

## Antifungal activity of native isolates of *Trichoderma* spp. against phytopathogens

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### Abstract

Diseases represent one of the primary causes of loss in agricultural production. Although chemicals are a common alternative for controlling them, they generate adverse effects on human health and the environment. Therefore, sustainable options are required, such as the use of soil microorganisms with biocontrol activity, such as *Trichoderma*, a fungus that has multiple mechanisms of action against phytopathogens, stimulates the soil microbiota, improves nutrient absorption, and activates plant defense mechanisms. This research aimed to evaluate the antagonism of eleven native *Trichoderma* species against *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* using the percentage of radial growth inhibition, the percentage of growth area, the degree of antagonism, and the presence of mycoparasitism. Dual confrontation was performed under *in vitro* conditions in the phytopathology laboratory of the Faculty of Agricultural Sciences of the Autonomous University of the State of Mexico. The isolates *Trichoderma atroviride* TH3 and *T. asperellum* Th11 stood out for their inhibition capacity, reduction of pathogenic growth, high degree of antagonism, and mycoparasitism. The degree of antagonism was assessed with coverage of up to two-thirds of the growth area over the pathogen. *Rhizoctonia solani* showed the lowest percentage of growth area and was the main target of mycoparasitism. The results suggest that *Trichoderma* species have potential for use in sustainable agricultural practices.

### Keywords:

antagonism, growth area, inhibition, mycoparasitism, pathogens, *Trichoderma*.



## Introduction

There is a serious risk of economic losses in agricultural production, as well as a major challenge to food security, due to the significant impact of pests, which cause global losses of between 17.2% and 21.5% in crops such as wheat, rice, corn, potatoes, and soybeans (Savary *et al.*, 2019; Skendžić *et al.*, 2021). The massive use of phytosanitary products in pest control has generated negative consequences, such as biodiversity loss, the development of resistance in pest organisms, and damage to human health (Vinchira-Villarraga and Moreno-Sarmiento, 2019).

In response, more sustainable alternatives have been sought, including the use of antagonistic microorganisms, which are considered a viable option to ensure the production of healthy food (Gutiérrez-Ramírez *et al.*, 2013; Companioni *et al.*, 2019; Vinchira-Villarraga and Moreno-Sarmiento, 2019). Microorganisms with biocontrol activity are characterized by rapid growth, high reproductive capacity, competitive efficiency, and environmental adaptation (Viera-Arroyo *et al.*, 2020).

Currently, there is a large number of microorganisms with profitable applications in agriculture as control agents, with *Trichoderma* spp. standing out, a cosmopolitan fungus highly competitive for space and nutrients and that produces bioactive metabolites (Amerio *et al.*, 2020; Andrade-Hoyos *et al.*, 2023; Cortés-Hernández *et al.*, 2023). More than 200 species of the genus *Trichoderma* have been described as biological control agents (Garrido *et al.*, 2019; Sood *et al.*, 2020; Allende-Molar *et al.*, 2022). In Mexico, 42 species have been reported, with *T. asperellum* and *T. viride* being the most common (Allende-Molar *et al.*, 2022).

Species such as *T. asperellum*, *T. atroviride*, *T. hamatum*, *T. harzianum*, *T. longibrachiatum*, *T. koningii*, and *T. viride*, among others, have shown antagonistic and mycoparasitic effects against soil phytopathogens such as *Phytophthora capsici*, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Phymatotrichopsis omnivora* (Andrade-Hoyos *et al.*, 2019; Camacho-Luna *et al.*, 2021; Allende-Molar *et al.*, 2022). Other species act on pathogens due to the presence of various secondary metabolites (Montes-Vergara *et al.*, 2022).

In the biotechnological area, *Trichoderma* species are of interest for the production of enzymes, generously used in the food industry (Allende-Molar *et al.*, 2022); in addition, other species are reported as bioinducers of plant growth (Hernández-Melchor *et al.*, 2019; Matas-Baca *et al.*, 2023) and as responsible for activating the systemic response in plants through the expression of genes such as *Epl1* and *Sm1* (Martínez-Canto *et al.*, 2021).

Although there are numerous studies on the biological control of pathogens by *Trichoderma* spp., it is necessary to implement studies on autochthonous strains, adapted to local environmental conditions (Amerio *et al.*, 2020). This research aimed to evaluate the antagonistic activity of eleven native *Trichoderma* species against the phytopathogens *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* under *in vitro* conditions.

## Materials and methods

### Biological material

The fungal strains used in this study belong to the collection of the Phytopathology Laboratory of the Faculty of Agricultural Sciences of the Autonomous University of the State of Mexico. The pathogens used were: *Botrytis cinerea* (strawberry), *Fusarium oxysporum* (avocado), *Rhizoctonia solani* (potato), and *Sclerotinia sclerotiorum* (sunflower). The antagonist strains of *Trichoderma* spp. were collected in different regions of the State of Mexico in 2023, from rhizosphere samples of various agricultural crops, and molecularly identified in 2024 (in the process of publication).

## Growth inhibition tests

The pathogen and antagonist strains were activated in PDA BD Difco™ medium (potato dextrose agar) for seven days at 25 °C, using the same medium for dual seeding. The confrontation assays consisted of antagonist-pathogen dual seeding, following the methodology of Dennis and Webster (1971). To do this, a 7 mm mycelial disc of the pathogen was placed on the inner margin of the Petri dish containing the culture medium, and the antagonist was seeded at the opposite end.

The control consisted of seeding the pathogen alone at the margin of the Petri dish. Petri dishes were incubated at 25 °C in darkness for 10 days, until the mycelia made contact and the control fully colonized the Petri dish. The mean values of the percentage of radial growth inhibition (PRGI) of the mycelium were calculated according to the formula:  $PRGI = [(R1 - R2) / R1] \times 100$  (Ezziyyani *et al.*, 2004). Where: PRGI= percentage of radial growth inhibition of mycelium (mm); R1= mycelial growth of the pathogen (control); R2= mycelial growth of the pathogen in confrontation with the antagonist.

The experiment was conducted under a completely randomized design with three replications per pathogen in dual seeding with eleven *Trichoderma* species.

## Growth area percentage (GAP)

The percentage of the pathogen's growth area due to the antagonist's effect in dual seeding was estimated using the AutoCAD® technological application, for use in engineering and architecture (Khoroshko, 2020). This software allows areas to be estimated, using dynamic commands, which is helpful with circular or irregular shapes, as in the case of fungal growth.

## Degree of antagonism (DA)

The degree of *Trichoderma* antagonism was determined using the scale by Bell *et al.* (1982), which describes the antagonist's colonization capacity on the surface of the culture medium in dual seeding with the pathogen, with five levels ranging from the antagonist's supremacy to its absence.

## Mycoparasitism activity

Semipermanent preparations stained with lactophenol blue (Corrales *et al.*, 2020) were used from dual cultures of 10 days of age. These samples were observed under the optical microscope at 40X and 100X magnifications. Ten fields from each sample were examined to determine the presence of mycoparasitism, following the methodology described by Andrade-Hoyos *et al.* (2019), with modifications. Mycoparasitism was considered when coiling of the antagonist's hyphae and deformation or fragmentation of the pathogen's mycelium were observed.

## Statistical analysis

The results of PRGI and GAP were transformed using the arcsine function, followed by an analysis of variance (Anova) and a multiple comparison of means with Tukey's test ( $p \leq 0.05$ ), under a completely randomized design with three replications. The statistical analysis was performed using InfoStat version 2020.

## Results and discussion

### Percentage of radial growth inhibition (PRGI)

The percentage of radial growth inhibition (PRGI) evaluated in dual seeding with the eleven native species of *Trichoderma* and the phytopathogens *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* showed inhibition of mycelial growth with significant differences ( $p \leq 0.05$ ) relative to the control (Table 1).

**Table 1. Percentage of inhibition of radial mycelial growth (PRGI) of *Trichoderma* spp. against phytopathogens in dual growth.**

Species	<i>Botrytis cinerea</i>	<i>Fusarium oxysporum</i>	<i>Rhizoctonia solani</i>	<i>Sclerotinia sclerotiorum</i>
<i>T. atroviride</i> Th1	63.25A	60.62A	56.07A	48.95A
<i>T. atroviride</i> Th2	64.98A	58.06AB	54.2A	49.35A
<i>T. atroviride</i> Th3	64.66A	58.73AB	57.51A	48.96A
<i>T. viridarium</i> Th4	49.37C	50.94CD	40.61B	41.66BC
<i>T. viride</i> Th5	48.67AB	48.21D	41.77B	37.32C
<i>T. hamatum</i> Th6	58.22AB	56.2ABC	52.47A	51.45A
<i>T. atroviride</i> Th7	58.45AB	59.3AB	55.23A	46.13AB
<i>T. atroviride</i> Th8	61.91AB	53.5BCD	55.41A	49.01A
<i>T. scalesiae</i> Th9	65.02A	58.39AB	55.88A	49.96A
<i>T. atroviride</i> Th10	63.08AB	57.89AB	54.56A	51.19A
<i>T. asperellum</i> Th11	54.95BC	52.91BCD	55.70A	47.53A

Means with different letters in the same column are statistically different.

The isolates that showed the highest inhibitory activity against pathogens were: *T. atroviride* Th3 (57.46%), *T. scalesiae* Th9 (57.31%), and *T. atroviride* Th1 (57.22%). As for the pathogens most affected in their mycelial development by the effect of the antagonist, they were *B. cinerea* (59.32%), *F. oxysporum* (55.88%), and *R. solani* (52.67%); in contrast, the lowest level of control was observed in *S. sclerotiorum* (47.41%).

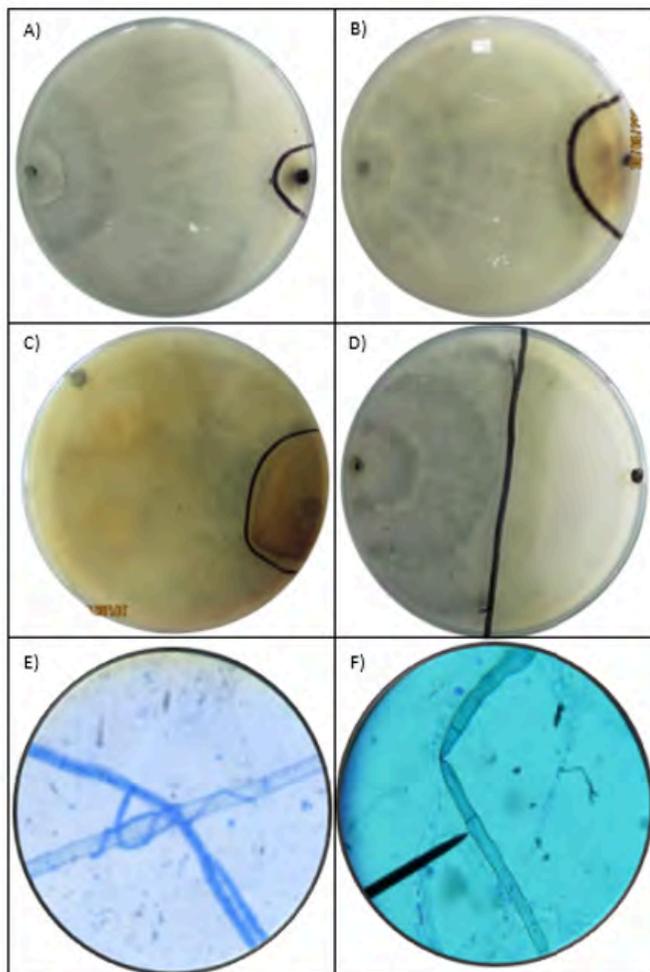
According to Matas-Baca *et al.* (2023), the use of native species with natural antagonistic activity is highly efficient in controlling various phytopathogens, and their use is recognized in sustainable agriculture plans (Companiononi *et al.*, 2019; Cortés-Hernández *et al.*, 2023). The *Trichoderma* species considered in this study showed biocontrol activity against soil phytopathogens, as indicated by Yao *et al.* (2023), with *T. viride* and *T. harzianum* limiting the growth of 29 species of phytopathogenic fungi, among which *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* stand out.

The inhibition of pathogens by *Trichoderma* isolates varies with the native strain and the phytopathogen species used. In this study, the response of the PRGI of *T. atroviride* Th3 on the pathogen *Rhizoctonia solani* was 57.51%, exceeding that reported by Pérez *et al.* (2020) for this same species. Similarly, Geng *et al.* (2022) report a significant inhibition of 68% and a rapid development of *T. harzianum* on *B. cinerea* in dual culture *in vitro*, a percentage comparable to those of the autochthonous isolates *T. scalesiae* Th9 (65.02%) and *T. atroviride* Th2 (64.98%) (Figure 1).



**Figure 1. View of the confrontation of pathogens with *Trichoderma* and types of mycoparasitism.**

A) *B. cinerea* in confrontation with *T. scalesiae*; B) *F. oxysporum* in confrontation with *T. atroviride*; C) *R. solani* in confrontation with *T. asperellum*; D) *S. sclerotiorum* in confrontation with *T. scalesiae*. E) *Trichoderma atroviride* coiling around *Rhizoctonia solani*; F) *Trichoderma atroviride* invades the mycelium of *Rhizoctonia solani*.



On the other hand, Silva *et al.* (2022) report a 12.2% inhibition of *S. sclerotiorum* growth when using *T. lentiforme*; in contrast, *T. hamatum* Th6 and *T. atroviride* Th10 quadrupled this percentage, reaching PRGI values of 51.45% and 51.19%, respectively. As for *F. oxysporum*, Sallam *et al.* (2019) report high PRGI using *T. atroviride* as an antagonist, a result that agrees with the findings of this study. The analysis of variance indicated a significant effect of treatments with different native *Trichoderma* species on the PRGI of the different pathogens, with a coefficient of determination  $R^2 = 0.98$ . Likewise, Tukey's multiple comparisons test ( $p \neq 0.05$ ) identified significant differences among the isolates, grouping them into different levels of antifungal effectiveness.

### Growth area percentage (GAP)

Using AutoCAD® software for the percentage of growth area (GAP) of phytopathogenic fungi and their respective antagonists allowed us to measure colony area ( $\text{cm}^2$ ) more efficiently than with manual methods (Khoroshko, 2020). The pathogen with the lowest GAP was *Rhizoctonia solani* (9.54%), and the highest was *Fusarium oxysporum* (16.95%), showing significant differences between isolates (Tukey,  $p \leq 0.05$ ), as described in (Table 2).

**Table 2. Percentage of growth area (GAP) of pathogens in dual growth with *Trichoderma* spp.**

Species	<i>Botrytis cinerea</i>	<i>Fusarium oxysporum</i>	<i>Rhizoctonia solani</i>	<i>Sclerotinia sclerotiorum</i>
<i>T. atroviride</i> Th1	11.26ABC	18.09B	8.22C	11.26ABC
<i>T. atroviride</i> Th2	11.07ABC	20.74A	54.2A	49.35A
<i>T. atroviride</i> Th3	12.04AB	19.65AB	7.55C	12.04AB
<i>T. viridarium</i> Th4	13.61A	8.58D	14.04A	13.61A
<i>T. viride</i> Th5	9.41C	11.06C	13.95A	9.41C
<i>T. hamatum</i> Th6	11.94BC	20.28AB	9.12C	10.13BC
<i>T. atroviride</i> Th7	11.94ABC	19.83AB	8.02C	11.94ABC
<i>T. atroviride</i> Th8	10.67BC	19.75AB	8.13A	10.67ABC
<i>T. scalesiae</i> Th9	10.83BC	20.09AB	8C	10.83BC
<i>T. atroviride</i> Th10	9.97C	19.54AB	8.2C	9.97C
<i>T. asperellum</i> Th11	12.02AB	8.84CD	11.72B	12.02AB

Means with different letters in the same column are statistically different.

The need to estimate the percentage of fungal growth area in inhibition studies is confirmed by Camacho-Luna *et al.* (2021), who evaluated the action of *Trichoderma* sp. on the percentage of growth area of *Fusarium oxysporum* and *F. proliferatum*, reaching values of 20.4% and 33.3%, respectively. In contrast, the results obtained in this study showed GAPs ranging from 10.9% to 12.9%. These results highlight the use of computational tools to estimate areas in biological phenomena, allowing precise and quantifiable data to be obtained.

### Degree of antagonism (DA)

The *Trichoderma* attributes recognized in antagonist success include its ability to compete for space and nutrients, rapid development, and mycoparasite ability (Martínez-Martínez, 2020). In general, the results showed that the native species of *Trichoderma* reached degree 2 on the pathogens evaluated, which represents the colonization of two-thirds of the culture medium, thereby limiting the growth of phytopathogens. This behavior was particularly evident against *Botrytis cinerea* and *Fusarium oxysporum*.

In contrast, the strains *Trichoderma viridarium* TH4 and *Trichoderma viride* TH5 showed low antagonistic activity against *Rhizoctonia solani*, presenting a degree 3, which indicated competition between both organisms; on the other hand, for *Sclerotinia sclerotiorum*, degrees 3 and 4 were observed, respectively, reflecting a limited effectiveness of the antagonist. However, Rodríguez and Flores (2018) reported values of 1 to 3 using *T. harzianum* against *Rhizoctonia solani*.

### Mycoparasitism

The genus *Trichoderma* has been recognized with mycoparasitism activity, defined as the ability to act on the host cell wall (Matas-Baca *et al.*, 2023). In this study, it was possible to document the type of mycoparasitism carried out by autochthonous strains of *Trichoderma* on the pathogen *Rhizoctonia solani*, because it presents a thick mycelium and dark coloration that contrasted with the antagonist. The *Trichoderma* species that showed mycelium coiled around the pathogen were *Trichoderma atroviride* Th2 and *Trichoderma viride* Th5; those with mycelium invading the pathogen internally were *T. atroviride* Th3, *Trichoderma scalesiae* Th9, and *Trichoderma asperellum* Th11; and the strains that fragmented the mycelium of the pathogen were *T. atroviride* Th7 and *T. atroviride* Th10 (Figure 1).

It has been documented that, after successful penetration, *Trichoderma* is able to degrade the cell wall of its host by producing enzymes on different pathogen species; it can even be located in

the lumen of the fungus once it has penetrated its interior (Sood *et al.*, 2020). Tyskiewicz *et al.* (2022) recognize the action of *Trichoderma* spp. by degrading the cell wall of fungi. In this sense, Fernández (2022) reports parasitic initiation with the activation of recognition genes, followed by coiling and, finally, the disintegration of hyphae.

## Conclusions

The potential of native species of *Trichoderma* spp. as biocontrol agents against the phytopathogens *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* is confirmed, given their PRGI, GAP, DA, and mycoparasitism characteristics, with the isolates *Trichoderma atroviride* Th3 and *Trichoderma asperellum* Th11 standing out. The mycoparasitic activity of *Trichoderma* was outstanding with *Rhizoctonia solani*, including mycelium coiling, penetration into the pathogen, and degradation of its cell wall.

Finally, this study contributed to the knowledge about the use of native *Trichoderma* spp. in the control of pathogens in *in vitro* cultures, providing valuable information for their future applications in disease management programs.

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