

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

Selection of *Metarhizium* spp. for the management of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) through inoculation in maize seeds and production of conidia and indole-3-acetic acid

Isabella Tavares de Oliveira Silva

Dissertation presented to obtain the degree of Master
in Science. Area: Entomology

**Piracicaba
2021**

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Bachelor in Biotechnology

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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Dissertation presented to obtain the degree of Master
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Piracicaba
2021

**Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP**

Silva, Isabella Tavares de Oliveira

Selection of *Metarhizium* spp. for the management of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) through inoculation in maize seeds and production of conidia and indole-3-acetic acid / Isabella Tavares de Oliveira Silva. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2021.

61 p.

Dissertação (Mestrado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

1. Fungos entomopatogênicos 2. Relação endofítica 3. Promoção de crescimento de plantas 4. Fitossanidade 5. *Spodoptera frugiperda* L.
Título

ACKNOWLEDGMENTS

I would like to thank the University of São Paulo (USP) and “Luiz de Queiroz” College of Agriculture (ESALQ) for all the academic support.

The Department of Entomology and Acarology and the Graduate Program in Entomology for excellence in learning and such qualified teachers.

And also to National Council for Scientific and Technological Development (CNPq) for the scholarship awarded.

I want to thank Prof. Ítalo Delalibera Jr. for his guidance and personal and professional contribution, including reception and trust during my work.

To Dr. Gabriel Moura Mascarin for his collaboration during the process, statistical analysis, and intellectual and moral support.

I'm very grateful to Solange Aparecida Vieira Barros, biologist and technician of the Laboratory of Pathology and Microbial Control of ESALQ-USP, for all the support and collaboration, sharing knowledge and information for the development of the research;

I thank all the friends of the Laboratory of Pathology and Microbial Control of ESALQ/ USP for the coexistence and learning, especially Dr. Ana Carolina Siqueira, who was by my side in several challenging times, Vitor Isaias, my friend for everytime who helped me inestimably in the assembly of experiments and Dr. Natasha Iwanick for all the support and friendship. As well as Joel, Camila, Lucas, Aline, Dani, Mari, Celeste, Isabella, Eliane for the partnership. And all the students and teachers of the Department of Entomology who assisted in my personal and professional construction.

And finally, I'd like to thank my family for being the base at all times. And specially my mother, Flávia Tavares, and my stepfather Saulo Barbosa for encouraging me to persist and never stop dreaming.

To my friends Laís, Ana Lúcia, André, Ariane, Aline, Mari, Ana Carolina, Miriam, Paulo, Junior Felipe, Carlos, and many others for the happy moments during the trajectory.

Thank you all, so much!

EPIGRAPH

“Aceitar o desafio do desconhecido, apesar de todos os medos, é coragem. A experiência de felicidade que o desconhecido produz, o grande êxtase que começa a acontecer com o desconhecido, te torna mais forte, lhe dá integridade e aguça a sua inteligência”

Osho

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RESUMO

Seleção de *Metarhizium* spp. para o manejo de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) através da inoculação em sementes de milho e produção de conídio e ácido 3-indol-acético

Fungos entomopatogênicos do gênero *Metarhizium* (Hipocreales: Clavicipitaceae) podem colonizar diferentes espécies de plantas endofiticamente e provaram ser uma estratégia promissora para promover o crescimento das plantas e controlar pragas e doenças. Entre as pragas agrícolas mais importantes do Brasil e de vários outros países está a lagarta-do-cartucho, *Spodoptera frugiperda*. Este estudo objetivou determinar o potencial de diferentes espécies de *Metarhizium* em relação a promoção do crescimento e colonização endofítica das plantas de milho e seu antagonismo contra *Spodoptera frugiperda* e duas doenças importantes desta cultura. A produção de conídios de *Metarhizium* spp. em fermentação sólida foi utilizada como primeira triagem revelando que isolados de *M. anisopliae* apresentaram melhor rendimento ($> 10^9$ conídios por grama de arroz), seguidos por isolados de *M. humberi* e finalmente *M. robertsii*. A produção de auxina (AIA) in vitro de 21 isolados de *Metarhizium* foi utilizada como uma segunda triagem, testando a hipótese de que a produção deste hormônio poderia estar relacionada ao crescimento da planta. Entretanto, não foi observada diferença estatística entre os isolados, com exceção de *Metarhizium* sp. indeterminada 4 ESALQ1684 (3,8 μ g IAA/mL), que difere estatisticamente das espécies *M. anisopliae* e *M. humberi*. Assim como a inoculação de sementes com *Metarhizium* spp. não apresentou promoção de crescimento das plantas de milho em comparação com plantas controle. O efeito da inoculação de 10^8 conídios via semente de *Metarhizium* spp. avaliado em lagartas *Spodoptera frugiperda* foi realizado com sete isolados. *M. humberi* ESALQ1781 e *M. robertsii* ESALQ2966 apresentaram efeitos mais significativos nas lagartas com mortalidade acima de 60% e tempo letal médio abaixo de 4,5 dias. Simultaneamente, apenas uma redução de 10% na sobrevivência de *S. frugiperda* foi registrada no controle. O efeito antagonista de *Metarhizium* spp. contra o patógeno de milho *Fusarium graminearum* se mostrou mais evidente em comparação com *Exserohilum turcicum*, com dois isolados de *M. robertsii* e um de *M. anisopliae* apresentando valores acima 35% de inibição contra *F. graminearum*. A colonização endofítica só foi confirmada em sete dos doze isolados de *Metarhizium* spp. em plantas de milho, independentemente dos métodos de recuperação testados, sendo o melhor macerando as raízes em placa de meio ágar seletivo com cristal violeta em comparação com raízes fragmentadas sem cristal violeta. O resultado mais promissor se mostrou com a baixa sobrevivência de lagartas *S. frugiperda* alimentadas com plantas inoculadas com *Metarhizium* spp., revelando o potencial desta nova estratégia para o uso de fungos entomopatogênicos.

Palavras-chave: Fungos entomopatogênicos, Relação endofítica, Promoção de crescimento de plantas, Fitossanidade, *Spodoptera frugiperda*

ABSTRACT

Selection of *Metarhizium* spp. for the management of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) through inoculation in maize seeds and production of conidia and indole-3-acetic acid

Entomopathogenic fungi of the genus *Metarhizium* (Hypocreales: Clavicipitaceae) can colonize different species of plants endophytically and have proved to be a promising strategy for promoting plant growth and controlling pests and diseases. Among the most important agricultural pests in Brazil and several other countries is the caterpillar, *Spodoptera frugiperda*, considered a key problem in maize. This study's objective was to determine the potential of different species of *Metarhizium* in promoting growth and endophytic colonization of corn plants and their antagonism against *Spodoptera frugiperda* and two important diseases of this crop. Conidia production of *Metarhizium* spp. in solid fermentation was used as the first screening of isolates and revealed that isolates of *M. anisopliae* showed better yield ($> 10^9$ conidia per gram of moist rice), followed by isolates of *M. humberi* and finally *M. robertsii*. Auxin production (IAA) in vitro of 21 *Metarhizium* spp. isolates was used as a second screening, testing the hypothesis that this hormone's production could be related to plant growth. However, no statistical difference was observed between the isolates, except for *Metarhizium* sp. Indeterminate 4 ESALQ1684 (3.8 μ g IAA/mL), which differed statistically from the species *M. anisopliae* and *M. humberi*. Likewise, the seed inoculation of *Metarhizium* spp. did not enhance the growth traits of maize plants grown compared to untreated control plants in a greenhouse. The effect of inoculation of 10^8 conidia of *Metarhizium* spp. by corn seed in *Spodoptera frugiperda* caterpillars was carried out with seven isolates. Inoculation by *M. humberi* ESALQ1781 and *M. robertsii* ESALQ2966 showed more significant effects on caterpillars with mortality above 60% and median lethal time below 4.5 days. Simultaneously, only a 10% reduction in *S. frugiperda* survival was recorded on the control. The antagonist effect of *Metarhizium* isolates against the maize pathogen *Fusarium graminearum* was more evident than for the *Exserohilum turcicum*. Two isolates of *M. robertsii* and one of *M. anisopliae* showed greater antagonism against *F. graminearum* with values above 35% inhibition. Endophytic colonization was only confirmed in seven of the twelve *Metarhizium* spp. isolates on maize plants regardless of the recovery methods tested. The best way to recover *Metarhizium* was to macerate the roots and plate in the selective agar medium with crystal violet compared to fragmented roots without crystal violet. The most promising result of these studies was the low survival of *S. frugiperda* caterpillars in plants inoculated with some *Metarhizium* isolates, revealing the potential of this new strategy for using entomopathogenic fungi.

Keywords: Entomopathogenic fungi, Endophytic relationship, Plant growth promotion, Plant health, *Spodoptera frugiperda*

1. INTRODUCTION

Metarhizium (Metschnikoff) Sorokin, are fungi of the phylum Ascomycota, family Clavicipitaceae. They are cosmopolitan and widely used as a biological control agent for arthropod pests of numerous crops. The *Metarhizium* complex includes several species such as *M. anisopliae*, *M. acridum*, *M. brunneum*, *M. globosum*, *M. guizhouense*, *M. humberi*, *M. lepidiotae*, *M. majus*, *M. pingshaense*, and *M. robertsii* (BISCHOFF; REHNER; HUMBER, 2009, LUZ et al., 2019).

Besides their pathogenicity, *Metarhizium* spp. are plant root colonizers (BEHIE et al., 2015). The colonization of *Metarhizium* spp. is more associated with plants' rhizosphere than the aerial part (SASAN; BIDOCHKA, 2012; BEHIE; JONES; BIDOCHKA, 2015; VEGA, 2018). The ability of *Metarhizium* spp. to infect soil insects and to establish roots endophytic association with plant allows tri-trophic interactions between plant, insect, and fungus. Previous studies reported that the fungus could translocate nitrogen from the dead insect to the plant, which in turn, provides carbon for the fungus (BEHIE; ZELISKO; BIDOCHKA, 2012; BEHIE et al., 2017). *Metarhizium* spp. can also act as antagonistic of phytopathogens (SASAN; BIDOCHKA, 2013; KEYSER et al., 2014).

The use of biological control methods has been boosted by increasing insect resistance to chemical insecticides and the need for more sustainable practices in agriculture. The susceptibility of caterpillar pests to *Metarhizium* spp. is widely known (HUMBER; HANSEN; WHEELER, 2011; COSTA et al., 2015; FRONZA et al., 2017; RAMOS et al., 2020). Actually, the first fungal-based microbial insecticide commercially registered in Brazil is based on *M. anisopliae* conidia (BETTIOL, 2011).

Maize (*Zea mays* L. subsp. *mays*) is the most cultivated crop globally, and Brazil is the third largest world producer of maize. The damages caused by pests in maize crops culminate in productivity losses of 5.7 million tons of grain per year in Brazil (OLIVEIRA et al., 2014). The maize pests cause damage to the roots, the aerial part of the plants and are taxonomically grouped in several orders of insects such as Lepidoptera, Coleoptera, and Hemiptera (KABALUK; ERICSSON, 2007). The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), a polyphagous insect, is considered a key pest in maize culture in Brazil.

This research aimed to determine: 1) the potential of different species of *Metarhizium* spp. in colonizing endophytically maize plants, 2) the *Metarhizium* plant

growth promotion through seed inoculation, and 3) to evaluate the effects of fungus-plant association in the antagonism to *Spodoptera frugiperda*. To address these objectives, we: i) Selected isolates of *Metarhizium* spp. with high conidia yield by solid fermentation (rice); ii) Evaluated the in vitro production of indole-3-acetic acid (auxin) by *Metarhizium* spp.; iii) Assessed the benefits of the endophytic association of *Metarhizium* spp. for maize plants including promoting aerial part and root growth; iv) Determined the in vitro growth inhibition by different species of *Metarhizium* against two phytopathogens, *Exserohilum turcicum*, and *Fusarium graminearum*; v) Determined if the endophytic colonization of maize by *Metarhizium* spp. results in repellency, changes in development, or mortality of *Spodoptera frugiperda*.

2. BIBLIOGRAPHIC REVIEW

2.1. *Spodoptera frugiperda* and maize crop (*Zea mays*)

According to estimates by the National Supply Company in Brazil, maize production exceeded 80 million tons per year (CONAB, 2018; CONAB, 2019). The great production of maize makes possible the exportation of grains, one of the primary commodities in Brazilian agriculture. The largest maize production region in Brazil is the Midwest, which contributes approximately 50% of production. The production of this region is concentrated in the second harvest or off-season (CONAB, 2020).

Despite the high maize production, Brazil's agriculture faces several losses due to direct and indirect damage caused by arthropod pests' attack. One of the key pests in maize is the fall armyworm, *Spodoptera frugiperda*. The fall armyworm feeds on maize leaves from the emergence of the seedling to the grains' formation (CRUZ, 1995; MOSCARDI et al., 2012).

S. frugiperda have high reproductive potential and can oviposit about 1600 eggs during their lifetime. The larvae stage comprises six instars lasting approximately 12 to 30 days, depending on climatic conditions, especially the temperature. The first instar caterpillars scrape one side of the leaf, causing opaque spots on the epidermis. After the second instar, the caterpillars show cannibal behavior; thus, it is common to find from that stage on, only one caterpillar per plant (ÁVILA; DEGRANDE; GOMEZ, 1997). From the third instar on, the caterpillars can cause more severe damage to the leaves and the cob maize (VALICENTE; TUELHER, 2009). Pupae, on the other hand, remain in the soil or inside the cob maize until the emergence of adults. Adults are nocturnal for feeding and mating purposes, and caterpillars remain hidden under the foliage with proximity to the ground during the day (CRUZ, 1995).

Many studies determined the susceptibility of *S. frugiperda* caterpillars to *Metarhizium* (HUMBER; HANSEN; WHEELER, 2011; COSTA et al., 2015; FRONZA et al., 2017, RAMOS et al., 2020). *Metarhizium rileyi*, for example, was found causing outstanding epizootics to caterpillars of this lepidopteran group (FRONZA et al., 2017). De Lira et al. (2020), for example, reported less than 45% *S. frugiperda* larvae survival in maize plants inoculated with *M. robertsii* and *M. humberi* inoculated in maize seed.

2.2. The *Metarhizium* genus and its use in biological pest control

Metarhizium (Hypocreales: Clavicipitaceae) has great importance in Brazil and the world. Species of the genus *Metarhizium* are present in tropical and temperate climate zones of the world (BISCHOFF; REHNER; HUMBER, 2009) and were found infecting (REZENDE et al., 2015) more than 300 insect species in several habitats around the world (ZIMMERMANN, 2007). They also are observed endophytically in the plant rhizosphere (SASAN; BIDOCHKA, 2012) and saprofitically in soils (HERNÁNDEZ-DOMÍNGUEZ; GUZMÁN-FRANCO, 2017). Some *Metarhizium* species, such as *M. robertsii* and *M. anisopliae*, are considered generalists with a large number of hosts, while *M. album* and *M. acridum* have narrow host ranges, generally limited to a single order of host insects (HU et al. 2014).

The infection cycle of *Metarhizium* spp. in insect hosts starts with the attachment of aerial conidia (asexual spores) on the insect body, followed by germination and penetration in insect cuticle. This process is mediated by specific adhesion proteins and non-specific hydrophobic interactions between epicuticular lipids and hydrophobic conidia (MOONJELY, et al., 2016; WANG and ST. LEGER, 2007). The success of conidia penetration in insect cuticle is explained by the mechanical pressure performed by appressoria and by chemical degradation of insect cuticle due to excretion of several hydrolytic enzymes such as proteases, lipases, and chitinases by fungi (ST. LEGER et al., 1987, 1996; MOONJELY et al., 2016; ORTIZ-URQUIZA and KEYHANI, 2013; SCHRANK and VAINSTEIN, 2010).

The process follows with hyphae changes into blastospores (IWANICKI et al. 2020; LEWIS et al., 2009; WANCHOO et al., 2009) yeast-like cells whose purpose is to increase at the insect hemolymph without being detected by the insect immune system. After colonizing the insect body, fungus promotes host death between 3 to 7 days, depending on insect species (SCHRANK and VAINSTEIN, 2010). The cycles end when hyphae outgrowth from mummified insect body and produce aerial conidia for dispersion (BARELLI et al., 2016; SCHRANK and VAINSTEIN, 2010; SMALL and BIDOCHKA, 2005).

The use of *Metarhizium* for pest control is done through the inundative application of fungal propagules, aiming to enhance the infectious conidia concentration in the field against insects. *Metarhizium anisopliae* (Metsch.) Sorokin, 1883 is the fungus species with the largest number of product registrations in Brazil,

an estimated 2 million hectares of land treated annually (PARRA, 2014; IWANICKI et al., 2019; MASCARIN, et al., 2019). This is the most commercialized entomopathogenic fungus by Brazilian companies. It is also produced on-farm at a large scale by the sugar cane industry for its sugarcane fields (ALVES, 1998). Besides, do not present minimal risk to the environment and humans and other vertebrates (ZIMMERMANN, 2007).

Although *Metarhizium* species have proven to be virulent to FAW larvae, the habits of the larvae to hide inside the maize whorl and the dispersal behavior that occurs into the lower extremity of the maize plant pose a challenge to chemical and biological control agents that act by contact as larvae keep protected (DE LIRA; MASCARIN; DELALIBERA, 2020).

Several studies on phylogenetics, distribution, and ecology of *Metarhizium* species have been conducted (ROCHA et al., 2013; REZENDE et al., 2015; BRUNNER-MENDOZA et al., 2017; HERNÁNDEZ-DOMÍNGUEZ and GUZMÁN-FRANCO, 2017; KRYUKOV et al., 2017; REHNER and KEPLER, 2017; MASOUDI et al., 2018; IWANICKI et al., 2019) to know more about this genus. New species are being discovered and described. A new lineage, not taxonomically characterized, *Metarhizium* sp. indet. 4, was found predominantly in the Caatinga (ZANARDO, 2015), and *M. humberi*, a genomically distinct new species, has been recently described from Brazil (LUZ et al., 2019). The diversity of *Metarhizium* species found in Brazil (IWANICKI et al., 2019) shows the importance of studying another species of *Metarhizium*, exploring its potential considering that *Metarhizium* species may also be for use in biological pest control.

2.3. Use of the entomopathogenic fungus *Metarhizium* spp. as a plant growth promoter and plant defense

Entomopathogenic fungi can be found endophytically colonizing different plant species (VEGA et al., 2009; PAVA-RIPOLL et al., 2011; BEHIE; JONES; BIDOCHKA, 2015; LACEY et al., 2015) without causing any apparent damage (CARROLL, 1986). These symbiotic microorganisms can also increase root proliferation (HUISMAN, 1982; GLICK, 1995). A phytohormone, indole-3-acetic acid, or auxin (IAA), regulates rhizogenesis and mediates the elongation, division, and differentiation of plant cells (WOODWARD and BARTEI, 2005).

The versatile lifestyle as a saprotrophic, endophytic, and entomopathogen of *Metarhizium* spp. is explained, in part, by its ability to express several proteins and enzymes in an environment that facilitate the acquisition of nutrients of a variety of substrates (STONE; BIDOCHKA, 2020). The ability of *Metarhizium* to colonize plant tissues varies by fungal species and strain, environmental conditions, like ultraviolet radiation, temperature, humidity, and host species (LOVETT and ST. LEGER, 2015).

Although most referred to as insect pathogenic fungi, the evidence indicates that phylogenetically *Metarhizium* spp. are more closely related to plant-associated fungi than animal pathogens (GAO et al., 2011; BARELLI et al., 2016). The ability of *Metarhizium* to colonize crop plants and translocate insect-derived nitrogen has been observed with great interest (STONE; BIDOCHKA, 2020). Interestingly, some studies indicate that similar mechanisms to infect insects are activated to colonize plant hosts, with several related processes facilitated by similar genes (WANG and ST. LEGER, 2007; STONE; BIDOCHKA, 2020).

Metarhizium robertsi, is the most common species found in Brazilian soil (BOTELHO et al., 2019; IWANICKI et al., 2019). This species establish mutualistic interactions with plants by colonizing its roots (HU and LEGER, 2002; LIAO et al., 2014), increase plant growth, leading indirectly insect death of those that fed on plant inoculated with the fungus (KABALUK and ERICSSON, 2007; DE LIRA et al., 2019), boost the absorption of nutrients by the roots (BEHIE et al., 2012) and provide protection against plant pathogens (OWNLEY et al., 2010). However, there is a scarcity of studies addressing these interactions' mechanisms (WANG and LEGER, 2007; LIAO et al., 2013; LIAO et al., 2014).

The new species, *M. humberi*, taxonomically described in 2019 (Luz et al. 2019), has gained increasing attention during the last years as a promising species to be used in biological control and as bioinoculant. *M. humberi* was highly virulent against the two-spotted spider mite (CASTRO et al., 2018) and is rhizosphere competent in sugarcane, strawberry, tomato, and bean plants. (LUZ et al., 2019; OLIVEIRA et al., 2020).

The endophytic colonization by mutualistic fungus can boost phytohormone production by plants, such as salicylic acid and jasmonic acid that play a role in the plant defense system. On the other hand, the fungus produces secondary fungi metabolites like destruxins and beauvericin (GOLO et al., 2014; SIDDAIAH et al., 2017; MALLEBRERA et al., 2018). After inoculation of *M. robertsii*, Golo et al. (2014)

detected destruxins in roots, stems, and leaves of cowpea plants (*Vigna unguiculata*) via seed inoculation. These compounds produced by the plants and the fungi can establish an antibiosis relationship, in which pest control occurs indirectly by inhibiting herbivory.

The lethal and sub-lethal effects of entomopathogenic fungi on natural enemies have been investigated. There are reports on the use of fungi as plant endophytic and its impact on parasitoids and predators (GATHAGE et al., 2016). The absence of significant deleterious effects in micro hymenopters and predators' life cycle was observed after contact with aphids on plants endophytically colonized by entomopathogens (JABER and ARAJ, 2017; GONZÁLEZ-MAS et al., 2019).

The antagonism of *Metarhizium* spp. against phytopathogens is another exciting ability of these fungi. An accomplished study with *M. robertsii* and the phytopathogen, *Fusarium solani*, which causes sudden death syndrome (SDS), showed the formation of a small zone of inhibition when both fungi were grown "in vitro" (SASAN; BIDOCHKA, 2013). Keyser et al. (2014) studied the antagonism of *M. brunneum*, *M. robertsii*, and *M. flavoviridae* against the phytopathogen *Fusarium culmorum* and observed the formation of different sizes of zones of inhibition in each species. Altogether these studies suggest that *Metarhizium* spp. may act as an antagonist against phytopathogens.

The ability of microorganisms to promote plant growth and control phytopathogenic fungi may be associated with the production of several compounds responsible for these characteristics. Among the mechanisms of antagonism related to phytopathogens' biological control is the production of lytic enzymes, such as β -1,3-Glucanase, which can degrade β -1,3-Glucan, a component of the fungal cell wall, as well as chitin (KAVINO et al., 2010).

2.4. *Metarhizium* spp. as a potential antagonist to *Exserohilum turcicum* and *Fusarium graminearum*

Maize diseases significantly decrease annual yield. *Exserohilum turcicum*, and *Fusarium graminearum* are the two most injurious phytopathogens of maize plants. Northern leaf blight or helminthosporiosis (NBL) of maize is caused by *E. turcicum* (Pass.) Leonard and Suggs (syn. *Helminthosporium turcicum* (Pass.) is a foliar fungal

disease, resulting in substantial yield losses (NKONYA et al., 1998; MURIITHI and MUTINDA, 2001; PRATT et al., 2003; RAMATHANI et al., 2011) that can be more than 50% if the symptoms show up before flowering (RAYMUNDO and HOOKER, 1981; TEFFERI et al., 1996; KUTAWA et al., 2017). Northern leaf blight causes premature death of blighted leaves leading to significant yield losses due to reduced photosynthetic leaf areas. (DE VRIES and TOENNIESSEN, 2001; VEERABHADRASWAMY et al., 2014). The control NBL have been recommended through seed treatment and application of fungicides (RAID, 1990, 1991; VEERABHADRASWAMY et al., 2014; WATHANEEYAWECH et al., 2015), tolerant genotypes (RAMATHANI et al., 2011), and crop rotation (PATAKY and LEDENCAN, 2006; LIPPS and MILLS, 2011). Despite these control measures, NBL disease continues to be a significant constraint in maize production worldwide.

Another important disease is the fusarium head blight (FHB) caused by the soil-borne fungus *F. graminearum* Schwabe (FG) (Teleomorph, *Giberella zeae* (Schwein) Petch. FHB has been reported in 23 countries, including the United States, Canada, India, and France (MARASAS et al., 1984). Several chemical fungicides used as seed coatings could not effectively control FHB, which can infect the maize at any growing stage and season (LI et al., 2016).

An alternative to chemical control and other methods is the use of biological control against phytopathogens. KHEDEKAR et al. (2010) and (SARTORI et al., 2017) showed the potential of biological control agents such as *Bacillus* spp. and *Trichoderma harzianum* in reducing disease severity of northern leaf blight caused by *E. turcicum* in the maize plant. Similarly, SARAVANAKUMAR et al. (2017) and Li et al. (2016) determined that maize plants inoculated via seed with *T. harzianum* and *T. asperellum* reduced the incidence of FHB disease by 70%. Recently RIVAS-FRANCO et al. (2019) showed that maize seed coating with *M. anisopliae* conidia resulted in up to 67% dead *Costelytra giveni* larvae and 44% reduction in *F. graminearum* symptoms.

The mechanisms by which *Metarhizium* provides plant protection against plant fungal pathogens include secretion of extracellular water-soluble metabolites, competition for nutrients, space, and antibiosis (QI; CHEN; LI, 2010; SASAN and BIDOCHKA, 2013).

Considering the potential of *Metarhizium* as an antagonist of phytopathogens, in this study, we evaluated *Metarhizium* spp. potential against *E. turcicum* and *F. graminearum*.

3. MATERIAL AND METHODS

All steps of this research were carried out at the Laboratory of Pathology and Microbial Control of Insects at ESALQ/ USP, Piracicaba, State of São Paulo, Brazil. We selected 25 isolates of *Metarhizium* spp. (Table 1), from 5 different Brazilian biomes, deposited in the collection of entomopathogens “Prof. Sérgio Batista Alves” from the Laboratory of Pathology and Microbial Control of Insects at ESALQ/ USP, Piracicaba / SP.

Table 1. List of *Metarhizium* spp isolates used in the investigations of endophytic colonization for conidia and indole-3-acetic acid production.

Number of isolates	Species	Place of origin
ESALQ1684	<i>Metarhizium</i> sp. indet. 4	Sugarcane rhizosphere – Itacemópolis (SP)
ESALQ1669	<i>Metarhizium anisopliae</i>	Sugarcane soil – Itacemópolis (SP)
ESALQ1641	<i>Metarhizium anisopliae</i>	Cercopidae – Boca-da-mata (AL)
ESALQ4133	<i>Metarhizium anisopliae</i>	Maize – Sinop (MT)
ESALQ4818	<i>Metarhizium robertsii</i>	Soybean – Rio Verde (GO)
ESALQ2364	<i>Metarhizium robertsii</i>	Banana – Sinop (MT)
ESALQ2966	<i>Metarhizium robertsii</i>	Maize – Sinop (MT)
ESALQ4927	<i>Metarhizium robertsii</i>	Maize – Sinop (MT)
ESALQ1887	<i>Metarhizium robertsii</i>	Maize – Sinop (MT)
ESALQ2450	<i>Metarhizium robertsii</i>	Maize – Rio Verde (GO)
ESALQ3959	<i>Metarhizium robertsii</i>	Native Vegetation – T. Vilela (AL)
ESALQ2322	<i>Metarhizium robertsii</i>	Native Vegetation – Aceguá (RS)
ESALQ5253	<i>Metarhizium robertsii</i>	Sugarcane soil – Itacemópolis (SP)
ESALQ5240	<i>Metarhizium robertsii</i>	Sugarcane soil – Itacemópolis (SP)
ESALQ3715	<i>Metarhizium humberii</i>	Maize – Sinop (MT)
ESALQ4637	<i>Metarhizium humberii</i>	Maize – Sinop (MT)
ESALQ4614	<i>Metarhizium humberii</i>	Maize – Sinop (MT)
ESALQ1657	<i>Metarhizium humberii</i>	Sugarcane soil – Itacemópolis (SP)
ESALQ4829	<i>Metarhizium humberii</i>	Soybean – Rio Verde (GO)
ESALQ4925	<i>Metarhizium humberii</i>	Native Vegetation – Sinop (MT)
ESALQ4207	<i>Metarhizium humberii</i>	Soybean – Rio Verde (GO)
ESALQ4544	<i>Metarhizium humberii</i>	Native vegetation – Rio Verde (GO)
ESALQ1781	<i>Metarhizium humberii</i>	Native vegetation – Rio Verde (GO)
ESALQ4764	<i>Metarhizium humberii</i>	Sugarcane – Rio Verde (GO)
ESALQ4287	<i>Metarhizium humberii</i>	Banana – Sinop (MT)
ESALQ3651	<i>Metarhizium humberii</i>	Native vegetation – Rio Verde (GO)

The choice of isolates was made in an attempt to cover some isolates of the species *M. anisopliae*, which is known worldwide for its great use as an effective

biological control agent. The isolates of the species of *M. robertsii* and *M. humberi* are species with high endophilic potential and represent a new species described, studying their potential, respectively.

The *Metarhizium* spp. isolates grown in PDA culture medium (Difco®) for 15 days in a Biochemical Oxygen Demand (BOD) climate chamber under controlled laboratory conditions (25 ± 1 °C, 70 ± 10 % RH, and a photoperiod of 12h12 [L:D]). The isolates that showed satisfactory growth, those who presented high conidia production, were again replicated in Petri dishes containing the same culture medium and under the same conditions (Figure 1). Isolates were preserved as sporulated agar chunks immersed in sterile 10% glycerol solution at -80 °C. (JARONSKI & JACKSON, 2012).



Figure 1. Sporulation of the 25 isolates of *Metarhizium* spp. in PDA medium (Difco®) after cultivated for 15 days in BOD at 25°C and 70% RH.

In addition to cryopreservation, pure spores of each isolate were stored without the addition of cryoprotectants in 1.5 mL microtubes (KASVI®) and kept at -40°C.

3.1. Conidia production of *Metarhizium* spp. by solid fermentation

The 25 isolates of the *Metarhizium* spp. (Table 1) were grown on PDA for 14 days. Conidial suspensions were obtained by washing the Petri dishes containing sporulated fungus with 10 mL of a sterile aqueous solution made with 0.05% Tween® 80. The dilutions were adjusted by counting the number of conidia under a Neubauer chamber on the phase-contrast microscope (400X magnification) to a concentration of 10^8 conidia/mL. An aliquot of each suspension was taken for determining conidial viability using the methodology described by Oliveira et al. (2015).

A total of 50 g of sterilized parboiled rice was placed in 250 mL Schott® flasks and inoculated with 5 mL of a 10^8 conidia/mL suspension of each isolate in a laminar flow hood. The experiment was repeated three times and consisted of three repetitions per isolate each time. All treatments were placed, for ten days, in a Biochemical Oxygen Demand (BOD) climate chamber under controlled laboratory conditions (25 ± 1 °C, 70 ± 10 % RH, and a photoperiod of 12h12 [L:D]).

The bottles of each treatment were washed with distilled water and taken to the shaker for 15 minutes to extract the conidia from the rice. The conidia concentration was quantified by the serial dilution method. An aliquot of 1 mL was removed from the inoculated vials with their respective isolate, followed by dilution in 9 mL of water + Tween® 80 (0.05%). Conidia counting was performed with a Neubauer chamber and light microscope (DM4000B, Leica®Microsystems, Wetzlar, Germany).

3.2. Production of indole-3-acetic acid by *Metarhizium* spp.

The indole-3-acetic acid (IAA) production was performed using the FAN medium (FAN, 2002) (Glucose: 20g; yeast extract: 3g; K_2HPO_4 : 0.6g; $MgSO_4$: 0.3g; 1 liter of distilled water and pH: 5.9). The FAN medium was supplemented with L-tryptophan (0.5 g/L) and poured on 125 mL flasks and sterilized in an autoclave for 20 minutes at 121°C. After that, flasks were inoculated with a conidia suspension of 10^6 conidia/mL. The inoculated culture media was kept under the agitation of 150 rpm on a Marconi® orbital shaker for seven days at 26°C. After seven days, 1.5 mL aliquots were removed from each repetition, centrifuged at 6000 rpm for 10 minutes in an Eppendorf 5415D® centrifuge, and the supernatant was collected to carry out the reaction. The experiment was repeated three times with one (flask) repetitions per isolate each time.

The reactions were performed using Salkowski's reagent (15 mL of H₂SO₄, 0.75 mL of FeCl₃, and 25 mL of distilled water) as it is extremely sensitive to indole compounds. The supernatant, which was 0.5 mL of each culture strain, was added to an Eppendorf tube with 0.5 mL of Salkowski's reagent. The reaction remained in the dark for 30 minutes, followed by the IAA measurement in µg/mL at 530 nm in a spectrophotometer. The standard curve was determined with 10 mg Sigma® commercial auxin, diluted in 10 mL acetone for the stock solution. Concentrations of 0, 1, 5, 10, and 20 µg/mL were used to read the samples.

3.3. Isolates screening for inoculation in maize plants

All the experiments were conducted in a greenhouse at the Department of Entomology and Acarology of the College of Agriculture “Luiz de Queiroz,” ESALQ/USP, using conventional maize seeds provided by the Department of Genetics of the same university.

3.3.1. Effects of *Metarhizium* spp. on corn plant traits treated by seed coating with conidia

Nine treatments were compared and consisted of *M. anisopliae* (ESALQ1669), *M. humberti* (ESALQ1781 and ESALQ4287), *M. robertsii* (ESALQ2450, ESALQ3959, ESALQ4927 and ESALQ5253), and *M. sp. Ind. 3* (ESALQ1684) and uninoculated control. The experiment was repeated at three different times. Seeds were inoculated via pipette with 1 mL of conidia suspension equivalent to 3.6×10^7 conidia/seed corresponding to 2×10^{12} conidia/ha. The control treatment consisted of seeds inoculated with distilled water contained 0.05% of Tween 80. The inoculation was carried out with ten plants for each treatment that remained for 30 days in a greenhouse under daily irrigation. Corn plants were grown in plastic pots (500 ml) on a mix of non-sterile soil obtained from ESALQ-USP, and from commercial substrate (Basaplant®) with a ratio of 1:1 under greenhouse conditions and providing water as needed; each pot hosted three seeds. When the seedlings were established, seven days after sowing, they were thinned to standard the number of planting.

Four parameters were evaluated to measure the plant growth promotion mediated by *Metarhizium* seed inoculation: shoot length (cm), stem diameter (mm) and dry weight of shoots, and roots (g). All parameters were measured 30 days after sowing.

Plants' height was measured from the beginning of the hypocotyl, the region just above the seed, to the last expanded leaf's most distant point. All measurements were performed with a millimeter ruler and a digital caliper. Five plants of each treatment were used to evaluate dry weight. Plants were removed from the pots containing substrate and soil and transported to the laboratory. The substrate surrounding the roots was removed with tap water. After cutting in the region above each plant's main root, the plant fragments were packed in paper bags (13×18 cm) and transferred to a hot air oven at 60 °C for 24 hours. After this period, dry plant fragments were weighed.

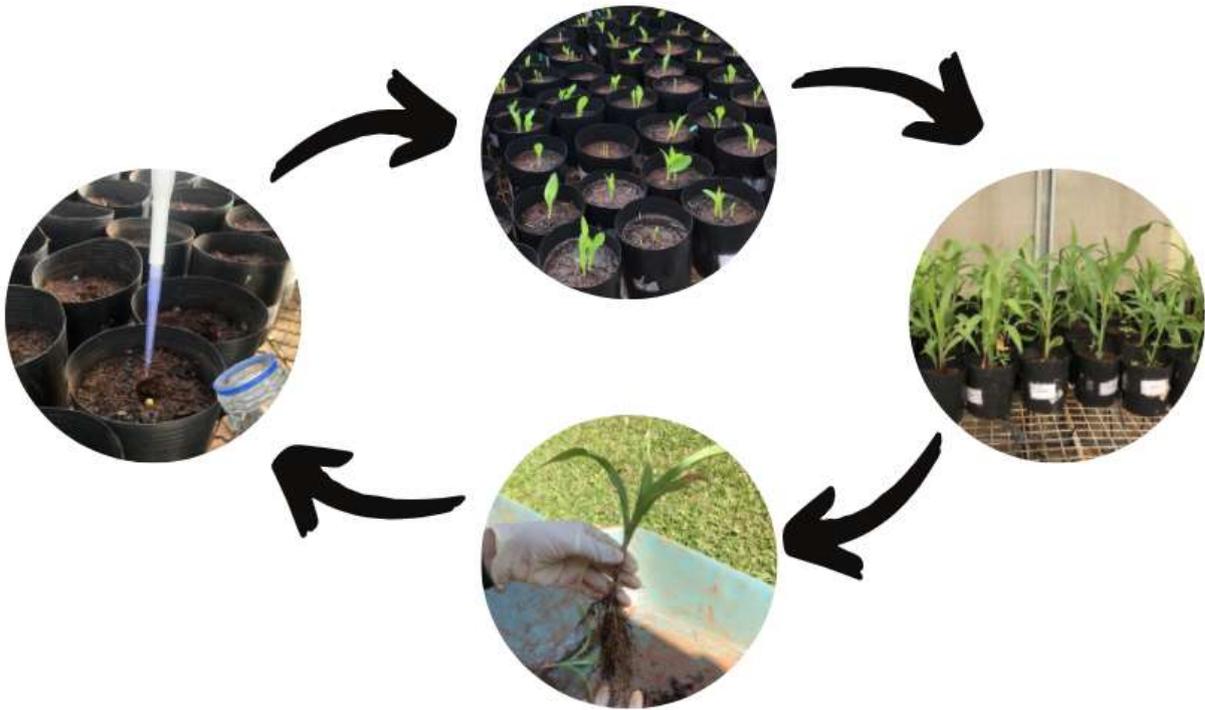


Figure 2. Maize plants inoculated via seed and plant growth promotion evaluation after 30 days of growth in a greenhouse.

3.3.2. Effects of seed inoculation with *Metarhizium* spp. on *S. frugiperda* mortality fed on maize plants

Spodoptera frugiperda 3rd instar caterpillars, previously fed on a natural diet composed of maize leaves, were transferred to inoculated corn plants treated via seed with *Metarhizium* spp. conidia. Based on the average production of conidia of the 21 isolates, seven isolates were selected, two producers of each species of *Metarhizium* spp. for the pest antagonism assays, according to Figure 1. The chosen isolates were: *M. anisopliae* (ESALQ1641 and ESALQ4133), *M. humberi* (ESALQ1781 and ESALQ4925), *M. robertsii* (ESALQ4927 and ESALQ2966), and *M. sp. indet. 4* (ESALQ 1684), and uninoculated control plants. The application was made via pipette, adding 1×10^8 conidia/seed, and plants were left to grow for 30 days in the greenhouse before transferring the insects. The control treatment consisted of seeds treated with distilled water contained 0.05% of Tween 80. The insects were confined in cylindrical-shaped cages made of transparent acrylic material (21 cm high and 8 cm diameter) sealed by voile at the upper end.



Figure 3. Cages with maize plants inoculated with *Metarhizium* spp. A) Caterpillar after feeding on plant treated with *Metarhizium* spp. B) Maize leaf scraped by *Spodoptera frugiperda* larvae.

Each treatment was composed of 20 repetitions (plants) each, with two *S. frugiperda* per plant, summing 40 larvae per treatment. The insect mortality was evaluated every 24 hours for seven days. To check for *Metarhizium* spp. outgrowth on dead larvae, they were transferred and individualized in Elisa plates with moist cotton

between the compartments (wells) to provide humidity for fungus sporulation. The experiment was maintained in an air-conditioned room at 26 ± 5 ° C with 12 hours of photophase and was repeated three times.

3.3.3. Endophytic colonization

All plants from experiments about growth promotion and effect on *S. frugiperda* by inoculation of *Metarhizium* spp. were checked for endophytic colonization. Preliminary tests were performed to select culture media and to adjust a methodology for accessing the ability of *Metarhizium* to colonize maize plants endophytically. Two selective medium and two methods were tested.

In the first experiment, we used root fragments. The fragments were washed in running water to remove soil particles and sterilized by immersion in 70% alcohol for 2 minutes and triple passage in sterile distilled water. For the second experiment, we used macerated roots. Seedlings were placed in 500 mL pots contained a mix of soil and substrate (Basaplant ®) and evaluated after 30 days of fungus inoculation. Roots were removed from the soil and washed in distilled water. The roots were cut into sections of 3 g weight. The sections were then placed into a sample crusher with 9 ml distilled water for one minute and homogenized using a rotary homogenizer (Greiner Scientific) for 30 minutes. Samples (100 µl) of roots macerated fragment were spread on selective media, containing: PDA, 0.5 g cycloheximide, 0.2 g chloramphenicol, 6 µl/L dodine, and 0.1 g crystal violet per liter.

The fragments and macerated roots were also plated in a selective culture medium for *Metarhizium* containing: 10 g/L peptone, 20g/L dextrose, 15g/L ágar, 0.5 g cycloheximide, 0.5 g/L tetracycline, 6 µl/L dodine, and 0.6 g/L streptomycin (KEPLER et al. 2014 half strength). Petri dishes were incubated at 25 ± 2 ° C and 14 hours of photophase for up to 20 days (BEHIE; JONES; BIDOCHKA, 2015). After the incubation period, *Metarhizium* spp. colony-forming units (CFU) were counted. Colonies of *Metarhizium* spp. were identified visually based on morphological characteristics such as the conidia and mycelium, confirmed under a phase-contrast microscope at 400X magnification.

3.4. Antagonism in paired comparison of cultures

The in vitro assay considered the inhibition of mycelial growth to determine the growth inhibition of *E. turcicum*, and *F. graminearum*, in agar medium inoculated with different species of *Metarhizium*. A total of seven isolates of *Metarhizium* spp. (Table 1) selected for the assay with *S. frugiperda* were tested (ESALQ1684, ESALQ1641, ESALQ4133, ESALQ2966, ESALQ4927, ESALQ4925, ESALQ1781).

The experimental unit consisted of a Petri dish (90 x 15mm) with Potato-Dextrose-Agar culture medium (PDA Difco USA) plus 0.5 g/L of antibiotic (Pentabiótico: 500 mg/L; composed of benzathine benzylpenicillin, procaine benzylpenicillin, benzylpenicillin potassium, dihydrostreptomycin base, and streptomycin base), inoculated with a mycelium disk with 0.7 cm in diameter of each *Metarhizium* isolate. The Petri dishes were then incubated in a B.O.D. at 26 ° C for five days. For each isolate, five experimental units were settled. After five days of incubation, Petri dishes were inoculated with a disk (0.7 cm) of the phytopathogens at the other end of the plate, according to methodology adapted from Sasan and Bidochka (2013). The fungus *T. harzianum* was used as a positive control, and the fungi growth without phytopathogens was also measured. After phytopathogen inoculation, the plates were kept in BOD (26°C) for ten days to observe the antagonism. The diameter of *Metarhizium* spp. and phytopathogen colonies were measured after 5, 7, and 10 days after the inoculation of the phytopathogen. The percentage of inhibition was calculated using the formula: % Inhibition = [(C-T)/C] x100, where, C denotes diameter of the radial growth of the pathogen towards opposite side and T denotes the radial growth of the pathogen towards the opponent antagonist (*Metarhizim* or *Trichoderma*), according to Mishra (2010).

3.5. Statistical analyses

All experiments followed a completely randomized design. The production of *Metarhizium* spp. conidia was analyzed using a generalized linear model (GLM) with a negative binomial distribution with a log link function. The quality of the fit of the model was assessed through the graphical analysis of residues using the “hnp” package (MORAL; HINDE; DEMÉTRIO, 2017) together with the Akaike information criterion (AIC), with smaller AIC indicating a better model fit. The multiple comparisons

of means were based on the Tukey HSD method at 5% significance using the “emmeans” package (LENTH, 2018).

Endophytic colonization was also evaluated by the general GLM model with binomial negative for the interaction of fungi, selective media, and plating methods. Linear mixed models (assuming a normal distribution) were adjusted to the IAA production data. The maize growth promotion data were analyzed using one-way ANOVA by a mixed linear model with normal distribution. Significant differences between means were determined by the Tukey HSD method at 5% significance.

The mortality data of *S. frugiperda* were adjusted to a generalized mixed linear model with binomial distribution and logit link function. The Tukey HSD contrast test separated the caterpillar mortality means at 5% significance. The Weibull model estimated the median lethal time (LT50) of the caterpillars. The probability of survival for the fall armyworm was monitored up to 10 days post-infestation and analyzed with the non-parametric Kaplan-Meier method. Survival curves were compared by the log-rank test and with *P*-value adjusted with Bonferroni-Holm.

In vitro antagonism by paired-colonies of *F. graminearum* and *E. turcicum* were analyzed with a linear mixed model fit by REML (“lmerMod”) and analysis of Deviance Table (Type II Wald chi-square tests). Means of both tests were compared with the Tukey HSD test at $P < 0.05$.

4. RESULTS

4.1. Screening isolates of the *Metarhizium* spp. for conidia production

We found a remarkable variation in conidia yield between the 21 isolates of different *Metarhizium* species ($\chi^2 = 204.04$, $df = 20$, $p < 0.05$). Conidia yield varies from 2.7×10^7 to 4.16×10^9 conidia per gram of moist rice. The majority of the isolates produced above 1×10^8 conidia/gr of moist rice after ten days of incubation. *M. anisopliae* isolate ESALQ4133 was the best producer attaining concentrations up to 2.8×10^9 conidia/ gr of moist rice, not differing statistically from *M. humberii* ESALQ1781 and ESALQ4925, *M. anisopliae* ESALQ1669 and ESALQ1641 (Fig. 4). Conversely, the *M. robertsii* ESALQ4818 was the worst producer, attaining concentrations of up to 6.5×10^7 conidia/ gr of moist rice. Despite conidia formation in the PDA medium, the *M. robertsii* ESALQ2364, and *M. humberii* ESALQ3715, ESALQ1657, and ESALQ4764 did not sporulate on rice.

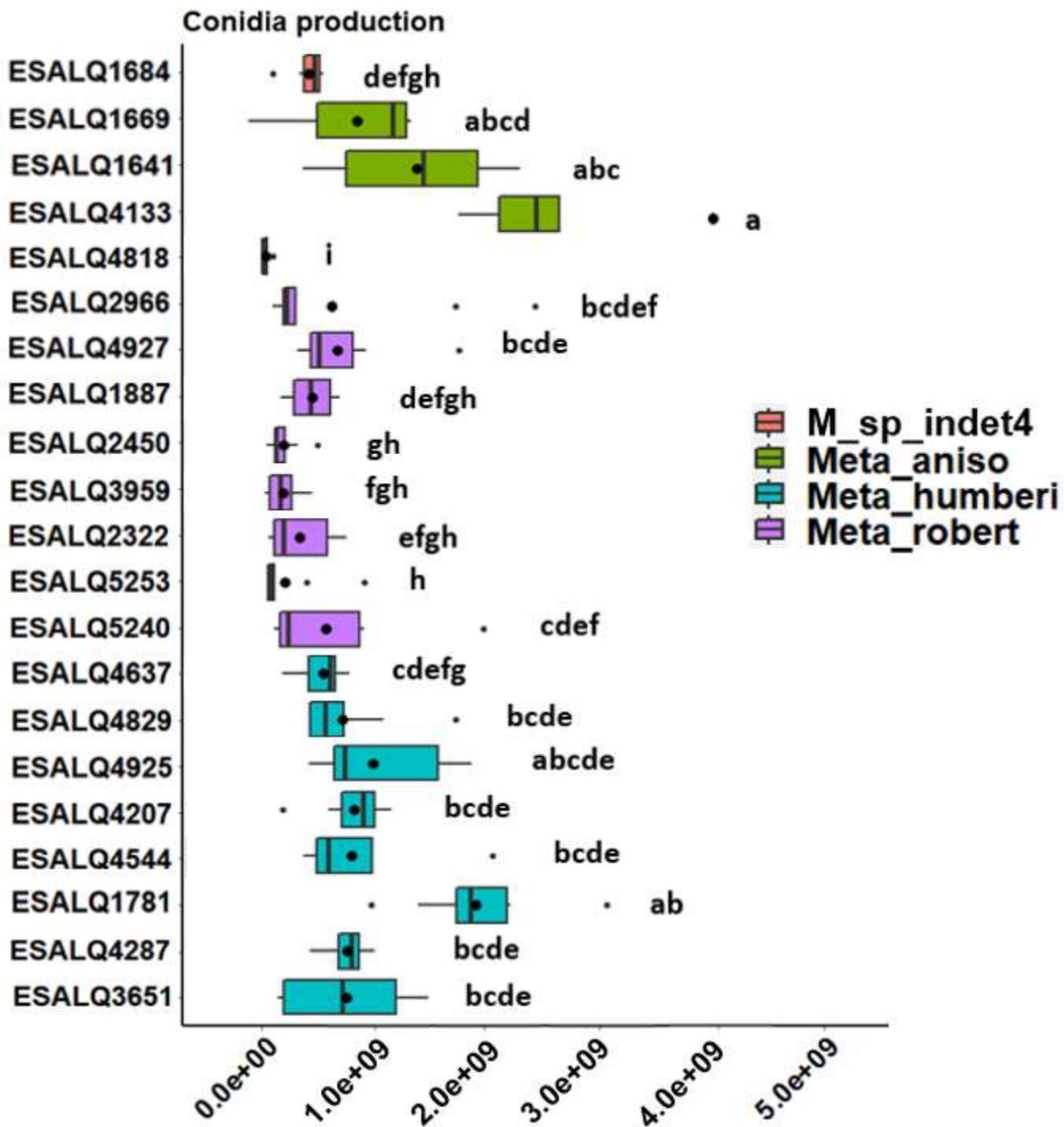


Figure 4. Conidia yield of 21 *Metarhizium* isolates grown on rice and incubated at 26 °C, 12h photoperiod during ten days. M_sp_indet4 = *Metarhizium* sp. indeterminate 4; Meta_aniso = *Metarhizium anisopliae*, Meta_humberi = *Metarhizium humberi*, Meta_robert = *Metarhizium robertsii*. Means (\pm SE) followed by different letters indicate significant differences according to Tukey's HSD test ($P < 0.05$). Boxes show the median, 25th, and 75th percentiles, error bars show 10th and 90th percentiles. A black dot (.) denotes the mean value of the data. The results illustrate data from three repetitions at different times.

We found a significant difference in conidia yield by species of *Metarhizium* ($\chi^2 = 211.19$, $df = 3$, $p < 0.05$) (Fig. 5). The highest conidia production was found for *M. anisopliae* isolates (average production of three isolates = 1.73×10^9 conidia/g of moist

rice). *M. sp. indet. 4* ESALQ1684 isolate (4.32×10^8 conidia/g of moist rice) did not differ from both *M. humberi* isolates (average production of eight isolates = 8.71×10^8 conidia/g of moist rice) and *M. robertsii* (average production of nine isolates = 3.42×10^8 conidia/g of moist rice) isolates.

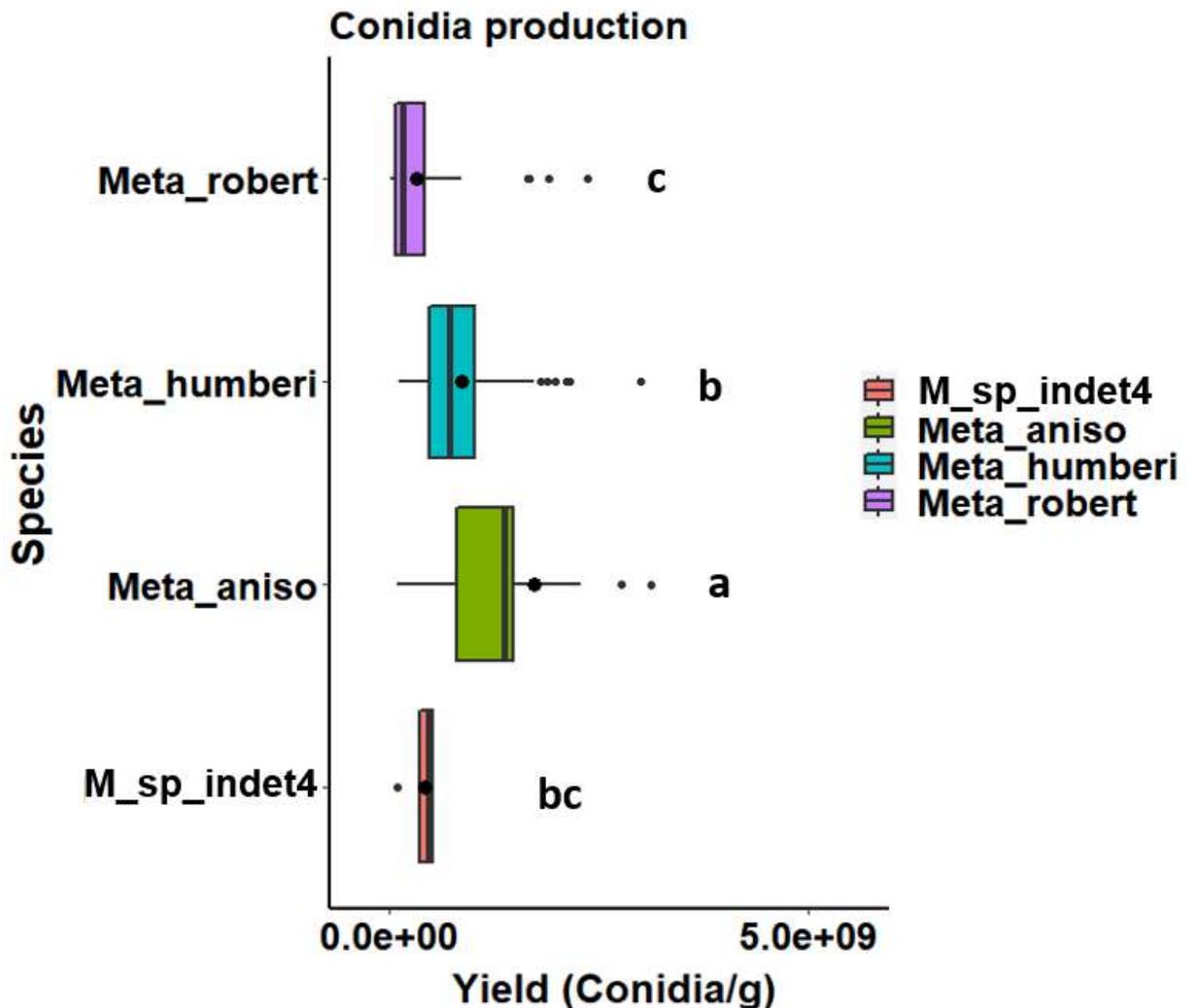


Figure 5. Conidia yield of *Metarhizium* spp. Isolates grown on rice and incubated at 26 °C, 12h photoperiod during ten days. M_sp_indet4 = *Metarhizium* species indeterminate 4; Meta_aniso = *Metarhizium anisopliae*, Meta_humberi= *Metarhizium humberi*, Meta_robert = *Metarhizium robertsii*. Means (\pm SE) followed by different letters indicate significant differences according to Tukey's HSD test ($P < 0.05$). Boxes show the median, 25th, and 75th percentiles, error bars show 10th and 90th percentiles. A black dot (.) denotes the mean value of the data. The results illustrate data from three repetitions at different times.

4.2. Production of indole-3-acetic acid (IAA)

Here we analyzed indole-3-acetic acid production by 21 isolates previously cultivated on rice (Fig. 6). We found no significant difference in IAA production among isolates ($\chi^2 = 32.968$, $df = 21$, $p < 0.05$). When isolates were compared by species, it was observed that the *M. sp. indet. 4* was superior to the other species ($\chi^2 = 19.218$, $df = 4$, $p < 0.05$). IAA production for *M. sp. Indet. 4* ESALQ1684 was 0.3837 ± 0.0725 $\mu\text{g/mL}$, whereas *M. anisopliae* ESALQ1669 produced 0.0093 ± 0.0005 $\mu\text{g/mL}$ and *T. harzianum* isolate 0.0307 ± 0.0068 $\mu\text{g/mL}$.

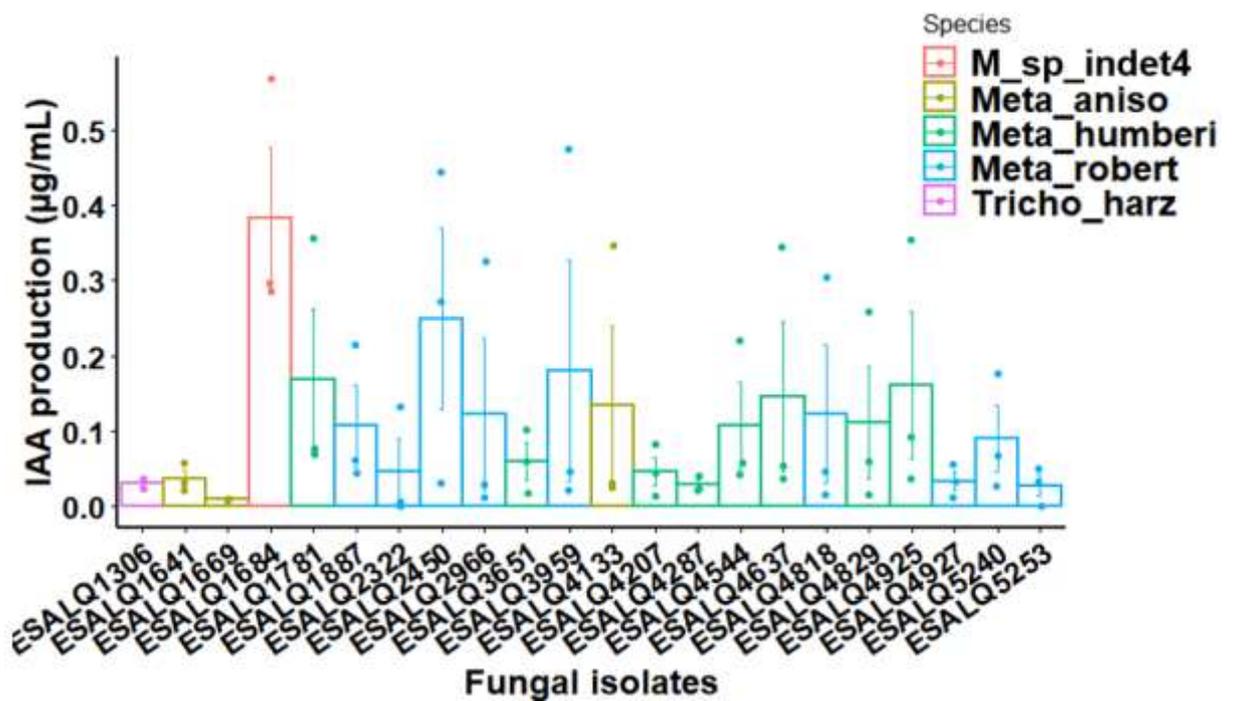


Figure 6. Production (Mean \pm SE) of indole-3-acetic acid ($\mu\text{g/mL}$) by isolates of *Metarhizium* spp. and *Trichoderma harzianum*. *M_sp_indet4* = *Metarhizium* sp. indeterminate 4; *Meta_aniso* = *Metarhizium anisopliae*, *Meta_humberi* = *Metarhizium humberi*, *Meta_robert* = *Metarhizium robertsii*, *Tricho_harz* = *Trichoderma harzianum*.). The results illustrate data from three repetitions on different times.

Table 2. IAA production (Means \pm SE). by *Metarhizium* spp. Isolates. Different letters in group columns indicate significant differences according to Tukey's HSD test ($P < 0.05$).

Fungal species	Mean production of IAA ($\mu\text{g/mL}$)	Lower Confidence Level (LCL)	Upper Confidence Level (UCL)	Group
<i>Metarhizium</i> sp. indet. 4	0.3837 \pm 0.0725	0.2372	0.530	a
<i>Metarhizium robertsii</i>	0.1090 \pm 0.0337	0.0122	0.206	b
<i>Metarhizium humberi</i>	0.1036 \pm 0.0347	0.0084	0.199	b
<i>Metarhizium anisopliae</i>	0.0601 \pm 0.0466	-0.0409	0.161	b
<i>Trichoderma harzianum</i>	0.0307 \pm 0.0725	-0.1157	0.177	b

4.3. Effects of *Metarhizium* spp. on maize plant traits treated by seed inoculation with conidia

Here we evaluated four phenotypic traits as indicators of growth promotion on maize plants inoculated with eight *Metarhizium* isolates. We found significant differences between isolates in all of them: plant height ($\chi^2 = 11.77$, $df = 8$, $p < 0.05$), stem diameter ($\chi^2 = 106.64$, $df = 8$, $p < 0.05$) aerial part dry weight ($\chi^2 = 0.45$, $df = 8$, $p < 0.05$) and root dry weight ($\chi^2 = 0.11$, $df = 8$, $p < 0.05$). However, none of the isolates differed from the control, except *M. humberi* ESALQ1781, which had lower plant weight and aerial part dry weight than the control. The plant height of maize inoculated with *M. robertsii* ESALQ3959 (34.6 cm) and ESALQ2450 (34.2 cm) was higher than treatments with isolates ESALQ1781, ESALQ5253, and ESALQ1669. On the other hand, maize plants inoculated with *M. humberi* ESALQ1781 had the lowest height (22.9 cm), differing significantly from all isolates and the control (Fig 7A).

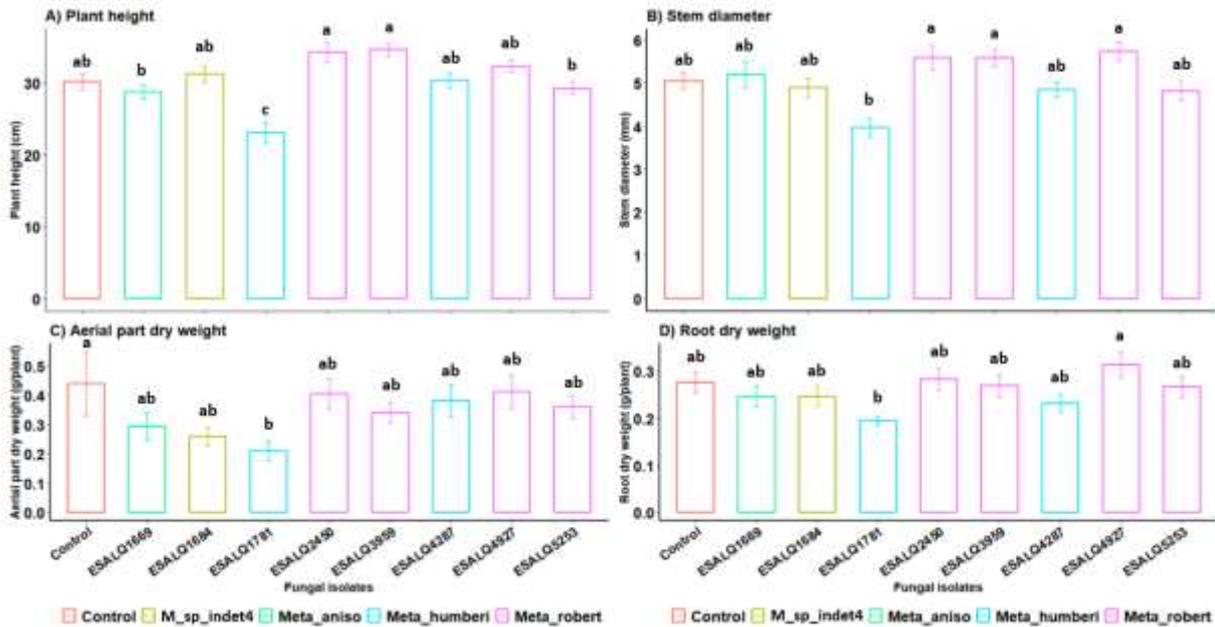


Figure 7. Phenotypic traits of maize plants 30 days after inoculation with *Metarhizium* spp. A) Plant height (cm); B) Stem diameter (mm); C) Aerial part dry weight (g/plant); D) Root dry weight (g/plant) M_sp_indet4 = *Metarhizium* sp. indeterminate 4; Meta_aniso = *Metarhizium anisopliae*, Meta_humberi = *Metarhizium humberi*, Meta_robert = *Metarhizium robertsii*. Means of three independent biological repetitions. Means (\pm SE) followed by different letters, indicate significant differences according to Tukey's HSD test ($P < 0.05$).

The stem diameters of maize inoculated with *M. robertsii* isolates ESALQ4927 (5.74 mm), ESALQ3959 (5.59 mm), and ESALQ2450 (4.85 mm) (Fig. 7B) were higher only compared with *M. humberi* (ESALQ1781) (3.44 mm). The aerial part dry weight (Fig. 7C) of *M. humberi* (ESALQ1781) was lower only than (0.18 ± 0.03 g) the control (0.39 ± 0.04 g) with half of the weight. *M. humberi* ESALQ1781 also had lower root dry weight (Fig. 7D) (0.19 ± 0.02 g), differing statistically only from *M. robertsii* ESALQ4927 (0.30 ± 0.02 g).

4.4 Endophytic colonization

A total of 1197 *Metarhizium* spp. CFUs. Of these, 12 and 1185 were recovered from fragmented and macerated roots respectively regardless of culture medium. We found a significant effect of media ($\chi^2 = 1494.1$, $df = 1$, $p < 0.05$), method (fragmented or macerated roots) ($\chi^2 = 773.4$, $df = 1$, $p < 0.05$), *Metarhizium* isolates ($\chi^2 = 1494.1$, $df = 11$, $p < 0.05$), interaction between media x isolates ($\chi^2 =$

173.1, $df = 11$, $p < 0.05$); isolates \times methods ($\chi^2 = 144.7$, $df = 11$, $p < 0.05$) and media \times methods ($\chi^2 = 97.7$, $df = 1$, $p < 0.05$) on the number of CFU recovered.

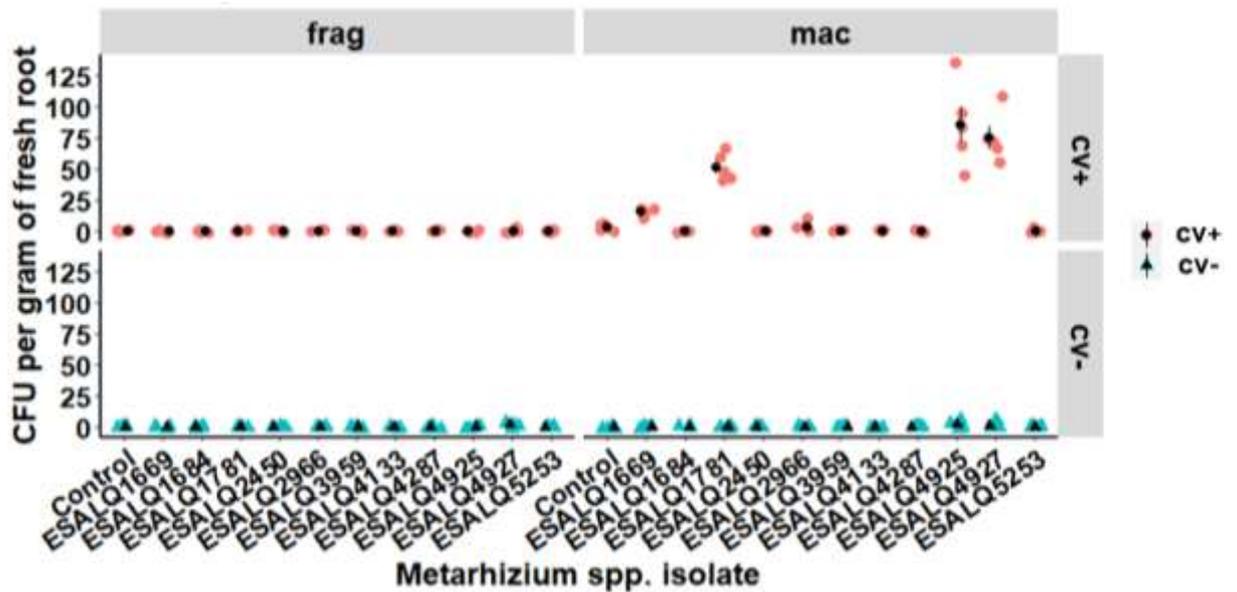


Figure 8. Colony Forming Units (CFU) of *Metarhizium* spp per gram of fresh maize root, recovered using two selective media (medium without crystal violet (= cv-) and medium with crystal violet (= cv+)) and two plating method (mac = macerated and frag = fragmented). Black triangles and circles represent the CFU average observed on five plates in dodine and crystal violet medium, respectively, per isolate and plating method.

The best method and medium to recover *Metarhizium* isolates seem to be macerated roots and medium with crystal violet. Indeed, we found a statistically higher number of CFU in roots inoculated with *M. robertsii* ESALQ4925, ESALQ1781, and *M. humeri* ESALQ4927 compared to the *M. robertsii* ESALQ5253 and the control by using macerated roots and medium with violet crystal (Table 3). The 12 CFU from fragmented roots were recovered from plants inoculated with *M. robertsii* ESALQ4927 isolate ($n = 12$ CFU). However, there was no statistical difference in CFU numbers between isolates considering this method regardless of medium. Nonetheless, we found no statistical difference in CFU numbers recovered from the medium without crystal violet between isolates irrespective of the method. No colonies were recovered from roots inoculated with *Metarhizium* sp. indet. 4 ESALQ1684, *M. anisopliae* ESALQ4133, ESALQ2450, ESALQ4287, and ESALQ3959 regardless of media and method (Fig. 8).

Table 3. Colony Forming Units (CFU) of *Metarhizium* isolates per gram of fresh maize root, recovered from macerated roots using the medium with crystal violet. CFU (means \pm SE), Lower and Upper Confidence Level. Group columns followed by different letters indicate significant differences according to Tukey's HSD test ($P < 0.05$).

Fungal isolates	CFU per gram of fresh root mean (\pm SE)	Lower Confidence Level (LCL)	Upper Confidence Level (UCL)	Group
<i>Metarhizium robertsii</i> ESALQ4925	51.0 \pm 32.1	14.8	175.3	a
<i>Metarhizium robertsii</i> ESALQ1781	43.8 \pm 19.5	18.3	104.9	a
<i>Metarhizium humberi</i> ESALQ4927	38.1 \pm 17.0	15.9	91.4	a
<i>Metarhizium anisopliae</i> ESALQ1669	15.6 \pm 9.9	4.5	54.34	ab
Control	3.0 \pm 2.0	0.8	11.3	bc
<i>Metarhizium robertsii</i> ESALQ5253	0.6 \pm 0.5	0.1	3.2	c

4.5 Effects of seed inoculation with *Metarhizium* spp. on *S. frugiperda* mortality fed on maize plants

All fungal isolates effectively reduced the survival likelihood of *S. frugiperda* larvae after seven days fed to inoculated maize plants compared to control uninoculated plants ($\chi^2 = 36.42$, $df = 7$, $p < 0.05$). Larvae survival varies from 35 and 70% in all fungal treatments than a nearly 85% survival found in the control (Fig 9). Remarkably, the isolates ESALQ1781, ESALQ2966 and ESALQ4925 were the most effective against *S. frugiperda* larvae with estimated median lethal time (LT_{50}) of 5,4; 5,5 and 6 days (Table 4) respectively, and mortalities ranged from 57 to 65% at day seven. Simultaneously, in the control, we recorded only 10% of mortality on the same day. The caterpillar cadavers were transferred to a humid chamber, and after 7-10 days, there was no *Metarhizium* growth.

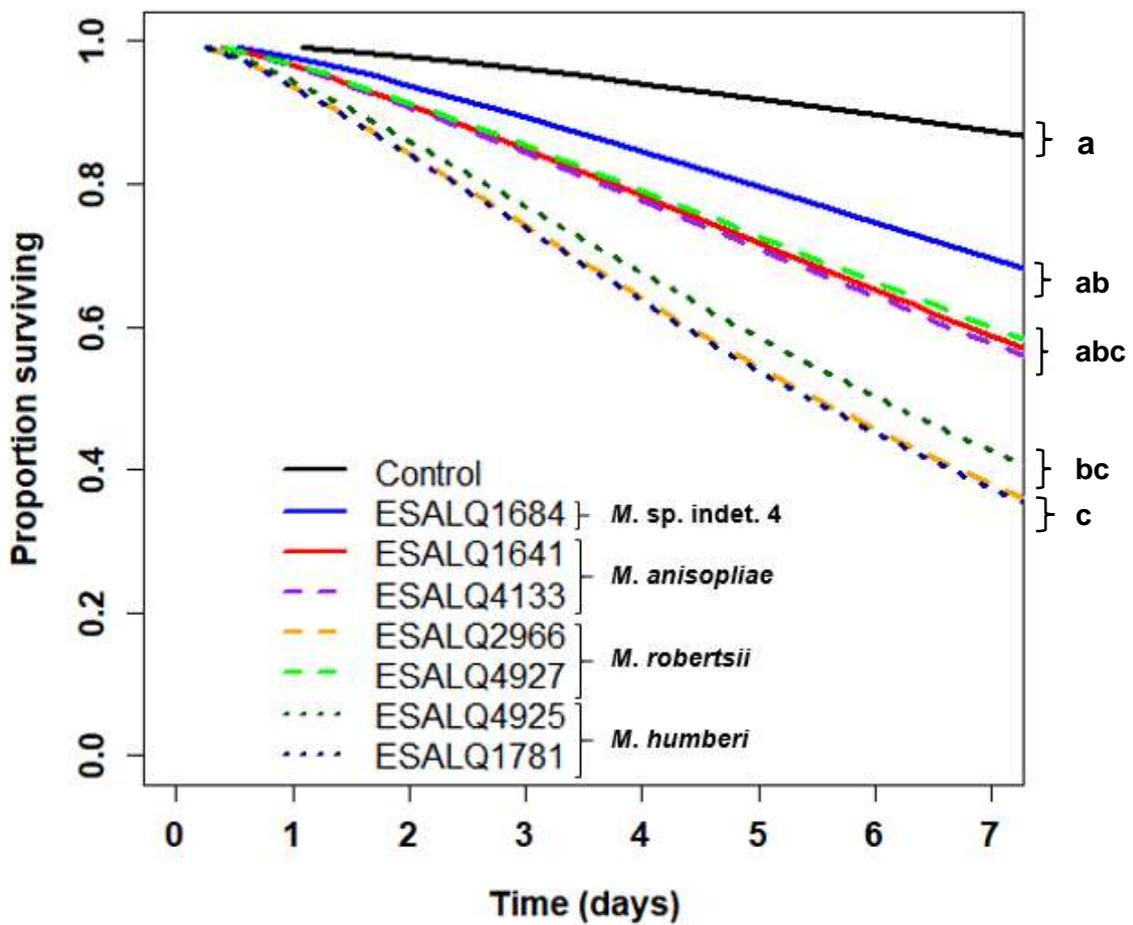


Figure 9. = Survival curves of 3rd instar *Spodoptera frugiperda* fed on maize plants inoculated with *Metarhizium* sp. indeterminate 4, *Metarhizium anisopliae*, *Metarhizium humberi*, and *Metarhizium robertsii* isolates and untreated plant. Distinct letters indicate significant differences between survival curves, according to the log-likelihood ratio test ($P < 0.05$). The results illustrate data from two repetitions at different times.

Table 4. Median lethal time (LT₅₀), in days, of *Spodoptera frugiperda* larvae fed with *Metarhizium* spp. inoculated maize plants. LT₅₀, Lower and Upper Confidence Level estimated by Weibull model evaluated for a survival curve.

Fungal isolates	LT ₅₀ = Median Lethal Time (days)	Lower Confidence Level (LCL)	Upper Confidence Level (UCL)
ESALQ1781	5.437595	4.099033	7.169679
ESALQ2966	5.497267	4.119621	7.428795
ESALQ4925	6.031603	4.498224	8.074735
ESALQ4133	8.287793	5.850863	11.7182
ESALQ1641	8.489102	5.912063	12.1342
ESALQ4927	8.735856	6.06609	12.64391
ESALQ1684	11.15941	7.384087	16.9346

4.6 Phytopathogen antagonism

The experiment was performed to determine whether and to which extent *Metarhizium* isolates inhibit the growth of the phytopathogens *Exserohilum turcicum* and *Fusarium graminearum* on PDA plates. As a positive control, we used the fast-growing fungus *Trichoderma harzianum* (ESALQ1306), a well-known antagonist to several phytopathogens. We found a significant inhibitory effect of isolates ($\chi^2 = 171.18$, $df = 6$, $p < 0.05$), time ($\chi^2 = 1244.59$, $df = 3$, $p < 0.05$), and interaction between treatment and time ($\chi^2 = 217.20$, $df = 12$, $p < 0.05$), on the growth of *F. graminearum* (Fig. 10 and 13A). The antagonist of *Metarhizium* to *F. graminearum* varied from 0% at day five for isolates ESALQ1641 and ESALQ1781 to 37% at day ten for isolate ESALQ4133 (Fig.13A). On day five, the *T. harzianum* isolate ESALQ1306 showed a higher inhibitory effect (27%) and was the only isolate that differs statistically from the others (Fig. 13A). On day seven, we noticed a higher difference in inhibitory effect between isolates than day five, being ESALQ1306 the most antagonist, with 27% of inhibition followed by ESALQ4133 (15%) the less antagonist, ESALQ1641 with only 7% of inhibition. The inhibitory effect of *T. harzianum* was kept at the same percentage from day five to day ten, remaining static during the ten days of the experiment, considering that this fungus covered almost all the plates on day five. On day ten, the isolates ESALQ4133, ESALQ2966, ESALQ4927 showed a higher inhibitory effect, being the best antagonists on this date.

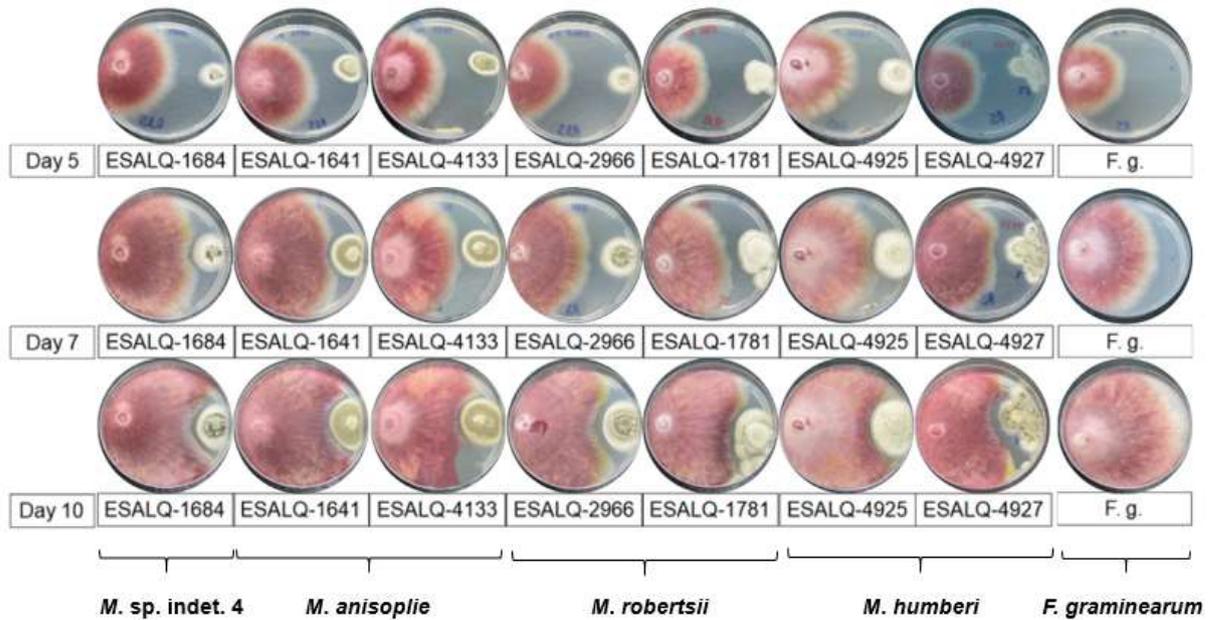


Figure 10. Antagonism effect of *Metarhizium* spp. (on right side of the plate) on mycelial growth of *Fusarium graminearum* (F. g.) (on the left side of the plate) in PDA medium (Difco®) at day five, seven, and ten after incubation at 25°C, 70% RH, and 12h photoperiod.

As expected, *T. harzianum* ESALQ1306 had an excellent antagonism against *E. turcicum*, as it has grown through all the plates (100% of inhibitory effect) in just five days. The antagonistic effect of *Metarhizium* spp. against this phytopathogen was less evident. We found a significant effect of isolates ($\chi^2 = 4378.62$, $df = 6$, $p < 0.05$), time ($\chi^2 = 7.33$, $df = 2$, $p < 0.05$), and interaction between treatment and time ($\chi^2 = 7.09$, $df = 12$, $p < 0.05$), on inhibitory effect on the growth of *E. turcicum* (Fig. 11 and 12). Considering only *Metarhizium* isolates, the antagonist effect on the growth of *E. turcicum* varied from 1% at day five for *M. humberi* ESALQ4925 to 18% at day 10 for *M. anisopliae* ESALQ4133. On day five, we found no statistical differences in the inhibitory effect on *E. turcicum* between *Metarhizium* spp. isolates (Fig. 13B) while on day seven, the differences were more evident, being ESALQ4133 the best antagonist (not differing from ESALQ1641 and ESALQ1781), with 15% of inhibition, and ESALQ1641 presented less antagonism, with only 7% of inhibition. On day ten, we found no statistical differences in the inhibitory effect of *Metarhizium* sp. isolates against *E. turcicum*.

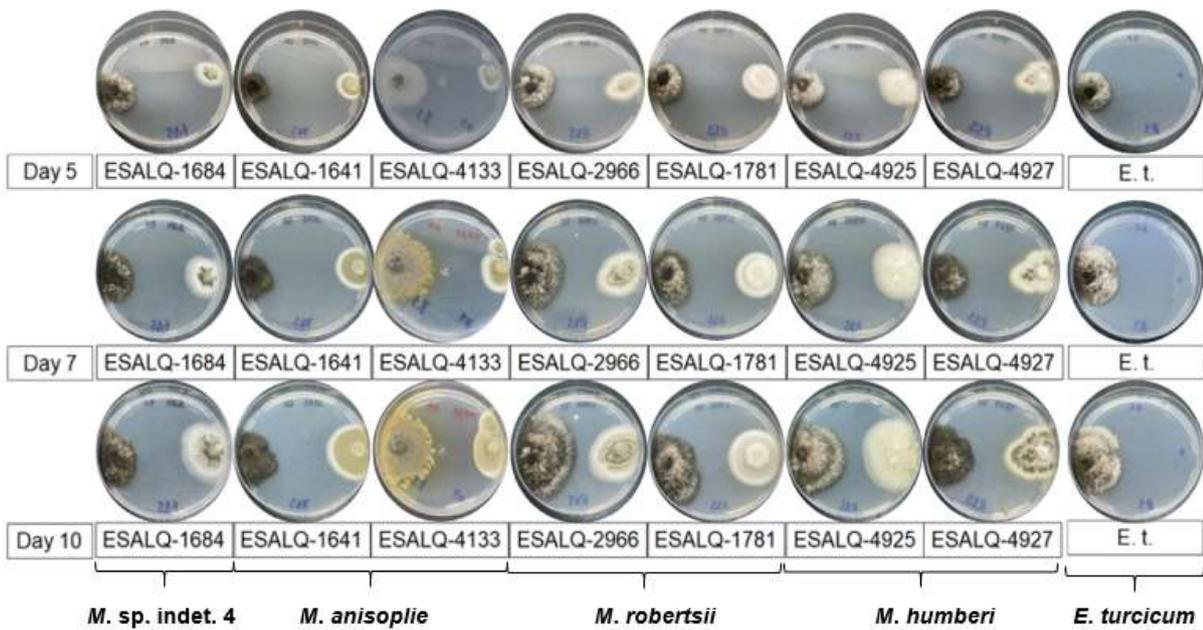


Figure 11. Antagonism effect of *Metarhizium* spp. (on right side of the plate) on mycelium growth of *Exserohilum turcicum* (E. t.) (on the left side of the plate) in PDA medium (Difco®) at day five, seven, and ten after incubation at 25°C, 70% RH, and 12h photoperiod.

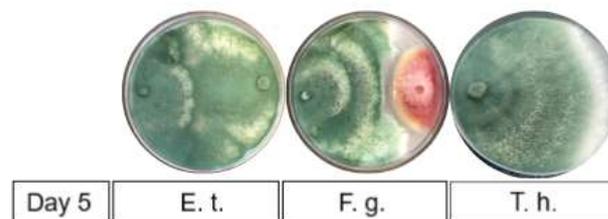


Figure 12. Antagonism effect of *Trichoderma harzianum* (T. h.) on mycelial growth of *Exserohilum turcicum* (E. t.) and *Fusarium graminearum* (F. g.) in PDA medium (Difco®) at fifth day after incubated at 25°C and 70% RH.

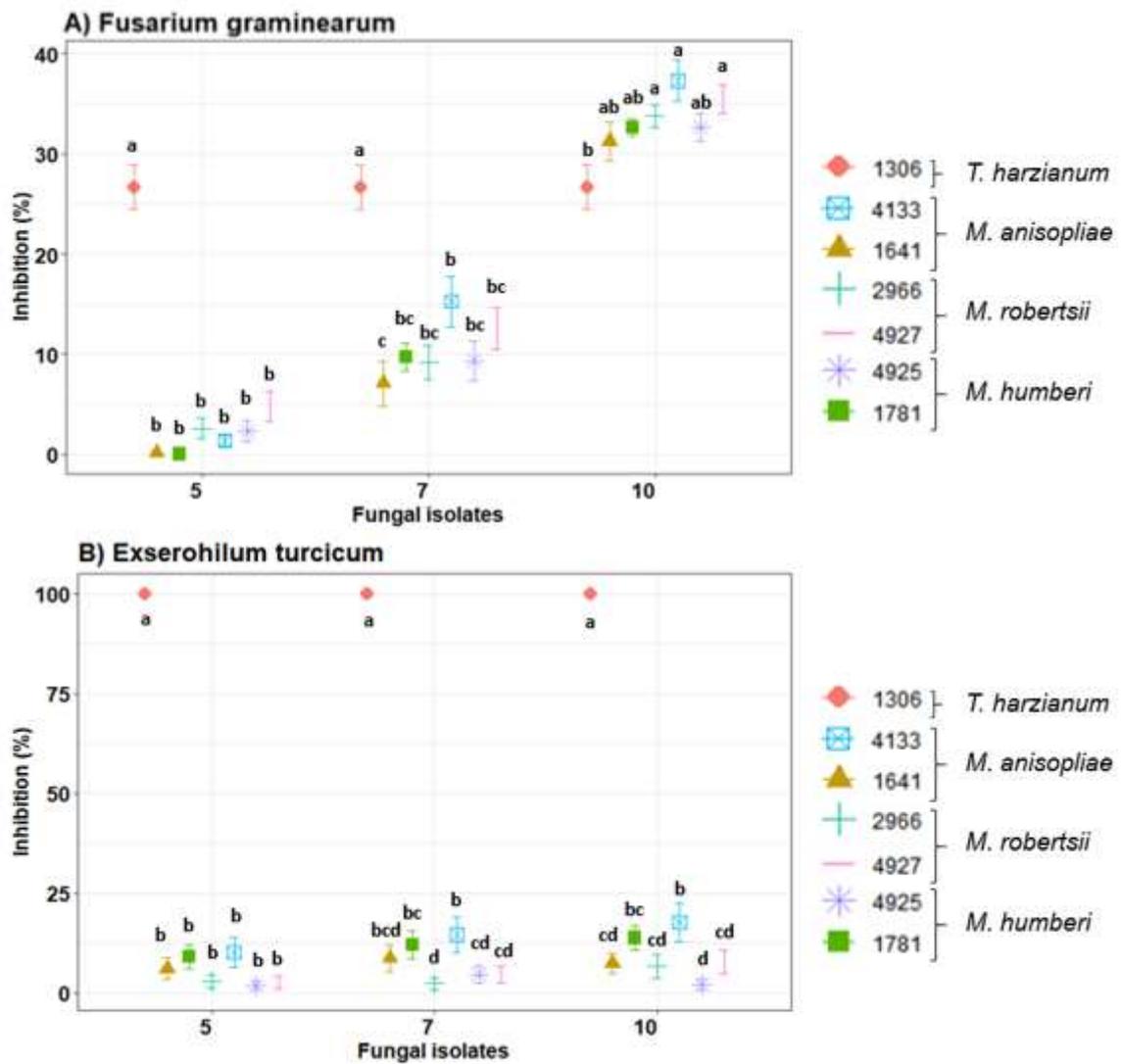


Figure 13. Antagonism of *Metarhizium* spp. on mycelial growth of two maize pathogens, A) *Fusarium graminearum* (F. g.) and B) *Exserohilum turcicum* (E. t.) grown on PDA medium (Difco®) and incubated at 25°C and 70% RH. The evaluation was carried on day 5, 7 and 10. *Trichoderma harzianum* (ESALQ1306) was used as a positive control. Averages followed by the same letter do not differ according to the Tukey test ($p \leq 0.05$).

5. DISCUSSION

This study investigated the biostimulant effect of *Metarhizium* species on maize growth parameters and the antagonist effects against *S. frugiperda* fed on maize plants grown from conidia-inoculated seeds and the in vitro effect on two maize phytopathogens, *F. graminearum*, and *E. turcicum*. We showed that *M. humberi* ESALQ1781 and ESALQ4925 and *M. robertsii* ESALQ2966 reduced in 65% *S. frugiperda* survival when fed on maize plants from inoculated seeds while those fed on untreated plants has its survival reduced by only 10%. Similar results were reported by De Lira et al. (2020) that noticed 55% of *S. frugiperda* mortality when fed on maize plants from seed inoculated with *Metarhizium* microsclerotia. This observed reduction in *S. frugiperda* survival suggests an indirect defensive strategy against herbivory modulated by *Metarhizium* spp, probably associated with mechanisms of plant defense responses.

Additionally, no *Metarhizium* sporulation was observed on the dead larvae. The absence of mycoses in dead insects that fed on plants inoculated with entomopathogens have been extensively reported in the literature (CHERRY et al., 2005; AKUTSE et al., 2013; CASTILLO LOPEZ and SWORD, 2015; MANTZOUKAS; CHONDROGIANNIS, GRAMMATIKOPOULOS, 2015), indicating that toxic biochemical compound(s) produced by the plant, by fungi or by both may be involved in mortality of *S. frugiperda* instead of fungi propagules.

Although it is well-documented in literature the ability of entomopathogenic fungi to secrete metabolites with harmful effects against insects such as dextrusins, beauvericin, and oosporins (AMIRI; IBRAHIM, BUTT, 1999; CHERRY et al., 2005; GOLO et al., 2014; TAIBON et al., 2015; RESQUÍN-ROMERO et al., 2016; LEFORT et al., 2016; GARRIDO-JURADO et al., 2017) it remains to be elucidated whether these metabolites are present in maize leaves and in which concentration that could lead to high mortality of *S. frugiperda*. Another approach would be to investigate metabolites produced by maize plants that could be harmful to insects. Although we determined that *Metarhizium* spp application through seed inoculation is a promising strategy for *S. frugiperda* control, its use as biostimulants was not demonstrated at conditions reported in this study.

The maize plants grown from seed inoculation with *M. robertsii*, *M. anisopliae*, *M. sp.* indet. 4, and *M. humberi* isolates did not enhanced growth traits compared to

untreated control plants. These results differed from that observed by De Lira et al. (2020) in seed-inoculated maize plants with microsclerotia of *Metarhizium* spp. The authors found that three isolates, one of each fungus: *M. robertsii*, *M. anisopliae*, and *M. humberi*, improved at least one of the growth parameters: leaf area, plant height, root length, and dry weight of plants compared to untreated plants. The results observed here for the growth parameters of corn plants can be explained in part by genetic diversity between isolates, plant cultivar, and inoculation methods.

De Lira et al. (2020) used dried granules of microsclerotia mixed with hydrogel, a crosslinked polyacrylic acid homopolymer, to effectively attach, coat the maize seed, and provide high humidity necessary for conidia production and germination. In our case, conidia suspension was poured on top of each seed, which may have increased the chances of losses due to the dispersion of conidia suspension to soil after sowing and lixiviation after watering before establishing primary roots. Therefore, the low concentration of inocula in contact with seeds and roots, necessary for establishing a symbiotic relationship and promoting maize growth, could explain the lack of biostimulation. These hypotheses may also explain why we found no correlation between IAA production by *Metarhizium* spp. *in vitro* and plant growth promotion.

We determined that all *Metarhizium* isolates investigated in this study produced IAA *in vitro* at different concentrations. *Metarhizium* sp. Indet. 4 was one of the best IAA producers, which led us to hypothesize that plants inoculated with *M. sp. indet. 4* would have more outstanding plant parameters than other *Metarhizium*-inoculated and untreated plants. However, this hypothesis has not been validated here. The IAA *in vitro* production observed for *Metarhizium* sp. indet. 4 in this study were was up to four times greater than concentrations found by Siqueira et al. (2020) (up to 0.1µg/mL). These authors also demonstrated that Brazilian *M. robertsii*, and *M. humberi* produced IAA in tomato plants.

Additionally, these authors showed that the number of flowers and the fresh weight of fruits in tomato plants was significantly increased by *M. robertsii* and *M. humberi* conidia-inoculated in tomato seedling. We did not find a correlation between IAA production *in vitro* and the biostimulant effect in maize plants. This may be related to the low concentration of inocula in contact with seeds and roots and the maize cultivar. Canassa et al. (2019) inoculated seedlings of two strawberry cultivars with *Metarhizium* spp. and *Beauveria bassiana* isolates and showed that, for one cultivar, several plant growth parameters were improved while for the other cultivar, only one

isolate promotes an increase in dry weight of roots. In our case, the maize hybrid maybe not responsive to fungal stimulus.

The genetic diversity between isolates plays a role in the different responses of inoculated plants. Maize plants inoculated with the *M. humberii* ESALQ1781 isolate had statistically lower plant height and less aerial part dry weight than untreated plants. Interestingly ESALQ1781 was one of the most promising isolates against *S. frugiperda*, which indicates that the increased plant metabolism to resist herbivory may have negatively affected plant growth, the trade-off strategy. These xenobiotic compounds produced by fungus may trigger the plants' detoxification systems displacing energy on this process rather than investing in biomass accumulation and growth, explaining lower plant height and less aerial part dry weight than untreated plants found in ESALQ1781 inoculated-plants.

A detailed analysis of bean and maize plant colonization patterns showed that, as an endophyte, *Metarhizium* preferentially colonized the hypocotyl. In the context of rhizoplane colonization, *Metarhizium* was found most prevalently in regions proximal to the hypocotyl (LAHEY et al. 2020). This may explain why macerated plants performed better than inoculated fragments in the dodine and crystal violet culture medium, providing a useful method for future studies.

Although mortality of *S. frugiperda* and beneficial effects on plants inoculated by the fungus were observed, endophytic colonization of *Metarhizium* in plant tissues 30 days after the inoculation of maize seeds was only confirmed in some isolates by the macerated method. Studies indicate that the endophytic action of entomopathogen and the re-isolation of the fungus from plant tissues are closely linked to the host plant species and the inoculated fungus, and the amount of this fungus is decreasing over time (AKUTSE et al., 2013; RUSSO et al., 2015; PARSA et al., 2016).

Similarly, Mutune et al. (2016) could not detect *Metarhizium* colonization in bean plants via seed treatment for conidia-inoculated. However, the fungus inoculation in these plants resulted in a population decrease in *Ophiomyia phaseoli*, a Dipteran pest of shoots. In the present work, the use of molecular techniques would be recommendable for detecting fungi not detected by the culturing method.

The second part of the experiments was conducted to study *Metarhizium* spp. antagonism against fungi pathogenic to maize crop. The results showed that all *Metarhizium* species tested were able to inhibit the growth of *E. turcicum* and *F. graminearum*, and the best species among the foreheads was *M. robertsii*, which

provided up to 54% inhibition for both phytopathogens. Only a few studies have been carried out to determine *Metarhizium* spp. antagonism against phytopathogens, including the species *M. anisopliae*, *M. robertsii*, *M. humberi*, *M. brunneum*, and *M. flavoviridae* (KEYSER et al. 2015; SASAN and BIDOCHKA, 2013; RIVAS-FRANCO et al. 2019; SIQUEIRA et al., 2020).

Li et al. (2016) evaluated the efficacy of seven *Trichoderma asperellum* strains against *Fusarium graminearum*, the causal agent of maize stalk rot of maize. Up to 71% disease reduction in inoculated maize plants was observed compared to the negative control. Effect of *Trichoderma harzianum* on maize rhizosphere microbiome and biocontrol of *Fusarium graminearum* in greenhouse experiments decreased 66% of disease and increased the plant growth (SARAVANAKUMAR et al., 2017). RIVAS-FRANCO et al. (2019) showed that seed coating with conidia of *Metarhizium anisopliae* resulted in up to 67% mycosis *Costelytra giveni* larvae and a reduction in *F. graminearum* symptoms around 44%. The hypothesis is that *Metarhizium* spp. enzymes degrade the cell walls of other fungi to compete with them. Further studies are needed to clarify the mechanisms involved in these interactions.

The lethal effect in *S. frugiperda* and antagonistic evaluation of phytopathogens indicate the potential use of isolates from *M. robertsii* (ESALQ3959, ESALQ4927) and *M. anisopliae* (ESALQ4133) via inoculation in seeds for plant protection. The benefits of *Metarhizium* spp. as a biostimulant suggest an alternative way to chemical insecticides or association with conventional control. The results obtained in the present study reinforce the need for further studies to investigate the endophytic colonization of the isolates under investigation, thus understanding the mechanisms involved in the relationship between plant, fungus, and insect.

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