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Inhibition of *Colletotrichum* spp. causing anthracnose in coffee (*Coffea arabica*) using native isolates of *Trichoderma* sp.

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ABSTRACT

Background/Objective. The objective of the study was to isolate and characterize native isolate of *Trichoderma* from organic crops of Arabica coffee (*Coffea arabica*) in Oaxaca state, as well as to evaluate their *in vitro* biocontrol potential against *Colletotrichum* spp., causal agent of anthracnose.

Materials y Methods. Soil and vegetative material samples were collected from coffee plant plots, from which fungal strains corresponding to the genera *Trichoderma* and *Colletotrichum* were isolated. Macroscopic and microscopic characterization was performed and the growth rate of each of the isolates was evaluated. Finally, molecular characterization was performed by sequencing the ITS region of rRNA. To evaluate the biocontrol potential, antagonism tests were performed between the isolates of the two genera.

Results. Seven different species were identified: *T. harzianum*, *T. pleuroticola*, *T. sulphureum*, *T. tomentosum*, *T. koningii*, *T. spirale* and *T. lentiformе*. The latter were the most abundant. Of these, *T. lentiformе* was selected and evaluated for its *in vitro* inhibition capacity against three *Colletotrichum* spp. It was observed that the growth of the fungus was inhibited by 20 to 80%.

Conclusion. The potential of *Trichoderma* as a biocontrol agent for *Colletotrichum* spp. is highlighted, acting in different ways against this phytopathogen. This contributes to the knowledge about the diversity of native *Trichoderma* species, to the coffee-growing region of the state of Oaxaca. In addition, this deeper knowledge contributes to enriching knowledge and choosing these species for future studies in the biocontrol of phytopathogens, in order to promote sustainable agricultural practices.

Keywords: Antagonist, Anthracnose, Biological Control, ITS

INTRODUCTION

The coffee plant (*Coffea arabica*) is cultivated in more than 80 countries across Latin America, Africa, and Asia (Coutiño *et al*., 2017). In Mexico, it was introduced around 1790 (Medina-Meléndez *et al*., 2016) and has since become a highly significant activity in terms of production, commercial value, and economic and social impact (Santiago-Santiago, 2020). Oaxaca is one of the leading coffee-producing states and a pioneer in organic coffee production (Valkila, 2009). Therefore, the application of bioinoculants with biocontrol properties is a top priority for clean and sustainable agricultural production (Vu and Tran, 2020).

Trichoderma is a cosmopolitan fungus (Herrera-Parra *et al*., 2017) that includes more than 100 species (Savín-Molina *et al*., 2021), some of which have been studied for use as biological control agents (BCAs) against plant pathogens (Kannangara and Dharmarathna, 2017), including *Hemileia vastatrix*, the causal agent of coffee rust (Gomez-De La Cruz *et al*., 2017). The significance of this genus lies in the ability of certain species to colonize roots (Contreras-Cornejo *et al*., 2009), where they compete for space and nutrients (Savín-Molina *et al*., 2021), promote plant growth, and induce systemic resistance (Andrade-Hoyos *et al*., 2019). Additionally, they produce secondary metabolites that protect plants from phytopathogenic fungi through mechanisms such as antibiosis and mycoparasitism (Yassin *et al*., 2022). On the other hand, anthracnose, caused by *Colletotrichum* sp., is one of the most common and significant diseases in coffee cultivation, as it leads to considerable economic losses (Luo *et al*., 2015).

Although *Trichoderma* has been studied as an antagonist for more than 70 years, its commercialization as an agricultural biocontrol agent began in the early 21st century (Pineda-Insuasti *et al*., 2017). Several studies have evaluated its antagonistic effects against *Colletotrichum* in economically important crops such as passion fruit (*Passiflora edulis*) (Niño and Mogollón, 2018), mango (*Mangifera indica*) (Díaz-Medina *et al*., 2019), and strawberry (*Fragaria* × *ananassa*) (Morales-Mora *et al*., 2020), among others. However, there are few studies on the antagonistic effect of *Trichoderma* against *Colletotrichum* in coffee plantations.

Additionally, Arias-Mota and Heredia-Abarca (2022) state that *Trichoderma* is an environmentally friendly biological control option that also supports the maintenance of organic crops. Therefore, the objective of this study was to characterize native isolates of *Trichoderma* spp. from Oaxaca and evaluate their biocontrol potential against *Colletotrichum* spp., the causal agent of anthracnose in coffee plants.

MATERIALS AND METHODS

Isolation of *Colletotrichum* and *Trichoderma* isolates. Soil samples were collected from organically managed *Coffea arabica* plots in the municipalities of San Bartolomé Loxicha, San Agustín Loxicha, and Aguacate Loxicha in the state of Oaxaca. Sampling followed the "five of diamonds" methodology to obtain a composite sample of 100 g of soil per study site (one site per municipality). In the laboratory, samples were processed using the soil dilution method on Petri dishes, following Waksman's (1927) methodology. A stock solution (10 mL sterile distilled water: 1 g soil) was prepared, from which a 10^{-2} dilution was obtained. A 0.1 mL aliquot was evenly spread onto Petri dishes

containing Potato Dextrose Agar (PDA). The plates were incubated at room temperature (25 °C) for 72 h, after which the number of fungal colonies formed (15) was quantified. Individual fungal colonies (12) were isolated from each plate to observe their colonial morphology and reproductive structures, enabling genus-level identification (Barnett and Hunter, 1998). Isolates with morphology similar to *Trichoderma* were purified by hyphal tip culture on PDA. Finally, the isolates were preserved in glycerol until further reactivation.

Simultaneously, in commercial organic *Coffea arabica* plantations in Oaxaca (San Agustín Loxicha Municipality) and Chiapas (Unión Juárez Municipality), coffee fruits and leaves of the *Bourbon* variety showing anthracnose symptoms, such as necrotic tissue, were collected. In the laboratory, two fragments (1 cm²) of diseased tissue were cut from each sample, sterilized with 1.5% NaOCl for 1 min, rinsed three times with sterile distilled water, and dried on sterile paper. The tissues were incubated on PDA at 25 °C for 48 h. Developed fungal colonies were transferred to new PDA plates to promote the formation of reproductive structures for genus-level identification (Cao *et al*., 2019; Hassan *et al.*, 2018; Cristóbal-Martínez *et al.*, 2017). Of the total isolates obtained (11), three with morphology similar to *Colletotrichum*, exhibiting the highest growth rate and conidia production per unit of mycelial growth, were selected for this study.

Macroscopic characterization. The selected fungal isolates were reactivated on PDA. After a seven-day incubation period, they were used to describe external colonial morphology. The evaluated characteristics included texture (cottony, flat) and mycelium color (white, green), the presence or absence of concentric ring formation (Granda-Mora *et al*., 2020; García-Núñez *et al*., 2017), and the reverse colony color (hyaline, yellow, brown) (López-Calva *et al*., 2018).

Microscopic characterization. From the selected isolates of both *Trichoderma* and *Colletotrichum*, temporary glycerol-based preparations were made. Using a compound microscope (Velab, model Ve-b2) at 40× magnification and a digital camera (QUASAR) attached to the microscope, spores from each isolate were photographed.

To observe the phialides of *Trichoderma*, microcultures were prepared following the method of Vu & Tran (2020). Under aseptic conditions, a 5 mm diameter PDA disk was placed on a sterile slide. Using an entomological needle, conidial masses from purified colonies were transferred onto the slide. A sterile coverslip was then placed over the preparation, which was placed inside a Petri dish containing moist sterile paper. The samples were incubated at 25 °C for 4 to 5 days. Subsequently, microscopic observations were made, and photographs were taken to examine the structures.

To determine conidial size, photographs of conidial groups from each isolate were taken using the AmScope software. The length and width of 100 conidia were measured, and the average size of the evaluated population was calculated.

Growth rate (GR). From each isolate, a 5 mm diameter mycelial growth disk was taken and transferred to the center of a new Petri dish containing PDA. The plates were then incubated at 25 °C, and diametric measurements were recorded every 24 hours until the mycelium completely covered the surface of the dish. At the end of the experiment, the growth rate (GR) was calculated using the formula proposed by Zadoks and Schein (1979).

Where: Cf = Final diametric growth (mm); Ci = Initial diametric growth (mm); Tf = Final time (days); Ti = Initial time (day one).

Molecular identification. The selected isolates were cultivated in a liquid potato sucrose medium for seven days at room temperature (25 °C) with continuous agitation. DNA was extracted from the mycelium of each isolate using 1 g of mycelium, following a modified version of the methodology by Castle *et al.* (1998). The integrity of the extracted DNA was determined by electrophoresis on a 1% agarose gel.

The PCR reaction mixture was prepared in a final volume of 25 µL, containing 50 pM of genomic DNA, 10X Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 1 U of *Taq* DNA polymerase, and 50 pM of the primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Aljanabi and Martínez, 1997). The amplification program consisted of one cycle at 94 °C for 6 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products were visualized on a 1.2% agarose gel in an electrophoresis chamber (Bio-Rad®) at 100 V for 30 min. The amplified DNA was purified using a commercial kit (Zymoclean™), and sequencing was performed using the Sanger method with the BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI 3130 sequencer.

Phylogenetic reconstruction. The bioinformatic analysis of the sequences was performed using MEGA11 software (Tamura et al., 2021) and aligned using the cluster W algorithm. The obtained sequences were compared with those of other *Trichoderma* species available in GenBank using the BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST). The sequences with the highest similarity were used to assign species identification to each isolated strain. The phylogenetic tree was constructed using the Bootstrap method (1000 replicates), incorporating sequences obtained from GenBank and molecular identification sequences referenced in the GeneBank database of the National Center for Biotechnology Information (NCBI).

Antagonism tests. To determine the antagonistic capacity of the isolates, dual confrontation tests were performed using a completely randomized design. Three *Trichoderma* isolates (T13, T14, and T15, selected for their higher growth rate and conidial production) and three *Colletotrichum* isolates (112, 108, and Z1) were used. Each confrontation was repeated three times. In 90 mm diameter Petri dishes containing PDA, a 5 mm diameter disk of *Trichoderma* (previously grown on PDA for five days) was placed at one end of the dish, while an equidistant 5 mm diameter mycelial disk of *Colletotrichum* (also previously grown on PDA for five days) was placed at the opposite end. The *Colletotrichum* disks were placed first, followed by the *Trichoderma* disks four days later. The plates were incubated at 25 °C, and radial growth was recorded every 24 hours to evaluate the percentage of mycelial growth inhibition using the formula proposed by Ezziyyani *et al*. (2007).

$$PICR\% = \left(\frac{R1 - R2}{R1}\right)100$$

Where: **PICR** = Percentage of inhibition of radial growth; **R1** = Radial growth (mm) of the pathogen without *Trichoderma* spp.; **R2** = Radial growth (mm) of the fungus with *Trichoderma* spp.

Statistical analysis. The data obtained from each experiment met the assumptions of normality and homogeneity and were subjected to an analysis of variance (ANOVA) and a Tukey's multiple comparison test ($p \leq 0.05$). The analysis was performed using the SAS statistical software, version 9.0 for Windows. For the growth rate (GR), the data were transformed using the square root +1 method to meet the assumptions of normality and homogeneity (Toutenburg and Shalabh, 2009), similar to the approach used by Savín-Molina *et al*. (2021).

RESULTS AND DISCUSSION

Macroscopic characterization. Twelve *Trichoderma* isolates were characterized, of which 41.6% exhibited green colonies (T2, T6, T13, T14, and T15), while 58.4% showed a white coloration (T3, T4, T5, T7, T9, T10, and T12). Half of the isolates developed flat mycelium (T2, T5, T6, T13, T14, and T15), while the other half presented cottony mycelium (T3, T4, T7, T9, T10, and T12). Additionally, 50% of the isolates did not display concentric rings (T2, T3, T4, T5, T9, and T10), 8.4% showed one concentric ring (T7), 25% exhibited two concentric rings (T6, T12, and T13), and the remaining 16.6% presented three rings (T14 and T15).

Arias-Mota and Heredia-Abarca (2022) state that of the 438 *Trichoderma* species described worldwide, 30 have been recorded in association with coffee plants, all exhibiting morphological heterogeneity. Therefore, Hermosa *et al*. (2000) emphasize the importance of conducting molecular studies for more precise taxonomic classification. The isolates T2, T4, T5, T6, and T12 exhibited hyaline mycelium (41.6%), while T3, T7, T9, and T10 displayed yellowish tones (33.4%), and T13, T14, and T15 showed brown coloration (25%) (Figure 1). The coloration of the isolates may be influenced by anthraquinones, which are the most abundant natural fungal pigments responsible for the coloration of developmental structures (Yu and Keller, 2005).

Isolate T6 exhibited an intense coconut-like aroma (8.4%), which may be associated with the presence of 6-penta-alpha-pyrone, a compound previously reported in *T. harzianum* (Rocha-Valdez *et al*., 2005). Regarding the *Colletotrichum* isolates, Z1 and 112 presented white colonies, while isolate 108 exhibited a gray coloration. All isolates had a circular shape. Cottony mycelium was observed in Z1 and 112, whereas isolate 108 displayed flat mycelium. The mycelium color was white in Z1 and 112, and gray in 108 (Figure 2).

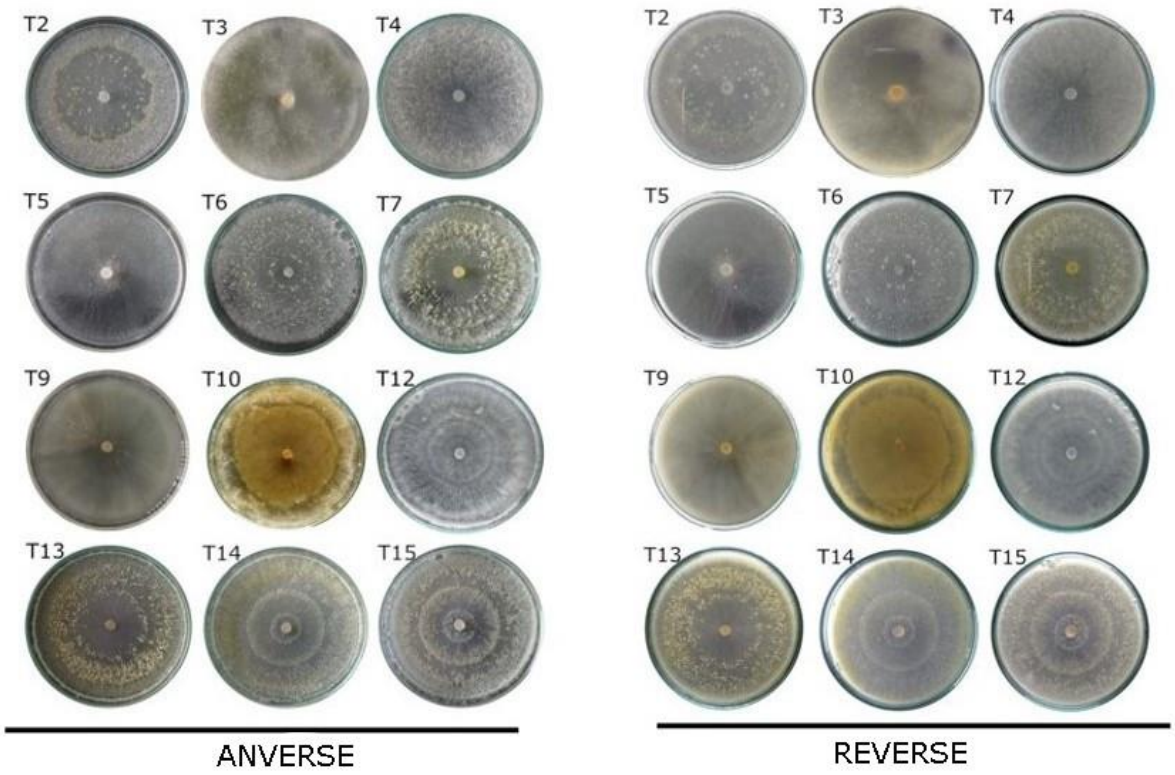

Figure 1. Macroscopic characterization of 12 *Trichoderma* isolates on PDA.

Figure 2. Macroscopic characterization of three *Colletotrichum* Isolates on PDA.

Microscopic characterization. Of the 12 *Trichoderma* isolates, 33.4% exhibited subglobose conidia (T2, T4, T7, and T12), 25% displayed an ellipsoidal shape (T5, T6, and T9), and another 25% had globose conidia (T13, T14, and T15). The conidial size ranged from 7.91 to 10.21 µm in length and 6.65 to 7.83 µm in width (Table 1). All isolates produced similar amounts of conidia, which can be attributed to their development under homogeneous environmental conditions (Osorio-Concepción *et al.*, 2013). However, some strains did not produce conidia, which may be due to their genetic condition (Martínez *et al.*, 2013).

Table 1. Characterization and growth rate of different *Trichoderma* spp. Isolates.

Isolate[z]	Conidium Shape	Conidium Lenght (µm)	Conidium width (µm)	Growth rate			
T. sulphureum (T4)	Subglobose	9.89	7.14	2.95	±	0.09	a
T. lentiforme (T15)	Globose	8.64	7.07	2.66	±	0.38	a
T. sulphureum (T5)	Ellipsoidal	9.93	7.51	2.60	±	0.30	a
T. spiralek (T3)	ND	ND	ND	2.51	±	0.06	ab
T. harzianum (T7)	Subglobose	8.05	6.69	2.45	±	0.06	abc
T. lentiforme (T13)	Globose	7.91	6.71	2.31	±	0.01	abcd
T. lentiforme (T14)	Globose	8.22	6.92	2.30	±	0.01	abcd
T. koningii (T6)	Ellipsoidal	9.53	7.83	2.28	±	0.06	abcd
T. spirale (T9)	Ellipsoidal	10.22	6.66	2.23	±	0.04	abcd
T. pleuroticola (T2)	Subglobose	9.06	7.72	1.78	±	0.01	bcd
T. tomentosum (T12)	Subglobose	9.84	7.5	1.74	±	0.01	dc
T. spirale (T10)	ND	ND	ND	1.69	±	0.02	dc

[z] Determined by sequencing of the ITS region of rDNA. ND: Not determined. Different letters indicate significant differences between treatments according to Tukey's test ($p \leq 0.05$).

Harman (1990) states that isolates belonging to the same species may differ in the production of fungal structures during multiplication, even when grown in the same culture medium. This variability could indicate the presence of different subspecies or ecotypes. The isolates obtained and selected from coffee tissues showing anthracnose symptoms had ovoid conidia, measuring between 10.3 and 13.3 µm in length and 3.3 to 4.5 µm in width. These characteristics, along with growth rate, colony color, and appressorium shape, are used to differentiate *Colletotrichum* species (Hu *et al*., 2015; Hyde et al., 2009). Another criterion for distinguishing or grouping potential species is primarily based on their host plant (Cano *et al*., 2004). However, of the 39 species reported by Sutton (1992) within this genus, the author notes that species delimitation and recognition remain unclear, though these traits provide an initial approach to identification.

Growth rate (GR). Among the isolates belonging to the *Trichoderma* genus, T4, T5, and T15 exhibited the highest significant growth rates ($p \leq 0.05$, Tukey). Isolate T4 showed the greatest diametric growth, with a difference of 1.3 mm day^{-1} compared to isolate T10, which had the lowest diametric growth among all isolates (Table 1).

The rapid growth characteristic of *Trichoderma* spp. enables it to compete for space and nutrients (Sánchez et al., 2015). Therefore, growth rate (GR) serves as a useful physiological tool for predicting the biocontrol potential of new *Trichoderma* spp. isolates (Uzunovic and Webber, 1998).

Regarding the growth rate of *Colletotrichum*, isolate Z1 exhibited significantly different values ($p \leq 0.05$, Tukey) compared to isolates 108 and 112. Isolate Z1 completely covered the 9 cm diameter Petri dish within seven days, whereas isolates 108 and 112 reached their maximum growth only after 11 days of inoculation.

Molecular identification. In this study, the amplification products had an average size of 620 bp (Figure 3A). By comparing the sequences with the GeneBank database, identity percentages ranged from 98.62% to 99.99%, identifying the species (Table 1) as *Trichoderma harzianum* (T7), *T. spirale* (T3, T9, and T10), *T. lentiforme* (T13, T14, and T15), *T. pleuroticola* (T2), *T. sulphureum* (T4 and T5), *T. tomentosum* (T12), and *T. koningii* (T6). *T. koningii* and *T. harzianum* have been reported in coffee agroforestry systems (Belayneh-Mulaw *et al.*, 2010). Ahedo-Quero *et al*. (2024) list 57 *Trichoderma*

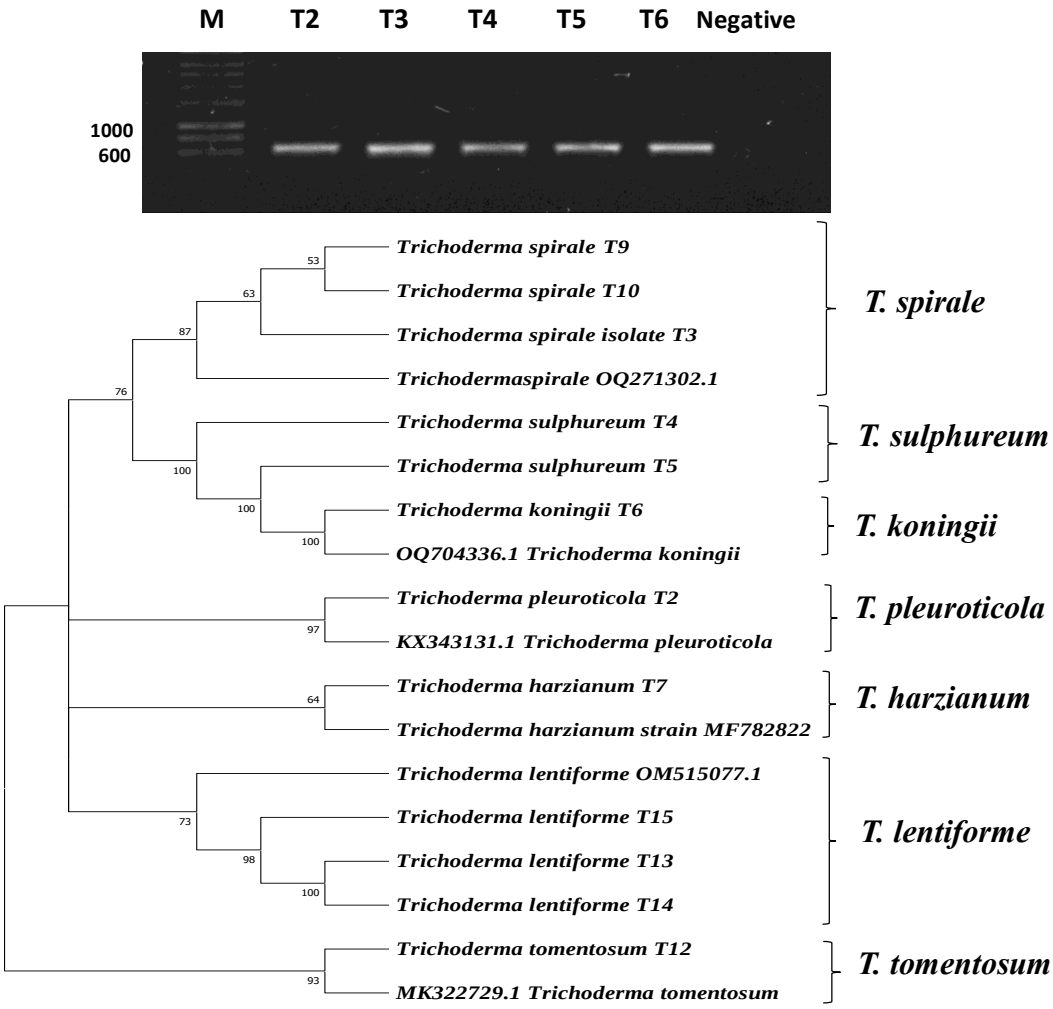

Figure 3. Phylogenetic tree. A) PCR amplification products of the ITS region of rDNA using the universal primers ITS1 and ITS4, yielding approximately 620 bp. B) Phylogenetic relationship of 12 Trichoderma isolates inferred from rDNA sequence analysis (ITS1, 5.8S, and ITS2). The numbers at the nodes represent the bootstrap percentage based on 1,000 replicates.

species reported in Mexico, 12 of which are associated with coffee cultivation, making it the crop with the greatest diversity. However, in this study, *T. lentiforme* and *T. sulphureum* are reported for the first time in Mexico in organically managed coffee soils.

Molecular markers play a crucial role in the molecular identification of *Trichoderma*. In our results, the sequences of the internal transcribed spacers (ITS) were suitable for differentiating the various *Trichoderma* isolates, consistent with other studies on this rRNA region (Islam *et al.*, 2022). The phylogenetic tree topology was generated using the neighbor-joining method with the isolate sequences, including the accession number of the GeneBank reference sequence with more than 98% similarity. The generated tree shows seven species-separated groups (Figure 3B), with an intraspecific relationship observed in two predominant groups: *T. spirale* (T3, T9, and T10) and *T. lentiforme* (T13, T14, and T15).

Beyond contributing to the knowledge of *Trichoderma* diversity, this study is particularly relevant for the biotechnological applications of the collected isolates in the biocontrol of *Colletotrichum* sp.

The importance of the genus *Trichoderma* as a biological control agent involves mechanisms of action such as substrate competition, mycoparasitism, and antibiosis (Revilla-Medina *et al.*, 2020; Rolz Asturias *et al*., 2013). For the species *T. spirale*, Baiyee *et al*. (2019) demonstrated that its biocontrol potential is due to its competition for space, volatile antifungal compounds, and enzymatic activities. In a recent study, Ye *et al*. (2023) reported that a compound extracted from *T. spirale* exhibits a strong inhibitory effect on *Candida albicans*, indicating that the role of these microorganisms is not limited to plant health and development. *Trichoderma koningii* is a highly versatile species that functions as a biocontrol agent against both soilborne and foliar phytopathogens, achieving 70–100% control under greenhouse conditions (Nina *et al*., 2011).

Molecular characterization revealed that the three *Colletotrichum* isolates belong to different species: *C. gloeosporioides* (Z1), *C. siamense* (108), and *C. aenigma* (112) (Figure 4). Two of these species (*C. gloeosporioides* and *C. siamense*) were previously reported as pathogens affecting leaves and/or fruits of Arabica coffee crops in Mexico (Cristobal-Martínez *et al.*, 2016), as well as in Asia (Damm *et al*., 2012) and Brazil (Prihastuti *et al*., 2009). In contrast, *C. aenigma*, a species within the *C. gloeosporioides* complex, has not been reported in Arabica coffee crops, suggesting this may be the first report of its occurrence. However, given the complexity of *Colletotrichum* species identification, we consider it necessary to conduct molecular characterization using multilocus sequences beyond ITS (Cristobal-Martínez *et al*., 2016; Zhu *et al*., 2023) to confirm the identification of this species.

Antagonism tests. The inhibition of *Colletotrichum* sp. mycelial growth was mostly observed on the fourth day of incubation, confirming the rapid growth of *Trichoderma* and its potential to inhibit the mycelial development of the phytopathogen (Fernández and Suárez, 2009). The highest percentage of *C. siamense* (108) mycelial growth inhibition was observed in its interaction with the *Trichoderma* isolate T15 (Tukey, 0.05). In the interaction of *Trichoderma* strains (T13, T14, and T15) with *C. gloeosporioides* (Z1), *C. siamense* (108), and *C. aenigma* (112), a color change in the culture medium was observed at the interaction zone of both microorganisms' mycelia. Subsequently, *Trichoderma* isolates overgrew the *Colletotrichum* mycelium, a response that serves as

an initial indication of their potential as hyperparasitic microorganisms (Hernández-Mendoza *et al*., 2011).

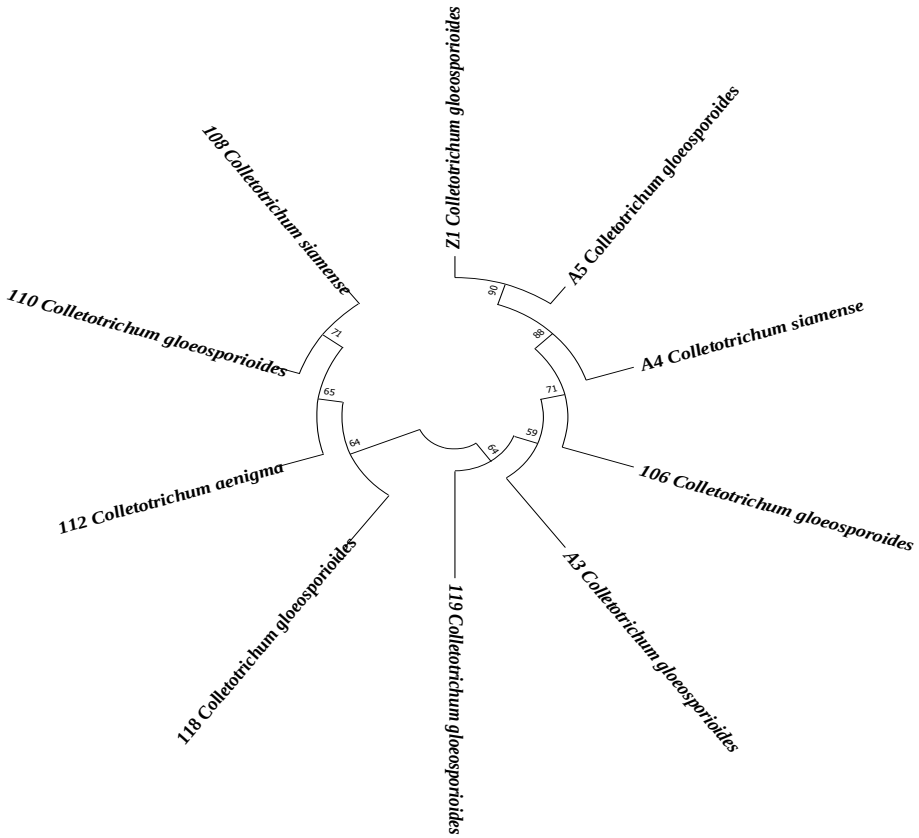

Figure 4. Phylogenetic relationship dendrogram of 10 *Colletotrichum* isolates.

Sporulation of *Trichoderma* on *Colletotrichum* mycelium was observed only in the *T. lentiforme* (T14) - *C. siamense* (108) interaction. The antagonistic capacity of *Trichoderma* to inhibit *Colletotrichum* growth has been previously reported (Morales-Mora *et al*., 2020). Figure 5 presents the different mycelial growth inhibition responses of *Colletotrichum* isolates when evaluated against our *Trichoderma* isolates. This growth inhibition is attributed to the production of metabolites such as chitinases and glucanases (Sánchez-García *et al*., 2017), as well as the secretion of antibiotics or other inhibitory substances produced by antagonistic isolates (Al-Mekhlafi *et al*., 2019).

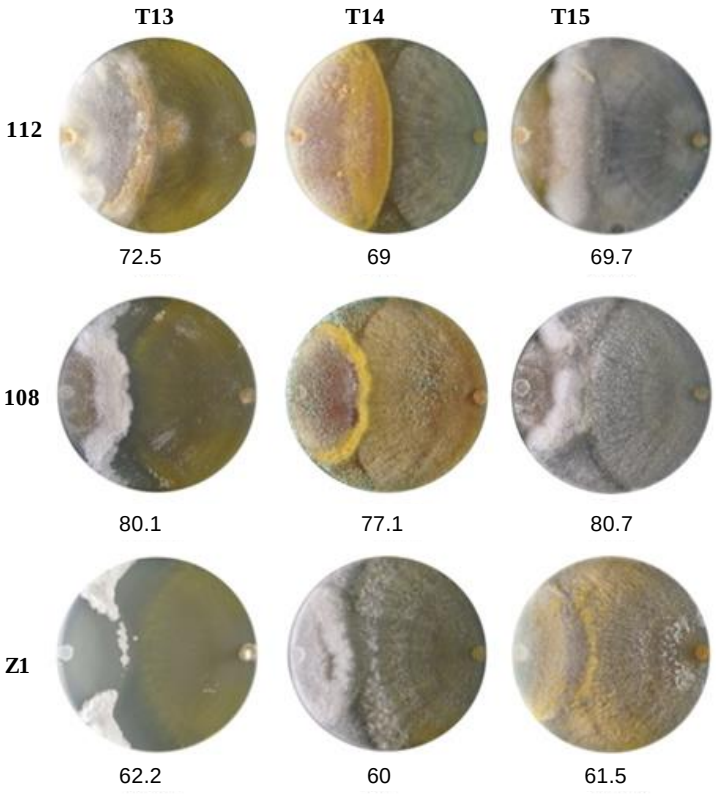

Figure 5. Dual confrontation between *Trichoderma* spp. isolates (T13, T14, and T15) and *Colletotrichum* spp. isolates (Z1, 108, and 112). The numbers below the isolates represent the PICR (percentage of radial growth inhibition).

CONCLUSIONS

Seven *Trichoderma* species were identified: *T. harzianum*, *T. spirale*, *T. lentiforme*, *T. pleuroticola*, *T. sulphureum*, *T. tomentosum*, and *T. koningii*. These species were distinguished by their rapid growth, displaying the characteristic green coloration of the genus and concentric rings in some isolates. Notably, *Trichoderma* demonstrated its potential as a biocontrol agent against *Colletotrichum*, acting through different mechanisms against this phytopathogen. This study contributes to the understanding of the diversity of native *Trichoderma* species in the coffee-growing region of Oaxaca. Furthermore, this deeper insight enhances knowledge and supports the selection of these species for future studies on the biocontrol of phytopathogens, aiming to promote sustainable agricultural practices.

Conflicts of interest

There are no conflicts of interest.

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Author Contributions

Conceptualization: Abimael Rubio Sosa; methodology: Abimael Rubio Sosa, Misael Martínez Bolaños; validation: Misael Martínez Bolaños, Salvador Lozano Trejo, Ernesto Castañeda Hidalgo, and Gustavo Omar Diaz Zorrilla; formal analysis: Abimael Rubio Sosa, Misael Martínez Bolaños; investigation: Abimael Rubio Sosa; data curation: Juan Florencio Gómez Leyva; writing—original draft preparation: Abimael Rubio Sosa; writing—review and editing: Misael Martínez Bolaños, Salvador Lozano Trejo, Ernesto Castañeda Hidalgo; visualization: Misael Martínez Bolaños, Salvador Lozano Trejo; supervision: Misael Martínez Bolaños, Salvador Lozano Trejo. All authors have read and approved the published version of the manuscript.

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