



Multi-Trait Biochemical Features of *Metarhizium* Species and Their Activities That Stimulate the Growth of Tomato Plants

Ana Carolina Oliveira Siqueira¹, Gabriel Moura Mascarin²,
Cassiana R. N. C. B. Gonçalves¹, Joelma Marcon³, Maria Carolina Quecine³,
Antonio Figueira⁴ and Ítalo Delalibera Jr.^{1*}

¹ Department of Entomology and Acarology, “Luiz de Queiroz” College of Agriculture/University of São Paulo (ESALQ/USP), Piracicaba, Brazil, ² Laboratory of Environmental Microbiology, Brazilian Agricultural Research Corporation, Embrapa Environment (EMBRAPA), Jaguariúna, Brazil, ³ Department of Genetics, “Luiz de Queiroz” College of Agriculture/University of São Paulo (ESALQ/USP), Piracicaba, Brazil, ⁴ Center for Nuclear Energy in Agriculture/University of São Paulo (CENA/USP), Piracicaba, Brazil

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*Correspondence:

Ítalo Delalibera Jr.
delalibera@usp.br

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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, in relation 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. humberii* could be explored as complementary plant growth promoters.

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

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 (Singh and Satyanarayana, 2011; Ghosh et al., 2015). 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 (0.5–1.5 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. humberii* 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. humberii* and *M. robertsii* induced endogenous IAA production. These findings support the commercial development of both indigenous isolates of *M. robertsii* and *M. humberii* as biocontrol agents and root colonizers for stimulating plant growth.

MATERIALS AND METHODS

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. humberii*, and *M. anisopliae* (Table 1) were used in this study. The

TABLE 1 | Description of fungal isolates used in the experiments.

Isolate code ^a	Fungal species	Crop/Isolation method	Location in Brazil (City—State) ^b	Geographical coordinates	Sampling date
ESALQ 1635	<i>Metarhizium robertsii</i>	Forest soil/Insect bait with <i>Tenebrio molitor</i>	Delmiro Gouveia, AL	9°25'0.12"S 37°57'8.49"W	Mar/2012
ESALQ 1638	<i>Metarhizium humberi</i>	Savanna soil/Selective media (PDAY)	Rio Verde, GO	17°29'49.3"S 51°13'40.7"W	Mar/2012
ESALQ 1669	<i>Metarhizium anisopliae</i>	Sugarcane soil/Insect bait with <i>T. molitor</i>	Iracemápolis, SP	22°36'10"S 47°33'17"W	Dec/2012
ESALQ 1306	<i>Trichoderma harzianum</i>	Soil/Selective media (PDAY)	Piracicaba, SP	22°42'06.7"S 47°38'44.2"W	Jul/2002

^aESALQ—Escola Superior de Agricultura “Luiz de Queiroz” —University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil. ^bBrazilian States: AL, Alagoas; SP, São Paulo, and GO, Goiás.

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.

Plant Material

The tomato miniature cultivar Micro-Tom (MT) employed is considered a suitable genetic model (Meissner et al., 1997; Scott 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 (Mart 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.

Plan 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 20 replicates. The average max and min daily temperatures during the course of this greenhouse experiment were 28.7 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 \times 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.

Biochemical Assays

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 mycelia 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 H₂SO₄, 0.75 mL FeCl₃, 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 (Etesami et al., 2015). 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).

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 NaH₂PO₄·7H₂O pH 7.0, 10 mM EDTA pH 7.0, 0.5 mM K₃Fe(CN)₆ pH 7.0, 0.5 mM

K₄[Fe(CN)₄].3H₂O 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.

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 × 15 mm) (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: $FSI = \frac{\text{Ø halo}}{\text{Ø colony}}$. This assay followed a completely randomized design with five replicates (plates) and was repeated three times on different occasions to ensure reproducibility.

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 NH₄NO₃; 0.5 g L⁻¹ of MgSO₄·7H₂O; 0.5 g L⁻¹ of KCl; 0.01 g L⁻¹ of FeSO₄·7H₂O; 0.01 g L⁻¹ of MnSO₄·4H₂O; 5 g L⁻¹ of calcium phytate (C₆H₁₈O₂₄P₆); 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: $PDI = \frac{\text{Ø halo}}{\text{Ø colony}}$. This assay was repeated three times and followed a completely randomized design with five replicates (plates).

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 FeCl₃·6H₂O 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 NH₄Cl, 0.3 g KH₂PO₄, 0.5 g NaCl, 1.2 g piperazine (C₄H₁₀N₂), 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).

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 (NH₄)₂SO₄; 2.0 g KH₂PO₄; 0.3 g MgSO₄; 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, 2012). 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).

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 Core Team (2018). Linear mixed models (assuming a normal distribution for the error) were fitted to the IAA production, including the effects of 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 et al., 2018).

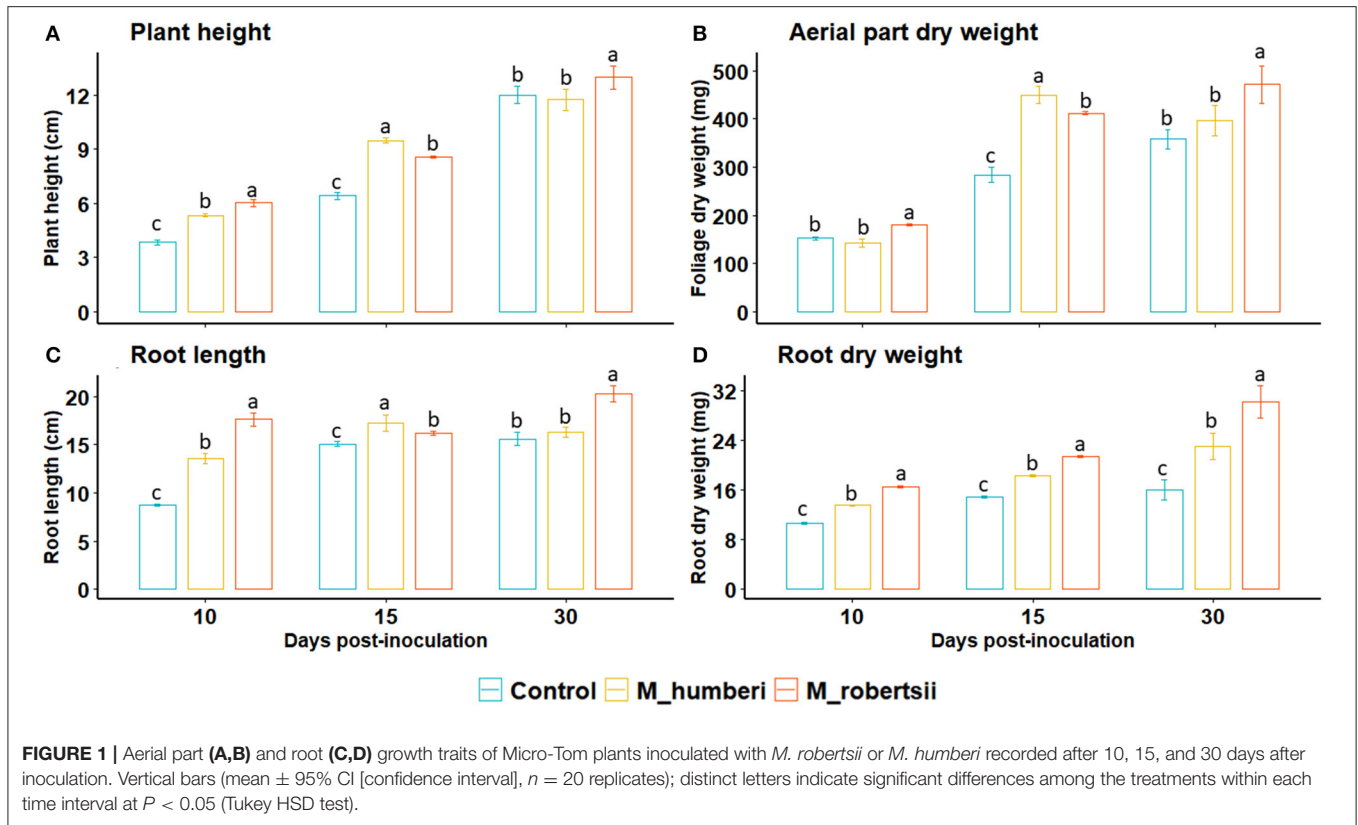
Finally, generalized canonical discriminant analysis for a multivariate linear model was performed with datasets from biochemical assays and plant growth traits to determine the correlation between treatments and response variables. A significant group effect was obtained with the type II MANOVA test 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).

RESULTS

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: $F = 10.61$, $df = 2, 57$, $P = 0.00012$) (Figure 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

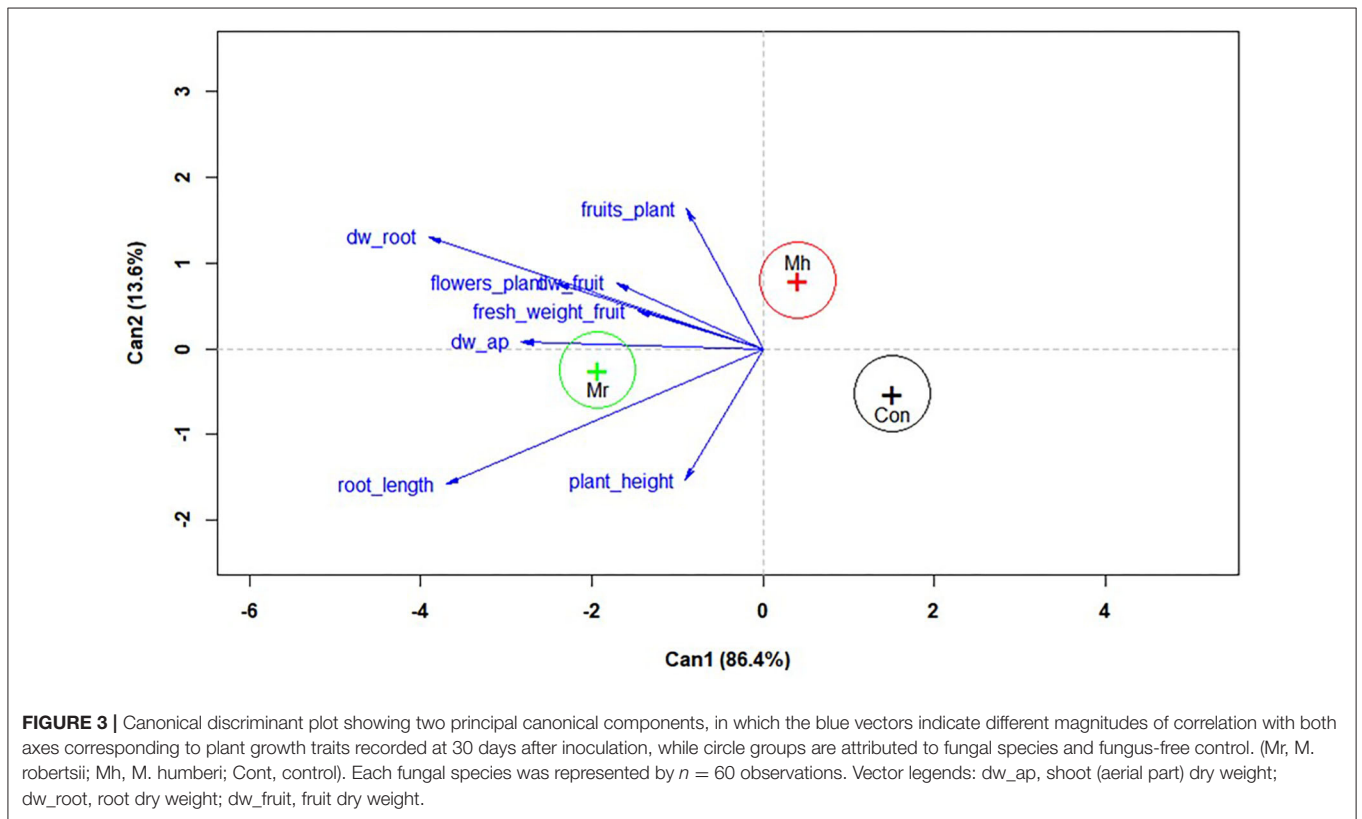
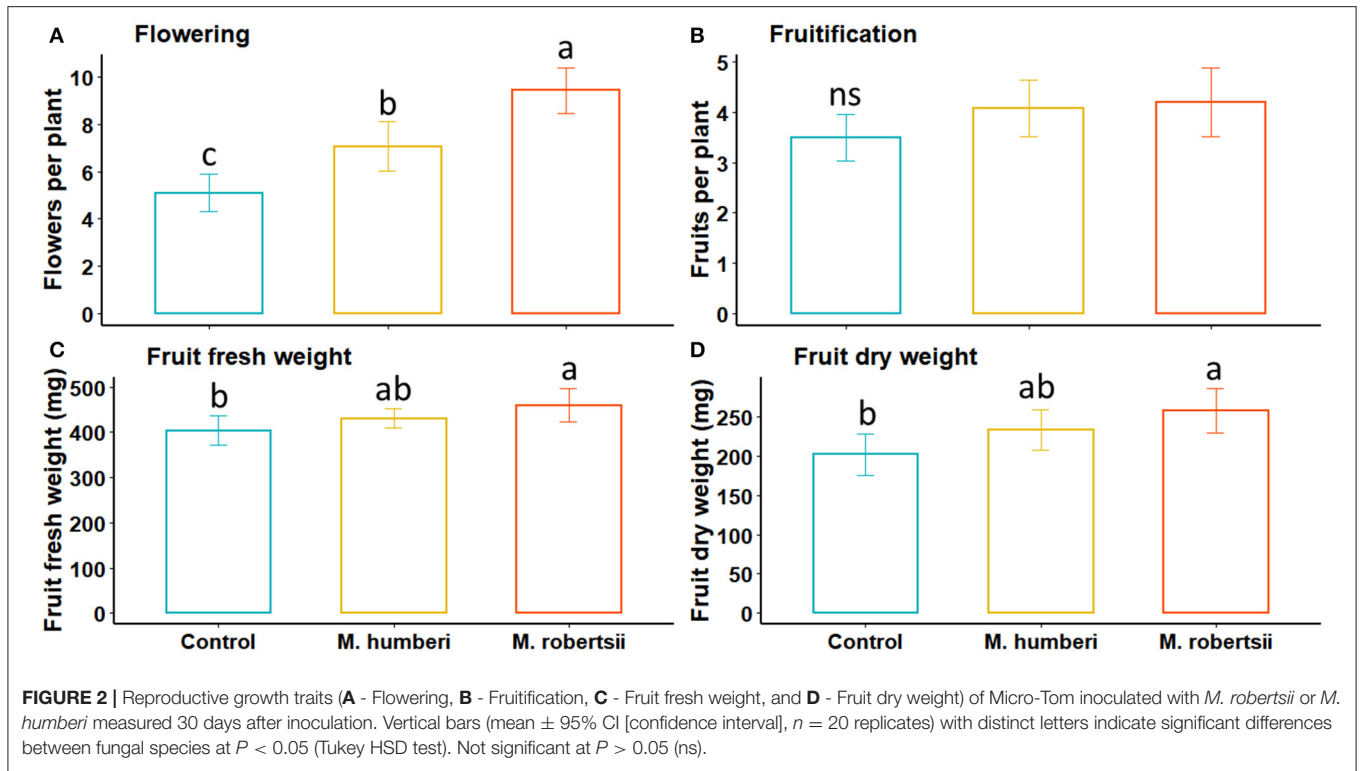


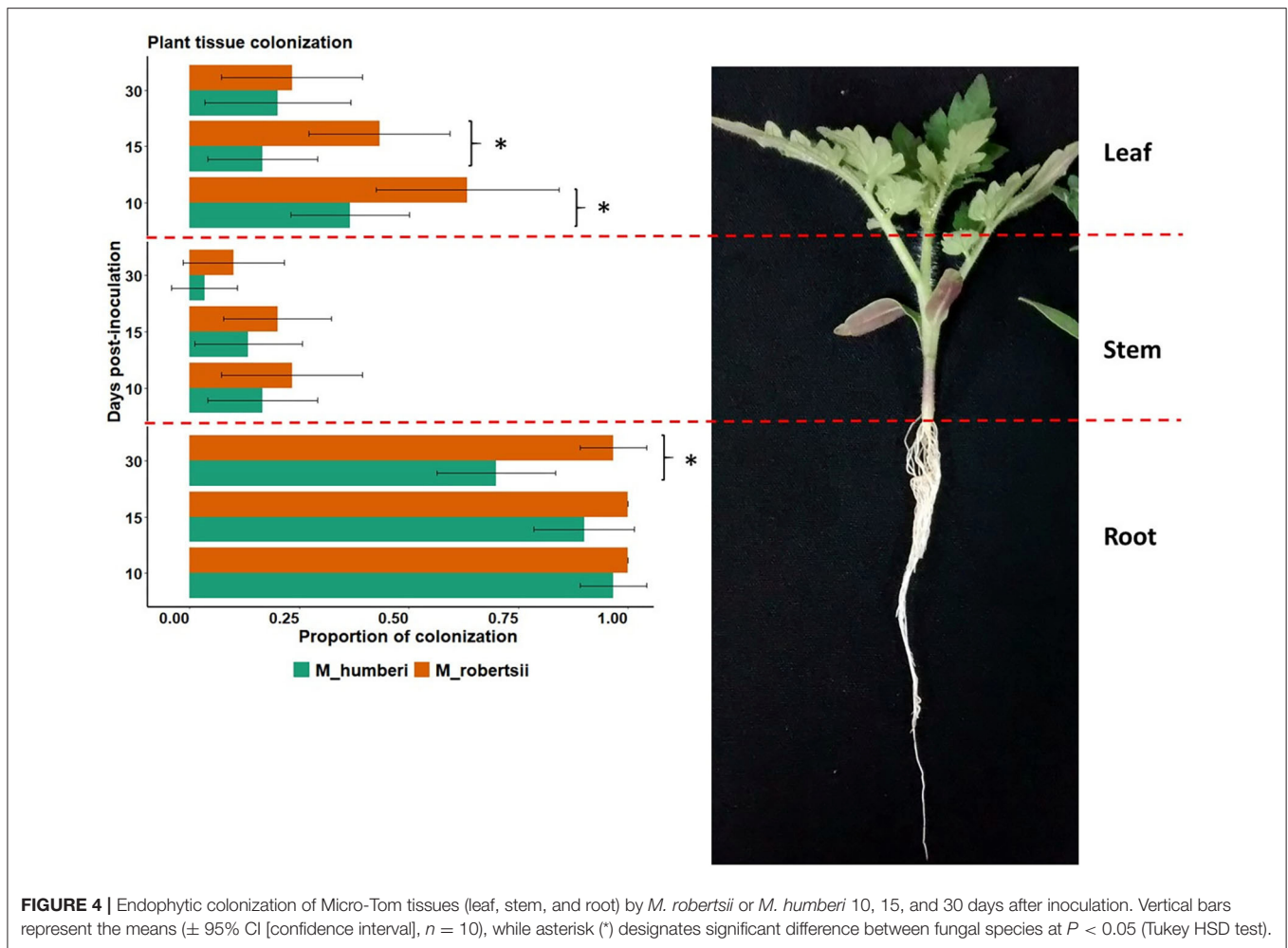
dry matter by day 30 ($F = 21.03$, $df = 2, 42$, $P < 0.0001$) (Figure 1D). Similarly, root dry matter was greater after 10, 15, and 30 DAI in treatments with either fungal species than the control plants ($F = 11.44$, $df = 2, 42$, $P = 0.00011$) (Figure 1D). In all evaluations, the plants inoculated with *M. robertsii* (ESALQ 1635) had more root dry weight than those inoculated with *M. humberi* (ESALQ 1638) (Figure 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 (Figure 2A). However, the number of fruits per plant was not significantly affected by the treatments ($\chi^2 = 2.95$, $df = 2$, $P = 0.23$) (Figure 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 (Figures 2C,D).

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) (Figure 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 aerial part 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.





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. humberii* (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 (Figure 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. humberii* (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. humberii* (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. humberii* (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.

Determination of *in vitro* IAA Production

M. robertsii (ESALQ 1635) and *M. humberii* (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.*

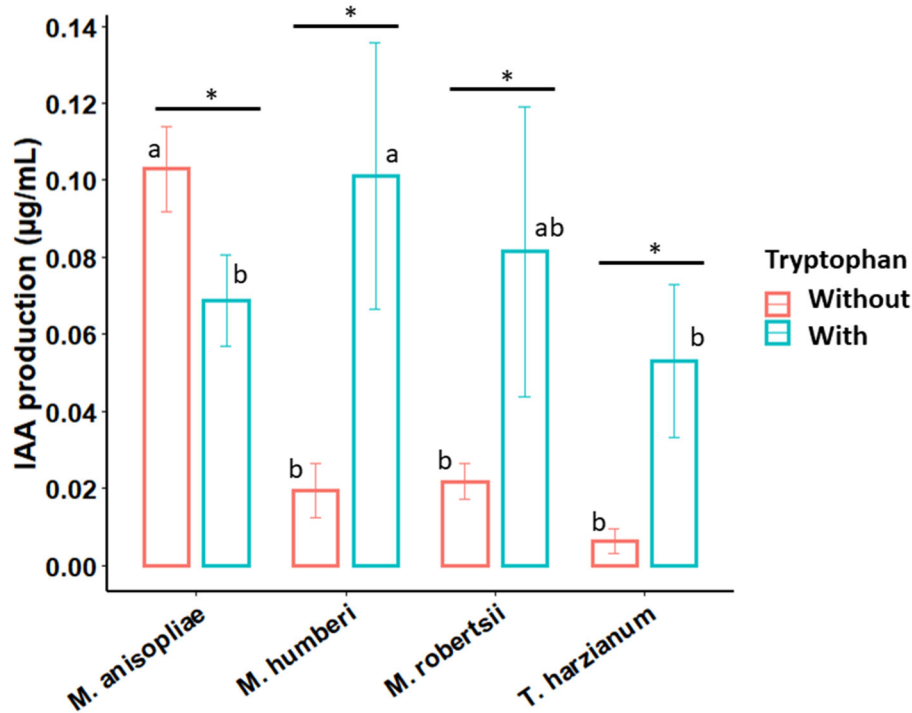


FIGURE 5 | Effect of phenotype and exogenous tryptophan on the ability of *Metarhizium* species to produce indole-3-acetic acid (IAA) under *in vitro* conditions. *Trichoderma harzianum* was used as a positive control for IAA production. Bars (means \pm 95% CIs [confidence intervals]) followed by distinct 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 $P < 0.05$ ($n = 9$).

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 (Figure 5). Thus, the presence of tryptophan was essential for achieving higher IAA synthesis by *M. humberii* (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. humberii* (ESALQ 1638) presented higher production of IAA than *M. anisopliae* (ESALQ 1669) and *T. harzianum* (ESALQ 1306) when cultured with this IAA precursor.

IAA Signaling by *Metarhizium* Inoculation

Compared to the non-inoculated control plants, both *M. robertsii* (ESALQ 1635) and *M. humberii* (ESALQ 1638) increased the detection of auxin-induced GUS expression in the roots at 10 and 30 DAI (Figure 6). The GUS staining was more intense and covered larger areas of the roots infected by *M. robertsii* (ESALQ 1635) than *M. humberii* (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.

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. humberii* (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) (Figure 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. humberii* (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 (Figure 7B).

In vitro production of siderophore was significantly different among the *Metarhizium* species ($F = 427.69$, $df = 2, 40$, $P < 2.2e-16$) (Figure 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

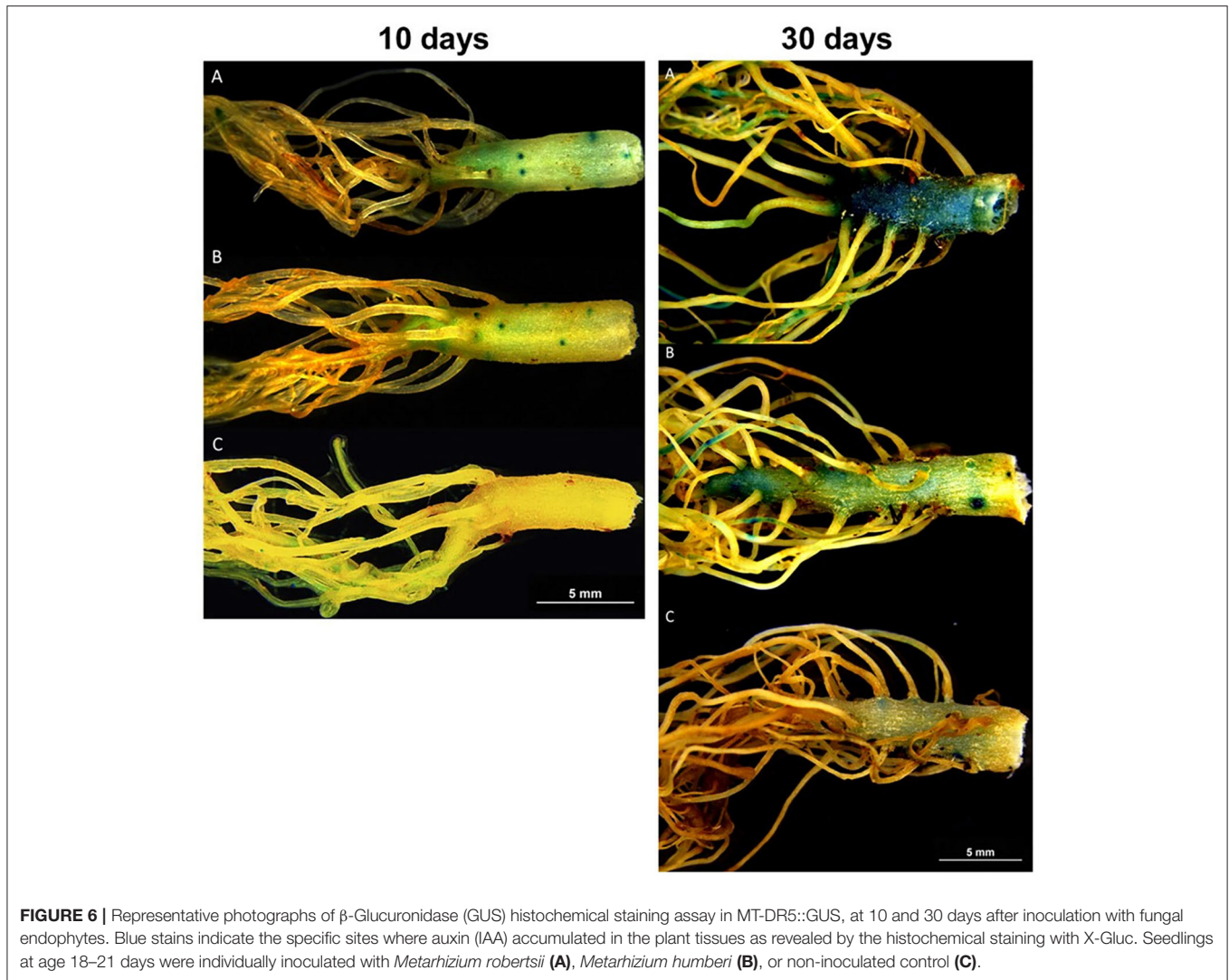


FIGURE 6 | Representative photographs of β -Glucuronidase (GUS) histochemical staining assay in MT-DR5::GUS, at 10 and 30 days after inoculation with fungal endophytes. Blue stains indicate the specific sites where auxin (IAA) accumulated in the plant tissues as revealed by the histochemical staining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

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 (Figure 7C).

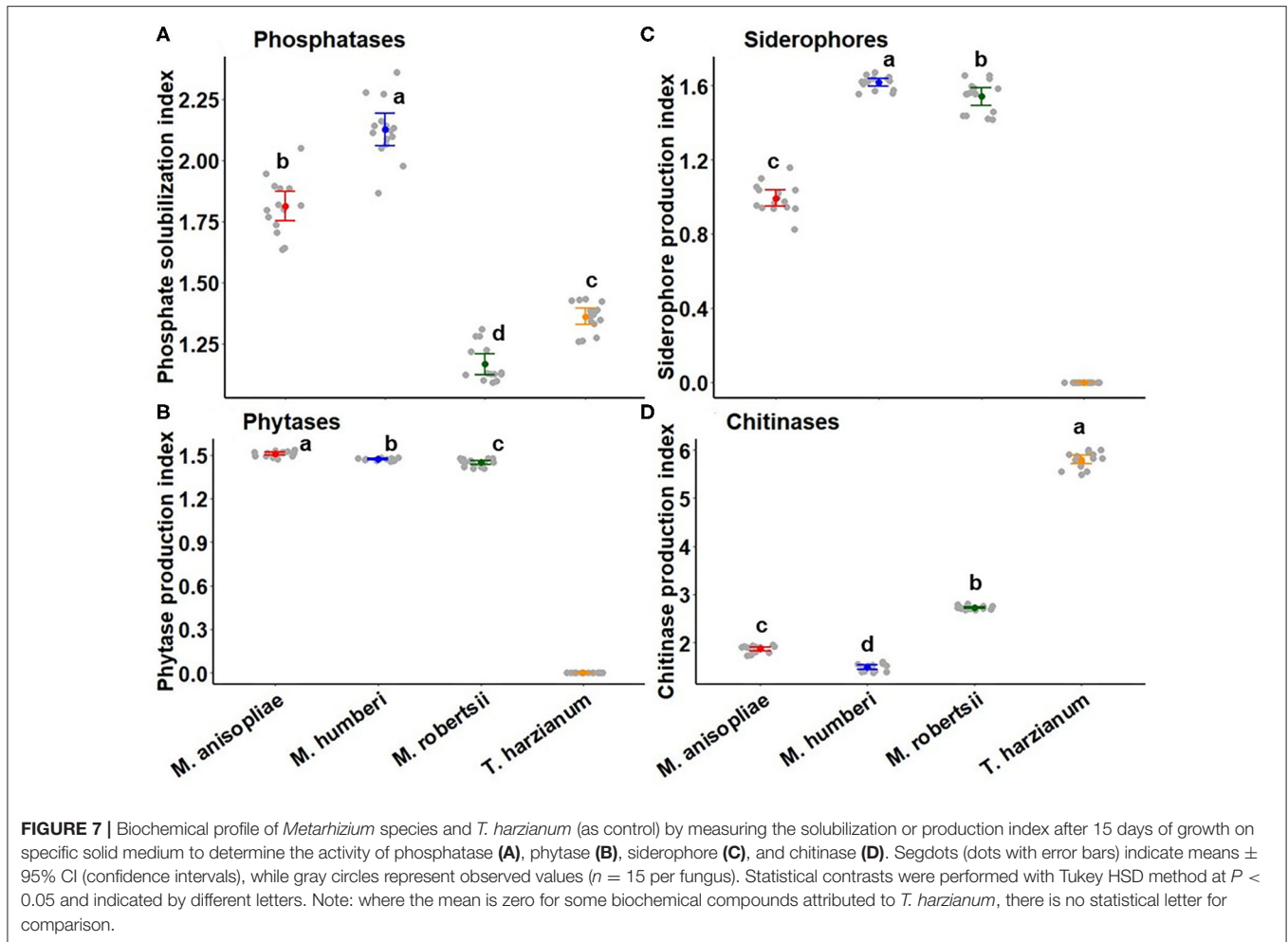
The ability to degrade colloidal chitin by chitinase recorded after 15 days of incubation was significantly affected by fungal species ($F = 7315.5$, $df = 3, 54$, $P < 0.0001$) (Figure 7D). *T. harzianum* (ESALQ 1306) exhibited higher chitinase activity than all three *Metarhizium* species. The second 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 (Figure 7D).

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 (Figure 8).

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 aerial part and roots compared to the non-inoculated plants. This observation agrees with similar studies using *M. anisopliae* (García et al., 2011).

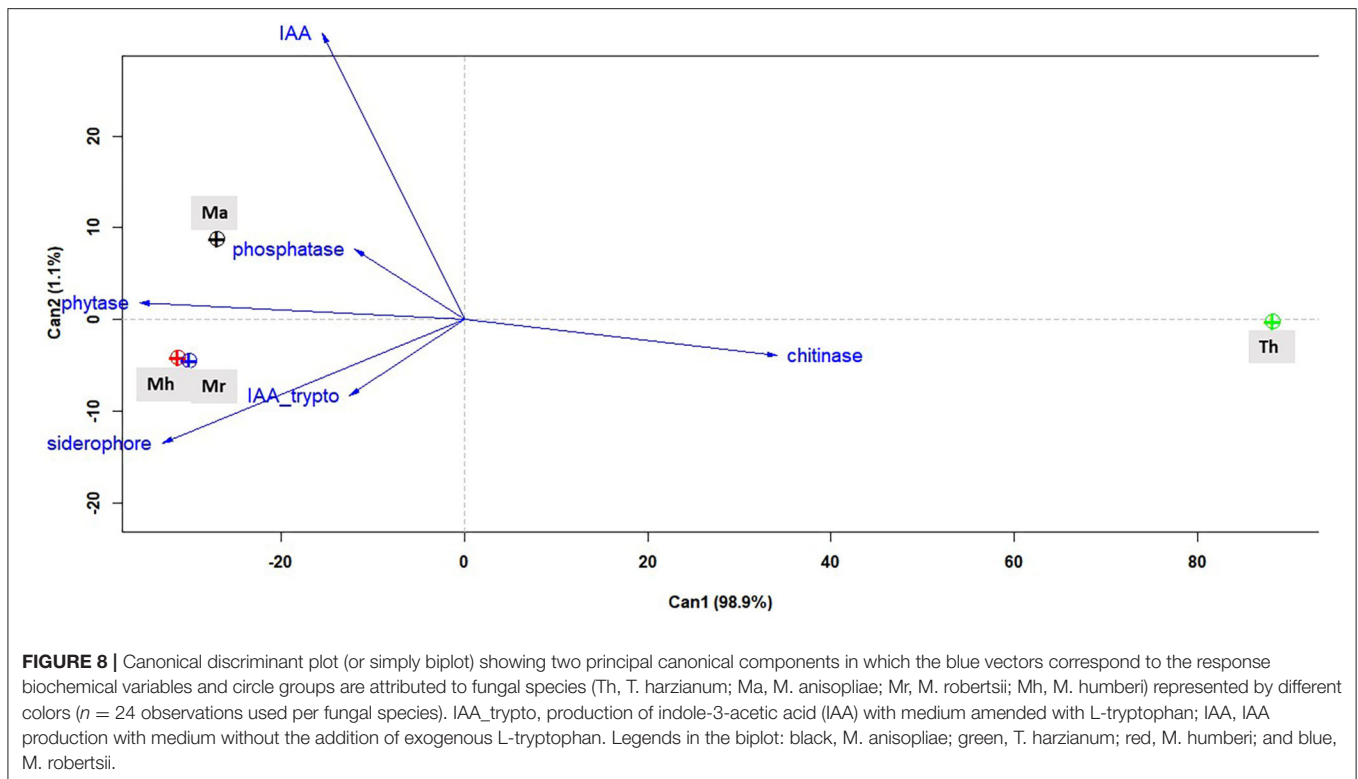


Both *M. robertsii* (ESALQ 1635) and *M. humberii* (ESALQ 1638) were able to endophytically colonize “Micro-Tom” tissues of roots, leaves, and stems at least 30 DAI. Endophytic fungi notably display preferential tissue colonization, and this preference is commonly attributed to fungal species and isolate, 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 (Harman et al., 2004; Behie and Bidochka, 2014). 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. humberii* (ESALQ 1638) in promoting tomato growth. It is interesting to note that *M. humberii* (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. humberii* (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 (Spaepen and Vanderleyden, 2011; Rana et al., 2019). 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 its plant growth stimulation. All three *Metarhizium* species tested here appeared to produce IAA *in vitro* 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). 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. humberti* (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; Jha 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. humberti* (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. humberti* (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 complementary 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.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AS, CG, and ÍD conceived the idea and designed the experiments. AS, CG, and JM performed the experiments and acquired data. GM analyzed the data. AS, GM, and ÍD 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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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.

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