



Production of Microsclerotia From Entomopathogenic Fungi and Use in Maize Seed Coating as Delivery for Biocontrol Against *Fusarium graminearum*

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The commercial use of the entomopathogenic fungi *Metarhizium* spp. in biopesticides has gained more interest since the discovery that several species of this genus are able to colonize roots. In general, commercial products with *Metarhizium* are formulated based on conidia for insect pest control. The process of mass production, harvesting, and formulation of infective conidia can be detrimental for conidial viability. Entomopathogenic fungi such as *Metarhizium* spp. are able to produce high concentrations of resistant structures, known as microsclerotia, when grown in liquid media. Microsclerotia are desiccation tolerant, with excellent storage stability, and are capable of producing high quantities of infective conidia after rehydration. The aim of this study was to evaluate microsclerotia production by different isolates of *Metarhizium* spp. and determine the effect of microsclerotia coated onto maize seeds on plant growth in the presence of soil-borne pathogen *Fusarium graminearum*. On average, $\sim 1 \times 10^5$ microsclerotia/mL were produced by selected isolates of *M. anisopliae* (A1080 and F672) and *Metarhizium robertsii* (F447). Microsclerotia were formulated as granules with diatomaceous earth and used for seed coating, after which propagules produced around 5×10^6 CFU/g of seeds. In the presence of the plant pathogen, maize plants grown from untreated seeds had the lowest growth, while plants treated with the *Metarhizium* microsclerotia had significantly greater growth than the control plants. Hyphae were observed growing on and in root tissues in all the *Metarhizium* spp. treatments but not in samples from control plants. *Metarhizium* hyphal penetration points' on roots were observed 1 month after sowing, indicating the fungi were colonizing roots as endophytes. The results obtained indicate that microsclerotia can be coated onto seeds, providing plant protection against soil plant pathogens and a method to establish *Metarhizium* in the ecto- and endo-rhizosphere of maize roots, allowing the persistence of this biocontrol agent.

Keywords: plant growth promotion, microsclerotia, plant protection, *Metarhizium* spp, *Fusarium graminearum*, seed coating, endophytic entomopathogenic fungi, biocontrol

INTRODUCTION

The commercial use of entomopathogenic fungi such as *Metarhizium* (Metschnikoff) Sorokin, 1883 (*Hypocreales: Clavicipitaceae*) or *Beauveria* (Balsamo) Vuillemin, 1912 (*Hypocreales: Clavicipitaceae*) as “mycoinsecticides” or “biopesticides” is generally practiced using the inundative biocontrol approach, where the environment harboring the insect pest is treated with high concentrations of infective fungal propagules (Eilenberg et al., 2001; Jackson et al., 2010). Generally, fungal antagonists of plant pathogens or fungal biopesticides for biocontrol purposes are produced using solid substrate fermentations on grains which are subsequently formulated as powders or liquids for topical applications or as granules for soil applications (Jackson et al., 2010; Tripathi et al., 2010; Jaronski, 2014). However, the different steps involved in mass production of infective conidia as well as during harvesting and formulation can be detrimental for conidia viability and survival. Additionally, proper application with standard farming equipment and homogenous distribution through the soil to target phytopathogens or soil dwelling insect pests, can be difficult to achieve (Jaronski and Jackson, 2008). Granular formulations are a practical alternative to agrochemicals for the application of fungal antagonists for plant protection targeting soil plant pathogens and soil-dwelling insect pests (Burgess, 1998; Jaronski and Jackson, 2008).

The development of mycoinsecticides as conidiogenic granules is preferred since fresh conidia are produced *in situ* where the target plant pathogens or insect pests dwell (Jackson and Jaronski, 2012). Using this propagule alternative, conidia avoid all the necessary steps from mass-production up to application, which can damage these fungal reproductive structures. When incorporated into soil, biocontrol agents formulated as conidiogenic granules have some additional advantages such as protection from the harmful effects of UV radiation or from unfavorable conditions such as high temperatures and low humidity to which microorganisms can be exposed after foliar applications. Another benefit is that in soil, moisture content and temperatures are generally within the optimal range for fungal survival and growth (Jackson and Jaronski, 2009).

Over the past decade, it has been discovered that entomopathogenic fungi belonging to *M. anisopliae*, *M. brunneum*, *Beauveria bassiana*, *B. brongniartii*, and *B. pseudobassiana* are able to produce high concentrations of microsclerotia (MS) when grown in liquid media (Jackson and Jaronski, 2009, 2012; Vega et al., 2009; Wang et al., 2011; Villamizar et al., 2018). These environmentally resistant structures are desiccation tolerant, have excellent storage stability, and are capable of producing high quantities of infective conidia suitable for management insect pests or as antagonists of phytopathogens (Jaronski and Jackson, 2008; Jackson and Jaronski, 2009; Vega et al., 2009; Song, 2018). Microsclerotia from *M. anisopliae* can be produced by culturing in liquid substrate fermentation for ~3 to 4 days in continuous agitation, while further fermentation up to 8 days allows the complete MS melanization. Subsequently, obtained MS are

harvested and formulated in diatomaceous earth (DE) and air-dried to produce MS-DE granules (Jackson and Jaronski, 2009). MS-DE granules can survive a moderate drying process without significant viability losses and can be safely stored at 4°C for up to 12 months. In appropriate conditions, MS germinate (hyphal development) and produce high quantities of conidia, in general above 1×10^8 conidia/g dried MS-DE (Jackson and Jaronski, 2009, 2012). As for conidiogenic granules, the soil environment is suitable for reactivation of MS, since humid conditions are favorable for the rehydration and germination process of these fungal resting structures. Consequently, MS obtained from entomopathogenic fungi have been recognized as a viable alternative for use in biocontrol programmes (Jaronski and Jackson, 2008).

Another interesting feature of some entomopathogenic fungi like *Metarhizium* and *Beauveria* is their natural ability to associate endophytically with plant roots and to colonize the rhizosphere (Hu and St. Leger, 2002; Vega et al., 2009; Rivas-Franco et al., 2019). This property not only allows entomopathogenic fungi to persist in the root system, but also fulfill additional ecological roles such as antagonism of plant pathogens, plant growth promotion, biofertilization, and promotion of the plant induced resistance (Goettel et al., 2008; Vega et al., 2009; Sasan and Bidochka, 2012; Rivas-Franco et al., 2020). In order to make use of these advantages, an alternative to MS formulated as granules (0.6–1.2 mm) and incorporated into soils at planting is to formulate the MS directly as a seed coating. Therefore, after sowing, MS coated onto seeds reactivated by soil humidity produce hyphae in a process known as MS germination. When nutrients in microsclerotia are scarce, hyphae develop phialides which, in turn, produce fresh conidia. These conidia are released in proximity to the nutrient-rich environment of the rhizosphere, where conidia soon germinate, and produce hyphae. Finally, either hyphae from microsclerotia or from conidia will interact and associate with the growing roots. Entomopathogenic fungal colonization of the rhizosphere allows fungal persistence, exerting biocontrol activity against the pests or plant pathogens threatening plant roots (Rivas-Franco et al., 2019).

Previous studies with seed treatments with different species of *Metarhizium*, resulted in 50% of larvae of *Anomala cincta* (*Coleoptera: Rutelidae*) or up to 60% of *Costelytra giveni* larvae (*Coleoptera: Scarabaeidae*) infected with the entomopathogenic fungi (Peña-Peña et al., 2015; Rivas-Franco et al., 2019). However, the biocontrol ability of *Metarhizium* is not limited only to insects but also against plant pathogens like *Fusarium*. Several studies have shown that *Metarhizium* spp. can reduce *Fusarium* root rot symptoms after seeds were coated with conidia obtained from this biocontrol agents (Sasan and Bidochka, 2013; Rivas-Franco et al., 2019). Additionally, maize roots provide support for *Metarhizium* rhizosphere colonization as well as increasing fungal persistence in the root system through endophytic growth (Keyser et al., 2014; Peña-Peña et al., 2015; Rivas-Franco et al., 2020). The ability of *Metarhizium* to colonize the rhizosphere and plant roots endophytically supports the bodyguard hypothesis (Elliot et al., 2000) where the host plant plays an important role driving this interaction in the presence of insect pests or phytopathogens threatening roots (Rivas-Franco et al., 2020).

Previously, we found that several species of *Metarhizium* coated on maize seeds showed a significant decrease in their ability to colonize the rhizosphere in the presence of larvae of *C. giveni*, while endophytic colonization by *Metarhizium* increased in the presence of the pathogen *Fusarium graminearum* (Rivas-Franco et al., 2020). In those studies, root colonization by *Metarhizium* spp. altered maize physiology modifying the content of the phytohormones salicylic acid and jasmonic acid, possibly leading to induction of the systemic response in maize (Rivas-Franco et al., 2020).

Numerous species of the genus *Fusarium* are well-known plant pathogens affecting yield, nutritive value and hygienic quality of agricultural crops in most countries from Africa, Asia, Europe, North America, Oceania, and South America (van der Lee et al., 2015; Oldenburg et al., 2017; Kazan and Gardiner, 2018). In particular, the *Fusarium graminearum* Schwabe species complex cause *Fusarium* head blight in several plant cereals leading to yield losses above 50% in some cases and compromising grain quality (van der Lee et al., 2015; Garmendia et al., 2018). Infections of maize with *Fusarium* spp. can cause other serious plant diseases such as seedling blight, seed rot, root and stem rot, ear and kernel rot, and rudimentary ear rot (Oldenburg et al., 2017; Zhou et al., 2018). *Fusarium graminearum* also produces a class of mycotoxins known as trichothecenes (deoxynivalenol, nivalenol, and their derivatives) and zearalenone, with acute toxicity for animals and humans (van der Lee et al., 2015; Kazan and Gardiner, 2018; Zhou et al., 2018). Maize plants are susceptible to *Fusarium* spp. infections throughout the cultivation period and mycotoxins can be accumulated in the affected tissues and grains, which represents a risk for human and animal health (Oldenburg et al., 2017; Kazan and Gardiner, 2018). Management of *F. graminearum* can be difficult because standard seed fungicide treatments are not always sufficient, while aerial applications are costly. Additional difficulties are associated with correct application timing, environmental risk and the development of *Fusarium* resistance to fungicides (Kazan and Gardiner, 2018; Zhou et al., 2018).

Seed coating with conidia from entomopathogenic fungi has proved to be a strong tool for the delivery of these biocontrol agents, promoting fungal persistence in the system after entomopathogenic fungal colonization of the rhizosphere or even roots endophytically (Rivas-Franco et al., 2019). The aim of this study was to evaluate MS production by different isolates of entomopathogenic fungi and determine the effects of MS coated onto maize seeds on plant growth performance in the presence of *F. graminearum*. The ability of the entomopathogenic fungal isolates, coated on seeds as MS, to associate with roots was determined through fluorescent and laser confocal microscopy.

MATERIALS AND METHODS

Plants

Maize seeds (*Zea mays*) of the hybrid 34H31 (US patent 6,897,360 B1) were used. This hybrid is characterized by a stable yield performance in different environments, early flowering and good drought tolerance.

Fungal Isolates

The isolates selected for this study (Table 1) are all held in the fungal collections of the Bio-Protection Research Center and AgResearch (both at Lincoln, New Zealand) and the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF-USDA). The plant pathogen *F. graminearum* 13083 was obtained from a maize root rot sample and held in the Manaaki Whenua Landcare Research culture collection (Auckland, New Zealand). For liquid culture fermentation studies, monosporic cultures of each fungal isolate were used. Monosporic cultures were obtained by transferring a sole conidium from each fungal isolate to a Petri plate containing potato dextrose agar (PDA, Difco) and incubating at $25 \pm 2^\circ\text{C}$ in light:dark conditions (12:12). After 3 weeks, a conidial suspension was prepared for each fungal isolate from the monosporic fungal colony grown on PDA. The resulting monosporic conidial suspension was aliquoted in tubes with 10% glycerol and stored at -80°C (stock cultures) until use. Conidial inoculants for liquid culture experiments were obtained by inoculating PDA Petri plates with 100 μL from fungal stock cultures. Petri plates were then incubated at $25 \pm 2^\circ\text{C}$ in light:dark conditions (12:12) for 2 weeks until complete sporulation of colonies. Subsequently, a 250 mL flask containing 90 mL of liquid medium was inoculated with 10 mL of a conidial suspension of 5×10^6 conidia/mL. All conidial suspensions were obtained by rinsing the fungal colony grown on a PDA plate with 3–5 mL of 0.01% Triton X-100 and transferring the resulting conidial suspension to a 15 mL Falcon tube. Conidial suspensions were quantified using a haemocytometer (Neubauer Improved).

Fungal Growth and Microsclerotia Production in Liquid Substrate Fermentation

Media Composition and Fermentation Conditions

Media composition and fermentation conditions were as described by Jackson and Jaronski (2009, 2012) with slight modifications in carbon and nitrogen content. The liquid media used to produce hyphal inoculum and MS from the fungal isolates were composed of a basal salts solution with trace metals and vitamins (Jackson and Jaronski, 2009). The pre-culture medium [C:N ratio 33:1; (C) = 40 g/L] used for producing the hyphal inoculum of fungal isolates contained the basal medium supplemented with 80 g/L glucose and 15 g/L acid hydrolysed casein (Casamino acids, Difco). Production medium for MS contained the same composition but with 25 g/L of hydrolysed casein [C:N ratio 23:1, (C) = 45 g/L]. Carbon concentration and C:N ratio calculations were based on 40% carbon in glucose and 53% carbon and 8% nitrogen in acid hydrolysed casein (Jackson and Jaronski, 2009). Glucose solutions (20% w/v) were autoclaved separately and added to the basal salts solution with trace metals and vitamins prior to inoculation. Pre-cultures were obtained by inoculating 90 mL pre-culture medium in baffled Erlenmeyer flasks (250 mL) with 10 mL conidial suspension of 5×10^6 conidia/mL. Production cultures were obtained by inoculating 90 mL of production medium in baffled Erlenmeyer flasks (250 mL) with 10 mL of 4 day-old pre-culture broth.

TABLE 1 | Voucher information for the specimens used in this study.

Isolate	Species	Origin	Source	Location	Collection ^a
A1080	<i>Metarhizium anisopliae</i>	Insect larvae	<i>Trichoplusia ni</i> (Lep: Noctuidae)	Florida, USA	ARSEF
Bb21	<i>Beauveria bassiana</i>	Endophyte from leaf husk	<i>Zea mays</i> (Poa: Poaceae)	Ashburton, Canterbury, NZ	BPRC
F16	<i>Metarhizium guizhouense</i>	Insect cadaver	<i>Costelytra giveni</i> (Col: Scarabaeidae)	Nelson, Marlborough, NZ	AgR
F327	<i>Trichoderma harzianum</i>	Endophyte from roots	<i>Mentha</i> spp. (Lam: Nepetoideae)	Waitakaruru, Waikato, NZ	BPRC
F447	<i>Metarhizium robertsii</i>	Endophyte from roots	<i>Actinidia deliciosa</i> (Eri: Actinidiaceae)	Avondale, Auckland, NZ	BPRC
F672	<i>Metarhizium anisopliae</i>	Endophyte from root	<i>Pinus radiata</i> (Pin: Pinaceae)	Taupo, Waikato, NZ	AgR
F99	<i>Metarhizium novozealandicum</i>	Insect cadaver from farm soil	<i>Costelytra giveni</i> (Col: Scarabaeidae)	Methven, Canterbury, NZ	AgR
BK41	<i>Metarhizium guizhouense</i>	Endophyte from leaves	<i>Actinidia deliciosa</i> (Eri: Actinidiaceae)	Nelson, Marlborough, NZ	BPRC
13083	<i>Fusarium graminearum</i>	Root rot	<i>Zea mays</i> (Poa: Poaceae)	Pukekohe, Auckland, NZ	ICMP

^aARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; BPRC, Bio-Protection Research Center Fungal Collection, Lincoln, Canterbury; AgR, AgResearch Fungal Collection, Lincoln, Canterbury; ICMP, International Collection of Microorganisms from Plants, Manaaki Whenua Landcare Research, Lincoln Canterbury.

Production cultures were grown for 8 days at 28°C in an orbital shaker incubator (Cocono TU 4540) at 300 RPM. Total fermentation time for pre-cultures plus production cultures was 12 days. All pre-cultures and production cultures were inoculated in duplicate. All experiments were carried out at least three times.

Quantification Methods and Sample Processing During Liquid Fermentations

Samples for the determination of biomass, blastospore and MS were taken after inoculation for pre-cultures (days 3 and 4) and for production cultures (days 3, 4, 6, 7, and 8). At each sampling day, 5 mL replicates of culture broth were taken from each isolate and processed for quantification of biomass, blastospore, and MS content determination. Biomass was determined as dry weight after incubating 1 mL of culture broth in a 1.5 mL Eppendorf tube for 96 h at 65°C. Before drying biomass, the medium culture was removed by two consecutive washes with 1 ml of 0.01% Triton X-100 solution. Blastospore concentration was determined by transferring 1 mL of culture broth to a tube containing 9 mL of distilled water and quantifying microscopically using a haemocytometer (Neubauer Improved). MS production was determined using 70 µL of sample placed onto a glass slide and covered with a large (50 × 24 mm) coverslip. All MS on the slide were counted. Only discrete hyphal aggregates larger than 50 µm in diameter and melanized were counted as MS (Jaronski and Jackson, 2012). All quantifications were done in duplicate. Culture broth was diluted as appropriate for quantifications and all broth suspensions were vortexed to ensure homogeneity before quantification. Microscopic analysis was done using a Leica DM 2500 microscope and images were taken with the Celsens Standard software (Olympus). Quantifications (biomass, blastospores, and MS) from each sampling day and fungal isolate were averaged. Data were analyzed by a two-way analysis of variance (ANOVA).

Microsclerotia Production for Characterization and Seed Coating

Microsclerotia for maize seed coating were produced following the procedure indicated above using the isolates of *M. anisopliae* A1080 and F672 and *M. robertsii* F447. These isolates exhibited

highest MS production among remaining *Metarhizium* isolates and were selected for the following studies. The pre-cultures for biomass and blastospore production were evaluated only on day 4 after inoculation, and production cultures were evaluated for biomass and MS production on harvest at day 6 after inoculation. MS production cultures were completed using two replicates per isolate. Experiments were repeated three times. Biomass and MS quantification from each fungal isolate at sampling day were averaged for data analysis.

Microsclerotia Harvest and Drying Process

After six fermentation days, total fungal biomass volume including MS was determined and 5% (W/V) of diatomaceous earth (DE, Hyflo Sigma-Aldrich) was added. The combined MS-DE were mixed, and vacuum filtered with a Büchner funnel using Whatman N°54 filter paper. The resulting filtered MS-DE solid block was broken up with a coffee blender by applying short pulses until small crumbs were obtained. The resulting crumbs were layered on glass Petri plates of 15 cm diameter and air-dried overnight in the air flow within a laminar-flow. The moisture content of the MS-DE granules was determined with an activity meter (Aqualab Lite V4). The granules were dried until the water content was between 3–5 % and then sealed in plastic bags and stored at 4°C until further processing. Moisture content (a_w) determination from the MS of each fungal isolate was completed in duplicates. Each experiment was repeated three times. Data were analyzed by a two-way ANOVA.

Viability and Conidiogenesis of MS-DE Granules

Microsclerotial viability and conidial production from MS were determined by sprinkling 25 mg of the air-dried MS-DE granules onto the surface of a water agar plate (Jackson and Jaronski, 2009). Two water agar plates were used for each of the MS-DE granules obtained from the corresponding *Metarhizium* isolate. After 24 hrs incubation at 28°C in light:dark conditions (12:12 h), a total of 100 MS-DE granules on each plate were examined with a stereo microscope (Olympus SZX 12) to determinate hyphal emergence (MS germination) as an indicator of viability. Conidial production was determined after further incubation of MS-DE granules on water agar plates in the same conditions for 8 days. Then, MS-DE water agar plates were

flooded with 7 mL of a sterile 0.01% Triton X-100 and agitated at 60 RPM in a rotary shaker platform for 30 min at room temperature. After agitation, granules were dislodged from agar with the help of a hockey stick cell spreader and the resulting suspension containing granules and conidia was transferred to a 15 mL Falcon tube, and the recovered volume was recorded. The concentration of conidia was determined microscopically using a haemocytometer (Neubauer Improved) and total conidia per gram of MS was calculated from duplicate samples. Experiments were repeated three times. Data were analyzed by a two-way ANOVA.

Maize Seed Coating With Microsclerotia

After MS characterization, isolates *M. anisopliae* F672 and *M. robertsii* F447 were selected for maize seed coating based on their high MS production. Seed coating was prepared as described in Rivas-Franco et al. (2019) with slight modifications as follows. The conidial suspension in the polymer gel was substituted by an amount of MS-DE estimated to provide, after MS germination and conidiation, a final conidial concentration of $\sim 1 \times 10^8$ conidia/mL per gram of seeds. The corresponding quantity of MS-DE was added to the polymer gel, mixed and then used for coating maize seeds with the same proportions of polymer with MS-DE, bentonite and talc as previously described (Rivas-Franco et al., 2019). Control seeds (CS) were coated with the polymer and the other corresponding ingredients without the microsclerotia. Germination of maize seeds with a MS-DE coating was determined using the between-paper method as described in Rivas-Franco et al. (2019). In this evaluation test four replicates, each consisting of 50 maize seeds with MS-DE coating or CS, were used. Experiments were repeated twice. Statistical design was a randomized block design with two blocks of the MS fungal seed coatings treatments. Data were analyzed with a one-way ANOVA.

MS-DE Coated Maize Seeds Propagule Viability

After maize seeds were coated with *Metarhizium* MS-DE granules, 10 g of coated seeds were placed in a Falcon centrifuge tube (50 mL) containing 30 mL of a solution of Triton x-100 (0.01%). The resulting mix composed of seeds and solution was agitated vigorously and incubated at 4°C overnight (mother suspension). After incubation, the mix was agitated vigorously and serial dilutions from mother suspension were prepared. From each dilution 0.1 mL was spread onto Petri plates containing half strength acidified PDA. To prepare half strength acidified PDA, two drops of Lactic acid were added to the sterilized medium before pouring in the Petri plates. This simple strategy of medium acidification reduces bacterial contamination with no effects on fungal development. Inoculated plates were incubated at 20°C with 12:12 h light:dark conditions. After 7 days, fungal colonies originated from MS were quantified as colony forming units (CFU). Data were analyzed by a one-way ANOVA.

Evaluation of Maize Plant Performance in the Presence of *Fusarium graminearum* After Seeds Were Coated With Microsclerotia of *Metarhizium* spp

Maize seeds coated with MS from *M. anisopliae* F672 and *M. robertsii* F447 were sown in 1 L pots containing 920 g of potting-mix with 0.5% (w/w) of *F. graminearum* 13083. Control seeds without MS treatment were grown in the absence (CS) or the presence of *F. graminearum* (CSfg). Plants were allowed to grow in the Biotron (controlled environment facility, Lincoln University), at $24.3 \pm 2.5^\circ\text{C}$, (referred as 25°C) in light:dark conditions (16:8 h), 65 ± 10 RH% and watered every fourth day with 300 mL of tap water. Three weeks after sowing, plants were harvested and dry weight of shoots and roots determined (Rivas-Franco et al., 2019). Each maize plant trial consisted of two randomized blocks with five pots in each block with the corresponding MS seed coating treatments (*M. anisopliae* F672 and *M. robertsii* F447) and CS. All pots contained the fungal pathogen *F. graminearum* 13083 incorporated in the soil potting mix. Additionally, there were two randomized blocks, also with five pots in each block, with the corresponding CS without the fungal pathogen in the potting mix. Experiments were repeated four times. Data were analyzed by a two-way ANOVA.

Fusarium Graminearum 13083 Biomass Production for Maize Trials With MS-DE Coated Seeds

Fusarium graminearum isolate 13083 was maintained in slant tubes on PDA at 4°C and grown on PDA plates at 25°C with 12:12 h light:dark conditions for experiments. For maize trials, *F. graminearum* 13083 biomass was obtained as follows. From the edge of a *Fusarium* colony grew in PDA for 14 days, 10 plugs of ~ 0.5 cm diameter were taken and transferred to 1 L Erlenmeyer flasks containing 500 mL of malt extract broth (MEB). Flask were incubated in an orbital shaker at 180 rpm and $25 \pm 2^\circ\text{C}$. After 7 days, biomass was harvested by centrifugation at 400 rpm for 5 min at 4°C. Following this procedure, up to 69.7 ± 3.4 g/L of *F. graminearum* 13083 biomass containing $\sim 1.3 \pm 0.5$ ($\times 10^8$) blastospores/mL was obtained. Then, the *F. graminearum* 13083 biomass was incorporated with the potting mix at 0.5% w/w and mixed by hand to homogenize. Immediately, the mix was used to fill pots and then CS and MS-DE coated maize seeds were sown. Filled pots were watered and maize plants let to grow for 3 weeks at 25°C before plant performance evaluation.

Determination of Fungal Endophytism by Confocal Fluorescent Microscopy After Maize Seed Coating With *Metarhizium* spp MS-DE Granules

Maize seeds were coated with MS from *M. anisopliae* F672 and A1080, and *M. robertsii* F447 as described above, while for CS the coating consisted of polymer gel without MS. After coating, maize seeds were sown in 1 L pots containing 600 g of vermiculite (fine grade 2). Pots were watered with 400 mL of tap water, and transferred to a growth chamber at 25°C with light:dark

conditions (12:12 h). Plants were watered every fifth day with 400 mL of distilled water. After 1 month, maize plants were gently pulled out, and maize roots, stems and leaf sheath were cleared and fluorescent dyed as described by Rivas-Franco et al. (2019). Maize samples of roots, stems and leaves from seeds coated with MS, and control plants were analyzed for the presence of fungal structures on the surface or internally as endophytes using confocal microscopy (LSM 510 META–Zeiss, Germany). Confocal fluorescence images were recorded on a multichannel confocal microscope (LSM 510 META–Zeiss, Germany) using the program ZEN 2009.

Statistical Analysis

For each individual trial data were analyzed by the ANOVA test that was appropriate for the experimental design. For treatment factors with several levels (e.g., isolates, trial, day), an unrestricted LSD procedure was used to compare means (Saville, 2015). To combine data over several identical trials, an ANOVA test was used, as above, followed by an unrestricted LSD procedure, with data being treatment means from individual trials, and with “trial” being specified as a blocking factor.

RESULTS

Fungal Growth and Microsclerotia Production in Liquid Substrate Fermentation

Biomass Production
All the fungal isolates had produced their highest biomass by day 3 (89.7 ± 16.5 mg/mL) and 4 (87.1 ± 15.1 mg/mL) after inoculation followed by a subsequent steady weight decrease ($F_{28/78} = 5.1$; $p < 0.01$; **Figure 1A**). By day 7, *Trichoderma harzianum* F327 and *B. bassiana* Bb21 had the lowest biomass among the isolates, 38.4 and 42.2 mg/mL, respectively, while *M. anisopliae* F672 and *M. novozealandicum* F99 had the highest 68.4 and 71.7 mg/mL also, respectively ($LSD_{5\%} = 0.075$; **Figure 1A**). At the end of the fermentation process on day 8, only *T. harzianum* F327 and *B. bassiana* Bb21 showed a significant increase in biomass compared to their biomass on day 7 ($LSD_{5\%} = 0.075$; **Figure 1A**).

Blastospore Production

Contrary to the development of fungal biomass, blastospore production of each isolate increased over the fermentation period but with almost no significant variation during consecutive sampling days ($F_{28/78} = 1.2$; $p = 0.288$; **Figure 1B**). Only *T. harzianum* F327 and *M. robertsii* F447 had a significant increase in the number of blastospores per mL by day 6 of the experiment, 1.4×10^7 and 1.9×10^6 blastospores/mL, respectively ($LSD_{5\%} = 0.557$). In contrast, the remaining isolates had the highest number of blastospores per mL by day 7. The highest production of blastospores was by *B. bassiana* Bb21 and *M. guizhouense* Bk41, both with 9.1×10^8 blastospores/mL, while the lowest was found in *T. harzianum* F327 with 9.6×10^6 blastospores/mL ($LSD_{5\%} = 0.557$; **Figure 1B**).

Microsclerotia Production

As well as the trend observed with blastospore production, the amount of MS increased with fermentation time for most of the isolates. The highest concentration, $7.3 \pm 7.9 (\times 10^4)$ MS/mL, was found around the sixth day after inoculation and the level remained stable until the last sampling day ($F_{28/78} = 7.0$; $p < 0.01$). The exception was *M. novozealandicum* F99 which showed little variation in MS content over the 8 days of fermentation ($LSD_{5\%} = 0.498$; **Figure 1C**). This isolate, together with *M. guizhouense* Bk41, produced, on average, the lowest number of MS, $9.9 \pm 4.3 (\times 10^2)$ MS/mL (**Figure 1C**). At the end of the fermentation process, most MS were melanised heterogenous round structures of differing sizes (**Figure 2**). The highest MS production was determined in *M. robertsii* F447 at day 8 with 2.3×10^5 MS/mL followed closely by *B. bassiana* Bb21 with 1.6×10^5 MS/mL ($LSD_{5\%} = 0.498$). The lowest production of MS was found in *M. guizhouense* Bk41 with 684 MS/mL at day 6, while the plant growth promotor *T. harzianum* showed its highest production with 9.7×10^4 MS/mL, also at 6 days after inoculation (**Figure 1C**). Microsclerotia formation during fermentation were first recorded on day 3, undergoing on the following days different developmental stages until complete melanization was achieved (**Figure 2**). At days 3 and 4 after inoculation, MS were still immature as aggregated hyphae were not completely melanized. By day 6, all isolates had slightly melanized compact hyphal structures, conforming to previous descriptions of MS structures. The stages in MS development differed among the isolates in rate of development, size and melanization. For example, MS from *T. harzianum* F327 were relatively small when compared to those from *Metarhizium* isolates. The MS in *M. guizhouense* melanized faster than those in *M. novozealandicum* F99 (data not shown). The longer the fermentation time, the greater melanization of the MS. Complete melanization of MS occurred after 7 days of fermentation culture (**Figure 2**).

Microsclerotia Production for Seed Coating and Characterization

The isolates *M. anisopliae* A1080 and F672, and *M. robertsii* F447 were selected for MS production and incorporation into a coating for maize seeds. These isolates produced the highest concentration of MS among the *Metarhizium* isolates. Interestingly, they were obtained from different sources, *M. anisopliae* A1080 from an insect and the other two isolates from plant samples as endophytes (**Table 1**). Following the procedure for MS production described above, cultures yielded on average around 80 mg/mL of biomass and $\sim 1 \times 10^5$ MS/mL (**Figure 3**).

All MS-DE granules obtained from the three isolates had germinations rates above 90% with no significant differences among isolates ($F_{2/13} = 1.59$; $p = 0.242$; **Table 2**). However, isolate-specific differences were found in the water content of MS-DE after drying. The lowest water content was found in *M. anisopliae* F672 granules (**Table 2**). Conidia production by the different MS-DE granules was always above 1×10^9 conidia/g,

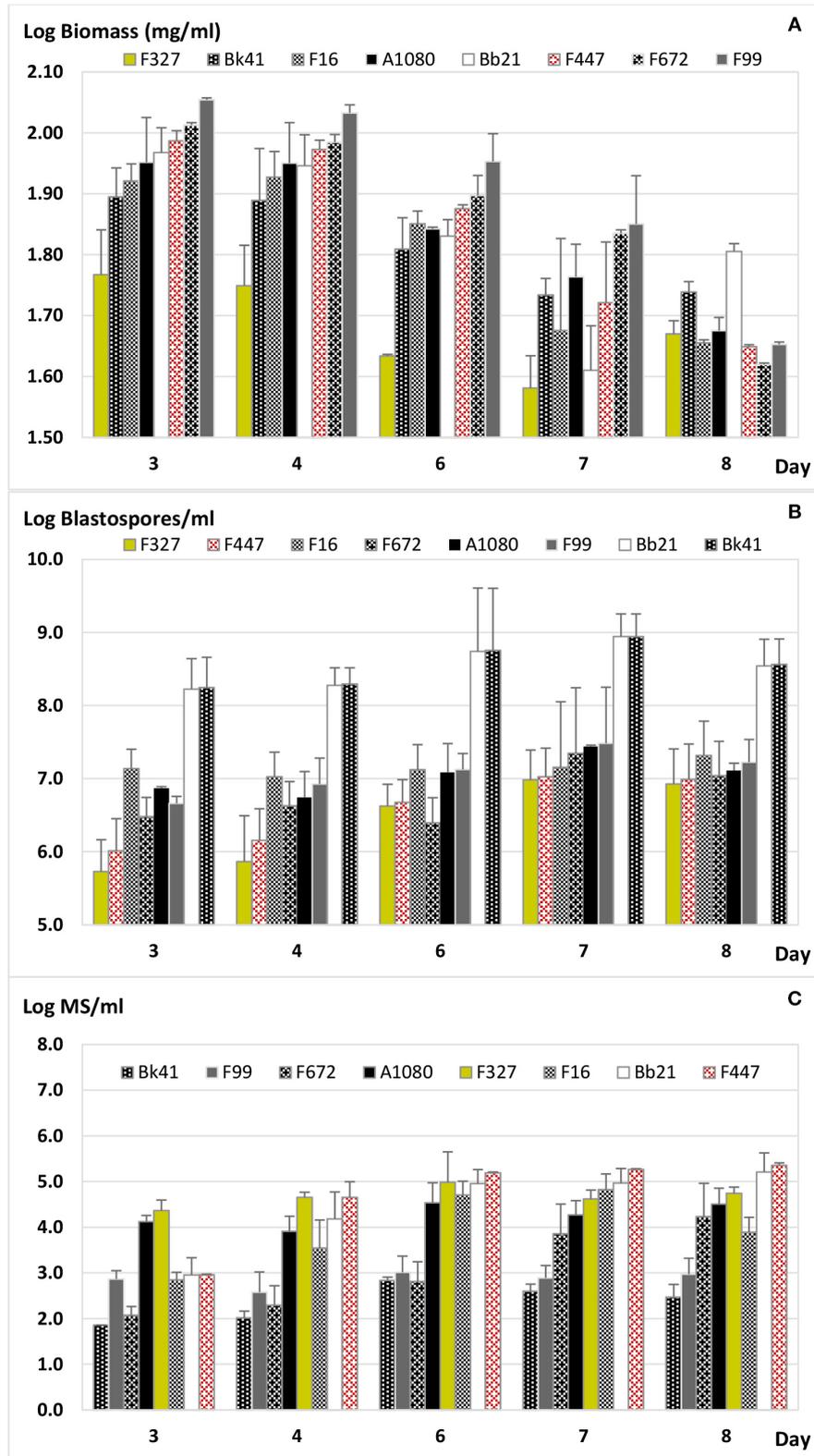


FIGURE 1 | Fungal growth in liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). **(A)** Fungal biomass (dry weight Log₁₀ mg/mL); LSD_{5%} = 0.075, *p* < 0.01. **(B)** Blastospore production (Log₁₀ number/mL); LSD_{5%} = 0.557, *p* = 0.288. **(C)** Microsclerotia production (Log₁₀ number/mL); LSD_{5%} = 0.498, *p* < 0.01. Error bars = standard error.

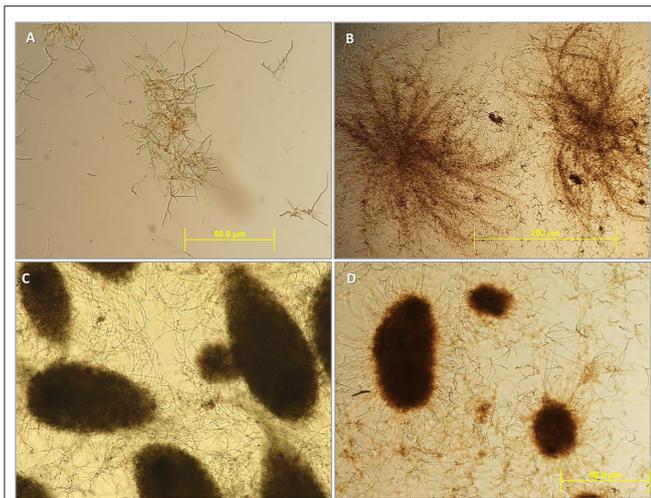


FIGURE 2 | Microsclerotia development during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). The initial process for the formation of microsclerotia (MS) started early in the fermentation under liquid conditions with the formation of hyphal aggregates [(A,B), 4 days after inoculation]. After 7 days of inoculation, the MS formed as round structures with compact pseudoparenchymatous cells layers highly melanised (C,D). Images from: *M. anisopliae* A1080 (A); *M. robertsii* F447 (B); *M. anisopliae* F672 (C); and from *M. novozelandicum* F99 (D).

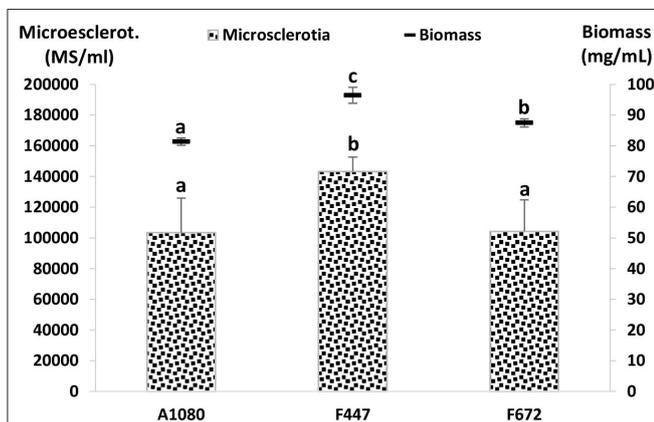


FIGURE 3 | Biomass and MS production by entomopathogenic fungi in basal salts solution with trace metals and vitamins (C:N ratio 23:1). Biomass (mg/mL); $LSD_{5\%} = 4.9$, $p < 0.01$. Microsclerotia (MS/mL); $LSD_{5\%} = 32,542.3$, $p < 0.05$. Error bars = standard error.

with the highest production achieved by *M. anisopliae* F672 ($p < 0.01$; Table 2).

Maize seeds were coated with an amount of MS-DE granules which provided an estimated final concentration of 1×10^8 conidia per gram of seeds. Control seeds (CS) were coated with the same amount of DE and the rest of the coating ingredients, but without fungal MS or conidia. Seeds coated with MS-DE from *M. anisopliae* F672 had a viable conidia production of 7.8×10^6 CFU per gram of maize seeds. This did not differ from *M. anisopliae* A1080 but was greater than for *M. robertsii*, which

produced only 2.0×10^6 CFU per gram of maize seeds (Figure 4). These CFU values were expected since previous results showed that CFU decreased an estimated two orders in relation to the initial conidial concentration coated onto maize seeds. From CS no fungal colonies were obtained.

Maize Plant Performance in the Presence of *Fusarium graminearum* After Seeds Were Coated With Microsclerotia of *Metarhizium* spp

Maize Plant Dry Weight

Maize growth, determined as total dry weight of shoots and roots, differed significantly among treatments ($F_{3/153} = 145.6$; $p < 0.01$). Maize dry weight was highest in CS in the absence of *F. graminearum*, while CS in the presence of this phytopathogen (CSfg) had a significantly lower dry weight (Figure 5; $LSD_{5\%} = 0.029$). In the presence of *F. graminearum*, maize plants with the *Metarhizium* spp. MS-DE coated seed treatments had a significantly greater dry weight than CSfg ($LSD_{5\%} = 0.029$). In the presence of *F. graminearum*, plants coated with the *M. anisopliae* F672 MS-DE treatment had, on average, a dry weight gain of 32% compared to the maize plants without the coating, while the dry weight of plants was almost 26% lower when compared to CS in the absence of *F. graminearum* (Figure 5; $F_{3/153} = 145.6$; $p < 0.01$).

The negative effect of *F. graminearum* on total plant dry weight was due to the phytopathogen's impact on both shoot and root dry weight, which was significantly lower in the CSfg compared to the CS treatment (Figure 5; $p < 0.01$). The *Metarhizium* spp. seed coating with MS-DE partially compensated this reduction in shoot dry weight caused by the plant pathogen. Compared to CS grew in the absence of *F. graminearum*, the dry weight of shoots from plants treated with *M. anisopliae* F672 was reduced by 27% ($F_{3/153} = 73.5$; $p < 0.01$). In plants treated with *M. robertsii* F447 a 37% shoot dry weight reduction occurred when plants were grown in the presence of *F. graminearum* (Figure 5; $LSD_{5\%} = 0.054$). In the presence of the phytopathogen maize plants with MS-DE treatments had a greater root dry weight when compared to plants without a fungal treatment (Figure 5; $LSD_{5\%} = 0.034$). However, in the presence of *F. graminearum*, dry weight of roots from plants with the MS-DE treatments with *M. anisopliae* F672 and *M. robertsii* F447 were 14 and 28%, respectively, lower than root dry weight in CS in the absence of *F. graminearum* (Figure 5; $F_{3/153} = 80.5$; $p < 0.01$).

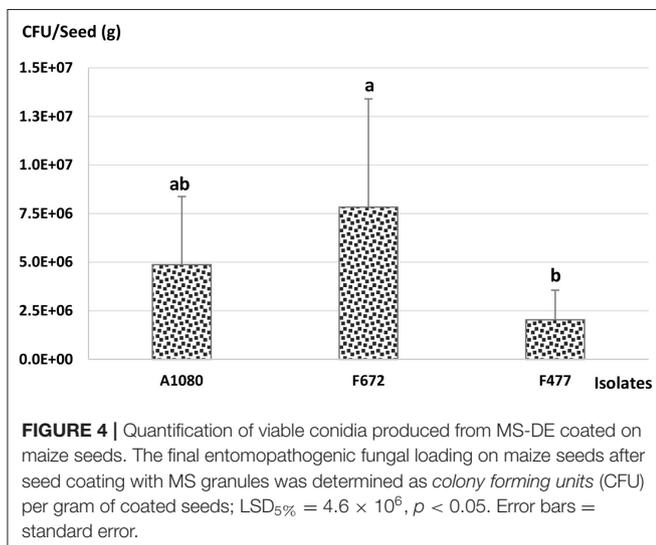
Determination of Fungal Endophytism by Confocal Fluorescent Microscopy After Maize Seed Coating With *Metarhizium* spp. MS-DE Granules

The presence of hyphae on and in root tissues was observed in all the *Metarhizium* MS-DE treatments but not in samples from CS without fungal treatment. Fungal hyphae growing on the surface of roots were observed growing in proximity to the root crown, in seeds remains and all along the length of the root, but not in root apices. The protrusion zone, where secondary

TABLE 2 | Characterization of *Metarhizium* spp. microsclerotia–diatomaceous earth granules.

Isolate	Species	Germination \pm SE (%)	$a_w \pm$ SE	Conidia production \pm SE (conidia/g MS-DE)
A1080	<i>M. anisopliae</i>	95.2 \pm 1.8 ^a	0.75 \pm 0.03 ^a	2.1 \pm 0.1 ($\times 10^9$) ^a
F447	<i>M. robertsii</i>	91.0 \pm 3.4 ^a	0.70 \pm 0.12 ^a	4.4 \pm 0.3 ($\times 10^9$) ^b
F672	<i>M. anisopliae</i>	95.2 \pm 1.9 ^a	0.58 \pm 0.01 ^b	8.8 \pm 0.3 ($\times 10^9$) ^c
LSD _{5%}		5.7	0.09	1.8 $\times 10^9$
<i>p</i> -value		= 0.236	< 0.01	< 0.01

Microsclerotia were obtained from production cultures and filtered with diatomaceous earth to obtain granules (MS-DE). The MS-DE granules after drying were evaluated for water content determined as water activity (a_w), hyphae production (germination) and conidiogenesis (conidia production). Average values with no superscript letters in common represent statistically significant differences at $p < 0.05$ within the same column (germination, a_w or conidia production).

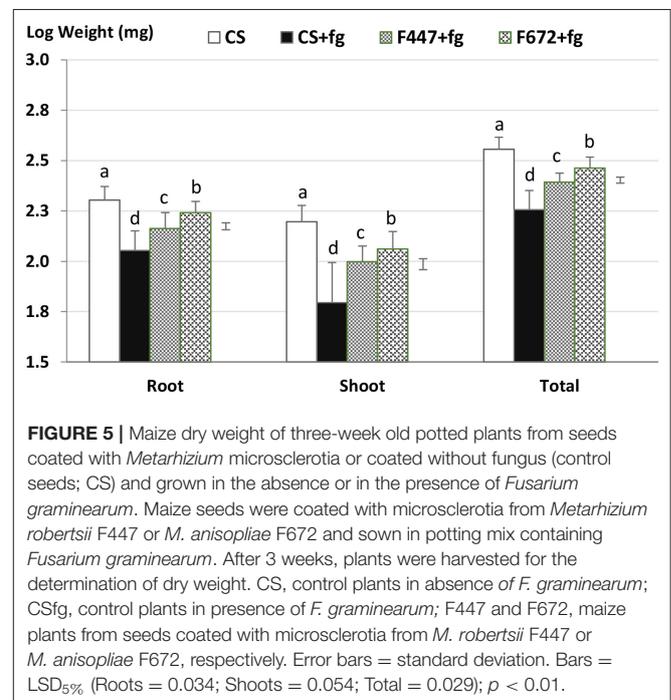


roots emerge, seemed to be one of the access points into the roots. In this area, extensive colonization by *M. anisopliae* A1080 was observed (Figures 6A–D). The presence of hyphae growing inside the cortical root cells was observed 1-month after sowing the MS-DE coated seeds (Figure 7). The lectin, CoA-AF688, binds to glycoproteins, α -mannopyranosyl, and/or α -glucopyranosyl residues allowing the identification of hypha penetration sites through the root cell wall which are visualized as red dots (Figure 7A, arrow heads). The constriction points in the hypha also confirmed these penetration zones (Figure 7B, arrow heads). At this plant stage, the roots showed extensive fungal colonization on the surface of the roots but also endophytic colonization (Figure 7D).

Samples of stems and leaves were also analyzed in all the MS-DE treatments by fluorescent and confocal microscopy, but no endophytic colonization was observed. At least 1 month after sowing *Metarhizium* MS-DE coated seeds, fungal colonization seemed to be limited only to roots.

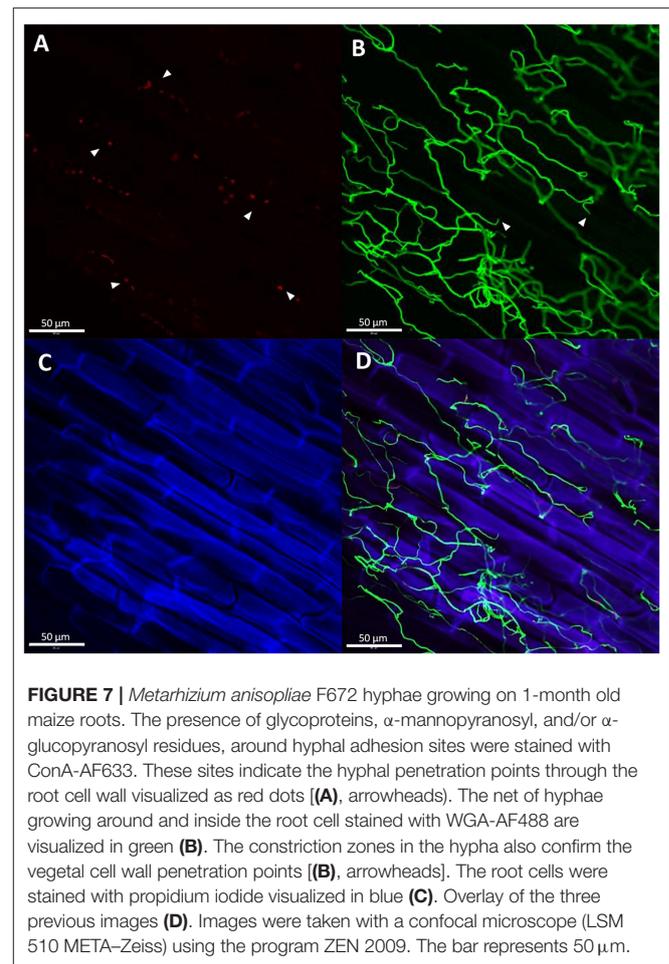
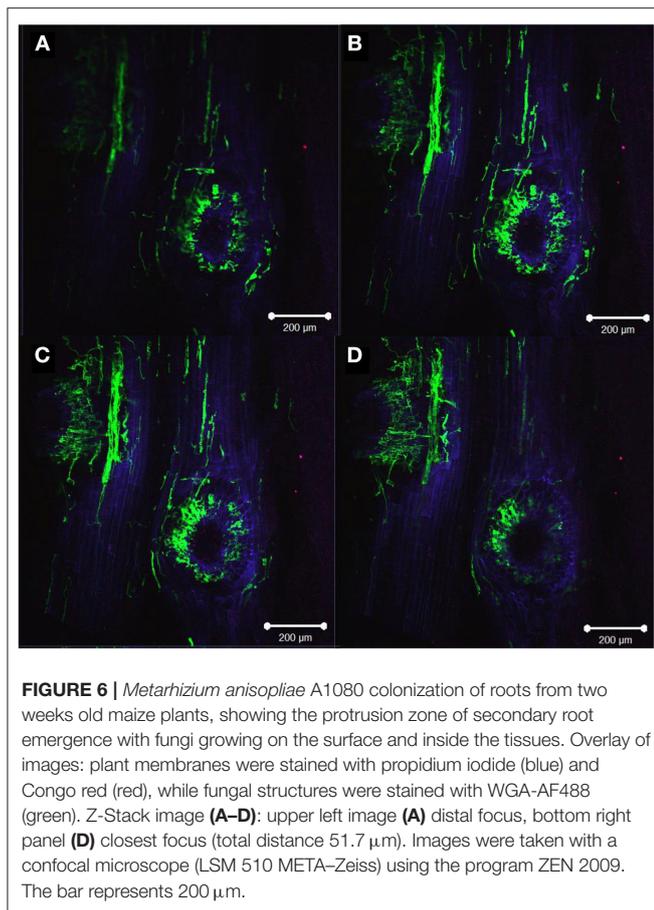
DISCUSSION

In this study the production and formulation of microsclerotia (MS) by isolates of *M. anisopliae*, *M. guizhouense*,



M. novozealandicum, *M. robertsii*, *B. bassiana*, and *T. harzianum* was determined. All isolates produced MS, compact hyphal aggregates that become pigmented with culture age, in addition to more typical blastospores and mycelia. Previous reports have reported the production of these resistant structures in *M. anisopliae*, *M. brunneum*, *M. humberi*, *M. rileyi*, *M. robertsii*, *B. bassiana*, *B. brogniartii*, *B. pseudobassiana*, and *T. harzianum* (Jaronski and Jackson, 2008; Jackson and Jaronski, 2009; Behle et al., 2013; Kobori et al., 2015; Villamizar et al., 2018; Lira et al., 2020). However, this study also reports on the production of MS in several *Metarhizium* species, *T. harzianum*, and *B. bassiana* simultaneously, and it is the first report of MS obtained from isolates of *M. guizhouense* and *M. novozealandicum*.

The biomass obtained by the New Zealand *Metarhizium* spp. isolates (42–55 mg/mL) was higher than values previously reported which vary between 7.0 and 33.0 mg/mL (Jackson and Jaronski, 2009; Mascarin et al., 2014). In a previous study with *M. rileyi*, with a biomass production of 40.7 mg/mL (Song et al., 2016), similar values were obtained as in the present



study. The highest biomass production was in *B. bassiana* Bb21 with 64 mg/mL. This yield was higher than in previous work under similar conditions where biomass weight obtained was between 15.3 and 20.5 mg/mL (Bidochka et al., 1987; Lohse et al., 2014). The variances in biomass yield might be due to differences in growth conditions and media [C:N ratio 23:1, (C) = 45 g/L], or to the different isolates used. The reasons behind the biomass increase observed in *Trichoderma* and *Beauveria* isolates compared to *Metarhizium* could be to their well-known fast growth and the ability to produce different types of fungal propagules such as submerged conidia, blastospores, blastoconidia, and budding hyphal cells from the developing hyphae (microcycle conidiation).

The production of blastospores by the *M. anisopliae* isolates varied between 6.5×10^5 and 1.3×10^7 blastospores/mL after six fermentation days. Blastospore concentration obtained was slightly lower than that reported by Jackson and Jaronski (2009) for *Metarhizium* spp., which was around 2.0 – 1.6×10^8 blastospores/mL after eight fermentation days in similar conditions. The differences in blastospore production might be based on the slight differences in C:N ratios but is more likely to be due to the intrinsic properties of the isolates used. *Beauveria bassiana* Bb21 blastospore production after 3 days of fermentation at 2.7×10^8 blastospore/mL was close to the values reported by Mascarin et al. (2015) of between 0.95 and

7.9×10^8 blastospores/mL in a similar study. However, in the present work, *B. bassiana* Bb21 maximum production, 4.3×10^9 blastospore/mL, was obtained after six fermentation days.

The quantity of MS obtained from both *M. anisopliae* isolates (3.5 – 4.0×10^4 MS/mL) was close to those reported previously by Jackson and Jaronski (2009) for *M. anisopliae* (1.8×10^4 – 6.4×10^4 MS/mL) using identical fermentation conditions and similar C:N ratios. Although in their study the highest production was by *M. brunneum* F52 (1.2×10^5 MS/mL), in this study the highest MS production was found in *M. robertsii* F447 (2.3×10^5 MS/mL). Mascarin et al. (2015) reported yields of around 6.1 – 7.3×10^6 MS/L for *M. anisopliae*, *M. acridum*, and *M. robertsii* isolates after growth in liquid fermentation for 3 days, with a maximum yield of 0.7 – 1.1×10^4 MS/mL after 5 days in liquid fermentation. In a different study with *M. rileyi* MS production of up to 9.7×10^4 MS/mL was reported (Behle et al., 2013). Lira et al. (2020) screened 48 isolates of different species of *Metarhizium* using only 3-day liquid fermentation obtaining a range from 1.3×10^2 to 3.6×10^3 MS/mL.

Evidently, all *Metarhizium* species tested are capable of producing MS although, as expected, the production of the resting structures varied between species and isolates, as reported previously (Lira et al., 2020). In this study, the lowest MS

production was by *M. guizhouense* Bk41 with 3.3×10^2 MS/mL, while for *M. guizhouense* F16, 9.5×10^3 MS/mL was achieved. However, *M. guizhouense* Bk41 produced higher amounts of blastospores (2.6×10^7 blastospores/mL) than *M. guizhouense* F16 (6.1×10^5 blastospores/mL). This fact highlights the importance of isolate characterization to determine potential use and applicability. The results obtained in the current study confirm that growth conditions required for formation of MS can vary among fungal species and even among isolates of a particular species as stated also by Wang et al. (2013).

Another factor to consider during MS production is maturation. Although the production of these resistant structures can be obtained at an early stage of fermentation, a complete melanization of MS did not occur until after at least 6 days of liquid fermentation. The longer the fermentation process, the higher the melanization of the MS, not only in entomopathogenic fungi but also in *T. harzianum* (Jackson and Jaronski, 2009; Mascarin et al., 2014; Kobori et al., 2015). On average, MS can be observed after 4 days of fermentation, but this period might not be sufficient for complete melanization. Further studies are needed to determine the relationship between melanization and subsequent performance of MS. If these resistant structures are not fully mature, an early harvest, and following downstream processing (drying and formulation) could result in MS viability losses. Melanization has been associated with prolonged persistence in soil and resistance to desiccation (stress tolerance) in various filamentous fungi (Jackson and Jaronski, 2009; Kobori et al., 2015). Melanin has also been reported as a natural barrier against the harmful effects of UV-B radiation as well as resistance to toxic substances (Eisenman and Casadevall, 2012).

The rehydration and incubation of air-dried MS-DE granules from the isolates *M. anisopliae* A1080 and F672, and *M. robertsii* F447, on water agar plates resulted in hyphal formation after just 24 h. The development of sporogenic structures able to produce high numbers of conidia was obtained in less than a week. This was also reported in previous studies for *M. brunneum* and *M. anisopliae* (Jackson and Jaronski, 2009; Mascarin et al., 2014), *Lecanicillium lecanii* (Wang et al., 2013), *T. harzianum* (Kobori et al., 2015), *M. robertsii*, and *M. acridum* (Mascarin et al., 2014). In the current study, the concentration of conidia/g of dried MS-DE reached were one log higher than those reported by Jackson and Jaronski (2009), but similar to those values reported by Mascarin et al. (2014).

Lira et al. (2020), showed that coating maize seeds with MS is a feasible method for delivery of entomopathogenic fungi for biocontrol and plant growth promotion purposes. In our study, we demonstrated that after coating, MS were viable, and provided protection to maize plants by counteracting the negative effects of *F. graminearum*. As stated by Partida-Martínez and Heil (2011) the plant gain from any beneficial microorganism is mainly noticed when challenged by plant pathogens. Thus, the potential benefits of MS coated to maize seeds on maize growth was observed in the presence of the plant pathogen *F. graminearum*. Control plants grown in the absence of *F. graminearum* (CS) had significantly greater dry weight than plants treated with *M. robertsii* F447 or *M. anisopliae* F672. Conversely, in the presence of this phytopathogen, shoot development in plants

grown from seeds coated with MS from *M. robertsii* and *M. anisopliae* was significantly better, when compared to maize plants from seeds without the MS coating.

Previous work found that bean or maize plants colonized by *Metarhizium* spp., and then exposed to *F. solani* or *F. graminearum*, had greater plant growth and lower disease indices compared with uncolonized plants (Sasan and Bidochka, 2013; Rivas-Franco et al., 2019). The mechanisms used by *Metarhizium* to counteract the negative effects of the presence of the plant pathogen could be due to multiple factors including competence for rhizosphere colonization, promotion of the plant induced response or production of *Metarhizium* secondary metabolites that inhibit *Fusarium* growth (Sasan and Bidochka, 2013; Ravindran et al., 2014). Thus, the overall results suggest that species of *Metarhizium* may be used as a control for plant pathogens as well as insect pests. The mechanisms behind the entomopathogenic fungi *Metarhizium* spp. ability to reduce the impact of this plant root pathogen represents an area that warrants further research.

Using laser confocal microscopy, we showed that the hyphae were closely associated with the ecto and endorhizosphere of maize roots. In the present work, two isolates, *M. anisopliae* F672, and *M. robertsii* F447, were obtained from plant material, *Pinus radiata* and *Actinidia deliciosa*, respectively, while *M. anisopliae* A1080 was isolated from a lepidopteran larva, *Trichoplusia ni*. The ability to produce MS as well as the capability to associate with maize roots seemed to be conserved in *Metarhizium* independent of origin. This reinforces the fact that *Metarhizium*, as soil dwelling fungi, have the capability to form resting structures to survive unsuitable conditions, and the ability to associate with roots which guarantees long term persistence.

Vega et al. (2009) stated “the ability of *Metarhizium anisopliae* to form sclerotia may be important for rhizosphere competence following a pattern seen in phytopathogenic fungi.” In this study, *M. anisopliae* and *M. robertsii* strongly colonized the differentiation and the root hair zones, inter- and intracellularly, and were infrequently detected in the elongation and meristematic zones. This colonization pattern was also observed in the plant pathogen *Piriformospora indica* (Zuccaro et al., 2011). However, the endophytic ability of *Metarhizium* and the ability to colonize cortical cell roots, places this genus closer to ecto- and arbuscular mycorrhizal fungi, which either grow intercellularly or predominantly colonize the deeper cortex layers of younger parts of the root (Zuccaro et al., 2011). This suggests that entomopathogenic fungi might also follow a pattern similar to that observed in *P. indica* and mycorrhiza. In *P. indica*, the failure of WGA-AF488 to stain the hyphae inside living cells strongly suggested that the fungus remained enveloped in an intact plant-derived membrane throughout intracellular growth (Zuccaro et al., 2011). *Metarhizium anisopliae* and *M. robertsii* were observed inside the cells stained with the WGA-AF488 which may indicate that entomopathogenic fungi growing inside the plant cell were not enveloped by an endomembrane as in *P. indica*. Whether the association pattern between hyphae of entomopathogenic fungi and roots resembles a similar process to that of phytopathogenic fungi or mycorrhizae, or even represents a novel process, requires further studies.

In summary, the results obtained provide insights into liquid culture production of MS from different species of *Metarhizium* and demonstrates the potential use of these structures when coated onto seeds for the biocontrol of plant pathogens and plant growth promotion. Additionally, this MS coating strategy places the fungus close to the roots which can be colonized both superficially and endophytically. This fungus-root association allows persistence of the biocontrol agent, and places it close to harmful plant challengers such as plant pathogens and soil-dwelling insect pests. Innovation in the development of *Metarhizium* as a biocontrol agent needs to consider all its capabilities including insect pathogenicity, antagonism of fungal phytopathogens, root colonization, and stimulation of the plant induced response, making *Metarhizium* a multifunctional bioinput for plant health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FR-F: conceptualization, methodology, investigation, formal analysis, and writing—original draft. JH, MR, TJ, MJ, and

TG: conceptualization, resources, writing—review & editing, and supervision. NA: resources, writing—review & editing, and supervision. JS: conceptualization and methodology. PW: conceptualization, resources, and supervision. DS: validation, formal analysis, and writing—review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: DS was employed by the institute Bio-Protection Research Centre, as a consultant to provide assistant in experimental design, statistics analysis and data processing involved in the different research lines carry on by the institute.

The remaining 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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