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Research Article

Application of *Trichoderma asperellum* in apple trees as a growth regulator and antagonist for the control of *Alternaria* sp.

Melissa MADRID-MOLINA[1], Sandra PÉREZ-ÁLVAREZ[2*], César M. ESCOBEDO-BONILLA[3], Crescencio URÍAS-GARCÍA[4]

[1]*Universidad Autónoma de Chihuahua, Facultad de Ciencias Agrotecnológicas, Campus 1. C.P. 31200. Correo postal 24, Chihuahua, Chihuahua, México; melimadrid92@hotmail.com*
[2]*Universidad Autónoma de Chihuahua, Facultad de Ciencias Agrícolas y Forestales, Km 2.5 carretera a Rosales, Campus Delicias, C. P. 33000, Delicias, Chihuahua, México; spalvarez@uach.mx (*corresponding author);*
[3]*Instituto Politécnico Nacional-CIIDIR Unidad Sinaloa, Juan de Dios Bátiz Paredes No. 250, CP 81101 Guasave, Sinaloa, México; cesar_escobedomx@yahoo.com*
[4]*Universidad Vizcaya de las Américas, Calz. Del Charro 301, Col. Centro, Campus Delicias, CD. Delicias Chihuahua, CP 33000, México; uriasgarciac@outlook.com*

Abstract

Chihuahua state is the main apple producer in Mexico. The present study aimed to evaluate the effectiveness of *Trichoderma asperellum* as a plant growth regulator and antagonist against *Alternaria* sp. Three treatments were used: T1 = control, T2 = 200 mL of *T. asperellum* per tree; T3 = 100 mL of *T. asperellum* per tree. Agronomic variables were evaluated including number of leaves, shoots and flowers, disease incidence, and trunk thickness. *Alternaria* sp. was isolated from apple leaves at the experimental site in Guerrero County, Chihuahua, Mexico, and it was grown on solid PDA medium for morphological characterization. The molecular characterization was done by PCR using primers ITS1 and ITS4 producing products of 700 bp which were sequenced, submitted to GenBank (acc. no. OQ344593) and used for further phylogenetic analysis through Bayesian inference approach. Three clades were identified and the polytome topography recovered from clade 2 indicates a high genetic similarity with *A. tenuissima* (100% similarity according to BLAST). The analysis of *T. asperellum* as growth regulator only showed significant differences in trunk thickness and displayed higher values with T3 ($p \leq 0.05$). The presence of *A. tenuissima* was only observed in the control, which indicated the ability of *Trichoderma* to control the fungus. In this study *T. asperellum* was not an efficient plant growth regulator, but it was a good antagonist, and hence it can be recommended to control *A. tenuissima*. This is the first record of *A. tenuissima* in apple trees in Mexico. These results indicate that *T. asperellum* showed no benefit as plant growth regulator when applied to apple trees of the 'Granny Smith' variety.

Keywords: *Alternaria tenuissima*; antagonism; biological control; chemical control; growth regulation; *Malus domestica*

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Introduction

The apple (*Malus x domestica* Borkh.) represents one of the most important crops in Mexico (Ramírez-Legarreta *et al*., 2011), the state of Chihuahua being the first national producer with a total of 490,669.59 t produced in 2021 (SIAP, 2023). Plant pathogens may induce crop losses around the world and they can affect the quality of the harvest (Thambugala *et al*., 2020). Apple trees are affected by several fungal pathogens such as *Rhizoctonia*, *Alternaria*, *Fusarium*, among others (Lin and Hand, 2019). *Alternaria* spp. has caused several losses not only in apple production but also in the apple industry being the main symptoms leaf blotch, severe premature tree defoliation and fruit spot (Filajdic and Sutton, 1992). Chemical control and cultural practices are the most common methods to control *Alternaria* spp. (Cooley and Autio, 2011) but the use of biological methods, specifically antagonistic microorganisms, are becoming more common because they are an efficient and environment-friendly method to control plant pathogens (Zhang *et al*., 2018).

Species of the genus *Trichoderma* have been widely studied as antagonists since they can be easily manipulated, are adaptable to various environments and they are versatil (Duarte-Leal *et al*., 2017). Tamandegani *et al*. (2020) reported that *T. asperellum* increased the production of peptaibol (trichotoxin) during *in vitro* confrontation with fungal plant pathogens such as *Alternaria solani*, *Fusarium oxysporum, F. moniliforme, F. graminearum, F. culmorum,* and *Rhizoctonia solani*. In this context, Cabrefiga *et al*. (2023) applied *T. asperellum* in the soil of a commercial apple orchard to control *Alternaria* spp., resulting in 50% to 80% reduction of fruit spots and 30% to 40% decrease of leaf blotch.

It has also been reported that some *Trichoderma* species have beneficial effects on crops, such as increased plant growth, seed germination and seedling emergence (Chagas *et al*., 2016). As plant growth regulator, *Trichoderma* spp. can synthetize phytohormones (Cai *et al*., 2016; Jaroszuk-Ściseł *et al*., 2019) such as indole 3 acetic acid (IAA) (Kumar *et al*., 2017; Sabre *et al.*, 2017) and gibberellic acid (AG3) (Turaeva *et al*., 2020; Díaz *et al*., 2020) which are necessary for plant growth, including improvement of root condition and structure, enhancement of seed germination and viability, along with increased photosynthesis efficiency, flowering and yield quality (Halifu *et al*., 2019). López-Valenzuela *et al*. (2022) found that two isolates of *T. asperellum* (TB and TM) produced IAA at 25.5 $\mu g \cdot mL^{-1}$ and 24.5 $\mu g \cdot mL^{-1}$, and AG3 (2.51 ± 0.127 $\mu g \cdot mL^{-1}$; 2.46 ± 0.104 $\mu g \cdot mL^{-1}$). Promwee and Intana (2022) reported that *T. asperellum* significantly stimulated the growth of *Lactuca sativa* L. by increasing plant height (8.62%), number of leaves (18.39%), root fresh weight (39.26%) and shoot fresh weight (25.71%). Further, Wang *et al*. (2021) reported that *T. asperellum* promoted plant growth in an apple orchard in Shandong Province, China. In view of these reports, the aim of this research was to evaluate the effectiveness of *T. asperellum* as plant growth regulator and antagonist against *Alternaria* sp. in an apple orchard in Chihuahua, Mexico.

Materials and Methods

Study area

This research was carried out at the orchard "La Escondida" located at Mesa de Miñaca, Guerrero County, Chihuahua, Mexico at 28° 28' 11.7" North and 107° 27' 02.6" West, at an altitude of 21.50 m.a.s.l. The apple trees used for the experiments were the 'Granny Smith' variety, with an age of 7 years. The plantation is found in sandy clay loam soil, with a moderately alkaline pH, with a medium texture free of carbonates and salts.

Trichoderma asperellum production

The production of the bioproduct was described by Andrzejak *et al*. (2022) and the ITS sequence of the *T. asperellum* isolate was submitted to GenBank with acc. no. MN950427 (Matas-Baca *et al*., 2022). The biofungicide used contained *T. asperellum* at a concentration of 1×10^{10} UFM mL^{-1}. The *T. asperellum* strain used in this work is part of the FCAyF strain collection and is registered in the GenBank under the accession number MN950427.

Experimental design and treatments to evaluate Trichoderma asperellum as plant growth regulator and as antagonistic

A fully randomized design was done using three replicates per treatment. Three treatments were done for the application of *T. asperellum* using 10 apple trees per treatment: T1 = control, T2 = 200 mL of *T. asperellum* per tree; T3 = 100 mL of *T. asperellum* per tree. Each treatment was applied three times to the trees by injection, making 4 perforations, close to the trunk, approximately 15 cm deep, considering their physiology. The first application was done in early April on pink button before flowering; the second application was done a month later in the petal fall stage; and the third application was done in July when the apple tree was in its development and filling phase. The agronomic variables evaluated at the end of the experiment were: number of leaves, shoots and flowers, disease incidence, and trunk thickness.

Statistical analysis

Normality of data was assessed with the Shapiro-Wilk test ($p \leq 0.05$) and the homogeneity of variances of the analysed variables was evaluated using Levene's test with significance value $p \leq 0.05$. Subsequently, an ANOVA analysis was carried out at a significance level of 0.05. The variables included were analysed with Duncan's test to compare the means of the two factor levels (dose and application time) at a level of significance ≤ 0.05. The statistical package IBM-SPSS version 25 was used (IBM Corp., 2017).

Alternaria sp. isolation

Leaves with visible symptoms of *Alternaria* sp. infection were collected and disinfected with 1% sodium hypochlorite and then washed three times with sterile distilled water to directly proceed to plating of leaf portions (half healthy and half diseased) in Potato Dextrose Agar (PDA) medium (BD Bioxon) at pH 5.0-5.5. The Petri dishes were incubated in the dark at 25 ± 2 °C for 7 d. The morphological and molecular characterization of the pathogen were done at Universidad Autónoma de Chihuahua, Facultad de Ciencias Agrícolas y Forestales (FCAyF), Delicias city.

Morphological characterization of Alternaria sp.

Macroscopic identification

The morphological characterization was done after obtaining a pure isolate of *Alternaria* sp. The isolate was identified following the method of Simmons (1993, 1999), considering colony characteristics (color, structure, aerial and substrate mycelia).

Microscopic identification

The microscopic identification was implemented by the description of reproductive organs of the isolate (conidia, conidiophores, chain formation, etc.) after 7 d of colony development on PDA. Once the fungal structures were visualized, it was identified using the codes and descriptions of Morales-Mora *et al*. (2020).

Molecular characterization of Alternaria sp.

A pure isolate of *Alternaria* was used for molecular characterization. Mycelial tissues were collected for total DNA extraction using the cetyltrimethyl ammonium bromide (CTAB) method (Zhang *et al*., 1998). The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') that amplify the internal transcribed spacer (ITS) region of fungi (White *et al*., 1990) were used. The PCR assay was done in a thermocycler (Dlab TC-1000G, China) in a reaction volume of 25 µL using the enzyme MyTaq (Bioline, USA). The PCR reaction contained ultrapure water (17.75 µL), 5X PCR buffer (5 µL), 10 µM ITS1 and ITS4 primers (0.5 µL each), 1-unit (0.25 µL) MyTaq DNA polymerase (Bioline, USA) and 1 µL of extracted total DNA. The amplification conditions were: an initial denaturation cycle at 95 °C for 3 min, then 35 cycles with a denaturation step (95 °C for 35 s), primer annealing (60 °C for 40 s) and extension step (72 °C for 90 s), with a final extension step at 72 °C for 7 min. The PCR product was resolved in a 1% agarose gel electrophoresis and visualized in a GelDoc XRS photodocumenter (Biorad, USA). The amplified PCR products were further purified using the Wizard SV Gel Kit and PCR Clean-Up System (Promega, USA) and sent for forward and reverse strand sequencing by the Sanger method at LANGEBIO - CINVESTAV Irapuato, Mexico.

The resulting sequences were analysed, and a consensus sequence was obtained and submitted to GenBank with the accession number OQ344593. It was compared through a Bayesian analysis with selected sequences of *Alternaria* species: *A. aconidiophora* (NR1666229), *A. breviramosa* (NR165504), *A. hyacinthi* (NR145168), *A. junci-acuti* (NR174907), *A. lawrencei* (NR166227), *A. lolii* (NR159632), *A. malorum* (NR145142), *A. mirabibensis* (NR171997), *A. obclavata* (NR165505), *A. pobletensis* (NR166226) and *A. tenuissima* (ON514230). Sequences of the related genera Bipolaris (NR147489) and Curvularia (NR130653) were used as outgroup. All sequences are available at GenBank® database. Sequences were aligned with ClustalW (Thompson *et al*., 1994) in BioEdit 7.0.9 (Hall, 1999) and the final matrix containing 472 nucleotide positions was used for a phylogenetic analysis through Bayesian inference performed in MrBayes 3.2.7 (Ronquist *et al*., 2012). Best fit model of sequence evolution was selected in jModeltest 2.1.4 (Darriba *et al*., 2012). The general time reversible with proportion of invariant sites (I) and gamma distribution (G) model of nucleotide substitution was selected according to the results using the corrected Akaike Information Criterion (Akaike, 1973). The parameters for the Bayesian analysis were set as two independent runs of four Metropolis-coupled Markov chain Monte Carlo (MC)3 (Hastings, 1970) for a total of 1×10^6 generations, sampling one every 100 trees and discarding the first 25% of the trees as burn-in. Posterior probabilities were assessed from the 50% majority rule consensus and the tree was edited in FigTree 1.4.4 (Rambaut, 2018).

Results

Morphological characterization of Alternaria sp.

Macroscopic identification

A pure culture was obtained, and the isolate showed yellowish-brown to black mycelium. On both sides of the Petri dish, the agar acquired a colour between brown and black (Figure 1).

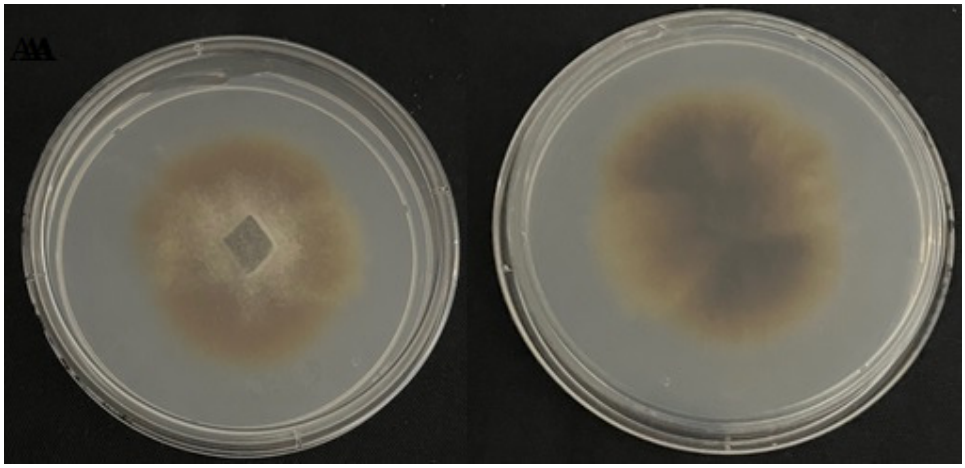

Figure 1. Colony of the native strain of *Alternaria* sp. in PDA medium: A) front colony, B) reverse colony

Microscopic identification

Results from the microscopic observation of *Alternaria* sp. isolate showed septated hyphae, oval shaped and hyaline conidia, longitudinally and transversally septated, with three to five divisions in the conidia (Figure 2).

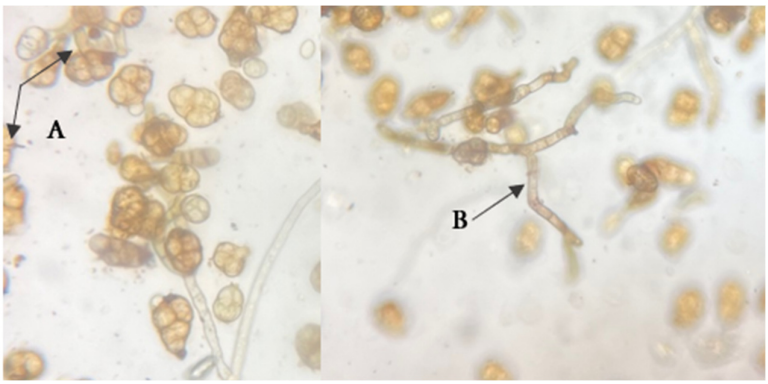

Figure 2. Microscopic structures of *Alternaria* sp. A) mature conidia (x100); B) septate hyphae (x100) Scale bars: 10 mm

Molecular characterization of Alternaria sp.

A fragment of around 700 bp was visualized after DNA extraction and PCR amplification. A consensus sequence was obtained for this region and deposited in GenBank with accession number OQ344593. The PCR amplification done with primers ITS1 and ITS4 are showed in Figure 3.

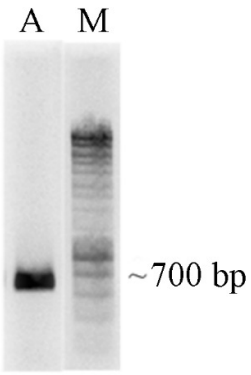

Figure 3. PCR amplification of *Alternaria* DNA with ITS1 and ITS4 primers resolved on 1% agarose gel electrophoresis
A, is the lane with *Alternaria* sp. PCR product; M, is the lane with the DNA molecular weight marker

A BLAST analysis (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) and the phylogenetic analysis showed that the ITS sequence of this isolate had 100% identity match with *Alternaria tenuissima* (GenBank acc. no. OQ344593); thus, confirming by molecular and bioinformatics means that the isolate corresponds to mentioned species (Figure 4).

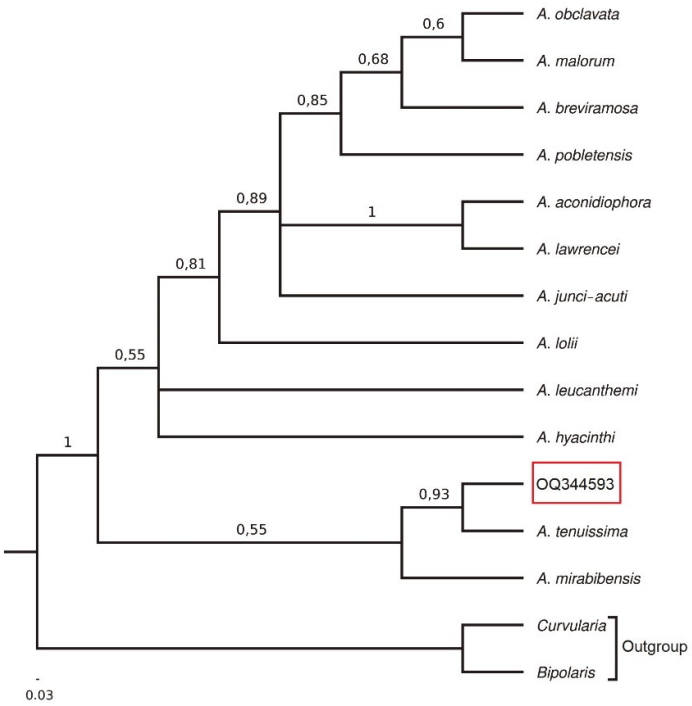

Figure 4. Neighboring tree of *Alternaria tenuissima* based on ITS sequences. The new isolate OQ344593 is marked with a red box. The numbers on the branches indicate Bootstrap compatibility (100%) and the scale bar indicates nucleotide substitutions per site

Using the ITS sequence information, three clades have been identified, and the polytomous topography suggests a high genetic similarity of the new isolate with *A. tenuissima* (Figure 4, clade 2). The group including OQ344593 also contains *A. tenuissima* and *A. mirabibensis*; however, the internal resolution of this clade

indicates that OQ344593 represents *A. tenuissima* with 93% probability. These molecular results also are supported by the morphological description of the isolate and confirms that the analyses used led to a precise and reliable taxonomic diagnosis. To our knowledge, this is the first report of *A. tenuissima* in apple trees in Mexico.

Evaluation of Trichoderma asperellum as plant growth regulator and as antagonistic

The results of the effects of the application of *T. asperellum* to control *A. tenuissima* and as plant growth regulator are shown in Table 1.

Table 1. Effect of treatments on agronomic variables and diseases incidence

Agronomic variables	T1	T2	T3
Number of leaves	98.3[a]	103.825[a]	103.65[a]
Number of shoots	7.55[a]	10.275[a]	10.075[a]
Number of flowers	16.23[a]	18.43[a]	15.5[a]
Trunk thickness (cm)	33[b]	35.8[b]	42.8[a]
Number of diseased trees	10	0	0

T1, control; T2, 200 mL of *T. asperellum*; T3, 100 mL of *T. asperellum*

Means followed by the same letter are not statistically different, according to the Duncan Multiple Range Test at P ≤ 0.05

The effect of the application of *T. asperellum* as plant growth regulator showed no significant differences between treatments. Only trunk thickness showed significant differences between treatments, being higher in T3 (100 mL of *T. asperellum*) ($p \leq 0.05$). These results indicate that *T. asperellum* showed no benefit as plant growth regulator when applied to apple trees of the Granny Smith variety.

Conversely, results showed that *T. asperellum* was an effective antagonist of *A. tenuissima* when applied to Granny Smith apple trees. Treatments T2 and T3 showed no signs of *Alternaria* infection, whereas T1 (control) displayed signs of the disease. These results indicate an effective role of *T. asperellum* as antagonistic of *A. tenuissima* in this apple variety (Figure 5).

Figure 5. Apple trees var. 'Granny Smith' A) necrotic damages on leaves caused by *A. tenuissima* (control); B) healthy leaves from T3 (100 mL of *T. asperellum*) (Source: Authors)

Early symptoms of *Alternaria* infection on leaves are described as circular necrotic lesions, that later become into wilting (Figure 5) and defoliation.

Discussion

The role of *T. asperellum* as antagonistic against different plant fungi pathogens such as *A. solani* (Ronnie-Gakegne and Martínez-Coca, 2018), *Fusarium oxysporum* (Hao *et al*., 2022, Rubio-Tinajero *et al*., 2022; Sharma *et al*., 2022), *Sclerotinia sclerotiorum*, *A. solani*, *Phytophthora sphave* (Sharma *et al*., 2022), *Puccinia striiformis* (Esmail *et al*., 2022) have been demonstrated. *Alternaria* comprise a group of species causing several damages on different plants, including apple trees, to which they can induce leaf blotch and fruit spot (Cabrefiga *et al*., 2023).

The species of *Alternaria* isolated from apple tree leaves showed macroscopic and microscopic features similar to those described by Simmons (1993, 1999) and Morales-Mora *et al*. (2020). Such characteristics included mycelia of dark, blackish brown to black coloration, presenting fluffy development and the presence of pear-shaped conidia with transverse and longitudinal septa (Benavides *et al*., 2019). These aspects are similar to those reported for the specie *A. tenuissima* (Rodríguez-Roa *et al*., 2013; Rodarte Díaz *et al*., 2020).

The ITS regions of fungi are frequently used for molecular identification. In the present study, molecular analyses showed a PCR product of 700 bp of the amplified ITS region of the *Alternaria* isolate. This amplicon size agrees with findings of several other studies. Rodarte-Díaz *et al*. (2020) identified *A. tenuissima* on carrot crops (*Daucus carota* L.) using the ITS region with 100% similarity in GenBank. Jasnić *et al*. (2011) also used

ITS regions to identify *A. tenuissima* from soybean crops (*Glycine max* (L.) Merr., 1917) with sequences ranging from 566 to 576 base pairs and 99% similarity in GenBank. Another study on tomato fruits (*Solanum lycopersicum* L.) reported sequence fragments of 547 (*A. alternata*, *A. brassicicola* and *A. tenuissima*), 542 (*A. citri*) and 554 bp (*A. radicina*), with a BLAST similarity of 97-98% in the NCBI GenBank database (Saleem and El-Shahir, 2022).

The neighbor-joining tree used in the present work supports the morphological data obtained from the isolate and allowed to determine the species as *A. tenuissima,* with full identity (100%) with *A. tenuissima* (ON514230). Similar results were reported by Iftikhar *et al*. (2021) who identified *A. tenuissima* in bitter gourd (*Momordica charantia* L.) using a BLAST analysis of the amplified ITS sequences, where *A. tenuissima* (LT670913) showed maximum identity (99% in GenBank) with *A. tenuissima isolates* JX205160, HQ647307, KX139157 and JX867218. Saleem and El-Shahir (2022) found that five *Alternaria* species (*A. alternata*, *A. brassicicola*, *A. citri*, *A. radicina*, and *A. tenuissima*) had 97-98% similarity in the NCBI GenBank database. In this regard Mohammadi and Bahramikia (2019) using the NCBI database, identified *A. alternata* from tomato crops with 99-100% similarity. Garganese *et al.* (2016) analysed 20 isolates of *Alternaria* from leaves and fruits of tangerines (*Citrus* × *nobilis* Lour) and several clades showed *Alternaria* species morphotypes, containing *A. tenuissima, A. limoniasperae, A. toxicogenica, A. alternata, A. arborescens* and *A. citri* with a partial match between morphotypes and molecular clades.

The results obtained from applying *T. asperellum* to apple trees indicated that this fungus did not improve most of the plant growth parameters evaluated. No significant differences between treatments were found in number of leaves, shoots or flowers, although slightly higher values of these parameters were recorded in Treatment 2 (200 mL of *T. asperellum*). Trunk thickness was the only growth parameter significantly different in T3 (100 mL of *T. asperellum*). Trunk thickness is physiologically important to trees because the thicker the trunk, the wider the xylem and the phloem tissues inside, and through these tissues water, nutrients and raw sap flow from the roots to the leaves. So, if the trunk diameter decreases, so does the transport of water and nutrients to the top of the tree (Castedo-Dorado *et al*., 2009; Kuliešis *et al*., 2010).

Conversely, Berlian *et al*. (2021) reported positive results using *T. asperellum* on mango seedlings, where the fungus accelerated the emergence of shoots and increased shoot length. According to Singh *et al*. (2018) and López-Bucioa *et al*. (2015) *Trichoderma* can accelerate the emergence and growth of shoots because it can produce secondary metabolites such as harzianolides and peptaibols. Several authors have presented the valuable effects of *Trichoderma* spp. on horticultural crops such as periwinkle, lettuce, cucumber and chrysanthemum using agronomic parameters such as seed germination, vegetative growth and flowering (Chang *et al*., 1986, Hermosa *et al*., 2012, Studholme *et al*., 2013).

The benefit of *Trichoderma* as plant growth regulator was also reported in wheat growing with *T. reesei,* with increased numbers of shoots, higher weight of dried and fresh shoots, higher root length and root numbers (Ikram *et al*., 2019). The *Trichoderma asperellum* strain 6S-2 promoted the growth of apple seedlings in greenhouse, and also enhanced branch elongation of young apple trees (Wang *et al*., 2022).

In the present study, it is possible that *T. asperellum* did not display function as growth regulator because the experiment was done in adult trees and the translocation of the product from the roots to the leaves may have been difficult due to the age of the orchard. The use of *T. asperellum* as antagonist of *A. tenuissima* in apple trees was very effective with both treatments used (200 mL and 100 mL).

This result is similar to that reported by Cabrefiga *et al*. (2023) where application of *T. asperellum* to the soil reduced between 30% to 40% leaf blotch, and fruit spot caused by *Alternaria* spp. was reduced between 50% and 80%.

Numerous studies also have demonstrated the antagonistic effect of *T. asperellum* against several fungal pathogens. Examples include a hydroponics experiment with 'Green Oak' lettuce (*Lactuca sativa* L.) in

Thailand, where *T. asperellum* NST-009 reduced leaf spot caused by *Cercospora lactucae-sativae* by 67.51% compared to the control (Promwee and Intana, 2022). Also, the mycelial growth of *A. porri* (causative agent of purple spot-on onion) was inhibited in 56% by *T. asperellum* in dual culture (Camacho-Luna *et al*., 2021). Tomato plants were treated *in vitro* with *T. atroviride* and *T. asperellum* against *A. solani, A. linariae* and *A. grandi.* Results showed a reduction of mycelial growth of *Alternaria* spp. by both *Trichoderma* species. Under *in vivo* conditions disease symptoms were reduced to 65.9% in pre-treated plants (Mohammedi *et al*. 2022).

Species of *Trichoderma* are widely used as biological control agents around the world, representing about 50 - 60% of the global market on the control over many fungal and oomycete pathogens, such as *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Collisletotnicios* spp., *Diplodia natalensis*, *Fusarium* spp., *Rhizoctonia solani*, *Pythium* spp., *Sclerotium* spp., *Rhizopus oryzae*, *Verticillium dahliae*, among others (Ketta and Hewedy, 2021; Verma *et al*., 2007).

Conclusions

The present study described morphologically and molecularly an isolate of *Alternaria tenuissima* affecting apple orchards in Chihuahua, Mexico. This is the first report of the species in the country. The isolate was registered at the National Center for Biotechnology Information (NCBI) database with the accession number OQ344593.

The application of *T. asperellum* as plant growth regulator did not improve growth parameters in adult apple trees. Nonetheless, under field conditions it showed an antagonistic effect against *A. tenuissima,* since it was able to effectively reduce disease symptoms.

Authors' Contributions

All the authors included in this article contributed equally to the work. S.P.A. was responsible for directing the project and for writing this article. M.M.M. was responsible for executing and monitoring the experimental work. J.R.S. was responsible for the interpretation of the data, while C.M.E.B. headed the molecular experimental work. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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