AN INTEGRATIVE ANALYSIS OF COTTON – FUNGAL – APHID INTERACTIONS

A Thesis

by

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ABSTRACT

Two genotypes of cotton DeltaPine 0912 and Phytogen 367, three species of fungi *Beauveria bassiana, Phialemonium inflatum, Chaetomium globosum,* and the cotton aphid *Aphis gossypii* were used in this study to test whether foliar treatments of fungal spores (viable or dead) affect the cotton plant and how the cotton aphid performs on these plants. Three-week-old plants were infested with two aphids and two weeks later the total number of aphids per plant was counted. Aphid population was significantly higher on Phytogen 367 plants treated with dead spores of *B. bassiana* or *C. globosum*, no other combination of plant genotype and spore status (viable or dead) were different than controls. Furthermore, plants of these two genotypes treated with the plant defense elicitors methyl jasmonate and Actigard 50WG yielded same results as fungal spore treatments denoting that changes in the plant defense profile were responsible for the observed aphid results. Foliar applications of Chitosan provided clues that chitin recognition might be the reason why Phytogen 367 plants responded to dead spore applications. Gene expression analysis corroborated the hypothesis that fungal spores induce changes in the plant defense hormone profile, however it was found (in consistency with the preceding experiments) that only one plant genotype showed significant differences in gene expression. Moreover, the gene expression from plants treated with viable spores was opposite to the one from dead spores.

As a follow up experiment, microscopic observations of the leaves were done to determine the behavior of spores on the leaf surface following treatment. No spore germination was observed at 1, 3 and 7 days post treatment, suggesting spores undergo a state of fungistasis upon landing on the leaves. However, it was found that enrichment of spore suspensions with molasses combined with high humidity induced the germination of all three species of fungi.

Moreover, upon germination, fungal leaf colonization through stomata openings was observed. Additionally, it was found that the fungus *B. bassiana* was able to reproduce on the surface of cotton leaves through the process of microcycle conidiation.

DEDICATION

I am dedicating this thesis to my beloved wife Karin for her constant and devoted efforts to encourage and inspire my work and research. This is proof together we can overcome all challenges in this life and hereafter.

I also dedicate this work to my mother Florita in heaven, I know somewhere in eternity you are so proud of all the things I achieved in the land of the free.

Next, my father Benigno whose example of hard work and perseverance have been a great example to follow in my professional and personal life. To my brothers Daniel, Eduardo “Lalo”, Carlos “Beto”, Martin y Jose “Pepe”, thank you for keeping in touch with me, our everyday communication makes me feel like I never left our hometown.

I also want to dedicate this thesis to my aunts Exilda and Amelia and to my grandma Donatila, Thank you for your love and prayers.

Last but not least I am dedicating this to my four nieces Lucero, Elida, Gia and Brunela and to my nephew Wilmer “chino”, I just raised the bar kiddos, however I know you have the potential to surpass me.

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CHAPTER I

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is a major global agricultural crop. Worldwide cotton production in 2021 is expected to reach 118.7 million 480 lb. bales, comprising an area of approximately 33.5 million hectares (USDA - NASS, 2020). Cotton plants are vulnerable to many species of herbivores, the cotton aphid *Aphis gossypii* Glover is one of them. Overall, the cotton aphid is a secondary pest attacking cotton and other crops around the world. It can damage cotton plants by feeding on phloem from leaves and stems, and damage fiber by staining it with excreted honeydew. With warm temperatures, the life cycle of *A. gossypii* can be completed in only seven days, and adult females are able to give birth (viviparous) to up to 80 nymphs (Capinera, 2007). Although a pest, the presence of aphids is also known for building up population of generalist predators in cotton fields such as lady beetles, spiders, lacewings and predatory hemipterans (Ali et al, 2016; Yao et al., 2016). Eventually, these generalist predators will feed on other insect species available to meet their nutritional demands (Symondson, 2002).

Modern sustainable agriculture aims to incorporate environmentally friendly approaches that minimize negative effects to humans and eventually the planet. The use of beneficial microbes for agricultural pest management could provide a sustainable way to augment or in some cases replace traditional approaches in conventional agriculture (Bhattacharyya et al., 2016). This involves the integration of many species of beneficial microbes as biofertilizers, biostimulants, or biopesticides (Busby et al., 2016). The latter includes many of the well-known fungal

entomopathogens such as *Beauveria bassiana*, *Metarhizium anisopliae* or *Bacillus thuringensis*, which have been used in agriculture for decades and are usually sprayed on crops to control different species of insects.

Although entomopathogens as biopesticides can be used as part of an integrated pest management (IPM) program, there are some constraints associated with the use of the beneficial fungi. For example, they all have a limited shelf life (Scholte et al., 2004) and are susceptible to degradation by UV light from the sun that can negatively impact the efficacy of these organisms in terms of insect control especially under field conditions (Fernandes et al., 2015).

Salicylic acid (SA), Jasmonic acid (JA) and Ethylene (ET) hormone pathways are active mechanisms of plant defense against biotic attacks by pathogens as well as insects (Bruce & Pickett, 2007). Moreover, these pathways do not function in isolation and communicate in a coordinated crosstalk to better target appropriate responses (Van Wees, et al. 2008). These plant hormones signaling pathways are the core of Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) defensive responses. SAR is mainly considered a response to biotrophs and involves SA signaling, while the ISR pathway is based on JA and ET signaling in response to necrotrophs (Leonetti et al. 2017; Smirnova et al., 2017). ISR is also associated with the term "defense priming" which is the ability of the plant to be better prepared to counteract subsequent biotic stressor attacks. Defense priming is considered a favorable defensive strategy against pathogen and insect attacks because the plant only mounts a response in the presence of the stressor, and incurs little cost in the absence of the particular stress (Martinez-Medina et al., 2017). Priming is indicated by a plant response at the cellular level that is either more rapid or produced to a greater extent (Pieterse et al., 2014). Crosstalk between SA and JA is well known in dicots, and can result in antagonistic effects where upregulation of one will downregulate the production

of the other (Tamaoki et al. 2013). However, when and how the plant will deploy these mechanisms of defense is complex and not as predictable as formerly assumed because our understanding of these processes is not yet sufficient (Verwaaijen et al., 2019; Ruan et al., 2019).

Allene Oxide Synthase (AOS) and Lipoxygenase (LOX) are key enzymes in JA biosynthesis (Ruan et al., 2019). AOS is involved in the biosynthesis of typically non-volatile oxylipins in plants. AOSs as part of the JA pathway are crucial in plant defense signaling and growth (Farmer & Goossens, 2019). In cotton specifically, AOS gene downregulation has been associated with increased vulnerability to a whitefly associated virus (Naqvi et al., 2019). The LOX gene family includes several genes associated with multiple important functions in the plant, some of which participate actively in plant defense (Hu et al. 2009). Among numerous metabolites being regulated by this family of genes, there are antimicrobial and antifungal molecules that are produced in response to biotic stressors (Feussner & Wasternack, 2002). Pathogenesis related PR proteins like β-1,3-glucanases (PR-2) are important components of the SAR response, and they have been used extensively in monitoring activation of defenses in plants against pathogens (Ali et al., 2018). β-1,3-glucanases are key players in many physiological processes in plants. In several species they play a role in fungal pathogen defense mainly by participating in pathogenesis-related protein synthesis (Balasubramanian et al., 2012). As causal evidence of their role in plant defense, downregulation of β-1,3 glucanase genes in cotton resulted in greater susceptibility to the fungus *Verticillium dahlia* (Xu et al., 2016).

Several molecules that have been shown to activate plant defenses. Methyl jasmonate (MeJA) and Chitosan are well known elicitors of plant defenses that work principally by activating the JA pathway in several plant species (Jia et al, 2018). The use of JA elicitors as a tool against aphids has been explored in different plants species including wheat (Bruce, 2003), tomato

(Cooper & Goggin, 2005; Boughton, 2006), potato (Sobhy, 2017), and soybean (Selig, 2016). All of these studies reported negative effects of the inducers on the aphid species being investigated. In cotton specifically, surprisingly few studies have investigated the effects of JA elicitors on *A. gossypii* performance and survival. In one study, cotton plants were treated with JA directly as an inducer, after which excised leaves were placed in Petri dishes then infested with two aphids. This resulted in fewer aphid nymphs per leaf (i.e., lower reproduction) compared to control treatments (Omer, 2001). In another study, applications of MeJA under field conditions did not translate into changes in the aphid population (Williams, 2017). Foliar application of Cis Jasmonate on cotton plants under field conditions in Turkey resulted in fewer wingless aphids per plant, but no difference in the number of winged aphids was found (Tonga, 2020).

Actigard® 50WG (Syngenta, Basel, CH) common name benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, (BTH), is known for activating the SAR pathway in many plants and has been on the market as a plant defense elicitor for more than a decade (Shrestha et al.,2018; Cooper & Horton, 2017; Pye et al., 2013). It has been used for control of aphids on tomato (Boughton et al., 2006; Cooper et al., 2004) and potato (Dupius et al., 2014). On melon plants, foliar applications of BTH against *A. gossypii* resulted in 100% control when plants were infested with aphids on the day of treatment, but had no effects on aphid populations 7 days after treatment (Moreno-Delafuente et al., 2020).

Although there are many examples of fungi altering plant defenses against insects, the specific mechanisms underlying these interactions are often not well understood (War, 2012; Desurmont, 2014). Plants are capable of recognizing several compounds from fungi as elicitors. Fungal elicitors like chitin, a major fungal cell wall component, are referred as microbe/pathogen associated molecular patterns (MAMP or PAMP) (Newman et al. 2013). These molecules are

recognized by the plant as potential danger which eventually triggers host defenses (Boller & Felix 2009). This is important because fungal spore sprays to control insects with commercially available products in field or greenhouse crops have been used for decades. Most of the time the efficacy of these treatments has been focused on insect mortality directly due to pathogen infection. However, the spores may also have indirect effects on herbivore pests by triggering plant defensive pathways. Thus, we may have overlooked how the plant itself responds to fungal spore treatments and the resulting indirect consequences on herbivores. For example, Hajek et al. (1987) used foliar sprays of the fungus *B. bassiana* with the sole purpose of controlling the Colorado potato beetle attacking potato plants under field conditions. More recently, Saenz-Aponte et al. (2020) used foliar sprays of *B. bassiana* and *M. anisopilae* to control *Plutella xyllostella* attacking broccoli under both greenhouse and field environments. In both cases, they focused on the percentage of damaged leaves as an indication of pest control and assumed the effects to be due to the pathogenic effects of the fungus on the insects.

With advances in molecular biology and gene sequencing, researchers have started to pay attention to the gene expression of plants exposed to several species of fungi. However, most of these studies are done with pathogenic fungi. Zhu et al. (2017) looked in to the apple transcriptome of leaves exposed to the fungal pathogen *Alternaria alternata*. Tian et al. (2019) also explored the changes in gene expression of apple leaves after being challenged with the fungal pathogen *Podosphaera leucotricha*. One of the few studies examining plant gene expression changes in response to non-pathogenic fungi such as the entomopathogen *B. bassiana* was done by Raad et al. (2019) using *Arabidopsis thaliana* as plant host. They assessed the changes in gene expression of plants that were dipped (roots) in a spore suspension of the fungus and found the fungus indeed changed the plant defense profiles of treated plants. Rondot & Reineke (2019) applied foliar sprays

of *B. bassiana* on grapes and then looked at the relative expression changes (RT-qPCR) of plant defense genes (ATPase, PR-1 and β-1,3 glucanase) and observed a significant downregulation of the PR-1 gene on the treated plants at one and seven days after treatment, with no effect of treatment on the other genes. They also reported that inoculation with the pathogen *Plasmopora viticola* at 7 days post-treatment with *B. bassiana* was the best timing for better downy mildew control since the plant had more time to interact with the beneficial fungus.

Fungal plant endophytes are described as species of fungi that live within plant tissues and produce no signs of apparent damage to the host. Moreover, in many cases they stablish a mutualist relationship with their host (Vega 2018). Endophytes as a group also include dormant pathogens and non-culturable species that cannot be isolated and grown on artificial media. True endophytes will accomplish satisfactory colonization and re-isolation from host as indicated by Koch's postulates (Lugtenberg et al., 2016).

Microscopic observation of fungal spores on leaves following fungal treatment is not a common practice among studies claiming active endophytic colonization by the organisms being studied. In the majority of cases, detection of fungal establishment in the plant is achieved by means of plant tissue plating on nutrient media following surface sterilization, PCR, or a combination of both techniques. Using such methods, endophytic colonization of different plant species by the fungus *B. bassiana* following foliar sprays has been reported for common bean (Parsa et al. 2013), tomato (wei et al., 2020), cucumber (Rajab et al., 2020), artichoke (Guesmi-Jouini et al., 2014), sugarcane (Donga et al., 2018), citrus (Bamisile et al., 2019), and cotton (Gurulingappa et al., 2010). However, the few studies involving microscopy techniques to confirm fungal establishment of assumed endophytes usually suggests that even when spores germinate on the surface or in some way reach the tissues below epidermis, extensive systemic colonization of

plant tissues rarely occurs, and endophytic fungal structures frequently vanish over short periods of time (Nishi et al., 2020; Kock et al., 2018; Landa et al., 2013, Ullrich et al., 2017).

The goal of this study was to test for the effects of foliar applications of potentially beneficial fungi to cotton on plant defensive responses and herbivore performance. Both viable and dead spores of three different fungi, *B. bassiana*, *P. inflatum* and *C. globosum*, were separately applied to two genotypes of cotton. The resulting treatment effects on cotton aphid population growth were quantified under laboratory conditions. We used gene expression analyses and microscopy to test hypotheses about the mechanisms involved in this plant-fungus-aphid interaction. This study was composed of two parts. In the first part (Chapter 2), we tested the hypothesis that foliar applications of fungal spores trigger plant defenses, and that this induction has an effect on cotton aphids. In the second part of the study (Chapter 3), I looked into the dynamics of fungal spores on the surface of cotton plants following inoculation using conventional clearing and staining techniques for microscopic observations.

CHAPTER II

COTTON GENOTYPE MEDIATES FOLIAR ELICITATION OF PLANT DEFENSES BY FUNGAL SPORES AND RESULTING EFFECTS ON COTTON APHIDS

Introduction

Cotton (*Gossypium hirsutum* L.) is a major crop grown worldwide mainly for its fiber which contributed $600 billion to the global economy with China, India and the USA being the three largest cotton producers (Khan et al., 2020). Nowadays, more than 90% of upland cotton varieties grown in the US are genetically engineered to be insect and herbicide resistant (USDA[1] 2020). Deltapine, Americot and Phytogen are among the top three commercial varieties of upland cotton planted in the US (USDA[2] 2020).

Bt transgenic cotton targets, for the most part, species of Lepidoptera, but there are many other species of non-target insects that attack cotton plants, the cotton aphid *Aphis gossypii* being one of them (Liu et al., 2005). *A. gossypii* is a phloem sap-sucking species that is well-known in cotton production areas as a troublesome pest (Jian et al., 2018). Honeydew from aphids can be detrimental to the quality of fiber when infestations occur at the end of the season (Chen et al., 2013). The cotton aphid completes its life cycle in about one month, and takes approximately one week to reach the adult stage, passing through four instars during this time (Chamuene et al., 2020). *A. gossypii* reproduces asexually during the growing season (spring and summer), and overwinters as eggs that come from sexual reproduction at the end of the season (Quan et al.,

2019). Although considered a pest, many species of generalist predators feed on cotton aphids and can contribute to augmentation of these natural enemies in cotton fields (Shrestha and Parajulee 2013).

The worldwide biopesticide market is currently estimated to be worth roughly $3.3 billion and accounts for about 8% of the entire pesticide market, with increasing growth expected in the future (Khun et al., 2020). Entomopathogenic fungi (EPF) like *Beauveria spp*., *Lecanicillium lecanii* and *Metarhizium spp.* by themselves or in combination with chemical pesticides can be used as biopesticides in cotton integrated pest management (IPM) programs (Sain et al., 2019). Even though these EPF have clear environmental advantages over chemical pesticides, they have yet to achieve widespread adoption by growers (Skimmer et al., 2014). Given that these fungal entomopathogens are living organisms, there are some drawbacks in their production and use as biopesticides. For instance, contamination during production, relative short storage life, and quality control (spore viability and vigor) are common issues in the biopesticides industry (Mascarin et al., 2019). Moreover, environmental stressors such as sunlight UV radiation, and hot/dry conditions can negatively affect the efficacy of these EPF under field conditions. Thus, there is a need to overcome these challenges (Zhao et al., 2016).

A crucial step in plant responses to pathogen attacks is the identification of microbe-associated molecular patterns (MAMP) by specialized receptors in the host that convey signals which eventually turn on pattern-triggered immunity (Klauser et al., 2013). Chitin and β-glucan are key components of fungal cell walls and are well-known examples of MAMPs (Newman et al., 2013). MAMP recognition is critical in plant defensive responses to pathogens (Nishad et al., 2020). Chitin and its modified version Chitosan are known to trigger plant defenses in many species of monocot and dicot plants (Pusztahelyi, 2018; Suarez-Fernandez et al., 2020). In fact,

treatment of grapevine fruits with Chitosan resulted in upregulation of JA gene expression which rendered fruits more resistant to a necrotrophic pathogen (Peian et al., 2021). Upon microbe recognition, plants may react in many ways including stomata closing to prevent pathogen penetration, biosynthesis of reactive oxygen species (ROS) (Ye & Murata, 2016), jasmonic acid production, activation of chitin and glucan enzymes, and upregulation of genes related to plant defense (Pusztahelyi, 2018).

In addition to live fungi, dead fungi can also be a source of MAMPs (Vishwanathan et al., 2020). Unintended plant defense elicitation with dead spores arising from the use of EPF as biopesticides could be more common than we think, with overlooked consequences on pest populations. For example, spores on the adaxial side of leaves may die off due to sunlight UV exposure following foliar applications of EPF under field conditions. Alternatively, inadvertent foliar applications could occur through use of expired or improperly stored EPF products.

Some species of EPF like the well-known *Beauveria bassiana* can, under certain circumstances, become plant endophytes following foliar applications (Parsa et al., 2013; Gurulingappa et al., 2010). Fungal endophytes are described as species of fungi that have the ability to live asymptomatically inside plant tissues. In addition to direct infection and mycosis of insects outside the plant, EPF as endophytes can provide plants with many other forms of host protection such as changes in the expression of JA and SA genes related to plant resistance (Raad et al, 2019), production of volatile organic compounds (Kaddes et al., 2019), production of fungal secondary compounds that negatively affect insect performance or survival, and competition with herbivores for available nutrients in the host plant (de Vries et al., 2018). Some beneficial microorganisms have evolved the capacity to repress or avoid plant immune responses, thereby enabling epiphytic or endophytic colonization (Zeilinger et al., 2016).

However, as colonization takes place and defensive phytohormone profiles are disrupted, the host could eventually become susceptible to herbivores. In soybean for example, endophytic colonization by *Metarhiium brunneum* and *B. bassiana* rendered the plants significantly more susceptible to the soybean aphid (Clifton et al., 2018), possibly due to disruptions in the phytohormone defense profile.

Plant induced defenses to biotic stressors are activated by different signal cascades led by the phytohormones salicilyc acid (SA) and jasmonic acid (JA). The SA-conditional pathway is acknowledged as the systemic acquired resistance (SAR) pathway. It is an important plant defense against attacks from biotrophic and hemi-biotrophic pathogens (Wenig et al., 2019) and phloem sap sucking insects (Thaler et al., 2012). The SAR pathway includes the upregulation of pathogenesis related (PR) defense genes and eventually PR proteins (Shah & Zeier, 2013). Another key defensive pathway is induced systemic resistance (ISR) that is led by jasmonate signaling including JA and related molecules along with ethylene (ET) (Choudhary et al., 2007). This JA-dependent pathway is induced upon with interaction with necrotrophic microbes (Proietti et al., 2018) as well as chewing and certain phloem sucking insects (Thaler et al., 2012). Plants are constantly challenged by a combination of biotic and abiotic stressors, thus a cascade of signals and phytohormones must be in permanent intercommunication. This is known as "crosstalk" (Liu et al., 2010). Crosstalk between SA and JA signal pathways has typically been though to result in reciprocal antagonism, but this is not always the case. Moreover, this crosstalk can be manipulated by plant foes to benefit themselves from the interaction (Thaler et al., 2012).

The goal of this study was to test the effects of foliar applications of either viable or dead fungal spores of *Beauveria bassiana, Phialemonium inflatum* and *Chaetomium globosum* fungi on two genotypes of cotton and how these treatments influence the growth of cotton aphid, *Aphis*

gossypi, populations under laboratory conditions. I also tested hypotheses into the possible mechanisms involved in this plant-fungus-aphid interaction using plant defense elicitors and gene expression analyses.

Materials and Methods

Plants

Delinted cotton seeds without pesticide treatments of two varieties, DeltaPine 0912 and Phytogen 367, were sown in 6.35 cm^2 black plastic pots (Greenhouse Megastore CA, US) containing 245 cc of C25 growing mix (Jolly Gardener). Pots were placed in 32 pocket plastic trays mounted in a 1020 tray (Greenhouse Megastore CA, US). Plants were grown indoors on shelving units equipped with a six tube, 1.22 m long T5 (6400 K) light fixture set to a 14:10 light:dark photoperiod. All plants were watered with same amount of water and rotated both vertically and horizontally on the shelves every day to control for differences in light and temperature. Plants were grown this way until they were ready for aphid infestation at the third true leaf stage.

Fungi

Three species of fungi were included in this study, *B. bassiana*, *P. inflatum* and *C. globosum*. *B. bassiana* strain GHA was obtained from the commercial product Botanigard 22 WP (BioWorks NY, US). A pure culture of the fungus was isolated by preparing 100 ml of commercial product suspension (as recommended on label), then diluted 100000 times and

plated on potato dextrose agar (PDA) media (Hardy Diagnostics CA, US). Individual colonies were subcultured on new PDA plates. *P. inflatum* (TAMU490) and *C. globosum* (TAMU520) are fungal species isolated from cultivated cotton plants in Texas (Ek-Ramos et al., 2013). *P. inflatum* was cultured on PDA media and *C. globosum* was cultured on 10% unclarified V8 agar media (Miller, 1995). Disposable 100 x 15 mm plastic Petri dishes (VWR International Co, PA, US) were used for all culturing. All culture media was prepared with 100X Penicillin-Streptomycin solution (Corning VA, US) and incubated at 26 °C in dark.

Mature cultures (approx. two weeks old) were used as a source of inoculum. Fungal conidia (spores) we harvested by adding 15 ml of sterile Tween 80 0.01% solution to the culture plate. A "L" shape cell spreader was used to free spores from agar making a suspension that was then passed through autoclaved sieves (500 mesh for *B. bassiana* and *P. inflatum* and 325 mesh for *C. globosum*). Cell concentration was calculated using a Neubauer hemocytometer. Spore viability was verified by making serial dilutions and plating 100 µl of suspension on PDA plates. Colony Forming Units (CFUs) were counted 3 days later for *C. globosum*, and 5 days later and for *B. bassiana* and *P. inflatum*. Dead spores for this study were prepared by placing glass beakers containing spore suspensions in a hot water bath at 70^{o}C for 2 hours. Spore unviability was confirmed by plating 100 µl of spore suspensions on PDA media.

Fungal Spore Treatments

Approximately three weeks after planting (second true leaf stage), pots containing individual cotton plants (n=16 per treatment) were covered at the base around the stem with premium plastic wrap (Walmart AR, US) to prevent spores from reaching contact with soil.

Then, all pots were color labeled for easier treatment identification later on. Cotton plants were sprayed with spore suspensions containing 1 x 10^6 spores per ml of either *B. bassiana*, *P. inflatum*, or *C. globosum* using handheld sprayers until the point of runoff. Sterile 0.01 % Tween 80 was sprayed on control plants. Care was taken to prevent cross contamination of plants during the foliar applications.

Plant Defense Elicitor Treatment

Methyl Jasmonate 90% (MeJA) (Tokyo Chemical Industry Co., LTD. Japan) at a rate of 85 mg/200ml dissolved in 2ml of acetone, Acibenzolar-S-methyl 75 mg/L (Actigard 50WG Syngenta, CH) and Chitosan 1g/L (Spectrum Chemical Corp. NJ, US), were used in this study. MeJA and Actigard were included to test whether eliciting JA/SA in cotton plants produced the same effects on aphid populations as the fungal spore treatments. Chitosan was used as a source of chitin to test if a Microbe Associated Molecular Pattern (MAMP) could generate same effect as fungal spore treatments on aphid populations. Assays using each elicitor were replicated twice.

Aphid Infestation and Counts

An *A. gossypii* lab colony was established in the spring of 2018 using aphids collected from cotton at the Texas A&M AgriLife Field Station in Burleson Co., TX. The colony was kept on caged transgenic cotton plants grown under artificial light in the laboratory. Aphid infestation was carried out seven days after foliar fungal treatments. Individual experimental cotton plants

were infested with two aphid nymphs of mixed developmental stages using a # zero sized paint brush. A 10X magnifier headband was used to visually ensure aphids were in good condition. Immediately after infestation, each plant was carefully caged within a 20 x 30 cm white organza pouch (Amazon WA, US). Individually caged plants were then placed on 10 cm diameter clear vinyl saucers (Growers Solution TN, US), arranged in a Latin square design on shelves, and maintained at 30 ºC and 22 ºC daytime and nighttime respectively with and average humid relativity of 60%. The total number of aphids per plant was counted two weeks after infestation using a 10X headband magnifier and the numbers recorded using a Denominator multi tally 4-counter (Fisher Scientific MA, US). Fungal spore-aphid assays were replicated 3 times using DP0912 and 4 times using PHY367. Aphid experiments testing for effects of phytohormone elicitors were conducted as above and replicated twice for each compound.

Gene Expression Analysis

Leaf tissue samples were taken 7 days after fungal treatments from plants grown at the same time as the aphid-infested plants. Samples (N=5 per treatment group) consisted of 0.1 g of fresh leaf tissue per plant inserted in a 2 ml Eppendorf safe-lock tube (Eppendorf Hamburg, Germany), immediately flash frozen in liquid nitrogen, and stored in a -80 ºC freezer. To test whether foliar applications of live and dead spores from either *B. bassiana*, *C. globosum*, or *P. inflatum* altered cotton plant defenses, we used real-time quantitative PCR to compare the expression of defensive genes known to be part of the inducible JA and SA pathways (Table 1) between treated and untreated plants. Total RNA was extracted using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, MO, USA), following the manufacturer's Protocol B. The total RNA

was treated with DNase and used for cDNA synthesis using an iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, USA). The protocol for cDNA synthesis was adapted from the manufacturer's instructions to increase the reverse transcription time to 60min. The cDNA template was amplified using SYBR green real-time quantitative PCR according to the manufacturer's instructions and the PCRs were performed on a CFX384™ Real-Time System (Bio-Rad). Actin was used as a housekeeping gene.

Table1 II-1: Primers pairs of JA and SA responsive genes used in qRT-PCR gene expression assays to test for induction of plant defensive pathways by fungal and elicitor treatments.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
JA responsive			
LOX1	ACATGCCGAAGCCGCTGCTT	GGGCGTATTCGGGGCCCTTG	Zhang et al., 2011
AOS	ATCATGTAATCCCCGAGTTCC	CCAGCTTGATCGTTAGCTGTC	Zebelo et al., 2017
SA responsive			
β1,3 glucanase	AATGCGCTCTATGATCCG	GATGATTTATCAATAGCAGCG	Zhang et al., 2011
Reference			
Actin	TTGCAGACCGTATGAGCAAG	ATCCTCCGATCCAGACACTG	Zhang et al., 2011

Statistical Analysis

R version 4.0.3 was used for statistical analysis. Normality was tested with Shapiro-Wilk test. Aphid data from replicate trials were combined for the analysis. Data were not normally distributed and over dispersed. The R "MASS" package was used in the analysis with the glm.nb function (Generalized linear /negative binomial model). The Emmeans package was used for post-hoc comparisons. When unable to combine data, we ran either Kruskal-Wallis or ANOVA for the analysis. Gene expression data were log10 transformed and analyzed using Student's t-

test for independent samples. Significance level was tested using an alpha level of $p < 0.05$. Figures were created using the ggplot2, ggpubr packages and JMP 15.

Results

Effect of Foliar Fungal Spore Treatments on the Aphid Populations

I tested the performance of *A. gossypii* on plants of genotypes DP0912 and PHY367 previously treated with foliar applications of viable or dead spores of either *B. bassiana*, *P. inflatum* or *C. globosum* versus controls. In the experiments with viable spores, I found significant main effects in the GLM model of genotype and trial. The treatment effect was not significant (Table 2). I also found that the genotype by trial interaction was significant. However, there were not significant interactions between genotype by treatment nor trial by treatment. similarly, the three-way interaction of genotype, trial and treatment was not significant.

Overall, following the foliar application of viable spores, no significant differences in the number of aphids were detected on either combination of genotype and treatments. In the PHY367 plants, no differences in aphid numbers were found between treatment and control plants ($p=0.0600$, $p=0.4558$, $p=0.9017$, $p=0.0942$ first, second, third and fourth trial respectively) (Fig. 1a). Likewise, for DP0912, aphid numbers on treated plants in all three trials were not different from controls ($p=0.1260$, $p=0.7659$, $p=0.1757$ first, second and third trial respectively) (Fig. 1c).

On the other hand, in the experiments with dead spores, I found significant main effects in the GLM model of genotype, trial and treatment (Table 3). There were also significant interactions between genotype and trial as well as genotype and treatment. Importantly, there

was not a significant interaction between trial and treatment. Nor was there a significant three-way interaction between genotype, trial and treatment. Because of the significant genotype interactions with treatment and trial, the results from four independent trials on PHY367 (Fig. 1 b) and three independent trials on DP0912 (Fig. 1d) are presented separately.

Overall, following foliar application of dead spores there were significant increases in the number of aphids on PHY367 plants treated with dead spores in every single trial ($p=0.0213$, $p=0.0087$, $p=0.0418$, $p=0.0327$ first, second, third and fourth trial respectively) (Fig. 1b). Pairwise comparisons between aphid numbers on treatment versus control plants (Tukey's HSD) were significant for *B. bassiana* ($p=0.0002$) and for *C. globosum* ($p=0.0004$), but not for *P. inflatum* ($p=0.2198$). However, in contrast to the response of PHY367 plants, there were no significant differences in the number of aphids on the DP0912 plants across any of the trials ($p=0.6703$, $p=0.9184$, $p=0.6504$ first, second and third trial, respectively) (Fig. 1d).

To summarize the effects of foliar spore treatments on aphid numbers, there was no difference in the way the DP0912 plants responded to either viable or dead spore treatments with respect changes in aphid numbers. The same also applies to PHY367 plants treated with viable spores. However, when PHY367 plants were treated with dead fungal spores, the number of aphids tended to increase on treated plants, though the magnitude of responses to specific fungi occasionally varied across trials (Fig. 1).

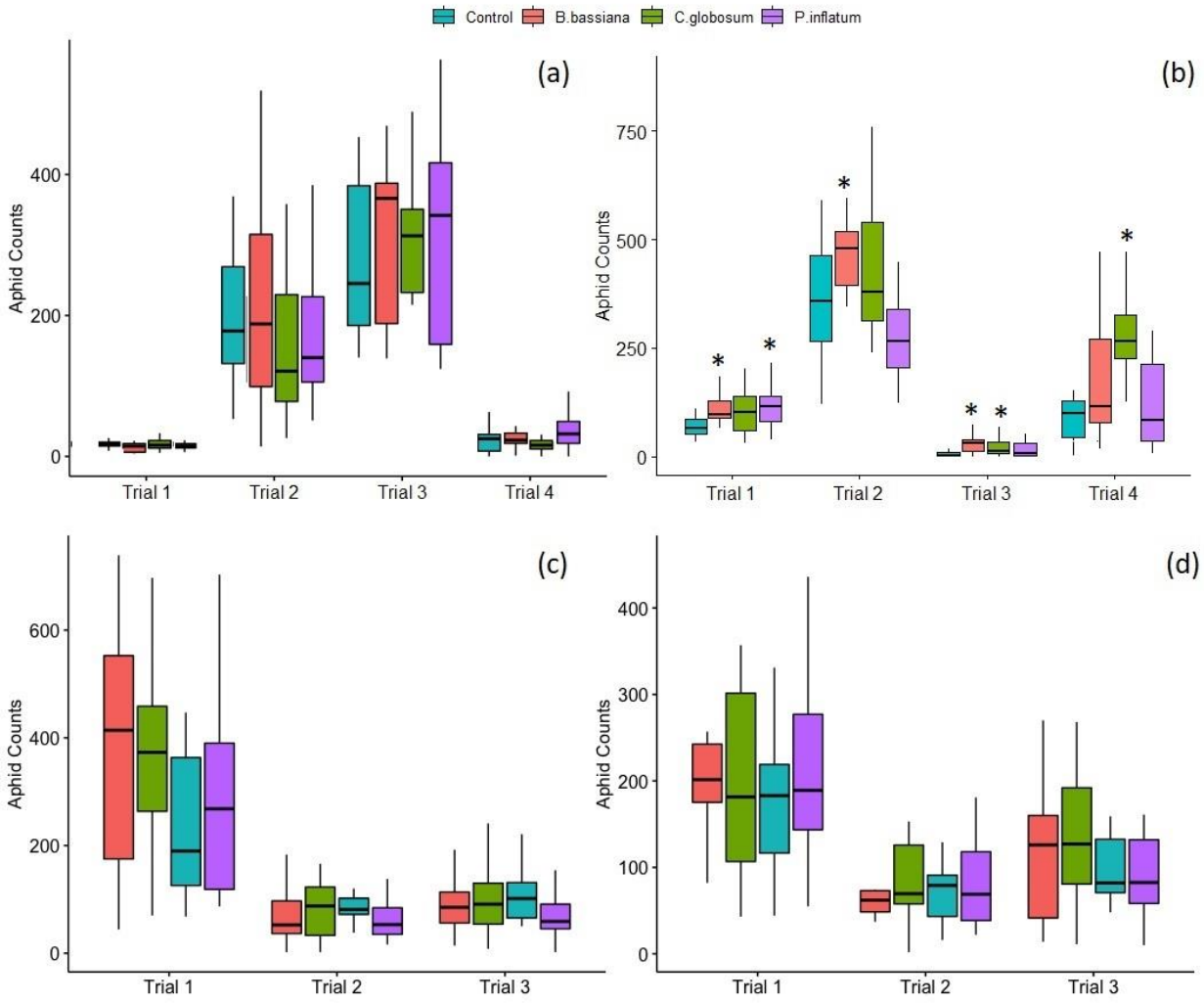

Figure II-1. Effects of viable and dead *B. bassiana, P. inflatum* and *C. globosum* treatments on the performance of *A. gossypii* in two genotypes of cotton across trials. Reproduction of *A. gossypii* on PHY367 plants treated with viable (a) or dead spores (b), and DP0912 plants treated with viable (c) and dead (d) spores. Significance denotated by asterisk ($p < 0.05$).

Table II-2. Analysis of deviance for viable spore treatments on DP0912 and PHY367.

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
Null			444	1728.33	
Genotype	1	21.180	443	1707.15	4.171e-06 ***
Trial	3	330.71	440	1376.44	< 2.2e-16 ***
Treatment	3	5.38	437	1371.06	0.14581
Genotype:Trial	2	863.13	435	507.93	< 2.2e-16 ***
Genotype:Treatment	3	5.77	432	502.15	0.12328
Trial:Treatment	9	9.44	423	492.71	0.39758
Genotype:Trial:Treatment	6	11.52	417	481.19	0.07351
Signif. codes: *** P < 0.001, ** P <0.01, * P< 0.05					

Results from generalized linear model (GLM) with negative binomial distribution.

Table II-3. Analysis of deviance for dead spore treatments on DP0912 and PHY367.

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
Null			370	1016.32	
Genotype	1	8.260	369	1008.06	0.0040526 **
Trial	3	229.226	366	778.84	< 2.2e-16 ***
Treatment	3	16.806	363	762.03	0.0007748 ***
Genotype:Trial	2	309.153	361	452.88	< 2.2e-16 ***
Genotype:Treatment	3	8.159	358	444.72	0.0428373 *
Trial:Treatment	9	10.604	349	434.12	0.3038257
Genotype:Trial:Treatment	6	5.783	343	428.33	0.4478912
Signif. codes: *** P < 0.001, ** P <0.01, * P< 0.05					

Results from generalized linear model (GLM) with negative binomial distribution.

Effect of MeJA and Actigard 50WG on the Aphid Populations

If the aphid responses to dead fungal spore treatments were the result of defensive phytohormone elicitation, then the application of a chemical plant defense elicitor should produce a similar effect on aphids. I tested this hypothesis by spraying cotton plants with two

well-known plant defense hormone elicitors, MeJA (ISR inducer) and Actigard 50WG (SAR inducer) and then assessed the performance of the *A. gossypii* on treated plants versus controls.

In the experiments with chemical plant defense elicitors, I found significant main effects in the GLM model of genotype and treatment, the trial effect was not significant. I also found significant interactions between genotype and trial as well as genotype and treatment. Notably, there was not a significant interaction between trial and treatment. Nor was there a significant three-way interaction between genotype, trial and treatment (Table 4). Once again, because of the significant genotype interactions with treatment and trial, we separately present the results from two independent trials on PHY367 (Fig. 2 a) and two independent trials done on DP0912 (Fig. 2 b).

Aphid numbers significantly increased on PHY367 plants that were previously treated with MeJA and Actigard 50WG in both replicate trials (Fig. 2 a). The calculated p values in the first trial were 0.0357 and 0.0012 for the MeJA and Actigard 50WG treatments, respectively. P values in the second trial were 0.0037 and < 0.0001 for MeJA and Actigard, respectively. Similar to the variation due to plant genotype observed in the dead spore experiment, DP0912 plants responded differently to the elicitor treatments than PHY367 plants. In contrast to PHY367, the number of aphids on DP0912 plants treated with either elicitor did not significantly differ from the controls in either trial (Fig. 2 b).

Table II-4. Analysis of deviance for MeJA and Actigard 50WG on DP0912 and PHY367.

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
Null			172	413.85	
Genotype	1	115.477	171	259.38	< 2.2e-16 ***
Trial	1	0.315	170	259.06	0.57477
Treatment	2	43.027	168	216.03	4.538e-10 ***
Genotype:Trial	1	17.022	167	199.01	3.695e-05 ***
Genotype:Treatment	2	7.164	165	191.85	0.02782 *
Trial:Treatment	2	4.929	163	186.92	0.08505
Genotype:Trial:Treatment	2	4.534	161	182.38	0.10361
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

Results from generalized linear model (GLM) with negative binomial distribution.

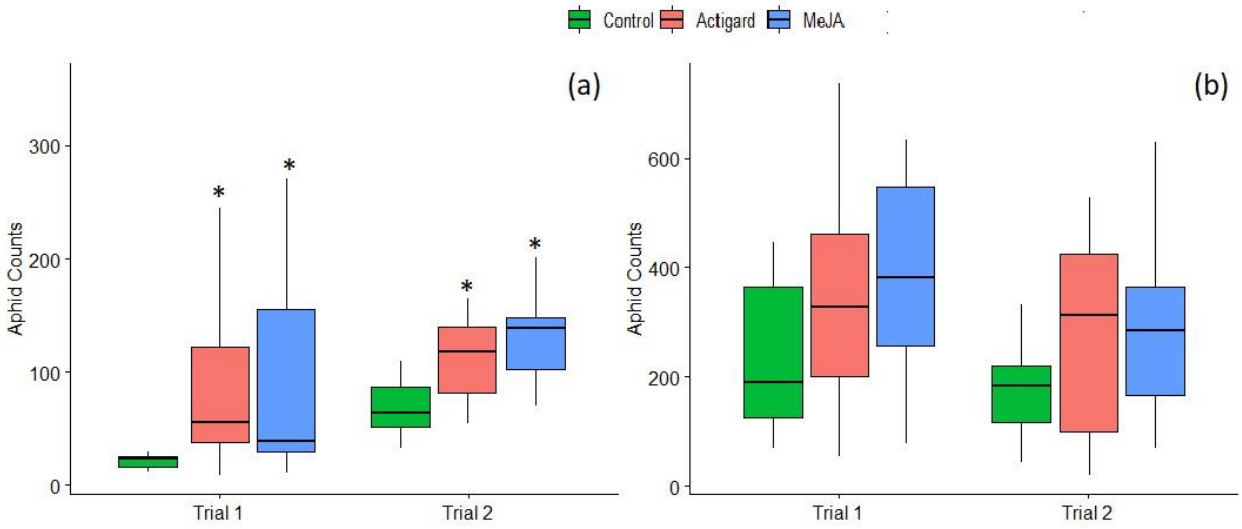

Figure II-2. Effects of MeJA and Actigard 50WG foliar plant treatments on the performance of *A. gossypii* on two genotypes of cotton. *A. gossypii* reproduction on PHY367 (a) was significatively higher on MeJA and Actigard treatments compared to control in both trials. On DP0912 (b) aphid numbers were not significantly different than control. Significance denotated by asterisk ($p < 0.05$).

Effect of Chitosan on the Aphid Populations

As a follow up experiment to test whether or not MAMP recognition is involved in the aphid response to fungal treatments, plants from both genotypes were treated with Chitosan (Fig 3) and infested with two aphids one week later. *A. gossypii* numbers were significantly higher than controls on PHY367 plants ($p = 0.0003$). As with the dead spore treatments and applications of the elicitors MeJA and Actigard, there was once again no significant effect of Chitosan treatment on the number of cotton aphids when applied to the DP0912 genotype.

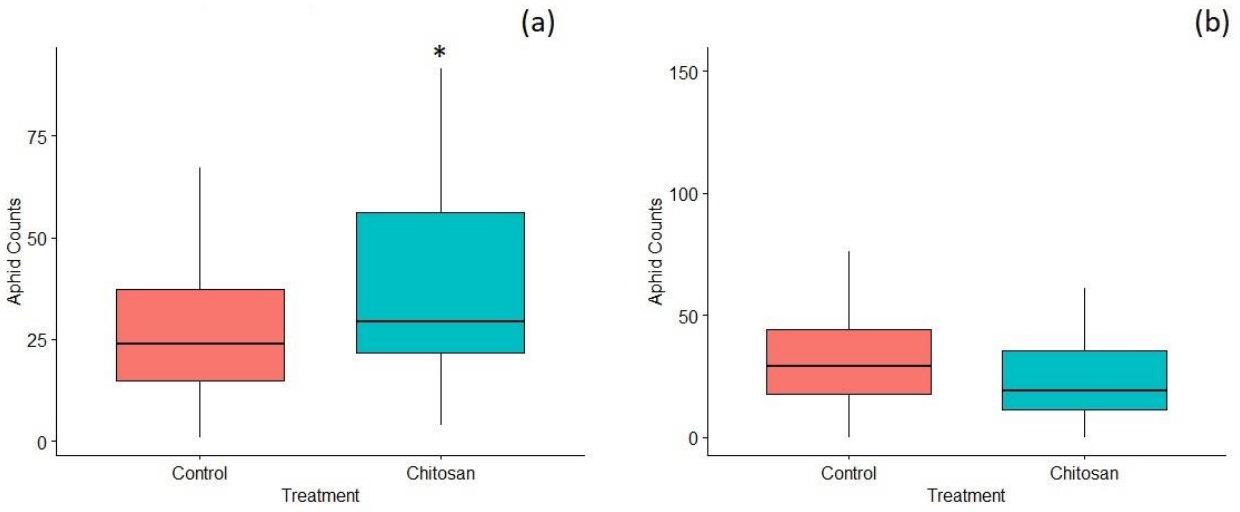

Figure II-3. Effects Chitosan foliar plant treatments on the performance of *A. gossypii* in two genotypes of cotton. *A. gossypii* reproduction on PHY367 (a) and DP0912 (b) plants. Each graph comprise data from two trials. Significance denotated by asterisk ($p < 0.05$).

Effect of Fungal Treatments on Gene Expression

Observed changes in aphid populations in response to fungal treatments and chemical hormone elicitors are consistent with underlying changes in plant defensive phytohormone signaling. To test this hypothesis, we performed RT-qPCR assays to compare the relative

expression of downstream genes involved in ISR (AOS and LOX1) and SAR (PR-2 β 1,3 glucanase) following treatments with viable and dead fungal spores. Changes in the expression of the AOS gene (Fig 4) were detected only in the PHY367 genotype, and these changes differed depending on the status of inoculum used in the foliar treatment (viable or dead). When PHY367 plants were sprayed with viable spores, AOS was downregulated for all three fungi tested, with the effect being significant for *P. inflatum* ($p = 0.0070$) and *C. globosum* ($p = 0.0146$) treatments. In contrast, foliar applications of dead spores resulted in the AOS gene being significantly upregulated in the *P. inflatum* ($p = 0.0391$) and *C. globosum* ($p = 0.0417$) treatments compared to control. AOS expression following treatment with either live or dead *B. bassiana* spores was not significantly different from control. However, expression patterns followed the same pattern of downregulation and upregulation when applied as viable and dead spore, respectively. In the DP0912 cotton genotype, no significant differences in AOS gene expression among viable or dead spore treatments were found, consistent with the lack responses observed in the aphid count and plant defense elicitor experiments.

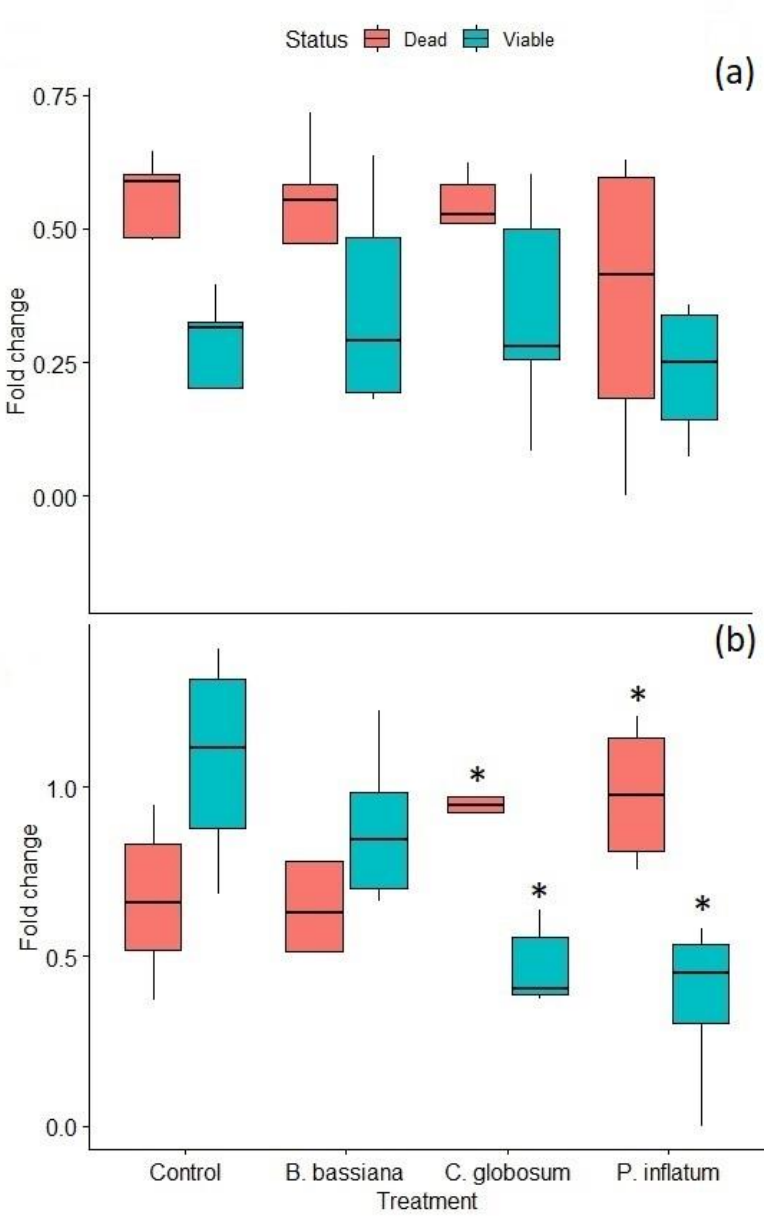

Figure II-4. Relative RNA expression of AOS gene by treatment, spore status and genotype. (a) DP0912 plants (b) PHY367 plants. Fold changes in Log10 scale. Significance denotated by asterisk ($p < 0.05$).

LOX1 gene expression in PHY367 was significantly downregulated in plants treated with dead spores of *P. inflatum* ($p < 0.0001$) and *C. globosum* ($p < 0.0001$) compared to control treatment (Fig 5). Dead *B. bassiana* spore treatments showed a similar trend of downregulation, but it was not significant. When viable spores of the same three species of fungi were used, there

was no effect on the relative expression of LOX1 gene. Coinciding with the AOS gene expression results, DP0912 plants treated with viable or dead spores did not respond with significant changes in the relative expression of LOX1 compared to control plants.

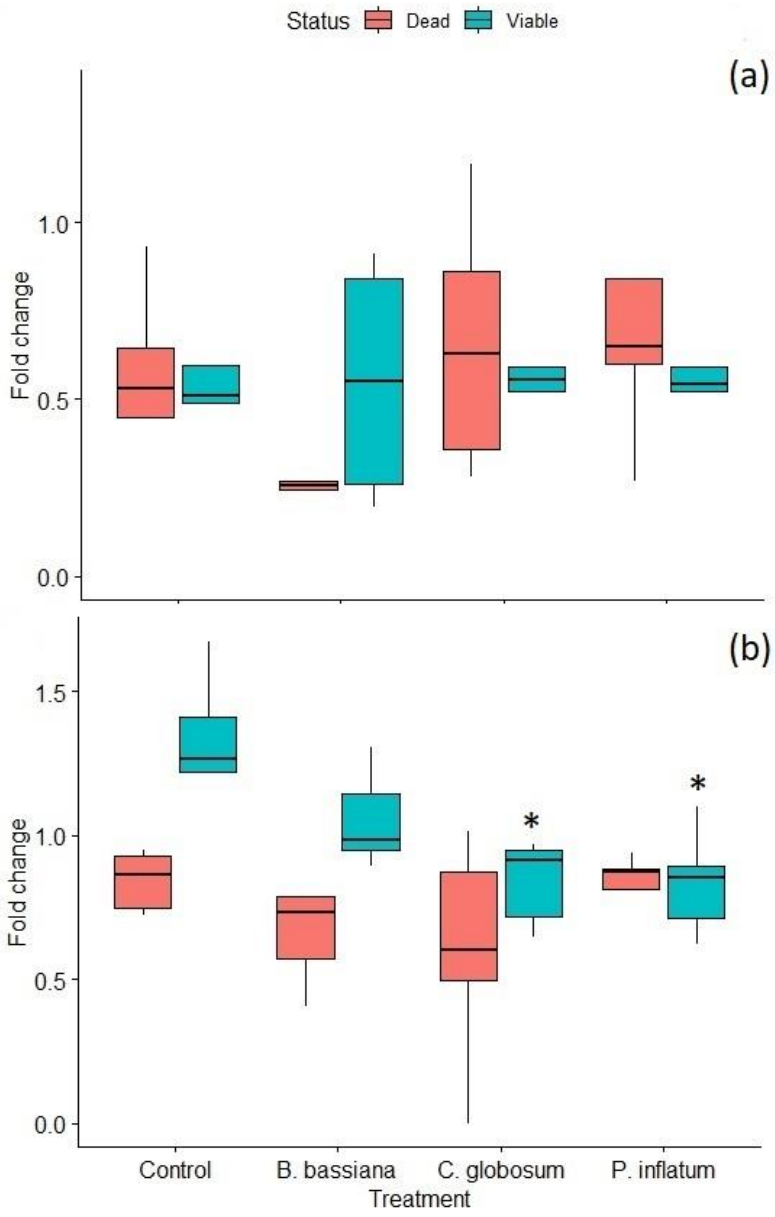

Figure II-5. Relative RNA expression of LOX1 gene by treatment, spore status and genotype. (a) DP0912 plants (b) PHY367 plants. Fold changes in Log10 scale. Significance denotated by asterisk ($p < 0.05$).

In addition to genes involved in ISR, we also analyzed expression of the Salicylic acid-responsive ß 1,3 glucanase gene involved in SAR in plants of both genotypes treated with either viable or dead spores (Fig 6). Significant differences in gene expression were detected only in PHY367 plants treated with viable spores of *P. inflatum* ($p = 0.0141$), resulting in this gene being downregulated in treated compared to control plants. No other combination of genotype, treatment or spore status was significantly different than controls.

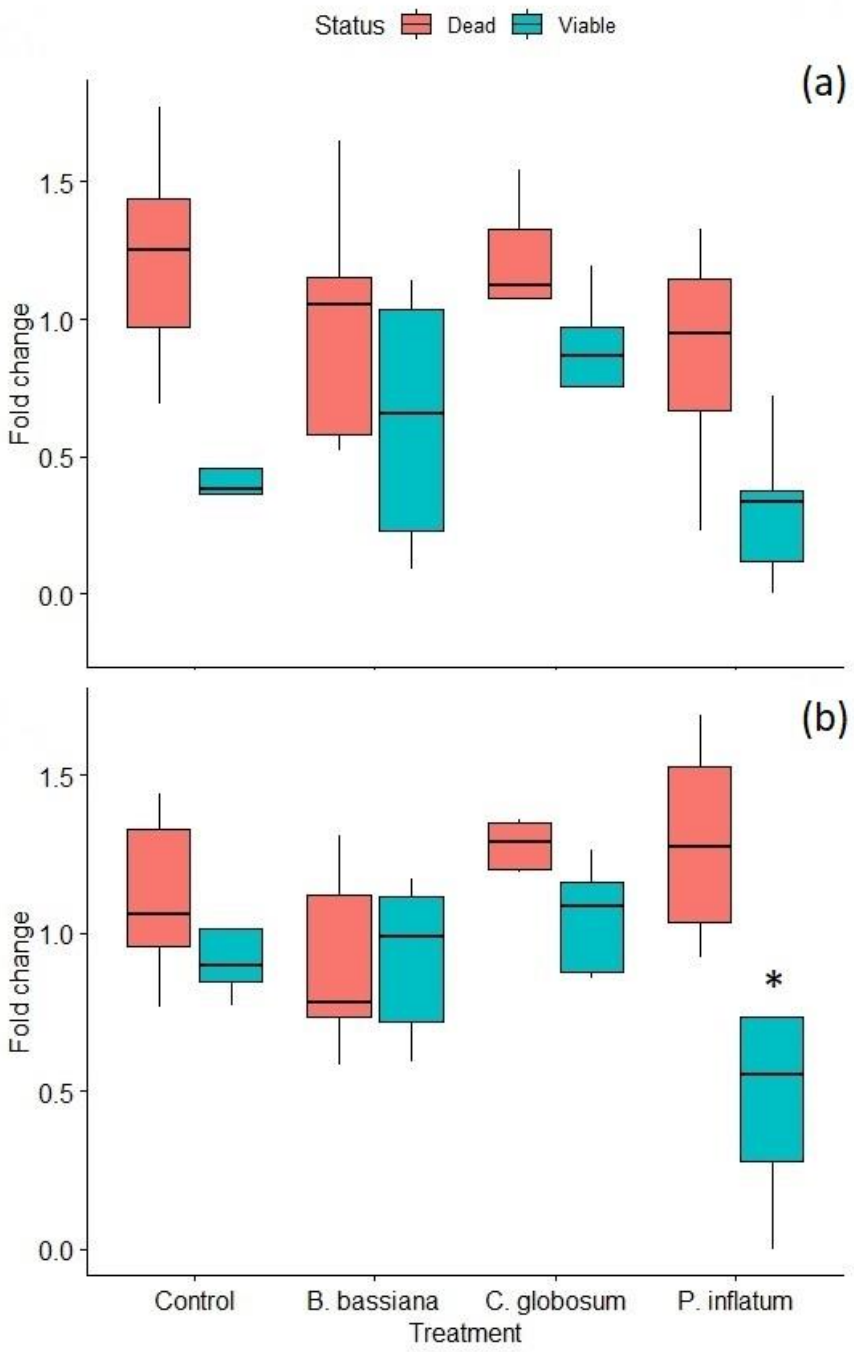

Figure II-6. Relative RNA expression of ß 1,3 glucanase gene by treatment, spore status and genotype. (a) DP0912 plants (b) PHY367 plants. Fold changes in Log10 scale. Significance denotated by asterisk ($p < 0.05$).

Effect of Foliar Treatments with Plant Defense Elicitors on Gene Expression

Since plant defense chemical elicitors showed a similar effect on aphids as fungal spore treatments, the corresponding gene expression patterns from plants treated with these chemicals should be comparable with the ones from spore treatments if the same plant defensive responses are involved. We tested this by assaying gene expression of AOS, LOX1 and β 1,3 glucanase genes on PHY367 and DP0912 cotton genotypes upon treatment with the plant hormone elicitors Actigard 50WG and MeJA (Fig 7). Treatment of PHY367 plants with MeJA resulted AOS gene upregulation, but it was not significant. The LOX1 gene on the other hand, was significantly upregulated ($p = 0.0388$) compared to controls. No treatment effects were detected in the expression of β 1,3 glucanase gene. Contrastingly, foliar treatments with Actigard 50WG had a clear significant upregulating effect on AOS ($p = 0.0036$), LOX1 ($p = 0.0079$, and ß1,3 glucanase ($p = <0.0001$) compared to controls. DP0912 plants treated with MeJA had significantly upregulated AOS ($p = 0.0179$) and LOX1 ($p = 0.0267$) gene expression. In contrast to PHY367, applications of Actigard 50WG to DP0912 resulted in a clear pattern of downregulation for AOS ($p = 0.0114$) and ß1,3 glucanase ($p < 0.0001$) genes. LOX1 showed the same trend, but it was not significant.

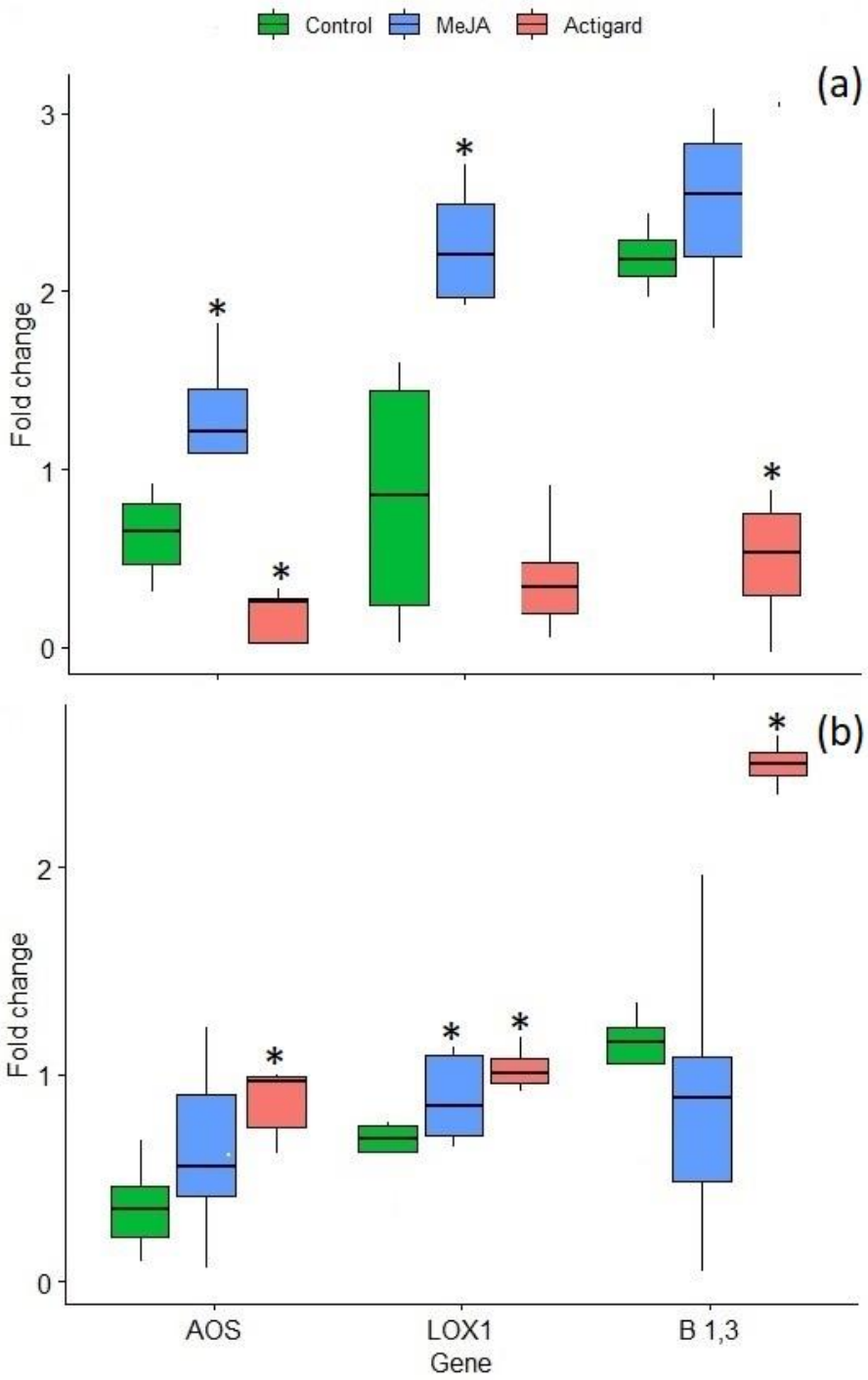

Figure II-7. Relative RNA expression of AOS, LOX1 and ß 1,3 glucanase genes by elicitor and genotype. (a) On DP0912 plants, MeJA significantly upregulated JA-responsive (ISR) genes, but had no effect on the SA-responsive ß 1,3 glucanase gene. Actigard 50WG significantly downregulated AOS and ß 1,3 glucanase genes. (b) On PHY367 plants, MeJA significantly upregulated the LOX1. Actigard 50WG significantly upregulated all three genes. Fold changes in Log10 scale. Significance denotated by asterisk ($p < 0.05$).

Discussion

Relatively little is known about how plants respond to the application of spores from potentially beneficial fungi (Jensen et al., 2020). Few if any studies have attempted to understand the role of plant genotype in mediating these interactions. This study clearly shows that two different varieties of transgenic upland cotton responded very differently to fungal spore treatments and chemical defense elicitors in terms of their effects on aphid population growth and defensive gene expression. The two cotton genotypes used in this study are commercial transgenic lines with both Bt and Round-up ready Flex technology traits that make plants resistant to lepidopterans and glyphosate, respectively. A key known ecological and agronomic difference between these two genotypes is that PHY367 is a variety considered as partially-resistant to root knot nematodes - RKN (Wheeler & Woodward, 2010). Although the specific mechanism of resistance is unknown, this attribute might result in these plants being more sensitive to microbe contact or plant phytohormone elicitation in general. Resistance to RKN in cotton is not vertical and comprises several defense related genes (Starr et al. 2007). It is also known that interactions between RKN resistance and fungal pathogen resistance usually results in a more effective plant protection profile against fungal disease (Wang & Roberts, 2006). Moreover, it has been reported that one of the mechanisms involved in nematode resistance is the active and elevated biosynthesis of chitinase to neutralize the initial nematode attack (Qtu et al., 1997 and Baker et al., 2020). Chitin is a mutual crucial component of both fungi and nematodes (Chen & Peng, 2019). Consequently, it seems reasonable to draw a connection between nematode resistance and increased sensitivity to fungi in PHY367.

Our results showed that PHY367 plants were more sensitive and responsive than DP0912 plants to the foliar application of fungal spores, suggesting this genotype is more prone to the induction of pattern triggered immunity (PTI) which is the way perceive and counterattack microbes (Szatmári et al., 2014). This also suggests that plant genotype might be more important to consider than previously thought when it comes to effects of microbial agents in agriculture. Indeed, genotype mediation of the establishment of microorganisms in a plant has been previously demonstrated in wheat (Samain et al., 2019) and cotton (Li et al., 2015). Our data also demonstrated that the response of PHY367 plants depended on whether spores were viable or dead. This resulted in a different pattern of defense gene expression and corresponding aphid performance. These observations suggest MAMP recognition when spores are dead could be suggested as a possible mechanism of the interaction.

To our knowledge, this study is the first to consider fungal spore viability as a variable in plant responses to foliar application with potentially beneficial fungi. Foliar treatments with viable spores on PHY367 resulted in a pattern of JA-responsive gene downregulation by all three fungi. However, in the case of the assayed SA-responsive gene, expression was only downregulated after treatment with *P. inflatum*. This change in defensive gene expression did not have a measurable corresponding effect on the population of cotton aphids. However, this pattern of defense gene downregulation when spores are viable may be an indication of plant colonization (Zeilinger et al., 2016). On the contrary, treatment with dead spores resulted in the JA-responsive AOS gene being upregulated and the cotton aphid populations on plants treated with dead spores were higher than those on untreated control plants. These findings suggest that the JA mediated ISR pathway might be involved in the positive response of aphids infested on plants treated with dead spores. Consistent with this, the aphid data showing an increase in

numbers on plants treated with MeJA, Actigard and Chitosan supports this assumption. Gene expression of PHY367 plants seven days after treatment with Actigard (a known SAR elicitor) showed that both JA and SA genes were significantly upregulated. In a recent study, the cross talk among different plant hormones was studied and concluded that JA plays a primary role and dominates the interaction (Yang et al., 2019). Thus, our hypothesis of JA induction and ISR being responsible for the positive aphid response still applies for the Actigard treatment.

Notably, several studies testing the effects of ISR and SAR inducers against aphids have reported lower number of aphids as consequence of the defense induction (Bruce, 2003; Cooper, 2005; Boughton, 2006; Sobhy, 2017; Selig, 2016; Dupius et al., 2014). In cotton specifically, Omer et al., (2001) reported negative effects on *A. gossypii* following foliar applications of JA. The main difference with our study is that we utilized whole plant in vivo assays and in the Omer paper they used detached leaves for their experiments. This means we tested aphid responses to whole plants with induced hormone profiles triggered solely by the elicitor treatment. When one excises the leaf from a plant, there is likely an amalgamation of induced responses due to the experimental treatments as well as the mechanical stress of excision (Heil et al., 2012). The cotton genotype in Omer paper was different as well. They used a conventional non-transgenic variety whereas both varieties used in this study were Bt and herbicide transgenic. As illustrated in this study, plant genotypic variation may able be an important variable to consider when it comes to comparing results among different studies.

Plants are always exposed to all kinds of biotic and abiotic stressors that may induce responses from the plant to defend itself. SA is typically associated with plant defense against biotrophs and phloem feeding insects (Costarelli et al., 2020). SA -JA cross talk has been widely studied and considered to be generally antagonistic (Koomneef et al., 2008). If these assumptions

applied to our study, then the activation of JA signaling by dead spores could undermine the plant defenses against aphids by the antagonistic effect JA on SA. However, our gene expression data does not suggest an antagonistic scenario based on the few genes we have investigated. Considering that the upregulation of AOS - JA gene by both *P. inflatum* and *C. globosum* in PHY367 treated with dead spores did not downregulate the expression of the SA gene in those plants. In the same way, the significant downregulation of both AOS and LOX1 in PHY367 plants treated with viable spores did not upregulated of the SA gene. In those plants. Perhaps crosstalk in this case is more complicated than what is assumed and a full transcriptome analysis may be necessary to better elucidate the complexity of these effects.

The DP0912 plants in this study did not respond to the application of fungal spores in the same way as PHY367 in terms of cotton aphid population performance or expression of plant defense related genes. No differences in the number of aphids were detected among treatments. Moreover, the foliar application of the plant defense elicitors MeJA, Actigard and Chitosan did not have an effect on aphid populations. Additionally, foliar fungal spore treatments on these plants did not change the expression of the defense related genes. However, the gene expression changes observed in DP0912 plants treated with both MeJA and Actigard showed that this genotype does indeed respond to the elicitors. In this case, MeJA upregulated JA-responsive genes, but Actigard downregulated both JA- and SA-responsive genes on plants of this genotype. Relative to the responses observed in PHY367, these observations suggest that differences in sensitivity to microbial recognition may underlie the lack of RKN tolerance in DP0912.

The sensitivity of PHY367 to the presence of dead fungal spores after foliar application led to a significantly higher number of aphids following treatments with dead spores of *B. bassiana* and *C. globosum*. *P. inflatum* treatment exhibited the same trend. The answer to why

dead spores produced the same effects as Chitosan treatment might reside in the process used to deactivate spores. In this study, viable spores were killed by raising the temperature of spore suspensions to 70ºC and maintaining it for two hours. This high temperature must produce some degree of protein denaturation, and this is exactly how the traditional process of chitin extraction begins when the product used as source of chitin is heated to a temperature of 62 to 100 ºC (Surinder & Dhillon 2015). Consequently, killing fungal spores with high temperature likely makes chitin from the fungal cell wall more readily available to be detected by the plant as a MAMP. The observed response of aphids to foliar applications of Chitosan supports our hypothesis that dead spores are being recognized as MAMPs (chitin) by cotton plants. Moreover, Chitosan promoted a positive effect on cotton aphid reproduction exclusively on PHY367 plants and not DP0912, which is exactly what happened when plants were treated with dead spores. The effects of dead fungi as plant defense elicitors have been recently explored by Vishwanathan et al., (2020). They found that soil treatments with hot water- killed fungi were enough to trigger ISR in Arabidopsis, thus demonstrating that dead fungi can be used as a source of MAMP to induce plant defenses.

Given that *B. bassiana* is a well-known entomopathogen, its failure to have a direct negative effect on aphid populations in this study deserves particular attention. One thing to consider is the timing of events. Plants in this study were infested with aphids seven days after fungal treatments with a moderate number of conidia per ml (1 x 10^6). Considering that the leaf surface is not a nutrient rich environment to support active growth of microbes (Lindow & Brandl, 2003), a gradual decline in spore viability is possible. Additionally, the way the isolates are maintained in the lab through many cycles of sub culturing in nutrient agar media might have

affected its virulence of this (Safavi, 2012). During the length of aphid assays, we did not observe any signs of mycosis affecting *A. gossypii* on plants previously treated with *B. bassiana*.

Fungal treatments as performed in this study did not reduce aphid populations; however, as Mortensen (2013) stated, plant defenses are not necessarily intended to negatively affect herbivores directly. Further investigation on different trophic levels might be necessary to more fully understand the plant – fungus – aphid interactions studied here and their ecological impacts on aphid populations.

CHAPTER III

MICROSCOPIC OBSERVATIONS OF FUNGAL SPORES ON LEAVES OF TWO GENOTYPES OF COTTON FOLLOWING FOLIAR APPLICATION

Introduction

Microscopy opens a new world of discoveries and expands the frontiers of our understanding of the most important life processes at the cellular level (Götz et al., 2020). Consequently, the use of microscopy is indispensable for the study of microbes including entomopathogenic fungi (EPF) (Li, 2013). In recent years, EPF such as *Beauveria bassiana* and *Metarhizium anisopliae* have been embraced as a more environment friendly approach to control of insect pest on different crops (Bamisile et al., 2020). EPF are usually applied as foliar sprays on different crops with the purpose of directly killing insect pests as a biological biopesticide. However, under the right circumstances and depending on the fungal species, this foliar treatment could lead to endophytic colonization of the host plant (Garrido-Jurado et al., 2020). Endophytes are defined as microorganisms that have the ability to live within plant tissues while producing no visible signs of negatively affecting their host (Bamisile et al., 2018)

Microscopic observations of fungal spores on leaves following foliar treatment are not a common practice among studies claiming active endophytic colonization of plants by the organisms being studied. In the majority of cases, detection of fungal establishment in the plant is achieved by means of plant tissue plating on nutrient media following surface sterilization of

tissues (Gurulingappa et al., 2010; Donga et al., 2018; Guesmi-Jouini et al., 2014; Rajab et al., 2020; Wei et al., 2020; Parsa et al., 2013). Fungal growth observed from these tissues is then reported to be the result of endophytic fungi colonization. However, the few studies utilizing microscopy to confirm fungal establishment of assumed endophytes typically find that when spores germinate on the surface of leaves, or in some way reach the tissues below the epidermis, there is rarely extensive systemic colonization of plant tissues, and often these fungal structures vanish over short periods of time (Nishi et al., 2020; Kock et al., 2018; Landa et al., 2013, Ullrich et al., 2017).

B. bassiana (Bals.-Criv) Vuill, is among the most common endophytic entomopathogenic fungus studied because of its widely-reported ability to colonize plants. Successful endophytic colonization by *B. bassiana* following foliar sprays has been reported in many different plant species including common bean (Parsa et al. 2013), tomato (Wei et al., 2020), cucumber (Rajab et al., 2020), artichoke (Guesmi-Jouini et al., 2014), sugarcane (Donga et al., 2018), citrus (Bamisile et al., 2019), and cotton (Gurulingappa et al., 2010). *B. bassiana* conidia are one-celled, of a rounded shape, colorless and range from 2 to 3 µm in size (Affandi et al., 2013). Conidia are attached to a structure called conidiophore (Pavlov et al., 2018).

Phialemonium inflatum (Burnside) Dania García, Perdomo, Gené, Cano & Guarro (= *Paecilomyces inflatus*), is a natural inhabitant of soil known to be involved in organic matter degradation (composting) by breaking down lignin and humic acid molecules (Kluczek-Turpeinen et al., 2003, 2005). It has been isolated as an endophyte from cotton (Ek-Ramos et al., 2013), red fescue (Santangelo & Kotanen, 2016), coffee berries (Vega et al., 2008), chili peppers (Paul et al., 2013) and tomatoes (Manzotti et al., 2020). Conidia from *P. inflatum* are usually

described as lemon-shaped, 3 to 4 µm in size, and form long, continuous chains connected to hyphae by a structure called the phialide (Paul et al., 2013).

Chaetomium globosum (Kunze), is a species of fungi recognized by its widespread distribution and because it thrives in an extensive number of living and inert substrates (Wang et al., 2016). *C. globosum* has been reported as a saprophytic fungus with quite remarkable activity in terms of degrading lignocellulose (Darshan et al., 2020; Wanmolee et al., 2016, Longoni et al., 2012; Di Prieto et al 1992). It has also been reported as a plant endophyte colonizing several species including *Artemisia desertorum* (Zhang et al., 2021), gingko (Li et al., 2014), cotton (Ek-Ramos et al., 2013), *Viguiera robusta* (Momesso et al., 2008), and barley (Vilich et al., 1998). Morphologically, it forms a macroscopic reproductive fruiting body called the ascoma (0.5 mm) which contains the ascospores (10 µm). The ascospores themselves resemble the shape of an American football (Wan et al., 2016).

Fungal species have evolved many different strategies to deal with environmental stressors and still complete their asexual cycles. Microcycle conidiation is one such strategy. Microcycle conidiation refers to the process of conidia being produced immediately after germination, with no formation of mycelia as there would be in a regular fungal life cycle (Hanlin, 1994). Microcycle conidiation can be triggered by unfavorable environmental conditions such as high temperature and has been observed in more than 100 fungal species (Jun et al., 2014). Some important entomopathogenic fungi have been reported to undergo microcycle conidiation such as *Hirsutella thompsoni* (Latgé et al., 1988), *Metarhizium acridum* (Zhang et al., 2010), and *B. bassiana* (Nishi et al., 2020). Additionally, fungal conidia can be subjected to fungistasis, a state of dormancy in which viable conidia do not germinate in the presence of other microorganisms (Blakeman and Fraser, 1971; Lockwood, 1977).

In this three-part study, we used microscopy to examine the fate of *B. bassiana*, *P. inflatum* and *C. globosum* spores on the surface of cotton plants following foliar inoculation. First, we looked at the spores on cotton leaves at three different time points following foliar applications. Second, we induced spore germination by increasing the relative humidity and adding molasses as a source of carbon to the spore suspension. Lastly, in-vitro assays were performed to test for the occurrence of microcycle conidiation of *B. bassiana* and *P. inflatum*. We used conventional leaf clearing, staining, and compound microscopy along with scanning electron microscopy (SEM) to observe fungal structures.

Materials and methods

Cotton Plants

Upland cotton (*Gossypium hirsutum*) seeds from two commercially available transgenic cultivars, Deltapine 0912 and Phytogen 367, were used in this study. Seeds were planted in 6.3 cm^2 disposable pots containing 250 cc of Pro-Line C25 growing mixture. Plants were grown indoors with artificial light (light fixture with 6 T5 6400K 1.2m tubes) under a 14:10 photoperiod of light and dark, respectively. Temperature and relative humidity in the growing room were 30 ± 2 ºC and 55 ± 5% respectively.

Fungal Isolates

Conidia (spores) of three species of fungi, *B. bassiana, P. inflatum* and *C. globosum* were used in this study. The *B. bassiana* GHA isolate was obtained from the commercial product

Botanigard (BioWorks). *P. inflatum* (TAMU 490) and *C. globosum* (TAMU 520) were obtained from a collection of fungal endophytes originally isolated from cotton (Ek-Ramos et al 2013). Fungal isolates were cultured on 100 x 15 mm plastic Petri dishes containing solid agar media. *B. bassiana* and *P. inflatum* were cultured on Potato dextrose agar (PDA) and *C. globosum* was cultured on an unclarified V8 media. *C. globosum* was cultivated on V8 media to shorten the time needed to yield conidia. Culture plates were placed in Styrofoam boxes and kept at room temperature in the dark. Mature cultures (14 days old) were the source of inoculum for these experiments. Conidia were harvested by adding 15 ml of 0.01% Tween 80 solution on top of the fungal culture. Then a sterile L-shaped spreader was used to dislodge all conidia from the surface, making a concentrated spore suspension that was transferred to a 50 ml sterile centrifuge tube. Additional 0.01% Tween 80 was added to obtain a final volume of 40 ml. Tubes were vortexed for two minutes and then the suspension was passed through a 500-mesh sieve (25 µm). The final, filtered spore suspension was then transferred to a new 50 ml centrifuge tube. A Neubauer improved hemocytometer was used to adjust the final spore concentration to 1×10^5 per ml. Spore viability was checked by making serial dilutions and plating 100 µl of spore suspension on PDA. These dishes were placed in an incubator and kept at 28 ºC. Colony forming units (CFU) were counted four days later.

Fungal Spore Treatment and Sampling

Spores of the three fungal isolates described above were sprayed on second true leaf stage cotton plants of both DP0912 and PHY367 genotypes. A 250 ml handheld sprayer was used to

cover plants with fungal spore suspension. Leaf samples for microscopic observation were taken at 24h, three days and seven days after treatment. Five plants per treatment and two leaves per plant were used at each sampling date. At the time of foliar application, 100 µl samples of each spore suspension were spread over PDA media and served as a reference of spore germination timeline. For this purpose, 1 cm^2 of culture media was taken 24h and 48h after plating, and placed on a glass slide. A drop of lactophenol cotton blue was placed on the media, and a coverslip laid on top. Spore germination was observed at 400x magnification.

Induced Germination of Fungal Spores

To promote spore germination on the leaf surface, spore suspensions were prepared with a sterilized 0.5% (v/v) aqueous solution of molasses (Grandma's original unsulphured). Spore suspensions were prepared and applied as described above. Treated cotton plants were then placed on a plastic tray and inserted into a large clear plastic bag to maintain high humidity. Plants remained in this set-up for 72 hours at room temperature, after which samples were taken to be processed for later microscopy. Preliminary observations indicated that 72 hour was enough time to promote germination.

Microcycle Conidiation Assays

Preliminary observations of *B. bassiana* development on glass slides with no extra sources of carbon suggested that microcycle conidiation could be induced on the leaf surface by

simply increasing the relative humidity. We tested this hypothesis for *B. bassiana* and *P. inflatum*. *C. globosum* does not undergo reproduction on live green tissue and was excluded from this part of the study. Plants were grown as previously described. Individual, fully developed leaves from both genotypes (n=10) were surface sterilized by placing the leaves into 70% ethanol for one minute, then transferring to a 10% bleach solution for two minutes. The leaves were then rinsed three times in sterile water. Following this surface sterilization, leaves were dried with sterile paper towels and kept under a laminar flow hood. Leaves were then individually transferred to non-nutrient 1.5% w/v agar dishes (100 x 15mm) with adaxial side facing down. A water-based spore suspension was used in this study and adjusted to 2 x 10^5 spores per ml (*B. bassiana*) and 2 x 10^4 spores per ml (*P. inflatum*). Leaf inoculation was done by placing 100 μl of spore suspension (divided into 10 small drops) on different parts of the leaf. Leaves were kept under a laminar flow hood until the droplets dried. Dishes containing leaves were covered with lids and sealed with Parafilm, then moved to an incubator and kept for five days at 28 ºC.

To test whether microcycle conidiation can increase the number of spores on a leaf surface, the number of viable spores was compared between the day of inoculation and five days later. To quantify the initial number of viable spores contained in the original 100 μl spore suspensions, aliquots of 100 μl were pipetted in 50ml sterile centrifuge tubes (n=6) containing 20 ml of 0.01% Triton 100x solution. Tubes were vortexed for two minutes, then 100 μl was taken from each tube, plated on PDA dishes and kept at 28 ºC in incubator. CFUs were counted five days later. To determine the number of CFUs per leaf at five days post inoculation, leaves were taken from agar dishes and transferred to 50ml sterile centrifuge tubes containing 20 ml of 0.01% Triton 100x solution, then vortexed for two minutes to remove all conidia from leaf

surface. A 100 μl aliquot of this suspension was taken from each tube and spread on two sets of PDA dishes (10 and 100 times diluted).

Leaf Sample Preparation for Microscopy

A 15mm hole diameter cork borer was used to take pieces of leaf for sample preparation. These samples were placed in individual 17ml wells of sterile, clear plastic 6-well cell culture plates and stained using the Trypan blue staining technique (Chung, et al 2010). The tissue clearing phase required 2.5 ml of clearing solution "A" containing acetic acid and ethanol (1:3, v/v). Plates were shaken at low speed on a Bellco orbital shaker for 24 hours. The first clearing solution was then removed and replaced with 2.5 ml of clearing solution "B" containing acetic acid, ethanol, and glycerol (1:5:1, v/v/v), and plates were shaken at low speed overnight. The second clearing solution was then removed and replaced with 2.5ml of a staining solution composed of 0.01% (w/v) trypan blue in lactophenol. After a 24-hour period, staining solution was removed and replaced with 2.5 ml of an acidified 60% glycerol solution. Plates containing stained samples were stored at room temperature until prior to microscopy. Sample preparation for scanning electron microscopy was done following Shively & Miller (2009) and Neinhuis & Edelmann (1996). Briefly, small pieces of the leaf (max 10x10mm) were dipped in 100% methanol for at least 30 min. Leaf tissues then went through a methanol/hexamethyldisilazane (HMDS) series (2:1, 1:1, 2:1) five minutes each, and then left in 100% HMDS overnight. The leaf sample was then allowed to dry for several hours, mounted on SEM stubs, sputter coated with gold, and imaged in the SEM.

Microscopic Observations

Bright field microscopic observations were made using a Motic BA410 microscope equipped with EC Plan strain-free objectives (10X/0.25, 20X/0.45 and 40X/0.65). Images were taken with a Moticam 2500 CMOS 5.0 MP live resolution capability digital camera. A calibration slide micrometer (0.01mm) was used to calibrate scale bars. SEM was done at the Texas A&M Microscopy and Imaging Center using the Tescan Vega SEM. Microscopic observations focused primarily on finding spores and any sign of fungal development such as germination tube formation and the presence of fungal hyphae inside or outside of plant tissues.

Statistical Analysis

JMP Pro 15 was used for statistical comparisons. We checked for normality with the Anderson-Darling test. Depending on data being normally distributed or not, either the student t-test for independent variables, or the Wilcoxon test was used to evaluate differences between treatment groups ($\alpha = 0.05$). Graphs we built with R ggplot package.

Results

Microscopic Recognition of Fungal Species

B. bassiana, P. inflatum, and *C. globosum* were cultured on nutrient agar media. Reproductive structures and spore germination patterns are shown in Fig. 1. Spore germination

time on PDA dishes at 26 ºC was 12 hours for *C. globosum*, 24 hours for *B. bassiana,* and 48 hours for *P. inflatum*. The typical size of the spores was approximately 4µm, 5µm and 10µm for *B. bassiana, P. inflatum* and *C. globosum,* respectively. *B. bassiana* spores presented more size variation (from 3 to 6 µm Fig. 1A), and the other two fungal species were more consistent in their dimensions. Bipolar germination tubes were more commonly observed in *P. inflatum* and *C. globosum* (Fig 1 E, F respectively).

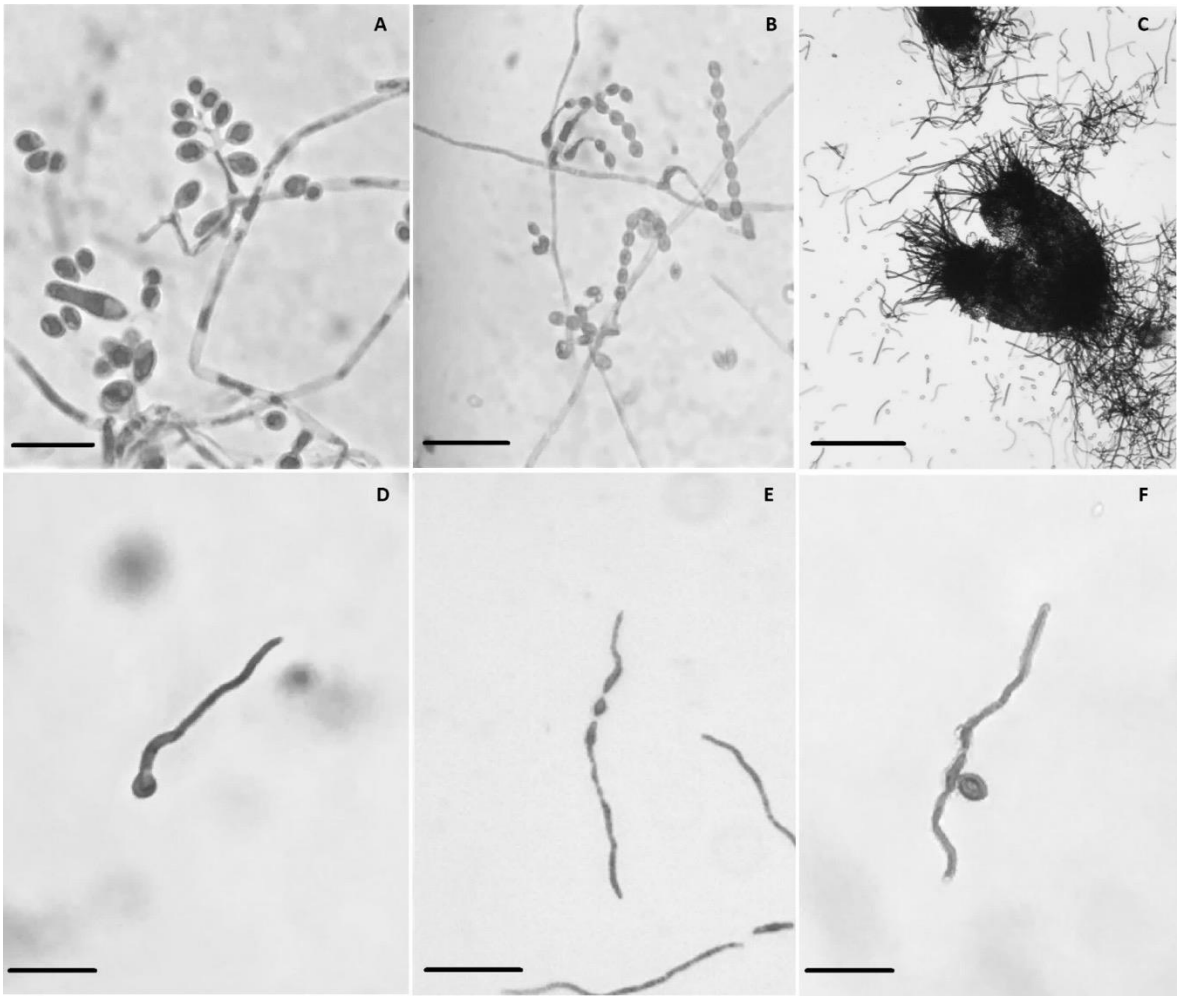

Figure III-1: *B. bassiana* conidiophores (A) and germinating spore (D), *P. inflatum* phialides (B) and bipolar germination spore pattern (E) *C. globosum* Ascoma (perithecium) (C) and germinating spore (F). Scale bares are: A= 10 µm, B= 20 µm, C= 0.2 mm, D= 15 µm, E= 25 µm, F= 30 µm.

Fungal Spores on Leaves Following Spray Application

Under the conditions plants were grown in this study, we found that no spore germination took place on the surface of cotton leaves at any of the three-time observation points following spore applications. All three tested fungi had the same results (Fig. 2).

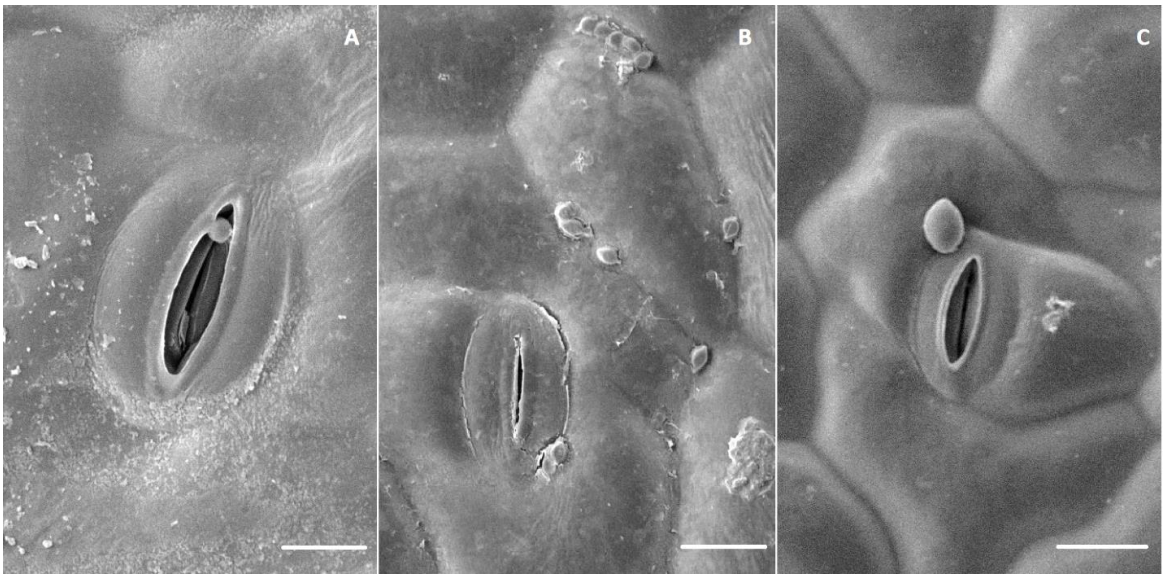

Figure III-2: Spores of *B. bassiana* (A), *P. inflatum* (B) and *C. globosum* (D) on cotton leaves as seen following spray applications. Photos taken with SEM. Scale bars: A= 15 µm, B= 10 µm, C= 20 µm.

Fungal Spore Development on Leaves when Germination was Induced

Given the lack of spore germination on the surface of leaves under ambient conditions, we induced spores to germinate by adding molasses to the spore suspensions and by increasing the RH to 100%. Under these conditions, germination tubes were observed within 48 hours for *B. bassiana* and *C. globosum*, and within 72 hours for *P. inflatum* on both plant genotypes (Fig. 3 A - C). In a few rare instances, we observed germination tubes making their way inside the plant

through stomata openings (Fig 3. D – F). In all cases, there rare events involved close spatial proximity between spores and nearby stomata by chance during foliar application.

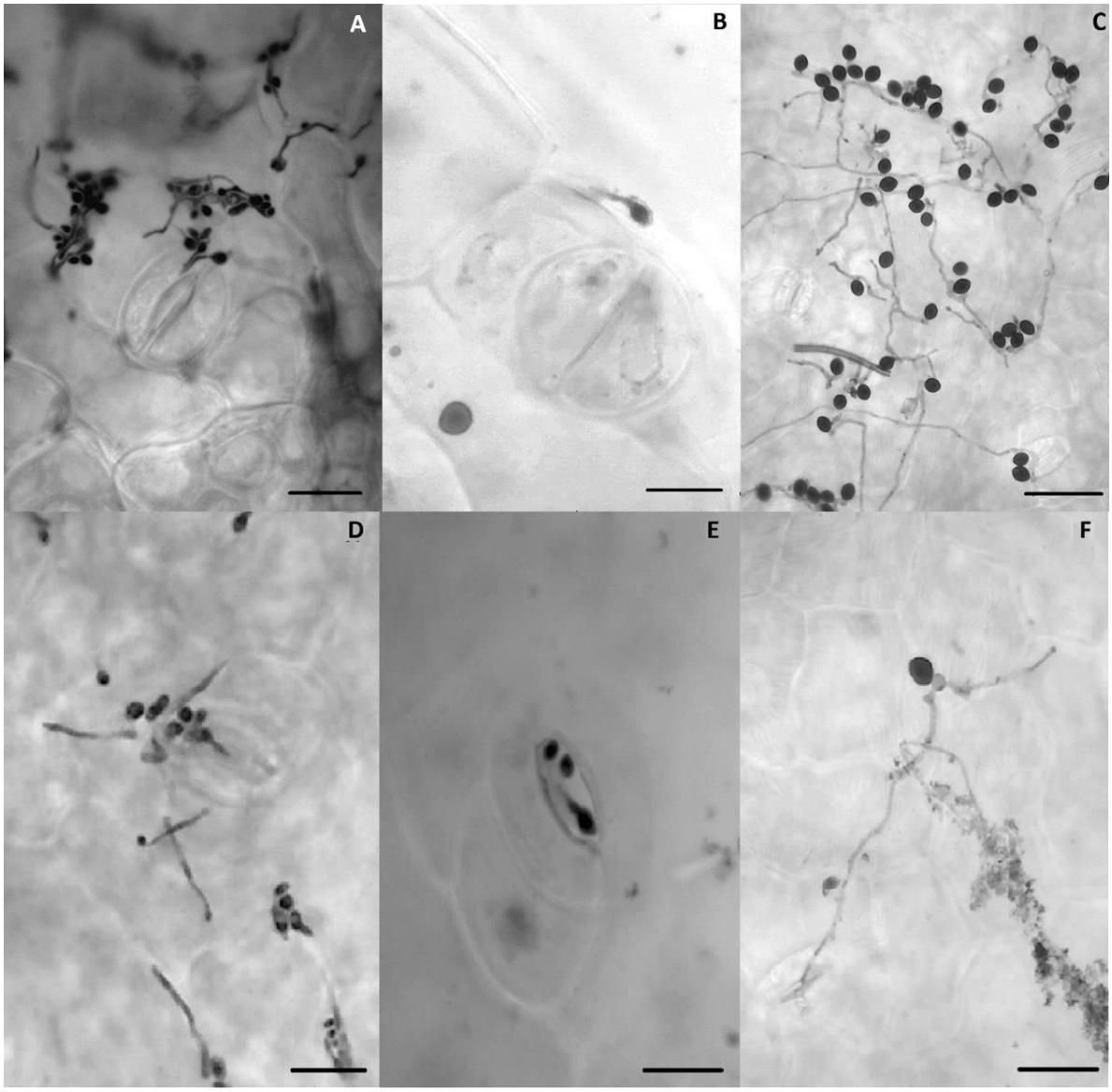

Figure III-3: Induced spore germination and signs of fungal colonization of cotton leaves for *B. bassiana* (A, D), *P. inflatum* (B, E), and *C. globosum* (C, F). St (stoma), Co (conidia). Scale bars: A= 20 µm, B= 25 µm, C= 50 µm, D= 15 µm, E= 10 µm, F= 30 µm

Spore germination induction with molasses and high relative humidity also led both *B. bassiana* and *P. inflatum* to undergo microcycle conidiation on the leaf surface (Fig. 4). This resulted in

these two fungal species reproducing asexually and potentially providing a mechanism to increasing the amount of inoculum on leaf surfaces.

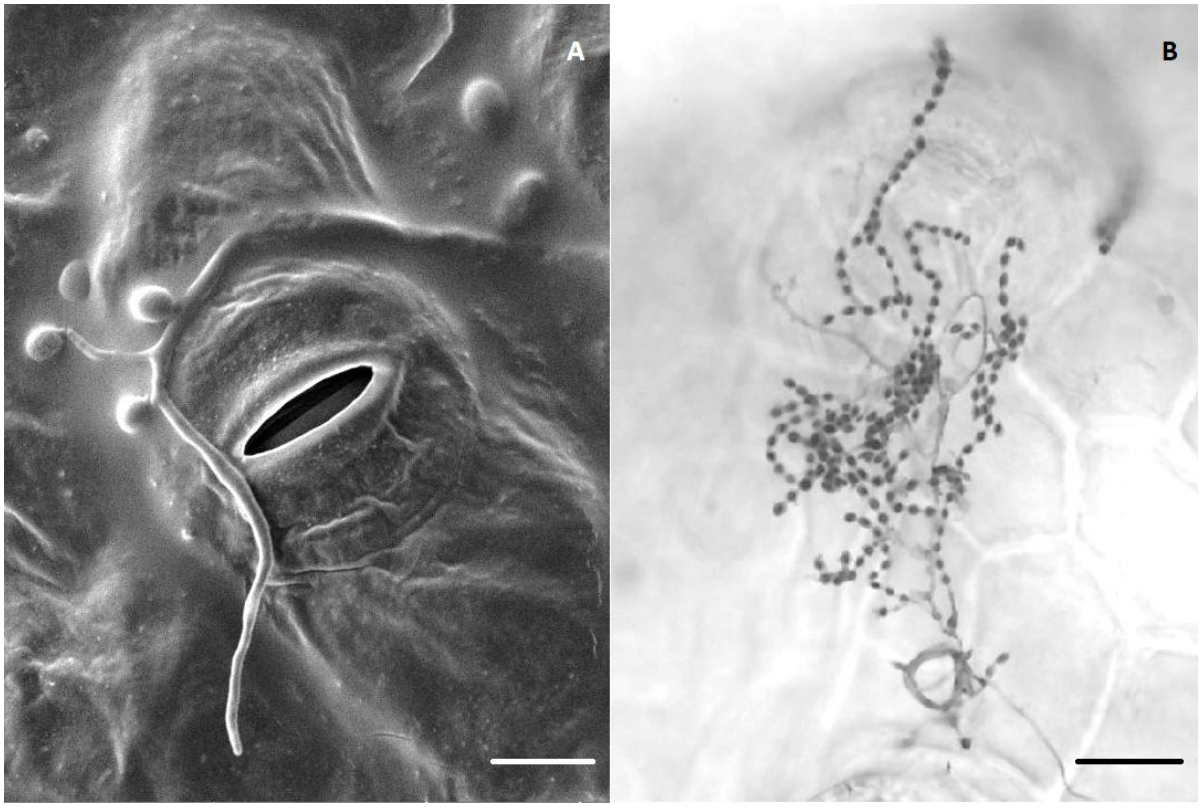

Figure III-4: Microcycle conidiation of *B. bassiana* as observed on the surface of a cotton leaf with SEM (A). *P. inflatum* microcycle conidiation on a cotton leaf as observed with a compound microscope (B). Scale bars: A= 10 µm, B= 20µm

Microcycle Conidiation Assays

Preliminary observations of *B. bassiana* development on glass slides with no extra sources of carbon, suggested that microcycle conidiation could occur on the leaf in the presence of sufficient relative humidity. Fig. 5 shows the number of CFUs (colony forming units) recovered from leaves on the day of inoculation (baseline) and five days post inoculation. *B. bassiana* went into active asexual reproduction, producing significantly more conidia on the

surface of leaves of both cotton genotypes in both trials (Phy367: p = 0.0024 and 0.0109 and DP0912: p = 0.0042 and 0.0081). *P. inflatum* did reproduce, but to a much lesser degree and not enough to be statistically different than control checks on either genotype.

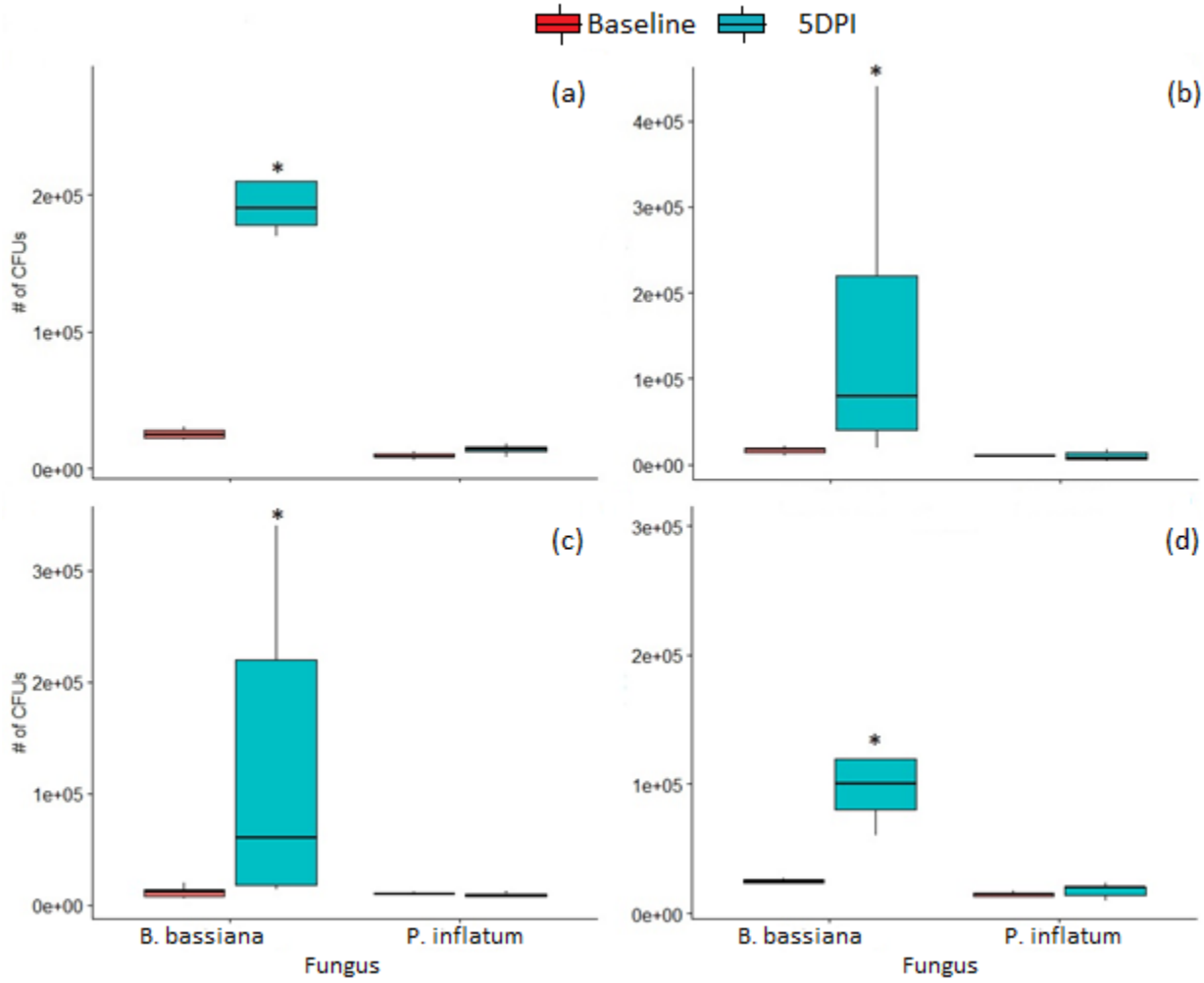

Figure III-5: In vitro microcycle conidiation assays of *B. bassiana* and *P. inflatum* by plant genotype. PHY367 genotype first and repeat assays (a, b). DP0912 genotype first and repeat assays (c, d). Error bars are SE from the mean. Significant differences are denoted by asterisk (p < 0.05).

Discussion

This study aimed to clarify whether endophytic colonization of cotton by *B. bassiana*, *P. inflatum* and *C. globosum* fungi was possible following foliar spore applications. Findings in this section are an attempt to test some of the assumptions from the previous chapter. We report that under the conditions the plants were grown in this study (55% and 30ºC average HR and temperature), endophytic colonization by the three fungal species was unlikely to occur given that no spore germination was observed on the surface of cotton leaves. However, since we did not screen 100% of leaf area treated with spores, we cannot rule out the possibility of a very low occurrence of colonization that was not detected in this study. This finding is in congruence with the results of Wagner and Lewis (2010) and Quesada et al. (2006) who reported (based on microscopic observations) that *B. bassiana* spore germination percentages on corn and opium poppy leaves were low and in the single digits. These studies did not provide any special conditions to promote spore germination. Thus, this study provides further evidence that fungal spore germination on the leaf surface will likely not occur if conducive conditions are not met (Sephton-Clark & Voelz, 2018). However, it is important to notice that after 7 days on the leaf, fungal conidia remained intact in shape and size on the leaf epidermis which may be an indicator of spore viability given that chitin, the component of fungal cell walls, is not very persistent and easy to break down (Fernandez & Koide, 2014). Additionally, it is possible that these spores could have been in a state of fungistasis on the leaves in the absence of suitable exogenous carbon for germination and growth. The leaf surface is well known for being an unfriendly setting microbe without resources to stimulate germination (Lindow and Brand, 2003). Hence,

extensive spore germination and colonization should not have been expected given the conditions the plants were grown in these experiments.

It was also found that modifying the environment of the leaf surface by enriching the carbon source with 0.5 % molasses and increasing the relative humidity created a more conducive setting for conidia of all three fungi species to germinate. This was consistent with breaking the state of fungistasis. This germination was followed by the proliferation of germination tubes on the epidermis of cotton that eventually can result in hyphal penetration through stomata openings. This agrees with previous studies that have reported that foliar applications of *B. bassiana* resulted in endophytic colonization of cotton (Gurulingappa et al., 2010; Gonzalez-Mas et al., 2021), citrus (Bamisile et al, 2019), common bean (Parsa et al. 2013), tomato (wei et al., 2020), and other plants. In the Gurulingappa (2010), Parsa (2013) and Gonzalez-Mas (2021) studies, they induced the germination of spores by covering the plant treated area with plastic wrap or bags. Thus, providing more favorable conditions (high levels of humidity) for initial fungal development. The extent of fungal colonization beyond the stoma opening could not be revealed with the light microscopy techniques used in this study. Fluorescence microscopy with GFP transformed fungi may be a better technique to further evaluate the extent of colonization below the epidermis. Active appressorium-like colonization involving penetration of plant tissues, as it occurs with specialized plant pathogens, was not observed.

The use of molasses as a source of carbon to supplement growing media for mass production of fungi has been described previously in several studies (Mascarin et al., 2010; Sughra et al., 2013; Wafaa et al., 2016). However, the use of molasses in foliar sprays as a means to intentionally promote fungal spore germination and development has not yet been described in

the literature to our knowledge. In this study, microscopic observations of fungal development on leaves treated with spore suspensions that had been enriched with molasses provided evidence of *B. bassiana* and *P. inflatum* going into an asexual reproductive stage known as microcycle conidiation. To our knowledge, microcycle conidiation of the *P. inflatum* as a consequence of carbon enriched spore suspension containing molasses has not been previously described in the literature. Importantly, this approach of enriching spore suspensions with a source of carbon could potentially be used to improve the performance of fungal entomopathogens applied under greenhouse or even field conditions; especially in situations with a high level of insect infestation, where boosted spore germination and on the leaf surface could improve insect control by increasing the number of infectious spores. Further investigation is needed to more fully evaluate this possibility and its impact, if any, on biological control efficacy.

As part of this study, we surface sterilized cotton leaves used in microcycle conidiation assays due to unwanted infections with fast growing fungi that had ruined experiments in the preliminary phase of this work. However, we did not achieve 100% control of undesirable fungal contaminants. This prevented us from having an even number of samples for processing on each trial. CFU counts to quantify spore production due to microcycle conidiation of *B. bassiana* were done by Nishi et al (2020). In that study they standardized samples by area, taking small leaf pieces of 1 x 1 cm and placing them in tubes with 0.05% Tween. We, on the other hand, standardized samples by volume of spore suspension placed on leaf. Thus, each leaf, regardless of size, received the same number of spores. Of course, we assume a non-significant variation based on the numbers of CFUs obtained to assess spore viability (baseline). In preliminary tests, we found it more convenient to use the whole leaves and 50 ml centrifuge tubes to carry out the experiments, as no tedious leaf cutting was needed. Regardless of size, our *B. bassiana*

microcycle reproduction data is consistent with the data reported in the Nishi et al (2020) paper. *P. inflatum* on the other hand, did not perform as expected. We observed this fungus reproducing successfully on cotton leaves when the spore suspension was carbon enriched with molasses and high humidity, but not with high humidity alone. These results suggest that the leaf surface is not conducive for microcycle conidiation of *P. inflatum*, and unlike *B. bassiana*, it needs and extra source of energy to meet its metabolic requirements for reproduction.

CHAPTER IV

CONCLUSIONS

This study explored the interactions of plant – fungus – insect in cultivated cotton. It was demonstrated that foliar applications of fungal spores on cotton undoubtedly affect plant defensive responses, however this response is greatly influenced by plant genotype and spore status (viable or dead). Moreover, it was showed that cotton aphids benefitted from changes in the plant defense hormone profile induced by dead fungal spores in the responsive genotype. Data from chemical plant defense elicitors and gene expression analysis was consistent with my initial findings, hence demonstrating that elicitation of plant defenses was responsible for the higher number of aphids on PHY367 plants. The results obtained from treatments with Chitosan suggest that MAMP recognition is the possible mechanism involved in the responses of PHY367 to dead spore treatments. Hence, heat-killed fungal spore treatments should be considered to explore the potential of this finding in agriculture. To my knowledge this is the first report of fungal dead spore foliar treatments as a means to trigger plant defenses.

In the third chapter, I provided photographic evidence that fungal spores applied as simple water-based foliar sprays on cotton leaves do not readily germinate under conditions where the relative humidity is not high. Moreover, we show that a combination of carbon enriched spore suspensions and high humidity conditions can boost germination and initial fungal development. Spore germination under these conditions led to a low incidence of fungal colonization of leaves through stomata and microcycle conidiation of both *B. bassiana* and *P.*

inflatum on the leaf surface. However, it appears like plant colonization is more the result of chance rather than an active process of penetration into plant tissues. It was also found that high humidity was the only requirement for *B. bassiana* to reproduce on cotton leaves through the process of microcycle conidiation.

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