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Development of a *Metarhizium*-based biostimulant for the sugarcane crop: effects on pests and crop yield

Ana Carolina Oliveira Siqueira

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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"Learning is the only thing that the mind never tires, never fears, and never regrets.

Leonardo da Vinci

SUMMARY

RESUMO

Desenvolvimento de um inoculante a base de *Metarhizium* para a cultura da cana-de-açúcar: efeitos na produtividade e pragas

O fungo entomopatogênico *Metarhizium anisopliae* tem sido usado com eficiência há décadas na cultura da cana-de-açúcar para o controle biológico de pragas. Algumas espécies de *Metarhizium* podem colonizar uma grande variedade de espécies de plantas endofiticamente, fornecendo proteção contra pragas de artrópodes, acelerando o desenvolvimento das plantas e agindo como antagonistas de fitopatógenos. O objetivo geral deste projeto foi avaliar o potencial de espécies de *Metarhizium* como inoculantes, visando promover o crescimento da cana-de-açúcar e, conseqüentemente, aumentar a produtividade das culturas e controlar importantes pragas de insetos e nematóides. Utilizando plantas de tomateiro (*Solanum lycopersicum* L.) da variedade “Micro-Tom” como modelo, conduzimos bioensaios *in vivo* e *in vitro* usando isolados brasileiros nativos de *Metarhizium robertsii*, *M. humberi* e *M. anisopliae* para compreender melhor os possíveis mecanismos relacionados a características de promoção de crescimento de plantas e colonização do sistema radicular. A produção de compostos que poderiam estar envolvidos na estimulação da promoção do crescimento vegetal foi avaliada *in vitro* pela medição da produção do hormônio auxina (ácido 3-indol-acético), fosfatase, fitase, sideróforos e quitinases. Os efeitos da inoculação da cana-de-açúcar com dois tipos de propágulos, duas concentrações de fungos e a inoculação combinada de duas espécies de fungos na promoção do crescimento das plantas foram avaliados em casa de vegetação. Os efeitos sobre as populações dos nematóides *Meloidogyne javanica* e *Pratylenchus zeae* foram avaliados *in vivo* e *in vitro*. Também avaliamos o desenvolvimento e a preferência alimentar do inseto *Diatraea saccharalis* em condições de laboratório. Por fim, foram realizados estudos de campo em cinco áreas comerciais de cana-de-açúcar em Iracemápolis-SP, Batatais-SP e Sertãozinho-SP. Plantas de cana-de-açúcar foram inoculadas e seu desenvolvimento e incidência de pragas foram avaliados até a colheita. Ensaios *in vivo* revelaram que *M. robertsii* (ESALQ 1635) ou *M. humberi* (ESALQ 1638) inoculados em mudas de tomate melhoraram as características vegetativas e reprodutivas. A inoculação com *M. robertsii* produziu plantas mais altas, raízes mais longas e mais massa seca de parte aérea e raiz do que *M. humberi*. As plantas inoculadas com *M. robertsii* ou *M. humberi* aumentaram a expressão de GUS induzida por auxina nas raízes por até 30 dias após a inoculação, confirmando que *Metarhizium* induz a expressão gênica regulada por auxina. As três espécies de *Metarhizium* cultivadas com ou sem triptofano exógeno produziram ácido indol-3-acético (IAA) em diferentes quantidades. Todos os isolados de *Metarhizium* secretaram fosfatases, fitases, sideróforos e quitinases. De particular importância, os isolados de *M. robertsii* e *M. humberi* exibiram perfis bioquímicos *in vitro* semelhantes, enquanto os isolados de *M. anisopliae* e *Trichoderma harzianum* demonstraram características distintas dos demais. Ambos os tipos de propágulos, conídios e blastosporos, de *M. robertsii* e *M. humberi*, colonizaram os tecidos de cana-de-açúcar; entretanto, maior colonização foi observada para conídios. A inoculação da cana-de-açúcar com suspensões 10^8 conídios / mL (correspondendo a uma aplicação de 10^{12} con / ha) de *M. robertsii* e *M. humberi* foi mais eficiente para aumentar o crescimento das plantas em comparação com 10^7 conídios / mL e o controle não inoculado. As inoculações de *M. robertsii* e *M. humberi* contribuíram para a redução de *M. javanica*

(até 45%), *P. zeae* (até 33%), em relação ao controle. A mortalidade de larvas de *D. saccharalis* em plantas colonizadas por *M. robertsii* e *M. humberi* foi de até 91,7% após 30 dias da inoculação. Em ensaios de escolha hospedeira, as larvas de *D. saccharalis* preferiram o controle não inoculado do que as plantas inoculadas com fungos. Nos estudos de campo, notavelmente, ambos os isolados de fungos aumentaram significativamente o comprimento do caule (em 4 de 5 locais) e aumentaram significativamente o volume do caule (m^3) em comparação com as parcelas do controle. Como resultado, o maior rendimento da cana-de-açúcar (toneladas / ha) foi alcançado com a inoculação de *Metarhizium*, principalmente nos locais A, D e E, e o lucro dos agricultores aumentou em até 22,9%. No geral, a inoculação de *Metarhizium* reduziu significativamente as principais pragas de insetos na maioria das parcelas testadas. Juntos, esses achados comprovam os múltiplos benefícios promovidos por *M. robertsii* e *M. humberi* quando empregados como bioinoculantes da cultura da cana-de-açúcar, aumentando a produtividade, bem como aliviando ataques de insetos-praga de raízes e caules. Os resultados promissores aqui obtidos culminaram com a solicitação de registro comercial de um produto baseado nos isolados de *M. robertsii* e *M. humberi* e trazem uma perspectiva única sobre a utilização de fungos endofíticos entomopatogênicos como bioestimulantes e biopesticidas.

Palavras-chave: Fungos endofíticos, Cana-de-açúcar, *Metarhizium*, Interação planta-inseto-fungo, Promoção de crescimento, Bioestimulante

ABSTRACT

Development of a *Metarhizium*-based biostimulant for the sugarcane crop: effects on pests and crop yield

The entomopathogenic fungus *Metarhizium anisopliae* has been used efficiently for decades in the sugarcane crops for biological pest control. Some *Metarhizium* species can colonize a wide variety of plant species endophytically, providing protection against arthropod pests, accelerating plant development, and acting as antagonists of phytopathogens. This project´s general objective was to evaluate the potential of *Metarhizium* species as inoculants, aiming to promote sugarcane growth and, consequently, increase crop production yields and control important insect and nematodes pests. We conducted *in vivo* and *in vitro* bioassays using native Brazilian isolates of *Metarhizium robertsii*, *M. humberi*, and *M. anisopliae* to better understand the possible mechanisms related to plant growth promotion traits and colonization of the root system in the model pot-grown tomato (*Solanum lycopersicum* L.) miniature cultivar 'Micro-Tom'. The production of compounds that could be involved in stimulating plant growth promotion was assessed *in vitro* by measuring the production of the hormone auxin (3-indole-acetic acid), phosphatase, phytase, siderophores, and chitinases. The effects of sugarcane inoculation using two types of propagules, two fungal concentrations, and the combined inoculation of two fungal species in promoting plant growth were evaluated at the greenhouse. Effects on populations of the nematode *Meloidogyne javanica* and *Pratylenchus zeae* were assessed *in vivo* and *in vitro*. We also evaluated the development and food preference of the insect *Diatraea saccharalis* under laboratory conditions. Finally, field studies were carried out in five commercial sugarcane areas in Iracemápolis-SP, Batatais-SP, and Sertãozinho-SP. Sugarcane plants were inoculated, and their development and pest incidence were evaluated until harvest. *In vivo* trials revealed that *M. robertsii* (ESALQ 1635) or *M. humberi* (ESALQ 1638) inoculated in tomato seedlings improved vegetative and reproductive traits. Inoculation with *M. robertsii* yielded taller plants, longer roots, and more shoot and root dry mass than *M. humberi*. Plants inoculated with either *M. robertsii* or *M. humberi* increased auxin-induced GUS expression in the roots for up to 30 days after inoculation, confirming that *Metarhizium* induces auxin-regulated gene expression. The three *Metarhizium* species grown with or without exogenous tryptophan could produce indole-3-acetic acid (IAA) at different titers. All *Metarhizium* isolates secreted phosphatases, phytases, siderophores, and chitinases. Of particular importance, the *M. robertsii* and *M. humberi* isolates exhibited similar *in vitro* biochemical profiles, whereas *M. anisopliae* and *Trichoderma harzianum* isolates demonstrated distinct traits from the others. Both types of propagules, conidia, and blastospores, of *M. robertsii* and *M. humberi,* colonized sugarcane tissues; however, higher colonization was observed for conidia. The inoculation of sugarcane with 10^8 conidia/mL (corresponding to an application of 10^{12} con/ha) conidia suspensions of *M. robertsii* and *M. humberi* was more efficient to increased plant growth compared to 10^7 conidia/mL and the uninoculated control. The inoculation *M. robertsii* and *M. humberi* contributed to reducing *M. javanica* (up to 45%), *P. zeae* (up to 33%), compared to the control. Mortality of *D. saccharalis* larvae on plants colonized of *M. robertsii* and *M. humberi* was as high as 91.7% after 30 days of inoculation. In host-choice assays, *D. saccharalis* larvae preferred the uninoculated control than the fungi inoculated plants. In the field studies, notably, both fungal isolates significantly enlarged stalk length (in

4 out of 5 locations) and markedly boosted stalk volume (m^3) in comparison to control plots. As a result, greater sugarcane yield (tons/ha) was achieved with *Metarhizium* inoculation, mainly at locations A, D, and E, and the farmers' profit was raised by up to 22.9%. Overall, *Metarhizium* inoculation significantly reduced key insect pests in most tested plots. Altogether, these findings prove the multiple benefits promoted by *M. robertsii* and *M. humberi* when employed as bio-inoculants of sugarcane crop by boosting yields as well as alleviating attacks of root and stalk insect pests. The promising results obtained here culminated in the request for commercial registration of a product based on the *M. robertsii* and *M. humberi* isolates and bring a unique perspective on the use of entomopathogenic endophytic fungus as biostimulants and biopesticide.

Keywords: Endophytic fungi, Sugarcane, *Metarhizium*, Plant-insect-fungus interaction, Growth promotion, Biostimulant

1. INTRODUCTION

1.1. Sugarcane crop

The sugarcane crop is a perennial plant, typical of tropical and subtropical climates, and belongs to the Poaceae family and the genus *Saccharum* (CLAYTON; DANIELS, 1975). The sugarcane cultivars currently cultivated are hybrids.

Sugarcane was introduced in Brazil in XV century being cultivated to this day. In 1975, the government created the Proálcool program to replace part of the imported oil and stimulate ethanol as biofuel. Besides, it launched credit lines stimulating sugarcane production and the expansion of the industrial plants (PLANALSUCAR, 1975). Currently, sugarcane occupies about 7.0 million hectares or about 2% of all arable land. It continues to grow, with the implantation of new areas in São Paulo, Minas Gerais, Goiás, Mato Grosso do Sul, Mato Grosso, and the North and Northeast of Brazil (ROSSETTO, 2008).

Sugarcane is a crop of extreme socioeconomic importance for Brazil. It has a large share in the GDP (Gross Domestic Product) and is responsible for approximately 25% of world production. The average Brazilian productivity in the 2020/2021 harvest was 76.4 kg/ha, resulting in the production of 39.3 million tons of sugar and 30.6 billion liters of ethanol, with the state of São Paulo being the largest Brazilian representative, both in area (4.230 million / ha) and in yield (335,554.5 TONS) (CONAB, 2020).

The sugarcane crop has undergone significant changes in this decade, both in technological and social spheres, in order to adapt to production demands with high productivity, competitiveness and respect for the environment. In this sense, State Decree-Law No. 42,056 / 9, which provides for the prohibition of the dispatch of cane to the industry by burning, meets the technological aspirations for the sustainable increase of the productivity of sugarcane in the State of São Paulo (ALMEIDA; BATISTA-FILHO; SANTOS, 2003).

The gradual elimination of the practice of using burning in the harvest of the raw material had, as a benefit, the improvement of the agroindustrial quality of the cane, and economic benefits, such as lower cultivation cost, improved water absorption, and soil conservation that contributes to increasing productivity (LEAL, et al.; 2008). On the other hand, pests previously considered of secondary importance had their population increased significantly (DINARDO-MIRANDA; FERREIRA, 2004).

1.2. Sugarcane pests

The sugarcane agro-ecosystem is home to numerous species that can cause severe economic losses. Among the main pests of this crop, we can mention the leafhopper complex (Hemiptera: Cercopidae), including *Mahanarva fimbriolata* (Stal, 1854) and *M. posticata* (Stal, 1855), *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae), *Telchin licus* (Drury, 1773) (Lepidoptera: Castniidae), *Sphenophorus levis* (Vaurie, 1978) (Coleoptera: Curculionidae), *Migdolus fryanus* (Westwood, 1863) (Coleoptera: Cerambycidae), termite complex (Blattodea order) of the genera *Syntermes* spp., *Nasutitermes* sp., *Neocapritermes* sp. and *Heterotermes* sp., leaf-cutting ants such as *Atta laevigata* (Fr. Smith, 1858) (Hymenoptera: Formicidae) and nematodes (*Meloidogyne javanica* and *M. incognita*).

Diatraea saccharalis is one of Brazil's most important sugarcane pests and is present in all regions where sugarcane is grown (DINARDO-MIRANDA, 2008). It's biological cycle lasts around two months, with a potential of four to five generations per year, depending on climatic conditions (GALLO et al., 2002). Right after hatching, the caterpillars feed on the leaf parenchyma, inside the leaf sheath. After the first ecdysis, they penetrate the softer part of the stem, puncturing it and opening galleries from the bottom up. In these galleries, they remain during the pupal phase, and when adults emerge, they seek new places for oviposition.

The damage caused by the caterpillars can be considered direct, through the opening of galleries, which lead to the death of the tillers and in young plants, drying of the pointers, which is popularly known as “dead heart,” and indirect damage when the holes favor the penetration of microorganisms. Pathogens inside the stem, especially the fungi *Colletotrichum falcatum* and *Fusarium moniliforme* that cause “red stem rot,” reducing the sugar content due to the inversion of the sucrose stored in the plant and contaminating the broth that hinders the fermentation process (BOTELHO; MACEDO, 2002; DINARDO-MIRANDA, 2008).

The nematode *M. javanica* penetrates the sugarcane root and begins to feed, inducing the formation of giant cells (hypertrophy) and multiplication of cells (hyperplasia), which are the known galls. The root system then becomes inefficient in absorbing water and nutrients, affecting the crop's development and production. Plants attacked by nematodes generally become more predisposed to attack by other pests

and diseases (ABAWI; CHEN, 1998). In Brazil, the losses caused by this pest are on the order of 20 to 30% reduction in production (DINARDO-MIRANDA, 2005).

1.3. A classic example of microbial control in sugarcane: the case of spittlebugs and *Metarhizium anisopliae*

The biological control of *M. posticata* with the fungus *Metarhizium* sp. began in 1969 (MARQUES et al., 1981). From 1970 to 1998, more than 40 tons of conidia were produced in Pernambuco state and applied to approximately 500,000 hectares of sugarcane. In Alagoas, about 670,000 hectares were sprayed with *Metarhizium* sp. from 1977 to 1991.

This entomopathogenic fungus is currently produced in Brazil almost exclusively, using autoclaved rice as a substrate and applied in several ways. Some producers wash the sporulated rice grains in water and apply the resulting syrup, while others directly distribute the rice grains in the infested places, practices that can be done even by plane (DINARDO-MIRANDA et al., 2004). Field tests proved the efficiency of some isolates in reducing the leafhoppers (BATISTA FILHO et al., 2003), reaching control values up to 80% (ALVES et al., 2007).

Due to the good handling that the fungus *M. anisopliae* has provided for sugarcane leafhoppers, extra official information estimated that in 2012 it was sprayed on approximately 2 million hectares. Taking into account the area cultivated with sugar cane in Brazil and still does not use the fungus, there is a great potential for increasing the application of this mycoinsecticide in the country.

The success of the control with this fungus can be expressed by the existence of almos 50 commercial products based on *M. anisopliae* registered for the management of the leafhopper and several farms that produce it in their laboratories for own consumption.

Despite the widespread use of *M. anisopliae* for the control of leafhoppers, little is known about the potential of other *Metarhizium* species as well as their endophytic colonization and effects on sugarcane growth promotion.

1.4. *Metarhizium* genus

Metarhizium (Hypocreales: Clavicipitaceae) species stands out as an important agent for the control of arthropods and have great importance in Brazil and the world, occurring naturally. It is naturally found in soils and it was reported infecting more than 300 insects species. its hability to colonize plant tissues was more recently discovered (ZIMMERMANN, 2007; BEHIE; ZELISKO; BIDOCHKA, 2012).

The *M. anisopliae* complex is composed of nine species that have been characterized using molecular techniques, with multi gene phylogeny that offer robust and reliable support. Bischoff, Rehner and Humber (2009) described two new species (*M. globosum* and *M. robertsii*), three varieties were raised to the species level (*M. majus*, *M. lepidiotae* and *M. acridum*), the name *M. brunneum* was reconsidered as a species and *M. guizhouense* was recognized as the anamorphic phase of *Metacordyceps taii*. Besides, two clades were recognized, PARB formed by the species *M. pingshaense*, *M. anisopliae*, *M. robertsii* and *M. brunneum* and MGT referred to as representing *M. majus* and *M. guizhouense* (= *M. taii*).

Seven species of this complex have already been identified in Brazil, using the region 5'-TEF (Translation Elongation Factor), which are *M. pingshaense*, *M. anisopliae ss*, *M. robertsii,* and *M. brunneum* belonging to the PARB clade and also, *M. lepidiotae*, *M. acridum* and *M. majus* (LOPES et al., 2013a, LOPES et al., 2013b, LOPES et al., 2014; REZENDE et al., 2015; ROCHA et al., 2013; ZANARDO, 2015). *M. pemphigi* (= *M. Flavoviride* var. Pemphigi), *M. rileyi,* and *M. brasiliense* also occur in Brazil (KEPLER et al., 2014; ROCHA et al., 2013). Several strains not taxonomically characterized were recognized in Brazilian soil and rhizosphere. One was recently described as a new species, *M. humberi* (*Metarhizium* sp. indet. 1 (LOPES et al., 2014; ROCHA et al., 2013; REZENDE et al., 2015; ZANARDO, 2015), and the others are referred to as *Metarhizium* sp. indet. 2 (REZENDE et al., 2015) and *Metarhizium* sp. indet. 3 (ZANARDO, 2015), probably in the MGT clade and finally, *Metarhizium* sp. indet. 4 (REZENDE, 2014).

Metarhizium anisopliae (Metsch.) Sorokin, 1883 is the species with the largest number of product registrations in Brazil to control the spittlebugs (Hemiptera: Cercopidae), including *Mahanarva fimbriolata* (Stal, 1854) and *M. posticata* (Stal, 1855) in sugar cane and *M. fimbriolata*, *Deois flavopicta* (Stal, 1854) and *Notozulia entreriana* (Berg, 1879) in pasture (ALVES et al., 2008). This fungus has provided

efficient levels in the control of leafhoppers, with an estimated annual application of more than 2 million hectares in sugarcane cultivation in 2011 (BETTIOL, 2011).

1.5. *Metarhizium* as endophytes: life soil and plant associates

The rhizosphere is the narrow zone of soil directly influenced by root secretions. It is a place where complex interactions occur between the plant and bacteria, fungi, protists, nematodes, and insects (BAIS et al., 2006). The activities in the rhizosphere of plants can stimulate or inhibit microbial populations and their activities (ST. LEGER, 2008).

The amount and composition of root exudates entering the soil depend on the plant species, age, and nutritional status. Rhizoplane (root surface) also provides a nutrient base for many species of bacteria and fungi. In exchange for the plant's nutrients, microorganisms can help the plant by solubilizing inorganic nutrients or as biocontrol agents against plant pathogens (ST. LEGER, 2008). Bruck (2010) defined that rhizosphere-competent are those microorganisms with more significant growth in response to the more remarkable development of plant roots.

Hu and St. Leger, in 2002, were the first researchers to demonstrate that the fungus *Metarhizium anisopliae* is rhizosphere-competent. The authors marked the fungus with a green fluorescent protein obtained from *Aequorea victoria*. They showed the persistence of the fungus over time in the field and its greater affinity with the plants' rhizosphere than with the soil.

The review by St. Leger (2008) shows that *M. anisopliae* expresses a different subset of genes to be able to persist in the soil, colonize insects, or plant tissues, suggesting that the ability to adapt to life in the soil and requires different subsets of genes to infect insect.

Another study showed that *M. anisopliae* produces two different types of proteins (MAD1 and MAD2) used to adhere to insects and plants' surfaces since MAD1 and MAD2 are differentially produced in response to insect hemolymph and plant root exudates, respectively (WANG; ST. LEGER 2007). The same authors showed that mutant fungi with MAD2 turned off were not able to colonize the plant rhizosphere.

Metarhizium anisopliae and *Beauveria bassiana* grow and sporulate very well in exudates of bean plants. Molecular studies showed the presence of genes expressed by fungi in the presence of exudates from these plants and are the same

involved in pathogenicity to insects (PR1A subtilisina Gene) (PAVA-RIPOLL et al., 2011). These results corroborate those of Wang and St. Leger (2007), where the authors also show that this fungus has a bifunctional lifestyle, having genes expressed in different ways in the presence of plants and insects.

Researchers studying *Metarhizium* demonstrated that sucrose and oligosaccharides from the family of raffinose present in plant exudates are essential in the rhizosphere-competence of this entomopathogenic fungus. In this study, it was possible to identify genes containing a raffinose transporter (*Metarhizium* rafinose transporter - MRT), which, when absent, reduced the fungus association with the grass rhizosphere, but did not decrease its virulence to insects. From this, the authors were able to verify that the expression of this MRT gene is exclusively regulated by galactosides and sucrose (FANG; ST. LEGER, 2010).

Kepler and Bruck (2005), when placing an insect in an olfactometer, noticed that they were moving towards roots colonized by *Metarhizium* sp. and not for roots without the association with the fungus. From an evolutionary point of view, this makes sense, since, in the soil, these entomopathogenic fungi can find and infect insects. It is possible that the fungus uses the plant's rhizosphere as a bridge to the insect, or that the plant, in association with the fungus, can produce attractive compounds to the pest, or that the fungus produces attractive compounds when it is in the different rhizosphere when it is not associated with plants (BRUCK, 2010).

Regarding the survival of these fungi in the soil, Bruck (2005) inoculated *Metarhizium anisopliae* in the soil and re-isolated it using selective medium and insect baiting for up to 342 days after treatment from the soil. For Bruck (2010) the use of entomopathogenic fungi applied to the soil could decrease by ten times the amount of fungus typically used for biological pest control. This could result in the more effective management of insects that feed on plant roots at no additional product or application cost. Pioneering work has shown that *Metarhizium* occurs naturally more frequently in agricultural habitats and that habitat and proximity to potential hosts are important in the population structure of *Metarhizium* and *Beauveria* (BRUCK, 2005).

The term "endophyte" refers to all organisms, especially fungi or bacteria, that occur within a plant tissue without causing apparent damage to it (BATTA, 2013).

Some species of entomopathogenic fungi have been isolated in the form of endophytes in certain plant species, some of which have been reported naturally

occurring, while others have been introduced into plants using different techniques (VEGA, 2008).

Wyrebek et al. (2011) isolated three different species of *Metarhizium* from the rhizosphere of the same wheat plant, *M. robertsii*, *M. anisopliae,* and *M. guizhouense*. *Metarhizium robertsii* (Bischoff)'s ability to form symbiotic associations with plant roots causing root hair proliferation was demonstrated by Sasan and Bidochka (2012). Batta (2013) successfully colonized *Brassica napus* plants by *Metarhizium anisopliae* re-isolating the fungus from tissues far from the fungus inoculation site, demonstrating that the entomopathogenic fungus occupied not only the region rhizospheric of the plant but also the petioles, leaves, and stem, showing that it is an endophyte.

1.6. Potential of *Metarhizium* spp. as plant inoculants and biostimulant

Mycorrhizal fungi and endophytic fungi are the two major fungi groups that maintain a beneficial association with plants. A characteristic that distinguishes mycorrhizal fungi from endophytic fungi is that mycorrhizal fungi cannot survive in the absence of their plant host (BEHIE; BIDOCHKA, 2014a).

Species of pathogenic fungi to insects, such as *Metarhizium anisopliae* and *Beauveria bassiana* can infect soil insects and subsequently transfer nitrogen derivatives to the insect, as demonstrated by Behie et al. (2012) and by Behie and Bidochka (2014B).

Metarhizium, *Beauveria,* and other entomopathogenic fungi have a similar habitat, specificity of hosts, and nutrient requirements. In the future, it will be necessary to classify them as endophytic that participate in nutrient cycling (BEHIE; BIDOCHKA, 2014).

The costs and the benefits of the endophytic association of fungi such as *Metarhizium* and *Beauveria* with plants have not yet been elucidated. As previously mentioned, some studies have already shown that plants can capture nitrogen through associations with endophytic entomopathogenic fungi. The research of the symbiotic transfer of nitrogen in the soil is primarily focused on nitrogen-fixing bacteria. Vascular plants can lose a substantial amount of nitrogen through insect herbivory.

Behie et al. (2012) and Behie and Bidochka (2014) showed that the plants could acquire nitrogen from insects through a partnership with the endophytic and entomopathogenic fungus *Metarhizium robertsii*. This result indicated that the

endophytic capacity and pathogenicity are coupled so that the fungus killed the insect in the soil and, through association with the plant, transferred the insect's nitrogen. *Metarhizium* and *Beauveria* species managed to transfer significant amounts of nitrogen from dead insects to the plants, even in field conditions (BEHIE; BIDOCHKA, 2014B), suggesting the creation of an additional branch to the nitrogen cycle in the soil and that the association of plants with these fungi may be important for plants that survive in nitrogen-poor soils.

Although arbuscular mycorrhizae, ectomycorrhizae, and endophytic fungi are present in the soil, specific applications of these fungi in agricultural areas to increase the plant's productivity are not widespread.

Entomopathogenic fungi, such as *Metarhizium* and *Beauveria*, which are also capable of colonizing plant tissues, are produced on an industrial scale worldwide to treat agricultural pest insects. In the future, these fungi may be inoculated as endophytes in plantations (BEHIE; BIDOCHKA, 2014).

Entomopathogenic fungi may have been primarily inhabitants of plant tissues and soil and, after contact with underground insects, became entomopathogenic, or on the contrary, has an entomopathogenic origin, starting to exhibit endophytic action as a way to find susceptible host insects that can infect (SASAN; BIDOCHKA, 2012).

Wyrebek and Bidochka (2013), through the analysis of the MAD1 and MAD 2 genes, showed that the fungus *Metarhizium* relationships with plants, instead of insects, have an essential role in the divergence of this genus.

The authors proposed in their study three probable models of evolution for the genus *Metarhizium*, however further studies will be necessary for a better understanding of the evolution of this group: (1) host insect has caused divergence between species; (2) host plant has caused divergence between species; (3) other abiotic or biotic factors have caused divergence and evolution among Metarhizium species.

Zhao et al. (2014) suggested that evolutionarily, *Metarhizium* was first a fungus with endophytic activity and probably acquired the pathogenic capacity many years later. Such authors studied horizontal transfer genes in this group of fungi to understand whether the evolution of pathogenicity occurred before or after the association with plants. *Metarhizum* shares 16% identity with endophytic plant fungi and phytopathogenic fungi (GAO et al., 2011). However, more studies are needed to

understand the evolution of both endophytic life and pathogenicity to insects of these fungi.

Kabaluck and Ericson (2007) applied *Metarhizium anisopliae* on corn seeds (*Zea mays* L.) as an alternative to control the attack of *Agriotes obscurus* L. As a result, the authors observed that the fields where seeds were treated with the fungus were left with larger plants and with a higher wet leaf weight than the areas that only received insecticide, which was attributed to the entomopathogenic fungus. Also, insect corpses with *Metarhizium* fungus growth were found, suggesting that the increase in the size of the plants and the decrease in their attack were due to the product's application. However, the authors did not attempt to find the fungus in the plant's tissues or its roots, since until then, there was no idea of the endophytic action of *Metarhizium*.

A study conducted testing three different *Metarhizium* isolates showed that they could significantly increase tomato plants' size, from the root to the stem, compared to controls not treated with the fungus. The authors also showed that the three isolates showed endophytic activity and were isolated from roots and the aerial part of the plant (GARCÍA et al., 2011).

Many recent studies have shown that the benefits derived from the association between endophytic entomopathogenic fungi and plants are numerous. An study showed that the fungus inoculated in the bean plants germinated and colonized the roots of them. The authors also showed that the roots of plants treated with *Metarhizium* grew much more in size and more quickly than control plants without fungus inoculation, in addition to having a greater density of root hair a few days after planting (SASAN; BIDOCHKA, 2012).

Sasan and Bidochka (2012) were able to verify that the fungus grew inside the cells of the plants' root cortex and in the intercellular spaces without causing any apparent damage. In addition to *Metarhizium*, a list of entomopathogenic fungi with endophytic activity has been reported, including species of *Acremonium, Beauveria, Cladosporium, Clonostachys,* and *Isaria* (VEGA, 2008).

The action of endophytic entomopathogenic fungi on abiotic stresses was demonstrated in soybean plants submitted to salt stress. *Metarhizium anisopliae* was isolated from the tissues of soybean plants and was subsequently identified molecularly. A filtrate of *M. anisopliae*, together with isolates of fungi of other species was applied to soybean plants, and the treatment with the entomopathogen had more

remarkable growth in length, biomass, chlorophyll content, photosynthetic transpiration rate, and leaf area, compared to plants without inoculation (KHAN et al., 2012).

Amounts of the abscisic acid hormone (ABA) show altered levels in plants inoculated with the fungus, indicating that this association can also act under the hormonal pathways of plants, which is yet to be investigated. In addition to this, Jasmonic acid (AJ), also had a high level, showing that the fungus also reduced the negative effects of salt stress that was induced in plants (KHAN et al., 2012).

1.7. Effects of *Metarhizium* inoculation on pest populations

The association of plants with entomopathogenic fungi can ward off repelling and protect the plant against herbivory attacks by insects. Krueger and Roberts in 1997 inoculated dry mycelium of *Beauveria bassiana* and *Metarhizium anisopliae* 15 cm deep in the soil to control a corn pest. After a few months, the concentration of the fungus propagules decreased in the soil. Still, it persisted for an extended period, and the authors observed that the caterpillars started to eat less of the plants, and there was a limitation in the emergence and size of adults. At this time, the authors associated these facts with the presence of conidia of the fungus in the soil that could be causing harmful changes in the pest's biological development.

Kabaluck and Ericson (2007) affirmed that farmers soaked the soil with suspensions of *Metarhizium* conidia to try to control the tobacco borer *Conoderus vespertinus*, causing the population density of the borer to drop drastically. Still, at that time, this was not yet attributed fact the colonization of roots or the antagonistic effect provided by the fungus.

For Elliot et al. (2000), plants' defense against herbivores mediated by entomopathogenic fungi is an indirect effect. This association increases the contact rate between insects and entomopathogens and may also increase the insect's susceptibility to the fungus. However, these factors still need to be further investigated.

Currently, Batta (2013), in addition to showing the internal colonization of *Brassica napus* plants by *Metarhizium* anisopliae, also demonstrated direct effects of the endophytic fungus against *Plutella xylostella* larvae. Larvae of this insect were fed with the leaves of the rapeseed plant, previously colonized by the entomopathogenic fungus. The results showed that this feeding caused insects' death in percentages

similar to a bioassay prepared with the direct application of the fungus on the caterpillars.

The antagonism of endophytic entomopathogenic fungi against phytopathogenic fungi was demonstrated by Sasan and Bidochka (2013), where bean plants inoculated with *Metarhizium robertsii* became more resistant to the fungus causing root rot (*Fusarium solani*). Antagonism was also demonstrated in *in vitro* tests that showed that *M. robertisii* is capable of inhibiting the phytopathogen. The authors relate this antagonism to competition for nutrition and space and antibiosis among fungi.

According to Fang and St. Leger (2010), some of the benefits of plants' association with fungi, whether mycorrhizal or entomopathogenic, are already known. These symbiotic associations between fungi and the plant rhizosphere can antagonize phytopathogens and herbivores, in addition to facilitating the absorption of nutrients by plants.

1.8. Objectives and hypotheses

Considering the importance of developing strategies that increase the crop production with minimal environmental impact by the inclusion of alternative control methods by farmers such as the use of biological control agents, the overall aim of this research was, therefore, to evaluate two *Metarhizium* isolates as inoculants in sugarcane crop for effects on pest control and plant productivity. The study focused on the inoculation of fungi in the field, and greenhouses and endpoint measurements were taken with a focus on sugarcane pest population parameters. At the same time, final plant biomass and yield were evaluated.

1.9. Obtained results and future perspectives

The use of entomopathogenic fungi, primarily aiming to take advantage of the endophytic capacity and act as plant rhizosphere colonizers, as growth promoters and antagonists to phytopathogens, opens up more possibilities for exploring their interactions with plants. Given this scenario, there is a need for more research aiming at understanding the colonization mechanisms of *Metarhzium* spp. in plants, their

persistence, the benefits that the fungus may offer to the host plant, and mainly to understand the fungus-plant-insect relationship, seeking to clarify the capacity of interactions like this applied to the biological control of pest insects.

The research focused on the inoculation of fungi in sugarcane plants by soil drench. Endpoint measurements were taken on plant growth promotion parameters and the effects on *D. saccharalis, S. levis, M. fimbriolata, M. janavica,* and *P. zeae* population parameters.

The first study of this thesis aimed to understand the possible mechanisms related to plant growth promotion traits and colonization of the root system by two Brazilian isolates of *M. robertsii* and *M. humberi* in the model pot-grown tomato (*Solanum lycopersicum* L.) miniature cultivar 'Micro-Tom'. The hypotheses of this study were, therefore: 1) *M. robertsii* and *M. humberi* can produce *in vitro* auxin, a plant growth hormone, in specific concentrations that can improve plant growth and increased auxin-induced GUS expression in the roots; 2) The *Metarhizium* isolates can produce key enzymes involved in plant growth and pathogen and pest control, like phosphatases, phytases, siderophores, and chitinases.

The second study's objective was to evaluate the sugarcane growth and tissue colonization at the greenhouse level after the inoculation of conidia or blastospore and different fungal concentrations and the combination of *M. robertsii* and *M. humberi* isolates. Besides that, we aimed to evaluate the effect of *Metarhizium* inoculation in sugarcane plants on nematodes *M. javanica* and *P. zeae* populations and *D. saccharalis* development, mortality, and behavior. The hypotheses of this study were, therefore: 1) plant inoculation with conidia was more promising than the blastospore inoculation, conferring plant development and plant tissue colonization; 2) plants inoculated with both *M. robertsii* and *M. humberi* isolates in combination will enhance the sugarcane plant growth when compared to control plants; 3) plants inoculated with higher fungal concentrations will show more growth and development than plants inoculated with a lower concentrations 4) inoculation with the *M. robertsii* and *M. humberi* isolates reduces the nematodes populations and cause mortality and development delay on *D. saccharalis* larvae; and 5) conidia of *M. robertsii* and *M. humberi* are pathogenic to eggs of *M. javanica* and *P. zeae.*

In the third study, the entomopathogenic fungi were implemented in field conditions as root inoculants of sugarcane plants. The objective was to evaluate the potential of two selected isolates of *M. robertsii* and *M. humberi* as inoculants of

sugarcane plants for above-ground pest management in five commercial sugarcane fields, during three years in Brazil. The central hypothesis was that the inoculation would provide long-term control of *D. saccharalis*, *S. levis,* and *M. fimbriolata* populations under field conditions. Besides, the expected additional benefits on sugarcane growth and productivity were measured.

Several research questions should be addressed in further studies, including:

• Laboratory and field studies for in-depth understanding of the antagonism towards plant pathogens caused by entomopathogenic fungi as inoculants;

• understand the mechanisms responsible for the effects caused by entomopathogenic fungi as plant inoculants on arthropod pests and plant growth promotion;

• how the inoculation effects may be influenced by abiotic factors, such as the temperature, relative humidity, UV radiation, type of soil/substrate;

• How to implement this strategy in production systems and IPM programs, ensure the efficacy through the development of formulations, appropriate application technology, and extension to and training of the producers, to benefit the most from the potential of the entomopathogenic fungi as plant inoculants.

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2. MULTI-TRAIT BIOCHEMICAL FEATURES OF *Metarhizium* SPECIES AND THEIR ACTIVITIES THAT STIMULATE THE GROWTH OF TOMATO PLANTS

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Abstract

The interaction between *Metarhizium* spp. and plant roots may induce host plant growth and help control below- and above-ground pests and diseases. We conducted *in vivo* and in vitro bioassays using native Brazilian isolates of *Metarhizium robertsii* (ESALQ 1635), *M. humberi* (ESALQ 1638), and *M. anisopliae* (ESALQ 1669) to better

understand the possible mechanisms related to plant growth promotion traits and colonization of the root system in the model pot-grown tomato (*Solanum lycopersicum* L.) miniature cultivar 'Micro-Tom'. *In vivo* trials revealed that *M. robertsii* (ESALQ 1635) or *M. humberi* (ESALQ 1638) inoculated in tomato seedlings improved vegetative and reproductive traits. Inoculation with *M. robertsii* yielded taller plants, longer roots, and more shoot and root dry mass than *M. humberi*. The number of flowers and the fresh weight of fruits was significantly increased by *M. robertsii* and *M. humberi* inoculation, compared to uninoculated control plants. Both fungal species endophytically colonized all tomato tissues within 30 days of inoculation. Plants inoculated with either *M. robertsii* or *M. humberi* increased auxin-induced GUS expression in the roots for up to 30 days after inoculation, confirming that *Metarhizium* induces auxin-regulated gene expression. We also explored the production of key compounds including enzymes, hormones, and metabolites involved in plant growth promotion. The three *Metarhizium* species grown with or without exogenous tryptophan were able to produce indole-3-acetic acid (IAA) at different titers. All *Metarhizium* isolates produced phosphatases, phytases, siderophores, and chitinases. Of particular importance, the *M. robertsii* and *M. humberi* isolates exhibited similar *in vitro* biochemical profiles, whereas *M. anisopliae* and *Trichoderma harzianum* isolates, demonstrated distinct traits from the others. Taken together, we argue that the *M. robertsii* isolate is more efficient than the *M. humberi* isolate to endophytically colonize tomato plants resulting in improved growth. However, *M. humberi* (ESALQ 1638) yielded a slightly better production of some metabolites *in vitro*. Thus, we propose that the isolates of *M. robertsii* and *M. humberi* could be explored as complementary plant growth promoters.

Keywords: endophytes; plant growth promotion; GUS; IAA; phosphate solubilization; siderophores; phytases; chitinases.

2.1 Introduction

Endophytes may benefit plants through protecting against insect pests and promoting growth. Attempts to use fungi as endophytes in plants have been tested as a novel strategy for pest control (Jaber and Ownley, 2017). Entomopathogenic fungi as endophytes stand out as a prospective approach for plant protection, inducing resistance against insect pests and consequently reduced pesticide use (Vidal and Jaber, 2015). *Metarhizium* spp., a group of soil-borne fungi, are biocontrol agents of insects, arachnids, and other arthropod pests. Beyond their well-established entomopathogenic lifestyle, *Metarhizium* are ubiquitous, free-living fungi that inhabit predominantly the soil and can colonize plant roots, which may improve plant growth (Roberts and St. Leger, 2004; Behie and Bidochka, 2014; Vega, 2018).

Some plant growth-promoting fungi (PGPF) can promote plant growth and health after inoculation (Elsharkawy and El-Khateeb., 2019) by stimulating several biological mechanisms, including the production of hormones, such as auxins (Bose et al., 2013), solubilization of phosphate (Barrow and Osuna, 2002) and the production

of siderophores (Bartholdy et al., 2001) or chitinases (Caldwell et al., 2000). These mechanisms possibly associated with plant growth promotion have been demonstrated for some PGPF, but have been overlooked for the entomopathogenic fungus *Metarhizium*.

Indole-3-acetic acid (IAA), the most common auxin, regulates plant growth and development. Some microorganisms possess the ability to synthesize IAA to boost plant growth (Nieto-Jacobo et al., 2017). Besides, IAA may improve the colonization efficiency of endophytic fungi. The PGPF can affect the endogenous levels of IAA, consequently improving plant nutrition and growth (Jha and Saraf, 2015).

Plant-growth-promoting microorganisms, such as fungi, can also mineralize and solubilize organic phosphorus into inorganic forms by producing enzymes called phytases and phosphatases, which then become available for plant uptake (Ghosh et al., 2015; Singh and Satyanarayana, 2011). P solubilization is fundamental for plant growth and development. Organic P reserve in soil ranges from 5% to 95% of the total soil P, of which phytate constitutes up to 50% (Dalai, 1977). Although phosphate is abundantly available in the soil, plants can only use inorganic phosphate, which is the soluble form (Khan et al., 2010).

Entomopathogenic fungi use chitinases to enter their hosts by directly penetrating the cuticle (Samuels et al., 1989). Chitinase is also linked to the process of endophyte colonization in plants (Kauss et al., 1983). Chitin is an important component of the eggshell of nematodes, and chitin synthesis is a process maintained across the fungal kingdom. Production of chitinase by PGPF can indirectly contribute to plant growth by reducing insects, plant pathogens, and nematode populations (Furtado et al., 2019).

Siderophores are low biomolecular weight (0.5 to 1.5 kDa) iron-chelating compounds, which are produced by microorganisms with a high affinity for Fe^{+3}, and can mediate iron (Fe) uptake into the microbial cells (Gupta et al., 2015). Siderophores can contribute to plant growth and development, as well as improve the acquisition of Fe and other essential micronutrients in plants (Raya-Díaz et al., 2017). The inorganic form of Fe in the soil is not readily assimilated by plants (Kobayashi and Nishizawa, 2012). However, siderophores can chelate Fe through a biochemical mechanism of reducing inorganic Fe^{+3} to Fe^{+2}, which then becomes available for root uptake (Grobelak et al., 2015). For plant nutrition and health, Fe participates as a component in many enzymatic systems, including those associated with plant defenses, and is

intrinsically involved in the major process of chlorophyll synthesis (Rout and Sahoo, 2015). PGPF can also indirectly affect plant growth through the chelating property of siderophores by making Fe less available to other competing microorganisms, and thus negatively affect their growth, including plant pathogens (Parmar and Chakraborty, 2016).

Therefore, our main objectives of this study focused on the biochemical characterization of Brazilian *Metarhizium* spp. isolates, including the production of IAA, siderophores, chitinases, phytases, and phosphatases for phosphorus solubilization, alongside the effects on tomato miniature cultivar Micro-Tom (MT), inoculated with two root colonizers, *M. robertsii* and *M. humberi* sp. nov. (Luz et al., 2019), on growth promotion, fruit yield, and auxin accumulation in roots. The MT transgenic reporter tomato line is a useful phenotype to investigate IAA signaling through visualization of root parts based on the activity of β-glucuronidase (Blanco et al., 1982), confirming that both *M. humberi* and *M. robertsii* induced endogenous IAA production. These findings support the commercial development of both indigenous isolates of *M. robertsii* and *M. humberi* as biocontrol agents and root colonizers for stimulating plant growth.

2.2 Materials and Methods

2.2.1 Fungal isolates

All assays were conducted in the “Insect Pathology and Microbial Control Laboratory” at the Entomology Department, in the “Prof. João Lúcio de Azevedo” Laboratory at the Genetics Department both at ESALQ/USP and at Plant Breeding Laboratory at Center for Nuclear Energy in Agriculture/University of São Paulo (CENA/USP). One isolate of each of the endophytic entomopathogenic fungi *M. robertsii*, *M. humberi,* and *M. anisopliae* (Table 1) were used in this study. The isolates were deposited in “Prof. Sérgio Batista Alves” collection at ESALQ/USP and had been previously identified to species level by molecular techniques (Rezende et al., 2015; Botelho et al., 2019; Luz et al., 2019). *M. robertsii* and *M. humberi*, which are registered as biopesticides in Brazil, were selected for their effects on growth promotion of

sugarcane plants (length of plants, increased roots, increase in chlorophyll content, and antagonism to sugarcane phytopathogens, and insect pests), which were demonstrated in our previous greenhouse and field studies (A.C.O. Siqueira, unpublished). The soil-borne and opportunistic plant symbiont *T. harzianum* isolate ESALQ 1306, deposited in the same collection at ESALQ/USP, was used as a standard control for comparisons in the biochemical assays (marketed by Koppert do Brasil Holding Ltd., Piracicaba, SP, Brazil). The access of these three *Metarhizium* species is registered at the Brazilian System for the Management of Genetic Heritage and Associated Traditional Knowledge – SisGen under the code RAC856E.

Table 1. Description of fungal isolates used in the experiments.

Isolate code[1]	Fungal species	Crop/Isolation method	Location in Brazil (City - State)[2]	Geographical coordinates	Sampling date
ESALQ 1635	*Metarhizium robertsii*	Forest soil/Insect bait with *Tenebrio molitor*	Delmiro Gouveia, AL	9°25'0.12"S 37º57'8.49"W	Mar/2012
ESALQ 1638	*Metarhizium humberi*	Savanna soil/Selective media (PDAY)	Rio Verde, GO	17º29'49.3"S 51º13'40.7"W	Mar/2012
ESALQ 1669	*Metarhizium anisopliae*	Sugarcane soil/Insect bait with *T. molitor*	Iracemápolis, SP	22°36′10″S 47°33′17″W	Dec/2012
ESALQ 1306	*Trichoderma harzianum*	Soil/Selective media (PDAY)	Piracicaba, SP	22°42'06.7"S 47°38'44.2"W	Jul/2002

[1]ESALQ - Escola Superior de Agricultura “Luiz de Queiroz” – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil. [2] Brazilian States: AL = Alagoas, SP = São Paulo, and GO = Goiás.

2.2.2 Plant material

The tomato miniature cultivar Micro-Tom (MT) employed is considered a suitable genetic model (Meissner et al., 1997; Scott and Harbaugh 1989). The transgenic line DR5::GUS on the MT genetic background contains the auxin-responsive promoter of *DR5* fused to the reporter gene *uid* encoding a β-glucuronidase (GUS), which allows enzymatic staining of sites where auxin accumulates (Martí et al., 2010). The use of this reporter line is valuable to elucidate the role of *Metarhizium* in root colonization and to screen the best root-competent isolates.

2.2.3 Plant growth promotion tests

Only *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) isolates were selected due to limitations of the experimental setup and considering the superior plant growth promotion by both isolates in previous studies in our laboratory (A. C. Siqueira et al., unpublished). DR5::GUS seeds were sown on cell trays containing non-autoclaved Tropstrato HT substrate + expanded vermiculite (1:1), according to Lima et al. (2009). Conidia production, viability reading, and concentration adjustment were performed as described by Oliveira et al. (2015). The seedlings were kept in a greenhouse and irrigated daily. After 18 days of sowing, the seedlings were transplanted to 150 mL plastic pots. Fungal spores were inoculated by applying 1 mL of a 10^8 conidia mL^{-1} suspension of *Metarhizium* spp. with 0.05% of Tween 80® (v/v) at the base of each seedling using a sterile pipette for each fungal species. As a control, 1 mL of distilled water prepared with 0.05% of Tween 80® (v/v) was applied to each plant. The experiment was conducted in a greenhouse and plants were exposed to the natural incidence of light. This entire assay was repeated independently twice and followed a completely randomized design. Within each assay, each treatment had twenty replicates. The average max and min daily temperatures during this greenhouse experiment were 28.7 °C and 12.3 °C, respectively. The max, mean, and min relative humidity (RH) were 96.1%, 71%, and 40.1%, respectively, inside the greenhouse. Then, 10, 15, and 30 days after inoculation (DAI), the length of the shoot and roots were evaluated using a 50 cm ruler, and the diameter of the shoot was assessed using a digital caliper (Western® PRO, SP, Brazil). Shoots and roots were separated and dried in paper bags using a drying oven (Marconi, MA033, Piracicaba, SP, Brazil) at 60 °C for 24 h. After drying, the samples were weighed.

To assess the endophytism of these fungal species, plant materials from independent samples comprised of leaves, stems, and roots were collected 10, 15, and 30 DAI, cut into 3-cm fragments and surface sterilized by immersion for 15 s in 70% ethanol, 50 s in 0.8% sodium hypochlorite, 15 s in 70% ethanol again, rinsed three times in sterile distilled water, and finally left to dry on sterile filter paper. The efficacy of the sterilization was confirmed by plating the last rinsing water (100 µL) onto Potato Dextrose Agar (PDA) (Kasvi®, Brazil) (Parsa et al., 2013). The plant samples were then placed in Petri dishes (90 × 15 mm) containing selective medium composed of PDA, supplemented with 500 mg L^{-1} cycloheximide, 200 mg L^{-1} chloramphenicol, 500 mg L^{-1} Dodine (65% w/v Dodex 450 SC® SIPCAM-AGRO, Brazil), 10 mg L^{-1} Crystal Violet (Dinâmica Analytical Reagents®, SP, Brazil) (Behie et al., 2015). The

cultures were incubated at 24 °C for 15 days in darkness. There were three independent replicate tissues per plate and five plates for each fungal species. The presence of colonies was detected according to morphological characteristics of the fungal outgrowth in each plant fragment, and the fungal colonization rate was given by the number of fungus-colonized plant fragments divided for the number of fragments plated on a selective culture medium, multiplied by 100.

In addition, the presence of the fungi in the soil was determined 10, 15, and 30 DAI by preparing suspensions of soil substrate from where the plants had grown. Soil samples were obtained from the region near the plant from each replicate. A soil sample (1.0 g) from each pot was transferred to 10 mL sterile water with 0.05% Tween 80® (v/v). The suspension was then vigorously vortexed and four consecutive ten-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were inoculated in Petri dishes (90 × 15 mm) containing the selective agar media described above. The plates were divided into four equal quarters by marking the bottom of the Petri dish with a permanent marker and one aliquot (20 µL) of each of the four dilutions was pipetted onto each quarter (Miles et al., 1938). The Petri dishes were incubated at 24 °C for 15 days in darkness and the presence of colonies was detected according to morphological characteristics of the fungal outgrowth.

2.2.4 Biochemical assays

2.2.4.1 IAA production *in vitro*

The *in vitro* production of IAA by *M. robertsii* (isolate ESALQ 1635), *M. humberi* (isolate ESALQ 1638), *M. anisopliae* (ESALQ 1669), and the *T. harzianum* (isolate ESALQ 1306 – as a positive control) was determined in Potato Dextrose Broth (PDB) medium, amended or not with L-tryptophan (0 and 0.1 g L^{-1}) (Labsynth®, Diadema, SP, Brazil). Fungal cultures were established in 50 mL of liquid PDB media inoculated with three mycelial plugs (Ø 0.7 cm). The media were maintained in an orbital shaker incubator (Marconi®, Piracicaba, SP, Brazil) at 150 rpm for 7 days and 25±1 °C. After this incubation time, 1.5 mL of fungal broth was centrifuged at 10,000 *g,* and the supernatant was collected. An aliquot 0.5 mL was mixed with 0.5 mL of Salkowski's reagent (15 mL H_2SO_4, 0.75 mL $FeCl_3$, and 25 mL distilled water) (Gordon and Weber, 1951). The reaction was performed in the dark for 30 min and subsequently read with

an optical density (O.D.) at 530 nm in a spectrophotometer (Amersham Pharmacia Biotech, Ultrospec 3000, Little Chalfont, UK). The standard curve was obtained by diluting 10 mg of a commercial synthetic auxin (CAS: 87-51-4, purity > 98%, Sigma®, Germany) in 10 mL acetone. Concentrations of commercial IAA were adjusted to 0, 1, 5, 10, and 20 µg mL^{-1} using PDB to determine the standard concentration-absorbance curve (Sarker and Al-Rashid, 2013). This assay followed a completely randomized design and was conducted three times using new fungal batches. In each independent assay, fungal treatments were evaluated in triplicates (i.e., Erlenmeyer flasks containing fungal liquid cultures).

2.2.4.2 IAA production *in vivo*

To confirm the role of auxin in growth stimulation by inoculation with the *Metarhizium* spp., and to locate the tissue with more effective auxin signaling and/or accumulation, the DR5::GUS transgenic line was inoculated by applying a conidia suspension at the base of the seedlings with the two *Metarhizium* isolates. Histochemical assays for β-glucuronidase (GUS) activity detection were performed according to Jefferson et al (1987) after 10 and 30 DAI with *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638). Roots of tomato plants inoculated and non-inoculated were separated from the plants and washed in running water to remove excess substrate, followed by immersion in the GUS buffer [100 mM $NaH_2PO_4.7H_2O$ pH 7.0, 10 mM EDTA pH 7.0, 0.5 mM $K_3Fe(CN)_6$ pH 7.0, 0.5 mM $K_4[Fe(CN)_4].3H_2O$ pH 7.0, 0.1% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl glucuronide - X-Gluc (Jersey Lab and Glove Supply, Livingston, NJ, USA)]. The samples immersed into GUS buffer were submitted to 600 bar vacuum for 5 min, followed by incubation at 37 °C with 100 rpm of orbital shaking in total darkness for 24 h. Images of the roots were captured using the Leica® stereomicroscope 205C (Leica Microsystems, Wetzlar, Germany) and analyzed using LAV4.5 software. The assay was conducted three times and in each independent assay, five plants per treatment were evaluated (biological replicates) for GUS activity.

2.2.4.3 Determination of *in vitro* phosphate solubilization

Phosphate solubilization activity was determined by a plate *assay* using the culture medium described by Pikovskaya (1948). The media were placed in Petri dishes (90 × 15mm) (20 mL) and inoculated at the center of the plate with a three-day old-growth disc (Ø 0.7 cm). The positive control was *T. harzianum* (ESALQ 1306), while *M. anisopliae* (ESALQ 1669) was used for comparison. Plates were incubated at 25 °C, 12 h photophase and 60% RH. Measurements of fungal colony diameter and the diameter of the surrounding transparent halo were taken at 15 DAI, and both parameters were used as indicative of phosphorus solubilization. The Phosphate Solubilization Index (FSI) was expressed by: FSI = Ø halo/Ø colony. This assay followed a completely randomized design with five replicates (plates) and was repeated three times on different occasions to ensure reproducibility.

2.2.4.4 Determination of phytase production

Fungal phytase production was evaluated using a modified phytate screening medium (Howson and Davis, 1983), containing 5 g L^{-1} of NH_4NO_3; 0.5 g L^{-1} of $MgSO_4.7H_2O$; 0.5 g L^{-1} of KCl; 0.01 g L^{-1} of $FeSO_4.7H_2O$; 0.01 g L^{-1} of $MnSO_4.4H_2O$; 5 g L^{-1} of calcium phytate ($C_6H_{18}O_{24}P_6$); 15 g L^{-1} of glucose; 15 g L^{-1} of agar. The medium pH was adjusted to 5.5. A fungal growth disc (Ø 0.7 cm) from three-day-old mycelia was inoculated onto the center of the Petri dishes (90 × 15 mm), containing 20 mL of the medium. Fungal cultures of the *M. robertsii* (ESALQ 1635), *M. humberi* (ESALQ 1638), and *M. anisopliae* (ESALQ 1669) isolates and the control *T. harzianum* (ESALQ 1306) were incubated at 25 °C, 12 h photophase, and 60% RH. Measurements were taken at 15 DAI and expressed as fungal diameter and the transparent halo diameter, as indicative of phytate solubilization. The phytase activity was determined according to the Phytase Degradation Index (PDI), calculated as: PDI = Ø halo/Ø colony. This assay was repeated three times and followed a completely randomized design with five replicates (plates).

2.2.4.5 Determination of siderophore production

To investigate the siderophore production of *Metarhizium* spp., the Chromoazurol S (CAS) analysis was used (Schwyn and Neilands, 1987). All glassware used during the analysis was previously immersed in a 6.0 M HCl solution overnight to ensure the removal of all traces of iron that could have been present. For the indicator solution, 60.5 mg of CAS was dissolved in 50 mL of ultra-pure water and mixed with 10 mL of Iron (III) (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). Under stirring, this solution was slowly added to 72.9 mg of HDTMA (Hexadecyltrimethylammonium) dissolved in 40 mL of ultra-pure water. These solutions were autoclaved separately. The iron-deficient culture medium MM9 (Payne, 1994) contained 1.0 g NH_4Cl, 0.3 g KH_2PO_4, 0.5 g NaCl, 1.2 g piperazine ($C_4H_{10}N_2$), and 18.0 g agar, completed to 1 L with ultra-pure water, with initial pH set to 5.6. The CAS solution was added, mixed into the MM9 medium, and placed in Petri dishes. A fungal growth disc (Ø 0.7 cm) from three-day-old fungal mycelia was inoculated at the center of the Petri dishes (90 × 15 mm). Fungal cultures of the three *Metarhizium* spp. isolates and the control *T. harzianum* were incubated at 25 °C, 12 h photophase, and 60% RH. The changing of the blue color of the CAS medium to yellow indicates the production of siderophores. Measurements were taken at 15 DAI based on the yellow halo and the fungal colony diameter. The Siderophore Production Index (SPI) was determined according to the SPI = Ø halo/Ø colony. This assay was repeated three different times and followed a completely randomized design with five replicates (plates).

2.2.4.6 Determination of chitinase production

To investigate the chitinase production by *M. robertsii* (ESALQ 1635), *M. humberi* (ESALQ 1638), *M. anisopliae* (ESALQ 1669), and the control *T. harzianum* (ESALQ 1306), the media contained 3.0 g $(NH_4)_2SO_4$; 2.0 g KH_2PO_4; 0.3 g $MgSO_4$; 1.0 g citric acid, 4.5 g Sigma® colloidal chitin; 15.0 g agar; in 1 L of distilled water, with pH adjusted to 4.8, and supplemented with 0.15 g of bromocresol purple (Agrawal and Kotasthane 2009). A fungal growth disc (Ø 0.7 cm) from three-day-old fungal mycelia was inoculated at the center of the Petri dishes (90 × 15 mm). Fungal cultures of the three isolates of *Metarhizium* spp. and the control *T. harzianum* (ESALQ 1306) were incubated at 25 °C, 12 h photophase, and 60% RH. The diameter of both the fungal colony and purple colored chitin degradation halo were each measured at 15 DAI, to

calculate the Chitin Degradation Index (CDI), expressed by: CDI = Ø halo/Ø colony. This assay was repeated three different times and followed a completely randomized design with five replicates (plates).

2.2.5 Data analysis

Goodness-of-fit was assessed using half-normal plots with simulation envelopes (Moral et al., 2017). All analyses were carried out in R (R Core Team, 2018). Linear mixed models (assuming a normal distribution for the error) were fitted to the IAA production, including the effects of the experiment and the interaction term between fungal species and amino acid precursor in the linear predictor, as well as with random replicates. Similarly, continuous data recorded for enzyme activities (phytases, phosphatases, chitinases, and siderophores) were separately fitted to linear mixed models including experiment and fungal species and random replicates in the linear predictor. When the fungus did not show any enzyme activity, i.e., the data consisted of only zeros and were not included in the analysis.

Linear mixed models (assuming a normal distribution for the error) were fitted to continuous data sets on foliage and root dry weights, fresh and dry weights of tomato fruits, plant height, and root length, with the inclusion of experiments and the interaction between fungal species and evaluation periods as fixed effects, along with random intercepts and slopes per each group of observations measured over time, given they are correlated. Generalized linear mixed models assuming Poisson distribution for the error were fitted separately to the number of flowers and the number of fruits per plant, including fungal species and experiments as the fixed effects and random replicates in the linear predictor.

Binomial generalized linear mixed models (McCullagh and Nelder, 1989) were fitted to the colonization data (soil, leaves, stems, and roots), including the effects of the experiment and the interaction between fungal species and evaluation periods, and the random effect for the observational level. A colonization success was recorded when fungal growth by either of the isolates occurred. When no colonization could be detected for all observations in a specific treatment, i.e., the data consisted of only zeros, the observations in all plants of the treatment were not included in the analysis. All multiple comparisons of means with their respective 95% confidence intervals (CIs)

were performed with Tukey's HSD test at a significance level of 5%, using the package 'emmeans' (Lenth, 2018).

Finally, generalized canonical discriminant analysis for a multivariate linear model was performed with datasets from biochemical assays and plant growth traits to determine the correlation between treatments and response variables. A significant group effect was obtained with the type II MANOVA test using Wilk's test statistic at $P < 0.05$, and then correlations between treatment groups and variable vectors were visualized in the biplot. This analysis was performed with the package 'candisc' (Friendly and Fox, 2013).

2.3 Results

2.3.1 Growth promotion traits induced by Metarhizium spp. in the Micro-Tom

The inoculation of MT seedlings with conidial suspensions of *M. robertsii* (ESALQ 1635) or *M. humberi* (ESALQ 1638) isolates increased plant height over non-inoculated control plants at 10 and 15 DAI (interaction between treatment and time: $F = 10.61$, df = 2, 57, $P = 0.00012$) (Fig. 1A). At 30 DAI, inoculation with *M. robertsii* (ESALQ 1635) significantly increased plant height in relation to plants inoculated with *M. humberi* (ESALQ 1638) or control plants. A similar result was observed for root length at 30 DAI, in which roots were longer when treated with *M. robertsii* (ESALQ 1635) than non-inoculated plants (interaction between treatment and time: $F = 12.02$, df = 2, 57, $P < 0.0001$)

Aerial part and root dry weight increased in inoculated treatments (Aerial part: $F = 214.79$, df = 1, 42, $P < 0.0001$; Root: $F = 142.58$, df = 1, 42, $P < 0.0001$). Both *Metarhizium* spp. promoted greater shoot biomass at 15 DAI, while only *M. robertsii* (ESALQ 1635) increased shoot dry matter by day 30 ($F = 21.03$, df = 2, 42, $P < 0.0001$) (Fig. 1D). Similarly, root dry matter was greater after 10, 15, and 30 DAI in treatments with either fungal species than the control plants ($F = 11.44$, df = 2, 42, $P = 0.00011$) (Fig. 1E). In all evaluations, the plants inoculated with *M. robertsii* (ESALQ 1635) had more root dry weight than those inoculated with *M. humberi* (ESALQ 1638) (Fig. 1).

Phenotypic characteristics related to the tomato reproductive stage were measured only at 30 DAI and all were positively impacted by *Metarhizium* inoculation. Inoculation with the fungi significantly increased the number of flowers ($\chi^2 = 25.34$, df

= 2, $P < 0.0001$), with the most flowers attained from plants inoculated with *M. robertsii* (ESALQ 1635), followed by *M. humberi* (ESALQ 1638), and inoculation with either fungus induced more flowering than control plants (Fig. 2A). However, the number of fruits per plant was not significantly affected by the treatments ($\chi^2 = 2.95$, df = 2, $P = 0.23$) (Fig. 2B). Both fruit fresh and dry weight were significantly increased by inoculation with *M. robertsii* (ESALQ 1635) in comparison to control plants (Fresh weight: $F = 3.60$, df = 2, 72, $P = 0.032$; Dry weight: $F = 4.79$, df = 2, 72, $P = 0.011$, respectively), but differences were detected between control and *M. humberi*-inoculated (ESALQ 1638) plants only for fruit fresh weight (Fig. 2C,D).

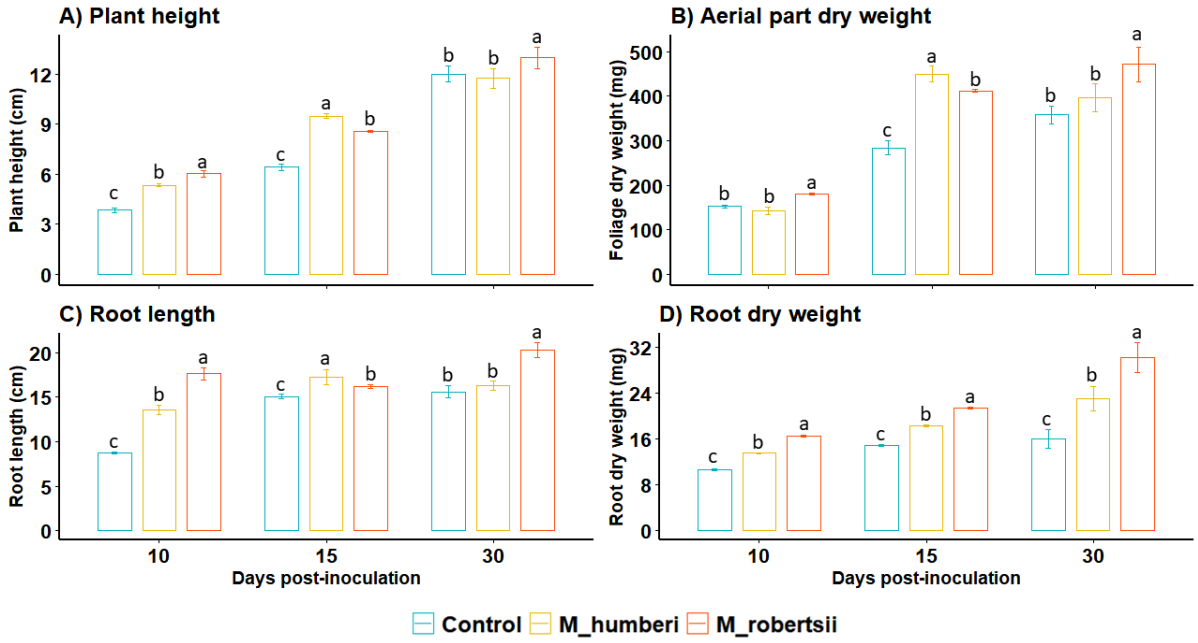

Figure 1. Shoot and root growth traits of MT plants inoculated with *M. robertsii* or *M. humberi* recorded after 10, 15, and 30 days after inoculation. Vertical bars (mean ± 95% CI [confidence interval], *n* = 20 replicates); distinct letters indicate significant differences among the treatments at $P < 0.05$ (Tukey HSD test).

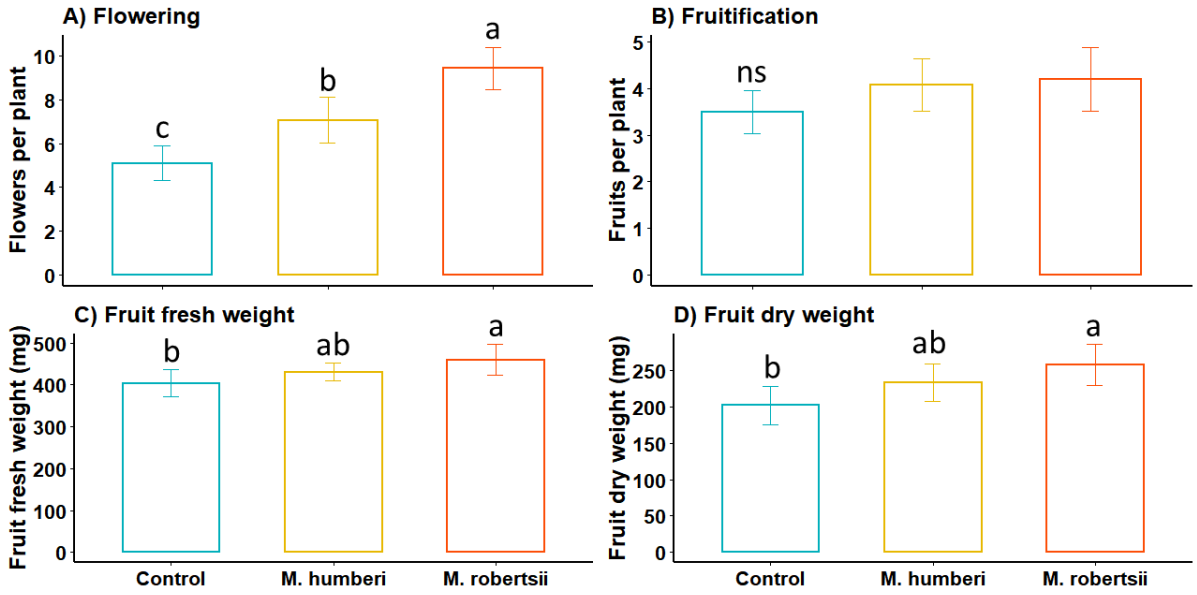

Figure 2. Reproductive growth traits of MT inoculated with *M. robertsii* or *M. humberi* measured 30 days after inoculation. Vertical bars (mean ± 95% CI [confidence interval], n = 20 replicates) with distinct letters indicate significant differences between fungal species at $P < 0.05$ (Tukey HSD test). Not significant at $P > 0.05$ (ns).

The multivariate linear analysis taking together all variables of tomato growth traits indicates significant variability in plant phenotypic responses mediated by fungal treatments and untreated control (treatment effect: $F = 10.42$, df = 16, 158, $P < 0.0001$). The canonical discriminant plot based on all tomato growth variables showed a cluster formed by *M. robertsii* (ESALQ 1635), spatially distant from single clusters formed by *M. humberi* (ESALQ 1638) and the control group (circles indicating the 95% confidence level of multivariate means (+) do not intersect) (Fig. 3). Both groups of *M. humberi* (ESALQ 1638) and control were placed on the opposite side of *M. robertsii* (ESALQ 1635) when viewing at the first canonical dimension, which corresponded to 86.4% of all data variability. Thus, this spatial pattern of these groups indicates that *M. robertsii* (ESALQ 1635) distinctly influenced tomato plant growth parameters in relation to *M. humberi* (ESALQ 1638) and control (untreated) plants. The length and direction of each vector in the biplot indicate the degree of association of the corresponding covariate with the canonical variables and demonstrate the overall superior performance of *M. robertsii* for all measured growth traits in tomato plants. The magnitude of each vector reveals the contribution or importance of each growth trait to each principal canonical

component in explaining the clusters formed across treatments. Hence, root dry weight, root length, and shoot dry weight produced longer vectors and were, therefore, the discriminant factors of these treatments. To a lesser extent, the other growth traits measured in this study also positively contributed to separate *M. robertsii* from the other treatments.

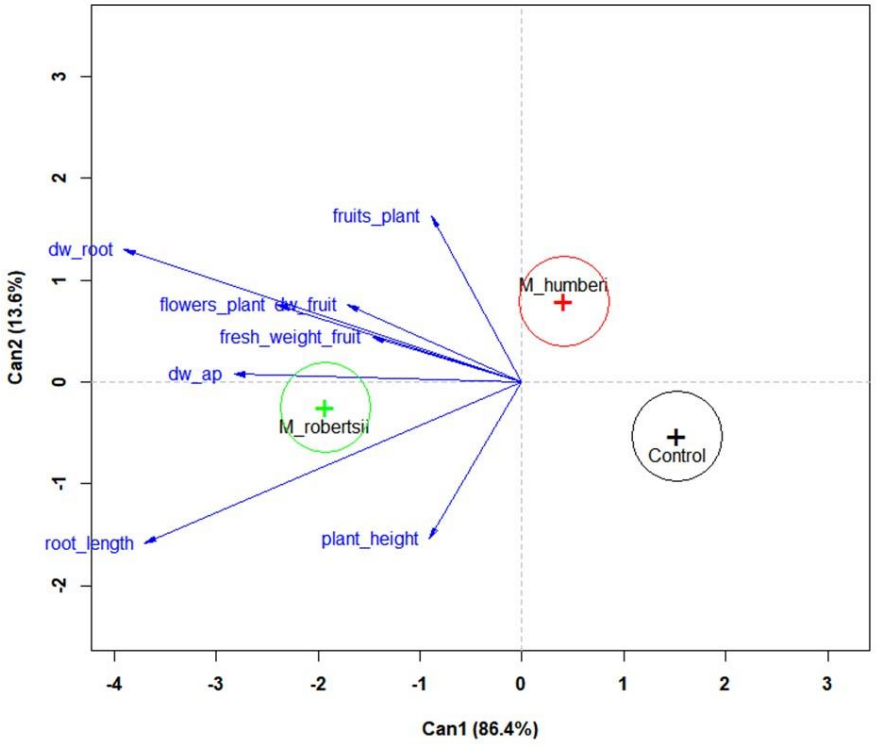

Figure 3. Canonical discriminant plot showing two principal canonical components, in which the blue vectors at different magnitudes of correlation with both axes corresponding to plant growth traits recorded at 30 days after inoculation, while circle groups are attributed to fungal species (labels: Mr = *M. robertsii*, Mh = *M. humberi,* and Cont = control). Each fungal species was represented by n = 60 observations. Vector legends: dw_ap = shoot dry weight; dw_root = root dry weight; dw_fruit= fruit dry weight.

2.3.2 Re-isolation of Metarhizium spp. from tissues of the Micro-Tom

To confirm the infection of MT by the *Metarhizium* spp., endophytic fungi were re-isolated from inoculated tissues and soil using selective media. Remarkably, both *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) were able to endophytically

colonize all the tomato tissues analyzed (root, stem, and leaf) 10–30 DAI using a conidial suspension delivered next to the roots of the tomato seedlings (Fig. 4). Our three-way factorial model found no interactions between plant tissues, fungal species, and evaluation periods ($P > 0.05$). However, the difference in colonization among the plant tissues was striking, in which the highest proportion of endophytic colonization was found in the roots, with 70–100% fungal recovery ($\chi^2 = 103.29$, df = 3, $P < 0.0001$). *M. robertsii* (ESALQ 1635) appeared to be more successful than *M. humberi* (ESALQ 1638) in colonizing root tissues evaluated at 30 DAI and leaf tissues at 10 and 15 DAI ($\chi^2 = 103.29$, df = 2, $P < 0.0001$); both fungi similarly colonized the stem. The proportion of tomato leaf fragments colonized by *M. robertsii* (ESALQ 1635) decreased by 63% with time from 15 to 30 DAI ($\chi^2 = 13.60$, df = 2, $P = 0.001$), whereas *M. humberi* (ESALQ 1638), despite its lower colonization levels than *M. robertsii* (ESALQ 1635), varied over time from 36.7% to 16.7%.

From the soil cultivated with tomato, both *M robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) were recovered at similar rates from soil samples ($\chi^2 = 0.69$, df = 1, $P = 0.40$), corresponding to inoculum densities of 4.4×10^6 CFU/g and 4.1×10^6 CFU/g of soil. The persistence of both species in the soil was unaltered over time ($\chi^2 = 0.69$, df = 2, $P = 0.71$), most likely due to the ubiquitous presence and pronounced colonization of the tomato root system by these fungi. None of the target fungi was retrieved from the plant tissue or soil substrate in the non-inoculated control plants, indicating no contamination of pot-grown tomato plants by the *Metarhizium* treatments or a natural occurrence.

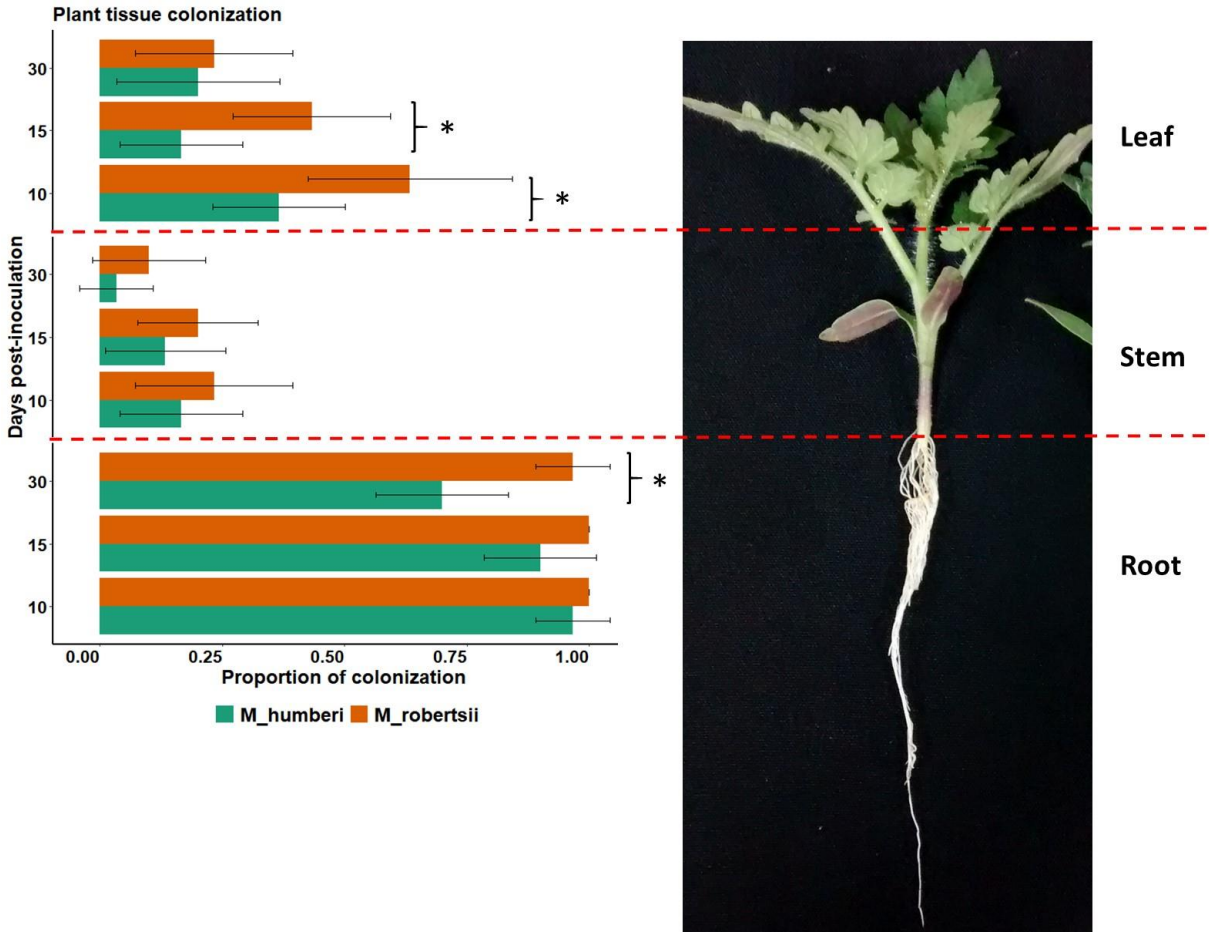

Figure 4. Endophytic colonization of Micro-Tom tissues (leaf, stem, and root) by *M. robertsii* or *M. humberi* 10, 15, and 30 days after inoculation. Vertical bars represent the means (± 95% CI [confidence interval], *n* = 10), while asterisk (*) designates significant difference between fungal species at $P < 0.05$ (Tukey HSD test).

2.3.3 Determination of *in vitro* IAA production

M. robertsii (ESALQ 1635) and *M. humberi* (ESALQ 1638) were able to produce IAA at different extents. The *in vitro* IAA production greatly varied with fungal species and with the presence or not of tryptophan ($F = 17.80$, df = 3, 60, $P < 0.00001$). Interestingly, IAA synthesis by *M. anisopliae* (ESALQ 1669) was significantly boosted without tryptophan and attained the highest concentration level, whereas the other *Metarhizium* species and *T. harzianum* (ESALQ 1306) had higher titers when grown in the presence of tryptophan (Fig. 5). Thus, the presence of tryptophan was essential for achieving higher IAA synthesis by *M. humberi* (ESALQ 1638), *M. robertsii* (ESALQ 1638), and *T. harzianum* (ESALQ 1306), but not for *M. anisopliae* (ESALQ 1669). On

the other hand, *M. anisopliae* (ESALQ 1669) cultures without tryptophan exhibited the highest IAA concentrations, whereas *M. humberi* (ESALQ 1638) presented higher production of IAA than *M. anisopliae* (ESALQ 1669) and *T. harzianum* (ESALQ 1306) when cultured with this IAA precursor.

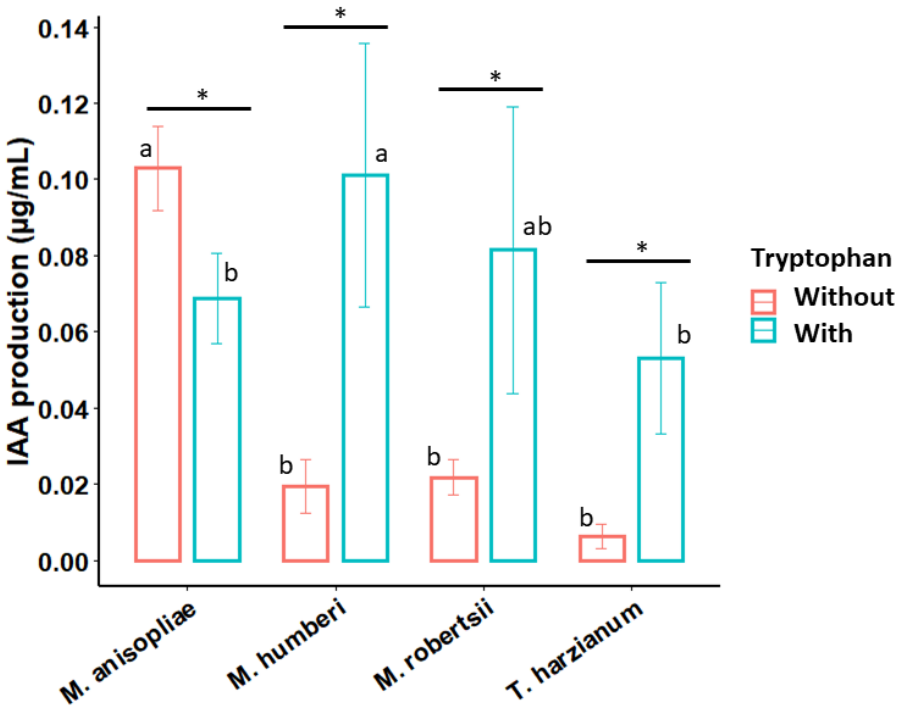

Figure 5. Effect of phenotype and exogenous tryptophan on the ability of *Metarhizium* species to produce indole-3-acetic acid (IAA) under *in vitro* conditions. *Trichoderma harzianum* was used as a positive control for IAA production. Bars (means ± 95% CIs [confidence intervals]) followed by distinct lowercase letters denote significant differences between fungal species for each culture medium (with or without tryptophan), while asterisk (*) indicates significant differences between culture medium supplemented with or without tryptophan within each fungal species. Statistical contrasts were performed with Tukey HSD method at $P < 0.05$ ($n = 9$).

2.3.4 IAA signaling by Metarhizium inoculation

Compared to the non-inoculated control plants, both *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) increased the detection of auxin-induced GUS expression in the roots at 10 and 30 DAI (Fig. 6). The GUS staining was more intense

and covered larger areas of the roots infected by *M. robertsii* (ESALQ 1635) than *M. humberi* (ESALQ 1638), suggesting that the former induced more auxin accumulation. Besides, auxin accumulation appeared to be more pronounced in the roots by 30 DAI in relation to day 10.

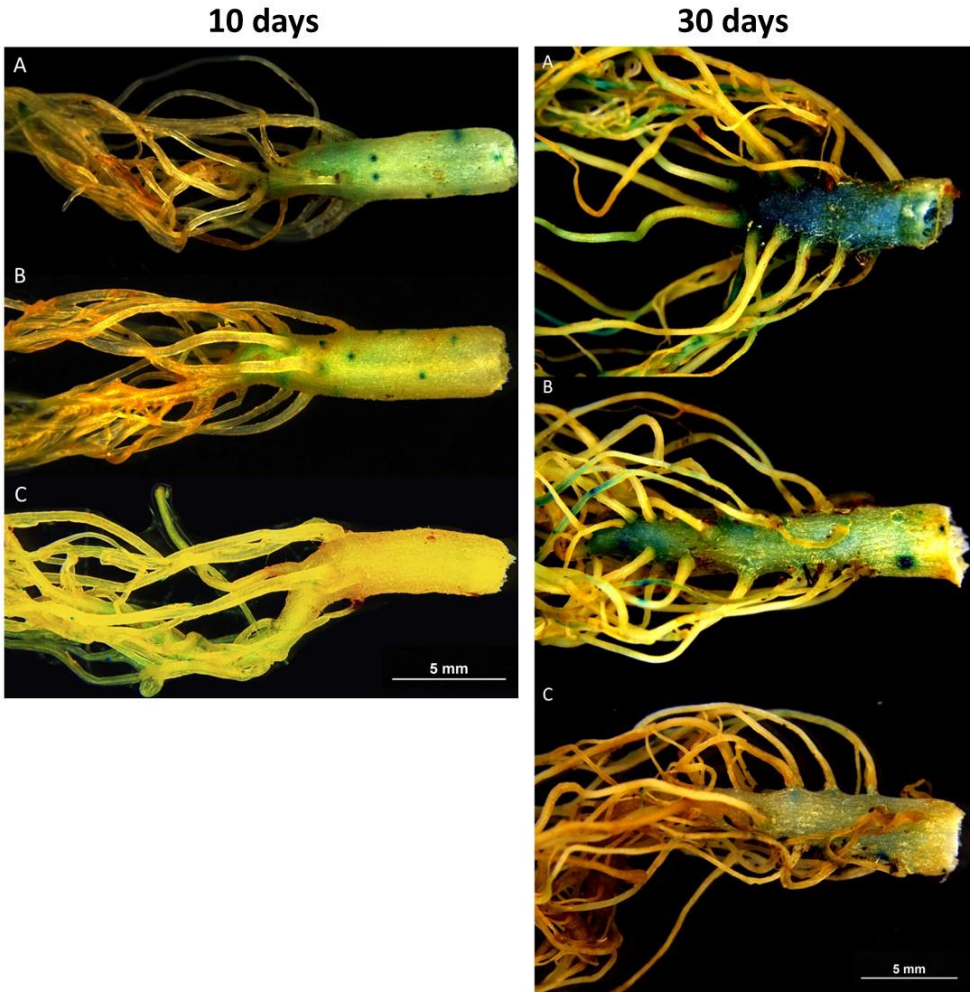

Figure 6. Representative photographs of β-Glucuronidase (GUS) histochemical staining assay in MT-DR5::GUS, at 10 and 30 days after inoculation with fungal endophytes. Blue stains indicate the specific sites where auxin (IAA) accumulated in the plant tissues as revealed by the histochemical staining with X-Gluc. Seedlings at age 18–21 days were individually inoculated with *Metarhizium robertsii* (A), *Metarhizium humberi* (B), or non-inoculated control (C).

2.3.5 Determination of enzyme activities and siderophores

The phosphate solubilization index was significantly different among *Metarhizium* spp. and *T. harzianum* (ESALQ 1306) (F = 322.56, df = 3, 54, P < 0.0001), where the *M. humberi* (ESALQ 1638) isolate exhibited the highest phosphatase activity, followed by *M. anisopliae* (ESALQ 1669), *T. harzianum* (ESALQ 1306), and *M. robertsii* (ESALQ 1635) after 15 DAI. The solubilization index of phosphate ranged from 1.16 to 2.12. The two species of *Metarhizium* outperformed *T. harzianum* (ESALQ 1306) in their ability to solubilize phosphate, with the lowest activity achieved for *M. robertsii* (ESALQ 1635) (Fig. 7A).

The index used to indirectly estimate phytase activity ranged significantly among the *Metarhizium* isolates (F = 38.255, df= 2, 40, P < 0.0001); the *M. anisopliae* isolate attained the higher phytase activity, followed by *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638). The solubilization index of phytate ranged from 1.47 to 1.51. The isolate of *T. harzianum* (ESALQ 1306) had null phytase activity, as the fungus did not show any halo zone when grown on medium amended with calcium phytate (Fig. 7B).

In vitro production of siderophore was significantly different among the *Metarhizium* species (F = 427.69, df = 2, 40, P < 2.2e-16) (Fig. 7C). The siderophore secretion index estimated by the size of halo per the size of the colony ranged from 0.99 to 1.62 across *Metarhizium* species after 15 days of growth. The isolate of *M. humberi* (ESALQ 1638) outproduced siderophores compared to the other *Metarhizium* species, whereas the *T. harzianum* (ESALQ 1306) isolate did not appear to produce such iron-chelating compounds (Fig. 7C).

The ability to degrade colloidal chitin by chitinase recorded after 15 days of incubation was significantly affected by fungal species (F = 7315.5, df = 3, 54, P < 0.0001) (Fig. 7D). *T. harzianum* (ESALQ 1306) exhibited higher chitinase activity than all three *Metarhizium* species. The second best chitinase producer was the isolate of *M. robertsii* (ESALQ 1635) followed by *M. anisopliae* (ESALQ 1669) and *M. humberi* (ESALQ 1638). Chitinase activity indexes varied from 1.49 to 5.80 (Fig. 7D).

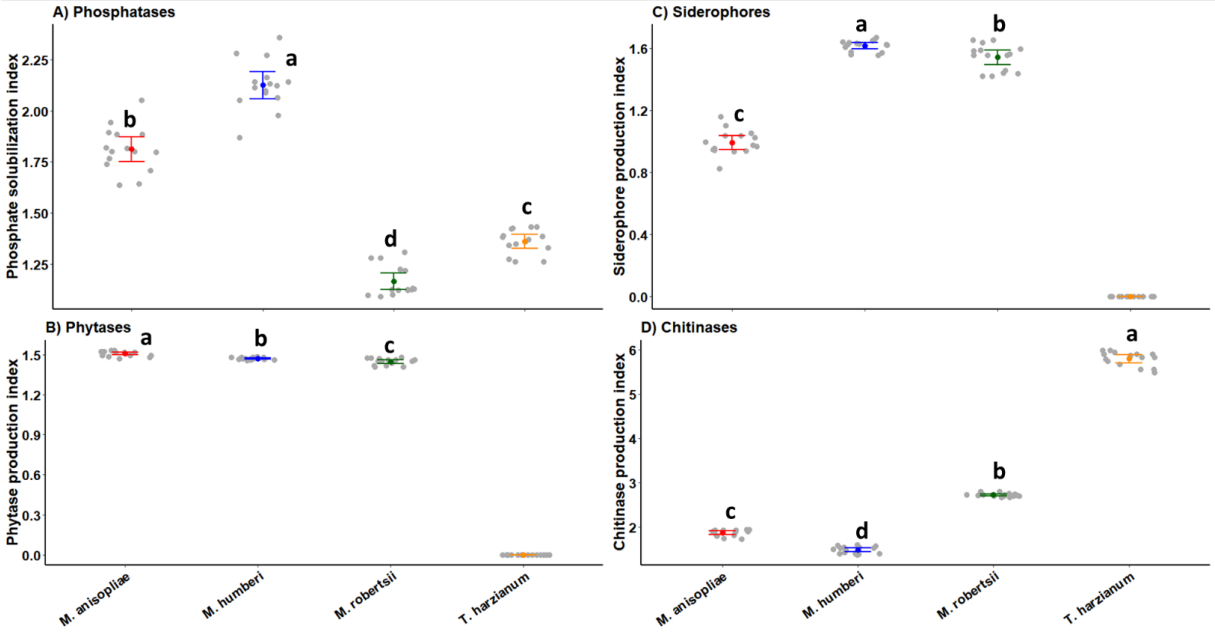

Figure 7. Biochemical profile of *Metarhizium* species and *T. harzianum* (as control) by measuring the solubilization or production index after 15 days of growth on specific solid medium to determine the activity of phosphatase (A), phytase (B), siderophore (C), and chitinase (D). Segdots (dots with error bars) indicate means ± 95% CI (confidence intervals), while gray circles represent observed values (n = 15 per fungus). Statistical contrasts were performed with Tukey HSD method at $P < 0.05$ and indicated by different letters. Note: where the mean is zero for some biochemical compounds attributed to *T. harzianum*, there is no statistical letter for comparison.

The multivariate linear analysis fitted to all the biochemical parameters measured for three isolates of *Metarhizium* spp. and *T. harzianum* as a positive control significantly captured data variation and revealed quite distinct biochemical profiles among these fungal isolates (treatment effect: F = 19.94, df = 12, 36, $P < 0.0001$). Biochemical traits examined with a canonical discriminant analysis closely grouped *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) (circles indicate that the 95% confidence level of multivariate means (+) intersect), mainly due to by the tryptophan-induced IAA and siderophore activities. *M. anisopliae* (ESALQ 1669) appeared isolated on the opposite side showing a strong positive correlation of IAA (without exogenous tryptophan) and to a less extent, with phosphatase activity. Chitinase activity by far was the strongest trait that separated *T. harzianum* (ESALQ 1306) from the *Metarhizium* species (Fig. 8)

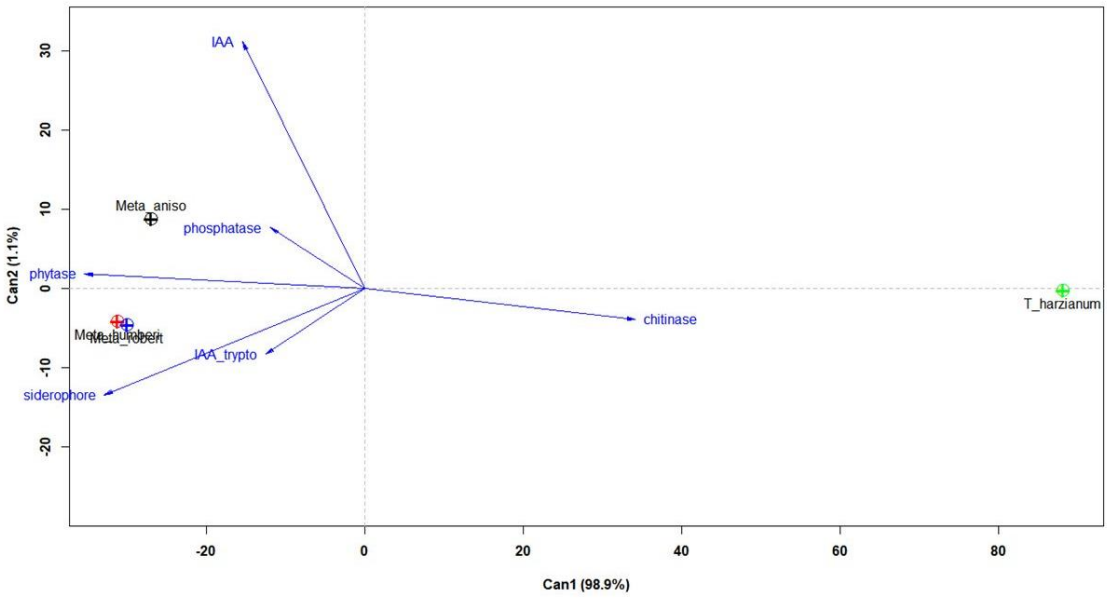

Figure 8. Canonical discriminant plot (or simply biplot) showing two principal canonical components in which the blue vectors correspond to the response biochemical variables and circle groups are attributed to fungal species (labels: Th = *T. harzianum*, Ma = *M. anisopliae*, Mr = *M. robertsii,* and Mh = *M. humberi*) represented by different colors (*n* = 24 observations used per fungal species). Legends: IAA_trypto = production of indole-3-acetic acid (IAA) with medium amended with L-tryptophan; IAA = IAA production with medium without the addition of exogenous L-tryptophan. Legends in the biplot: black = *M. anisopliae*, green = *T. harzianum*, red = *M. humberi*, and blue = *M. robertsii*.

2.4 Discussion

Overall, our data underpin the endophytic colonization of tomato by both *M. robertsii* and *M. humberi* that remarkably boosted plant growth, demonstrated by the increase in plant height, root length, and dry weight of shoots and roots compared to the non-inoculated plants. This observation agrees with similar studies using *M. anisopliae* (García et al., 2011).

Both *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) were able to endophytically colonize ‘Micro-Tom’ tissues of roots, leaves, and stems at least at 30 DAI. Endophytic fungi notably display preferential tissue colonization, and this preference is commonly attributed to fungal species and isolate, host species, and cultivar, as well as inoculation method and competition by natural soil microbiota.

Similarly, García et al. (2011) found a higher ratio of *M. anisopliae* colonization in roots and shoots, but the incidence was limited in leaves. Krell et al. (2018) recently showed the endophytic establishment of *M. brunneum* in stems of tomato plants following delivery of mycelia to the roots, which prompts future studies to address the type of inoculum propagule as a source of variation in the colonization success of plant hosts by endophytic pathogenic fungi.

Several mechanisms underlying the endophytic lifestyle of *Metarhizium* spp. associated with beneficial effects to plant hosts have been proposed (Behie and Bidochka., 2014; Harman et al., 2004). Nevertheless, the association between endophyte-induced plant growth promotion and biochemical features of *Metarhizium* involved in plant growth and antagonism toward other fungal pathogens has not been explored. The present study highlights the superior performance of *M. robertsii* (ESALQ 1635) over *M. humberi* (ESALQ 1638) in promoting tomato growth. It is interesting to note that *M. humberi* (ESALQ 1638) showed better performance than *M. robertsii* (ESALQ 1635) in the assays for the activity of phosphatases and phytases and the production of siderophores, but the difference between isolates was significant only for phosphatases. *M. robertsii* (ESALQ 1635) achieved superior performance to *M. humberi* (ESALQ 1638) only for the production of chitinases and *in vivo* IAA expression estimated by the GUS histochemical assay. A hypothesis is that the higher colonization ability of *M. robertsii* (ESALQ 1635) in tomato MT, revealed by the higher proportion of re-isolation from infected tissues, might be one of the main factors responsible for the stronger growth response of vegetative and reproductive traits.

Indole-3-acetic acid (IAA) is known to regulate plant growth, stimulating seed germination; increasing root development, controlling vegetative growth processes, and affecting photosynthesis and biosynthesis of various metabolites (Rana et al., 2019; Spaepen and Vanderleyden., 2011). The ability to produce IAA is an attribute of several microorganisms, including both plant growth promoters and some plant pathogens (Duca et al., 2014). A recent study was the first to report about IAA production by *M. robertsii,* which promoted root growth in *Arabidopsis,* and this phenomenon underpinned the importance of auxin in the ability of *Metarhizium* to stimulate plant growth (Liao et al., 2017). Our study reveals that Brazilian *Metarhizium* isolates can produce IAA *in vitro* and this production is higher than for the *T. harzianum* isolate, a species known for their plant growth stimulation. All three *Metarhizium* species tested here appeared to produce IAA *in vitr*o in the presence or absence of its

precursor tryptophan. Tryptophan is an amino acid found in root exudates and is the main precursor molecule for IAA biosynthesis by both fungi and bacteria (Gupta et al., 2015). Tryptophan appears to be essential for *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) IAA production, but it is not required for *M. anisopliae* (ESALQ 1669), which curiously produced more IAA in the absence of tryptophan. The coexistence of both tryptophan-dependent and tryptophan-independent IAA-biosynthetic pathways has been documented for *Azospirillum brasilense* (Prinsen et al., 1993) and *Saccharomyces cerevisiae* (Rao et al., 2010). IAA induced hyphal growth in the human pathogen *Candida albicans* and thus may function as a secondary metabolite signal that regulates virulence traits, such as hyphal transition in pathogenic fungi (Rao et al., 2010). Such behavior displayed by *M. anisopliae* (ESALQ 1669) for IAA production without the need for tryptophan and its relationship with the plant and hyphal growth warrants further investigation.

The *in vitro* data on IAA production by the isolates corroborate our findings in the biochemical GUS bioassay, in which the inoculation of DR5::GUS with our isolates of *Metarhizium* induced auxin-regulated gene expression. Both *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) increased auxin-induced GUS expression in primary and lateral roots, as well as in root hairs and root tip. These observations suggest that both *Metarhizium* species induced auxin-regulated gene expression in tomato plants at different extents. Liao et al. (2017) observed that both exogenous synthetic IAA application and *M. robertsii* culture filtrates increased GUS expression in *Arabidopsis* seedlings, but only in the root tips. The GUS activity varied for the different *Metarhizium* species, and the highest levels were recorded in roots at 30 DAI. The DR5 system has been widely used, as it provides a high level of accuracy concerning the sensitivity of auxin concentration and is a reliable evaluation for the presence of auxin (Chen et al., 2013). Although *M. humberi* is a sister lineage of the *M. anisopliae* s.str (Luz et al., 2019), the results obtained indicated that its performance is more similar to *M. robertsii* than to *M. anisopliae* in many aspects, including the production of key biomolecules that could be associated to endophytic colonization of plants and the antagonism of plant pathogens.

The phosphorus solubilization potential of *Metarhizium* has only been demonstrated *in vitro* for *M. anisopliae* by Mishra et al. (2014) and Shukla and Vyas (2014). We also demonstrated that the Brazilian isolates of *M. humberi* (ESALQ 1638) and *M. robertsii* (ESALQ 1635) can solubilize tricalcium phosphate, an insoluble

source of P. The greatest phosphate solubilization was attributed to *M. humberi* (ESALQ 1638) followed by *M. anisopliae* (ESALQ 1669), while the solubilization index of *M. robertsii* (ESALQ 1635) was very close to *T. harzianum* (ESALQ 1306). The microbial phosphorus solubilization helps to make phosphorus available for plants to uptake. After nitrogen (N), phosphorus (P) is the most important element in plant nutrition and acts in major plant metabolic processes, such as photosynthesis and respiration (Khan et al., 2010). Plants absorb phosphate only as monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{-2}) ions, but most of the P present in the soil is insoluble, immobilized, or precipitated, and thus these P forms are unavailable for plants (Gouda et al., 2018). Soil microorganisms can solubilize insoluble phosphorus by producing organic exudates, organic acids, acid phosphatases, or enzymes, such as phytases, making P available to be acquired by plants (Jha and Saraf., 2015).

The *Metarhizium* isolates tested can produce phytase, an enzyme able to decompose phytic acid. Between 60–80% of organic phosphate in the soil is inositol-hexakisphosphate, also known as phytic acid or phytate (Schachtman et al., 1998). This enzyme has been detected in microorganisms like fungi but had never been identified in *Metarhizium* species. Our study proved for the first time that *M. anisopliae* (ESALQ 1669)*, M. robertsii* (ESALQ 1635)*,* and *M. humberi* (ESALQ 1638) produced high titers of phytase. The isolate of *T. harzianum* (ESALQ 1306) did not produce this enzyme. These data highlight the potential of *Metarhizium* to improve the P content and to enhance plant growth, but further studies should be developed to check the importance of phytases in plant growth promotion by entomopathogenic endophytic fungi in the field.

Iron (Fe) is one of the most abundant elements on earth and an essential micronutrient for several living organisms. Siderophores play an important role in iron uptake by plants and siderophore-secreting microorganisms inside plant tissues act in the transport of Fe^{3+} for the synthesis of ATP, chlorophyll, and DNA, contributing to plant growth and yield (Schwyn and Neilands, 1987; Neilands, 1995; Beneduzi et al., 2012). Indirectly, siderophore production by plant growth promoting microorganisms can avoid the proliferation of plant pathogens through the sequestration of Fe^{3+} around the rhizosphere (Gupta et al., 2015; Jah and Saraf 2015). Some studies indicated that the siderophore production by *M. robertsii* can play an important role during insect infection and fungal virulence, as well as alleviate the sensitivity of conidia and microsclerotium to oxidative stress and sustain their development under iron-limited

conditions (Krasnoff et al., 2014; Donzelli et al., 2015; Li et al 2016; Sbaraini et al., 2016; Raya-Díaz et al., 2017). Our study was the first to report siderophore synthesis by *M. anisopliae* (ESALQ 1669) and *M. humberi* (ESALQ 1638), and our results indicate that siderophore production by some *Metarhizium* species could be involved in plant growth promotion.

The complexity of chitinases isolated from *Metarhizium* has been extensively studied. Several studies have shown that this fungus produces extracellular chitinases and correlate their importance during the fungal invasion in insect hosts (St. Leger et al., 1991, 1993). However, the role of chitinases is not clearly understood in *Metarhizium* plant colonization and growth. Chitinases are produced in a large number of microbial endophytes and can contribute to plant growth promotion, both directly and indirectly. This group of enzymes has been studied for their potential for biocontrol of phytophagous nematodes and plant pathogenic fungi (Gan et al., 2007). Few studies have examined the potential of chitinases involved in the antagonistic effects of *Metarhizium* to plant pathogens or even plant-parasitic nematodes. A large range of Brazilian *Metarhizium* isolates can antagonize *in vitro* the sugarcane plant pathogens *Fusarium moniliforme* and *Colletotrichum falcatum* (A.C. Siqueira, unpublished data). Thus, chitinase and siderophore activities displayed by *Metarhizium* isolates may be good proxies for the selection of good antagonists against other fungal plant pathogens. However, it is important to point out that the biochemical characterization of fungal isolates determined *in vitro* does not always reflect the outcomes for *in situ* antagonism or plant growth promotion.

The two isolates of *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) are currently under registration in Brazil as the active ingredients of one biopesticide product. Here, were demonstrated that both isolates have interesting and complimentary beneficial attributes, such as the biochemical metabolic traits, endophytic colonization, and stimulation of plant growth. This study envisions the first endophytic *Metarhizium*-based multifunctional bioproduct as an innovative strategy for plant stimulation and pest biocontrol in agriculture, which may ultimately lead to increased crop productivity and less reliance on chemical inputs.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

AS, CG, and IDJ conceived the idea and designed the experiments. AS, CG and JM performed the experiments and acquired data. GM analyzed data and AS, GM, and IDJ wrote the manuscript. JM, MQ, and AF contributed to the manuscript write and revisions. All authors have read and approved the final manuscript.

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3 EFFECTS OF *Metarhizium* ENDOPHYTIC ESTABLISHMENT ON SUGARCANE GROWTH AND INSECT PEST POPULATIONS

Abstract

The entomopathogenic fungi *Metarhizium* spp. can colonize the plant rhizosphere and, consequently, promote plant growth and affect below- and above-ground pests. We conducted a series of *in vivo* bioassays using the isolates of *Metarhizium robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) originating from Brazil to select the best propagule type and concentration for the promotion of sugarcane plant (*Saccharum* L.) growth and for reducing of the sugarcane nematodes, *Meloidogyne javanica* and *P. zeae* and the sugarcane borer, *Diatraea saccharalis* in the greenhouse. Plants grown in soil inoculated with conidia of both *Metarhizium* isolates presented larger aerial part after 30 days and the inoculation of *M. robertsii* conidia improved the stem diameter. No differences were observed on the dry weight of both the aerial part and the plant roots among treatments. Both types of propagules, conidia, and blastospores, of *M. robertsii* and *M. humberi,* colonized sugarcane tissues; however, higher colonization was observed for conidia. The inoculation of sugarcane with 10^8 conidia/mL (corresponding to an application of 10^{12} con/ha) conidia suspensions of *M. robertsii* and *M. humberi* increased the length of the aerial part over non-inoculated plants at 45 and 70 days after inoculations (DAI) compared to 10^7 conidia/mL and the uninoculated control. The re-isolation of both *M. robertsii* and *M. humberi* was confirmed in all plant tissues, independently of the conidia concentration, time of evaluation, or inoculation method (individually or both fungi combined). The inoculation *M. robertsii* and *M. humberi* contributed to reducing *M. javanica* (up to 45%) and *P. zeae* (up to 33%), compared to the control. *In vitro* studies revealed that cultures filtrates of both *M. robertsii* and *M. humberi* had a higher effect on the nematodes egg hatch than conidia. Mortality of *D. saccharalis* larvae on plants colonized by *M. robertsii* and *M. humberi* was as high as 91.7% after 30 days of inoculation. In host-choice assays, *D. saccharalis* larvae preferred the uninoculated control than the fungi inoculated plants. These results bring a new perspective on *Metarhizium* spp. as an inoculant for plant growth and plant protection.

Keywords: Endophytes; Plant growth promotion; *Metarhizium*; sugarcane; insects

3.1 Introduction

Sugarcane, *Saccharum officinarum* L, is a crop of great socioeconomic importance for Brazil, as it has a large share of our country's GDP (Gross Domestic Product) and is responsible for approximately 25% of world production and reached a production of 642.7 million tons in the 2019/2020 (CONAB, 2020).

However, pests' occurrence is one of the limiting factors for the productivity of sugarcane and the industrial use of its by-products. In this sense, biological control using entomopathogens is a safer way to keep populations in balance, limiting their

rapid multiplication, causing a low impact on non-target organisms and the soil (ALVES, 1998).

Entomopathogenic fungi inhabit the soil and have a cosmopolitan distribution in a wide range of arthropod hosts (ZIMMERMANN, 2007). These fungi and their hosts live in a complex landscape influenced by multi-trophic relationships in a community modulated by abiotic factors.

Until now, the most significant emphasis on the study of entomopathogens has been centered on the development of biological control agents while basic research on their ecology, which is essential for its successful use in biological control has been neglected (BRUCK, 2005; MEYLING; EILENBERG, 2007; VEGA et al., 2009).

To fill this knowledge gap, recent studies have been dedicated to the ecology of these fungi in their natural habitats and the ecosystems they are applied to. These studies have shown that there are genotypic groups of *Metarhizium* spp., some can be closely associated with host insects (natural habitat) or with plants (IWANICKI et al., 2019; WYREBEK et al., 2011). Competence with the rhizosphere is a microorganism's ability to increase in the microhabitat (ST LEGER, 2008) and was observed in some *Metarhizium* spp. (BRUCK, 2005; HU; ST LEGER, 2002).

Also, recent studies show that *Metarhizium* spp. inoculated in the plant rhizosphere benefits plant growth and aids in the acquisition of nutrients, such as nitrogen, by the plant (BEHIE; BIDOCHKA, 2014; BEHIE et al., 2012; KHAN et aL., 2012; SASAN; BIDOCHKA, 2012). Among other benefits, *Metarhizium* spp. antagonize phytopathogenic fungi (SASAN; BIDOCHKA, 2013, KEYSER et al., 2016) and reduce pest insects (KEYSER et al., 2014; BATTA, 2013), conferring plant protection.

To our knowledge, no studies have reported the effect of sugarcane inoculations with different *Metarhizium* species, propagules, or fungal concentration on plant productivity and plant pests. The objectives of the present study were, therefore, to evaluate the effect of inoculations of sugarcane with *M. robertsii* and a new species, *Metarhizium humberi* (LUZ et al., 2019), comparing conidia or blastospores, two different fungal concentrations, and the combination of both species on sugarcane growth, *D. saccharalis* behavior, development, and mortality and the effects on two nematodes, *M. javanica* and *P.* zeae under greenhouse conditions. The present research aimed to identify promising candidate fungal isolates for pest management and crop biostimulant in sugarcane production in Brazil.

3.2 Material and Methods

3.2.1 Fungal isolates

All studies were performed at the Laboratory of Insect Pathology and Microbial Control of Insects and the Laboratory of Nematology, both at ESALQ/USP (Piracicaba/SP). Two isolates of the entomopathogenic fungus *Metarhizium* spp. (Table 1) was selected from the collection of entomopathogens “Prof. Sérgio Batista Alves” from the Laboratory of Pathology and Microbial Control of Insects at ESALQ/USP, Piracicaba, State of São Paulo. These isolates had been previously identified to species level by molecular techniques (REZENDE et al., 2015; BOTELHO et al., 2019; LUZ et al., 2019).

The isolates were selected based on preliminary results obtained previously, which confirmed the ability to promote the growth of sugarcane plants and reduce pests (A. C. SIQUEIRA, unpublished), besides the production of auxin, phosphate solubilization, and the secretion of key enzymes such as chitinases, siderophores, phytases (SIQUEIRA, et al., 2020). The access of these two *Metarhizium* species is registered at the Brazilian System for the Management of Genetic Heritage and Associated Traditional Knowledge – SisGen under the code RAC856E.

Table 1. Isolates of the entomopathogenic fungus *Metarhizium* spp. used in this research.

Isolate code[1]	Fungal species	Crop/Isolation method	Location in Brazil (City - State)[2]	Geographical coordinates	Sampling date
ESALQ 1635	*Metarhizium robertsii*	Forest soil/Insect bait with *Tenebrio molitor*	Delmiro Gouveia, AL	9°25'0.12"S 37º57'8.49"W	Mar/2012
ESALQ 1638	*Metarhizium humberi*	Savanna soil/Selective media (PDAY)	Rio Verde, GO	17º29'49.3"S 51º13'40.7"W	Mar/2012

[1]ESALQ - Escola Superior de Agricultura “Luiz de Queiroz” – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil. [2] Brazilian States: AL = Alagoas, SP = São Paulo, and GO = Goiás.

3.2.2 Fungal suspensions

The isolates ESALQ-1635 (*Metarhizium robertsii*) and ESALQ-1638 (*Metarhizium humberi*) (Table 1) were used in the tests to promote the growth of sugarcane in the greenhouse. We selected both conidia and blastospore propagule, to verify which presents the best endophytic colonization and promotion of plant growth. Conidia were produced in parboiled rice, according to the methods described by Alves (1998). After production, the fungus conidia were extracted, the conidia concentration per gram of rice and the conidia viability were determined. Blastospores were obtained from the fungus fermentation in liquid medium nº 4 described by Jackson and Jaronski (2009). For the fermentation, 250 ml BELCO ® baffled flasks were used, shaken for five days in a refrigerated Shaker incubator (MARCONI®, Model: MA 830) at 26 ± 1 ° C and 300 rpm to obtain the required blastospores concentration.

Both conidia and blastospores were estimated in a Neubauer chamber using a dilution of the original suspension. An aliquot of the original suspension was removed to verify the propagules' viability according to the method described by Oliveira (2010). For this, 150 µL of the fungal suspensions were inoculated into Rodac ™ Petri dishes (60x10mm; Falcon) containing 5 ml of PDA culture medium (Difco®) with the Pentabiotic antibiotic (5 mg / L) and 10 µL / L of the fungicide Derosal®, covering the four central quadrants. The plates were kept in a B.O.D incubator (25 ± 1 ° C, 14 hours of photophase) for 24 hours. A feasibility analysis was performed by direct counting under a light microscope at 400x magnification, focusing on the plate's center totaling 200 conidia or blastospores per plate. Propagules that presented a germ tube with a size equal to or greater than its diameter were considered viable. Suspensions with less than 95% viability were not used.

3.2.3 Sugarcane inoculation with *Metarhizium*: propagule and concentration selection

Different experiments were carried out to select the best propagule for the inoculation of sugarcane plants, comparing conidia with blastospores. After choosing the type of propagule, additional experiments were conducted to verify which was the

best fungal concentration for inoculation in plants and test the effect of the combination of the two *Metarhizium* species selected for the research.

3.2.3.1 Sugarcane inoculation with two different propagules of *Metarhizium:* conidia and blastospores

The experiment was carried out in a greenhouse at the Entomology and Acarology Department of the “Luiz de Queiroz” Superior College of Agriculture (ESALQ/USP), using the RB96-6928 variety and substrate Tropstrato HT. Sugarcane buds were planted in 180 cm^3 tubes, and inoculation was based on the adapted methodology of Lanza et al., (2004), where the fungus conidia or blastospores were applied directly on the seedlings and the substrate. Five treatments were used with 30 repetitions each (2 isolates of *Metarhizium* x 2 types of propagules + Control). A treatment using only sterile distilled water + Tween® 80 (0.05%) was used as a negative control. The plants were maintained in a greenhouse irrigated daily for 30 days. After this time, the plant growth and endophytic colonization by the fungi were assessed. The experiment was repeated three times.

3.2.3.2 Determination of conidia concentrations for sugarcane inoculation with two *Metarhizium* species

The experiment was carried out in a greenhouse at the Entomology and Acarology Department of “Luiz de Queiroz” College of Agriculture ESALQ / USP, using sugarcane buds of the CTC-9001 variety. *Metarhizium* conidia concentrations, $1x10^{11}$ ($1x10^7$ conidia/mL) and $1x10^{12}$ conidia/hectare ($1x10^8$ conidia/mL) were selected to determine the plant's fungus's best colonization and the best seedling growth. Also, both fungal species co-inoculation was tested in the same concentrations mentioned above to verify whether the co-inoculation could increase plant growth. The application method consists of inoculating 1 mL of each suspension directly over sugarcane buds and soil, and 35 repetitions were made per treatment. The seedlings were planted in 1L pots and kept under daily drip irrigation in a greenhouse. The experiment was evaluated 45, 70, and 90 days after fungal inoculations, and it was repeated three times.

3.2.4 Effect of *Metarhizium* inoculation on sugarcane plant growth

Plant growth promotion was evaluated by measuring the aerial part's length using the methodology described by Khan (2012) with some modifications. The stem diameter was measured in millimeters using a digital caliper at the base of the seedling stem. Additionally, the dry weight of the aerial part was determined. The plants' stems and leaves were cut, placed in paper envelopes (20x20 cm), and kept in an oven at 60°C until constant weight (approximately 24 hours). After that period, it was weighed on a precision digital scale. Equally, the roots were detached from the planting sheet and then washed for complete removal of the adhered soil. After washing, they were individually placed in paper envelopes (20x20 cm) and kept in an oven at 60 ° C until constant weight (approximately 30 hours). After that period, it was weighed on a precision digital scale.

3.2.5 Colonization of entomopathogenic fungi in sugarcane tissues

The recovery of isolates from plant tissues was assessed in each evaluation time after inoculation (30 days after inoculation for propagule selection assay and 45, 70, and 90 days after inoculation for conidia concentration on the co-inoculation assay). Samples of the roots, stem, and leaf were taken from the plants and washed under running water. The samples were cut into fragments of approximately 5 cm and superficially sterilized by immersion in 70% alcohol for 30 seconds, 1% sodium hypochlorite for 1 minute, 70% alcohol again for 30 seconds, and finally, in sterile distilled water three times. This water was inoculated in a Petri dishes to confirm de sterilization process. The plant fragments were chosen at random and transferred to Petri dishes containing 15 mL of culture medium described by Behie et al. (2015), totaling fiften replicates (fragments) per treatment. The culture medium consists of potato (200g/L), dextrose (20g/L), agar (15g/L), Cycloheximide (0.5g/L), Chloramphenicol (0.2g/L), Dodine 65% (0.5g/L), Violet crystal (0.01g/L) and deionized water (1,000 mL). The plates were kept in an incubator at 25 ± 2 ° C and 14 hours of photophase for 30 days. After this period, the colonies of *Metarhizium* that grew on the plant fragments were visually identified based on the morphological characters (colony color) and microscopic observations. The percentage of recovery (PR) was calculated by the number of fragments colonized by the entomopathogenic fungus (*Metarhizium*

spp.) divided by the total number of fragments inoculated according to Araújo et al., (2002).

3.2.6 Effect of *Metarhizium* inoculation on *M. javanica* and *P. brachyurus* development

An experiment was carried out to verify whether the inoculation of *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) in sugarcane plants, in addition to promoting growth, could assist in reducing infestations by two important nematode species that cause several damages in sugarcane crop. For this, sugarcane, variety CTC-9001, were planted in 1L pots containing a mixture of soil and fine sand. *Metarhizium* conidia concentration $1x10^{12}$ conidia/hectare were selected, and the sugarcane seedlings were inoculated with 1 mL of this suspension in each plant distributed in the soil. After 30 days of planting and fungal inoculation, 1,000 individuals (eggs and juveniles) of *Meloidogyne javanica* or *Pratylenchus zeae* were inoculated per potted plant. The experiment consisted of 10 replicates of each treatment and was evaluated after 70 days after inoculation for *M. javanica* and 90 days after inoculation for *P. zeae*.

For evaluation, the plants had their aerial portion removed, and the roots were washed and stored. After that, the material was transported to the Laboratory of Nematology, located at ESALQ/USP, where the analysis was carried out. After this process, the nematodes were extracted following Coolen and D'Herde (1972) method, which consists of washing the roots with tap water, cut in 1cm pieces, and blended with commercial sodium hypochlorite (0.5%), used to dissolve the egg masses. The resultant suspension was poured through a 60 and 500 mesh sieve (60 mesh – 0.260mm aperture – to retain the coarse root particles; and 500 mesh – 0.025mm aperture – to keep fine particles, tiny roots pieces, and nematodes), being collected on 250ml beaker. The suspension was then submitted to the centrifugal-flotation technique for the isolation and concentration of nematodes, with a sucrose solution. The nematodes in the samples were killed under low heat and stored on flasks for counting under a Peters' slide. At the end of the evaluation, the variables, the Final population (Pf), the Percentage of reduction in final population (% Pf reduction), and Nematodes per gram of root (Nem/g) were obtained.

3.2.7 Effect of *Metarhizium* on nematodes *in vitro*

The direct effect of culture filtrates and conidia of *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) (table 1) on nematodes were evaluated in this study. Conidia were obtained using the methodology described above (3.2.2) (ALVES, 1998). For filtrates cultures preparation, a suspension of fungi was adjusted at 5 x 10^6 conidia/mL, and 1 mL of this suspension was transferred to Erlenmeyer flasks (250 mL) containing 100 mL of PD broth media (Kasvi®), previously autoclaved (120°C at 1ATM, for 20 minutes). The cultures were kept on a shaker for five days at 25°C, 225 rpm, in the dark. After that, the cultures were filtrated on Kitasato flask with Whatman® filter (n°40) using a vacuum pump and centrifugated at 10,000 g. The supernatants were transported to a flow chamber and filtered through paper n°40. Millipore filter 0,22µm (Merck®) to remove the reminiscent cells. The filtrates were immediately transferred for sterilized Falcon tubes (50ml) stored at -10ºC until usage.

For the experiment, 500 µl of a conidia suspension at 1x10^8 conidia/mL was poured inside sterilized Eppendorf microtubes, with 1,5mL capacity with 500 eggs of *M. javanica* and *P. zeae*, obtained from Nematology laboratory at ESALQ/USP and consisted of 500 nematode eggs per repetition. The same was performed using 500 µl of the *Metarhizium* cultures filtrates, with 500 nematode eggs per repetition. Another treatment was performed with a mixture of 250 µl of conidia + 250 µl of cultures filtrates + 500 nematode eggs per repetition. The experiment had two control treatments, one using deionized water + Tween 80 and another using only PD broth media with 500 nematode eggs per repetition. The tubes were sealed and kept on a shaker, to aerate the nematode suspension, in the dark and maintained at 25°C. The evaluation was carried out seven days after inoculation, and the number of eggs and J2 were counted, and the hatching percentage was estimated. The experiment contained ten repetitions (Eppendorf tubes), and it was repeated three times.

3.2.8 Effect on *D. saccharalis*

The objective was to verify whether the inoculation of *Metarhizium* in sugarcane plants could affect the development or result in mortality of *Diatraea saccharalis* caterpillars, considering that this is the phase that causes the most damage to sugarcane. For this, sugarcane plants, Var. CTC-9001, were planted in 1L pots

containing commercial substrate Tropstrato HT (Vida Verde®). The cane seedlings were inoculated with 1 mL of $1x10^8$ conidia/mL of the isolates of *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) and distributed in the soil. *D. saccharalis* eggs were originated from the Laboratory of Insect Biology at the Department of Entomology and Acarology of ESALQ/USP. After the newborn caterpillars' hatching, they were fed on sugarcane leaves for acclimatization to the natural diet until the 3rd larval instar.

For the experiment, 3rd instar caterpillars were fed in stalks of sugarcane plants after 30, 60, and 90 days after inoculation with *Metarhizium* and control plants (without fungus inoculation). The caterpillars were confined and kept under controlled conditions (25 ± 1 ° C, 12 hours of photophase). Mortality was also assessed every 48 hours at the time of weighing. Each treatment contained 30 repetitions, represented by 30 caterpillars, and the experiments were evaluated for 30 days, and it was repeated three times.

3.2.9 Host-choice experiment with *Diatraea saccharalis* in sugarcane plants inoculated with *Metarhizium*

The host-choice of sugarcane borer larva was assessed to determine its preference for uninoculated or *Metarhizium* inoculated plants. The experiment was carried out at the Laboratory of Chemical ecology of insects, and *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) (Table 1) were selected for this bioassay. Sugarcane plants of CTC-9001 variety were previously inoculated with 1 mL of a suspension on each isolate with $1x10^{12}$ conidia/ha, as described at 3.2.3.2 item. Plants were daily drip irrigated in a greenhouse for 30 days and after that, were placed in a double choice box, confronting: T1 – a plant without inoculation (control) x plant inoculated with *M. robertsii* (ESALQ 1635), T2 – a plant without inoculation (control) x plant inoculated with *M. humberi* (ESALQ 1638) and T3 – a plant inoculated with *M. robertsii* (ESALQ 1635) x plant inoculated with *M. humberi* (ESALQ 1638). One 3rd larval instar of *D. saccharalis* was confined in a double choice box, and 24 hours after the plants were inspected, the treatment chosen was recorded. Each treatment contained ten repetitions (boxes), and the experiment was repeated three times.

3.3 Statistical analysis

The data obtained in plant growth promotion experiments described at 3.2.3 was first submitted to the Shapiro Wilk normality tests. If the data attended the normality assumption, it was then submitted to analysis of variance (ANOVA) by using the R statistical software (R Core Team 2013). All multiple comparisons of means were performed with Tukey's HSD test at a significance level of 5%, using the package "Agricolae". The endophytic colonization data were analyzed using the Chi-square statistic ($\chi 2$), considering the total number of plant fragments evaluated and the number of pieces colonized by fungus using the PAST 3.22 software (Hammer et al., 2001). Additionally, all experiments with insects and nematodes (number of nematodes per gram of roots, the final population of nematodes, number of J2, eclosion percentage, larvae's weight, and percentage of larvae mortality) were analyzed using the R statistical software. First, the data were submitted to normality tests, as described above, and then to the analysis of variance (ANOVA). Multiple comparisons were performed with Tukey's HSD test at a significance level of 5%, using the same package described above. On the other hand, the host-choice data were analyzed using the Chi-square statistic ($\chi 2$), considering the total number of larvae that chose one or another treatment to establishment the PAST 3.22 software Hammer et al., (2001).

3.4 Results

3.4.1 Sugarcane inoculation with two different propagules of *Metarhizium*: conidia and blastospores

The plants that received the treatments with the application of conidia of both tested *Metarhizium* isolates (ESALQ 1635 and ESALQ 1638) presented a larger size of the aerial part after 30 days, comparing to the control ($F= 9.03$, $d.f= 4$, $P < 0.0001$) (Table 1). Regarding the stem diameter, however, only the treatment that received the inoculation of the conidia of *M. robertsii* (ESALQ 1635) differed statistically from the control ($F= 6.45$, $d.f= 4$, $P < 0.0001$) (Table 2).

The dry weight parameters of both the aerial part ($F= 0.94$, $d.f= 4$, $P= 0.4407$) none of the treatments applied differed from the control or each other, indicating no influence on this parameter (Table 2). The roots' dry weight was marginally significant

(F= 2.41, d.f= 4, P= 0.05281), being the weight in treatments with conidia 1,28 times the weight of the control plants.

Table 2. Sugarcane plant growth parameters (Means ± SE) at 30 days after conidia or blastospores inoculation with the entomopathogenic fungi *Metarhizium robertsii* (ESALQ 1635) and *Metarhizium humberi* (ESALQ 1638).

	Assessment[1]			
Treatment	Length of Aerial part (cm)	Diameter of culm (mm)	Dry weight Aerial part (mg)	Dry weight Roots (mg)
M. robertsii - conidia	30.5 ± 0.9 a	5.1 ± 0.1 a	1269.1 ± 100.9	331.4 ± 31.7
M. robertsii - blastospores	22.3 ± 0.5 b	4.1 ± 0.2 b	1074.1 ± 60.1	258.2 ± 17.5
M. humberi - conidia	27.9 ± 1.3 a	4.7 ± 0.2 b	1189.3 ± 87.2	333.8 ± 24.5
M. humberi - blastospores	25.1 ± 1.4 ab	4.6 ± 0.1 b	1164.5 ± 92.3	317.5 ± 26.6
Control - Tween 80	21.9 ± 0.9 b	4.2 ± 0.1 b	1077.4 ± 74.7	259.5 ± 19.9
F	9.03	6.45	0.94	2.41
d.f	4	4	4	4
P-value	P < 0.0001	P < 0.0001	0.4407	0.05281

[1]Data (mean ± SE) followed by different letters within a column are significantly different (ANOVA, followed by post hoc Tukey test, P < 0.05).

Both *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) were able to colonize sugarcane tissues, regardless of the type of propagule that was inoculated (Figure 1). The inoculation with conidia resulted in better endophytic colonization on the tissues. Concerning roots colonization, the treatments that received conidia inoculation presented 60-80% of fungal recovery from *M. humberi* and *M. robertsii*, respectively, showing significant colonization percentage compared to treatments receiving blastospores inoculation and the control, differing statistically ($\chi 2 = 10.87$, df = 5, P= 0.028). The same occurs for the aerial part colonization parameter; the treatments with conidia inoculation showed significant endophytic colonization for both *Metarhizium* isolates (27-40% fungal recovery) differing from blastospores treatments and control ($\chi 2 = 126.50$, df = 5, P < 0.0001) (Figure 1). No samples colonized by fungus were found in the Control treatment, indicating success in surface sterilization of plant tissues. There was no cross-contamination between treatments during the experiment.

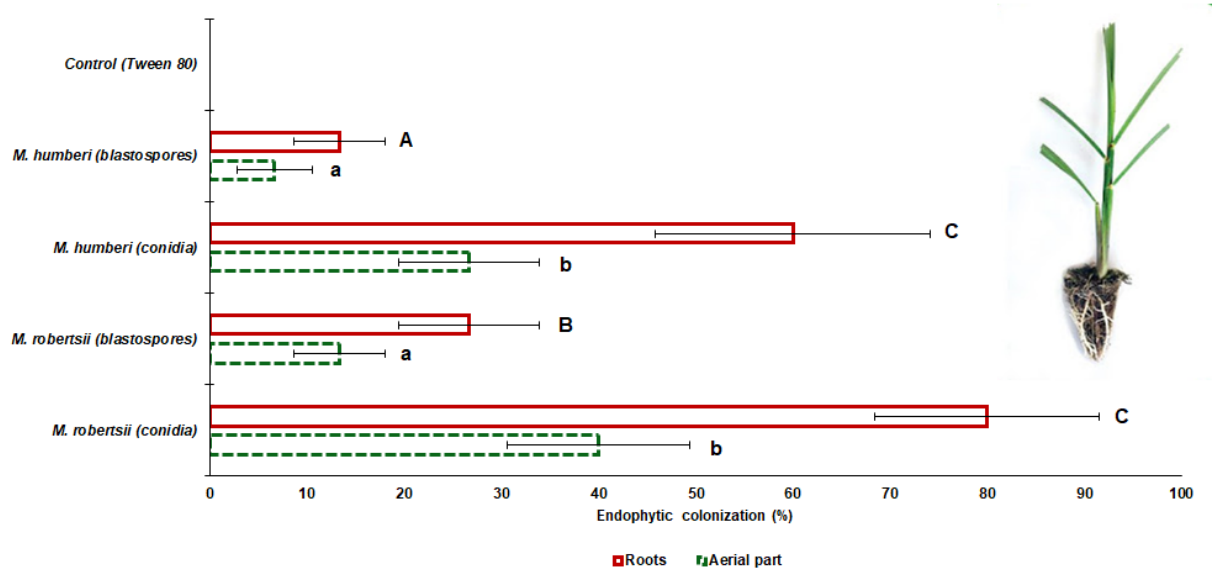

Figure 1. Endophytic colonization of sugarcane (var. CTC-9001) tissues (aerial part and roots) by *Metarhizium robertsii* or *M. humberi* 30 days after inoculation with conidia or blastospores. Horizontal bars with different letters are significantly different at $P < 0.05$ (Tukey).

3.4.2 Determination of conidia concentrations for sugarcane inoculation with two *Metarhizium* species

The inoculation of sugarcane with conidia suspensions of *M. robertsii* and *M. humberi* increased the length of aerial part over non-inoculated plants (control) at 45 (F= 4.32, d.f= 6, P < 0.0001) and 70 days (F= 25.28, d.f= 6, P < 0.0001) after inoculations (DAI). The treatments that result in greater length were the ones with the higher concentration of conidia ($1x10^{12}$ con/ha). At 45 DAI, the higher concentration treatments, both individually or in combination by two *Metarhizium* species, differed statistically to the control treatment. The same occurs at 70 DAI; however, no difference was found between the treatments applied compared to the length of the aerial part of the sugarcane plants 90 DAI (Table 3). The treatment with *M. robertsii* (ESALQ 1635) in the lower concentration ($1x10^{11}$ con/ha) presented a greater diameter of the stalks, only at 70 days, compared to the other treatments with *Metarhizium* inoculation and the uninoculated control (F= 2.33, d.f= 6, P < 0.037) (Table 3).

The aerial part's dry weight was similar at all times evaluated, with no differences between treatments. In contrast, the dry weight of roots was significantly greater in the treatments with *Metarhizium* inoculation (Table 4).

At 70 DAI, the dry weight of sugarcane roots was increased in the treatments with the inoculation of the two *Metarhizium* species with the higher conidia concentration ($1x10^{12}$ con/ha), individually or in combination (F= 6.31, d.f= 6, P= 0.0004). At 90 DAI, only the treatments with *M. robertsii* (ESALQ 1635) in the highest conidia concentration ($1x10^{12}$ con/ha), and the combination of *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) in the highest conidia concentration ($1x10^{12}$ con/ha), presented an increase in the dry weight of roots (F= 4.21, d.f= 6, P= 0.0038) (Table 4).

Table 3. Sugarcane plant growth parameters (Means ± SE) 45, 60, and 90 days after inoculation with the entomopathogenic fungi *Metarhizium robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) in different concentrations. 10^7 conidia/mL corresponds to an application of 10^{11} conidia/ha, and 10^8 conidia/mL corresponds to an application of 10^{12} conidia/ha

		Treatments							
		***M.r* (10^7)**	***M. r* (10^8)**	***M.h* (10^7)**	***M.h* (10^8)**	***M.r+M.h* (10^7)**	***M.r+M.h* (10^8)**	**Control**	**P-Value**
45 d.a.i	**Length (cm)**	17.2 ± 0.6 b	18.3 ± 0.6 a	17.7 ± 0.6 b	18.8 ± 0.4 a	17.4 ± 0.5 b	18.4 ± 0.4 a	15.8 ± 0.3 b	*P* < 0.0001
	Diameter (mm)	9.1 ± 0.3 a	8.9 ± 0.4 a	8.7 ± 0.4 a	8.3 ± 0.4 a	8.6 ± 0.5 a	8.9 ± 0.3 a	7.6 ± 0.2 a	*P*= 0.094
	R weight (mg)	561.0 ±156.4	746.0 ± 17.8	541.2 ± 146.7	697.0 ± 136.3	638.2 ± 11.2	695.1 ± 135.1	506.8 ± 14.6	*P*= 0.778
	A.P weight (mg)	1,164.4 ± 127.1	1,584.0 ± 255.1	1,225.5 ± 299.1	1,496.0 ± 81.1	1,338.0 ± 118.6	1,474.8 ± 136.1	1,100.4 ± 111.7	*P*= 0.516
70 d.a.i	**Length (cm)**	18.3 ± 0.6 b	23.5 ± 0.6 a	18.8 ± 0.6 b	23.1 ± 0.5 a	18.9 ± 0.4 b	22.4 ± 0.5 a	17.5 ± 0.2 b	*P* < 0.0001
	Diameter (mm)	11.3 ± 0.4 a	10.7 ± 0.5 b	10.6 ± 0.3 b	10.2 ± 0.4 b	10.7 ± 0.4 b	10.7 ± 0.4 b	9.9 ± 0.4 b	*P*= 0.037
	R weight (mg)	804.4 ± 1.7 b	1,020.0 ± 11.9 a	670,6 ± 2.8 b	989,1 ± 11.5 a	723,2 ± 7.7 b	847,7 ± 11.4 a	523.2 ± 133.6 b	*P*= 0.0004
	A.P weight (mg)	1,682.1 ± 420.8	2,020.8 ± 259.3	1,573.0 ± 68.1	1,772.0 ± 282.5	1,746.6 ± 6.5	1,946.5 ± 212.7	1,342.4 ± 263.6	*P*= 0.645
90 d.a.i	**Length (cm)**	19.23 ± 0.3	20.0 ± 0.6	19.3 ± 0.5	19.6 ± 0.5	19.6 ± 0.4	20.0 ± 0.3	18.6 ± 0.2	*P*= 0.249
	Diameter (mm)	11.4 ± 0.4	11.6 ± 0.6	11.1 ± 0.5	10.7 ± 0.5	11.0 ± 0.4	10.7 ± 0.4	10.2 ± 0.1	*P*= 0.4293
	R weight (mg)	1,540.0 ± 146.9 b	2,289.8 ± 30.3 a	1,400.0 ± 22.5 b	1,600.0 ± 311.4 b	1,520.0 ± 115.7 b	1,680.0 ± 58.3 a	1,340.0 ± 116.6 b	*P*= 0.0038
	A.P weight (mg)	2,412.4 ± 29.7	3,440.0 ± 802.3	2,500.0 ± 384.7	3,180.0 ± 468.4	2,560.0 ± 332.6	2,760.0 ± 500.6	2,382.4 ± 26.6	*P*= 0.526

[1]Data (mean ± SE) followed by different letters within the lines are significantly different (ANOVA, followed by post hoc Tukey test, *P* < 0.05).

R weight = Dry weight of roots

A.P weight = Dry weight of the aerial part

The re-isolation of both *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) was confirmed in all plant tissues, independently of the conidia concentration, time of evaluation, or inoculation method (individually or co-inoculation) (Figure 2). The recovery of *Metarhizium* in the aerial part tissues varied between 33% at 45 DAI, 13,3% at 70 DAI, and 6% at 90 DAI; there are no differences between the inoculated treatments to the control. However, the recovery from roots tissues of inoculated sugarcane with *Metarhizium* presented significant differences. At 45 DAI, the recovery of fungi in the treatments with *Metarhizium* inoculation, independently of the concentration, was higher compared to both control and treatment with combined inoculation ($\chi 2 = 55.20$, $df = 6$, $P < 0.0001$). At 70 and 90 DAI the treatments with the major fungi recovery from sugarcane tissues, were those with inoculation in the highest conidia concentration, in comparison to the treatments with lower concentrations or fungal combination (70 DAI: $\chi 2 = 39.97$, $df = 6$, $P < 0.0001$) (90 DAI: $\chi 2 = 4.69$, $df = 6$, $P < 0.0001$) (Figure 2).

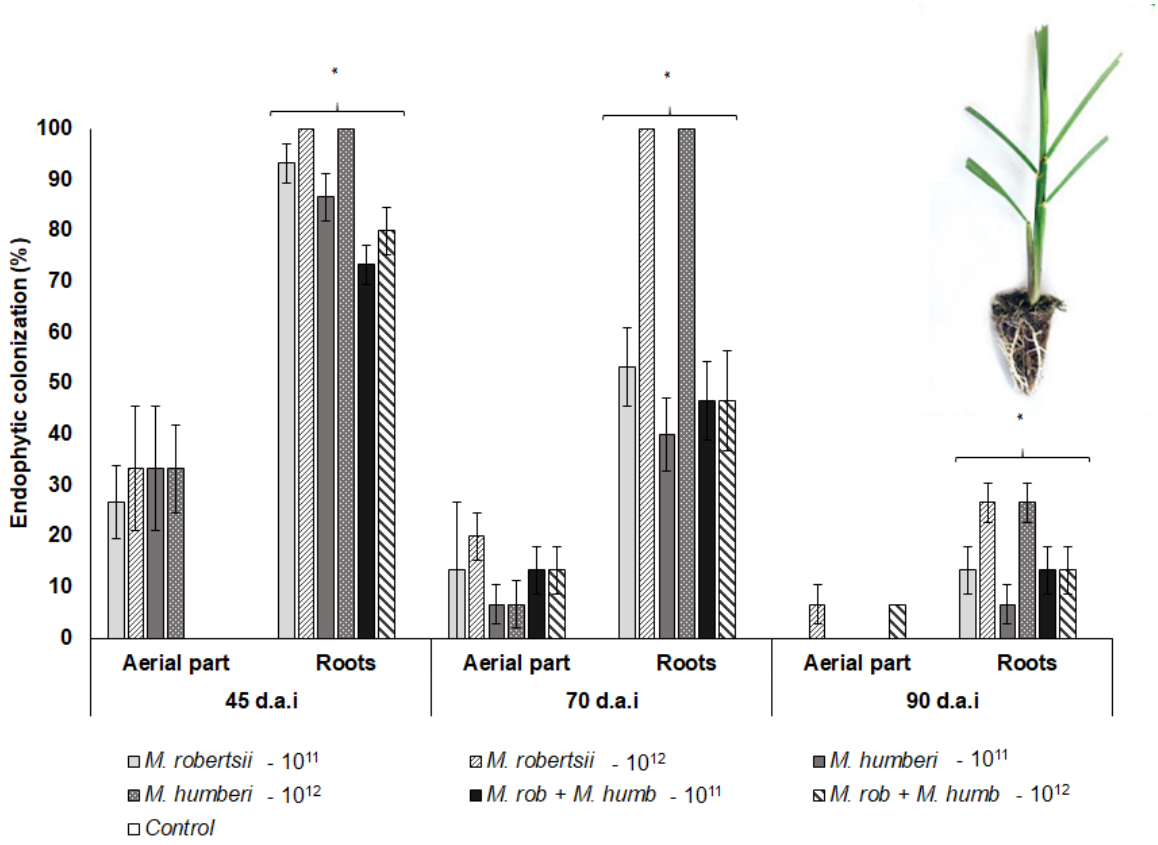

Figure 2. Endophytic colonization of sugarcane (var. CTC-9001) tissues (aerial part and roots) by *Metarhizium robertsii* and *M. humberi* 45, 70, and 90 days after inoculation with different conidia concentrations. Vertical bars represent the means while asterisk (*) designates significant difference among fungal species at $P < 0.05$ (Tukey).

3.4.3 Effects of *Metarhizium* inoculation on *M. javanica* and *P. brachyurus* development

The inoculation of both *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) contributed to the reduction of *M. javanica* per gram of roots (Nem/g of roots) (Figure 3). We verified that both *Metarhizium* isolates reduced up to 78% the number of nematodes 70 days after *M. javanica* infestation ($F= 24.87$, d.f= 4, $P < 0.0001$), differing to the control treatment (Control: 377.06 individuals/g of the root; ESALQ 1635: 206.50 individuals/g of root and ESALQ 1638: 82.35 individuals/g of the root). Different results were found to *P. zeae*. The isolate of *M. humberi* (ESALQ 1638) cause 15% of reduction in the number of *P. zeae* per gram of roots, but not differing to the control and *M. robertsii* treatment (Control: 141.62 individuals/g of the root; ESALQ

1635: 136.18 individuals/g of root and ESALQ 1638: 120.95 individuals / g of the root) (Figure 3).

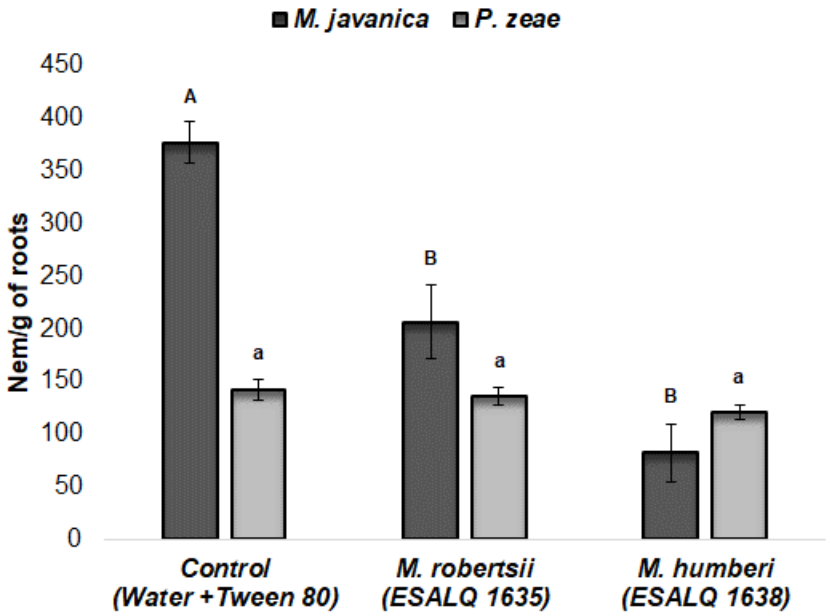

Figure 3: Nematodes per gram of roots (Nem/g) obtained for sugarcane plants (variety CTC-9001) inoculated with the entomopathogenic fungi *Metarhizium robertsii* or *Metarhizium humberi* and infested with 1000 individuals of *Meloidogyne javanica* and *Pratylenchus zeae*. Bars with different letters for each nematode species differ statistically (Tukey 0.05%).

The final population of *M. javanica* per plant was reduced by up to 45% using both *Metarhizium* species, compared to the control. However, no statistical differences between treatments were observed (F= 2, d.f= 3.45, P = 0.07).

The same result was observed in the experiment with the nematode *P. zeae*, where we found that inoculation with both species of fungus also reduced the nematode population in the treatment with ESALQ 1638, followed by ESALQ 1635, compared to the control (Control: 1500 individuals; ESALQ 1635: 1207.7 individuals and ESALQ 1638: 1005 individuals), but no statistical differences were found between treatments (F= 2, d.f= 1.65, P = 0.245).

3.4.4 Effect of *Metarhizium* on nematodes *in vitro*

The nematodes' eggs development was affected after the exposure of both conidia and filtrates for *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638). The application of the cultures filtrates of both isolates of *Metarhizium* reduced the number of J2 juveniles of *M. javanica* (F= 13.22, d.f= 7, p < 0.0001) and P. zeae (F= 15.55, d.f= 7, p < 0.0001) significantly after seven days of the application, in comparison to conidia suspension treatment. The treatments with the combination of conidia and filtrates were the best ones resulting in the lowest nematode populations (Figure 4).

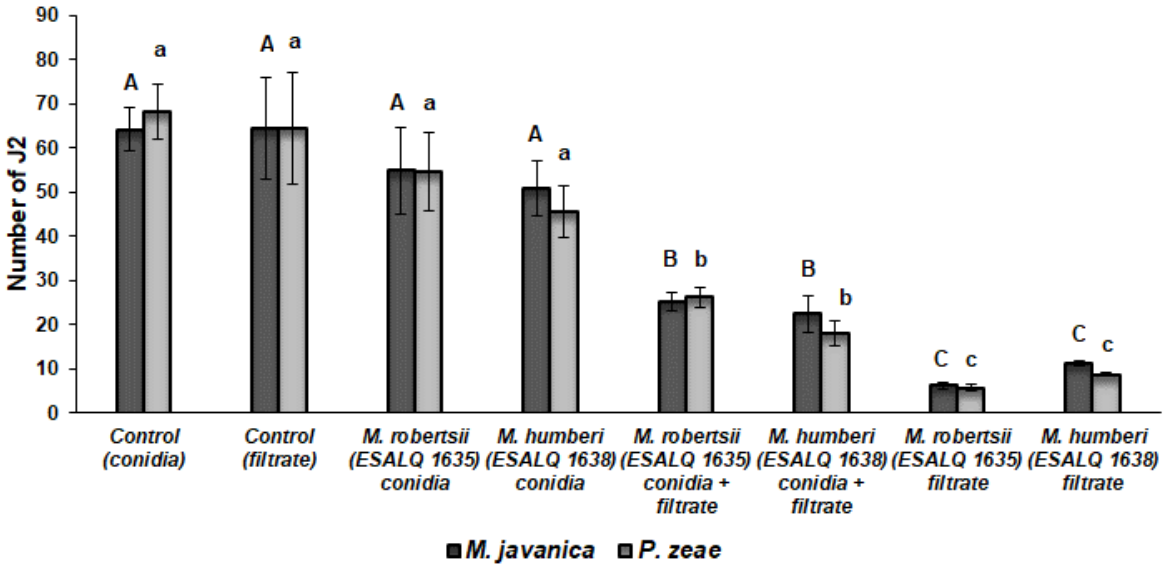

Figure 4. Number of juveniles J2 found seven days after inoculation with the entomopathogenic endophytic fungi *Metarhizium robertsii* (ESALQ 1635) and *Metarhizium humberi* (ESALQ 1638) using conidia, filtrates of the culture media, or the combination of both. Tubes were inoculated with 500 eggs of *Meloidogyne javanica* or *Pratylenchus zeae*. Bars followed by different letters are significantly different (ANOVA, followed by post hoc Tukey test, P < 0.05. Capital letters indicate *M. javanica,* while small letters indicate *P. zeae*.

The percentage of eggs hatching of *M. javanica* and *P. zeae* (% eclosion) was reduced in the treatments where *M. robertsii* (ESALQ 1635) was applied, both in the form of conidia, as a filtrate, or in the combination of both (Figure 5). The application of *M. robertsii*, using the combination between conidia and filtrates, reduced up to 97% of *M. javanica* eclosion (F= 25.48, d.f= 7, P < 0.0001) and up to 98% of *P. zeae* eclosion

(F= 16.57 d.f= 7, P < 0.0001). The application of *M. humberi* also reduced the eclosion of both nematodes' species, differing to the control. *In vitro* results indicate that these fungi' filtrates have a great potential to control eggs and juveniles J2 of nematodes.

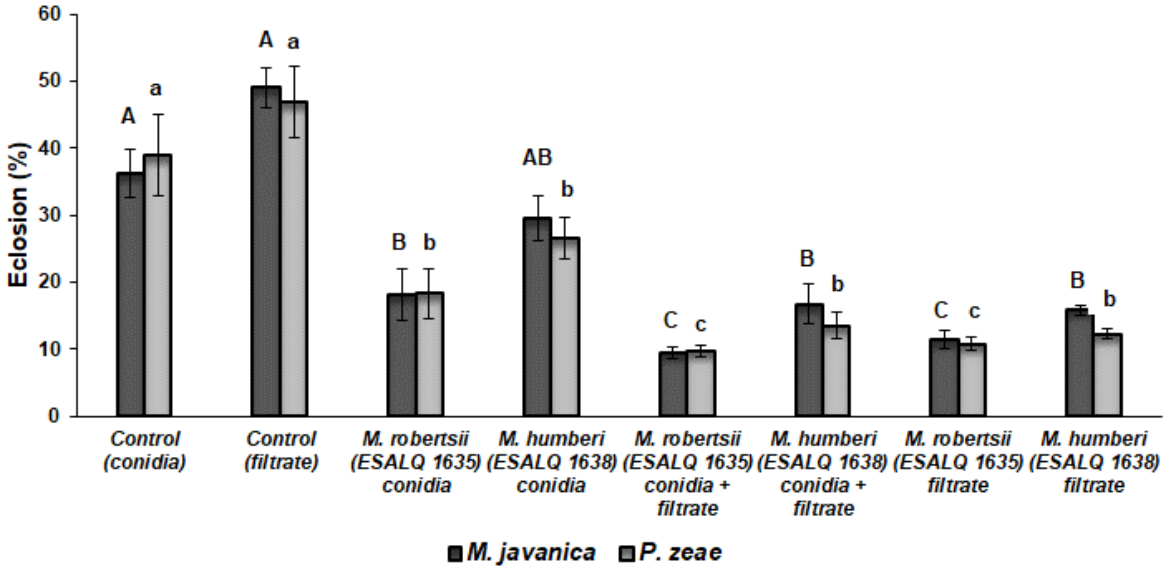

Figure 5. Percentage (%) of eclosion found in the cultures seven days after inoculation with the entomopathogenic fungi *Metarhizium robertsii* (ESALQ 1635) and *Metarhizium humberi* (ESALQ 1638) using conidia, filtrates, or the combination with both. Tubes were inoculated with 500 eggs of *Meloidogyne javanica* and *Pratylenchus zeae*. Bars followed by different letters are significantly different (ANOVA, followed by post hoc Tukey test, P < 0.05).

3.4.5 Effects on *D. saccharalis*

The inoculation of *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) affected significantly *D. saccharalis* larvae for up to 90 days after inoculation in sugarcane plants. The fungi inoculation caused a reduction in the weight of *D. saccharalis* larvae that fed on the inoculated tissues for 20 days (Table 4).

The treatment with *M. robertsii* (ESALQ 1635) reduced significantly the weight of larvae in comparison to control and to *M. humberi* (ESALQ 1638) treatments after 30 (F= 693.7, d.f= 2, P < 0.0001), 60 (F= 295.2, d.f= 2, P < 0.0001) and 90 DAI (F= 765, d.f= 2, P < 0.0001). Similarly, the treatment with the same *Metarhizium* isolate cause a considerably mortality to *D. saccharalis* ranging from 91.7% mortality after 30

days of inoculation (F= 135.5, d.f= 2, P < 0.0001) to 70.8% mortality after 90 days after fungal inoculation (F= 34.79, d.f= 2, P = 0.0005) (Table 4), differing to the control and the *M. humberi* (ESALQ 1638) treatments. The mortality resulted are similar to the weight data, since the same treatment that caused weight reduction also caused higher mortality in *D. saccharalis* caterpillars.

Table 4. Weight (mg) and mortality of sugarcane borer (*D. saccharalis*) larvae after 20 days of feeding sugarcane plants (var. CTC-9001) inoculated with the entomopathogenic endophytic fungi *Metarhizium robertsii* (ESALQ 1635) or *Metarhizium humberi* (ESALQ 1638) and control with 0.05% Tween 80. Plants were used in this bioassay 30, 60, and 90 days after fungal and control inoculation.

	Assessment[1]					
	30 d.a.i*		60 d.a.i*		90 d.a.i*	
Treatment[2]	Weight of larvae (mg)	Mortality (%)	Weight of larvae (mg)	Mortality (%)	Weight of larvae (mg)	Mortality (%)
M. robertsii (ESALQ 1635)	40.9 ± 0.5 a	91.7 ± 2.4 a	38.5 ± 0.2 a	81.9 ± 5.0 a	41.0 ± 0.1 a	70.8 ± 7.2 a
M. humberi (ESALQ 1638)	44.7 ± 0.2 b	69.4 ± 3.7 b	42.8 ± 0.1 b	70.8 ± 2.4 a	43.4 ± 0.1 b	62.5 ± 2.4 a
Control -Tween 80	52.2 ± 0.1 c	30.6 ± 1.4 c	49.5 ± 0.1 c	20.8 ± 4.8 b	45.5 ± 0.1 c	19.4 ± 2.8 b
F	693.7	135.5	295.2	58.9	765	34.8
d.f	2	2	2	2	2	2
P-value	P < 0.0001	P < 0.0001	P < 0.0001	P = 0.00011	P < 0.0001	P = 0.0005

[1]Data (mean ± SE) followed by different letters within a column are significantly different (ANOVA, followed by post hoc Tukey test, P < 0.05).

3.4.6 Host-choice experiment with *Diatraea saccharalis* in sugarcane plants inoculated with *Metarhizium*

The host-choice assay showed that *Metarhizium* inoculation on sugarcane plants could affect the feeding behavior of *D. saccharalis* larvae. When the control *vs. M. robertsii* (ESALQ 1635) or control *vs. M. humberi* (ESALQ 1638) treatment were compared, plants without fungus were always more attractive to larvae (X^2= 14.00, d.f= 1, P = 0.0072) (Figure 6).

Curiously, when plants inoculated with both fungal treatments were offered at the same time in a double choice box, the larvae were attracted to plants inoculated with *M. humberi* (ESALQ 1638) and try to avoid the plants inoculated with *M. robertsii* (ESALQ 1635), indicating that there may be a higher repellency to plants inoculated with *M. robertsii* (X^2= 14.14, d.f= 1, P = 0.008) (Figure 6). The collected results indicate that there may be changes in the volatile profile of plants inoculated and not inoculated with *Metarhizium.* Regardless of the isolate, *D. saccharalis* larvae always prefer to feed on uninoculated plants over plants with fungus.

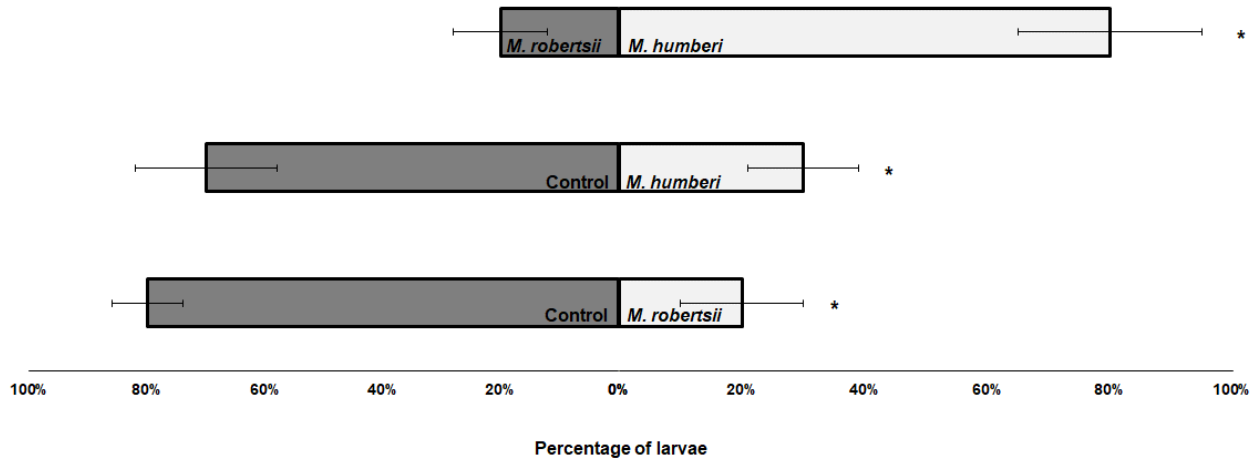

Figure 6. Percentage of sugarcane borer (*D. saccharalis*) larvae (mean ± SE) found in sugarcane plants (var. CTC-9001) after 24 hours of exposure. Plants were inoculated with the entomopathogenic endophytic fungi *Metarhizium robertsii* (ESALQ 1635), *Metarhizium humberi* (ESALQ 1638), and Control (Water +Tween 80). Vertical bars represent the means while asterisk (*) designates a significant difference between combined treatments at P < 0.05 (Tukey).

3.5 Discussion

In this study, we showed that *Metarhizium* inoculation in sugarcane plants could improve some aspects of plant growth and affected insects and nematodes development when plant tissues were colonized. The experiment comparing the inoculation with conidia *vs.* blastospores confirmed our hypothesis that the first was better able to colonize plant tissues and improve plant growth. This is the first study about using blastospores of entomopathogenic fungi to stimulate plant growth promotion and colonization. Conidia are a structure of greater resistance, as they need to survive in the environment under adverse several biotic and abiotic factors. Hence, as expected, treatments with conidia resulted in better performance compared to treatments with blastospores.

The length of the aerial part and stalk diameter was greater than the control when conidia of both *Metarhizium* species tested was inoculated. Still, no differences were observed in plant dry weight (aerial part or roots). The increase in this parameter indicates the existence of interactions between the fungus and the plant. The interaction between plant and fungus allows an increase in host plants' vegetative development (BEHIE ET AL., 2015; JABER; ENKERLI, 2016; CANASSA et al., 2019). Studies have shown the existence of nutrient translocation between plants and entomopathogenic fungi (BEHIE; ZELISKO; BIDOCHKA, 2012) and that the increase in biomass and the vegetative growth of plants inoculated by the fungus may be dependent on the availability of nutrients in the cultivated soil (TALL; MEYLING, 2018). A recent study performed in our laboratory reveals that *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) produces *in vitro* compounds and enzymes like phosphatases, phytases, chitinases, and siderophores that can act improving plant growth and development (SIQUEIRA et al., 2020). In addition to the nutrition, this study and others show that *Metarhizium* can produce and regulate the growth hormone auxin in plants (SIQUEIRA et al., 2020; LIAO et al., 2017).

Our results do not prove that a combined inoculation with two fungal species can improve the plant growth more than a single inoculation. In our study, the inoculation of combined *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) did not show good results compared to a single inoculation of the same isolates in sugarcane plants. Similar results were found by Canassa et al. (2019) with beans plants inoculated with *M. robertsii* and *B. bassiana*. The authors observed that a single inoculation produces

more benefits than the combined inoculation of these two species. Few studies investigated the effects of combined inoculations of entomopathogenic endophytic fungi in plants. Still, It has already been demonstrated that plants treated with combinations of beneficial microbes show limited additional effects on plant growth than single species additions (GADHAVE et al., 2016).

We determined the best fungal concentrations for inoculation. The aerial part's length was greater in the treatments with *M. robertsii* and *M. humberi* inoculation in the conidia concentration of 10^{12} after 45 and 70 days after inoculation in comparison to 10 times lower concentrations. No differences were verified in the stalk diameter and the dry weight of the aerial part, but we can observe that *Metarhizium* inoculation increases the dry weight of roots after 90 days after inoculation.

The two fungal isolates of *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) were able to colonize sugarcane plants. Interestingly the inoculation of blastospores also resulted in colonization of sugarcane tissues; however, the most significant colonization was obtained in the inoculation of conidia. In this study, fungi' recovery was up to 80% in roots and 40% in aerial part (leaves and stalk) 30 days after conidia inoculation. Similarly, in the experiment where we tested different concentrations and co-inoculation of fungi, the best colonization was obtained in the treatments with the inoculation with the highest concentrations. The major colonization also occurred in roots. Behie et al. (2015) demonstrated that *M. robertsii* was restricted to the roots of haricot bean plants (*P. vulgaris*) under laboratory and field conditions. Likewise, Akello and Sikora (2012) reported that an isolate of *M. anisopliae* only colonized plants' roots. Differential colonization rates of plants by fungal isolates could have various causes, such as innate characteristics of the fungal isolate (POSADA et al., 2007); host plant genetics (ARNOLD; LEWIS, 2005); leaf surface chemistry (POSADA et al., 2007); and competition with other endophytes naturally occurring within plants (POSADA et al., 2007; SCHULZ et al., 2015; JABER; ENKERLI, 2016). In our study, it is possible to verify that the fungi are present in the aerial part tissues and the roots, with a variation of the percentage of recovery depending on the date of evaluation. These differences may occur due to the change in fungi' location in plant tissues over time and by the methodology used, because depending on the location from which the fragment is removed, there may be variations.

The reduction of insect herbivore population on *Metarhizium* inoculated plants has been reported in a few studies. Our data showed a decrease in larvae weight and

high mortality levels of *D. saccharalis* fed on sugarcane plants inoculated with M. robertsii (ESALQ 1635) and M *humberi* (ESALQ 1638). Additionally, we observed that these insects prefer to feed on non-inoculated plants in a double host-choice assay than in plants inoculated with fungi. Still, additional assays as the collection and identification of the possible volatiles emitted by the plants can be carried out to elucidate the presented results.

Canassa et al. (2019) showed that the inoculation of *M. robertsii* and *B. bassiana* in bean plants reduced *Tetranychus urticae* population. Jaber and Araj (2018) reported that the inoculation of *M. brunneum* in sweet pepper resulted in fewer aphids, *Myzus persicae*, including prolonged development time and reduced reproduction compared to aphid populations on control plants. The inoculations of *M. anisopliae* in bean reduced the *Ophiomyia phaseoli* (MUTUNE et al., 2016). The inoculation of *M. robertsii* in sweet sorghum against the *Sesamia nonagrioides* suppressed tunneling by 87% and caused 100% mortality (MANTZOUKAS et al., 2015). Reductions on nematode populations after exposure to inoculated plants have not yet been reported. In a preliminary study, we observed that sugarcane inoculation with isolates of *M. robertsii*, *M. humberi,* and *M. anisopliae* also reduced the number of egg mass of *M. javanica* (SIQUEIRA et al., unpublished) after 30 days of the inoculation. In this study, we found that the sugarcane inoculation with *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) resulted in a reduction in the number of *M. javanica* per gram of roots, but not for *P. zeae*, due to the establishment mode of each nematode specie. Besides that, fungal inoculation reduced up to 45% of the *M. javanica* and 35% of *P. zeae* populations but did not differ statistically. The effects observed in the tests with nematodes are promising for the use of *Metarhizium* in the control of nematodes pests of the sugarcane culture. We believe that the reduction in nematode populations occurs due to the production of chitinases by fungi. This hypothesis could be proven in our study of the entomopathogenic effect of *Metarhizium* under nematodes development. Our study showed that fungi inoculation reduced de number of juveniles (J2) and the eclosion percentage seven days after conidia and filtrates application on eggs of *M. javanica* and *P. zeae*.

The insects killed in the bioassays did not show growth and sporulation of *Metarhizium* in his cadavers. Other authors have reported the absence of mycoses in dead insects fed on plants inoculated by entomopathogens (AKUTSE et al., 2013; LOPEZ; SWORD, 2015; MANTZOUKAS et al., 2015). The absence of fungal

colonization in caterpillars suggests that mortality results from the interaction of the fungus with the plant.

The mechanisms behind the adverse effects caused by plant-associated *Metarhizium* spp. remain largely unknown. Some authors suggested that the plant's compounds or associated fungus are causing the reported sub-lethal adverse effects (VIDAL; JABER, 2015; MCKINNON et al., 2017). The plant colonization by inoculated fungi can at first be recognized by the plant as potential invaders leading to the triggering of immune responses with the synthesis of specific regulatory elements, such as transcription factors involved in resistance against herbivores (BROTMAN et al., 2013; MCKINNON et al., 2017). Production of secondary plant metabolites may also be considered; for example, terpenoids have anti-herbivore properties (VEGA, 2018). Alternatively, the production of fungal secondary metabolites in plants could also be a possible mechanism for observed negative effects against herbivores (JABER; OWNLEY, 2018).

The sugarcane treatment with conidia of the entomopathogenic fungal isolates *M. robertsii* (ESALQ 1635) and *M. humberi* (ESALQ 1638) in a concentration of 10^{12} con/ha can contribute to plant growth. These treatments are expected to contribute to the reduced population growth of *M. javanica* and *P. zeae* and cause developmental delay and mortality in *D. saccharalis*. The results bring a new perspective on the use of plant-associated *Metarhizium* spp., revealing that using entomopathogenic fungi as plant inoculants may be a promising strategy.

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4 FIELD INOCULATION OF TWO *METARHIZIUM* SPECIES IMPROVES SUGARCANE TRAITS AND CONCOMITANTLY PROMOTES PEST CONTROL: TOWARDS THE MULTIFUNCTIONAL BIOPESTICIDE

Abstract

Brazil ranks as the world leader in the commercial utilization of *Metarhizium* for biological control of insect pests, mainly to managing spittlebugs (Hemiptera: Cercopidae) in sugarcane. Nevertheless, field application and studies addressing the multiple benefits mediated by *Metarhizium* spp. are scarce. In this sense, we conducted the first field research to measure the benefits derived from the numerous roles exerted by *Metarhizium robertsii* (ESALQ-1635) and *Metarhizium humberi* (ESALQ-1638) in plant growth traits alongside the management of key arthropod pest after single fungal application in five commercial sugarcane areas. These five commercial fields were located in São Paulo State, encompassing “cane plant” and “ratoon cane” production systems. Fungal applications remarkably increased sugarcane yield and concomitantly reduced damages caused by insect pests. The numbers of germinated plants and the numbers of tillers per plant (tillering) were not affected by these fungal isolates. Notably, both fungal isolates significantly enlarged stalk length (in 4 out of 5 locations) and markedly boosted stalk volume (m3) in comparison to control plots. As a result, greater sugarcane yield was achieved with *Metarhizium* inoculation, mainly at locations A, D, and E. This result also raised the farmers’ profit by up to 22.9%. The quality components assessed in sugarcane after harvesting, such as BRIX, TRS, Purity, and Fiber in most locations remained unaffected by fungal inoculation, except at location D, where both fungi improved these attributes. Overall, *Metarhizium* inoculation significantly reduced key insect pests in most tested plots. The presence of the root spittlebug *Mahanarva fimbriolata* was considerably lower at location D treated with *M. robertsii* (20%) and *M. humberi* (26%) than in control plots (53%). Similarly, the sugarcane borer, *Diatraea saccharalis* incidence, appeared to be less frequently detected at location C in *M. robertsii* (6%) and *M. humberi* (20%) inoculated plots compared to control plots (40%). The number of sugarcane weevil, *Metamasius hemipterus*, was significantly suppressed by up to 83% at location C relative to the control. Altogether, these findings prove the multiple benefits promoted by *M. robertsii* and *M. humberi* when employed as bio-inoculants of sugarcane crop by boosting yields as well as alleviating attacks of root and stalk insect pests. These outcomes pave a way to use entomopathogenic endophytic fungi as inoculant and biopesticide in Brazil's sugarcane plantations.

Keywords: Microbial control; Entomopathogenic endophytic fungi; Plant growth promotion; Integrated pest management (IPM)

4.1 Introduction

Sugarcane is an important commodity crop in Brazil and worldwide, as it is cultivated in 110 countries for sugar and ethanol production, besides the electricity co-generation (ISOSUGAR, 2020). Sugarcane crop, Saccharum spp., belongs to the

Poaceae family and the sugarcane currently grown is hybrid varieties, where *S. officinarum* L. and *S. spontaneum* L. comprise the most important species that contribute to the genetic breeding of sugarcane (MATSUOKA et al., 2005). Brazil is the largest producer and exporter of cane sugar globally (FAOSTAT, 2020) and reached production of 642.7 million tons in the 2019/2020 harvest (CONAB, 2020).

Despite adapting to Brazil's climate, sugarcane has a series of phytosanitary problems; among them, many insect pests decrease productivity and cause severe economic losses for growers (BOIÇA JR. et al., 1997). The main insect pests of sugarcane crop include the root spittlebug, *Mahanarva fimbriolata* (Stal, 1854) (Hemiptera: Cercopidae), the sugarcane stem borer, *Diatraea saccharalis* (Fabr., 1794) (Lepidoptera: Crambidae) and the cane weevil, *Sphenophorus levis*, (Vaurie, 1978) (Coleoptera: Curculionidae), in addition to others like nematodes and termites (GALLO et al., 2002). These insects cause severe damage to the crop that can result in productivity losses of up to 2.30% due to sugarcane borer attack (ARRIGONI, 2002), 85% because of root spittlebug infestation (LEAL et al., 2008), and up to 40% owing to sugarcane weevils attack (PRECETTI & ARRIGONI, 1990). Different control methods are employed for the management of these insects, such as cultural control, the use of resistant varieties, chemical control, and biological control. The integrated Pest Management (IPM) program of sugarcane pests in Brazil has a long history, but its success generally varies with the sector economy.

The use of biological control with the entomopathogenic fungus *Metarhizium* sp. in Brazil's sugarcane crop is a classic example of biological control program with relative success when adequately practiced. This program started in the 1960s (MARQUES et al., 1981) and lasts until today. *Metarhizium* is an Ascomycete fungus belonging to the order Hypocreales and the family Clavicipitaceae. Due to the good management that the fungus *M. anisopliae* has provided towards *M. fimbriolata* and to a lesser extent towards *D. saccharalis*, this entomopathogen has been applied up to 4 million hectares in Brazil for root spittlebug control currently and the estimated growth in its use can be up to 2% per year due to the broad area of sugarcane crop that still does not use this control agent (PINTO, A. S extra official information). Hence, Brazil undoubtedly leads the most extensive microbial control program worldwide using *Metarhizium* to control spittlebugs in sugarcane and pasture (Mascarin et al., 2019).

Currently, this entomopathogenic fungus is produced in Brazil and worldwide almost exclusively using autoclaved (pre-cooked) rice grains as a substrate and then

applied in many ways, including the release of colonized rice grains directly on field ground, application of conidia formulated or not in syrups and spraying aqueous or oily suspensions of spores using tractors or airplanes. (ALMEIDA et al., 2004; DINARDO-MIRANDA et al., 2004).

Recently, the discovery of the endophytic association of *Metarhizium* genus with many plant species has been explored as a bioinoculant or plant bioenhancer (BEHIE; ZELISKO; BIDOCHKA, 2012) due to their attributes in promoting plant growth (SASAN; BIDOCHKA, 2012), facilitating the acquisition of nutrients (BEHIE; ZELISKO; BIDOCHKA, 2012 BEHIE; BIDOCHKA, 2014; FANG; ST. LEGER, 2010), affording protection against phytopathogens (KEYSER; JENSEN; MEYLING, 2016; SASAN; BIDOCHKA, 2013), herbivory (BATTA, 2013; KEYSER; THORUP-KRISTENSEN; MEYLING, 2014; PARSA; ORTIZ; VEGA, 2013) and abiotic stress (KHAN, 2012). This new ecological role played by *Metarhizium* spp. adds value to its status and make them more valuable to agriculture due to their multifaceted benefits to crop health and protection.

Although the use of *Metarhizium* for the control of root and leaf spittlebugs in sugarcane has been historically explored in Brazil, knowledge concerning the interactions of this fungus with the sugarcane rhizosphere and the benefits of this symbiotic relationship for Integrated Pest Management (IPM) is still scarce and overlooked. Results obtained in our laboratory (SIQUEIRA et al., in preparation) showed that the inoculation of *Metarhizium* in sugarcane in greenhouse improved plant growth (i.e., increased aerial size part and dry weight of roots and leaves). Besides that, our group has demonstrated that two isolates of *Metarhizium robertsii* and *Metarhizium humberi* sp. nov. (LUZ et al., 2019) can produce several biochemical traits, including indole acetic acid (IAA) and a plethora of compounds such as siderophores, chitinases, phytases which are involved in the plant defense mechanisms and nutrition aspects (SIQUEIRA et al., 2020).

In this context, this study's primary goal focused on revealing the beneficial attributes of two native isolates belonging to *Metarhizium robertsii* and the recently described *Metarhizium humberi* after field inoculation in sugarcane plants from five different commercial areas and conducted during three consecutive years. To this end, treated sugarcane plots were evaluated for plant growth, productivity, and pest control, compared with plots treated with a standard chemical insecticide based on fipronil, which has been extensively used in sugarcane plantations around Brazil. The findings

gathered in this unprecedented long-term field research represent a step onward the commercial development of *M. robertsii* and *M. humberi* as inoculants and biostimulant through root colonization, alongside their potential in inducing pest control and for increasing productivity in sugarcane crop systems. Therefore, this paper advances and expands our knowledge on the real benefits mediated by plant-*Metarhizium* mutualism interactions in the field and opens a new avenue for the development of biostimulant mycoinsecticides in compliance with the sustainability principles applied to sugarcane crop systems

4.2 Material and Methods

4.2.1 Fungal strains

The entomopathogenic fungal isolates were selected for their effects on the growth promotion of sugarcane plants in our previous studies in the greenhouse (A.C.O. Siqueira, unpublished). We chose the endophytic entomopathogenic fungi *M. robertsii* (ESALQ 1635) and *M. humberi* sp. nov. (Luz et al., 2019) (ESALQ 1638). Isolates were kept at -80°C in the entomopathogen collection "Prof. Sérgio Batista Alves" in the "Laboratory of Insect Pathology and Microbial Control" at Escola Superior de Agricultura “Luiz de Queiroz” – University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil. The isolate *M. robertsii* - ESALQ 1635 was originated from the soil of native forest in Delmiro Gouveia (9°25'0.12" S37º57'8.49"W), Alagoas State, Brazil, and the *M. humberi* - ESALQ 1638 isolate was obtained from the soil of savanna in Rio Verde (17º29'49.3"S 51º13'40.7"W), Goiás State, Brazil. These isolates were identified to species level by molecular techniques, and both are under registration of a biopesticide product in Brazil.

4.2.2 Experimental setup

The experiments were conducted in three different municipalities, totalizing four fields in São Paulo state, Brazil (Figure 1).

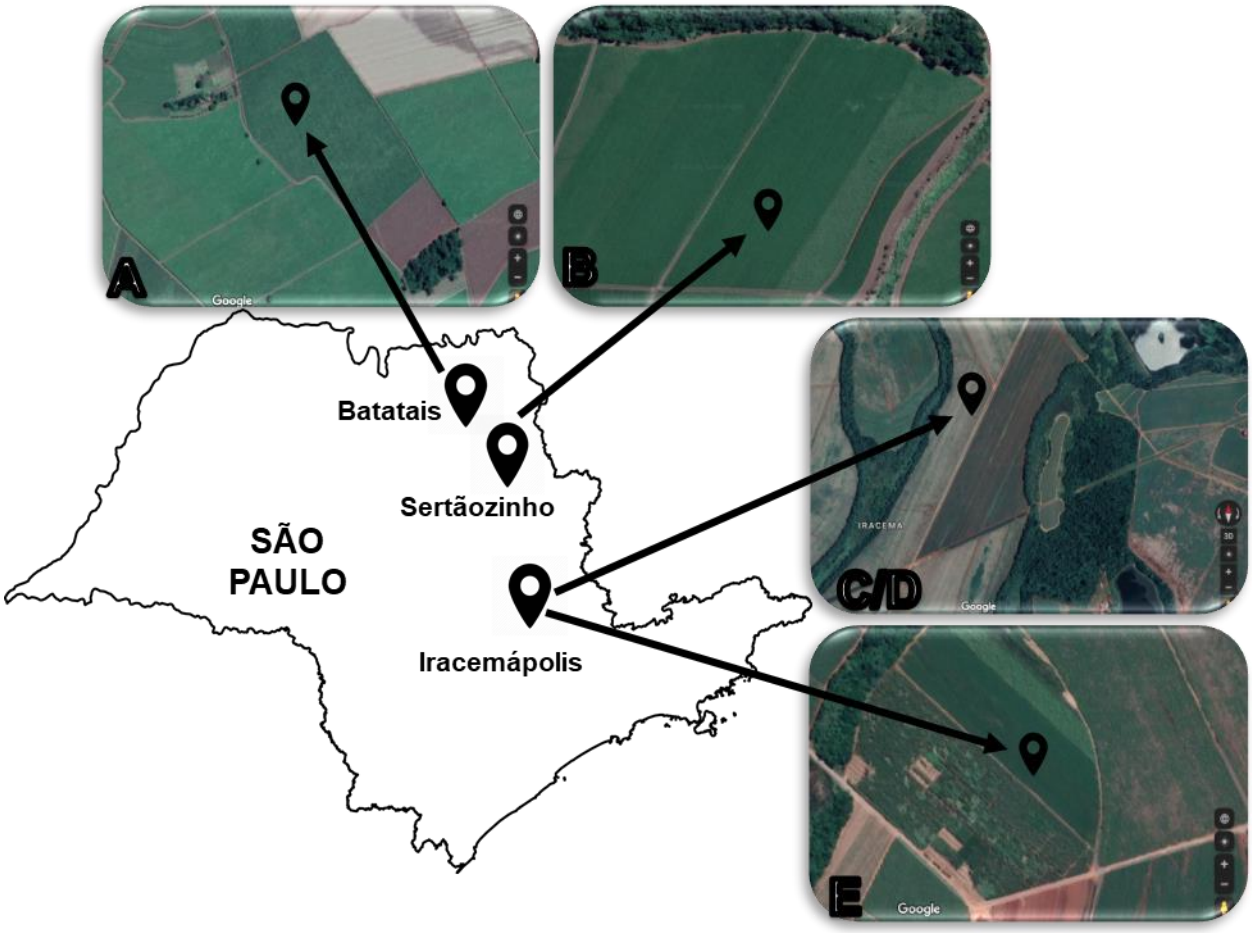

Figure 1. Experimental field set up in Open Field locations A in Batatais (20°52'56.3"S 47°45'21.9"W), B in Sertãozinho (21°04’35.1’’S 47°50’42.4’’W), and C and D in Iracemápolis (C: 22°33'27.4"S 47°30'26.6"W and D: 22°35'22.3"S 47°33'20.3"W). The area used for the experiment is marked with a dot on the map.

Sugarcane seedlings grown in greenhouses were inoculated with the following treatments before transplanting to the field: 1) *M. robertsii* ESALQ 1635 conidia suspended in water + 0.05% Tween 80; 2) *M. humberi* ESALQ 1638 in conidia suspended in water + 0.05% Tween 80; 3) Fipronil (alpha-cypermethrin 120g/L and Fipronil 180g/L) (positive control) and 4) Water + 0.05% Tween 80 (blank control). It is important to note that some fields did not receive all treatments mentioned above. A randomized block design was set up in all five-field experiments conducted during three consecutive years.

We evaluated two sugarcane crop types named “sugarcane plant” and “sugarcane ratoon”, which consist of the first plantation before the first cut (harvest), while the other regards the crop with more than one cut after planting, respectively.

The first experiment was conducted in the “Grupo Balbo” mill at Batatais, São Paulo, Brazil, from March/2017 to April/2018, cultivated with the commercial variety IAC95-5000 of “sugarcane plant” (location A). The total area was represented by ten hectares, and it was split into two big blocks with five hectares per treatment, and each block was divided into four plots with 100 square meters each. The treatments consisted of *M. humberi* - ESALQ 1638 (2) and the commercial insecticide Fipronil (3).

The second experiment was also conducted in the “Grupo Balbo” mill, in Sertãozinho, São Paulo State, Brazil, from November/2017 to December/2018 in commercial sugarcane variety CTC-4 of “sugarcane plant” (location B). We selected an area with 1 hectare split into three blocks containing three plots measuring 22.5 m x 50 m for each block's treatment. The treatments consist into *M. robertsii* (ESALQ 1635) (1) + Fipronil (3), *M. humberi* (ESALQ 1638) (2) + Fipronil (3) and only the insecticide Fipronil (3) (control).

Third, fourth and fifth experiments were performed in the “Grupo São Martinho S/A” mill in Iracemápolis, São Paulo State, Brazil. Two experiments were conducted in an open commercial sugarcane variety IAC95-5000 of ‘ratoon sugarcane’ (location C and D), both from November/2017 to November/2018. The location C encompassed 1 hectare divided into three blocks containing three plots with 22.5 m x 50 m for each block's treatment. The location D had 18.6 ha divided into three blocks containing 6.2 ha for each treatment. The treatments consisted of *M. robertsii* (ESALQ 1635) (1) + Fipronil (3), *M. humberi* (ESALQ 1638) (2) + Fipronil (3) and only the insecticide Fipronil (3) (control). The other experiment in the same mill factory was conducted in commercial sugarcane variety CTC-9001 from March/2018 to June/2019 of “sugarcane plant” (location E) grown in 1 hectare and split into three blocks containing three plots with 22.5 m x 50 m for each treatment in each block. The treatments consist of *M. robertsii* (ESALQ 1635) (1), *M. humberi* (ESALQ 1638) (2), and only water + Tween 80 0.05% (4) (control), consisting of an organic farming experiment.

4.2.3 Characterization of climate in each location

The climate data at each location was obtained from a weather station during the experiments. The temperature data was obtained from AGRITEMPO – Agrometeorological Monitoring System, and rainfall data were obtained from CIIAGRO - Integrated Agrometeorological Information Center (Figure 2).

We observed minimal variation in temperatures and average rainfall in all locations. In general, the drought periods were between April and August, and average temperatures ranged from 20 to 30 degrees. The spot with the hottest climate was Sertãozinho (Location B), while the rainiest was Iracemápolis (Locations C, D, and E) (Figure 2).

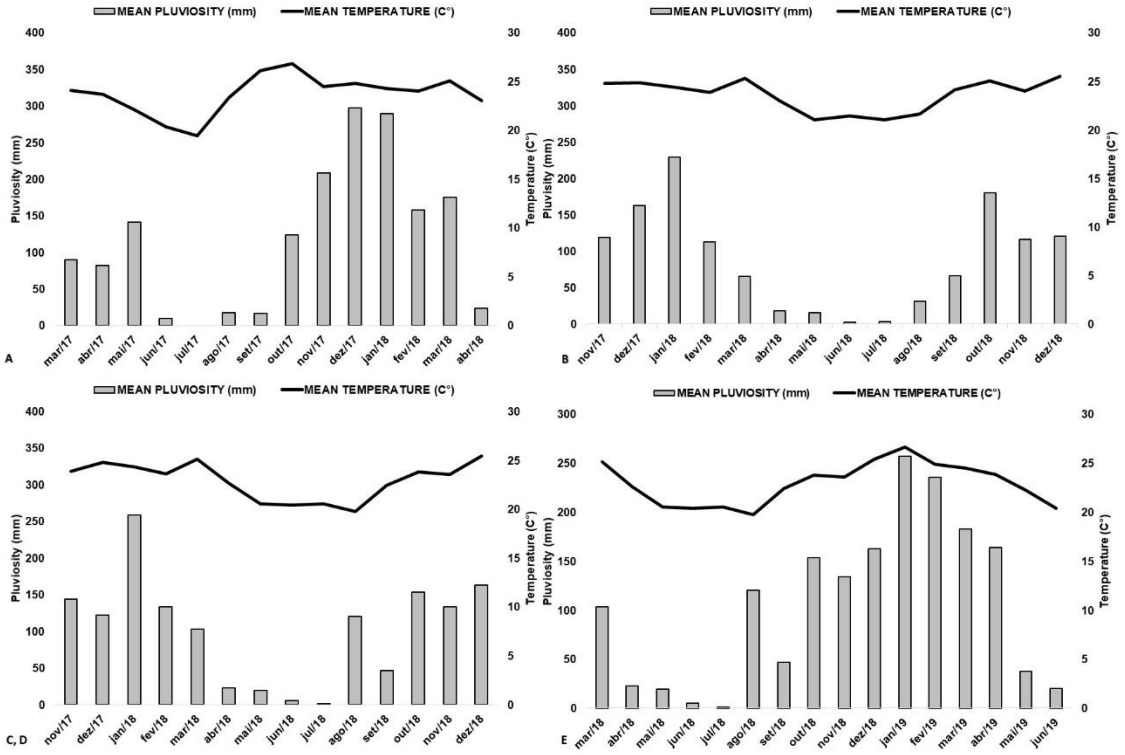

Figure 2. Climate data monitoring at experimental locations A (Batatais/SP), B (Sertãozinho/SP), C and D: (Iracemápolis/SP), and E (Iracemápolis/SP), from 2017 to 2019. Bars indicate rainfall average, and the black line indicates the average temperature in each location. The temperature obtained from https://www.agritempo.gov.br/agritempo/jsp/PesquisaClima/index.jsp?siglaUF=SP&lang=pt_b and rainfall from www.ciiagro.sp.gov.br.

4.2.4 Fungal inoculum preparation

The two fungal isolates (*M. robertsii* ESALQ 1635 and *M. humberi* ESALQ 1638) were retrieved from the -80 °C culture collection and cultivated in Petri dishes (90 x 15 mm) containing 20 ml of Potato Dextrose Agar (PDA; Difco™, Livonia, USA).

The cultures were kept in total darkness at 25 ºC for ten days until harvesting of conidia. Conidial suspensions were prepared by adding 10 ml of a sterile aqueous solution of 0.05% Tween 80 (Oxiteno, São Paulo, Brazil) to sporulated cultures and subsequently scraping off conidia with a sterile spatula. Conidial concentrations were estimated using a Neubauer hemocytometer (Merck, Darmstadt, Germany) and adjusted to 1 x 108 conidia ml-1. After that, an aliquot of 10 ml of each conidial suspension was inoculated with a pipette into individual polypropylene bags (35 cm length x 22 cm width) containing 300 g of autoclaved (121 ºC, 20 min) parboiled rice to scale-up the production of aerial conidia using a solid-state fermentation method (Alves, 1998).

The fungal inoculated rice kernels were incubated in total darkness at 25 ºC for ten days. The bags were gently shaken every two days to ensure evenly distributed fungal growth on rice kernels. The conidial viability was checked by preparing a conidial suspension by adding 1 g of fungus-colonized rice into 10 ml of sterile 0.05% Tween 80. From the third dilution, 150 µL of the conidial suspension was transferred with a pipette onto PDA. The percentage of conidial germination was then evaluated according to Oliveira et al. (2015). Fungal suspensions were only used in experiments when germination levels were higher than 95%.

4.2.5 *Metarhizium* inoculation in sugarcane on field

Rice kernels colonized with each fungal isolate were added into aqueous 0.05% Tween 80 solution, as described below. The concentration was then adjusted, and the suspensions were continuously mixed during the sugarcane inoculation to ensure homogeneous concentrations.

For the experiments at location A, the fungal suspension was applied inside the furrow by mechanical planting. For spray application, we prepared a 100 L ha-1 syrup with 2 x 1012 conidia ha-1 of *M. humberi* (B), and the insecticide Fipronil (C) (control) was applied according to manufacturer's instructions with 100 L ha-1.

In the experiment at location B, the treatments consisted of *M. robertsii* (A) and *M. humberi* (B) application at 1 x 10^{12} conidia ha-1 inside the furrow and overhead sugarcane before planting. The insecticide Fipronil (C) (control) was applied in the total area according to the manufacturer's instructions.

In experiments of locations C and D, the treatments consisted of *M. robertsii* (A) and *M. humberi* (B) application at 1 x 10^{12} conidia ha-1 and the insecticide Fipronil

(control) was applied into total areas. Both fungi and insecticide were applied inside the ratoon using a ratoon cutter method, which consists of the fungi or insecticide injection into the plants' rhizome.

Finally, the experiment's application at location E, *M. robertsii* (A), and *M. humberi* (B) was applied at 1 x 10^{12} conidia ha-1 inside the furrow and overhead of the sugarcane before planting. The control (D) was performed without fungus or insecticide (Water + 0.05% Tween 80).

4.2.6 Evaluations of initial stages and development of plants

After 120 days of planting and fungi inoculation, we evaluated the initial stages of the plants. The average number of sugarcane clumps and tillers in all experimental areas was assessed. At location A, we evaluated six lines with 10 meters for each treatment. At locations B, C, and E, we considered three lines with 30 meters for each plot, totalizing 270 meters for each treatment. The plants were evaluated individually for each parameter.

4.2.7 Monitoring of insect pests

Some evaluations were conducted during the crop cycle in all areas. The surveys were carried out within the previously mentioned delimited plots. They consisted of the assessment of the presence of root spittlebug nymphs (*Mahanarva fimbriolata*) as indicated by the production of foam right by the sugarcane stem at the soil level, the number of adults of sugarcane weevil (*Sphenophorus levis*), number of adults of sugarcane silky weevil (*M. hemipterus*) and the presence of sugarcane stem borer (*Diatraea saccharalis*). The insect monitoring occurred between January to April, months with these insects' highest occurrence and corresponding to the rainy season in all experimental locations.

Three lines of 10 meters per treatment plot were considered for evaluating the spittlebug, totaling 90 m of evaluation per treatment. The straw was removed from the clumps, and the characteristic foam produced by the spittlebug nymphs (non-destructive sample) was observed in all clusters that totaled the plot.

For the evaluation of the sugarcane weevils, we used sugarcane baits. The baits were 30 cm long, transversely cut, covered with straw, and placed at the clump base. Baits were placed on all lines of the plot. After seven days of placement, the straw was removed, and the bait turned to the count of adults attracted by the baits.

The evaluation of the sugarcane borer, as it is destructive, occurred only during the harvest. Fifteen stalks from each plot, totaling 45 stalks per treatment, were opened vertically to check the presence of the immature forms of sugarcane borer.

4.2.8 Biometric analysis, productivity and technological quality

The pre-harvest evaluations took place 14 months (Location A), 16 months, (Location B), 14 months (Location C and D), and 12 months (Location E) after planting. The stalk length of fifteen stalks per plot was measured, totalizing 45 stalks per treatment. The average diameter of the stalks was evaluated using 15 plants per plot in three different positions: basal part of the stalk, the median portion of the stalk, and the upper tip, and based on this was calculated the stalk volume of this plants.

The weight assessment occurred manually (locations A and B) and mechanically (locations C, D, and E) and consisted of cutting all plot plants in each treatment. The cut plants were weighed in a truck instrumented with a scale, and based on the size of the harvest area, we calculated the weight obtained in tons of sugarcane per hectare.

The technological analysis was carried out at AFOCAPI (Associação dos Fornecedores de Cana de Piracicaba) and consisted of three samples from the plots harvested. The production estimates were calculated with the average production and the ATR of the technological analysis

4.2.9 Statistical analysis

For binomial variables represented by incidence (presence vs. absence) of *D. saccharalis* in stalks and *M. fimbriolata* nymphs in plants were fitted to GLM with a binomial distribution, including treatment block as fixed terms in the linear predictor. For counting data expressing density of *S. levis* and *M. hemipterus*, GLM with Poisson or quasi-Poisson distribution for errors with log link function including treatment and

block as fixed effects in the linear predictor were implemented. Once the treatment showed significance at $P < 0.05$, multiple pairwise comparisons based on Tukey HSD method with FDRFDR P-value adjustment were employed to contrast means within each field trial.

Continuous variables encompassing sugarcane growth traits (stalk length, stalk volume, stalk weight, number of plants per 10 linear meters, and tillering) and post-production technological parameters (reduced sugars, Brix, fiber, and purity) were separately fitted to the generalized linear model (GLM) with Gaussian distribution for errors and overdispersion parameter accounted in the model, whenever necessary, including treatment and block as fixed effects in the linear predictor.

The principal component analysis was performed, taking into account 13 variables measured under field conditions and was correlated with treatments tested. This approach is quite useful to reduce the number of correlated variables based on the selection of main variables accounting for more than 70% of total data variability. The PCA was carried out with the “FactomineR” package and was set to scale = 1 to normalize data across all variables (Lê et al., 2008). After selecting the eight most significant variables contributing to the first three principal components in the PCA, a multivariate linear regression analysis followed by a canonical discriminant analysis was conducted to identify similar or distinct profiles between treatments. The package “candisc” in R was employed for this purpose (Friendly and Fox, 2013).

Heatmaps accompanied by vertical and horizontal dendrograms using averaged values of each variable were built to visualize clusters of treatments and variables and demonstrate their relationships, according to Euclidian distances and Ward’s method of clustering. The package “heat2.map” was used to perform this analysis. All statistical analysis were performed using the free environment R software (R Core Team, 2016).

4.3 Results

4.3.1 Number of plants (clumps) in each experimental location of plants per area

The number of plants sampled in the experimental areas was very close, with no significant differences between treatments in all locations (Figure 3). At location A,

the inoculation of sugarcane with *M. humberi* isolate (ESALQ1638) was very close to the number of clumps in the area with control (Fipronil) application, with 14.2±0.7 and 15±0,6 clumps in 10 linear meters, respectively. In the experimental site in location B, the number of plants was 16±0.81 in the treatment Control with Fipronil, 17±0,94 in the treatment with *M. robertsii* isolate (ESALQ 1635) + Fipronil, and 17±0.7 in the treatment with *M. humberi* isolate (ESALQ 1638) + Fipronil.

The number of plants at location C was not different too. We found 6±0.50, 5±0.67, and 5±0.90 plants at the treatments Control with Fipronil, *M. robertsii* isolate (ESALQ 1635) + Fipronil and *M. humberi* isolate (ESALQ 1638) + Fipronil, respectively. Finally, at the location E, the number of plants in 10 linear meters was 17±0.92 at Control treatment (organic area), 16±0.93 in the treatment with *M. robertsii* isolate (ESALQ 1635), and 16±0.76 plants in the treatment with *M. humberi* isolate (ESALQ 1638).

Considering the experimental locations, we observed that the sites with a greater number of plants were at locations E, B, and A, respectively. In these three places, planting was carried out using the inoculation method in the planting furrow (cane plant) and results in a major number compared with the ratoon cane inoculation method (location C).

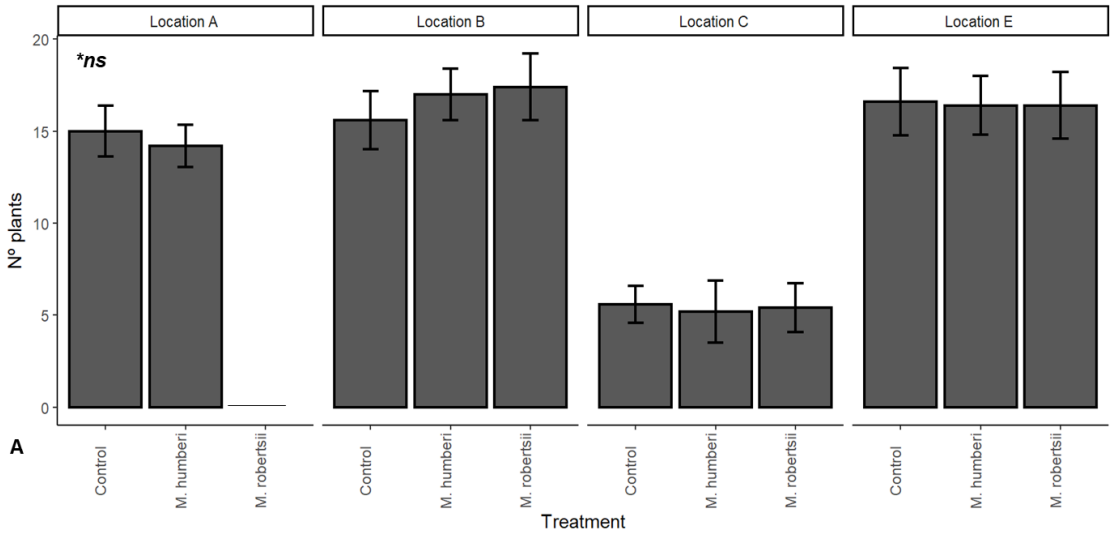

Figure 3. Average number of plants (clumps) of sugarcane counting in 10 linear meters 120 days after inoculation with Control, *M. humberi,* or *M. robertsii* at four different locations. Vertical bars (mean ± 95% CI [confidence interval]); there are no differences among the treatments at P < 0.05 (Tukey HSD test).

4.3.2 Number of tillers per plant at each location

The number of tillers per area also showed no statistical difference between treatments, independently of the locations (Figure 4). At location A, the average of tillers per plants was 8.5±0.40 and 8.0±0.52 in the Control (Fipronil) and *M. humberi* isolate (ESALQ1638), respectively. At location B, the number of tillers per plants was 6.7±0.42, 6.8±0.55, and 6.6±0.45 in the treatments when was applied Control (Fipronil), *M. robertsii* isolate (ESALQ 1635) + Fipronil and *M. humberi* isolate (ESALQ1638) + Fipronil, respectively.

At locations C and E, although there were numerical differences, there were also no significant differences in the number of tillers per plant. The average number of tillers at local C was 11.4±0.90 in the Control (Fipronil) treatment, 12.8±0.74 in the *M. robertsii* isolate (ESALQ 1635) + Fipronil, and 12.7±1,03 in the *M. humberi* isolate (ESALQ1638) + Fipronil. At the location E, the number of tillers was 8.4±0.65, 8.8±0.72, and 9.3±0.49 in the treatments with Control (organic area), *M. robertsii* isolate (ESALQ 1635), and *M. humberi* isolate (ESALQ1638), respectively.

When we consider all the experimental fields, we found that the experimental area with the most massive tiller production was location C (Figure 4). This corroborates the information that the cultivation of ratoon cane produces a much faster and greater number of tillers than cane plant areas.

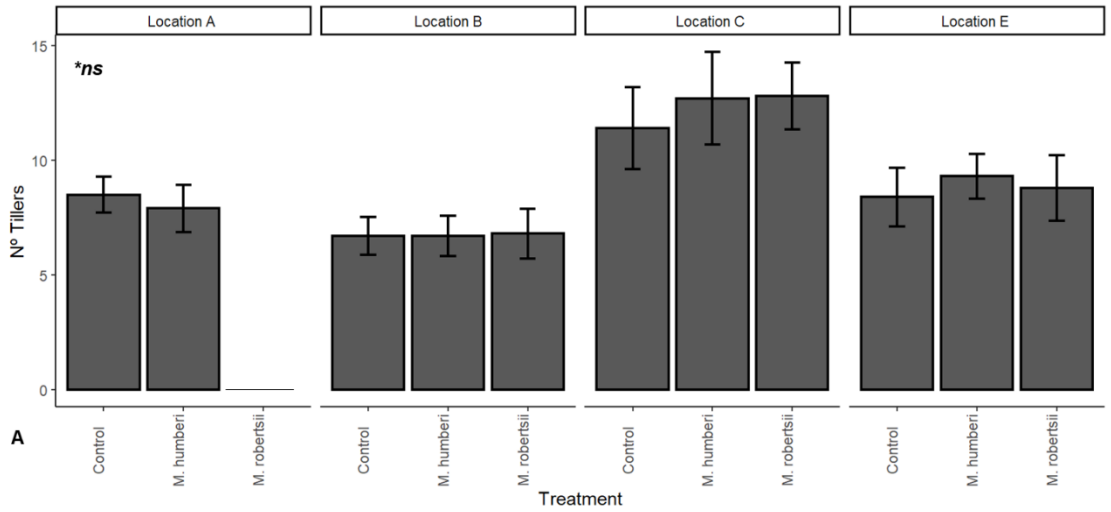

Figure 4. (A) Average number of tillers in sugarcane plants, 120 days after inoculation with Control, *M. humberi,* or *M. robertsii* at locations. Vertical bars (mean ± 95% CI [confidence interval]); there are no differences among the treatments at P < 0.05 (Tukey HSD test).

4.3.3 Monitoring of the sugarcane insect pest complex

The inoculation of sugarcane with conidial suspensions of *M. robertsii* isolate (ESALQ 1635) or *M. humberi* isolate (ESALQ 1638) generally reduced the presence and the insect pest attack in the five experimental fields (Table 1). The presence of spittlebug nymphs, *M. fimbriolata*, was decreased in all locations after treatment with entomopathogenic endophytic fungus *Metarhizium* but not differ statistically. At location A, the presence of spittlebugs in the Control (Fipronil) treatment was 13±2.3% while treatment with *M. humberi* isolate (ESALQ 1638) was 6±1.7% and represented 77% of reduction. Reductions in spittlebug attacks were also observed at location B. We found an 80±2.7% presence in the Control (Fipronil) treatment. In contrast, in the treatment with inoculation of *M. robertsii* isolate (ESALQ 1635) + Fipronil, we found 46±3.4% and 60±3.34% in the treatment with *M. humberi* isolate (ESALQ 1638) + Fipronil, with a reduction of 43% and 25% of spittlebugs, respectively in these treatments.

Spittlebug presence was reduced at location C, too. We found 46±3.4% of attack in the Control (Fipronil) treatment. In comparison, in the treatment with *M. robertsii* isolate (ESALQ 1635) + Fipronil, the percentage was 13±2.32%, and in the *M. humberi* isolate (ESALQ 1638) + Fipronil treatment was 6±1.73%, resulting in a

71.7 and 87% percent of population reduction the. At location D, the reduction of spittlebug was statistically different in the treatments with *Metarhizium* inoculation. In the Control treatment, we observed 53±3.3% of attack while in the treatments with *M. robertsii* isolate (ESALQ 1635) + Fipronil and *M. humberi* isolate (ESALQ 1638) + Fipronil, we observed 20±2.60% and 26±3.02%, respectively, resulting in 62 and 50.9% percent of the reduction. No spittlebugs were found, nor were there any signs of presence or damage caused by this insect at location E.

Considering the sampling in all areas, it is possible to see that location B is the location with a major spittlebug attack, differing statistically from other sites sampled.

Metarhizium inoculation reduced up to 85 percent of the sugarcane borer's damage in tested areas (Table 1).

The sugarcane borer larvae were found in both treatments at location A, with a percentage of 6±2.35% the in sugarcane stalks in the Control (Fipronil) treatment and 6±1.70% of damage in the treatment with *M. humberi* isolate (ESALQ 1638). At location B, the sugarcane borer *D. saccharalis* was found in 13%±2.40 of the stalks sampled in the Control (Fipronil), 11±2.00% in the treatment with the application of *M. robertsii* isolate (ESALQ 1635) + Fipronil and 6±1.65% in the treatments with the application of *M. humberi* isolate (ESALQ 1638) + Fipronil, resulting in 15 to 54% of reduction in the sugarcane borer damage.

At location C, the sugarcane borer damage differed statistically between Control and the treatments with fungus inoculation. The damage was found in 40±3.35% of the stalks sampled in the Control with Fipronil application. In the treatment with the application of *M. robertsii* isolate (ESALQ 1635) + Fipronil, the damage was 6±1.71%, reducing 85% of this insect's presence. The application of *M. humberi* isolate (ESALQ 1638) + Fipronil reduced by 50% the attack of the sugarcane borer in the stalks (20±2.73% of damage). At location D, we observed 26±3.02%, 13±2.32%, and 6±2.20% of sugarcane borer damage in the Control (Fipronil) treatment, *M. robertsii* isolate (ESALQ 1635) + Fipronil and *M. humberi* isolate (ESALQ 1638) + Fipronil, respectively. The reduction of damage was 50 to 76,9% in the treatments with fungal inoculation. At location E, the sugarcane borer was found in 53±3.41% of the stalks sampled in the Control treatment, followed by 40±3.35% in the treatment where the *M. humberi* isolate (ESALQ 1638) was applied and then by 41±3.39 %of sugarcane borer damage where the *M. robertsii* isolated (ESALQ 1635), representing a reduction of 24% and 22.6%.

The percentage of the bore into sugarcane stalks was different when we observed all experimental areas. At location E, we found a greater occurrence of this insect, followed by location C. In other areas, de presence of sugarcane borer did not differ statistically.

Metarhizium inoculation in sugarcane resulted in a reduction of up to 24% of the sugarcane weevil (*S. levis*) and up to 83% of the silky sugarcane weevil (*M. hemipterus*) (Table 1). In sugarcane weevil sampling at location A, we found only 1±0.5 and 0±0 *S. levis* in the Control (Fipronil) and *M. humberi* isolate (ESALQ 1638) treatments, respectively. However, we found 36±0.71 adults of *M. hemipterus* in Control (Fipronil) treatment and 6±0.29 adults in the treatment *M. humberi* isolate (ESALQ 1638), differing statistically. At location B, the reduction of sugarcane weevils was up to 24%. Were captured 102±4.57, 78±3.93, and 89±2.36 adults of *S. levis* in the treatment inoculated with Control (Fipronil), *M. robertsii* isolated (ESALQ 1635) + Fipronil and *M. humberi* isolate (ESALQ 1638) + Fipronil, respectively. The number of silky weevils differed statistically, being 33±0.85, 24±0.41, and 11±0.84 adults in the Control (Fipronil), *M. robertsii* isolate (ESALQ 1635) + Fipronil and *M. humberi* isolate (ESALQ 1638) + Fipronil, respectively. It represents up to a 66% reduction in the number of this insect.

At location C, the number of sugarcane weevils captured showed no significant differences between the treatments evaluated. We found 197±7.66 insects in the Control (Fipronil), 158±3.48 in *M. robertsii* isolate (ESALQ 1635) + Fipronil, and 188±2.20 in *M. humberi* isolate (ESALQ 1638) + Fipronil. At this location, we observed a reduction of this pest of up to 18%. At the same place, the number of silky weevils was lower in the *M. robertsii* isolate (ESALQ 1635) + Fipronil (107±2.81), differing statistically from the Control (Fipronil) (160±3.34), but not to the *M. humberi* isolate (ESALQ 1638) + Fipronil (152±2.68). This reduction represents up to 33% less *M. hemipterus* in this area. It was not possible to sample location D as for the presence of *S. levis* and *M. hemipterus*. At location E, *S. levis* was not found in any of the evaluations, and we observed a 50% of reduction in the number of silky weevils in the *M. robertsii* isolate (ESALQ 1635) (21±1.11), differing statistically to the Control (42±1.26) but not to the *M. humberi* isolate (ESALQ 1638) (38±1.04).

Table 1. Percentage of spittlebugs nymphs, sugarcane borer attack, and the number of sugarcane weevils captured in the insect pest monitoring after *Metarhizium* inoculation at five different experimental locations from 2017 to 2019.

Insect	Location	Control	*M. robertsii*	*M. humberi*	Reduction (%) *
Sugarcane Spittlebug (*M. fimbriolata*) (%)	Location A	13±2.32% a	-	6±1.70% a	77%
	Location B	80±2.73% a	46±3.40% a	60±3.3%4 a	43/25%
	Location C	46±3.40% a	13±2.32% a	6±1.73% a	71.7/87%
	Location D	53±3.30% a	20±2.60% b	26±3.0%2 b	62/50.9%
	Location E	**	**	**	**
Sugarcane borer (*D. saccharalis*) (%)	Location A	6.0±2.35% a	-	6±1.70% a	0%
	Location B	13±2.40% a	11±2.00% a	6±1.65% a	15/54%
	Location C	40±3.35% a	6±1.71% b	20±2.73% b	85/50%
	Location D	26±3.02% a	13±2.32% a	6±2.20% a	50/76.9%
	Location E	53±3.41% a	40±3.35% a	41±3.39% a	24/22.6%
Sugarcane Weevil (*S. levis*) (nº)	Location A	1±0.25 a	-	0±0 a	1%
	Location B	102±4.57 a	78±3.93 a	89±2.36 a	24/13%
	Location C	197±7.66 a	158±3.48 a	188±2.20 a	18/2.1%
	Location D	***	***	***	***
	Location E	**	**	**	**
Sugarcane Weevil (*M. hemipterus*) (nº)	Location A	36±0.71 a	-	6±0.29 b	83%
	Location B	33±0.85 a	24±0.41 a	11±0.84 b	27/66%
	Location C	160±3.34 a	107±2.81 b	152±2.68 ab	33/5%
	Location D	***	***	***	***
	Location E	42±1.26 a	21±1.11 b	38±1.04 ab	50/9.5%

Lines followed by different letters indicating statistical differences (ANOVA). *Compared to the control treatment. **This insect species was not found in this area. ***The site has not been sampled for this pest.

4.3.4 Stalk length of sugarcane in experimental fields

A significant effect of treatments on the stalk length was observed. At location A the average size of the stalks was greater in the treatment where the isolate *M. humberi* ESALQ1638 was applied compared to the average length of the stalks where the insecticide Fipronil was used, being 3.07±0.31 and 2.72±0.28 m, respectively (Figure 5). The average stalk length at location B was statistically equal in the Control (Fipronil), and where isolate ESALQ 1635 (M. robertsii) + Fipronil was applied 2.25±0.19 and 2.27±0.21 m, respectively. The treatment where isolate ESALQ 1638 (*M. humberi*) + Fipronil was used was lower than the other treatments, for this parameter was of 2.15±0.18 m (Figure 5).

No statistical differences were found between treatments in sugarcane stalk length at location C (ratoon sugarcane). The length was 1.76±0.23 m for the Control (Fipronil), 1.81±0.14 m where was applied ESALQ 1635 (*M. robertsii*) + Fipronil and 1.74±0.19 m where the isolated ESALQ 1638 (*M. humberi*) + Fipronil was inoculated (Figure 5). At the commercial ratoon sugarcane area (location D), the treatment inoculated the isolate ESALQ 1638 (M. humberi) + Fipronil was statistically higher than the others 1.66±0.16 m. No differences were observed between Control (Fipronil) and ESALQ 1635 (*M. robertsii*) + Fipronil, being 1.41±0.12 and 1.52±0.11 m, respectively (Figure 5).

Finally, at location E the average length of the stalks of the treatments where the ESALQ 1635 isolate (*M. robertsii*) was inoculated had a significant difference when compared to the Control and the treatment with the ESALQ 1638 isolate (*M. humberi*), being 2.72±0.45, 2.41±0.45 and 2.54±0.43 m, respectively (Figure 5). When we compare all 5 locations, we found that the sites with the longest stalk lengths were at cane plant locations A (variety IAC 5000) and E (CTC 9001 variety), both differing mainly from the ratoon cane cultivation (Figure 5).

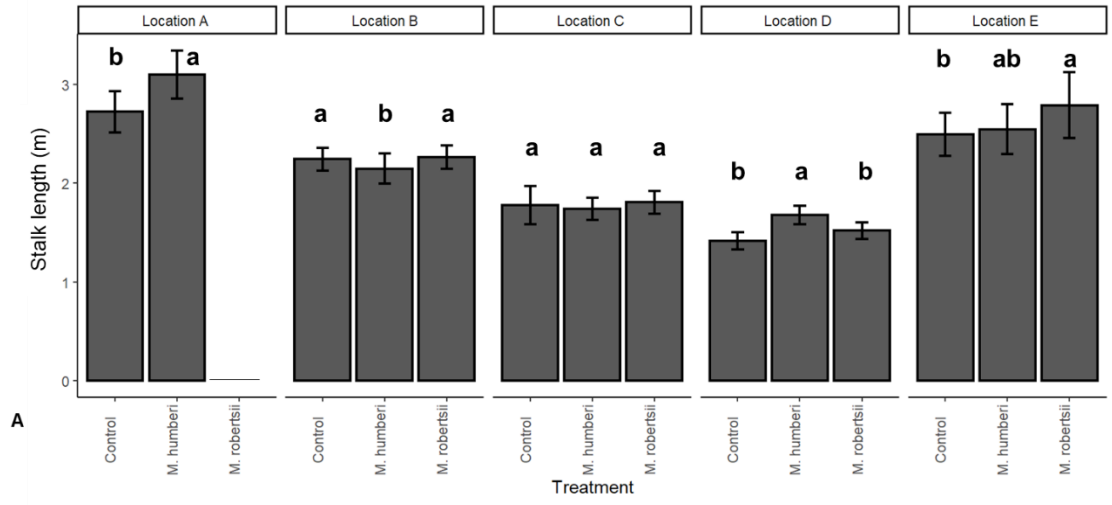

Figure 5. (A) Average length of sugarcane stalks (meters), after inoculation with Control, *M. robertsii,* or *M. humberi* at locations. Vertical bars (mean ± 95% CI [confidence interval], n = 45 replicates); distinct letters indicate significant differences among the treatments at P < 0.05 (Tukey HSD test).

4.3.5 Stalk volume of sugarcane in experimental fields

Based on the stalk length and the diameter of data (base, middle and tip diameter), it was possible to estimate the total stalk volume using this formula: *base/middle*^{v} = 3,1415*(length/2)/3*((base diameter + base diameter^2)*(middle diameter + middle diameter^2)) + *middle/tip*^{v} = 3,1415*(length/2)/3*((middle diameter + middle diameter^2)*(tip diameter + tip diameter^2)).

The plants inoculated with the fungal treatments showed an increase in the volume of sugarcane stalks in all tested areas. At location A, the stalk volume was significantly higher in the treatment with *M. humberi* isolate (ESALQ 1638) (29,223 ± 5,113 m^3) than the Control (Fipronil) (23,550 ± 708.15 m^3) (Figure 6A). The average stalk volume at location B was significantly higher in the treatment with inoculation of *M. robertsii* isolate (ESALQ 1635) + Fipronil (12,909 ± 1,055.62 m^3) than the Control (Fipronil) (11,582 ± 756 m^3) and the *M. humberi* isolate (ESALQ 1638) + Fipronil (11,151 ± 914.67 m^3) (Figure 6).

At location C, the *M. robertsii* isolate (ESALQ 1635) +Fipronil showed the higher stalk volume. The average volume was significantly different in this treatment (49,478 ± 4,165.46 m^3) than the Control (Fipronil) (38,096 ± 3,538 m^3), and the treatment with *M. humberi* isolate (ESALQ 1638) + Fipronil inoculation (39,353 ± 6,292.49 m^3) (Figure

6). There were no significant differences between the stalk volume treatments at location D, despite numerical differences (Figure 6). At location E, the stalks inoculated with fungal treatments were significantly different from the Control. The stalk volume was higher in the *M. robertsii* (ESALQ 1635) treatment (50,972 ± 7,777 m^3), followed by *M. humberi* isolate (ESALQ 1638) (43,995 ± 6,711.64 m^3) and the Control (37,153 ± 5,624.01 m^3) (Figure 6).

When we considered all field locations, the stalk volume was higher at location E (CTC 9001 variety) and location C (variety IAC 5000) differing to other sites (Figure 6).

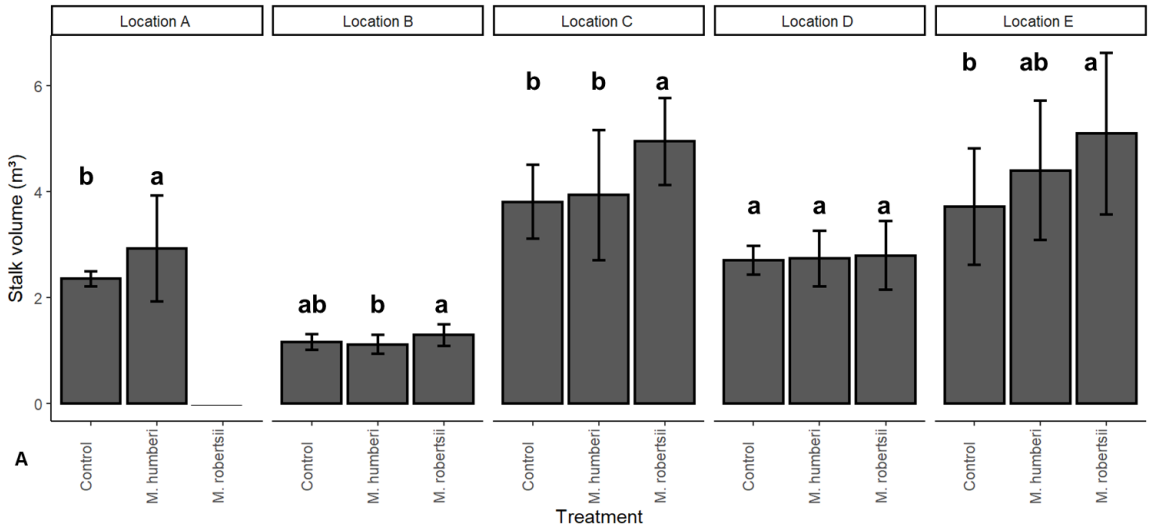

Figure 6. (A) Average volume (m^3) of sugarcane stalks, after inoculation with Control, *M. robertsii,* or *M. humberi* at locations. Vertical bars (mean ± 95% CI [confidence interval], n = 45 replicates); distinct letters indicate significant differences among the treatments at $P < 0.05$ (Tukey HSD test).

4.3.6 Sugarcane yield at different experimental fields

The productivity of sugarcane was higher in inoculated plants than in non-inoculated plants (Figure 7). At location A, the sugarcane production was 124.86 tons/ha in Control (Fipronil) and 138.06 tons/ha in *M. humberi* (ESALQ 1638), differing statistically. At location B, we did not see significant differences between treatments. The productivity was 40 tons/ha in Control (Fipronil), 39.92 tons/ha in *M. robertsii* (ESALQ 1635) + Fipronil, and 37.8 tons/ha in *M. humberi* (ESALQ 1638) + Fipronil.

The same occurs at location C; the productivity was 56.53, 56.80, and 51.20 in the treatments inoculate with Control (Fipronil), *M. robertsii* (ESALQ 1635) + Fipronil and *M. humberi* (ESALQ 1638) + Fipronil, respectively and showed no statistical differences.

At location D, the weight in the *M. humberi* (ESALQ 1638) + Fipronil treatment was significantly different from other treatments, being 82 tons/ha of sugarcane. The Control (Fipronil) productivity was 70.66 tons/ha and *M. robertsii* (ESALQ 1635) + Fipronil 70 tons/ha. Finally ate location E, both fungal treatments increased sugarcane productivity. The *M. robertsii* (ESALQ 1635) produced 140.40 tons/ha, *M. humberi* (ESALQ 1638) 139.33 tons/ha, and Control 122.8 tons/ha, differing statistically.

When we consider all areas, we observed that the areas with higher yield were Location A (IAC-5000 variety) and Location E (CTC-9001 variety), both sugarcane plants (Figure 7).

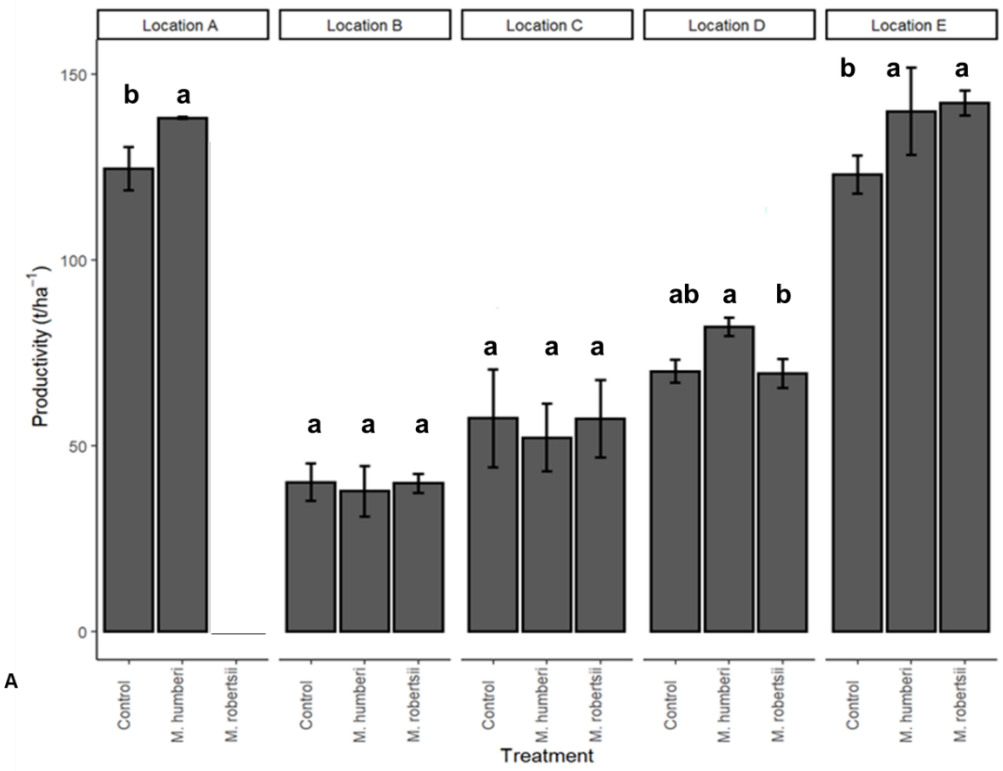

Figure 7. Productivity (tons/ha) of sugarcane obtained at harvest after inoculation with Control, *M. robertsii,* or *M. humberi* at locations. Vertical bars (mean ± 95% CI [confidence interval]); distinct letters indicate significant differences among the treatments at $P < 0.05$ (Tukey HSD test).

4.3.7 Technological analysis of sugarcane

For the sugarcane quality analysis, it is critical to analyze two types of factors that affect the quality of the raw material intended for the industry, the intrinsic characteristics (related to the composition of sugarcane) and extrinsic factors (related to foreign materials found in sugarcane). The technological analysis shows the main indicators of sugarcane quality. In general, few significant differences were found in sugarcane's quality parameters in our experiments (Table 2). The Brix parameter differs only at location D, and in this case, the treatment with *M. humberi* (ESALQ 1638) + Fipronil showed the highest Brix. The total reducing sugars (TRS) was also statistically higher in the *M. humberi* (ESALQ 1638) + Fipronil treatment than the others at location D. Fiber is another parameter that differs statically only at location D, and was higher in *M. humberi* (ESALQ 1638) + Fipronil than the others treatments.

Table 2. Technological analysis of sugarcane culm after 14 months of planting and inoculation with *M. humberi* or Fipronil treatments. Sugarcane plant experiment at location A in Batatais/SP.

Parameter	Location	Control	*M. robertsii*	*M. humberi*
BRIX[1]	Location A	20.84±0.061	-	19.6±0.093
	Location B	15.71±0.621	15.46±0.621	16.11±0.507
	Location C	20.73±0.527	20.19±0.232	20.64±0.056
	Location D	19.45±0.282 b	19.74±0.038 b	20.56±0.124 a
	Location E	17.89±0.465	18.26±0.392	18.10±0.211
TRS[2]	Location A	158.42±0.33	-	148.87±0.25
	Location B	117.43±7.34	116.45±2.05	121.94±4.01
	Location C	149.75±3.21	143.75±2.15	146.55±2.27
	Location D	146.65±0.60 b	148.15±0.09 b	155.29±0.72 a
	Location E	130.00±3.96	134.75±5.03	134.49±2.26
Fiber[3]	Location A	11.60±0.05	-	11.15±0.01
	Location B	11.96±0.33	11.86±0.21	11.66±0.16
	Location C	13.63±0.32	13.48±0.03	13.69±0.20
	Location D	11.47±0.02 b	11.95±0.01 a	11.51±0.04 b
	Location E	11.28±0.14	11.56±0.15	11.20±0.08
Purity[4]	Location A	89.91±0.03	-	89.20±0.05
	Location B	87.46±2.38	88.36±0.12	88.72±0.75
	Location C	88.36±0.34	86.33±0.50	86.59±1.01
	Location D	88.95±0.02	88.92±0.01	89.47±0.28
	Location E	83.90±0.88	86.12±2.11	86.34±0.82

[1] *BRIX:* Proximate graduation that corresponds to the sucrose content in pure solutions. When it comes to sugarcane juice, which is an impure sucrose solution, the sucrose content is apparent since it contains other dissolved solids. Brix grade corresponds, in terms of practical, the percentage, by mass, of soluble solids in the broth.

[2] *TRS (Total Reducing Sugars):* an indicator represents the total amount of sugarcane (sucrose, glucose, and fructose). The ATR is determined by the POL / 0.95 ratio plus the content of reducing sugars. The concentration of sugars in the cane varies, in general, within the range of 13 to 17.5%. However, it is essential to remember that very rich canes with a low percentage of fibers are more subject to physical damage and attack by pests and microorganisms. Studies show that in the first 14 hours of sugarcane deterioration, 93% of sucrose losses were due to microorganisms' action, 5.7% by enzymatic reactions, and 1.3% by chemical reactions, resulting from acidity.

[3] *Fiber:* reflects the efficiency of the extraction of the mill; that is, the higher the sugar cane fiber, the lower the extraction efficiency. On the other hand, it is necessary to consider that sugarcane varieties with low fiber content are more susceptible to mechanical damage caused by cutting and transport, which favors contamination and losses in the industry. When the cane is low in fiber, it also falls and breaks with the wind, which causes it to lose more sugar in the washing water.

4 Purity: determined by the POL / Brix x 100 ratio. The greater the cane's purity, the better the quality of the raw material to recover sugar. All substances with optical activity can interfere with POL, such as reducing sugars (glucose and fructose), polysaccharides, and some proteins.

4.3.8 Production estimates of sugarcane

Based on the productivity and the TRS parameter, we estimate the profit obtained from selling the cane produced in each area. We concluded that the inoculation of *Metarhizium* spp. increased producers' profit by up to 22.9% (Table 3). At location A, the most significant gain was obtained in the treatment with *M. humberi* (ESALQ 1638) inoculation (R$58,278.01) and generated a 3.9% increase compared to the control. According to our projection, at location B, there was a 0.6% increase in profit for the producer in the *M. robertsii* (ESALQ 1635) + Fipronil site (R $ 9,421.61). At location C, *M. robertsii* (ESALQ 1635) + Fipronil was the treatment with the great profit (R$16,675.94), resulting in a 4.7% increase. The highest increase in the profit was obtained at location D with *M. humberi* (ESALQ 1638) + Fipronil inoculation, that generate R$45,861.73 of profit (22.9% the increase). Finally, at location E *M. robertsii* (ESALQ 1635) showed the most significant gain (R$39,415.40) with an 18.5% increase.

Table 3. Production estimates by area after 14 months of planting and inoculation with *M. humberi* or Fipronil treatments. Sugarcane plant experiment at location A in Batatais/SP.

Parameter	Location	Control	*M. robertsii*	*M. humberi*	
Tons of sugarcane / ha[1]	Location A	124.86	-	138.06	
	Location B	40.01	39.92	37.80	
	Location C	56.53	56.80	51.20	
	Location D	70.66	70.00	82.00	
	Location E	122.80	140.40	139.33	
Kg of TRS/ton of cane[2]	Location A	19,780.32	-	20,552.99	
	Location B	4,659.16	4,687.81	4,609.33	
	Location C	8,126.19	8,505.80	7,503.36	
	Location D	10,362.29	10,370.50	12,733.78	
	Location E	15,964.00	18,918.90	18,738.49	
TRS (value R$)[3]	Location A	11,217.42	-	11,655.60	
	Location B	2,774.53	2,791.59	2,744.86	
	Location C	4,720.50	4,941.02	4,358.70	
	Location D	6,019.45	6,024.22	7,397.05	
	Location E	9,854.58	11,678.64	11,567.27	**Increase (%)***
Profit obtained in the area (R$)[4]	Location A	56,087.10	-	58,278.01	3.9%
	Location B	9,364.05	9,421.61	9,263.89	0.6%
	Location C	15,931.70	16,675.94	14,710.62	4.7%
	Location D	37,320.61	37,350.19	45,861.73	22.9%
	Location E	33,259.20	39,415.40	39,039.54	18.5%

[1]*Tons of sugarcane/ha:* The productivity obtained per hectare after the harvesting.

[2] *Kg of TRS/ton of cane:* (Total of recoverable sugar) We multiply the number of tons of cane per hectare harvested by TRS's value obtained in the technological analysis of cane quality.

[3] *TRS (value R$):* (Total of recoverable sugar) We multiply the factor 2 (Kg of TRS/ton of cane) by the price paid for TRS on the harvest date. TRS value in April/2018 (location A): R$0.5671/kg. TRS value in December/2018 (location B): R$0.5955/. TRS value in November/2018 (location C): R$0.5809/kg. TRS value in November/2018 (location D): R$0.5809/kg. TSR value in June/2019 (location E): R$0.6173/kg. TRS values were obtained from https://www.udop.com.br/ (UDOP - União dos Produtores de Bioenergia).

[4] *Profit obtained in the area (R$):* We multiply the value obtained in factor 3 (TRS value R$) by the experimental area's size.

4.3.9 Multivariate analysis

The treatments exhibited different performances in sugarcane fields according to their plant traits (pre- and post-harvest parameters) and key pests assessment

(Fig. 8). The multivariate principal component analysis indicated grouping of data into three main components that accounted for that 77.83% of the total variance (Fig. 8; Eigenvalue 10.11). In more detail, the variables *Metamasius*, *Sphenophorus*, tillering, fiber, total reduced sugars, Brix, and stalk weight (productivity) were positively correlated with PC1 ($P < 0.0001$), indicating that treatments encompassing insecticide, *M. robertsii* + insecticide, and *M. humberi* + insecticide were positioned in positive scores of PCQ had significantly higher values for these variables. In contrast, the treatments used in the organic sugarcane field, comprising *M. robertsii*, *M. humberi,* and untreated control, were located at the opposite side of PC1 and PC2, assuming negative scores, indicating they exhibited higher values for the plant stand and stalk length but the lower density of *Sphenophorus* and *Metamasius*. When examining PC2, a positive correlation was strongly seen with *Mahanarva* infestation level and sugarcane purity, while the *Diatraea* infestation level was negatively loaded to PC2 ($P < 0.0001$). In more detail, fungal treatments applied in the organic sugarcane field had a low incidence of *Mahanarva* and reduced sugarcane purity, but rendered a high incidence of *Diatraea*. Conversely, fungus + insecticide treatments and insecticide alone showed higher values for *Mahanarva* incidence and sugarcane purity.

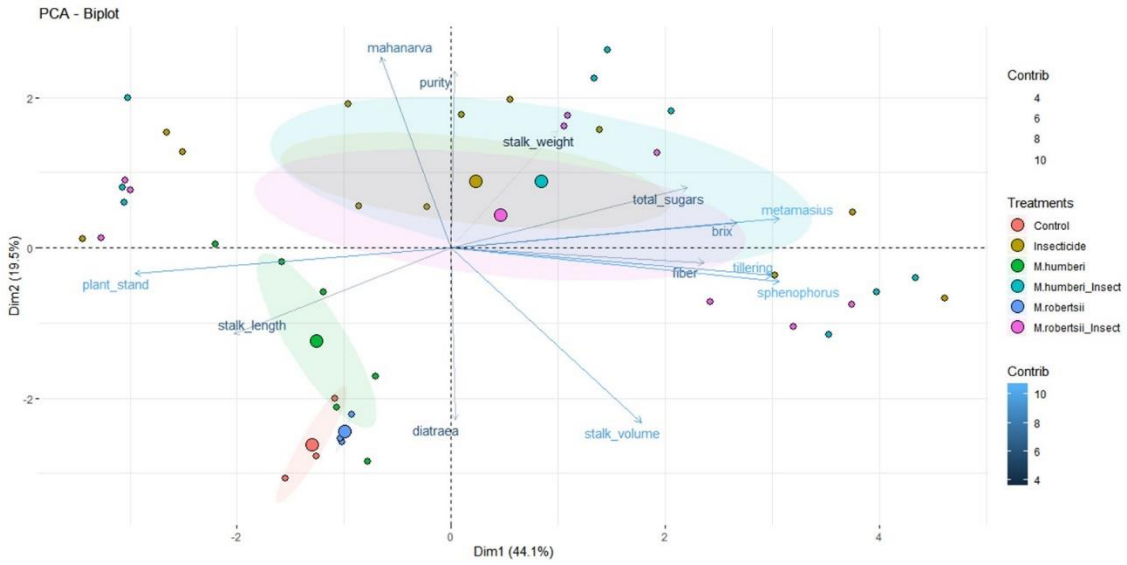

Figure 8. Relationship between sugarcane traits/pests and biological or chemical treatments. Biplot of principal component analysis showing the importance of the sugarcane technological parameters and key pests for class dispersion (*Metarhizium* spp. strains, insecticide applied together with each *Metarhizium* species, insecticide alone and control) across two principal components (PC1 vs. PC2). Each point represents each treatment's weighted sum in its respective component, while larger circles denoting the average followed by its respective 95% confidence interval ellipse. The arrows (vectors) indicate the direction and magnitude of the variables' correlation with the extracted components (the longer the arrow, the higher its contribution explaining data variability).

In multivariate linear analysis followed by canonical discriminant analysis, the fungal treatments and control were located on the opposite side, assuming negative scores for Canonical axis 1. In contrast, fungi combined with insecticide and insecticide alone assumed positive scores as they positioned to the right side, showing higher values of total reduced sugars, Brix, *Sphenophorus,* and *Metamasius* densities, and tillering. In contrast, stalk length, plant stand, and stalk volume were more related to single fungal treatments and control in the organic sugarcane field, in which *M. robertsii* diverged from control and *M. humberi*.

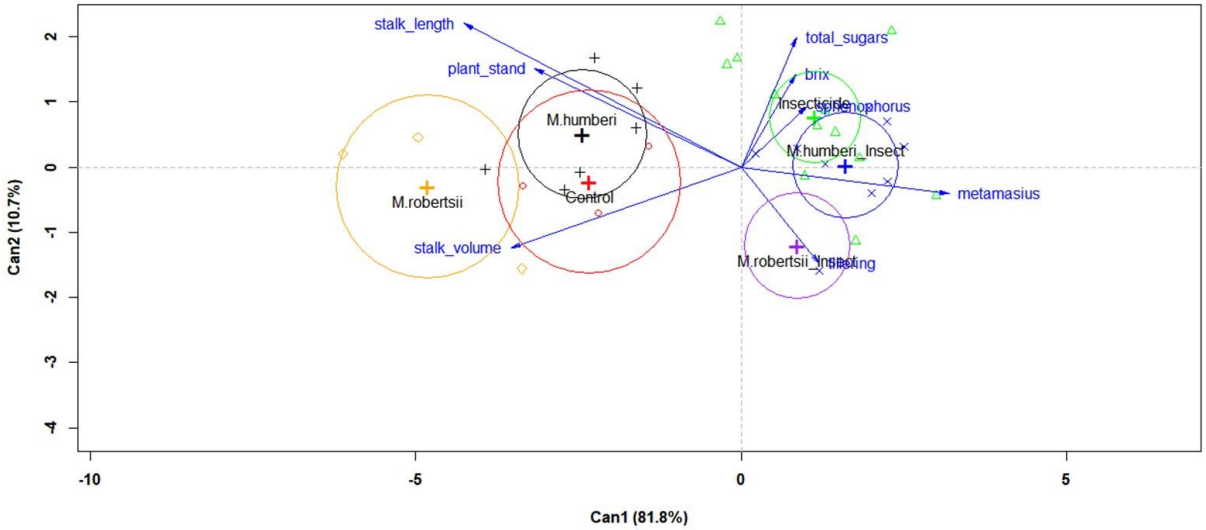

Figure 9. Canonical discriminant biplot of the main variables selected by principal component analysis and their relationship with fungal treatments, insecticide, and control categories. Large crosses in the center of 95% confidence interval circles denote average values, while symbols represent data points. The arrows or vectors indicate the direction and magnitude of the variables' correlation with the extracted components (the longer the arrow, the higher its contribution to explaining data variability).

4.4 Discussion

Sugarcane inoculation with *M. robertsii* and *M. humberi* resulted in reducing pests and increasing productivity on the field. A small number of studies have investigated the effects of endophytic entomopathogenic fungi inoculation on plants under natural field conditions (JABER; OWNLEY, 2018; VEGA, 2018; CANASSA et al., 2019). The present study is the first report of the effect of sugarcane inoculation with *M. robertsii* and *M. humberi* on insect pest populations and productivity under open field cultivation regimes. The two fungal isolates were previously found to increase sugarcane growth and cause mortality in sugarcane pests in the laboratory and greenhouse (SIQUEIRA, et al. in preparation). Similar effects were observed under field conditions, indicating broad host plant indirect impacts of these isolates against sugarcane insect pest complex.

The use of *Metarhizium* in sugar cane has been carried out for decades and has a history of positive results and high levels of control, especially for root spittlebugs

(ALMEIDA, 2019). Also, recent studies have revealed that there is a high molecular diversity of *Metarhizium* in dead insects, roots, and soil in sugarcane crops in Brazil (REZENDE, et al. 2015; IWANICKI et al. 2019), but we still do not know that there are the effects of inoculation of endophytic *Metarhizium* in sugarcane for growth, development, and productivity, in addition to the indirect effects on the main pests of this crop.

Propagules of the fungus *Metarhizium* spp. are widely present in the soil, and their distribution is more associated with the environment than with host insects (BEHIE; BIDOCHKA, 2014; MEYLING et al., 2011; VEGA et al., 2012; BIDOCHKA et al., 2001; WYREBEK et al., 2011). Several studies have shown that *Metarhizium* spp. is distributed in the soil, but exhibits associations with the plant rhizosphere (HU and St. LEGER, 2002; BRUCK, 2005; St. LEGER, 2008; BRUCK, 2010; PAVA-RIPOLL ET AL., 2011) indicating that *Metarhizium* is more than just an entomopathogenic fungus, but it is also involved in other forms of propagation for example in interaction with plants.

A large number of recent studies have shown the capacity for endophytic colonization of *Metarhizium* in several plants and the effects of this relationship for both, such as promoting plant growth (KABALUK AND ERICSSON, 2007; GARCIA ET AL., 2011; SASAN AND BIDOCHKA, 2012; LIAO ET AL., 2014; LOPEZ AND SWORD, 2015; JABER AND ENKERLI, 2016, 2017) and reducing damage caused by insects (CASTILLO-LOPEZ ET AL., 2014; GOLO ET AL., 2014; MUVEA ET AL., 2014; MANTZOUKAS ET AL., 2015; GARRIDO-JURADO ET AL., 2017; LEFORT ET AL., 2016; MUTUNE ET AL., 2016; RÍOS-MORENO ET AL., 2016).

The potential of *Metarhizium* inoculation as an endophyte and the benefits of this relationship has been widely reported in the greenhouse, but few studies have investigated the pest control and plant growth promotion potential of entomopathogenic fungi as inoculants of plants under field conditions (JABER AND OWNLEY, 2018; VEGA, 2018). Our study demonstrates that *Metarhizium* inoculated on sugarcane plants improves plant development and productivity and reduces the number of insect pests on field conditions.

Kabaluck and Ericson (2007) treated corn seeds with *M. anisopliae* conidia, and they observed significant increases in stand density, stock, and foliage area of maize plants on the field. Besides that, they found high wireworm mortality, suggesting that the increase in yield may have been due to wireworm control. Canassa et al. (2019)

studying *M. robertsii* inoculation on strawberry plants, observed a significant reduction in *T. urticae* adults' numbers compared to non-inoculated control plants. In another study, Ramanujam et al. (2017) observed that *M. anisopliae* inoculation on maize in the field reduces maize stem borer damages (dead hearts, stem tunneling, and exit holes) and its contributed to higher yields. Li et al. (2020) also showed beneficial effects on Peanut inoculation with *M. anisopliae* on the field to both grain yield and few densities of the white grub larvae.

Our results demonstrate that *M. robertsii* and *M. humberi* inoculation in sugarcane reduce the number of insects and the damage caused by these pests in five different locations. We suggested that this reduction is responsible in part for the higher yield found in these areas.

The use of *Metarhizium* as an endophyte for pest management or pest reduction is already known. However, the mechanisms involved in this effect are still unknown since no fungal outgrowth is observed from the cadavers of insects after being exposed to plants endophytically colonized by this fungus (GARRIDO-JURADO ET AL., 2017; RÍOS-MORENO ET AL., 2016). The production of insecticidal compounds within the plant as one of the possible mechanisms (RESQUÍN-ROMERO ET AL., 2016; RÍOS-MORENO ET AL., 2016; ROHLFS & CHURCHILL, 2011), and also secondary fungal metabolites produced in colonized plant tissues, such as destruxins A, detected in tissues of potato plants and melon leaves colonized by *Metarhizium brunneum* (GARRIDO-JURADO ET AL., 2017; RÍOS-MORENO ET AL., 2016). According to Quesada-Moraga (2020), the utilization of entomopathogenic endophytic fungi as endophytes has the benefit of contacting the pest inside the plant.

The growth promotion process was investigated in some studies, and the most likely mechanisms are that entomopathogenic endophytic fungi inoculation can improve plant nutrition (BEHIE ET AL., 2012; BEHIE & BIDOCHKA, 2014), to promote root development (LIAO ET AL., 2014; SASAN & BIDOCHKA, 2012; WYREBEK ET AL., 2011), relief from abiotic stresses, such as salinity or Fe deficiency (KHAN et al., 2011; SÁNCHEZ-RODRÍGUEZ ET AL., 2015, 2016).

Other mechanisms can be involved in plant growth by entomopathogenic endophytic fungi inoculation, such as Indol Acetic Acid production, phosphate (P) solubilization, and the production of other key enzymes and another compounds, like siderophores and chitinases that can contribute to plant health and development (LIAO et al., 2017; MISHRA et al., 2014; DONZELLI et al., 2015; SIQUEIRA et al. 2020).

The use of entomopathogenic endophytic fungi as inoculants requires lower amounts of inoculum than those used in inundative applications to the soil, since it is confined and protected against abiotic and biotic factors inside the plant (AKELLO et al., 2008; BACKMAN & SIKORA, 2008).

The inoculation of isolates ESALQ 1635 (*M. robertsii*) and ESALQ 1638 (*M. humberi*) brought benefits when applied to cane, reducing the main sugarcane incidence pests. In conclusion, the present study demonstrates that entomopathogenic fungi can be used as inoculants in commercial sugarcane fields to control important pests and improve productivity simultaneously.

These results are promising for developing an inoculant based on these fungi, which, besides being a biological product and environmentally correct, also has persistence in the soil and can be economically viable.

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