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Plant Growth Promoting Activity Of Trichoderma Spp. And Its Potential Biocontrol Against Colletotrichum Gloesporioides

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Abstract

Anthracnose caused by Colletotrichum gloesporioides is one of the main diseases that has limited the production of Dioscorea spp. yam in the Caribbean region. To control the pathogen requires the application of agrochemicals, but this has caused environmental problems. Trichoderma spp. has properties in promoting plant growth and controlling phytopathogens, being proposed as an alternative to replace chemical fertilizers. Currently little is known about the defense mechanisms that Trichoderma spp. species can confer when applied to crops of agricultural interest for protection against pathogens. In turn, in the department of Sucre the diversity of Trichoderma spp. has not been exhaustively explored and how these can be used to increase the yield and development of crops in the region. To evaluate the antagonistic activity of Trichoderma spp. against C. gloesporioides and its potential plant growth promoter in vitro. Samples were taken from yam cultivation in the municipalities of Ovejas and Chalán. Serial diluteons were performed for the isolation of Trichoderma spp. Once the strains were purified, the antagonism test against C. gloesporioides was performed in PDA culture medium. For the promotion of growth, the SRS medium for phosphate solubilization and the CAS medium for the production of siderophores were used. DNA extraction and identification of the isolates was performed using the tef1 gene. The species Trichoderma harzianum, T. atroviride and T. asperellum 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 it to die or inhibit its

growth by producing secondary metabolites. The application of Trichoderma spp. in crops confers protection against pathogens and stimulates plant growth in order to obtain a good yield.

Keywords: Antagonism; Mycoparasitism; Production of siderophores; Phosphate solubilization.

1. Introduction

The yam crop is considered one of the most important major crops in tropical climates (Obidiegwu & Akpabio, 2017), 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 shown an increase in consumption due to its 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 Ministry of Agriculture and Rural Development, there are approximately 30,000 yam-producing families in Colombia, 70% of which grow creole varieties, and only 14% and 16% of which grow 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 constraints that affect the production of yam crops, among which is the anthracnose disease caused by the fungus Colletotrichum gloeosporioides. Symptoms of the disease are characterized by necrotic lesions on the stem, leaf and petiole, resulting in a decrease in the photosynthetic rate of the plant and generating yield 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 used 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 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. The fungus also has the potential to be used in bioremediation processes (Guoweia et al., 2011). It can be easily captured and replicated in the laboratory due to its rapid growth. Thanks to their biocontrol and plant growth promoting potential they are currently marketed as biofertilizers, their inoculation in plants allows the uptake of macronutrients and micronutrients allowing in crop yields and soil quality (Guoweia et al., 2011; Sandheep et al., 2013). For this reason, the aim of this study was to evaluate the antagonistic activity of Trichoderma spp. against C. gloesporioides and its plant growth promoting potential in vitro.

2. Materials and methods

Study area. The study was carried out in yam crops in the municipalities of Ovejas and Chalán, sub-region Montes de María, department of Sucre, Colombia. It corresponds to an area of tropical dry forest 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).

Isolation 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 placed in zip lock bags and taken to the Microbiological Research Laboratory of the University of Sucre for processing. The soil samples were placed in an Erlenmeyer flask containing 90 mL of sterile water, which was left in constant agitation for homogenization. After the time elapsed, 1 mL of the homogenate was taken with the help of a micropipette and inoculated into test tubes containing 9 mL of saline solution (Camargo & Ávila, 2014). From this solution, serial dilutions were made in triplicate. From each of these dilutions, 10 µL were taken to be plated on PDA medium and incubated for 72 hours at a temperature of 32 °C (Astorga et al., 2014; Rivera et al., 2016).

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

DNA extraction 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 the primers EF1-728F 5'-CATCGAGAAGTTCGAGAAGAAGG-3' Tef1-Llevrev 5'-AACTTGCAGGCAGGCAATGTGG-3', following the methodology proposed by Druzhinina (2009). 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 (figure 1). 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 on the culture medium.

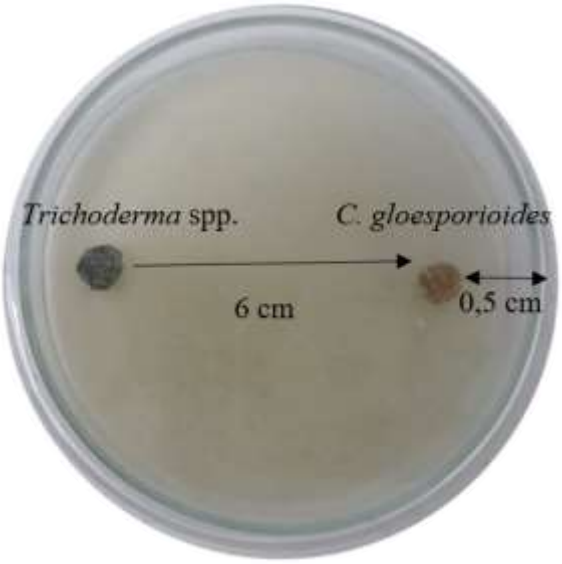

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Figure 1. Schematic diagram of the antagonistic activity of Trichoderma spp. against C. gloesporioides.

The following formula proposed by Rivera et al. (2016) was used to assess antagonistic capacity.

$$\mathrm{PICR} = \frac{R_1 - R_2}{R_1} \mathrm{x}\ 100 \quad \text{(equation 1)}$$

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

Siderophore production. Qualitative siderophore production was determined using the chromium azurol-S (CAS) medium proposed by Schwyn and Neilands (1987).

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

Statistical analysis

A completely randomized design was applied for the antifungal activity of Trichoderma spp. against C. gloesporioides. Also, 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 InfoStat software was used. The assays were performed in triplicate.

3. Results and discussion

A total of 10 strains of Trichoderma spp. were isolated, 6 from the municipality of Ovejas and 4 from the municipality of Chalán. Likewise, 1 strain from Chalán (C7CHLIM) and 2 strains from Ovejas (C8OVLIM, C11OVLIM) showed an in vitro antagonistic effect against the pathogen. Furthermore, it is observed how the different Trichoderma spp. strains rapidly occupy the space in the Petri dish (figure 2). The Duncan multiple range test showed significant statistical differences ($p < 0.05$) in the percentage of inhibition of each of the Trichoderma spp. strains. In addition, the C7CHLIM and C8OVLIM strains showed the highest percentages of inhibition and no significant statistical differences against the pathogen ($p > 0.05$) (figure 3).

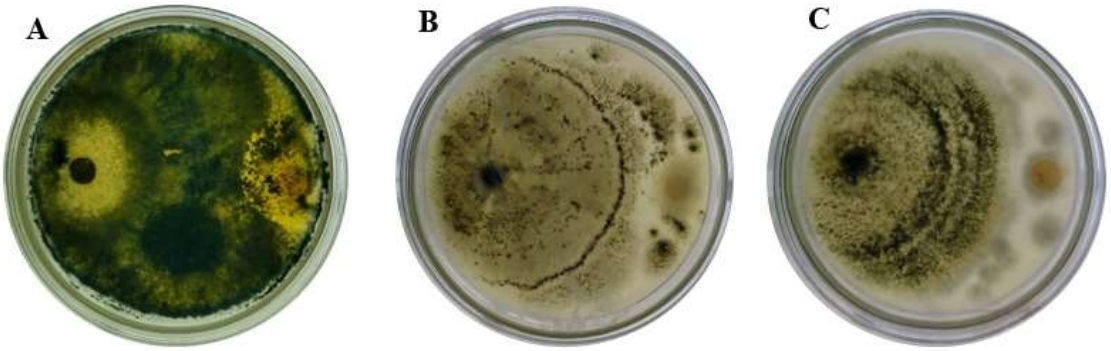

Figure 2. In vitro inhibition of Trichoderma spp. against C. gloesporioides in PDA culture medium. (A) C7CHLIM, (B) C8OVLIM, (C) C11OVLIM, (C): strain; (OV): Sheep; (CH): Chalan; (LIM): Microbiological Research Laboratory.

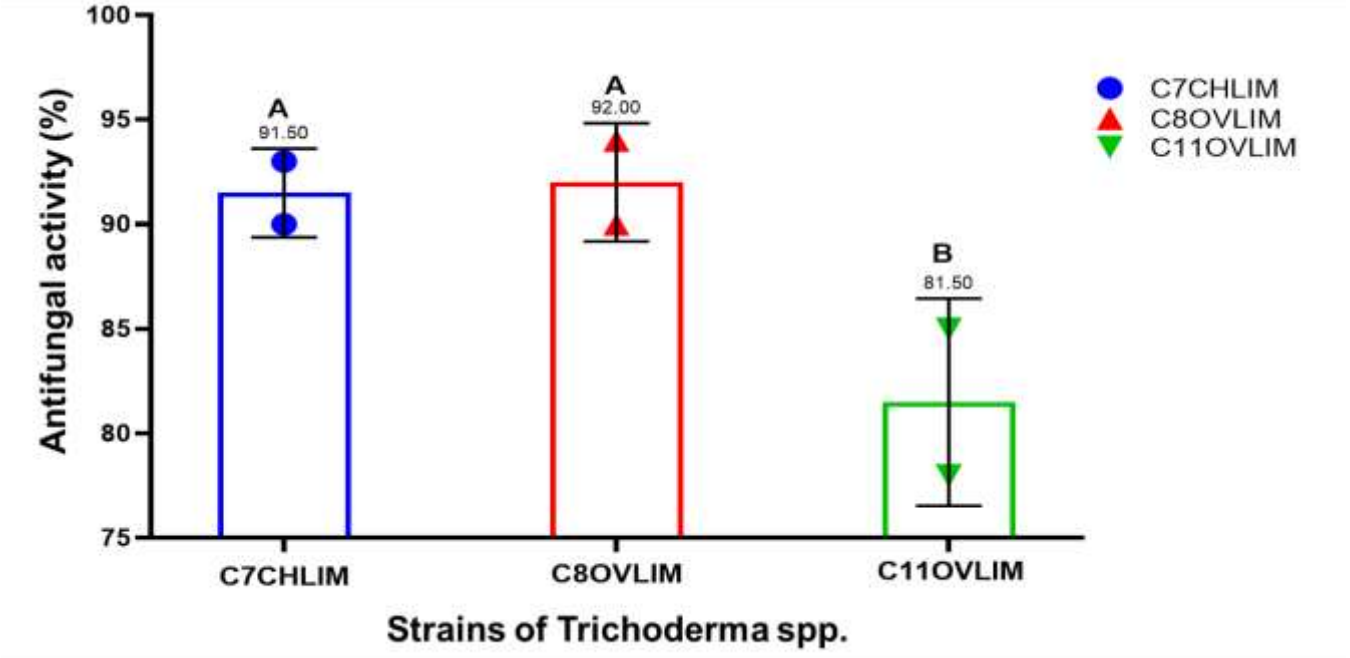

Figure 3. Percentage inhibition of Trichoderma spp. strains against C. gloesporioides. Means with a common letter are not significantly different ($p > 0.05$).

According to the results obtained from the sequence analysis of the tef1 gene of the Trichoderma spp. strains, strain C11OVLIM was identified as Trichoderma harzianum, C8OVLIM as T. atroviride and C7CHLIM as T. asperellum (figure 4).

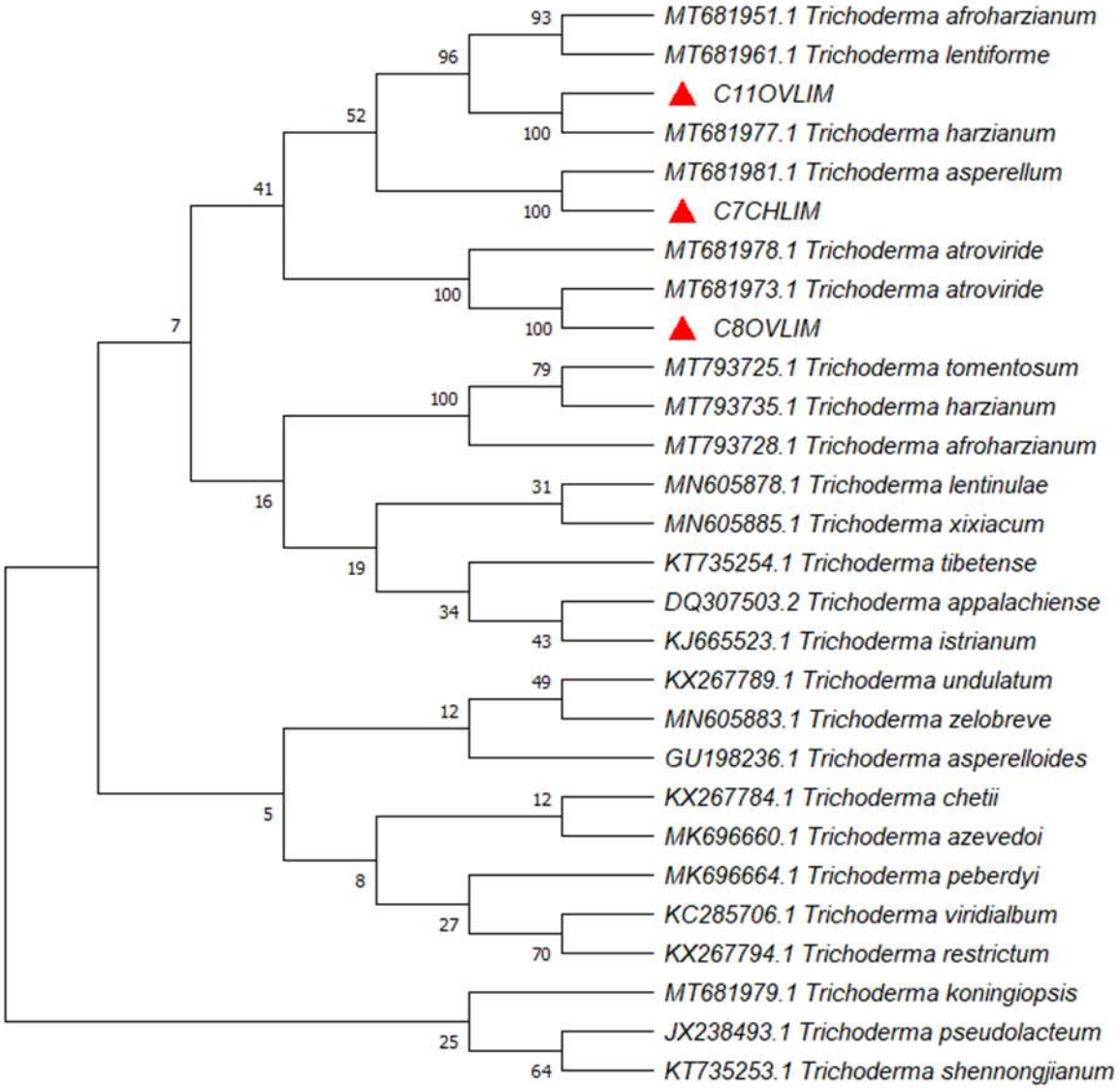

Figure 4. Sequence dendrogram of the tef1 gene sequence of Trichoderma spp. isolated from avocado cultivars from the Maria Mountains.

On the other hand, the results of the qualitative activity of plant growth promotion in vitro show that for phosphate solubilization there is a color change from purple to yellow, which is an indication that the Trichoderma spp. species is possibly solubilizing the phosphorus in the medium by releasing enzymes such as phosphatases or organic acids, which cause a change in the pH of the medium. The same is true for the CAS medium. In this medium, iron is found forming a stable 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 siderophores, capture the iron from the complex, it loses its blue color and the medium turns orange or transparent. This color change is used as a response indicator in siderophore-producing microorganisms (Mahmoud & Abd-Alla, 2001) (figure 5).

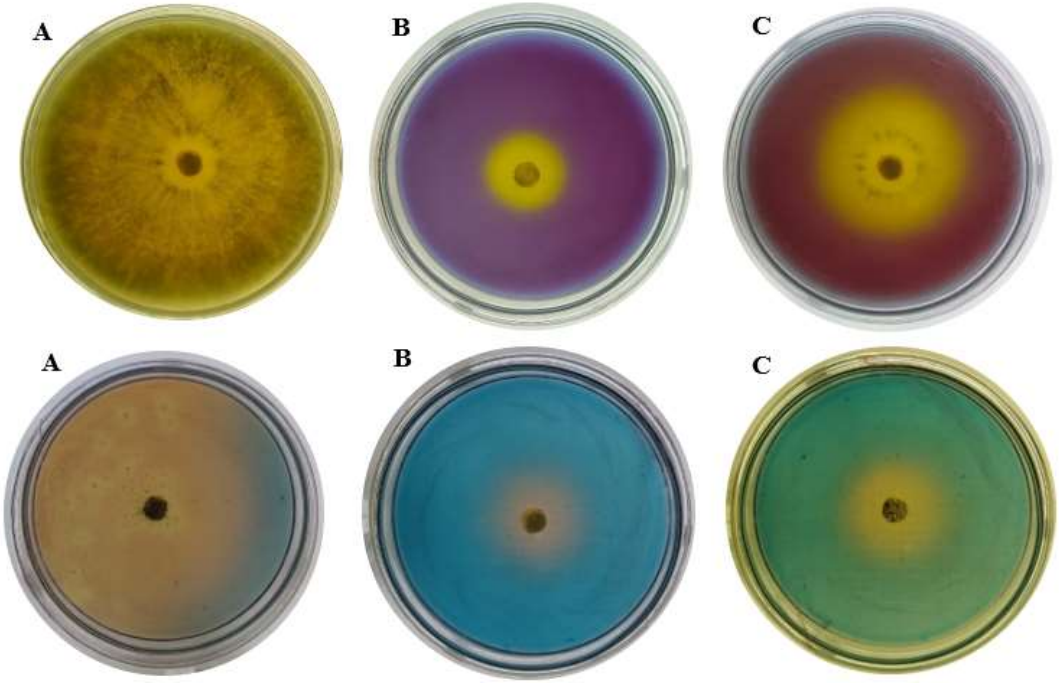

Figure 5. Plant growth-promoting activity: (A) T. harzianum, (B) T. atroviride, (C) T. koningiopsis. The first row corresponds to phosphate solubilization and the second row to siderophore production.

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 cultivated in vitro (Brotman et al., 2010). They have proven to be a biotechnological tool because they have great benefits for agriculture, including plant growth promoting activity from siderophore production, 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 soil-borne phytopathogens.

On the other hand, Trichoderma spp. are fast-growing, which helps them to compete for space and nutrients in their environment (figure 2). In addition, it has the ability to release enzymes such as chitinases and glucanases that cause damage to the cell wall of the pathogen, allowing its hyphae to enter and causing a process known as mycoparasitism. This mechanism was evidenced in this study, where Trichoderma spp. has the ability to control and suppress pathogen growth by producing volatile secondary metabolites and releasing 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. 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. Showing that these two aforementioned species are promising biological control agents for the control of grey mould disease on tomato. It has been shown that T. harzianum has the ability to colonize Arabidopsis thaliana roots and induce induced systemic host 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, 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 have the potential to be used for the control of plant

pathological 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. Besides showing 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, the most important of which are phosphate solubilization, siderophore production and indole acetic acid. For example, Shang et al. (2020) stated that T. asperellum has the ability 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.

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. The fungus also induced indole acetic acid production, 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, an aetiological agent of maize stalk rot, in greenhouses. In addition, the expression of genes related to defense response and signal 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).

On the other hand, T. 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 above is confirmed by Li et al. (2020) that the combination of metabolites produced by Bacillus subtilis and T. atroviride inhibited the growth of F. graminearum. Furthermore, 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 showed 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 also produced lignin. This indicates that inoculation of T. atroviride can stimulate plant growth through lignin production and phosphate solubilization.

T. harzianum is one of the main fungi widely used in agriculture for its efficiency in the recovery of degraded soils and uptake of plant-assimilable nutrients. The results of this research are in agreement with several authors who demonstrate the inhibition of T. harzianum against different pathogens. For example, in a study by Braun et al. (2018) on the inhibition process of T. harzianum against mycotoxin-generating fungi, it was shown that this species can act as a mycoparasite and release hydrolytic enzymes such as chitinases and laccases that have the ability to disintegrate the cell wall of the pathogen. This shows that this species is considered a good biological control. Moreover, 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). The combination of certain biostimulants with T. harzianum has brought great results which 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. The fungus also associates with the roots of the plant, giving it greater vigour and growth (Santana et al., 2016).

The application of T. harzianum to crops has become a sustainable alternative that has allowed a decrease in the purchase of chemical

fertilisers. 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 resulted in a total yield of 40% compared to untreated tomato seedlings. This indicated that the application of this combination improved the marketable 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 yield is that T. harzianum protects the plant by producing 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 an increased uptake of nutrients and minerals.

4. Conclusion

In this study, T. harzianum, T. atroviride and T. asperellum showed control over the growth of C. gloesporioides and 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 has become so dependent on agrochemicals.

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