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# Quantitative and Qualitative Estimation of Moroccan *Trichoderma* Isolates Capacity to Solubilize Rock Phosphate

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Thirty *Trichoderma* isolates isolated from compost, various crops and soil with roots of adjacent sites to the phosphate mines of Morocco, were tested *in vitro* for their potential to solubilize phosphorus from phosphate rock. The qualitative assessment of phosphate solubilization by *Trichoderma* isolates was performed on Modified Pikovskaya Agar (MPA) solid medium. The visual observation of the 3 and 6 -days old cultures didn't show any clear zone around the colony. However, all the isolates were able to grow on the culture medium 3 days after incubation, the maximum recorded diameter was 58.6 mm for isolate TR-B 98 (3) and the minimum value was 34.8 mm for isolate TS-EM-98 (2). After 6 days, they showed good radial growth that exceeded 79.8 mm with variable appearance of the mycelial density such as the isolates TS-B 98, TS-EM-98 (1) and TR-CB 2000 (1) that presented respectively high, regular and low mycelial density. Also, the *Trichoderma* isolates produced variable number of conidia on MPA medium. Quantitative estimation on the Modified Pikovskaya Broth (MPB) liquid medium showed a variable potential of the *Trichoderma* isolates to solubilize phosphate when the amount of soluble phosphorus remained low in the liquid medium without the fungus (0.26 $mgL^{-1}$). The maximum concentration of soluble phosphorus was 11.92 $mgL^{-1}$ with percentage of soluble phosphorus equal to 95.39% recorded by the isolate TR-TB 2000 after 9 days of incubation, followed by the isolates TR-B 98 (3), TS-B 98 and TR-EM 2 respectively 11.20, 10.47 and 9.61 $mgL^{-1}$ and 89.6, 83.76 and 76.38%. In addition, treatments with *Trichoderma* isolates provided a lower final broth pH which varied between 6.81 for TOL isolate and 3.40 for TS-B-2000 (2) compared to initial pH (7.2). The isolates that proved potent for phosphate solubilization displayed the highest fresh and dry weights such as TR-TB 2000 (FW = 4.11 g and DW = 2.56 g), while the lowest fresh and dry weight were noted in the weakest isolates for phosphate solubilization such as T27 (FW = 1.025 g and DW = 0.58 g).

The high solubilization potential of *Trichoderma* isolates can be exploited for the solubilization of fixed phosphorus present in the soil, thus improving soil fertility and plant growth.

**Keywords:** *Trichoderma* isolates, *in vitro* solubilization, phosphorus; phosphate rock.

Phosphorus (P) is the second most limiting macroelement to plant growth after nitrogen (Wang et al., 2009; Balemi and Negisho, 2012), representing about 0.2% of the dry matter (Schachtman et al., 1998). It is an essential constituent of phospholipids, adenosine triphosphate (ATP) and nucleic acids (Schachtman et al., 1998), and intervenes in

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various key metabolic processes such as division and cell development, energy transport, transduction, macromolecular biosynthesis, photosynthesis, plant respiration and a large number of signaling processes via protein phosphorylation and dephosphorylation (Shenoy and Kalagudi, 2005; Ahemad et al., 2009; Khan et al., 2009).

Unlike other elements, phosphorus is found only in soil with a soluble P concentration ranging from 0.05 to 10 ppm. But most of the phosphorus in the soil is insoluble (Fernández et al., 2007), more than 80% of the P becomes unavailable and can not be absorbed by plants because of its attachment to other elements such as calcium to give $Ca_3 (PO_4)_2$ in neutral or alkaline soils, iron and aluminum to give $FePO_4$ and $AlPO_4$ in acidic soils (Altomare et al., 1999) and precipitation or its conversion into organic forms (Holford, 1997).

Solubilization of insoluble phosphorus can be achieved by root phosphatases, but microorganisms also play a significant role, with organic acids or chelates they excrete (Davet, 1996). Many studies have reported that there is a high proportion of phosphate solubilizing microorganisms (PSMs), including bacteria, fungi and Actinomycetes, which live in the plant rhizosphere and play an important role in phosphate solubilization, converting phosphate into soluble compounds for plants (Sujatha et al., 2004; Gravel et al., 2007; Lang et al., 2016).

Fungi of the genus *Trichoderma* are among the most frequently studied microorganisms as biological control agents and promoters of plant growth (de Terogoff and Ricard, 1976; Kelley, 1976; Gindrat et al., 1977; Davet, 1979; Rishbeth, 1979; Dumitras and Fratilescu-Sesan, 1980; Elad et al., 1981; Kapgate and Rane, 2016; Yedidia et al., 1999; Harman, 2000; Harman, 2006; Gravel et al., 2007; Vinalea et al., 2008; Achá, 2008; Santos et al., 2010; Oliveira et al., 2012). Root colonization by the genus *Trichoderma* frequently increases root growth, plant development, nutrient uptake, abiotic stress resistance, and consequently productivity (Harman et al., 2004).

Soil microorganisms have been reported to alter soil pH and the balance of many chemical and biochemical reactions (de Santiago et al., 2013). Fungi, and probably all living organisms, synthesize a number of acidic and alkaline phosphatases in the soil, each of which activates in a distinct pH range. These are secreted in response to the signals of the absence of P (Peleg et al., 1996).

Studies have demonstrated the ability of *Trichoderma* species to promote the growth of several types of plants and protect them against plant pathogens (Kapri and Tewari, 2010, Santos et al., 2010, Machado et al., 2011, Hannan et al., 2013) and to improve the bioavailability of phosphorus by reducing the need for inorganic phosphates. This capacity is favored by the production of phytases and certain organic acids such as citric acid, lactic acid, and succinic acid by different strains of *Trichoderma* spp. (Promwee et al., 2014).

The present study was undertaken to estimate *in vitro* the capacity of 30 *Trichoderma* isolates to solubilize the crude phosphate rock originating from the phosphate mines of Khouribga (Morocco).

# Materials and Methods

*Fungal material*

Seven *Trichoderma* isolates belonging to the Botanical Laboratory, Biotechnology and Plant Protection Laboratory (LBBPP) (originating from compost and different crops, two isolates of *Trichoderma asperellum* were registered in NCBI database) and twenty-three isolates newly isolated from sites adjacent to the phosphate mines of Morocco (Table 1) were grown on PSA (Potato sucrose Agar) medium (potato 200 g, Sucrose 20 g, Agar-agar: 15 g, distilled water 1000 ml) and incubated at 28 ± 1 °C in the dark.

*Inorganic phosphate*

Crude Phosphate rock (BPL* = 68 (*bone phosphate of lime with 31,12% $P_2O_5$ content)) from the phosphate mines of Khouribga (Morocco) was ground in a porcelain mortar and washed 3 times with tap water to remove soluble phosphorus).

*Study of the capacity of Trichoderma isolates to solubilize phosphate*

*Qualitative estimation*

The ability of *Trichoderma* isolates to solubilize inorganic phosphate was tested on the MPA medium (Modified Pikovskaya Agar): Phosphate rock powder: 2.5 g; Glucose: 13 g; $(NH_4)$ $SO_4$: 0.5 g; NaCl: 0.2 g; $MgSO_4$, $7H_2O$: 0.1 g; KCl: 0.2 g; Yeast extract: 0.5 g; $MnSO_4$ : 0.0002 g; $FeSO_4$,$7H_2O$; 0.0002 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 (Pikovskaya, 1948).

A 5-mm mycelial disk from the 7-day old culture of each *Trichoderma* isolate was placed in the center of the agar plate and incubated at 28 °C. After 3 and 6 days of incubation, the colony and the halo zone diameters were measured by a double decimeter. The phosphate solubilization index (PSI) was calculated according to the following formula (Alam et al., 2002; Afzal and Asghari, 2008):

$$\text{PSI} = \frac{\text{The colony diameter} + \text{The halo-zone diameter}}{\text{The colony diameter}}$$

To determine the number of the conidia produced, three 5-mm discs were taken from the 7 days old cultures on MPA for each *Trichoderma* isolates, put in a test tube containing 1 ml of sterile distilled water and shaken for 5 min using an orbital shaker at 30 rpm. The conidia concentration was measured using a Malassez slide.

The density of mycelia was identified by visual observation after 7 days when *Trichoderma* isolates complete colonization of the Petri dishes of MPA using a scale of Sobal et al. (2007): (High density: + + + ; Regular density: + + ; Low density: + ).

*Quantitative estimation*

*Trichoderma* isolates were tested for their ability to solubilize inorganic phosphate in Modified Pikovskaya Broth (MPB): Phosphate rock powder: 2.5 g; glucose: 13 g; $(NH_4)$ $2SO_4$: 0.5 g; NaCl: 0.2 g; $MgSO_4$, $7H_2O$: 0.1 g; KCl: 0.2 g; Yeast extract: 0.5 g; $MnSO_4$: 0.0002 g; $FeSO_4$, $7H_2O$: 0.0002 g; the pH was adjusted to 7.2 and the components were dissolved in 1000 mL of distilled water (Pikovskaya, 1948).

Five 5-mm mycelial disks from each *Trichoderma* isolate were inoculated into a 250 mL Erlenmeyer flask containing 100 mL broth and incubated at 28 °C in a shaker

**Table 1**

Origin and sources of isolation of *Trichoderma* isolates tested

| *Trichoderma* isolates | 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 / My Bouselham / Morrocco |
| **T 30** | Strawberry agriculture, Sabrina variety | Gnafda / My Bouselham / Morocco |
| **TOL** | roots of an olive tree | Sidi Kacem/ Morocco |
| **TY** | rhizosphere of the roots of an olive tree | Sidi Kacem/ 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 | |

(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 spores and mycelium of *Trichoderma* isolates.

The pH of each culture was measured using a pH meter. The phosphorus concentration in the supernatant was estimated spectrophotometrically (Fiske and Subbarow, 1925; Saravanakumar et al., 2013). 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% (w / v), sulfuric acid solution ($H_2SO_4$) 5.5% (v / v) and 2.7% (w / v) ferrous sulphate solution ($FeSO_4$) and then measured by a UV-visible spectrophotometer at 600 nm. The level of phosphorus concentration was determined using the standard potassium dihydrogen phosphate curve ($KH_2PO_4$) and expressed as equivalent phosphorus in mg-$P.L^{-1}$.

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

$$\%\ \text{of soluble P} = \frac{\text{Soluble phosphorus concentration in filtrate} \times 100}{\text{Initial phosphate concentration}}$$

*Measurement of 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 through 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 calculated using a precision weighting scale using the following formula:

$$\text{Dry weight} = (\text{weight of filter paper} + \text{mycelium}) - (\text{weight of filter paper})$$

*Statistical analysis*

All the experiments were performed in triplicate for each isolate and the Blanc. Statistical data processing included analysis of variance using ANOVA I and LSD test at 5% level.

# Results

*Study of the capacity of* Trichoderma *isolates to solubilize phosphate*

*Qualitative estimation*

After 3 days of incubation at 28 °C, all the *Trichoderma* isolates were able to grow on MPA solid medium but no clear zone around the mycelial colony was observed to estimate Phosphate solubilization (Fig. 1). The cultures were also monitored after 6 days

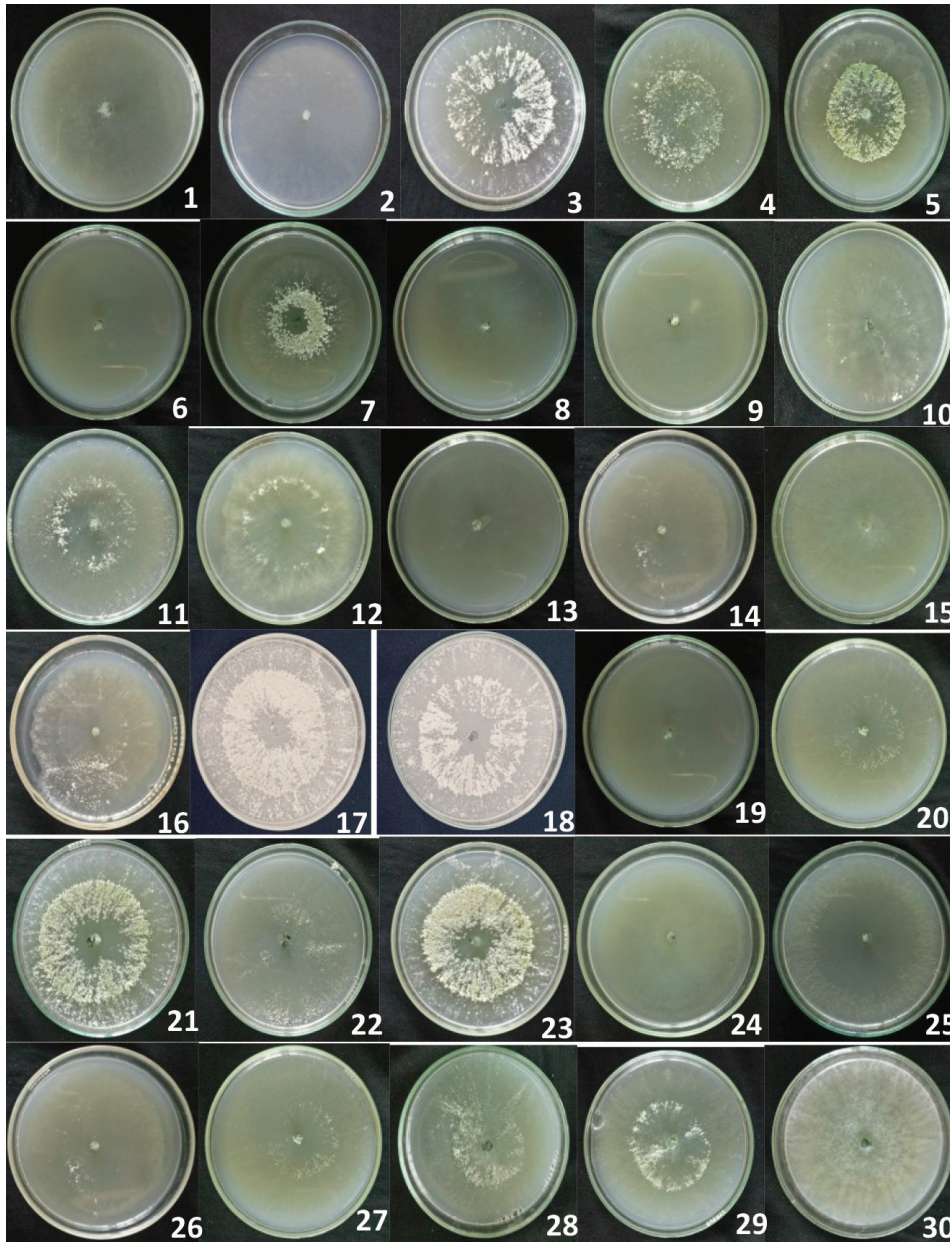


Fig.1: Absence of the halo-zone around the culture
of *Trichoderma* isolates on modified Pikovskaya agar medium (MPA)

**1** : TR-EM 2 ; **2** : TR-CB 2000 (1) ; **3** : T27 ; **4** :TR-B- 98 (2); **5** : TH2 ; **6** : TS-B 98/2002 (1); **7** : TR OL (1) ; **8** : TY ; **9** : Tol ; **10** : TR-TB 2000 ; **11** : TS-H ; **12** :TS-BG ; **13** : T30 ; **14** : TR-EM 1; **15** : TR-B 98 (1) ; **16** : T1 ; **17** : TR-B 98/2002 (2) ; **18** : TROL 2 ; **19** : TR-CB 2000 (2) ; **20** : TS-B 2000 (2) ; **21** : TR-B 98 (3); **22** : TS-EM 98 (1) ; **23** : TS-B 98 ; **24** :TS-B 2000 (1); **25** : 1TH ; **26** : TS-RP; **27** : TS-B 98/2002 (2) ; **28** :TS-ML; **29**: TS-EM 98 (2) ; **30** : TR-B 98/2002 (1).

without the observation of any halo - zone, so the phosphate solubilization index was not calculated.

The isolates TR-B 98 (3); TS-B-2000 (2); T30; TR-B 98/2002 (1); 1TH; TR-B 98/2002 (2); TR-OL 2; TR-EM 1; TR-TB 2000; TS-BG; TR-EM 2; TS-B 2000 (1) showed a radial colonies that diameters varied between 51.0 and 58.6 mm on 3 -days old cultures, followed by T1; $TH_2$ ; TOL; TY; TS-ML; TS-H; TS-B 98/2002 (2); TR-CB 2000 (2); TR-CB 2000 (1) ; TS-RP ; TR-B 98 (1); TR-B 98 (2) with colony diameters

**Table 2**

Growth and conidia production of *Trichoderma* isolates in the modified Pikovskaya agar (MPA) supplemented with rock phosphate (RP)

| Isolates | Colony diameter (mm) | | Mycelium Density | Conidia production (Conidia/mm$^2$) |
|---|---|---|---|---|
| | After 3 days | After 6 days | | |
| **T1** | 43.3$^{d}$ | 85$^{b}$ | + + | 8492.55$^{e}$ |
| **1TH** | 54.1$^{b}$ | 90$^{a}$ | + | 0$^{i}$ |
| **$TH_2$** | 47.5$^{d}$ | 90$^{a}$ | + + + | 8492.55$^{e}$ |
| **TOL** | 42.5$^{de}$ | 80$^{bc}$ | + | 0$^{i}$ |
| **T27** | 50.5$^{c}$ | 90$^{a}$ | + + + | 10191.06$^{d}$ |
| **T30** | 57.8$^{a}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TY** | 46$^{d}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TS-ML** | 43.3$^{d}$ | 90$^{a}$ | + + | 8492.55$^{e}$ |
| **TS-H** | 45.3$^{d}$ | 90$^{a}$ | + + + | 8492.55$^{e}$ |
| **TS-B 2000 (1)** | 51.0$^{c}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TS-B 98/2002 (1)** | 54$^{b}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TS-B 98/2002 (2)** | 45.6$^{b}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TR-B 98/2002 (1)** | 55.1$^{b}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TR-B 98/2002 (2)** | 54.1$^{b}$ | 90$^{a}$ | + + + | 44161.26$^{b}$ |
| **TR-OL 2** | 54.0$^{b}$ | 90$^{a}$ | + + + | 33970.2$^{c}$ |
| **TS-EM-98 (1)** | 39.8$^{e}$ | 90$^{a}$ | + + | 8492.55$^{e}$ |
| **TR-CB 2000 (2)** | 46.6$^{d}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TS-B-2000 (2)** | 58.5$^{a}$ | 90$^{a}$ | + + | 10191.06$^{d}$ |
| **TR-CB 2000 (1)** | 41.1$^{e}$ | 83.3$^{b}$ | + | 8492.55$^{e}$ |
| **TS-EM-98 (2)** | 34.8$^{e}$ | 90$^{a}$ | + + | 3397.02$^{g}$ |
| **TR-TB 2000** | 51.8$^{c}$ | 90$^{a}$ | + + | 3397.02$^{g}$ |
| **TS-BG** | 51.6$^{c}$ | 90$^{a}$ | + + + | 5095.35$^{f}$ |
| **TR-OL 1** | 39.5$^{e}$ | 85$^{b}$ | + + + | 8492.55$^{e}$ |
| **TS-RP** | 40.6$^{e}$ | 79.8$^{c}$ | + | 1698.51$^{h}$ |
| **TR-EM 1** | 53.3$^{c}$ | 90$^{a}$ | + + | 3397.02$^{g}$ |
| **TS-B 98** | 57.3$^{a}$ | 90$^{a}$ | + + + | 59447.85$^{a}$ |
| **TR-EM 2** | 51.3$^{c}$ | 90$^{a}$ | + | 0$^{i}$ |
| **TR-B 98 (1)** | 45.0$^{d}$ | 90$^{a}$ | + + | 0$^{i}$ |
| **TR-B 98 (2)** | 46.3$^{d}$ | 90$^{a}$ | + + + | 13588.08$^{c}$ |
| **TR-B 98 (3)** | 58.6$^{a}$ | 86.6$^{b}$ | + + + | 59447.85$^{a}$ |

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

**Table 3**

Phosphorus concentrations solubilized by *Trichoderma* isolates in the modified Pikovskaya broth (MPB) supplemented with rock phosphate (RP), the pH of the broth, the fresh and dry weight of the mycelium

| Isolates | The concentration of phosphorus ($mgL^{-1}$) | | | | Percentage of soluble P (%) | Final pH | Fresh weight of mycelium (g) | Dry weight of mycelium (g) |
|---|---|---|---|---|---|---|---|---|
| | 3 days | 6 days | 9 days | 12 days | | | | |
| Blank | $0.22^{f}$ | $0.26^{g}$ | $0.26^{g}$ | $0.28^{h}$ | $0^{J}$ | $7.22^{a}$ | _____ | _____ |
| **T1** | $0.77^{ef*}_{b*}$ | $1.37^{f}_{b}$ | $1.96^{e}_{a}$ | $2.46^{g}_{a}$ | $15.68^{e}$ | $6.44^{b}$ | $1.66^{d}$ | $0.752^{c}$ |
| **1TH** | $0.44^{f}_{c}$ | $1.20^{f}_{b}$ | $1.97^{e}_{a}$ | $2.15^{g}_{a}$ | $15.76^{e}$ | $6.24^{c}$ | $1.69^{d}$ | $0.78^{c}$ |
| **$TH_2$** | $0.95^{e}_{c}$ | $2.62^{e}_{b}$ | $8.01^{b}_{a}$ | $7.81^{c}_{a}$ | $64.08^{b}$ | $4.91^{g}$ | $3.22^{b}$ | $1.38^{b}$ |
| **TOL** | $0.37^{f}_{b}$ | $1.59^{f}_{a}$ | $0.99^{f}_{a}$ | $1.19^{gh}_{a}$ | $7.92^{f}$ | $6.81^{a}$ | $1.57^{d}$ | $0.68^{c}$ |
| **T27** | $0.41^{f}_{a}$ | $0.70^{g}_{a}$ | $0.67^{f}_{a}$ | $0.60^{h}_{a}$ | $5.36^{f}$ | $6.53^{b}$ | $1.025^{d}$ | $0.58^{c}$ |
| **T30** | $3.96^{c}_{b}$ | $4.30^{e}_{ab}$ | $4.92^{d}_{a}$ | $4.67^{e}_{a}$ | $39.36^{d}$ | $6.10^{c}$ | $2.73^{c}$ | $1.08^{bc}$ |
| **TY** | $4.76^{b}_{ab}$ | $4.\ 36^{e}_{b}$ | $5.15^{d}_{a}$ | $4.26^{e}_{b}$ | $41.2^{d}$ | $4.90^{g}$ | $2.84^{bc}$ | $1.15^{b}$ |
| **TS-ML** | $0.63^{f}_{c}$ | $0.78^{g}_{b}$ | $0.98^{f}_{a}$ | $1.23^{gh}_{a}$ | $7.84^{f}$ | $6.77^{a}$ | $1.56^{d}$ | $0.64^{c}$ |
| **TS-H** | $0.54^{f}_{c}$ | $2.30^{e}_{b}$ | $7.06^{c}_{a}$ | $7.79^{c}_{a}$ | $56.48^{c}$ | $5.47^{e}$ | $3.1^{b}$ | $1.27^{b}$ |
| **TS-B 2000 (1)** | $1.15^{e}_{b}$ | $1.09^{f}_{a}$ | $1.25^{ef}_{a}$ | $1.15^{gh}_{a}$ | $10^{ef}$ | $6.49^{b}$ | $1.62^{d}$ | $0.71^{c}$ |
| **TS-B 98/2002 (1)** | $0.91^{e}_{c}$ | $2.47^{e}_{b}$ | $7.88^{b}_{a}$ | $7.33^{c}_{a}$ | $63.04^{b}$ | $4.51^{h}$ | $3.15^{b}$ | $1.3^{b}$ |
| **TS-B 98/2002 (2)** | $0.86^{e}_{b}$ | $1.14^{f}_{b}$ | $5.70^{d}_{a}$ | $5.39^{e}_{a}$ | $45.6^{d}$ | $5.64^{de}$ | $2.98^{bc}$ | $1.2^{b}$ |
| **TR-B 98/2002 (1)** | $0.40^{f}_{b}$ | $0.58^{g}_{b}$ | $2.60^{e}_{a}$ | $0.85^{h}_{b}$ | $20.8^{e}$ | $5.73^{d}$ | $1.86^{d}$ | $0.88^{c}$ |
| **TR-B 98/2002 (2)** | $0.40^{f}_{c}$ | $0.67^{g}_{bc}$ | $0.99^{f}_{b}$ | $2.08^{g}_{a}$ | $7.92^{f}$ | $6.06^{c}$ | $1.58^{d}$ | $0.67^{c}$ |
| **TR-OL 2** | $0.50^{f}_{c}$ | $1.31^{f}_{b}$ | $3.13^{de}_{a}$ | $2.97f^{g}_{a}$ | $25.04^{de}$ | $5.52^{e}$ | $1.86^{d}$ | $0.884^{c}$ |
| **TS-EM-98 (1)** | $0.27^{f}_{b}$ | $5.49^{d}_{a}$ | $6.45^{cd}_{a}$ | $6.39^{d}_{a}$ | $51.6^{cd}$ | $5.01^{fg}$ | $3.08^{b}$ | $1.221^{b}$ |
| **TR-CB 2000 (2)** | $4.04^{c}_{b}$ | $4.99^{d}_{b}$ | $7.89^{b}_{a}$ | $7.11^{cd}_{a}$ | $63.84^{b}$ | $4.16^{i}$ | $3.22^{b}$ | $1.32^{b}$ |
| **TS-B-2000 (2)** | $2.66^{d}_{b}$ | $2.77^{e}_{b}$ | $8.01^{b}_{a}$ | $6.95^{d}_{a}$ | $64.08^{b}$ | $3.40^{k}$ | $3.26^{b}$ | $1.44^{b}$ |
| **TR-CB 2000 (1)** | $0.87^{e}_{c}$ | $7.11^{c}_{b}$ | $8.63^{a}_{a}$ | $6.91^{d}_{b}$ | $69.04^{a}$ | $4.1^{ij}$ | $3.42^{b}$ | $1.53^{b}$ |
| **TS-EM-98 (2)** | $2.24^{d}_{c}$ | $3.13^{e}_{bc}$ | $5.11^{d}_{a}$ | $4.30^{e}_{ab}$ | $40.88^{d}$ | $4.27^{i}$ | $2.76^{c}$ | $1.107^{b}$ |
| **TR-TB 2000** | $3.22^{d}_{c}$ | $8.97^{a}_{b}$ | $11.92^{a}_{a}$ | $11.76^{a}_{a}$ | $95.39^{a}$ | $4.18^{i}$ | $4.11^{a}$ | $2.56^{a}$ |
| **TS-BG** | $2.73^{d}_{b}$ | $7.79^{b}_{a}$ | $7.88^{b}_{a}$ | $6.49^{d}_{a}$ | $63.04^{b}$ | $5.12^{f}$ | $3.19^{b}$ | $1.31^{b}$ |
| **TR-OL 1** | $4.56^{b}_{b}$ | $8.85^{a}_{a}$ | $8.62^{a}_{a}$ | $6.95^{d}_{a}$ | $68.96^{a}$ | $5.13^{f}$ | $3.41^{b}$ | $1.51^{b}$ |
| **TS-RP** | $5.00^{b}_{a}$ | $2.96^{e}_{b}$ | $4.91^{d}_{a}$ | $3.46^{f}_{b}$ | $39.28^{d}$ | $6.14^{e}$ | $2.73^{c}$ | $1.06^{bc}$ |

**Table 3 cont.**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **TR-EM 1** | $4.94^{b}{}_{b}$ | $7.55^{b}{}_{a}$ | $7.06^{c}{}_{a}$ | $5.37^{e}{}_{b}$ | $56.48^{c}$ | $5.10^{f}$ | $3.13^{b}$ | $1.28^{b}$ |
| **TS-B 98** | $4.28^{b}{}_{c}$ | $6.68^{c}{}_{b}$ | $10.47^{a}{}_{a}$ | $7.18^{c}{}_{b}$ | $83.76^{a}$ | $4.09^{ij}$ | $3.66^{b}$ | $1.63^{b}$ |
| **TR-EM 2** | $4.77^{b}{}_{b}$ | $6.62^{c}{}_{a}$ | $9.61^{a}{}_{a}$ | $8.54^{b}{}_{a}$ | $76.88^{a}$ | $3.97^{j}$ | $3.42^{b}$ | $1.51^{b}$ |
| **TR-B 98 (1)** | $2.71^{d}{}_{b}$ | $5.29^{d}{}_{ab}$ | $8.56^{ab}{}_{a}$ | $8.54^{b}{}_{a}$ | $68.48^{ab}$ | $4.16^{i}$ | $3.4^{b}$ | $1.49^{b}$ |
| **TR-B 98 (2)** | $6.02^{a}{}_{c}$ | $8.18^{ab}{}_{a}$ | $7.89^{b}{}_{ab}$ | $6.80^{d}{}_{bc}$ | $63.12^{b}$ | $5.13^{f}$ | $3.2^{b}$ | $1.36^{b}$ |
| **TR-B 98 (3)** | $2.46^{d}{}_{b}$ | $8.22^{a}{}_{a}$ | $11.20^{a}{}_{a}$ | $8.52^{b}{}_{a}$ | $89.6^{a}$ | $3.46^{k}$ | $3.89^{ab}$ | $1.68^{b}$ |

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

ranging from 46.3 to 43.3 mm and the isolates TS-EM-98 (1); TS-EM-98 (2); TR-OL1 with radial growth ranging from 34.8 to 39.5 mm. After 6 days, the *Trichoderma* isolates continued to grow on MPA medium reaching 90 mm except isolates T1; TR-CB 2000 (1); TR-OL 1; TS-RP; TR-B 98 (3) that colony diameters varied between 79.8 mm and 86.6 mm (Table 2).

The mycelial density of *Trichoderma* isolates was variable on the MPA solid medium after 7 days of incubation. 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 (3); TR-B 98 (2) displayed high mycelial density, followed by T1 ; TS-ML ; TS-EM-98 (1) ; TS-B-2000 (2) ; TS-EM-98 (2) ; TR-TB 2000 ; TR-EM 1 ; TR-B 98 (1), showing a regular mycelial density. However, the isolates 1TH ; TOL ;T30 ; TY ; TS-B 2000 (1) ; TS-B 98/2002 (1) ; TS-B 98/2002 (2); TR-B 98/2002 (1) ; TR-CB 2000 (2) ; TR-CB 2000 (1) ; TS-RP ; TR-EM 2 exhibited low mycelial density (Table 2).

The conidia production by the *Trichoderma* isolates was estimated on the 7-days old cultures. The isolates TR-B 98 (2), TR-OL 2, TR-B 98/2002 (2), TS-B 98, TR-B 98 (3) produced an important number of conidia with values ranging from 13588.08 to 59447.85 conidia.mm$^{-2}$, succeeded by the isolates T1, $TH_2$, T2, TS-ML, TS-H, TS-EM-98 (1), TS-B-2000 (2), TR-CB 2000 (1), TS-EM-98 (2), TR-TB 2000, TS-BG, TR-OL 1, TS-RP, TR-EM 1, with a conidia number varying between 3397.02 and 10191.06 conidia. mm$^{-2}$. Finally, the other isolates were unable to produce conidia (Table 2).

*Quantitative estimation*

A gradual increase with time of the soluble phosphorus concentrations was observed in culture filtrates of the 30 *Trichoderma* isolates tested after 3, 6 and 9 days of incubation but decreased slightly the 12$^{th}$ day when the amount of soluble phosphorus remained low in the liquid medium without the fungus (Table 3).

Three days after inoculation, the *Trichoderma* isolates TR-B98 (2) and TS-RP gave a best soluble phosphate concentrations respectively 6.02 and 5 mgL$^{-1}$ compared to the blank (0,22 mgL$^{-1}$), followed by TR-EM (1), TR-EM 2, TY, TR-OL 1, TS-B 98 and TR-CB 2000 (2), with phosphorus concentrations ranging from 4.04 to 4.94 mg.L$^{-1}$. T30 (3.96 mgL$^{-1}$) and TR-TB 2000 (3.22 mgL$^{-1}$). The isolates TS-BG, TR-B 98 (1), TS-B 2000 (2), TR -B 98 (3) and TS-EM 98 (2) showed moderate phosphorus concentrations ranging from 2.24 to 2.73 mg.L$^{-1}$ when the other isolates not exceeding 1.15 mgL$^{-1}$.

After the 6$^{th}$ day of incubation, the isolates TR-TB 2000; TR-OL (1); TR-B 98 (3); TR-B 98 (2); TR-CB 2000 (1); TR-EM (1); TS-BG revealed a very good ability to solubilize rock phosphate by increasing soluble phosphorus concentrations in culture filtrates varying from 7.11 to 8.97 mgL$^{-1}$. The blank's concentration remained low, equal to 0.26 mgL$^{-1}$.

All *Trichoderma* isolates continued to solubilize rock phosphate even after 9 days of incubation. The isolates that performed best were TH2; TS-H;TS-B 98/2002 (1); TR-CB 2000 (2); TS-B 2000 (2); TR-CB 2000 (1); TR-TB 2000; TS-BG; TR-OL1; TR-EM (1), TS-B 98; TR-EM2; TR-B98 (1); TR-B98 (2); TR-B98 (3), with concentrations exceeding 7 mgL$^{-1}$. But an insignificant decrease in phosphorus concentrations was noted after 12 days of incubation.

The concentrations of soluble phosphorus in the liquid medium without *Trichoderma* isolates remained low after 9 and 12 days incubation not exceeding 0.28 mgL$^{-1}$.

The *Trichoderma* isolates exhibited also variable soluble phosphorus percentages in 9 –days culture filtrates. The isolates TR-TB 2000, TR-B 98 (3), TS-B 98 and TR-EM 2 isolates showed high soluble phosphorus percentages equal to 95.39, 89.6, 83.76 and 76.88%, followed by TH2, TS-H, TS-B 98/2002 (1), TS-EM-98 (1), TR-CB 2000 (2), TS-B-2000 (2), TR-CB 2000 ( 1), TS-BG, TR-OL 1, TR-EM 1, TR-B 98 (1), TR-B 98 (2) with soluble phosphorus percentages ranging from 51.6 to 69.04%. Then the T30, TY, TS-B 98/2002 (2), TR-B 98/2002 (1), TR-OL 2, TS-EM-98 (2), TS-RP isolates showed varying percentages between 20.8 and 45.6%. Finally the 1TH, TH2, TOL, T27, TS-ML, TS-B 2000 (1), TR-B 98/2002 (2) isolates proved to be very weak for the solubilization of rock phosphate with percentages less than 15.76%.

Treatments with *Trichoderma* isolates provided a lower final pH compared to the initial pH of the broths (7.2). The culture filtrates of the *Trichoderma* isolates TS-B 2000 (2), TR-EM (2) and TR-B98 (3) displayed a lower final pH less than 4, followed by TH2, TS-B 98/2002 (1), TY, TR-CB 2000 (2), TR-CB 2000 (1), TR-TB 2000, TS-B 98, the pH was under 5. The other isolates gave a final pH of 5.01 and 6.81 (Table 3).

The fresh and dry weights of the mycelium were estimated after the 12th day of incubation. The highest fresh and dry weights were noted in TR-TB 2000 isolate (FW = 4.11 g and DW = 2.56 g) followed by the isolates $TH_2$ ;TS-H ;TS-B 98/2002 (1);TS-EM-98 (1) ;TR-CB 2000 (2);TS-B-2000 (2);TR-CB 2000 (1);TS-BG; TR-OL 1;TR-EM 1;TS-B 98;TR-EM 2 having fresh weights ranging from 3.89 to 3.08 g and dry weights ranging from 1.68 to 1.221 g. While other isolates have fresh weights ranging from 1.025 to 2.98 g and dry weights ranging from 0.58 to 1.2 g (Table 3).

## Discussion

The *Trichoderma* isolates isolated from compost, various crops and soil with roots of adjacent sites to the phosphate mines of Morocco, were tested *in vitro* for their potential to solubilize phosphorus from phosphate rock in liquid and solid Pikovskaya medium. Thus, the qualitative estimate of the ability to solubilize phosphate by *Trichoderma* isolates revealed that all isolates didn't show any clear zone around colonies on the agar plate but gave good mycelial growth with variable mycelial density and conidia production. Recently, many studies have been carried out on the microbial solubilization of phosphates as a substitute for chemical fertilizers. Indeed, most studies use agar plate screening as the initial strategy for selecting phosphate-solubilizing microorganisms based on the formation of a halo-zone around colonies. According to Nautiyal (1999), the criterion for the isolation of phosphate-solubilizing microorganisms based on the formation of a visible halo-zone on the Pikovskaya Agar medium is not a reliable technique because many isolates of microorganisms solubilize the phosphate (PSM) showing no clear area on agar plates, may be able to solubilize insoluble inorganic phosphates in a liquid medium. In addition, the studies of Rawat and Tewari (2011) and Promwee et al. (2014) reported that *Trichoderma* species showed good mycelial growth, but no formation of a halo-zone on the solid medium containing an insoluble inorganic phosphorus source. In contrast, the Zeroual et al. (2012) study has revealed that *Aspergillus niger* was able to form the halo-zone on the agar plate.

Thus, the appearance of a clear zone on a solid medium should not be the only method to be tested for phosphate solubilization, additional tests should be undertaken simultaneously to evaluate the solubilization of phosphate in liquid medium. This may be because of the varying diffusion rates of different organic acids secreted by an organism (Johnston, 1952).

Quantitative estimation has shown that all the isolates used in this study have a capacity to solubilize phosphate in the liquid medium but at different concentrations during the 9 days. An increase in the concentration of soluble phosphorus in the culture filtrates was observed. This seems to correspond to phosphate sequestration by *Trichoderma* mycelium (Altomare et al., 1999; Nautiyal, 1999).

Kapri and Tewari (2010) suggested the disappearance of TCP after 96 h of incubation in some isolates of *Trichoderma harzianum* as demonstrated by a 100% solubilization percentage. This indicates the high potential of *Trichoderma harzianum* isolates for the solubilization of inorganic bound phosphate (TCP). In addition, in natural habitats, some phytopathogenic fungi such as *Pythium* and *Rhizoctonia* have been shown to be incapable of solubilizing phosphates and can be easily suppressed by the high competitive efficiency of *T. harzianum* by P absorption (Altomare et al., 1999). While other fungi, case of *Aspergillus niger*, have shown a high performance to solubilize different sources of phosphate (Zeroual et al., 2012). The solubilization of phosphates by *Trichoderma* species has been reported in several studies (Akintokun et al., 2007, Saravanakumar et al., 2013). Also, Silva et al. (2002), Alam et al. (2002), Soushie et al. (2007) worked on the study of fungal phosphate solubilization capacity in different solid and liquid culture media.

But the soluble phosphorus concentration in the broth began to decrease after the 12$^{th}$ day of incubation. Kapri and Tewari (2010) who correlated the decrease of soluble phosphorus in culture broths with its sequestration in *Trichoderma* mycelium to be released in readily available form near the roots after mycelium lysis with age suggest the same results.

The addition of *Trichoderma* isolates to the MPB broth resulted in a lowering of the pH of the broths. Thus, Illmer and Schinner (1992) also reported that *Penicillium* and *Pseudomonas* have a tendency to decrease pH four days after culturing followed by a gradual increase during solubilization in liquid cultures. This appears to be consistent with phosphorus sequestration by *Trichoderma* mycelium (Altomare et al., 1999; Nautiyal, 1999). Kpomblekou and Tabatabai (1994) also show that microorganisms that tend to lower the pH of the medium during growth are effective solubilizers of phosphate. Similarly, the drop in pH in broth cultures has been reported in several studies that support lowering pH in this study (Vazquez et al., 2000, Alam et al., 2002, Rashid et al., 2004, Pradham and Sukla, 2005; Akintokun et al., 2007, Yadav et al., 2011, Saravanakumar et al., 2013, Promwee et al., 2014).

## Conclusion

In addition to its ability in biological control against plant pathogens and the promotion of plant growth. *Trichoderma* spp. has also succeeded in showing its potential in the solubilization of phosphate rock.

All Trichoderma isolates didn't formed clear zone around the colonies during qualitative assessment and the most newly isolated fungi showed high solubilization percentages up to 95.39% in TR-TB 2000 isolate during quantitative estimation of phosphate solubilization.

There is a negative correlation between the pH level and the percentage of phosphate solubilization. The lowest pH values are found in *Trichoderma* isolates which gave the highest solubilization percentages.

Isolates with high performance in solubilizing rock phosphate showed higher fresh and dry mycelium weights.

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