CHILEAN JOURNAL of AGRICULTURAL RESEARCH

CHILEANJAR
CHILEAN JOURNAL of AGRICULTURAL RESEARCH

RESEARCH ARTICLE

Exploring the in vitro bioefficacy of *Trichoderma* spp. against a fungal complex associated with chickpea wilt

María B. Contreras-Soto[1], Kamila C. Correia[2], Elizabeth García-León[3], Guadalupe A. Mora-Romero[4], Milagros Ramírez-Soto[5], Juan M. Tovar-Pedraza[1], and Carlos I. Cota-Barreras[5*]

[1]Centro de Investigación en Alimentación y Desarrollo, Coordinación Regional, Culiacán, 80110, Sinaloa, México.
[2]Universidade Federal do Cariri, Centro de Ciências Agrárias e da Biodiversidade, Crato, 63.133-610, Ceará, Brasil.
[3]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental Valle del Fuerte, Sinaloa, México.
[4]Universidad Autónoma de Occidente, Unidad Regional Los Mochis, Unidad de Investigación en Ambiente y Salud, Los Mochis, 81223, Sinaloa, México.
[5]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental Valle de Culiacán, Sinaloa, México.
[*]Corresponding author (carlosi.cotab@gmail.com).
Received: 26 August 2024; Accepted: 23 October 2024, doi:10.4067/S0718-58392025000200256

ABSTRACT

In Mexico, chickpea (*Cicer arietinum* L.) crop production is extensively limited and reduced by a disease known as wilt, caused by a complex of soil-borne plant pathogenic fungi. The objective of this study was to evaluate the antagonistic capacity of 31 isolates of *Trichoderma* spp. against the fungi *Macrophomina phaseolina, Agroathelia rolfsii, Rhizoctonia solani, Neocosmospora falciformis, Fusarium languescens, F. nirenbergiae*, and *F. verticillioides*. *Trichoderma* spp. isolates were obtained from rhizospheric soil and chickpea roots collected in the municipalities of Angostura and Salvador Alvarado, in Sinaloa, Mexico. Dual confrontations between the pathogen isolates and antagonistic agents were evaluated on PDA culture medium, using a completely randomized two-factor experimental design with three replicates. The recorded variable was the percentage of inhibition of radial mycelial growth. Of the 31 *Trichoderma* isolates evaluated, 22 induced > 55% inhibition of the plant pathogens; meanwhile, 7 *Trichoderma* isolates showed the highest antagonistic effect on each of the plant pathogens, causing > 59% inhibition. These 7 *Trichoderma* isolates were identified through multilocus phylogenetic analysis using internal transcribed spacer (ITS), *EF-1a*, and *rpb2* sequences, distinguishing 5 isolates as *T. afroharzianum* and 2 isolates as *T. afarasin*. These *Trichoderma* spp. isolates represent a biological control alternative against the complex of fungi causing chickpea wilt in Sinaloa.

Key words: Biological control, *Cicer arietinum*, *Trichoderma afarasin, Trichoderma afroharzianum*, phylogeny.

INTRODUCTION

Globally, chickpea (*Cicer arietinum* L.) stands out among the most cultivated legumes, following soybeans (*Glycine max*), faba beans (*Vicia faba*), common beans (*Phaseolus vulgaris*), and peas (*Pisum sativum*). Chickpea holds significant economic importance worldwide, with India leading production and Mexico ranked as the eighth-largest producer, with Sinaloa being the primary state, producing 128 884 t (SIAP, 2023).

Chickpea cultivation faces challenges due to biotic and abiotic factors. Among the biotic factors are fungal diseases such as root rot and wilt, associated with a complex of soil-borne plant pathogenic fungi, resulting in reduced grain production (Jiménez-Díaz et al., 2015; Oliva-Ortiz et al., 2017; Cota-Barreras et al., 2022; 2024). Chemical seed treatment is an alternative for reducing the incidence and severity of the disease; however, this practice can harm the ecosystem. Some chemicals can have phytotoxic effects and eliminate beneficial organisms, leading to a reduction in soil microfauna and pathogen resistance to fungicides (Oliva-Ortiz et al., 2017).

Despite other practices for controlling chickpea wilt, such as using tolerant varieties, these have not been sufficient to control the disease (Fierros et al., 2017). An alternative for disease management is seed inoculation with biological agents, promoting biocontrol and benefiting the ecosystem (Harman, 2006). Among these biological agents, *Trichoderma* species are the most used in the biological control of soil-borne plant pathogens. These biocontrol fungi employ antagonistic action mechanisms such as mycoparasitism, lysis, antibiosis, competition for nutrients and space, or induction of resistance, or a combination of these, in the host (Błaszczyk et al., 2014; Contreras-Cornejo et al., 2016; Guzmán-Guzmán et al., 2019). Many *Trichoderma* species have been used as biocontrol agents in a wide range of crops (Saravanakumar et al., 2017; Badaluddin et al., 2018; Rivera-Méndez et al., 2020). Saxena et al. (2015) and Andoji (2021) reported that various *Trichoderma* isolates obtained from chickpea-cultivated soils in India showed high antagonistic activity against isolates of *Fusarium solani, Sclerotinia sclerotiorum*, and *Agroathelia rolfsii*. Additionally, it has been reported that *Trichoderma* species have the potential to inhibit the growth of the fungi *A. rolfsii* and *Fusarium oxysporum* in chickpea plants (Saxena et al., 2015; Khare et al., 2018).

It is important to correctly characterize and identify *Trichoderma* species through multilocus phylogenetic analysis to consider the possible use of these isolates in the biological control of agriculturally significant plant pathogens, as mentioned in various studies (Chaverri et al., 2015; Zhang et al., 2022). Therefore, the objective of this study was to characterize *Trichoderma* isolates from soils and roots of chickpea plants by phenotypic and molecular approaches, as well as to evaluate the in vitro antagonistic activity of *Trichoderma* spp. isolates as biocontrol agents against the complex of fungi associated with chickpea wilt.

MATERIALS AND METHODS

Study site and sampling

During the 2021-2022 cycle, a total of 31 *Trichoderma* spp. isolates were obtained from rhizospheric soils and healthy chickpea (*Cicer arietinum* L.) roots in commercial fields located in the municipalities of Angostura and Salvador Alvarado, in the state of Sinaloa, Mexico. The geographical coordinates are shown in Table 1.

Isolation and conservation of *Trichoderma* isolates

For rhizospheric soil samples, *Trichoderma* isolates were obtained using the method proposed by Karthikeyan et al. (2008). A 50 g portion of each homogenized soil sample was added to a flask containing 450 mL sterile distilled water and shaken for 20 min. Serial dilutions were then performed to reach a 10^{-5} dilution. From each dilution, 200 µL were spread evenly on Petri dishes containing potato dextrose agar (PDA, Difco, Franklin Lakes, New Jersey, USA), with three replicates of each dilution. The Petri dishes were incubated at 28 °C with 12 h light-dark cycles for 7 d. After the incubation period, colonies displaying *Trichoderma* characteristics were transferred. The isolates were purified using the single-spore culture technique.

For the roots, small fragments were cut and disinfected with 2% sodium hypochlorite for 1 min, followed by two rinses with sterile distilled water for 1 min each, and then dried with sterile absorbent paper. The sterilized fragments were plated on Petri dishes with PDA medium, placing five fragments per plate. Each plating was done in duplicate. The plated dishes were incubated at 27 °C under a 12 h photoperiod for 48 to 72 h. The monosporic isolates obtained were deposited in the Culture Collection of the Phytopathology Laboratory of the Centro de Investigación en Alimentación y Desarrollo, Culiacán, México, under accession numbers CCLF370–CCLF400.

Morphological and cultural characterization of *Trichoderma* isolates

To compare colony growth, appearance, and morphological characteristics, *Trichoderma* cultures were transferred to Petri dishes with PDA and incubated at 25 °C with a 12 h photoperiod. After 6 d, colony growth characteristics, including color and conidiophore and conidia masses, were recorded. For each isolate, the conidia, phialides, and chlamydospores were examined, and qualitative and quantitative characters were recorded using an Axio Imager M2 microscope (Zeiss, Oberkochen, Baden-Wurtemberg, Germany) with an Axiocam 305 camera (Zeiss).

Table 1. Geographical coordinates of the sampling points of *Trichoderma* sp. isolates in northwestern Mexico.

Number of isolates	Code	Site	Origin (Municipality, State)	Latitude	Longitude
1	CCLF370	1	Angostura, Sinaloa	25.21703	-108.15205
2	CCLF371	1	Angostura, Sinaloa	25.21703	-108.15205
3	CCLF372	1	Angostura, Sinaloa	25.21703	-108.15205
4	CCLF373	1	Angostura, Sinaloa	25.21703	-108.15205
5	CCLF374	1	Angostura, Sinaloa	25.21703	-108.15205
6	CCLF375	2	Salvador Alvarado, Sinaloa	25.398669	-108.25765
7	CCLF376	2	Salvador Alvarado, Sinaloa	25.398669	-108.25765
8	CCLF377	2	Salvador Alvarado, Sinaloa	25.398669	-108.25765
9	CCLF378	2	Salvador Alvarado, Sinaloa	25.398669	-108.25765
10	CCLF379	2	Salvador Alvarado, Sinaloa	25.398669	-108.25765
11	CCLF380	2	Salvador Alvarado, Sinaloa	25.398669	-108.25765
12	CCLF381	2	Salvador Alvarado, Sinaloa	25.398669	-108.25765
13	CCLF382	3	Angostura, Sinaloa	25.29225	-108.08654
14	CCLF383	3	Angostura, Sinaloa	25.29225	-108.08654
15	CCLF384	3	Angostura, Sinaloa	25.29225	-108.08654
16	CCLF385	3	Angostura, Sinaloa	25.29225	-108.08654
17	CCLF386	3	Angostura, Sinaloa	25.29225	-108.08654
18	CCLF387	4	Angostura, Sinaloa	25.35776	-108.25403
19	CCLF388	5	Angostura, Sinaloa	25.09938	-107.91904
20	CCLF389	5	Angostura, Sinaloa	25.09938	-107.91904
21	CCLF390	6	Angostura, Sinaloa	25.06370	-107.89862
22	CCLF391	6	Angostura, Sinaloa	25.06370	-107.89862
23	CCLF392	6	Angostura, Sinaloa	25.06370	-107.89862
24	CCLF393	6	Angostura, Sinaloa	25.06370	-107.89862
25	CCLF394	6	Angostura, Sinaloa	25.06370	-107.89862
26	CCLF395	6	Angostura, Sinaloa	25.06370	-107.89862
27	CCLF396	6	Angostura, Sinaloa	25.06370	-107.89862
28	CCLF397	7	Angostura, Sinaloa	25.09542	-107.93767
29	CCLF398	8	Angostura, Sinaloa	25.22292	-108.10011
30	CCLF399	9	Salvador Alvarado, Sinaloa	25.40162	-108.20279
31	CCLF400	9	Salvador Alvarado, Sinaloa	25.40162	-108.20279

Antagonism of *Trichoderma* spp.

To evaluate the antagonistic effect of the 31 *Trichoderma* isolates, dual confrontations were performed on Petri dishes against seven economically important chickpea pathogens (*Agroathelia rolfsii, Fusarium nirenbergiae, F. languescens, F. verticillioides, Macrophomina phaseolina, Neocosmospora falciformis,* and *Rhizoctonia solani*). Isolates of these pathogens were sourced from the Culture Collection of Phytopathogenic Fungi at Centro de Investigación en Alimentación y Desarrollo, Culiacán, Sinaloa, Mexico.

Trichoderma and plant pathogens isolates were grown on PDA medium (Difco). A mycelial plug (6 mm diameter) of *Trichoderma* was placed 1 cm from the edge of the Petri dish, and a mycelial plug of the pathogen was placed 1 cm from the opposite edge. This procedure was performed for each combination of *Trichoderma* isolate and pathogen, ensuring all isolates were confronted. A Petri dish with PDA containing only a pathogen mycelial plug served as a control. All Petri dishes were incubated for 72 h at 25 °C with 12 h light-dark cycles.

The antagonistic capacity of *Trichoderma* isolates was determined by the percentage of radial growth inhibition using the formula: I% = (C - T)/C × 100, where C represents the radial growth of the pathogen (mm) alone (control), and T represents the radial growth of the pathogen (mm) in the presence of *Trichoderma* isolates (Khare et al., 2018).

DNA extraction, PCR amplification, and sequencing

The *Trichoderma* isolates that showed the best results in dual confrontation were selected for phylogenetic analysis, which were CCLF375, CCLF387, CCLF374, CCLF370, CCLF394, CCLF398, and CCLF386. For each isolate, mycelium was scraped from the culture medium with a sterile spatula and placed in 2 mL microtubes. Genomic DNA from *Trichoderma* isolates was extracted from 5-d-old colonies using the CTAB method (2% cetyltrimethylammonium bromide, 100 mM Tris HCL pH 8.0, 20 mM EDTA, and 1.4 M NaCl) (Doyle and Doyle, 1990). DNA concentrations were determined using a NanoDrop One (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The PCR amplification was performed on the ITS region and fragments of the *EF-1α* and *rpb2* genes using the primer pairs ITS1/ITS4 (White et al., 1990), EF-1/EF-2 (O'Donnell et al., 1998), and RBP2-5F/RPB2-7R (Liu et al., 1999), respectively. The PCR mixture contained 1 µL (100 ng) DNA, 1X reaction buffer, 1 mM $MgCl_2$, 0.5 mM each primer, 500 µM deoxynucleotide triphosphate, and 0.5 U Taq DNA polymerase (Invitrogen, Carlsbad, California, USA) in a total volume of 25 µL. Amplification conditions were an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 57 °C for 40 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCRs were performed in a C1000 thermal cycler (Bio-Rad, Hercules, California, USA). The PCR products were separated by electrophoresis in 1% agarose gel stained with ethidium bromide and imaged using a Gel Doc XR+ system (Bio-Rad). The amplified PCR products were purified and sequenced in both directions by Macrogen Inc. (Seoul, South Korea).

Phylogenetic analysis

Sequences of ITS, *EF-1α*, and *rpb2* markers were assembled using the Staden Package (Staden et al., 1998). Multiple sequence alignments for each locus were independently performed using ClustalX v.1.83 (Thompson et al., 1997), manually adjusting alignments when necessary. Reference *Trichoderma* isolates sequences were obtained from GenBank of National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and included in the analyses. The maximum likelihood (ML) method was used to infer phylogenetic relationships between individual and concatenated molecular marker sequences. Consensus sequences were generated using SequenceMatrix (Vaidya et al., 2011). Phylogenetic relationships were analyzed for each gene and concatenated sequences, employing the GTR + G + I nucleotide evolution model and executing 1000 rapid bootstrap replications in the MEGA 7 program (Kumar et al., 2016).

Statistical analysis

The experimental design was a completely randomized two-factor design. Data were subjected to ANOVA followed by Fisher's LSD tests. Data were processed and analyzed using the SAS v.9.4 statistical package (SAS Institute, Cary, North Carolina, USA). The experiment was performed three times.

RESULTS

The results indicated that all 31 *Trichoderma* isolates exhibited antagonistic activity against the plant pathogens affecting chickpea crops. However, 7 *Trichoderma* isolates (CCLF375, CCLF387, CCLF374, CCLF370, CCLF394, CCLF398, and CCLF386) induced an inhibition percentage greater than 59% on the mycelial growth of each plant pathogen. Fifteen isolates caused inhibition percentages between 55% and 59%, and 9 isolates caused inhibition percentages ≤ 55% (Table 2; Figure 1).

Rhizoctonia solani was the most inhibited plant pathogen by the effect of all 31 *Trichoderma* isolates, with an inhibition percentage of 61.4%, followed by *F. languescens* with 60.7%, and *M. phaseolina* with 56.8%. The pathogens *F. nirenbergiae, F. verticillioides, A. rolfsii*, and *N. falciformis* showed the lowest levels of mycelial growth inhibition with 54.6%, 53.9%, 52.2%, and 51.2%, respectively.

Table 2. *Trichoderma* sp. isolates against the major chickpea soil-borne pathogens in northwestern Mexico. Data is presented by columns. Mean values followed by different letters in the same column indicate significant differences among treatments according to LSD test ($P \leq 0.05$).

Isolates of *Trichoderma* sp.	Inhibition of mycelial growth (%) for each pathogen							
	Macrophomina phaseolina	*Agroathelia rolfsii*	*Rhizoctonia solani*	*Fusarium nirenbergiae*	*Fusarium languescens*	*Fusarium verticillioides*	*Neocosmospora falciformis*	Combined
CCLF375	53.5^{b-h}	65.2^{a}	68.8^{ab}	60.5^{ab}	63.0^{b-e}	56.8^{a-c}	55.0^{a-f}	60.4^{a}
CCLF387	59.4^{b-h}	57.9^{h-k}	68.0^{a-e}	55.5^{a-e}	61.6^{a-b}	57.0^{a-d}	57.6^{a-c}	59.6^{a-g}
CCLF374	61.2^{a-d}	62.1^{ab}	60.5^{b-f}	53.5^{a-f}	64.0^{b-e}	55.7^{a-g}	58.9^{a-b}	59.4^{ab}
CCLF370	60.7^{a-d}	55.7^{b-g}	68.4^{a-c}	56.0^{a-g}	63.5^{b-e}	56.4^{a-d}	54.9^{a-g}	59.4^{a-d}
CCLF394	60.4^{a-d}	51.3^{e-k}	71.9^{a}	57.1^{a-f}	63.1^{b-e}	51.8^{d-j}	59.3^{a}	59.4^{a-d}
CCLF398	60.2^{a-d}	52.4^{c-k}	61.2^{a-f}	59.2^{a-e}	69.8^{a}	59.0^{a}	53.8^{b-i}	59.4^{a-d}
CCLF386	56.2^{a-g}	58.4^{a-c}	68.1^{a-c}	56.1^{a-f}	58.9^{e-h}	58.9^{a-b}	57.8^{a-c}	59.3^{a-e}
CCLF390	54.9^{b-h}	56.7^{b-f}	67.5^{a-d}	57.4^{a-f}	63.5^{b-e}	56.8^{a-c}	53.4^{c-j}	58.7^{a-f}
CCLF395	60.7^{a-d}	50.1^{f-k}	63.3^{a-e}	60.1^{a-c}	65.1^{a-c}	56.1^{a-e}	52.3^{d-k}	58.3^{a-g}
CCLF376	54.4^{a-e}	48.1^{b-e}	62.6^{a-c}	59.0^{a-h}	67.5^{c-f}	56.7^{a-c}	58.8^{a-d}	58.2^{bc}
CCLF377	62.0^{a-c}	55.0^{c-g}	68.1^{a-c}	50.9^{f-i}	63.3^{b-e}	55.8^{a-f}	51.6^{e-k}	58.1^{b-g}
CCLF391	60.8^{a-d}	57.6^{b-e}	59.5^{b-f}	61.1^{a}	61.7^{c-f}	55.9^{a-f}	49.9^{f-l}	58.1^{a-g}
CCLF400	66.5^{a}	53.0^{c-k}	62.9^{a-e}	55.3^{a-h}	62.5^{b-e}	51.1^{g-j}	48.2^{j-n}	57.0^{a-h}
CCLF388	56.8^{a-g}	50.6^{f-k}	66.9^{a-d}	57.5^{a-f}	64.3^{b-d}	54.6^{a-h}	47.7^{k-o}	56.9^{a-h}
CCLF393	56.5^{a-g}	53.5^{c-i}	68.5^{a-c}	59.8^{a-d}	59.4^{d-g}	51.8^{d-j}	47.5^{k-o}	56.7^{b-i}
CCLF371	62.5^{ab}	55.4^{b-g}	61.6^{a-f}	55.1^{a-h}	56.7^{f-i}	53.7^{c-i}	49.5^{g-l}	56.4^{b-i}
CCLF380	63.9^{ab}	55.4^{c-h}	57.6^{c-f}	53.9^{b-h}	53.5^{i}	52.3^{c-j}	56.2^{a-e}	56.1^{d-i}
CCLF384	54.8^{b-h}	51.4^{e-k}	63.7^{a-e}	53.8^{b-h}	62.4^{b-e}	56.7^{a-c}	49.9^{f-l}	56.1^{c-i}
CCLF392	56.9^{a-f}	46.6^{j-k}	66.4^{a-e}	53.3^{c-h}	59.2^{d-h}	56.7^{a-c}	53.5^{c-j}	56.1^{e-i}
CCLF373	55.8^{b-h}	54.0^{c-i}	67.1^{a-d}	55^{a-h}	61.0^{c-f}	49.3^{h-j}	48.5^{i-m}	55.8^{e-i}
CCLF396	57.9^{a-e}	51.6^{d-k}	60.8^{b-f}	52.5^{e-i}	63.7^{b-e}	54.6^{a-h}	48.8^{h-m}	55.7^{f-i}
CCLF389	62.3^{ab}	55.2^{c-g}	57.7^{c-f}	51.2^{f-i}	53.7^{i}	51.2^{f-j}	54.4^{a-h}	55.0^{g-i}
CCLF385	63.9^{ab}	49.5^{g-k}	54.3^{e-f}	56.1^{a-g}	60.0^{d-f}	53.9^{c-i}	43.6^{m-o}	54.5^{h-j}
CCLF382	60.4^{a-d}	56.0^{b-g}	59.3^{b-f}	48.9^{h-i}	54.2^{h-i}	51.4^{e-j}	50.0^{f-l}	54.3^{h-j}
CCLF381	48.9^{e-h}	53.1^{c-j}	58.8^{b-f}	45.9^{i}	59.8^{d-g}	54.2^{b-h}	54.5^{o}	53.6^{h-k}
CCLF397	51.4^{d-h}	47.4^{i-k}	59.2^{b-f}	57.8^{a-f}	61.5^{c-f}	53.7^{c-j}	42.5^{a-g}	53.3^{i-k}
CCLF372	50.8^{d-h}	46.2^{k}	51.2^{f-g}	48.4^{h-i}	63.7^{b-e}	51.3^{e-j}	47.9^{k-o}	51.4^{j-l}
CCLF379	46.4^{f-h}	33.2^{l}	66.3^{a-d}	49.1^{g-i}	57.0^{f-i}	51.9^{d-j}	49.8^{k-o}	50.5^{k-m}
CCLF383	45.4^{h}	58.4^{b-d}	41.3^{g-h}	50.9^{f-i}	54.7^{g-i}	50.1^{h-j}	47.1^{k-o}	49.7^{l-m}
CCLF378	46.2^{g-h}	35.3^{l}	56.8^{d-f}	51.9^{e-i}	56.8^{f-i}	48.9^{j}	44.7^{l-o}	48.7^{l-m}
CCLF399	51.5^{c-h}	47.3^{i-k}	37.1^{h}	52.7^{d-i}	52.7^{i}	50.1^{h-j}	42.9^{n-o}	47.8^{m}

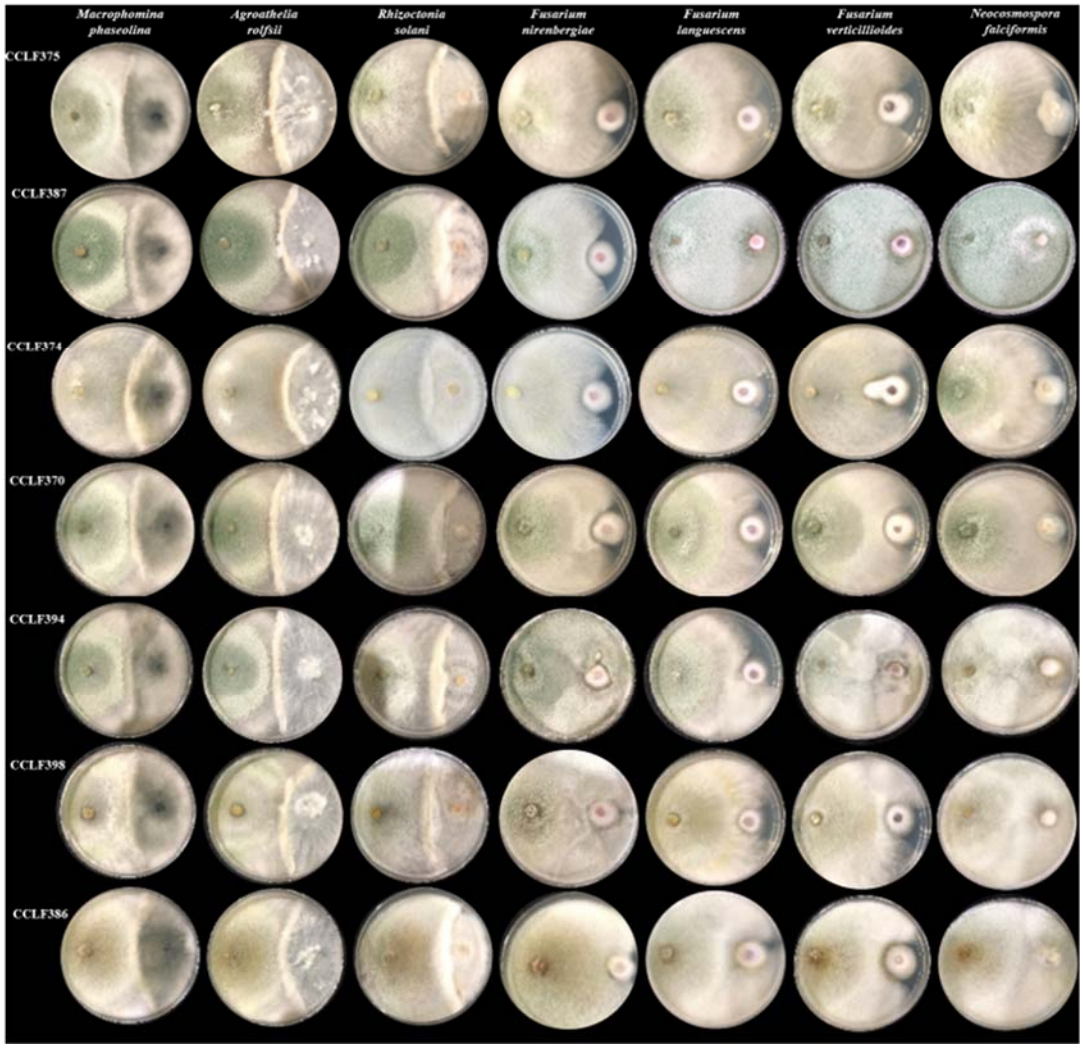

Figure 1. Dual Confrontation of seven *Trichoderma* spp. isolates (left side of the Petri dish) against seven major plant pathogenic fungi (right side of the Petri dish) affecting chickpea cultivation in Sinaloa, Mexico. Horizontal Columns represent *Trichoderma* spp. isolates (CCLF375, CCLF387, CCLF374, CCLF370, CCLF394, CCLF398, and CCLF386).

Phylogenetic analyses, based on concatenated ITS, *EF-1α*, and *rpb2* sequences (Figure 2), identified five isolates as *Trichoderma afroharzianum* (CCLF370, CCLF375, CCLF387, CCLF394, and CCLF398) and two isolates as *T. afarasin* (CCLF374 and CCLF386). The GenBank accession numbers for the sequences analyzed in this study are shown in Table 3.

The seven *Trichoderma* isolates (CCLF370, CCLF374, CCLF375, CCLF386, CCLF387, CCLF394, and CCLF398) that showed the best results *in vitro* assays retained the typical characteristics of the *Trichoderma* genus, with variations in color, starting white and then turning yellow or green. Branched conidiophores in a tree-like form and the formation of conidia in phialides were observed, with dense sporulation in the seven colonies. Conidia were oval, hyaline, and smooth, measuring 2.85-3.41 × 2.24-2.79 µm for *T. afarasin* and 2.97-4.22 × 2.33-2.81 µm for *T. afroharzianum*. Regarding the formation of chlamydospores, all seven isolates presented these resistance structures (Figures 3 and 4).

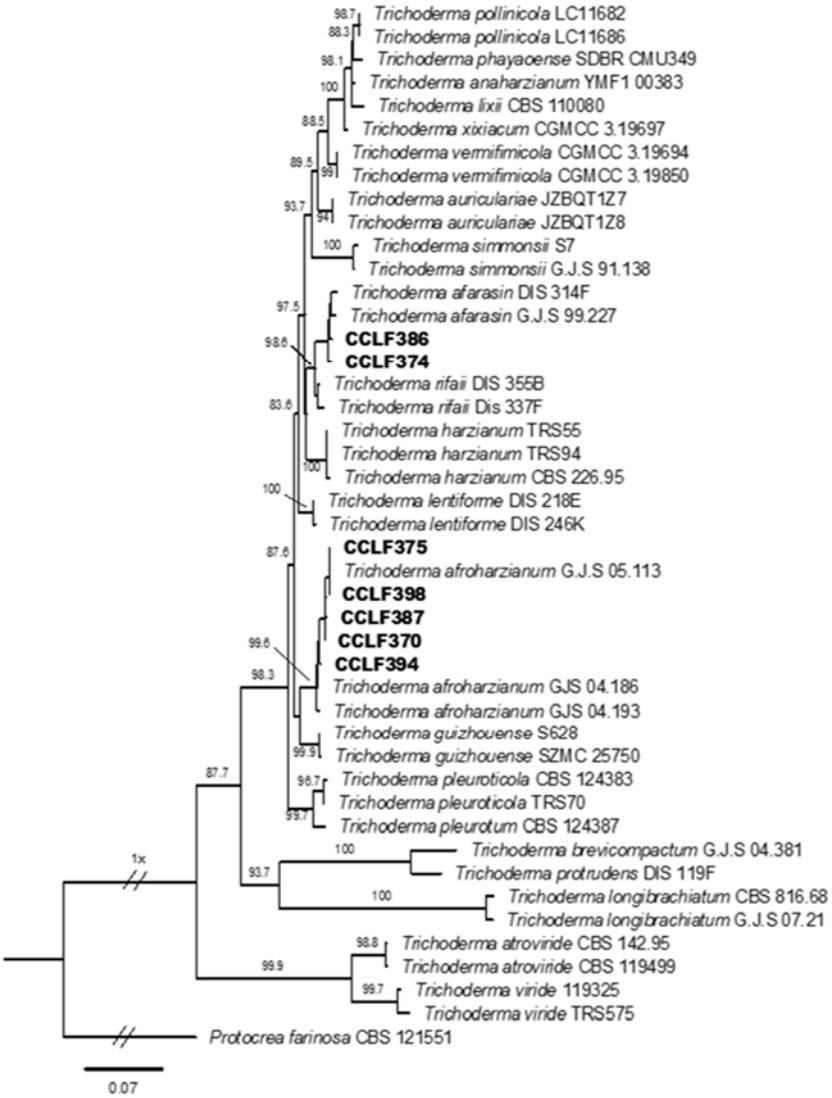

Figure 2. Maximum likelihood tree generated from the analysis of concatenated internal transcribed spacer (ITS), *EF1-α* y *rpb2* dataset sequences. Bootstrap support values (> 80%) for maximum likelihood are shown at the nodes. The tree is rooted with *Protocrea farinose* CBS 121551. Isolates characterized in this study are highlighted in bold.

Table 3. GenBank accession numbers of DNA sequences of *Trichoderma* spp. included in the phylogenetic study. Newly deposited sequences are shown in bold. ITS: Internal transcribed spacer.

Species	Isolate	GenBank accession numbers		
		ITS	*tef1-α*	*rpb2*
Protocrea farinosa	CBS 121551	MH863119	EU703889	EU703935
T. afarasin	GJS 99.227	NR_137301	AF348093	-
T. afarasin	DIS 314F	FJ442259	FJ463400	FJ442778
T. afarasin	**CCLF374**	**PQ136033**	**PQ182128**	**PQ156041**
T. afarasin	**CCLF386**	**PQ136035**	**PQ182130**	**PQ156043**
T. afroharzianum	**CCLF375**	**PQ136034**	**PQ182129**	**PQ156042**
T. afroharzianum	**CCLF398**	**PQ136038**	**PQ182133**	**PQ156046**
T. afroharzianum	**CCLF387**	**PQ136036**	**PQ182131**	**PQ156044**
T. afroharzianum	**CCLF370**	**PQ136032**	**PQ182127**	**PQ156040**
T. afroharzianum	**CCLF394**	**PQ136037**	**PQ182132**	**PQ156045**
T. afroharzianum	GJS 05.113	FJ442235	FJ463378	-
T. afroharzianum	*GJS 04.186*	FJ442265	FJ463301	FJ442691
T. afroharzianum	*GJS 04.193*	FJ442233	FJ463298	FJ442709
T. anaharzianum	YMF1 00383	NR_174890	MH183182	MH158995
T. atroviride	*CBS 142.95*	AY380906	AY376051	EU341801
T. atroviride	CBS 119499	FJ860726	FJ860611	FJ860518
T. auriculariae	JZBQT1Z7	ON653396	ON649896	ON649949
T. auriculariae	JZBQT1Z8	ON653397	ON649897	ON649950
T. brevicompactum	*GJS 04.381*	EU330941	EU338299	EU338317
T. guizhouense	*S628*	-	KJ665511	KJ665273
T. guizhouense	*SZMC 25750*	-	MZ773438	MZ773417
T. harzianum	CBS 226.95	AJ222720	AF348101	AF545549
T. harzianum	TRS55	KP009211	KP008803	KP009121
T. harzianum	TRS94	KP009250	KP008802	KP009120
T. lentiforme	DIS 218E	FJ442220	FJ463310	FJ442793
T. lentiforme	DIS 246K	FJ442617	FJ463353	FJ442758
T. lixii	CBS 110080	NR_131264	FJ716622	KJ665290
T. longibrachiatum	*CBS 816.68*	MH859229	AY865640	DQ087242
T. longibrachiatum	GJS 07.21	-	JN175569	JN175513
T. phayaoense	SDBR CMU349	MT995122	MW002073	MW002074
T. pleuroticola	*CBS 124383*	HM142362	HM142381	HM142371
T. pleuroticola	TRS70	KP009264	KP008951	KP009172
T. pleuratum	*CBS 124387*	NR_134421	HM142382	HM142372
T. pollinicola	LC11682	MF939592	MF939619	MF939604
T. pollinicola	LC11686	MF939593	MF939620	MF939605
T. protrudens	*DIS 119F*	EU330946	EU338289	EU338322
T. rifaii	DIS 355B	FJ442621	FJ463321	FJ442720
T. rifaii	DIS 337F	FJ442621	FJ463321	FJ442720
T. simmonsii	G.J.S 91.138	AF443917	AF443935	FJ442757
T. simmonsii	S7	-	KJ665719	KJ665337
T. vermifimicola	CGMCC 3.19694	MN594473	MN605882	MN605871
T. vermifimicola	CGMCC 3.19850	MN594472	MN605881	MN605870
T. viride	CBS119325	DQ677655	DQ672615	EU711362
T. viride	TRS575	KP009372	KP008931	KP009081
T. xixiacum	CGMCC 3.19697	MN594476	MN605885	MN605874

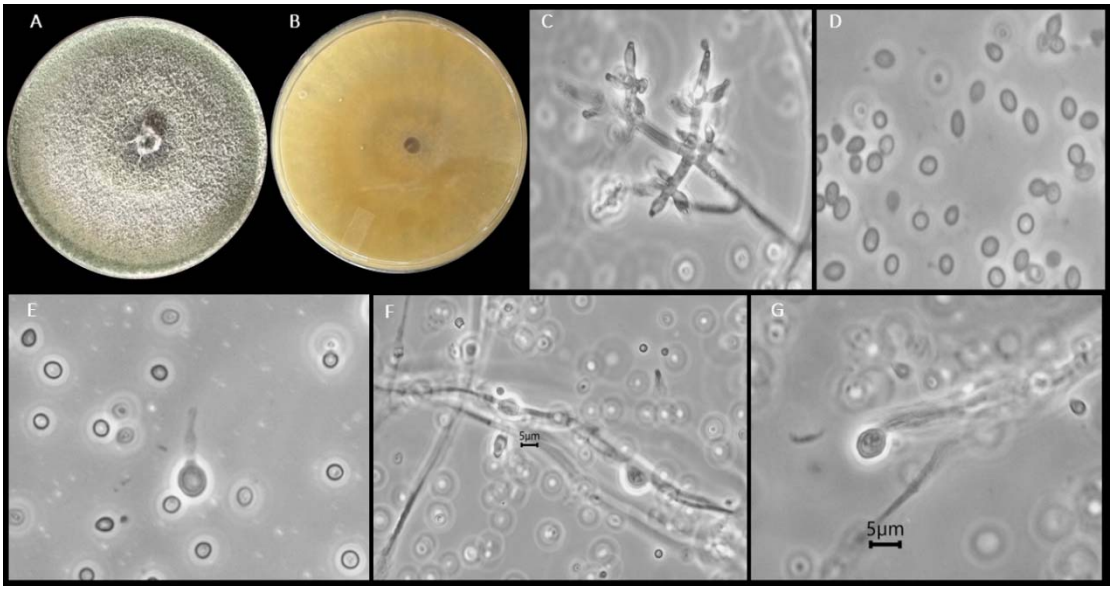

Figure 3. Morphology of *Trichoderma afroharzianum*: Cultures on PDA, front and reverse at 7 d of growth (A and B), phialide (C), conidia (D), terminal chlamydospores (E and G), intercalary chlamydospore (F).

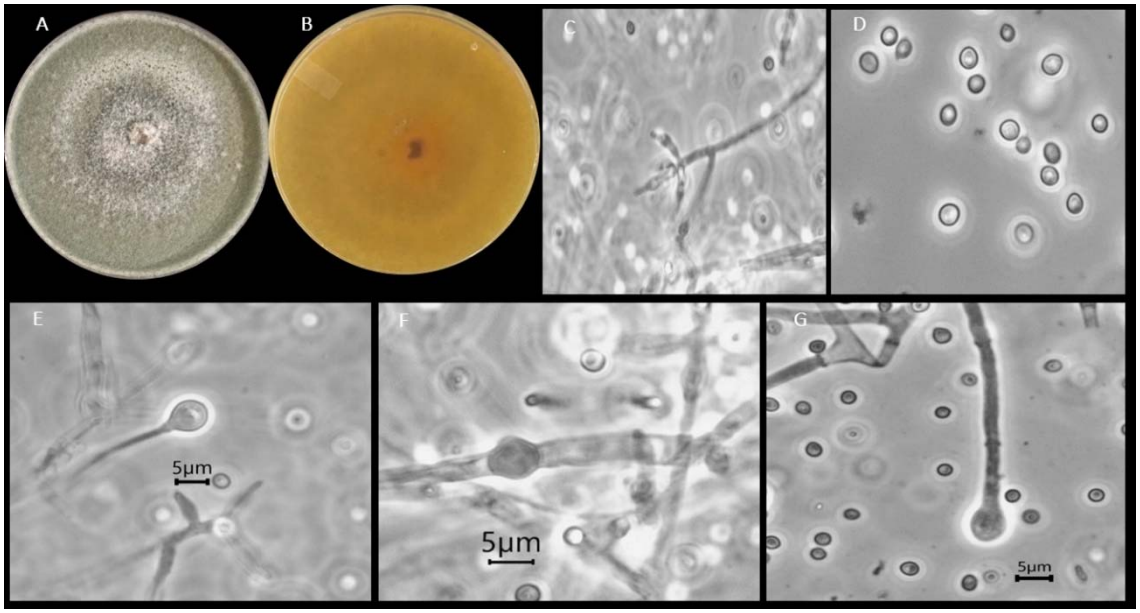

Figure 4. Morphology of *Trichoderma afarasin*: Cultures on PDA, front and reverse at 7 d of growth (A and B), phialide (C), conidia (D), terminal chlamydospores (E and G), intercalary chlamydospore (F).

DISCUSSION

*Trichoderm*a spp. is an effective antagonist agent, extensively studied worldwide to combat pathogenic fungi plants in various crops. In Mexico, its research has increased in crops such as cacao (Torres-De la Cruz et al., 2015), maize (Mendoza et al., 2015), tomato (Ruiz-Cisneros et al., 2018), chili pepper (Herrera-Parra et al.,

2017), and avocado (López-López et al., 2022). In other countries such as Brazil, the use of *Trichoderma* to improve crop yields has also been reported (Gonçalves et al., 2023). However, it has been little studied in chickpea cultivation (Ortiz et al., 2016; Martínez-Martínez et al., 2020). In soil, *Trichoderma* spp. has been widely documented as a biocontrol agent to suppress plant diseases. This process is considered multifaceted, involving the synergistic collaboration of various mechanisms, including the activation of plant defense systems. For example, *T. harzianum* (Harzianum clade) can induce defense mechanisms against phytopathogens in plants, as demonstrated in various studies in cucumber (Yedidia et al., 1999) and bell peppers (Ezziyyani et al., 2004). The antibiotic mechanism through the production of volatile compounds exerted by *T. azevedoi* can complement other mechanisms, such as parasitism and competition, thereby contributing to greater efficiency in controlling white mold caused by *S. sclerotiorum* in bean plants (da Silva et al., 2023).

Our study analyzed the capacity of 31 *Trichoderma* isolates to inhibit the mycelial growth of seven soil-borne plant pathogens, including *M. phaseolina, A. rolfsii, R. solani, N. falciformis*, *F. languescens, F. nirenbergiae*, and *F. verticillioides*. All 31 *Trichoderma* isolates inhibited the mycelial growth of the seven pathogens *in vitro* with values ranging from 47% to 61%.

The biocontrol effect of *Trichoderma afroharzianum* has been widely demonstrated. Bouanaka et al. (2021) reported inhibition percentages of 77% to 81% against *Fusarium culmorum* and a reduction in the severity of spike fusarium and crown rot in wheat by 50.0% to 63.3%. Similarly, the isolate TM24 was effective in controlling gray mold in tomato by inhibiting the mycelial growth of *Botrytis cinerea* and increasing the production of enzymes such as glucanase and chitinase (Zhao et al., 2021). It has also been indicated that metabolites produced by *T. afroharzianum* inhibit the growth of the pathogen *Alternaria alternata* and induce defense-related enzymes in tomato plants (Philip et al., 2024). In another study through the prediction of biosynthetic gene clusters, it was reported that *T. afroharzianum* (ThT22) encoded at least 64 natural products (Han et al., 2023). Furthermore, *T. harzianum* (Harzianum clade) showed antagonism against different chickpea pathogens and enhanced plant strength and grain yield (Martínez-Martínez et al., 2020).

Our results are consistent with another study conducted with *Trichoderma* spp. isolates from maize plants, where inhibition zones were less intense against *Fusarium verticillioides* (28% to 47%) compared to *Rhizoctonia solani* (44.9% to 60.0%) (Rodríguez and Flores, 2018). Other research has mentioned that various *Trichoderma* isolates obtained from chickpea field soils in India showed significant antagonistic activity against *Fusarium solani, S. sclerotiorum*, and *A. rolfsii* isolates (Saxena et al., 2015; Andoji, 2021).

The Harzianum clade is a complex of at least 95 species, including *T. afarasin* and *T. afroharzianum* (Cao et al., 2022). Therefore, species previously reported as *T. harzianum* belonged to different species within the same clade (Barrera et al., 2021; Xiao et al., 2023). This clade commonly presents the formation of chlamydospores (Zhu and Zhuang, 2015), which is consistent with our results, as these resistance structures favor the persistence of *Trichoderma* spp. under adverse conditions, enhancing the use of these isolates as biocontrol agents for soil-borne plant pathogens.

CONCLUSIONS

The results of this study indicate that all 31 isolates of *Trichoderma* spp. exhibited antagonistic activity against chickpea pathogens, with seven isolates showing particularly high inhibition percentages (> 59%). These isolates demonstrated the formation of chlamydospores, granting them the advantage of persisting for prolonged periods under adverse conditions until they encounter optimal conditions to exert biocontrol. This characteristic highlights their potential as an effective and sustainable alternative for the control of pathogenic fungi plants in agricultural fields.

Author contribution

Conceptualization: M.B.C.S., J.M.T.P. Methodology: C.I.C.B., M.R.S., M.B.C.S. Software: M.B.C.S. Validation: J.M.T.P. Formal analysis: J.M.T.P., K.C.C., E.G.L. Investigation: M.B.C.S. Resources: G.A.M.R., J.M.T.P. Data curation: M.B.C.S. Writing-original draft: M.B.C.S. Writing-review & editing: J.M.T.P. Visualization: K.C.C. Supervision: C.I.C.B. Project administration: J.M.T.P. Funding acquisition: J.M.T.P. All co-authors reviewed the final version and approved the manuscript before submission.

Acknowledgements

A special thank for Comité Estatal de Sanidad Vegetal del Estado de Sinaloa (CESAVESIN) and Bs. Jorge Luis Manjarrez Vázquez for their technical support.

References

Andoji, Y.S. 2021. *Invitro* mycoparasitism activity of *Trichoderma* spp against *Fusarium solani* inciting root rot of chickpea (*Cicer arietinum* L.) World Journal of Biology and Biotechnology 6(3):1-4. doi:10.33865/wjb.006.03.0430,

Badaluddin, N.A., Jamaluddin, S.N.T., Ihsam, N.S., Sajili, M.H., Khalit, S.I., Mohamed, N.A. 2018. Molecular identification of isolated fungi from Kelantan and Terengganu using internal transcriber spacer (ITS) region. Journal of Agrobiotechnology 9(1):222-231.

Barrera, V.A., Iannone, L., Romero, A.I., Chaverri, P. 2021. Expanding the *Trichoderma harzianum* species complex: Three new species from Argentine natural and cultivated ecosystems. Mycologia 113(6):1136-1155. doi:10.1080/00275514.2021.1947641.

Błaszczyk, L., Siwulski, M., Sobieralski, K., Lisiecka, J., Jędryczka, M. 2014. *Trichoderma* spp. -Application and prospects for use in organic farming and industry. Journal of Plant Protection Research 54(4):309-317.

Bouanaka, H., Bellil, I., Harrat, W., Boussaha, S., Benbelkacem, A., Khelifi, D. 2021. On the biocontrol by *Trichoderma afroharzianum* against *Fusarium culmorum* responsible of Fusarium head blight and crown rot of wheat in Algeria. Egyptian Journal of Biological Pest Control 31:1-13. doi:10.1186/s41938-021-00416-3.

Cao, Z.J., Qin, W.T., Zhao, J., Liu, Y., Wang, S.X., Zheng, S.Y. 2022. Three new *Trichoderma* species in Harzianum clade associated with the contaminated substrates of edible fungi. Journal of Fungi 8:1154. doi:10.3390/jof8111154.

Chaverri, P., Branco-Rocha, F., Jaklitsch, W., Gazis, R., Degenkolb, T., Samuels, G.J. 2015. Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. Mycologia 107(3):558-590. doi:10.3852/14-147.

Contreras-Cornejo, H.A., Macías-Rodríguez, L., del-Val, E., Larsen, J. 2016. Ecological functions of *Trichoderma* spp. and their secondary metabolites in the rhizosphere: Interactions with plants. FEMS Microbiology Ecology 92(4):fiw036. doi:10.1093/femsec/fiw036.

Cota-Barreras, C.I., García-Estrada, R.S., León-Félix, J., Valenzuela-Herrera, V., Mora-Romero, G.A., Leyva-Madrigal, K.Y., et al. 2024. Phylogeny, distribution, and pathogenicity of fusarioid fungi associated with chickpea wilt in Sinaloa and Sonora, Mexico. Tropical Plant Pathology 49:622-632. doi:10.1007/s40858-024-00663-3.

Cota-Barreras, C.I., García-Estrada, R.S., Valdez-Torres, J.B., León-Félix, J., Valenzuela-Herrera, V., Tovar-Pedraza, J.M. 2022. Molecular detection, virulence, and mycelial compatibility of *Macrophomina phaseolina* isolates associated with chickpea wilt in Sinaloa and Sonora, Mexico. Canadian Journal of Plant Pathology 44(6):849-857. doi:10.1080/07060661.2022.2084642.

da Silva, L.R., de Barros Rodrigues, L.L., Botelho, A.S., de Castro, B.S., Muniz, P.H.P.C., Moraes, M.C.B., et al. 2023. Colony age of *Trichoderma azevedoi* alters the profile of volatile organic compounds and ability to suppress *Sclerotinia sclerotiorum* in bean plants. Plant Pathology Journal 39(1):39-51. doi:10.5423/PPJ.OA.08.2022.0106.

Doyle, J.J., Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Ezziyyani, M., Sánchez, C.P., Ahmed, A.S., Requena, M.E., Castillo, M.E.C. 2004. *Trichoderma harzianum* como biofungicida para el biocontrol de *Phytophthora capsici* en plantas de pimiento (*Capsicum annuum* L.) Anales de Biología 26:35-45.

Fierros, L.G., Ortega, M.P., Acosta, G.J., Padilla, V.I., Valenzuela, H.V., Jiménez, H.Y., et al. 2017. Respuesta del rendimiento de genotipos de garbanzo blanco a la sequía terminal. Revista Mexicana de Ciencias Agrícolas 8(5):1143-1154. doi:10.29312/remexca.v8i5.114.

Gonçalves, G.C., Ferreira Ferbonink, G., Hemkemeier, C., Caione, G., Yamashita, O.M., Rocha Luiz, S.A., et al. 2023. Vegetative and productive characteristics of soybean under doses of boron and inoculation of *Trichoderma atroviride*. Chilean Journal of Agricultural Research 83:159-167. doi:10.4067/S0718-58392023000200159.

Guzmán-Guzmán, P., Porras-Troncoso, M.D., Olmedo-Monfil, V., Herrera-Estrella, A. 2019. *Trichoderma* species: Versatile plant symbionts. Phytopathology 109(1):6-16. doi:10.1094/PHYTO-07-18-0218-RVW.

Han, W., Wu, Z., Zhong, Z., Williams, J., Jacobsen, S.E., Sun, Z., et al. 2023. Assessing the biosynthetic inventory of the biocontrol fungus *Trichoderma afroharzianum* T22. Journal of Agricultural and Food Chemistry 71(30):11502-11519.

Harman, G.E. 2006. Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 96:190-194. doi:10.1094/PHYTO-96-0190.

Herrera-Parra, E., Cristóbal-Alejo, J., Ramos-Zapata, J.A. 2017. *Trichoderma* strains as growth promoters in *Capsicum annum* and as biocontrol agents in *Meloidogyne incognita*. Chilean Journal of Agricultural Research 77:318-324. doi:10.4067/S0718-58392017000400318.

Jiménez-Díaz, R.M., Castillo, P., Jiménez-Gasco, M.M, Landa, B.B., Navas-Cortés, J.A. 2015. Fusarium wilt of chickpeas: Biology, ecology and management. Crop Protection 73:16-27. doi:10.1016/j.cropro.2015.02.023.

Karthikeyan, B.C., Jaleel, A., Lakshmanan, G.A., Deiveekasundaram, M. 2008. Studies on rhizosphere microbial diversity of some commercially important medicinal plants. Colloids and Surfaces B: Biointerfaces 62(1):143-145. doi:10.1016/j.colsurfb.2007.09.004.

Khare, E., Kumar, S., Kim, K. 2018. Role of peptaibols and lytic enzymes of *Trichoderma cerinum* Gur1 in biocontrol of *Fusarium oxysporum* and chickpea wilt. Environmental Sustainability 303(1):39-47. doi:10.1007/s42398-018-0022-2.

Kumar, S., Stecher, G., Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874.

Liu, Y.J., Whelen, S., Hall, B.D. 1999. Phylogenetic relationships among ascomycetes: Evidence from an RNA polymerase II subunit. Molecular Phylogenetics and Evolution 16:1799-1808. doi:10.1093/oxfordjournals.molbev.a026092.

López-López, M.E., Del-Toro-Sánchez, C.L., Gutiérrez-Lomelí, M., Ochoa-Ascencio, S., Aguilar-López, J.A., Robles-García, M.A., et al. 2022. Isolation and characterization of *Trichoderma* spp. for antagonistic activity against avocado (*Persea americana* Mill) fruit pathogens. Horticulturae 8(8):714. doi:10.3390/horticulturae8080714.

Martínez-Martínez, T.O., Guerrero-Aguilar, B.Z., Pecina-Quintero, V., Rivas-Valencia, P., González-Pérez, E., Angeles-Núñez, J.G. 2020. *Trichoderma harzianum* antagonism against chickpea fusariosis and its biofertilizing effect. Revista Mexicana de Ciencias Agrícolas 11(5):1135-1147.

Mendoza, J.L.H., Pérez, I.S., Prieto, J.M.G., Velásquez, J.D.Q., Olivares, J.G.G., Langarica, H.R.G. 2015. Antibiosis of *Trichoderma* spp. strains native to northeastern Mexico against the pathogenic fungus *Macrophomina phaseolina*. Brazilian Journal of Microbiology 46(4):1093-1101.

O'Donnell, K., Kistler, H.C., Cigelni, E., Ploetz, R.C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences of the United States of America 95:2044-2049. doi:10.1073/pnas.95.5.2044.

Oliva-Ortiz, L.C., Velázquez-Alcaraz, T.J., Sosa-Pérez, R., Partida-Ruvalcaba, L., Díaz-Valdés, T., Arciniega-Ramos, J., et al. 2017. Control de la fusariosis vascular del garbanzo (*Cicer arietinum* L.) por microorganismos nativos de Sinaloa, México. Agrociencia 51:683-695.

Ortiz, L.D.C.O., Alcaraz, T.D.J.V., Pérez, R.S., Ruvalcaba, L.P., Valdés, T.D., Ramos, J.A., et al. 2016. Morphology and Biochemistry of microbial antagonists and their effect in the growth of garbanzo and control of fusariosis. Open Access Library Journal 3(12):1-19. doi:10.4236/oalib.1103226.

Philip, B., Behiry, S.I., Salem, M.Z., Amer, M.A., El-Samra, I.A., Abdelkhalek, A., et al. 2024. *Trichoderma afroharzianum* TRI07 metabolites inhibit *Alternaria alternata* growth and induce tomato defense-related enzymes. Scientific Reports 14:1874.

Rivera-Méndez, W., Obregón, M., Morán-Diez, M.E., Hermosa, R., Monte, E. 2020. *Trichoderma asperellum* biocontrol activity and induction of systemic defenses against *Sclerotium cepivorum* in onion plants under tropical climate conditions. Biological Control 141:104145.

Rodríguez, I.C., Flores, J. 2018. Capacidad antagónica *in vitro* de *Trichoderma* spp. frente a *Rhizoctonia solani* Kuhn y *Fusarium verticillioides* Nirenberg. Bioagro 30:1.

Ruiz-Cisneros, M.F., Ornelas-Paz, J.D.J., Olivas-Orozco, G.I., Acosta-Muñiz, C.H., Sepúlveda-Ahumada, D.R., Pérez-Corral, D.A., et al. 2018. Effect of *Trichoderma* spp. and phytopathogenic fungi on plant growth and tomato fruit quality. Revista Mexicana de Fitopatología 36(3):444-456.

Saravanakumar, K., Li, Y., Yu, C., Wang, Q.Q., Wang, M., Sun, J., et al. 2017. Effect of *Trichoderma harzianum* on maize rhizosphere microbiome and biocontrol of Fusarium Stalk rot. Scientific Reports 7:1771.

Saxena, A., Raghuwanshi, R., Singh, H.B. 2015. *Trichoderma* species mediated differential tolerance against biotic stress of phytopathogens in *Cicer arietinum* L. Journal of Basic Microbiology 55(2):195-206.

SIAP. 2023. Panorama Agroalimentario 2023. Available at Servicio de Información Agroalimentaria y Pesquera (SIAP), Ciudad de México. Available at https://www.gob.mx/siap/acciones-y-programas/panorama-agroalimentario-258035.

Staden, R., Beal, K.F., Bonfield, J.K. 1998. The Staden Package, 1998. p. 115-130. In Misener, S., Krawetz, S. A. (eds.) Bioinformatics methods and protocols. The Humana Press, New York, USA.

Thompson, J.D., Gibson, T., Plewniak, F., Jeanmougin, F., Higgins, DG. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25(24):4876-4882.

Torres-De la Cruz, M., Ortiz-García, C.F., Bautista-Muñoz, C., Ramírez-Pool, J.A., Ávalos-Contreras, N., Cappello-García, S., et al. 2015. Diversidad de *Trichoderma* en el agroecosistema cacao del estado de Tabasco, México. Revista Mexicana de Biodiversidad 86(4):947-961. doi:10.1016/j.rmb.2015.07.012.

Vaidya, G., Lohman, D.J., Meier, R. 2011. SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27:171-180.

White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W. 1990. Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal DNA genes. p. 315-322. In Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (eds.) PCR protocols: A guide to methods and applications. Academic Press, San Diego, California, USA.

Xiao, Z., Zhao, Q., Li, W., Gao, L., Liu, G. 2023. Strain improvement of *Trichoderma harzianum* for enhanced biocontrol capacity: Strategies and prospects. Frontiers in Microbiology 14:1146210.

Yedidia, I., Benhamou, N., Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology 65(3):1061-1070.

Zhang, G.Z., Yang, H.T., Zhang, X.J., Zhou, F.Y., Wu, X.Q., Xie, X.Y., et al. 2022. Five new species of *Trichoderma* from moist soils in China. MycoKeys 87:133-157. doi:10.3897/mycokeys.87.76085.

Zhao, J., Liu, T., Zhang, D., Wu, H., Zhang, T. 2021. Biocontrol potential of *Trichoderma afroharzianum* TM24 against grey mould on tomato plants. Current Microbiology 78:4115-4126. doi:10.1007/s00284-021-02671-x.

Zhu, Z.X., Zhuang, W.Y. 2015. *Trichoderma* (Hypocrea) species with green ascospores from China. Persoonia 34(1):113-129. doi:10.3767/003158515X686732.