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Inhibitory Activity of Native Strains of Trichoderma Sp. Isolated from Yam Crops with Inhibitory Activity Against Phytopathogenic Fungi

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Abstract

Colletotrichum gloesporioides is one of the main phytopathogens causing limiting diseases in the production of Dioscorea spp. yam (Dioscoreaceae) in the Caribbean region. The objective of the study was to evaluate the antagonistic activity of Trichoderma spp. against C. gloesporioides and its in vitro plant growth promoting potential. Trichoderma harzianum, T. atroviride and T. asperellum species showed inhibition against the pathogen ($p<0.05$) and promoted plant growth. These species release enzymes that can degrade the cell wall of the pathogen causing its death or inhibit its growth through the production of secondary metabolites. The application of Trichoderma spp. mounts in crops confers protection against pathogens and stimulates plant growth in order to obtain a good yield.

Keywords: *Yam Cultivation, Disease, Fungus, Biological Control, Growth Promotion.*

Introduction

According to Fróna et al., (2019), agricultural activity affects the environment due to the excessive use of phytosanitary products such as fertilizers and pesticides. Several researches warn of a dynamic population growth in the next 30 years, so that agricultural emissions will increase by more than 50% so that people can acquire food in adequate quantity and quality.

As inferred by what was stated by Obidiegwu and Akpabio, (2017), the yam crop is considered one of the main products of major importance in tropical climates providing a staple food source for millions of people in Africa, South America, Asia and the Pacific (Sukal et al., 2017). In recent years, yam cultivation has presented an increase in its consumption due to its contribution of calcium, phosphorus, iron, vitamins B and C (Thomas et al., 2017). Likewise, this tuber is used in traditional medicine in order to cure arthritis, traumatic injuries and respiratory conditions (Vega, 2012; Andrés et al., 2015; Sun et al., 2017; Siddiqui et al., 2018). According to the stipulations of the Ministry of Agriculture and Rural Development, in Colombia, there are approximately 30,000 yam producing families of which 70% grow creole varieties, and only 14% and 16% correspond to the diamond and hawthorn varieties. Yam production is found specifically in the Caribbean region, where the crop can be found in backyard areas for consumption (MADR, 2020).

There are different limiting factors that affect yam crop production, among which is the anthracnose disease caused by the fungus *Colletotrichum gloeosporioides*. The symptoms of the disease are characterized by necrotic lesions on the stem, leaf and petiole, which produce a decrease in the photosynthetic rate of the plant and generate production losses of up to 95% (Dos Passos Braga et al., 2019; Orlando & De Jesús, 2020; Rincón et al., 2006; Sánchez et al., 2020). Chemical fungicides are applied to control anthracnose, but they have caused environmental problems and their excessive use can generate fungicide-resistant strains (Gaviria et al., 2013; Lobo et al., 2020).

Biological control has become an alternative to replace agrochemicals. The use of fungi and bacteria capable of inhibiting the growth of C. gloesporioides has been proposed for the control of anthracnose. Among these microorganisms is the fungus Trichoderma spp. which are able to control pathogens that cause phytosanitary problems through the production of volatile and non-volatile secondary metabolites, mycoparasitism and the ability to compete for space and nutrients in their habitat (Bae et al., 2016; Contreras

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et al., 2016; Harman et al., 2004; Pakora et al., 2018; Vinale et al., 2008; Zin et al., 2020). In recent years Trichoderma spp. fungi have been applied in crops of economic interest such as rice, maize and tomato. Likewise, the fungus has the ability to be used in bioremediation processes (Guoweia et al., 2011).

The indiscriminate use of agrochemicals has induced the release of harmful compounds that have contributed to the increase in temperature and global warming of the planet. However, as stated by Pineda-Insuasti et al. (2017), this leads to a pressure towards extreme climate change, which represents a threat to food supply. Therefore, there is a need to develop and implement new alternatives for agricultural production that generate less damage to the environment or, in the best case scenario, reverse to some extent the damage already caused. One alternative is the use of microorganisms capable of controlling diseases and organic fertilizers. Such as the filamentous fungus Trichoderma spp. this genus of saprophytic fungi coexists in different types of substrates, are important for its role in ecosystems to be decomposers of organic matter and are fundamental in the cycle of nutrients in the soil. It can be easily captured and replicated in the laboratory due to its rapid growth. Thanks to its potential biocontrol and plant growth promoter are currently marketed as biofertilizer, its inoculation in plants allows the uptake of macronutrients and micronutrients allowing in crop yield and soil quality (Guoweia et al., 2011; Sandheep et al., 2013).

Based on this situation, the present study had the purpose of isolating fungal strains belonging to the genus Trichoderma sp. in rhizosphere of yam crops and to evaluate *in vitro* the inhibition capacity against *Colletotrichum gloesporioides* and the capacity to produce siderophore and solubilize phosphate.

Materials and Methods

Study area. The study was carried out in yam fields in the municipalities of Ovejas and Chalán, Montes de María subregion, department of Sucre, Colombia. It corresponds to a tropical dry forest zone and its characteristic landscape is mountainous. It has an average temperature of 27 °C and rainfall that can vary between 1,000 and 1,200 milliliters per year (Aguilera, 2013; Hernández, 2013).

Isolation of native strains of Trichoderma spp. With the help of a previously sterilized auger, a soil sample was taken at a depth of 30 cm at the base of the yam plant. This sample was deposited in zip lock bags and taken to the Microbiological Research Laboratory of the University of Sucre for processing. The soil samples were deposited in an Erlenmeyer flask containing 90 mL of sterile water, which was left in constant agitation for homogenization. After the time elapsed, with the help of a micropipette, 1 mL of the homogenate was taken and inoculated in test tubes containing 9 mL of saline solution (Camargo & Avila, 2014). From this solution, serial dilutions were performed in triplicate. From each of these dilutions, 10 µL were taken to be plated on PDA medium and placed in incubation for 72 h at a temperature of 32 °C (Astorga et al., 2014; Cubillos et al., 2014; Rivera et al., 2016).

The isolation process in rhizosphere soil of yam crop was performed as described in Figure 1.

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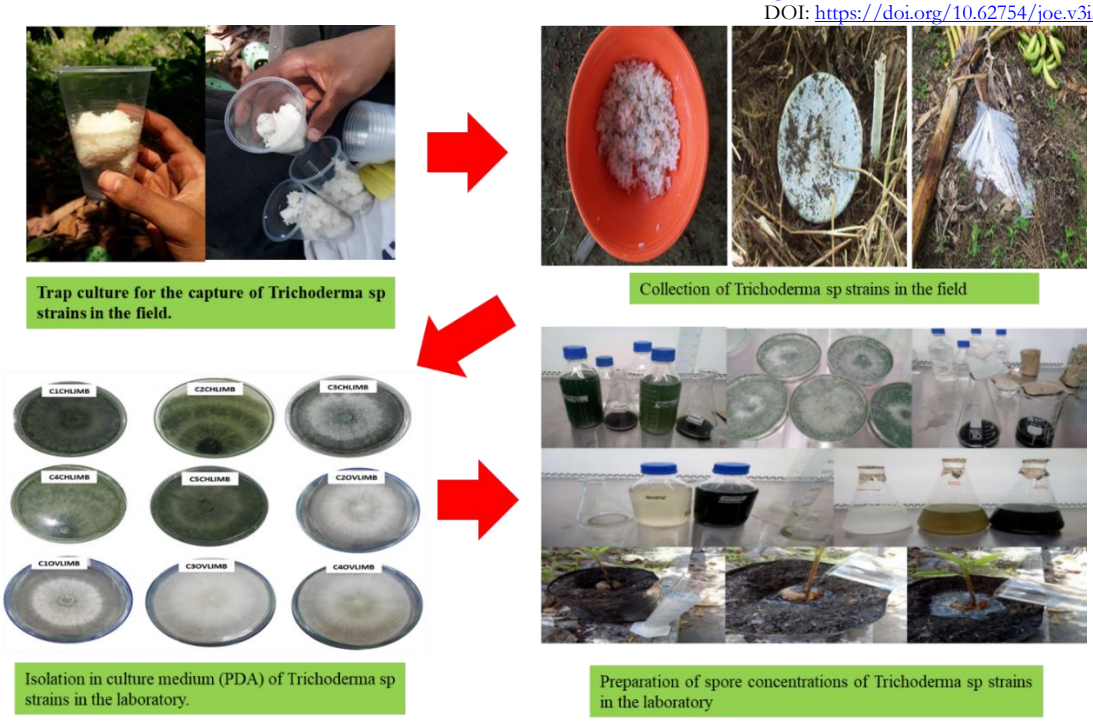

Figure 1. Scheme of procedure for the capture, isolation and preparation of native strains of Trichoderma sp.

Morphological identifications of Trichoderma sp. strains. The Petri dishes that presented growth of microorganisms with characteristics belonging to the genus Trichoderma were purified in PDA culture medium. The strains were incubated for 10 days at a temperature of 32 °C for optimal development. Once the fungus showed growth in the culture medium, the morphological structures such as conidia, conidiophores and phialides were observed under the microscope using the paper tape technique. Taxonomic identification at the genus level was carried out using the keys proposed by Barnett and Hunter (1998). In the case of *Colletotrichum gloesporioides*, it was taken from the microorganism bank of the Agricultural Bioprospecting group of the University of Sucre and activated in PDA culture medium, which was molecularly identified as *C. gloesporioides* as shown in Figure 2.

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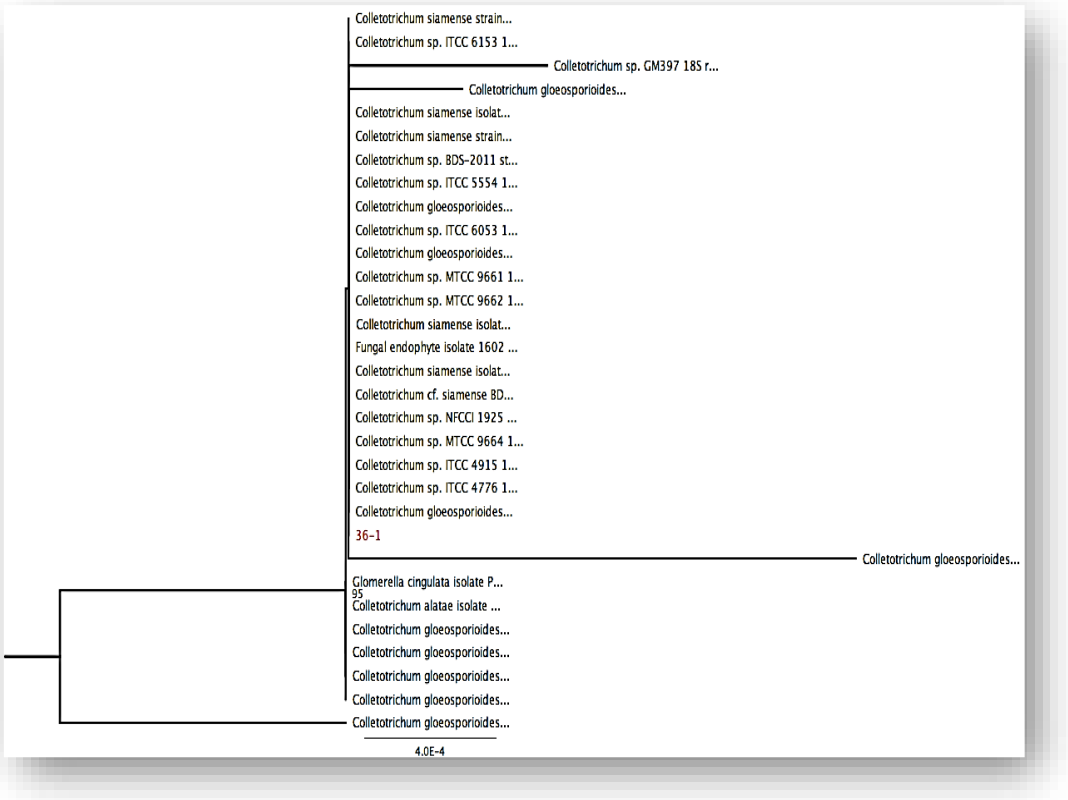

Figure 2. Phylogenetic distance tree constructed from the closest sequences available in the NCBI NR/NT database.

Extraction of genomic DNA from Trichoderma spp. rDNA extraction was performed using the DNeasy Plant Mini® kit following the manufacturer's protocol (Gamarra et al., 2017). The extracted DNA was subjected to polymerase chain reaction (PCR) to amplify the tef1 gene using primers EF1-728F 5'-CATCGAGAAGTTCGAGAAGAAGG-3' Tef1-Llevrev 5'-AACTTGCAGGCAGGCAATGTGG-3', following the methodology proposed by Druzhinina (2009) and Maniscalco and Dorta (2015). The amplified products were sent for sequencing to Macrogen. The nucleotide sequence entities obtained were compared with those stored in GenBank. The MEGA X program was used to perform sequence alignment and phylogenetic inferences applying the Neighbor Joining method based on the kimura-2-parameter model with bootstrap test 1,000 replicates.

In vitro antagonism test of Trichoderma spp. against *C. gloesporioides*. With the help of a punch, a mycelial block of both pathogen and antagonist was taken and placed at a distance of 6 cm from the PDA culture medium. The Petri dishes were incubated for 3 days at a temperature of 30 °C. The positive result of antagonistic activity is evidenced by the growth of the antagonist in the culture medium.

To evaluate the antagonistic capacity, the following formula proposed by Rivera et al. (2016) was applied.

$$PICR = \frac{R_1 - R_2}{R_1} x\ 100$$

Where R1 is the radius of the control pathogen and R2 is the radial growth of the phytopathogen exposed to the antagonist.

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Siderophore production. The qualitative production of siderophores was determined using the chromium azurol-S (CAS) medium proposed by Schwyn and Neilands (1987).

Phosphate solubilization. The phosphate solubilizing ability of each strain was determined using SRS culture medium. Color change from purple to yellow in the medium is considered positive for phosphate solubilization (Sundara & Sinha, 1963).

Statistical analysis. A completely randomized design (CRD) was applied for the antifungal activity of Trichoderma spp. against C. gloesporioides. Likewise, the Duncan rank multiple test was applied to establish significant statistical differences ($p<0.05$) in terms of the percentage of inhibition. The student version of the InfoStat program was used. The assays were performed in triplicate.

Results and Discussion

A total of 10 strains of Trichoderma spp. were isolated, of which 6 were isolated from the municipality of Ovejas and 4 from the municipality of Chalán. Likewise, 1 strain from Chalán identified as (C7CHLIM) and 2 strains from Ovejas (C8OVLIM, C11OVLIM) presented an antagonistic effect *in vitro* against the pathogen. In addition, it is observed how the different strains of Trichoderma spp. rapidly occupy the space in the Petri dish (Figure 3). The Duncan rank multiple test yielded significant statistical differences ($p < 0.05$) in the percentage of inhibition of each of the strains of Trichoderma spp. In addition, strain C7CHLIM and C8OVLIM presented the highest percentages of inhibition and did not present significant statistical differences against the pathogen ($p > 0.05$) (Figure 4).

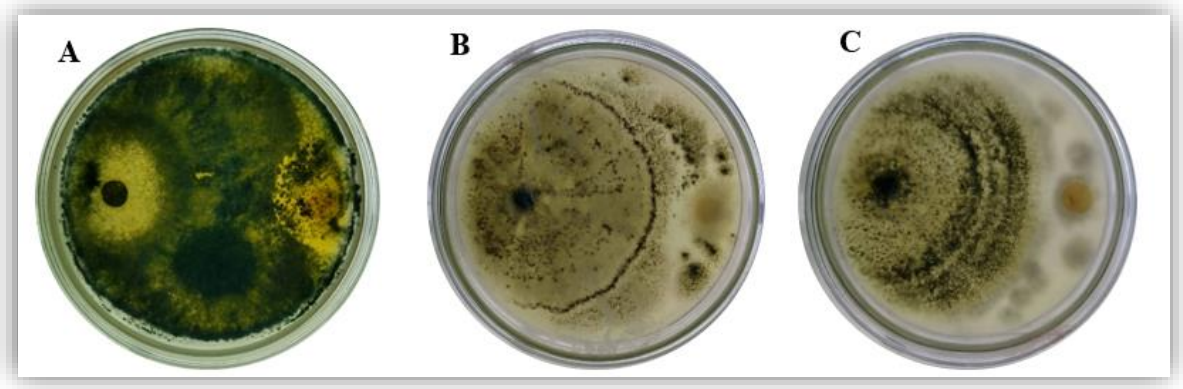

Figure 3. Results of *in vitro* inhibition test of native strains of Trichoderma spp. against the phytopathogenic fungus *C. gloesporioides* isolated in PDA culture medium. (A) C7CHLIM, (B) C8OVLIM, (C) C11OVLIM, (C): strain; (OV): Sheep; (CH): Chalan; (LIM): Microbiological Research Laboratory.

According to the results obtained from the sequence analysis of the tef1 gene of the Trichoderma spp. strains, it showed that strain C11OVLIM was identified as *Trichoderma harzianum*, C8OVLIM as *T. atroviride* and finally C7CHLIM as *T. asperellum* (Figure 5).

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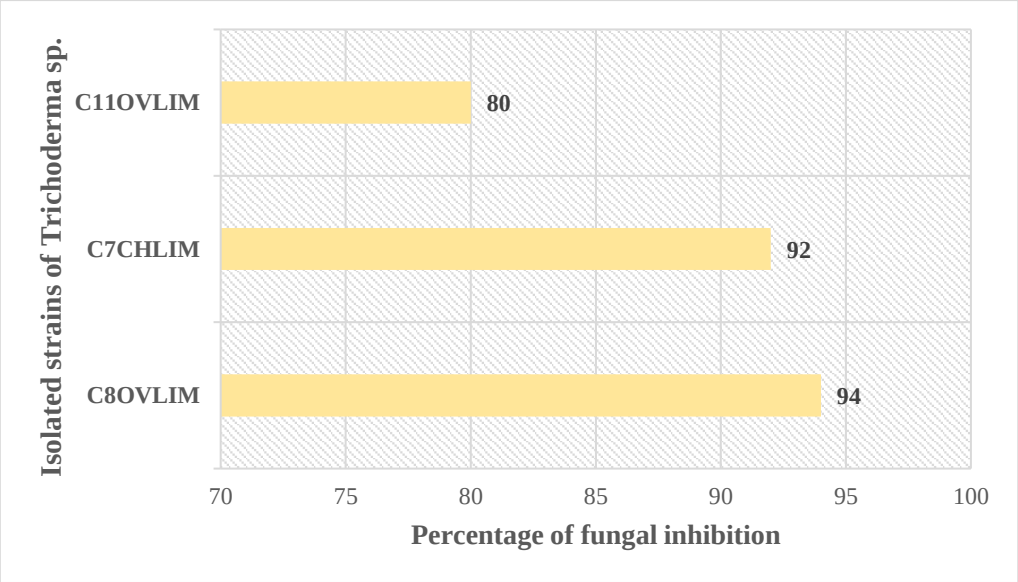

Figure 4. Percent inhibition of Trichoderma spp. strains against Colletotrichum gloesporioides.

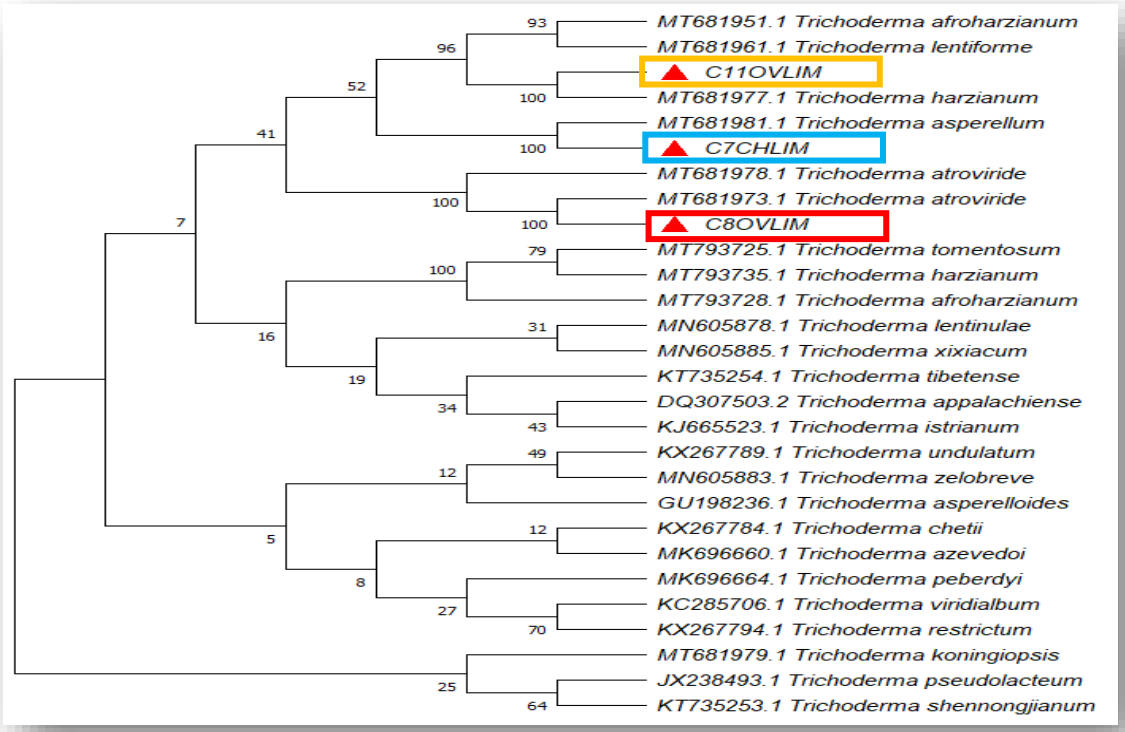

Figure 5. Dendogram from the sequence of the *tef1* gene of *Trichoderma* spp. isolated from avocado cultivars of the María Mountains.

On the other hand, the results of the qualitative activity of plant growth promotion *in vitro* show that for the solubilization of phosphate there is a change of color from purple to yellow,

which is an indication that the species of Trichoderma spp. is possibly solubilizing the phosphorus of the medium through the release of enzymes such as phosphatases or organic acids, which cause a change of pH of the medium. The same occurs with CAS medium. In this medium, iron is found forming a stable

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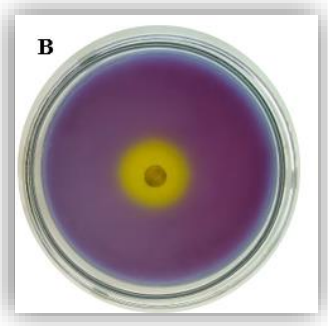
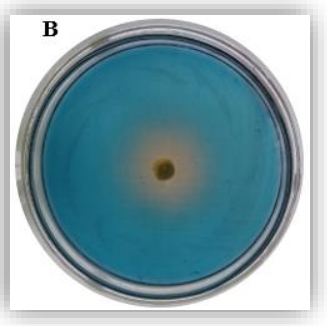
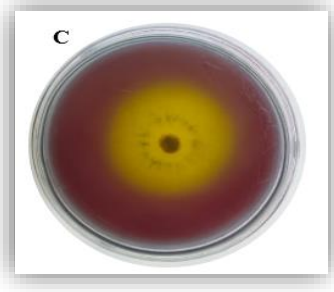
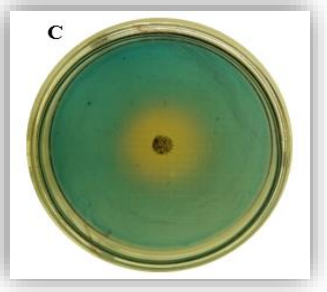
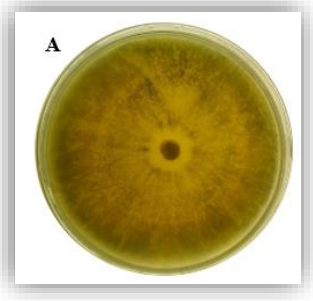
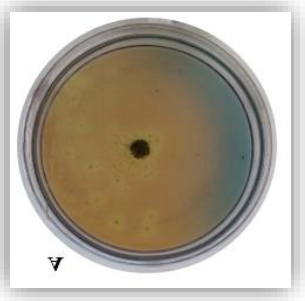
complex with Chrome Azurol Blue (CAS) and HDTMA (Hexadecyltrimethyl Ammonium Bromide), being this complex responsible for the blue color of the medium, when a strong chelator is produced, in this case the siderophores, capture the iron from the complex, it loses its blue color and the medium becomes orange or transparent. This color change is used as a response indicator in siderophore-producing microorganisms (Mahmoud & Abd-Alla, 2001) (Table 1).

Table 1. *In Vitro* Plant Growth Promotion Activity: (A) T. Harzianum, (B) T. Atroviride, (C) T. Aperellum. the First Row Corresponds to Phosphate Solubilization and the Second Row To Siderophore Production.

In vitro antagonistic activity of Trichoderma spp. against C. gloesporioides. The genus Trichoderma comprises a wide variety of filamentous fungi that are present in most ecosystems. These fungi have been isolated from soils and can be easily cultured *in vitro* (Brotman et al., 2010).

Stains	**Species**	***In vitro* growth promotion activity**	
		Phosphate solubilization	**Production of siderophore**
C8OVLIM	*Trichoderma atroviride*		
C7CHLIM	*Trichoderma asperellum*		
C11OVLIM	*Trichoderma harzianum*		

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They have proven to be a biotechnological tool because they present great benefits for agriculture, including plant growth promoting activity from the production of siderophores, phosphate solubilization, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole acetic acid (IAA), phytase and acid phosphatase activity under biotic or abiotic stress (Babu et al., 2014) and for their biological control against phytopathogens present in the soil.

On the other hand, Trichoderma spp. presents a fast growth which favors it to compete for space and nutrients in its environment (figure 2). Besides, it has the capacity to be able to release enzymes like chitinases and glucanases that cause damages in the cellular wall of the pathogen, which allows the entrance of its hyphae and cause a process called mycoparasitism. This mechanism was evidenced in this study, where Trichoderma spp. has the ability to control and suppress the growth of the pathogen through the production of volatile secondary metabolites and release of hydrolytic enzymes that will cause irreversible damage to the structure and metabolism of the pathogen, leading to its death (Bulgari et al., 2020; Rajani et al., 2021; Mukherjee et al., 2022). For example, in a study by Kuzmanovska et al. (2018) evaluated T. asperellum and T. harzianum species against 18 genetically different strains of Botrytis cinerea, one of the main pathogens attacking tomato crop.

Translated with www. The results showed that T. harzianum and T. asperellum exhibited inhibition against all Botrytis cinerea isolates, both mycelial and conidial germination through the production of volatile secondary metabolites and mycoparasitism. Demonstrating that these two species mentioned above are promising biological control agents for the control of gray mold disease on tomato. It has been demonstrated that T. harzianum has the ability to colonize Arabidopsis thaliana roots and induce host-induced systemic response to counteract B. cinera infection, in turn releasing enzymes such as β-glucosidase, endochitinases, proteases and mannosidases, being the main enzymes involved in the mycoparasitism process (Amira et al., 2017; Poveda et al., 2019). In turn, Yassin et al. (2021) stated that T. harzianum and T.viride controlled *in vitro* the mycelial growth of Fusarium verticillioides and F. proliferatum. Also, these two species released bioactive compounds such as palmitic acid and acetic acid, which are able to control pathogen growth. This shows that Trichoderma species are an essential source of biological fungicides which can be an alternative to replace chemical control.

The genus Trichoderma includes species widely used as biocontrol agents in agriculture, this is due to their ability to inhibit the growth of soil pathogens through the production of hydrolytic enzymes, volatile secondary metabolites, antibiosis and mycoparasitism (Kosanovic et al., 2020; Zou et al., 2021; Oliveira-Mendonça et al., 2022). For this reason, they are a potential to be used for the control of phytopathological diseases. In a research conducted by De la cruz et al. (2018) determined that T. harzianum, T. longibranchiatum, T. yunnanense, T. asperellum had the ability to control the *in vitro* growth of Phytophthora capsici and C. gloeosporioides by mycoparasitism which was evidenced at 100%, where the antagonist grew on top of the pathogenic fungus. In addition to presenting antagonistic activity against C. gloeosporioides (de los Santos et al., 2013), T. asperellum is characterized by stimulating the growth of its host by different mechanisms, among which phosphate solubilization, production of siderophores and indole acetic acid stand out.

As indicated by Shang et al. (2020) stated that T. asperellum has the ability to be able to reduce disease severity by 58.37% in Camellia sinensis plants compared to the control treatment. It increased plant height (7.5%), stem diameter (34.09%), shoot fresh weight (81.18%), root fresh weight (93.75%), shoot fresh weight (93.75%), dry weight (85.71%) and root dry weight (115.38%) at 45 days after inoculation under greenhouse conditions.

According to Ortega et al. (2015) evaluated the growth of onion bulbs when inoculated with T. asperellum. According to the results, the authors state that after 150 days after inoculation with the fungus, the onion bulb increased its diameter and increased the content of flavonoids and phenolic compounds compared to the control treatment. At the same time, the fungus induced the production of indole acetic acid, phosphate solubilization and siderophore production, which favored plant growth. Recent studies have shown that T. asperellum has the ability to control stem and root growth of Fusarium graminearum, the etiological agent of corn stalk rot, in greenhouses. In addition, the expression of genes related to defense response and signal

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transduction in T. asperellum maize plants may be aiding the increased expression of plant peroxidase III (POD) gene, salicylic acid (SA) pathway-related gene, pathogenesis-related protein 5 activation (PR5), and jasmonic acid (JA) (Karuppiah et al., 2022).

Another species, Trichoderma atroviride, has shown good qualities in activating host genes and inducing plant growth. In A. thaliana the fungus induced the production of volatile metabolic compounds and diffusible molecules such as indole acetic acid. It inhibited the growth of B. cinerea (Contreras et al., 2022). The combination of microorganisms has brought excellent results for disease control which has favored crop production. The aforementioned is confirmed by Li et al. (2020) that the combination of metabolites produced by Bacillus subtilis and T. atroviride inhibit the growth of F. graminearum. In addition, the metabolites improved plant growth parameters. Similarly, Bello et al. (2022) evaluated the ability of volatile organic compounds produced by T. atroviride to control B. cinerea in blueberries after harvest. The results obtained from this research allowed to know that T. atroviride released the compound 6-pentyl-α-pyrone (6PP), which is involved in the vacuolization and death of the pathogen. Zanoni et al. (2019) determined that T. atroviride had the ability to produce phytases through solid-state fermentation and likewise produced lignin. Indicating that inoculation of T. atroviride can stimulate plant growth through lignin production and phosphate solubilization.

The *Trichoderma harzianum* species is one of the main fungi widely used in agriculture due to its efficiency in the recovery of degraded soils and uptake of assimilable nutrients for the plant. The results of this research agree with several authors that demonstrate the inhibition generated by T. harzianum against different pathogens. For example, in a study by Braun et al. (2018) about the inhibition process of T. harzianum against mycotoxin-generating fungi, it showed that this species can act as a mycoparasite and release hydrolytic enzymes such as chitinases and laccases that have the ability to be able to disintegrate the cell wall of the pathogen. This demonstrates that this species is considered a good biological control. On the other hand, T. harzianum has the ability to promote plant growth due to its phosphate solubilizing activity, production of siderophores and production of plant growth hormones, such as indole acetic acid, which induces plant root system growth (Bader et al., 2020; Alvarez et al., 2022; Shukla et al., 2022).

According to Santana et al. (2016). The combination of certain biostimulants with T. harzianum has brought great results that have favored an increase in the rate of germination and growth in tomato seedlings, which generates an increase in the diameter of the stem, root mass of the plant and gives it a greater advantage at the time of transplanting. Likewise, the fungus is associated with the roots of the plant, giving it greater vigor and growth.

The use and application of T. harzianum as a biofertilizer to crops has become a sustainable alternative that has allowed a decrease in the purchase of chemical fertilizers. In a research conducted by Carillo et al. (2020) they applied the combination T. harzianum + biopolymers + 6-pentyl-α-pyrone (pyrone) to tomato seedlings in greenhouses. The result of this combination caused a total yield of 40% compared to untreated tomato seedlings. This indicated that the application of this combination improved commercial yield, in terms of number of fruits and average fruit weight when compared to the control. One of the possible causes of this increase in production is that T. harzianum protects the plant through the production of volatile secondary metabolites, enzyme production and siderophores that have the ability to limit the growth of the pathogen. Also, by stimulating root growth, the plant has a greater absorption of nutrients and minerals.

Conclusion

The findings in this study infer that the fungal species identified as T. harzianum, T. atroviride and T. asperellum presented a control in the growth of C. gloesporioides and in turn had the ability to promote plant growth through the production of siderophores and phosphate solubilization. This makes them an excellent alternative to combat phytopathogens that cause crop losses and to improve crop production, which have become so dependent on agrochemicals.

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Author Contribution. Alexander Perez Cordero: experiment execution, data analysis. Donicer Montes V and Yelitza Aguas M, conceptualization, writing - revision and editing. All authors have read and approved the manuscript.

Conflict of Interest. All the authors of the manuscript declare that they have no conflict of interest.

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