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Rock Phosphate Solubilization by Two Isolates of *Aspergillus niger* and *Penicillium* sp. and their Promotion to Mung Bean Plants

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Abstract: Isolation and identification of rock phosphate (RP) solubilizing fungi were studied under laboratory conditions. Fungal isolates that displayed the highest ratio of clear zone/colony diameter on plates of phosphate solubilization medium, were selected and identified as *Aspergillus niger* and *Penicillium* sp. The optimum condition for RP solubilization were found to be at the 6th (*A. niger*) and 7th (*Penicillium* sp.) day of incubation with shaking (150 rpm) at 30°C and pH ranging from 5.6 to 6.0. Glucose followed by fructose and xylose supported the RP solubilization process in the presence of 2.5 g L^{-1} RP as the optimum concentration. The overall soluble P after optimization studies on RP were 99.7 (*A. niger*) and 77.5 mg L^{-1} (*Penicillium* sp.). During the fermentation process, there was remarkable reduction in the final culture pH. The titratable acidity was positively correlated with RP solubilization. Under NaCl salt stress both fungi were able to solubilize RP, in which, *A. niger* was more tolerant than *Penicillium* sp. The dual and individual cultures of fungi solubilized sources of phosphate commonly exist in soil and also, possessed phytase activity. Under *in vivo* conditions, the inoculation of mung bean seeds with *A. niger* and/or *Penicillium* sp. in the presence of RP or calcium superphosphate (CSP), increased significantly the growth (except for branches No. $plant^{-1}$), seed yield and P-uptake, as well as, improved the nodulation status and population of total and phosphate dissolving fungi in the rhizospheric soil of mung bean. These inoculations saved about 1/3 phosphate fertilizer dose. Hereby, these combined effects encourage the potential use of the isolated fungi in the biosolubilization of RP in soil plant system.

Key words: *Aspergillus niger*, *Penicillium* sp., rock phosphate, solubilization, mung bean, biofertilization

INTRODUCTION

Phosphorus (P) is one of the major nutrient elements limiting agricultural production in the world. Phosphorus entering in the composition of adenosine triphosphate (ATP) which is considered by biologists to be the energy currency of life and essential for all the physiological mechanisms that require energy for different operations. Phosphorus, however, plays an important role in N_2-fixation process, as the reduction of atmospheric nitrogen

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requires large amount of energy. It is estimated that 16 molecules of ATP are required for the reduction of one molecule of atmospheric nitrogen (Krishnan and Bennett, 2006). On average, soil contains 0.02 to 0.5% total P. It is added to the soil in the form of phosphate fertilizers, a part of which (1%) is utilized by plants and the rest is rapidly converted into insoluble complexes e.g., calcium phosphate, iron phosphate and aluminum phosphate in the soil. This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and environmentally undesirable. Therefore, the necessity to develop economical and eco-friendly technologies is steadily increasing (Vassileva *et al*., 1998; Reddy *et al*., 2002; Chuang *et al*., 2007).

Natural phosphate rocks have been recognized as a valuable alternative for P fertilizers. In recent years, the possibility of practical use of rock phosphates (RP) as fertilizers has received significant interest. Unfortunately, RP is not plant available in soils with a pH greater than 5.5 to 6.0 and, even when conditions are optimal, plant yields are lower than those obtained with soluble phosphate (Khasawneh and Doll, 1978). Conventionally, RP is chemically processed by reacting with sulphuric acid or phosphoric acid into soluble phosphate fertilizer. The process increases fertilizer cost and makes the environment worse (Reddy *et al*., 2002; Chuang *et al*., 2007; Xiao *et al*., 2008).

An alternative, has been the use of microorganisms with the capability to solubilize RP and release soluble P through the production of organic acids, chelating oxo acids from sugars, reduction of pH and production of enzymes. Several reports have indicated that some microorganisms are capable of solubilizing insoluble RP and releasing soluble P. However, few reported microorganisms represent a high potential to release soluble P from RP and this seriously restrains the biosolubilization of RP and its use as biofertilizer, hence, isolation and application of new and potential phosphate solubilizing microorganisms are significant and necessary (Son *et al*., 2006; Achala *et al*., 2007; Xiao *et al*., 2008). Filamentous fungi are widely used as producers of organic acids, particularly black *Aspergilli* and some species of *Penicillium*, these species have been tested for solubilization of RP and have been reported for various properties of biotechnological importance, such as, biocontrol, biodegradation, phosphate solubilization and P fertilizer (Narsian and Patel, 2000; Reddy *et al*., 2002; Chuang *et al*., 2007; Richa *et al*., 2007; Pandey *et al*., 2008).

Mung bean (*Vigna radiata* L.) is a new introduced summer pulse crop in Egypt with short growing season and high nutritive value, grown principally for its protein rich edible seeds, this crop can be used for both seed and forage production. It plays an important role not only in human diet, but also in improving the soil fertility by fixing atmospheric nitrogen with the association of *Rhizobium* species present in the nodules of its roots (Thalooth *et al*., 2006).

Therefore, this study was conducted to isolate fungi capable of solubilizing RP and studying the biosolubilization process, especially, under stress conditions as well as testing the response of mung bean plants to inoculation by these isolates.

MATERIALS AND METHODS

Media

National Botanical Research Institute's phosphate growth medium (NBRIP) contained L^{-1}: glucose, 10 g; $Ca_3(PO_4)_2$ (TCP) 5 g; $MgCl_2.6H_2O$, 5 g; $MgSO_4.7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g.; distilled water, 1000 mL; pH 7.0 was used (Nautiyal, 1999). Plates were prepared by adding agar (1.5%) to NBRIP medium.

Rock Phosphate and Mung Bean Cultivar

RP was kindly obtained from Soils, Water and Environment Research Institute, Agric. Res. Center, Giza, Egypt and analyzed for its P content (7.97%) and added to the growth medium instead of TCP in the amount equivalent to 50 mg P_2O_5 100 mL^{-1} (Reddy *et al*., 2002). Mung bean cultivar; Kawmy 1 was kindly obtained from Legumes Dept., Crop Res. Institute, Agric. Res. Center, Giza Egypt.

Isolation and Identification of Phosphate Solubilizing Fungi (PSF)

Soil samples characterized with high percentage of RP were incorporated in NBRIP agar medium and incubated at 30°C for 5 days. PSF were shown by the formation of a clear zone around fungal colonies. Colonies with clear zones were further purified by replanting on medium mentioned above. Comparisons of phosphate solubilizing capabilities were carried out on the base of ratio of clear zone diameter/colony diameter of each isolate. Two PSF were thus screened and identified as *A. niger* and *Penicillium* sp. (Domsch *et al*., 1980) and used for further studies. The isolates were maintained on PDA slants and sub-cultured weekly.

Solubilization Conditions of RP

The abilities of the tested fungi to dissolve the RP were determined in 250 mL Erlenmeyer flask with 50 mL of the sterile NBRIP broth medium described above and using RP (50 mg P_2O_5 100 mL^{-1}) as sole P source. Spore suspensions of the isolates were adjusted by a haemocytometer to approximately 2.5×10^6 spores mL^{-1}. Each flask was inoculated with the spore suspension at 10% (v/v) and incubated at 30°C on a rotary shaker (120 rpm).

The Tested Parameters

The investigated parameters were carried out in NBRIP broth medium as follows: (1) time course of RP solubilization (1 to 9 days), (2) shaking speeds (60 to 180 rpm), (3) incubation temperature (20 to 45°C), (4) initial culture pH (4.5 to 8, using HCl or NaOH), (5) carbon sources at 1% (Xylose, Glucose, Galactose, Fructose, Maltose, Sucrose and Starch), (6) RP concentration (0.5 to 3.5 gL^{-1}), (7) NaCl stress (1 to 7%) and (8) solubilization of different common sources of P i.e., $Ca_3(PO_4)_2$, aluminium phosphate, ferrous phosphate, sodium phytate in addition to RP. It is worthy to mention that, before using the dual culture of both fungi, an antagonism test was carried out on PDA plates.

After incubation, suspended mycelium was carefully harvested from the medium by centrifugation (4000 rpm), washed several times with distilled water and dried in an oven at 105°C to constant weight. The supernatant was collected to be analyzed for pH (HI 9321 microprocessor pH-meter), titratable acidity (TA) by the method of Cerezine *et al*. (1988) and soluble phosphate (Jackson, 1967). The uninoculated autoclaved medium with phosphate substrate was incubated under similar conditions to serve as the control.

Assay of Phytase

Phytase activity was assayed in the supernatant of *A. niger* and *Penicillium* sp. using sodium phytate as substrate (El-Sawah *et al*., 2001). One unit of phytase activity was defined as the amount of enzyme releasing one μ mole of inorganic phosphorus/mL/min.

Field Experiment

Field experiment was conducted at Tag El-Ezz Agric. Res. Station, Dakahlia, Egypt, during the summer season of 2008, to study the effect of inoculation by PSF on mung bean

Table 1: Physical and chemical characteristics of experimental soil

Soil character	Value
Physical properties	
Particle size (%)	
Coarse sand	2.44
Fine sand	5.82
Silt	35.20
Clay	56.54
Texture class	Clayey
$E.C_e$ dS m^{-1} soil paste	5.93
pH 1:2.5, Soil : Water suspension	7.90
ESP (%)	7.54
Chemical analysis	
Soluble anions meq L^{-1}	
CO_3^{--}	0.00
HCO_3^{--}	0.27
Cl^-	1.53
SO_4^-	2.90
Soluble cations meq L^{-1}	
Ca^{++}	1.40
Mg^{++}	0.70
Na^+	2.56
K^+	0.04
Some available nutrients (mg kg^{-1})	
Iron	10.66
Manganese	3.50
Zinc	1.45
Cupper	1.96
Nitrogen	40.45
Phosphorus	10.01
Potassium	268.00
Organic matter (%)	1.67
$CaCO_3$ (%)	2.31
Fungal phosphate dissolvers (log cfu g^{-1} dried soil)	1.51
Total fungi (log cfu g^{-1} dried soil)	2.87

plants cultivated in salinity affected soil (E.C. 5.93) (Table 1). Phosphate fertilization in the form of calcium superphosphate or RP was added to the soil at 1/3, 2/3 and full-recommended dose, on the base of P_2O_5 content, before sowing. Mung bean seeds were inoculated by soaking in the spore suspension (2.6×10^6 spore mL^{-1}) of *A. niger*, *Penicillium* sp. or both (*A. niger* and *Penicillium* sp., half from each) for 15 min in the presence of 16% Arabic gum as adhesive agent, air dried and then sowed immediately. Seeds treated with spore free solution are considered as control treatment. Treatments were arranged in three replicates. All recommended agricultural practices for mung bean crop were carried out. After two weeks of germination, plants were checked for nodulation.

After 30 and 60 days from sowing, plant and rhizospheric soil samples were collected for determination of numbers and dry weight of nodules $plant^{-1}$ and counts of total fungi and phosphate solubilizers on PDA and NBRIP agar media, respectively. At the end of cultivation period, plant height (cm), number of branches and pods $plant^{-1}$, seed index (100-seed weight in gram) and seed yield (kg $feddan^{-1}$) were recorded. The P content of the seeds was determined by the method of Jackson (1967) after digestion with H_2SO_4.

Statistical Analysis

The study design was fully randomized blocks. Duncan's new multiple range test were used to compare means. Simple correlation coefficient (r) was performed to examine the relationships between individual properties using the statistical analysis software; CoStat v6.4.

RESULTS AND DISCUSSION

Isolation and Identification of PSF

Fungal isolates with the ability to solubilize insoluble P were isolated from soil characterized with high level of RP. The two isolates that displayed the highest ratio of clear zone/colony diameter were selected and identified as *A. niger* and *Penicillium* sp. The zone of P biosolubilization appeared on third day of incubation on NBRIP agar. Continuous observation of the halo zone formation indicates phosphate solubilizing ability which was in increasing order up to the 7th day (Fig. 1). The appearance of a clear halo zone around the colony indicated phosphate solubilization by the fungus (Kang *et al*., 2002; Gupta *et al*., 2007). The advantage of using natural phosphate solubilizers over the genetically manipulated or ones that have been isolated from a different environmental sets-up is the easier adaptation and succession when inoculated into the medium containing RP (Xiao *et al*., 2008).

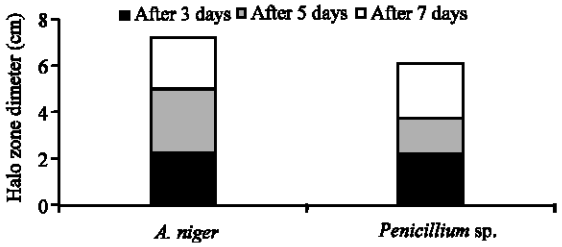

Fig. 1: Comparing the efficiency of the selected fungi in releasing P by halo zone formation

Time Course of RP Biosolubilization

The results (Fig. 2a, b) of the periodic solubilization of RP show that the solubilization of RP was achieved earlier at the third day of incubation on NBRIP broth medium supplemented with RP as a sole P source. Maximum solubilization of RP was observed after 6 and 7 days of incubation by *A. niger* (67.0 mg L^{-1}) and *Penicillium* sp. (46.2 mg L^{-1}), respectively. After that, there was no additional solubilization of RP. Maximum P solubilization by *A. niger* occurred at the end of logarithmic growth phase, whereas, it occurred at the beginning of stationary phase of *Penicillium* sp. growth.

Vyas *et al*. (2007) found significant increase with the prolongation of incubation period from 3 to 9 days, followed by a significant decline after 12 day of incubation. Narsian and Patel (2000) reported maximum release of P from China and Udaipur RPs and Sonrai and Hirapur RPs by *A. aculeatus* after 8 and 14 days of incubation, respectively, they added that

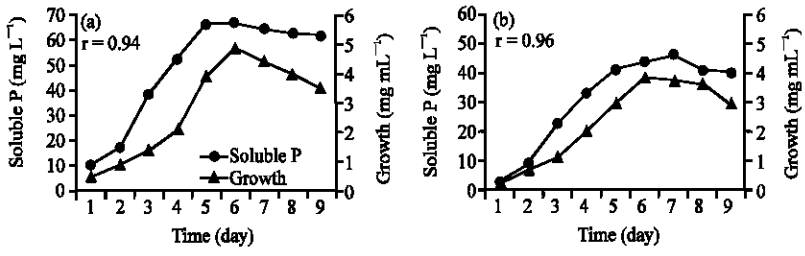

Fig. 2: Time course of RP solubilization by the isolated fungi. (a) *A. niger* and (b) *Pencillium* sp.

variation of time in biosolubilization of RP may have been due to the nature and quantity of organic acids secreted in the medium.

Data of the analysis of correlation coefficient revealed significant positive correlation between RP solubilization and growth of both *A. niger* ($r = 0.94$, $p \leq 0.01$) and *Penicillium* sp. (0.96, $p \leq 0.01$). Although, Reddy *et al.* (2002) mentioned that this correlation is not always showed positive trend.

Biosolubilization of RP at Different Shaking Speeds

Data of the content of soluble P released by the two fungal isolates as affected by different shaking speeds are shown in Fig. 3. The content of soluble P increased with the increase of shaking speed from 60 up to 150 rpm. The maximum contents of soluble P released were 70.5 mg L^{-1} (*A. niger*) and 57.2 mg L^{-1} (*Penicillium* sp.). Additional shaking speed led to obvious decline in RP solubilization. These results are in consistent with the findings of Xiao *et al.* (2008), who reported that at the excessive shaking speeds, the growth of the isolates was often weakened by the shear stress resulting from the strong stirring, that was why the released soluble P decreased when the shaking speed increased from 160 to 200 rpm.

At the different shaking speed, decrease in the final pH of the medium of both isolates showed variable values (Fig. 3a, b). As, *A. niger* recorded slightly lower pHs than that recorded by *Penicillium* sp. Such decrease in final pH was accompanied by increasing P solubility. The fungus of *Penicillium* sp. recorded significant correlation between final pH and soluble P ($r = -0.92$, $p \leq 0.05$), whereas, in *A. niger*, this correlation did not reach to the level of significancy ($r = -0.85$, ns). Many authors (Cerezine *et al.*, 1988; Vassilev *et al.*, 1995; Vassileva *et al.*, 1998; Barroso and Nahas, 2005) reported reduction in the final culture pH with the increasing of soluble P. However, no significant relationship could be established between the quantities of phosphate solubilized and drop in pH (Narsian and Patel, 2000).

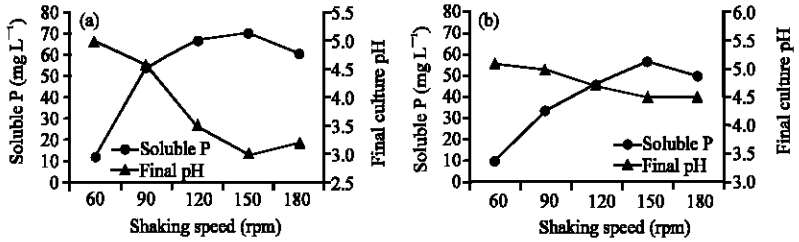

Fig. 3: Effect of shaking speed on biosolubilization of RP. (a) *A. niger* and (b) *Penicillium* sp.

Optimum Temperature and pH for Biosolubilization of RP

The optimum temperature and initial pH for RP solubilization were investigated as shown in Figures 4 and 5. Temperature and initial pH had significant effects on the biosolubilization

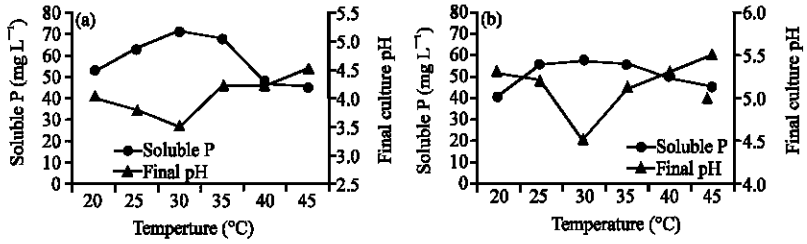

Fig. 4: Effect of temperature on the biosolubilization of RP. (a) *A. niger* and (b) *Penicillium* sp.

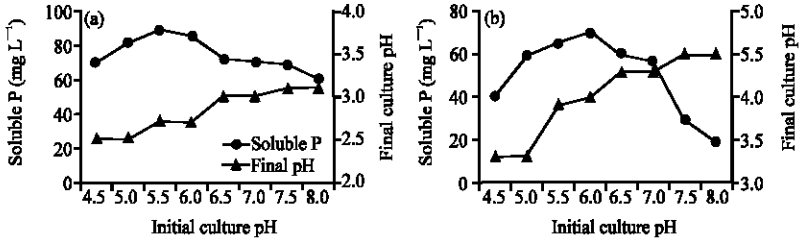

Fig. 5: Effect of initial culture pH on the biosolubilization of RP. (a) *A. niger* and (b) *Penicillium* sp.

of RP (Figures 4a, b and 5a, b). The maximum content of soluble P was recorded at 30°C, which were 70.5 and 57.2 mg L^{-1} released by *A. niger* and *Penicillium* sp., respectively (Fig. 4a, b). Vyas *et al.* (2007) reported maximum biosolubilization of RP at 36°C. As far as initial pH is concerned, there were differences between the two fungi (Fig. 5a, b). The maximum content of soluble P was recorded at 89.3 mg L^{-1} released by *A. niger* at initial pH 5.5, while it was recorded at 70.2 mg L^{-1} released by *Penicillium* sp. at initial pH 6.0. Higher or lower than the optimal temperature and initial pH, the content of soluble P decreased. In addition, there was remarkable reduction in final pH for both isolates during the investigations on temperature and initial pHs. The results are in similar to those of Barroso and Nahas (2005) and Xiao *et al.* (2008).

Biosolubilization of RP as Affected by Carbon Sources

To find out the best source of carbon that can achieve the highest phosphate solubilization, different sources of carbon were added individually in growth medium inoculated with *A. niger* or *Penicillium* sp. (Fig. 6). The obtained results showed that glucose was the best followed by fructose and xylose for the two tested fungi in phosphate solubilization. While, the other tested carbon sources showed lower values. According to Cerezine *et al.* (1988), glucose and fructose are the most frequent and abundant sugars detected in plant exudates that possibly affect the microbial population which solubilizes insoluble phosphates.

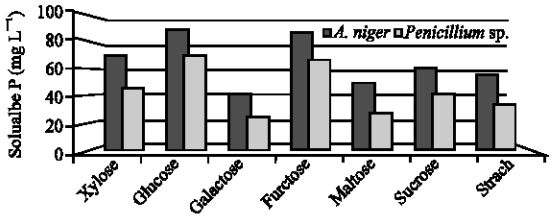

Fig. 6: Biosolubilization of RP in the presence of different carbon sources

Biosolubilization of Different Concentrations of RP

It is clearly obvious from data shown in Table 2 that both *A. niger* and *Penicillium* sp. showed positive responses in solubilization efficiency to RP concentration but with various degrees. Generally, a continuous increase in RP solubilization was observed by increasing the concentration of RP added to the growth medium until 2.5 g L^{-1}. This concentration was the optimum in yielding the maximum soluble P. The corresponding values were 99.7 mg L^{-1} for *A. niger* and 77.5 mg L^{-1} for *Penicillium* sp., above this level of RP (2.5 g L^{-1}) no further

Table 2: Releasing of soluble P by the tested fungi at different RP concentrations

	A. niger			*Penicillium* sp.		
RP ($g\ L^{-1}$)	Final 1 pH	TA (mg NaOH mL^{-1})	Soluble P (mg L^{-1})	Final 1 pH	TA (mg NaOH mL^{-1})	Soluble P (mg L^{-1})
0.5	3.2	0.64	44.3	5.3	0.16	39.8
1.0	3.0	0.73	60.7	5.0	0.66	59.9
1.5	2.8	1.79	76.6	4.7	1.35	66.5
2.0	2.7	2.3	88.3	4.3	1.90	68.2
2.5	2.7	2.11	99.7	4.0	1.97	77.5
3.0	3.5	2.00	83.7	5.1	1.34	50.1
3.5	4.8	1.13	60.8	5.5	1.00	42.7

Table 3: Correlation coefficients between every pairs of RP concentration, final culture pH, TA and soluble P, in culture media of *A. niger* and *Penicillium* sp.

Fungi	RP concentration	Final pH	TA
A. niger			
Final pH	0.59^{ns}		
TA	0.49^{ns}	-0.36^{ns}	
Soluble P	0.48^{ns}	-0.43^{ns}	0.94^{**}
***Penicillium* sp.**			
Final pH	0.00^{ns}		
TA	0.53^{ns}	-0.81^{*}	
Soluble P	0.00^{ns}	-0.95^{**}	0.79^{*}

^{ns}: Not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$

increases in solubilization rate was observed. The solubilization of RP have been reported to depend on their structure complexity, particle size and quantity of organic acid secreted by microorganisms (Pradhan and Sukla, 2005). Consequently, P is released from mineral phosphate by proton substitution from Ca_2^+ (Goldstein, 1994), while, Illmer and Schinner (1995) mentioned that RP solubilization is the result of release of protons accompanying respiration or NH_4^+ assimilation.

At all concentrations of RP, the two tested fungi showed reduction in the final pH, *A. niger* showed priority in this respect. The titratable acidity (TA) of the supernatant reached its maximum at RP concentration of 2 g L^{-1} (*A. niger*) and 2.5 g L^{-1} (*Penicillium* sp.). Analysis of correlation coefficient (Table 3) revealed that RP concentration was not related to pH, TA or soluble P, but the later was positively correlated with TA, with respect to pH, results were apparently inconsistent. The results are in accordance with those obtained by Reddy *et al.* (2002), who reported that phosphate solubilization was not always accompanied by a drop in pH, but always showed the largest production of acids.

Abdel-Hafez (1966), Vassilev *et al.* (1995), Vassileva *et al.* (1998) and Barroso and Nahas (2005) explained the biosolubilization of phosphate on the base of acid production. The significant relationship detected between pH and TA shows that the fall in pH may possibly have been the consequence more of acid production than of selective ion absorption by the fungus (Cerezine *et al.*, 1988; Gupta *et al.*, 2007). Indeed, *A. niger* is characterized by the production of large amount of acids such as oxalic and citric acids (Vassilev *et al.*, 1995; Rashid *et al.*, 2004). Such organic acids have been also, recognized for phosphate solubilization by several species of *Penicillium*, namely, *P. bilaii*, *P. citrinum*, *P. janthinellum*, *P. oxalicum* and *P. purpurogenum* (Cunningham and Kuiack, 1992; Pandey *et al.*, 2008).

Biosolubilization of RP Under Field Simulated Conditions

Aiming to apply the isolated fungi to soil-plant system, the following section was to study their efficiencies in phosphate solubilization in conditions similar to those of field, especially in salinity-affected soils.

RP Solubilization by Isolated Fungi Under NaCl Stress Conditions

The results of the effect of NaCl stress on solubilization of RP (Table 4) indicate that *A. niger* solubilized RP under salt stress up to 5%, at this concentration the reduction in soluble P did not exceed 1.2%, however, this fungus was still able to keep 52% of its efficiency at 7% NaCl. *Penicillium* sp. was less efficient, where; the reduction in soluble P reached 3.2 % at 4% of NaCl and lost 86.5% of its efficiency at 7% NaCl. Pandey *et al.* (2008) isolated RP solubilizing species of *Penicillium* tolerated salt concentration up to 20%. On the other side, Kang *et al.* (2002) observed enhancement of RP solubilization in the presence of 1% NaCl by *Fomitopsis* sp. PS 102. Higher concentrations of salt are likely to possess mechanisms for survival under stressed environments. Occurrence of such fungal communities may play an important ecological role in low nutrient status and low decomposition rates (Pandy *et al.*, 2008).

Both fungi showed reduction in pH and increasing in TA with the increasing of soluble P. Rajankar *et al.* (2007) reported that the application of the biofertilizer prepared by fungi may helpful to reduce the salinity of soil by neutralization phenomenon, because these microorganisms release acids in very minute quantity during phosphate solubilization.

Table 4: Variation in final culture pH, soluble P and TA by *A. niger* and *Penicillium* sp. grown under salt stress conditions

	A. niger				*Penicillium* sp.			
			Soluble P				Soluble P	
NaCl ($g\ L^{-1}$)	Final pH	TA (mg NaOH mL^{-1})	($mg\ L^{-1}$)	Reduction (%)	Final pH	TA (mg NaOH mL^{-1})	($mg\ L^{-1}$)	Reduction (%)
0	2.7	2.11	99.7	0.0	4.0	1.97	77.5	0.0
1	2.8	2.10	99.7	0.0	4.0	1.97	77.5	0.0
2	3.1	2.10	99.5	0.2	4.2	1.97	76.1	1.8
3	3.4	2.15	99.1	0.6	4.5	1.95	76.5	1.3
4	3.7	2.00	99.0	0.7	4.5	1.80	75.0	3.2
5	4.4	2.10	98.5	1.2	5.2	0.89	34.9	55.0
6	4.8	1.55	85.3	14.4	6.0	0.51	16.3	79.0
7	5.0	0.78	51.8	48.0	6.2	0.11	10.5	86.5

Biosolubilization of Common P Sources

As the initial investigations indicated the potentiality of the two tested fungi in solubilization of RP, it was considered important to extend the study to determine the phosphate dissolving ability of those fungi on some common phosphate forms exist in soil. Data of Table 5 show that, the two isolates were capable of solubilizing all the forms of insoluble P. Solubilization of $Ca_3(PO_4)_2$ (TCP) was found to be easier than other sources. However, the solubilization levels varied with the two isolates. The released soluble P from the common phosphate sources in descending order was in presence of TCP (202.3 and 211.1 mg L^{-1}), followed by aluminium phosphate (Al-P) (104.7 and 82.6 mg L^{-1}), RP (99.7 and 77.5 mg L^{-1}), sodium phytate (S-P) (89.1 and 50.4 mg L^{-1}) and ferrous phosphate (Fe-P)

Table 5: The efficacy of the tested fungi in solubilization of some common sources of phosphate

	A. niger			*Penicillium* sp.		
P source	Final pH	TA (mg NaOH mL^{-1})	Soluble P ($mg\ L^{-1}$)	Final pH	TA (mg NaOH mL^{-1})	Soluble P ($mg\ L^{-1}$)
$Ca_3(PO_4)_2$	3.1	2.74	202.3	5.1	2.44	211.1
RP	2.7	2.11	99.7	4.0	1.97	77.5
$AlPO_4$	4.1	2.10	104.7	5.3	2.03	82.6
$Fe_3(PO_4)_2$	5.1	0.67	33.7	5.5	0.55	19.9
Sodium phytate	5.0	1.90	89.1	5.7	1.81	50.4

(33.7 and 19.9 mg L^{-1}) by *A. niger* and *Penicillium* sp., respectively. Interestingly, *A. niger* showed a higher soluble P releasing ability than *Penicillium* sp. with all the P sources except TCP.

The microbial solubilization of complex phosphate is not restricted to calcium salts as microorganisms also act upon iron, aluminium and other phosphates (Kang *et al*., 2002). The majority of phosphate solubilizing microorganisms are able to solubilize calcium-phosphorus complexes but only a few can solubilize iron-phosphorus and aluminium-phosphorus complexes (Kucey *et al*., 1989). Kang *et al*. (2002) found that TCP was maximally solubilized by *Fomitopsis* sp. PS 102 compared with RP and Al-P. In addition, Xiao *et al*. (2008) concluded that the capability of P solubilization was positively correlated with the grade of P sources, the higher the grade, the higher the content of soluble P released.

The maximum production of acids (Table 5) was observed with TCP (2.74 and 2.44 mg NaOH mL^{-1}) followed by RP (2.11 and 1.97 mg NaOH mL^{-1}) and Al-P (2.1 and 2.3 mg NaOH mL^{-1}), for *A. niger* and *Penicillium* sp., respectively. These results are similar to those obtained by Barroso and Nahas (2005), who reported significant positive correlation between the contents of released P and TA.

Antagonism and Biosolubilization of Phosphate Using Dual Culture

The present experiment was initiated to study the interactions between the two tested fungi, aimed at clarifying their antagonistic effect and their solubilization efficiency on different sources of mineral phosphate when applied in a form of dual inoculum. On PDA plates results obtained herein indicated no visible antagonism between *A. niger* and *Penicillium* sp. On contrary, a clear compatibility had been confirmed, although, the members of the genus *Penicillium* are known to be potent producers of antibiotics. Hence, the dual inoculation was applied in solubilization of different P sources.

As illustrated in Fig. 7, the presence of both fungi in the same culture medium increased the efficiency of solubilization of different phosphate sources. The pH and TA of dual inoculation lied within moderate levels between cultures inoculated with individual *A. niger* or *Penicillium* sp. as shown previously in Table 5. TCP was the most favorable as P source by the mixed cultures. One very attractive approach for RP solubilization is the application of microorganisms able to excrete low-molecular-mass organic acids, which can strongly increase the concentration of phosphorus by mechanisms involving chelation and exchange reactions (Vassilev *et al*., 1995; Rashid *et al*., 2004).

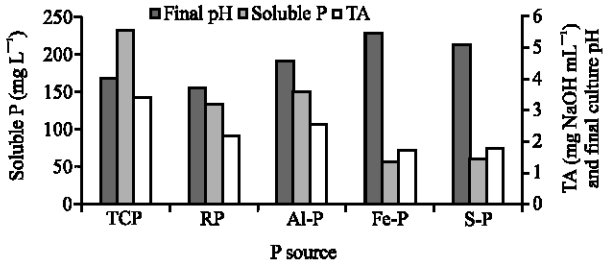

Fig. 7: Solubilization of different P sources by mixed culture of *A. niger* and *Penicillium* sp.

Phytase Activity of the Individual and Mixed Fungal Cultures

Data in Fig. 8 show that the tested isolates were active in phytase production, either in individual or in dual culture. Sodium phytate was the most favorable source than the other P forms for phytase activity. Releasing soluble P from different P sources suggest that

the enzyme is constitutive and the solubilization process by these isolates is also due to the activity of phytase and acid phosphatase (Achala *et al*., 2007; Vassilev *et al*., 2007).

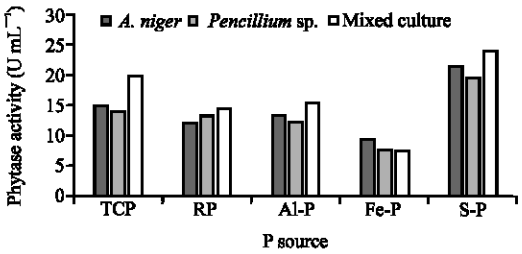

Fig. 8: Phytase activity of *A. niger*, *Penicillium* sp. and their mixed culture on different P sources

Application of PSF in Soil-Mung Bean System

Nodulation Status of Mung Bean Plants

Data presented in Table 6 show that inoculation by the two tested fungi in the form of single or dual inoculum significantly increased the number of nodules at all levels of mineral phosphate fertilizer. The stimulation varied however, according to the level of P applied. This was true at early and later stage of growth. The improvement of nodulation was further strengthened, by the dual inoculation, especially, at 2/3 dose of CSP or full dose of RP. This could be observed from checking the differences between those two treatments and uninoculated control after 60 days of planting time. The average increase was about 67.05 and 60.57%, respectively. It is worthy to mention that the dry weight nodular tissues showed similar trend to that observed in case of nodule number. Such results reflect the role of mode of action of the two tested fungi in solubilization efficiency of mineral phosphate through

Table 6: Nodulation status of mung bean at 30th and 60th day from sowing

Treatment		Number of nodules plant^{-1}		Dry weight of nodules (mg plant^{-1})	
P fertilizer	Inoculation	30 days	60 days	30 days	60 days
Calcium superphosphate					
1/3 P dose	A	49.0fg	109.7fg	40.0de	105.3f
	P	49.0fg	94.7h	41.7cd	81.3i
	AP	52.7de	112.7ef	41.3d	113.5e
2/3 P dose	A	50.3d-g	112.3f	37.0ef	148.7b
	P	56.7c	103.3g	45.7ab	153.3b
	AP	67.7a	147.0a	49.0a	163.0a
Full dose	A	51.3d-g	116.7d-f	27.3g	151.3b
	P	50.7d-g	104.0g	34.3f	122.3d
	AP	61.0b	137.3b	47.0ab	164.8a
Rock phosphate					
1/3 P dose	A	52.0d-f	121.0c-e	29.0g	142.7c
	P	51.7d-g	114.0ef	44.7bc	99.3g
	AP	49.7e-g	124.0cd	39.7de	148.5b
2/3 P dose	A	53.0d	123.37cd	30.3g	119.0d
	P	45.0h	110.0fg	34.3f	102.0fg
	AP	52.3de	129.0c	46.7ab	142.3c
Full P dose	A	58.0c	115.0ef	36.0f	112.9e
	P	48.7g	114.3ef	37.7ef	92.7h
	AP	56.7c	141.3ab	47.3ab	153.3b
Control[1]		32.7i	88.0h	21.0h	63.0j

[1]Full dose of recommended phosphate fertilizer (Calcium superphosphate) without inoculation. A: *A. niger*, P: *Penicillium* sp., AP: Dual inoculation. Means within the column followed by the same letter(s) are not significantly differed at $p \leq 0.05$

production of a large amount of organic acids in plant rhizosphere, which in return increasing the level of available nutritional elements (Hauka *et al*., 1996) required at trace level both by the plant and nodule system.

Mung Bean Growth and Yield

Concerning the effect of phosphate solubilizing fungi on the growth and yield of mung bean plants under levels of calcium superphosphate (CSP) or RP, data (Table 7) indicated that dual inoculation with *A. niger* and *Penicillium* sp. increased significantly plant height in the presence of 2/3 dose of CSP compared to control. Similarly, the dual inoculation showed the same trend in plant height in the presence of full dose of RP. Meanwhile, no significant increase was observed in the inoculation with *Penicillium* sp. in the presence of 1/3 dose of CSP. In addition, no significant variation among treatments in number of branches per plant. However, the significant effect of the used organisms on plant growth can be attributed not only to nutrient availability (Hauka *et al*., 1996) but also, to the production of growth substances (Sundaro, 1988).

Concerning the seed yield, the main and final expression of many interacting factors, it could be concluded that the dual inoculation still showed the superiority in achieving seed yield. The beneficial effect of mineral phosphate fertilization in combination with dual inoculation of *A. niger* and *Penicillium* sp. could be arranged in descending order (percentage of increase over the control) as follows: 2/3 dose of CSP (18.7%) > full dose CSP (17.8%) > full dose of RP (13.1%) >2/3 dose of RP (10.1%) >1/3 dose of CSP (5.2%) >1/3 dose of RP (3.7%). These results provide sufficient basis to recommend the use of 2/3 or full dose of CSP or RP in combination with dual inoculation with *A. niger* and *Penicillium* sp. as successful and proper management of mung bean production.

Table 7: Effect of inoculation with phosphate solubilizing fungi on growth and yield of mung bean grown under levels of different phosphate fertilizers

Treatment		Plant height	Branches No.	Pods No.	Seed index	Seed yield
P fertilizer	Inoculation	(cm)	plant^{-1}	plant^{-1}	(g)	(kg feddan^{-1})
Calcium superphosphate						
1/3 P dose	A	77.4bc	3	20.3gh	4.17g-i	742.2h
	P	73.9d	3	19.3i	4.17g-i	759.4g
	AP	76.7c	3	20.7fg	4.40ef	797.3d
2/3 P dose	A	80.6a	3	22.7bc	4.67bc	854.2b
	P	79.7a	3	22.0cd	4.78b	839.5c
	AP	81.2a	3.7	23.7a	5.20a	899.3a
Full dose	A	78.1b	3	21.7de	4.23gh	856.8b
	P	79.7a	3	20.3gh	4.57cd	840.9c
	AP	80.6a	3	21.3d-f	4.53c-e	892.3a
Rock phosphate						
1/3 P dose	A	80.6a	3	20.7fg	4.18g-i	773.9f
	P	80.2a	3	19.7hi	4.05i	756.6g
	AP	80.9a	3	21.0e-g	4.56c-e	785.5d-f
2/3 P dose	A	80.3a	3	22.7bc	4.40ef	788.9de
	P	78.0b	3	20.3gh	4.17g-i	747.6gh
	AP	80.7a	3	21.3def	4.22gh	834.2c
Full P dose	A	79.8a	3	23.0ab	4.47de	784.6ef
	P	80.5a	3	22.7bc	4.10hi	749.6gh
	AP	80.0a	3.3	22.7bc	4.67bc	857.2b
Control[1]		75.1d	3.3	21.0e-g	4.28fg	757.7g

[1]Full dose of recommended phosphate fertilizer (Calcium superphosphate) without inoculation, A: *A. niger*, P: *Penicillium* sp., AP: Dual inoculation. Means within the column followed by the same letter(s) are not significantly differed at $p \leq 0.05$

Phosphorus Content of Mung Bean

Data of the P content and P uptake of mung bean seeds (Table 8) show that the dual inoculation with 2/3 dose of CSP or RP recorded the highest significantly increment of P content in the seeds of mung bean, compared to control. These treatments also, recorded higher P uptake. These results are inline with the findings of Richa *et al.* (2007) on maize plants. It is generally accepted that the mechanisms of phosphate solubilization in soils is closely related to the complex forming properties of low molecular weight organic acid (Kucey *et al*., 1989). Soil microorganisms are deeply involved in this process and their role in solubilization of phosphate bearing materials has been the subject of an interesting number of studies in soil plant system. The attractive approach of microbial mediated solubilization of RP has successfully proved using many filamentous fungi (Vassilev *et al*., 1995; Reddy *et al*., 2002). Moreover, Richa *et al.* (2007) recommended phosphate solubilizing fungi along with RP in alkaline soil (as the soil in the present study), since, the pH of the soil lowered compared to the initial pH. The presence of high P content in mung bean plants may explain the improvement in its growth and yield. Since, P entering in the composition of ATP.

Table 8: P content of mung bean as influenced by inoculation with PSF and phosphate fertilization treatments

Treatment		Phosphorus in mung bean seed	
P fertilizer	Inoculation	Content (%)	Uptake (mg plant seeds^{-1})
Calcium superphosphate			
1/3 P dose	A	0.197fg	20.09hi
	P	0.189h	19.75i
	AP	0.200f	22.24f
2/3 P dose	A	0.246a-e	29.90bc
	P	0.243c-e	29.27c
	AP	0.251a	31.52a
Full dose	A	0.244b-e	29.68bc
	P	0.247a-d	30.09bc
	AP	0.249a-c	31.46a
Rock phosphate			
1/3 P dose	A	0.189h	20.89gh
	P	0.193gh	21.25g
	AP	0.196fg	22.37f
2/3 P dose	A	0.240e	27.34d
	P	0.248a-d	25.97e
	AP	0.249a-c	29.40bc
Full P dose	A	0.242de	27.17d
	P	0.243de	25.97e
	AP	0.250ab	30.42b
Control[1]		0.183i	19.52I

[1]Full dose of recommended phosphate fertilizer (Calcium superphosphate) without inoculation, A: *A. niger*, P: *Penicillium* sp., AP: Dual inoculation. Means within the column followed by the same letter(s) are not significantly differed at $p \leq 0.05$.

Population of Total and Phosphate Dissolving Fungi

Data in Table 9 show the incidence of both total and phosphate dissolving fungi in the rhizospheric soil of mung bean plants as affected by inoculation with *A. niger* and/or *Penicillium* sp. Obtained results showed increment in both counts with the increasing of time for all inoculated plants as compared to uninoculated control. These increases in both fungal populations may be due the ability of the isolated fungi to survive under NaCl stress (Table 4) (the experimental soil (Table 1) is saline) and their potential activity in solubilization of different phosphorus forms (Table 5 and Fig. 7) that may commonly exist in the soil, as well as, their phytase activity (Fig. 8) which hydrolysis phytate compounds in the plant residues in the soil.

Table 9: Counts of total and phosphate dissolving fungi in the rhizospheric soil of mung bean plants inoculated with *A. niger* and/or *Penicillium* sp.

Treatment		Total fungal count (logarithm of cfu g^{-1})		Fungal phosphate dissolvers (logarithm of cfu g^{-1})	
P fertilizer	Inoculation	30 days	60 days	30 days	60 days
Calcium superphosphate					
1/3 P dose	A	5.568	5.595	3.529	3.685
	P	5.288	5.517	3.238	3.385
	AP	5.666	5.714	3.452	3.636
1/32 dose	A	5.663	5.718	3.611	3.788
	P	5.556	5.626	3.361	3.553
	AP	5.616	5.650	3.633	3.793
Full dose	A	5.572	5.615	3.538	3.721
	P	5.516	5.609	3.220	3.424
	AP	5.710	5.756	3.414	3.621
Rock phosphate					
1/3 P dose	A	5.495	5.526	3.548	3.705
	P	5.613	5.672	3.113	3.204
	AP	5.467	5.504	3.334	3.458
23 P dose	A	5.615	5.658	3.364	3.55
	P	5.425	5.501	3.429	3.647
	AP	5.74	5.75	3.496	3.693
Full P dose	A	5.648	5.695	3.555	3.742
	P	5.517	5.572	3.333	3.545
	AP	5.687	5.700	3.461	3.710
Control[1]		5.097	5.451	2.418	2.648

[1]Full dose of recommended phosphate fertilizer (Calcium superphosphate) without inoculation. A: *A. niger*, P: *Penicillium* sp., AP: Dual inoculation

Based on the present data, the amendment of soil with RP along with PSF improves mung bean growth, yield and P-uptake and saves about 1/3 of phosphate fertilizer dose. Thus, it could be recalled that, *A. niger* and *Penicillium* sp. could serve as phosphate solubilizers in RP amended soil.

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