PLANT-ASSOCIATED FUNGI EFFECTS ON INSECT HERBIVORES AND ON A PREDATOR

A Dissertation

by

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ABSTRACT

This dissertation analyzed plant-associated fungal effects on two insect pests of cotton (*Gossypium hirsutum*), the boll weevil (*Anthonomus grandis grandis*) and cotton aphid (*Aphis gossypii*), as well as on the predatory convergent lady beetle (*Hippodamia convergens*). In chapter 2, I tested whether cotton plants grown from seeds treated with plant-associated fungi affect boll weevil behavior, fecundity, and development. In chapter 3, the direct pathogenicity of different plant-associated fungal isolates towards cotton aphids was tested. In chapter 4, I tested whether olfactory cues from cotton plants seed-treated with plant-associated fungi and infested with cotton aphids affect convergent lady beetle behavior.

Boll weevil behavior towards fungal-treated cotton squares was strain-specific, in some cases making them avoid squares from fungal-treated cotton plants. Regarding boll weevil fecundity, fewer larvae hatched, and fewer adults emerged from fungal-treated plants. In addition, developmental time to the adult stage was prolonged in fungal-treated plants. These results indicate the potential for fungal cotton treatments as a new tool for boll weevil management that can repel adults, reducing offspring numbers and affecting their performance. Consequently, fungal treatments could negatively affect the population size of subsequent generations in the field.

Spore suspensions of four plant-associated fungal strains originally isolated as endophytes from cotton were shown to be entomopathogenic to cotton aphids and lowered survival to levels comparable to the commercially-available entomopathogen,

Beauveria bassiana, in all the treatments tested. Mycosis was confirmed from the cadavers, establishing the complete infection cycle for all tested isolates. These results highlight the potential to develop these fungi as novel bioinsecticides.

Lastly, cotton aphids infesting fungal seed-treated plants were used in olfactory assays to assess predatory convergent lady beetle behavioral responses. Minor strain-specific responses were found, with one isolate having mild negative effects on convergent lady beetle host selection behavior while another had no effect. This evidence suggests that lady beetle behavioral responses to plants might vary according to fungal treatment but would not strongly impact their use as part of an insect pest management strategy.

DEDICATION

This dissertation is dedicated *in memoriam* to my grandmother Gisele Cunha, who always encouraged me never to stop studying and could not see the conclusion of this degree in life.

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Contributors

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All the work and analysis conducted for this dissertation were completed by the student independently. Chapter 4 is a manuscript submitted and under revision.

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1. INTRODUCTION

1.1. Cotton as a crop

Gossypium belongs to the Malvaceae family and is in the tribe Gossypieae with four subgenera and 50 species distributed in the tropics and subtropics (Wendel, Brubaker et al. 2010). Species in this genus exhibit substantial morphological variability ranging from herbaceous perennials to small trees (Wendel and Cronn 2003). Among *Gossypium* species, only four are used as crops (*G. arboreum* L., *G. herbaceum* L., *G. hirsutum L.*, and *G. barbadense* L.), with the last two representing over 90% of annual fiber production all over the world (Wendel, Brubaker et al. 2010, Wang, Tu et al. 2019). *G. hirsutum*, Upland cotton, is the species most widely cultivated worldwide due to its high yield. The cotton crop represents a large portion of global trade, and it is one of the most valuable fiber crops in the world.

In the 2021/2022 growing season, 33 million hectares of cotton are expected to be cultivated worldwide (ICAC 2021). Globally, the crop generates about US$ 12 billion and employs 350 million people in all production processes (ABRAPA 2016). Around 70 countries cultivate cotton worldwide, producing approximately 25 million metric tons of cotton lint this season. Five countries stand out as the leading cotton producers (India, China, United States, Brazil, and Pakistan ranked in order of production), three of which were top exporters (United States, Brazil, and India ranked in order of exportation) (ICAC 2021).

Given the international importance of cotton as a crop for the global economy, it is a crucial plant system for studies of agroecosystem ecology. The economic and ecological sustainability of cotton production is directly related to the efficiency of combating agricultural pests and minimizing adverse environmental effects. Various pests attack cotton by feeding on its above- and belowground structures such as roots, stems, leaves, squares (flower buds), flowers, and bolls. The damage caused by these pests can directly affect cottonseed and fiber production when insects feed directly on the squares and bolls or may indirectly affect boll production when insects damage the foliage or roots causing adverse effects on plant development (Santos 2001, Bastos and Torres 2006, Silva and da Silva 2015).

Integrated Pest Management (IPM) is a pest control strategy that uses a combination of measures to reduce pest damage (Pedigo and Rice 2014). According to the US Department of Agriculture, IPM is a sustainable, science-based, decision-making approach that combines different tools to identify, manage and reduce pest damage in a way that minimizes economic, health and environmental risks (USDA 2018). IPM utilizes a combination of approaches, including biological, cultural, chemical control, plant resistance, and genetic engineer (Dhaliwal, Koul et al. 2004, Kos, van Loon et al. 2009, Anderson, Ellsworth et al. 2019). Thus, the overall goal of utilizing these techniques is to maintain pest populations at a low level to prevent economic damage to crops.

Farmers widely use chemical control, but the unnecessary or excessive use of insecticides may cause insect resistance in pests and have negative unintended

environmental and health consequences (Rani, Thapa et al. 2021). Thus, it is essential to better understand how pests and their natural enemies (micro-and macroscopic organisms) interact to manipulate these interactions to produce cotton crops more efficiently (Miranda 2010).

1.2. Plant-associated fungi as an IPM tool

The phytobiome represents the interactions between plants, environment, and organisms that may also be manipulated as a vital tool for IPM in agricultural systems (Hawkes and Connor 2017, Leach, Triplett et al. 2017). Some microbes can be used to increase natural enemies' efficiency in multitrophic interactions as well as to control insect pests directly (Jaber and Araj 2018). Endophytic microorganisms, mainly bacteria and fungi, live within most parts of a host plant without causing any noticeable harm, and in some cases, can be beneficial to plants (Porras-Alfaro and Bayman 2011, Bamisile, Dash et al. 2018). Endophytic fungi can colonize plant tissues by different mechanisms such as spores' penetration through natural openings or wounds, vertical transmission of hyphae into seeds and seedlings, or direct spores' inoculation by insects (Tintjer, Leuchtmann et al. 2008, Vieira 2010). The relationship of endophytes with plants differs from phytopathogenic microorganisms, which cause disease, and epiphytes that only live on plant external surfaces (Azevedo, Maccheroni Jr. et al. 2000, Santos and Varavallo 2011).

There are many potential benefits of the relationship between host plants and endophytic microorganisms. Endophytic fungi receive food (carbon source) and

protection from their host plant and, in turn, can provide a source of nitrogen, increased resistance to abiotic and biotic stressors, and enhanced plant growth to their host (Behie, Zelisko et al. 2012, Behie and Bidochka 2014, Behie, Moreira et al. 2017). The ecological results of these plant-associated microbes and the symbiosis with their host plants make them potentially valuable for improving crop performance in agriculture. Little is known about the specific mechanisms involved in these beneficial interactions but, in some cases, secondary metabolites produced by microbes have been shown to promote plant growth and development and have been found to possess antimicrobial and antioxidant compounds (Sadrati, Daoud et al. 2013, Dutta, Puzari et al. 2014). Microbial insecticides (or biopesticides) are biological preparations often sprayed or delivered similarly to conventional insecticides (Pedigo and Rice 2014). At least 170 fungal biopesticide products have been developed worldwide, and about half of them come from Central and South America (de Faria and Wraight 2007). As shown in **Error! Reference source not found.**, the most used products are based on the fungi, *B eauveria bassiana* or *Metarhizium anisopliae* (Chandler, Bailey et al. 2011). The most widespread use of commercial biopesticides based on *M. anisopliae* takes place in Brazil, where 750,000 ha of sugarcane and 250,000 ha of grassland are treated with biopesticide annually (Li, Alves et al. 2010).

Table 1.1 Summary of major fungi species applied in the field as biological control and their target insect pest species.

Fungus species	Insect Pest Species	Crop	Location	References
Beauveria bassiana	*Spodoptera frugiperda*, *S. cosmioides*, and *S. eridania* (Lepidoptera: Noctuidae)	Cotton	Brazil	(Miranda 2010)
	Diatraea saccharalis (Fabricius) (Lepidoptera: Pyralidae)	Sugarcane	Brazil	(Botelho and Monteiro 2011)
	Hypothenemus hampei (Coleoptera: Scolytidae)	Coffee	Brazil and Colombia	(Faria and Magalhães 2001, Posada-Flórez 2008)
	Orthezia praelonga (Hemiptera: Ortheziidae)	Citrus	Brazil	(Faria and Magalhães 2001)
	Cosmopolites sordidus (Coleoptera: Dryophthoridae)	Banana	Brazil	(Mascarin and Pauli 2010)
	Metamasius hemipterus (Coleoptera: Curculionidae)	Banana	Brazil	(Mascarin and Pauli 2010)
	Dendrolimus spp. (Lepidoptera: Anthelidae)	Rubber and Thorny trees	China	(Li, Alves et al. 2010)
	Ostrinia furnacalis (Lepidoptera: Pyralidae)	Corn	China	(Li, Alves et al. 2010)
	Myzus persicae (Hemiptera: Aphididae)	Tea orchards	China	(Li, Alves et al. 2010)
	Empoasca flavescens (Hemiptera: Cicadellidae)	Tea orchards	China	(Li, Alves et al. 2010)
	Plutella xylostella (Lepidoptera: Plutellidae)	Crucifer	India and Benin	(Srinivasan, Sevgan et al. 2019)
	Monochamus alternatus (Coleoptera: Cerambycidae)	Pine	China	(Li, Alves et al. 2010)

Table 1.1. (continued)

Metarhizium anisopliae	*Mahanarva fimbriolata* (Hemiptera: Cercopidae)	Sugarcane and forage grasslands	Brazil	(Li, Alves et al. 2010, Mascarin and Pauli 2010, Botelho and Monteiro 2011)
	Mahanarva posticata (Hemiptera: Cercopidae)	Sugarcane	Brazil	(Faria and Magalhães 2001, Mascarin and Pauli 2010)
	Notozulia entreriana (Hemiptera: Cercopidae)	Forage grasslands	Brazil	(Li, Alves et al. 2010)
	Deois flavopicta, *D. incompleta*, and *D. schach* (Hemiptera: Cercopidae)	Forage grasslands	Brazil	(Li, Alves et al. 2010)
Metarhizium acridum	*Locusta orientalis* (Orthoptera: Tettigoniidae)	Grasslands	China	(Li, Alves et al. 2010)
Isaria fumorosea	*Bemisia tabaci* and *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)	Greenhouse	Europe	(Faria and Wraight 2001)
"Sporothrix insectorum"	*Leptopharsa heveae*	Rubber-tree	Brazil	(Faria and Magalhães 2001, Li, Alves et al. 2010)
	Vatiga manihotae (Hemiptera: Tingidae)	Rubber tree	Brazil	(Faria and Magalhães 2001)
Verticillium lecanii	*Bemisia tabaci* and *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)	Greenhouse	Europe	(Faria and Wraight 2001)

In this dissertation, the goal was to investigate the effects of plant-associated fungi applied to cotton plants on the behavior and development of boll weevil, how they affect cotton aphids when topically applied, and their effects on a multitrophic interaction between cotton aphids, their host plant and predatory convergent lady beetles.

1.3. Boll weevil biology and ecology

The genus *Anthonomus* (Coleoptera: Curculionidae) contains species that are typically host plant specialists that utilize closely related plant taxa, rarely using hosts in different families. One example is *Anthonomus grandis* that develops only on the fruiting bodies of Gossypieae plants (family Malvaceae) (Gabriel 2016). The adult can have a wide range of hosts inside the Malvaceae family as foraging resources (Hardee, Jones et al. 1999). Thus, having a secondary or alternate host plays an essential role in boll weevil survivorship during cotton-free periods (Hardee, Jones et al. 1999, Azambuja and Degrande 2014). Its length is around 4 to 9 mm, including the rostrum – the elongated snout is half of the total body length. This beetle has a dark-brown, mahogany, or almost black coloration with small and thin hairs along the body and elytra with parallel grooves (Gravena 2001, Azambuja and Degrande 2014, Gabriel 2016).

After the first report of cotton boll weevil *Anthonomus grandis grandis* Boheman (CBW) in the US (Texas) in 1892 (presumably due to expanding its geographic range from Mexico), this pest rapidly spread throughout the US Cotton Belt, and its elimination became the main aim of the US cotton industry (Howard 1894, Raszick 2021). In some places in the US Cotton Belt, due to high and uncontrolled boll weevil

infestations, some growers started to abandon cotton production and invest in other more profitable crops such as peanuts, sugarcane, potatoes, tobacco, and sorghum (Boissoneault 2017). The National Boll Weevil Eradication Program was launched by USDA and APHIS in 1971 with a pilot project to assess the technical and operational feasibility of eradicating a CBW population in a determined area (southern Mississippi, Alabama, and Louisiana) (Pedigo and Rice 2014, NCCA 2019). The experiment included in-season insecticide applications, diapause and reproduction control late-season, pheromone trapping in spring, insecticide applications at the bud stage in spring, and release of sexually sterile males (Pedigo and Rice 2014). In 1983, CBW was declared eradicated from the southeast region of the US, and in 1985 eradication efforts were initiated the southwestern US (Pedigo and Rice 2014). The program was expanded to include 15 states across the southern US (Alabama, Arizona, Arkansas, California, Florida, Georgia, Kansas, Louisiana, Mississippi, Missouri, New Mexico, Oklahoma, South Carolina, Tennessee, and Texas), and parts of northern Mexico near the border (APHIS 2013). The CBW is estimated to have cost the US cotton industry more than US$ 23 billion in economic losses, including control costs and yield losses (APHIS 2013).

The CBW is still considered one of the most destructive cotton pests in the Americas (Azambuja and Degrande 2014). The methods used for boll weevil control include cultural control such as burning or plowing cotton stalks, earlier flowering varieties, collecting decayed bolls, and crop rotation, chemical control, and biological control (Bélot, Barros et al. 2016, Gabriel 2016). These methods can be costly and time-

consuming, highlighting a growing need for more efficient methods. This dissertation analyzed whether cotton plants grown from seeds treated with plant-associated fungi negatively affect boll weevil behavior, growth, and development.

1.4. Cotton aphids and Convergent lady beetles

Another destructive pest of cotton is the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), also known as the melon aphid. This piercing-sucking pest is widespread worldwide, with many host plants across multiple plant families (Blackman and Eastop 2000). Aphids are critical plant pests that can directly damage plants through phloem sap consumption, negatively affecting the availability of carbohydrates, proteins, and amino acids available to support plant growth, resulting in crop yield losses and/or quality. Aphids can also indirectly damage plants through virus transmission or promotion of sooty mold growth on honeydew (Godfrey, Fuson et al. 1997, Gildow, Shah et al. 2008, Campolo, Chiera et al. 2014). They can cause physiological and morphological modifications on plants such as chlorosis, water stress, necrosis, gall formation, leaf curling, wilting, and stunting (Goggin 2007, Jaouannet, Rodriguez et al. 2014).

Broad-spectrum insecticides are widely used to control aphids in cotton, such as neonicotinoids (acetamiprid, imidacloprid, and thiamethoxam), organophosphates (clothianidin, dicrotophos, and dimethoate), and pyridines (flonicamid) (Stewart and McClure 2019). However, intense chemical control commonly results in insecticide resistance, highlighting the importance of studying natural enemies that can be used to control this pest using biocontrol (Lu, Wu et al. 2012). Some fungal entomopathogens

have been developed as biopesticides or mycoinsecticides and used to control herbivores as alternatives to chemical insecticides. Some examples of entomopathogens used to control aphids are *Beauveria bassiana* for the green peach aphid *Myzus persicae* (Sulzer) in tea orchards (Li, Alves et al. 2010), and *Verticillium lecanii* to control the mustard aphid *Lipaphis erysimi* (Kaltenbach) in mustard and rapeseed (Ramanujam, Rangeshwaran et al. 2014). Several examples of commercially available mycoinsecticides target the family Aphididae (Table 1.2).

Table 1.2 Mycoinsecticides used to control hemipterans of the family Aphididae.

Fungus	Trade Name	Registration	Source
Beauveria bassiana	Bb Plus	South Africa	(Maina, Galadima et al. 2018)
	Botanigard & Mycotrol	Denmark, Italy, Japan, Mexico, Spain, Sweden, USA	(Kabaluk and Gazdik 2007)
	Naturalis	Greece, Italy, Mexico, Spain, Switzerland, USA	(Kabaluk and Gazdik 2007)
	Trichobass-L	Spain	(Kabaluk and Gazdik 2007)
	Beaublast & Beaugenic	New Zealand	(Mascarin and Jaronski 2016)
B. bassiana + *Metarhizium anisopliae*	Bometil	Brazil	(Michereff Filho, Faria et al. 2021)
Conidiobolus thromboides	Vektor 25SL	South Africa	(Maina, Galadima et al. 2018)
Isaria fumorosea	PFR-97	Mexico, USA	(Faria and Wraight 2007)
	Fumosil	Colombia	(Faria and Wraight 2007)
Lecanicillium spp	Micro Germin Plus	Switzerland	(Faria and Wraight 2007)
	Verzam	Honduras, El Salvador, Jamaica, Nicaragua	(Reddy 2020)
M. anisopliae	Metanat	Brazil	(Michereff Filho, Faria et al. 2021)
	Kalichkra	India	(Thakur, Joshi et al. 2021)
Verticillium lecanii	BioCatch	India	(Reddy 2020)
	Green Basivert	India	(Reddy 2020)
	Multiplex Varsha	India	(Reddy 2020)
	Verti-Sin	Mexico	(Faria and Wraight 2007)

Key natural enemies of cotton aphids are parasitoids, coccinellid beetles, lacewings, and spiders (Ma, Liu et al. 2006). In a study conducted in China, natural enemies showed significant aphid population suppression in *Bt* cotton (Ali, Desneux et al. 2016). Beetles in the family Coccinellidae (Coleoptera) are aphid predators (Hodek and Evans 2012) and agriculturally crucial biological control agents (Koch and Costamagna 2017, Riddick 2017). The species *Hippodamia convergens* (Guérin-Méneville) is a generalist predator, known as the convergent lady beetle. Despite being a generalist predator, it is a common aphidophagous species native to North America found from southern Canada to South America (Flint and Dreistadt 2005). Due to its efficacy in controlling aphids in alfalfa in California, such as the pea aphid *Acyrthosiphon pisum* (Harris) and the blue aphid *A. kondoi* (Shinji), *H. convergens* has been introduced to other continents, including South America (Hodek 1973, Minks, Harrewijn et al. 1987, Villegas, Verdugo et al. 2013).

Convergent lady beetles have a body slightly elongated, ranging from 4 to 7 mm in length with two characteristic converging white lines behind the head (Hoffmann and Frodsham 1993). In one to three months, convergent lady beetle females can oviposit from 200 to more than 1,000 eggs, and after hatching, third and fourth instar larvae may eat between 30 to 50 aphids per day (Hoffmann and Frodsham 1993, Aristizábal and Arthurs 2014). Before oviposition, females need to consume the right amount of carbohydrates and proteins to increase the viability of their eggs (Aristizábal and Arthurs 2014). When aphid numbers are low, convergent lady beetles can feed on nectar, pollen, and honeydew secreted by aphids and other sucking insects to supplement their diet

(Hoffmann and Frodsham 1993, De Clercq, Bonte et al. 2005). Because of their predaceous habit and distribution among crops attacked by aphids, this species is a key part of many multitrophic agroecosystems and an important biological control agent.

In this dissertation, I investigated the direct pathogenicity of different plant-associated fungal isolates towards cotton aphids. I also investigated whether cotton treated with plant-associated fungi affected the behavior of convergent lady beetle in multitrophic interactions with cotton aphids.

2. PLANT-ASSOCIATED FUNGI AFFECT COTTON BOLL WEEVIL BEHAVIOR AND DEVELOPMENT

2.1. Introduction

The cotton boll weevil, *Anthonomus grandis grandis* Boheman (Coleoptera: Curculionidae), is a major cotton pest that directly attacks cotton flower buds (squares) and bolls by feeding and development of larvae inside cotton reproductive structures (Showler 2004). The cotton boll weevil (CBW) reproduces sexually and has a high reproductive capacity leading to rapid infestations that can reduce cotton production or quality and may even cause 100% crop loss (Santos, Monnerat et al. 2002, Monnerat and dos Santos 2015, Gabriel 2016).

After oviposition, the hatching time can be 3 to 4 days. The development time from larva to adult can last from 10 to 17 days, with adult longevity ranging from 20 to 40 days during which females can oviposit ~ 100 to 300 eggs (Gallo, Nakano et al. 2002). Depending on temperature and humidity, the CBW can reproduce from three to seven generations during the crop cycle (Gabriel 2016). In other words, an initial population of just 50 boll weevils at the beginning of the season can potentially grow into 500,000 adults by harvest time (Praça 2007). Thus, plant damage and yield losses are proportionally related to weevil reproductive intensity because the insects use the plant's reproductive structures for larval feeding and development (Miranda and Rodrigues 2015). The critical phase of CBW attack occurs during the cotton reproductive stage (between 40 and 90 days after germination) (Gabriel 2016). CBW

feeding can also be a causal factor in boll rot because it provides a means for fungi and bacteria penetration into the developing fruits when they puncture cotton bolls (Azambuja and Degrande 2014).

Despite the constraints (e.g., pesticide resistance and environment and human health hazards), chemical control has been the dominant pest control strategy in many agricultural systems (Rani, Thapa et al. 2021). The cotton boll weevil is no different; chemical control remains the most used method. However, it is costly and can be environmentally unsafe. In addition, since the CBW immature stages are protected inside the plant reproductive structures, the use of contact insecticides is not always practical (Neves, Colares et al. 2014, Pimenta, Mata et al. 2016). Although the United States has conducted a successful and intensive eradication program, the boll weevil is not yet completely eradicated in Texas. The southern border with Mexico presents a roadblock to eradication due to various ecological and social factors (Showler 2007, Semple 2017, Shipman 2017). CBW remains the most destructive cotton insect pest in the Americas, generating environmental concerns and economic expenses for cotton production (Azambuja and Degrande 2014). Thus, new environmentally friendly practices need to be developed to minimize or eliminate the impact of the boll weevil on cotton production, thereby helping the economy and promoting cotton sustainability.

Several fungi are entomopathogenic and could substitute for chemical pesticides in insect control (Bale, Lenteren et al. 2008). These entomopathogens are essential for plant protection against insects because they can indirectly help by repelling insects or may directly help by affecting insect survivorship (Saxena and Sharma 2008). Some

entomopathogens can also be plant-associated microbes that can influence plant' defenses, consequently modifying pest behavior and fitness (Shikano, Rosa et al. 2017). In cotton, multiple fungal genera were found naturally occurring in the leaves, stems and reproductive structures that could promote a healthier crop. Some examples include *Acremonium alternatum*, *Alternaria* spp., *Bipolaris spicifera*, *Botryosphaeria* spp., *Cercospora* spp., *Chaetomium* spp., *Cladosporium* spp., *Dichomera* spp., *Fusarium* spp., *Phialemonium* sp., *Phomopsis* spp. (McGee 2002, Wang, Priest et al. 2007, Ek-Ramos, Zhou et al. 2013).

Fungal seed treatments of cotton with *Beauveria bassiana* and *Phialemonium inflatum* negatively affected survivorship and development of the cotton bollworm *Helicoverpa zea* (Boddie) (Castillo Lopez, Zhu-Salzman et al. 2014, Lopez and Sword 2015), as well as the host selection behavior of two hemipteran pests, the western tarnished plant bugs *Lygus hesperus* Knight and the southern green stink bugs *Nezara viridula* (Linnaeus) (Sword, Tessnow et al. 2017). Belowground, *P. inflatum* demonstrated adverse effects on galling and reproduction of root-knot nematodes *Meloidogyne incognita* (Kofold & White) in cotton (Zhou, Wheeler et al. 2018). When similarly applied to cotton, the fungus *C. globosum* inhibited infection, and reduced reproduction of root-knot nematodes belowground and negatively affected the fecundity of cotton aphids and beet armyworms *Spodoptera exigua* (Hubner) feeding aboveground (Zhou, Starr et al. 2016, Zhou, Verma et al. 2020). A number of other plant-associated fungi have also been shown to include nematode galling when inoculated back to cotton, including *Curvularia spicifera*, *Cladosporium antropophilum*, *Epicoccum nigrum*,

Alternaria eichorniae, Purpureocillium lavendulum, Chaetomium coarctatum, Gibellulopsis piscis, and *Cladosporium cladosporioides* (Zhou, Verma et al. 2020).

Examples of well-known plant-associated entomopathogens are the fungal genera *Beauveria* and *Metarhizium* that can infect boll weevils and many other insects by entering through the insect's gut, spiracles, and tegument, followed by mycosis, and insect death (Bastos, Pereira et al. 2005). The species *B. bassiana* and *M. anisopliae* have previously been shown to directly affect boll weevil feeding behavior and mortality (Nussenbaum and Lecuona 2012). However, the potential indirect effects of treating cotton plants with these fungi on boll weevil behavior, performance and survival have not been previously tested. Therefore, a better understanding of indirect boll weevil responses to cotton plants when associated with these and other plant-associated fungi can potentially contribute to developing improved IPM strategies to control this pest. In this study, we used a commercial strain of *B. bassiana* and four strains of fungi previously isolated from cotton in the field. Our goals were to test whether cotton plants grown from seeds treated with plant-associated fungi (i) negatively affect cotton boll weevil behavior, reproduction, or development, and (ii) if the plants were also more resistant to CBW damage.

2.2. Materials and Methods

2.2.1. Cotton seed treatment

Seeds used for all experiments were chemically-untreated and non-transgenic *Gossypium hirsutum* variety LA122 from All-Tex Seed Inc., Levelland, TX. For the fungal

treatments, we used *Acremonium alternatum* (TAMU 505), *Chaetomium globosum* (TAMU520 and TAMU559), and *Phialemonium inflatum* (TAMU490), all of which were initially isolated as endophytes from field-collected cotton in Texas, USA (Ek-Ramos, Zhou et al. 2013). The fungal entomopathogen *Beauveria bassiana,* cultured from a commercially-obtained strain (Botanigard, BioWorks Inc, Victor, NY), was also tested. All fungi were cultured in Petri dishes on potato dextrose agar (PDA) media in the dark. For the fungal seed treatments, spore suspensions of each fungus were made following the method used in Sword, Tessnow et al. (2017) and filtered through autoclaved 25mm sieves. We used a Neubauer hemocytometer (Thomas Scientific, Philadelphia, PA, USA) to quantify spore concentrations and diluted them with sterile water to a final working concentration of 1×10^7 spores/ ml^{-1}. Cotton seeds were first surfaced-sterilized (Posada, Aime et al. 2007), dried in autoclaved paper towels for 30 min, and inoculated by saturating 200 seeds in a glass beaker with 1 ml of the fungal spore suspensions plus 1 ml of 2% methylcellulose as a sticker to bind the spores. Untreated control seeds were saturated with only the 2% methylcellulose solution. Treated and untreated seeds were spread out in autoclaved plates and air-dried for at least 3 hours under sterile conditions before packing to send to Weslaco, TX, for planting.

We planted five seeds per treatment (5 fungi strains and control) in 5-gallon plant pots containing unsterilized Berger BM7 soil (KBW Supply, Inc., Donna, TX). After germination, we removed the extra germinated seeds to have only one plant per pot. All plants were grown in a greenhouse at 25 °C with natural photoperiod. Each pot position

was randomized. Pots were watered as needed, and no fertilizer was added. Plants were grown, and all assays were conducted at the Texas A&M AgriLife Research and Extension Center in Weslaco, Texas (USA).

2.2.2. Cotton boll weevil rearing

Infested cotton squares (flower buds) and bolls were collected in commercial cotton fields in the Lower Rio Grande Valley, TX, in July and August 2019 and July 2020. The field-collected squares, bolls and emerging insects were held in cages in a controlled environment room at the Texas A&M AgriLife Research and Extension Center in Weslaco, TX at 29 °C, 60% RH, and 13:11 L:D photoperiod (Pires, da Mata et al. 2011, Spurgeon, Suh et al. 2018). Boll weevil pupae were collected daily as they emerged from infested squares. Five pupae were placed in 10 cm Petri dishes with a thin layer of moistened vermiculite (Figure 2.1A) and transferred to different cages maintained under the same environmental conditions as above. Dishes were checked twice a day for newly molted adults that were then sexed using the tergal-notch method (Sappington and Spurgeon 2000, Sappington and Spurgeon 2000, Spurgeon, Suh et al. 2018).

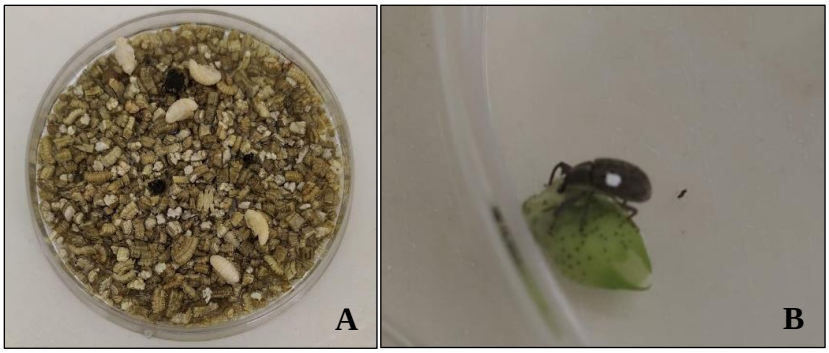

Figure 2.1 Cotton boll weevil rearing methods, keeping five pupae in moistened vermiculite (A) and marked female left elytron with a non-toxic white pen.

After adult emergence, insects were kept in cages and fed daily with clean, undamaged, 5-9 mm diameter bracteole free squares (1 square/5 weevil) and provided with a cotton wick soaked in water (Spurgeon and Suh 2017, Magalhaes, Borges et al. 2018, Spurgeon, Suh et al. 2018). Females were marked on the left elytron using a non-toxic fine tip white paint pen (Speedball Painters, Hunt Manufacturing, Statesville, NC) (Figure 2.1B) to separate them easily after mating (Spurgeon, Suh et al. 2018). For mating, 20 mixed-sex weevils were kept in 15 cm Petri dishes with a meshed lid (Greenberg, Sappington et al. 2003) and starved for 48 h to maximize the possibility of mating and fertilization. For both behavior and development assays, experimental weevils were randomly selected from the lab population.

2.2.3. Behavioral assays

We conducted no-choice and choice behavioral assays to assess CBW responses to squares from untreated and fungal-treated cotton plants. The squares (5 to 10 mm) used

were fresh, clean, undamaged, and bracteole-free to better observe the insects' activity. The assays were conducted in 10 cm diameter Petri dishes. All the squares were removed from source plants in the greenhouse and transferred to the laboratory for experiments.

CBW individuals were observed for 6 hours. The insects were observed continuously until they made a choice to record the latency time. A choice was considered when the individual stayed for more than 1 min on a square (Nussenbaum, Devescovi et al. 2018) to record the first choice for choice assays. The behavioral states of feeding, resting on or off the square, and walking were recorded every 30 minutes, except for the first 30 minutes, when observations were made every 10 minutes (Sword, Tessnow et al. 2017). All individuals were starved 24 h before assays. Thus, we recorded latency (time to make the first contact) and the activity intervals (Showler 2004, Grigolli, Souza et al. 2012, Sword, Tessnow et al. 2017) for no-choice and choice assays.

2.2.3.1. No-choice assay in Petri dish

For no-choice behavioral assays, 10 Petri dishes per treatment were used (Sword, Tessnow et al. 2017) with a total of N=120 individuals tested. In each no-choice trial, 10 females and 10 males were observed for each fungal treatment group (five fungi and one control). One mated female or one mated male was placed in a Petri dish with one square from a fungal-treated plant or one square from an untreated control plant in the center, and the dishes' positions were randomized to avoid positional bias (Figure 2.2A).

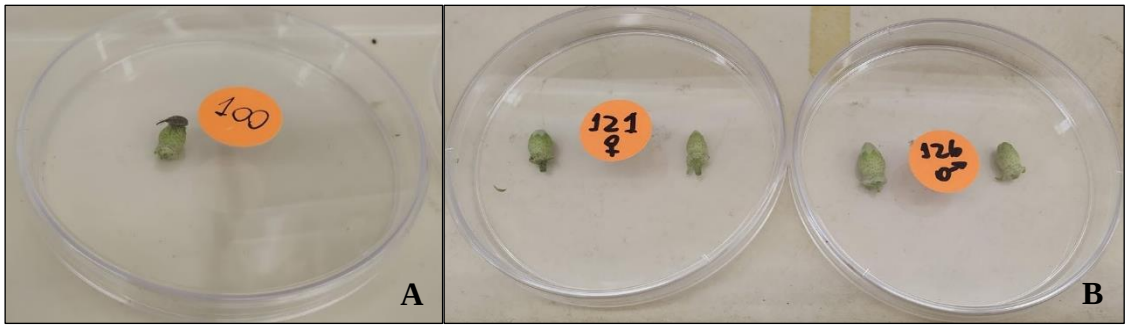

Figure 2.2 Examples of how the behavioral assays were conducted in Petri dishes with *Anthonomus grandis grandis* with one flower bud (square) of fungal-treated or untreated control plants in the no-choice assay (A), and one fungal-treated and one control square in the choice assay (B).

2.2.3.2. Choice assay in Petri dish

In the choice behavioral assays, 10 Petri dishes per combination (treatment vs. control) by sex were used (Sword, Tessnow et al. 2017) with a total of N = 100 individuals were tested, with 10 females and 10 males observed per each fungal treatment and control combination. One mated female or one mated male was released into each Petri dish with two equal-sized squares placed 4 cm apart in the center of the dish, one from treated plants and one untreated square (Figure 2.2B) (Showler 2004, Sword, Tessnow et al. 2017). The positions of treated and untreated squares (left and right) inside the Petri dish and the dishes' location were randomized to avoid position bias.

2.2.4. Greenhouse assay

The greenhouse assay consisted of treated and control plants individually infested with single weevils in cages (61 cm x 61 cm x 91 cm) maintained under the same environmental conditions as previously described. At the beginning of the experiments,

cotton plants were about 50-70 days old and monitored for 14 days after weevil infestation to record plant resistance and insect performance data. One mated female was placed in each cage with a total of N = 18 females (cages) per treatment tested across six replicated trials (only three cages per treatment per trial could be tested at once due to space constraints). We used only females in this assay because male feeding could destroy plant reproductive structures used for oviposition (Rolim, Barros et al. 2019).

The cages were checked daily to mark the damaged structures using a non-toxic pen, check if the female was alive, and collect abscised cotton reproductive structures (squares, flowers, and bolls). The abscised and/or infested reproductive structures were collected to record insect performance and plant resistance data. Although some oviposition is found in unsealed punctures (Esquivel 2007), we considered the sealed punctures by the presence of a sealing wax and/or frass plug as an oviposition puncture (Everett and Ray 1962, Greenberg, Sappington et al. 2003, Nussenbaum, Devescovi et al. 2018).

2.2.4.1. Female reproduction

From the oviposited cotton reproductive structures, we recorded fecundity (n. of eggs = oviposition punctures), the fertility rate (n. of larvae/oviposition punctures x 100), and emergence rate (n. of adults emerged/larvae x 100). In this study, an oviposition puncture was considered representative of one egg laid (Greenberg, Sappington et al. 2008, Nussenbaum, Devescovi et al. 2018). If a pupa did not emerge from the oviposited

cotton reproductive structures collected, we carefully dissected them to check for the presence of a larva and confirm oviposition (Greenberg, Jones et al. 2009).

2.2.4.2. Progeny

To collect the progeny, we kept squares and bolls separated according to the cage from which they were collected in 50 ml cups with a moistened filter paper to avoid dissection that would interfere with immature development (Pires, da Mata et al. 2011). After pupal emergence, pupae were placed in Petri dishes with a layer of moistened vermiculite (Spurgeon, Suh et al. 2018) and recorded the developmental time to adult in days. After adult emergence, we weighed, measured the size under a microscope, and sexed them using the tergal-notch method (Sappington and Spurgeon 2000, Spurgeon, Suh et al. 2018). Lastly, we fed them with one square from the treatment they emerged from and recorded the death date as a measure of survival. Thus, we recorded the developmental time (pupa and adult), pupa weight (mg), adult body size (mm) and weight (mg) at emergence, sex, and survival (larvae, pupae, and adults). Also, we calculated the growth rate for pupa and adult, equal to weight divided by developmental time (mg/day).

2.2.4.3. Plant resistance

From the collected cotton reproductive structures (abscised or not), we counted the punctures on cotton reproductive structures under a dissecting microscope. We differentiated between feeding and oviposition punctures by the presence of sealing structure, as mentioned previously. To evaluate plant resistance conferred by each

treatment, we recorded the time the structures were damaged, the number of abscised reproductive structures and the number of feeding and oviposition punctures on them. The number of feeding and oviposition punctures related to the cotton reproductive structures did not show a treatment effect; therefore, data are not presented in the chapter.

2.2.5. Statistical analysis

2.2.5.1. Behavioral assays

In the no-choice assays, we calculated the proportion of weevils per treatment on the square at each observation interval (10, 20, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min), yielding a value between 0 (no activity) to 1 (all weevils on a square) (Sword, Tessnow et al. 2017). For the choice assays, we calculated the response ratio at each interval by dividing the total of individuals that selected the untreated control squares by the total that chose either the treatment or control at each observation interval. The response ratio yields a value between 0 and 1, with 1 representing 100% of individuals choosing the control square and 0 indicating that 100% chose the fungal-treated squares (Martin, Bateson et al. 1993, Sword, Tessnow et al. 2017).

In both no-choice and choice assays, we compared the proportion and response ratio using the Wilcoxon Signed-Rank test with 0.5 representing the null hypothesis of no difference in responses to squares from fungal-treated versus control plants (Sword, Tessnow et al. 2017). For the latency time (minutes), we used a generalized linear model (*glm* function in R) with a quasi-Poisson model distribution (Pachú, Macedo et al. 2021),

and to assess the goodness of fit of the model, we used a half-normal envelope using the hnp package in R (Moral, Hinde et al. 2017). For the first choice in choice assays, we used Pearson's chi-squared (*chisq.test* function in R) to test the proportion of responses in each comparison with the expected proportion of 0.5 for a null hypothesis of non-preference (Sokame, Ntiri et al. 2019, Xiu, Dai et al. 2019) (Table 2.1).

2.2.5.2. Greenhouse assay

For the greenhouse assay, we used a Multivariate Analysis of Variance (MANOVA) to test the main effects of the treatments on the female reproduction and progeny dependent variables (Table 2.1). No transformation was performed since our data exhibited a normal distribution. For the MANOVA, Hotelling-Lawley Trace, Pillai's Trace, and Wilks'Lambda tests were used to assess significance at $\alpha = 0.05$ (*manova* function in R) (Malaquias, Ramalho et al. 2017). Following significant MANOVA tests, we conducted follow-up univariate ANOVAs to compare each dependent variable across the treatments (Figure 2.3). In cases where we found significance in the ANOVA among the treatments for a specific variable, we conducted follow-up pairwise comparisons between the fungal treatments and the control (Table 2.1). Since we did not find any sex treatment effects, analyses were conducted with males and females combined.

Table 2.1 Dependent variables in behavioral and greenhouse assays and the respective statistical test performed.

Experiment	Dependent Variable		Statistical Test
Behavioral assays			
No-choice	Latency		Quasi-Poisson model - GLM
	Proportion = $\frac{n.of\ cotton\ boll\ weevil\ choosing\ the\ treatment}{total\ of\ individuals\ responding}$		Wilcoxon Signed-Rank
Choice	First choice		Pearson's chi-squared
	Latency		Quasi-Poisson model - GLM
	Response ratio = $\frac{n.of\ cotton\ boll\ weevil\ choosing\ control}{n.choosing\ control + n.choosing\ fungal\ treatment}$		Wilcoxon Signed-Rank
Greenhouse assay			
Female reproduction	Fecundity (n. of oviposition puncture/female)		Poisson model - GLM
	Fertility or Hatch rate (n. of larva/puncture %)		Poisson model - GLM
	Emergence rate (n. of adult/larva %)		Poisson model - GLM
Progeny	Larva	Survival time	Kaplan-Meier analysis
	Pupa	Weight (mg)	Gaussian model - GLM
		Growth rate (mg/day)	Gaussian model - GLM
		Developmental time	Kaplan-Meier analysis
	Adult	Survival time	Kaplan-Meier analysis
		Weight (mg)	Gaussian model - GLM
		Body size (mm)	Gaussian model - GLM
		Growth rate (mg/day)	Gaussian model - GLM
		Developmental time	Kaplan-Meier analysis
		Survival time	Kaplan-Meier analysis
		Sex ratio	ANOVA
Plant resistance	Proportion of abscised cotton reproductive structures %		Quasi-Poisson model - GLM

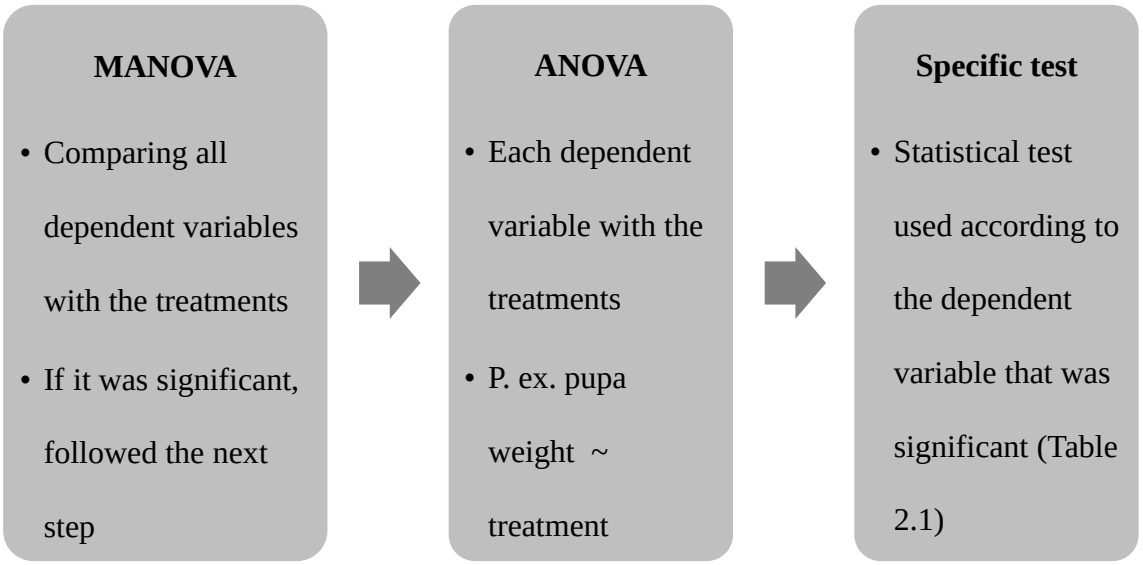

Figure 2.3 Statistical analysis workflow.

We used a generalized linear model (*glm* function in R) with a Poisson model distribution to compare the female reproduction traits. We performed a Kaplan-Meier analysis with log-rank and a pairwise comparison with FDR *p*-value adjustment to compare the developmental times between the fungal treatments and the control with a percentage scale. For the survival data, we also performed the same analysis but with survival probability. Both Kaplan-Meier analyses were conducted using the packages survival (Therneau 2015) and survminer (Kassambara, Kosinski et al. 2017) in R. We also performed a generalized linear model with a Gaussian model distribution for the progeny variables that were significant in the ANOVA (pupa weight, pupa growth rate and adult body size) (Table 2.1).

Lastly, for plant resistance, we calculated the proportion of abscised cotton reproductive structures by the total per plant and analyzed using *glm* with a quasi-

Poisson distribution (Table 2.1). Naturally abscised structures (no punctures) were not included in this analysis. All data analyses were conducted using R version 3.6.3 (R Core Team 2020) with an $\alpha = 0.05$. Descriptive statistics were used to compute means and standard error for each dependent variable with the Rmisc package in the R program (Hope 2013). For the graphs, we used the ggplot2 package (Wickham 2016).

2.3. Results

2.3.1. Cotton boll weevil behavior

2.3.1.1. No-choice behavioral assay

In no-choice behavioral assays, cotton boll weevils did not show significant differences in latency (time to make first contact) between squares from fungal-treated versus control plants ($F = 1.119$, $p = 0.345$) (Table 2.2, Figure 2.4A). However, there was a significant difference in the proportion of CBW contacting a square over time in two fungal treatment groups compared to untreated control. A significantly lower proportion of CBW was observed on squares over time from plants treated with *C. globosum* TAMU559 and *P. inflatum* compared to control (Table 2.2, Figure 2.4B).

Table 2.2 Petri dish no-choice behavioral assay statistical analyses and means (± SE) of latency to the first contact time, and proportion of response for *Anthonomus grandis grandis* when exposed to different cotton flower buds from fungal-treated or untreated control plants. For each treatment, N = 10♀ and 10♂. * $p < 0.05$

Treatment	**Latency time** (min)		**Proportion**		Non-responding Individuals
	Mean ± SE	*p*	Mean ± SE	*p*	
A. alternatum	9.09 ± 4.48	0.496	0.81 ± 0.03	0.151	0
B. bassiana	23.42 ± 11.24	0.572	0.75 ± 0.04	0.287	1
***C. globosum*520**	26.52 ± 8.95	0.439	0.75 ± 0.03	0.562	1
***C. globosum*559**	38.85 ± 15.77	0.142	0.69 ± 0.03	0.032*	0
P. inflatum	24.91 ± 11.89	0.509	0.48 ± 0.02	0.001*	2
Control	16.11 ± 6.90	-	0.77 ± 0.03	-	0

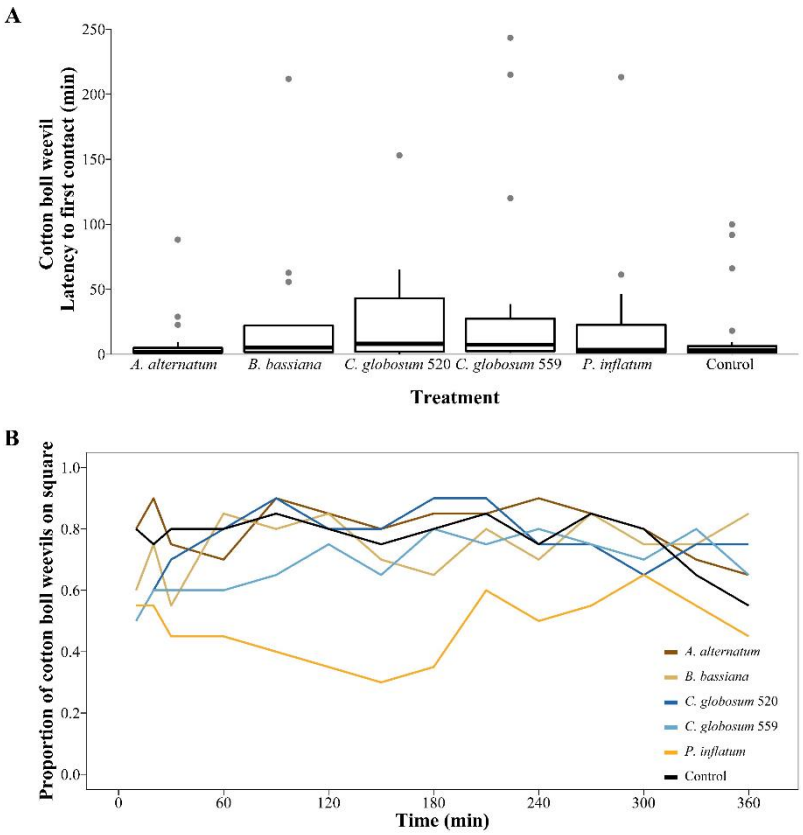

Figure 2.4 *Anthonomus grandis grandis* latency (minutes) to contact cotton flower buds (squares) from untreated control or fungal-treated in Petri dishes (A), and proportion of individuals on squares (n. of weevils responding/total) (B) in no-choice assays. Each individual had 360 minutes (6 hours) to stay in the Petri dish. The beetles that did not respond were not included in the analysis.

2.3.1.2. Choice behavioral assay

When CBW was offered a simultaneous choice between a fungal-treated square and an untreated square in the same Petri dish, weevils first selected squares from *B. bassiana* treated plants significantly more often than the control squares (Table 2.3, Figure 2.5A), but there were no differences in the first choice for any of the treatment comparisons. No significant differences were found in latency time to first contact between any of the treatment comparisons (fungal-treated square vs. untreated control) (Table 2.3, Figure 2.5B).

In contrast, all the fungal treatments affected the response ratio of weevils on squares, with CBW preferring more often to be on squares from untreated control plants than those from plants treated with either *A. alternatum*, *C. globosum* TAMU520 or *P. inflatum*. A significant effect in the opposite direction was observed when offered squares from *B. bassiana* and *C. globosum* TAMU559 treated plants, with the weevils more often in contact with squares from fungal-treated plants compared to control (Table 2.3, Figure 2.5C).

Table 2.3 Simultaneous choice behavioral assay statistical analyses and means (± SE) of the first choice, latency to first contact, and response ratio for *Anthonomus grandis grandis*. Tests were conducted in Petri dishes providing individuals with a choice between flower buds (squares) from fungal-treated and untreated cotton plants. Sample sizes for each comparison were N = 10♀ and 10♂. * $p < 0.05$

Treatment	First choice		Latency time (min)		Response ratio		Non-responding Individuals
	χ^2	*p*	Mean ± SE	*p*	Mean ± SE	*p*	
***A. alternatum* vs. Control**	0.474	0.491	22.36 ± 6.21 23.26 ± 5.55	0.908	0.57 ± 0.02	0.007*	1
***B. bassiana* vs. Control**	4.263	0.039*	11.54 ± 2.85 4.28 ± 2.04	0.219	0.29 ± 0.27	0.001*	1
***C. globosum*520 vs. Control**	2.579	0.108	4.79 ± 1.68 4.89 ± 1.24	0.977	0.63 ± 0.02	0.001*	1
***C. globosum*559 vs. Control**	0.8	0.371	6.23 ± 2.05 15.87 ± 9.51	0.067	0.33 ± 0.03	0.001*	0
***P. inflatum* vs. Control**	0.053	0.818	13.32 ± 3.79 12.81 ± 4.04	0.930	0.62 ± 0.01	0.001*	1

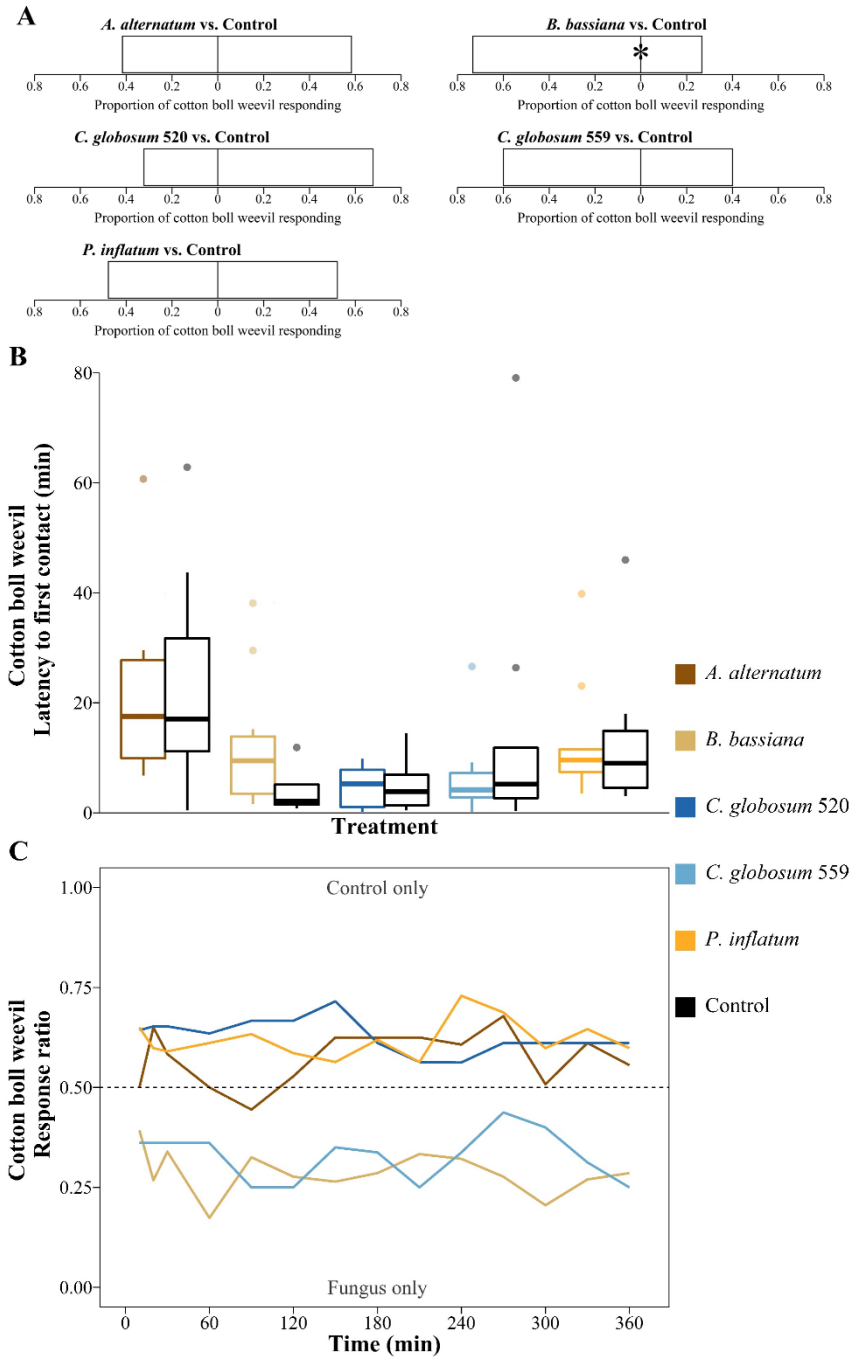

Figure 2.5 Responses of *Anthonomus grandis grandis* to cotton plant flower buds (squares) from untreated control and fungal-treated plants in simultaneous choice assays. First choice (A), latency (minutes) to make first contact (B), and response ratio (n. of weevils choosing control/n. of choosing control + fungus) (C). The beetles that did not respond were not included in the analysis. * $p < 0.05$ (Pearson's chi-squared test).

2.3.2. Cotton boll weevil performance

2.3.2.1. Female fecundity multivariate analysis

All the multivariate statistical tests Pillai's Trace, Wilk's Lambda, and Hotelling-Lawley Trace showed significant effects of fungal treatments on CBW female fecundity parameters (df = 5; $p > F < 0.05$) (Table 2.4).

Table 2.4 Multivariate analysis (MANOVA) results related to the effects of fungal treatments on female reproduction variables of the cotton boll weevil. W = Wilk's Lambda, V = Pillai's Trace, and HL = Hotelling-Lawley Trace tests.

Source	df	Multivariate statistics		
Treatment	5	W = 0.9801; F =1.855; p = 0.023	V = 0.01993; F = 1.853; p = 0.023	HL = 0.02016; F = 1.857; p = 0.023

2.3.2.2. Female fecundity univariate analysis

In the ANOVA, fecundity (n. of oviposition punctures/female) significantly differed among all treatments (Table 2.5). We also found a significant difference among treatments for fertility or hatch rate (n. of larvae/oviposition punctures %) (Table 2.5). Adult emergence (n. of adults/larvae) also differed significantly across all treatments (Table 2.5).

Table 2.5 Univariate analysis (ANOVA) results related to the effects of fungal treatments on female reproduction variables of the cotton boll weevil.

Female reproduction	df	Treatment	
		F	p
Fecundity	5	4.759	<0.001
Fertility or Hatch rate	5	16.006	<0.0001
Emergence rate	5	28.385	<0.0001

2.3.2.3. Female fecundity, fertility, and adult emergence

In *C. globosum* TAMU520, CBW females had significantly higher ($p = 0.014$) fecundity than in control plants (Table 2.6). CBW females laid more eggs (mean ± SE) on *C. globosum* TAMU520 treated plants (16.39 ± 2.79 oviposition puncture/female) compared to the number of eggs laid on control plants (13.22 ± 1.66 oviposition puncture/female) (Table 2.6, Figure 2.6A).

Hatch rates for eggs laid in all fungal-treated plants were all significantly lower compared to control (Table 2.6). All females from fungal-treated plants had fewer larvae hatching from their oviposition punctures relative to control females (Figure 2.6B). All fungal-treated plants had significantly lower adult emergence rates than control plants (Table 2.6, Figure 2.6C).

Table 2.6 Statistical analyses of fecundity, fertility (hatch rate), and emergence rates for *Anthonomus grandis grandis* female progeny grown in cages of fungal-treated or untreated control cotton plants. Sample sizes for each treatment were N = 18♀. *$p < 0.05$ (Poisson model – Generalized linear model).

Treatment	**Fecundity** (n. of oviposition punctures/female)		**Fertility rate %** (larvae/oviposition punctures)		**Emergence rate %** (adults/larvae)	
	Mean ± SE	*p*	Mean ± SE	*p*	Mean ± SE	*p*
A. alternatum	13.72 ± 3.19	0.683	57.08 ± 7.99	<0.0001*	53.98 ± 7.74	<0.0001*
B. bassiana	12.44 ± 2.48	0.515	49.96 ± 7.81	<0.0001*	50.63 ± 8.85	<0.0001*
***C. globosum*520**	16.39 ± 2.79	0.014*	57.48 ± 7.27	<0.0001*	67.59 ± 7.94	0.004*
***C. globosum*559**	11.78 ± 1.68	0.221	61.72 ± 7.04	<0.001*	61.69 ± 7.52	<0.0001*
P. inflatum	11.00 ± 2.34	0.056	57.30 ± 8.61	<0.0001*	52.78 ± 8.71	<0.0001*
Control	13.22 ± 1.66	-	72.11 ± 4.39	-	75.83 ± 5.21	-

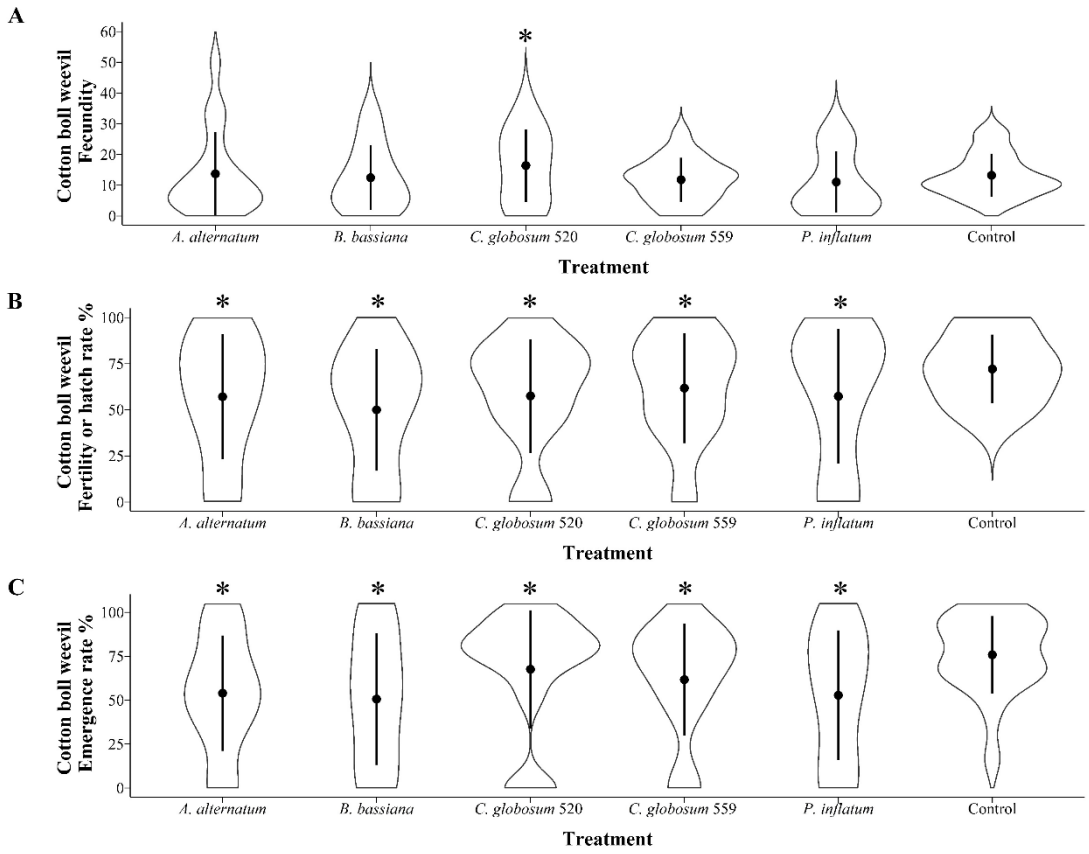

Figure 2.6 *Anthonomus grandis grandis* female fecundity (n. of oviposition punctures/female) (A), fertility or hatch rate% (larvae/oviposition punctures) (B), and emergence rate % (adults/larvae) (C) from untreated control and fungal-treated cotton plants. Violin plots combine a density plot and a boxplot with a point range in the middle representing the mean and standard deviation. *$p < 0.05$ (Poisson model – Generalized linear model in a pairwise comparison between the fungal-treated plants and the control).

2.3.2.4. Progeny multivariate analysis

All the multivariate statistical tests Pillai's Trace, Wilk's Lambda, and Hotelling-Lawley Trace showed significant effects of fungal treatments on CBW progeny (pupa and adult) performance variables ($df = 5$; $p > F < 0.05$) (Table 2.7).

Table 2.7 Multivariate analysis (MANOVA) results of the effects of fungal treatments on progeny (larva, pupa, and adult) variables of the cotton boll weevil. W = Wilk's Lambda, V = Pillai's Trace, and HL = Hotelling-Lawley Trace tests.

Source	df	Multivariate statistics		
Treatment	5	W = 0.75935; F = 3.381; $p < 0.0001$	V = 0.2589; F = 3.2616; $p < 0.0001$	HL = 0.29441; F = 3.4941; $p < 0.0001$

2.3.2.5. Progeny univariate analysis

In the ANOVA comparing each variable across all treatments, for pupa dependent variables, weight, growth rate, developmental time, and survival showed significant differences across treatments (Table 2.8). Body size, developmental time, and survival showed significant differences among treatments for adults. However, adult weight, growth rate, and sex ratio did not show a significant difference across the treatments (Table 2.8). The following sections separately report the significantly different variables in follow-up comparisons between progeny reared on fungal-treated and untreated control plants.

Table 2.8 Univariate analysis (ANOVA) results of the effects of fungal treatments on progeny (larva, pupa, and adult) performance variables of the cotton boll weevil.

Progeny	df	Treatment	
		F	*p*
Larva			
Survival (days)	5	1.416	0.215
Pupa			
Weight (mg)	5	2.769	0.018*
Growth rate (mg/day)	5	2.154	0.045*
Developmental time (days)	5	8.676	<0.001*
Survival (days)	5	3.2351	0.002*
Adult			
Weight (mg)	5	1.102	0.358
Body size (mm)	5	2.200	0.048*
Growth rate (mg/day)	5	0.967	0.438
Developmental time (days)	5	9.566	<0.001*
Survival (days)	5	2.257	0.047*
Sex ratio	5	1.427	0.212

2.3.2.6. Progeny developmental and survival times

Median developmental time (= 50% of the population reaching the stage) of CBW to pupation was 10 days for control and ranged between 12 and 15 days for the fungal-treated plants (Table 2.9). Developmental times to pupation when reared on plants treated with *A. alternatum*, *B. bassiana*, and *P. inflatum* were significantly longer than the control. CBW reached the pupa stage earlier on control plants at a mean of 12.4 days, while for the fungal treatments, the mean was around 14 days (Table 2.9, Figure 2.7A). Developmental times to adult emergence were significantly longer than the control for all fungal treatments, with control individuals reaching the adult stage earlier on average in about 13.2 days versus 17.1 days for *A. alternatum*, and for the remaining fungal treatments around 15 days (Table 2.9, Figure 2.7B).

Table 2.9 Developmental time in days (mean ± SE) of *Anthonomus grandis grandis* progeny to pupation and adult emergence from infested cotton reproductive structures of fungal-treated or untreated control plants. *$p < 0.05$ (Pairwise comparisons using log-rank test with FDR *p*-value adjustment).

Treatment	Pupation – developmental time			Adult emergence – developmental time		
	Mean ± SE	*p*	n	Mean ± SE	*p*	n
A. alternatum	14.43 ± 0.39	<0.0001*	167	17.06 ± 0.34	<0.0001*	112
B. bassiana	14.31 ± 0.64	0.011*	125	15.69 ± 0.42	<0.0001*	80
***C. globosum*520**	12.89 ± 0.33	0.088	188	15.65 ± 0.31	<0.0001*	154
***C. globosum*559**	13.15 ± 0.45	0.600	131	15.84 ± 0.34	<0.0001*	101
P. inflatum	14.32 ± 0.59	0.014*	136	15.87 ± 0.43	<0.0001*	98
Control	12.44 ± 0.65	-	133	13.19 ± 0.25	-	96

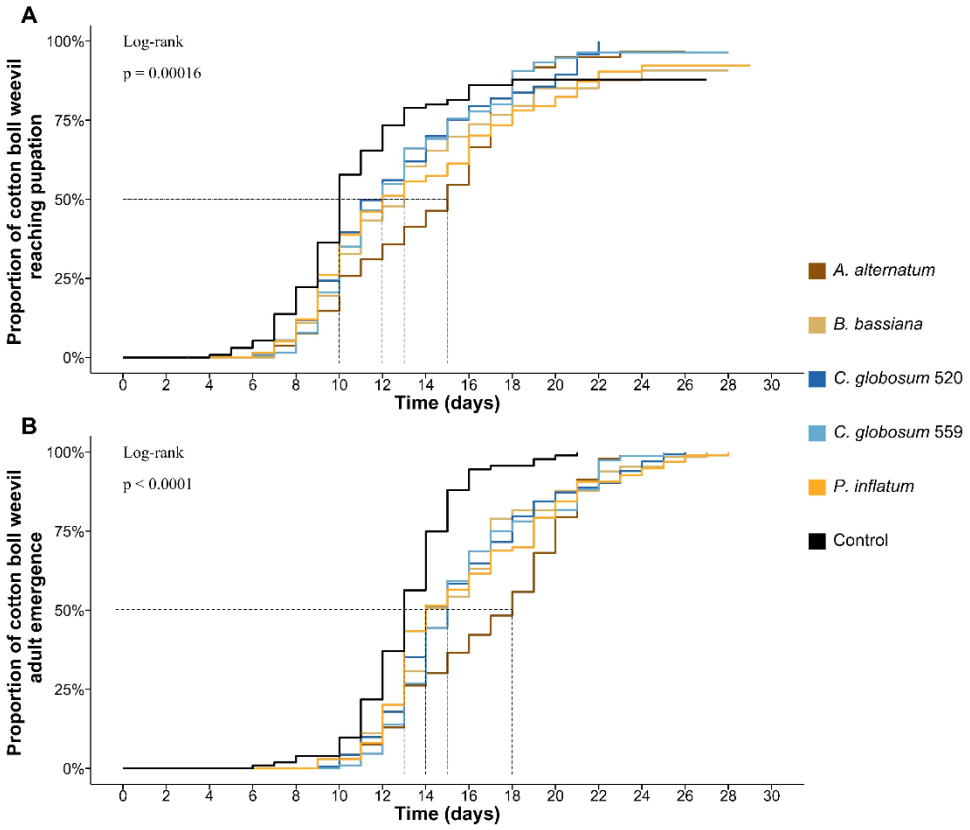

Figure 2.7 Curves of *Anthonomus grandis grandis* progeny from fungal-treated and untreated control plants for the developmental time to pupation (A) and adult emergence (B) using Kaplan-Meier method and compared using the log-rank test. Dashed lines represent the median survival time.

The median duration of total lifetime survival until adult death for control was 20.5 days, and for *A. alternatum*, *B. bassiana*, *C. globosum* TAMU520, *C. globosum* TAMU559 and *P. inflatum*, the median values were 22, 22, 21, 20, and 20.5 days, respectively (Figure 2.8B). Although we found significant differences among all different fungal treatments in total lifetime survival time (Table 2.8), there were no significant pairwise differences between the fungal treatments and the control for any of the fungi (Table 2.10). Since significant differences were found between fungal-treated plants but not between them with the control, these results are not shown here.

Table 2.10 Survival in days (mean ± SE) of *Anthonomus grandis grandis* progeny (pupa and adult) from infested cotton reproductive structures of fungal-treated or untreated control plants. *$p \leq 0.05$ (Pairwise comparisons of fungal treatments versus control using the log-rank test with FDR *p*-value adjustment).

Treatment	Lifetime adult survival		
	Duration (days)		n
	Mean ± SE	*p*	
A. alternatum	22.61 ± 0.37	0.648	112
B. bassiana	23.04 ± 0.55	0.471	80
***C. globosum*520**	21.71 ± 0.38	0.791	154
***C. globosum*559**	20.99 ± 0.39	0.416	101
P. inflatum	22.21 ± 0.50	0.791	98
Control	22.07 ± 0.71	-	96

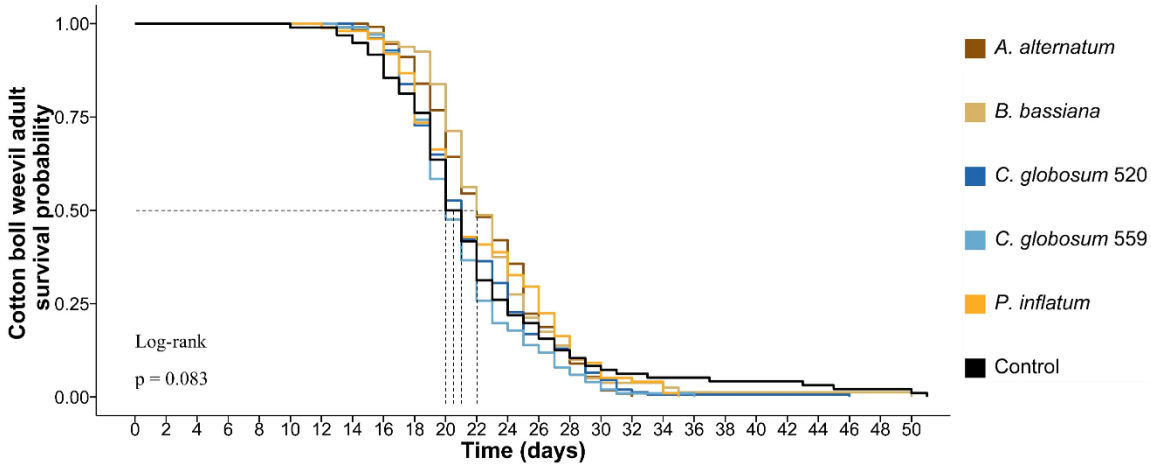

Figure 2.8 Curves of *Anthonomus grandis grandis* progeny from fungal-treated and untreated control plants for the total lifetime survival calculated based on survival probability to adult death using Kaplan-Meier method and compared using the log-rank test. Dashed lines represent the median survival time.

2.3.2.7. Progeny life history traits

Pupae from *C. globosum* TAMU520 were significantly heavier than those from untreated control plants (p = 0.001) (Table 2.11, Figure 2.9A). However, we did not see a difference due to this treatment when we compared the growth rate (weight/developmental time) (Table 2.11). A significant difference in pupa growth rate was found on *A. alternatum* (p = 0.002) compared to control with the pupae from the fungal-treated plants having a lower rate (Figure 2.9B). For CBW adults, the only significant trait was body size, with individuals reared on plants treated with *A. alternatum*, *B. bassiana*, *C. globosum* strains TAMU520 and TAMU559 being larger than the control (Table 2.11, Figure 2.9C).

Table 2.11. Statistical analyses of pupa weight (mg), pupa growth rate (mg/day), and adult body size (mm) of *Anthonomus grandis grandis* progeny grown in cages of fungal-treated or untreated control cotton plants. *$p < 0.05$ (Gaussian model – Generalized linear model).

Treatment	**Pupa weight** (mg)		**Pupa growth rate** (mg/day)		**Adult body size** (mm)	
	Mean ± SE	*p*	Mean ± SE	*p*	Mean ± SE	*p*
A. alternatum	13.27 ± 0.51	0.109	1.08 ± 0.05	0.002*	6.00 ± 0.10	0.018*
B. bassiana	12.93 ± 0.55	0.304	1.17 ± 0.06	0.081	6.06 ± 0.14	0.011*
***C. globosum*520**	14.40 ± 0.45	0.001*	1.33 ± 0.05	0.915	6.08 ± 0.08	0.002*
***C. globosum*559**	13.54 ± 0.55	0.059	1.25 ± 0.07	0.751	5.98 ± 0.09	0.032*
P. inflatum	13.29 ± 0.54	0.127	1.22 ± 0.06	0.199	5.89 ± 0.11	0.107
Control	12.13 ± 0.45	-	1.34 ± 0.07	-	5.64 ± 0.12	-

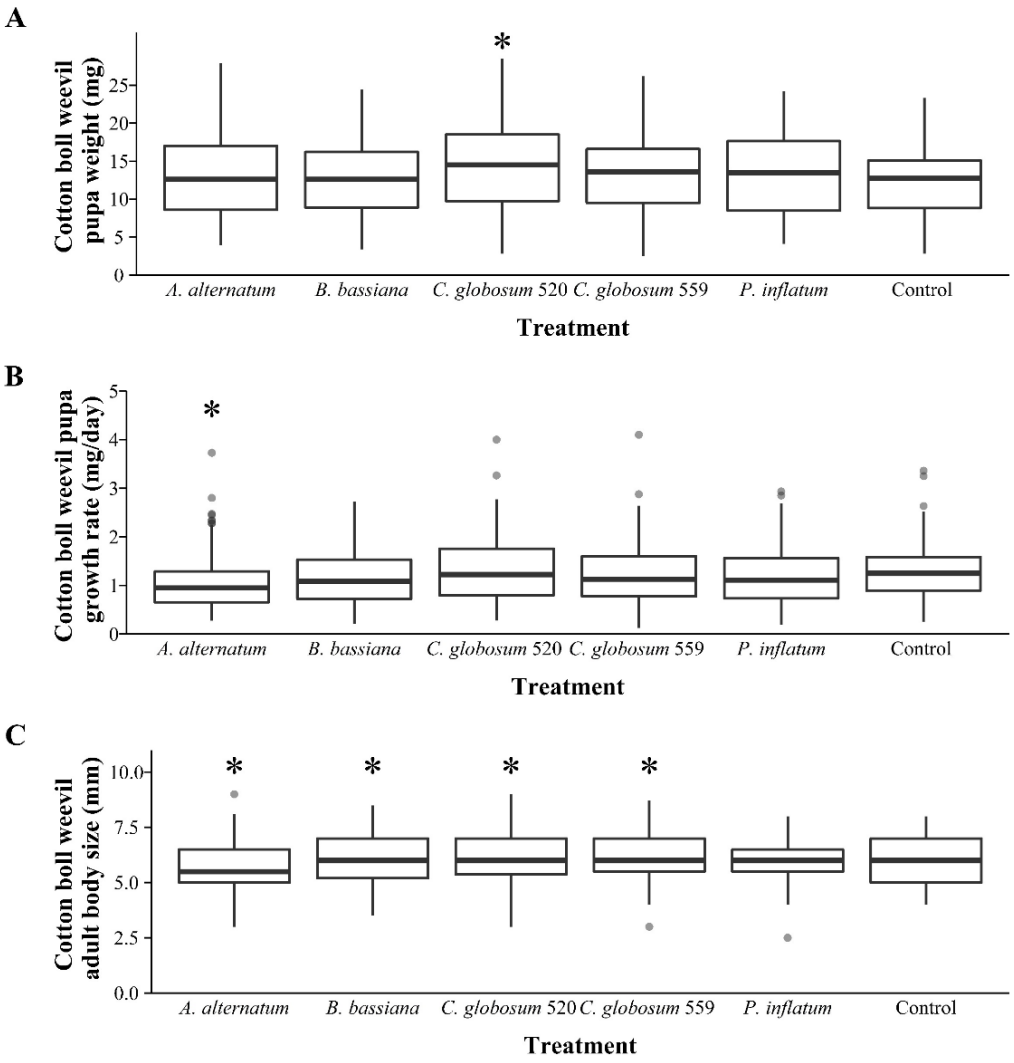

Figure 2.9 *Anthonomus grandis grandis* pupa weight (mg) (A), pupa growth rate (mg/day) (B), and adult body size (mm) (C) from untreated control and fungal-treated cotton plants in cages. *$p < 0.05$ (Gaussian model – Generalized linear model comparison between fungal-treated and control plants).

2.3.3. Abscised cotton reproductive structures

The percentage of abscised cotton reproductive structures was significantly different among all treatments (F = 2.668, p =0.023), with *B. bassiana*-treated plants dropping significantly fewer (p = 0.005) (31.93 ± 7.00 %) squares compared to the control

(57.52± 6.35 %) (Figure 2.10) with around 78% of *B. bassiana* abscised squares having both oviposition and feeding damage and control 73%. However, neither the flowers (F = 0.603, p = 0.698) nor the bolls (F = 0.667, p = 0.652) showed a significant difference among treatments in the percentage of abscised cotton structures in the comparison between the fungal-treated over the control plants, therefore not presented here.

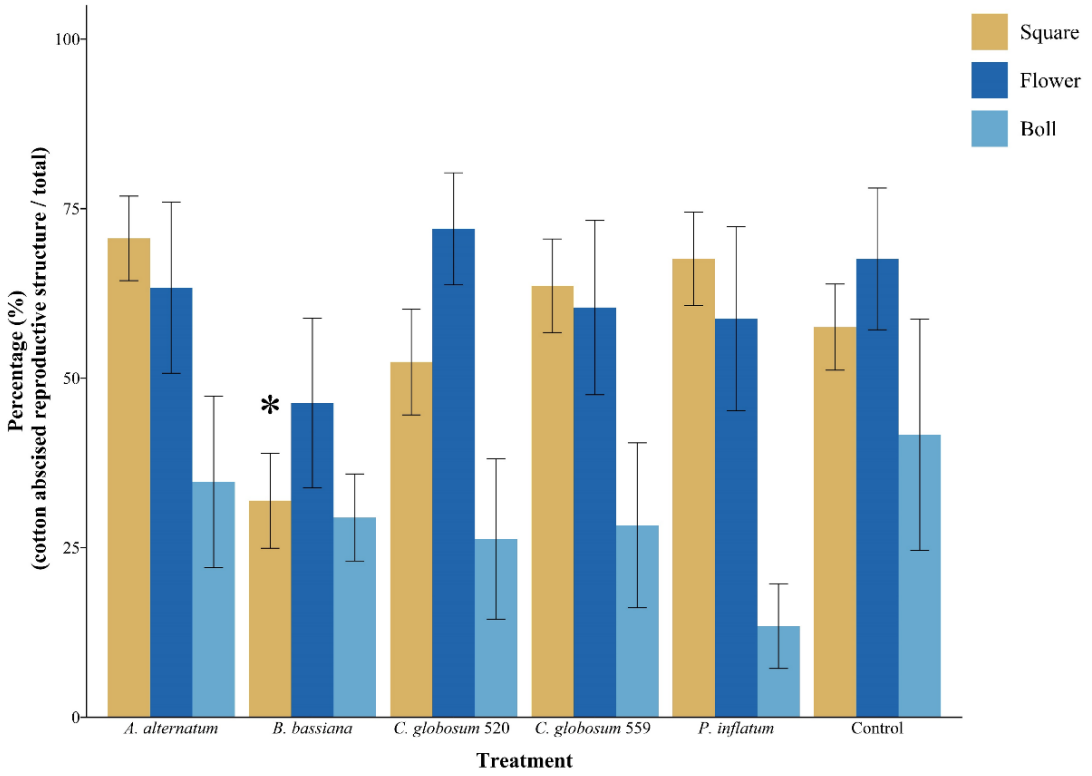

Figure 2.10 Percentage (±SE) of abscised cotton reproductive structures (square – flower bud, flower, and boll) from fungal-treated plants and untreated controls. * p < 0.05 (Quasi-Poisson distribution – Generalized linear model in comparison to control plants).

2.4. Discussion

This study shows for the first time that treating cotton seeds with different strains of plant-associated fungi can have wide-ranging effects on boll weevil behavior, reproduction, and development. The observed effects of fungal treatments on host selection behaviors were relatively minor and strain-specific. However, the treatment effects on weevil reproductive and developmental traits were much more apparent, and in many cases, consistent across the multiple fungal strains tested. These results provide insight into how microbes can mediate plant-insect interactions and highlight the potential for microbial treatments as new IPM tools to help manage a devastating agricultural pest.

Fungi associated with plants can produce or induce the production of secondary metabolites in plants (Rohlfs and Churchill 2011, Pusztahelyi, Holb et al. 2015), which are molecular compounds not involved in essential life processes but with many ecological roles (Scharf, Heinekamp et al. 2014). Many fungi, like *Muscodor vitigenus*, *Penicillium expansum*, *B. bassiana*, *Neotyphodium lolli*, *Epichloë typhina*, and *Epichloë fetuscae* are known to produce secondary metabolites that can be detected within the plant (Rowan, Dymock et al. 1990, Wilkinson, Siegel et al. 2000, Daisy, Strobel et al. 2002, Potter, Tyler Stokes et al. 2008, Xu, Orozco et al. 2008, Xu, Orozco et al. 2009, Azeem, Rajarao et al. 2013). These secondary metabolites can be insecticidal and can have indirect effects on insects by affecting their behavior (Rohlfs and Churchill 2011, Shikano, Rosa et al. 2017). For example, fungal secondary metabolites like peramine can change insect behavior by repelling them (Rowan, Dymock et al. 1990), thereby

protecting the plant from herbivory. Microbial volatile organic compounds (MVOCs) are volatile compounds produced by microbes (e.g., bacteria and fungi) that insects can use as olfactory cues (Davis, Crippen et al. 2013). MVOCs can have insect repellency properties via behavior changes (e.g., feeding or oviposition avoidance) (Davis, Crippen et al. 2013). *Muscodor vitigenus* is an endophytic fungus naturally occurring in flowering plants that produces naphthalene in high quantities, which was shown to be repellant to wheat stem sawfly adult in Y-tube olfactometer assays (Daisy, Strobel et al. 2002). Other research suggests that pine weevils avoided pine twig odor and the volatile styrene produced by the fungus *Penicillium expansum* in multichoice arena tests (Azeem, Rajarao et al. 2013).

Fungal secondary metabolites have also been shown to provide protection to plants through direct negative effects on herbivore survival, development, and reproduction (Rohlfs and Churchill 2011, Shikano, Rosa et al. 2017). *B. bassiana* produces several toxic secondary metabolites such as bassiatin, beauvericin, beauverolide, oosporein, tenellin, and bassianolides that can reduce survival in lepidopteran herbivores like the wax moth, the corn earworm, and the fall armyworm (Xu, Orozco et al. 2008, Xu, Orozco et al. 2009). Fungi associated with grasses are well known to produce mycotoxins (e.g., alkaloids) that are antifeedant and toxic to both vertebrates and invertebrates (Rowan 1993, Schardl, Florea et al. 2013). A hybrid between *N. lolli* and *E. typhina* associated with ryegrass can produce ergot alkaloids that affect the black cutworm by delaying the development and decreasing larvae weight (Potter, Tyler Stokes et al. 2008). Another fungus associated with grass, *E. fetuscae*,

produces loline alkaloids that reduce the progeny of the bird cherry-oat aphids and the greenbug (Wilkinson, Siegel et al. 2000).

Although the fungal taxa tested in this study were originally isolated as endophytes from commercial cotton (Ek-Ramos, Zhou et al. 2013), endophytic colonization following fungal seed treatments was not tested in this study. As such, we cannot conclusively determine whether the many phenotypic effects of the fungal treatments we observed were due to microbial activity as endophytes versus other epiphytic or rhizospheric effects. When seeds are treated with fungi, deleterious effects on herbivores feeding on the resulting plants can be due to several non-mutually exclusive processes. Endophytic colonization and subsequent production of fungal metabolites is but one possibility. Alternatives that are difficult to causally distinguish include induction of the plants own defensive responses, production of elicitors in the rhizosphere that alter plant responses even in the absence of colonization, or competition-mediated changes in the community composition of other microbes that also affect the plant (Rasool, Vidkjær et al. 2021). For example, fungi have been shown to alter secondary metabolite production in plants (Pusztahelyi, Holb et al. 2015, Cachapa, Meyling et al. 2021, Papantoniou, Vergara et al. 2021). The fungi *Rhizophagus irregularis* and *Trichoderma harzianum* associated with tomato plants increased levels of steroidal glycoalkaloids, and these compounds were highly accumulated in the tobacco hornworm larval gut (Papantoniou, Vergara et al. 2021). This interaction can interfere with insect development by increasing the number of unhealthy pupae, decreasing adult emergence, and having a more male-based sex ratio (Papantoniou,

Vergara et al. 2021). Cauliflower planted with *M. brunneum*-inoculated rice grains showed decreased leaf consumption by the diamondback moth larvae in conjunction with higher myrosinase activity, a plant secondary metabolite responsible for activating defensive glucosinolates (Cachapa, Meyling et al. 2021).

2.4.1. Different behavioral responses in no-choice and choice assays

This study, to our knowledge, is the first to evaluate the behavioral responses of cotton boll weevils towards squares from fungal-treated and untreated cotton plants in no-choice and choice assays. Based on similar assays conducted with other cotton pest insects (Sword, Tessnow et al. 2017), we hypothesized that the weevils would respond differently to squares from fungal-treated plants by avoiding the fungal-treated squares. In both the no-choice and choice assays, there were no significant differences in latency to first contact with squares from fungal-treated versus control plants. This suggested a lack of response to any cues that might affect the speed at which weevils made a decision to contact a square. The only observed pre-contact behavioral effect that did suggest a differential response of the weevils to treatment versus control plants was when weevils were offered a choice between squares from *B. bassiana*-treated versus untreated plants. In this case, the weevils selected squares from *B. bassiana* treated plants significantly more often. The other fungal treatments did not significantly affect the weevils' first choice.

Insect herbivores can use volatile organic compounds (VOCs) as olfactory cues to avoid or accept hosts (Thorsteinson 1960, Bruce, Wadhams et al. 2005), and these

blends can be influenced by plant-associated microbes (Yue, Wang et al. 2001, Grunseich, Thompson et al. 2020). VOCs blends are known to be an important factor in CBW host location/selection (Magalhães, Borges et al. 2012, Magalhães, Borges et al. 2018). Generally, above- and belowground herbivores can detect volatiles from plant-associated fungi and avoid microbe-inoculated plants (Bultman, Pulas et al. 2006, Crawford, Land et al. 2010, Rostás, Cripps et al. 2015). Thus, we expected CBW adults to avoid squares from fungal-treated plants like other herbivores, but we observed the opposite effect for *B. bassiana* in the first choice of choice behavioral assays. Similar examples in which fungal treatments result in plants that are seemingly more attractive to herbivores have been reported. The cotton bollworm, *Helicoverpa armigera* (Hübner), had a higher acceptance for fungal-treated tomato plants than control (Jallow, Dugassa-Gobena et al. 2008), and arbuscular mycorrhizal fungi changed the VOC blend of broad bean plants, making them more attractive to pea aphids (Babikova, Gilbert et al. 2014).

In the no-choice assays, we found that CBW was less frequently in contact with squares from *C. globosum* TAMU559 and *P. inflatum* plants than controls, which corroborates an expected repellency behavior towards fungal-treated plants (Meyling and Pell 2006). In the analysis of the response ratio during the choice assays, we found mixed results. CBW preferred *A. alternatum*, *C. globosum* TAMU520, and *P. inflatum* squares less frequently than untreated controls but preferred squares from *B. bassiana* and *C. globosum* TAMU559 plants significantly more often over the course of the assays. The behavioral responses of CBW to squares from *P. inflatum* treated plants relative to controls were consistently negative in both the no-choice and choice assays.

However, the behavioral responses to squares from *C. globosum* TAMU559 treated plants were inconsistent across the different assays. Using the same type of assays, *B. bassiana* and *P. inflatum* had previously been shown to affect host selection behavior of two hemipteran pests negatively, western tarnished plant bugs *Lygus hesperus* Knight and southern green stink bugs *Nezara viridula* (Linnaeus), with the individuals preferring squares from untreated plants over those from fungal-treated plants (Sword, Tessnow et al. 2017). Our study found the same pattern of avoidance for *P. inflatum* but not for *B. bassiana*.

Some secondary metabolites produced by fungi associated with plants can interfere with insect behavior (Rohlfs and Churchill 2011). One example of a secondary metabolite is peramine, a fungal alkaloid that acts as a feeding deterrent in both no-choice and choice assays of adults and larvae of the Argentine stem weevil (Rowan, Dymock et al. 1990). In our study, weevils were observed in contact with squares from fungal-treated plants less frequently in the no-choice assays, but there was no preference pattern for untreated versus fungal-treated squares in the choice assays. Our mixed preference results clearly illustrate that fungal treatments of cotton can affect boll weevil host selection behavior. The results were strain-dependent and inconsistent across different assays, making their biological relevance challenging to interpret.

2.4.2. Influence on female reproduction and progeny performance

For our female reproduction hypothesis, we expected that the fungal-treated plants would negatively affect fecundity, fertility or hatch rate, and emergence rate, thereby

affecting the number of individuals in the next generation. For fecundity (n. of oviposition punctures/female), only *C. globosum* TAMU520 showed a significant effect, with the females laying more eggs or making more oviposition punctures (higher fecundity) on this treatment than in control. Even though the number of oviposition punctures was the same across fungal treatments (or higher in the case of *C. globosum* TAMU520), hatching (n. of larvae/oviposition punctures) and emergence rates (n. of adults/larvae) were both lower across all fungal treatments relative to untreated controls. Thus, fewer larvae and fewer adults emerged from eggs oviposited in fungal-treated plants.

Although lower fecundity has been commonly observed in association with fungal-treated plants, some fungal treatments can show a different pattern with more eggs laid on treated plants (Jensen, Enkegaard et al. 2019, Agbessenou, Akutse et al. 2020). The *A. alternatum*, *B. bassiana*, *C. globosum* TAMU520 and TAMU559, and *P. inflatum* strains tested here have also previously been shown to have adverse effects on the performance of several herbivores such as cotton aphids, cotton bollworms, beet armyworms, and root-knot nematodes (Castillo Lopez, Zhu-Salzman et al. 2014, Lopez and Sword 2015, Zhou, Starr et al. 2016, Zhou, Wheeler et al. 2018, Zhou, Verma et al. 2020). Our results corroborated other studies that found lower fertility and lower adult emergence of herbivores feeding on fungal-treated plants over control, thereby reducing herbivore fitness (Van Bael, Valencia et al. 2009, Akutse, Maniania et al. 2013, Castillo Lopez, Zhu-Salzman et al. 2014, Russo, Scorsetti et al. 2019).

We hypothesized that the progeny (pupae and adults) reared on fungal-treated plants would take longer to develop and exhibit lower survival relative to those fed on control plants. Our results showed that the time to pupation was significantly longer for insects from *A. alternatum* and *P. inflatum*-treated plants and that the time to adult emergence was higher for insects emerging from all fungal-treated plants relative to controls. The prolonged development time observed for weevils reared on the fungal-treated plants corresponds to what has been found in other studies (Jaber and Araj 2018). Based on other studies, we also expected lower survival rates for insects reared on fungal-treated plants (Jaber and Vidal 2010, Russo, Scorsetti et al. 2019). However, we did not find a significant difference in total lifetime survival between insects from the control and fungal-treated plants. Similarly, Raps and Vidal (1998) found no significant effects on lifetime survival for diamondback moths, *Plutella xylostella* (Linnaeus), reared on fava bean treated with *A. alternatum* as an endophyte, and *Neotyphodium*-infected plants did not affect the survival of cereal leaf beetle adults compared to control plants (Clement, Hu et al. 2011).

Lastly, we expected that detrimental effects of the fungal treatments on weevil growth and development would negatively affect pupal weights, growth rates and adult body sizes (Wu, Youngman et al. 2016). The data partially supported these effects. The only effect on pupal weights was for insects reared on *C. globosum* TAMU520 treated plants, which were significantly heavier than the controls. However, the growth rate of these same insects to pupation was not significantly different from the controls. The only significant effect on observed growth rates was for weevils reared on *A. alternatum,*

which had lower growth rates than the controls. Yet, despite having significantly lower growth rates to pupation, the body size of adults from the same treatment group was significantly larger than that of control insects. In fact, adult body sizes were significantly larger than control insects for four out of the five fungal treatment groups (Table 2.11; Figure 2.9). This seemingly contradictory result in which larger adults were observed in many fungal treatment groups despite having similar or lower growth rates than the control insects can be explained by their extended development times. Even though growth rates were similar (or even lower) in the fungal treatment groups, weevils achieved significantly larger adult body sizes in the same treatment groups because they had correspondingly longer development times to both pupation and adult emergence (Table 2.9; Figure 2.7).

Fungal-mediated changes in plant defense are a likely mechanism to help explain the observed effects on boll weevil reproduction, growth, and development. Plant-induced defensive responses are grouped into two main types, induced systemic resistance (ISR) and systemic acquired resistance (SAR). SAR is a response to necrotrophic pathogens and some sucking insects that involves the salicylic acid (SA) pathway leading to the expression of pathogenesis-related (PR) proteins. ISR is related to the jasmonic acid (JA) and ethylene (ET) pathways and is induced mainly by biotrophic pathogens and chewing insects (Tripathi, Kamal et al. 2008). Beneficial microbes, such as plant-associated fungi, can induce host defenses, triggering induced resistance pathways that can negatively affect insect fitness (Rohlfs and Churchill 2011, Pieterse, Zamioudis et al. 2014). For example, in a laboratory study inoculating maize

seeds with *M. robertsii* fungus, they found that the fungus promoted plant growth, altered JA pathway defensive gene expression, and suppressed the growth rate of herbivorous black cutworm larvae (Ahmad, Jiménez-Gasco et al. 2020).

Moreover, as mentioned at the beginning of this chapter discussion, some fungal secondary metabolites can interfere with insect herbivores' development (Potter, Tyler Stokes et al. 2008). One example is *N*-formyl loline, a major component of loline alkaloids produced by *Neotyphodium uncinatum*, endophytic fungus, which is naturally associated with meadow fescue shown to be toxic to Argentine stem weevil, negatively affecting larval growth and development (Popay, Tapper et al. 2009). Given that neither endophytic colonization nor the production of fungal secondary metabolites was examined in this study, their possible effects in delaying boll weevil developmental times will require additional investigation.

2.4.3. Abscised cotton reproductive structures

CBW oviposition and feeding damage are known to induce squares' abscission, which affects yield and production (Coakley, Maxwell et al. 1969, Neves, Showler et al. 2013). If the fungal treatments reduced boll weevil feeding and oviposition on the plants, or otherwise made cotton more resistant to weevil damage, we expected that fungal-treated squares damaged by CBW would abscise less frequently than the untreated control squares. This effect was observed only for the *B. bassiana* treatment plants. Despite having slightly more feeding and oviposition damage than controls, cotton squares from *B. bassiana* plants were abscised significantly less than the control. The persistence of

CBW inside abscised cotton reproductive structures after post-harvesting operations is one of the problems for the growers (Showler 2003, Showler 2007). Thus, a simple method such as fungal-treated seeds could help minimize squares abscission due to cotton boll weevil damage and improve cotton production.

B. bassiana is a well-known entomopathogen widely used in biological control in many crops. Some strains have previously been shown to have a high virulence against boll weevil (Behie, Zelisko et al. 2012, Behie, Moreira et al. 2017, Branine, Bazzicalupo et al. 2019). In the current study, *B. bassiana* treatment appeared to make squares more attractive to CBW adults, but it was also detrimental to female reproduction (lower hatch and emergence rates) and progeny development. Such an effect in the field would be predicted to reduce population growth over successive generations and highlight the potential of such fungal seed treatments as novel IPM tools. In terms of the adaptive significance of these three-way insect-microbe-plant interactions, it remains possible that this fungus might be luring the weevils to the plant and negatively affecting their performance. This could be related to endophytes evolving a pathogenic relationship with insects, penetrating the insect cuticle using proteases, colonizing, and acquiring the nutrients needed by the plants (Barelli, Moonjely et al. 2016).

2.5. Conclusion

All plant-associated fungi originally isolated from cotton along with the commercial *B. bassiana* (Botanigard ®) isolate tested in these experiments affected boll weevil behavior, reproduction, or development to varying degrees. This study highlights the

potential for fungal seed treatments of cotton as new tools for boll weevil IPM. We showed that squares from *A. alternatum* TAMU505, *C. globosum* TAMU520, and *P. inflatum* TAMU490 seed-treated plants could repel the insects. When the adult insects were more attracted to the squares from fungal-treated plants, like in *B. bassiana* and *C. globosum* TAMU559, feeding on the same plants, they had detrimental effects on the progeny. This suggests that the fungal treatments may be useful as an "attract and kill" strategy, luring adult weevils to the fungal-treated plants but negatively affecting the next generation.

Moreover, all fungal seed treatments used in this study affected the CBW life cycle, reducing egg viability (fewer larvae hatching and fewer adult emergence) and prolonging the developmental time in adults. According to the slow-growth high-mortality hypothesis, herbivores with slower growth can subsequently suffer higher mortality (Feeny 1976, Chen and Chen 2018). Because the herbivores would have a prolonged developmental time, they would be more vulnerable to mortality factors such as abiotic conditions and natural enemies. Thus, this observed effect could negatively affect population size in subsequent generations by reducing the number of generations per growing season. For future investigations, population dynamics models could help explore the effect of extending development time on the number of generations in the field and better understand the application of fungal treatments as new IPM tools in an agroecosystem.

3. TO BE OR NOT TO BE A PATHOGEN: PLANT-ASSOCIATED FUNGI CAN DIRECTLY INFECT APHIDS

3.1. Introduction

Fungi associated with plants have been classified in multiple ways according to ecological functions, host specificity, evolution, or other aspects of their biology. One classification is based on their source of nutrition, either as biotrophs or necrotrophs (Bamisile, Dash et al. 2018). Necrotrophs, such as *Botrytis cinereal*, kill plant cells and obtain nutrients from dead tissues (van Kan, Shaw et al. 2014). Biotrophic fungi obtain nutrients to support survival, growth and reproduction from a living organism (Pusztahelyi, Holb et al. 2015). One example of biotrophic fungus is the dark septate endophyte (DSE) *Harpophora oryzae* that lives asymptomatically within plant tissue (Su, Mao et al. 2013). However, some species can switch from one lifestyle to another due to environmental conditions, such as *Leptosphaeria maculans,* which become a pathogen when the plant is under stress (Junker, Draeger et al. 2012).

Some fungi can utilize both live plants and insects as hosts. These endophytic insect pathogenic fungi (EIPF) can have a multifunctional lifestyle that might be adopted according to abiotic and/or biotic conditions (Barelli, Moonjely et al. 2016, Stone and Bidochka 2020). Also, herbivores can have a symbiotic relationship with plant-associated fungi that interfere with natural enemies' efficiency. The black cutworm (*Agrotis ipsilon*), when feeding on *Neotyphodium lolii*-infected grasses, exhibited greater defense to the endoparasitic nematode, *Steinernema carpocapsae*, acquiring alkaloids

produced by the fungi to protect them, reducing nematode infectivity rates and decreasing the growth of symbiotic nematode bacteria (Kunkel and Grewal 2003, Kunkel, Grewal et al. 2004). Thus, the herbivore coevolved with the plant and fungi complex can still benefit despite the potentially adverse effects of plant-associated fungi because they acquire some resistance against a natural enemy.

Functional and phylogenetic analysis of the entomopathogenic genera *Metarhizium* and *Beauveri*a suggests that the relationship between EIPF and plants originally began with ancestral saprotrophic fungi that first evolved a plant pathogenic lifestyle. This was followed by the transition to symbiosis potentially due to selection pressure for plant-fungal mutualistic relationships (Barelli, Moonjely et al. 2016). The evolutionary transition to using insects as hosts is a relatively recent adaptation, with plant symbiotic fungi acquiring the ability to infect and kill insects. The proteases, lipases, and chitinases that enable insect cuticle penetration and host use were either co-opted from genes involved in plant colonization or obtained via horizontal gene transfer (Barelli, Moonjely et al. 2016, Stone and Bidochka 2020). As a result, the genomes of the EIPF genera *Metarhizium* and *Beauveria* contain elements necessary for both plant and insect use that enable their multifunctional lifestyles (Moonjely, Barelli et al. 2016). It has been proposed that the use of insects as hosts by these fungi is an adaptation that provides fungal access to insect nutrients such as nitrogen that can then be available to plants as part of a nutritional symbiosis (Barelli, Moonjely et al. 2016).

All plants are associated and interact with a diversity of microorganisms as part of what is referred to as the phytobiome (Hawkes and Connor 2017, Leach, Triplett et al.

2017). In most cases, the ecological consequences of the vast majority of these interactions are uncharacterized (Porras-Alfaro and Bayman 2011, Wani, Ashraf et al. 2015, Bell, Hockett et al. 2019). Zhou, Verma et al. (2020) screened a diversity of fungal isolates originally collected associated with cultivated cotton in Texas, USA, for potential negative effects against root-knot nematodes. They found that a surprising number of fungi had negative effects on nematodes when inoculated back to the plant, many of which had not been previously reported to have any known ecological effects on nematodes. Similarly, Anwar, Nawaz et al. (2021) recently screened fungi associated with dead hemipteran insects that feed on cotton and found that they had been colonized by a wide variety of plant-associated fungi, many of which are not typically considered insect pathogens.

A study conducted in Korea recovered a fungal isolate from pinecones and identified it as *Simplicillium lanosoniveum*, a well-known mycoparasite. However, after inoculation of this fungus to silkworm larvae and pupae, they found a new role for the fungus as a pathogen capable of killing insects similar to well-known entomopathogens like *B. bassiana* (Lim, Lee et al. 2014). Niu, Xie et al. (2019) collected soil samples in plant-covered areas to analyze the diversity of fungi, tested the pathogenicity of 15 isolates against B-biotype whitefly, and found two new entomopathogens, *Nectria mauritiicola* and *Scopulariopsis brumptii*.

Based on these observations of plant-associated fungi that can also use live on insects as hosts, we set out to test whether several fungal isolates originally isolated from cotton were capable of infecting and killing cotton aphids, *Aphis gossypii* Glover, as

entomopathogens. The cotton aphid is one of the major pests of cotton with a nearly global distribution and the potential to feed on a broad range of host plant species (Blackman and Eastop 2000). This aphid can cause severe economic losses in cotton fields mainly because of its short lifecycle and asexual reproduction during the cropping season that enables aphids to reach large population sizes in a short period (Kersting, Satar et al. 1999, Lu, Wu et al. 2012). Notably, the honeydew produced by the aphids contaminates cotton (sticky cotton), increasing cotton lint stickiness due to the honeydew's sugars, negatively affecting fiber production, and generating economic losses (Slosser, Parajulee et al. 2002). It is known to be susceptible to direct infection by several fungal entomopathogens, some of which are also commonly associated with plants, including *B. bassiana* and *Phialemonium inflatum* (Loureiro and Moino Jr 2006, Vu, Hong et al. 2007, Castillo Lopez, Zhu-Salzman et al. 2014, Jandricic, Filotas et al. 2014).

Knowledge about pests and plant-associated fungi is essential for developing and implementing novel pest management approaches in cotton. Microorganisms can be used in IPM to inhibit pest activity or even make them more vulnerable to predation (Miranda 2010). It is critically important to understand how the manipulation of these plant-associated microbes affects these crop pests. Thus, the present study objective was to investigate the pathogenicity of different fungal isolates, including some presumably non-pathogenic species (plant-associated fungi), towards the cotton aphid.

3.2. Materials and Methods

3.2.1. Cotton aphid rearing and host plants

A cotton aphid colony originally isolated from field-collected insects was maintained for multiple generations in the Sword Lab at Texas A&M University under controlled conditions (25 ± 1 ℃, RH: 60 ± 10% and 16L:8D). The non-transgenic variety of upland cotton *Gossypium hirsutum* LA122 (All-Tex Seed Inc., Levelland, TX) was used for colony rearing and all experiments reported here. All plants were grown by planting one seed per pot in 515ml pots with unsterilized Metro-Mix 900 soil (Sun Gro Horticulture Canada Ltd.) and maintained in greenhouses at approx. 25°C with natural light. Pots were watered as needed, and no fertilizer was added. Aphid infested plants were kept in cages (39,88 x 39,88 x 60,96 cm) in the same greenhouse. The fourth-true leaf of non-infested plants used in the spray bioassays described below was similarly grown in the greenhouse but kept prior to use in experiments for 48h in a plant growth chamber under the same conditions as the aphid colony.

3.2.2. Fungal isolates spore suspensions

Plant-associated fungal isolates tested for pathogenicity against cotton aphids were *Acremonium alternatum* (TAMU 505), *Chaetomium globosum* (TAMU520, TAMU559), and *Phialemonium inflatum* (TAMU490), all of which were previously isolated as endophytes from field-grown cotton in Texas, USA (Ek-Ramos, Zhou et al. 2013). As a positive control entomopathogen, we also used *Beauveria bassiana* cultured from a commercial strain (Botanigard®, BioWorks Inc, Victor, NY). All isolates were cultured

in 10 cm Petri dishes on potato dextrose agar (PDA) in the dark at 25°C.

Spore suspensions of each fungus were made by adding 2 ml of 0.1% sterile Tween-80 diluted with sterile distilled water to the fungal spore plates, scraping them with a sterile metal spatula, filtering through autoclaved 0.25 mm sieves into a sterile beaker, and placing them in 50 ml centrifuge tubes (Sword, Tessnow et al. 2017). Suspensions were mixed on a vortex, centrifuged for 10 minutes in a Cole-Parmer Fixed-speed Centrifuge at 3000 rpm, and excess water was removed by pouring out the supernatant. We used a Neubauer hemocytometer (Thomas Scientific, Philadelphia, PA, USA) to quantify spore concentrations, and final treatment concentrations for experiments were diluted with sterile water to reach 10^2 - 10^7 spore/1 ml solutions (Hassan, Abdullah et al. 2019). Lastly, all the spore suspensions were rinsed three times with sterile distilled water to remove the Tween-80 from the suspensions to avoid potentially confounding effects of the surfactant on cotton aphid survival. We rinsed the spores because preliminary trials revealed a significant difference in aphid survival between those treated with sterile distilled water only versus the 0.1% Tween-80 solution used to isolate the spores (Tween mean ± SE survival time 4.30 ± 0.69 compared to water 10.10 ± 1.88, a *p*-value of 0.05 for the pairwise comparison using Log-rank test).

3.2.3. Pathogenicity bioassays

3.2.3.1. Dipping bioassays

We used a dipping assay to evaluate the pathogenicity of all isolates by immersing 2nd

instar aphids in 1ml of each 10^2 - 10^7 spore/1 ml spore suspensions along with 1 ml of sterile water as a control for 5 seconds (Castillo Lopez, Zhu-Salzman et al. 2014). Individual aphids were submerged in the solutions using a #2/0 Daler-Rowney paintbrush, then placed on cotton leaf fragments (2 x 2 cm^2) in 10 cm Petri dishes with a moistened 5.5 cm diameter filter paper disc (Whatman® Cellulose Filter Papers) sealed with parafilm (Bemis™, Neenah, WI) (Figure 3.1A).

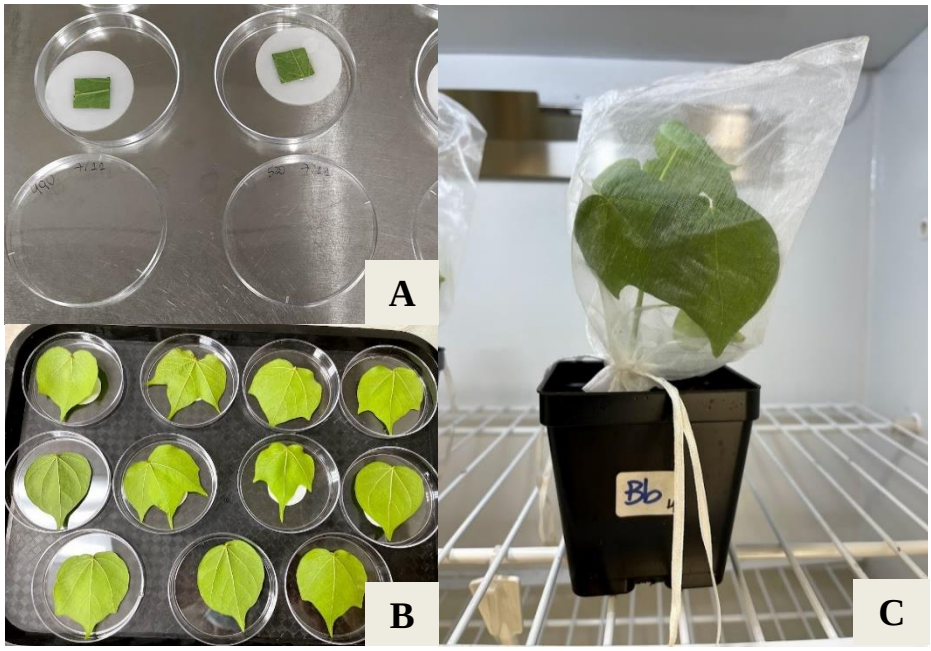

Figure 3.1 Examples of cotton aphid *Aphis gossypii* survival bioassays conditions following inoculation with one of five fungal strains or water control in (A) dipping assays, (B) spray bioassays in Petri dishes, and (C) whole plant assays.

We placed 10 aphids per leaf square (10 aphids/plate), replicated three times (3 replicates/treatment/concentration), and incubated plates under the same controlled conditions as the aphid rearing colony. A total of 360 individuals were tested in the

experiment (30 aphids/treatment/concentration plus 30 aphids/control). Plate positions were randomized daily to avoid position effects inside the incubator. For 14 days, we recorded the number of surviving aphids daily. Following aphid deaths, cadavers were sterilized by immersion in 1% sodium hypochlorite (NaOCl) for 30 s and 70% ethanol for 30 s, followed by three rinses in sterile water to test for mycosis (Ondiaka, Maniania et al. 2008, Jordan, dos Santos et al. 2021) and placed on PDA plates in the dark at 25 °C (Figure 3.2). The mycosis was confirmed by cadaver sporulation on PDA plates and microscopic analysis of the hyphae (Ondiaka, Maniania et al. 2008). Though all the assays could not be conducted simultaneously for logistical reasons, we conducted separate trials containing all fungi within a single concentration level along with their corresponding control group.

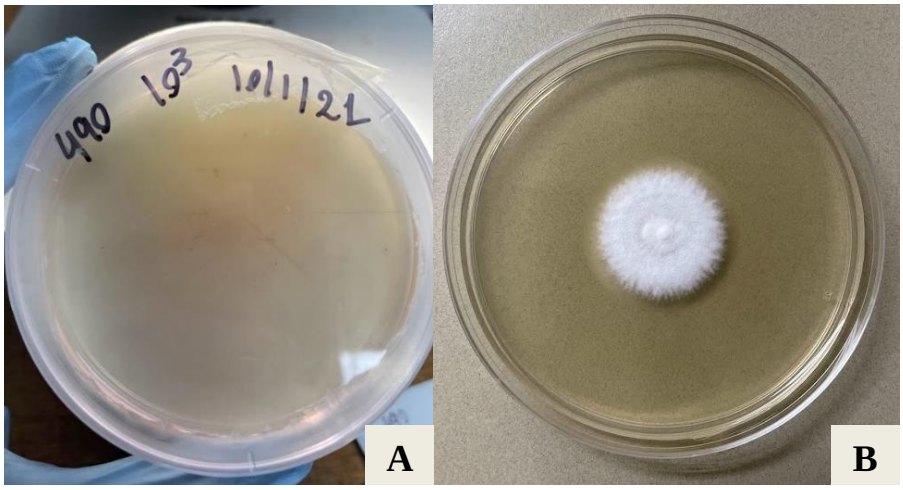

Figure 3.2 Examples of PDA (potato dextrose agar) plates used after sterilizing cotton aphid *Aphis gossypii* cadavers to confirm mycosis in dipping and spray bioassays with (A) no-mycosis and (B) positive mycosis confirmation following treatment with *B. bassiana*.

3.2.3.2. Spray bioassays

Fourth true-leaf cotton plants were manually infested using a paintbrush with 10 cotton aphids 24 hours before the bioassays to ensure aphids were not killed during transfer. We sprayed the cotton aphid-infested plants to run off with 2 ml of each fungal spore concentration using a 59.1 ml cosmetic hand spray (Fingu-Mabola, Bawin et al. 2021) sterilized with 70% ethanol and 5 minutes on UV light after each application. Aphids were sprayed with sterile water as the control.

After spraying, we kept 5 aphids on the plant and transferred 5 aphids with a paintbrush to 10 cm Petri dishes containing one unsprayed equally sized cotton leaf with a moistened filter paper and sealed with parafilm (Anderson, McGee et al. 2007) (Figure 3.1B). The detached leaves for the spray bioassays were from the same cohort of cotton plants but were not sprayed. The plants were individually covered with organza mesh bags (23 x 16 cm) to prevent aphid escape and kept in an incubator (Figure 3.1C), along with the Petri dishes, under the same controlled conditions as the rearing colony. The spray bioassays were replicated twice. A total of 120 aphids were treated, with 60 individuals (10 aphids/treatment/concentration plus 10 aphids/control) kept on whole plants and 60 individuals kept in Petri dishes. We recorded the aphid survival daily for 14 days and analyzed cadaver mycosis as described for the dipping bioassays.

3.2.4. Statistical analysis

All data analyses were conducted using R version 3.6.3 (R Core Team 2020) with an $\alpha = 0.05$. We performed a Kaplan-Meier analysis with log-rank and a pairwise comparison

with FDR *p*-value adjustment using the function pairwise_survdiff to compare the cotton aphid survival between the fungal treatments and the control in each concentration and bioassay method (dipping, spray-plant, and spray-plate). The Kaplan-Meier analyses were conducted using the R packages survival (Therneau 2015) and survminer (Kassambara, Kosinski et al. 2017). To test for a handling effect of moving sprayed aphids on their survival, we also compared the mean survival time between the control spray aphids left on the plant versus those moved to Petri dishes.

We performed a generalized linear model (*glm* function in R) with Gaussian model distribution (Pachú, Macedo et al. 2021) for the cadavers' mycosis confirmation in the bioassays to test for differences between the spore concentration treatments and controls. We used a half-normal envelope in R's hnp package (Moral, Hinde et al. 2017) to assess the model's goodness of fit. We calculated the percentage of confirmed mycosis by dividing the number of confirmed mycosis cases by the total cadavers within each spore concentration treatment group x 100. When significant treatment effects were found in the glm, we conducted a Tukey HSD analysis at 5% significance ($p < 0.05$) using the Tukey HSD function in R. Spore concentration treatment groups in which no mycosis was observed were not included in the analysis, nor were they represented in the bar graphs. For the graphs, we used the Rmisc package in the R program (Hope 2013) to compute means and standard error for the cadaver mycosis confirmation and the ggplot2 package for the graphs (Wickham 2016).

3.3. Results

3.3.1. Cotton aphid survival

In the dipping bioassays, aphid survival times were significantly shorter than controls for every fungal spore treatment at every concentration tested (Table 3.1, Figure 3.3). The median survival time ranged from 2 to 3 days for all fungal treatment groups, whereas it was not possible to calculate a median for the control aphids because >50% of the aphids did not die during the experimental period (Table 3.1). The mean survival time for the controls ranged from 8.93 ± 1.07 days to 10.80 ± 0.98 days, while the fungal treatment means ranged from a low of 1.87 ± 0.11 for *B. bassiana* at 10^7 spore/ml to a high of 4.70 ± 0.80 for *P. inflatum* at 10^4 spores/ml (Table 3.1)

Table 3.1 Survival analysis comparisons of Aphis gossypii in dipping bioassays (n = 30/treatment) and spray bioassays with aphids kept either on plants (n = 10/treatment) or in Petri dishes (n = 10/treatment) treated with five fungi (*A. alternatum*, *B. bassiana*, *C. globosum* 520, *C. globosum* 559, and *P. inflatum*) and water control in six concentrations ranging from 10^2 to 10^7 spore ml^{-1}. *$p < 0.05$ (Pairwise comparisons with log-rank test and FDR p-value adjustment).

Fungus and suspension concentration	Dipping			Spray (Plant)			Spray (Plate)		
	Median (95% CI)	Mean ± SE	*p*	Median (95% CI)	Mean ± SE	*p*	Median (95% CI)	Mean ± SE	*p*
A. alternatum									
10^7	2.0 (0.075 – 0.371)	1.93 ± 0.15	**<0.0001***	4.0 (0.269 – 0.929)	8.00 ± 1.91	0.132	3.5 (0.116 – 0.773)	4.50 ± 1.06	**0.0003***
10^6	2.0 (0.258 – 0.620)	2.27 ± 0.23	**<0.0001***	NA	9.30 ± 1.85	0.920	4.5 (0.016 – 0.642)	4.40 ± 0.72	**0.0004***
10^5	2.0 (0.201 – 0.553)	2.07 ± 0.19	**<0.0001***	NA	11.90 ± 1.33	0.440	7.0 (0.187 – 0.855	6.70 ± 0.95	**0.0008***
10^4	2.5 (0.147 – 0.483)	2.77 ± 0.36	**<0.0001***	NA	12.80 ± 1.14	0.980	7.0 (0.187 – 0.855)	7.80 ± 1.49	0.054
10^3	3.0 (0.229 – 0.587)	3.00 ± 0.29	**<0.0001***	NA	13.00 ± 0.95	1.000	NA	9.00 ± 1.94	0.652
10^2	2.0 (0.258 – 0.258)	2.87 ± 0.47	**0.0001***	NA	14.00 ± 0.00	1.000	NA	12.90 ± 1.04	0.696
B. bassiana									
10^7	2.0 (0.054 – 0.332)	1.87 ± 0.11	**<0.0001***	4.5 (0.269 – 0.929)	7.00 ± 1.88	0.074	2.5 (0.116 – 0.773	3.40 ± 0.71	**<0.0001***
10^6	2.0 (0.288 – 0.652)	2.37 ± 0.16	**<0.0001***	5.0 (0.269 – 0.929)	8.50 ± 1.77	0.920	2.0 (0.187 – 0.855)	4.10 ± 1.26	**0.0025***
10^5	2.0 (0.229 – 0.587)	2.43 ± 0.24	**<0.0001***	NA	9.70 ± 1.67	0.220	2.5 (0.187 – 0.855)	4.90 ± 1.51	**0.0035***
10^4	2.0 (0.288 – 0.652)	2.93 ± 0.42	**0.0001***	NA	10.80 ± 1.57	0.730	5.5 (0.187 – 0.855)	6.30 ± 1.35	**0.0172***

Table 3.1 (continued)

10^3	2.0 (0.288 – 0.652)	2.57 ± 0.23	**<0.0001***	NA	12.10 ± 1.22	1.000	6.0 (0.187 – 0.855)	7.20 ± 1.62	0.234
10^2	3.0 (0.174 – 0.518)	2.93 ± 0.26	**0.0001***	NA	14.00 ± 0.00	1.000	6.0 (0.269 – 0.929)	8.70 ± 1.74	0.363
*C. globosum*520									
10^7	2.0 (0.147 – 0.483)	1.97 ± 0.17	**<0.0001***	4.0 (0.187 – 0.855)	7.40 ± 1.74	0.074	3.0 (0.058 – 0.691	3.10 ± 0.52	**<0.0001***
10^6	2.0 (0.201 – 0.553)	2.40 ± 0.26	**<0.0001***	NA	9.70 ± 1.70	0.920	5.5 (0.116 – 0.773	4.40 ± 0.77	**0.0007***
10^5	2.0 (0.098 – 0.409)	1.97 ± 0.14	**<0.0001***	NA	10.00 ± 1.59	0.220	3.5 (0.187 – 0.855)	5.40 ± 1.49	**0.0035***
10^4	3.0 (0.122 – 0.446)	2.70 ± 0.21	**<0.0001***	NA	10.30 ± 1.79	0.730	8.0 (0.187 – 0.855)	8.00 ± 1.27	**0.0302***
10^3	2.0 (0.318 – 0.684)	2.43 ± 0.21	**<0.0001***	NA	11.70 ± 1.46	1.000	6.0 (0.269 – 0.929	8.90 ± 1.63	0.541
10^2	2.5 (0.075 – 0.371)	2.70 ± 0.31	**<0.0001***	NA	12.90 ± 1.04	0.530	NA	12.30 ± 1.05	0.735
*C. globosum*559									
10^7	2.0 (0.018 – 0.254)	1.93 ± 0.16	**<0.0001***	4.0 (0.187 – 0.855)	7.00 ± 1.83	0.074	2.5 (0.187 – 0.855)	3.20 ± 0.53	**<0.0001***
10^6	2.0 (0.288 – 0.652)	2.47 ± 0.27	**<0.0001***	NA	10.00 ± 1.63	0.920	3.5 (0.187 – 0.855)	4.70 ± 1.07	**0.0008***
10^5	2.0 (0.201 – 0.553)	2.00 ± 0.18	**<0.0001***	NA	10.60 ± 1.64	0.340	5.5 (0.116 – 0.773)	5.90 ± 1.42	**0.0038***
10^4	2.0 (0.018 – 0.254)	2.33 ± 0.16	**<0.0001***	NA	10.40 ± 1.74	0.730	7.0 (0.016 – 0.642)	6.20 ± 0.54	**0.0019***
10^3	2.0 (0.288 – 0.652)	2.60 ± 0.22	**<0.0001***	NA	11.70 ± 1.46	1.000	8.0 (0.187 – 0.855)	8.20 ± 1.44	0.234

Table 3.2 (continued)

10^2	2.0 (0.201 – 0.553)	2.37 ± 0.21	**<0.0001***	NA	12.00 ± 1.27	0.370	NA	11.90 ± 1.33	0.976
P. inflatum									
10^7	2.0 (0.229 – 0.587)	2.33 ± 0.25	**<0.0001***	6.5 (0.269 – 0.929)	7.50 ± 1.85	0.074	3.0 (0.116 – 0.773)	2.70 ± 0.35	**<0.0001***
10^6	3.0 (0.201 – 0.553)	3.43 ± 0.34	**<0.0001***	NA	9.70 ± 1.69	0.920	3.0 (0.187 – 0.855)	3.20 ± 0.31	**<0.0001***
10^5	3.0 (0.174 – 0.518)	2.73 ± 0.25	**0.0001***	NA	11.50 ± 1.58	0.440	5.0 (0.187 – 0.855)	4.00 ± 0.68	**0.0006***
10^4	3.0 (0.229 – 0.587)	4.70 ± 0.80	**0.0096***	NA	11.90 ± 1.33	0.860	3.0 (0.016 – 0.642)	3.50 ± 0.68	**0.0006***
10^3	3.0 (0.174 – 0.518)	3.17 ± 0.45	**<0.0001***	NA	11.50 ± 1.58	1.000	5.5 (0.116 – 0.773)	5.40 ± 0.83	**0.0120***
10^2	2.5 (0.098 – 0.409)	2.63 ± 0.22	**<0.0001***	NA	11.80 ± 1.39	0.370	6.0 (0.058 – 0.691	6.70 ± 1.27	0.061
Control (water)									
7	NA	10.80 ± 0.98	-	NA	13.00 ± 0.95	-	NA	13.40 ± 0.57	-
6	NA	9.57 ± 1.01	-	NA	12.30 ± 1.09	-	NA	11.90 ± 1.02	-
5	NA	8.93 ± 1.07	-	NA	14.00 ± 0.00	-	NA	13.20 ± 0.76	-
4	NA	9.43 ± 1.03	-	NA	12.90 ± 1.04	-	NA	12.60 ± 0.89	-
3	NA	9.50 ± 1.02	-	NA	12.90 ± 1.04	-	NA	11.60 ± 1.27	-
2	NA	9.43 ± 1.10	-	NA	14.00 ± 0.00	-	NA	12.20 ± 1.17	-

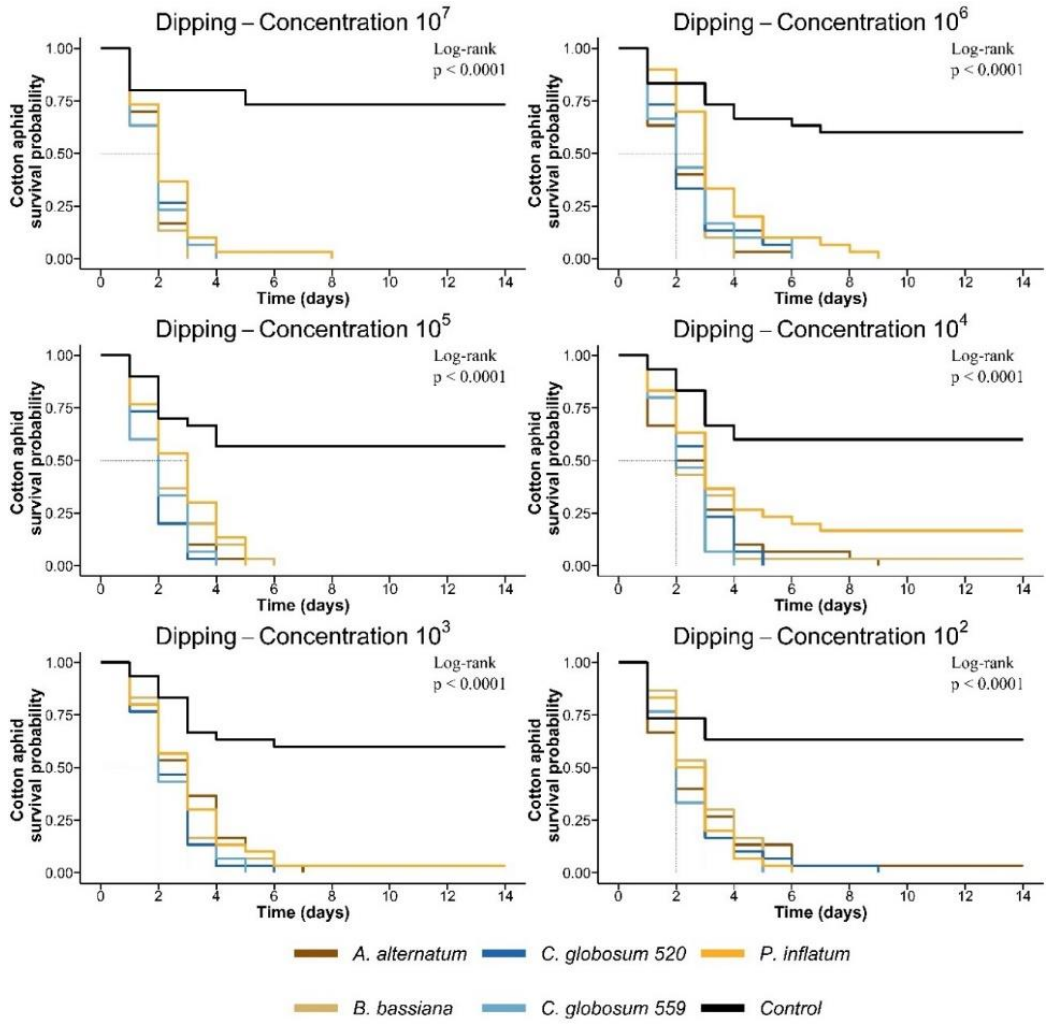

Figure 3.3 Kaplan–Meier survival curves of *Aphis gossypii* 2nd instars in dipping bioassays. Individuals were immersed in 10^2-10^7 spore/ml suspensions of five fungi and monitored for survival for 14 days. Log-rank *p*-values, medians (95%CI), and means ±SE for all pairwise fungal treatment versus control comparisons are presented in Table 3.1.

When aphids were sprayed with varying spore concentrations of the different fungi and maintained on whole plants, aphid survival times did not vary from those of control insects for any of the fungal treatment groups at any concentration (Fig 3.4; Table 3.1). There was a non-significant trend for lower survival times versus control in

all fungal treatment groups at the highest spore concentration of 10^7 spores/ml, particularly for *B. bassiana*, *C. globosum* strains TAMU520 and TAMU559 and *P. inflatum*, each with P values of 0.074. However, the trend diminished as spore concentrations were reduced (Fig. 3.4, Table 3.1).

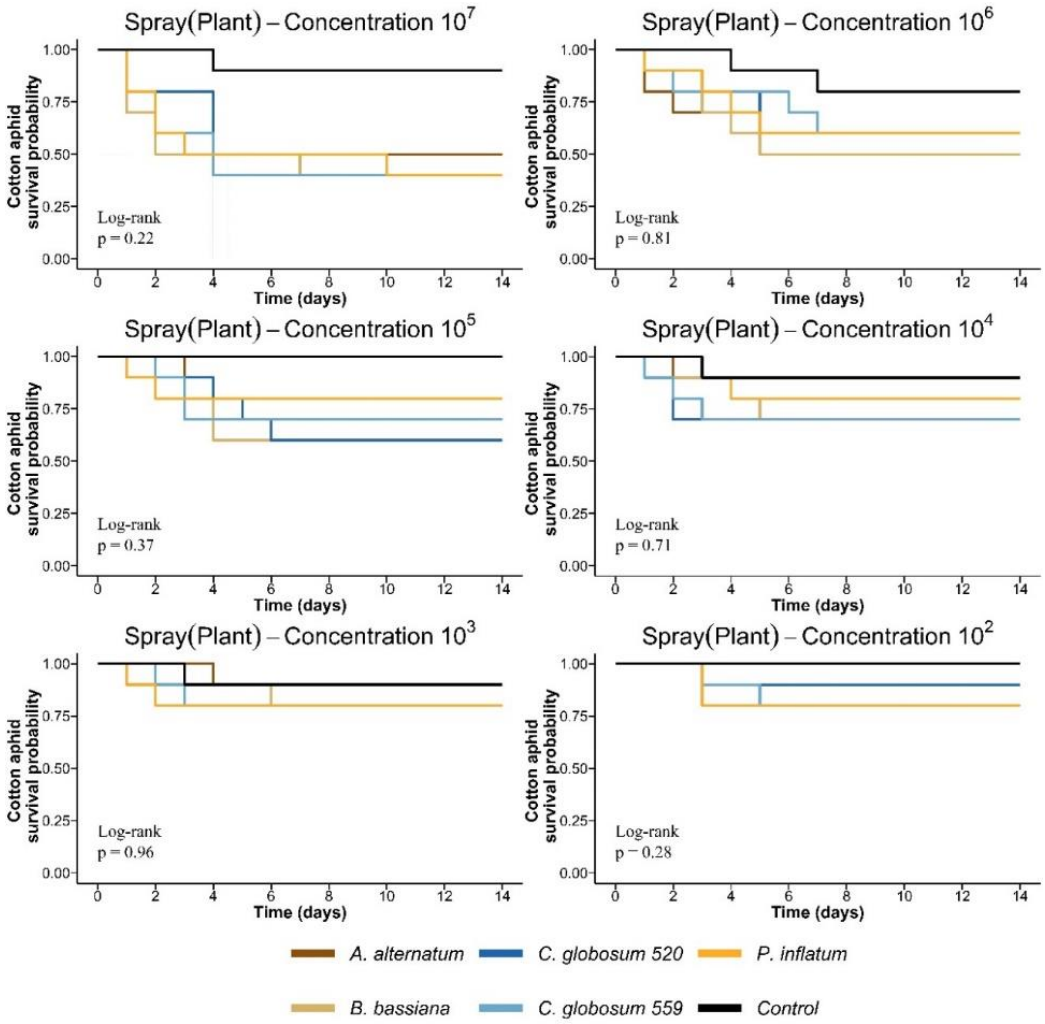

Figure 3.4 Kaplan–Meier survival curves of *Aphis gossypii* 2nd instars in spray (plant) bioassays. Individuals were sprayed with 10^2-10^7 spore/ml suspensions of five fungi and monitored for survival on cotton plants for 14 days. Log-rank *p*-values, medians (95%CI), and means ±SE for all pairwise comparisons of fungal treatments versus control are presented in Table 3.1.

In sharp contrast to the spray bioassays in which survival of treated aphids was monitored on whole plants, those aphids that were transferred to leaves and maintained in Petri dishes had significantly reduced survival times relative to control insects at most of the spore concentrations tested (Table 3.1; Figure 3.5). The decrease in survival was dose-dependent for all fungal treatments, with lower survival times at higher spore concentrations and vice versa (Table 3.1; Figure 3.5).

To test whether a handling effect could account for different survival patterns in response to the fungal treatments when sprayed insects were maintained on whole plants versus moved to Petri dishes, the survival times of control aphids were compared between the two treatment groups. There was no significant difference in survival time between the control insects that were sprayed with water only and then maintained either on whole caged plants or in Petri dishes (means ±SE on Table 3.1; *p*-values ranged from 0.312 to 0.983).

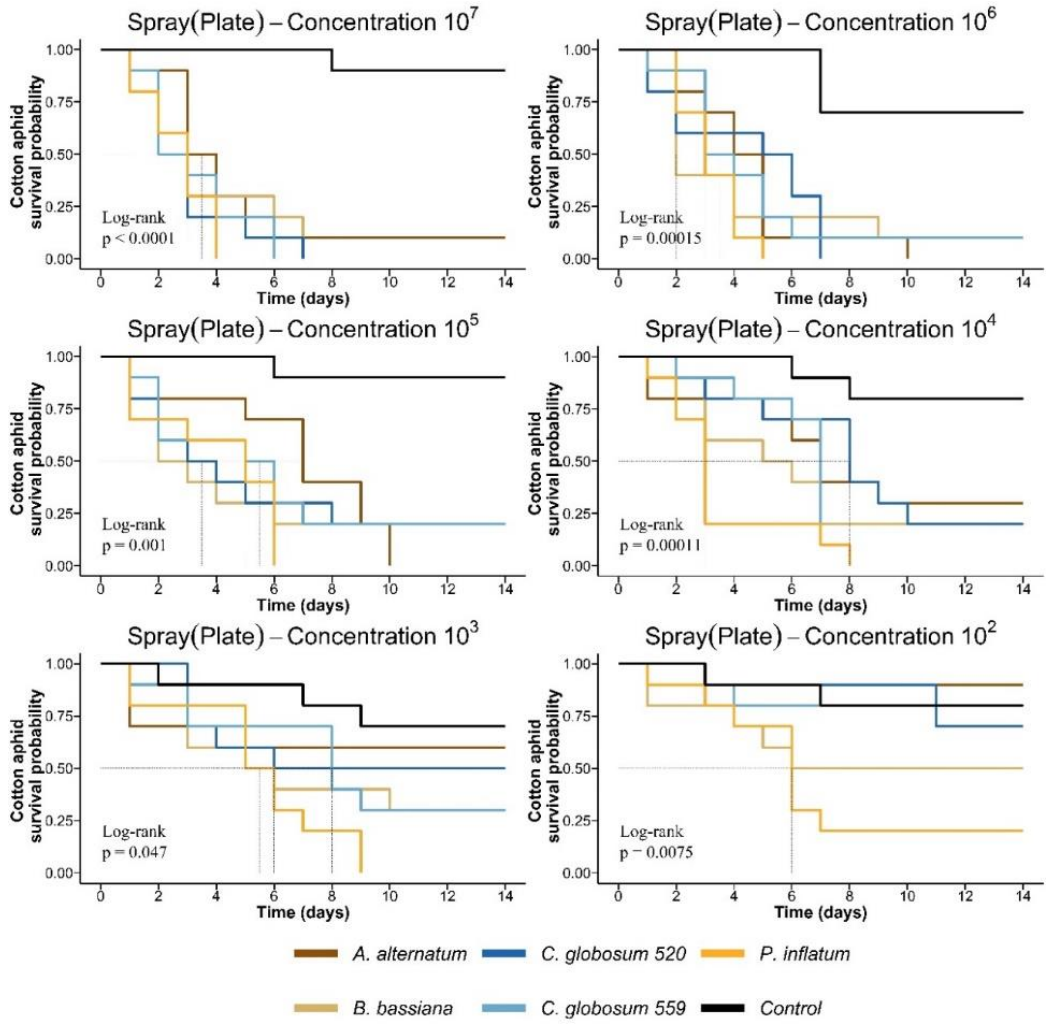

Figure 3.5 Kaplan–Meier survival curves of *Aphis gossypii* 2nd instars in spray (plate) bioassays. Individuals were sprayed with 10^2-10^7 spore/ml suspensions of five fungi and monitored for survival in Petri dishes for 14 days. Log-rank *p*-values, medians (95%CI), and means ±SE for all pairwise comparisons of fungal treatments versus control are presented in Table 3.1.

3.3.2. Mycosis

Mycosis from aphid cadavers was confirmed for all the fungi tested and was observed across all the bioassays conducted. The percentage of aphid cadavers exhibiting mycosis following fungal treatment did not vary across the three experimental groups of dipping,

spray (plant) and spray (plate) ($F = 1.985$, $df = 2$, $p = 0.140$). However, there was a significant difference among the concentrations tested ($F = 7.739$, $df = 5$, $p < 0.0001$) with the highest concentration of 10^7 spore/mL having the highest level of mycosis confirmation at 64.9% ± 4.5. Mycosis percentage observed at 10^7 spores/ml was not significantly different from the 54.9% ± 4.9 found at 10^6 ($z = 1.530$, $p = 0.126$). However, it was significantly different from the other spores/ml concentrations of 10^5 (50.1% ± 4.5 confirmation, $z = 2.218$, $p = 0.027$), 10^4 (47.6 % ± 5.0, $z = 2.595$, $p = 0.009$), 10^3 (30.7% ± 4.9, $z = 5.040$, $p < 0.0001$), and 10^2 (29.7% ± 5.5, $z = 4.873$, $p < 0.0001$).

In the dipping bioassays, the mycosis confirmation percentage varied among the fungal treatments ($F = 5.549$, $df = 4$, $p = 0.0005$), with *C. globosum* TAMU559 and *A. alternatum* having the highest mycosis percentages of 60.5% and 59.3%, respectively, compared to the other treatments (Table 3.2). We also found significant differences in mycosis among the fungal strains in the spray (plant) ($F = 2.944$, $df = 4$, $p = 0.032$) and spray (plate) ($F = 3.165$, $df = 4$, $p = 0.021$) bioassays, with *B. bassiana* showing the highest confirmation percentages of 70% and 69.6%, respectively, compared to the other treatments (Table 3.2).

Table 3.2 Summary of the mean (± SE) mycosis confirmation percentages calculated by dividing the number of confirmed mycoses by the total cadavers in dipping, spray (plant), and spray (plate) bioassays. Data were fitted to a generalized linear model with Gaussian distribution. The letters represent the differences between the treatments in each bioassay separated by the Tukey HSD test at $p < 0.05$.

Fungus treatment	Dipping	Spray (Plant)	Spray (Plate)
	Mean ± SE	Mean ± SE	Mean ± SE
A. alternatum	59.26 ± 5.31 a	47.62 ± 13.82 a	42.67 ± 6.51 b
B. bassiana	32.96 ± 5.16 c	70.00 ± 13.33 c	69.58 ± 4.13 a
*C. globosum*520	51.11 ± 5.36 ab	26.67 ± 12.96 ab	35.56 ± 8.81 b
*C. globosum*559	60.56 ± 6.13 a	60.56 ± 6.13 a	52.88 ± 9.39 ab
P. inflatum	39.44 ± 14.74 bc	39.44 ± 14.74 bc	55.00 ± 7.02 ab

In the dipping bioassays, *B. bassiana* was the only treatment that showed a significant difference in mycosis among the concentrations (F = 1.146, df = 5, p = 0.039). The highest mycosis percentage was 53.3% at 10^7 spores/ml, and the lowest was 16.7% at 10^2 spores/ml (Figure 3.6A). For spray (plant) bioassays, no significance differences were found among the concentrations in each treatment (Figure 3.6B). In spray (plate) bioassays, *B. bassiana* (F = 4.083, df = 5, p = 0.050) and *P. inflatum* (F = 2.346, df = 5, p = 0.017) showed significant differences among their concentrations. For *B. bassiana*, the significant differences were between 10^7 with the highest mycosis of 90% and 10^4, 10^3, and 10^2 with the lowest mycosis percentages of 63.3%, 55%, and 58.3%, respectively (Figure 3.6C). For *P. inflatum*, the highest mycosis percentages were 80%, 70%, and 60% at the highest concentrations 10^7, 10^6, and 10^5, respectively, and the lowest was 20% at 10^2 spore/mL (z = 3.133, p = 0.002), 10^6 vs. 10^2 (Figure 3.6C).

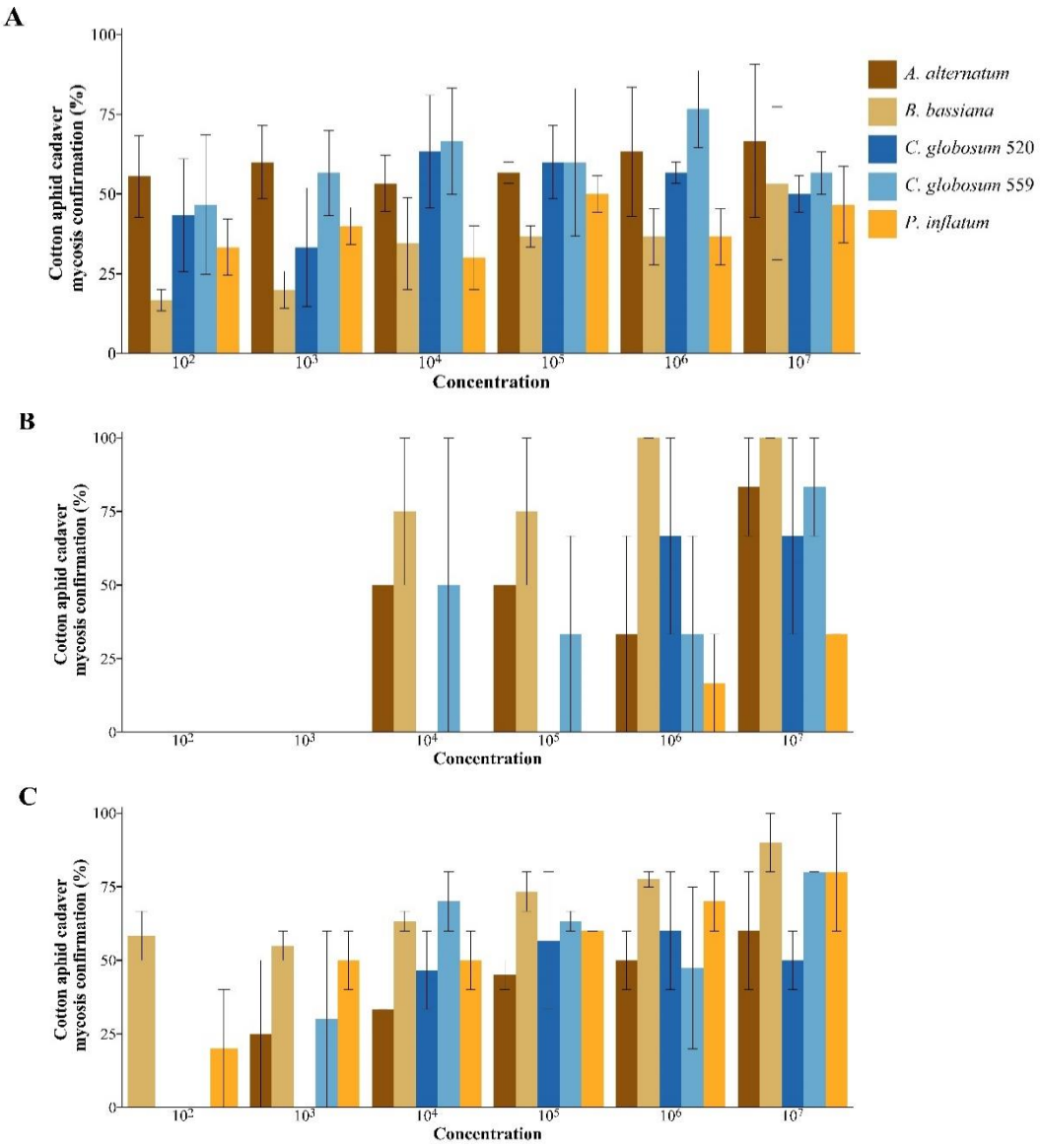

Figure 3.6 Mycosis confirmation of sterilized cotton aphids *Aphis gossypii* cadavers after inoculation with six different concentrations (10^2 to 10^7 conidia mL^{-1}) of five fungi (*A. alternatum*, *B. bassiana*, *C. globosum* 520, *C. globosum* 559, and *P. inflatum*) after plating in PDA (potato dextrose agar). Percentage (%) calculated by dividing the number of confirmed mycoses by the total cadavers in the dipping bioassays (A), spray bioassays with individuals kept on whole plants (B), and spray bioassays with individuals kept in Petri dishes (C). The absence of bars is due to the absence of cadavers at the respective concentration. Control data were not included in the graph because, after sterilization, no fungi were recovered from any control cadavers.

3.4. Discussion

These results demonstrate that some fungi previously found *in-planta* in cotton can infect and kill cotton aphids as entomopathogens when topically applied. The fungi reduced the survival of cotton aphids, showing similar levels of mortality and subsequent mycosis as the well-known entomopathogen, *B. bassiana*. This study provides new insight into the multifunctional lifestyles of fungi that can use both plants and insects as hosts and further elucidates the tripartite ecological interactions between plants, fungi, and insect herbivores as part of the phytobiome. Showing that naturally occurring plant-associated fungi can negatively affect cotton aphid survival as entomopathogens open the door to the potential discovery of many new biological agents to control this pest.

Many fungal taxa previously reported as endophytes are considered entomopathogens (Vega 2008). One of these well-known taxa is *Beauveria*, more specifically the species *B. bassiana*. *Beauveria* (Hypocreales, Cordycipitaceae) is one of the most well-known entomopathogen genera, including ecologically and economically important species (Ownley, Griffin et al. 2008). *B. bassiana* is a naturally soil-inhabiting facultative necrotrophic fungus (Rehner, Minnis et al. 2011). It has been previously found in endophytic association with multiple crops occurring mainly in the leaves and stems (Vega 2008, Behie, Jones et al. 2015). In addition to being an entomopathogen that can directly kill insects (Loureiro and Moino Jr 2006, Ek-Ramos, Mantzoukas et al. 2021), several studies have shown that *B. bassiana* can have an indirect effect on insect herbivores when endophytically associated with plants, including decreasing cotton

aphid fecundity (Castillo Lopez, Zhu-Salzman et al. 2014, Lopez and Sword 2015). Due to its proven efficacy, *B. bassiana* is one of the most used fungi for mycoinsecticides (Faria and Wraight 2007) with many types of formulations (Mascarin and Jaronski 2016). Several commercially available mycoinsecticides made with *B. bassiana* target the family Aphididae, such as Botanigard®, Mycotrol®, and Naturalis® registered in Europe and North America (Kabaluk and Gazdik 2007), Bb Plus in South Africa (Maina, Galadima et al. 2018), and Trichobass-L in Spain (Kabaluk and Gazdik 2007). Given that its biology as an entomopathogen is well characterized, *B. bassiana* was used as a positive control to generate baseline pathogenicity data for comparisons with the other less well-known plant-associated fungal isolates tested in the current study.

Acremonium (Hypocreales, Hypocreaceae) is a soil-borne fungus known to be endophytic mainly in plant roots as well as entomopathogenic (Jallow, Dugassa-Gobena et al. 2008, Vega, Posada et al. 2008, Jaber and Vidal 2010). *A. alternatum* as an endophyte in bean showed indirect negative effects in the diamondback moth, increasing larval mortality and reducing growth rate (Raps and Vidal 1998), and collected from cadavers of palm leafhopper (Aminaee, Zare et al. 2010). This species is also utilized as a mycoparasite that can reduce plant pathogen growth rate and disease development (Romero, Rivera et al. 2003, Kasselaki, Shaw et al. 2006, Jäschke, Dugassa-Gobena et al. 2010). For the first time, our results showed that *A. alternatum* strain TAMU505 can function as an entomopathogen capable of infecting and killing cotton aphids.

Chaetomium (Sordariales, Chaetomiaceae) is a saprophytic and commonly endophytic fungal genus with many strains known to be antagonists of plant pathogens

(Soytong, Kahonokmedhakul et al. 2021). Flavipin, a secondary metabolite produced by *C. globosum*, reduced egg hatching of root-knot nematode and soybean cyst nematode (Nitao, Meyer et al. 2002) and reduced gall numbers of the root-knot nematode as an endophyte (Zhou, Verma et al. 2020). *C. globosum* negatively affected the fecundity of cotton aphid and beet armyworms as plant-associated fungi in cotton (Zhou, Starr et al. 2016) and a recombinant of the fungus expressing insecticidal lectins within rape reduced green peach aphid growth and reproduction (Qi, Lan et al. 2011). Mohammed, Kadhim et al. (2018) sprayed cucumber plants with different fungal isolates, including *B. bassiana* and *C. globosum,* to find the most virulent isolate against the green peach aphid and the cotton aphid, which was *B. bassiana* and not *C. globosum*. In the current study, both of the tested *C. globosum* strains TAMU520 and TAMU559 had strong pathogenic effects on cotton aphids when either as dipping or spray treatments. Mortality levels caused by both *C. globosum* strains were on par with that of the well-known pathogen, *B. bassiana*.

Phialemonium (Sordariales, Cephalothecaceae) is commonly found in the environment and has been isolated from air, soil, sewage, and plants (Perdomo, García et al. 2013, Stępniewska, Uzarowicz et al. 2020). The same *P. inflatum* TAMU490 isolate tested here suppressed penetration, galling and reproduction of the root-knot nematode when applied to cotton as a seed treatment (Zhou, Wheeler et al. 2018) and affected the host selection behavior of sucking bugs by deterring them from cotton reproductive structures (Sword, Tessnow et al. 2017). When applied to cotton as a seed treatment, *P. inflatum* TAMU490 has also been shown to reduce cotton aphid fecundity and increase

aphid mortality when applied directly in dipping bioassays (Castillo Lopez, Zhu-Salzman et al. 2014). The current study confirms the ability of this strain to directly infect and kill cotton aphids as an entomopathogen.

Fungi can have multiple lifestyles, making it possible to be either a plant symbiont or an entomopathogen. One study exploited the bifunctional lifestyle of *Metarhizium* fungi as insect pathogens and plant symbionts to analyze the evolution of adhesins to determine whether plant or insect association drove diversification in the genus. Their results suggested that *Metarhizium* relationships with plants were more likely to have driven divergence than insect hosts (insect pathogenicity) (Wyrebek and Bidochka 2013). The colonization of both insects and plants by fungal hyphae can have different ecological effects. Using radioactive isotopes, one study showed that *M. robertsii* could take nitrogen from insect tissue and transfer it to the plant, and the plant moves carbon to the fungus as part of a 3-way symbiosis (Behie, Zelisko et al. 2012, Behie, Moreira et al. 2017, Branine, Bazzicalupo et al. 2019). Many entomopathogenic fungi can invade the insect cuticle through the use of enzymes and mechanical pressure (Barelli, Moonjely et al. 2016). For example, *M. robertsii* uses adhesins (MAD1 – insect and MAD2 – plant) to colonize host tissue, proteases are used for penetration (degrading insect cuticle and plant cell wall), and it then establishes and proliferates inside the host (Wang and St Leger 2007, Barelli, Moonjely et al. 2016). These studies illustrate the similar yet distinct mechanisms underlying how the fungus can interact with both plants and insects as hosts.

3.4.1. Differences in survival time across bioassays

This chapter evaluated the survival of 2nd instar cotton aphids in three bioassays (dipping, spray-plant, and spray-plate) with five fungi strains and control (water). We hypothesized that the fungi isolates would decrease the aphid survival in all the bioassays. Although the pathogenicity of all the isolates tested was observed in all the bioassays, we did find differences in survival across the bioassays. In dipping bioassays, we found a faster reduction in survival across all concentrations and fungal treatments. The dipping assay was also the only assay in which significant differences in survival from control were observed for all fungi at all of the tested concentrations.

In contrast, we found longer survival times in the spray (plant) and spray (plate) bioassays, with the spray (plant) having the highest survival of all bioassays. We suggest that the increased efficacy observed in the dipping assays is because the entire aphid body surface has a higher chance of contacting the spores leading to subsequent infection than in the spray bioassays. The spray assays depended on the fungal spores reaching the aphid body from the spray mist, with the dorsal surface of the insect likely receiving the most inoculum.

Previous studies involving immersion bioassays of *M. persicae* aphids on leaves dipped in filtrates, and conidial suspensions of *B. bassiana* and *L. lecanii* led to high mortality (Javed, Javed et al. 2019). Aphid dipping bioassays in conidial suspensions and keeping the insects in Petri dishes were used to show mortality differences across more or less virulent *B. bassiana* isolates (Todorova, Coderre et al. 2000). In the current study, the fungal treatments in the spray (plate) assays showed significant differences in

survival at almost all concentrations, but survival using the same treatments in spray (plant) bioassay were not significantly different from control. However, the fungal treatments in spray (plant) bioassay showed some decrease in survival, and the absence of statistical significance could be due to the smaller sample size (low power). The large differences in survival between the spray (plant) and spray (Petri dish) assays could be related to differences in the humidity experienced by the aphids during the 14 days after spray inoculation. Despite the incubator-controlled temperature and humidity (~25 ºC and RH ~60%), the sealed plates could be an environment with higher moisture resulting in aphid higher mortality than on the plants. High humidity is known to promote spore germination and increases fungal virulence against herbivores (Sivasankaran, Easwaramoorthy et al. 1998, Mishra, Kumar et al. 2015). In cadavers of the desert locust, entomopathogen conidial sporulation increased proportionally to relative humidity with an optimal RH of 96% (Arthurs and Thomas 2001). However, in a study with *L. lecanii* and the oat-bird berry aphid, the sporulation on apterous cadavers did not differ between 76% and 100% RH (Hsiao, Bidochka et al. 1992). In palm weevil, using a solid formulation of *B. bassiana* with insects maintained in Petri dishes or simulated field conditions on plants showed some survival fluctuations according to the month of the year in plant bioassays and lower survival time in Petri dishes (Ricaño, Güerri-Agulló et al. 2013). Importantly, since we did not find significant differences between the survival time of the untreated control insects in the spray (plant) and spray (plate) assays, we can discard the possibility of a handling effect accounting for the differences in the efficacy of the fungal treatments between the two similar assays.

3.4.2. Sporulation on aphid cadavers

We recorded mycosis from sterilized cotton aphid cadavers to determine if we could recover the same fungus with which they had been inoculated. We expected to recover the fungus after infecting the insects, completing the fungal life cycle and sporulating from the cadaver, thereby confirming the pathogenicity of the isolates tested. Following inoculation, we observed mycosis with all of the fungal isolates tested, with the highest frequency of confirmed mycosis events following exposure to the highest inoculum concentration (10^7 spore/ml), consistent with more spores reaching the aphids' bodies. A similar dose-dependent relationship between spore treatment concentration and the extent of mycosis has been observed in other studies (Iwanicki, Mascarin et al. 2021). In dipping bioassays, we found that *C. globosum* TAMU559 and *A. alternatum* 505 had the highest mycosis percentage compared to the other fungal treatments. *B. bassiana,* the well-known entomopathogen, had the lowest percentage in the dipping bioassay but had the highest in spray (plant) and spray (plate) bioassays.

Some strains with the highest virulence across all bioassays, *B. bassiana*, *C. globosum* TAMU559 and *P. inflatum*, also had the highest mycosis percentage. In a study testing the virulence of various strains of *Beauveria* spp. and *M. anisopliae* in the Eucalyptus snout beetle, they also found variation in mycosis frequency among fungal strains and observed more mycosis on the most virulent strains (Jordan, dos Santos et al. 2021). In some cases, for some isolates, the frequency of mycosis from the sterilized cadavers had low mycosis confirmation as has been observed in other studies (Anderson,

McGee et al. 2007), but it did not systematically differ between the different strains or bioassays.

3.5. Conclusion

This study confirms that several strains of plant-associated fungi originally isolated from cotton can act as pathogens that directly affect the survival of cotton aphids. Aphid mortality was observed following topical application of spores in both dipping and spray bioassays, even when applied in low concentrations. Levels of mortality of all the plant-associated fungal isolated in all the assays were on par with mortality caused by *B. bassiana,* an established and widely-used entomopathogenic biological control agent. In dipping bioassays, fungal treatments significantly reduced aphid survival time across all treatments and all concentrations. The efficacy of topical sprays for all the fungal strains depended on the environmental condition the aphids were exposed to the following inoculation. In the spray (plate) bioassays in which the insects were maintained in Petri dishes with higher humidity, survival was significantly lower than controls in the highest spore concentration treatments for all the isolates testes. Survival was not significantly lower in the spray (plant) bioassay with the insects maintained on plants in open-air cages, but some strains still tended to decrease aphid survival. Thus, the plant-associated fungi species *C. globosum* strains TAMU520 and TAMU559 and *P. inflatum* tended to decrease aphid survival in spray (plant) bioassay, and therefore have potential for use as novel aphid biocontrol tools.

Post-mortem production of spores (mycosis) is crucial to understanding disease establishment and pathogenicity. Following topical application, we confirmed mycosis

from the aphid cadavers for all of the fungal isolates tested, indicating novel insect pathogenicity from plant-associated fungi strains not typically considered direct insect pathogens. Since we clearly found established pathogenicity of these fungal strains in the cotton aphid, future studies should investigate the effects of the same fungal isolates on other herbivores' survival along with testing their efficacy against pests in the field. Additionally, much more information is needed about these strains' virulence under different temperature and relative humidity combinations to better understand the potential for fungal infections with these isolates under field conditions and whether they hold potential as novel naturally-occurring tools for insect IPM.

4. CONVERGENT LADY BEETLE (COLEOPTERA: COCCINELLIDAE) BEHAVIORAL RESPONSES TO OLFACTORY CUES FROM APHID-INFESTED COTTON PLANTS TREATED WITH PLANT-ASSOCIATED FUNGI

4.1. Introduction

A phytobiome is the association between plants, the environment, and micro- and macroscopic organisms influencing plant growth, health, and productivity (Hawkes and Connor 2017, Leach, Triplett et al. 2017). A wide variety of studies have shown that plant-associated microbes, including fungi, can enhance plant resistance or tolerance against biotic and abiotic stressors such as insect herbivores, pathogens, plant-parasitic nematodes, drought, and heat (Gao, Dai et al. 2010, Porras-Alfaro and Bayman 2011, Castillo Lopez, Zhu-Salzman et al. 2014, Hubbard, Germida et al. 2014, Lopez and Sword 2015, Zhou, Starr et al. 2016, Sword, Tessnow et al. 2017, Bamisile, Dash et al. 2018, Zhou, Wheeler et al. 2018, Dini-Andreote 2020, Zhou, Verma et al. 2020).

Many plant-associated microbes can induce plant host defenses through Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) (Ownley, Griffin et al. 2008, Porras-Alfaro and Bayman 2011, Shikano, Rosa et al. 2017). For example, a laboratory study inoculating maize seeds with an endophytic fungus showed the fungus promoted plant growth, altered the expression of defensive genes belonging to the JA (jasmonic acid) pathway, and suppressed herbivore larvae growth rate (Ahmad, Jiménez-Gasco et al. 2020). Moreover, microbes can affect the production of various chemicals by the plant, including volatile organic compounds (VOCs), thereby modifying plant

defenses (Newcombe, Shipunov et al. 2009, Davis, Crippen et al. 2013, Wani, Ashraf et al. 2015). These altered volatile profiles can affect herbivore host-selection behavior (Daisy, Strobel et al. 2002, Jallow, Dugassa-Gobena et al. 2008, Rostás, Cripps et al. 2015). Consequently, these changes in volatile chemical bouquets could also affect the attraction of natural enemies, such as predators and parasitoids (McCormick, Unsicker et al. 2012, Xiao, Wang et al. 2012).

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a well-known pest that can cause severe economic losses in cotton fields (Kersting, Satar et al. 1999, Lu, Wu et al. 2012). Some plant-associated fungi have been shown to negatively affect cotton aphid reproduction and alter feeding behavior (Gurulingappa, Sword et al. 2010, Castillo Lopez, Zhu-Salzman et al. 2014, Zhou, Starr et al. 2016, Gonzalez-Mas, Quesada-Moraga et al. 2019). Diverse species in the family Coccinellidae (Coleoptera) are voracious aphid predators (Hodek and Evans 2012) and agriculturally valuable biological control agents (Koch and Costamagna 2017, Riddick 2017). One example is the generalist predator *Hippodamia convergens* (Guérin-Méneville), commonly known as the convergent lady beetle. This aphidophagous species is found broadly across the Western Hemisphere (Flint and Dreistadt 2005). Due to their predaceous habit and distribution among crops attacked by aphids, this species is often considered an essential part of many agroecosystems as an essential biological control agent (Bjørnson 2008). However, most studies assessing the effects of plant-associated fungi on lady beetles have only been limited to grasses (de Sassi, Muller et al. 2006, Fuchs, Krischke et al.

2013, Saari, Richter et al. 2014) despite its presence in many other crops, including cotton (Prasifka, Heinz et al. 2004, Bastola, Parajulee et al. 2016).

In order to better manage insect pests, we may be able to manipulate a plant's phytobiome to increase the efficiency of natural enemies (Miranda 2010, Jaber and Araj 2018). Knowledge about pests and their natural enemies is crucial for developing and implementing sustainable pest management strategies in cotton (Miranda 2010). Cotton plants treated with some beneficial plant-associated fungi have previously been shown to negatively affect aphid reproduction (Castillo Lopez, Zhu-Salzman et al. 2014, Zhou, Starr et al. 2016). However, whether these plant-associated fungi might also affect the behavior of an aphid predator in a multitrophic interaction has not been investigated to date. As such, the goal of this study was to investigate the effects of plant-associated fungi applied to cotton plants on convergent lady beetle behavior.

4.2. Materials and Methods

4.2.1. Fungal treatment of cotton seeds

Chemically untreated *Gossypium hirsutum* seeds of the non-transgenic variety LA122 were obtained from All-Tex Seed Inc., Levelland, TX. The fungal strains used were *Phialemonium inflatum* (TAMU490) and *Chaetomium globosum* (TAMU520), which were first isolated as endophytes from surface-sterilized cultivated cotton as part of a field survey in Texas, USA (Ek-Ramos, Zhou et al. 2013). The fungal inoculum for all trials was cultured in 100 x 15 mm Petri dishes on potato dextrose agar (PDA) in the dark at 25°C. Spore suspensions of each fungus were made by adding 2 ml of 0.1%

Triton X-100 solution to the fungal conidia plates, scraping them with a sterile metal spatula, filtering through autoclaved 0.25 mm sieves into a sterile beaker, and placing them in 50 ml centrifuge tubes (Sword, Tessnow et al. 2017). The suspensions were mixed on a vortex and then centrifuged for 10 minutes in a Cole-Parmer Fixed-speed Centrifuge at 3000 rpm. Excess water was removed by pouring out the supernatant. We used a Neubauer hemocytometer (Thomas Scientific, Philadelphia, PA, USA) to quantify the spores' concentration. Final treatment concentrations were diluted with sterile water to reach 1 x 10^8 spores/ml (Sword, Tessnow et al. 2017).

Cotton seeds were surface sterilized by immersion in 3% sodium hypochlorite (NaOCl) for 3 min, and 70% ethanol for 2 min, followed by three rinses in sterile water (Posada, Aime et al. 2007). Before applying the fungal treatment, surface-sterilized seeds were dried on sterile paper towels for 30 min. The seeds were inoculated with spore suspensions (approximately 200 seeds/1 ml) plus 1 ml of a 2% methylcellulose sticker to bind the spores to the seeds. We treated the control seeds with 1 ml of 2% methylcellulose only. Treated seeds were dried for at least three hours after inoculation before planting. Five treated seeds per treatment were plated in Petri dishes containing PDA to confirm inoculation with viable fungi (Sword, Tessnow et al. 2017). Three seeds per treatment were planted in 515ml pots with unsterilized Metro mix 900 soil (Borlaug Institute, Texas A&M University). For the duration of the experiment, all plants were grown in a greenhouse at approx. 25°C with natural photoperiod. Pots were randomized and watered as needed at least once per week.

4.2.2. Insect rearing and experimental design

Using a dual-choice Y-tube olfactometer, we assessed lady beetle behavioral responses to olfactory stimuli emitted by cotton plants grown from seeds treated with plant-associated fungi (described below). Third true-leaf plants from each fungus and untreated control treatments were infested two weeks before the trials with cotton aphids from a colony maintained in the Sword Lab at Texas A&M University to prepare aphid-infested plants for the behavioral assays. A total of 18 infested plants per treatment were maintained inside multiple insect mesh cages and housed in a greenhouse at 25°C with natural light. Plants from all three treatment groups that were not infested with cotton aphids were maintained in the same environmental conditions as the infested plants.

Convergent lady beetle, *Hippodamia convergens*, adults were obtained from ARBICO Organics® (Oro Valley, AZ) originally collected from overwintering aggregations in California, USA (Rodrigues, Ruberson et al. 2013, Barbosa, Michaud et al. 2016). Prior to use in the trials, the beetles were sexed and maintained in reproductive diapause in 44ml plastic cups at 3°C (Michaud and Qureshi 2005, Michaud and Qureshi 2006). We placed ten individuals per cup and arranged the cups randomly on trays inside an incubator at 25°C, 50–60% RH, and 16:8 L:D photoperiod (Barbosa, Michaud et al. 2016). Convergent lady beetles were also field collected from sorghum plants at the Texas A&M AgriLife Research Farm in Burleson County, TX, randomized and maintained under the same conditions.

Adult lady beetles were starved ~24 hours before the behavior assays (Schaller and Nentwig 2000, Adedipe and Park 2010, Xiu, Xu et al. 2019). Because the lady

beetles came from two different sources, we conducted a control experiment to test for differences between the commercial and wild-caught individuals in their preference for stimuli from cotton plants with and without aphids in the absence of any fungal treatments. No difference was observed (see Results). We then tested for the effects of fungal cotton seed treatments on lady beetle behavior by conducting choice tests between stimuli from one untreated control versus one fungal-treated plant (TAMU490 or TAMU 520). We conducted this comparison between untreated and fungal-treated plants using plants that were either aphid-free or infested with aphids in two separate series of trials. We used a total of 960 adults, 120 for the initial comparison between commercial and wild-caught populations (60 males and 60 females per population), 120 for the untreated control aphid-infested and non-infested plants, 240 in each comparison of fungal-treated plants aphid-infested and non-infested plants, and 240 in each of the two separate comparisons between fungal-treated and untreated plants in either the presence or absence of aphids. After every five individuals, the plants' position in the olfactometer was changed, alternating between the 30 individuals. Thus, 30 females and 30 males had one treatment on the left side, plus 30 females and 30 males with the same treatment on the right side. 10 females and 10 males had the same plant in both positions then, new plants were added. Each adult was tested only once and discarded after the experiment.

4.2.3. Y-tube olfactometer

The olfactometer consisted of a Y-shaped glass tube with a trunk measuring 15.2 cm and each arm 12.7 cm (Figure 4.1). Two 2 L mason jars were attached to the outside as chambers for each plant. The jars' lids were sealed inside with clay to avoid air escaping. A filter was connected in series to a water bubbler to humidify the incoming air pulled from a DOA Series Oilless Diaphragm Vacuum Pump (Thomas Scientific). The filters were attached to silicone tubes, and the flow was measured with an Acrylic Flowmeter (Cole-Parmer Scientific Experts, Illinois, USA). The olfactometer was positioned horizontally on a countertop (Borges, Millar et al. 2007, Magalhães, Borges et al. 2012) inside a dark room. The light source came from a flexible LED strip light equidistantly placed to provide uniform light to both arms of the olfactometer. Carbon filtered humidified air was pumped in at ~2.0 L min^{-1}, and a single adult convergent lady beetle was introduced at the base of the Y-tube olfactometer.

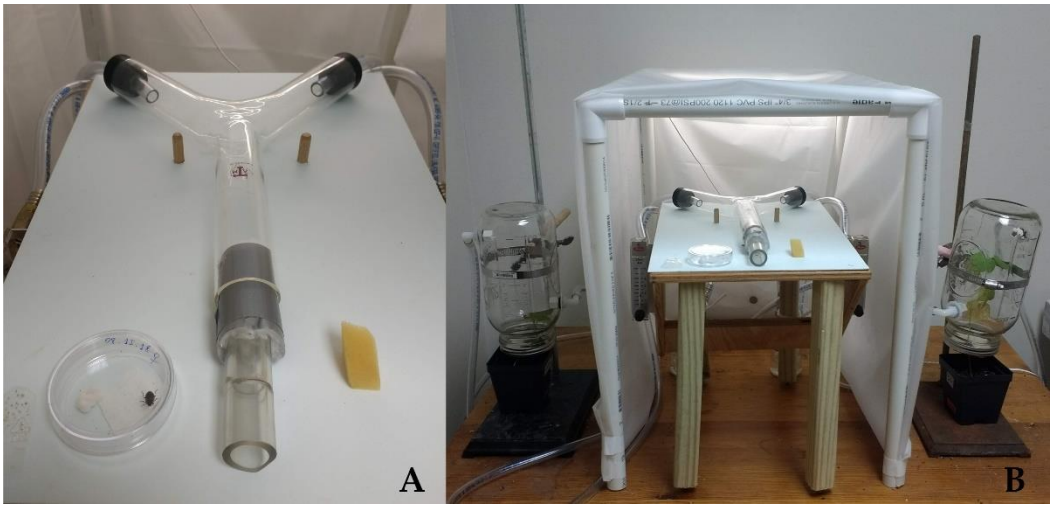

Figure 4.1 Y-shaped glass tube with the acclimation chamber (A) and the entire olfactometer setup showing the plant chambers (B).

After five individuals were tested, we changed the Y-tube, the jars, and the treatment sides to avoid positional bias (Magalhaes, Borges et al. 2018, Magalhães, Borges et al. 2018). Jars were cleaned with fragrance-free soap, rinsed with water, and dried in an oven at 80°C to sterilize and avoid residuals from the previous treatment (Borges, Millar et al. 2007). Adult lady beetles were gently introduced into the release chamber with a #2/0 Daler-Rowney paintbrush and allowed to acclimate for five minutes (Borges, Millar et al. 2007). Consistent with previous olfactometer studies, the insect had 10 minutes to choose between the different stimuli (Bahlai, Welsman et al. 2008, Choate and Lundgren 2013, Duffy, Hughes et al. 2018). We recorded the insect responses as a choice when they entered into one arm of the Y-tube and remained there for at least 20 seconds (Magalhães, Borges et al. 2012). Within the 10 minutes, we also recorded the first choice, latency (time to make a choice), and residence time (spent time in an arm) (Magalhaes, Borges et al. 2018, Magalhães, Borges et al. 2018). If an individual did not choose within five minutes, it was recorded as "no choice" and excluded from the statistical analysis (Rim, Uefune et al. 2018, Barloggio, Tamm et al. 2019, Xiu, Dai et al. 2019).

4.2.4. Statistical analysis

We recorded the number of responding lady beetles (females and males) and expressed it as a response ratio calculated as the number of individuals that chose a given treatment divided by the total number of individuals that selected either the treatment or control stimulus. The proportions of responding individuals yield a value between 0 and 1, with

1 representing 100% of individuals choosing the control stimulus versus the fungus (Martin, Bateson et al. 1993, Sword, Tessnow et al. 2017). We analyzed the proportions using Pearson's chi-squared test using the *chisq.test* function in *R* (R Core Team 2020), testing the null hypothesis that *H. convergens s*howed no preference for either arm, and the expected proportion was equal to 0.5 (Sokame, Ntiri et al. 2019, Xiu, Dai et al. 2019). The latency and residence time data were transformed to satisfy the assumptions of normality using log (x + 1) (Sarkar, Mukherjee et al. 2015) and compared the means of each sex between treatments using Welch's two-sample *t*-test using the *t.test* function in *R* (Magalhaes, Borges et al. 2018, Michereff, Magalhães et al. 2019, R Core Team 2020). We used linear model ANOVA using the *aov* function in the R program to analyze a difference between wild and commercial lady beetle responses. All analyses were done using R version 3.6.3 (R Core Team 2020) with a 5% significance level ($\alpha = 0.05$), and we used the ggplot2 package for graphs (Wickham 2016).

4.3. Results

4.4. Wild and commercial *H. convergens* responses comparison

We found no differences in the behavior of wild versus commercially obtained lady beetles in response to aphid-infested and non-infested cotton plants in the absence of any fungal treatments. The first-choice responses from *H. convergens* wild and commercial females and males were not significantly different ($F_{3, 4} = 3.576$, $p = 0.125$). Moreover, there was no significant difference in either latency ($F_{3, 89} = 2.01$, $p = 0.1182$) or residence time ($F_{3, 89} = 0.3669$, $p = 0.777$) between wild and commercial individuals of

both sexes. Since we did not find a significant difference between responses of wild and commercial *H. convergens*, their responses in subsequent experiments were pooled for analysis.

4.4.1. First choice

In the Y-tube olfactometer, *H. convergens* females did not show a significant preference for stimuli emitted by untreated cotton plants that were either infested or not infested with aphids. However, when the females were exposed to stimuli from *P. inflatum* fungal-treated plants with or without aphids, they preferred stimuli from non-infested plants significantly more often. There were no significant differences in the first choices between stimuli from plants that had been treated with either fungus versus untreated control plants, regardless of whether the plants were infested or not with aphids (Table 4.1, Figure 4.2). In contrast, *H. convergens* males did show a significant preference for aphid-infested plants over non-infested plants in the absence of any fungal treatments, but the fungal treatments did not affect their responses regardless of whether aphids were present or absent (Table 4.1, Figure 4.2).

Table 4.1 Statistical analyses of the first choice, latency, and residence time in seconds for female and male *Hippodamia convergens*. Tests were conducted in a Y-tube olfactometer providing individuals with a choice between stimuli emitted by fungal-treated or untreated cotton plants in the presence or absence of aphids. Sample sizes for each comparison were N=60 for each sex. * $p \leq 0.05$

	Pearson's chi-squared test First choice				**Welch's Two-Sample *t*-test Latency time (sec)**				**Welch's Two-Sample *t*-test Residence time (sec)**				**Nonresponding individuals**	
	♂		♀		♂		♀		♂		♀		♂	♀
	χ^2	*p*	χ^2	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*		
Fungus absent														
Aphids vs. No aphids	4.57	**0.03***	1.77	0.18	1.18	0.26	1.44	0.17	-1.98	0.07	2.13	**0.05***	23	24
C. globosum														
Aphids vs. No aphids	2.08	0.15	0	1	0.78	0.45	-0.45	0.66	1.04	0.32	-0.23	0.82	21	16
P. inflatum														
Aphids vs. No aphids	3.27	0.07	4.92	**0.02***	0.74	0.48	-1.44	0.17	-0.03	0.98	-0.39	0.69	23	8
Fungus present/Aphids absent														
C. globosum vs. control	1.12	0.29	0.23	0.63	1.7	0.1	-1.38	0.19	-0.6	0.55	1.14	0.27	28	21
P. inflatum vs. control	0	1	0.23	0.63	2.56	**0.02***	-0.11	0.91	-1.9	0.07	-0.01	0.1	38	21
Fungus present/Aphids present														
C. globosum vs. control	0.53	0.47	2.27	0.13	-0.79	0.43	-1.13	0.28	-1.15	0.26	1.35	0.20	8	11
P. inflatum vs. control	0	1	2.08	0.15	-1.07	0.29	-0.97	0.35	-1.01	0.32	1.17	0.25	8	12

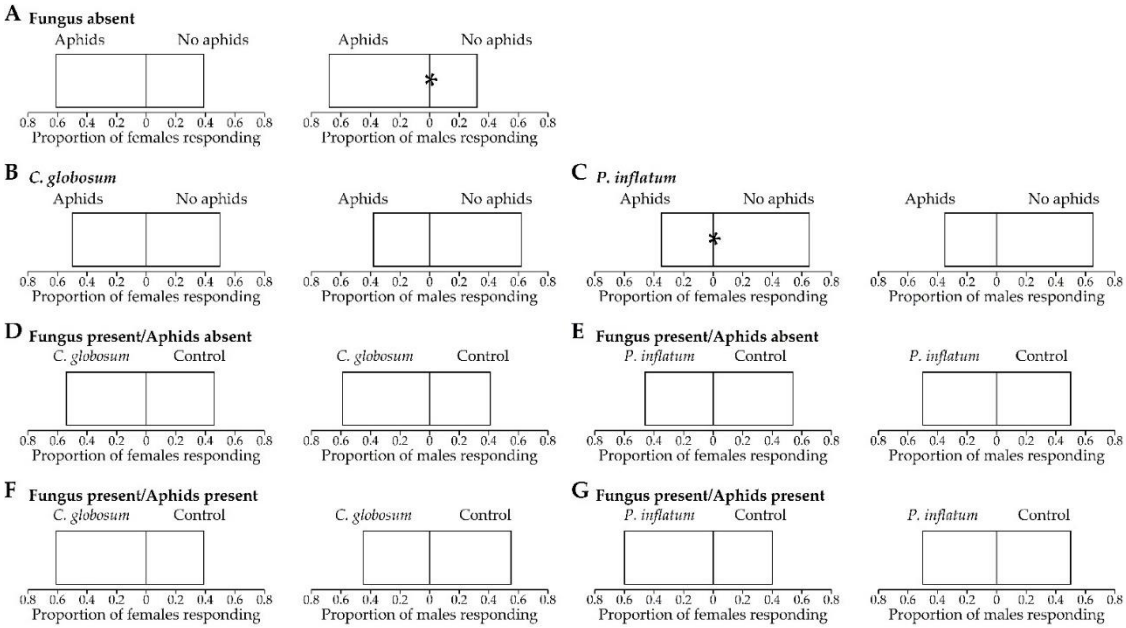

Figure 4.2. Proportion of *Hippodamia convergens* females and males responding to untreated control and fungal-treated (*Chaetomium globosum* and *Phialemonium inflatum*) cotton plants in a dual-choice Y-tube olfactometer. (A, B, C) Untreated plants, *C. globosum,* and *P. inflatum* treated plants with aphids vs. no aphids, respectively. (D, E) Fungal-treated plants vs. untreated plants, both without aphids. (F, G) Fungal-treated plants vs. untreated plants, both with aphids. Each individual had 300 seconds (five minutes) to make a choice, and the beetles that did not respond were not included in the analysis. * $p < 0.05$ (Pearson's chi-squared test).

4.4.2. Latency to first choice

For the *H. convergens* females, no significant differences were found in the latency to their first choice among any treatment pairs (Table 4.1, Figure 4.3). However, the males exhibited a significant difference in the absence of aphids in cotton plants treated with *P. inflatum*, taking more time to choose the stimuli emitted by the fungal-treated plants relative to untreated control plants. No other significant differences in latency to the first choice were observed in any other treatment comparisons (Table 4.1, Figure 4.3).

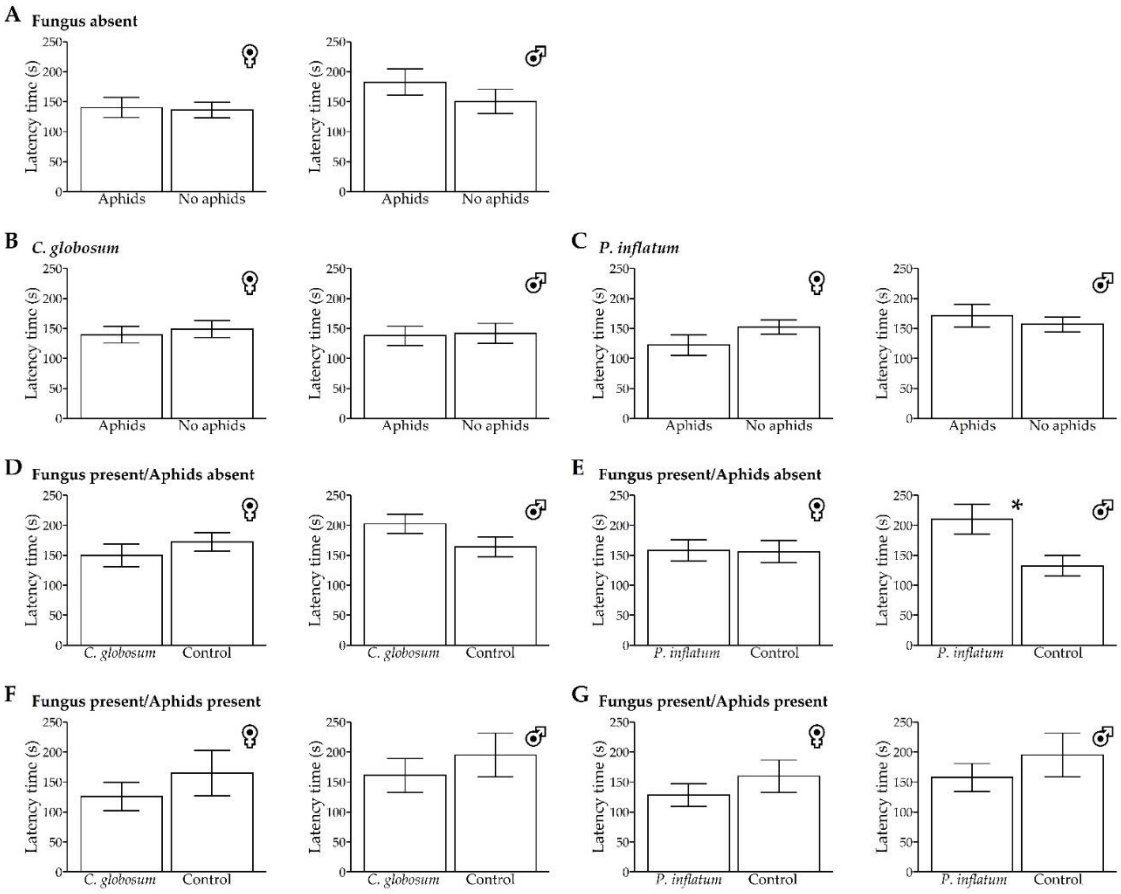

Figure 4.3. Means (±SE) of *Hippodamia convergens* female and male latency (seconds) to make a choice between olfactory stimuli emitted from untreated control and fungal-treated (*Chaetomium globosum* and *Phialemonium inflatum*) cotton plants in a dual-choice Y-tube olfactometer. (A, B, C) Untreated plants, *C. globosum,* and *P. inflatum* treated plants with aphids vs. no aphids, respectively. (D, E) Fungal-treated plants vs. untreated plants, both without aphids. (F, G) Fungal-treated plants vs. untreated plants, both with aphids. Each individual had 300 seconds (five minutes) to make a choice, and the beetles that did not respond were not included in the analysis. * $p < 0.05$ (Welch's two-sample *t*-test).

4.4.3. Residence time

In the absence of any fungal treatments, *H. convergens* females spent more time in association with the stimuli emitted by aphid-infested plants, whereas the response of

males was not significantly different. Fungal treatments had no effects on the residence time of the insects in response to stimuli aphid-infested versus non-infested plans, nor were there any differences between fungal-treated and untreated plants, regardless of whether aphids were present or absent (Table 4.1, Figure 4.4).

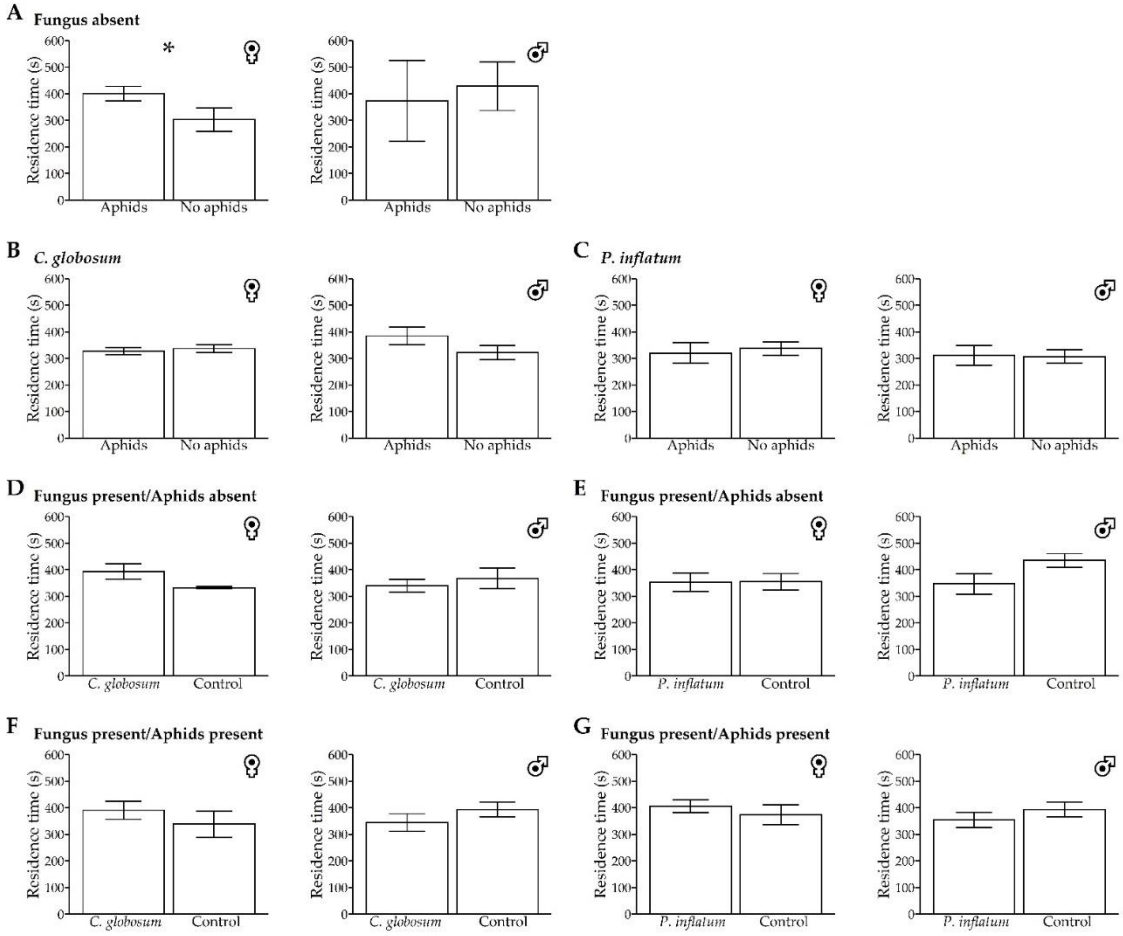

Figure 4.4. Means (±SE) of *Hippodamia convergens* female and male residence times (seconds) associated with olfactory stimuli emitted from untreated control and fungal-treated (*Chaetomium globosum* and *Phialemonium inflatum*) cotton plants in a dual-choice Y-tube olfactometer. (A, B, C) Untreated plants, *C. globosum,* and *P. inflatum* treated plants with aphids vs. no aphids, respectively. (D, E) Fungal-treated plants vs. untreated plants, both without aphids. (F, G) Fungal-treated plants vs. untreated plants, both with aphids. Each individual had 600 seconds (10 minutes) to stay in the Y-tube arm, and the beetles that did not respond were not included in the analysis. * $p < 0.05$ (Welch's two-sample t-test).

4.5. Discussion

Commonly, aphid-damaged plants are more attractive to lady beetles than non-infested plants (Obrycki, Harwood et al. 2009, Norkute, Olsson et al. 2020). *H. convergens* have previously been shown to be strongly attracted to the odor emitted by plants infested with aphids (Elliott, Kieckhefer et al. 2011) and to the aphid alarm pheromone (Verheggen, Fagel et al. 2007). Other predatory beetle species have shown a similar preference for plants infested with aphids, such as *Coleomegilla maculata* (De Geer) females that significantly preferred fava bean plants infested with pea aphids (Choate and Lundgren 2013). Moreover, some piercing-sucking insects, such as aphids, induce salicylic acid (SA) signaling mediated by feeding, triggering systemic acquired resistance (SAR) in the plant. SAR is primarily thought of as a defense against plant pathogens and can involve plant volatiles (Sloggett, Magro et al. 2011, Leroy, Schillings et al. 2012, Song and Ryu 2013). Thus, herbivore-induced plant volatiles (HIPVs) and semiochemicals from aphids are potentially available in the environment as olfactory cues for predators and have been shown to affect electroantennogram activity, foraging behavior, and attractiveness of prey to coccinellids (Guerrieri, Lingua et al. 2004, Verheggen, Fagel et al. 2007, Schausberger, Peneder et al. 2012, Fuchs and Krauss 2019, González-Mas, Cuenca-Medina et al. 2019).

For our first hypothesis, we expected *H. convergens* females and males would prefer olfactory stimuli from aphid-infested cotton plants in the absence of any fungal treatments. We partially supported this hypothesis because only the males showed a clear first choice for plants infested with aphids. The females did not exhibit the first-

choice preference in these trials. We expected that both males and females would have lower latency times and higher residence times associated with stimuli from aphid-infested plants in the absence of fungal treatments. However, we did not find a significant difference in latency for either males or females. For residence time, the females spent more time associated with stimuli emitted by aphid-infested plants, but there was no effect on male residence time.

H. convergens is a generalist predator, but aphids are its primary food source, and the presence of cotton aphids can increase convergent lady beetle feeding and egg viability (Prasifka, Heinz et al. 2004, Obrycki, Harwood et al. 2009). Thus, we expected that males and females would have a higher residence time associated with stimuli from aphid-infested plants, but only females showed this pattern in untreated control plants. *Coccinella septempunctata* females showed a higher attractiveness to aphid-infested plants (Norkute, Olsson et al. 2020), which could explain the higher residence time for females. Elliott, Kieckhefer et al. (2011) found that the high density of aphids influenced the foraging behavior of the convergent lady beetle with increased residence time in both females and males. The possible explanation for this attractiveness was the influence of chemicals (e.g., volatile sesquiterpenes and alkaloids) on prey and habitat location (Verheggen, Fagel et al. 2007, Sloggett, Magro et al. 2011). Both alarm and sexual pheromones of aphids attract the Asian lady beetle *Harmonia axyridis* (Pallas), indicating that these components influence the beetles' behavioral responses (Leroy, Schillings et al. 2012). Thus, the preference for aphid-infested plants could be related to the difference in the volatile blends from damaged plants.

For the second hypothesis, we predicted that the fungal treatment of cotton plants would affect the beetles' behavioral responses. The only two significant behavioral responses to fungal treatments observed in these assays involved *P. inflatum*-treated plants. First, females initially chose stimuli from *P. inflatum* treated plants that were not infested with aphids over those that were infested. Secondly, in the absence of any aphids, males took longer to respond to stimuli from *P. inflatum*-treated versus untreated control plants. For the first choice, neither females nor males preferred stimuli from untreated control cotton plants versus those treated with either *C. globosum* or *P. inflatum* regardless of whether aphids were present or absent. We also did not find any significant differences in the residence times of either males or females in the presence or absence of aphids.

We initially predicted the lady beetles would prefer fungal-treated plants if the VOCs emitted by these plants acted as a cue for host finding that increased plant attractiveness to natural enemies (Song and Ryu 2013). Plant-associated fungi have previously been shown to cause plants to emit different volatile organic compounds (VOCs) profiles, that by attracting natural enemies, can indirectly act as a plant defense mechanism (Guerrieri, Lingua et al. 2004, Schausberger, Peneder et al. 2012, Fuchs and Krauss 2019, González-Mas, Cuenca-Medina et al. 2019). Other predators, such as *Chrysoperla carnea* (Stephens), preferred feeding on cotton aphids when plants had been treated with fungi (González-Mas, Cuenca-Medina et al. 2019). One possibility for the observation of males taking more time to respond to stimuli from fungal-treated plants in the absence of aphids is that the stimuli from the fungal-treated plants repelled

them. The well-known fungus-plant complex *Neotyphodium lolii* and *Lolium perenne* showed adverse effects on the aphidophagous *C. septempunctata* fed on cereal aphids, extending larval development, reducing survival and adult fecundity, and reducing the reproductive performance (de Sassi, Muller et al. 2006) that could lead to a repellency behavior in the presence of fungal-treated plants. In the study conducted with the *Neotyphodium*-Arizona fescue complex and the bird cherry-oat aphids, *C. septempunctata* avoided feeding on aphids from plant hybrids with endophyte, showing a preference for other treatments (Saari, Richter et al. 2014). However, the lady beetles in our experiment had no prior experience with aphids fed on fungal-treated plants, so this seems unlikely as an explanation for the increased latency of males to respond to fungal-treated plants that we observed.

The lady beetle females tended to first select stimuli from *P. inflatum*-treated plants without aphids over stimuli from aphid-infested plants treated with the same fungus. Although we do not know the mechanism underlying this response in our experiments, some herbivores can use secondary metabolites resulting from microbe-plant associations to defend themselves against natural enemies (Kunkel and Grewal 2003, Kunkel, Grewal et al. 2004), making them avoid these plants. In parasitoids, some fungal endophytes can alter the plant alkaloids produced, affecting herbivore susceptibility to natural enemies (Bultman, McNeill et al. 2003). Some secondary parasitoids can be negatively influenced by endophytes reducing their lifespan, with experienced females learning to avoid hosts arising from the endophyte-aphid-primary parasitoid interaction (Härri, Krauss et al. 2008).

If the fungi played a significant role in natural enemy attraction, we would have expected more robust behavioral responses in *H. convergens* towards stimuli from fungal-treated plants, but we did not observe any strong evidence for this in our results. The only observed response to fungal treatment that might have implications for biological control was when plants were treated with *P. inflatum*, females first preferred stimuli from plants without aphids over those infested. If this same response occurred under field conditions, we might expect reduced lady beetle predation of aphids on *P. inflatum*-treated plants. However, this same strain of fungi has previously been shown to reduce aphid population growth on cotton when applied in the same manner (Castillo Lopez, Zhu-Salzman et al. 2014). Thus, although the potential exists for a negative trade-off of *P. inflatum* treatment in terms of predation, its impact on population dynamics in the field would be expected to be moderated by the direct adverse effects of the same fungal treatment on aphid reproduction. Notably, the same potential for a negative trade-off was not suggested in any of the trials involving *C. globosum* treatments, highlighting the taxonomic specificity in effects on the next trophic level and the need for more studies investigating the ecological consequences fungal treatments as an aphid control strategy in the field.

To the best of our knowledge, this is the first study assessing the effects of plant-associated fungi on the behavioral responses of convergent lady beetles. Despite the relatively minor effects observed across the experiments, we did observe some *H. convergens* responses associated with both aphid infestation and fungal treatments of cotton plants. For future work, these effects should be assessed under field conditions to

determine whether the attractiveness patterns observed here are different at the spatial scales found in agricultural ecosystems. Moreover, the responses of different species of lady beetles and other predators should be investigated to understand better whether the patterns we observed apply to predators in general or are specific responses of *H. convergens*. Expanding our understanding of natural enemies' responses to cotton treated with plant-associated fungi might improve our ability to utilize fungal treatments as part of IPM strategies.

5. CONCLUSIONS

A major issue facing crop production is the expense required to control agricultural pests. The overall goal of this dissertation research was to better understand the ecological roles of plant-associated fungi and their potential use as new tools to manage insect pests. My specific goals were to test for the effects of plant-associated fungi (1) on boll weevil behavior and development when applied to cotton as a seed treatment, (2) as entomopathogens when applied directly to cotton aphids, and (3) on convergent lady beetle behavior as part of a multitrophic plant-aphid-microbe system. Insights from these studies into the effects of fungal treatments relationships between insects and plants can help improve their use as part of an efficient strategy in insect pest management. Such as strategy based on naturally-occurring microorganisms can be easy to apply and more environmentally friendly, minimizing the impact of pests and promoting cotton sustainability, thereby helping the economy and the agroecosystem. In addition to benefits for insect management, this work also provided new insights into insect biology, ecology, and behavior as part of interactions between plants, microbes, and insects.

5.1. Plant-associated fungi as a strategy in boll weevil control

Growers heavily rely on chemical control for boll weevil management but developing new efficient techniques to avoid insecticide resistance and environmental degradation is essential. In addition to the well-known adverse effects of insecticides, boll weevil immature stages develop inside the cotton reproductive parts, decreasing insecticide

efficiency. Chapter 2 evaluated whether cotton plants grown from seeds treated with one of five different isolates of plant-associated fungi affected boll weevil survival, reproduction, and development in lab assays and greenhouse experiments.

The experiments showed that fungal treatments of cotton have the potential for use as a new strategy in IPM for boll weevil control because they can negatively affect the boll weevil's behavior, reproduction, or development. The fungal treated squares affected boll weevil behavior, making them avoid or, in some cases, prefer squares from treated plants. It can be questioned if attracting weevils to plants should be considered a good thing, but the results showed that even the fungal isolates that attracted boll weevil to squares had other negative on its biology. Fungal treatments could potentially be used as an "attract and kill" strategy in IPM.

With respect to boll weevil development and reproduction, a major effect of the plant-associated fungi was to extend weevil developmental time, consistent with the Slow Growth – High Mortality hypothesis. This extended developmental time would be expected to result in fewer generations per growing season and increase insect vulnerability to abiotic and/or biotic mortality factors due to prolonged growth. The fungal treatments also negatively affected boll weevil reproduction, with fewer larvae hatching and adults emerging from treated plants. This effect would also be expected to reduce population size in the field.

5.2. Entomopathogens and cotton aphid control

Chapter 3 tested if some plant-associated fungi can act as entomopathogens that directly affect cotton aphid survival. Aphids were topically treated with fungal spore solutions in six concentrations (10^2 to 10^7 spore/ml) using three different bioassays (dipping, spray–plant, and spray–plate bioassays). The fungal treatments reduced aphid survival in all the trials even when applied at low concentrations, but there were different survival times in the three methods. In dipping bioassays, all fungal strains in all concentrations strongly reduced the cotton aphid survival, with half of the population dying around 2.5 days after fungi topical application. For spray bioassays, all fungal treatments in the highest concentrations (10^5 to 10^7 spores/ml) negatively affected cotton aphid survival when the treated aphids were maintained in Petri dishes with relatively high humidity. However, the effects were not statistically significant when the treated aphids were maintained on plants in open-air cages. Nevertheless, some fungal strains tended to decrease cotton aphid survival in the same manner.

The same fungus applied to healthy aphids could subsequently be recovered from their cadavers, confirming their effects as entomopathogens. Mycosis from the cadavers following fungal treatment was confirmed regardless of whether the spores were applied in dipping or spray (in-plant and Petri dishes) bioassays. Notably, the plant-associated fungal isolates tested here that are not widely considered as insect pathogens completed the infection cycle and exhibited similar mortality levels as a well-known commercial biopesticide strain of *B. bassiana*. These results demonstrate the potential for developing new bioinsecticides from naturally-occurring fungal strains that are currently not available for aphid control.

5.3. Multitrophic associations including plant-associated fungi, cotton aphid and convergent lady beetle

The attraction of more natural enemies to cotton treated with plant-associated fungi could provide another way of reducing herbivore populations. Some fungal treatments are known to reduce cotton aphid reproduction. Thus, it was important to understand if these cotton aphids could send different olfactory cues when reared on those treated plants that decrease their fecundity. For that reason, Chapter 4 main goal was to investigate the effects of cotton aphids reared on fungal seed-treated plants on the behavior of convergent lady beetle in a y-tube olfactometer.

However, our results showed that reducing the aphid population when reared on fungal-treated plants did not strongly affect the generalist predator convergent lady beetle (next trophic level) attractiveness or repellency in this multitrophic system. We found some different behavior in males and females. Generally, males and females preferred aphid-infested plants in the absence of fungal treatments, but they had mixed responses in the presence of one fungal isolate in the presence or absence of aphids.

It is crucial to understand better the insect (herbivore and predator) ecology in a multitrophic system involving cotton plants and fungal treatments. Our results showed that despite both isolates that we used in this study previously reduced aphid reproduction, they had different effects on the next trophic level. One isolate did not affect the lady beetle behavior, and the other had different made the males respond slowly and the females preferring this isolate in the absence of aphids. However, these are not strong effects that would interfere with aphid control by this natural enemy (lady

beetle). Thus, knowing the consequences of a potentially beneficial microbe-plant-herbivore interaction at higher trophic levels is essential to fully understand how plant-associated fungi might function in the context of IPM.

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