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QUALITATIVE AND QUANTITATIVE ESTIMATION OF THE ABILITY OF *Trichoderma spp.* MOROCCAN ISOLATES TO SOLUBILIZE TRICALCIUM PHOSPHATE

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ABSTRACT

The present study was undertaken to estimate *in vitro* the capacity of twenty-three isolates of *Trichoderma*, isolated from soil and roots collected from adjacent sites to the Khouribga and Benguerir Phosphate mines (Morocco), and seven isolates from the laboratory's mycoteca to solubilize the Tricalcium phosphate. All these isolates showed, after three days of incubation, on NBRIP medium, amended by $Ca_3(PO_4)_2$, colony diameters which vary between 41.7 and 59.4 mm. Six days later, all isolates reached maximum diameters (90 mm), but no clear halo-zone was observed around the colonies. Mycelial densities of all isolates were medium to high on NBRIP agar. After 7 days of incubation, isolates TS-B 98; TR-B 98 (2); TR-B 98 (3) were found to be able of producing a large number of conidia with respective concentrations of 38.10^5, 39.10^5 and 42.10^5 conidia.mm^{-2} while others have poorly sporulated or not-forming conidia. A simultaneous increase in the concentration of soluble phosphorus was recorded in the culture filtrates. Peak concentrations are noted at ninth day after incubation in TR-B 98/2002 (2), TS-B-2000 (2), TR-CB 2000 (1), TR-TB 2000, TR-OL 1; TS-B 98, TR-EM 2, TR-B 98 (1), TR-B 98 (3), with values exceeding 10.15 µg.mL^{-1} and reaching a maximum value of 12.42 µg.mL^{-1} compared to the blank (0.52 µg.mL^{-1}). pH values decreased to 4.12 in TR-TB 2000 compared to control (6.12). The highest fresh and dry weights were recorded in TR-TB 2000 isolates (FW = 4.1 g, DW = 2.78 g) and TR-B 98 (3) (FW = 3.9 g, DW = 3.00 g). TR-TB 2000, TS-B 98, TR-EM 2, TR-B 98 (1) isolates proved to be the most successful in phosphate solubilization, with maximum phosphorus concentrations.

Keywords: *Trichoderma* spp.; *in vitro*; solubilization; Tricalcium phosphate; soluble phosphorus.

INTRODUCTION

Soil is a substrate rich in chemical elements such as nitrogen, potassium and phosphorus which are a source of nutrients for plants. Phosphorus (P) is of vital importance for metabolic needs, growth and reproduction and therefore for crop production [1,2] However, phosphorus is only absorbed by plants and microorganisms in the form of the orthophosphate ion (Pi), represented by H_2PO^{4-} and HPO_4^2. Due to its strong ability to form complexes with cations [3] and its low mobility, the free P concentrations in the soil solution are generally low, estimated between 1 and 10 µM [4] and vary depending on the type of soil [5]. Much of the phosphate used as fertilizer is

immobilized after application and becomes inaccessible to plants [6,7]. In general, the deficiency of phosphorus (P) is one of the major constraints to agricultural production. To remedy this deficiency, the addition of inorganic fertilizers in quantities greater than the amount commonly used to overcome this effect can cause environmental problems such as groundwater contamination and eutrophication of waterways [8].

Many microorganisms, especially those associated with roots, can increase plant growth and productivity [9,10]. Better use of accumulated soil phosphorus is possible through to biofertilising microorganisms able of solubilizing insoluble phosphorus and transforming it into soluble forms. A large number of microorganisms, including bacteria, fungi, actinomycetes and algae, exhibit phosphorus solubilization and mineralization capacity [11].

Microorganism interactions with plant roots profoundly affect the nutritional status of plants and their resistance to pathogens [12]. It has been reported that soil fungi are able to roam long distances in the soil more easily than bacteria and produce organic acids such as glucuronic, citric or fumaric acids that acidify the soil, thus promoting the solubilization of phosphates, some micronutrients and cations such as iron, manganese and magnesium essential for plants [13,14].

Fungi can solubilize natural phosphates and make phosphorus available to plants. In fact, there are plants in the soils and rhizosphere, species of *Trichoderma* genus able of dissolving the different forms of insoluble phosphates [15]. Some strains of *Trichoderma* have been shown to be able to naturally solubilize insoluble powdered mineral phosphate (PM) to make it available for plant growth. Other strains of *Trichoderma* are also able to provide iron for cucumber plants in soil [16]. Oliveira et al. [17] reported that strains of *Trichoderma* were able to solubilize Calcium phosphate. The interaction between a plant species and *Trichoderma* involves systemic modifications in different levels of phytohormones that have physiological consequences on the growth and resistance of plant species to diseases [18]. Most strains of the *Trichoderma* genus tested by Oliveira et al. [17] produced auxin, indole acetic acid (AIA), with or without the precursor of L-tryptophan.

Given the low level of soil fertility, the main objective of this study is to evaluate *in vitro* the solubilization capacities of Tri-calcium phosphate by different *Trichoderma* isolates, which could be an important way to stimulate and promote growth and development of cultivated plant species.

MATERIALS AND METHODS

Fungal Material

Thirty isolates of *Trichoderma* spp., Seven isolates from the Laboratory of Botanic Biotechnology and Plant Protection (LBBPP) mycoteca, isolated from compost and from different crops (two isolates of *Trichoderma asperellum* are registred in the database NCBI) and twenty-three of these newly collected isolates from sites adjacent to the phosphate mines were maintained on PSA medium (Potato-Saccharose Agar: 200 g of potatoes, Saccharose: 20 g, Agar-agar: 15 g and 1000 mL of distilled water) in the dark at 28°C (Table 1).

Study of the Ability of *Trichoderma* Isolates to Solubilize Phosphate

Qualitative estimation

The ability of *Trichoderma* isolates to solubilize inorganic phosphate was evaluated *in vitro* on NBRIP medium: glucose : 10.0 g; Tricalcium phosphate (TCP): 10.0 g; $MgCl_2$, $6H_20$: 5.0 g; $MgS0_4.7H_20$: 0.25 g; KCl : 0.2 g; $(NH_4)2\ SO_4$: 0.1 g; Agar-agar: 15 g. The pH was adjusted to 7.2 using a pH meter and the components were dissolved in 1000 mL of distilled water [19,20].

A 5 mm - mycelial disk from the 7-day old culture of each isolate of *Trichoderma* spp. was placed in the center of the agar plate and incubated in the dark at 28°C. After 3 and 6 days of incubation, the colonies and halo-zones diameters were measured by a double decimeter. The phosphate solubilization index (PSI) was measured and

calculated according to the following formula [21,22]:

PSI = [The colony diameter + The halo-zone diameter] / The colony diameter

The number of conidia produced is determined by taking three 5 mm - mycelial disks from a seven-day old culture of each *Trichoderma* isolate. They are placed in a test tube containing 1 ml of sterile distilled water and stirred for 5 minutes using a vortex. The conidia concentration was estimated using a Malassez slide.

The mycelia density was performed by visual observation after 7 days of incubation at the time when the *Trichoderma* isolates completely colonized the Petri dishes using the scale of Sobal et al. [23]: (Hight density: + + +; Regular density: + +; Low density: +).

Quantitative Estimation

Trichoderma spp. isolates was tested for their ability to solubilize inorganic phosphate in NBRIP broth: Tricalcium Phosphate (TCP): 10.0 g; glucose: 10.0 g; $MgCl_2$, $6H_20$: 5.0 g; $MgSO_4.7H_20$: 0.25 g; KCl: 0.2g; (NH_4) $2SO_4$: 0.1 g. The pH is adjusted to 7.2 using a pH meter and the components are dissolved in 1000 mL of distilled water [19]. Five 5 mm-mycelial disks from each isolate of *Trichoderma* spp. were inoculated into a 250 mL Erlenmeyer flask containing 100 mL broth and incubated at 28°C in a shaker (GFL 3020) at 120 rpm for 7 days. The broths were filtered through Whatman N°1 paper (0.45 µm) and centrifuged at 5,000 rpm for 10 min to remove conidia and mycelium from *Trichoderma* isolates.

Table 1. Origin and sources of isolation of tested *Trichoderma* spp.

Isolates of *Trichoderma* spp.	Sources of isolation	Locality (country)
T1 (BankIt1902509 SMis1 KU987252) *Trichoderma asperellum*	TTC Compost	Missour/ Morocco
1TH	Bananas agriculture/ Mnasra	Kenitra region / Morocco
TH2	Bananas agriculture /Mnasra	Kenitra region / Morocco
T27(BankIt1902509 SDLA27 KU987250)*Trichoderma asperellum*	Strawberry agriculture, Festival variety	Dlalha / MyBouselham / Morrocco
T 30	Strawberry agriculture, Sabrina variety	Gnafda / MyBouselham / Morocco
TOL	Roots of an olive tree	Sidi kasem/ Morocco
TY	Rhizosphere of an olive tree's roots	Sidi kasem/ Morocco
TS-BG	Soil of Bengurir region,	Bengurir region /Morocco
TS-ML	Soil of Mrah Lahrech site	
TS-H	Hattan site soil	
TS-RP	Pure phosphate rock	
TR-OL 1	Rhizosphere of the roots of an olive tree	
TR-OL 2	Rhizosphere of the roots of an olive tree	
TR-CB 2000 (2)	Root rhizosphere of a *Crucifera* agriculture, sludge 2000	
TR-TB 2000	Roots of *Tamarix*, sludge 2000	
TS-B 98	Sludge soil 1998	
TS-EM 98 (1)	Sludge soil 1998	
TS-EM 98 (2)	Sludge soil 1998	
TR-EM 1	Roots of mixed plant samples	Khouribga region /Morocco
TR-EM 2	Roots of mixed plant samples	
TR-B 98 (1)	Sludge roots 1998	
TR-B 98 (2)	Sludge roots 1998	
TR-B 98 (3)	Sludge roots 1998	
TS-B 98/2002 (1)	Sludge roots 1998/2002	
TS-B 98/2002 (2)	Sludge roots 1998/2002	
TR-B 98/2002 (1)	Sludge roots 1998/2002	
TR-B 98/2002 (2)	Sludge roots 1998/2002	
TS-B 2000 (1)	Sludge soil 2000	
TS-B 2000 (2)	Sludge soil 2000	
TR-C B 2000 (1)	Sludge cruciferous roots 2000	

The pH of each culture was measured using a pH meter. The phosphorus concentration in the supernatant was estimated spectrophotometrically [24,25]. An aliquot of 750 µL of culture supernatant was mixed with 750 µL of the colored reagent containing ammonium molybdate ($(NH_4)6Mo_7O_{24}, 4H_2O$) 1.5% (p / v), sulfuric acid solution (H_2SO_4) 5.5% (v / v) and solution of ferrous sulphate ($FeSO_4$) 2.7% (p/v). The optical density was measured by a UV-visible spectrophotometer at 600 nm. The level of phosphorus concentration was determined using the standard potassium phosphate dihydrogen curve (KH_2PO_4) and expressed as equivalent phosphorus in µg-P.mL^{-1}.

The percentage of phosphorus soluble in the culture filtrates was estimated at the 9th day of incubation, when the soluble phosphorus concentration reached the maximum for all isolates using the formula:

% PS = (Concentration of soluble phosphorus in the filtrates / Initial phosphate concentration) x 100

Measurement of the Mycelial Biomass of Different *Trichoderma* Isolates in Broth Cultures

The fungal mycelium was harvested after 12 days of incubation and separated from the culture liquid by filtration on Whatman No. 1 filter paper. The fresh weight of the mycelium was measured using a weighting scale. Then the mycelial pellet was dried at 70°C for 24 h and the dry weight of the fungus was also calculated using a precision weighting scale using the following formula:

Dry weight = (weight of filter paper + mycelium) - (weight of filter paper)

Statistical Analysis

Statistical data processing included analysis of variance using the 5% Anova and LSD test.

RESULTS

All of the *Trichoderma* isolates used in this study showed good mycelial growth on $Ca_3(PO_4)_2$ NBRIP agar but there was no clear zone around the colonies to estimate phosphate solubilization (Table 2). The cultures were monitored for 7 days without any appearance of a halo-zone around the colonies. The solubilization index of phosphate was therefore not calculated.

Three days after incubation, good mycelial growth was observed on NBRIP agar (Table 2) for all isolates studied, with a slight difference between isolates for colony diameters ranging from 41.7 mm to 59.4 mm. Six days later, the colony diameter reaches the maximum (90 mm) for all isolates.

In NBRIP medium, the isolates TH_2; T27; TS-H; TR-B 98/2002 (2); TR-OL 2; TS-BG ;TR-OL 1 ;TS-B 98; TR-B 98 (2); TR-B 98 (3) showed very high mycelial densities on the agar plate and the other tested isolates showed regular mycelial densities.

The isolates TS-B 98 (3) produced a large number of conidia, 7 days after incubation of cultures; this number varies from 38.10^5 to 42.10^5 conidia.mm^{-2} (Table 2). But, the isolates TOL; T30; TY; TS-B 98/2002 (2);TR-B 98/2002 (1); TR-EM 2; TR-B 98 (1) didn't formed conidia (Table 2).

All cultures showed variable mycelial growth in the modified NBRIP broth, with a simultaneous increase in soluble phosphorus concentration reaching a maximum value at the 9th day in all isolates (Table 3). The concentration of soluble phosphorus increased gradually from the 3th day to the 9th day and then showed a slight decrease at the 12th day in the culture filtrates of the isolates (Table 3). The soluble phosphorus concentrations in the filtrates of the different tested isolates ranged from 1.57 µg.mL^{-1} to 12.05 µg.mL^{-1} at the first evaluation, three days after adding *Trichoderma* to the solution compared to the blank (0.4 µg.mL^{-1}). On the sixth day of incubation, soluble phosphorus concentrations ranged from 9.62 µg.mL^{-1} to 11.18 µg.mL^{-1} relative to Blank (0.46 µg.mL^{-1}), from 11.41 µg.mL^{-1} to 24.88 µg.mL^{-1} on the ninth day compared to Blank (0.52 µg.mL^{-1}), and from 16.01 µg.mL^{-1} to 20.73 µg. mL^{-1} compared to the Blank at a concentration not greater than 0.59 µg.mL^{-1} in the last assessment on the twelfth day.

Table 2. Growth and conidia production of *Trichoderma* isolates on NBRIP $Ca_3(PO4)_2$ Agar

Isolates	Colony diameter (mm)		Mycelium density	Number of conidia ($x10^5.mm^{-2}$)
	After 3 days	After 6 days		
T1	52.2[b*]	90[a]	++	22[b]
1TH	48.4[b]	90[a]	++	5[d]
TH_2	51.7[b]	90[a]	+++	10[c]
TOL	51.6[b]	90[a]	++	0[e]
T27	51.9[b]	90[a]	+++	14[c]
T30	44.3[c]	90[a]	++	0[e]
TY	41.7[c]	90[a]	++	0[e]
TS-ML	49.4[b]	90[a]	++	12[c]
TS-H	53.3[b]	90[a]	+++	16[c]
TS-B 2000 (1)	46.9[b]	90[a]	++	2[d]
TS-B 98/2002 (1)	47.6[b]	90[a]	++	2[d]
TS-B 98/2002 (2)	47.5[b]	90[a]	++	0[e]
TR-B 98/2002 (1)	49.1[b]	90[a]	++	0[e]
TR-B 98/2002 (2)	56.4[a]	90[a]	+++	24[b]
TR-OL 2	57.3[a]	90[a]	+++	12[c]
TS-EM-98 (1)	42.8[c]	90[a]	++	3[d]
TR-CB 2000 (2)	47.9[b]	90[a]	++	2[d]
TS-B-2000 (2)	51.2[b]	90[a]	++	10[c]
TR-CB 2000 (1)	46.7[bc]	90[a]	++	12[c]
TS-EM-98 (2)	49.8[b]	90[a]	++	9[d]
TR-TB 2000	48.9[b]	90[a]	++	11[c]
TS-BG	59.4[a]	90[a]	+++	23[b]
TR-OL 1	49.1[b]	90[a]	+++	26[b]
TS-RP	42.6[c]	90[a]	++	14[c]
TR-EM 1	56.5[a]	90[a]	++	12[c]
TS-B 98	59.2[a]	90[a]	+++	42[a]
TR-EM 2	45.4[c]	90[a]	++	0[e]
TR-B 98 (1)	47.5[b]	90[a]	++	0[e]
TR-B 98 (2)	54.3[b]	90[a]	+++	38[a]
TR-B 98 (3)	59.3[a]	90[a]	+++	39[a]

*Two values on the same column show no significant difference at the 5% level if they are affected by the same letter

The soluble phosphorus concentrations in the filtrates of the different isolates varied from 1.57 $\mu g.mL^{-1}$ for the TOL isolate to 6.73 $\mu g.mL^{-1}$ for the TH2 isolate at the first evaluation three days after the addition of *Trichoderma* to the solution (Table 3). Isolates that perform well for solubilizing Tricalcium phosphate six days after incubation are T1; TR-CB 2000 (1); TR-TB 2000; TR-OL 1 ; TR-EM 2 ; TR-B 98 (3), with concentrations exceeding 8 $\mu g.mL^{-1}$. Nine days after incubation, the highest soluble phosphorus concentrations were recorded in isolates TR-B 98/2002 (2), TS-B-2000 (2), TR-CB 2000 (1), TR-TB 2000; TR-OL 1, TS-B 98, TR-EM 2, TR-B 98 (1), TR-B 98 (3), with values exceeding 10.15 $\mu g.mL^{-1}$ and reaching a maximum value of 12.42 $\mu g.ml^{-1}$. Concentrations of soluble phosphorus remain stable without any significant difference between 9th and 12th day of incubation.

A remarkable decrease in pH values was recorded differentially in all isolates. The pH values decreased to varying levels up to 4.12 in the isolate (TR-TB 2000) compared to the control whose pH is equal to 6.12.

Fresh and dry weights of the mycelium of *Trichoderma* spp. in culture filtrates were estimated after the 12^{th} day of incubation. The highest fresh and dry weights are found in TR-TB 2000 isolates (FW = 4.1 g, DW = 2.78 g) and TR-B 98 (3) (FW = 3.9 g; DW = 3.00 g) which are the best performing isolates for the solubilization of inorganic phosphate. In contrast, isolates 1TH, TOL and TS-EM-98 (1) showed the lowest fresh and dry weights, respectively (DW = 0.954 g, DW = 0.657 g, FW= 0.854 g, DW = 0.522 g, DW = 1.06 g, DW= 0.45 g) and also showed poor performance for solubilization of phosphorus.

Table 3. Solubilization of Tricalcium phosphate by thirty isolates of *Trichoderma* spp. in the NBRIP broth

Isolates	Phosphorus concentration (µg.mL^{-1})				Soluble phosphorus (%)	Final pH	Fresh weight of mycelium (g)	Dry weight of mycelium (g)
	3 days	6 days	9 days	12 jours				
Blank	0,40^{i*}	0,46^{g}	0,52^{f}	0,59^{f}	5.77^{f}	6 .12^{a}	-------	-------
T1	6,65^{a}	8,12^{bc}	9,46^{b}	9.22^{b}	63.03^{b}	4.94^{c}	2.631^{b}	1.729^{b}
1TH	2,18^{g}	2.20^{f}	2.97^{e}	2.9^{5e}	18.6^{e}	5,86^{b}	0.954^{c}	0.657^{c}
TH_2	6.73^{a}	7.62^{c}	8.10^{c}	8.07^{c}	54^{c}	4 .39^{c}	2.129^{b}	1.565^{b}
TOL	1.57^{h}	2.09^{f}	2.75^{e}	2,63^{e}	18 .33^{e}	5.93^{b}	0.854^{d}	0.522^{c}
T27	6.13^{b}	6.72^{c}	8.19^{c}	7.09^{c}	54.60^{c}	4.75^{c}	2.278^{b}	1.893^{b}
T30	5.69^{c}	6.03^{c}	6.89^{c}	7.72^{c}	45.93^{c}	5.76^{b}	1.965^{c}	1.307^{b}
TY	5.36^{c}	6.12^{c}	8.23^{c}	7,12^{c}	54.86^{c}	4.54^{c}	2.457^{b}	1.842^{b}
TS-ML	4,54^{de}	6.28^{c}	7.86^{c}	7.03^{c}	52.40^{c}	5.09^{bc}	2.154^{b}	1.731^{b}
TS-H	4,06^{d}	6 ,22^{c}	8.61^{c}	7.88^{c}	57.40^{c}	4.81^{c}	2.695^{b}	2 .01^{ab}
TS-B 2000 (1)	2.52^{g}	6.93^{c}	9.87^{b}	10.84^{b}	65.80^{b}	4. 47^{c}	2.81^{b}	2 .12^{a}
TS-B 98/2002 (1)	3.23^{f}	3.46^{e}	4.15^{d}	4.17^{d}	27.66^{d}	6.11^{a}	1.2^{c}	0 .98^{c}
TS-B 98/2002 (2)	2.46^{g}	4.56^{e}	7.17^{c}	6.40^{c}	47.80^{c}	6.03^{ab}	2.105^{b}	1.831^{b}
TR-B 98/2002 (1)	4.13^{e}	4.83^{e}	6.05^{c}	6.02^{c}	40.33^{c}	5.98^{b}	1.654^{c}	1.05^{bc}
TR-B 98/2002 (2)	3.74^{e}	4 .76^{e}	10.49^{b}	9.93^{b}	69.93^{b}	4.55^{c}	3. 159^{a}	2.513^{a}
TR-OL 2	2.42^{g}	3.96^{e}	4.54^{d}	4.37^{d}	30.26^{d}	6 .17^{a}	1.206^{c}	0.940^{c}
TS-EM-98 (1)	2,3^{g}	2,67^{f}	3.12^{d}	2.85^{d}	21.20^{d}	6.12^{a}	1.06^{c}	0.45^{c}
TR-CB 2000 (2)	3.43^{f}	6.65^{c}	9.27^{bc}	8.89^{bc}	61.80^{bc}	4 .85^{c}	2.523^{b}	2.06^{ab}
TS-B-2000 (2)	4.16^{e}	6.43^{c}	10.71^{b}	10.32^{b}	71.40^{b}	4 .62^{c}	3. 387^{a}	2.95^{a}
TR-CB 2000 (1)	3.65^{ef}	8.32^{b}	10.15^{b}	9.93^{b}	67.66^{b}	4.36^{c}	3.059^{ab}	2.62^{a}
TS-EM-98 (2)	4.73^{d}	5.39^{de}	7.44^{c}	6.79^{c}	49.60^{c}	6.09^{a}	2.198^{b}	1.86b
TR-TB 2000	6.60^{a}	9.54^{b}	12.50^{a}	11.89^{a}	83.3^{a}	4.12c	4.1a	2.78a
TS-BG	3.12^{f}	5.36^{de}	6.65^{c}	6.51^{c}	44.33^{c}	5.86b	1.766c	1.15b
TR-OL 1	5.42^{c}	9.18^{b}	10.42^{b}	10.01^{b}	69.46^{b}	4 .54^{c}	3. 216^{a}	2.87^{a}
TS-RP	4.19^{e}	5.63^{d}	8.84^{c}	8.15^{c}	56.26^{c}	5.64^{b}	2.854^{b}	2.74^{a}
TR-EM 1	5.72^{c}	7.86^{c}	8.13^{c}	8.02^{c}	54.20^{c}	5.43^{b}	2.254^{b}	1 .83^{b}
TS-B 98	5.45^{c}	9.73^{b}	11.73^{a}	10.00^{a}	78.20^{a}	4.23^{c}	3.4^{a}	2 .54^{a}
TR-EM 2	6.12^{b}	10.51^{a}	11.85^{a}	10.49^{a}	79^{a}	4.36^{c}	3.4^{a}	2.43^{a}
TR-B 98 (1)	4.53^{de}	8.72^{b}	10.34^{b}	10.28^{b}	68.93^{b}	4.18^{c}	3.20^{a}	2.27^{a}
TR-B 98 (2)	4.63^{d}	6.45^{c}	8.18^{c}	7.92^{c}	54.53^{c}	5.87^{b}	2.262^{b}	1.85^{b}
TR-B 98 (3)	5.64^{c}	9.87^{b}	12.42^{a}	11.23^{a}	82.80^{a}	4.2^{c}	3.9^{a}	3.00^{a}

*Two values on the same column show no significant difference at the 5% level if they are affected by the same letter

DISCUSSION

Among the filamentous fungi that solubilize phosphate, species of the genera *Aspergillus, Penicillium* [26, 27, 28, 29] and *Trichoderma* [15] are the most cited. *Rhizoctonia solani* has also been reported as a species capable of solubilizing P [30].

Fungi of the genus *Trichoderma* are among the most commonly studied microorganisms as biological control agents and as promoters of plant growth [31, 32, 33, 17]. Several studies have been conducted to study another mechanism used by *Trichoderma* spp. which is the solubilization of phosphates. The qualitative study of the solubilization capacity of inorganic phosphate by *Trichoderma* isolates showed that all isolates showed good growth on modified NBRIP agar plates but showed no clear area around colonies. Nautiyal [20] suggested that the criterion of halo formation around colony for the selection of phosphate solubilizing microorganisms is not a reliable technique since many phosphate solubilizing (PSM) isolates do not form a single zone clear on the agar plates. Rawat and Tewari [34] and Promwee et al. [35] also reported that *Trichoderma* species showed good mycelial growth but did not form any halo zone on the solid medium containing an insoluble inorganic phosphorus source. França et al. [36] noted that it is the *Trichoderma* isolate that solubilized most tricalcic phosphate that showed very important mycelial growth and sporulation.

Other studies have reported that some bacteria, unable to produce clear areas around their colonies, can solubilize inorganic phosphates in a

liquid medium [37,38]. According to these authors, the composition of culture media has been called into question and it has become important to know the elements that are essential for the solubilization of phosphates of those who are not, but also, to determine at what concentrations of these elements, the solubilization is maximal. Nautiyal [20] reported, for example, that glucose and $Ca_3(PO4)_2$ are essential components of the culture medium, whereas yeast extract and ammonium sulphate ($(NH4)_2SO_4$) do not are not.

In this study, all *Trichoderma* isolates showed an ability to solubilize phosphate by increasing the concentration of soluble phosphorus in the culture filtrates. This increase in soluble phosphorus concentration was observed during the first 9 days followed by a progressive decrease after 12 days. This corresponds to the sequestration of phosphate by the mycelia of *Trichoderma* [15, 20, 39]. According to Borges et al. [39], the decrease in phosphate concentration observed over time may be correlated with the use of P to facilitate mycelial growth of *Trichoderma* to be available later for plant roots, located near the mycelium.

P-solubilizing fungi produce more acids than bacteria and therefore exhibit greater P solubilization activity [40]. These organic acids have the role of chelating the cationic counterpart of P ions and releasing inorganic phosphate into the medium [41,42,43]. In addition, fungi in soils are able to travel long distances more easily than bacteria and therefore may be more important for solubilization of P in soils [44].

The tested *Trichoderma* isolates in this study showed a decrease in pH during the solubilization process. Thus, the most basic pH values are noted in the best performing isolates for phosphate solubilization. This result is in agreement with the conclusions of Illmer and Schinner [45], which also noted a decrease in pH up to four days, followed by a gradual increase during solubilization of P by *Penicillium* and *Pseudomonas* in liquid cultures. According to Kpomblekou and Tabatabai [46], microorganisms that tend to decrease the pH of the liquid medium during the growth of microganisms are solubilizers of phosphates.

Several authors have associated the solubilization of phosphates with a lowering of the pH of the medium [47,48]. In liquid medium, a correlation between pH and the amount of solubilized P in liquid medium has been reported by Kucey et al. [44]. Banana [38] has also reported a correlation between lower pH and P solubilization, between acid production and titratable acidity, and between increasing titratable acidity and solubilization of P. These significant relationships highlight the important role of acids in the solubilization of phosphates.

However, a drop in pH is not the only factor to be taken into account for the solubilization of inorganic phosphates [20]. Other authors [49] have reported that there is no correlation between pH and the amount of solubilized phosphorus in a liquid medium.

CONCLUSION

In this study, thirty isolates of *Trichoderma* spp. were tested for their ability to solubilize Tricalcium phosphate on solid and liquid NBRIP medium. The qualitative estimate on solid medium showed that all the isolates studied show good growth on this medium but do not develop a halo-zone around the colonies. The qualitative estimation on liquid medium showed that all the isolates tested solubilize the Tricalcium phosphate but at different levels. The soluble phosphorus concentration gradually increases in the filtrates during the first 9 days after the addition of *Trichoderma* to the culture medium. TR-TB 2000 isolates; TS-B 98; TR-EM 2; TR-B 98 (1) proved to be the most successful in phosphate solubilization, with maximum phosphorus concentrations.

Similarly, the pH of the culture filtrates decreased during the solubilization process, the lowest pH values are recorded in isolates that showed high performance in solubilizing phosphate. A positive correlation was observed between the fresh and dry weight of the mycelium of the tested isolates and the solubilization level of the tricalcic phosphate, the weight of the mycelium is higher in the best performing isolates for phosphate solubilization.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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