2 calculated from four internodes per plant with 6 biological

replicates each; error bars indicate ± SE. Pairwise statistical differences between treatments and the control group were compared using Dunnett's test (*$P < 0.05$).

Fungal symbiont effects on plant development of maize under field conditions

Effects of fungal symbionts on germination of maize seeds:

In College Station in 2018, seed treatment showed no effect on germination ($P = 0.3482$), however genotype ($P < 0.0001$) as well as genotype x seed treatment interaction did have a significant effect ($P = 0.0008$) (Fig.20). Therefore, seed treatment means were analyzed within genotypes and revealed that *E. nigrum* 89 increased germination significantly in Sp596 from 83.2% in control to 92.3% ($P = 0.0165$).

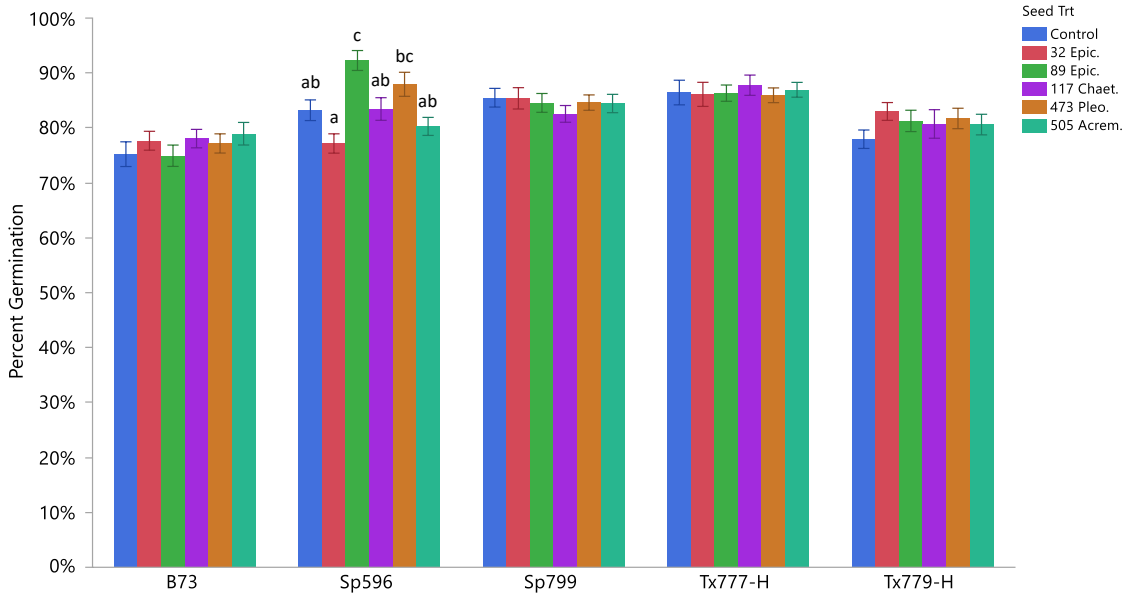

Figure 20 Germination of seeds treated with fungal symbionts in College Station in 2018 shows *E. nigrum* 89 increased percent germination in Sp596. Bars are average percent seed germinated out of total seeds planted of 18 biological replicates; error bars indicate ± SE. Results were converted to proportions and exp transformed before statistical analysis. Differences in letters indicate significant differences between means determined by Tukey's HSD ($P < 0.05$).

Germination data in Lubbock 2018 was analyzed by ANOVA with eight biological replicates and showed a significant difference between genotypes ($P < 0.0001$) and genotype x seed treatment interaction ($P = 0.0235$) but not among seed treatments ($P = 0.4916$) (Fig. 21). Thus, seed treatments means were separated by Tukey's HSD by genotype. Although *A. alternatum* 505 demonstrated a trend of increasing germination rate, there was no significant differences between seed treatments ($P = 0.1820$).

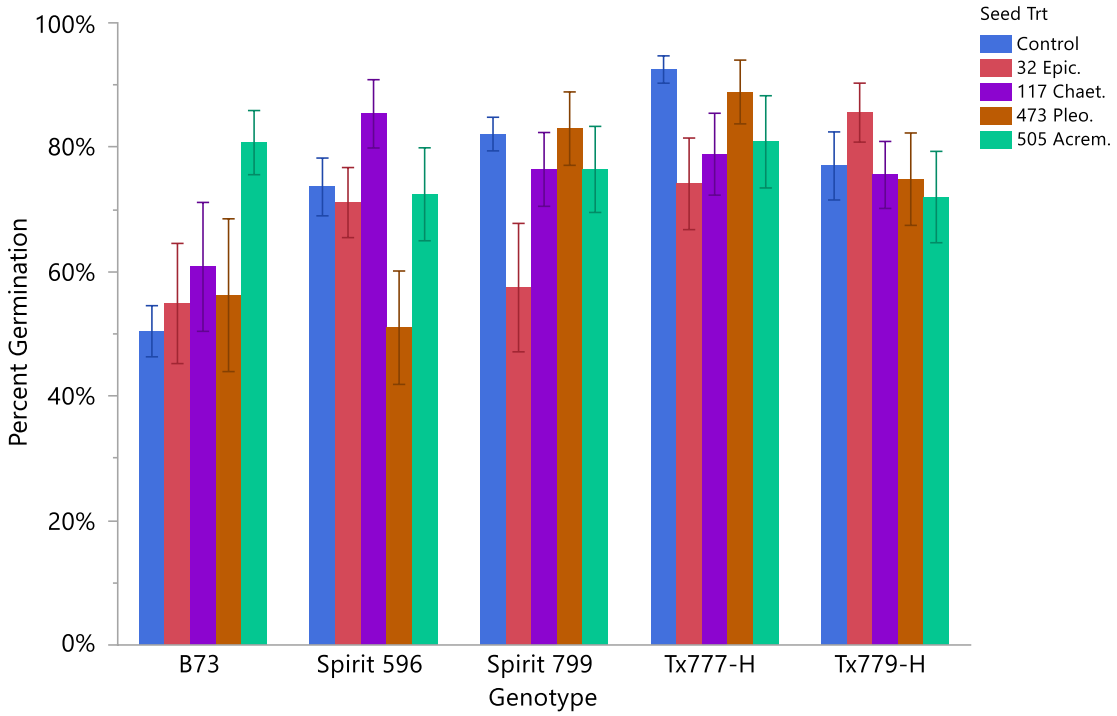

Figure 21 Germination of seeds treated with fungal symbionts in Lubbock in 2018 showed no differences in seed treatments. Bars are average percent seed germinated out of total seeds planted of 9 biological replicates; error bars indicate ± SE. Results were converted to proportions and exp transformed before statistical analysis. Means were compared by post hoc analysis with Tukey's HSD ($P < 0.05$).

Interestingly, if *A. alternatum* 505 and control seed treatments were compared only to each other in each genotype, *A. alternatum* 505 treatment markedly increased germination rate from 50.4% to 80.8% ($P = 0.0383$) (Fig. 22). ANOVA revealed no difference in seed treatments ($P = 0.6383$) whereas there were differences between genotypes ($P = 0.0104$) and genotype x seed treatment ($P = 0.0127$).

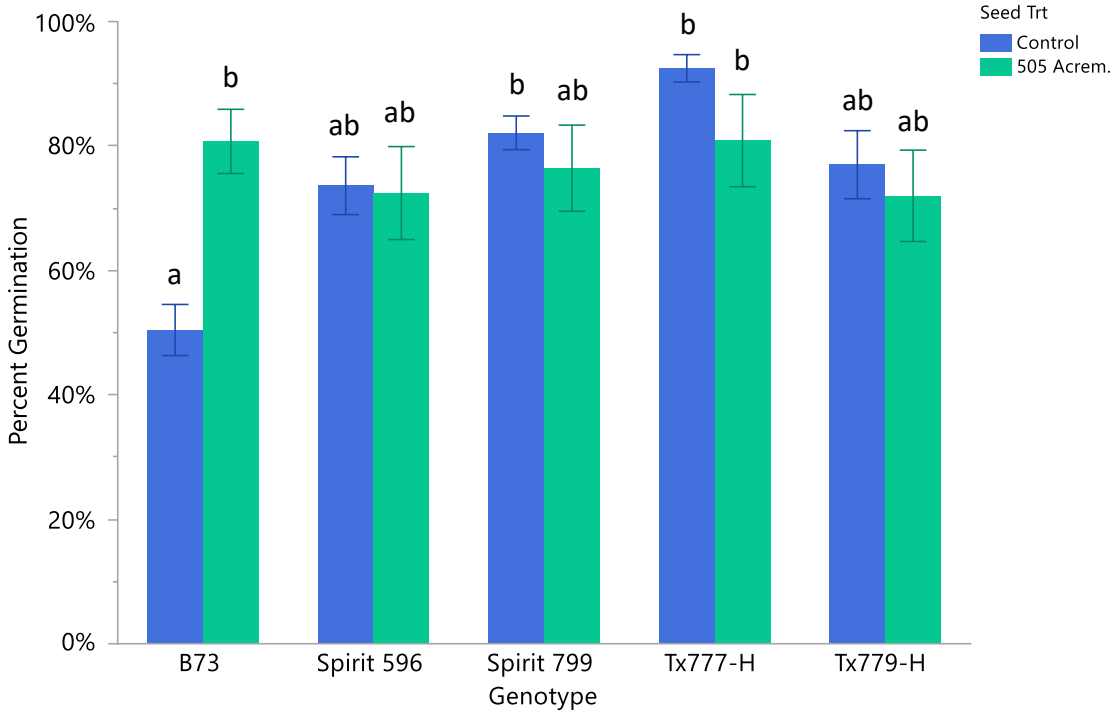

Figure 22 Germination of seeds treated with control or *A. alternatum* 505 in Lubbock in 2018 showed *A. alternatum* 505 improved percent germination in B73 inbred. Bars are average percent seed germinated out of total seeds planted of 9 biological replicates; error bars indicate ± SE. Results were converted to proportions and exp transformed before statistical analysis. Differences in letters indicate significant differences between means determined by Tukey's HSD ($P < 0.05$).

Results from 2019 showed no significant differences in germination between locations, genotypes, or seed treatments (Fig. 23).

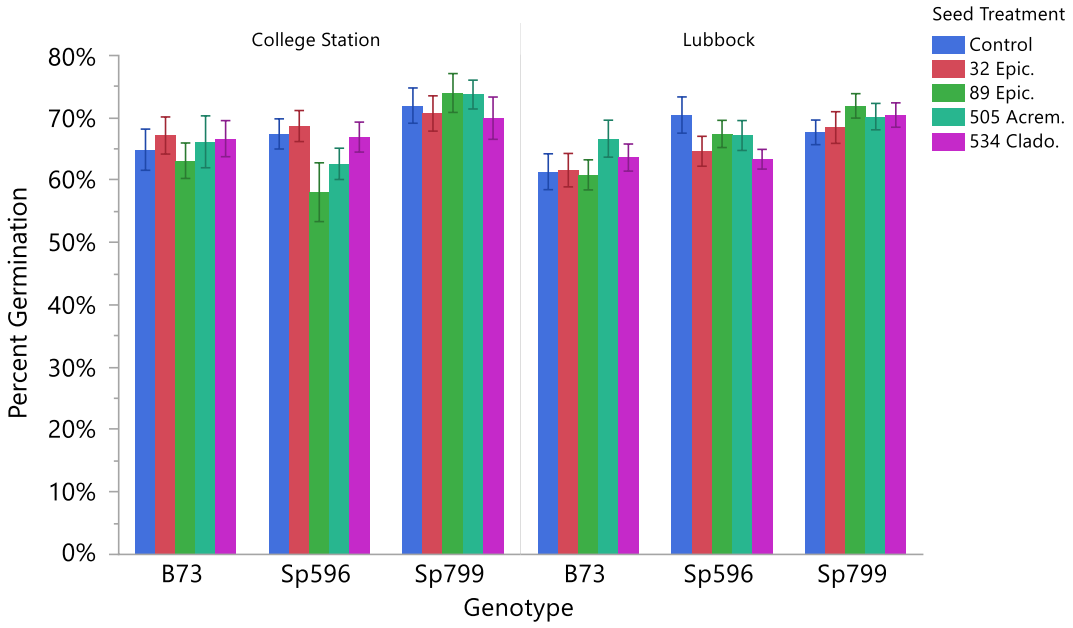

Figure 23 Germination of seeds treated with fungal symbionts in College Station and Lubbock had no differences between treatments in 2019. Bars are average percent seed germinated out of total seeds planted of 6 biological replicates; error bars indicate ± SE. Results were converted to proportions and exp transformed before statistical analysis. Differences in letters indicate significant differences between means determined by Tukey's HSD ($P \leq 0.05$).

Effects of fungal symbionts on plant heights:

Plant height in 2018 field trials had to be analyzed by location separately as seed treatment *E. nigrum* 89 was not included in Lubbock due to limited seed availability and plants were measured at different times (~1 week later in Lubbock). Even if *E. nigrum* 89 was excluded from analysis of combined results from both locations, the effect of

location ($P < 0.0001$), genotype ($P < 0.0001$), and location x genotype ($P < 0.0001$) were all significant, but there was no difference between seed treatment ($P = 0.2301$) (Fig. 24). There was, however, a significant effect in location x genotype x seed treatment interaction ($P < 0.0001$). Therefore, seed treatment means were separated with Tukey's HSD by location and genotype. In College Station plant height of Sp596 was reduced from 62.2 cm in control to 56.6 cm in *A. alternatum* 505-treated plots, though not significantly ($P = 0.0714$). In Lubbock, plant height was reduced in Tx779 by *C. globosum* 117.

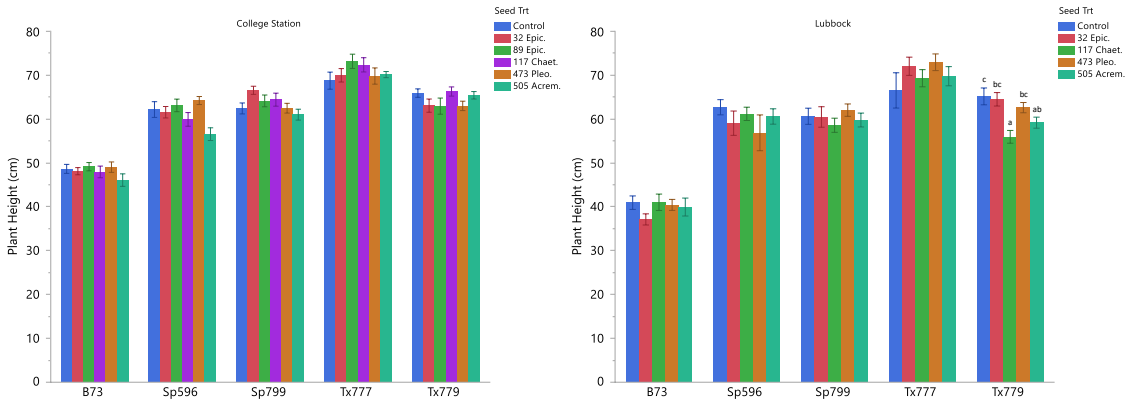

Figure 24 Plant heights of College Station and Lubbock in 2018 showed only *C. globosum* 117 seed treatment reduced plant growth. *E. nigrum* 89 was not tested in Lubbock. Bars are average plant height of three plants per plot recorded at 4 weeks after planting from 18 biological replicates. Differences in letters indicate significant differences between means determined by Tukey's HSD ($P < 0.05$).

In the 2019 field trials, there was a significant effect of genotype in College Station ($P < 0.0001$) and Lubbock ($P < 0.0001$) but not in seed treatment or genotype x seed treatment interaction (Fig. 25). The two locations could not be directly compared as they were measured at different time points. In Lubbock, blocks had a significant effect

on plant height ($P < 0.0001$) but seed treatment and rep x seed treatment effects were not significant ($P = 0.98$ and 0.99, respectively).

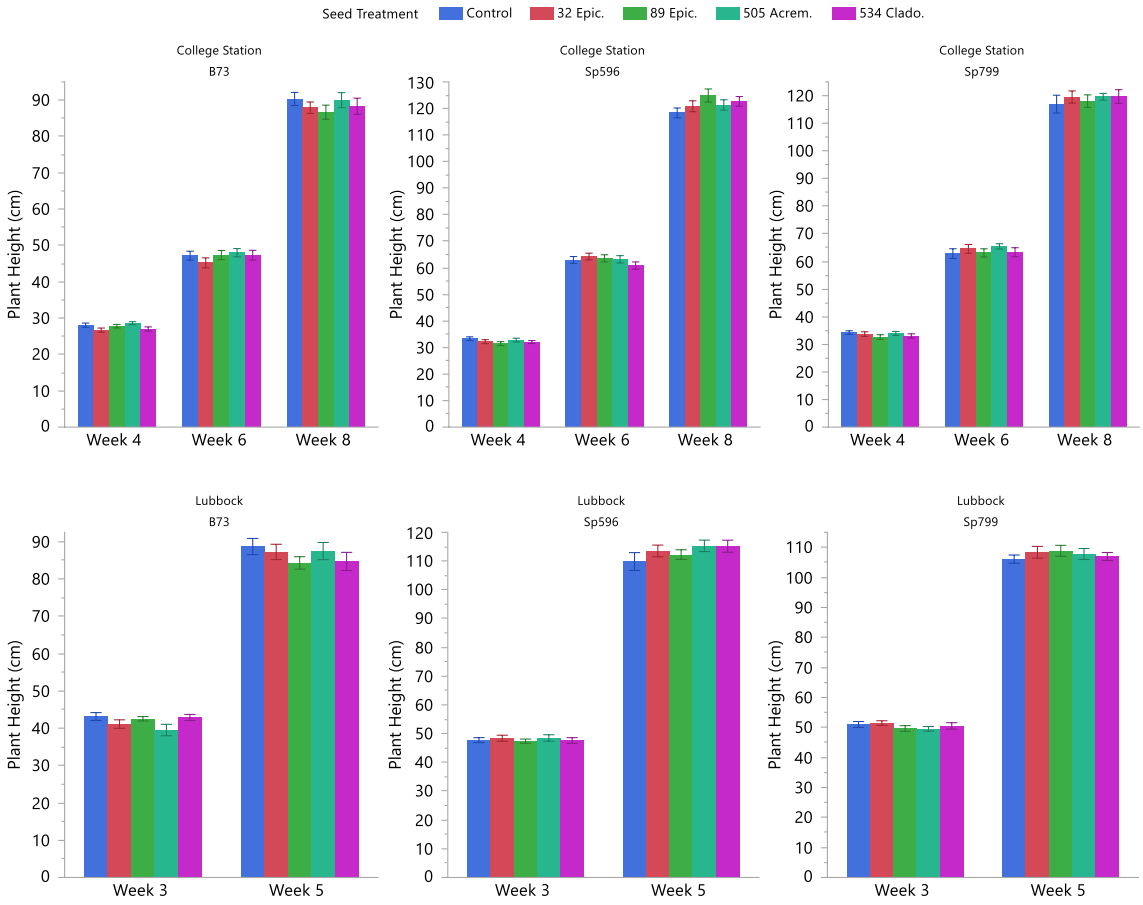

Figure 25 Plant heights of College Station and Lubbock in 2019 showed no significant differences between seed treatments. Bars are average plant height of three plants per plot recorded at 4, 6, and 8 weeks in College Station and 3 and 5 weeks in Lubbock after planting from 16 biological replicates. Means were compared post hoc by Tukey's HSD ($P < 0.05$).

Effects of fungal symbionts on time to midsilk

In order to accurately inoculate ears 10-14 days past midsilking, silking was recorded at multiple time points and differences between treatments were analyzed.

Days to silking were significantly different between locations ($P < 0.0001$), genotypes ($P = 0.0196$), and location by genotype interaction ($P < 0.0001$), but not among seed treatments ($P = 0.2676$) (Fig. 26).

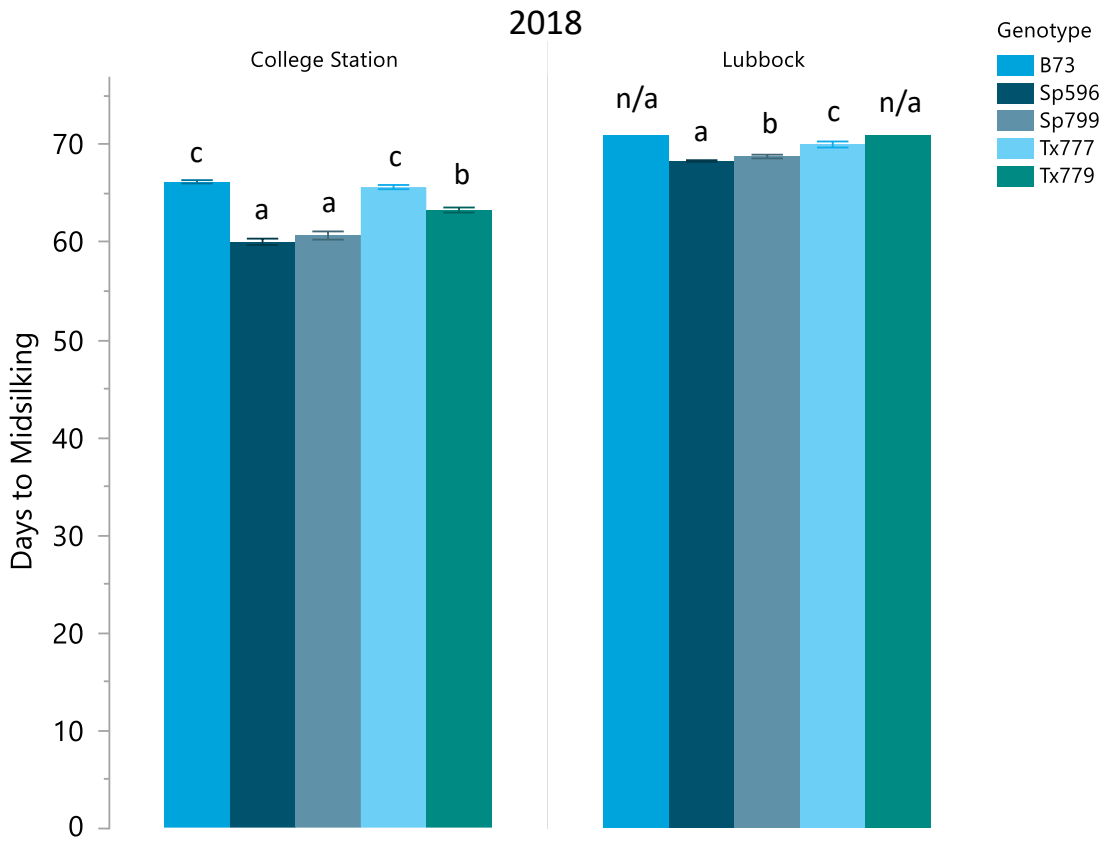

Figure 26 Days to midsilking showed significant differences between B73 inbred and hybrid genotypes. Bars represent average days to midsilking of 10-16 biological replicates except for B73 and Tx779 in Lubbock which included only 1 rep. Differences in letters indicate significant differences between means determined by Tukey's HSD ($P < 0.05$).

As locations and genotypes were significantly different, silking results were analyzed within location and then within genotypes as well. Results from Lubbock in 2018 showed a significant difference on days to silking among seed treatments

(ANOVA; $P = 0.0155$) and Dunnett's test showed a significant delay in days to silking in plots treated with *P. herbarum* 473 compared to control ($P = 0.0269$) (Fig. 27).

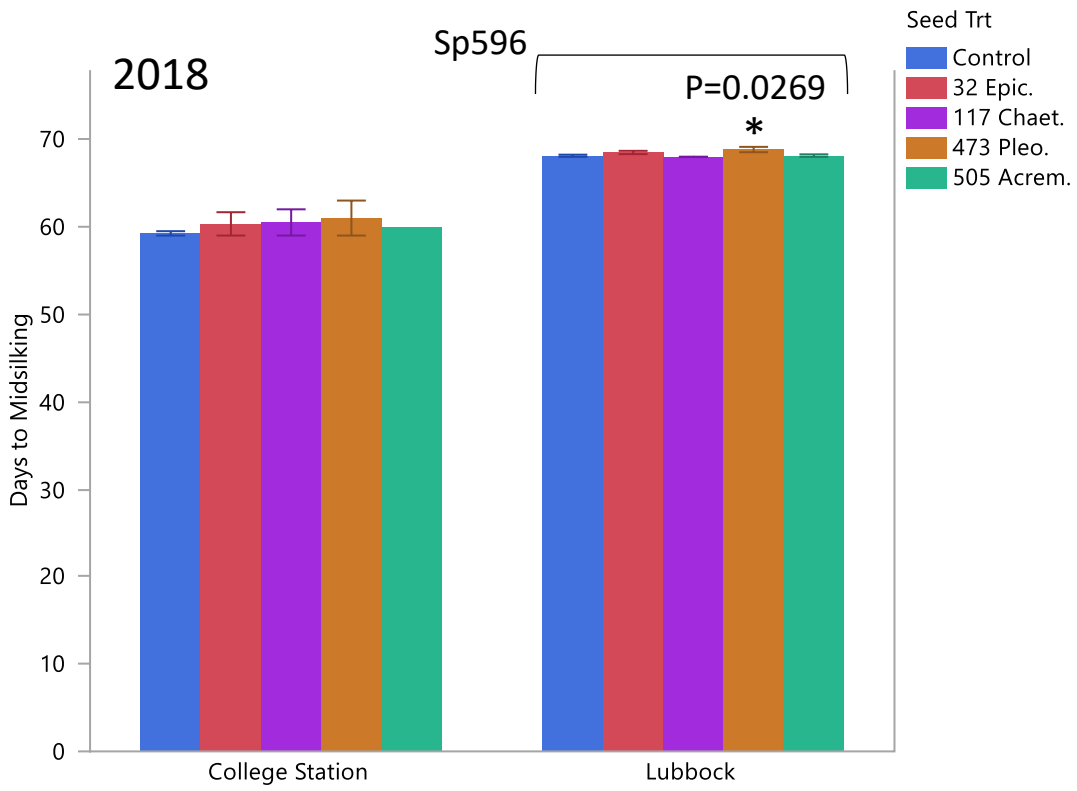

Figure 27 Time to midsilking was delayed in 2018 in plants seed-treated with *P. herbarum* 473. Bars represent average days to midsilking in days, determined by counting the silked vs. unsilked ears per plot on multiple days and estimating the day at which 50% of ears reached midsilking. Average and significance were calculated from 8-12 biological replicates; error bars indicate ± SE. Pairwise statistical differences between treatments and the control group were compared using Dunnett's test (*$P <$ 0.05).

In 2019, silking was recorded later due to time constraints, thus data were not converted to days after midsilking as I was unable to estimate it accurately for Sp596 (Fig. 28). Results from Lubbock showed an overall trend of delayed silking in plots

treated with *E. nigrum* 32. In particular, *E. nigrum* 32 delayed the rate of silking significantly compared to control in Sp596 on Aug. 10.

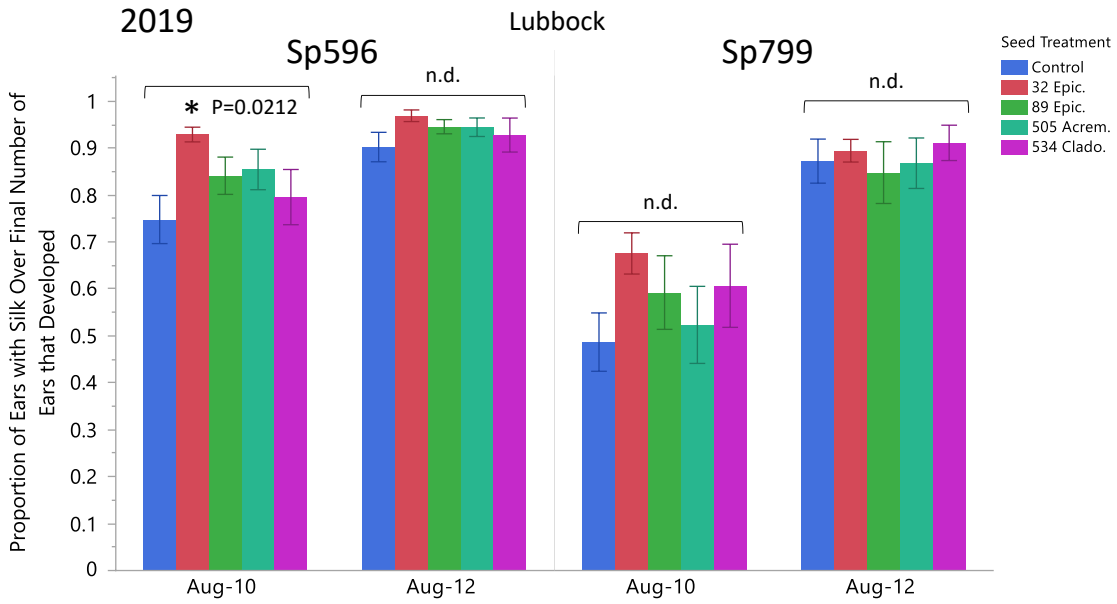

Figure 28 Time to midsilking was delayed in 2019 in plants seed-treated with *P. herbarum* 473. Bars represent average days to midsilking in days, determined by counting the silked vs. unsilked ears per plot on multiple days and estimating the day at which 50% of ears reached midsilking. Average and significance were calculated from 9 biological replicates; error bars indicate ± SE. Pairwise statistical differences between treatments and the control group were compared using Dunnett's test (*$P < 0.05$).

Effects of fungal symbionts on yield:

In order to evaluate the potential of these fungal symbionts for improvement of maize yield, yield per plot, thousand kernel weight, and weight of kernels per ear were

measured. Seed treatment with *E. nigrum* 89 led to a significant increase in yield in Sp596 (Fig. 29).

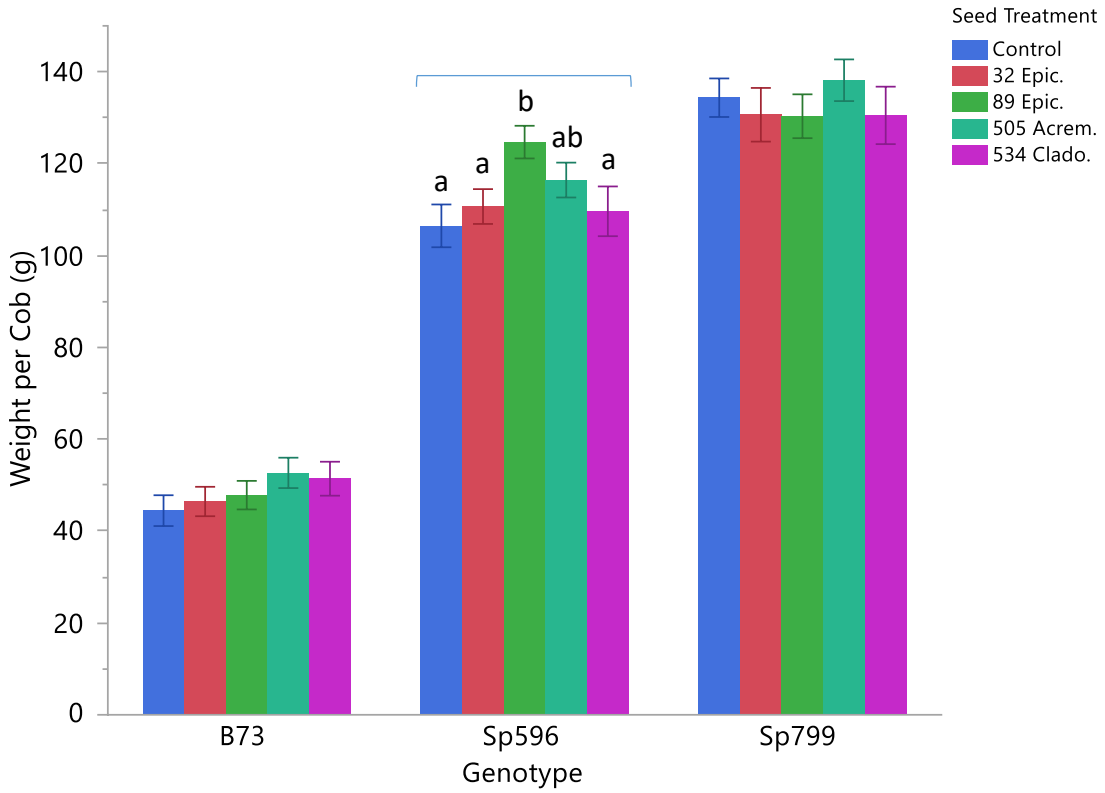

Figure 29 Weight of kernels per ear was increased by *E. nigrum* 89 in Sp596 hybrid maize in College Station field trial in 2019. Bars represent average weight of kernels in g per ear. Different letters indicate significant differences between means determined by Tukey's HSD ($P < 0.05$).

Fungal symbiont-mediated defense against insect herbivory on maize

Incidence of insect damage on leaves:

In order to help determine if beneficial fungal symbionts led to increased resistance against insect herbivory, incidence of insect damage was recorded at multiple time points. In 2018 damage was recorded only for plants grown in Lubbock as insect

pressure was negligible in College Station that year. Data were collected from two of the three blocks for a total of 6 reps per genotype-seed treatment combination, and as the data were not normally distributed they were log transformed before analysis to improve normality. There was a significant difference between genotypes ($P < 0.0001$) but not between seed treatments ($P = 0.1156$) or genotype x seed treatment interactions ($P = 0.3362$) (Fig. 30). When symbiont treatments were analyzed alone, ANOVA showed no significant difference across all genotypes ($P = 0.0940$) but did show differences within B73 ($P = 0.0243$), wherein *A. alternatum* 505 reduced insect damage from 70.6% in control plots to 20.8% in the treated plots ($P = 0.0155$). *C. globosum* 117 and *P. herbarum* 473 also showed similarly reduced insect damage, but were not statistically significant.

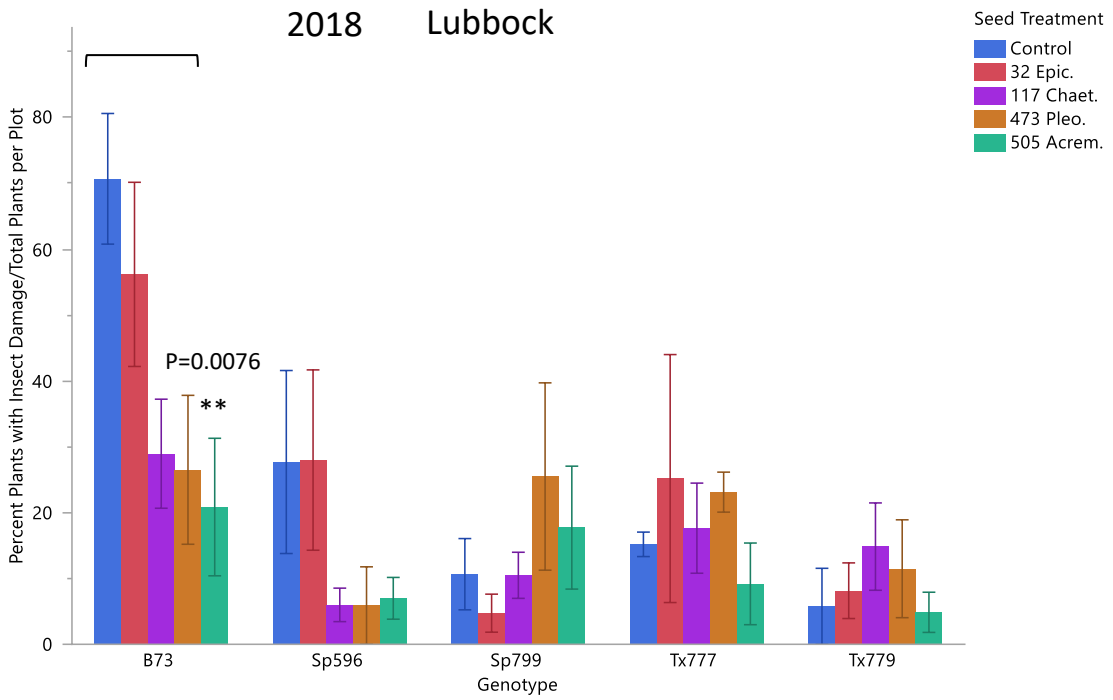

Figure 30 Incidence of insect damage in Lubbock in 2018 showed *A. alternatum* 505 significantly reduced the occurrence of insect damage in field plots. Bars represent average number of plants with insect damage over total number of plants per plot from two blocks for a total of 6 biological replicates; error bars indicate ± SE. Pairwise statistical differences between treatments and the control group were compared using Dunnett's test (*$P < 0.05$; **$P < 0.01$).

In 2019, no significant effects were found between averages of seed treatments. *E. nigrum* 32, *A. alternatum* 505, and *C. oxysporum* 534 treatments had trends of lower occurrence of insect damage but were not significant by Dunnett's tests (Fig. 31).

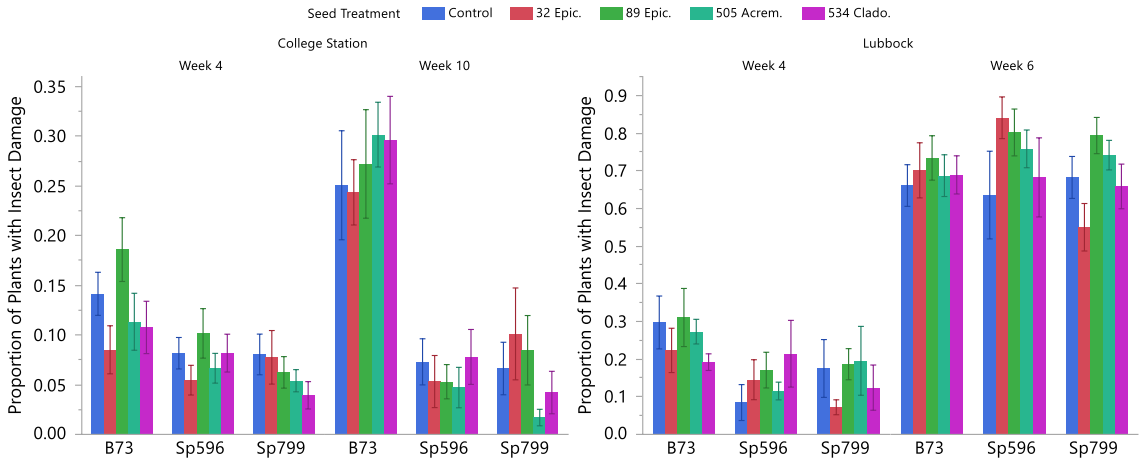

Figure 31 Incidence of insect damage on maize leaves in 2019 showed only non-significant effects by fungal symbionts in field plots. Bars represent average number of plants with insect damage over total number of plants per plot from two blocks for a total of 6 biological replicates; error bars indicate ± SE. Asterisks indicate significant differences between means determined by Dunnett's test of log transformed data ($P < 0.05$).

Intriguingly, a potential difference in the two strains of *E. nigrum* was detected in Lubbock in week 6. Insect damage was recorded by random selection from blocks 1, 3, and 5 and were significantly different ($P < 0.0001$); specifically block 5 was different from 1 and 3 (Tukey's HSD, $P < 0.05$) (Fig. 32). Further testing would be needed to confirm *E. nigrum* 32 may improve maize resistance to insect damage.

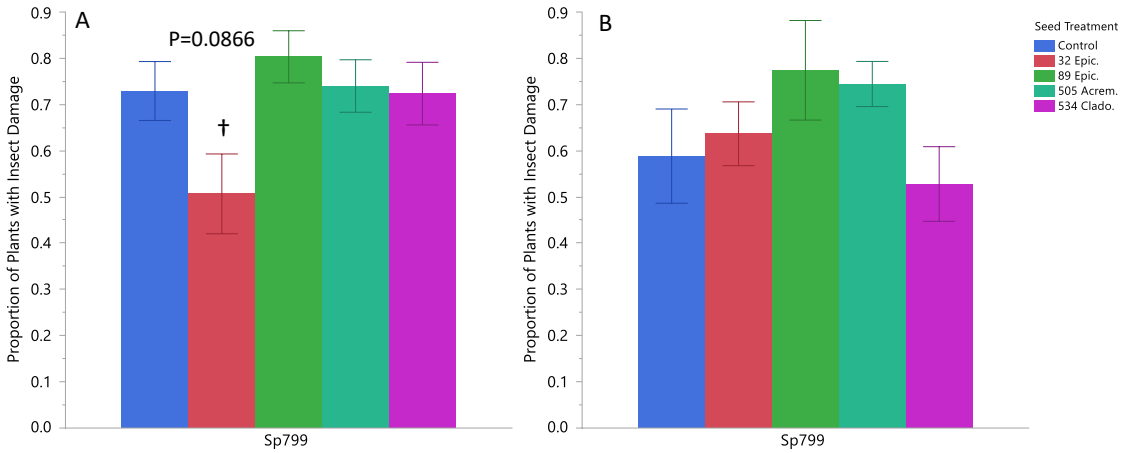

Figure 32 Incidence of insect damage in Lubbock in 2019 showed two strains of *E. nigrum* had significantly different effects on insect damage in field plots. Bars represent average number of plants with insect damage over total number of plants per plot from A) blocks 1 and 3 and B) block 5 with a total of 3 biological replicates; error bars indicate ± SE. Differences in letters indicate significant differences between means determined by Tukey's HSD of log transformed data ($P < 0.05$).

Summary and correlations of results from field trials:

To summarize the findings of the field trials in 2018 and 2019, Table 2 provides all significant results and non-significant trends found in maize treated with the selections of beneficial fungal symbionts.

Table 2 Summary of trends and significant results from field trials of *E. nigrum* 32, *E. nigrum* 89, and *A. alternatum* 505 for College Station and Lubbock from 2018 and 2019.

Variable	Location	*Epicoccum nigrum* 32		*Epicoccum nigrum* 89		*Acremonium alternatum* 505	
Year		2018	2019	2018	2019	2018	2019
Aflatoxin	College Station				↓ *in B73 mock		↓ † in B73 mock
	Lubbock		↓ *in B73 mock	↑ *in Tx779 *A. flavus*	↑ † in Sp799 mock	↓ † in Tx777 *A. flavus.* ↑ *in Tx779 *A. flavus.*	
Fumonisin	College Station				↓ *in Sp596 *F. vert*		↓ † in Sp799 *F. vert.*
	Lubbock						
Stalk Rot Lesion Area	College Station	↑ *** In Sp596		↑ *** In Sp596		↑ *** In Sp596 and Tx777	↓ † in B73, * in combined geno.
Insect Damage	Lubbock					↓ * in B73	

Results from both locations in 2018 were analyzed by principal component analysis and points colored by seed treatment (Fig. 33). PCA indicated a negative relationship between aflatoxin levels and both 1000 kernel weight and days to midsilking, while fumonisin was inversely related to yield and number of cobs per plot. There were no clear groupings found in fungal seed treatments in relation to the variables included.

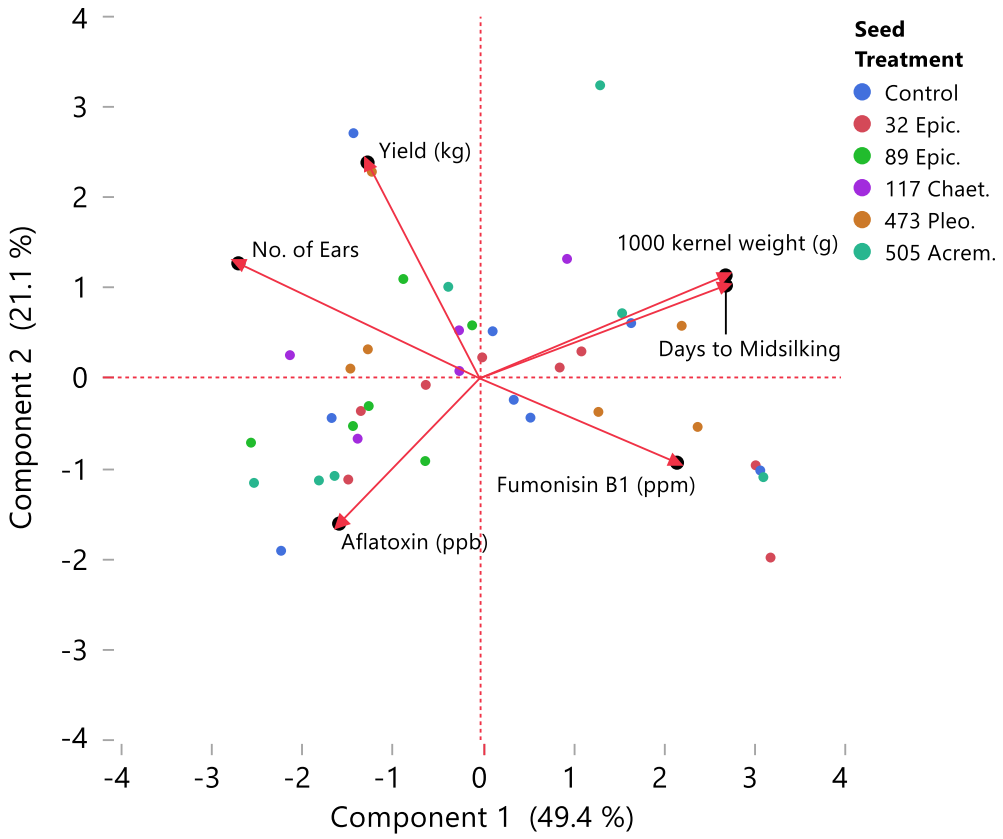

Figure 33 Principal component analysis of the relationships in maize of multiple traits: yield per plot, number of cobs, 1000 kernel weight, days to midsilking, fumonisin, and aflatoxin, excluding the susceptible inbred B73 from analysis.

DISCUSSION

The current changing climate and expected growth of global population to 9.8 billion by the year 2050 (United Nations, 2019) necessitates the development of new approaches for improving global food production and food safety. Furthermore, new concerns about the ecological sustainability of conventional agriculture are making additional methods of biocontrol for insects and pathogens and increased yield all the more necessary.

In this study I evaluated the potential for selected fungal symbionts to inhibit infection in maize of one or both of the ear rot pathogens, *A. flavus* and *F. verticillioides*, from both naturally occurring and artificially-treated inoculum sources, as well as the fungal pathogen *Colletotrichum graminicola*, the causal agent of Anthracnose stalk rot. Towards these goals, I first tested for biocontrol activity of two fungal symbionts in a maize kernel bioassay. Both *P. inflatum* 490 and *C. globosum* 554 were able to reduce the aflatoxin production of *A. flavus* by 52.1% and 42.2%, respectively, when embryo-wounded seeds were treated with the symbionts and then inoculated two days later with *A. flavus* (Fig. 11). This decrease in aflatoxin was less than that observed for two atoxigenic strains of *A. flavus*, which showed a decrease of 11.6 to 2.3 and 2.5 ng/mL (80% and 78%, respectively) (Degola et al., 2011). However, those reductions were observed on unwounded kernels simultaneously co-inoculated, limiting direct comparison. Bacon et al. (2001) showed that *Trichoderma* sp. could reduce fumonisin production in maize kernels when co-inoculated and even inhibit fumonisin accumulation by 72% when *F. verticillioides* was allowed to grow for 7 days first. In addition, *P. inflatum* 490 showed a significant reduction in aflatoxin in ears grown a field trial in Corpus Christi when *P. inflatum* 490 was applied to seed and ears were inoculated with *A. flavus* (Fig. 12) which led me to test additional fungal symbionts in field trials.

As field conditions can inherently lead to highly variable and sometimes neutral outcomes when conducting trials of new biocontrol agents, I next evaluated the field performance of selected beneficial fungi seed treated on maize silk-channel inoculated

with *A. flavus* and *F. verticillioides* in field trials in College Station and Lubbock over two years. I observed a pattern of genotype-specific interactions in maize with the fungal symbionts, evidenced by certain genotypes treated with a given seed treatments displaying a significant difference in aflatoxin levels. In 2018, there was no significant reduction in aflatoxin concentration by the treatments with fungal symbionts, although *P. herbarum* 473 and *A. alternatum* 505 treatments led to aflatoxin reductions (Fig. 13). In 2019 field trials, however, *E. nigrum* 32 significantly reduced aflatoxin by 89.1% to below the most stringent federal aflatoxin limit of 20 ppb (FDA, 2019) in mock-treated B73 plots in Lubbock (Fig.14). In addition, all seed treatments applied in College Station in 2019 showed a trend of reduced aflatoxin in mock-inoculated B73, suggesting these fungal symbionts hold potential for increasing plant systemic defense against the native *A. flavus* inoculum found in the field depending on maize genotype and location.

The ability of the fungal symbionts to prime immunity towards inhibition of aflatoxin production also appeared to be dependent on the inoculum load applied to ears. *E. nigrum* 89, *A. alternatum* 505, and *C. cladosporioides* 534, all lowered aflatoxin in mock-inoculated B73 in College Station, but not in *A. flavus*-inoculated ears (Fig.13), suggesting a tripartite interaction, in which fungal symbionts can improve resistance to low levels of inoculum that growers would experience naturally, but are overwhelmed when maize ears are artificially challenged with inoculum levels much greater than would otherwise occur in nature. This indicates that fungal symbionts can provide protection against naturally occurring sources of ear rot pathogens, and further testing should include other treatment methods such as the common method of sowing of *A.*

flavus spores on infected grain husks between plots. The amount of *A. flavus* inoculum that silks, and thus ears, experience could be much lower than the amount of spores applied as silk-channel inoculum. Bothast et al. (1978) conducted an airborne mold survey from Maryland, Virginia, South Carolina, and Georgia and found that culture plates left exposed in the field at ground and ear level detected only 13 colonies of *A. flavus* recovered on 260 exposed plates, suggesting $3x10^6$ spores per ear is more than adequate for testing natural exposure to *A. flavus*.

Fumonisin accumulation showed no significant differences between symbiont treatments in 2018 (Fig.15). Intriguingly, all fungal symbionts led to varying levels of decreased fumonisin in Tx777 inoculated with *F. verticillioides* in Lubbock. *P. herbarum* 473 showed the same reduction of fumonisin, to a lesser extent, for Tx777 in College Station. Similar effects by the two symbionts were also observed in terms of aflatoxin levels for Tx777, suggesting these fungal symbionts could improve resistance to the higher *A. flavus* and *F. verticillioides* inoculum levels equivalent to those provided by silk-channel inoculation. When repeated in 2019, there were again no immediate differences detected between symbiont treatments and controls (Fig.16). However, when means from mock and *F. verticillioides* ear treatments were combined, *E. nigrum* 89 showed a significant reduction of fumonisin in Sp596 (Fig. 17A). Potentially effective biocontrol agents may be overlooked when comparing higher numbers of treatments to each other, making it worthwhile to compare each symbiont treatment separately to controls across genotypes. For instance, comparing symbiont treatments individually to controls across genotypes, *A. alternatum* 505 showed a trend of reducing fumonisin

across genotypes (Fig.17B). These trends by *A. alternatum* 505 were also seen in aflatoxin results in both 2018 and 2019 depending on genotype (Figs. 13, 14C). Together, these results provided evidence that these selected fungal symbionts can provide varying levels of induced resistance that limit the accumulation of both aflatoxin and fumonisin. On the other hand, further testing involving greater number of replicates may be required to increase the statistical power of these observed beneficial effects.

The Tx777 and Tx779 hybrids, which were bred specifically for resistance to aflatoxin in Texas conditions (Murray et al., 2019) displayed trends of increasing aflatoxin content in plots treated with fungal symbionts (Fig. 14D, 14E). This suggests that while these two hybrids are innately immune to *A. flavus* under normal conditions, defense pathways that provide resistance to *A. flavus* were attenuated in these genotypes upon treatment with fungal symbionts, leading to reduced resistance to this specific pathogen. Intriguingly, this effect was largely not observed in *F. verticillioides*-inoculated plots, suggesting the defenses against these two pathogens are separate and, at least in some cases, decoupled. This phenomenon was seen by Abbas et al. (2006), wherein certain maize hybrids showed high levels of both mycotoxins, leading to the conclusion that highly susceptible hybrids may not follow the pattern of an exclusively antagonistic relationship between the two pathogens observed for highly resistant lines.

Regarding the effects of the fungal symbionts themselves on the accumulation of mycotoxins in maize, it is not uncommon for promising biocontrol treatments to not only show no effect on resistance to pathogens, but in some cases even induce greater

susceptibility depending on environment or season (Campanile et al., 2007; Martin et al., 2015). Ridout et al. (2019) found that while fungal antagonists, including *Pichia membranifaciens*, *Penicillium griseolum,* and co-occurring *Fusarium* species *F. temperatum,* reduced sporulation of *F. verticillioides* or fumonisin production when dual-cultured with *F. verticillioides,* under field conditions these treatments showed no reductions of fumonisin. In fact, in the case of the treatment with *Penicillium* sp. nov. WPT which inhibited sporulation of *F. verticillioides,* it led to 6 times greater fumonisin levels than in ears treated with just *F. verticillioides*. In a similar case, treatment with *Trichoderma harzianum* strain INAT11 greatly reduced *F. verticillioides* mycelial growth *in vitro* and disease symptoms in stalks under greenhouse conditions but led to no reduction of fumonisin contamination under field conditions (Ferrigo et al., 2020).

The infection of maize by these two ear rot fungi can result in highly variable levels of infection and subsequent accumulation of aflatoxin and fumonisin, making it challenging to evaluate effective control methods. Various studies have identified multiple possible factors that influence the relationship between these pathogens and maize. These factors include the inoculum load in the field and, in the case of *F. verticillioides*, systemically in the plant, the plant and fungal genotypes, the nutritional state of the plants, and the environmental conditions (Pereira et al., 2010; Cantalejo et al., 1998; Miller, 2001; Fandohan et al., 2003). Since the trials were conducted at a later than optimal planting date, drought and heat stress occurring during flowering and grain fill stages likely had a significant impact on many genotypes, even those of tropical origin (Wahl et al 2016). A study of harvested maize samples in Texas that tested

negative for fumonisin contamination were shown to still contain *F. verticillioides*, and all ten fungal isolates cultured from those samples were able to produce high levels of fumonisin when inoculated into kernels (Ortiz et al., 2015), suggesting environmental conditions can suppress fumonisin production in maize ears that are infected with *F. verticillioides*.

Delays in time to midsilking have been correlated with reduced aflatoxin contamination of maize (Wahl et al., 2017). Zavala-Gonzalez et al. (2016) found a root-colonizing endophyte, *Pochonia chlamydosporia*, similarly affected time to flowering, however, treatment by this endophyte reduced time to bolting and flowering and increased number of inflorescences and siliques in *A. thaliana*. In this study, time to midsilking was negatively correlated with aflatoxin accumulation, but not with fumonisin (Fig. 33). Results showed that in Lubbock in 2018, time to midsilking was the shortest in Sp596 and Sp799, followed by Tx777 and Tx779 (Fig. 26). Aflatoxin concentrations in control-treated plots generally followed an opposite pattern of highest accumulation in Sp596 and the lowest in Tx779, while B73 inbred was the most susceptible though it flowered the latest (Figs. 13, 26). A significant delay was observed in 2018 among plants of Sp596 in Lubbock treated with *P. herbarum* 473 (Fig. 27) and the same plots also exhibited a trend of reduced aflatoxin contamination in mock- and, to a lesser extent, in *A.flavus*-inoculated ears, though reductions were not significant (Fig. 13B). On the other hand, in 2019 *E. nigrum* 32 led to significantly earlier silking in Sp596 in Lubbock (Fig. 28) which follows the effects seen with *P. chlamydosporia* (Zavala-Gonzalez et al., 2016), but there was no corresponding increase in aflatoxin

levels (Fig. 14B) as the negative correlation in days to silking and aflatoxin seen in PCA analysis would suggest (Fig. 33). Thus, a correlation between aflatoxin levels and delayed or accelerated silking mediated by treatment with fungal symbionts may not be ruled out, as in the case of *P. herbarum* 473, but in most cases there was little influence of fungal symbionts on time to silking and the relationship of aflatoxin to time to silking is likely due primarily to genotype-specific effects. Additionally, this implies that increases or decreases of mycotoxins seen in fungal symbiont-treated maize are likely due to other effects of the symbionts on maize. More testing is needed to evaluate fungal symbiont-mediated effects on maize resistance to mycotoxins while including analysis of effects on time to silking.

C. graminicola infection of maize is a pathosystem widely used for evaluating the induction of ISR in maize by various beneficial microbes or chemical treatments. Here, I infected maize stalks in both years in College Station and evaluated the ability of these fungal symbionts to inhibit *C. graminicola* infection and lesion growth in symbiont-treated plants. In 2018, plots of Sp596 and Sp799 showed unexpected suppression of resistance to Anthracnose stalk rot (Fig. 18). Lesion areas in Sp596 were, on average, doubled in seed-treated plots compared to control, and in Sp799 *A. alternatum* 505 increased lesion area by 26%. However, when assessed again in 2019, the same fungal symbiont treatments in Sp596 and Sp799 showed no increased susceptibility (Fig. 19). On the contrary, *A. alternatum* 505 led to a significant decrease in lesion areas, suggesting it led to the induction of ISR under field conditions in that year. The mechanisms by which the fungal symbionts tested here induce resistance to

insects and pathogens are currently unknown, however there are a number of potential mechanisms by which resistance may be enhanced. Endophyte mediated resistance can be conferred even by endophytic strains of normally pathogenic fungi, as in the case of Fo47 and CS-20, two endophytic strains of *Fusarium oxysporum* that lack certain effector genes and induce immune signaling against the pathogenic *F. oxysporum* (de Lamo and Takken, 2020). Ascomycete endophytes such as *Harpophora oryzae* or the phylogenetically distant basidiomycete *S. indica* are known to trigger localized cell death upon root colonization (Deshmukh et al., 2006; Su et al., 2013). Cell death in maize stalks upon the initiation of infection could explain the smaller lesion size, as this would limit the ability of the early biotrophic stage of *C. graminicola* to successfully infect and spread in the internode. De Lamo and Takken (2020) speculated that a required characteristic of endophyte mediated resistance (EMR) may be host cell death, which would inhibit spread of biotrophic and hemibiotrophic pathogens.

The ability of any beneficial microbes applied as biocontrol agents to also improve, or at least avoid causing detriment to, plant growth parameters and ultimately yield, is of importance when developing new biocontrol agents. Towards that end, germination was measured and rates were affected in certain cases by fungal symbionts, suggesting a few possibilities including the outcompeting of soil pathogenic microbes in the rhizosphere of the germinating seedlings or the induction of ISR in the seedlings. In 2018, *E. nigrum* 89 led to a 9% increase in germination of Sp596 (Fig. 20), but this effect was not seen in 2019 (Fig. 23). *A. alternatum* 505 also increased germination in 2018 for B73 in Lubbock and slightly increased it in College Station (Figs. 22, 20). The

variation seen in these effects is unsurprising given the difference in environment between locations and variability due to weather. Fungal symbiont treatments showed generally minimal effects on plant growth as determined by height measurements taken at 1-3 time points during developmental stages V3-V6 when plants have 3-6 true leaves, respectively (Figs. 24, 25). Although plant heights were not recorded at later growth stages, yield was recorded and is ultimately the more relevant parameter to growers for quantifying beneficial effects of these treatments. In College Station in 2019, *E. nigrum* 89 treated plots had increased weight per cob in Sp596, which could have been due to the decrease in germination of these plots leading to higher availability of nutrients for each plant.

Finally, insect damage provides an additional parameter for determining the efficacy of beneficial microbes at inducing ISR. In 2018, insect damage was recorded in Lubbock after high numbers of plants with insect damage were noticed in field plots. Insect damage in B73 was much higher than in any of the hybrids, and all symbiont treatments except *E. nigrum* 32 reduced insect damage significantly (Fig. 30). The ability of certain fungal treatments to increase resistance to insect damage is well studied (Pieterse et al., 2014), and in the case of this project, the fungal symbiont *P. inflatum* 490, initially screened and found to inhibit aflatoxin accumulation, has been previously shown to increase plant resistance to insects. *P. inflatum* 490 treatment in cotton led to suppression of nematode infestations and subsequent reproduction (Zhou et al., 2016; Zhou et al., 2018) and led to decreased attraction of western tarnished plant bugs (*Lygus hesperus*) and southern green stink bugs (*Nezara viridula*) to host cotton plants in choice

and no-choice assays (Sword et al., 2017). This deterrence of insects suggests volatiles released upon colonization by the endophytic fungus play a role in deterring insects from laying eggs on treated plants. While *P. inflatum* 490 was not tested in 2018/19 field trials, it is tempting to speculate the symbionts tested here, *C. globosum* 117, *P. herbarum 473*, and *A. alternatum* 505, showed decreased occurrence of insect damage due likewise to deterrence of gravid herbivores. Insect damage was not recorded in College Station in 2018 as observed insect damage was negligible or not detected in most plots due to pesticide applications following standard agronomic practices for the area.

The ultimate aim of this project being to identify one or more fungal symbionts that can inhibit mycotoxin levels while either not affecting or improving other parameters such as plant growth, yield, and resistance to anthracnose stalk rot and insect herbivory, it is essential to evaluate the fungal symbionts many interactions with maize. *A. alternatum* 505 treatment showed clear reductions in the occurrence of insect damage in B73 and Sp596 in Lubbock in 2018, and simultaneously did not affect aflatoxin while, on the other hand, led to an increase in fumonisin contamination in Sp596, though not statistically significant. The following year *A. alternatum* 505 showed no clear effects on fumonisin in Lubbock but did show a distinct trend of reducing fumonisin in B73 and both Sp596 and Sp799.

CHAPTER V

SUMMARY

Major progress has been in maize production towards protecting producers from yield losses due to biotic stresses and consumers from harmful mycotoxin contamination, but there remains much to be done to attain reliable food security in maize. The rapidly evolving climate of today can contribute to already variable and hard-to-predict infestations and infections by herbivores and fungal pathogens. The ear rot pathogens of maize, *A. flavus* and *F. verticillioides*, lead to highly variable levels of contamination by aflatoxin and fumonisin, and the occurrence of these mycotoxins depend on many factors such as precipitation, temperature, genotypes of the plant hosts and pathogens, insect herbivory, and agronomic practices. Infection of seed with these two major ear rot pathogens of maize leads to high yield losses and detrimental health effects to humans and animals. These carcinogenic compounds are some of the most agriculturally important mycotoxins, consumption of which in food and feed can lead to cancers, liver damage, and stunted growth. The major goals of this research were to determine if atoxigenic strains of *F. verticillioides* could be found from diverse locations across Texas, to evaluate their ability to induce plant growth of maize and inhibit the accumulation of aflatoxin in greenhouse and field conditions, and to evaluate selected fungal symbionts for the ability to improve maize resistance to aflatoxin and fumonisin accumulation as well as insect herbivory, while maintaining or improving plant growth and promoting increased yield. Collectively, this study has provided important information on the presence of atoxigenic *F. verticillioides* occurring in Texas and

insights into the complexity of fungal symbiont-mediated effects on maize resistance to mycotoxins, insect herbivory, and to effects on yield, as well as provide evidence for the potential use of *Acremonium alternatum* TAMU505 for the reduction of mycotoxin contamination and insect herbivory depending on maize genotype being used.

I have provided molecular and chemical evidence that maize kernel-derived fungal isolates 302-A6 and PAL 401 are isolates of *F. verticillioides* and are unable to produce fumonisin in two medias including maize kernels. Other isolates of *F. verticillioides* and unidentified fungi were able to produce fumonisin in a substrate-dependent manner while *F. verticillioides* 302-A6 and PAL 401 remained non-fumonisin producing and thus were atoxigenic. These isolates were shown to significantly inhibit aflatoxin production when pre-treated in a maize kernel bioassay. However, they did not show suppression of aflatoxin or fumonisin production when seed treated in maize plants in greenhouse or field trials. This suggests that either these strains do no colonize the plant systemically and cannot contribute to disease resistance by excluding *A. flavus* or naturally occurring toxigenic *F. verticillioides*, or treatment by these isolates does not lead to resistance to these pathogens through induced systemic resistance. The experimentally untested potential remains, however, that these isolates could be applied as treatments sprayed on maize silks or spread throughout fields as soil treatments, in the same manner as atoxigenic *A. flavus*, to competitively exclude the infection and fungal growth of toxigenic *F. verticillioides*. Additionally, these isolates showed an inconclusive effectiveness at improving maize plant growth and yield, though PAL 401

indicated a potential for increasing plant height and yield in lab and greenhouse conditions.

The mycotoxins aflatoxin and fumonisin remain major problems for maize production, and maize growers have few effective and reliable methods of control for fumonisin levels in particular. Towards identifying and evaluating additional methods of disease control, specifically biocontrol agents, a selection of fungal symbionts were tested in two locations across two years and in multiple maize genotypes of varying susceptibility to these mycotoxins. The goals of this project were to determine if one or more fungal symbionts could provide fungal symbiont-mediated plant resistance to these ear rot pathogens and their resulting production of aflatoxin and fumonisin. Additionally, their ability to inhibit stalk rot due to *Colletotrichum graminicola* and to inhibit the occurrence of insect damage from naturally occurring leaf herbivores was evaluated to further understand how these fungal symbionts may improve plant health and to elucidate the mechanisms by which these do so, such as the potential for induction of ISR. Here, results of multiple measured parameters demonstrated that a selection of beneficial fungal symbionts applied in two locations hold potential for reducing mycotoxin contamination in maize ears as well as improving resistance to insect herbivory, while showing variable effects on Anthracnose stalk rot levels due to environmental conditions. Considering the fungal symbiont treatments that were repeated across both locations and years (excluding *Epicoccum nigrum* TAMU89 which was not tested in Lubbock the first year of field trials), seed treatment with *Epicoccum nigrum* TAMU32 led to statistically significantly reduced aflatoxin in the susceptible

maize inbred B73 in one location and year, but showed increased lesions of Anthracnose stalk rot following inoculation with *C. graminicola* in one year. *E. nigrum* 89 seed treatment led to increases in aflatoxin in one susceptible line and significant increases in one resistant line of maize hybrids in Lubbock, while significantly reducing both mycotoxins tested in different susceptible maize lines in College Station. This further emphasizes that environmental conditions greatly effect plant-microbe interactions and symbiont-mediated resistance to pathogens. Intriguingly, seed treatment with *Acremonium alternatum* TAMU505 led to the highest number of reductions in disease measurements while additionally showing reduced occurrence of insect damage. *A. alternatum* 505 demonstrated trends of reduced aflatoxin levels in B73 inbred and a resistant hybrid maize line, as well as a trend of inhibited fumonisin in susceptible maize in College Station in one year. Additionally, treatment with this symbiont led to greatly reduced occurrence of herbivory from naturally occurring insect pressure but increased diseased lesions of Anthracnose stalk rot. These results, together with suppression of *F. verticillioides*, suggests an induction of defense-related pathways that warrant further study. Treatments with *E. nigrum* 89 and *Pleospora herbarum* 473 increased aflatoxin in the already resistant Tx779 hybrid, which emphasized the need for rigorous testing of all fungal treatments before marketing to avoid increasing contamination of maize by mycotoxins. Seed treatment with *A. alternatum* 505 before planting led to promising reductions of aflatoxin and fumonisin in College Station and Lubbock, and showed reduced occurrence of insect damage, suggesting it may improve resistance to both mycotoxins and herbivores depending on genotype and location.

Collectively, these results provide insight into the discovery, application, and potential for employing atoxigenic strains of *Fusarium verticillioides* and cotton-derived beneficial fungal symbionts for the reduction of fungal diseases and mycotoxin contamination, insect herbivory, and for the promotion of plant growth and yield in maize. Importantly, this study identified atoxigenic *F. verticillioides* isolates occurring in Texas and found potential for improving maize growth. Investigations of their potential for inhibiting mycotoxins indicated the methods tested here were not successful while not ruling out other methods and applications as biocontrol agents. Lastly, the second project of this study found important evidence that selected fungal symbionts hold potential for use as inducers of maize resistance to mycotoxins, while making clear that maize genotype and environment play a key role in mediating fungal symbiont interactions with the maize host.

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