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Full Length Research Paper

Root colonization and association of phosphate-solubilizing bacteria at various levels of triple supper phosphate in aerobic rice seedlings

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Phosphate-soubilizing bacteria (PSB) have the ability to solubilize substantial amount of insoluble phosphorus from native soil. An experiment was conducted *in vitro* condition at Universiti Putra Malaysia, Malaysia, to study the effect of different doses of triple supper phosphate (0, 30 and 60 kg P_2O_5 ha^{-1}) on colonization and association of two *Bacillus* spp. (PSB9 and PSB16 strains) in aerobic rice system. Root colonization was examined under scanning and transmission electron microscope (SEM and TEM). Bacterial association as well as, its effects on plant growth with different rates of P fertilizer was determined during 30 days of growth. There was a significant interaction effect found in PSB × TSP doses × days for plant P uptake. Significantly, the highest available P (31.75 mg kg^{-1}) and P uptake (0.78 mg P Pot^{-1}) was found with PSB16 inoculated treatments at 60 kg P_2O_5 ha^{-1}, where as, highest biomass (82.25 mg $plant^{-1}$) and root growth obtained at 30 kg of P_2O_5 ha^{-1}. The SEM and TEM micrograph proved inoculated PSB successfully colonized on the surface as well as root interior. The bacterial population survived during the planting period and there was no significant population difference found between the two strains. While, the highest PSB16 rhizosphere population (8.16 log_{10} cfu g^{-1}) was recorded after 30 days of planting at 30 kg of P_2O_5 ha^{-1}. Hence, present study proved the PSB inoculation along with the TSP fertilizer improved association, P uptake, available soil P and growth of aerobic rice.

Key words: Biomass, P uptake, rhizosphere, scanning electron microscope, transmission electron microscope.

INTRODUCTION

Phosphorus is an important element and is involved in different functions like root development and metabolic activities, particularly in synthesis of protein (Tanwar and Shaktawat, 2003). Phosphorus (P) affects both the growth of P-solubilizer micro-organisms and domination of enzyme system concerned with the solubilization mechanism (Nahas, 2003). Different levels of P are recognized to enhance many changes in plant metabolism such as, carbohydrate content (Rychter and Randall, 1994), total respiration rate (Wanke et al., 1998), amino acids concentration and enzyme activities (Almeida et al., 2000). While, P deficient soils limit plant growth in many agricultural soils especially aerobic soil environment. Generally P is applied in the forms of chemical fertilizers and single super phosphate, mono or

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Abbreviations: MARDI, Malaysian agricultural research and development institute; **NBRIP** national botanical research institute of phosphate; **PSB** phosphate-solubilizing bacteria; **P,** phosphorus/phosphate; **SEM,** scanning electron microscope; **TEM,** transmission electron microscope; **TSP,** triple super phosphate.

diammonium phosphate fertilizers are used, which is relatively soluble compared to raw phosphate rock fertilizer. Unluckily, a considerable part of soluble phosphate can be vanished in runoff, or become fixed to tropical soils where, Fe and Al ions are dominant (Choudhury et al., 2007). In these circumstances, soil microorganisms play a crucial role in P solubilization and simultaneously increase plant uptake. Many soil microorganisms have ability to convert insoluble P compounds into soluble for the crops (Pradhan and Sukla, 2005). Soil fungi and bacteria can solubilize inorganic P (Singal et al., 1994). On the other hand, microorganisms (Myccorrhiza) influenced at the higher rates of P (Mehrvarz et al., 2008), while the majority of crops are positively affected by rhizospheric micro-organisms under P deficiency condition (Khan et al., 2009). Among micro-organisms, phosphate-solubilizing bacteria play an important role to convert fixed P into plant available forms (Ahmed et al., 2008) and allowing a sustainable use of P fertilizers (Gyaneshwar et al., 1998).

Phosphate-solubilizing bacteria (PSB) are able to colonize in the roots. During colonization process large cell aggregation was found by *Bacillus megaterium* C4 and *Azospirillum brasilense* (Bahat-Samet et al., 2004; Liu et al., 2006). The majorities of plant growth promoting rhizobacteria are associated with rice roots (Li and You, 1991) and colonize the root surface and some are able to colonize endophytically (Naher et al., 2009). The PSB survive in association with the rice roots as they get oxygen from the atmosphere through rice aerenchymatous tissues (Purakayastha and Chhonkar, 2001). The association and colonization of PSB on surface of roots involve direct competition with other rhizosphere micro-organisms, while endophytic population of PSB may give more beneficial effects for the plants due to less competition of other micro-organisms. The micro-organisms particularly PSB, are affected by the various processes like mineralization, solubilization or immobilization (Oliveira et al., 2009). PSB act as a bio-enhancer and stimulate chemical phosphatic fertilizers availability to the plants. However, the efficacy of PSB can be increased or decreased with increasing rates of chemical fertilizers applied to the soil. Hence, the present study was undertaken with the aim to observe the potential of PSB colonization and association at different doses of phosphate fertilizer (TSP) in aerobic rice seedlings.

MATERIALS AND METHODS

Phosphatic fertilizer treatments

The experiment was conducted *in vitro* (growth chamber) condition. Two *Bacillus* sp. PSB9 and PSB16 were selected and aerobic rice line MR 219-9 mutant (M-9) obtained from Malaysian Agricultural Research and Development Institute (MARDI). Seeds were surface sterilized by method modified from Amin et al. (2004). Uniform 7 days old seedlings were transplanted to pots (17 × 11 cm) containing 500 g of sterilized sandy clay loam (alluvial soil). The treatments were as follows: control, bacterial strains PSB9 and PSB16 with phosphatic fertilizer triple superphosphate (TSP) at 0, 30 and 60 kg P_2O_5 ha^{-1} which was applied before one week of transplanting. The experiment was laid out in a factorial completely randomized design (CRD) with 4 replications. Seedlings were grown for 30 days in sterile condition and plant sampling was done at intervals of 10 days.

Preparation of inocula and rice seedlings inoculation

The PSB9 and PSB16 (*Bacillus* sp.) strains were previously isolated from the aerobic rice field of Penang Malaysia. The bacterial strains were cultured in NBRIP broth for 72 h. At exponential growth stage, bacterial cells were harvested by centrifugation and washed with distilled water. Three days after transplanting, approximately 5×10^9 ml^{-1} of live washed bacterial cells were used as inoculums in each bacterial treatment. The population was confirmed by cell enumeration in drop plate method on NBRIP agar plate (Somasegaran and Hoben, 1985).

Determination of non-rhizosphere, rhizosphere and root-endosphere population

At each sampling, 10 g of soil was taken from each pot and diluted 10-fold up to 10^{-7} dilution. Aliquot of 0.1 ml from each dilution was dropped onto NBRIP agar plates and populations were determined following the drop plate count method. To determine rhizosphere population, plants were harvested and roots were gently washed with sterile water and placed in Erlenmeyer flask containing 99 mL distilled water. The content in flasks was shaken for 15 min and a series of 10-fold dilutions were prepared and bacterial populations determined.

At each sampling date, the roots (1.0 g) were washed, blotted dry and surface sterilized with 70% ethanol for 5 min and then treated with 3% Clorox for 30 s. The roots were meshed using a sterilized mortar and pestle (Gyaneshwar et al., 2001). A 10-fold series of dilution were prepared up to 10^{-8} and the endosphere PSB populations were determined.

Determination of soil available P, plant root development and plant biomass

The soil available P was determined by the Bray II (Bray and Kurtz, 1945) extraction method and P in the tissue was analyzed by wet digestion method (Havlin and Soltanpour, 1980). The root morphology was studied at harvest using root scanner, model Epson Expression 1680 with root scanning analysis software, version Win-Rhizo 2007d. Fresh roots were washed with distilled water and placed in the root scanner. Total root length (cm), total surface area (cm^2) and total volume (cm^3) were quantified using a scanner (Expression 1680, Epson) equipped with a 2 cm depth plexiglass tank (20 × 30 cm) filled with up H_2O (Hamdy et al., 2007). After each harvest plant samples were carefully washed to remove all soil particles and dried in oven at 70°C for 5 days until constant weight was achieved.

Visual observation of root colonization using SEM and TEM

Rice seedlings were prepared by transplanting five days old germinated rice plantlets in a sterilized 2 L glass tube containing sterilized 50 ml of plant growth culture medium (Naher et al., 2009). The seedlings were placed on a stainless steel net and lowered down into the tube with roots touching the solution. Each seedling

was subsequently inoculated with 5 mL of washed PSB inoculums at 5 × 10^9 cfu mL^{-1}. Plants were grown for five days in growth chamber with 12 h light / dark cycle at 29 ± 1°C. The bacterial colonization on roots was observed using scanning electron microscope (SEM) and transmission electron microscopy (TEM). For SEM, roots of 5 days old seedlings inoculated with PSB were cut 1 cm^3 and root samples were pre-fixed with 4% glutaraldehyde overnight and washed with 0.1 M sodium cacodylate buffer for 3 changes at 30 min each. Osmium tetraoxide buffer (1%) was used for post fixation. After series of dehydration in acetone (35, 50, 75, 95 and 100%) the samples were dried in a critical point dryer and mounted on aluminum stubs, sputter coated in gold and viewed under Scanning Electron Microscope (JEOL JSM-6400 attached with OXFORD INCA ENERGY 200 EDX). For TEM, observation roots were cut 1 mm^3 and were infiltrated with acetone and resin mixture and embedded. After the polymerized steps, samples were sectioned, coated with gold and observed under TEM (PHILIPS HMG 400).

Data analysis

All data were statistically analyzed using the SAS Software Program (Version 9.2), and treatment means were compared using Tukey's test ($P < 0.05$).

RESULTS

Population of PSB at different P rates

The average populations of the recovered PSB strains were 4.46 and 8.16 log_{10} cfu g^{-1}. The higher population was found in rhizosphere while lower was in non-rhizosphere. The bacterial population was affected by the application of phosphatic fertilizer at various rates in both strains (Figure 1). Non-rhizosphere population was showed slightly decrease with the increase of time period, while rhizosphere and endosphere population showed reciprocal trend at various rates of P. Among two strains, there were no differences found in PSB populations while PSB16 produced significantly highest non-rhizosphere (6.09 log_{10} cfu g^{-1}) and endosphere (8.00 log_{10} cfu g^{-1}) population after 10 days of planting, where as in rhizosphere (8.16 log_{10} cfu g^{-1}) after 30 days of planting at the rate of 30 kg of P_2O_5 ha^{-1} (Figure 1b, d and f).

Effect of PSB on available P and plant P uptake in aerobic rice

The amount of P from soil and plant was determined after harvest. The amount of available P and plant P uptake was increasing with the increase of P rates and planting period in aerobic rice seedlings. Comparatively higher available P and Plant P uptake values were observed with PSB inoculated treatments. Significantly highest available P (31.75 mg kg^{-1}) and P uptake (0.78 mg P Pot^{-1}) was found with PSB16 inoculated treatments at 60 kg P_2O_5 ha^{-1} respectively (Tables 1 and 2). There were significant positive interaction found in the PSB, P dose and days for available soil P and as well as in plant tissue P.

Effect of PSB on root development of aerobic rice

The root development of rice plants was affected with the different treatments. Higher root length, root surface area and volume were observed in inoculated treatments. The highest root length (43.96 cm), root surface (8.50 cm^2) and root volume (0.273 cm^3) was recorded with PSB16 at 30 kg followed by 60 kg P_2O_5 ha^{-1} (Table 3).

Effect of PSB on plant biomass production in aerobic rice

The PSB strains significantly increased plant biomass of aerobic rice (Table 4). The PSB16 inoculated plants produced significantly highest biomass (82.25 mg $plant^{-1}$) at 30 followed by (61.33 mg $plant^{-1}$) 60 kg of P_2O_5 ha^{-1} treatment. The PSB strains showed better results with phosphatic fertilizer applications.

Visual observation of root colonization

The inoculated PSB isolates were able to colonize M9 rice plant roots and non-inoculated roots were found free of bacteria after 5 days of inoculation, on the surfaces of the primary and lateral roots, root hair zone, lateral root junction, and the root tips (Figure 2). Aggregations of cells were observed on the root tips, at the elongation and discrimination zones. The PSB strains (*Bacillus* spp.) were observed to form bunches on the root surfaces (Figure 2b), in crevices (Figure 2c) and covered with gummy material (Figure 2f). Using TEM, transverse sections of the roots were viewed and found that PSB survived on cell walls (Figures 3b and c) and intercellular spaces of cortical parenchyma, within the epidermis (Figure 3d and e).

DISCUSSION

The application of different rates of TSP fertilizer positively influenced PSB population and plant association. Within 30 days of *in vitro* study period, the PSB strains found survive at non-rhizosphere, rhizosphere and endosphere of the inoculated plants. A higher population was found in the rhizosphere and endosphere compared to non-rhizosphere. This is due to the rhizosphere activity, as releases of root exudates supplies required carbon compounds and attracted the heterotrophic soil biota (Naher et al., 2008). The addition of P sources positively affected PSB population growth in

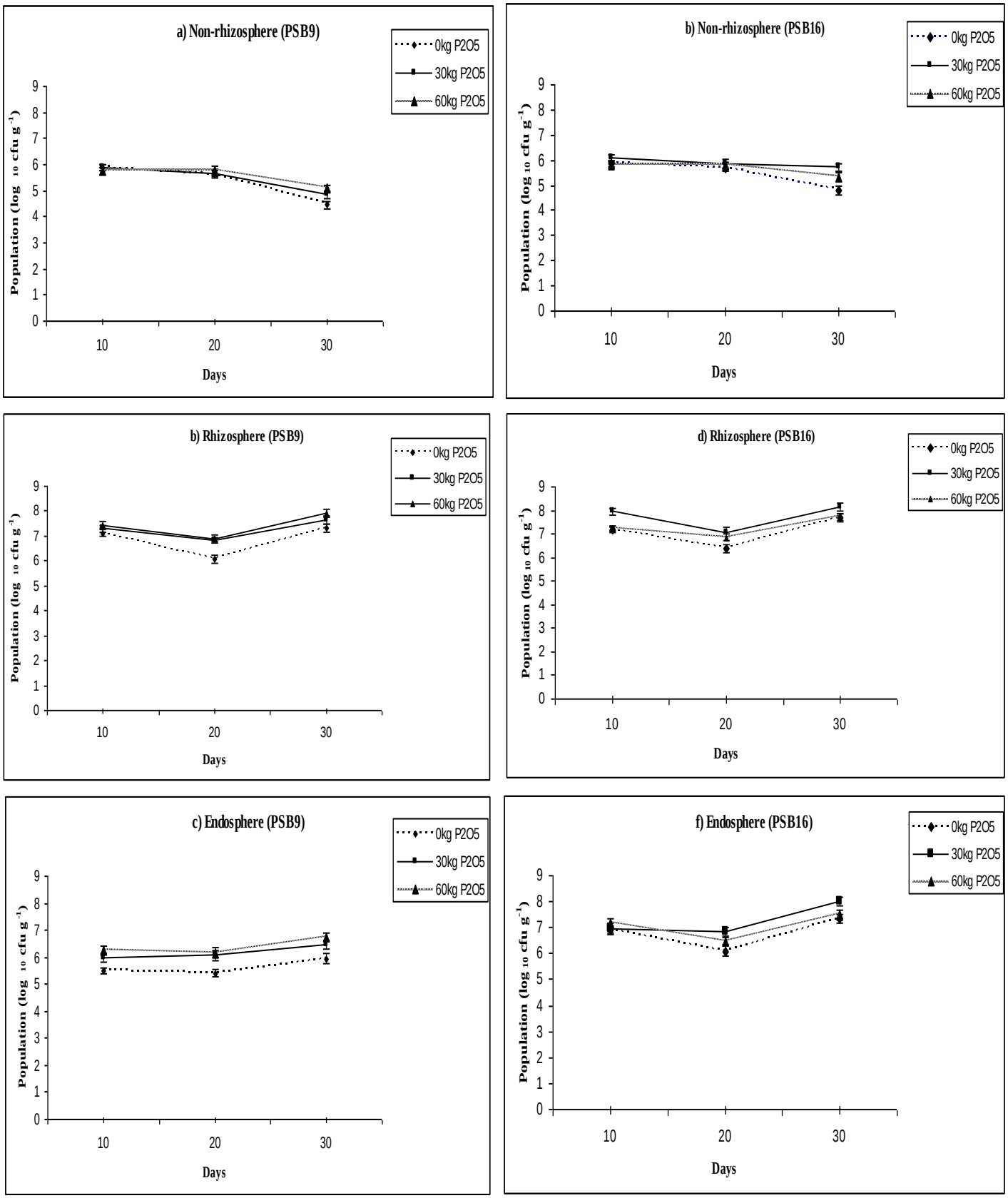

Figure 1. Bacterial population in non-rhizosphere, rhizosphere and endosphere of inoculated M9 rice at different P rates Bars indicate standard error, n = 5.

Table 1. Effect of PSB and different rates of P_2O_5 on the soil available P in aerobic rice seedlings.

Treatments	Days	Extractable P in soil (mg kg^{-1})		
		0 kg P_2O_5 ha^{-1}	30 kg P_2O_5 ha^{-1}	60 kg P_2O_5 ha^{-1}
B0	10	9.2^{ef}	15.73^{g}	24.15^{e}
	20	9.39^{e}	18.91^{e}	25.26^{d}
	30	9.43^{e}	19.36^{de}	24.86^{e}
PSB9	10	10.31^{d}	18.43^{f}	26.65^{cd}
	20	12.24^{cd}	19.91^{d}	26.91^{c}
	30	13.86^{b}	22.71^{c}	27.28^{c}
PSB16	10	11.78^{d}	28.18^{ab}	30.17^{b}
	20	12.96^{c}	27.69^{b}	30.54^{b}
	30	14.88^{a}	28.75^{a}	31.75^{a}
P dose			***	
Bacteria			***	
Days			***	
P dose × bacteria			NS	
P dose × days			***	
Bacteria × days			**	
P dose × bacteria × days			NS	

Means with in the same column followed by the same letters are not significantly different at P=0.05; ** = 0.01, and *** = 0.001, at $p \leq 0.05$.

Table 2. Effect of PSB and different rates of P_2O_5 on the P uptake of aerobic rice seedlings.

Treatments	Days	Plant uptake (mg P Pot^{-1})		
		0 kg P_2O_5 ha^{-1}	30 kg P_2O_5 ha^{-1}	60 kg P_2O_5 ha^{-1}
B0	10	0.20^{g}	0.22^{g}	0.23^{f}
	20	0.23^{f}	0.43^{d}	0.46^{d}
	30	0.24^{e}	0.47^{d}	0.49^{d}
PSB9	10	0.26^{e}	0.29^{f}	0.34^{e}
	20	0.33^{d}	0.56^{c}	0.59^{c}
	30	0.41^{b}	0.64^{b}	0.71^{b}
PSB16	10	0.36^{c}	0.32^{e}	0.37^{e}
	20	0.42^{b}	0.63^{b}	0.65^{c}
	30	0.53^{a}	0.71^{a}	0.78^{a}
P dose			***	
Bacteria			***	
Days			***	
P dose × bacteria			*	
P dose × days			***	
Bacteria × days			***	
P dose × bacteria × days			***	

Means within the same column followed by the same letters are not significantly different at P=0.05,** = 0.01, and *** = 0.001, at $p \leq 0.05$.

Table 3. Effect of PSB on root development of aerobic rice.

Treatments	Days	Root length (cm)			Root surface (cm^2)			Root volume (cm^3)		
		0	30	60	0	30	60	0	30	60
		---------- kg P_2O_5 ha^{-1}----------								
B0	10	11.45^{f}	14.64^{f}	20.18^{e}	2.86^{f}	3.21^{h}	3.37^{g}	0.043^{f}	0.057^{g}	0.070^{f}
	20	16.04^{e}	20.55^{e}	22.35^{d}	3.55^{e}	4.17^{g}	4.25^{f}	0.050^{f}	0.080^{f}	0.083^{e}
	30	19.54^{d}	23.57^{d}	27.76^{c}	4.49^{d}	5.23^{e}	6.54^{c}	0.090^{e}	0.103^{d}	0.113^{d}
PSB9	10	22.05^{d}	24.76^{d}	26.10^{c}	3.83^{e}	4.45^{f}	5.05^{e}	0.083^{e}	0.093^{e}	0.113^{d}
	20	24.68^{c}	29.58^{c}	26.63^{c}	4.69^{d}	5.62^{d}	5.26^{d}	0.103^{d}	0.110^{d}	0.127^{c}
	30	28.54^{b}	31.33^{c}	37.06^{a}	6.45^{b}	7.00^{b}	7.38^{a}	0.127^{c}	0.160^{c}	0.203^{a}
PSB16	10	25.88^{c}	28.47	26.35^{c}	5.16^{c}	6.70^{c}	5.41^{d}	0.123^{c}	0.163^{c}	0.143^{b}
	20	29.19^{b}	37.20^{b}	32.49^{b}	6.21^{b}	7.17^{b}	6.88^{b}	0.140^{b}	0.177^{b}	0.153^{b}
	30	33.00^{a}	43.96^{a}	37.57^{a}	6.90^{a}	8.50^{a}	7.23^{a}	0.190^{a}	0.273^{a}	0.203^{a}

Means with in the same column followed by the same letters are not significantly different at P=0.05.

Table 4. Effect of PSB inoculation on plant biomass at the different levels of P in aerobic rice.

Treatments	Days	Plant biomass (mg pl^{-1})		
		0 kg P_2O_5 ha^{-1}	30 kg P_2O_5 ha^{-1}	60 kg P_2O_5 ha^{-1}
B0	10	5.67^{g}	8.33^{f}	12.00^{f}
	20	12.00^{f}	18.00^{e}	20.67^{e}
	30	25.00^{c}	31.33^{c}	34.67^{c}
PSB9	10	14.67^{e}	16.00^{e}	15.67^{g}
	20	21.00^{d}	22.67^{d}	26.67^{d}
	30	41.00^{b}	53.00^{b}	54.33^{b}
PSB16	10	15.67^{e}	21.33^{d}	18.00^{e}
	20	25.67^{c}	31.00^{c}	28.00^{d}
	30	69.33^{a}	82.00^{a}	61.33^{a}

Means with in the same column followed by the same letters are not significantly different at P=0.05.

the rice seedlings, especially at the optimum P levels rather than higher P rates. Moreover, the higher plant P uptake was found at higher levels (60 kg P_2O_5 ha^{-1}) but high plant biomass were obtained at optimum P levels (30 kg P_2O_5 ha^{-1}). This also proved that optimum level provides a friendly environment for plant growth and PSB activity. The incidence of amount of P fertilizer reduction and plant growth were observed with PSB inoculation. A study with sugarcane by Sundara et al. (2002) found that *Bacillus megatherium* var. *phosphaticum* reduced the required P dosage by 25% when applied with phosphatic fertilizer in sugarcane. In their study the maximum PSB population and soluble P was found in PSB + fertilizer treatment compared to fertilizer treatments alone. In the present study similar findings also observed where, PSB with optimum TSP doses produced maximum rhizosphere population and soluble P.

The PSB with TSP fertilizer treatment produced higher root growth over fertilizer treatments. There might be several factors; one of them is P solubilization, uptake and another production of growth hormone (IAA). Although, TSP is immediate soluble in soil after application and plant available form of phosphorus might be fixed with Fe and Al or Ca before plant uptake. In these circumstances, PSB play a role to make available insoluble fixed P to soluble form Previous studies also showed that inoculation with PSB strains (*S. marcescens* EB 67 and *Pseudomonas* sp. CDB 35), in maize crop solubilize P and showed potential for growth promotion of the crop (Hameeda et al., 2008). Similarly, a number of studies have shown the ability for bacteria to colonize in the root surfaces and survive as endophytes, thereby,

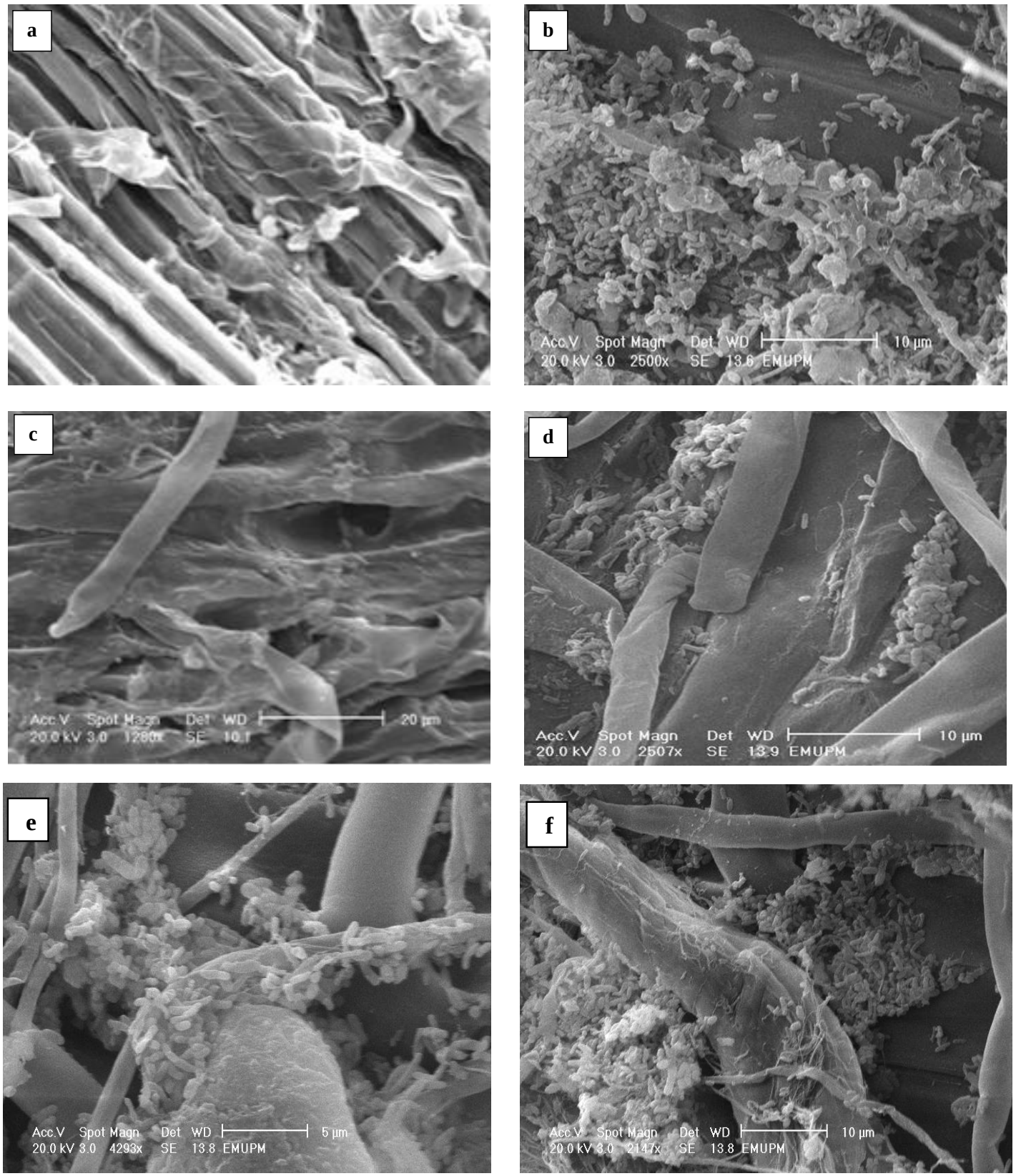

Figure 2. SEM micrographs showing the location of PSB colonization inoculated on aerobic rice roots: a) control, b) on the lateral roots and in disrupted zones of the mucigel, c) in the crevices and junction of lateral root meristem, d) on the root hair zone, e) in the emerging zones of secondary roots, and f) bacterial cell aggregation on the root hair zone covered by mucilage material.

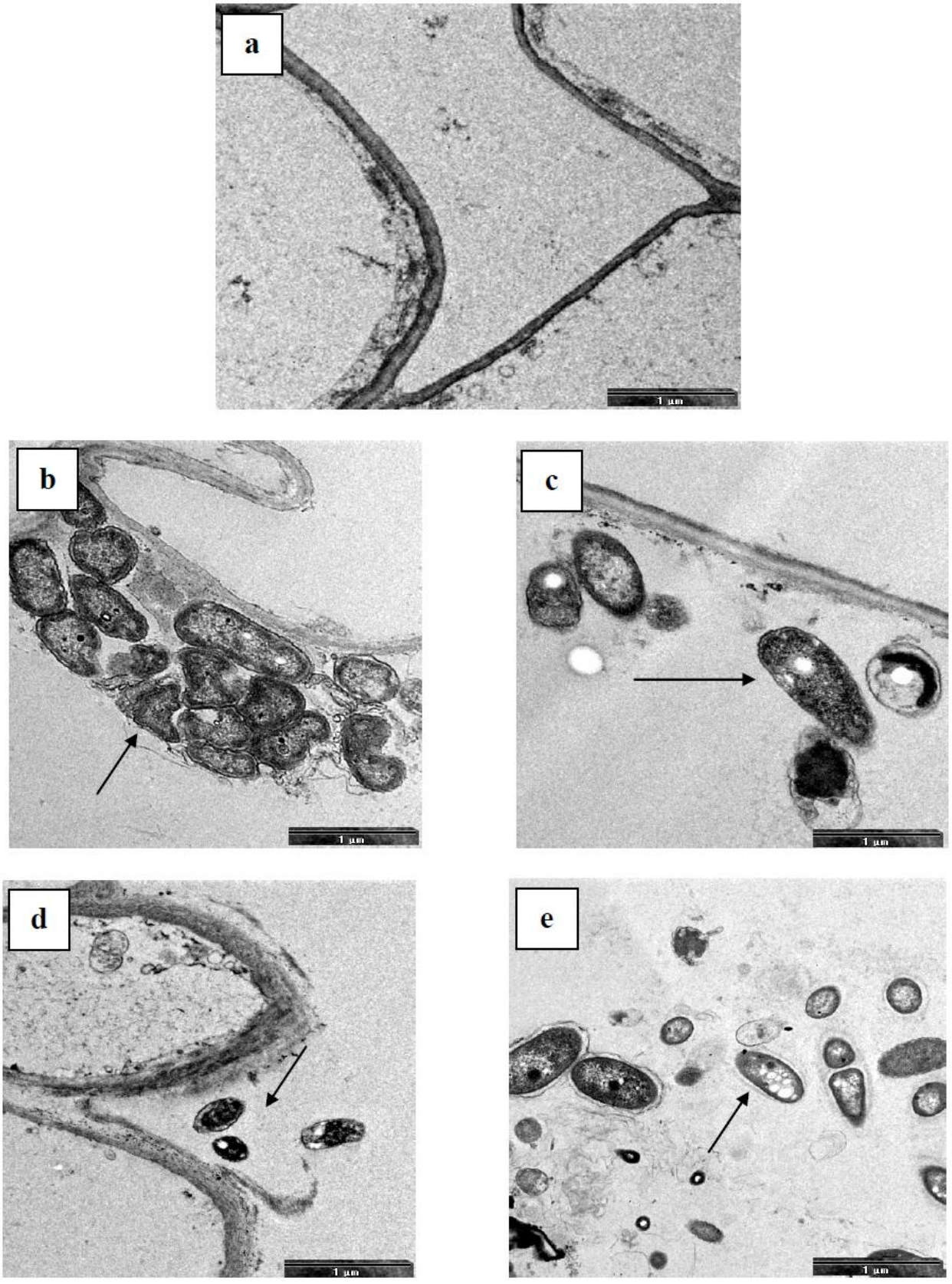

Figure 3. TEM micrographs showing the PSB colonization inoculated on aerobic rice roots of interior spaces: a) control, b and c) bacterial cells intra cellular cell wall, d & e) bacterial cells in inter cellular spaces and within epidermis. Scale bars: 1 µm.

subsequently improving plant growth (Naher et al., 2009; Whipps, 2001; Chanway et al., 2000). Some endophytic bacteria including PSB such as *Bacillus, Enterococcus, Paenibacillus* and *Methylobacterium*, are moved from seeds to seedlings, rendering benefits to the host plant (Ferreira et al., 2008).

Both inoculated PSB isolates were associated with the plant; colonize in the rhizosphere and grow endophytically. Micrographs showed the bacterial colonization and cell aggregation on the root surface, elongation zones, root hair, lateral root junctions, root tips and in crevices. TEM micrographs illustrated widespread colonization of the PSB from intra and intercellular spaces and extending into the cortex. Several study showed isolated bacteria from rice roots in paddy soil had close association with rice; accumulated on the rice root surface and enter into the rice roots. They can grow in the intercellular spaces, especially inside the root cells and formed aggregated cells and mucilaginous materials that may be involved in their attachment to the roots (You et al., 1991; Naher et al., 2009). According to Islam et al. (2007), inoculation of PSB with rice seedlings were determined under SEM and found intense colonization on surface of the roots most probably using fimbriae on the bacterial cells. Similar findings were observed by Achouak et al. (1994). These studies were in agreement with the present study.

Conclusion

The inoculated strains produced significantly highest plant biomass; root development and higher population with their beneficial association at 30 kg of P_2O_5 ha^{-1}. The SEM and TEM view proved PSB strains formed natural association with plant roots and colonize in root interior tissues and growing near the vascular tissues. In conclusion, the study proved that the locally isolated *Bacillus* sp. strains PSB9 and PSB16 were able to colonize the rhizosphere, non-rhizosphere and endosphere of aerobic rice plant and enhanced growth at optimum P fertilizer rates.

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