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Trichoderma harzianum mutants enhance antagonism against phytopathogenic fungi, phosphorus assimilation and drought tolerance in Jalapeño pepper plants

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ABSTRACT

The *Trichoderma harzianum* fungus is one of the most widely used biological control agents in agriculture. A new *T. harzianum* THITR01 strain was isolated and their spores were mutagenized with ethyl methane sulfonate obtaining 174 mutants. M7, M14, M21 and M24 are mutant strains that showed 97.4%-100% antagonist effect against *Sclerotium rolfsii*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* compared to the wild strain. Under potato dextrose agar (PDA) medium with either NaCl, sorbitol or $NaHCO_3$ there were nonsignificant growth rate differences between the mutants and the wild strain. M14 and M21 mutant strains were inoculated on 'Jalapeño' pepper plants (*Capsicum annuum* L. var. *annuum*) with unavailable P promoted a significant increase in root fresh weight (54% and 40%, respectively) and dry weight (28% in both strains), compared to plant inoculation with the wild strain. The M14 strain presented the highest P solubilization ability (13.4 μg g^{-1}) in the substrate and promoted a change on root architecture. There was a higher relative water content (82.9%) in drought stressed plants inoculated with the M24 mutant than in plants that were inoculated with the wild type strain (76.3%), and also higher levels of proline in chili pepper plants inoculated with the M24 mutant (939.5 μg g^{-1} dry weight) than in plants inoculated with the wild type strain (419.8 μg g^{-1} dry weight). Therefore, M14, M21 and M24 mutant strains could potentially be used as biocontrol agents and plant protector from abiotic stress.

Key words: Abiotic stress, fungi, mutants, phosphorus, *Trichoderma*.

INTRODUCTION

Chemical pesticides have generated resistance, environmental pollution, and health problems due to their toxicity. For many years, biocontrol alternatives have been sought, to fight pests and plant diseases without damaging the environment, humans and crops. The *Trichoderma* genus includes species that adapt to different habitats, which have high specific growth rate and antagonist ability against phytopathogenic fungi such as *Phytophthora*, *Rhizoctonia*, *Sclerotium*, *Pythium* and *Fusarium* among others (Zin and Badaluddin, 2020). *Trichoderma* spp. inhibits pathogen growth by coiling around the host their hyphae with appressorium-like structures and triggering abundant secretion of lytic enzymes such as glucanases, chitinases, proteases and lipases causing cell wall breakdown of host fungi (Druzhinina et al., 2011; Wu et al., 2017).

The genus *Trichoderma* is of great interest due to its direct-action mechanisms against phytopathogenic fungi (Baron et al., 2019). The fungus is able to control plant pathogens acting as antagonist using different mechanisms, such as competition for space and nutrients, mycoparasitism, antibiosis and secretion of hydrolytic enzymes, which cause vacuolization, cytoplasm disintegration and cell lysis of phytopathogenic fungi (Keswani et al., 2014; Zeilinger et al., 2016). In addition, *T. harzianum* has the ability to facilitate the solubilization and absorption of nutrient compounds for the plant such as Cu, P, Fe, Mn, Na and N (Vinale et al., 2013). Therefore, *T. harzianum* is widely used as a biocontrol and biofertilizer agent due to its beneficial interactions with plants, since it induces systemic resistance to pathogens and release compounds that promote plant growth and root development (Cai et al., 2015). Plant colonization by *T. harzianum* modulates endogenous plant hormones and antioxidants, compatible solutes, phytoalexins and phenolic compounds (Carvalhais et al., 2015).

There are reports of *Trichoderma* strains that improve tolerance to abiotic stress in plants such as cold, drought and salinity, through stimulation of root growth, improving photosynthetic efficiency, nutrient absorption and protecting the plant from oxidative damage more efficiently by eliminating reactive oxygen species (ROS) (Ahmad et al., 2015; Guler et al., 2016).

Several *Trichoderma* spp. can solubilize P for plant growth. This is a key element for plant nutrition and productivity and is mainly present in nucleic acids, phospholipids and ATP. The P is the second most limiting nutrient for crop production and is a non-renewable resource (Ham et al., 2018). Although total soil P is high, only 0.1% is available to plants due to rapid orthophosphate reactivity with soil minerals and bacteria conversion to organic forms that cannot be assimilated by plants, thus inorganic fertilizers supply this crop deficiency (Alori et al., 2017). However, excessive applications of fertilizers have led to water pollution and eutrophication (Hobbie et al., 2017). As an alternative, *Trichoderma* spp. have the ability to solubilize the soil P (Rawat and Tewari, 2011; Li et al., 2015).

In order to improve fungi performance, random mutagenesis experiments with ethyl methanesulfonate (EMS) have been successfully carried out to increase the secretion of extracellular enzymes in several filamentous fungi (Ribeiro et al., 2013). *Trichoderma* mutants have been described to improve their antagonism to phytopathogenic fungi using chemical mutagens (Singh et al., 2016), or T-DNA mutagenesis to promote tolerance to abiotic stress in plants (Guo et al., 2018).

The aim of the present study was to isolate *T. harzianum* mutants generated by EMS that improve their antagonist ability against plant pathogens, and also enhance their effect on plant stress tolerance and P solubilization. To accomplish this goal, various physiological and biochemical parameters in the isolated mutants and in the inoculated 'Jalapeño' pepper plants were determined. Our data provide novel mutants that could contribute to the use of field applications of *Trichoderma* fungus as a biocontrol agent.

MATERIALS AND METHODS

Isolation of a new strain of *Trichoderma harzianum*

Soil samples were collected from Comonfort (20°46'43.0" N, 100°49'34.1" W), Celaya (20°39'08.1" N, 100°51'47.4" W), Juventino Rosas (20°39'21.7" N, 101°02'12.9" W) and Apaseo el Alto (20°26'38.7" N, 100°37'40.9" W) on the state of Guanajuato, Mexico. The method of serial dilutions and sowing on potato dextrose agar (PDA, BD Bioxon, Cuautitlán Izcalli, Mexico) supplemented with 0.48 mg mL^{-1} lactic acid and morphological identification, were used for the isolation of four strains of *Trichoderma* spp. The choice of the best antagonist strain was made by dual confrontation test against *Fusarium oxysporum* and *Sclerotinia sclerotiorum* selecting the strain with higher mycoparasitism ability, which led to the selection of a *Trichoderma* spp. strain labelled as THITR01. This strain is cryopreserved in the microbial collection of the División de Estudios de Posgrado e Investigación, Tecnológico Nacional de México/I.T. Roque, Celaya, México.

Molecular identification by an internal transcribed spacer (ITS) sequence was performed (Feitosa et al., 2019). For this purpose, the *Trichoderma* spp. strain THITR01 was grown in flasks containing 40 mL potato dextrose broth (PDB, BD Bioxon, Cuautitlán Izcalli, Mexico) medium for 72 h with constant stirring at 28 ºC. The supernatant was eliminated, and the DNA extraction was carried out by the sodium dodecyl sulfate (SDS)-proteinase K method (Carpi et al., 2011). For the amplification of the internal transcribed regions the primers ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') were used. The amplification was carried out in a thermocycler (Techne, FTGENE5D, Cambridge, UK) by PCR under a 35 cycles program, in which each cycle consisted of the initial

denaturation for 30 s at 94 °C, the alignment of 1 min at 56 °C and of 2 min at 72 °C for the extension. Sequencing results were aligned with the reported ITS sequence using NCBI BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome; National Center for Biotechnology Information [NCBI], Bethesda, Maryland, USA).

Isolation of *T. harzianum* THITR01 mutants

Trichoderma harzianum THITR01 spores were collected and their concentration was adjusted to 1×10^7 spores mL^{-1} and treated with 5%, 10%, 15% and 20% ethyl methanesulfonate (EMS) for 1, 2 and 3 h. Then, the spores were washed twice with sterile distilled water and serial dilutions were prepared. From the 10^{-3} dilution 200 µL were taken and inoculated on PDA supplemented with sorbitol as a selection agent (0.2, 0.4 and 0.6 M), incubating at 28 ºC. Fungal growth was monitored every 24 h under a microscope to observe spores that had the ability to germinate to be subcultured thereafter.

Growth rate and antagonist potential of *T. harzianum* mutants

To determine if the mutant's growth and sporulation ability were affected, six consecutive subcultures were carried out in PDA, discarding the strains with undesirable characteristics. From the normal growth and sporulation ability strains, the growth rate was determined placing a 0.5 cm disk in the center of PDA dishes and their radial growth was measured every 24 h after incubating at 28 °C. The determination of the mutant's antagonist potential was determined by dual confrontation against *Fusarium oxysporum*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, placing at one end of the 9 cm diameter Petri dish with PDA a 0.5 cm disk with active fungal growth of each of pathogen, and at the opposite end a disk with well-grown selected mutants and incubated at 28 °C. The growth rate of the phytopathogenic fungi was evaluated until 120 h, and then the inhibition percentage of phytopathogens growth was calculated in triplicate.

Growth of *T. harzianum* mutants under osmotic and alkaline stress

From the antagonism tests previously described, five mutants were selected to determine tolerance to saline (NaCl), alkaline ($NaHCO_3$) and osmotic (sorbitol) stress, and as control the wild strain (*T. harzianum* THITR01) and a *T. harzianum* commercial strain (Natucontrol-Biokrone, Mexico) were used. Mycelium discs of 0.5 cm diameter were taken from the edges of the fungal growth area and placed at the center of PDA dishes with 0.5, 0.75 or 1.25 M NaCl to assay salt stress; with 0.1%, 0.2% and 0.3% $NaHCO_3$ to test alkaline stress; or placed on with 0.75, 1.0 or 1.5 M sorbitol to analyze osmotic stress, and incubated at 28 °C. Colony diameter was recorded every 24 h. The experiment was a randomized complete block with five replicates.

Phosphorus solubilization ability of mutants

To determine the *T. harzianum* mutant's ability to solubilize unavailable P, the Pikovskaya (PVK)-agar culture medium containing 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.002 g $MnSO_4{\cdot}H_2O$, 0.2 g NaCl, 10 g glucose and 0.002 g $FeSO_4{\cdot}7H_2O$, supplemented with 0.5 g yeast extract and 20 g agar was used (Maurya et al., 2019). To this medium, 5 g $Ca_3(PO_4)_2$ were added as a source of unavailable P and 0.002 g bromocresol blue as an indicator of color change. For the test, 0.5 cm diameter discs with mycelium from the edges of the growth area of the five mutants were taken, including the wild type and the commercial strains as a control, and were placed in the center of the Petri dish and incubated at 28 °C during 12 d.

Phosphorus solubilization *in planta*

Chili pepper (*Capsicum annuum* L. var. *annuum*) 'Jalapeño' seedlings were transplanted from the seedbed into pots with 300 g vermiculite and were immediately inoculated with 1 mL 1×10^7 spores of *T. harzianum* M5, M7, M14, M21 and M24 mutants, THITR01 wild type (WT), and commercial strain (C) on the plant stem close to the soil area, and watered at field capacity. A pot without microorganism (NT) was also included in the experiment. Fertilization was 20 mL of 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.002 g $MnSO_4{\cdot}H_2O$, 0.002 g $FeSO_4{\cdot}7H_2O$ and in 5 g $Ca_3(PO_4)_2$ as unavailable P. After 15 d plants were re-inoculated with the all-mentioned strains using the same spore concentration. For control (F) with available orthophosphate, the plants were watered with 50 mL of a solution containing 0.3 g NPK (at 16-10-12 + microelements, Diamond mixture, ISAOSA, Jalisco, Mexico). The plant height was measured every week. At the end of

the experiment, the vermiculite substrate of the different plant treatments was collected for the determination of available P with the Olsen method (Koralage et al., 2015), which consists of extraction of soil with 0.5 M $NaHCO_3$ solution (pH adjusted to 8.5) for 30 min before filtration and thereafter the phosphate concentration is measured by atomic absorption spectrometry.

Drought stress treatment in plants inoculated with mutant strains

The chili pepper seedlings were transplanted from the seedbed into pots with a peat moss-vermiculite substrate mixture (3:1). M5, M7, M14, M21, M24 mutants, WT and C were inoculated at a concentration of 1×10^7 spores mL^{-1} (fertilized plant control [F] was not inoculated). All plants were watered at field capacity, and 15 d after transplanting they were re-inoculated with the same spore concentration as mentioned above except for F. After 2-mo, five plants from each experimental unit were randomly selected to determine agronomic parameters such as plant height, stem diameter, and plant and root fresh and dry weight. Thereafter watering was hold for 9 d and a sample was taken to determine chlorophyll and carotenoids (Guler et al., 2016), soluble phenols (Hura et al., 2012), relative water content (RWC) and proline (Salgado-Aguilar et al., 2020) and catalase activity (Singh et al., 2020).

Data analysis

The statistical analysis was performed with the SAS-University-Edition program (SAS Institute, Cary, North Carolina, USA), which consisted of ANOVA for experimental units in a completely randomized design with three replicates of two independent experiments, and comparison of means was determined by Tukey's test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Morphological and molecular identification of *T. harzianum* THITR01

As a first step, four *Trichoderma* spp. strains identified by morphological and taxonomic characters were isolated and subjected to dual confrontation against *F. oxysporum* and *S. sclerotiorum*, selecting the strain THITR01 as the best antagonist (data not shown). By ITS PCR amplification, a DNA 535 bp fragment was obtained and a 99% nucleotide sequence similarity with *T. harzianum* was found in an analysis by Blast (NCBI). The strain was registered in the NCBI with number MH282575 as *T. harzianum* THITR01.

Selection of *T. harzianum* mutants

Although currently site-directed mutagenesis is increasingly used in filamentous fungi, random mutagenesis continues to be a genome variation source that allows to broaden the desired phenotype of several fungi (Alfiky, 2019). Mutation with EMS is an empirical method where the higher the concentration and exposure time to the mutagenic agent, the greater the negative effect on the germination ability of *T. harzianum* spores. The 5% EMS treatment of *T. harzianum* THITR001 during 1 h, allowed the isolation of 174 mutants, which only 70 had radial growth. Other mutants had undesired morphological and sporulation changes. From these mutants only 25 had the ability to produce spores, the remaining 104 mutants had yeast-like thallus growth habit and were not considered for further testing.

Mutants of *T. harzianum* have enhanced antagonist ability

The *T. harzianum* potential as antagonist against different phytopathogens was evaluated by the growth rate and the dual confrontation test allowing the identification of 25 mutants that had a greater antagonist ability against pathogenic fungi. Initially, we measured *T. harzianum* mutants and the wild type strain growth rate (*T. harzianum* THITR001) as shown in Table 1. Most of them showed 2.13 cm d^{-1} growth rate except for M13, M15 and M20 that displayed 1.78, 1.77 and 0.70 cm d^{-1} growth rate decrease respectively (Table 1).

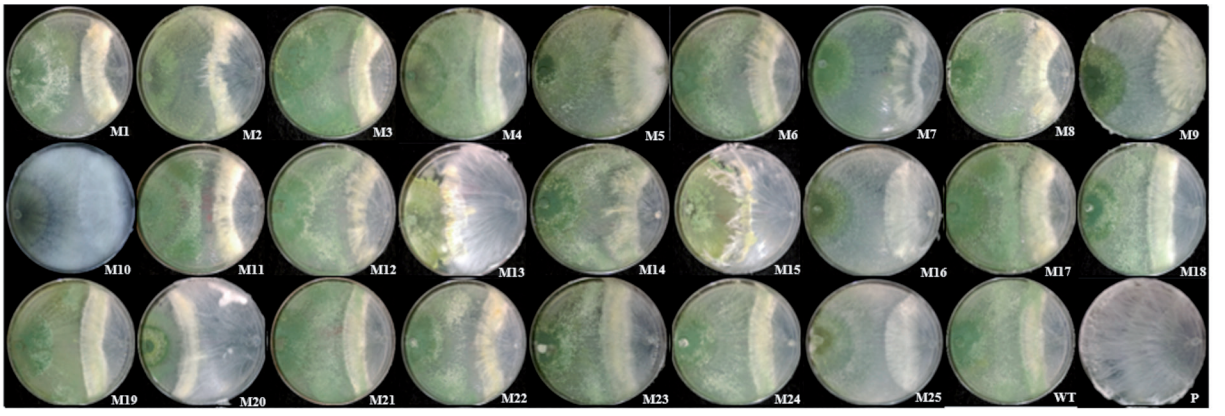
When *Sclerotium rolfsii* was used to confront the M3, M7, M5, M14 and M21 mutants they inhibited 97.6%, 97.4%, 96.8%, 95.9% and 95.6% pathogen growth rate respectively, compared to the WT which promoted 95.0% growth rate inhibition. However, M13, M15 and M20 lost about half their antagonist ability against *S. rolfsii*, since they inhibited only 45.0%, 50.3% and 50.3% pathogen growth rate respectively.

Table 1. Antifungal growth inhibition by *Trichoderma harzianum* mutants.

Mutant	Growth rate	Growth inhibition			
		Sclerotium rolfsii	*Sclerotinia sclerotiorum*	*Rhizoctonia solani*	*Fusarium oxysporum*
	cm d^{-1}	%			
1	2.13c	90.0bcd	80cd	75.6ab	53.1b
2	2.13c	71.8abcd	83.3cd	64.7ab	51.3b
3	2.13c	97.6d	73.3bcd	75.3ab	50.0b
4	2.13c	93.8cd	86.7cd	90.0ab	50.9b
5	2.13c	96.8d	95.0d	75.0ab	54.4b
6	2.13c	95.9cd	75.0bcd	78.5ab	50.9b
7	2.13c	97.4d	100.0d	71.8ab	51.6b
8	2.13c	93.5cd	66.7bcd	60.6a	52.5b
9	2.13c	80.8cd	76.7bcd	72.4ab	51.3b
10	2.13c	94.1cd	65.0abcd	62.6a	50.0b
11	2.13c	90.9cd	78.3cd	64.1a	49.7ab
12	2.13c	89.1bcd	91.7d	64.7ab	52.2b
13	1.78b	45.0a	25.0ab	79.9ab	54.7b
14	2.13c	95.9d	100.0d	63.8a	51.6b
15	1.77b	50.3ab	13.3a	61.8a	53.1b
16	2.13c	81.3cd	100.0d	65.3ab	51.3b
17	2.13c	90.3cd	95.0d	68.5ab	55.3b
18	2.13c	93.8cd	90.0d	87.1ab	51.3b
19	2.13c	90.0cd	85.0cd	67.6ab	50.6b
20	0.70a	50.3abc	36.7abc	64.7ab	42.2a
21	2.13c	95.6d	100.0d	72.1ab	53.4b
22	2.13c	90.3cd	96.7d	91.2ab	53.8b
23	2.13c	91.5cd	65.0abcd	100.0b	50.3b
24	2.13c	91.8cd	95.0d	100.0b	55.6b
25	2.13c	90.6cd	85.0cd	82.4ab	50.6b
WT	2.13c	95.0cd	93.3d	77.1ab	52.2b

WT: *Trichoderma harzianum* THITR01 wild type strain.
Values are means of five biological replicates. For each column, values followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$).

Figure 1. Dual confrontation of *Trichoderma harzianum* mutants against *Sclerotium rolfsii* after 7 d incubation at 28 °C on PDA medium. M1 to M25: *T. harzianum* mutants; WT: *T. harzianum* THITR01 wild type strain; P: *S. rolfsii*.



From the dual confrontation test of *T. harzianum* mutants against *S. rolfsii*, a clearly identified hyphae and sporulation zone of the different mutants and *S. rolfsii* hyphae could be observed (Figure 1). Concerning M13, M15 and M20 mutants, the pathogen mycelium grew over the mutant's mycelia as shown in Figure 1.

When *S. sclerotiorum* was confronted with the *T. harzianum* M5, M7, M12, M14, M16, M17, M18, M21, M22 and M24 mutants, they inhibited 90%-100% the pathogen growth, which is comparable to WT 93.3% growth rate inhibition and all them belonged to the same statistical group ($P \leq 0.05$). In contrast, M13, M15 and M20 decreased their antagonist ability against *S. sclerotiorum* to 25.0%, 13.3% and 36.7% growth rate respectively (Table 1). When *R. solani* was confronted against M23 and M24 mutants, it showed 100% growth rate inhibition, compared to 77.1% growth rate inhibition against WT. If *F. oxysporum* was used against all the *T. harzianum* mutants, they displayed 49.6% antagonistic ability compared to 52.2% for the WT and belong to the same statistical group ($P \leq 0.05$) except for M20 mutant which inhibited 42.2% pathogen growth rate. These described mutants had a higher growth rate inhibition ability against pathogens than the *Trichoderma* mutants reported by Alfiky (2019), where spore mutagenesis by ultraviolet light radiation of *Trichoderma virens* and *T. asperellum* allowed the selection of *T. virens* mutants that inhibited 76.6% and 78.3% growth rate of *S. rolfsii* and *R. solani* respectively, whereas *T. asperellum* mutants displayed 56.7% and 56.3% growth rate inhibition ability against those phytopathogen fungi 7 d after confrontation (Alfiky, 2019). Similarly, *T. harzianum* mutants obtained by gamma radiation displayed 48.13% and 88.40% antagonist ability against *R. solani* and *S. sclerotiorum*, respectively, after 3 d of confrontation (Abbasi et al., 2016). These two reports showed a lower antagonist ability than the mutant described in the present work.

Trichoderma harzianum mutant's growth under osmotic and alkaline conditions

The 25 isolated mutants in this study were subjected to different NaCl concentrations to test their possible salt-stress tolerance (Table 2). We assayed *T. harzianum* growth rate at 0.50, 0.75 and 1.25 M NaCl for the different strains, however there were nonsignificant ($P \leq 0.05$) differences between the mutants and WT and C at any of the mentioned NaCl concentrations. We observed that the mycelium radial growth remained constant; however, the spore production capacity was inhibited under the different NaCl concentrations. Poosapati et al. (2014) found natural isolates of *Trichoderma* strains that could tolerate up to 1 M NaCl.

To further characterize *Trichoderma* mutants, they were subjected to osmotic stress. All mutant grew at 0.75 and 1.0 M sorbitol, however the growth rate did not show significant ($P \leq 0.05$) differences compared to WT but it was higher than C. At 1.5 M sorbitol there were nonsignificant ($P \leq 0.05$) differences between the mutants, WT and C (Table 2).

Lastly, alkaline tolerance was tested. It was found that at 0.1%, 0.2% and 0.3% $NaHCO_3$ all mutants showed a significantly ($P \leq 0.05$) similar growth rate compared to WT (Table 2). Guo et al. (2018) reported the construction of a T-DNA insertion mutant library, yielding 65 *T. asperellum* mutants, the T59 mutant could tolerate up to 1.7 M NaCl and T3 and T5 mutants could grow on 0.4% $NaHCO_3$. Alkaline soils are common in arid and semi-arid regions accounting around 25% of our planet's surface.

Trichoderma harzianum mutants solubilize P

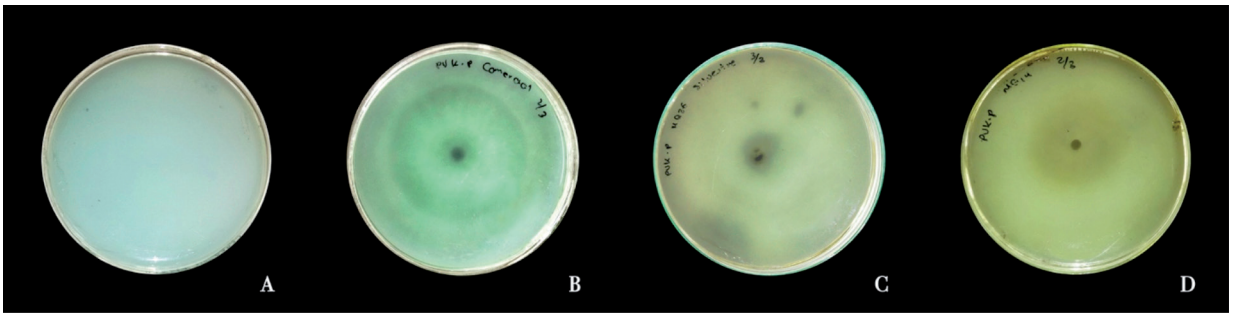
Trichoderma harzianum is an efficient biocontrol agent that promotes plant growth and enhances nutrients uptake by plants (Rawat and Tewari, 2011; Li et al., 2015). Therefore, it was decided to evaluate the ability of *T. harzianum* mutants to solubilize $Ca_3(PO_4)_2$ as a source of unavailable P. The M14 mutant and THITR01 WT showed $Ca_3(PO_4)_2$ solubilizing capacity 7 d after growth in PVK-agar, since a color change was observed due to the excretion of organic acids from a blue to yellow tone because of a change in pH visualized by the bromocresol green dye (Figure 2). In contrast, C was unable to

Table 2. Growth rate of *Trichoderma harzianum* mutants under salt, osmotic and alkaline stress.

	NaCl			Sorbitol			$NaHCO_3$		
Strains	0.5 M	0.75 M	1.25 M	0.75 M	1.0 M	1.5 M	0.1%	0.2%	0.3%
	cm d^{-1}			cm d^{-1}			cm d^{-1}		
M5	1.28b	0.82bc	0.23bc	1.42b	1.35b	0.58a	1.48c	1.36c	1.14d
M7	1.28b	0.93c	0.24bc	1.42b	1.35b	0.64a	1.43c	1.37c	0.98cd
M14	1.33b	0.72ab	0.23bc	1.42b	1.32b	0.63a	1.43c	1.27c	1.11d
M21	1.07a	0.86bc	0.21b	1.42b	1.37b	0.64a	1.27ab	0.87b	0.73bc
M24	1.15ab	0.64a	0.13a	1.40b	1.29b	0.60a	1.37bc	0.89b	0.56b
WT	1.34b	0.86bc	0.27b	1.42b	1.30b	0.67a	1.46c	1.28c	0.88bcd
C	1.28b	0.84bc	0.31c	1.26a	1.04a	0.60a	1.15a	0.62a	0.19a

M5, M7, M14, M21, M24: Mutant strains; WT: THITR01 wild type strain; C: commercial strain.
Values are means of five biological replicates. For each column, values followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$).

Figure 2. Phosphorus solubilization by *Trichoderma harzianum* strains on PVK-agar media. Growth medium without fungus (A), commercial strain (B), THITR01 wild type strain (C), and M14 mutant (D).



promote P solubilization (Figure 2). The M21 mutant strain also showed this solubilization ability, however it was lower compared to M14; whereas M5, M7 and M24 mutants did not show P solubilization (data not shown). Similar $Ca_3(PO_4)_2$ solubilization effect was previously reported in another *T. harzianum* strains (Alori et al., 2017).

Mutant strains solubilize P in chili pepper plants

The effect of *T. harzianum* mutants on the agronomic parameters of inoculated plants with unavailable P was determined. There were nonsignificant differences ($P \leq 0.05$) in shoot elongation (plant height, plant fresh and dry weight) after inoculation of chili pepper plants with the mutant and WT. Plant responses to low P availability are displayed at root architecture level (Niu et al., 2013). It was found that the M7 and M24 mutants, C and NT had significant ($P \leq 0.05$) capacity to induce root elongation displaying 26.4 and 29.0, 25.3 and 28.4 cm respectively after 2-mo, whereas inoculation with the other mutants and WT did not promote significantly root elongation (Table 3). However, regarding plant biomass analysis it was observed that inoculation with the M14 and M21 mutants led to significant ($P \leq 0.05$) increases of 2.6 and 2.0 g respectively of root fresh weight and 0.28 g dry weight corresponding to both mutants, compared to plant inoculation with WT (Table 3). These data are consistent with previous reports where root development and P solubilization are induced by *T. harzianum* inoculation (Li et al., 2015).

Another important effect on chili pepper plants inoculated with *T. harzianum* mutants and grown with unavailable P, was a change in the root architecture (Figure 3). As shown in Table 3, there were found significant ($P \leq 0.05$) changes in root length on plants inoculated with the mutant strains compared to inoculation with WT. An increase in lateral root formation and root hairs elongation compared to treatment with chemical fertilization and treatment without *T. harzianum* was observed (Figure 3). These root morphological changes were more evident on plants inoculated with M14 compared to inoculation with WT or C (Figure 3). Similar alterations in root architecture in *Pinus sylvestris* seedlings promoted by *Trichoderma* spp. inoculation have already been described and correlate with higher P solubilization (Halifu et al., 2019).

Table 3. Effect of *Trichoderma harzianum* mutants on the agronomic parameters of inoculated 'Jalapeño' pepper plants with unavailable P.

Treatments	Fresh weight		Dry weight		Length	
	Shoot	Root	Shoot	Root	Shoot	Root
	g		g		cm	
M5	1.7a	1.3bc	0.22a	0.17bc	14.4cd	21.7ab
M7	2.1ab	1.6cd	0.25ab	0.18bcd	12.8bc	26.4cde
M14	3.0d	2.6e	0.40d	0.28e	13.5bcd	24.4abc
M21	2.8cd	2.0d	0.35c	0.28e	15.1d	25.1bcd
M24	2.2ab	1.4bc	0.26ab	0.16bc	12.8bc	29.0e
WT	3.1d	1.2bc	0.40d	0.20cd	13.5bcd	23.3abc
C	2.5bc	1.0b	0.28b	0.15b	12.2ab	25.3bcde
NT	3.2d	1.5bcd	0.39cd	0.21d	12.9bc	28.4de
F	3.3d	0.4a	0.36cd	0.09a	10.6a	20.8a

M5, M7, M14, M21, M24: Mutant strains; WT: THITR01 wild type strain; C: commercial strain; NT: uninoculated plants; F: fertilized control.
Values are means of five biological replicates. For each column, values followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$).

Figure 3. Effect of *Trichoderma harzianum* mutants on root architecture of 2-mo-old 'Jalapeño' chili pepper plants grown with unavailable P.

M5, M7, M14, M21, M24: Mutant strains; WT: THITR01 wild type strain: C: commercial strain; NT: uninoculated plants; F: fertilized control. Photograph shows representative plants from each treatment.

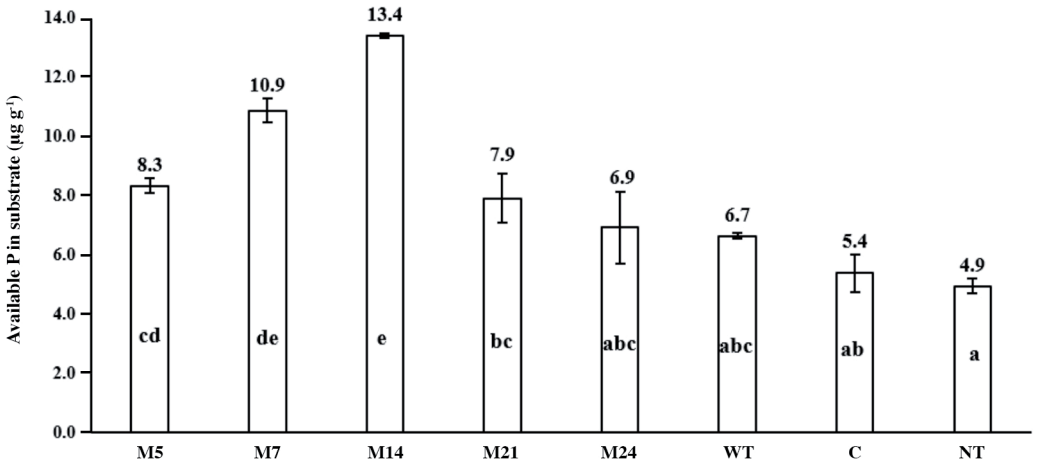
Also, we determined the available P in the vermiculite substrate of inoculated plants to assess the mineral solubilization by *Trichoderma* mutants. There were significant ($P \leq 0.05$) differences between mutants and WT, the M7 and M14 mutant solubilized 10.9 and 13.4 μg g^{-1} P in the substrate respectively compared to the other mutants and controls (Figure 4). The P solubilization improves the biological N fixation, the phytohormones synthesis and the availability of other nutrients (Alori et al., 2017).

Mutant strains mitigate drought stress in chili pepper plants

First of all, the effect of inoculating chili pepper plants with *T. harzianum* mutants was evaluated regarding various agronomic parameters. It was found that there were nonsignificant ($P \leq 0.05$) differences in plants inoculated with the different *T. harzianum* strains (mutants, WT or C), compared to the plants without inoculation with *T. harzianum*, regarding shoot or root growth and stem diameter (Table 4). These data indicate the *T. harzianum* strains used in this work do not function as promoters of plant growth, in contrast to other reports (Contreras-Cornejo et al., 2014; Ahmad et al., 2015).

In addition to the role of *T. harzianum* as a biocontrol agent against fungal diseases in plants, it has recently been reported that this fungus is also able to protect plants from cold, heat, salinity or drought stress (Ahmad et al., 2015; Guler et al., 2016). Therefore, this study analyzed whether there was a greater tolerance to drought stress in chili pepper plants

Figure 4. Quantification of available P in substrate of 'Jalapeño' pepper plants grown with unavailable P and inoculated with *Trichoderma harzianum* strains.



M5, M7, M14, M21, M24: Mutant strains; WT: THITR01 wild type strain; C: commercial strain; NT: uninoculated plants. The values represent the mean ± SE (n = 5). Different letters indicate significant differences between treatments according to Tukey's test ($P \leq 0.05$).

Table 4. Effect of *Trichoderma harzianum* inoculation on 'Jalapeño' pepper plants growth.

Strains	Fresh weight		Dry weight		Length		Diameter
	Shoot	Root	Shoot	Root	Shoot	Root	
	g		g		cm		cm
M5	10.52a	4.43a	1.34a	0.47a	36.4a	26.1a	0.7a
M7	9.77a	4.30a	1.28a	0.50a	33.6a	27.9a	0.7a
M14	10.07a	4.07a	1.21a	0.54a	37.8a	24.7a	0.8a
M21	10.42a	3.85a	1.37a	0.54a	38.3a	25.3a	0.8a
M24	10.85a	4.51a	1.27a	0.54a	35.0a	26.0a	0.7a
WT	10.47a	3.20a	1.28a	0.47a	39.0a	26.5a	0.7a
C	11.6 a	3.75a	1.43a	0.54a	34.2a	26.9a	0.8a
NT	12.13a	3.56a	1.45a	0.51a	40.8a	24.5a	0.7a

M5, M7, M14, M21, M24: Mutant strains; WT: THITR01 wild type strain; C: commercial strain; NT: uninoculated plants. Values are means of five biological replicates. For each column, values followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$).

inoculated with *T. harzianum* mutants. After 9 d without irrigation, a significant ($P \leq 0.05$) higher relative water content (RWC) in plants inoculated with the M24 mutant was found, compared to plants inoculated with WT or C (Table 5). Also, there was a higher RWC in plants inoculated with all the strains than in plants without fungus inoculation (Table 5). Our data with the *T. harzianum* mutants is similar to previous reports where plants inoculated with *Trichoderma* strains improved their stress tolerance (Guler et al., 2016). Barros et al. (2018) described that *Cinophalla flexuosa* inoculated with mycorrhiza subjected to 7 d of drought stress had a higher RWC than without mycorrhiza.

Interestingly, after 9 d of drought stress chlorophyll a, chlorophyll b and total chlorophyll contents showed a significant ($P \leq 0.05$) increase of 14.5, 10.5 and 25.0 mg g^{-1} dry weight, respectively, in plants inoculated with the *T. harzianum* WT compared to the inoculation with C or the mutant strains (Table 5). We speculate that EMS-induced mutation in *T. harzianum* triggers down regulation of chlorophyll biosynthesis or salicylic acid (SA) signaling genes in inoculated chili pepper plants. Salicylic acid is a phytohormone that is involved in regulating photosynthetic reactions, transpiration rate and chlorophyll content, which decline under abiotic stress (Singh and Gautam, 2013). Inoculation of wheat plants with *Trichoderma longibrachiatum* enhances salt tolerance and chlorophyll contents (Zhang et al., 2016). Regarding carotenoids determination, nonsignificant ($P \leq 0.05$) differences were found between treatments (Table 5).

To gain further understanding of the biochemical compounds involved in stress tolerance and signaling, the content of total phenols in chili pepper plants inoculated with *T. harzianum* mutants was determined 9 d after drought stress (Table 5). Nonsignificant ($P \leq 0.05$) differences in total plant phenols content were found when inoculating with all the different mutant strains. It is well established that phenol accumulation is closely related to phenylpropanoid induction pathways and tolerance to drought stress (Hura et al., 2012).

Table 5. Physiological and biochemical tests of 'Jalapeño' pepper plants under drought stress inoculated with *Trichoderma harzianum*.

Strains	RWC %*	RWC	Chlorophyll			Carotenoids	Phenols	Proline	Catalase
			a	b	T				
		%	mg g^{-1}			mg g^{-1}	mg g^{-1}	µg g^{-1}	U mg^{-1} protein
M5	84.6a	78.3b	5.0a	3.0a	8.1a	1.5ab	286.2b	399.8a	0.0a
M7	80.2a	78.1b	6.3ab	4.3a	10.6ab	1.3a	358.3b	439.8ab	0.3a
M14	83.4a	77.0b	9.6bc	6.4ab	15.9b	2.1ab	368.8b	519.7ab	0.2a
M21	85.0a	78.6bc	9.3bc	5.9a	15.2b	1.7ab	169.8a	739.6c	0.1a
M24	85.2a	82.9c	11.0c	5.2a	16.2b	2.7b	315.7b	939.5d	0.0a
WT	84.5a	76.3b	14.5d	10.5b	25.0c	2.2ab	254.7ab	419.8ab	0.1a
C	84.1a	77.1b	7.3ab	4.4a	11.7ab	1.7ab	294.2b	379.8a	0.3a
NT	78.8a	71.1a	9.2bc	7.2ab	16.4b	1.6ab	297.8b	599.7bc	0.1a

M5, M7, M14, M21, M24: Mutant strains; WT: THITR01 wild type strain; C: commercial strain; NT: uninoculated plants.
*All measured parameters were conducted 9 d after subjecting 'Jalapeño' plants to drought stress, except for relative water content (RWC) which was also determined before drought stress. Chlorophyll (a, b or total, T), carotenoids, phenols and proline are related to dry plant material (g).
Values are means of five biological replicates. For each column, values followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$).

As first attempt to dissect the mechanism used by the chili pepper plant to adapt to drought stress when it is inoculated with the *T. harzianum* mutants, the proline content was determined. After 9 d without watering, plants inoculated with the M24 mutant accumulated 939.5 μg g^{-1}, which represents a significant ($P \leq 0.05$) increase compared to all other inoculated plants (Table 5). Proline can play a role as an osmoprotectant molecule and is widely distributed in plants. Finally, the catalase enzymatic activity was measured to assess if a detoxification mechanism of reactive oxygen compounds was involved (Ahmad et al., 2015). Nonsignificant ($P \leq 0.05$) differences were found between the plants inoculated with different *Trichoderma* strains (Table 5). The molecular pathway that the chili pepper plant uses to adapt to drought stress when inoculated with the mutants described in this work is unknown and could be investigated using a transcriptomic study.

CONCLUSIONS

Trichoderma harzianum THITR01 mutagenized with ethyl methanesulfonate (EMS) generated strains that have an enhanced antagonist ability in vitro against different soil fungal pathogens. Although these mutant strains are not plant growth promoters, under unavailable P growth conditions the M7 and M14 mutants had the ability to solubilize unavailable P in the substrate. Only the M14 promoted lateral root formation and root hair elongation in chili pepper plants, which are important to increase absorptive surface to facilitate nutrient uptake. M14 and M21 mutants led to a plant increase in their root fresh and dry weight. On the other hand, the M24 mutant induced proline accumulation in chili pepper plants subjected to drought stress, and display a higher relative water content, important parameters to mitigate drought stress. Therefore, the M14, M21 and M24 mutants could potentially be used as a biocontrol agent in the field for crops that are subjected to fungal diseases and abiotic stress. Further molecular characterization of these mutants might allow its genetic mapping and unravel its signaling mechanism.

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