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The dual nature of *Metarhizium*: integrating endophytic plant benefits and entomopathogenic pest control

Jéssica Letícia Abreu Martins
Doctor Scientiae

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Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Madelaine Venzon

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Jason M Schmidt

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Looking deeper through the telescope, you can see that your home is inside of you.
Jason Mraz

ABSTRACT

MARTINS, Jéssica Letícia Abreu, D.Sc., Universidade Federal de Viçosa, February, 2025. **The dual nature of *Metarhizium*: integrating endophytic plant benefits and entomopathogenic pest control**. Adviser: Madelaine Venzon. Co-advisers: Fernando Hercos Valicente, Simon Luke Elliot, Wânia dos Santos Neves and Jason M Schmidt.

Entomopathogenic fungi perform important functions in the ecosystem, being able to act as decomposers of organic matter, and plant growth promoters, in addition to their potential to control insects. Because they are highly diverse organisms, they can establish themselves in equally distinct systems and can occur naturally in soils in natural or highly managed environments, as is the case with crops. Considering the different compositions of the environment they inhabit, these microorganisms often need to adapt to stressful situations, especially in high temperatures and UV radiation. In this way, fungi that have multiple life cycles end up obtaining adaptive advantages, as they can remain in the environment for longer periods, effectively colonizing insects, associated with corpses, and even living in symbiosis with plants. Some of the classic examples of multifunctional organisms are fungi of the genus *Metarhizium*, which have already shown themselves capable of associating with plants from different cultures as endophytes. When this relationship occurs, these fungi become capable of manipulating direct and indirect defenses, promoting, for example, the production of secondary metabolites or even fungal toxins capable of reducing insect herbivory. It is even known that the fungus can relocate these defenses to specific organs of the plant, depending on the attack of the pests associated with them. Furthermore, there are still studies that explore the ability of these microorganisms to modulate the production of volatiles in plants, promoting attraction or repellency not only of herbivores but also of potential predators capable of controlling pests. In this work, we began to explore the fungal composition of soils from coffee cultivation systems in the Cerrado Mineiro, under diversified systems (i.e., with the insertion of multifunctional plants) and under conventional crops where monoculture was established. The isolates used for molecular characterization came from a pre-established system in 2019, with three samplings carried out until 2021. Although we did not find significant differences in the composition of fungal genera in both systems, we noticed that the dominant genus was *Metarhizium*, possibly due to its previously mentioned high adaptability characteristics. Therefore, we chose to evaluate in Chapter II the effect of the species *M. robertsii* and *M. brunneum* on the control of the coffee leaf miner (*Leucoptera coffeella*),

with their inoculation in coffee seeds treated with fungicides. We observed that these fungi were able to prolong the life cycle of insects, promoting a reduction in the total number of individuals. Furthermore, they also reduced the number of insects in the second generation, in addition to promoting the growth of coffee plants. Given these results, we also decided to evaluate in Chapter III the effects of this group of microorganisms on a hemimetabolous pest, a pest of a short-cycle crop. We then chose whiteflies (*Bemisia tabaci*) and cotton plants, and inoculated *M. anisopliae* by seed inoculation and also by drench in the soil. We observed a reduction in the number of insect eggs and adults when they fed on plants inoculated via seeds, in addition to a greater number of leaves in cotton plants that were subjected to the same treatment. To begin exploring higher trophic levels, we also carried out olfactometer choice experiments with the whitefly and its predator, the ladybug *Hippodamia convergens*. Our results demonstrated a repellency effect when subjected to the choice of plants inoculated via seed, and a predator attraction effect was observed with the same treatment. In this way, our work seeks to explore the potential benefits of using *Metarhizium* for sustainable pest management, considering its insect control potential and its endophytic action, which has also been shown to be beneficial for plants.

Keywords: entomopathogenic fungi; sustainable management ; biological control

RESUMO

MARTINS, Jéssica Letícia Abreu, D.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **A dupla natureza do *Metarhizium*: integrando os benefícios endofíticos às plantas e ao controle entomopatogênico de pragas**. Orientadora: Madelaine Venzon. Coorientadores: Fernando Hercos Valicente, Simon Luke Elliot, Wânia dos Santos Neves e Jason M Schmidt.

Os fungos entomopatogênicos desempenham importantes funções no ecossistema, podendo atuar como decompositores de matéria orgânica, promotores de crescimento de plantas, além do seu potencal de controle de insetos. Por se tratarem de organismos altamente diversos, eles conseguem se estabelecer em sistemas igualmente distintos, podendo ocorrer naturalmente em solos de ambientes naturais ou altamente manejados, como é o caso dos cultivos agrícolas. Considerando as diferentes composições do ambiente nos quais habitam, estes microrganismos muitas vezes precisam se adaptar a situações de estresse, especialmente no que diz respeito a temperatura e radiação UV. Desta forma, os fungos que possuem ciclos de vida múltiplos acabam obtendo vantagens adaptativas, pois são capazes de permanecer no ambiente por períodos mais longos, sejam efetivamente colonizando insetos, ou associados a cadáveres e até mesmo vivendo em simbiose com plantas. Alguns do clássicos exemplos de organismos multifuncionais são os fungos do gênero *Metarhizium*, os quais já se mostraram capazes de se associarem a plantas de diversas culturas como endofítico. Quando esta relação ocorre, estes fungos tornam-se capazes de manipularem defesas diretas e indiretas, promovendo, por exemplo, a produção de metabólitos secundários ou até mesmo toxinas fúngicas capazes de diminuírem a herbivoria de insetos. Sabe-se, inclusive, que o fungo é capaz de realocar estas defesas para órgãos específicos da planta, a depender do ataque das pragas a elas associadas. Além disso, ainda existem estudos que exploram a capacidade destes microrganismos de modularem a produção de voláteis nas plantas, promovendo atração ou repelência não só de herbívoros, mas também de potenciais predadores capazes de controlarem pragas. Neste trabalho, nós começamos a explorar a composição fúngica de solos de sistemas de cultivos de café do Cerrado Mineiro, sob sistemas diversificados (i.e com inserção de plantas multifuncionais) e de cultivos convencionais onde a monocultura foi estabelecida. Os isolados utilizados para a caracterização molecular foram provenientes de um sistema pré estabelecido em 2019, com três coletas realizadas até 2021. Embora não tenhamos encontrado diferenças significativas na composição de gêneros de fungos em ambos os sistemas,

nós percebemos que o gênero recuperado com maior frequência foi o *Metarhizium*, possivelmente por suas características de alta adaptabilidade anteriormente mencionadas. Desta forma, optamos por avaliar no Capítulo II o efeito das espécies *M. robertsii* e *M. brunneum* no controle do bicho-mineiro do cafeeiro (*Leucoptera coffeella*), com uma inoculação dos mesmos em sementes de café tratadas com fungicidas. Nós observamos que estes fungos foram capazes de prolongar o ciclo de vida dos insetos, promovendo uma redução no número total de indivíduos. Além disso, eles também reduziram o número de insetos na segunda geração, além de promoverem crescimento de plantas de café. Diante destes resultados, também avaliamos no Capítulo III os efeitos deste grupo de microrganismos em um inseto hemimetábolo, praga de uma cultura de ciclo curto. Escolhemos então a mosca-branca (*Bemisia tabaci*) e plantas de algodão, e realizamos a inoculação de *M. anisopliae* por inoculação de sementes e por drench no solo. Nós observamos uma redução no número de ovos e de adultos do inseto quando estes se alimentaram das plantas inoculadas via semente, além de um maior número de folhas em algodoeiros que foram submetidos ao mesmo tratamento. Para iniciar a exploração de níveis tróficos superiores, realizamos também experimentos de escolha em olfatômetro com a mosca-branca e com seu predador, a joaninha *Hippodamia convergens*. Nossos resultados demonstraram um efeito de repelência da mosca-branca quando submetida à escolha de plantas inoculadas via semente, e um efeito de atração do predador foi observado em relação ao mesmo tratamento.

Palavras-chave: fungos entomopatogênicos; manejo sustentável; controle biológico

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Chapter I

Chapter II

system fresh mass; (E) root system dry mass; and (F) shoot system dry mass. Bars with the same letters are not statistically different via the Tukey method ($p < 0.001$).

Chapter III

Figure 1: Representation of the percentage of fungi confirmed in each plant under seed and soil drench fungi inoculation. The control treatment represents plants untreated by fungi (i.e clean ones). The number in the bubbles represents the percentage of *Metarhizium* confirmation in each of the plant parts after DNA extraction

Figure 2: Number of *B. tabaci* eggs after fungi inoculation by SI (Seed Inoculation), SD (Soil Drench), compared to control (untreated seeds). $p<0.01$

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General introduction

Hypocrealean is an important group of entomopathogenic fungi, and the genera *Metarhizium* (Hypocreales: Clavicipitaceae) is one of the most common ones, naturally found in the soil, and it can occur all over the globe (Zimmerman, 2007, Quesada-Moraga et al., 2007). This microorganism has one of the most abundant lifestyles, capable of switching between saprophytic, endophytic, insect pathogens, and plant rhizosphere-associated (Behie & Bidochka, 2014; Hu & St. Leger, 2002; Liao et al., 2013). These fungi can be established in the environment in diverse ways aiming to stay alive and available to spread (Wang et al., 2011). Environmental conditions such as temperature, humidity, and host availability are normally related to inducing the fungi to switch between lifestyles (Inglis et al., 2001, Ment et al., 2010). This multifunctional action leads the studies in pest management to understand the potential and limitations of each fungi association, improving the options for exploring its relation (Roberts & St. Leger, 2004).

The ability to survive in variable conditions leads to a diversity of species of *Metarhizium* genus able to colonize many areas, even in the soil of crops (St. Leger & Wang, 2020). They normally need humidity and temperature conditions to stay alive and available to infect, and the diversification of the agricultural landscape can improve the likelihood of colonization (Hesketh et al., 2010; Hu & St. Leger; 2002). Nonetheless, *Metarhizium* genera can be considerably adaptive and change their metabolism to survive under extreme circumstances such as nutrition deficiency (Rangel et al. 2008; Shahid et al., 2012; Loong et al., 2013; Singh et al., 2017). In addition, studies show their capability to adapt to suboptimal temperature and UV exposure (Ekesi et al., 2003; Rangel et al., 2008; Liao et al., 2014). Together, given the environmental tolerances observed, the adaptability of *Metarhizium* shows continued potential for commercial purposes to improved insect control and plant protection even under variable or extreme environmental conditions (Lovett & St Leger, 2015).

As an entomopathogen, *Metarhizium* can kill by contact, and this use as a biopesticide is common (Kan et al., 2012; Perring et al., 2018). The fungi can access the insect hemolymph through the cuticle combining enzymes and mechanical pressure by appressoria structures, providing the fungi reproduction and posterior spread in the environment (Charnley 2003; Ortiz-Urquiza et al., 2013; Gul et al., 2014). One of the limitations of this process is related to the necessary time between infection and insect death, which is considered to be long. Besides, this process is considered one adaptative characteristic that permits the pathogen to absorb the maximum quantity of nutritional compounds from its host (Hajek & St Leger, 1994; Leger &

Wang, 2020). To improve the performance of these fungi in insect control, it is possible to use this genus as a plant inoculate, complementing the action and performing some advantages to the plant (Liao, 2014.

In an endophytic association, the fungi and plant are intrinsically related, which can induce a plant response to insect attack, for example, composing secondary metabolites or volatiles that could decrease insect feeding or even attract natural enemies (Peñaflor et al,., 2024; Pec et al., 2024). This interaction can also increase plant yield, once fungi can increase root volume and length, allowing even nitrogen absorption from dead insects to the plant (Kabaluk &, Ericsson, 2007; Sasan & Bidochka, 2012; Behie and Bidochka 2014). The effects of fungi inoculation on plants can be evaluated in perennial or annual crops and their performance in pest control is studied under different species. In coffee, some studies reported the influence of fungi association with the plants by seed inoculation and seedling drench in pest suppression (Franzin et al. 2022; Martins et al, 2024). In cotton, most studies evaluate insect control with spray applications of fungi (Malsam et al., 2002; Islam et al., 2016; Monga et al., 2019). Both methods are considered efficient and safe, but the choice between each of them depends especially on the available technology (Zimmerman, 2007).

To conduct my work, in Chapter I, we evaluate the presence of common fungal genera in the soil of contrasting coffee systems under conventional and diversified management. A living baiting protocol was used to recover all potential fungi from soil samples. In this work, we aimed not only to start the description of the genera found in our fields but also to start their potential exploration in future bio inputs development. As we found a considerable amount of *Metarhizium* genera and knew its capacity to infect and kill insects, we decided to conduct our experiments testing its pathogenicity in two different crops: coffee crops and cotton crops.

Chapter II was based on exploring the endophytic potential of *Metarhizium* when associated with a perennial crop: coffee. For this experiment, we decided to evaluate the efficiency of fungi seed inoculation in *Leucoptera coffeella* suppression. We also had the opportunity to evaluate the compatibility of the fungi with a fungicide commonly used on those seeds and to assess the effect of endophytic association in plant development, measuring plant growth parameters.

In Chapter III, to test the inoculation technique further, during my CAPES program at the University of Georgia, we replicated the methodology and inoculated cotton plants. To contract approaches again, we used seed and soil drench fungal inoculation treatments, and evaluate not only the effect of the endophytic association on an herbivorous pest genus species (commonly referred to as sweet potato whitefly) control and plant growth but also the influence

of the fungi association on plant communication with whiteflies and their predators to estimate fungal influences on higher trophic interactions.

Thus, this thesis aimed to clarify the potential and possibilities of integrated *Metarhizium* genera into cost-effective pest management solutions that could provide safer alternatives to synthetic chemistries and potentially synergize with to use of other biological control options in crop protection programs.

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Chapter I: Composition of fungi genera from the soil of coffee crops in the Brazilian Cerrado: an initial glimpse and its applications

Abstract

Hypocreales fungi are one of the most important groups of entomopathogens that naturally habitat the soil. Some of them, like *Metarhizium* genera, can control insects and act as endophytes in plants. This last lifestyle not only benefits the plant by improving its nutrient absorption but also provides a place for fungi to grow and stay available under stress conditions. This genus can also be associated with dead insects as a saprophytic one in the absence of a traditional host. Fungi normally need high humidity and UV protection to survive, but *Metarhizium* developed structural and molecular mechanisms to survive under extreme temperatures and solar radiation conditions. In this work, we evaluated the fungal composition in soil from coffee crops under conventional and diversified crop management. For the diversified ones, we insert between the coffee plants some multifunctional plants: *Inga edulis*, *Senna macranthera*, *Varronia curassavica,* and non-crop plants. We used fungi recovered from dead *Tenebrio molitor* baits and made a DNA extraction followed by PCR amplification. We made the identification of each of the isolates to the genus level. We found 10 different fungal genera in total, considering both areas. Our results didn't show a difference in the total number of isolates recovered between the two coffee areas, but *Metarhizium* was the most recovered one. Our studies demonstrated that *Metarhizium* genus is probably one of the most adaptable groups in coffee crops, and further investigations on the species level and long-term sampling evaluations can explain the relation between crop management and the plant species associated with coffee.

1.0 Introduction

When evaluating soil diversity and composition, fungi are among the most important microorganisms associated with these environments (Brown et al., 2020). These organisms play a crucial role in decomposition, promoting plant growth, and can also function as entomopathogens for insect control (Mendonça, 2005; Sassan & Bidochka, 2020; Singh et al., 2020). They are commonly found in the rhizosphere, an active zone surrounding plant roots, where root exudates enhance microbial activity (St Leger, 2008; Sasan and Bidochka, 2012). This process varies depending on plant species, soil composition, and crop management practices, which can either promote or suppress microbial abundance (Keller et al., 2003; Meyling, 2005; Frac et al., 2018; Botelho et al., 2019).

This zone, which encompasses fungi and crop cultures, is highly significant for plants, as these microorganisms can enhance nutrient availability by absorbing inorganic nutrients previously solubilized by fungi (Alam, 1999; Behie & Bidochka, 2014). Additionally, plants may receive protection from herbivore attacks through secondary compounds produced by entomopathogenic agents (Liu, & Tzeng, 2012;). In this relationship, microorganisms also gain advantages: they absorb plants' exudates and can persist for extended periods in association with plants (Govindarajulu, 2005; Fang & St. Leger; 2010). Due to their commercial and innovative potential, the organisms in these zones are extensively studied, particularly for their role in plant defense, plant growth, and insect control.

When considering the habitat structure of a cropping system, increasing plant diversity can enhance fungi abundance, as fungi rely on specific environmental conditions, such as high humidity and favorable temperatures for growth (Lovett & St. Leger, 2015). One effective strategy to promote these conditions is the introduction of plants into the environment to protect the soil and reduce exposure to extreme UV radiation (Lin, 2007). A diversified crop system improves soil protection by conserving moisture and maintaining more stable microclimatic conditions (Jose, 2009). In Brazil, coffee crops have been successfully diversified, with studies demonstrating the influence of agroforestry on fungi diversity and its potential for pest suppression (Moreira et al., 2018). Further exploring this relationship, research suggests that different fungi strains exhibit variable responses to temperature fluctuations, with each strain having an optimal range for development (Fargues et al., 1997; Inglis et al., 2001).

Nonetheless, some fungi genera can stabilize in the soil even under stress promoted by high temperatures and drought conditions. One of these is the *Metarhizium* genera, one of the most ubiquitous entomopathogenic fungi known (Fang et al., 2010). Some species in this group

are reported as capable of surviving in various temperatures, and some particularities related to their morphology and gene expression helped in this process (Freimoser et al., 2005). The greenish aspect of the conidia is related to high UV protection and also heat stress (Roberts and St. Leger, 2004). They also can change their genetic parameters, such as inducing HSP25 protein expression to increase fungal mycelia growth in high temperatures (Liao et al., 2014).

In this context, entomopathogenic fungi with multifunction lifestyles tend to be recovered more frequently than highly specialized species (Lovett & St Leger, 2014). Studies aimed at identifying new isolates for pest management focus on species capable of reproducing and remaining viable in the field for extended periods. In environments such as the Cerrado Mineiro, a vast tropical savannah in the state of Minas Gerais - Brazil, weather conditions typically include high temperatures and dry periods, which can present challenges for fungi survival. Under these conditions, fungi persistence in the soil depends on adaptations that enable survival in adverse environments (Qayyum et al., 2021). These adaptations are expected on entomopathogenic agent strains that effectively reduce pest populations below economic thresholds, either through direct contact or as an endophyte. If a bio-based input supports this dual capacity, the microorganism and its benefits will persist longer in the environment.

Considering this scenario, we aim to identify and quantify the most prominent genus of entomopathogenic fungi established in coffee crops in the Cerrado Mineiro region and compare their occurrence in conventional and diversified crop systems. These results will allow us to determine whether certain genres are more prevalent in both environments and facilitate the establishment of a fungi isolate bank for further exploration of bio-based inputs for pest management.

2.0 Material and methods

2.1 Coffee systems

The experimental area was established in Patrocinio - MG, at the Experimental Research Station of Agriculture and Livestock Research Enterprise of Minas Gerais (EPAMIG) (18º59’48’’S and 46º59’00’’W). This site served as the pilot area for diversified coffee system projects in Cerrado Mineiro, managed by EPAMIG’s Biological Control Laboratory. Tree soil sampling was conducted from 2019 to 2021, to determine the number of fungi species in the areas.

The sampling area consisted of three blocks in two plots - one conventional and one diversified coffee crop – each measuring 1,080 m² (30 m x 36 m) and separated by 200 m. In

the diversified plot, we introduced 4 plants of *Inga edulis*, 2 plants of *Senna macranthera*, and 12 plants of *Varronia. curassavica*, while spontaneous vegetation was maintained in intercrop lines. These plants were already studied as an important strategy to improve the biodiversity of beneficial insects in coffee crops (Venzon et al, 2021). In contrast, the conventional system consisted exclusively of coffee plants "Catuaí Vermelho IAC 99". No pesticides were applied in the diversified system, whereas in the conventional system, mineral fertilization and pesticide applications followed the farm's standard management schedule.

2.2 Soil sampling in coffee areas

The sampling process was conducted by Franzin et al., 2021. Three samplings were made in both coffee systems, and they were conducted at a depth of 20 cm, with 15 soil samples taken per plot per year between 2019 and 2021 in both coffee systems. To prevent cross-contamination, the core soil sampler was cleaned with water, 70% ethanol, and distilled water between each sample. After collection, the soil samples were placed in plastic bags and stored in a cold chamber at 10°C until they arrived at the Entomology Laboratory at EPAMIG, where the assessment processing began.

2.3 Assessment of fungi

To access fungi with potential entomopathogenic activity, we used mealworm larvae (*Tenebrio molitor*) as live bait, following the method described by Moreira et al., 2019. The insects were obtained from the rearing colony of the Entomology Laboratory from EPAMIG Sudeste. We selected five larvae with two months old, measuring between 1.3-1.5 cm in length. Each group of larvae was placed in a 200ml sterilized plastic pot containing 200g of a soil sample. These pots were sealed with a pierced lid to optimize the ventilation and incubated in a climate-controlled room at 25±5 ºC and 12:12 (L:D) photoperiod. To allow insect movement within the soil, the pots were periodically shaken and inverted.

Dead larvae were sterilized by immersion in a solution of 70% alcohol and 5% sodium hypochlorite, followed by rinsing in distilled water. After sterilization, the dead insects were incubated in sterile humidity chambers, consisting of a sterile microtube containing a cotton ball soaked in distilled water to promote fungi sporulation. These microtubes were maintained in a climate chamber (25±5 ºC).

2.4 Fungi isolation and grown

We evaluated fungi presence in all dead larvae daily. Any external fungal growth was transferred to a plate containing PDA (Potato Dextrose Agar) supplemented with 0.05g/l chloramphenicol and subsequently incubated at 25 ºC. This process facilitated the growth of micelles and spores, and the resulting material was stored for DNA extraction. After sufficient growth, the material was replicated on three more plates, with the same culture medium to obtain a larger fungi volume.

2.5 Fungi DNA extraction

We placed 100mg of conidia and mycelia in three growth plates and crushed them with liquid nitrogen. Then, we added 700ul of the extraction buffer and incubated the mixture in a water bath at 55°C for 40 minutes. After incubation, the removed was removed and allowed to cool for 3 minutes. It was then centrifuged at 6000g for 10 minutes, and the supernatant was collected and transferred to a 2ml microtube.

Next, we added 600ul of chloroform-alcohol isoamyl (24:1) and gently inverted the tube for 3 minutes. The sample was centrifuged for 10 minutes, and 500ul of the supernatant was transferred to a new 2ml tube. Then, we added 500ul of chloroform-alcohol isoamyl (24:1) and gently inverted the tube for 3 minutes. Subsequently, the material was centrifugated at 6000g for 10 minutes. We collected 450ul of the supernatant, transferred it to a new 1.5ml tube, added 300ul of cold isopropanol, and gently inverted the tube 15 times.

The tubes were placed in a freezer at -20°C for 30 minutes. After this, they were centrifuged at 12000g for 10 minutes, and the liquid was discarded, keeping the pellet. We then added 500ul of 70% ethanol, let it rest for 3 minutes, and centrifuged for at 10000g for 10 minutes. The liquid was discarded, and the pellet was allowed to dry at environmental temperature inside the microtube for 15 minutes. Finally, 100ul of DNA buffer was added to store the material at -20°C. Before storage, 3ul of each sample was run on an agarose gel to evaluate DNA quality.

2.6 PCR conduction

DNA extractions were amplified using the primers EF1 (Rehner and Buckley, 2005), Beta-tubulin (Tretter et al., 2014; Keeling et al., 2000), Ma-rIGS (Mayerhofer et al., 2019), Mb-MzFG546 (Mayerhofer et al., 2019), Mp-MzIGS2 (Mayerhofer et al., 2019) Mr-rIGS (Mayerhofer et al., 2019), ITS-Full (White et al, 1990), RPB1 (Simmons et al., 2015; Simmons et al., 2015; Wraight et al., 2018; Johnson et al., 2005), BT22 (Kepler et al. 2017), BT1_Clav

(Kepler et al. 2017). The primer sequences, cycle determinations, and temperature conditions for each primer are represented in supplementary materials (S1).

2.7 Statistical analyses

The two coffee systems were evaluated to determine their effect on the quantity of identified fungi genera. To evaluate the data, the Generalized Linear Model (GLM) with negative binomial distribution was used and analyzed by ANOVA with $\chi 2$ tests (Crawley, 2015). The count of individuals was considered as the response variable, and the production system (conventional and diversified) was the explanatory variable. The sampling site and Fungi ID were also tested as random factors. We then tested the significance of the random factors through model simplification using the 'bbmle' package (Bolker, 2022) and determined the minimum suitable model(s) by comparing the corrected Akaike Information Criterion (AICc) values with all analyses and the ANOVA function of R. Contrasts for the different genera were produced by simplifying the model gradually and grouping the genera that were not significantly different (Figure 3).

3.0 Results

3.1 Number of fungal genera

Fungi identification was performed using five different primers. Genera-level identification was considered valid when consensus was achieved for at least one of these primers. Across all identified fungal genera, a total of 154 isolates were recovered from both systems, with 48 isolates in the conventional system and 106 in the diversified system (Table 1). Conventional systems presented five different genres while conventional systems were compound by ten (Figure 1b)

Comparing conventional vs diversified systems, we didn't find differences in the genera amount (Df=1, Chisq= 42.308, p=0.4192) (Figure 1a). The number of *Metarhizium* isolates was higher compared to the other genres found in coffee crops. ($p<0.001$; Figure 2). However, there's no difference in the number of *Metarhizium* isolates found in conventional systems when compared to diversified ones (p=0.6491; Figure 3).

4.0 Discussion

This study provides an initial understanding of the natural occurrence of fungi genera in coffee crops of Cerrado Mineiro, comparing conventional and diversified cropping systems. We did not find a significant difference between the overall number of fungi genera between

these two systems, nevertheless, *Metarhizium* had the highest number of identified isolates. Several studies have demonstrated that *Metarhizium* is naturally found in agricultural soils (Botelho et al., 2019; Iwanicki et al, 2024). Our findings support this, indicating that this genus also occurs in coffee soils, likely due to its wide ecological range, diverse adaptability, and opportunistic characteristics. Previous studies have reported the ability of coffee plants to host *Metarhizium* as an endophytic in their rhizosphere, suggesting that this association may contribute to its increased presence and growth. This symbiotic relationship can be mutually beneficial, potentially enhancing both fungi persistence and plant health (Franzin et al., 2023; Martins et al., 2024).

Some ecological advantages of *Metarhizium* have been shown over the years. St Leger et al. 2008 described the ability of *M. anisopliae* to adapt its MAD1 and MAD2 adhesins in response to environmental stress (Wang and St Leger, 2007). MAD1 is normally induced by insect hemolymph colonization, whereas MAD2 responds to plant exudates. Their study reported that fungus can strategically regulate protein production based on the availability of environmental nutrients. Lovett & St Leger (2014) further reviewed *Metarhizium's* capacity to enhance its stress tolerance under UV radiation and high temperatures. It's reasonable to consider that similar adaptations could occur in coffee crops in the Cerrado, a naturally diverse ecosystem characterized by high temperatures, prolonged periods of dry weather, and seasonal pest infestations. These combined factors may generate stress conditions sufficient to induce *Metarhizium* to activate its “survival mode” and colonize different hots.

Metarhizium is known to be associated not only with plants but also as a saprophytic agent in the environment (Gao et al., 2011). Coffee crops support a diverse invertebrate fauna and are seasonally visited by at least two key pests: *Leucoptera coffeella* (Guérin-Mèneville) (Lepidoptera: Lyonetiidae) and *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae) (Johnson et al., 2020; Dantas et al., 2021). The latter comes into contact with the soil surface for at least one period of its life searching for cherry coffee fruits or sexual partners (Baker et al. 1999; Benavides et al., 2010). These insects spend their entire lives on this perennial plant and eventually die nearby. Specifically, *H. hampei* can also remain in coffee fruits on the soil between harvest seasons, potentially contributing to fungi persistence in the field. This insect has already been reported as an inoculum for *Beauveria. bassiana* and *M. anisopliae,* enhancing fungi recovery from the soil (Bustillo, Bernal, Benavides, & Chaves, 1999).

Metarhizium is widely distributed and can be found in environments ranging from tropical to arctic regions (Zimmerman et al., 2007; Vega et al, 2009). Agricultural landscapes

do not appear to limit their presence. Although plant diversification systems can enhance microorganism diversity, the time required to observe these effects may be longer than anticipated. In a previous study, Franzin et al (2021) showed an increase in enzymatic action in diversified soil systems, which serve as a primary indicator of fungi activity in the soil. Given the survival strategies employed by this fungus, strategic plant diversification is decisive in maintaining fungi populations. While long-term evaluations are necessary to assess chemical and physical soil changes, the nutritional potential provided by multifunctional plants may gradually increase fungi diversity (St. Leger & Wang, 2020) and potentially enhance their ability as pest pathogens.

Although *Metarhizium* was the most frequently identified genus, other fungi groups were also identified in the soil complex. Among the entomopathogenic fungi, we found *Beauveria*, *Metarhizium*, *Purpureocillium,* and *Simplicillium* (Valero-Jiménez, 2016; Wang et al., 2019; Chen et al., 2024; Wei et al., 2019). The saprophytic and potential organic decomposer groups included *Acremonium*, *Cladosporium*, *Delitschia, Penicillium, and Fusarium.* The latter has been reported as a secondary pathogen in insects that were initially killed by entomopathogenic fungi such as *Beauveria* and *Metarhizium* (Globe et at, 2010, Moreira et. al, 2019). *Aspergillus* appears as a potential contaminant. The presence of these fungi provides insights into soil dynamics, as the leaf litter and ground cover promoted by multifunctional plants can contribute to increased organic matter content. However, this process occurs gradually, and long-term evaluations are necessary to assess its full impact.

The relationship among the reported fungi groups could be further explored through species-level identification and phylogenetic analysis. Within the Hypocreales group, diversity varies depending on the biome and agricultural practices applied to ecosystems, particularly concerning the presence of native species and the type of crop production (Moreira et al., 2018; Botelho et al., 2019). Under Cerrado biome conditions, future evaluations could consider factors such as plant developmental stages and season variations, which are likely to influence soil activity.

This study represents an initial step in exploring fungi occurrence and its implications in diversified coffee crops in a highly productive region such as the Cerrado Mineiro. The primary objective was achieved, though certain limitations could be addressed in future research to improve fungi identification. One future direction involves species-level identification and cataloging the number of promising isolates recovered from the soil, with a particular focus on their potential use in bioproducts for pest management. *Metarhizium* appears

to be a strong candidate for this application, primarily due to its persistence and multifunctional lifestyle.

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Table 1: Number of isolates from each fungal genus identified from conventional and diversified coffee crops

Fungi genera	**Conventional System**	**Diversified System**	**Total**
Acremonium	0	2	2
Aspergillus	0	1	1
Beauveria	1	1	2
Cladosporium	0	1	1
Delitschia	0	1	1
Fusarium	3	5	8
Metarhizium	39	89	128
Penicillium	1	2	3
Purpureocillium	4	3	7
Simplicillium	0	1	1
General total	48	106	154

Figures:

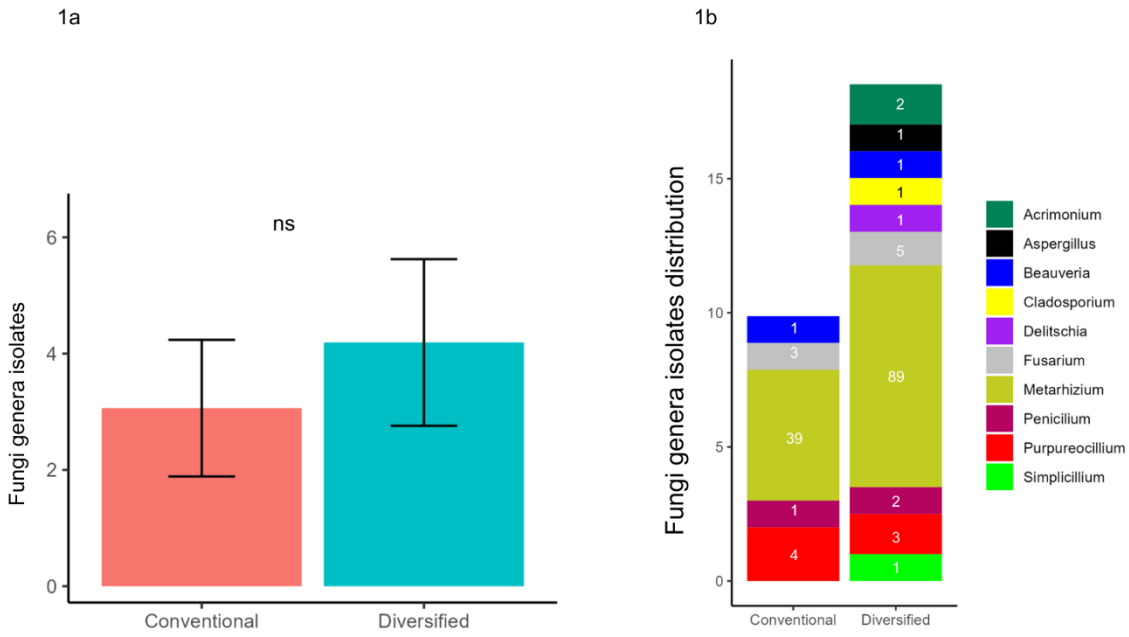

Figure 1: Media number of isolates from each identified fungi genera from diversified and conventional coffee crops (mean ± standard error) (1a, p=0.4192) and its distribution (1b). ns - not significant.

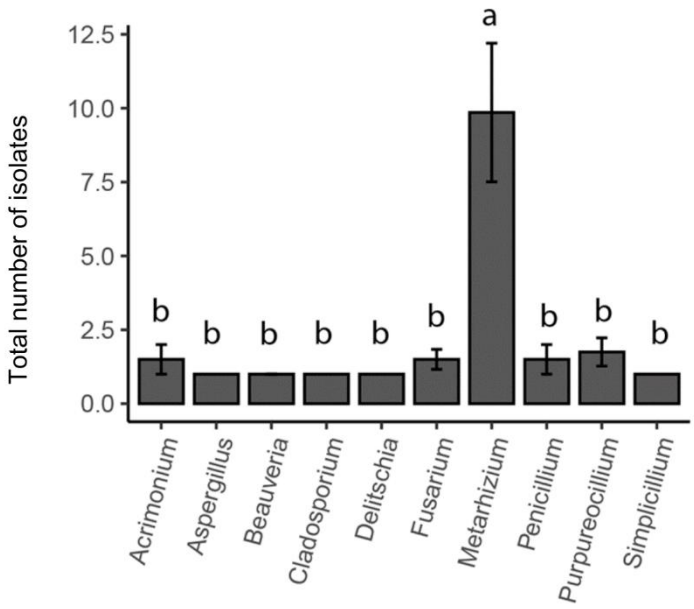

Figure 2: Comparison of the total number of fungi isolates identified from coffee crops ($p<0.001$). Bars with the same letter have no significant differences.

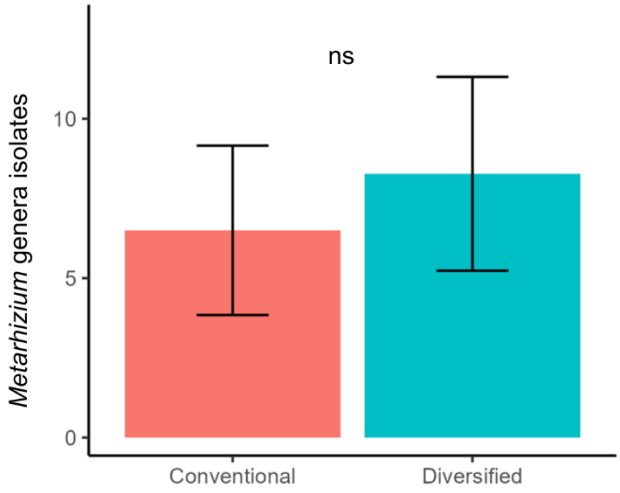

Figure 3: Number of *Metarhizium* isolates genus from conventional and diversified coffee crops ($p=0.6491$) ns - not significant.

Supplementary material

Table S1: PCR conditions and parameters used in the analysis for DNA amplification.

Size (bp)	Primer Name	Primer Sequence (5'-3')	Cycles	Init. Den.		Denaturation		Annealing		Extension		Final extension	
				Temp (C)	Time (min	Temp	Time	Temp	Time	Temp	Time	Temp	Time
1000	EF1-983F (FWD)	GCY CCY GGH CAY CGT GAY TTY AT	36	94	02:00	94	00:30	56	01:00	72	01:00	72	10:00
1000	EF1-2218R (REV)	ATG ACA CCR ACR GCR ACR GTY TG											
700-800	BTub-FWD	GCC TGC AGG ICA RTG YGG IAA YCA	45	95	02:00	95	00:30	46	00:45	72	02:00	72	10:00
700-800	BTub-REV	GGC CTC AGT RAA YTC CAT YTC RTC CAT											
328	Ma-rIGS-1648-F	ACG GTC GCA CAC AAA TC	38	95	05:00	94	00:30	63	00:30	72	00:30	72	10:00
328	Ma-rIGS-1975-R	CAG CCT ACC CGG TAC											
115	Mb-FG546-422-F	TAG TCA GTC GTT GAC GC	38	95	05:00	94	00:30	63	00:30	72	00:30	72	10:00
115	Mb-FG546-536-R	TCC TGT GTC GAC TGT GTC GA											
535	Mp-IGS2-240-F	ACG GCA TGG ACA TGC CC	38	95	05:00	94	00:30	63	00:30	72	00:30	72	10:00
535	Mp-IGS2-774-R	GCC TCT CGT TAC CTA CGA											
638	Mr-rIGS-444-F	ATT ACC AAG TCC AAA ATA CTG G	38	95	05:00	94	00:30	63	00:30	72	00:30	72	10:00
638	Mr-rIGS-1081-R	CAT ATA CCC ACC AAC TAC CC											
580	ITS1-Fwd	TCC GTA GGT GAA CCT GCG G	35	98	00:30	98	00:10	54,5	00:30	72	00:20	72	10
580	ITS4-Rev	TCC TCC GCT TAT TGA TAT GC											
700	RPB1-313F	CTG GAA ATT GTC TGC CAC AAC	39	94	02:00	94	00:30	47	01:00	72	02:00	72	03:00
700	RPB1-Cr	CCG GCA ATG TCG TTG TCC ATA TA											
1000	BT22-R	TCTGGATGTTGTTGGGAATCC	35	94	00:35	94	00:35	52	00:55	72	02:00	72	02:00
1000	BT1_Clav-F	AACATGCGTGAGATTGTRAGT	35	94	00:35	94	00:35	52	00:55	72	02:00	72	02:00

Chapter II: *Metarhizium*-Inoculated Coffee Seeds Promote Plant Growth and Biocontrol of Coffee Leaf Miner

Abstract

Metarhizium (Hypocreales: Clavicipitaceae) has a multifunctional life cycle, establishing as a plant endophyte and acting as entomopathogenic fungi. *Metarhizium robertsii* and *Metarhizium brunneum* can be associated with coffee plants and provide enhanced protection against a major pest of coffee, the coffee leaf miner (CLM) (*Leucoptera coffeella*). This association would be an easily deployable biological control option. Here we tested the potential of inoculating coffee seeds with *M. robertsii* and *M. brunneum* collected from the soil of coffee crops in the Cerrado (Brazil) for control of the CLM and the enhancement of plant growth with a commonly used fungicide. We conducted the experiment in a greenhouse, and after the seedlings grew, we placed them in a cage with two pairs of CLMs. We evaluated the CLM development time, reproduction, and plant growth traits. We observed a longer development time of CLMs when fed on plants inoculated with both isolates. In addition, the CLMs laid fewer eggs compared to those fed on plants without fungal inoculation. Plant growth was promoted when seeds were inoculated with fungi, and the fungicide did not affect any evaluated parameters. Coffee seed inoculation with *M. robertsii* and *M. brunneum* appears to provide protection against CLMs and promote growth improvement.

Keywords: sustainable pest management; biological control; *Leucoptera coffeella*

1.0 Introduction

Entomopathogenic fungi are important allies in the biological control of crop pests [1], and, besides their efficiency, they have a lower contamination risk associated with sprays [2], pest selectivity [3], and feasibility of being acquired in low-cost substrates for mass production by companies and farmers [4]. In this way, financial investments fostered by public and private initiatives for the development of new products based on fungi are promising, and initiatives for implementation are growing worldwide [5].

There is currently a diversity of entomopathogenic fungi available for pest control, and three genera are presently the most used for plant protection: *Beauveria* (Hypocreales: Cordycipitaceae), *Cordyceps* (Hypocreales: Cordycipitaceae) and *Metarhizium* (Hypocreales: Clavicipitaceae) [1,6,7]. In particular, *Metarhizium* is reported to control many insect pests across multiple agricultural systems including sugarcane, soybean, corn, and coffee crops [1,8–10]. Studies suggest its compatibility with pesticides and specificity to common pests [11–14]. The *Metarhizium* genus is also known for having a multifunctional life cycle. It acts as an entomopathogenic fungi and is capable of establishing in the rhizosphere of plants or engaging in endophytic relationships with plants [15–18]. The mutualist interactions of *Metarhizium* and plants can promote plant nutrient uptake and induce plant defenses to abiotic and biotic stresses [19–22].

The coffee leaf miner (CLM) or *Leucoptera coffeella* (Guérin-Mèneville) (Lepidoptera: Lyonetiidae) is one of the most important pests in coffee crops in Brazil [23]. The CLM significantly damages leaves and reduces photosynthetic rates, which drastically decreases coffee productivity [24]. Unfortunately, CLM control is mainly reliant on an array of pesticides that are not always successful and have led to pest resistance and resurgence [25–27]. Moreover, coffee produced under intensive chemical pest control can lead to violations of acceptable levels of pesticide residues allowed in the international market [28]. Encouragingly, recent work provides evidence that biocontrol and plant growth can be enhanced with root drenches of *Metarhizium* because these fungi can associate with coffee roots, which promotes plant growth, resistance to water stress, and protects against CLMs [9,29].

Ideally, endophytic associations that can be established early in the development of coffee plants and provide extended control could promote biological control throughout the growth from seed to production plants. Here, we hypothesize that inoculating coffee seeds with *Metarhizium* will generate seedlings with accelerated growth and enhanced protection against CLMs. We tested this hypothesis using two field-collected isolates of *Metarhizium brunneum*

Petch [30] and *Metarhizium robertsii* [31] both previously recovered from the soil by Franzin et al. [9]. We also tested whether this new inoculation methodology, in conjunction with the use of fungicide, would be viable for (i) protecting the plant against CLM; (ii) promoting vegetative growth; and (iii) reducing the number of insects from the CLM second-generation.

2.0 Materials and Methods

2.1. Fungal Isolates and Suspensions for Seed Inoculations

We selected two isolates from the Agriculture and Livestock Research Enterprise of Minas Gerais isolate bank (EPAMIG Sudeste), *M. robertsii* RD-20.114 and *M. brunneum* RD-20.120. These were originally obtained from the Coffee arabica variety "IAC 44" roots in 2020, collected in the Cerrado's savanna-like biome (18◦9′48″ S and 46◦59′00″ W) using a bait system of Tenebrio molitor larvae (Coleoptera; see Franzin et al. 2022 [9]). We inoculated the fungi on Petri dishes with potato dextrose agar (PDA) culture medium plus 0.05 g of chloramphenicol. The dishes were placed in an incubator in darkness at 26 ◦C for 14 days. By the end of this period, the fungi had sporulated and were ready for multiplication in a solid substrate. For this, we autoclaved 100 g of type 1 rice with 30 mL of distilled water for 20 min. After cooling, we placed the *Metarhizium* conidia obtained from three Petri dishes the size of 9 × 15 mm into each plastic bag with rice and incubated them at 26 ◦C for 14 days, by which time the fungi had sporulated on the rice grains and could be used to make spore suspensions.

To make spore suspensions, we added 50 g of rice grains with sporulating fungus in 1 L of sterile Tween solution at 0.05%. We added the Tween solution and stirred the mixture via inversion for 1 min to disperse the grains in the liquid. We filtered this through sterile gauze to remove rice grains and hyphal fragments. The suspension obtained was vortexed for 30 s. We adjusted the concentrations to 1.0 × 108 conidia with the aid of a Neubauer chamber. To assess conidial germination, we transferred 150 µL of the suspension onto Petri dishes (9 cm diameter) with PDA + chloramphenicol (0.05 g L−1) and incubated these at 26 ◦C for 24 h. We then count the germinated conidia in a 100 × stereomicroscope; we consider them to have germinated when their germ tube had grown at least twice as long as the conidia diameter.

2.2. Coffee Seed Inoculation

We selected 60 *C. arabica* seeds of the Catuaí Vermelho 144 variety. From these, to represent our control groups, 30 seeds were not treated with any fungicide and another 30 were treated with Tecto SC® (active ingredient thiabendazole) at a concentration of 1 mL kg−1 of

seed. Thus, we used 10 seeds in each proposed treatment. The fungicide treated seeds were commercially obtained with the fungicide application carried out by the seller. The use of this fungicide is normally carried out to increase the shelf life of coffee seeds that are stored in cold chambers. Before being obtained, those seeds were stored in a cold chamber for about 45 days. Before the inoculation, we sterilized all the seeds in 0.5% sodium hypochlorite solution and 70% ethanol for 2 min. After that, all 60 seeds were submerged in a 100 mL suspension of conidia of either *M. robertsii* or *M. brunneum* at concentrations of 1.0 × 108 conidia mL−1. We kept the seeds in this suspension for two hours, following the methodology of Canassa et al. [32].

2.3. Coffee Seedling Cultivation

We sowed the seeds in polyethylene plastic bags (20 × 30 × 20 cm) filled with commercial substrate and kept these in a greenhouse. During cultivation, we irrigated the plants according to their water needs. We did not use any pesticides at any time during the experiment, and we fertilized the plants monthly with 10 mL of 4 g L−1 ammonium sulfate per seedling. For 240 days, we maintained plants inside wooden gauze-sided cages (60 × 30 cm) to avoid insect infestations. The development time of the seedlings was prolonged due to the low temperatures recorded during the growing time. They were below 18 ◦C for about 100 days, between May 2021 and January 2022, when normally temperatures range from 18 ◦C to 23 ◦C. At the latest, the time required between seed germination and the formation of the seedlings is about 180 days [9].

2.4. Insect Rearing

For CLM rearing, we collected CLM-infested coffee leaves from experimental fields of the Diogo Alves de Mello Experimental Station at the Federal University of Viçosa, Viçosa, state of Minas Gerais, Brazil (20◦45′14″ S; 42◦52′55″ W). Insect rearing was conducted at the Biological Control laboratory of EPAMIG Sudeste following the methodology of Martins et al. [33]. We kept mined leaves inside acrylic cages (40 × 40 × 40 cm), and to keep the leaves turgid, petioles were immersed in sterile sponges in plastic containers (20 × 20 cm) of water and covered with polyurethane foam. As the insects emerged, we transferred them to new cages with clean coffee plants, allowing the insect life cycle to continue.

2.5. Effect of *M. robertsii* and *M. brunneum* Inoculation on CLM Development

We conducted the experiment in a greenhouse and each treatment had 10 replicate coffee seedlings. We established the following treatments: C1 (untreated seeds); C2 (fungicide treated seeds); T1 (untreated fungicide seeds plus *M. robertsii*); T2 (fungicide-treated seeds plus *M. robertsii*); T3 (untreated fungicide seeds plus *M. brunneum*); and T4 (fungicide treated seeds plus *M. brunneum*). The potted seedlings with two pairs of leaves, originated from seeds that had been inoculated as described above, were kept in cages measuring 60 × 30 cm with metal rods on the side and gauze on the faces [9]. In each cage we added a newly emerged CLM couple obtained from the CLM laboratory rearing and left them for 48 h. Next, we removed the adult CLM couples and counted, with the aid of a magnifying glass, the number of eggs the females laid on each seedling. From these eggs, we began our daily evaluations of every single CLM development in the plants inside of the cages (i.e., time from egg to adult) and continued until all the adults from the laid eggs had emerged. After the evaluations, we checked for the presence of fungi in all plants and plant parts. We placed leaf, steam, and root fragments in a potato dextrose agar (PDA) culture medium.

2.6. Effect of *M. robertsii* and *M. brunneum* Inoculation on CLM Second-Generation

To verify the effects of *M. robertsii* and *M. brunneum* on the CLM second-generation, we collected 15 adult couples from each of the previous treatments, including the control. After that, we placed each couple into a 500 mL plastic container, along with a new coffee leaf with its petiole inserted in a 50 mL plastic container of water to maintain leaf turgor until the end of the evaluations. We evaluated the survival times of the males and females and the number of daily deposited eggs until both adult insects were dead.

2.7. Effect of *M. robertsii* and *M. brunneum* Inoculation on Plant Development

After the period of CLM evaluations, we removed all plants from the pots and cleaned the roots with a brush. With a measuring tape, we measured the shoot and root system lengths. For the shoot, we measured the distance from the first secondary root to the apical region of the plant. For the root, we measured from the first secondary root down to the root cap. After that, we cut the shoot and root systems and weighed their fresh and dry masses with a precision scale. Their dry weight was obtained after 24 h in a drying oven at 65 °C.

2.8. Statistical Analysis

We used the response variables including the number of laid eggs, the time of CLM development, the survival of emerged adults, and the plant parameters (i.e., root and shoot length, root and fresh dry weight) to investigate whether the two species of *Metarhizium* and their combination with fungicide affected the growth of coffee plants and CLM survival and reproduction. We analyzed the insect development times and the survival of the emerged males and females using ANOVA and survival analyses with censored Weibull distributions.

Data on the number of eggs from the second generation of CLM were analyzed with generalized linear mixed models (GLM) adjusted to a Poisson distribution. To analyze the CLM number of eggs we used ANOVA tests and pairwise comparisons with the emmeans package (v 1.10.4) [34]. For plant development parameters we used an analysis of deviance (F-tests) assuming a normal distribution. In case of significant results, pairwise comparisons were performed with the emmeans R-package (v 1.10.4) (adjustment method: Tukey) [35]. For the analyses, we considered the plants that had fungi in the tissue, and we found them in all the plants used in the experiments, except in the control ones.

3.0 Results

3.1. Fungi Recover from Plants and Identification

We recovered the fungi from all the plants and we found *Metarhizium* in all the plant roots, except the control ones. We performed visual identification of the fungi based on the structure of their conidia, thus validating the inoculation and persistence of the fungi in the plants. We cut 2 mm root fragments, performed superficial sterilization through immersing them in 70% alcohol and 5% sodium hypochlorite, then rinsed them in distilled water and dried them on sterile filter paper. We then placed them on Petri dishes with a potato dextrose agar medium to allow the growth and development of the endophytic fungi present in the plants. After the fungal structures grew, we identified them under a microscope at 40× magnification (Figure S1).

3.2. Effect of *M. robertsii* and *M. brunneum* Inoculation on CLM Development

When evaluating the total duration of the CLM life cycle (i.e., from egg to adult) in response to *Metarhizium* seed treatments, we observed that both isolates caused an increase in the total time of the CLM life cycle, when compared to the controls ($X2 = 404.32$, $p < 0.05$) (Figure 1).

3.3. Effect of *M. robertsii* and *M. brunneum* Inoculation on CLM Second-Generation

The inoculation of the coffee seeds with the *Metarhizium* isolates did not affect the survival of second-generation males and females ($X2 = 0.61378$, $p = 0.9874$) (Figure 2A), which did not differ from the controls ($X2 = 1.3255$, $p = 0.9323$) (Figure 2B). Regarding the total number of eggs laid per female, a significantly lower oviposition was found for females that emerged from plants, whose seeds were treated with the fungi compared to the controls ($X2 = 786.77$, $p < 0.05$) (Figure 3). Furthermore, the presence of the fungicide was also unable to affect the development of CLM males and females (Tables S2 and S3).

3.4. Effect of *M. robertsii* and *M. brunneum* Inoculation on Plant Development

The treatment of coffee seeds with the two isolates increased the height of the plant shoot system (Figure 4A) ($F = 13.133$, $p = 0.005$). The plants from the fungicide-treated seeds had longer roots (Figure 4B) ($F = 14.359$ $p < 0.001$), heavier fresh mass shoots (Figure 4C) ($F = 12.470$, $p < 0.001$), heavier root fresh mass (Figure 4D) ($F = 4.2923$, $p = 0.0021$), heavier shoot dry mass (Figure 4E) ($F = 19.287$, $p < 0.001$) and heavier root dry mass (Figure 4F) ($F = 1.7818$, $p = 0.0007$) compared to the control treatments.

4.0 Discussion

The isolates *M. robertsii* and *M. brunneum* used in our experiments as coffee seed inoculants lengthened the development time of the CLM adults. We also found a reduction in the number of eggs obtained from the CLM females developed on plants from fungal inoculated seeds, which suggests fungal effects on subsequent generations of the pest. One of the possible explanations for the impaired development and reproduction of CLMs is the presence of destruxins, which are peptides produced by the secondary metabolism in some entomopathogenic fungi [36]. The action of destruxins synthesized by *M. robertsii* was previously linked to causing food poisoning in herbivorous insects and herbivore repellency [37]. In the case of *M. brunneum*, the presence of destruxins has also been reported to suppress insect immunity, making them more susceptible to entomopathogens [38]. Thus, it is possible that the destruxins reduced foliar consumption by the herbivores and indirectly affected their reproductive performance, an effect reported by Ahmad et al. [15] for *Agrotis ipsilon*. In addition to destruxins, some secondary fungal compounds such as saponins, terpenoids, and phenolic acids may have contributed to an anti-feeding effect on the CLMs, as demonstrated in other herbivores [19,39].

Our results suggest that *M. robertsii* and *M. brunneum* promoted an increase in dry and fresh mass of the shoot system in coffee plants. Therefore, the species can be used as growth promoters, possibly due to the increase in nutrient absorption through the roots, which can influence the health status of the plant and thereby its resistance to pest attacks [40]. Another interesting point demonstrated by Schenkel et al. [41] is that the mutualism between fungi and the host plant can modulate the emission of some plant volatile compounds. This alteration in volatile emissions can affect the choice of insect pests, the architecture of their root system, and even the ability of these fungi to manipulate insect–plant interactions. In our studies, we were not able to test this effect, but future works will test the effectiveness of *Metarhizium* in this choice process on coffee systems.

Besides the endophyte relation with plants and its negative effect on herbivores, the presence of *Metarhizium* in the rhizosphere of the plant can also contribute to pest management [42,43]. One application of this effect was studied by Franzin et al. [9]. They tested the same isolates used in this work and revealed that when the fungi were applied via drenching the soil, CLMs developed slower, with a lower percentage of mined leaf area on leaves, and in the case of *M. robertsii*, the number of adults per coffee seedling and the number of eggs of the progeny were reduced. Therefore, *Metarhizium* fungal associations with coffee roots are able to negatively affect CLMs and promote plant growth parameters. Our studies show that inoculation can successfully be accomplished at the seed stage and has the potential to facilitate a cost-effective inoculation process for the producers.

Coffee seeds are frequently treated with a post-harvest fungicide and dried to prepare for seedling production and to protect them during storage. Our experiments were also designed to assess the compatibility of *Metarhizium* seed inoculation with the commonly used thiabendazole-based fungicide seed treatment. Promisingly, thiabendazole presence apparently did not inhibit the development of *M. robertsii* or *M. brunneum*. One of the factors that may explain this absence of negative effects on the development of the entomopathogenic fungus may be linked to the short safety interval registered for seed treatment, which is two days. The thiabendazole is registered in Brazil to control fungi of the genus *Colletrotrichum* (Phyllachorales), *Penicillium* (Eurotiomycetidae), and *Fusarium* (Hypocreales) [44]. Although *Fusarium* sp. belongs to the same order as the genus *Metarhizium*, the period between seed fungicide application and the inoculation with entomopathogenic fungi (i.e., approximately 45 days) was sufficient for the fungicide residues to degrade, and *Metarhizium* was unaffected. Another favorable factor for the persistence of *Metarhizium* in the soil may also be associated

with its saprophytic capacity (i.e., ability to survive in dead plant or animal material), which would help with its maintenance for so long in the soil [45,46].

Fungi inoculation of seeds to enhance crop protection is reported for other plant species, such as beans, soybeans, and corn [47,48]. However, these are annual crops, and the interaction between the fungi and the root of the plants is limited to a shorter period, compared to a perennial crop such as coffee. Thus, the results presented here are novel and promising for application in coffee crops. Applications of entomopathogenic fungi have potential in high-value crops such as coffee and can be of great value to seedling producers, helping the plants acquire protection even in the nurseries, reducing the costs of pesticides, and additionally promoting plant growth. Studies on the persistence of this interaction over time, the need to re-inoculate the fungus in the plant throughout the cycle, and its effects on other key pests of the coffee crop deserve to be studied to optimize the pest management plan with these organisms.

Another point to be emphasized was the influence of fungal isolates in the reduction in egg production by CLM females, which directly affected the number of individuals from these insects. The mechanisms involved in this effect, however, need to be better elucidated to understand the long-term effect of *Metarhizium* on pest control.

5.0 Conclusions

It is reasonable to conclude that the inoculation of entomopathogenic fungi of the genus *Metarhizium* in coffee seeds may be a promising strategy for not only the management of coffee leaf miners; apparently, its association with plant roots helps reduce the CLM population, increases its time of development, and also increases the development of coffee plants.

6.0 References

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Figures:

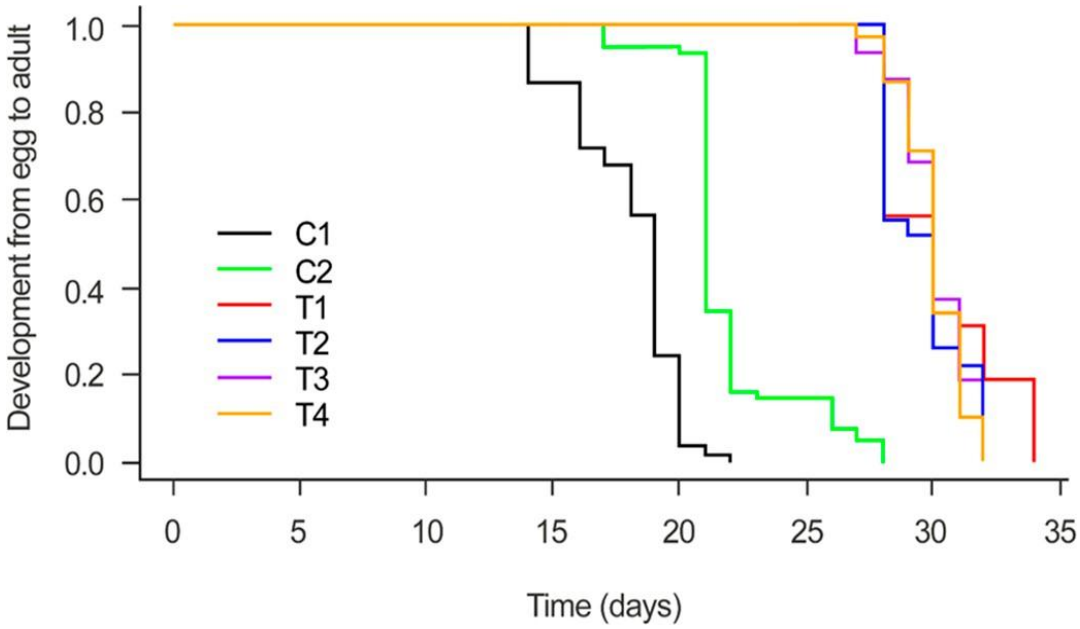

Figure 1. Developmental time from egg to adult of *Leucoptera coffeella* from coffee seeds inoculated with the treatments: C1 (untreated seeds); C2 (fungicide-treated seeds); T1 (untreated seeds plus *M. robertsii*); T2 (fungicide-treated seeds plus *M. robertsii*); T3 (untreated seeds plus *M. brunneum*); and T4 (fungicide-treated seeds plus *M. brunneum*).

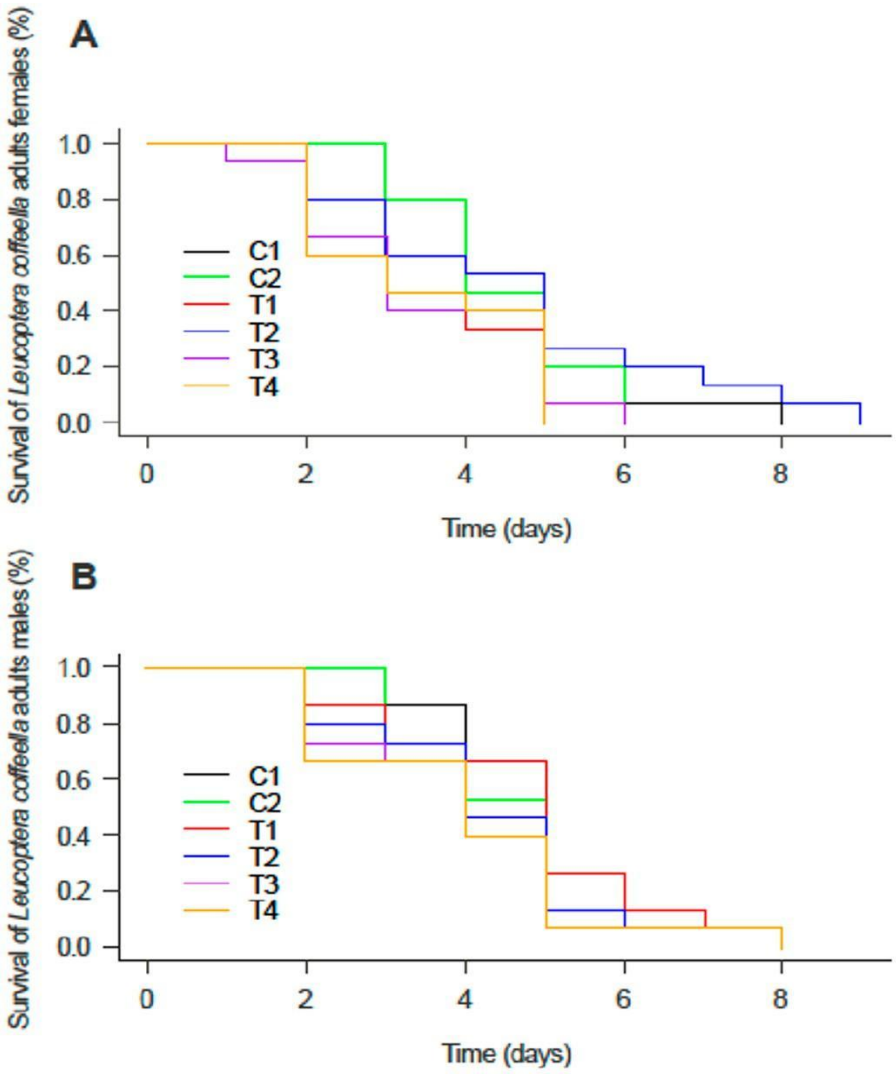

Figure 2. Survival of adult females (A) and males (B) of *Leucoptera coffeella* when fed on coffee plants following the treatments: C1 (untreated seeds); C2 (fungicide-treated seeds); T1 (untreated seeds plus *M. robertsii*); T2 (fungicide-treated seeds plus *M. robertsii*); T3 (untreated seeds plus *M. brunneum*); and T4 (fungicide-treated seeds plus *M. brunneum*).

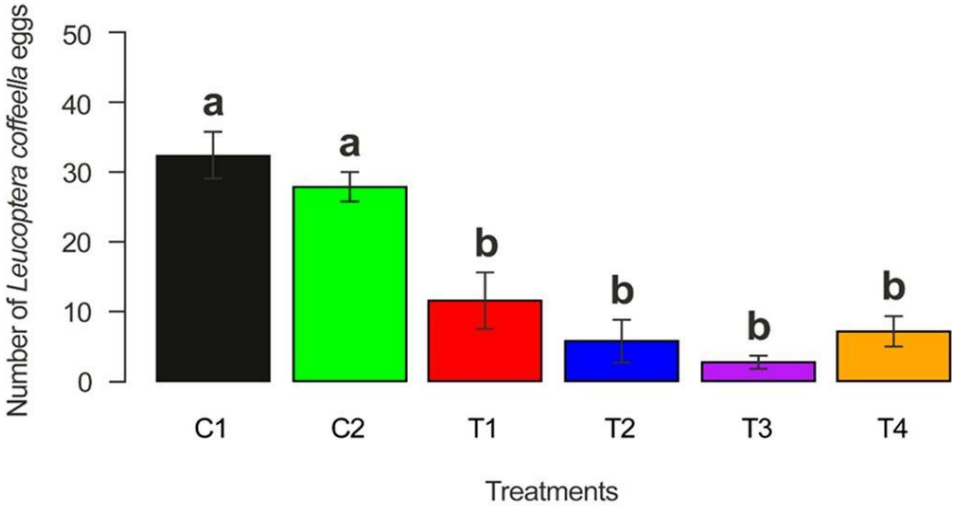

Figure 3. Number of eggs per female of Leucoptera coffeella that emerged from coffee seedlings grown from seeds inoculated with the treatments: C1 (untreated seeds); C2 (fungicide-treated seeds); T1 (untreated seeds plus *M. robertsii*); T2 (fungicide-treated seeds plus *M. robertsii*); T3 (untreated seeds plus *M. brunneum*); and T4 (fungicide-treated seeds plus *M. brunneum*). Bars with the same letters are not statistically different via the Tukey method ($p < 0.001$).

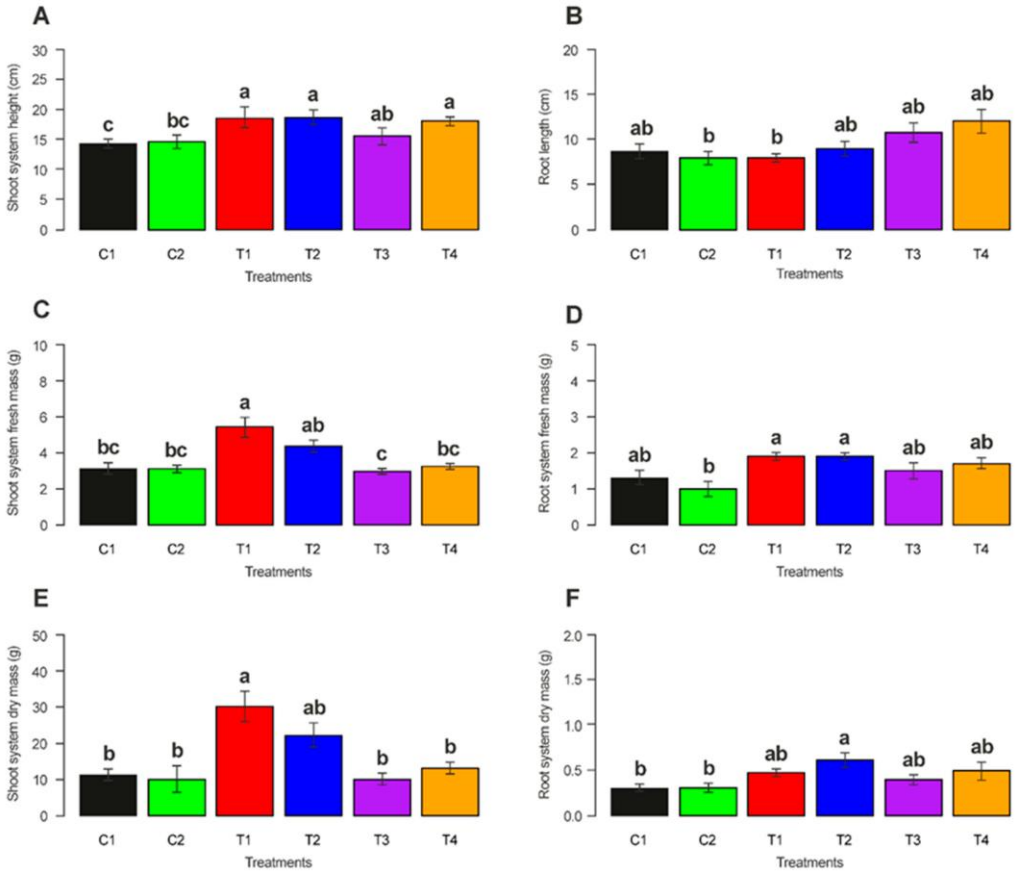

Figure 4. Growth variables of plants from coffee seeds: C1 (untreated seeds); C2 (fungicide-treated seeds); T1 (untreated seeds plus *M. robertsii*); T2 (fungicide-treated seeds plus *M. robertsii*); T3 (untreated seeds plus *M. brunneum*); and T4 (fungicide-treated seeds plus M. brunneum). (A) root length; (B) shoot system height; (C) shoot system fresh mass; (D) root system fresh mass; (E) root system dry mass; and (F) shoot system dry mass. Bars with the same letters are not statistically different via the Tukey method ($p < 0.001$).

Chapter III: Endophytic colonization of cotton seedlings by *Metarhizium* affects whitefly-plant interactions and improves plant development

Abstract

Endophytic relations between plants and entomopathogens are constantly studied. Their effects on plant development and pest suppression are valuable to pest management since they can realize efficient and sustainable insect control. One of the most studied fungi genres that can perform this process is *Metarhizium*. Some evolutionary characteristics of this fungi favor its relation with the plants, especially in the absence of an insect host or environmental stress conditions. Once associated with the plant, the fungi can grow through plant tissues or even stimulating plants' secondary metabolites. The endophytic relation can occur naturally but is also inoculated in plants by seed or soil drench. Both processes can colonize several crops, improving their resistance to insect attack. In our work, we investigate if the *Metarhizium anisopliae* inoculation by seed and soil drench can colonize plants' roots, stem, and root; if they can promote protection against the Sweet potato whitefly *Bemisia tabaci* reducing its number of eggs and adults, their effect on plant leaves grown, and its effect on *Hippodamia convergens* (i.e. a whitefly predator) foraging choice, evaluated under an olfactometer. Our results indicated that the seed inoculation process promotes whitefly control, increases cotton leaf number, affects negatively whiteflies' preference for cotton plants, and also increases predator preference. Our study demonstrated the potential of *M. anisopliae* as an endophytic entomopathogenic fungus on whitefly control, and further investigations about its efficiency in field conditions are necessary.

1.0 Introduction

Facilitating endophytic associations between plants and entomopathogens is becoming a method to improve plant defenses in managed systems (Vega et al., 2008; Jaber & Bonnie, 2018; Mantzoukas et al., 2020). This process is a sustainable alternative that is constantly evaluated to control key pests on crops, but the choice of inoculation process and their effects on plants needs constant evaluation (Greenfield et. al, 2016; Saragih et al., 2019). Generally, these are described as when fungi or bacteria are capable of living within or in symbiosis with plants and do not cause apparent harm to the plant (Wilson 1995). For example, *Metarhizium* fungi promote beneficial effects through endophytic relationships in several important crops (Elena et al., 2011; Ahmad et al., 2020; Stone and Bidochka, 2020). In particular, *Metarhizium* developing endophytic relationships can improve pest control by enhancing the production of plant secondary metabolites (Golo et al., 2014) and their chemical communication, thereby modulating interactions among pathogens and different insect trophic levels, such as herbivorous and predatory insects (Ahmad et al., 2022; Pec et al., 2024, Salaza-Mendoza, 2024). For instance, these studies further indicate that even with certain endophytic fungi genera, the plant can keep signaling when attacked by an herbivorous insect or even promote a major attraction of a natural enemy (Morawo and Fadamiro, 2016; González-Masl., 2021). This association can be established during the early stages of plant growth, such as through seed or seedling inoculation (Franzin et al., 2022; Martins et al., 2024; Panwar and Szczepaniec, 2024). Therefore, establishing endophytic relationships has the potential to be integrated into many pest management systems to improve plant responses and protection to pests.

A current worldwide pest challenge, the Sweet potato whitefly, *Bemisia tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae) is a major pest of many crops globally and a significant threat to cotton production (De Barro, 2011; Stansly and Naranjo 2010). This pest damages the plants by direct feeding, transmitting viruses, and producing honeydew, which supports the growth of sooty mold and reduces photosynthesis (Milenovic et al., 2019; Brown et al., 2002; Arjen Van Doorn et.al, 2015). Whitefly management relies on chemical pesticides with growers often opting for intensive pesticide frequent applications during periods of high infestation (Hasnain et al., 2011). However, the use of pesticides is increasingly discouraged due to their role in accelerating pest resistance and their potentially harmful environmental effects (Muhammad 2018, Perrier et al., 2022). To change this scenario, improving plant resistance and biological control can shift reliance on pesticides (Faria and Wraight, 2001; Togni et al., 2019). Currently, several microorganisms are used for whitefly control, most of them based on entomopathogenic fungi (EPF) genera such as *Beauveria*, *Cordyceps*, and

Metarhizium (Zafar et al., 2016; Shaohui et al., 2023; Faria and Wraight, 2001). Interestingly, some entomopathogen genera, such as *Metarhizium*, have been studied not only as a contact-control agent but also as endophytes (Clifton et al., 2018; Sasan and Bidochka, 2017). Studies also show positive effects of this endophytic fungi on pest control in vegetables (Elena et al., 2011; Batta 2013; Shaalan et al., 2021) promising a viable alternative to explore this use in cotton crops. Besides, Garrido et al. 2017 reported a transient status of endophytic of *Metarhizium* fungus in whitefly control, which lead us to start thinking if *M. anisopliae* strategic inoculations can increase plant defense and affect the survival parameters of whiteflies as an endophytic control strategy.

Given the potential of *Metarhizium* as an entomopathogen capable of developing as endophytes to protect plants, we aimed to investigate the potential of *Metarhizium* for a cost-effective treatment for improved crop protection of cotton. We study the interactions among cotton plants, whiteflies, and a whitefly predator in response to a commercial fungus *M. anisopliae*, inoculated either via seeds or through soil drench in seedlings. Cotton (*Gossypium hirsutum* L) is a crop worldwide on large areas of land, especially in the United States and Brazil, which produced approximately 117.4 million bales and 3.6 million tons during the 2024 harvest, respectively (USDA, 2024; MAPA, 2024). Therefore, finding cost-effective ways to reduce insecticide use could lead to lower management costs and less land exposure to pesticides. The objectives of this study were: i) to determine whether *M. anisopliae* can colonize cotton plants as an endophyte through seed and soil drench inoculation; ii) to evaluate whether *M. anisopliae* promotes cotton leaf growth; iii) to assess whether *M. anisopliae* reduces the number of *B. tabaci* eggs and adults; iv) to examine whether *M. anisopliae* influences *B. tabaci* and *H. convergens* plant choices.

2.0 Material and Methods

2.1 Seed preparation for inoculation and soil drench

We selected 36 conventional cotton seeds (Deltapine®) to use in all trials. These seeds were sterilized in a 0.5% sodium hypochlorite solution and 70% ethanol for 2 minutes. After that, we placed the seeds on absorbent paper and allowed them to rest for 10 minutes until their surface dried. We used the sterilized cotton seeds to proceed to fungi inoculation. For the control treatment, we placed 12 seeds in a container with distilled water, where the seeds remained for 2 hours. The remaining seeds were split into two groups 12 for the seed inoculation and 12 for the soil drench treatment. For inoculation with a *Metarhizium*

anisopliae formulation (Met Master®) at a concentration of 10^8 conidia per ml. The seeds were submerged in this solution for 2 hours. The remaining 12 seeds were selected for planting and subsequent soil drench treatment. For the soil drench treatment, we applied a solution of 30 ml of *M. anisopliae* solution at a concentration of 10^8 conidia per ml on the 7th, 14th, and 21st days after planting (following Ramakuwela et al. 2020).

2.2 Plant care and insect rearing

We moistened and placed the plant substrate in a soil sterilizer for 4 hours, turning it every 2 hours to ensure complete disinfection. During this process, the soil was exposed to a temperature of 77°C. This procedure was carried out to avoid contamination from other microorganisms and isolate only the effects of the selected fungi on plant development. Then, we sowed the seeds in plastic pots (7,5×10×7cm) filled with the substrate and kept them in growth chambers for 21 days at 28°C with a 12/12h light/dark cycle. During cultivation, we irrigated the plants according to their water needs. No pesticides were used.

Bemisia tabaci adults were obtained from populations of the Vegetable Entomology Lab at the University of Georgia, Tifton *campus*. The insects were transferred to clean cotton plants allowing their reproduction and population growth. The insects and plants were kept inside cages to avoid other insect infestations. The plants were watered daily. For the experiments, newly emerged insects were collected and identified as males and females under the microscope. For predator tests, we bought *Hippodamia convergens* adults from Nature's Control®.

2.3 Effect of *M. anisopliae* Inoculation on Whiteflies and Plant Leaf Production

To evaluate the effects of *M. anisopliae* inoculation on whitefly we performed the experiment in a growth chamber, with each treatment having 12 replicates. The following treatments were established: C1 (untreated seeds); T1 (inoculated seeds); and T2 (soil drench inoculation). We kept the plants in cylindrical plastic cages (19x45 cm) with gauze on top. In each cage, we added a newly emerged whitefly couple from a laboratory colony. After 48 hours, the adults were removed, and the number of eggs laid by the females on each treatment was counted under a magnifying glass. We continued the evaluations until the eggs turned into adults.

After the whitefly evaluation period, we counted the number of leaves on each plant to estimate the plant growth (Franzin et al., 2012). The count did not include the cotyledonary

leaves, only the fully expanded ones. We counted the leaves of all the plants involved in the experiment twice, considering the two trials made.

2.4 Confirmation of plant inoculation by *M. anisopliae*

By the end of all experiments, we removed fragments from each plant part (i.e. leaf, stem, and roots) from all the plants used in the experiments. In the leaves, we removed fragments with approximately $1cm^2$, in the stems, we sectioned pieces of 1cm and in the roots, we also used the same length. We made a surface sterilization by soaking those fragments, one by one, in a solution with 0.5% sodium hypochlorite for 2 min, followed by immersion in 70% ethanol for the same period. After this process, we placed the plant fragments in a Potato Dextrose Agar culture medium. We incubated plates at 25°C in darkness for 10 days, and after this period, we took all the grown mycelia and conidia and proceeded to molecular evaluation (Figure 1).

Furthermore, we applied a molecular approach to confirm inoculation. The fungi re-isolated associated with the plant fragmented were processed to confirm the presence of *M. anisopliae*. We placed 100mg of each sample in a tissue disruption tube and used a TissueLyser at 24Hz for 4 minutes. For the positive control, we used the commercial product. We used the DNeasy Plant Pro Kit to extract the DNA from plant fragments and their respective growth fungi following its specific protocols. The obtained DNA was stored at -20°C and after we made the PCR. The fungi DNA extracted from *M. anisopliae* in plant parts (i.e. roots, stems and leaves) was processed using Multiplex kit (QIAGEN, Germantown, MD), and the primer pairs TW81 -GTTTCCGTAGGTGAACCTGC- and AB28 -ATATGCTTAAGTTCAGCGGGT- (Curran et.al, 1994). After that, the DNA products were submitted to an automated gel electrophoresis run in QIAxcel connect and the DNA concentrations were recorded. With the results, we build a graphic representation to show the presence of the fungi in different plant organs (Figure 1).

2.5 Olfactometer to Determine Preference

To perform our choice tests, we used a glass Y-tube (18cm in length in each arm and 3.5cm in diameter) connected to two plastic cages (12cm in length and 4,8cm in diameter) with the odor source (i.e. control or infested plants). The base of the Y-tube was connected to a vacuum pump that conducted the air to both the Y-tube arms. Between the vacuum pump and the plants, we placed a charcoal container to clean the air. To control the airflow, we

used a flowmeter adjusted to permit 50 ml/s (Saad et al., 2013). For the whitefly and ladybeetle choice tests, we selected one of each insect and placed it in the base of the tube. We considered that the insect chose one of the odor sources when they passed through 1/3 of the arm length. We only accounted the responses within 5 minutes, and if they didn't make it in this time, we considered them unresponsive. After five responses, we changed the odor source position (Botti et al., 2019). We cleaned all the Y-tube olfactometer after each insect release with 70% ethanol.

To carry out the whitefly choice tests, we selected cotton plants inoculated by soil drench, seed inoculation, and clean ones that were cultivated using the same methodology used in cotton cultivation for whitefly development. To validate all the following tests considering the olfactometer performance, we first made one test using two clean cotton plants (i.e. control plants) in each odor source container. After this process, we performed the following treatments: clean cotton plants vs seed inoculation cotton plants; clean cotton plants vs soil drench plants; seed inoculation cotton plants vs soil drench cotton plants. Each test was performed three times (i.e. replicates) and we evaluated 15 responses, totaling 45 releases of whiteflies per treatment.

To conduct the ladybeetle tests, we used the same Y-tube system and method, but with different treatments. To investigate ladybeetle responses, we divided the treatment into two groups. The first group considered control plants vs seed inoculation plants; control plants vs soil drench plants, and seed inoculation plants vs soil drench plants. In the second group, we infested all the plants with 10 early emerged adults of *B. tabaci* and left them in the plants inside a clip-cage (20x40mm) for 48h and then we performed the following treatments: control plants + whiteflies vs control plants + whiteflies; control plants + whiteflies vs seed inoculation plants + whiteflies; control plants + whiteflies vs soil drench plants + whiteflies, and, seed inoculation plants + whiteflies vs soil drench plants + whiteflies.

2.6 Statistical analysis

The two methods of fungi inoculation (soil drench and seed inoculation) were used to investigate if they can reduce the number of whitefly eggs and adults; improve the sprouting of cotton leaves and modulate plant odors to attract or repulse whiteflies and ladybugs. For the number of eggs, adults, and cotton leaves we analyzed the data with generalized linear mixed models (GLM) adjusted to Poisson distribution. We used Analysis of Deviance with $\chi 2$ tests and with pairwise comparisons as above. In all olfactometer tests, we considered a completely

randomized design. We evaluated the whitefly and ladybug choices with a Chi-squared test for categorical data. All the analyses were performed in the R 4.4.2 version (R Development Core Team).

3.0 Results

3.1 Confirmation of inoculation

We evaluated the fungi grown on plates to verify if *M. anisopliae* can colonize cotton plants. Considering the 24 plants used (each treatment was repeated twice) we sampled the leaves, stems, and roots of each of them to evaluate the presence and absence of fungi. The clean plants (those not exposed to soil drench or seed inoculation) did not present a significant DNA concentration when compared to the positive control. In seed inoculation, 6% of leaves, 66% of stems, and 25% of roots had fungi DNA. In soil drench ones, 11% of leaves, 64% of stems, and 28% of roots had fungi associations (Figure 1). In addition, the clean plants (those not exposed to soil drench or seed inoculation) did not present a high fungi DNA concentration when compared to the positive control. Our results showed DNA concentrations under 0.3ng/ul for the control samples, compared to values between 13.28 ng/ul and 25.0 ng/ul of DNA on inoculated plants by soil drench and seed inoculation. So, for the percentage of recovered fungi in plant organs, we considered the control ones as zero.

3.2 Effect of *M. anisopliae* seed and soil drench on Whitefly eggs and adults

Plants inoculated by seed with *Metarhizium anisopliae* decreased the number of eggs laid by the whiteflies that fed on them ($X^2 = 180.99$, $p= 0.03315$; Figure 2). Soil drench inoculation did not reduce the number of eggs ($z= 1.628$, $p=0.2338$; Figure 2). We observed a reduction in the number of eggs laid by whiteflies treated with seed inoculation compared to clean plants ($z= 2.453$, $p=0.0376$). For the adults, fewer adults were observed on plants inoculated with fungi ($X^2= 417.53$, $p=0.001$; Figure 3). Soil drenching did not reduce the number of adults compared to clean plants ($z=2.180$, $p=0.0747.$; Figure 3) but the seed inoculation method promoted a significant reduction of whitefly adults ($z=3.721$, $p=0.0006$).

3.3 M. anisopliae Influence on Cotton Leaves Growth

Overall, plants inoculated with *M. anisopliae* resulted in a greater number of cotton leaves produced over 21 days ($X^2=16.129$, $p=0.001$; Figure 4). However, the seed inoculation method promoted a significant increase in the number of cotton leaves ($z=-3.977$, $p=0.0002$),

soil drenching did not increase the number of cotton leaves compared to the control (z=0.446, p=0.8962; Figure 4).

3.4 Whitefly and Ladybeetle olfactometer preference

Whiteflies avoided plants inoculated by seed inoculation when compared to the control (X^2=8.022, df=1, p= 0.04621) and the soil drench treatments (X^2= 5.0, df=1, p=0.02535). Considering the ladybeetle, this predator did not discriminate from clean plants, soil drench, and seed-inoculated seeds without any whitefly infestation. However, when we infested those plants with 10 nearly emerger whiteflies they were attracted to plants inoculated by seed inoculation (X^2=9.8, df=1, 0.001745).

4.0 Discussion

We can find a large amount of publications that evaluate the potential of endophytic fungi in pest control, and most of them consider the effect on the pests and their natural enemies. Here, we decided to evaluate their effect on pest control, predator-plant interaction, and their capacity to improve plants' leaf growth and we reached promising results. Considering the potential of *M. anisopliae* as an endophyte in cotton, it is essential to explore all the functional effects that this association may promote. Endophytic fungi can successfully colonize plant tissues without causing any deleterious effects on the host plant (Panwar and Szczepaniec, 2024). Nonetheless, when the *Metarhizium* endophytic colonization starts, the initial process begins in the rhizosphere. Barelli et al., 2018 demonstrated that *Metarhizium* can establish as part of a competent rhizosphere and persist for the entire life cycle of *Phaseolus vulgaris*. This accumulation may be attributed to the high concentration of excreted nutrients in the rhizosphere (Mwajita et al., 2013). This region is also an intensive nutrient transfer (Smith and Read, 2008), and considering the dynamic interaction between endophytic and rhizosphere-competent locations (Kobae and Fujiwara, 2014), plant roots were likely the most commonly observed site of fungi recovery in our cotton plants (Figure 1).

Our results also demonstrate that plants inoculated with *M. anisopliae* by seed inoculation can significantly reduce the number of *B. tabaci* eggs and adults (Figure 2 and Figure 3). In the literature, most studies investigating the influence of *Metarhizium* endophytic fungi on plants consider their effect on insect development and reproduction (Batta et al., 2013; Rudden et al., 2013; Ratzinger et al., 2014; Franzin et al., 2022). This

effect can be explained by the capacity of *Metarhizium* fungi to produce toxins classified as Destruxins, that can reduce feeding activity, directly affecting insect development. Destruxins A, B, and E were already reported in cowpeas inoculated with *M. robertsii* (Golo et al., 2014). In *B. tabaci* nymphs that were fed on melon plants with *M. brunneum* was reported the presence of Destruxins A (Garrido-Jurado et al., 2017). Furthermore, Destruxins A and E were found in *M. anisopliae* strains and they have insecticidal action (Wang et al., 2004). The authors defend that this toxin production and degradation depends on the insect hosts, temperatures, and dtxS1 gene presence (Skrobek et al., 2007; Wang et al., 2012) but the presence of any of them are signs of a probable insect fitness reduction.

When associated with plants, *Metarhizium* can also use the association with plant growth as a competitive advantage to even exclude other microorganisms for plant photoassimilates (Rodriguez et al., 2009). Together with its opportunist characteristics, this genus can act as a facultative endophyte, depending on environmental conditions (Hardoim et al., 2015) and the possibility of a more stabilized life cycle promoted by the plant. Normally, when associated with a dead insect, the relation with fungi is shorter. However, when associated with plants, *Metarhizium* can promote plant protection but also facilitates the exchange of nitrogen for carbon (Behie and Bidochka, 2014; Behie et al., 2017; St Leger and Wang, 2020). This beneficial interaction may explain the observed increase in cotton leaf development in plants treated by seed inoculation (Figure 4).

Our results also demonstrated that *M. anisopliae* did not influence ladybeetle preference when cotton plants were not infested with whiteflies (Figure 6). However, after herbivore infestation, plants infected with *M. anisopliae* by seed inoculation showed an increase in ladybug preference (Figure 7). On the other hand, *M. anisopliae* decreased whitefly preference (Figure 5). This variation in preference may be attributed to changes in plant volatile composition caused by endophytic colonization, which could enhance plant resistance to herbivory and/or attract natural enemies (Gonzales-Mas et al., 2021). Endophytic fungi can also alter phytohormone concentrations, such as jasmonic acid (JA) and salicylic acid (SA) as reported for *B. bassiana* in association with maize, cotton, and melon (Gonzales-Mas, 2021; Zhu et al., 2023). These effects often vary depending on the fungi species and the interacting insect. Pec et al., 2024 suggest that the *M. robertsii* associated with plants can modulate the reallocation of defense resources in aerial plant parts. This hypothesis could explain the observed repellency of whiteflies by plants inoculated by

M. anisopliae and the attraction of ladybugs by plants infested with the whitefly and inoculated by *M. anisopliae*.

It is reasonable to consider that *Metarhizium* endophytic relationship with plants overcomes the costs of the interaction, particularly through the exchange of photoassimilates and nitrogen, which may sustain the association for a considered period (St Leger and Wang, 2020). The colonization process begins with the modulation of phytohormones and the detoxification of constitutive plant defense metabolites, maintaining a functional balance in the interaction (Brader et al., 2017). However, when comparing the two inoculation methods, soil drenching may overload the plant with fungi inoculums, disrupting this balance and potentially leading to endophytic mortality (Yan et al., 2018). Consequently, in the two experiments in this study involving soil drench inoculation, the effect on whiteflies and ladybugs (i.e. number of eggs, number of adults, and olfactometer choices) were lower when compared to those observed with seed inoculation. The proposed inoculation process also needs to be considered depending on the availability of the grower technology for applying the inoculant and needs to be carefully considered.

The endophytic action of *Metarhizium* fungi presents a promising option for farmers, as its multifunctional capabilities enhance both plant health and resistance to pests while reducing application costs. Additionally, this fungus can persist in the field as a saprophytic in dead insects, promoting longer soil retention. However, several gaps remain to be addressed to fully understand the effectiveness of this fungi, especially regarding the duration of its association with plants. In our study, we assessed the presence of the fungus in cotton plants only during the vegetative stage. Further research is required to determine whether this association persists during reproductive stages. For instance, during flowering, it will be important to examine whether non-targeted insects will be affected by fungi toxins when feeding on nectary compounds. Moreover, gaining a deeper understanding of the potential effects of secondary compounds and volatile compounds in inoculated plants represents an important avenue for future research. Nevertheless, we have demonstrated that the presence of *Metarhizium* as an endophyte can significantly impact plant-insect interactions. Further exploration of these interactions could be instrumental in developing an additional strategy for sustainable pest management in cotton fields.

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Figures:

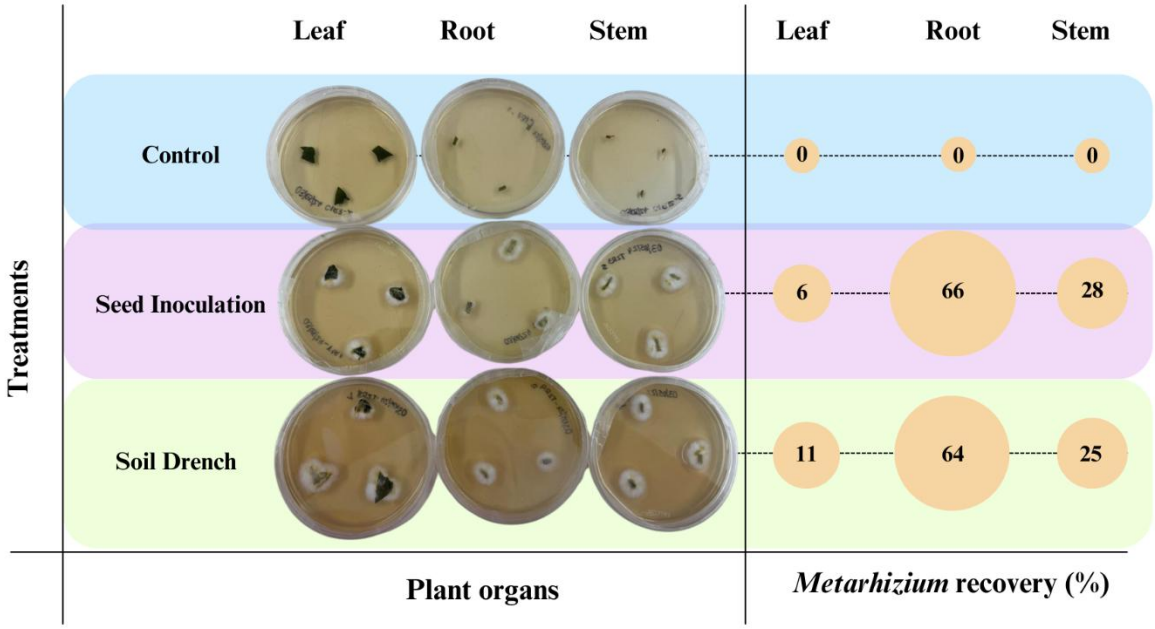

Figure 1: Representation of the percentage of fungi confirmed in each plant under seed and soil drench fungi inoculation. The control treatment represents plants untreated by fungi (i.e clean ones). The number in the bubbles represents the percentage of *Metarhizium* confirmation in each of the plant parts after DNA extraction

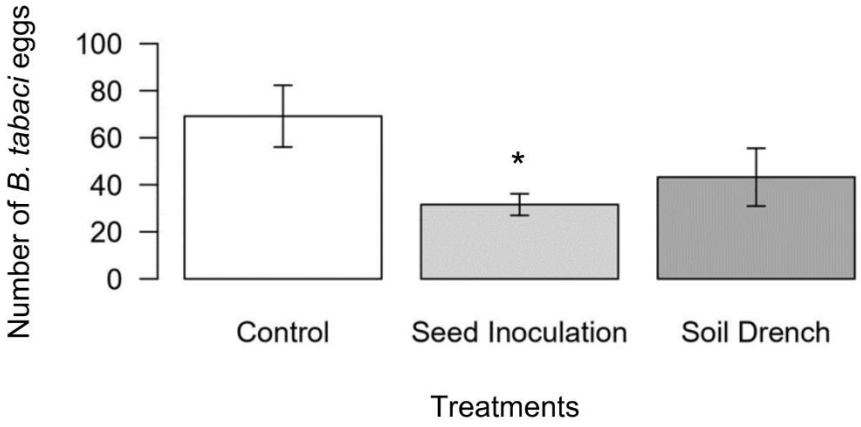

Figure 2: Number of *B. tabaci* eggs after fungi inoculation by SI (Seed Inoculation), SD (Soil Drench) compared to control (untreated seeds). $p<0.01$

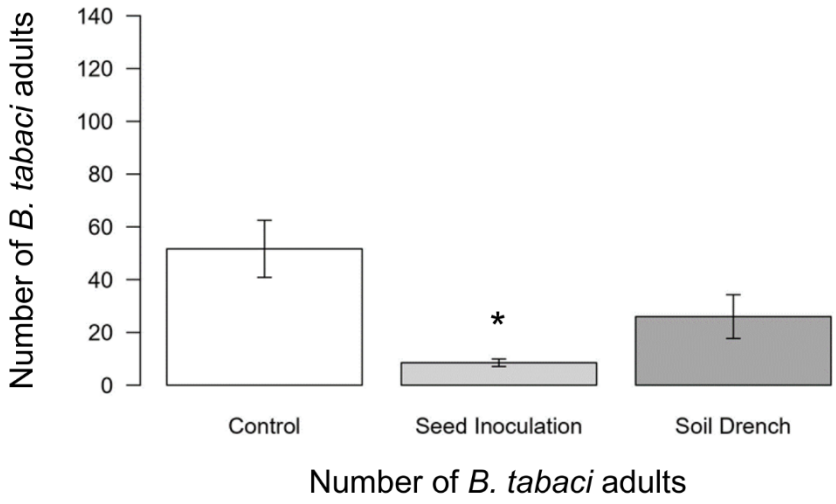

Figure 3: Number of *B. tabaci* adults after fungi inoculation by SI (Seed Inoculation), SD (Soil Drench) compared to control (untreated seeds). $p<0.01$

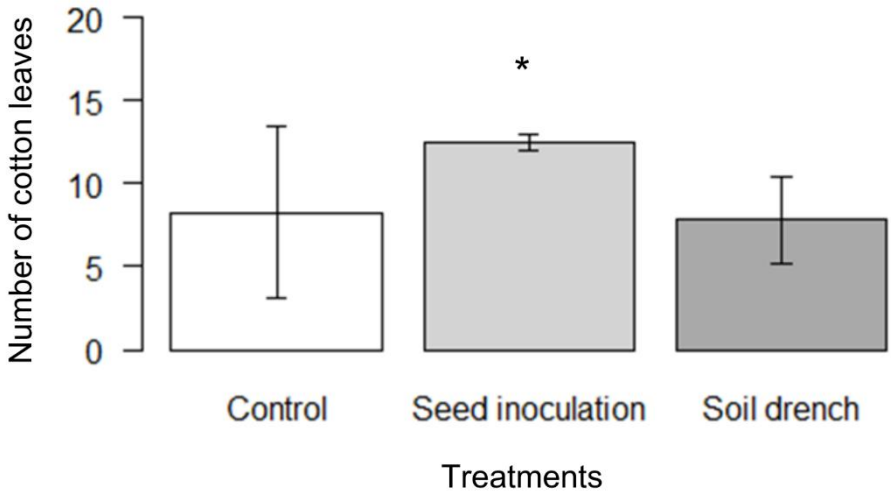

Figure 4: Number of cotton leaves after fungi inoculation by SI (Seed Inoculation), SD (Soil Drench) compared to control (untreated seeds).

Figure 5: Preference of whitefly in Y-choice olfactometer test when offered clean, seed inoculation and cotton plants. No response number = 8

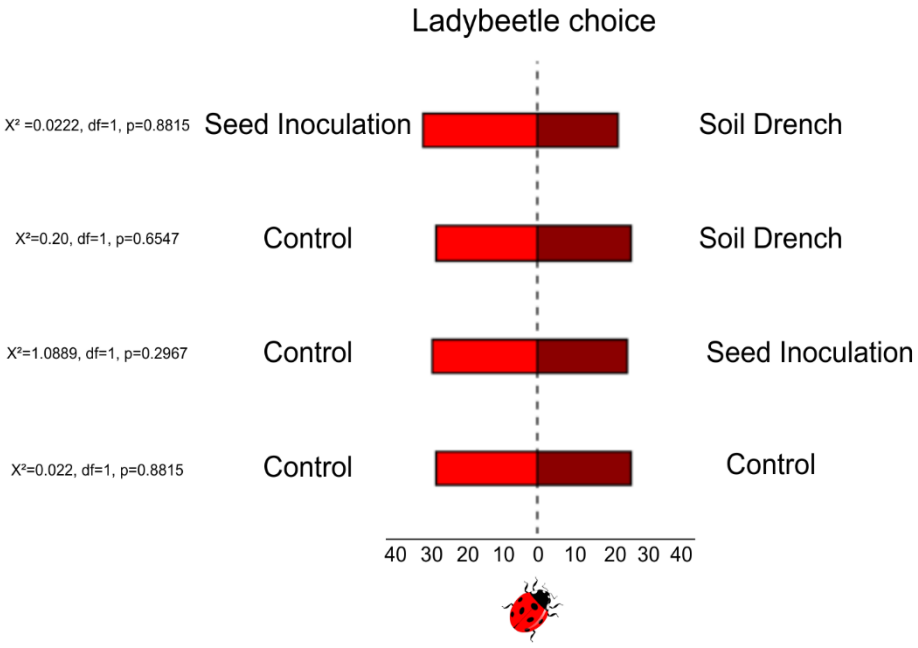

Figure 6: Preference of ladybeetle in Y-choice olfactometer test when offered clean, seed inoculation and cotton plants. No response number = 4

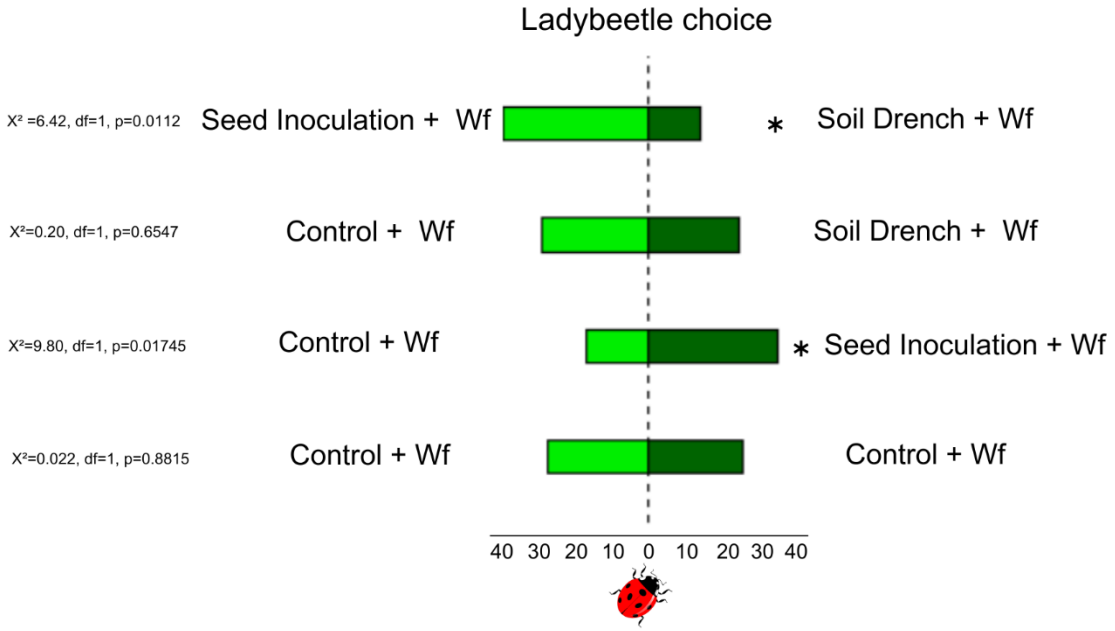

Figure 7: Preference of ladybeetle in Y-choice olfactometer test when offered clean, seed inoculation and cotton plants after a 48h previous inoculation with 10 whiteflies. No response number = 3

General conclusion

Metarhizium is naturally found on coffee crops in both conventional and diversified systems, and apparently, it is a resilient genus that can establish itself even in high-temperature environments. The *Metarhizium* genus, especially the species *M. robertsii, M. brunneum*, and *M. anisopliae*, seems to be a good option for pest management, acting as endophytes.

We evaluated their effects on holometabolous and hemimetabolous insects, which expands the alternatives for using these organisms on the most important pests of coffee and cotton. This fungal genus is capable of extending *Leucoptera coffeella* development time, and reducing the number of eggs and adults of *B. tabaci.*

They also do not seem to negatively affect the predator *H. convergens*. Additionally, the effect of these fungi on plant development needs to be considered, once they increase coffee and cotton development, it could lead to an increase in yield and the development of a sustainable alternative for insect control.