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Effect of Phosphate Solubilizing Fungi on Growth and Nutrient Uptake of Soybean (*Glycine max* L.) plants

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Abstract: *Aspergillus niger* and *Penicillium italicum* were isolated from the soil and rhizosphere of different plants. They were tested for their efficacy to solubilize tri-calcium-phosphate (TCP) *in vitro* as well as their effect *in vivo* to promote the growth of soybean (*Glycine max* L.) plants grown in soil amended with TCP. The results showed high solubilizing index in agar plates. Also, they effectively solubilized TCP in Pikovskaya's liquid medium (PVK) and released considerable amounts of P into medium. The efficacy of *Penicillium italicum* to solubilize and release the inorganic P was 275 µg P ml^{-1} whereas *Aspergillus niger* showed better efficiency and produced 490 µg P ml^{-1} after seven days of incubation. Drop in pH during growth was more prominent in absence of TCA in liquid medium. This indicated that absence of soluble P in media induces the acid production. The addition of TCP to the broth media produced an increment in fungal biomass. Pot experiment showed that the dual inoculation of phosphate-solubilizing fungi (*A. niger* and *P. italicum*) significantly increased dry matter and yield of soybean plants compared to the control soil. Significant increment in percentage of protein and oil was also recorded. There was an increase in the percentage of N and P content of the plant. It was significantly resulted with N levels of soybean plants but this increase was non-significant with the percentage of total phosphorus, under the experimental conditions. Soil analysis showed that the available P, organic carbon levels were significantly increased when compared to the initial soil. The pH was also lowered compared to the initial pH of the soil.

Key words: *Aspergillus niger*, *Penicillium italicum*, phosphate-solubilizing fungi, tricalcium-phosphate, soybean

INTRODUCTION

Compared with the other major nutrients, phosphorus is by far the least mobile and available to plants in most soil conditions. Although phosphorus is abundant in soils in both organic and inorganic forms, it is frequently a major or even the prime limiting factor for plant growth[18]. Phosphorus is added in the form of phosphatidic fertilizers, part of which is utilized by plants and the remainder converted into soluble fixed forms[21]. To circumvent phosphorus deficiency, phosphate-solubilizing microorganisms (PSM) could play an important role in supplying phosphate to plants in a more environmentally-friendly and sustainable manner[18].

Important genera of phosphate solubilizing bacteria are *Bacillus* and *Pseudomonas*[15,20]. Certain strains of *Rhizobium* can also solubilize both organic and inorganic phosphates[1]. Filamentous fungi are widely used, mainly of *Aspergillus* and *Penicillium* genus, to solubilize phosphates under in vitro conditions[23,31,34]. This ability is generally associated to the release of organic acids, decreasing the pH[31]. Moreover, these organic acids can increase considerably P in the soil solution through the quelation of Ca, Fe and Al, change reactions and solubilization of low soluble salts[13]. The inoculation of P-solubilizing microorganisms is a promising technique because it can increase P availability in soils fertilized with rock phosphates[26]. Several authors reported yield increasing on wheat[35], onion[32], alfalfa[29] and soybean[2] through simple inoculation of P-solubilizing fungi (PSF). Inoculation of phosphate solubilizing fungi and mycorrhizal fungi improves the physio-chemical, biochemical and biological properties of rock-P amended soil[8]. Beyond the phosphate solubilization, many P-solubilizing microorganisms increase the mycorrhizal root colonization by production of specific metabolites as vitamins, amino acids and hormones[5]. It has also been reported that the available P and aggregate stability levels, higher soil C levels, enzyme activities and lower soil pH were also reported due to inoculation of these fungi[8]. Therefore, there is a demand on studies on this subject, in order to improve the functional knowledge of compatibility of those microorganisms aiming at their co-inoculation to increase the nutrition and growth of plant species.

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The objective of this work was to evaluate solubilization ability of insoluble phosphates by several fungal isolates and their effect upon growth of soybean plants grown in soil amended with tri-calcium-phosphate.

MATERIALS AND METHODS

Fungal Strains, Isolation and Identification: Fungal strains were isolated from the soil and rhizosphere of roots of plants growing in fields, Sharkia district, Egypt, after serial dilution of soil solution on potato dextrose agar (PDA) plates. Distinct colonies present on the plates were selected, purified by repeated culturing and maintained on PDA slants at 4ºC. Isolates were identified according to Fisher and Cook[12].

Solubilization Index on Solid Media, Growth Condition: Preliminary screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium supplemented with tri-calcium phosphate (TCP)[24]. The medium contained l^{-1}: glucose, 10 g; $Ca_3(PO)_4$, 5 g; $(NH_4)SO_4$. 0.5 g; NaCl, 0.2 g; MgSO4.7H2O, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4.7H_2O$, 0.002 g; $FeSO_4.7H_2O$, 0.002 and agar 15 g. The pH of the media was adjusted to 7.0 before autoclaving. Sterilized PVK media was poured into sterilized Petri plates after solidification of the media, a pinpoint inoculation of fungal strains was made onto the plates under aseptic conditions. They were incubated at 28±2ºC for 7 days with continuous observation for colony diameter. The halo zone formations around the growing colony showing phosphate solubilization. Solubilization index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diameter[10].

Solubilization Capacity of Tricalcium Phosphate in Liquid Cultures: Solubilization activity was carried out in 100 ml PVK broth medium amended with 0.5% tricalcium phosphate. Then, after sterilization 1.0 ml suspension of each fungal culture (10^7 CFU/flask) was added to the broth in triplicate. A control without any inoculation was maintained. Media alone without TCP was also prepared for determination of mycelial biomass. The cultures were incubated on a rotary shaker at 30ºC for 7 days. Cultures were harvested after growth periods in order to record the change in pH, concentration of P released in the medium and dry fungal mats. Available phosphorus in broth cultures was estimated by the paramolybdate blue method[22]. It was expressed in terms of µg/ml phosphorus released in culture medium.

Soil Experiment: Seeds of soybean were surface sterilized[33], rinsed 6 times with sterile water and dried. The surface disinfected seeds were coated by soaking seeds in liquid culture medium for 2 h using 10% gum Arabic as adhesive to deliver 10^8 cells $seed^{-1}$ *Bradyrhizobium*. It was added as a biofertilizer in all treatments except the control. Spore suspensions (4 ml) of 2×10^6 ml^{-1} of *A. niger* and *P. italicum* was added to soils 48 h before sowing. The uninoculated seeds served as a control treatment for comparison. Tri-calcium phosphate (27.0%, P_2O_5), obtained from Abu Zaabal phosphate fertilizer Co. It was added as phosphatidic P (20 mg kg^{-1}) to the soil before seeding and was common in all treatments, except the control, which had 20 mg kg^{-1}N (urea) and 40 mg kg^{-1} P (single super phosphate).

The inoculated seeds were sown in earthern pots (10 seeds pot^{-1}) having 12.5 kg of unsterilized sandy soil collected from South El-Tahrir region , El-Behaira Governorate. The soil contains 78% sand, 10% silt, and 12% clay, pH 7.6, EC mmohs/cm 0.12, organic matter % 0.60, calcium carbonate % 3.5, total N ppm 72.0, and available P ppm 4.0. Seedlings were thinned to 3 plants per pot after 5 days of emergence. The plants were irrigated with tap water as and when required. The pots with different treatments were arranged in a randomized complete block design with triplicates of each treatment. The treatments were as follows: control (uninoculated seeds); plant + *Aspergillus niger*; plant + *Penicillium italicum* and plant + *Aspergillus niger* + *Penicillium italicum*. At 90 days plant age, a random sample was taken from each treatment to determine some growth parameters as follows: plant height (cm), dry weight/plant (g). At harvesting time the yield components were estimated as follows: number of pods/plant and 100-seed weight (g). Nitrogen (N) was extracted from plants with sulfuric acid using the semi-micro Kjeldahl method[17]. Phosphorus (P) was extracted by nitric-perchloric acid digestion and measured using the vanadono-molybdophosphoric colorimetric method[16]. The protein content of plant tissues were estimated according to Bradford[7]. Oil % in dry seeds was extracted with petroleum ether using Soxhlet apparatus according to A.O.A.C.[3].

Statistical Analysis: The statistical analysis done by using SPSS program (Statistical Package for the Sciences System). The variables were subjected to ANOVA (significance was set at $*P<0.05$ and $**P<0.01$).

RESULTS AND DISCUSSION

Phosphate Solubilization Index (SI) on Solid Agar Plates: Fig. (1) showed the solubilization Index (SI) of the tested phosphate-solubilizing fungal strains ranged between 2.42 to 3.15 in the present study. Data recorded high SI for *Aspergillus niger* strain than the *Penicillium italicum*. Sometimes abrupt changes or no consistent pattern for P-solubilization occurred in these values. Similar observations have been reported by several workers[19,11].

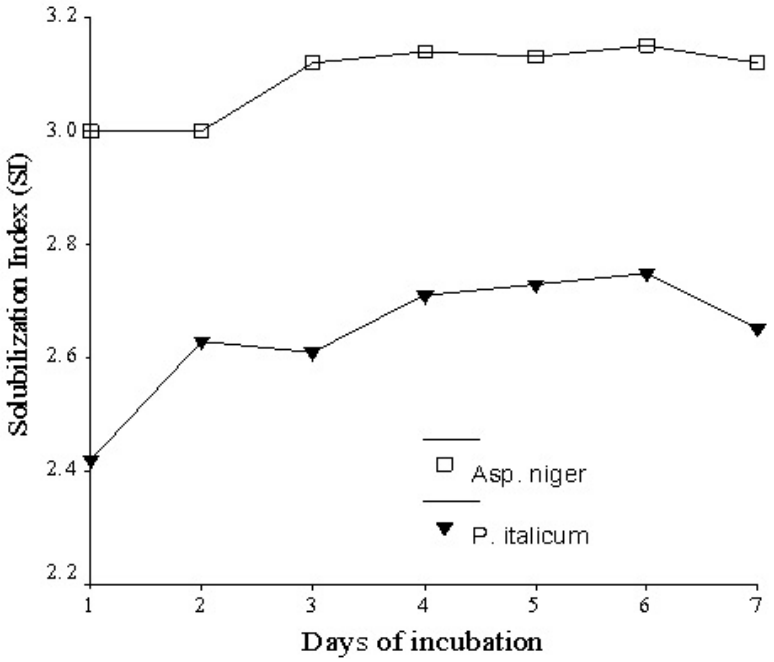

Fig. 1: Solubilization Index (SI) of the tested P-solubilizing fungal strains during seven days of incubation.(each value is a mean of three replicates).

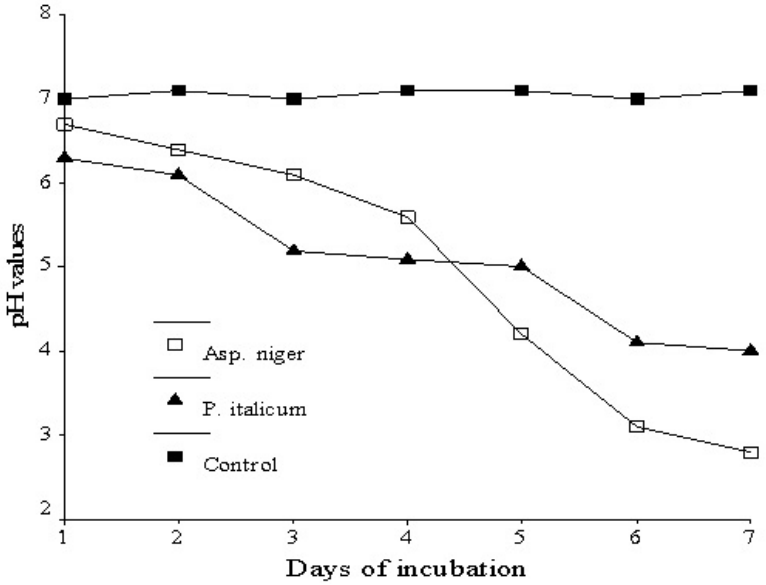

Fig. 2a: Change in pH by the tested P-solubilizing fungal strains during seven days of incubation. (each value is a mean of three replicates).

Phosphate Solubilization in Broth Medium: Fungi were sampled daily to determine the change in pH (Fig. 2a) and the solubilized released-P in liquid broth (Fig. 2b). The pH of the cultural broth samples dropped significantly as compared to the control where it remained constant around pH 7.0. The *Aspergillus niger* strain caused decrease in pH from 6.7, at the beginning, to 2.8, while *Penicillium italicum* decreased the pH from 6.3 to 4.0 (Fig. 2a). This was attributed to the varying diffusion rates of different organic acids secreted by the two tested organisms. The pH drop in P-solubilizing fungi liquid cultures resulted in present study was supported by El-Katatny,[11] who stated that P solubilization of *Aspergillus niger* and *Penicillium italicum* was accompanied by reduction in pH during incubation. Also, Pradhan and Sukla[25] stated that

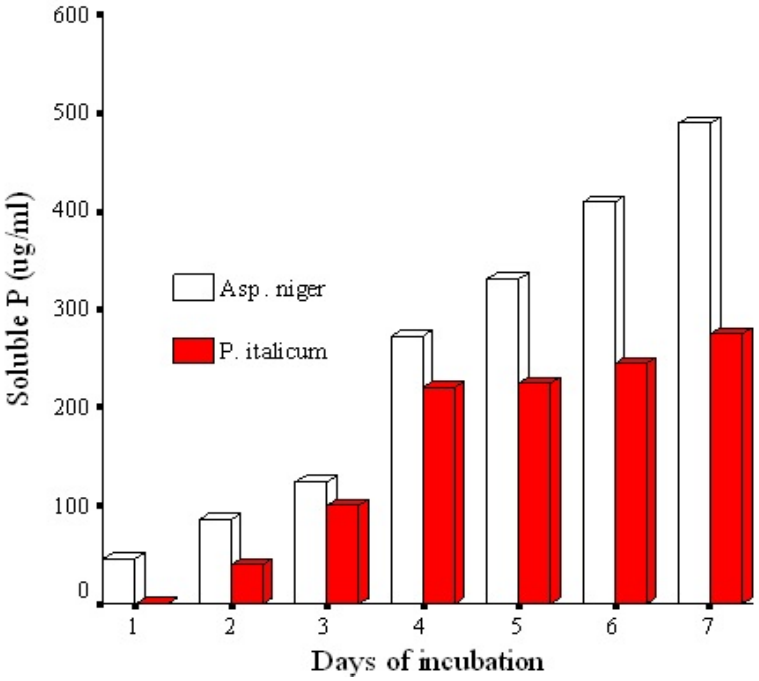

Fig. 2b: Released-P by the tested P-solubilizing fungal strains during seven days of incubation. (each value is a mean of three replicates).

Table 1: Effect of Co-inoculation with phosphate solubilizing fungi on some growth parameters, yield , P and N percentage in soybean plants.

Treatments	Height (cm)	Dry weight of plant (g)	Number of pods/plant	100-seed weight (g)	Protein %	Oil %	N %	P %
Control	62.1	10.7	15.7	1.27	35.9	20.1	6.3	0.40
Soil + TCP	76.5	15.4	25.9	1.51	42.6	23.8	7.1	0.46
Soil + TCP + *A. n.*	97.5	18.9	31.6	1.79	43.8	25.3	7.6	0.53
Soil + TCP + *P. i.*	89.7	16.7	33.5	1.63	43.9	24.9	7.4	0.49
Soil+TCP+ *A.n.* + *P. i.*	112.5**	21.9**	35.9**	2.0**	46.5**	26.3**	7.9*	0.57
Mean	87.66	16.72	28.52	1.64	42.54	24.08	7.26	0.49

* P value <0.05 (significant difference)
** P value <0.01 (highly significant difference)

Table 2: Characteristics of TCP amended soil after inoculation with tested fungi.

Treatments	pH		Available P (ppm)		Organic carbon (%)	
	Initial	Final	Initial	Final	Initial	Final
Control	7.6	7.5	4.0	5.4	0.60	0.62
Soil + TCP	7.5	6.9	4.4	6.2	0.70	0.74
Soil + TCP + *A. n.*	7.5	6.4	4.4	6.3	0.70	0.77
Soil + TCP + *P. i*	7.6	6.6	4.4	6.3	0.70	0.76
Soil+ TCP + *A. n.*+ *P. i.*	7.6	6.2**	4.4	7.1**	0.70	0.78**
Mean	7.56	6.72	4.32	6.26	0.68	0.73

* P value <0.05 (significant difference)
** P value <0.01 (highly significant difference)

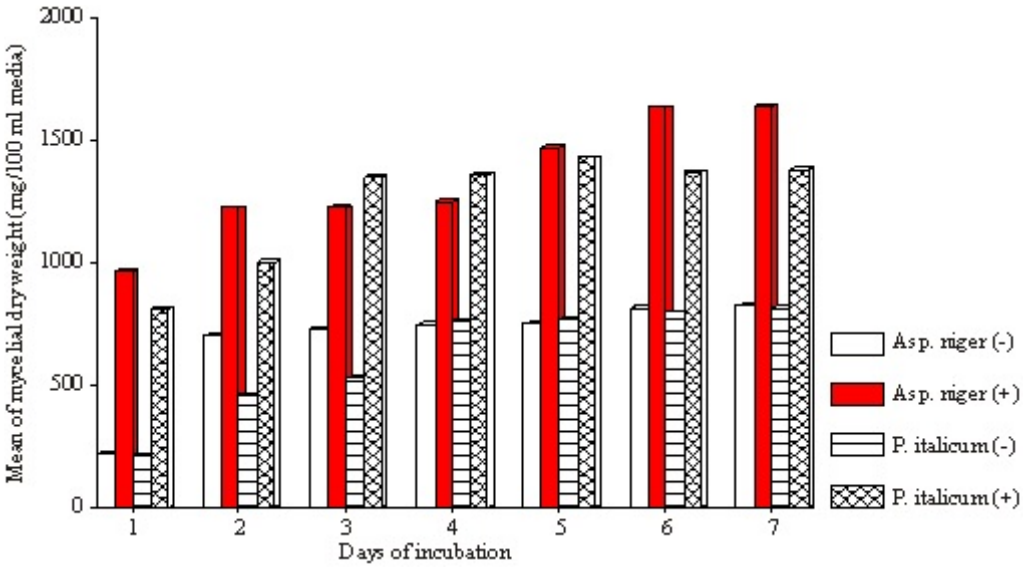

Fig. 3: Mycelial dry weight of the tested P-solubilizing fungal strains during seven days of incubation in absence (-) and presence (+) of TCP in liquid media. (each value is a mean of three replicates).

Aspergillus sp. showed much higher drop in pH and high P solubilization when compared to *Penicillium* sp.

Fig. (2b) showed P slubilization activity of the fungal isolates when tested on Pikovskaya liquid medium. *Penicillium italicum* solubilized and released 275µg P ml^{-1} whereas *Aspergillus niger* showed better efficiency of TCP solubilization and produced 490µg P ml^{-1} after seven days of incubation. The decrease in P concentration at the beginning stages of the experiment is consistent with the findings of Seshadri *et al.,*[30] who stated that the existing P is utilized for growth and development of the organism during this period. Soluble P apparently was removed from the cultures at the early periods as rapidly as it was solubilized. The increase of P concentration in the later stages might be due to the action of the fungi on the substrate for demands of nutrients, thus releasing more P from insoluble sources. Increasing the released P during the later stages was also attributed to cell lysis and P precipitation brought about by organic metabolites[15].

The addition of TCP to the PVK liquid media produced an increment in fungal biomass (Fig. 3). Gharieb[14] reported correlation between biomass production by *Aspergillus niger* and production of oxalic acid under in vitro conditions. The two tested isolates reached their maximum biomass level after seven days of incubation. Such result indicated the ability of the fungal strains to solubilize P and change it to available form. Culture media with no TCP produced poor growth. Obtained results are in accordance with those of Asea *et al.,*[4], who reported that the growth of two *Penicillium* spp. were improved by the addition of rock phosphate.

Soil Experiment: A pot experiment was undertaken to evaluate the effectiveness of *Aspergillus niger* and/or *Penicillium italicum* in TCP amended soils to enhance the growth of soybean and improve the physico-chemical characteristics of the soil. Table (1) showed that inoculation with the fungal strains, separately; improve the height and dry weight of plant. Dual inoculation of the fungal strains significantly increased the height up to 81% and the dry weight of plant up to 105%, respectively, compared to the non-inoculated TCP soil or that amended with super-P.

Significant increase in number of pods/plant and the weight of 100 seeds were also recorded with the application of single or dual inoculation of the tested strains. Thus the application of P solubilizing fungi is recommended as a sustainable way for increasing crop yield, under all experimental conditions. Many reports had shown the improvement in plant growth using P-solubilizing fungi[35].

The increase in protein and oil percentage of soybean plants was mainly attributed to the beneficial effect of inoculation with the two experimental fungal strains to the TCP amended soil, particularly in sand soil lacking enough nutrients (Table 1). This significant increase reached 57.5% and 29.5% for protein and oil contents, respectively, comparing to the control.. In this study, there was an increase in the percentages of N and P in plant. It was found that organic acids added to the soils increased the plant uptake of P from

a water soluble P[6]. Also, the release of organic acids that both sequester cations and acidify the microenvironment near the roots is thought to be a major mechanism of P-solubilization, as well as Mn, Fe and Zn by plants and non-vesicular mycorrhizal fungi[9]. An increase significantly resulted in N levels of soybean plants but was non-significant with the percentage of total phosphorus, under the experimental conditions. With this respect, Richardson[28] suggested that the plant growth promotion, as consequence of the microbial inoculation, can not necessarily be associated with the P solubilization, commonly observed under laboratory conditions. Mechanisms such as production of phyto- hormones, vitamins or amino acid can be involved in the P-solubilizing micro-organisms effect[5].

The soil properties were also improved after inoculation of the tested fungi. The available P levels and organic carbon were significantly improved in all treatments compared to the initial values. Also this application reported a drop in pH values of the soil compared to the control. The improvement of physio-chemical and biochemical properties of the soil amended with rock-P inoculated with *A. niger* and mycorrhizal fungi was reported by[8,27]. They reported higher available P, soil total carbohydrates, water soluble C and lower soil pH compared to control soils.

Conclusion: Phosphate-solubilizing fungi are an important contributor in microbial P-mobilization and would be important possible way to increase available P for plant. Accordingly, From the present work we can conclud that the amendment of soil with TCP along with the application of P-solubilizing fungi is suggesting as a sustainable way for increasing crop yield and also improve the physio-chemical properties of the soil.

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