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Solubilization of Hardly Soluble Phosphates and Growth Promotion of Maize (*Zea mays* L.) by *Penicillium oxalicum* Isolated from Rhizosphere

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Abstract *Penicillium oxalicum* strain CBPS-3F-Tsa, an efficient phosphate solubilizing fungus, was evaluated for its production of organic acid *in vitro* and effect of inoculation on the growth promotion of Maize under greenhouse conditions. The fungus solubilized 129.1, 118.8, and 54.1 mg P/l of tri-calcium phosphate [$Ca_3(PO_4)_2$], aluminum phosphate ($AlPO_4$), and ferric phosphate ($FePO_4$), respectively, after 72 h of incubation. Malic acid, gluconic acid, and oxalic acid were detected in the flasks supplemented with various phosphate sources [240, 146, 145 mM/l $AlPO_4$, $FePO_4$, and $Ca_3(PO_4)_2$, respectively] together with a large amount of malic acid followed by the other two. The effects of inoculation of *P. oxalicum* CBPS-3F-Tsa on maize plants were studied under pot culture conditions. *P. oxalicum* CBPS-3F-Tsa was inoculated to maize plants alone or together with inorganic phosphates in the form of fused phosphates (FP) and rock phosphates (RP). Inoculation of *P. oxalicum* CBPS-3F-Tsa increased the plant growth and N and P accumulation in plants, compared with control plants, and also had positive effects when applied with RP. The results of this study show that the fungus *P. oxalicum* strain CBPS-3F-Tsa could solubilize different insoluble phosphates by producing organic acids, particularly malic acid, and also improved the efficiency of RP applied to maize plants.

Key words: *Penicillium oxalicum*, phosphate solubilization, organic acids, maize, N and P accumulation

Phosphorus (P), one of the major plant nutrients whose availability is conditioned by various factors, is acquired from soil solution as phosphate anions (predominantly HPO_4^{2-} and $H_2PO_4^-$), and these anions are extremely reactive and may be immobilized through precipitation. The availability of phosphate anions in soil is attributed to the presence and activity of soil microorganisms. The existence of soil microorganisms capable of transforming soil P to forms available to the plants has earlier been reviewed [32]. A microbial plant inoculant able to sparingly dissolve soluble inorganic soil P during plant growth would have both economic and environmental advantages. One such soil fungus is the *Penicillium* fungus, which is considered to be a key group of soil microflora involved in P cycling [68]. Several isolates of phosphate solubilizing *Penicillium* spp. were commonly found both on and in the roots of wheat [64]. Isolates of phosphate-solubilizing fungi with high Fe-P, Al-P, and Ca-P solubilization capability were also obtained in pasture soils of Brazil [10].

The solubilization of soil phosphates in many organisms is due to excreted organic acids [11, 32, 52]. In *Penicillium rugulosum*, solubilization of a variscite (Al-Phosphate) mineral is due to production of citric acid, whereas gluconic acid appears to be responsible for the solubilization of apatite mineral or rock phosphate deposits [45]. In most of the studies regarding P-solubilizers, some types of calcium phosphate have been used, predominantly in the form of fluorapatite and hydroxyapatite [13, 40, 50, 63], tri-calcium phosphate [54], calcium hydrogen phosphate ($CaHPO_4$) [17], and freshly precipitated hydroxyapatite [39, 51]. However, a few studies have demonstrated the presence and the ability of fungi to solubilize Fe or Al phosphates [8, 6]. Successful release of *Penicillium bilaiae* (JumpStart; Philom Bios, Saskatoon, Canada) and *Penicillium radicum* (PR-70 RELEASE; Bio-Care Technology, Somersby, Australia) as commercial inoculants has proved the efficiencies of these fungi in crop P nutrition. *P. radicum* Hocking and Whitelaw, sp. nov., [23] was found to be good colonizers of wheat roots and also significantly increased the yield in both glasshouse and field conditions [61].

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Recently, the mineral phosphate solubilizing activity of *Penicillium oxalicum* CBPS-3F-Tsa, a soil fungus isolated from rice rhizosphere from Youngnam Province in Korea, has been reported [30]. In this study, we examined (i) the *in vitro* mineral phosphate solubilizing (MPS) activity of this fungus and possible mechanisms of solubilization in terms of organic acid production and (ii) its impact on growth and nitrogen (N) and phosphorous (P) accumulation in maize plants in the presence of fused and rock phosphates.

MATERIALS AND METHODS

Medium and Culture Conditions

Penicillium oxalicum strain CBPS-3F-Tsa (CABI accession number: IMI 387080) was isolated from rice rhizosphere soils of Youngnam Province, Korea [30]. Phosphate-solubilization and organic acid production were evaluated in minimal salts medium containing (per liter of distilled water): NH_4Cl 0.4 g; KNO_3 0.78 g; NaCl 0.1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $CaCl_2 \cdot 2H_2O$ 0.1 g; $FeSO_4 \cdot 7H_2O$ 0.5 mg; $MnSO_4 \cdot H_2O$ 1.56 mg; $ZnSO_4 \cdot 7H_2O$ 1.4 mg; glucose 10 g; and agar 20 g. Phosphorous was added to the medium in the form of $Ca_3(PO_4)_2$ at 4 g/l, and the pH was adjusted to 6.5. Pure cultures were maintained at 25°C in petri plates containing potato dextrose agar supplemented with streptomycin sulfate (100 μg/ml) to inhibit any potential bacterial growth [46].

Mineral Phosphate Solubilizing Activity

Strain *P. oxalicum* CBPS-3F-Tsa was subcultured in petri plate containing NBRIY medium [amounts per liter of distilled water: $Ca_3(PO_4)_2$ 5 g; $(NH_4)_2SO_4$ 0.5 g; NaCl 0.2 g; $MgSO_4 \cdot 7H_2O$ 0.1 g; KCl 0.2 g; $MnSO_4 \cdot H_2O$ 0.002 g and $FeSO_4 \cdot 7H_2O$ 0.002 g] [40] with sucrose (10 g/l) as the sole carbon source. Tri-calcium phosphate (TCP) was dry autoclaved and added immediately to the cooled molten agar medium at 5 g/l prior to plating. Petri plates were incubated at 25°C in the dark for up to 14 days and inspected every second day for the presence of clear zone around the colony margin, signifying P solubilization.

The solubilization rate of $Ca_3(PO_4)_2$ was measured by determining the amount of soluble P present in the culture filtrate and by its pH change. Aliquots of 100 ml media with $AlPO_4$, $FePO_4$, and $Ca_3(PO_4)_2$ were apportioned into a 250 ml-Erlenmeyer flask and autoclaved at 121°C for 15 min. As inoculum, 4 mm diameter culture blocks picked with a sterile cork borer from the periphery of actively growing fungal colony on potato dextrose agar plates were inoculated into flasks. For each treatment, triplicate flasks were incubated at 30°C on a rotary shaker set at 120 rpm. At regular intervals, the flasks were withdrawn, and the contents were filtered through a 0.2 μm filter. The contents of soluble phosphate and organic acids and pH of the filtrate were recorded.

Inorganic P concentration was colorimetrically determined by the molybdenum-blue method of Murphy and Riley [38]. The amount of P solubilized was obtained by subtracting the soluble P concentration of the uninoculated control (i.e. P released by autoclaving of the P suspension) from that of the corresponding inoculated culture.

HPLC Analysis of Organic Acid Production

Production of organic acid was determined using a Waters™ 600S Model HPLC fitted with 486 Tunable Absorbance Detector. The mobile phase was H_2SO_4 (0.1%) at a flow rate of 0.5 ml/min [42]. UV absorption was measured at 210 nm. Standard organic acids for identification in culture extract included acetic, citric, formic, fumaric, galacturonic, gluconic, glucuronic, glycolic, ketogluconic, lactic, malic, maleic, oxalic, propionic, pyruvic, succinic, and tartaric acids. Gluconic acid in combination with Ca^{2+}, Al^{3+}, or Fe^{3+} were also used separately as standards to identify peaks.

Greenhouse Trial

The soil used for the study was collected from Cheongju Agriculture Research Center, Cheongju. The soil was air-dried, sieved (5 mm), and transferred to pots (22.5×20.5×14 cm) at 6 kg/pot. The sandy loam soil had 0.54% organic matter, 0.016% N, and 12.7 mg P_2O_5/kg at pH 7.07. The experiment was arranged in a randomized design with 6 treatments and three replications per treatment. Surface-sterilization of the seeds was carried out, and the seeds were sown at 2 cm depth in each pot, initially maintaining with 2 plants per pot and thinned to 1 plant per pot 9 days after seeding. The 100% recommended level of P (3 kg/10a) was applied. Two phosphate sources, rock phosphate (RP) and fused phosphate (FP), were used throughout the study. Irrespective of the treatments, nitrogen and potassium were applied to a recommended level of 17.4 and 6.9 kg/10a, respectively [5].

Inoculum Preparation

The fungus was grown in 100 ml of potato dextrose agar in 500-ml flasks. One week after inoculation, the flasks were flooded with sterile distilled water, and the spores were collected aseptically. The spore concentration in distilled water, containing 1% Triton X-100, was adjusted to 6×10^8 cfu/ml. The plants were inoculated at near root regions (2 cm depth) at 10 ml/plant after 9 days of seeding.

Data Acquisition

The plants were harvested after 60 days of seeding. The soil particles adhering to the roots were carefully removed by gentle shaking. After recording the plant height, the plants were dried in an oven at 70°C for 120 h and weighed for dry matter analysis.

N and P Analysis

Nitrogen (N) concentrations in maize plant root and shoot were measured after digestion with sulfuric acid and

potassium sulfate, following Kjeldahl Auto1030 analyzer [28]. Concentrations of P in root and shoot were measured as per the method described by Jackson [28]. Briefly, powdered plant material (0.5 g), digested with 1 ml of concentrated sulfuric acid and 5 ml of 50% perchloric acid in a micro Kjeldahl flask, was filtered through Whatman No.6 filter. Five ml of the filtrate and 5 ml of ammonium metavanadate reagent were mixed, and the reaction mixture was allowed to stand for 15 min after vigorous shaking. The intensity of yellow color developed was measured at 470 nm using a spectrophotometer. Standards were prepared with potassium dihydrogen phosphate (Sigma, St. Louis, MO, U.S.A.).

RESULTS

In Vitro Phosphate Solubilization and Organic Acid Production

The fungus studied, *P. oxalicum* CBPS-3F-Tsa, was capable of solubilizing all the three phosphate sources, accompanied with significant changes in the amount of soluble phosphate and organic acid concentrations with concurrent reduction in pH (Fig. 1). The soluble phosphate contents of the medium after 72 h of incubation were 129.1 mg P/l for $Ca_3(PO_4)_2$, 54.1 mg P/l for $FePO_4$, and 111.8 mg P/l for $AlPO_4$, respectively (Fig. 1D–1F). The pH of the culture filtrate turned acidic with all treatments. pH drops from 6.5 to 3.61, 3.96, and 3.76 were observed for $Ca_3(PO_4)_2$, $FePO_4$, and $AlPO_4$, respectively.

The culture filtrate was analyzed for organic acids such as malic, oxalic, and gluconic acids as well as smaller concentrations of other unknown substances found. Although the malic acid concentration was found to be high in all the flasks, the concentrations of the three acids varied, depending on P source and incubation time. A rapid increase in malic acid concentration was observed in all the amendments until the final analysis. Its concentration in the $Ca_3(PO_4)_2$ amended media increased from 10 mM at 7 h to the maximum of 145 mM at 72 h. Similarly, the concentration ranged from 21 to 146 mM for $FePO_4$ and 17 to 240 mM

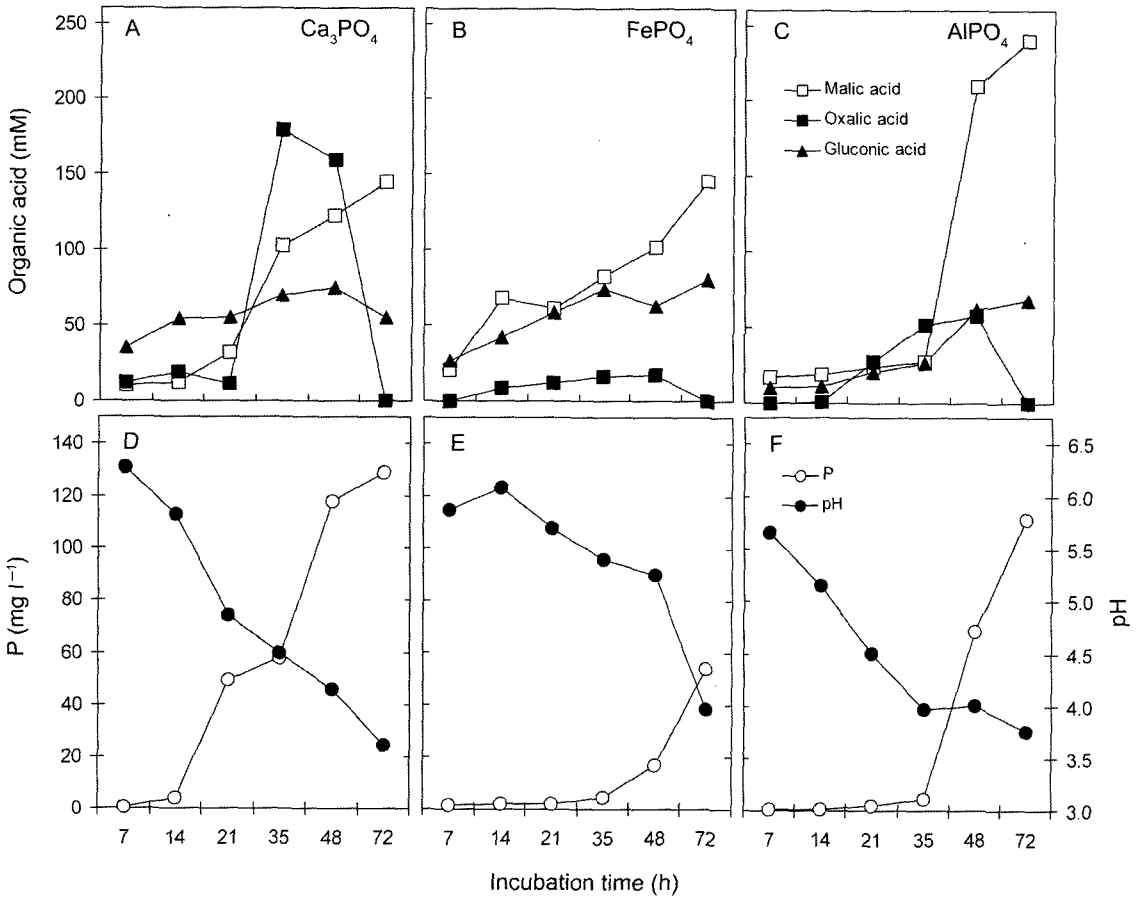

Fig. 1. Organic acid concentration (A-C) and the corresponding variation in the soluble P and pH (D-F) of the minimal salt medium, resulting from solubilization of the insoluble phosphates by *P. oxalicum* CBPS-3F-Tsa.

Experiments were carried out at 30°C using $Ca_3(PO_4)_2$, $FePO_4$, and $AlPO_4$ as the sole P source. Each data point represents the mean of three replicates. The control treatment was inoculated with sterile agar plugs only.

for $AlPO_4$ amended media. Next to malic acid, the gluconic acid concentration also increased rapidly (up to 48 h) in all the three amendments, with a gradual decline at the end of 72 h in the $Ca_3(PO_4)_2$ amendment. In general, the concentration of oxalic acid in the culture filtrate was low: After achieving a maximum production at 48 h, its concentration in the $Ca_3(PO_4)_2$ amendment declined to zero at the end of incubation. Its concentration was also very low in the $FePO_4$ and $AlPO_4$ amendments.

Nutrient Uptake and Plant Growth Evaluation

Under pot culture conditions, inoculation of *P. oxalicum* CBPS-3F-Tsa significantly increased the growth and N and P accumulation of maize plants, compared with a control. Inoculation of *P. oxalicum* CBPS-3F-Tsa with RP increased the plant height and dry matter content of maize plants, compared with individual application of RP (Fig. 2). However, with the application of FP, plant growth increased significantly irrespective of *P. oxalicum* CBPS-3F-Tsa inoculation.

Plants treated with *P. oxalicum* CBPS-3F-Tsa/RP and FP showed increased N and P uptake, with FP performing better among the treatments. The accumulation of P in maize

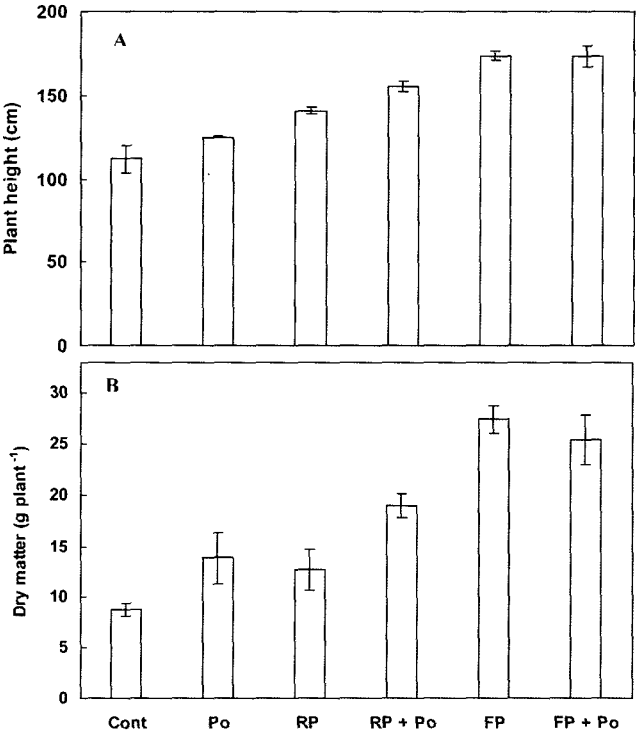

Fig. 2. Effect of *P. oxalicum* CBPS-3F-Tsa on plant height (A) and dry matter production (B) of maize at 60 days of seeding.
Each data point represents the mean of three replicates. The error bar indicates±S.E. For treatment details, Cont, uninoculated control; *Po*, *P. oxalicum* strain CBPS-3F-Tsa; RP, 100% - rock phosphate; RP+*Po*, 100% rock phosphate+*P. oxalicum* strain CBPS-3F-Tsa; FP, 100% fused phosphate; FP+*Po*, 100% fused phosphate+*P. oxalicum* strain CBPS-3F-Tsa.

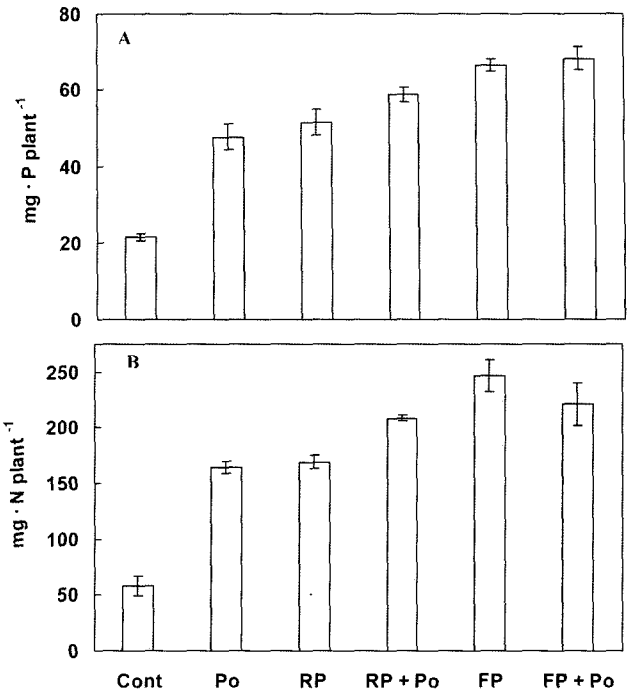

Fig. 3. Effect of *P. oxalicum* CBPS-3F-Tsa on (A) N and (B) P accumulation of maize at 60 days of seeding.
Each data point represents a mean of three replicates. The error bar indicates±S.E. For treatment details, see Fig. 2.

plants treated with *P. oxalicum* CBPS-3F-Tsa increased two-fold, when compared with a control, and no significant difference could be observed with RP-treated plants. In addition, the accumulated P in the plants increased, when RP was applied along with *P. oxalicum* CBPS-3F-Tsa (Fig. 3A).

When N uptake by the plants was measured, varied results could be observed. The treatments recorded significantly higher N uptake in the plants than the control (Fig. 3B). Significant differences could not be observed in FP treatments, when applied individually or together with *P. oxalicum* CBPS-3F-Tsa. However, enhancements of the total N accumulation of the maize plants could be observed, when inoculated along with RP.

Discussion

A number of reports have compared microbial communities in soil-root systems and the exploitation of plant growth promoting microorganisms has been gaining momentum in recent years. The importance of fungi can be realized, since their occurrence and plant growth promotion even in endangered vegetations have recently been reported. Several species of *Penicillium* have been shown to solubilize phosphates [24, 49, 57, 62] and promote growth of wheat, canola, and maize crops [32, 44, 61]. In the present study,

a gradual increase of soluble P concentration with a corresponding decrease in pH of the culture filtrate was observed in all the amendments. P solubilization was always found to be related to reduction of pH in the culture media. It was reported that the solubility of phosphate compounds in solution increases as pH decreases below 5 [53]. Whitelaw *et al.* [62] associated the *P. radicum*-mediated phosphate solubilization with the acidification of media to an increase in H^+ ions and to a lower pH at which the compounds are more soluble. Production of organic acids by fungi during phosphate solubilization in liquid media was also reported to cause acidification of media [17, 25, 27, 32, 46, 57, 62]. In this study, oxalic, malic, and gluconic acids were detected in the culture media. The pH reduction observed in this study could be related to the increase of H^+ ions in the culture medium for a given phosphate compound; however, the action of organic acids produced by the fungus could not be neglected in mediating the process. The excretion of organic acids, the intermediates of the tricarboxylic acid cycle, has been reported to take place in every culture of a filamentous fungus growing in a medium with a high C:N ratio and during the growth phase [15, 31, 37, 62]. While studying the citric acid production by *Penicillium simplicissium*, Gallmetzer and Burgstaller [19] observed overproduction of oxalate, citrate, malate, and succinate in the glucose-rich nitrogen-limited medium. Production of large quantities of organic acids in this study could be related to a large amount of carbon source in the medium [46]. The decline in oxalic acid at later stages of incubation could also be related to the above, where rich nutrient media with higher carbon (30 g/l) and less nitrogen (0.5 g/l) were used. This situation has been reported to induce nitrogen starvation in an environment of prolonged incubation [20], and would trigger reutilization of organic acids, especially oxalic acid, as an energy source by the fungi.

The mechanism of phosphate solubilization by several fungi such as *P. chrysogenum*, *P. notatum*, *P. rogulosum*, *P. radicum*, *P. aurantiogriseum*, *P. biliaji*, and *A. niger* was attributed to gluconic acid production [7, 25, 41, 43, 62]. In the present study, malic acid was detected at quantities higher than gluconic acid in the presence of all three phosphate sources. Malic acid was also reported to be secreted from the roots of plants in response to Al toxicity [12, 18, 35] and is known to have high complex forming ability with Al [35]. Results on the production of higher quantities of malic acid indicate overproduction of malic acid by *P. oxalicum* CBPS-3F-Tsa to solubilize phosphates including $AlPO_4$. Molecular level studies to identify and clone genes responsible for malic acid overproduction in the fungus should be pursued in future.

All plants, including maize, require a higher phosphate concentration at the early developmental stage for better root development [2, 29, 48, 56], which in turn can influence shoot growth [9, 26, 33]. Experiments with maize under rain-fed conditions have shown that a delay in crop fertilization results in yield reduction [1]. It has also been shown that, in soils with low and moderate P content, crops utilize P more efficiently, when fertilizer is applied in small volumes close to the seed [3, 4, 14, 21, 34]. In the present study, maize was found to respond well to the application of P during eight weeks after seeding. Leaf area and plant height are known to be indicators of plant response to fertilization of corn [47]. The present results confirmed the above notion that the effects of inoculation on plant growth varied with the source of P applied.

The increase of dry weight and N and P accumulation in shoots and roots highlight the importance of adding P at seeding time and the influence of the fungal isolate in playing a role in the release of phosphates in soil. These results are in consonance with earlier findings that increase of growth and productivity by various fungi occurred in maize, wheat, sorghum and chickpea [44, 50, 55, 61]. *P. oxalicum* CBPS-3F-Tsa actively solubilized inorganic RP and increased the accumulated P in maize plants. However, this effect could not be seen when applied with FP, and this could most likely be due to the repression of P solubilization in microbes, when exposed to phosphate-rich environments [16, 22]. It is also evident that the fungus requires an optimum amount of P available soil for it to function efficiently. Further studies on the root colonization and yield improvement would shed more light on the prospects of fertilizing maize crops with *P. oxalicum* CBPS-3F-Tsa.

The presently described results clearly suggest that, under greenhouse conditions, it is possible to benefit from the phosphate-solubilizing activity of *P. oxalicum* CBPS-3F-Tsa when rock phosphate is used. More field trials are required before recommendation of fungi as a beneficial bioinoculant for maize crop can be realized.

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