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Effect of phosphate-solubilizing bacteria and oxalic acid on phosphate uptake from different P fractions and growth improvement of aerobic rice using ^{32}P technique

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Abstract

Using the isotope dilution ^{32}P technique, a study was conducted to evaluate the P uptake and growth improvement of aerobic rice genotype (M9) inoculated with phosphate-solubilizing bacteria (PSB16, *Bacillus* sp.) and applied with Christmas Island Phosphate Rock (CIPR), and oxalic acid (OA). An absence of PSB and OA was considered as control treatment. The inoculation of PSB16 strain and OA application produced lower specific activity (12.57 Bq mg P^{-1}) in aerobic rice. The PSB16 and OA were able to release (82.72%) P either from the added CIPR or from the fixed, less available native soil P. Inoculation of PSB16 along with CIPR and OA enhanced P uptake and simultaneously increased P use efficiency (1.12%). A significantly higher photosynthesis rate (9.29 µmol CO_2 m^{-2}-s^{-1}) and indoleacetic acid (0.26 mg kg^{-1}) concentration in soil was found in the PSB16, OA and PR applied treatments. The highest amounts of water soluble P, Ca-P, Fe-P and Al-P were found in PSB inoculated samples along with the PR and oxalic acid treatments, whereas higher soluble P from all fractions was found in PSB16 inoculated and PR applied treatments. Among all of the fractions, the concentration of Fe-P was comparatively higher (237.67 mg kg^{-1}) than the other fractions and the P fractions were ranked in the order of Fe-P > Al-P > Ca-P > soluble P. In conclusion, PSB16 and organic acid have the ability to solubilize sparingly soluble phosphatic fertilizer and mobilize different fractions of fixed P from soil to the plant. The continuous supply of soluble P in soil P pool and phytohormone in the root environment increased the P uptake and improved the growth of aerobic rice.

Keywords: biomass; enzymes; indoleacetic acid; photosynthesis; solubilization.
Abbreviation: CIPR- Christmas Island phosphate rock; dpm- disintegrations per minute; mM- milli mole; OA- oxalic acid; PSB- phosphate-solubilizing bacteria; SA-specific activity; Pdfl- phosphate derived from labile fertilizer; µCi- micro curie.

Introduction

Phosphatic fertilizer management in aerobic rice is critical because 75-90% of the applied phosphate (P) fertilizers are precipitated by complexes of Fe, Al and Ca in the soils immediately after application. A suitable alternative that can mobilize this bio-element is therefore required (Stevenson, 1986). It is known that phosphate-solubilizing bacteria (PSB) have the ability to increase P availability to meet the requirements for plant metabolism and uptake of P (Panhwar et al., 2011; Tao et al., 2008). There are several mechanisms such as organic acid and enzyme production, the release of H^+, chelation, and respiratory H_2CO_3 production that are recognized for P solubilization. However, it is established that the main mechanism used to mineralize inorganic P is the production of organic acids (Rodriguez et al., 2004; Rodríguez and Fraga, 1999). The application of oxalic acid has been proven to be effective for the solubilization of P and is more capable of solubilizing P than other organic acids (Asea et al., 1988; Wei et al., 2009). Strong binding abilities of oxalic and citric acids have led to these acids being established as the most competent agents to solubilize soil P (Jones, 1998). The addition of organic acids increased the solubility of P from PR and enhanced the effectiveness up to 88% during the planting period (Bolan et al., 1994). Besides P solubilization different organic acids play a vital role in the soil removal of Al toxicity in the soil (Ma and Furukawa, 2003). Therefore, in plant and microbial nutrition, organic acids have been assumed to participate in the mobilization and solubilization of weakly soluble nutrients (Micales, 1997). There are evidences of significant and constant quantities of organic acids in the soil solution (Shen et al., 1996). However, the amount of organic acids has been found to be very low in the soil solution, usually from 1-50 μM (Strobel, 2001). Organic P forms can be mineralized into inorganic P by phosphatase and phytase enzymes. The considerable amount of phosphatase activity in soil has been documented (Sarapatka and Kraskova, 1997) and different levels of microbial phosphatase activity have been observed in various types of soils (Kucharski et al., 1996). It has also been recorded that soil enzymes directly are involved in soil P cycle (Gil Sotres et al., 2005). Moreover, enzymes play an important role in P release and simultaneously improve crop yield (Wyszkowska and Wyszkowski, 2010). Phytases are known to be hydrolyzing phytates to a series of lower phosphate esters of myo-inositol and phosphate; this also a contribution in plant nutrient cycle. Phosphorus is known as an essential element for plants and an expensive fertilizer. In tropical soils less P availability is a common problem and most of the soil P remains as a fraction of Fe or Al-P. The

Table 1a. Characteristics of experimental soil and PSB16 (*Bacillus* sp.) strain.

Physical and chemical properties of soil		*PSB16 (Bacillus sp.)*	
Texture	Sandy clay loam	Host	Rice (root endophytes)
pH	5.0	Gram reaction	+ive
% OC	1.01	IAA production (mg l^{-1}):	6.78 (broth culture)
%N	0.12	Siderophore production	+ive
P (mg/kg):	9.6	% P solubilization in CIPR broth:	3.90 (48 h)
K (cmol (+) kg^{-1}):	0.54	Acetylene reduction assay	-ive

†IAA, indole aceticacid; CIPR, Christmas Island Phosphate Rock.

Table 1b. Organic acid production by the PSB16 in different media

Organic acids production by the PSB16 *(Bacillus sp.)*			
Acids	Broth (mg L^{-1})	Soil (uM)	Roots (uM)
Oxalic	0.01	10.0	115
Malic	0.04	0.77	6.90
Succinic	0.25	0.16	8.40
Propionic	0.02	0.01	4.12

†PSB, phosphate-solubilizing bacteria.

Table 1c. Characteristics of Christmas Island Phosphate Rock

Size of CIPR	Total P %	P_2O_5 %	Total Ca%	Solubility (% P_2O_5)			
				H_2O	2% formic acid	2% Citric acid	2% Nent Amm. Citrate
<100 µm	14.0	32.2	24.2	0.01	11.6	9.3	3.6

†CIPR, Christmas Island Phosphate Rock; P, phosphorus.

comparatively cheap source of phosphate rock is less soluble; however, it has been proven that the bio-availability of PR can be increased by applying PSB (Panhwar et al., 2011; Zapata and Axmann, 1995). The ^{32}P isotope tracer technique is an important tool in agricultural phosphate research. It gives more reliable and accurate information on different aspects of phosphate behavior in the soil and soil-plant systems. The use of the isotope ^{32}P has made it possible to differentiate the available P from the original soil and the fertilizer P source. Radioactive P (^{32}P) can be used to evaluate the exchange rates between the P in the soil solution and solid phases of the soil (Fardeau, 1993). It is assumed that all of the 'labile' P in this fraction, will achieve isotopic exchange within a short experimental period (Fardeau, 1993). PSB can release P (^{31}P), either from the addition of PR or as a result of low available indigenous P sources. Using the ^{32}P isotope can confirm the origin of P in plants, i.e. whether they have utilized indigenous soil P or P from the PR released by the microbial involvements (Toro et al., 1997). The PSB has the ability to mobilize P from sparingly soluble P fertilizers in a better environmental friendly manner and the ^{32}P isotopic dilution is the best technique for assessing the agronomic effectiveness of the PR fertilizer in soil. In addition, the external application of OA may alter inorganic P availability in the soil. With these hypotheses therefore, the present study was undertaken to determine the P solubilization by phosphate solubilizing bacteria and oxalic acid from phosphate rock and different soil P fractions using the ^{32}P technique. The effect of PSB inoculation and oxalic acid application on P uptake and growth improvement of aerobic rice was also studied.

Results

Phosphate fractionation in soil

Total P content of the different treatments in aerobic rice soil varied from 266 to 408 mg kg^{-1} (Table 2). The variability in

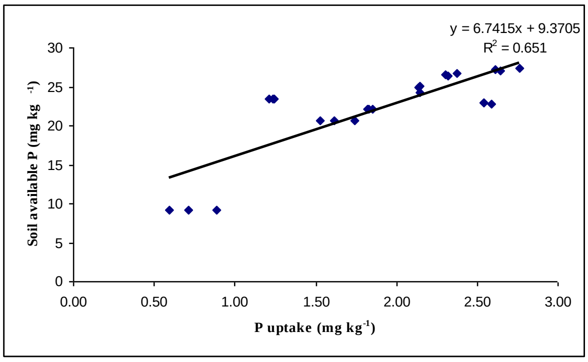

Fig 1. Relationship between plant P uptake and soil available P in aerobic rice

the inorganic P fractions appeared to be related to the different treatments. The amount of P fractions (Ca-P, Al-P Fe-P and organic-P) comprised the main fraction of the total P. The easily soluble P_w (1 M NH_4 Cl extractable) varied widely between the treatments and the values ranged from 7.35 to 18.87, whereas, the highest P_w was found in PSB inoculated treatments with the application of PR and OA. The Aluminum P (Al-P) varied from the 14.33 to 77.97 mg kg^{-1}, with the highest Al-P obtained by PSB with PR and OA (77.97 mg kg^{-1}). The iron P (Fe-P) varied with higher values from 27.63 to 237 mg kg^{-1}. The highest Fe-P (237.67 mg kg^{-1}) was observed in PSB inoculated with PR and OA. The calcium P (Ca-P) was found in the range of 7.27 to 73.43 mg kg^{-1}. The highest Ca-P was recorded by PSB inoculation with PR and OA application. Organic-P had a range of 73 to 209 mg kg^{-1}. Among the inorganic fractions higher amounts of P came from the Fe-P followed by Al-P. The simple correlation co-efficient (r) between inorganic P fractions revealed that soluble P was highly positively correlated with total P to Pw (r = 0.937; P<0.001), Al-P (r = 0.908; P<0.001), Fe-P (r =

0.960; <0.001), Ca-P (r = 0.775; P<0.001) and organic-P (r = 0.974; P<0.001) in the experimental soil (Table 3).

Effects of PSB, oxalic acid and P fertilizer on P uptake using ^{32}P technique

The PSB inoculation with PR and oxalic acid in aerobic rice produced lower specific activity (^{32}P) than their comparable non-inoculated treatments in aerobic rice plants did (Table 4). The lowest specific activity (12.57 Bq mg P^{-1}) was found in PSB inoculated with PR and oxalic acid followed by PSB with PR treatments (32.1 Bq mg P^{-1}). The PSB inoculation also showed significantly ($P<0.05$) higher values in plant P uptake and the amount of P derived from the unavailable sources. The highest plant P uptake (1.20 mg P Pot^{-1}) and released P from low-available P sources (82.72%) were found in the inoculated PSB, PR and oxalic acid treatments (Table 4). The highest P use efficiency was obtained in PSB inoculated with CIPR and OA (1.12%) and followed by PSB with PR (1.04%) treatments (Table 4). A significantly positive relationship was observed in P uptake and available soil P in the aerobic rice (Fig. 1).

Phosphatase and phytase enzyme activities in rice roots

Enzyme activities increased due to the inoculation of PSB. There were two enzymes (phosphatase and phytase) with activities determined in the aerobic rice roots. The PSB inoculation produced higher values of both enzymes as compared to non-inoculated plants. The highest phosphatase (10.68 µg p-NP g^{-1} root dry wt ha^{-1}) activity (Fig. 2) and phytase (25.71 U mg^{-1}) activity (Fig. 3) were found with the addition of PSB combined with PR and oxalic acid.

Production of indoleacetic acid in soil

The PSB inoculated treatments produced higher amounts of indoleacetic acid (IAA) in the soil of aerobic rice compared to the non-inoculated (Fig. 4). The production of IAA varied within the treatments. The highest amount of IAA (0.26 mg kg^{-1}) was observed in soil inoculated with PSB with the combination of PR and oxalic acid.

Leaf photosynthesis

The PSB inoculation with PR application significantly increased leaf photosynthesis in rice genotype M9. Significant differences were observed for leaf photosynthetic rate among treatments with time (Table 5). The highest leaf photosynthesis (9.29 µmol CO_2 m^{-2}-s^{-1}) was found in PSB inoculated treatments combined with oxalic acid and PR fertilized 60 days after planting.

Grain yield

The inoculation of PSB and CIPR along with OA application had a positive effect on the aerobic rice yield (Table 6). The PSB inoculation showed better grain and straw yield than the non-inoculated treatments. The data regarding the PSB inoculation, CIPR and oxalic acid application on the percentage of unfilled or non-ripened grains $panicle^{-1}$ are shown in Table 6. The PSB, CIPR and oxalic acids reduced the percentage of imperfectly ripened grains. The lowest percentage of unfilled grains was recorded in PSB with PR and OA (16.30%) followed by (16.84%) in PSB with CIPR application. The results also revealed that there were differences in the seed index of different treatments. Higher grain (1000) weight was found in PSB-inoculated treatments with the application of PR and OA compared to non-inoculated treatments. However, there was no significant grain weight difference observed between PSB inoculation with and without OA application. The data on the number of panicles $plants^{-1}$ revealed that PSB with CIPR and OA application on rice crop significantly increased the production of panicles $plant^{-1}$ (Table 6). The highest number (4.04) of panicles was found in PSB, CIPR and OA treatment. In contrast, there were no significant differences found in the number of panicles $plant^{-1}$ between PSB with CIPR and OA application. PSB inoculation and the application of PR and OA significantly increased the grain weight and plant biomass of aerobic rice (Table 6). The highest grain weight (16.83 g) was found in PSB-inoculated treatments combined with CIPR and OA, followed by PSB combined with PR (16.76 g). The highest plant biomass (18.36 g) was found in the PSB-inoculated treatment along with CIPR and OA application which was statistically identical to PSB and PR application treatment. The non-inoculated treatments produced lower grain yield and biomass of rice. The PSB inoculation only enhanced grain weight by 24-38% and plant biomass by 108-146%, whereas the, application of OA produced 22 - 29% grain weight and 37-112% plant biomass increments respectively, over the control. Higher grain weight increment and higher plant biomass increments were obtained with both the PSB-inoculated and the OA applied treatments.

Nutrient concentration in plant tissue, grain and total percent protein P in aerobic rice

The highest concentrations of macro-nutrients in plant tissues were found in PSB inoculated treatments. Significantly high plant and grain tissue N, P and K contents were found in PSB-inoculated and oxalic acid applied treatments (Table 7). The total protein content was not significantly different between the treatments. However, the highest protein content (6.33) was observed in PSB-inoculated samples with the application of CIPR and OA followed by in CIPR with OA applied treatments (Table 7). There was a significant ($P< 0.05$) positive relationship between P uptake and the grain yield of aerobic rice (Fig. 5).

Soil pH, total bacterial population and organic acids residues after harvesting

The application of oxalic acid (20 mM) did not change the soil pH and had no adverse effect on the total soil bacterial population (data not shown). The amount of organic acids from soil samples determined at harvesting show that there were no residual oxalic acids.

Discussion

Inoculation of phosphate solubilizing bacteria and the application of oxalic acid increased P uptake in aerobic rice. The lower specific activity (^{32}P) in the aerobic rice tissue proved a positive effect of PSB inoculation or OA application to make the bio-available P from phosphate rock and native soil sources. This result is in concurrence with the earlier findings of Bolan (1991), who found lower values of ^{32}P in the inoculated treatments. The PSB treatments showed effectiveness at releasing ^{31}P from sparingly soluble sources and the total amount of P derived from either the available (labeled) soil fraction (Pdfl) or from the added PR (PdfCIPR) in plants. In fact, PSB released P from the low-available P

Table 2. Fractions of soluble P, aluminum-P, iron-P, calcium-P, organic-P, and total P (mg kg^{-1}) in the soils collected from aerobic rice.

Treatments	Pw		Al-P		Fe-P		Ca-P		Organic-P		Total P	
	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$
PSB_0+OA_0	7.3f	14.9d	14.3f	55.4d	27.6g	143.6e	7.2g	33.5e	209.4b	147.3d	266f	395d
PSB_0+OA_{20}	13.6e	16.6c	45.5e	56.9d	106.6f	159.0dc	23.3f	43.2c	187.8c	122.2a	377e	498b
$PSB_{16}+OA_0$	16.1c	17.2b	55.5d	73.7b	149.3de	190.0b	33.4e	61.0b	135.5e	73.9g	390d	416c
$PSB_{16}+OA_{20}$	16.8c	18.8a	62.8c	77.9a	170.6b	237.6a	38.4d	73.4a	120.3f	78.0g	409c	476a
PSB_{16}	***		***		***		***		***		***	
OA	***		***		***		***		***		***	
PR	***		***		***		***		***		***	
PSB_{16}×OA	***		***		***		*		***		***	
PSB_{16}×PR	***		***		*		**		***		*	
OA×PR	***		**		***		***		***		*	
PSB_{16}×OA×PR	***		***		***		***		***		***	

*P < 0.05; **P < 0.01; ***P<0.001. †CIPR=Phosphate rock, PSB_{16} = *Bacillus* sp., PSB_0 = non-inoculated, OA = oxalic acid and PUE = phosphate use efficiency ‡Means with in the same column followed by the same letters are not significantly different at P<0.05.

Table 3. Correlation co-efficient between different P fractions with total P.

	Total P	Organic P	Soluble P	Al-P	Fe-P	Ca-P
Total P	-	0.974**	0.937**	0.908**	0.960**	0.775**
Organic P		-	0.742**	0.775**	0.670*	0.760**
Soluble P			-	0.981**	0.928**	0.669*
Al-P				-	0.949**	0.760**
Fe-P					-	0.808**
Ca-P					-	-

*P < 0.05; **P < 0.01.

Table 4. Specific activity of ^{32}P and concentration of P in plant tissue of aerobic rice.

Treatments	Specific activity ^{32}P		Pdfl (%)		PdfPR (%)		P uptake (mg P pot^{-1})		PUE(%)	
	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$
PSB_0+OA_0	152.9a	84.8b	55.4d	91.6a	8.3g	44.5d	0.34f	0.84d	0	0.43f
PSB_0+OA_{20}	83.4b	48.0dc	38.4e	78.5b	21.4f	61.5c	0.66d	0.99c	0.16g	0.77d
$PSB_{16}+OA_0$	54.1c	32.1de	30.9f	73.3b	26.6f	69.0b	0.75e	1.09b	0.56e	1.04b
$PSB_{16}+OA_{20}$	35.3d	12.5e	17.2g	67.1c	32.8e	82.7a	0.95c	1.20a	0.89c	1.12a
PSB_{16}	***		***		***		***		***	
OA	***		***		***		***		***	
PR	***		***		***		***		***	
PSB_{16}×OA	***		***		***		***		***	
PSB_{16}×PR	**		***		***		***		**	
OA×PR	NS		NS		NS		***		*	
PSB_{16}×OA×PR	***		***		***		***		***	

*P < 0.05; **P < 0.01; ***P<0.001†CIPR=Phosphate rock, PSB16 =(*Bacillus* sp.), OA = oxalic acid and PUE = phosphate use efficiency
‡Means within the same column followed by the same letters are not significantly (NS) different at P<0.05.

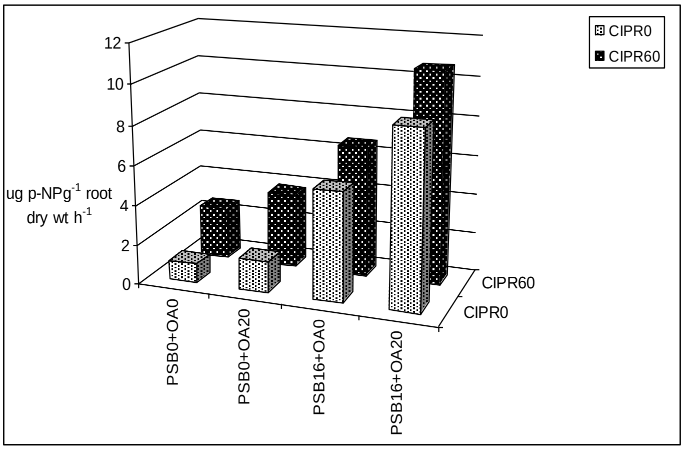

Fig 2. Effect of PSB and PR with OA on phosphatase activity in rice roots PSB16 = *Bacillus* sp., PSB 0 = non-inoculated, CIPR= phosphate rock, OA = oxalic acid.

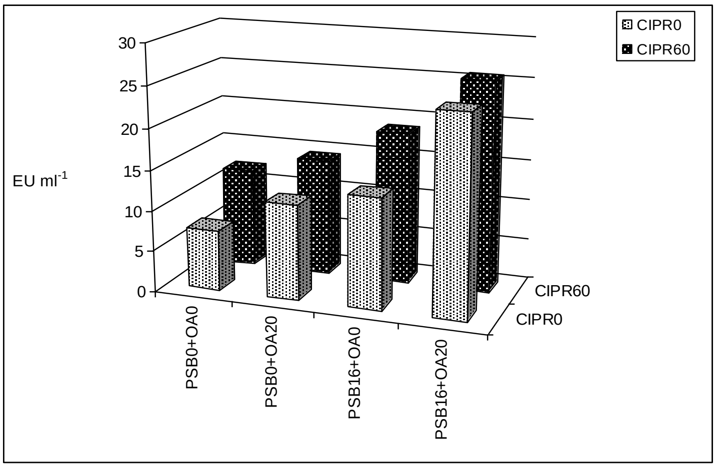

Fig 3. Effect of PSB and PR with OA on phytase activity in rice roots PSB16 = *Bacillus* sp., PSB 0 = non-inoculated, CIPR= phosphate rock, OA = oxalic acid.

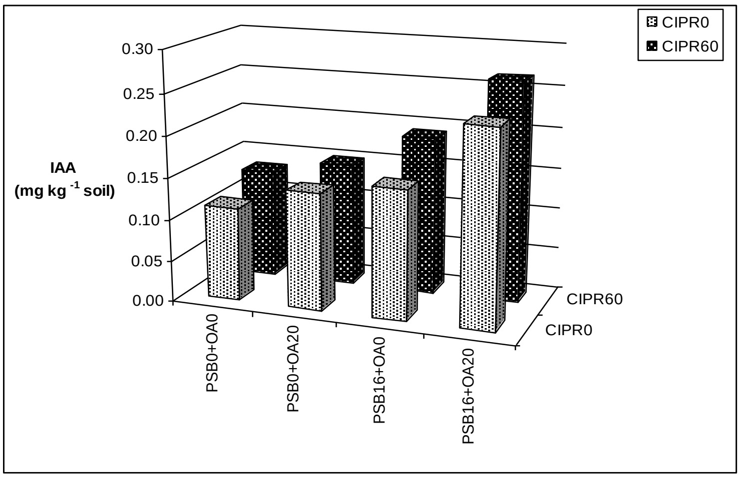

Fig 4. Effect of PSB, PR and OA on indoleacetic acid production in soils of aerobic rice. PSB (*Bacillus* sp.) = PSB 16, CIPR= phosphate rock, OA = oxalic acid).

sources. The inoculation of PSB with CIPR and OA showed higher values in plant P uptake and the amount of P derived from the unavailable sources. Our results are in agreement with the findings of Toro et al. (1997), which showed that plant total P and ^{32}P activity were lowered due to the dilution effects from the P solubilized by the PSB. Thus, this decreased the ^{32}P activity as compared to the control, where, no P was solubilized from the added PR. After harvesting, the experimental soil was analyzed to determine the different amount of P fractions present in the soil and the contribution of PSB for P solubilization from different fractions. The variability in the inorganic and organic P fractions seems to be caused by the influences of the treatments. Significantly, the highest amount of water soluble P, Ca-P, Fe-P and Al-P were found in PSB inoculated samples along with Christmas Island Phosphate Rock and oxalic acid application. In contrast, lower amounts of organic-P were found in PSB inoculated treatments which prove that the phosphatase and phytase enzymes may dissolve the soil organic P. Higher soluble P from all fractions was found in PSB-inoculated and PR applied treatments. Oxalic acid application along with PR or without inoculation also produced a small amount of soluble P in the different treatments, which indicated that an increase in oxalic acid concentration either from PSB or any other sources can enhance soluble P in the soil P pools. The application of PSB and OA increased all of the P fractions in the soil compared to the application of only OA, which indicated that the applied PSB strain was capable of solubilizing PR and immediately solubilized P form Fe, Al and Ca bound complexes. The higher amounts of Al-P, Fe-P and Ca-P in the fraction also proved that P was solubilized from PR by the PSB and OA that immediately formed complexes to the existing soil Fe, Al and Ca ions. Similar findings were observed by Barroso and Nahas (2005) who analyzed P limitation in Brazilian soils and found that the majority of the P formed complexes by P fractionation with iron, aluminium and calcium. Among all inorganic fractions, the concentration of Fe-P was comparatively higher than the other fractions followed by Al-P in the soil. This may be due to the higher amounts of P fixed with the Fe-P and Al-P in acidic soil. However, there was an accountable amount of Ca-P detected from the soil which indicates that this portion of P might have been released due to the presence of Ca in the applied PR fertilizer (Ca = 24.2%) in the soil. However, the correlation coefficient between P fractions was positively correlated with the total P. The addition of inorganic P pools in the fraction studies and the management of P may be helpful in terms of better consideration of P dynamics in soils. The PSB inoculation with the application of CIPR increased photosynthesis in aerobic rice. Similar findings were reported by the Zhang et al. (2009) who found that the PSB strain (*Bacillus subtilis* GB03) was able to increase physiological parameters like photosynthesis through modulation of ABA signaling in *Arabidopsis*. Besides photosynthesis, PSB was able to perform other activities in the rhizosphere like production of phytohormones (IAA), phosphatase and phytase enzymes. The results of these activities led to a significant effect on the plant growth of aerobic rice. The plant-growth promotion was influenced by the PSB inoculation as the bacteria can produce plant growth promoting hormones like, indole-3-acetic acid (IAA), cytokinins, and gibberellins (Bloemberg and Lugtenberg, 2001; Bottini et al., 2004). In the present study, it was found that the inoculated treatments produced significantly higher amounts of phosphatase enzyme. It is known that this enzyme excreted from roots can hydrolyse a wide range of organic P compounds in the soil and release Pi for plant

Table 5. Effect of PSB, OA and PR on photosynthesis (μmol CO_2 m^{-2} S^{-1}) in aerobic rice.

Treatments	20 days		40 days		60 days	
	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$
PSB_0+OA_0	4.18f	4.61e	4.58e	5.35d	4.76e	7.64c
PSB_0+OA_{20}	4.34e	5.52c	4.62e	6.24c	5.26d	8.23b
PSB_{16}+OA_0	5.10d	5.70b	6.04c	7.84a	7.72c	9.14a
PSB_{16}+OA_{20}	5.76 b	6.29a	6.58b	7.95a	8.70b	9.29a
PSB_{16}			**			
OA	**					
PR	***					
PSB_{16}×OA	**					
PSB_{16}×PR	***					
OA×PR	***					
PSB_{16}×OA×PR	***					

*P < 0.05; **P < 0.01; ***P<0.001. †CIPR=Phosphate rock, PSB_{16} = *Bacillus* sp., PSB_0 = non-inoculated, OA = oxalic acid
‡Means within the same column followed by the same letters are not significantly (NS) different at P<0.05.

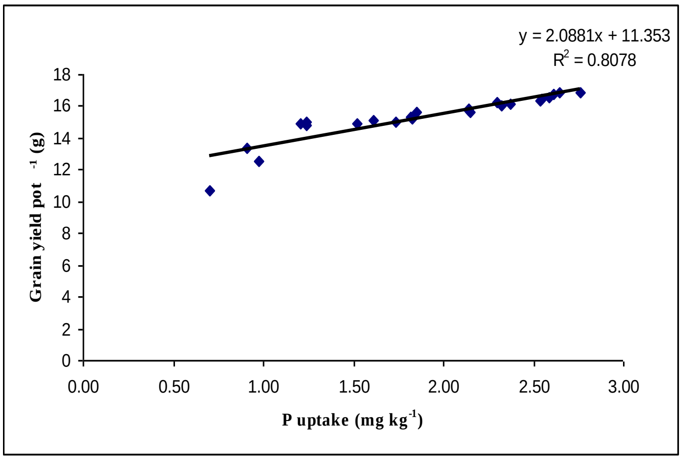

Fig 5. Relationship between plant P uptake and grain yield of Aerobic rice.

uptake (George et al., 2002). In the present study, the organic P pool proved the contribution of inoculated PSB strains to solubilized P from organic substances. A synergistic effect was observed with increased P uptake and plant biomass production. Barea et al. (2003) also found a similar trend in plants P uptake with the inoculation of PSB. The PSB inoculation (*Pseudomonas putida*) enhanced plant growth in canola (Lifshitz et al., 1987). The production of IAA has a positive effect on root architecture (Naher et al., 2009), an extensive root system improved nutrient uptake from the surroundings which produced higher plant biomass. The IAA exudation can increase carbon fixation through increased nutrient uptake. Moreover, inoculation with PSB (*Azospirillum lipoferum* 34H) has improved rice seeds P ion content and showed a significant improvement in root length, fresh and dry shoot weights (Murty and Ladha, 1988). Concurrently, increases in P uptake and crop yields have been observed after PSB inoculation with *Bacillus firmus* (Datta et al. 1982), *Bacillus polymyxa* (Gaur and Ostwal, 1972) and *Bacillus cereus* (Fernández et al., 1984). Inoculation of PSB enhanced P uptake and simultaneously increased the yield of aerobic rice. Plant P uptake, P use efficiency (PUE %), total biomass and total protein content increased with the inoculation of PSB with CIPR and OA. There was a positive relationship found between the plant P uptake and grain yield in aerobic rice. The PSB inoculation with PR and oxalic acid produced the significantly highest grain yield (37.87% over control) including other yield parameters, such as the number of panicles, 1000-grain weight and plant biomass. There were no significant differences in yield (Grain wt pot^{-1}) between the PSB with PR and OA, and PSB with only PR application. Thus, it was shown that OA increased P solubility but did not exert much of an effect on plant growth-promoting substances. The application of only inoculated PSB also produced higher yields than the control. These findings are consistent with the work of Kumar et al. (2001) who reported that the PSB strain showed a higher increase in grain of wheat (12.6%) and straw (11.4%) yield over the control and the strains also showed an increase in biological yield, spike length, spikelets $spike^{-1}$, 1000 grain weight and biomass as compared to the control. In general, PSB inoculation in aerobic rice ensured significant yield improvement by influencing P uptake and phytohormone production.

Materials and methods

Effect of PSB and P fertilizer on P uptake using ^{32}P technique

The experiment was conducted at glasshouse of Universiti Putra Malaysia, Serdang, Malaysia. The treatments were: with and without *Bacillus* sp. (PSB16), two levels of oxalic acids (0 and 20 mM) and Christmas Island Phosphate Rock (0 and 60 kg P_2O_5 ha^{-1} equivalent). The treatment of the experiment was arranged in a completely randomized factorial design (CRD) with 4 replications. Before the experiment started, different doses of oxalic acids were tested in the soil-plant system at growth chamber and 20 mM of oxalic acid was selected from that result of the study. PSB16 was isolated from aerobic rice rhizosphere (Panhwar et al., 2012) and identified using 16S rRNA gene sequencing. The sequences obtained were deposited in the European Molecular Biology Laboratory data bank (accession number JX103827). The detail information on soil, bacterial strain and CIPR properties are shown in Tables 1a, 1b & 1c. The oxalic acid was diluted in 100 ml sterile distilled water and mixed thoroughly with the soil before planting. Flat doses of nitrogen (100 kg N ha^{-1}) from urea and K (60 kg K_2O ha^{-1}) in the form of muriate of potash were applied 7 days before planting.

Inoculum preparation, seed inoculation

Pure cultures of PSB16 strains were grown in the nutrient broth for 48h. The bacterial cells were harvested by centrifugation at 13500 rev min^{-1} for 10 min in an eppendorf

Table 6. Effect of PSB, CIPR and OA on different yield parameters after harvesting aerobic rice.

Treatments	Number of panicle plant^{-1}		Number of unfilled grains (%)		1000 grain weight (g)		Wt of grains pot^{-1}(g)		Increment (%) of grain yield over control		Plant biomass (g pot^{-1})		Increment (%) of biomass over control	
	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$
PSB_0+OA_0	1.31d	2.30bc	25.9a	22.97b	15.65e	17.53c	12.21e	15.03cd	-	23.10c	7.48f	12.87e	-	72.10d
PSB_0+OA_{20}	1.87cd	3.37ab	24.94ab	19.18dc	16.46de	17.81b	14.89de	15.74b	21.98cd	28.91b	10.26e	15.91c	37.20e	112.08c
$PSB_{16}+OA_0$	1.94bcd	3.55ab	20.18c	16.84de	16.68cd	18.43ab	15.39b	16.76a	26.04b	34.81a	15.54d	17.82a	107.90c	138.30a
$PSB_{16}+OA_{20}$	3.58a	4.04a	18.71dc	16.30e	18.09ab	19.81a	15.92b	16.83a	32.05b	37.87a	16.39b	18.36a	119.30b	145.60a
PSB_{16}	***		***		***		***		-		***		-	
OA	***		**		**		***		-		***		-	
PR	NS		*		*		***		-		***		-	
PSB_{16}×OA	**		**		NS		***		-		*		-	
PSB_{16}×PR	***		*		***		*		-		NS		-	
OA×PR	***		**		**		**		-		**		-	
PSB_{16}×OA×PR	***		*		***		**		-		***		-	

*$P < 0.05$; **$P < 0.01$; ***$P<0.001$, †PR=Phosphate rock, PSB = PSB16 (*Bacillus* sp.), OA = Oxalic acid , ‡ Means within the same column followed by the same letters are not significantly (NS) different at $P<0.05$.

Table 7. Effect of PSB, OAs and CIPR on nutrients in plant tissue, grain tissue, and total protein content of aerobic rice

Treatments	N (%)				P (%)				K (%)				Total protein content	
	Plant		Grain		Plant		Grain		Plant		Grain			
	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$	$CIPR_0$	$CIPR_{60}$
$PSB0+OA_0$	0.458g	0.570e	0.94d	0.95cd	0.12de	0.14bc	0.16e	0.18de	1.35f	1.49d	0.24d	0.26c	5.58e	5.62de
$PSB0+OA_{20}$	0.549f	0.615d	0.95cd	1.00bc	0.12de	0.13cd	0.17de	0.29ab	1.41e	1.50d	0.24d	0.26c	5.66de	5.72ab
$PSB16+OA_0$	0.634c	0.692a	0.95cd	1.01b	0.13cd	0.14ab	0.22dc	0.30a	1.50d	1.51c	0.25cd	0.30a	5.68cd	5.93a
$PSB16+OA_{20}$	0.651b	0.701a	0.96cd	1.06a	0.13cd	0.15a	0.25bc	0.33a	1.53b	1.56a	0.27b	0.30a	5.98bc	6.33a
PSB_{16}	***				***				***				***	
OA	NS				***				***				*	
PR	***				*				***				***	
PSB_{16}×OA	**				***				**				*	
PSB_{16}×PR	*				**				*				**	
OA×PR	***				NS				***				**	
PSB_{16}×OA×PR	***				***				***				***	

*$P < 0.05$; **$P < 0.01$; ***$P<0.001$, †CIPR=Phosphate rock, PSB =PSB16 (*Bacillus* sp.), OA = Oxalic acid, PUE= P use efficiency, ‡Means with in the same column followed by the same letters are not significantly (NS) different at $P<0.05$.

tube and washed with 0.85% sterilized phosphate buffer saline (PBS). The optical densities (OD_{600}) of washed cells were checked and adjusted accordingly. Bacterial population applied, was confirmed by using drop plate method on nutrient agar (NA). Approximately 10^9 cfu ml^{-1} live bacterial cells were inoculated to rice seeds for 2 hours before seeding. The non-inoculated seeds received the same amount of killed cells (autoclaved for 30 min at 121°C).

Soil preparation and ${}^{32}P$ labeling

The air dried alluvial sandy clay loam soil was ground and passed through a 2 mm sieve and 5 kg of sieved soil was packed into plastic pots (28 cm diameter × 22 cm height). The isotope dilution technique (Zapata and Axmann 1995) was used for ${}^{32}P$ studies. The soil in the pots was wetted by adding distilled water to be conditions of saturation and the ${}^{32}P$-labelled carrier solution containing 250 µCi ${}^{32}P$ pot^{-1} (5 kg soil) was added to get enough activity in the plant material. The ${}^{32}P$-labelled carrier solution was prepared by taking 250 µCi ${}^{32}P$ carrier materials and applied to a known volume of KH_2PO_4 carrier solution with 2 mg P. Labeling was done by mixing the soil thoroughly with a 25 ml pot^{-1} of the solution containing ${}^{32}P$ phosphate. The soil was allowed to equilibrate for one week before rice seedlings were planted. Isotopic parameters such as, specific activity (SA), i.e., radioactivity per amount of P (becquerel/mg P), and the P percentage (%) in plant derived from the bioavailable (labeled) P fraction (Pdfl), were determined by using isotope dilution concepts (Zapata and Axmann, 1995) as follows:

$$\%\,Pdfl = \frac{SA\ plant\ in\ presence\ of\ PR}{SA\ plant\ in\ absence\ of\ PR} \times 100$$

Where % Pdfl is the P in the plant derived from the labeled fertilizer and SA is the specific activity, that is determined experimentally in the plant material and the labeled fertilizer, expressed in disintegrations per minute (dpm) ${}^{32}P$ mg P^{-1}.
The % Pdf PR is:

$$\%Pdf\ \ PR = (100 - \%\ Pdfl)$$

Planting of rice and determination of specific activity

Three healthy, seven days old aerobic rice (M9) seedlings were transplanted into each pot and plants were grown for 60 days in order to study the ${}^{32}P$ specific activity. The dry weight of each sample was recorded and ashed in the furnace. About 20 ml 2N HCl was added to samples filtered and collected in the plastic vials before 10 ml of supernatant was taken for Cerenkov counting to obtain the specific activity of each sample for P determination.

Phosphate fractionation

Phosphorus fractionation was determined by the sequential extraction procedure as per the method of Chang and Jackson (1957) modified by Hartikainen (1979). One gram (<2 mm) of soil and 50 ml of 1M NH_4Cl were placed into a 100 ml centrifuge tube and shaken for 30 min to extract the soluble and loosely-bound P. The procedure extracted inorganic P fractions-labile P with NH_4Cl, followed by Al-P with NH_4F, Fe-P with NaOH and Ca-P with H_2SO_4. Phosphorus concentrations in the various solutions were determined using the ascorbic acid method (Murphey and Riley, 1962). Standards of P were prepared and the amount of P in each fraction was calculated using the following equation:

$$P\ conc\ in\ \ fraction\,(mg/kg) = Conc.of\ \ P\,(mg/l) \times \frac{Vol\ of\ \ ext(l)}{mass\ of\ \ soil(kg)}$$

Total Phosphorus was determined using the digestion method according to the method of Olsen and Sommers (1982).

Determination of phosphatase and phytase activity in aerobic rice roots

The phosphatase activity was determined according to the method of Tabatabai and Bremner (1969). The roots were washed with distilled water and immersed in a plastic vial with 120 ml of 1mM $CaCl_2$ and left for 24 h. Three ml of aliquot, 1 ml modified universal buffer (MUB) and 1 ml of 0.115M *p*-NPP were pipetted into a 20 ml reagent vial. The mixture was incubated at 37 °C for 1 h and the reaction was stopped by the addition of 20 ml 0.5 N NaOH. The mixture of solution was transferred to a 50 ml volumetric flask and made up to the final volume with distilled water. Later, the roots used in extraction were excised and dried in an oven at 70 °C for 3 days before the dry weight was recorded.
Determination of indoleacetic acid from soil of aerobic rice
Indoleacetic acid (IAA) was determined at 60 days of growth. Three grams of soil was collected and the sample was prepared for IAA determination according to the method outlined by Sarwar et al. (1992). The concentration of IAA was determined using a spectrophotometer at 535 nm.

Determination of photosynthesis, yield and nutrient concentrations

The single-leaf net photosynthesis rate (A_{max}) was determined 20, 40 and 60 days of planting from the youngest expanded leaf (YEL) of each treatment using LI-6200 Portable photosynthesis system, LI-COR Inc. Lincoln, Nebraska, USA. Measurements were taken under full sunlight at noon with a constant CO_2 of 380 µmol CO_2 mol^{-1} in the chamber.
The rice yield parameters including both grain and straw were determined after harvest. The filled grains were separated from unfilled ones by using salt solution of 1.06 specific gravity (Seizo, 1980). Agronomic parameters and phosphorus use efficiency (PUE %) were calculated according to the procedure detailed by Dobermann and Fairhurst (2000). Total nitrogen in the plant sample was determined by the Kjeldahl method (Bremner and Mulvane, 1982) and K was determined by dry-ashing (Ryan et al., 2001). Protein in rice grains were calculated using Jones' factor (Protein % = N × 5.95) modified from Merrill and Watt (1973).

Determination of soil pH, total soil bacterial population and residual oxalic acid from the soil solution

Soil pH was checked every 15 days up to 60 days during the plant growth period. The total bacterial population was determined following the spread plate count method on nutrient agar plate (NA). Residual OA was determined after harvesting. Soil samples were extracted and purified with the extracting procedure described by Baziramakenga et al. (1995). The aliquot was analysed for residual OA by injecting 20 µL of sample into a high performance liquid chromatography.

Statistical data analysis

Data were analyzed for analysis of variance (ANOVA) using SAS statistical program version 9.1. The treatments means were compared by Tukeys' test at a 5% level of confidence using the Statistical Analysis System (SAS) software.

Conclusions

The results of the ^{32}P study showed that the effects and mechanisms accounting for the increased use of sparingly soluble phosphate rock in PSB-inoculated plants is the resultant effect of released phosphate ions from the used low-available CIPR sources. The plant P uptake and P use efficiency were improved by the PSB inoculated with phosphate rock and the application of oxalic acid. The inoculation of PSB and application of OA solubilized any insoluble inorganic bound P, while, enzymes solubilized organic P. Phosphorus fractionations from the total soil P showed the effectiveness of inoculation and oxalic acid application. The bio-available P from CIPR was immediately fixed with native soil Fe and Al fractions. The obtained rank of different P fractions was as follows: Fe-P > Al-P > Ca-P > soluble P. In general, the inoculation of PSB solubilized any insoluble organic and inorganic bound P, and produced phytohormones that developed a synergistic effect, which increased P uptake, growth and yield improvement of aerobic rice.

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