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Utilizing *Trichoderma* spp. as an alternative for the promotion of plant growth and the control of anthracnose in avocado crops

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Abstract

Avocado is a major crop of economic interest in the Montes de María region, known for its significant nutritional benefits. In recent years, avocado cultivation in Montes de María has been affected by the fungus *Colletotrichum gloeosporioides*, which causes substantial crop losses. To control this pathogen, agrochemical applications are typically required, but these can disrupt the soil microbiota. As an alternative, *Trichoderma* spp. is a fungus capable of controlling pathogens through the production of metabolites and also stimulates plant growth. The objective of this study was to evaluate the *in vitro* antifungal activity of native strains of *Trichoderma* spp. against *C. gloeosporioides* and their growth-promoting effects on avocado plants. Seven strains of *Trichoderma* spp. were molecularly identified as *Trichoderma harzianum*, *T. viride*, *T. reesei*, *T. atroviride*, *T. longibrachiatum*, *T. peberdyi*, and *T. koningiopsis*. These strains showed significant statistical differences in *in vitro* antagonism tests against *C. gloeosporioides*. Additionally, they demonstrated statistically significant ($p<0.05$) growth-promoting effects, with *T. harzianum* and *T. viride* being the most effective at enhancing plant height, stem diameter, and fresh root weight compared to commercial *Trichoderma*. *Trichoderma harzianum* and *T. viride* exhibited inhibitory activity against *C. gloeosporioides* in vitro and increased the biomass of avocado plants, making them a viable alternative for the integrated management of anthracnose in avocado plantations in the Montes de María.

Keywords – Mycoparasitism – Phosphate solubilization – Siderophores – *Trichoderma.*

Introduction

Avocado is considered one of the most important crops in tropical and subtropical climates worldwide (Bill et al. 2014). In recent years, its consumption has increased due to its high content of monounsaturated fatty acids, minerals, fibre, and antioxidants (Pedreschi et al. 2019). Additionally, this fruit is used in traditional medicine to treat cardiovascular diseases, control blood pressure, and for its significant antimicrobial potential against microorganisms (Ochoa-Zarzosa et al. 2021).

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According to the Ministry of Agriculture and Rural Development (MADR), approximately 75% of the avocado-cultivating area in Colombia is planted with Creole or West Indian varieties. Regionally, Antioquia, Bolívar, Caldas, Cauca, Quindío, Risaralda and Tolima account for 86% of the country's production (MADR 2020). However, avocado crop production in the country faces several constraints, including anthracnose, a disease caused by the fungus *Colletotrichum gloeosporioides* (Ramírez-Gil & Peterson 2019, Peralta-Ruiz et al. 2023, Ángel-García et al. 2023, Colín-Chávez et al. 2024). This disease results in approximately 60% loss of production due to fruit abortion in the field and post-harvest rots (Gañán et al. 2015, Kimaru et al. 2020). Symptoms include dark, sunken necrotic lesions with large orange conidial masses when the plant stem is fully colonized (Trinidad et al. 2017). Additionally, fruits in the field develop brown or black spots that enlarge, leading to fruit rot and limiting their commercialization or export (Fuentes-Aragón et al. 2020, Moral et al. 2021). While chemical fungicides have been used to control anthracnose, they have caused environmental problems, and their excessive use can lead to the development of resistant strains (Lobo et al. 2020).

For the aforementioned reasons, there is a growing interest in utilizing rhizospheric microorganisms and endophytes associated with their host plants, capable of suppressing the growth of *C. gloeosporioides* (Chaudhary et al. 2022). Among these microorganisms, *Trichoderma* spp. stands out as one of the primary endophytic fungi known for its beneficial effects on host plants, including the promotion of plant growth, competition for nutrients in the rhizosphere, and antagonistic activity against pathogens (Lacava et al. 2022, Chowdhary et al. 2024). *Trichoderma* spp. are renowned for their biocontrol activity, attributed to their ability to mycoparasitize phytopathogens by secreting hydrolytic enzymes that degrade fungal cell walls (Gruber & Seidl-Seiboth 2012, Mandujano et al. 2016). Notably, proteases among these hydrolytic enzymes seem to play a crucial role in regulating the mycoparasitic process (Deng et al. 2018). Additionally, *Trichoderma* spp. have been employed in conjunction with arbuscular mycorrhizae for biological control purposes (El-Sharkawy et al. 2018).

Certain strains of *Trichoderma* exhibit systemic endophytic properties (Druzhinina et al. 2011), contributing to the promotion of plant growth through the release of phytohormones, such as Indole Acetic Acid (IAA), phosphate solubilization, and metal chelation via siderophore production (Deng et al. 2018). Moreover, *Trichoderma* spp. have demonstrated utilities in bioremediation by effectively degrading various pollutants, including hydrocarbons, chlorophenolic compounds, polysaccharides, and xenobiotic pesticides commonly used in agriculture (Verma et al. 2007, Vinale et al. 2008, Hoyos et al. 2009). Therefore, the present study aims to assess the *in vitro* inhibitory activity of *Trichoderma* spp. against *C. gloeosporioides* and to investigate their potential for promoting plant growth both *in vitro* and *in vivo* in avocado plants.

Materials & Methods

Study area and Capture of *Trichoderma* spp.

The study area is located in the municipalities of Chalán and Ovejas, belonging to the department of Sucre, subregion Montes de María. It is characterized as a tropical dry forest zone, where most of the avocado-cultivating areas of the Caribbean region are located. Containers with precooked rice sealed with gauze were placed in each avocado crop plot. Each rice trap was placed at the base of the stem of each avocado tree at a depth of 25 cm. A total of 24 traps were set, which were left for a period of 10 days to allow the fungus to colonize the substrate. After this period, the traps were collected and transported to the Microbiological Research Laboratory of the University of Sucre for subsequent fungal purification and identification (Acurio & España 2017). Meanwhile, *C. gloeosporioides* was obtained from the strain bank of the Microbiological Research Laboratory of the University of Sucre.

Isolation and identification of *Trichoderma* spp.

Using a sterile loop, a portion of rice showing characteristics similar to the growth of *Trichoderma* spp. was taken and inoculated onto Potato Dextrose Agar (PDA) medium, then

incubated at 31 °C for 10 days for optimal growth. After this time, the Petri dishes showing the growth of *Trichoderma* spp. were purified using the successive subculturing technique on the same medium. The process included both macroscopic and microscopic analyses until identification at the genus level was achieved, following the keys proposed by Barnett & Hunter (1998).

In vitro antagonism test of *Trichoderma* spp. against *Colletotrichum gloeosporioides*

Approximately 5 mm in diameter mycelial discs of both the antagonist and the pathogen were taken and inoculated at opposite ends of 90 mm Petri dishes containing PDA medium, approximately 6 cm apart. The Petri dishes were incubated for 10 days at 30 °C. To evaluate the antagonistic capacity of the *Trichoderma* strains against the pathogen, the percentage of mycelial growth inhibition was calculated using the formula proposed by Yassin et al. (2021):

$$\text{Inhibition percentage} = \frac{A-B}{A}\ X\ 100 \quad \text{Ecu 1.}$$

where A is the radius of the control pathogen and B is the radial growth of the phytopathogen exposed to the antagonist.

Trichoderma strains that demonstrated both antagonistic activity and plant growth-promoting activity *in vitro* were selected for molecular identification and *in vivo* evaluation of plant growth parameters in avocado plants.

DNA extraction of *Trichoderma* spp. isolates

Pure mycelia of each strain of *Trichoderma* were obtained from a PDA culture medium and incubated at 25±2 °C for three days. A 100 mg of fresh mycelium was weighed and macerated in liquid nitrogen using a mortar and pestle. DNA was extracted using the commercial DNeasy Plant Mini® kit, following the manufacturer's protocol.

The extracted DNA of each strain was subjected to polymerase chain reaction (PCR) to amplify the translation elongation factor 1 complex (*tef1*) gene region. A final volume of 25 µL of the reaction mixture was prepared as follows for the PCR reaction: 2.5 µL of buffer; 1.5 µL of 25 mM $MgCl_2$; 1.5 µL of 10 mM dNTP; 0.75 µL of EF1-728F primer (5'-CATCGAGAAGTTCGAGAAGAAGG-3') at 0.2 mM and 0.75 µL of Tef1-LlevR primer (5'-AACTTGCAGGCAGGCAA-TGTGG-3') at 0.2 mM; and 0.5 µL of Taq DNA polymerase in 12.5 µL of sterile ultrapure water (Maniscalco & Dorta 2015, Bustamante et al. 2024). The amplified products were sent for sequencing at the Macrogen (Seoul, South Korea) on an automated sequencer with a 3730XL capillary. The obtained nucleotide sequences were compared with those stored in the databases of the National Center for Biotechnology Information (NCBI). Sequence alignment was performed using ClustalW, and phylogenetic inferences were obtained using the Neighbor-Joining method based on the Kimura-2-parameter model with a bootstrap test of 1,000 replicates using the MEGA X program (Bustamante et al. 2024).

In vitro plant growth promotion of *Trichoderma* spp.

Phosphate solubilization

Using a cork borer, 5 mm plug of mycelium was taken and inoculated into Síndrome Rickettsial Salmonídeo (SRS) culture medium. The fungi were incubated for seven days at 30 °C. A color change from purple to yellow in the medium was considered positive for phosphate solubilization (Sundara & Sinha 1963).

Siderophore production

Siderophore production was determined using the chromium azurol-S (CAS) medium proposed by Schwyn & Neilands (1987). A 5 mm plug of mycelium was taken from each fungus and inoculated into the culture medium using cork borer. These cultures were incubated for seven days at 30 °C. The ability of the fungus to produce siderophores was evidenced by the formation of a transparent halo around colonies.

In vivo plant growth promotion of *Trichoder*rma spp. in avocado plants

Preparation and disinfection of the Seed

The methodology proposed by Alvarado (2017) was followed with modifications in the disinfection process. This involved extracting the avocado seed when the fruit was at commercial maturity. The seeds were then washed and dried for 30 minutes. Subsequently, seeds that were of better quality and free of pests or diseases were selected. The tegument covering the seed surface was removed, and a 2 to 3 mm cut was made at the apex to promote germination. Finally, the seeds were placed in a benomyl solution for 20 minutes to complete the disinfection process. Once disinfected, the seeds were sown with the apex pointing upwards in previously sterilized soil contained in 4 kg capacity polyethylene bags.

Inoculation of *Trichoderma* spp. on avocado plants.

Trichoderma strains were grown on PDA agar at 28 °C under 12-hour light-dark cycles. A stock PDA broth solution was prepared and inoculated with 5 mL of *Trichoderma* spp. culture. After 10 days, 1 mL of the culture was collected and placed in a Neubauer chamber to determine the CFU/mL count under a microscope. After 10 days, 1 mL of the sample was taken and added to a Neubauer chamber. Using a microscope, the number of CFU/mL was determined, and different concentrations were adjusted through dilutions: 1×10^6, 1×10^7, and 1×10^8 CFU/mL. A commercial control was used at the concentration provided by the commercial company, which was 1×10^6 CFU/g. Each plant was inoculated after germination with 500 mL of each concentration every 15 days for a period of 3 months in a net house. The following growth parameters were measured: plant height, stem diameter, and root fresh weight. Measurements were taken every 8 days over a period of 95 days.

Statistical analysis

The data were analyzed statistically by one-way analysis of variance (ANOVA). Likewise, Tukey's test was applied to establish significant differences ($p<0.05$) in the antagonistic activity of *Trichoderma* spp. against C. *gloeosporioides* and the effect of *Trichoderma* spp. on avocado plant growth. The statistical program Infostat Student version was used for the analysis (Di Rienzo et al. 2016), and the R program version 3.4.1 (Ihaka R. & Gentleman 1996) was used for graph editing.

Results

Antagonism test of *Trichoderma* spp. against *C. gloeosporioides*

In this contribution, 24 strains of *Trichoderma* spp. were isolated from avocado crops in the municipalities of Chalán and Ovejas. Of these, three strains from Ovejas (OVC1, OVC2 and OVC3) and four from Chalán (CHC1, CHC4, CHC5 and CHC7) demonstrated the ability to inhibit the growth of *Colletotrichum gloeosporioides in vitro*, showing statistically significant differences ($p<0.05$) in the percentage of inhibition (Fig 1). Notably, the strains CHC5, CHC7 and OVC2 exhibited the highest inhibition averages of 59,6%, 91.5% and 92.6%, respectively (Fig 2).

Many species of *Trichoderma* demonstrate remarkable versatility when competing for space or nutrients. This is evident in our study, as the different strains were able to occupy all the space in the Petri dish and even grow over the pathogen (Fig 1).

Promotion of plant growth *in vitro* of *Trichoderma* spp.

The strains CHC7 and OVC2 were also effective in promoting plant growth *in vitro* through phosphate solubilization and siderophore production. Strains that exhibited a transparent halo in the CAS medium were identified as siderophore producers, while phosphate solubilization was indicated by a color change in the SRS medium from purple to yellow. Although other strains showed at least one growth-promoting activity, they were not considered for *in vivo* evaluations of avocado plant growth parameters (Fig. 3).

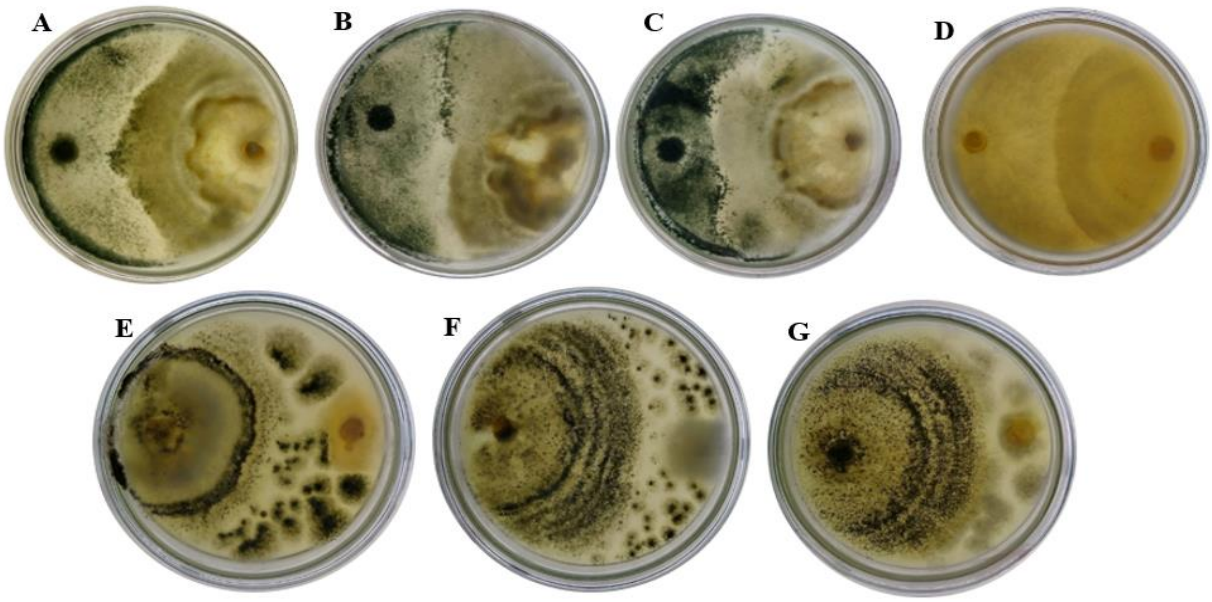

Fig. 1 – *In vitro* antagonistic activity of *Trichoderma* spp. against *C. gloeosporioides* on PDA culture medium. A OVC1, B OVC3, C CHC4, D CHC1, E OVC2, F CHC7 and G CHC5. C: cepa, CH: Chalan, OV: Ovejas.

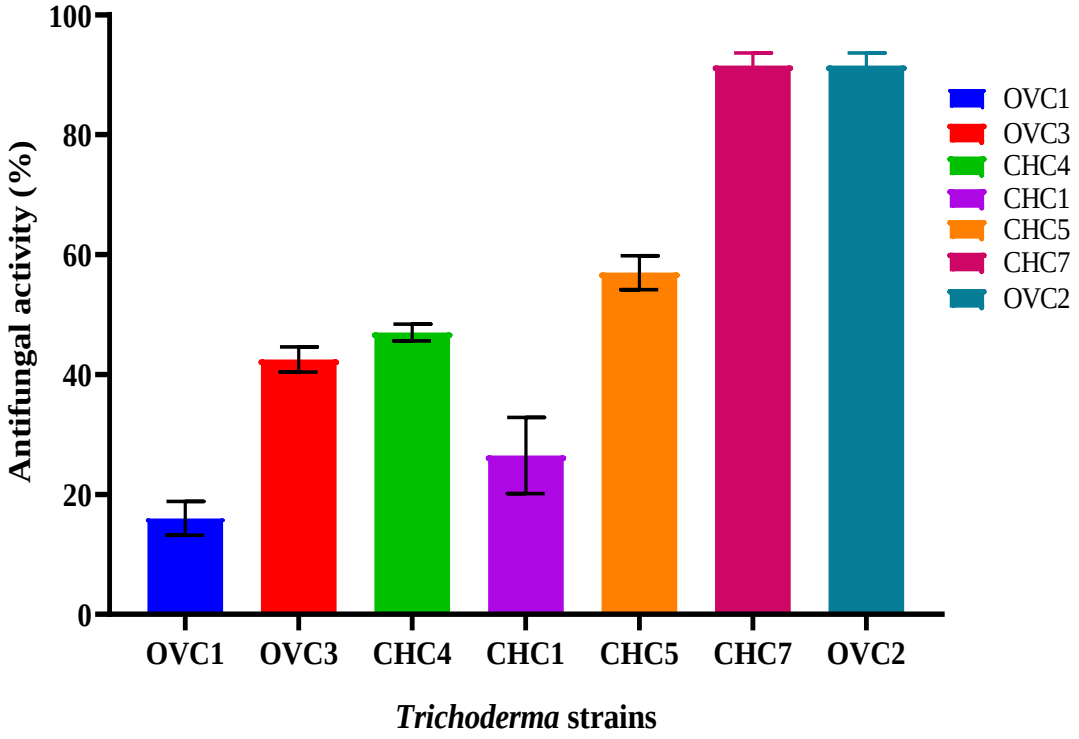

Fig. 2 – Percentage of *in vitro* inhibition of *Trichoderma* spp. strains against *C. gloeosporioides*. Different letters indicate that values were significantly different ($p<0.05$).

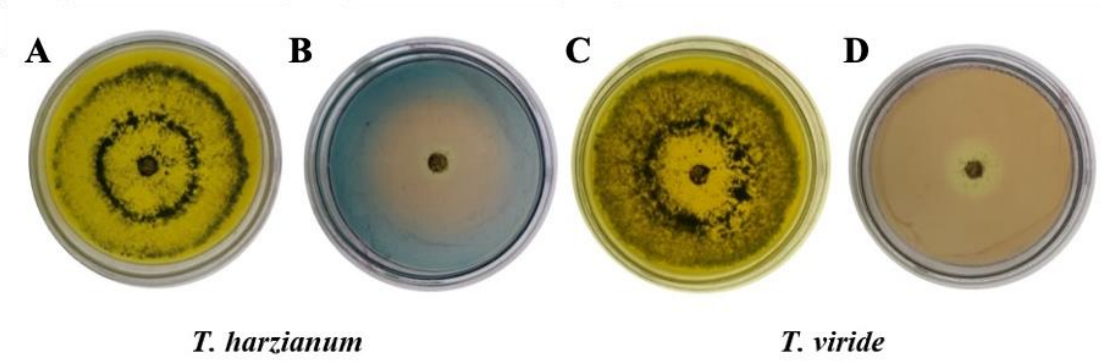

Fig 3. – *In vitro* plant growth promotion of *Trichoderma* strain CHC7 and OVC2. A phosphate solubilization CHC7 strain. B siderophore production CHC7 strain. C phosphate solubilization OVC2 strain. D siderophore production OVC2 strain.

Molecular identification of *Trichoderma* spp. isolates

The genomic DNA amplification using primers EF1-728F and Tef1-Llevrev produced a 1200 bp fragment, which was visualized as well-defined bands on a 1% agarose gel. Sequence analysis identified CHC5 as *Trichoderma reesei*, OVC1 as *T. atriviride*, CHC7 as *T. harzianum*, OVC2 as *T. viride*, OVC3 as *T. longibrachiatum*, CHC4 as *T. peberdyi*, and CHC1 as *T. koningiopsis*. All these strains play a significant role in controlling the *in vitro* growth of *C. gloeosporioides* (Fig 4).

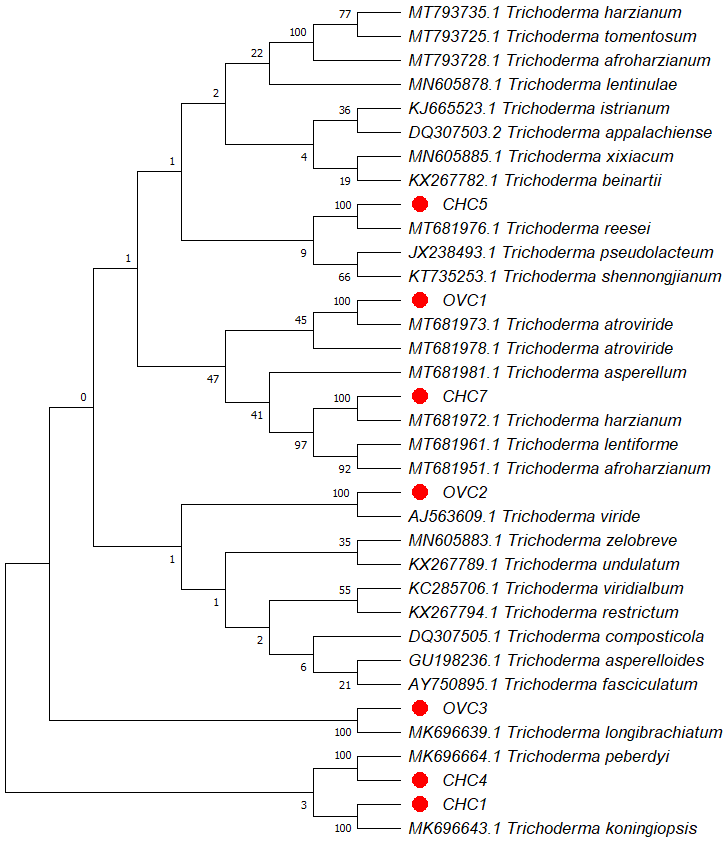

Fig 4. – Phylogenetic analysis from the sequence of the *tef 1α* gene of *Trichoderma* spp. isolated from avocado cultivars of the Montes de María. The tree is unrooted.

***In vivo* growth promotion of *Trichoderma* spp. in avocado plants**

Analysis of variance on plant growth promotion activity revealed statistically significant differences ($p<0.05$) between commercial conidia concentrations and strains of *Trichoderma* spp.

Enhanced growth was observed in avocado plants inoculated with *T. harzianum* and *T. viride* compared to the commercial *Trichoderma* strain (Fig 5). Additionally, the Tukey multiple range test indicated that *T. harzianum* achieved the highest growth at 1×10^8 CFU/mL, resulting in plant heights of 74.66 cm, stem diameters of 58 mm, and root fresh weights of 70 g. *Trichoderma viride* promoted plant growth with heights of 65.66 cm, stem diameters of 56 mm, and root fresh weights of 66.66 g. The commercial *Trichoderma* showed the lowest plant growth values (Fig 6).

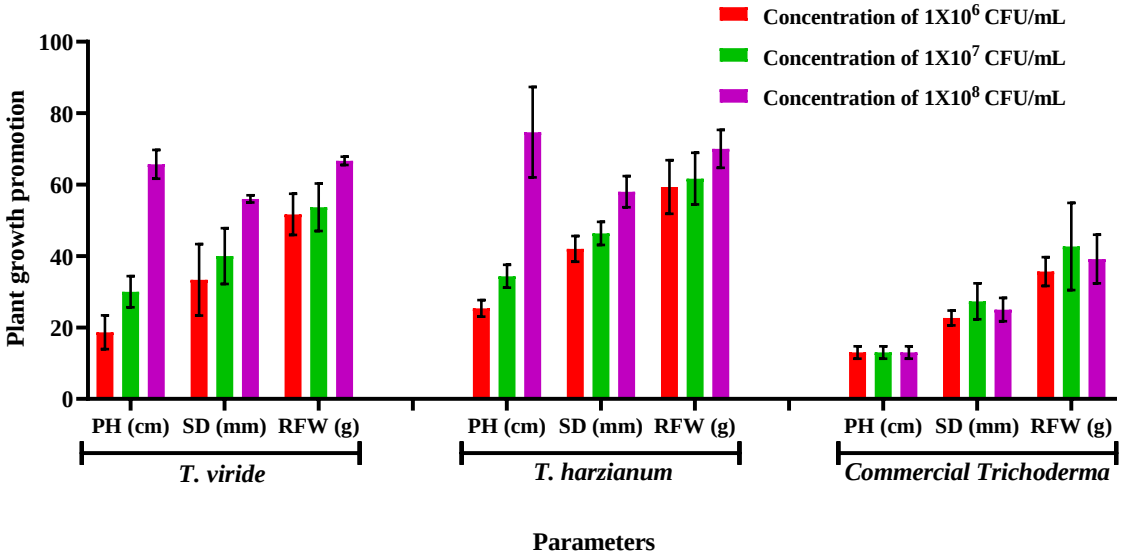

Fig. 5 – Effect of *in vivo* plant growth promotion of *Trichoderma harzianum*, *T. viride* and commercial *Trichoderma* on avocado plants. *PH: plant heights, SD: stem diameters, RFW: root fresh weights. The commercial *Trichoderma* was used as the standard at a concentration of 1 x 10^6 CFU.

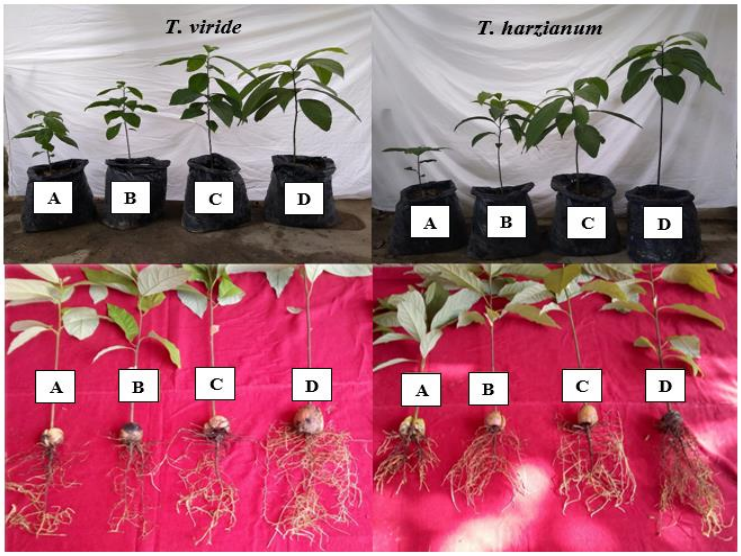

Fig 6. – Plant biomass increase of *T. harzianum*, *T. viride* and commercial *Trichoderma*. A Commercial *Trichoderma* a $1x10^6$ CFU/g and **B, C, D** *T. viride, T. harzianum* at $1x10^6$, $1x10^7$, $1x10^8$ CFU/mL concentrations.

Discussion

Fungi that live endogenously within plant tissues, known as endophytic fungi, play a crucial role in plant development by providing beneficial traits such as protection against pathogens, including viruses, bacteria, parasites, and even other fungi (Chaudhary et al. 2022, Chowdhary et al. 2024). In the case of *Trichoderma*, this mycelial fungus has been considered one of the most effective

in enhancing plant growth, comparable to the growth-promoting activities observed in rhizospheric microbes (Tyśkiewicz et al. 2022). In our study, the strains *T. harzianum* and *T. viride* efficiently contributed to the improved growth of avocado plants compared to the untreated control and commercial spore treatments.

Akbari et al. (2024) proposed that the application of *Trichoderma* significantly improves the growth and germination of rice. These findings may be relevant to our study, where we observed that siderophore production could be related to the growth promotion of avocado plants. However, the fungi obtained from the same ecological niche in the soil where avocado plants are cultivated showed a greater capacity to enhance plant growth. This could be attributed to the native characteristics of the soil that favor plant development due to pre-colonization that occurs without physical contact between fungal mycelia and the root cells or plant tissues (Wahab et al. 2023). In this process, soil-diffusible metabolites and volatile organic compounds coordinate the plant-fungus dialogue, promoting growth (Yao et al. 2023). It can also be attributed to tissue colonization by fungal hyphae, which includes the involvement of fungal hydrolytic enzymes to facilitate colonization by *Trichoderma* (Contreras-Cornejo et al. 2024).

Another important feature in plant growth facilitated by microorganisms is the solubilization of phosphates, which has led to their use as biofertilizers in crops. Various studies have shown that species of *Trichoderma* are effective in phosphate solubilization, even in soils contaminated with heavy metals (López-Bucio 2015, Silva et al. 2023). Do Prado et al. (2019) identified that *Trichoderma atroviride* can produce phytases by lignin production and phosphate solubilization. Tandon et al. (2020) found that *Trichoderma koningiopsis* uses different mechanisms to solubilize phosphates under abiotic conditions. It produces organic acids in alkaline media and accumulates polyphosphate under drought conditions, releasing phosphatases to make this compound stored in the mycelium available. Additionally, *Trichoderma* spp. can acidify their environment by releasing organic acids such as citric acid and gluconic acid, products of sugar metabolism such as glucose, which enhance the absorption of nutrients and minerals (Benítez et al. 2004).

In our *in vivo* experiments, *T. harzianum* and *T. viride* showed better plant growth results than commercial fungi at various concentrations of CFU/mL (Figs 5, 6). This advancement is significant, as it allows the use of native fungi to increase production and protect avocado plants in regions affected by *C. gloeosporioides*. Biotechnological advances help us understand the beneficial interaction between plants and fungi, improving crop quality and potentially replacing chemical fertilizers. Nykiel-Szymańska et al. (2020) demonstrated that spores of *T. koningii*, *T. citrinoviride*, *T. harzianum, T. viride,* and *T. viriens* increased root and shoot length in *Brassica napus* seedlings. These strains promoted growth through the production of siderophores, phosphate solubilization, and 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity, as well as reducing the toxic effect of the chloroacetanilide herbicide after seven days of application.

According to Camargo & Avila (2014), the application of commercial *Trichoderma* at concentrations of 10^7 and 10^8 spores/mL in pea seedlings (*Pisum sativum* L.) improved germination, leaf area, and dry and fresh weight of roots and aerial parts. In comparison, our research showed that concentrations of 10^6, 10^7, and 10^8 CFU/mL of *T. harzianum* and *T. viride* in avocado plants were superior to those of commercial *Trichoderma* (Figure 6), suggesting that native isolates may be more effective due to their active metabolic phase, observable in their *in vitro* growth or medium colonization. Similar results were reported by Cubillos et al. (2009), who compared the growth parameters of *Trichoderma* strains isolated from seedling growth sites and commercial *Trichoderma* evaluated under greenhouse conditions on passion fruit seedlings, under greenhouse conditions on passion fruit seedlings. The results showed that concentrations of 1×10^6 and 1×10^8 conidia/mL yielded superior results compared to the commercial concentration. Recent studies have shown that the application of *T. harzianum* has become a sustainable alternative, reducing the need for chemical fertilizers. Carillo et al. (2020) showed that the combination of *T. harzianum* with biopolymers and 6-pentyl-α-pyrone in tomato seedlings in greenhouses increased yield by 40% compared to untreated plants.

Conclusions

In this study, *T. harzianum* and *T. viride* demonstrated greater efficacy in promoting avocado plant growth compared to the commercial *Trichoderma* strains. Additionally, these species exhibited antagonistic effects against *C. gloeosporioides*. Their diverse mechanisms for pathogen control make them valuable biotechnological tools for managing diseases that currently constrain crop production. This approach has the potential to enhance crop yields and manage the endemic diseases affecting these regions.

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