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Research Article

Genetic Polymorphisms of Seven *Trichoderma Spp* Strains, their Potential as Biological Control Agent and Growth Promoter in Tomato

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ABSTRACT

Currently, many conventional methods for crop management have been insufficient to cope with the effects of climate change on food production such as droughts, heat, soil nutrient deficiency, and the occurrence of new pathogen strains. Utilizing biological inputs, such as *Trichoderma spp*, has proved useful in improving crop production and food safety. However, in Central American countries there is not enough expertise to accelerate, in a practical way, its use on a broader scale. Here, we report for the first time the study of a collection of *Trichoderma spp* strains from Nicaragua and Honduras with the purpose of analyzing their genetic diversity and their potential as biological control agents and growth promoters in tomatoes. Genetic diversity was estimated by sequencing the nuclear ribosomal Internal Transcribed Spacer (ITS) region. After, bifactorial experiments for testing the potential of two strains (TN1C and TC01) and two structures (conidia and microsclerotia) for controlling a harmful strain of *Fusarium solani* were conducted, followed by bifactorial experiments, considering the same factors, but analyzing their effects as a growth promoter in greenhouse conditions. The DNA sequences amplified from ITS regions (1–F and 4) indicate that there are two species, *T. asperellum* and *T. harzianum*, instead of one, consistent with morphological observations. Bayesian and parsimony modeling clustered *Trichoderma* strains by species providing novel insights about phylogenetic relationships and nucleotide polymorphisms. The strains TN1C and TC01 showed a reduction in the percentage of damage caused by *F. solani*. In addition, these strains increase the percentage of seed germination, plant height, stem diameter, and the number of leaves in tomato seedlings and plants. Root length and volume increased only in seedlings. Microsclerotia had a better performance with respect to conidia on plant growth development. These results strengthen ongoing research projects and incipient biological control programs oriented to benefit tomato farmers.

Keywords: Biological control; Conidia; DNA sequencing; Microsclerotia; *Trichoderma spp*.

ABBREVATIONS

HA: Hot Air; HW: Hot Water; RH: Relative Humidity; MEA: Malt Extract Agar.

INTRODUCTION

The improvement of crop management techniques has been a constant challenge for agricultural scientists since the Green Revolution. Even though the global statistics traced between 1997 to 2017 suggest that there has been significant progress in increasing yields of economically important crops such as maize, rice, soybean and wheat (FAOSTAT, 2019), conventional methods and technologies have not succeeded in coping with recent variable climate events, causing alarm in many food systems [1,2]. The Intergovernmental Panel on Climate Change reported that temperature increases are expected to accelerate to 1.5°C between 2030 and 2052, affecting habitats of around 6% of insects and 8% of plant species [3]. When high temperature coincides with abnormal intervals of rain precipitations, damages and losses on field production are even more profuse. Additionally, under that combination of factors, the dynamics of pest and disease populations change, inverting levels of incidences and promoting the occurrence of more severe strains without any possibility of control because of lacking previous experiences [4,5]. Chemical

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control by means of using synthetic pesticides has provided a feasible solution for crop protection in economic terms, but its continued and increased use threatens food safety and contributes to creating resistance in pests and pathogens over time [6]. Thus, it is important to identify novel alternatives that fulfil this purpose, but with an integrated crop management vision. In this sense, the development and utilization of biological inputs in agriculture has been identified as one of the eight principles of integrated pest management [7]. These compounds, based on the use of beneficial microorganisms, have received special attention in recent years because its impacts and practicality for small and even large farming systems. The endophytic fungi species from the genus *Trichoderma* encompasses several species with variable biotechnological applications, being the most important its use as biological agent for the control of a diversity of plant pathogens [8]. In nature, *Trichoderma* acts as symbiont, colonizing plant root systems and secreting proteins that interact with host genome, this relationship induces significant effects on plant health and production [9]. Numerous studies demonstrate the multiple benefits of inoculating crop plants with *Trichoderma spp*. strains; among them the induced tolerance to abiotic stresses such as soil salinity, water scarcity, anoxic conditions and heat shock [10,11]. The presence of *Trichoderma* in roots induces the up regulation of genes and certain pigments expressions that improve photosynthetic capability of plants by activating biochemical pathways that reduce reactive oxygen species to less destructive molecules [12]. Also, this symbiosis optimizes nutrient uptake and consumption by increasing root density and providing tolerance to diverse plant pathogens [13-17]. In the Central American countries, some projects have taken advantage of the existing knowledge concerning *Trichoderma* to propose practical solutions to many challenges in small-scale agriculture. Currently, seven strains have been suggested for agronomic management of tomato. However, the lack of scientific basis on those proposals has reduced the possibilities of scaling up to larger areas, limiting the impacts on tomato production. Most of the strains produced at laboratory level and used on fields are classified as *Trichoderma* harzianum, but there is no certainty of the real taxonomic identity of the species using molecular tools. Also, the antagonist level of those strains in the presence of important vegetable pathogens such as *Fusarium solani* has not been tested before, followed by robust greenhouse experiments that demonstrate the additional effects as growth parameters. The present study aimed to analyze the genetic diversity and phylogenetic relationships among seven *Trichoderma spp*. strains used in Central America, to evaluate the antagonist level before a *F. solani* strain and the effects as growth promoter in tomato.

MATERIALS AND METHODS

Trichoderma strain selection

Seven *Trichoderma spp* strains: TZ01, TC01, TC02, TN1A, TN1B, TN1C y TN2C from Zamorano University, Honduras were selected for this study, considering their availability and its significance as product in Central America. DNA from eight additional strains: R5BF2M2, TRIIIFIM2, R6CREF4M5, TRIIF3M2, R5KHFIM5A, R5TFIMI, R5IPF2MIB, and MMRI from the Nicaraguan Institute of Agricultural Technology (INTA, Spanish acronym) were included in molecular characterization as well as an isolate of *F. solani* collected and conserved by the Laboratory of Plant Pathology, Diagnosis and Molecular Research of Zamorano University. This strain of *F. solani* was stated as outgroup and quality control of the sequences in the phylogenetic tree.

Molecular characterization

Molecular characterization was performed in the Molecular Biology Center of the University of Central America.

DNA extraction and PCR amplification

The DNA of *Trichoderma spp* and *F. solani* strains from Zamorano were extracted from pure mycelium cultures, using the Wizard® Promega "Genomic DNA Purification Kit" with modifications of the factory protocol. DNA samples of strains from INTA were kindly provided by that institute. After DNA extracting, quality (260/280) and concentrations (µg/mL) with 320 corrections were estimated using a PerkinElmer *Lambda* 25 brand spectrophotometer. DNA integrity was measured by electrophoresis in 0.75% agarose gel stained with 0.5 µg/mL of ethidium bromide [18-20]. Primers ITS1–F, ITS4 and ITS4B were used for DNA amplification. PCR reactions were performed using a Phusion High-Fidelity PCR Master Mix with HF Buffer following manufacturer protocol at quarter of reaction (Thermo Fisher Scientific, n.d.-b). The thermocycling conditions were: initial denaturation at 98 °C for 30 seconds (s), 35 cycles of 98°C for 5 s, 53°C for 30 s and 72°C for 30 s, and a final extension of 72°C for 7 minutes (min). The product band uniformity, integrity and expected size were confirmed in 2% agarose gel stained with ethidium bromide at a concentration of 0.5 µg/ml. Dimers, oligos and any residual component were removed of the reaction by adding 0.4 µL of distilled and autoclaved water, 0.4 µL of rSAP and 0.2 µL of Exo I for each 4 µL of PCR product. The thermal cycler set up consists of 30 min hold at 37°C to activate the enzyme and 15 min at 85°C to denature it [21,22].

Capillary electrophoresis and analysis of sequencing electropherograms

Sequencing samples were prepared for cycle sequencing in 5.5 µL reactions, composed of 0.75 µL of Big Dye Terminator v3.1, 3 µL of distilled and autoclaved water, 0.75 µL of primers, either forward (ITS 1–F) or reverse (ITS 4) and 1 µL of clean PCR product. Reaction conditions were subjected to the manufacturer thermocycling protocol with minor modifications consisting of 30 cycles of 96°C for 10 s, 50°C for 15 s and 60°C for 180 s [22]. Prior to sequencing, the DNA pellet of the previous reaction was precipitated and cleaned using the EDTA protocol for BDT version 3.1 with minor variants [22]. Each PCR product (5.5 µL) was mixed with 1.25 µL of EDTA and 15 µL of 100% ethanol, vortexing at 1,000 revolutions per minute (rpm). Subsequently, the total mixture was transferred to a 1.5 mL microcentrifuge tube and incubates at room temperature for 15 min. Then, it was centrifuged at 18,985 relative centrifugal force (rcf) and 4°C for 15 min, discarding the supernatant and with a spin down at 14,000 rpm for 10 s. Samples were cleaned by adding 15 µL of 70% ethanol and centrifuged in the same way at 18,985 rcf for 15 min, discarding the supernatant and letting it dry for 15 min at room temperature. Finally, 10 µL Hi-Di Formamide was added to the bottom of the tube mixing by lightly pipetting. DNA sequencing was performed using a SeqStudio ABI 3200 machine (Applied Biosystems). In the plate, 10 µL of the previous mix for each sample were added and the run conditions were as manufacturer configuration. Initially, the quality of electropherograms of the sequences were analyzed in Sequencing Analysis Software version 6. Then, sequences were trimmed and detail viewed in the program Sequencing Scanner

version 2. Variant Reporter Software version 2 was used to review discrepancies between both, forward and reverse sequences, from the same sample replicates. These three software packages are integrated into SeqStudio ABI 3200 machine. Sequences were aligned in MEGAX creating a unique FASTA sequence per sample [23]. Unique sequences were blasted in the nucleotide database of NCBI (National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), The Barcode of Life Data Systems (BOLD) (http://www.boldsystems.org/index.php/IDS_OpenIdEngine) and TrichOkey (http://www.isth.info/tools/molkey/) in order to identify the species.

Phylogenetic modelling

Files containing DNA sequences previously aligned and edited were converted into*.nex format for phylogenetic analyses. A heuristics searching for best model was conducted by means of Computation of Likelihood Scores and Akaike Information Criterion (AIC) calculations using software JModelTest version 2.1.10 [24,25]. The best model was used to set up parameters for Bayesian analyses, MCMC (Markov Chain Monte Carlo) runs, applying 1,000,000 generations, a frequency of sampling of 100, four runs and chains respectively, all these analyses were performed using software MrBayes version 3.2.7b [26]. Additionally, a bootstrapping with 3,000 pseudo-replicates was performed followed by a simple search with 3,000 repetitions by Parsimony. This analysis was conducted using software POY version 5 [27].

In vivo experiments

***In vivo* antagonism tests using a *F. solani* strain:** Previously, the seven *Trichoderma* strains were confronted by means of *in vitro* dual tests against *F. verticilloide*, *F. delphinoide* and *F. solani* strains (data not shown). After those tests, the most promising strains *T. harzianum* (TN1C) and *T. asperellum* (TC01) were chosen to set *in vivo* evaluations against a *F. solani* strain which behaved as the most harmful in previous evaluations using tomato plants. For the production of conidia and microsclerotia of *Trichoderma spp*, a liquid medium was prepared containing: sucrose, yeast extract, KH_2PO_4, $CaCl_2$ and $MgSO_4$ that was inoculated with the strain from pure culture. The inoculum was stirred at 250 rpm for 72 h for the production of conidia and 120 h for the production of microsclerotia. For the production of conidia of *F. solani*, the inoculum was prepared in Sabouraud liquid medium, by mixing it with pure culture (168 h old). This mixture was stirred at 250 rpm for 72 h. After, 400 mL of *F. solani* suspension with a minimum concentration of 9×10^6 conidia mL^{-1} were applied per each kg of substrate seven days before seedling transplanting. During seedling transplanting, 250 mL of conidia and 250 mL of microsclerotia of strains TN1C and TC01 were applied at a concentration of 1×10^6 conidia mL^{-1} and 1.3×10^5 microsclerotia mL^{-1}, respectively. The experiment was established in pots with a diameter of 18 cm, a depth of 15 cm and a capacity volume of 2 kg. One seed per pot was sown. The substrate used was composed of soil, compost and rice husks (2:1:1) and sterilized with steam. Fertilizer 18-46-0 (N, P, K) was applied seven days after transplanting and 0-0-60 30 days after (3 g per pot). Weeds were manually removed every seven days. Irrigation was applied with an interval of 24 h to maintain humidity at field capacity. No disease control was conducted. A bi-factorial experiment (2 × 2) was established in a Randomized Complete Block Design (RCBD) with four repetitions and four plants per repetition for a total of 96 experimental units. The treatments evaluated correspond to the combination of two *Trichoderma* strains: TN1C (*T. harzianum*) and TC01 (*T. asperellum*) and two reproductive structures (conidia and microsclerotia), a positive (only *F. solani*) and a negative (sterile water) control were included. At 41 days after transplanting, the percentage of incidence and severity of diseases were measured.

***In vivo* growth promoter tests:** The experiment was stablished in 50-celled trays, sowing one tomato seed per cell, each tray was considered a repetition. The substrate used was based on soil, compost and rice husk (in a ratio 2:1:1), and sterilized with steam. In this trial, no nutrition or disease control management were performed. Weed control was carried out manually every seven days. Water applications were carried out 2-3 times per day to keep humidity at field capacity. The production of conidia and microsclerotia of both *Trichoderma* species was conducted in the same way as describe in previous tests. Prior to sowing the seed, 250 mL of the treatments were applied per tray at a concentration of 1×10^6 conidia mL^{-1} and 1.3×10^5 microsclerotia mL^{-1}, respectively. A bi-factorial experiment (2 × 2) was established in a RCBD with three repetitions for a total of 15 experimental units. The treatments evaluated correspond to the combination of two *Trichoderma* strains: TN1C (*T. harzianum*) and TC01 (*T. asperellum*) and two reproductive structures (conidia and microsclerotia). A negative control (sterile water) was also included. The number of germinated seeds was counted during the first 10 days after sowing. To determine the germination percentage (GP), the following formula was used:

$$GP=[\ n/N\ ] \times 100$$

Where "n" is the number of normal seedlings and "N" is the number of established seeds. At 27 and 41 days after sowing, the plant height (cm) was measured from the base of the stem to the youngest leaf of the plant; stem diameter (mm) was measured at 1 cm from the base of the plant using a vernier caliper; and number of total leaves of five random seedlings per repetition for a total of 15 seedlings per treatment. At 41 days, the root system of five random seedlings per repetition (15 seedlings per treatment) was evaluated, using the WinRHIZO® program where the root length (cm) and volume (cm^3) were estimated.

Statistical analyses: The analyses of the variables disease incidence and severity were merely descriptive. On the other hand, variables: plant height, stem diameter, number of seedling leaves, root length and root volume of plants were analyzed through Analysis of Variance (ANOVA). The percentage of germination, plant height, stem diameter and number of leaves were also analyzed in repeated measures over time, using the program Statistical Analysis System (SAS version 9.4®). For the variables that found significant differences, the means comparisons were conducted by Duncan's test (α=0.05).

RESULTS

Species identification and phylogenetic relationship among strains

The DNA amplification using ITS1–F and ITS4 primers had better yield and a single band in the ~600–650 bp range. On the other hand, the combination ITS1–F and ITS4B amplified with multiple non-specific bands. This was expected since ITS4B is

specific for Basidiomycota fungal samples. KB basecaller generates quality values from 1 to 99 per base and overall sequence, typical high quality mixed bases will have QV 10 to 50 and high quality pure bases will have QV 20 to 50. Only sequences with quality values of 30 (99.9% of confidence) at minimum were accepted in the downstream bioinformatic processes. The minimum overall sequence quality value was 34 (>99.9%) and maximum was 50 (99.999%) in all replicates per sample. Blast results in NCBI nucleotide collection as well as BOLD provided score values higher than 99.9% and E-values of zero at species level identification. In the oligonucleotide barcode program TrichOkey the identification reliability was high for all FASTA sequences submitted, which give a high level of certainty that each of the species was assessed correctly, resulting in two species, *Trichoderma asperellum* (10 strains) and *T. harzianum* (five). Most *Trichoderma* strains from INTA were classified as *T. asperellum* with exception of MMRI identified as *T. harzianum*. In contrast, strains from Zamorano were classified in both species. TC01, TC02 and TZ01 were *T. asperellum* while TN2C, TN1B, TN1C and TN1A were *T. harzianum*. The differences between these two species were located at 64 different nucleotide (nt) positions. Bayesian parameters for simulations were set up using model F81+G (lset snt=1, rates=gamma) obtained from heuristics searching. Both, Bayesian and Parsimony analyses conducted using haplotypes showed similar results, clustering strains within identified species. The results obtained from phylogenetic analyses are showed in (Figure 1A). Cluster I includes *T. asperellum* strains: TC01, TC02, R5BF2M2, TRIIIFIM2, R6CREF4M5, TRIIF3M2 and reference for *T. asperellum* (101201). Four *T. asperellum* strains: TZ01, R5KHFIM5A, R5TFIMI and R5IPF2MIB did not cluster at this branch due to a thymine (T) deletion at 180 nt and transversion of T to guanine (G) at 438 nt (Figure 1B). Consequently, they are shown as a sub-group within Cluster I with Bayesian posterior probability of 100 and a maximum parsimony bootstrap value of 1. Cluster II encompasses reference strains for *T. longibrachiatum* (5548) and *T. citrinoviride* (58853) species, strongly supported (100/0.99). Cluster III gathered *T. harzianum* strains TN2C, TN1B, TN1C, MMRI, TN1A and reference for *T. harzianum* species (5544) with Bayesian posterior probability of 100 and a maximum parsimony bootstrap value of 0.99. At this cluster, MMRI showed a deletion of T at 144 nt and TN1C exhibited a transversion of G to cytosine (C) at 576 nt. Finally, reference sample of *F. solani*, used as outgroup to identify the tree root was clearly classified as cluster IV. References sequences were included to robust phylogenetic inference.

In vivo antagonism tests using a *F. solani* strain

The incidence and severity data obtained at 41 days after transplanting showed that in general the use of *Trichoderma* strains effectively reduces the damage of wilt disease caused by *F. solani* in comparison with the positive control. The incidence of the disease in the positive control was 100%, which indicates that there was a successful inoculation. There were differences between *Trichoderma* strains, but not for reproductive structures. The incidence in *Trichoderma* treatments was 28.13% (strain TC01 (*T. asperellum*)) and 53.13% (strain TN1C (*T. harzianum*)), which would mean a decrease of 71.87% and 46.87% with respect to the positive control. The negative control (without *F. solani* inoculum) presented an incidence of 37.50%. The positive control presented the highest percentage of damage caused by *F. solani* in tomato plants with 41.25%, which was different with respect to *Trichoderma* treatments (7.50% for TC01 (*T. asperellum*) and 14.38% for TN1C (*T. harzianum*)) and the negative control 12.50%. The application of TC01 (*T. asperellum*) decreased the severity of the disease by 33.75% and the application of TN1C (*T. harzianum*) by 26.87%. A summary of these qualitative results is presented in (Table 1).

In vivo growth promoter tests

Statistical analysis on the germination percentage showed that there was a significant difference only in the strain factor (P=0.0001) and time of counts (P<0.0001). Although the germination percentage of these seeds was low, probably due to seed aging, it is evidenced that the use of *Trichoderma* increases the seed germination percentage between 17.60% (TN1C (*T. harzianum*)) and 18.85% (TC01 (*T. asperellum*)) with respect to the control (Table 2; Figure 2). The results obtained from growth variables at 27 days after sowing showed that the strains had a significant difference in the three variables. The use of *Trichoderma* regardless of the strain promotes an increase of plant height (TC01 (*T. asperellum*) 5.11 cm and TN1C (*T. harzianum*) 4.88 cm), stem diameter (TC01 (*T. asperellum*) 2.18 mm and TN1C (*T. harzianum*) 2.12 mm) and number of leaves (TC01 (*T. asperellum*) 2.86 and TN1C (*T. harzianum*) 2.93) (P=0.0001; P=0.0104; P<0.0001, respectively). The reproductive structure showed a significant difference in the variables plant height (P=0.0100) and number of leaves (P=0.0304). There were no significant differences in the species × reproductive structure interaction. The use of microsclerotia (5.30 cm) had a better performance increasing 1.42 cm the seedling height with respect to the control (3.88 cm) and 0.60 cm with respect to the use of conidia (4.70 cm). In the case of leaf production, the use of both conidia (2.80 leaves per seedling) and microsclerotia (3.00 leaves per seedling) increased leaf production between 0.27 and 0.47 leaves per seedling in relation to the control (2.53) (Table 3). The results of the biometric variables, at 41 days after seedling transplanting, showed that the use of *Trichoderma* has an effect on the growth of the crop in plant height (TC01 (*T. asperellum*) 34.50 cm and TN1C (*T. harzianum*) 34.55 cm), stem diameter (TC01 (*T. asperellum*) 6.21 mm and TN1C (*T. harzianum*) 6.19 mm) and number of leaves ((*T. asperellum*) 9.39 and TN1C (*T. harzianum*) 9.43 leaves per plant) compared to controls. The reproductive structure has a minor influence, showing a significant difference only in the plant height (P=0.0002), being the conidia (34.98 cm) statistically superior with respect to the microsclerotia (34.07 cm) and the control (32.69 cm) (Table 4). The interactions species × reproductive structures over time showed significant differences in plant height (P<0.0001) and diameter (P=0.0018). In these variables, the treatments began to differ after 13 days showing that the interactions that involve conidia as a reproductive structure (*T. harzianum*+conidia and *T. asperellum*+conidia) have a better performance compared to microsclerotia in time. The results from the variables of the root system at 28 days after seedling transplanting showed significant differences for strains and reproductive structure in all variables, but not so for the interaction species × reproductive structure. In plants 41 days after seedling transplanting, there was no significant difference in any variable, that is, the application of these *Trichoderma* strains and its reproductive structures did not have an effect on tomato root development. The use of Trichoderma promotes root growth by increasing the length of roots by 70.95% (TC01, *T. asperellum*, 278.27 cm) and 68.87% (TN1C, *T. harzianum*, 274.88 cm) and root volume by 60.00% (TC01, *T. asperellum*, 0.32 cm^3) and 45.00% (TN1C, *T. harzianum*, 0.29 cm^3) with respect to the control (162.77 cm of root length and 0.20 cm^3 of root volume) (Table 5; Figure 3). Regarding the reproductive structure, the effect of microsclerotia is statistically different in root length (323.71 cm)

and volume of roots (0.33 cm^3) increasing by 41.08% and 22.22%, respectively compared to conidia (length 229.44 cm and volume 0.27 cm^3) and with the control (length 162.77 cm and volume 0.20 cm^3) (Table 5).

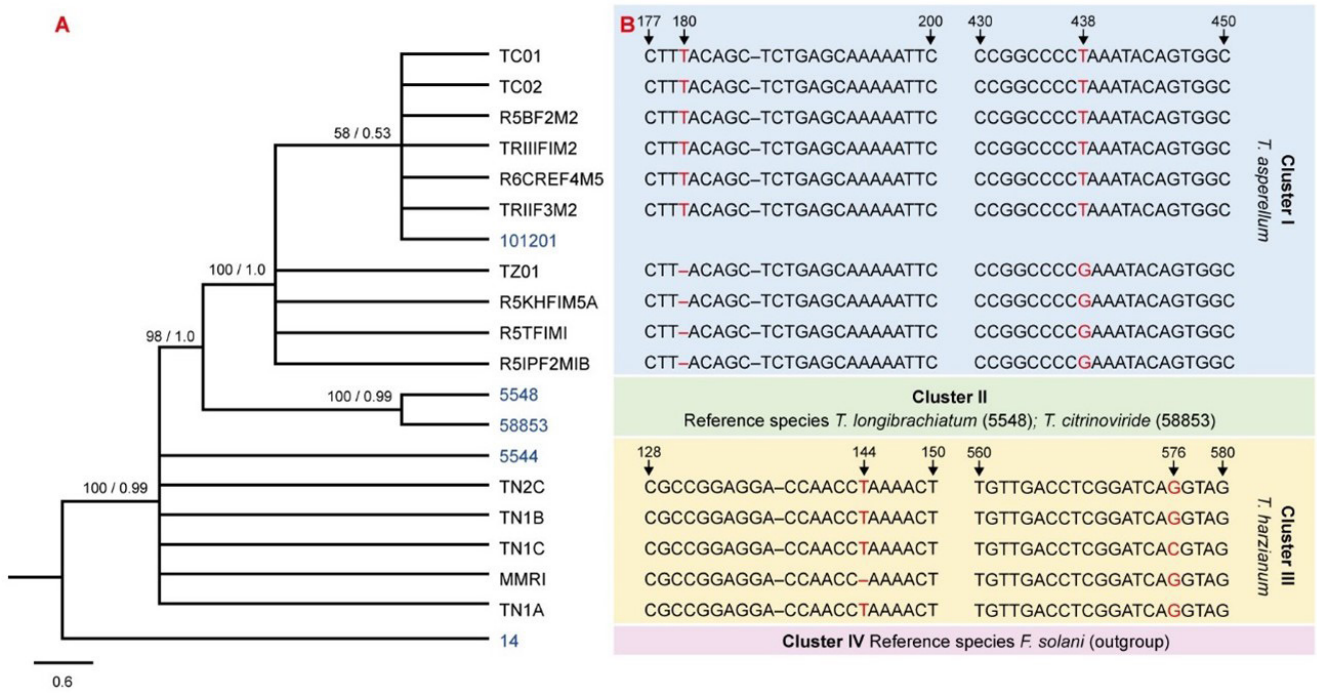

Figure 1: A: Phylogenetic tree inferred from ITS (1F and 4) sequences. Bayesian posterior probability values are followed by maximum parsimony bootstraps, both values are shown in each branch. Taxa in blue are reference sequences for identified species. The bar represents 0.6 substitutions per site. B: Nucleotide polymorphisms detected within each species (red coloration).

Table 1: Effect of *Trichoderma spp* strains TC01 and TNC1 on the incidence and severity of *F. solani* in tomato plants.

Treatment	Variables	
	Incidence (%)	Severity (%)
TC01 (*T. asperellum*)	28.13	7.50
TN1C (*T. harzianum*)	53.13	14.38
Negative control	37.50	12.50
Positive control	100	41.25

Table 2: Effect of two strains of *Trichoderma* (TN1C and TC01) and two reproductive structures (conidia and microsclerotia) on the germination of tomato seeds.

Treatments	Germination (%)z*
TN1C (*T. harzianum*)	41.77 ± 2.87 a
TC01 (*T. asperellum*)	43.02 ± 4.13 a
Control	24.17 ± 3.56 b
P value (model)	<0.0001
Pr>Strain (S)	0.0001
Pr>Reproductive structure (RE)	0.1658
Pr>Time (T)	<0.0001
Pr>Interaction (S × RE × T)	0.5914
R2	0.6440
CV (%)	36.7628

Note: z*Means in columns with the same letter are not significantly different at the significance level of P ≤ 0.05 according to Duncan's test.

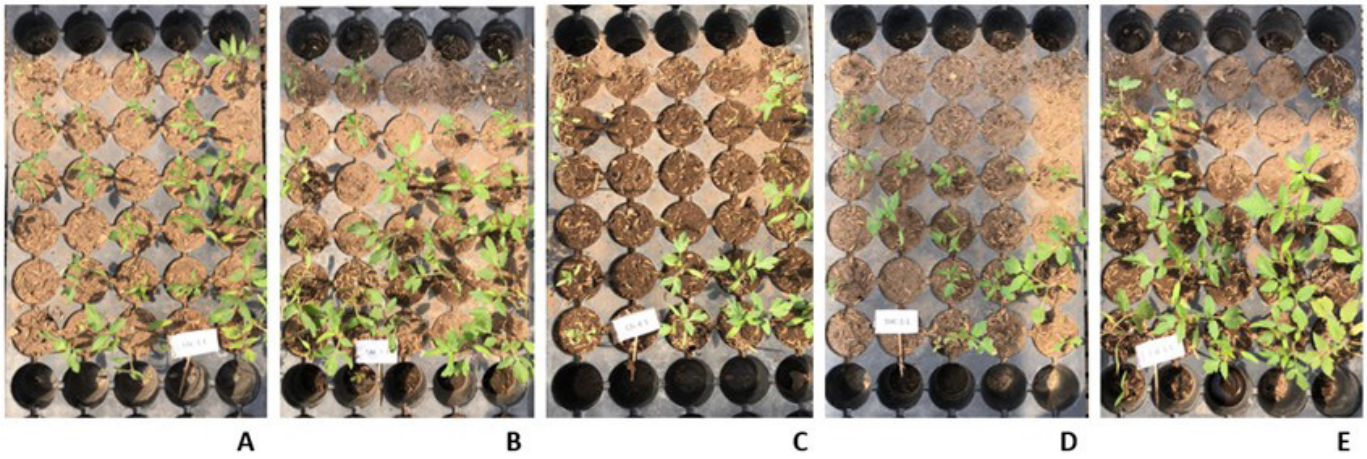

Figure 2: Germination of tomato seedlings with application of two *Trichoderma spp* strains and two reproductive structures. A: TC01 (*T. asperellum*)+conidia; B: TC01 (*T. asperellum*)+microsclerotia; C: Control; D: TN1C (*T. harzianum*)+conidia; E: TN1C (*T. harzianum*)+microsclerotia.

Table 3: Effects of two strains and two reproductive structures of *Trichoderma spp* on plant height stem diameter and number of leaves in tomato plants 27 days after sowing.

	Variables		
Treatment	**Plant height (cm) z***	**Stem diameter (mm) z***	**Number of leaves z***
Strain			
TN1C (*T. harzianum*)	4.88 ± 0.19 a	2.12 ± 0.06 a	2.93 ± 0.08 a
TC01 (*T. asperellum*)	5.11 ± 0.17 a	2.18 ± 0.04 a	2.86 ± 0.06 a
Control	3.88 ± 0.20 b	1.70 ± 0.06 b	2.53 ± 0.13 b
Reproductive structure			
Conidia	4.70 ± 0.15 b	2.07 ± 0.04 a	2.80 ± 0.07 a
Microesclerotia	5.30 ± 0.19 a	2.23 ± 0.06 a	3.00 ± 0.06 a
Control	3.88 ± 0.20 c	1.70 ± 0.06 a	2.53 ± 0.13 b
Pr>Strain (S)	0.0001	0.0104	<0.0001
Pr>Reproductive structure	0.0100	0.0658	0.0304

Note: z*Means in columns with the same letter are not significantly different at the significance level of P≤ 0.05 according to Duncan's test.

Table 4: Effects of two strains and two reproductive structures of *Trichoderma spp* on plant height stem diameter and number of leaves in tomato plants 41 days after sowing

Treatment	Variables		
	Plant height (cm)z*	**Stem diameter (mm)z***	**Number of leavesz***
Strain			
TN1C (*T. harzianum*)	34.55 ± 1.90 a	6.19 ± 0.23 a	9.43 ± 0.50 a
TC01 (*T. asperellum*)	34.50 ± 1.89 a	6.21 ± 0.23 a	9.39 ± 0.50 a
Control +	32.71 ± 2.65 b	5.94 ± 0.32 b	9.28 ± 0.71 ab
Control -	32.67 ± 2.54 b	5.80 ± 0.30 b	9.00 ± 0.68 b
Reproductive structure			
Conidia	34.98 ± 1.92 a	6.27 ± 0.23 a	9.44 ± 0.50 a
Microesclerotia	34.07 ± 1.86 b	6.14 ± 0.23 a	9.39 ± 0.50 a
Control +	32.69 ± 1.83 c	5.87 ± 0.22 a	9.14 ± 0.49 a
Pr>Strain (S)	<0.0001	<0.0001	0.0413
Pr>Reproductive structure	0.0002	0.0504	0.7182

Note: z*Means in columns with the same letter are not significantly different at the significance level of P≤ 0.05 according to Duncan's test

Table 5: Effects of two strains and two reproductive structures of *Trichoderma spp* on root length (cm) and root volume (cm^3) in tomato plants 28 days after sowing.

Treatment	Variables	
	Root length (cm)z*	Root volume (cm^3)z*
Strain		
TN1C (*T. harzianum*)	274.88 ± 18.54 a	0.29 ± 0.01 a
TC01 (*T. asperellum*)	278.27 ± 16.43 a	0.32 ± 0.01 a
Control	162.77 ± 16.58 b	0.20 ± 0.00 b
Reproductive structure		
Conidia	229.44 ± 12.70 b	0.27 ± 0.01 b
Microesclerotia	323.71 ± 17.30 a	0.33 ± 0.02 a
Control	162.77 ± 16.58 c	0.20 ± 0.00 c
Pr>Strain (S)	<0.0001	0.0003
Pr>Reproductive structure	<0.0001	0.0051

Note: z*Means in columns with the same letter are not significantly different at the significance level of P≤ 0.05 according to Duncan's test

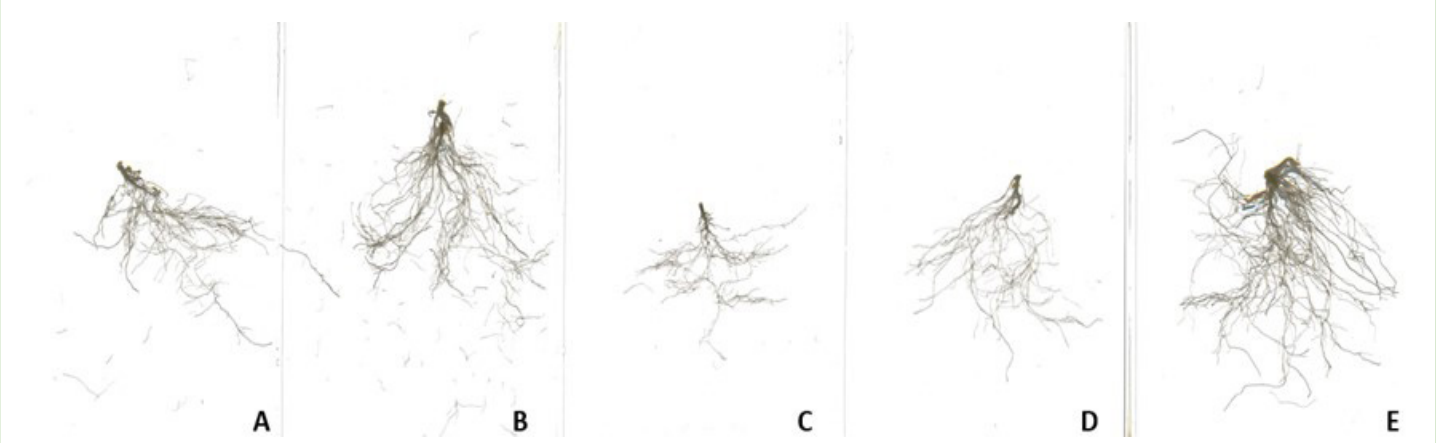

Figure 3: Root development of tomato plants treated with two *Trichoderma spp* strains and two reproductive structures at 28 days after sowing. A: TC01 (*T. asperellum*)+conidia; B: TC01 (*T. asperellum*)+microsclerotia; C: Control; D: TN1C (*T. harzianum*)+conidia; E: TN1C (*T. harzianum*)+microsclerotia.

DISCUSSION

A complete understanding of the taxonomical identity, molecular polymorphisms, antagonism level, and potential as a plant growth promoter of a collection of *Trichoderma spp* strains is essential for any biological control program. Here, we provided key information that could strengthen current strategies at the same time that optimizes inputs use in tomato cultivation.

There are two different *Trichoderma* species with polymorphisms among strains

Most previous efforts have been focused on describing genetic and phylogenetic characteristics of novel *Trichoderma* isolates from different origins and their potential as biocontrol agent in a broad number of pathogen species such as *Alternaria spp.*, *Botryodiplodia theobromae*, *Curvularia sp.*, *Macrophomina spp.*, *Mucor racemosus*, *Candida albicans*, *Plasmopara viticola*, *Pseuperonospo cubensis*, *Rhizopus spp.*, *Sphaerotheca fusca*, *Rhizoctonia spp.*, *Aspergillus spp.*, *Botrytis spp.*, *Colletotrichum spp.*, *Pseudomonas syringae*, *Sclerotinia spp.*, *Xanthomonas campestris*;, *Fusarium spp.*, *Pythium spp.*, *Phytophthora spp.*, *Sclerotium rolfsii* and *Rhizoctonia* [28-38]. But, there are no references of studies that describe the genetic diversity and polymorphisms of strains under production and promotion in tomato crop. According to DNA sequence analyses, strains were classified into two species *T. asperellum* and *T. harzianum*, correcting previous perception of having only one species. Indeed, some reports indicate that several isolates of genus *Trichoderma* have been initially misclassified because of their similarity, scarcity of morphological characters, and lacking molecular tools needed to confirm taxonomic identity [39]. Because *T. harzianun* is the most abundant species in diverse niches, it is very common to attribute any novel isolate to that species. Usually, DNA sequences are compared to reference databases in order to achieve taxonomic identification, a study of the assessment of the main public repositories of DNA Barcode sequences, BOLD and GeneBank, accuracy and reliability outlined that both databases performed comparably for fungi identification, and described BOLD as a curation tool that also stores sequences and GenBank just as a sequence repository with basic quality checks [40]. With the purpose of having a high level species identification confiability, we used a third database repository, TrichOkey, which is a method for molecular identification of *Trichoderma* at the genus and species levels, using a combination of several genus specific hallmarks and species clades specifically allocated within the internal transcribed spacer 1 and 2 (ITS1 and 2) sequences

of rDNA repeat. All top hits from blasts in GenBank and BOLD provided an E-value of 0.0, with expected identity scores higher than 99.9% and were an exact match with TrichOkey results. Hence, DNA sequences obtained using ITS1–F and ITS4 primers were capable of disaggregating strains into two species with high level of confidence (>99.9%) without the need of sampling more genome regions for this purpose. However, it would probably be necessary to analyze at least two additional gene regions to detect more nucleotide polymorphisms for each strain, for traceability purposes or to explore more secondary fungal DNA barcodes such as the stated optimal for secondary DNA barcoding in Ascomycota, DNA topoisomerase I (TOPI) and phosphoglycerate kinase (PGK) [40,41]. All these options are promising for fingerprinting all the *Trichoderma* strains. Phylogenetic analyses suggested a consensus tree (considering both approaches, Bayesian and Parsimony) desegregating strains into two main clusters according to species. This result was expected considering the reduced number of taxa and species. There was no apparent correlation between origin and phylogenetic structure as reported in other studies where geographic origin and host crop influence the genetic structure of *Trichoderma* populations. On the other hand, it was interesting that our results confirmed that strain TZ01 was clearly different from strains TC01 and TC02, something that has been suggested by researchers at Zamorano University based on morphological information (personal observation). Within cluster I, *T. asperellum* strains were divided into two sub-clusters, differences are due to a T deletion at 180 nt and a transversion (T–G) at 438 nt. As these two nucleotide changes were consistent in two groups of *T. asperellum* strains, phylogenetic algorithms conducted in both approaches divided strains into two sub-clusters. On the contrary, in cluster III, strains TN1C and MMR1 showed two separate changes in nucleotide sequences, a T deletion at 144 nt and a transversion (G–C) at 576 nt, respectively. Although, these variations were not significant for sub-branching those taxa during phylogenetic analyses. However, these polymorphisms are useful for strain barcoding. These kind of mutations are reported as the main cause of genetic differentiation within *Trichoderma* species, affecting in some cases gene functionality and exposing sub-clustering in phylogenetic analyses under diverse approaches [42,43]. It was interesting that during the growth of the strains in PDA media, we observed variations in morphology that suggest that more than one strain could be present in the pure culture. Although it was not confirmed in the present study, it is something to consider in future research. This would make a lot of sense if we consider the complex relationships between microorganisms and plants, as studied in sugarcane where the community plays a key role in the success of the symbiosis. This could change the perception of the "genetic purity of the strains" in the future and adopt the concept of "community-based collections" instead [44,45].

Trichoderma spp reduces incidence and severity of *F. solani* on tomato plants

The fungus *F. solani*, causative agent of the wilt disease, is one of the most harmful plant pathogens around the world, acting in some cases in complex with other pathogens, for instance, Pythium spp and Rhizoctonia spp in the damping-off disease in vegetable seedlings. In tomato, wilt disease causes important losses that could be estimated between 20% and 50% depending on environmental conditions, variety and pathogen's strain. The identification of biological alternatives that control *F. solani* is of great importance for the improvement of tomato productivity, its safety and the reduction of production costs, something important under current global context. In this study, the results of incidence and severity of *F. solani*, considering the positive control, demonstrated that *Trichoderma spp* is an effective alternative for reducing the damage caused by this pathogen. The endogenous presence of *F. solani* (37.50%) in the positive control is not surprising since it is a seed-borne pathogen frequently found in seeds [46-48]. However, despite of this, the supply of *Trichoderma spp* improves the health of plants expose to high concentration of inoculum. If we consider another scenario where there is no *F. solani* inoculum on the field, only the one present in the seed, even so, strain TC01 (*T. asperellum*) seems to suppress the incidence of the disease by 9.37% and its severity by 5%, improving the performance of infected seeds in nurseries. Indeed, *Trichoderma* antagonistic effects, such as substrate competition, mycoparasitism, and antifungal antibiotic production, are useful to inhibit the mycelial growth of pathogens [49]. Some hydrolytic enzymes and metabolites could play a key role in controlling Fusarium wilt. Sundaramoorthy and Balabaskar (2013) confirmed that under *in vitro* conditions the isolate ANR-1 (*T. harzianum*) was found to effectively inhibit the radial mycelial growth of this pathogen. In the meantime, under greenhouse conditions, this isolate exhibited the least disease incidence (by 15.33%) in the experiment. It is suggested that *Trichoderma* is able to trigger a long-lasting up-regulation of the salicylic acid pathway even without any pathogen infection, probably stimulating a priming mechanism in the plant [50]. Also, it has been observed the *Trichoderma* can induce genes involved in the jasmonic and ethylene transduction pathways confirmed by microarray and qRT-PCR analyses, suggesting a transitory increment of plant defense [51].

The use of *Trichoderma spp* improves the seed germination and growth of tomato plants

Although the physiological quality of the seed lot used in this study was very low, the use of these seeds in these experiments was an opportunity to test the benefits of using *Trichoderma spp*. It was impressive how *Trichoderma spp*, regardless of the strain, almost doubled the germination of tomato seeds compared with the control. This demonstrates the potential of these strains used as biological seed protectants, which could provide added value to seed inputs that are often very scarce for agriculture based on less use of inputs. Some studies suggest that *Trichoderma spp* could modify mRNA levels of 45 genes, 41 in roots and 4 in leaves promoting more growth in tomato seedlings expressed as an increasing in plant height, stem diameter and number of leaves, root length, dry matter parameters [52,53]. In our experiment, even at 27 days after sowing, the variables plant height, stem diameter and number of leaves was still higher in plants inoculated with *Trichoderma spp* compared to the control, showing that plant growth is effectively promoted beyond germination period. This same trend was observed when analyzing the same variables at 41 days after sowing. In addition of creating a biological protection against pathogens during seed germination, *Trichoderma spp* establishes an effective symbiosis with tomato plants, steadily promoting plant growth due to the stimulation of the secretion of growth hormones. In this respect, Chowdappa et al. (2013) found that the use of *T. harzianum* in tomato seedlings increased the levels of indoleacetic acid by 54.34% and gibberellic acid by 67.59% in the root tissue. That study reported increases in shoot length (32.04%), leaf area (62.68%) and fresh weight of shoots (28.87%) in tomato plants. Similar results were obtained by Nzanza et al., (2011) who evaluated

the performance of tomato seedlings supplied with *T. harzianum* at sowing stage or two weeks after sowing during two years. In that study, the application of *T. harzianum* at both times increased plant height of tomato in 63.41% and 74.29% respectively in 2008 and by 33.67% and 35.65% in 2009, respectively. Other authors report increases in the growth rate of tomato, maize, tobacco, radish [54], beans, cucumber, pepper and certain ornamental species as a result of the application of *Trichoderma* [55-59]. Although not addressed in depth in this study, it is known that *Trichoderma spp* can induce an improvement in photosynthetic capacities in plants [60], promoting greater generation of carbohydrates and therefore increased yields of many crops. These benefits can be further enhanced when added to other management actions that improve the availability of nutrients in the soil and the use of sunlight. The supply of *Trichoderma spp* influenced root growth only in plants 28 days after sowing. The improvement expressed as root length and root volume was similar for both strains, but significantly superior to the control. In average, *Trichoderma spp* strains improve root length by 41.15% and root volume by 34.43% compared with the control. Nzanza et al. (2011) reported that tomato seedlings inoculated with *T. harzianum* increased plant root length by 23.20% when *T. harzianum* was inoculated at planting time and 39.46% when it was applied two weeks after planting obtained similar results. In another experiment, Chowdappa et al. (2013) reported increases of 38.53% in root length and 36.21% in fresh weight of tomato root due to the use of *T. harzianum* compared to the control. Other authors report average increases of 66% in shoots and roots of sweet corn as well as increases of 75% in root length and 100% in root volume in cucumber. The application of *Trichoderma* as a seed treatment colonizes the roots of the seedlings and induces their growth and development, which allows them to reach greater depths of soil, improving the dynamics with the microbiota, tolerance to drought, the performance in compacted soils and the yields [61-63]. However, the host plant, pH, temperature and other microorganisms present in the rhizosphere could influence the growth and development of these populations [64]. For example, Nzanza et al. (2011) reported 90% colonization by *T. harzianum* in tomato seedlings at nursery and 85% in open field conditions. Thus, it is important together with *Trichoderma* to provide a proper crop management that improve the performance of the binomial *Trichoderma*–plant, such as incorporation of organic matter to the soil, crop/varieties rotations, and minimum soil tillage [65-70]. The response of tomato plants to the application of *Trichoderma* was different between the seedling stage and the stages after seedling transplanting. In the early stages of seedlings, *Trichoderma* directly influenced the increase in root length and volume, an effect that did not occur during the productive stages. This behavior agrees with the results obtained by Nzanza et al. (2012) who evaluated the effect of *T. harzianum* on the dry weight of tomato roots in the open field without presenting significant differences with respect to the control in two productive cycles [71-78]. However, Nzanza et al. (2011) previously found an average increase of 53.44% of the dry weight of seedlings roots under greenhouse conditions. This behavior is because plants at early stages of development that is during seed germination and seedling growth, they experience the highest growth rates in their life cycle, and then *Trichoderma* is able to influence significantly the hormone machinery producing the results expressed here and in other studies. The efficiency of the reproductive structure was different depending on the experiments. In the plants that were transplanted, the conidia stood out statistically over the microsclerotia in the interaction over time, as the independent factor. This is contrary to what happened in tomato seedlings at 28 days in which the microsclerotia had a better performance with respect to conidia at plant height. This performance could be due to the effect of environmental factors, in time and space. The temperature and relative humidity values recorded during both experiments (not shown) presented values above those registered in the antagonism experiment (*in vivo*) under greenhouse conditions. This would be reflected in an increase in the evaporation rate. Then, the soil moisture would be affected, creating stress conditions for both the plant and the *Trichoderma* strains, influencing the performance of the reproductive structures. In this case, the microsclerotia showed a better behavior under conditions of water stress compared to the conidia, which agrees with the description by Coley-Smith and Cooke (1971).

CONCLUSION

This first approach provided important insights about genetic diversity, genetic structure and nucleotide polymorphisms among fifteen *Trichoderma* strains which may be of value for biological control programs in Nicaragua and Honduras in order to detect duplicates in the collections, purity testing or traceability. On the other hand, the use of strains TN1C and TC01 effectively reduced the incidence and severity of *F. solani*, a harmful tomato pathogen which produced important economic losses around the world. Also, these strains influenced a positive effect on the growth and development of tomato plants, improving the germination of seeds, and increasing plant height, stem diameter and number of leaves in tomato seedlings and plants. Root length and volume increased only in seedlings. The evaluation of the reproductive structures only showed a better performance at the level of seedlings, being the microsclerotia the one with the best performance than the conidia. These results motivate the use of *Trichoderma* as a key input in tomato nurseries for improving the health and yield of plants. According to climate scenarios, the superior performance of microsclerotia could suggest its use considering the stressing conditions where tomato seedlings could be planted. All these results provided crucial information on the *Trichoderma spp* as a biological control agent, with practical and economic implications for tomato cultivation.

DECLARATION

CRediT authorship contribution statement

Samuel Baca: Conceptualization, Methodology, Formal analysis, Investigation, Writing–Original Draft. Oswalt R. Jiménez: Methodology, Formal analysis, Writing–Original Draft, Supervision. Dorian González: Methodology, Formal analysis, Writing–Original Draft, Supervision on molecular methods. Jorge A. Huete-Pérez: Writing–Review and Editing. Rogelio Trabanino: Writing–Review and Editing, Supervision. M. Carolina Avellaneda: Writing–Review and Editing, Supervision.

Competing interest

Authors express non-competing interests in this study.

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