Selection of fungal isolates from Buenos Aires, Argentina, as biological control agents of *Botrytis cinerea* and *Sclerotinia sclerotiorum*

Selección de aislados fúngicos de Buenos Aires, Argentina, como agentes de control biológico de *Botrytis cinerea* y *Sclerotinia sclerotiorum*

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Abstract

This work aimed to select promising microorganisms as biological control agents (BCA). Forty-one soil samples were obtained from florihorticultural farms located in Buenos Aires, Argentina. Insect trap techniques and soil serial dilutions were used to obtain isolates of entomopathogenic fungi and fungi of genera *Trichoderma,* respectively. A total of 20 isolates included five *Metarhizium* and 15 *Trichoderma*. The isolates were lyophilized and deposited as reference cultures in the Mycological Collection of the Centro de Estudios Parasitológicos y de Vectores (CEPAVE). We performed dual culture studies of the isolates collected against the pathogens *Botrytis cinerea* Pers. (1797) and *Sclerotinia sclerotiorum* (Lib.) de Bary (1884). Eleven isolates were selected for growth promotion studies in tomato plants (*Solanum lycopersicum* L.). The isolates of *Metarhizium taii* Liang & Liu (1991) CEP-722, CEP-723 *Trichoderma afroharzianum* Chaverri, Rocha, Degenkolb & Druzhinina (2015) CEP-753 and CEP-754, molecularly identified by amplification of the ITS and TEF1α zones, presented the best results in the dual culture and growth promotion tests. Subsequent studies will evaluate virulence of fungal strains in insects.

Keywords

entomopathogenic fungi • biological control agents • molecular identification • dual culture • plant growth promotion

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Resumen

El objetivo de este trabajo fue seleccionar microorganismos promisorios como agentes de control biológico (ACB). Se visitaron predios florihortícolas ubicados en Buenos Aires, Argentina, de los cuales se obtuvo un total de 41 muestras de suelo. Se utilizaron las técnicas de insecto trampa y diluciones seriadas de suelo para la obtención de aislados de hongos entomopatógenos y hongos del género *Trichoderm*a respectivamente. Se obtuvieron un total de 20 aislados, cinco pertenecientes al género *Metarhizium* y 15 aislados correspondientes al género *Trichoderma*. Los aislados fueron liofilizados y depositados como cultivos de referencia en la Colección Micológica del Centro de Estudios Parasitológicos y de Vectores (CEPAVE). Se realizaron estudios de cultivos duales de los aislados recolectados frente a los patógenos *Botrytis cinerea* Pers. (1797) y *Sclerotinia sclerotiorum* (Lib.) de Bary (1884). Se seleccionaron 11 aislados para la realización de estudios de promoción de crecimiento en plantas de tomate (*Solanum lycopersicum* L.). Los aislados de *Metarhizium taii* Liang y Liu (1991) CEP-722, CEP-723 y de *Trichoderma afroharzianum* Chaverri, Rocha, Degenkolb y Druzhinina (2015) CEP-753 y CEP-754, identificados molecularmente por medio de la amplificación de las zonas ITS y TEF1α, presentaron los mejores resultados en las pruebas de cultivo dual y promoción de crecimiento. Se espera avanzar en estudios posteriores que evalúen la virulencia de cepas de hongos en insectos.

Palabras clave

hongos entomopatógenos • agentes de control biológico • identificación molecular • cultivo dual • promoción de crecimiento de las plantas

Introduction

Stem “wet rot” caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, (1884) and “grey rot” caused by *Botrytis cinerea* Pers. (1797) stand among the economically most important diseases in tomato (*Solanum lycopersicum* L.) Control of these diseases has relied on benzimidazole and dicarboximide fungicides. However, dicarboximide-resistant isolates are commonly detected (3). In this regard, biological control programs sustained by isolation and subsequent selection of antagonists (4) constitute a valuable alternative. Among beneficial biota, nutrient-fixing and solubilizing microorganisms produce *plant growth-promoting* substances, induce plant resistance to diseases or behave as antagonists to phytopathogenic agents (32).

The genus *Trichoderma* dominates the mycobiome of various ecosystems (10) with the ability to colonize the rhizoplane, rhizosphere and roots, producing numerous metabolites with antimicrobial and biostimulant activity. The plant growth stimulating effect is probably generated by the interaction among growth hormones synthesized by *Trichoderma* spp. and plant defense hormones (8). Some entomopathogenic fungi act as fungal growth inhibitors of phytopathogens (11, 12). The genus *Metarhizium* is composed of diverse common soil fungi with multifunctional lifestyles and different nutrient acquisition modes, either saprophytes, endophytes, and/or insect pathogens (37). Classically, studies have focused on their entomopathogenic characteristics, but their ability to inhibit phytopathogens was recently determined (13). Some studies have evaluated the endophytic capacity and colonization methods of this genus (2).

Metarhizium is a genetically diverse taxon, and colony color and conidial measurements of different species are not reliable identification factors (22). Alternatively, the molecular identification of *Trichoderma* is abundant, with no standard process, except the recently proposed gene standardization system for molecular identification (7). This study intends to develop biological inputs based on native and/or naturalized strains of *Trichoderma* and entomopathogenic fungi for agricultural pest management.

Materials and methods

Collection of soil samples

Six agroecological productions located in the province of Buenos Aires, Argentina were visited. Agroecological production of Bernardo Castillo (Street 519, El Pato, Buenos Aires. -34.905505, -58.200043); Organization 1610 (Street 1610, La Capilla, Buenos Aires. -34.9046857, -58.2666433); Agroecological production Santa Elena (Road Parque Pereyra Iraola, Pereyra, Buenos Aires. -34.83699, -58.093384); M. G. Agroecológica (Esteban Echeverría, Buenos Aires. -34.8672710, -58.4608800); Cooperative UTT Jaúregui. (Luján, Buenos Aires. -34.6204249, -59.1764168); and the experimental plot of Cátedra de Horticultura, Facultad de Agronomía de la Universidad de Buenos Aires (Av. San Martín 4453, C.A.B.A. -34.594101, -58.484467). Fourty-one soil samples were obtained from cultures of cabbage (*Brassica oleracea* var. *capitata*); basil (*Ocimum basilicum*); corn (*Zea mays*); lettuce (*Lactuca sativa*); tomato (*Solanum lycopersicum*); zucchini (*Cucurbita pepo*); bell pepper (*Capsicum annuum*); cherry tomato (*Solanum lycopersicum* var. *cerasiforme*); chives (*Allium fistulosum*); leek (*Allium ampeloprasum* var. *porrum*); fennel (*Foeniculum vulgare*); beets (*Beta vulgaris*); broccoli (*Brassica oleracea* var. *italica*); brussels sprouts (*Brassica oleracea* var. *gemmifera*); chard (*Beta vulgaris* var. *cicla*); carrot (*Daucus carota*); artichoke (*Cynara cardunculus* var. *scolymus*); broad bean (*Vicia faba*); turnip (*Brassica rapa* subsp. *rapa*); kale (*Brassica oleracea* var. *sabellica*); peas (*Pisum sativum*); and arugula (*Eruca vesicaria*). Sample number and species varied by establishment. As sampling criterion, soil from more vigorous plants within the same plot was also sampled, for later obtention of growth-promoting microorganisms (20, 39, 42). Five random subsamples within a crop row were collected for each sample from the first 20 cm below ridge surface with crop roots. Then, they were mixed into a single homogeneous sample with approximately 500 g from each crop, soil and rhizosphere, holding the greatest biodiversity (24). Fungal greatest abundance is found in the superficial layers or soil horizons (21). Samples were arranged in plastic bags indicating date, culture and origin, later transported to the laboratory in a closed expanded-polystyrene container and processed within 24 hours (table 4, page 78 and table 5, page 79).

Isolation of *Metarhizium, Trichoderma, Botryris cinerea* and *Sclerotinia sclerotiorum* fungi

From the collected soil samples, the insect trap technique was used with larvae of *Tenebrio molitor* L. from stage L3 to L4 as bait insects (1). Samples were sieved and 300 g were placed in 500 ml plastic containers with five larvae each, moistened with 20 ml of sterile distilled water and incubated at 18°C 65% relative humidity and 14:10 h light-darkness photoperiod. *T. molitor* carcasses prospected after seven days. Dead larvae with external mycosis were washed with sterile distilled water and placed in a humid chamber to increase sporulation. External mycelium present in *T. molitor* corpses (figure 5A, page 82) was obtained via direct isolation from the sporulated corpses, using a previously sterilized loop and subsequent sowing in Sabouraud Dextrose Agar culture medium, SDYA (Merck, Germany) with the addition of 5% chloramphenicol inside a 90 mm diameter *Petri* dish. *Trichoderma*, fungi were isolated via serial dilution. Five grams of each soil sample were suspended in 100 ml of sterile distilled water in an Erlenmeyer and vortexed for one hour. Serial dilutions were made until reaching $x10^6$ spores/ml. Concentrations were determined with a Neubauer chamber, with each dilution inoculated in 90 mm diameter *Petri* dishes with Potato Glucose Agar, APG (Merck, Germany), and 2% streptomycin. The plates were incubated at 20-22°C for 72 hours. When fungal colonies developed, they were replicated in *Petri* dishes with APG (Merck, Germany) until purification.

Phytopathogenic fungi isolates from *B. cinerea* and *S. sclerotiorum* were obtained from the mycological bank of phytopathology (Facultad de Agronomía de la Universidad de Buenos Aires), with identification code BC18 and SS18 and pathogenicity tested on tomato (*S. lycopersicum*). After isolation and before bioassays, visual prospection of the isolates was carried out under a microscope (OLYMPUS BX51, Japan), identifying fungal types.

Monosporic isolates were preserved on sterile filter paper and were lyophilized, deposited and preserved (14) as reference cultures in the mycological collection of the "Centro de Estudios Parasitológicos y de Vectores" (CEPAVE) (CONICET-UNLP), La Plata, Argentina.

Laboratory tests in dual cultures of *Metarhizium* and *Trichoderma* isolates against *B. cinerea* and *S. sclerotiorum*

Ninety mm diameter *Petri* dishes were filled with 12 ml of APG (Merck, Germany) or 12 ml of SDYA (Merck, Germany) for *Trichoderma* and *Metarhizium* trials, respectively. Once culture medium solidified, two 10 mm diameter discs with seven-days mycelial growth were placed on the medium, 70 mm apart, one containing *Trichoderma* spp. or *Metarhizium* spp. and the other containing *B. cinerea* or *S. sclerotiorum* according to each treatment. A disk of each isolate (pathogens and antagonists) was inoculated as control against a disk of APG and/or SDYA without microorganisms. *Petri* dishes were incubated at 22°C and maintained under fluorescent lights with a 14:10 h light-darkness photoperiod in a completely randomized design, with eight replicates per treatment. Growth radius of colonies considering *Trichoderma* isolates against phytopathogenic fungi were measured with a millimeter ruler, at 1, 2, 3, 4, 5 and 6 days of trial with *B. cinerea* and at 1, 2, 3, 4 and 5 days with *S. sclerotiorum*. Considering *Metarhizium* isolates against phytopathogenic fungi, colony radius was measured at 4, 5, 6 and 7 days in both cases. The number of measurement days per trial differs according to growth rate in phytopathogen control treatments. Pathogen percentage inhibition (I) was calculated using the following equation:

$$\mathbf{I= (C-T) / C \times 100.}$$

where:
(I) = Percentage of mycelium growth inhibition
C = Pathogen growth on control plates
T = Pathogen growth in dual culture plates (19).

The data were analyzed by ANOVA and Tukey test ($p> 0.05$) with InfoStat software (version 2016e) (9).

Growth promotion assays in tomato plants var. platense (*S. lycopersicum*) inoculated with a spore suspension of isolates of the genera *Metarhizium* and *Trichoderma*

Tomato plants (*S. lycopersicum*) var. platense were inoculated with a spore suspension of *Metarhizium* sp. CEP-722, CEP-723, CEP-724, CEP-725 and CEP-726 or the *Trichoderma* sp. CEP-745, CEP-749, CEP-751, CEP-752, CEP-753 and CEP-754, selected after *in vitro* growth inhibition tests of *B. cinerea* and *S. sclerotiorum*. The test was conducted in a biotherium chamber under controlled conditions at an average temperature of 24°C, average relative humidity of 70%, and a 18-6 h light-darkness photoperiod, using high-pressure sodium vapor lamps (Philips Son T Agro 250 W, China). Seeds of tomato (*S. lycopersicum*) var. platense were sown in commercial substrate (Grow Mix Multipro, Argentina) in seedling trays with 50 x 50 mm holes and irrigated with sterile distilled water. Spore suspensions of *Trichoderma* spp. and *Metarhizium* spp. isolations were obtained in sterile distilled water standardized to a concentration of 1×10^7 spores/ml. Tomato plants (*S. lycopersicum*) were inoculated at 7, 21 and 35 days after being sown (31 days in the case of the genus *Metarhizium*) with 2 ml of conidial suspension on the substrate. Measurements were made 49 days after sowing in *Trichoderma* spp. and 37 days in *Metarhizium* spp. Plants were watered to field capacity with sterile distilled water throughout the study. The completely randomized block design had seven repetitions (seven plants) for each treatment (spore suspension of the *Metarhizium* sp. and *Trichoderma* sp. isolates selected). The variables analyzed were stem length (mm), stem diameter at cotyledon height (mm) and aerial fresh weight (g), using a millimeter rule, graduated metal caliper and precision balance, respectively. An ANOVA and means comparison with Tukey test ($p > 0.05$) were performed with InfoStat (2016e version) (9).

Morphological characterization, molecular identification and phylogenetic analysis of the isolates CEP-722, CEP-723, CEP-753 and CEP-754

Four isolates, two of the genus *Metarhizium* and two of the genus *Trichoderma,* were selected for best results on dual culture and promotion growth assays. Isolates were identified at genus level based on microscopic traits contrasted with taxonomic keys (5, 18). Once the material was mounted in lactophenol/cotton blue (0.01% w/v), shape and size of conidia, conidiogenous cells (phialide), mycelium and other traits were observed under an optical microscope (OLYMPUS BX51, Japan) and photographed with a digital camera (Sony DSCP73, Japan). Measurements were based on 25 observations per microstructure (conidia, phialide and chlamydospore) and average calculations. Molecular analysis and identification of the isolates included mycelium production in three 90 mm diameter *Petri* dishes with APG (Merck, Germany) as culture medium for *Trichoderma* and SDYA (Merck, Germany) for *Metarhizium*, kept at 23° ± 1°C for seven and 14 days, respectively. Then, mycelium was placed in 1.5 ml Eppendorf tubes. Tubes containing fungal material were placed in a container with liquid Nitrogen for eight minutes. DNA extraction was performed using the DNeasy extraction kit from Qiagen (Germany) according to manufacturer instructions. Extracted DNA was quantified using a micro-volume spectrophotometer (Nanodrop, Thermo Fisher Scientific, United States) and stored in a freezer. PCR was performed to amplify 2 DNA regions: 1) The ribosomal DNA region comprising the 3' end of the 18S gene (small ribosomal subunit, SSU). The ITS1 internal spacer sequence (internal transcribed spacer 1), the 5.8S gene, the internal transcribed spacer 2 (ITS2) sequence and the 5' end of the 28S gene (long ribosomal subunit), using the universal primers ITS4 (5' -TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (30). 2) The 5' region of the Elongation Factor 1-Alpha (TEF1α) gene with the primers EF1 983F (5'-ATGGGTAAGGARGACAAGAC-3') and EF" 2218R (5'-ATGGGTAAGGARGACAAGAC-3') (23). Amplification reactions were carried out in a final volume of 50 µl, containing 25 µl Mastermix Promega 2x (GoTaq, USA), 17 µl nuclease-free water, 2 µl PRIMER-F, 2 µl PRIMER- R and 4 µl of DNA for each isolate. Table 1 shows the thermocycling processes for the ITS1 and TEF1α regions.

Table 1. Thermocycling processes for the ITS1 and TEF1α regions.
Tabla 1. Procesos de termociclados para las regiones ITS1 y TEF1α.

ITS - 32 ciclos	CEP-722	CEP-723	CEP-753	CEP-754
Initial denaturation	ITS: 95°C 5 min. TEF1α: 94°C 2 min.	ITS: 95°C 5 min. TEF1α: 94°C 2 min.	ITS: 95°C 5 min. TEF1α: 94°C 2 min.	ITS: 95°C 5 min. TEF1α: 94°C 2 min.
Denaturation	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.	ITS: 94°C 1,30 min. TEF1α: 94°C 30 seg.
Alignment	ITS: 60°C 1 min. TEF1α: 53°C 40 seg.	ITS: 56°C 1 min. TEF1α: 54°C 40 seg.	ITS: 58°C 1 min. TEF1α: 54°C 40 seg.	ITS: 60°C 1 min. TEF1α: 54°C 40 seg.
Elongation	ITS: 72°C 50 seg. TEF1α: 72°C 30 seg.	ITS: 72°C 1,30 min. TEF1α: 72°C 30 seg.	ITS: 72°C 1,3 min. TEF1α: 72°C 30 seg.	ITS: 72°C 50 seg. TEF1α: 72°C 30 seg.
Denaturation 35 cycles*	TEF1α: 94°C 30 seg.	TEF1α: 94°C 30 seg.	TEF1α: 94°C 30 seg.	TEF1α: 94°C 30 seg.
Final elongation	ITS: 72°C 5 min. TEF1α: 72°C 10 min.	ITS: 72°C 5 min. TEF1α: 72°C 10 min.	ITS: 72°C 5 min. TEF1α: 72°C 10 min.	ITS: 72°C 5 min. TEF1α: 72°C 10 min.
Hold	ITS: 10°C TEF1α: 8°C.	ITS: 10°C TEF1α: 8°C.	ITS: 10°C TEF1α: 8°C.	ITS: 10°C TEF1α: 8°C.

* TEF1α region only.
* Solo región TEF1α.

Electrophoresis was performed in 1% agarose gels (UNQ Biological Products, Argentina) stained with Ethidium bromide in 0.5x Buffer TBE (Roti-Gelstain, Germany), applying a voltage of 90 V for 50 min. Five µl of each reaction was mixed with 1 µl of loading buffer (Productos Biológicos UNQ, Argentina). A molecular weight marker was added (Ladder 100 bp, PB-L), to determine PCR product size. Gels were visualized with a UV transilluminator (Analytik-Jena, Alemania). The ribosomal region was expected at 530 bp, while the TEF1α gene was 620 bp. Positive reactions were stored at -20°C. Samples were then sent to Macrogen (South Korea) for purification and sequencing. The free software "Chromas" (38)

cleaned the obtained sequencing then aligned using the online software "Clustal-Omega" (35). Agreement between the base pairs replicated by the forward and reverse primers of the four isolates analyzed was verified using the free software "Genedoc" (25). The sequences obtained were compared with those available in Genbank for each molecular marker (41). Sequences were aligned with 16 homologues and contrast of reference strains of *Metarhizium* with the sequencing of the ITS and TEF1α areas of Gutierrez *et al*. (2019) (table 2) and with 15 homologous species and a contrast obtained from the "Trichokey" data software (7), for *Trichoderma* (table 3, page 78). The phylogenetic tree was constructed with "Mr. Bayes" (version 3.2.7) (17), "Tracer" (version 1.7.2) (30) and "FigTree" (version 1.4.4) softwares (29).

Table 2. ITS and TEF1α gene sequences used for molecular identification of isolates CEP-722 and CEP-723.
Tabla 2. Secuencias de genes ITS y TEF1α utilizadas para la identificación molecular de los aislados CEP-722 y CEP-723.

Species	Strain	ITS	TEF1α
Metarhizium taii.	CEP-722	OP709693	OP792040
Metarhizium taii.	CEP-723	OP709705	OP792039
Metarhizium sp.	NHJ11597	HQ165703/AY646375	HQ165683
Metarhizium sp.	NHJ11618	HQ165704/AY646376	HQ165684
Metarhizium sp.	MY00896	HQ165697	HQ165678
Metarhizium argentinense	CEP414	MF784813	MF966620
Metarhizium argentinense	CEP424	MF784814	MF966624
Metarhizium blattodeae	IP414	KU182915	KU182917
Metarhizium flavoviride	ARSEF2025	AF138269	KJ398804
Metarhizium frigidum	ARSEF4124	HM055448	DQ464002
Metarhizium minus	ARSEF1764	HM055453	KJ398800
Metarhizium album	ARSEF1942	HM055452	KJ398802
Metarhizium taii	ARSEF5714	JN049829	AF543775
Metarhizium owariense	NBRC33258	JN049883	JF416017
Metarhizium kusanagiense	TNS-F18494	JN049873	JF416014
Metarhizium martiale	HMAS197472	JN049881	JF416015
Metarhizium pseudoatrovirens	TNS-F16380	JN049870	KJ398785
Metarhizium koreanum	ARSEF2038	HM055431	KJ398805
Beauveria bassiana	ARSEF751	AY532045	AY531954

Table 3. ITS and TEF1α gene sequences used for molecular identification of isolates CEP-753 and CEP-754.
Tabla 3. Secuencias de genes ITS y TEF1α utilizadas para la identificación molecular de los aislados CEP-753 y CEP-754.

Species	Strain	ITS	TEF1α
Trichoderma afroharzianum	CEP-753	OP700049	OP792041
Trichoderma afroharzianum	CEP-754	OP709539	OP792042
Trichoderma afroharzianum	GJS04-186/TRS835	FJ442265	KP008787.1
Trichoderma arundinaceum	GJS05-180/MSX70741	EU330928.1	KY630170.1
Trichoderma asperellum	CBS433.97/TRS705	AY380912	KP009011.1
Trichoderma atroviride	693.94 UTHSC08-2443	Z48817	KJ786838.1
Trichoderma bissettii	1158	KJ174235.1	MH249948.1
Trichoderma dorothopsis	HZA8/HZA5	MH624143	MK850827.1
Trichoderma gamsii	GJS04-09/TW20050	DQ315459	KU523895.1
Trichoderma harzianum	CBS226.95/T18	AY605713	KX632606.1
Trichoderma koningiopsis	GJS93-20/18ASMA001	NR_131281	MT671922.1
Trichoderma longibrachiatum	CBS816.68/S328	NR120298	JQ685867.1
Trichoderma ochroleucum	CBS119502/GJS01-265	NR134401	DQ835494.1
Trichoderma pollinicola	LC11682/LC11686	MF939592	MF939620.1
Trichoderma reesei	ATCC26921/QM6a	KU729028	XM006963994
Trichoderma virens	CBS249.59/Tvien3	MH857855	MT081441.1
Trichoderma viride	CBS119327/GJS89-127	DQ677657	AF534585.1
Hypomyces aurantius	GJS74-69/TFC95-171	FJ442642.1	FN868743.1

Results

Isolates with access numbers CEP-722, CEP-723, CEP-724, CEP-725 and CEP-726 for *Metarhizium* spp. (table 4) and CEP-745, CEP-747, CEP-748, CEP-749, CEP-750, CEP-751, CEP-752, CEP-753, CEP-754, CEP-755, CEP-756, CEP-757, CEP -758, CEP-759 and CEP-760 for *Trichoderma* spp. (table 5, page 79) were obtained and admitted to the mycological bank of the "Centro de Estudios Parasitológico y de Vectores" (CEPAVE) (CONICET-UNLP), La Plata, Argentina.

Table 4. *Metarhizium* isolates obtained from soil samples.
Tabla 4. Aislados del género *Metarhizium* obtenidos de las muestras de suelo.

N°	Establishment	Geographic reference	Code	Crop on soil
1	Coop. UTT Jauregui	-34.6204249, -59.1764168	CEP-722	*Beta vulgaris*
2	Coop. UTT Jauregui	-34.6204249, -59.1764168	CEP-723	*Beta vulgaris* var. *cicla*
3	Prod. Bernardo C.	-34.905505, -58.200043	CEP-724	*Cynara cardunculus* var. *scolymus*
4	Prod. Bernardo C.	-34.905505, -58.200043	CEP-725	*Vicia faba*
5	Prod. Bernardo C.	-34.905505, -58.200043	CEP-726	*Brassica rapa* subsp. *rapa*

Table 5. *Trichoderma* isolates obtained from soil samples.
Tabla 5. Aislados del género *Trichoderma* obtenidos de las muestras de suelo.

N°	Establishment	Geographic reference	Code	Crop on soil
1	Coop. UTT Jauregui	-34.6204249, - 59.1764168	CEP-745	*Allium ampeloprasum* var. *porrum*
2	FAUBA	-34.594101, -58.484467	CEP-747	*Solanum lycopersicum*
3	Org. 1610	-34.9046857, -58.2666433	CEP-748	*Brassica oleracea* var. *capitata*
4	Coop. UTT Jauregui	-34.6204249, - 59.1764168	CEP-749	*Lactuca sativa*
5	Sta. Elena Agroecológica	-34.83699, -58.0933384	CEP-750	*Pisum sativum*
6	Sta. Elena Agroecológica	-34.83699, -58.0933384	CEP-751	*Lactuca sativa*
7	FAUBA	-34.594101, -58.484467	CEP-752	*Solanum lycopersicum*
8	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-753	*Lactuca sativa*
9	Org. 1610	-34.9046857, -58.2666433	CEP-754	*Lactuca sativa*
10	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-755	*Zea mays*
11	Org. 1610	-34.9046857, -58.2666433	CEP-756	*Beta vulgaris*
12	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-757	*Ocimum basilicum*
13	Coop. UTT Jauregui	-34.6204249, - 59.1764168	CEP-758	*Beta vulgaris*
14	FAUBA	-34.594101, -58.484467	CEP-759	*Solanum lycopersicum*
15	M. G. Agroecológico	-3.8672710, -58.4608800	CEP-760	*Solanum lycopersicum*

Percentage inhibition of growth of *B. cinerea* and *S. sclerotiorum* caused by fungi of the genera *Trichoderma* and *Metarhizium*

Considering growth speed and physical conditions of the *Petri* dishes, results on dual culture trials are presented for day 4 for *Trichoderma* and day 7 for *Metarhizium*. Percentage growth inhibition of the pathogens stabilized after mycelial contact with the treatments or when an inhibition halo was generated. *Trichoderma* isolates with the highest inhibition percentages at day four of measurement in the dual culture tests against the pathogen *B. cinerea* were CEP-745, CEP-749, CEP-751, CEP-754 and CEP-756, these being 46.04; 51.75; 45.40; 45.40; and 43.18%, respectively. Inhibition percentage of *S. sclerotiorum* in *Petri* dishes on day 4 of measurement reached 55.88; 56.75; 58.25; 52.13; and 51.75% for isolates CEP-749, CEP-752, CEP-754, CEP-755 and CEP-758 respectively, presenting the highest mean values (figure 1, page 80). No significant differences were observed in growth percentage of *B. cinerea* at day seven among the different treatments of spores suspension *Metarhizium* isolates, but the highest mean values were recorded for CEP-722 and CEP-723, being 32.97 and 32.19%, respectively. The CEP-722 isolate presented the highest values in inhibition percentage of *S. sclerotiorum* at day seven, reaching a mean value of 27.34%. This was the only treatment with statistically significant differences concerning treatment CEP-726, which presented the lowest inhibition percentages (23.59%) against S. *sclerotiorum* (figure 2, page 80).

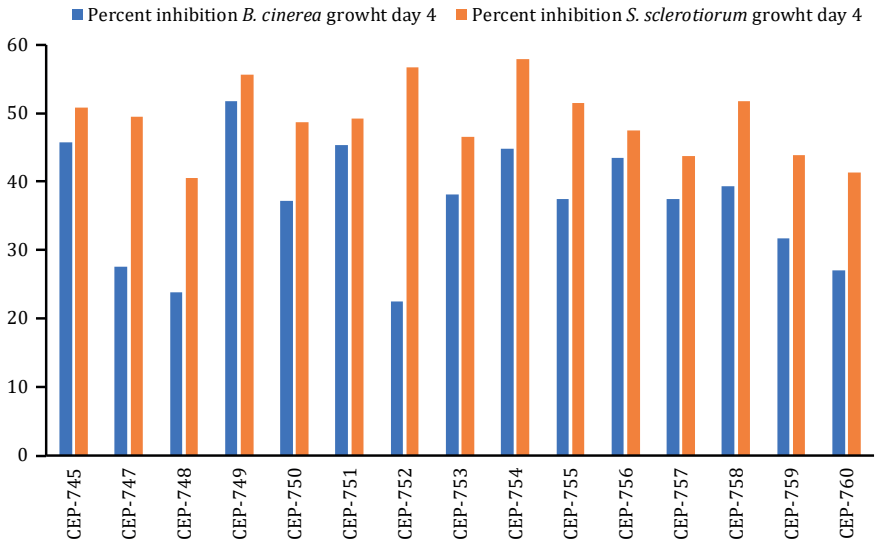


Figure 1. Growth inhibition (%) of *B. cinerea* and S. *sclerotiorum* by isolates of the genus *Trichoderma* at day 4 of measurement.
Figura 1. Medias del porcentaje de inhibición del crecimiento de *B. cinerea* y *S. sclerotiorum* generado por aislados del género *Trichoderma* al día 4 de medición.

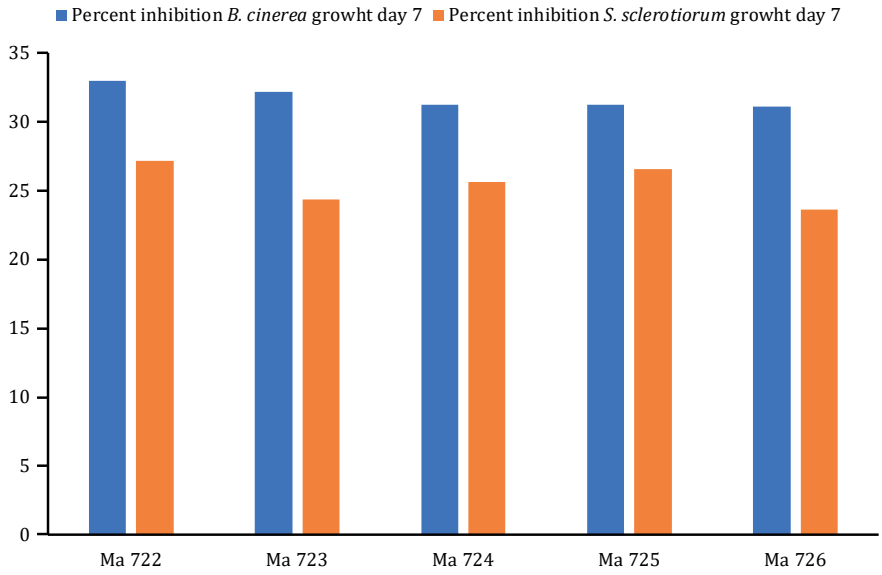


Figure 2. Growth inhibition (%) of *B. cinerea* and S. *sclerotiorum* by isolates of the genus *Metarhizium* at day 7 of measurement.
Figura 2. Medias del porcentaje de inhibición del crecimiento de *B. cinerea* y *S. sclerotiorum* generado por aislados del género *Metarhizium* al día 7 de medición.

Growth promotion of tomato plants (*S. lycopersicum*) var. platense inoculated with six isolates of *Trichoderma* and five isolates of *Metarhizium*

Considering all variables studied in the *Trichoderma* assays, control treatment presented the lowest mean values after application. Regarding stem diameter, the plant inoculated with spore suspension of the isolates CEP-751, CEP-752, CEP-753 and CEP-754 presented higher mean values than the plant inoculated with CEP-745, CEP-749 and the control. Stem length reached the highest mean value (21.37 cm) for the plant inoculated with spore suspension of the isolates CEP-754. Aerial weight mean was highest for the plants inoculated with spore

suspension of CEP-753 and CEP-754, reaching 13.02 and 12.59 g, respectively (figure 3). Stem diameter, stem length and aerial weight of all treatments with *Metarhizium* fungi differed from the control. Regarding stem diameter, treatments inoculated with a spore suspension of the isolates CEP-722, CEP-724 and CEP-726 presented differences from the control, with mean values of 5.71, 5.50 and 5.57 mm respectively. Stem length of tomato plants inoculated with CEP-724 stood out with a mean of 33.54 cm followed by treatments inoculated with CEP-723 and CEP-726, with mean values of 32.84 and 32.44 cm respectively. Regarding aerial weight, the treatment with CEP-726 presented 12.15 g, the highest mean value (figure 4, page 82).

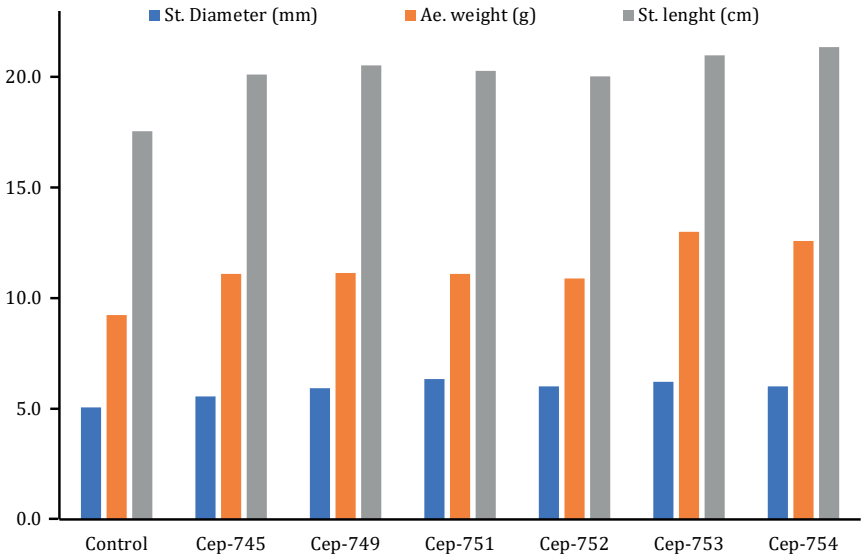


Figure 3. Mean values of stem diameter, aerial weight and stem length of tomato plants (*S. lycopersicum*) inoculated with spore suspensions of *Trichoderma* isolates in growth promotion assays.
Figura 3. Medias registradas en el diámetro de tallo, peso aéreo y longitud de tallo de plantas de tomate (*S. lycopersicum*) inoculadas con suspensiones de esporas de aislados del género *Trichoderma* en los ensayos de promoción del crecimiento.

Morphological characterization, molecular identification and phylogenetic Analysis of the isolates CEP-722, CEP-723, CEP-753 and CEP-754

Figures 5D, 5E and 5F (page 82), show microscopic traits of the isolates CEP-722 and CEP-723. Conidiogenesis occurs in a dense hymenium; conidiophores branch repeatedly at wide angles resembling candelabra; conidiogenous cells are clavate or cylindrical, with a rounded to conical apex, no obvious neck; the apical wall thickens progressively as conidia are produced in long chains, adhering laterally to form prismatic (palisade) columns. The CEP-722 isolate was the only one presenting chlamydospores (figure 5F, page 82). Microscopic measurements and morphological traits of CEP-722 and CEP-723 coincide with *Metarhizium* taxonomic keys (18). Microscopic traits of isolates CEP-753 and CEP-754 are hyaline conidiophores, smooth-walled, up to 5 µm wide near the base, gradually tapering to about 2 µm wide near the apex, with relatively conspicuous septa distant; side branches borne at right angles, singly or in whorls of 2-3, gradually increasing in length. Phialides occur in whorls of 2-5, solitary and alternate, or more irregularly arranged, particularly towards the apex of the conidiophore. Terminals are more elongated and generally not constricted at the base. Conidia are unicellular, diluted green in color, smooth-walled, short cylindrical and almost oblong, with obtusely rounded apex (figures 5B and 5C, page 82). Microscopic measurements and morphological traits of CEP-753 and CEP-754 coincide with *Trichoderma* taxonomic keys (5).

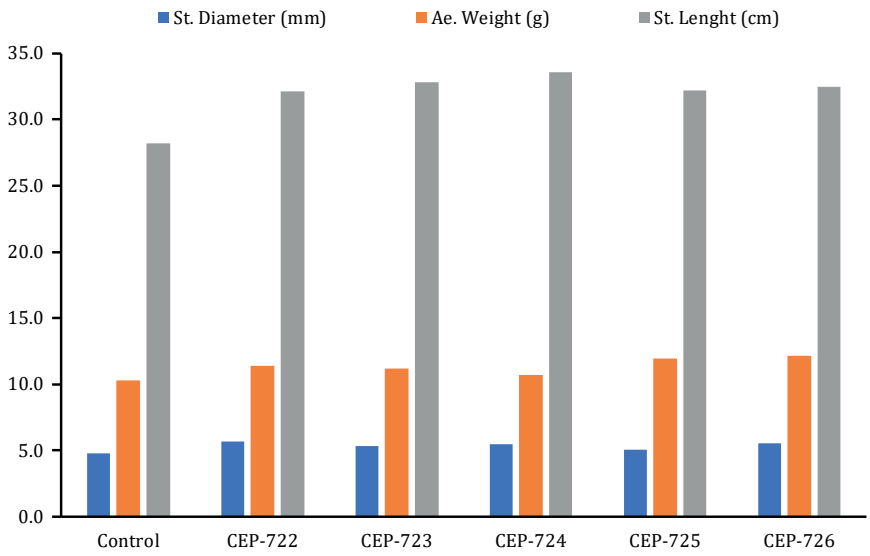


Figure 4. Mean values of stem diameter, aerial weight and stem length of tomato plants (*S. lycopersicum*) inoculated with spore suspensions of *Metarhizium* isolates in growth promotion assays.
Figura 4. Medias registradas en el diámetro de tallo, peso aéreo y longitud de tallo de plantas de tomate (*S. lycopersicum*) inoculadas con suspensiones de esporas de aislados del género *Metarhizium* en los ensayos de promoción del crecimiento.

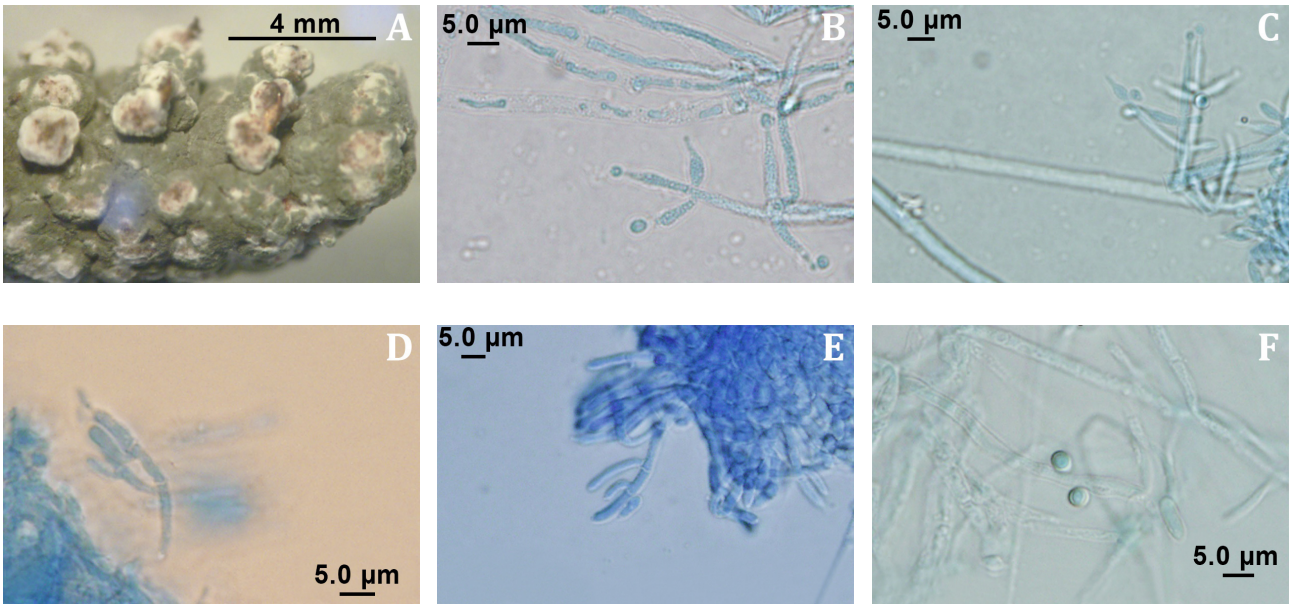


Figure 5. A) Detail of *T. molitor* larva corpse with sporulation of isolate CEP-722; B) Conidiophores, phialides and conidia of isolate CEP-753; C) Conidiophores, phialides and conidia of isolate CEP-754; D) Conidiophores, phialides and conidia of isolate CEP-722, E) Conidiophores, phialides and conidia of isolate CEP- 723; and F) Mycelium, conidia and chlamydospores of the isolate CEP-722.
Figura 5. A) Detalle de cadáver de larva de *T. molitor* con esporulación del aislado CEP-722; B)Conidióforos, fiálides y conidios del aislado CEP-753; C) Conidióforos, fiálides y conidios del aislado CEP-754; D) Conidióforos, fiálides y conidios del aislado CEP-722; E) Conidióforos, fiálides y conidios del aislado CEP-723; y F) Micelio, conidio y clamidosporas del aislado CEP-722.

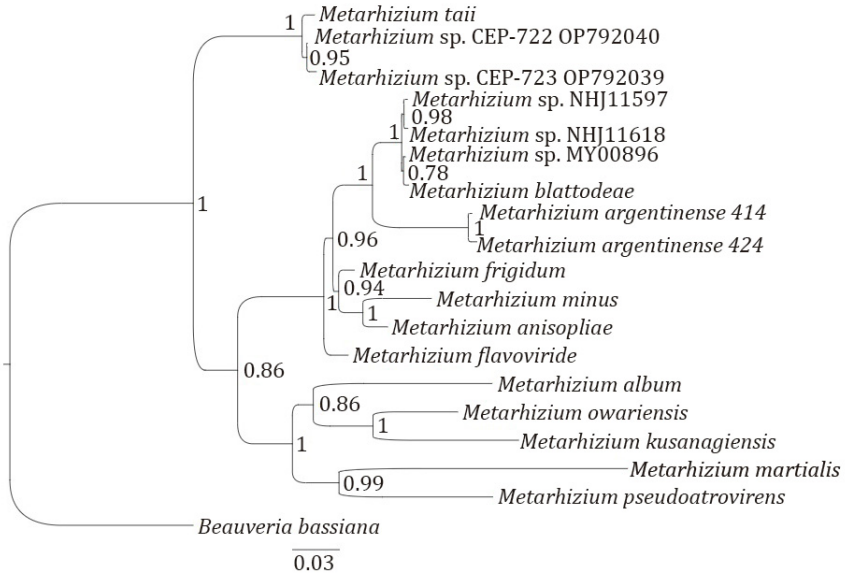
Table 6, shows average measurements of each microstructure.

Isolates CEP-722 and CEP-723 had 100% homology to each other, considering the sequences used for ITS and TEF1α markers of *Metarhizium* spp. Therefore, CEP-722 and CEP-723 correspond to the genus *Metarhizium* and present 0% genetic variability with the species *Metarhizium taii* (Genbank access code ARSEF5714). The taxonomic classification for CEP-722 and CEP-723 with GenBank reference codes ITS: OP709693/TEF1α: OP792040 and ITS: OP709705/TEF1α: OP792039, is Fungi; Ascomycota; Pezizomycotina; Sordariomycetes; Hypocreales; Clavicipitaceae; *Metarhizium* (Sorokin, 1883): *Metarhizium taii*. Isolates CEP-753 and CEP-754 presented 100% homology to each other considering reference sequences used for ITS and TEF1α markers of *Trichoderma* spp. Phylogenetic analysis shows CEP-753 and CEP-754 correspond to *Trichoderma* and present 0% genetic variability with the species *Trichoderma afroharzianum*, Genbank access code GJS04-186/TRS835. The taxonomic classification for CEP-753 and CEP-754 with reference codesITS:OP700049/TEF1α:OP792041andITS:OP709539/TEF1α:OP792042,respectively, is Fungi; Ascomycota; Euascomycetes; Hypocreales; Hypocraceae; *Trichoderma* and *Hypocrea* (Rifai, 1969): *Trichoderma afroharzianum*. Analytical "runs" of the sequences of selected microorganisms were carried out with MrBayes software (version 3.2.7). Databases were prepared according to published references. Figure 6 and figure 7 (page 84) show the phylogenetic trees for CEP-722 and CEP-723 isolates of *Metarhizium* and for CEP-753 and CEP-754 isolates of *Trichoderma*, respectively.

Table 6. Average measurements of reproductive structures of isolates CEP-722, CEP-723, CEP-753 and CEP-754.

Tabla 6. Promedios de mediciones de estructuras reproductivas de los aislados CEP-722, CEP-723, CEP-753 y CEP-754.

Strain	Average conidium size (length/width) (µm)	Average phialide size (length/width) (µm)	Average diameter of chlamydospore (µm)
CEP-722	6.45/2.83	9.12/2.66	3
CEP-723	6.99/3.07	8.76/2.20	-
CEP-753	3.16/2.58	10.24/2.88	-
CEP-754	3.22/2.52	11.04/2.78	-



Node values represent maximum likelihood probabilities.

Los valores entre las diagonales representan el valor de probabilidad de máxima verosimilitud.

Figure 6. Phylogenetic tree of ITS and TEF1α regions of CEP-722 and CEP-723 isolates.

Figura 6. Árbol filogenético de las regiones ITS y TEF1α de los aislados CEP-722 y CEP-723.

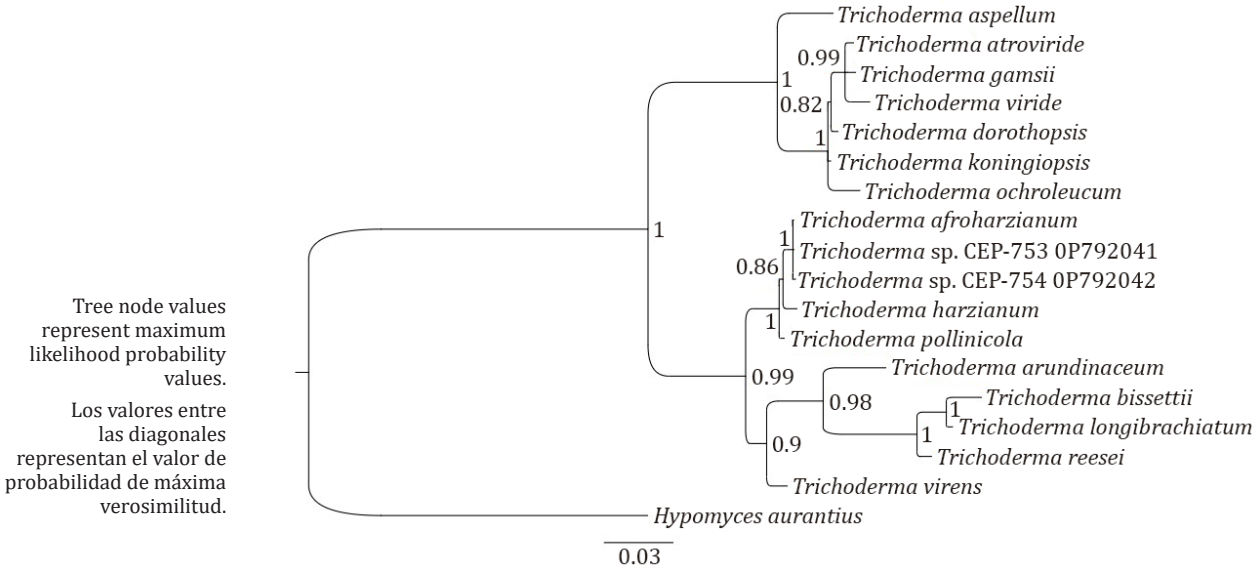


Figure 7. Phylogenetic tree of ITS and TEF1α regions of CEP-753 and CEP-754 isolates.
Figura 7. Árbol filogenético de las regiones ITS y TEF1α de los aislados CEP-753 y CEP-754.

Discussion

Trichoderma spp. and *Metarhizium* spp. isolates evaluated against the phytopathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum* in this study showed varying effects according to strain and isolate, as previously found (16, 34). In our *in vitro* studies, the *Metarhizium taii* strains CEP-722 and CEP-723, and the *Trichoderma afroharzianum* strains CEP-753 and CEP-754 presented different inhibition levels against different phytopathogens. This, because biological control agents of *Trichoderma* use varied mechanisms, like antifungal compounds, competition for nutrients, parasitism or pathogen inhibition, antibiosis, lytic enzymes (23) and systemic resistance (26, 28). Growth promotion of tomato plants (*S. lycopersicum*) inoculated with spore suspensions of *Metahrhizium* and *Trichoderma* fungi constitutes an important background when designing fertilization strategies in the cultivation of tomatoes (*S. lycopersicum*). The selected strains CEP-753 and CEP-754 of *T. afroharzianum* could be considered for nutritional management of crops. Our results agree with previous studies reporting a growth promotion in tomato plants (*S. lycopersicum*) var. platense inoculated with entomopathogenic fungi (33). Strains CEP-722 and CEP-723 of *M. taii* inhibit *B. cinerea* and *S. sclerotiorum*, in agreement with studies on the interaction of entomopathogenic and phytopathogenic microorganisms (11). Employing indigenous microorganisms could be a promising alternative to external inoculants, potentially reducing production costs and without introducing foreign microorganisms into the environment (6).

Conclusion

Metarhizium taii strains CEP-722 and CEP-723 and *Trichoderma afroharzianum* CEP-753 and CEP-754 were best candidates as biological control agents against *Botrytis cinerea* and *Sclerotinia sclerotiorum*. These strains constitute valuable tools for disease management and interesting ingredients for nutritional management of tomato (*S. lycopersicum*).

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