

In vitro biocontrol of phytopathogenic fungi isolated from the rhizosphere of multiple crops by a native *Trichoderma* strain

Alondra Santos Villegas,¹ Nuria Jiménez-Juárez,² Minerva Rosas Morales,³ Dalia Castillo-Hernández³

¹Biology School of the Technological Institute of Zacapoaxtla, Puebla; ²Monterrey Institute of Technology and Higher Education, Puebla;

³Research Center in Applied Biotechnology of the National Polytechnic Institute, Tlaxcala, México

Abstract

Phytopathogenic fungi associated with roots and leaves can cause significant losses in crops of commercial interest due to alterations in the growth and development of the host plants. In addition, they could contaminate fruits in the postharvest stage, provoking significant economic damage. In this regard, biocontrol by antagonistic fungi such as *Trichoderma* sp. (Peerson, 1974) has been shown as a viable eco-friendly solution. Accordingly, in this study,

four genera of native phytopathogenic fungi, namely *Fusarium* (Link, 1809), *Botrytis* (Micheli & Peersoon, 1729), *Alternaria* (Nees, 1817), and *Colletotrichum* (Corda, 1831)], as well as of native postharvest fungi, namely *Rhizopus* (Anton de Bary, 1886), *Mucor* (Saccardo, 1887), *Penicillium* (Friedrich, 1809), and *Aspergillus* (Micheli, 1728), were isolated and identified from the rhizosphere of multiple crops of an unstudied autochthonous region in Puebla, Mexico. The isolated phytopathogens were tested in dual confrontation assays against a native *Trichoderma* strain with presumable antagonistic activity, finding a significant growth inhibition, reported for the first time. For the phytopathogenic fungi, the highest percentage of inhibition of radial growth (PIRG) was observed in *Fusarium* sp., followed by *Alternaria* sp., and *Colletotrichum* sp.; for the post-harvesting fungi, the best PIRG was found in *Penicillium* sp. (2), followed by *Aspergillus* sp., *Rhizopus* sp., *Mucor* sp., and *Penicillium* sp. (1).

Correspondence: Dalia Castillo-Hernández, Research Center in Applied Biotechnology of the National Polytechnic Institute. Ex Hacienda San Juan Molino, Carretera Estatal Tecuexcoma-Tepetitla Km. 1.5, Tlaxcala C.P. 90700, México.
E-mail: dcastillohe@ipn.mx

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Introduction

Phytopathogenic fungi can cause multiple diseases in crops. It is estimated that around 8,000 fungi species are phytopathogens,¹ affecting crop quality and yield and diminishing their useful lifetime, causing economic losses to producers and marketers worldwide. Some of the most affected crops worldwide include rice, wheat, corn, potatoes, and soybean,² with production losses of 20 to 40%,³ and up to 10% in developed countries.¹ The most common phytopathogenic fungi isolated from the soils associated with horticultural crops are *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, and *Fusarium oxysporum*,³ this last one responsible for reducing the yield of tomato production in Mexico by 60%; while *Alternaria* sp. does it by 20–30%.⁴

Over the last few years, control strategies, such as chemical fungicides and integrated disease management, have been successfully applied. Nevertheless, treating crops with chemical fungicides has been associated with harmful effects, such as soil erosion, water pollution, and destruction of beneficial fungi and other rhizospheric microorganisms.⁵ Currently, as an environmentally friendly alternative to prevent collateral damage, commercial *Trichoderma* sp. products (soil amendments, biopesticides, and biofertilizers) are applied as biocontrol agents to suppress crop diseases and increase yields.⁶ Fungal species of the genus *Trichoderma* have shown antagonistic effects on phytopathogens like *Pythium*, *Phytophthora*, *Sclerotium*, *Rhizoctonia*, and *Fusarium*.^{7–11} Accordingly, in this study, the antagonistic effect of one native *Trichoderma* sp. strain isolated and identified from soils associated with the rhizosphere of infected crops of commercial interest (from an autochthonous region in Puebla, Mexico), was evaluated by dual confrontation assays against seven genera of phytopathogenic and postharvest

fungi, and results are reported here for the first time. In addition, an analysis of soils associated with these fungal strains is presented.

Materials and methods

Study area and fungi collection

All fungal strains were collected from the farmland of Ayototxo de Guerrero, Puebla, México (at 20°05'50.3 north latitude and 97°24'18.5" west longitude). The sampling was carried out in August 2022. The study area was selected based on the presence of different crops damaged by phytopathogenic fungi (studies were conducted to confirm damage to the stem and leaves). In total, fifty soil samples associated with the rhizosphere of diseased plants by phytopathogens were collected with the help of a shovel by digging 15 cm deep and placing 25 g/each into paper bags.

Isolation and identification of native fungi

The isolation of fungi was carried out by mixing 1 g of soil with 9 mL of sterile distilled water, to later make 1:10 and 1:100 dilutions.¹² Of these, 300 microliters were plated by the streak method on potato dextrose agar (PDA) supplemented with chloramphenicol (0.05 g/L). Samples were incubated at 28°C for seven days and subcultured several times until obtaining pure strains. The identification of fungi was conducted by optical and scanning electron microscopy, analyzing microscopic morphological characteristics (e.g., color of the colony, appearance, margin, texture, etc.) and type of conidiogenesis. For that, fungi were micro-cultured as described by López *et al.*¹³ After three days, grown mycelium was removed and placed between slide and cover slip, stained with Lactophenol cotton blue, covered with a cover slip and visualized with a VELAB optical microscope (model VE-M4) at two magnifications, 40× and 100×. Additionally, fungi were imaged with an Environmental Scanning Electron Microscope (ESEM-FEI Quanta250) at the Center for Nanoscience and Micro and Nanotechnologies (CNMN) of the Instituto Politécnico Nacional (IPN). Finally, taxonomic keys by López *et al.* and Ellis *et al.*, were utilized to determine genera and species.^{13,14}

Confrontation assays

First, to determine the growth rate of all fungi, strains were cultured individually on PDA at 28°C, and their radial growth (RG) was measured with a caliper every day for fifteen days. Then, from a plate containing one strain of grown phytopathogenic fungi and from another with grown *Trichoderma* sp., small pieces (5 mm) were cut with a borer and placed at opposite ends of a PDA plate, with a separation of 4 cm between them and 2.5 cm away from the edge of the Petri dish. Control treatments consisted of a piece of each phytopathogenic fungus with equal dimensions in the center of a PDA plate. The experimental setup was random, plates were set up in duplicate and incubated for six days at 28°C. Finally, every day the fungal radial growth was measured with a caliper. The percentage of inhibition of radial growth (PIRG) was estimated in data collected on day six by applying the Farkhrunnisa formula:

$$\text{PIRG \%} = (\text{R1}-\text{R2})/\text{R1} \times 100$$

where R1 represents the diameter (in mm) of the individual strains inoculated in the absence of *Trichoderma* sp. (controls), and R2 is the diameter from the inoculation point towards the antagonist *Trichoderma* sp. in the treatments.¹⁵ According to PIRG, phytopathogenic strains were classified from 1 to 5 on Bell's scale.¹⁶ Finally, PIRG data were subjected to a one-factor variance analysis

(ANOVA) with a $p < 0.05$ and Tukey's test with the program MegaStat 2007.

Soil analysis

Soil tests were done according to the Official Mexican Standard NOM-021-RECNAT-2000, which establishes the specifications of fertility, salinity, and soil classification, studies, sampling, and analysis.¹⁷ The following studies were performed: i) the AS-07 method by Walkley and Black to determine the content of organic matter in soil samples; ii) the AS-09 Bouyoucos procedure to determine soil texture; and iii) the AS-02 method for pH measurements in soil. In addition, to determine the levels of nitrogen (N), phosphorus (P), and potassium (K), a soil test chemical kit for NPK from HANNA Instruments (Cat. No. HI3896) was applied by following product specifications.

Results

Altogether, fifty-two soil samples associated with fourteen diseased crops were analyzed, of which one hundred and five fungi strains were isolated. The isolates were identified based on macroscopic and microscopic features analyzed by optical and scanning electron microscopy (Figure 1). From the isolates, eighty-one were identified at the genus level, finding eleven different genera (six of them are shown in Figure 1), and twenty-four of them were classified to the species level, finding four species (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus fumigatus*). The richest soil was the one associated with soursop, where were recovered the highest variety of fungi genera, while the poorest soil was the one associated with noni, with few fungi genera isolated (Figure 2A). Of the eleven genera identified, the most representative were *Penicillium*, *Aspergillus*, *Rhizopus*, and *Fusarium*, covering 85% of the total isolates (Figure 2B).

In total, twelve phytopathogenic strains of seven different genera were selected to perform confrontation assays: *Fusarium* sp. (1), sp. (2), and sp. (3); *Alternaria* sp. (1) and sp. (2); *Colletotrichum* sp.; *Penicillium* sp. (1) and sp. (2); *Aspergillus niger* and *A. flavus*; *Mucor* sp.; *Rhizopus* sp., and one potential antagonistic fungus, *Trichoderma* sp. To do that, first, the growing rate of the selected strains was determined as described previously, finding the fastest growing rate in *Trichoderma* sp., which entirely colonized the Petri plate in four days (Figure 3, red line) followed by *Mucor* sp., *Fusarium* sp. (1) and sp. (3). Then, dual confrontation assays were set up among the native *Trichoderma* strain and twelve fungi isolates, providing a two-day growing advantage to the slowest strains. As a result, it was observed an inhibitory effect of *Trichoderma* sp. against all the phytopathogenic isolates, getting significant inhibition against seven strains: *Fusarium* sp. (2) and sp. (3); *Alternaria* sp. (2), *Colletotrichum* sp., *Fusarium* sp. (1), *Penicillium* sp. (2), and *Aspergillus niger*, according to the Tukey test (Figure 4 A to G and Table 1). Likewise, the variance analysis confirmed that native *Trichoderma* sp. is suitable for biocontrol of phytopathogenic fungi since the obtained standard deviation in its absence was 1.2, while in its presence was 0.54, with a p-value equal to 0.0010. Besides, the degree of the antagonistic capacity of *Trichoderma* sp. was confirmed by following the scale of Bell *et al.*, where *Trichoderma* sp. showed a degree equal to 1 over five strains (*Fusarium* sp. (2) and sp. (3), *Colletotrichum* sp., *Fusarium* sp. (1), and *Mucor* sp.), meaning its overgrowing completely covering the medium surface; a degree of 2 over six strains (overgrowing in two-thirds of the medium surface); and a degree of 3 over one of them, (spreading over half of the medium surface)¹⁶ (Figure 4, Table 1).

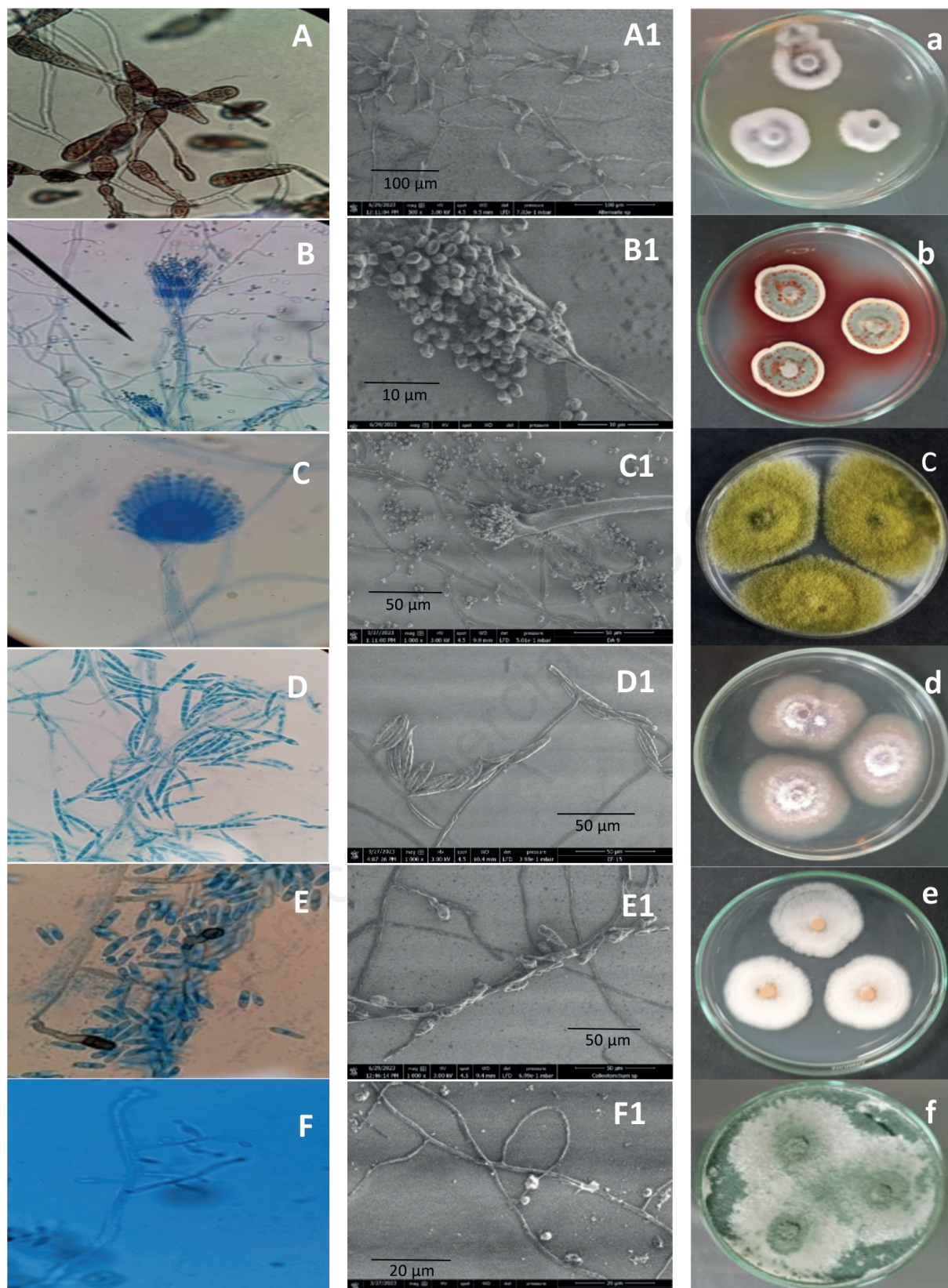


Figure 1. Optical microscope micrographs (100× magnification), scanning electron microscope micrographs, and pictures of cultured fungi. Optical microscope micrographs of: A) *Alternaria* sp., B) *Penicillium* sp., C) *Aspergillus flavus*, D) *Fusarium* sp. (1), E) *Colletotrichum* sp., and F) *Trichoderma* sp. Scanning electron microscope micrographs of A1) *Alternaria* sp., B1) *Penicillium* sp., C1) *Aspergillus* sp., D1) *Fusarium* sp., E1) *Colletotrichum* sp., and F1) *Trichoderma* sp. Following the same order and indicated in lowercase letters, pictures of fungi cultured on PDA.

Likewise, the fifty soil samples were analyzed with the HANNA kit to determine their pH, organic matter; texture type, nitrogen, potassium, and phosphorus content. Regarding the pH, results showed moderately acidic soils with a tendency to neutral pH, with an average value of 6.57, appropriate for phytopathogenic fungi growth.¹⁸ About the organic matter, samples consisted of non-volcanic soils in a medium range of 1.6-3.5%, making it a proper environment for the development of microorganisms.¹⁹ Concerning the texture, all soil samples were classified as sandy soils. Lastly, results indicated a high content of nitrogen (0.15%), phosphorus (1.1%), and potassium (0.06%) in all soil samples; the first two previously

reported as a condition favoring pathogenic over mutualistic fungi in grassland soils.²⁰

Discussion

This study reports for the first time the isolation of one hundred and five fungi strains from the soils associated with fourteen crops from a farmland field in Puebla, Mexico. Four genera were classified as phytopathogenic (*Fusarium*, *Alternaria*, *Botrytis* and *Colletotrichum*) and six other isolates corresponded to postharvest

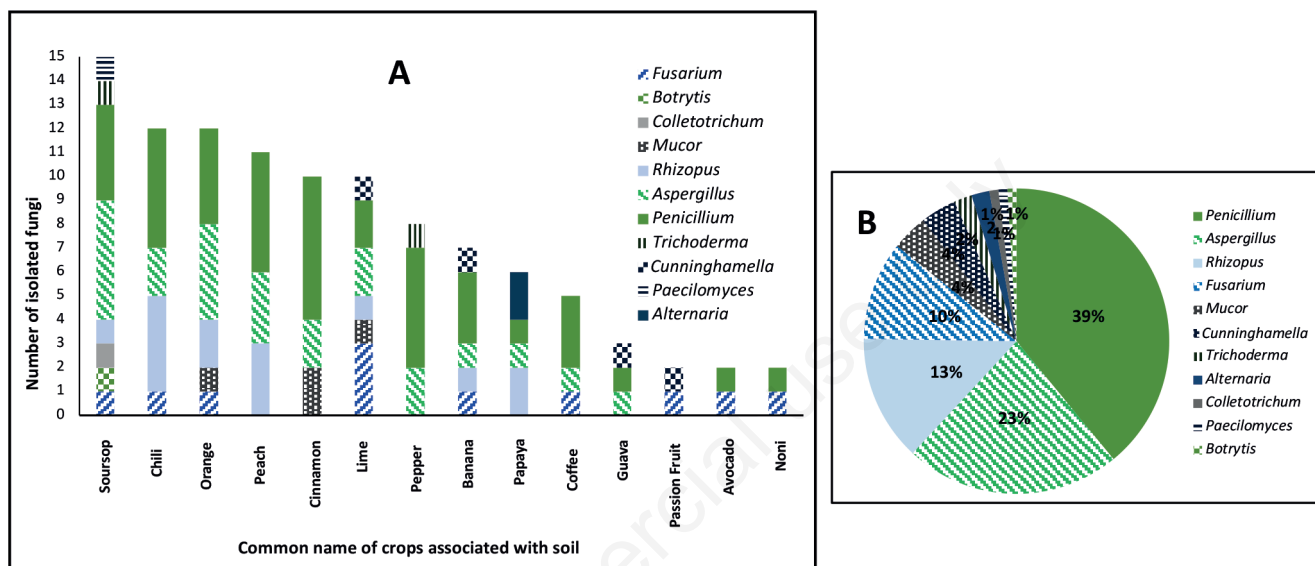


Figure 2. A) Number of isolated strains ordered by genera, identified from soil samples associated with the rhizosphere of fourteen different diseased crops. B) Distribution and abundance of genera of the isolated strains.

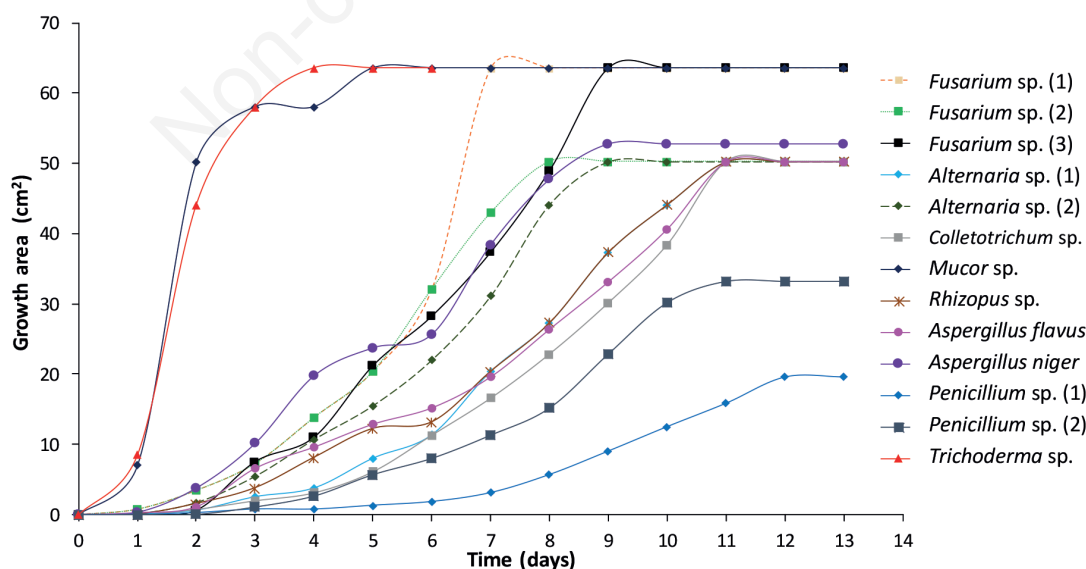


Figure 3. Growth curves of phytopathogenic fungi strains, *Fusarium* sp. (1), *Fusarium* sp. (2), *Fusarium* sp. (3), *Alternaria* sp. (1), *Alternaria* sp. (2), *Colletotrichum* sp., *Mucor* sp., *Rhizopus* sp., *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* sp. (1), *Penicillium* sp. (2); and a presumable antagonistic fungus *Trichoderma* sp.

fungi (*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Rhizopus* sp., *Mucor* sp., and *Penicillium* sp.).

Due to identification done at the macroscopic and microscopic, but not at the molecular level, most fungi were classified only with their genus and not the species. Nevertheless, the characteristics observed in all genera and species agreed with the identification keys published by López *et al.*¹³ One of the strains, identified as

Trichoderma sp., caught our attention because this genus groups around twenty-five species applied for biocontrol of plant fungal diseases, significantly controlling over a hundred plant pathogens worldwide.²¹

Therefore, dual confrontation assays were performed, confirming the native *Trichoderma* sp. significantly inhibited the growth of seven isolates. As mentioned, *Trichoderma* sp. had PIRGs from 81

Table 1. Percentage of inhibition of radial growth (PIRG) indicating significant results (P=0.001) according to Tukey test; and Antagonistic Capacity (AC) of fungi based on the scale of Bell *et al.* 1982.¹⁶

<i>Trichoderma</i> sp. against	PIRG (% ±SD)	AC	<i>Trichoderma</i> sp. against	PIRG (% ±SD)	AC
A <i>Fusarium</i> sp. (2)	81.0±6.1*	1	G <i>Aspergillus niger</i>	65.2±11.8*	3
B <i>Fusarium</i> sp. (3)	80.0±1.4*	1	H <i>Rhizopus</i> sp.	58.1±7.8	2
C <i>Alternaria</i> sp. (2)	74.0±7.5*	2	I <i>Alternaria</i> sp. (1)	58.0±0.2	2
D <i>Colletotrichum</i> sp.	73.7±0.0*	1	J <i>Aspergillus flavus</i>	52.3±0.1	2
E <i>Fusarium</i> sp. (1)	66.3±1.8*	1	K <i>Mucor</i> sp.	46.7±9.4	1
F <i>Penicillium</i> sp. (2)	65.6±4.4*	2	L <i>Penicillium</i> sp. (1)	27.8±15.8	2

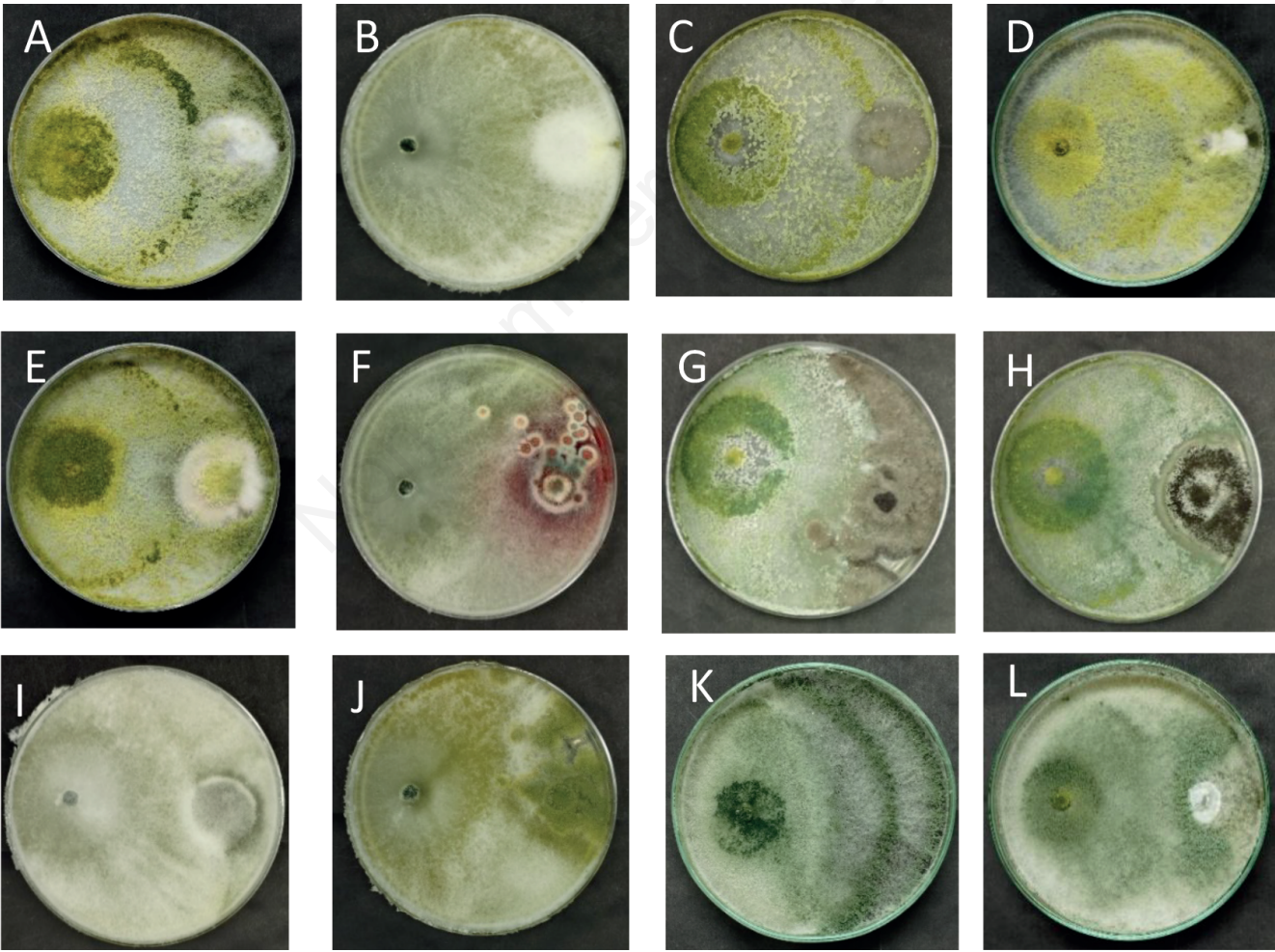


Figure 4. Dual confrontation assays after six days of incubation of native *Trichoderma* sp. against: A) *Fusarium* sp. (2), B) *Fusarium* sp. (3), C) *Alternaria* sp. (2), D) *Colletotrichum* sp., E) *Fusarium* sp. (1), F) *Penicillium* sp. (2), G) *Aspergillus niger*, H) *Rhizopus* sp., I) *Alternaria* sp. (1), J) *Aspergillus flavus*, K) *Mucor* sp., and L) *Penicillium* sp. (1).

to 63% over three strains of *Fusarium*; this reflects a variable range already reported in previous works, depending on the *Trichoderma* species.²²⁻²⁶ A similar variation in the antagonistic effect was observed between native *Trichoderma* spp. and *Alternaria* sp., where *T. harzianum* inhibited in 67% of *A. alternata*, while *T. viride* inhibited the pathogen in 67%.²⁷ Gajera and Vaknaria carried out *in vitro* antagonism studies evaluating twelve isolates of *Trichoderma* spp. against *Aspergillus niger*, finding an inhibition of up to 86.2%,²⁸ in agreement with results found in this study after evaluating the effect of *Trichoderma* sp. against *Fusarium* sp. (80.98% of inhibition). In another work, *T. harzianum* showed from 54 to 83% growth inhibition vs. *A. alternata*.²⁹ About the antagonistic effect of native *Trichoderma* sp. over *Colletotrichum* sp., several publications confirmed PIRG values close to 73%; for example, *T. harzianum* and *T. asperellum* showed PIRGs of 75 and 73%, respectively;³⁰ similarly to *T. orientale*, *T. longibrachiatum*, *T. koningiopsis*, and other unidentified *Trichoderma* isolates.³¹ In the particular case of *Penicillium* sp., little information is available; there is a report where *T. harzianum* significantly controlled the growth of *P. digitatum* in dual confrontation assays.³² Finally, for *Aspergillus niger*, several reports showed a different range of PIRG depending on the *Trichoderma* species; for instance, in one report from twelve *Trichoderma* species tested against *A. niger*, PIRGs varied from 86 to 50%;²⁸ in other works, *T. viride* and *T. harzianum* reduced the radial growth of *A. niger* (80.4%);³³ and *T. viride* had a higher inhibition on *A. niger* (78.2%) than *T. harzianum* (72.5%).³⁴

Conclusions

In this work, the native *Trichoderma* sp. isolated from soil associated with soursop proved to biocontrol phytopathogenic and postharvest fungi in dual confrontation assays. At the same time, it highlighted the importance of analyzing soils associated with diseased crops as a rich source of phytopathogens suitable for biocontrol (e.g., *Trichoderma*, *Penicillium*, *Alternaria*, *Aspergillus*, *Botrytis*, *Rhizopus*, *Colletotrichum*, and *Fusarium*). Thus, this study may contribute in a significant way to the scientific and social sectors since the isolation and identification of the phytopathogens were performed for the first time in an autochthonous area of Mexico, contributing to elucidate its unknown biodiversity and most importantly, offering a viable method of biocontrol, available to locals. In this way, when applying *Trichoderma* sp. as a bio fungicide agent, producers could reduce their economic losses by being able to control different diseases caused by phytopathogenic fungi. Also, they would obtain organic crops without environmental impact, allowing them to improve their income.

Additionally, this work contributes to the knowledge of microbial diversity associated with crops of a specific region studied for the first time. It also could be useful in further studies on plant-pathogen interactions, relevant to developing strategies for the control of phytopathogens in crops of agricultural importance.

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