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Original Article

Effects of root colonization by zinc-solubilizing bacteria on rice plant (*Oryza sativa* MR219) growth

Nur Maizatul Idayu Othman,[a, *] Radziah Othman,[a, d] Halimi Mohd Saud,[b] Puteri Edaroyati Megat Wahab[c]

[a] Department of Land Management, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[b] Department of Agriculture Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[c] Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[d] Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

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ABSTRACT

Two experiments were conducted using gnotobiotic conditions and sand culture treatment to determine the effects of root colonization by zinc-solubilizing bacteria (ZSB) on rice growth. Both experiments were designed as complete randomized designs. The first experiment was conducted in a growth chamber under gnobiotic conditions. Five-day-old rice plantlets (MR219) were inoculated with bacterial isolates—*Acinetobacter* sp. (TM56) and *Serratia* sp. (TM9). The roots were cut for analysis using scanning electron microscopy and transmission electron microscopy. The second experiment was also conducted in a growth chamber under sand culture conditions. The treatments consisted of the control, two bacterial isolates—*Acinetobacter* sp (TM56) and *Serratia* sp. (TM9), two types of zinc sources—$ZnSO_4$ and ZnO and three zinc rates—0 mg/L, 0.2 mg/L and 0.4 mg/L. Data were subjected to analysis of variance and means comparison. *Acinetobacter* sp. and *Serratia* sp. were able to colonize and penetrate rice plant roots. Bacterial populations of *Serratia* sp. were affected by different rates of zinc for endophytes and the rhizosphere. From the study, there were significant differences among bacterial inoculation and the different rates and sources of zinc. However, inoculation with *Acinetobacter* sp. at 0.2 mg/L of $ZnSO_4$ produced the highest rice plant growth and root development. It was concluded that rice plant growth was affected by ZSB inoculation, zinc sources and the rate of zinc.

Introduction

Plant growth promotion requires zinc as an essential micronutrient as it is an important constituent of various metabolic enzymes and its poor mobility in plants suggests the need for a constant supply of available zinc for optimum plant growth (Saravanan et al., 2007). Chemical fertilizers have been applied to rice to provide essential macro nutrients like nitrogen, phosphorous and potassium (Gandhi and Muralidharan, 2016). However, the importance of micronutrient application has not been considered much in rice, especially with regard to zinc that has a vital role in enzyme formation (Gandhi and Muralidharan, 2016). Most Zn fertilizers dissolve relatively slowly in soil, which in some cases may be too slow to supply adequate amounts required for vigorous plant growth (Rengel, 2015). However, a flooded soil has lower Zn availability for plant uptake than a non-flooded soil (Impa and Johnson-Beebout, 2012). Zinc introduced to any soil and the treatment applied might also affect plant growth. Findings have shown that the shoot zinc concentration increased in *Brassica rappa* linearly with increasing treatment levels for hydroponic culture treatment (Coolong et al., 2004). In sand culture, a study proved that rice varieties affect growth performance, dry-matter production, biochemical constituents and the enzymatic activity of rice which were measured employing graded levels of zinc, with the optimum level for rice being 0.2 mg/L using Hoagland nutrient solution (Malik et al., 2011). These authors also reported that increasing the level of zinc up to 200 ppm increased rice plant growth, while further input of zinc decreased plant growth. The optimum level of zinc fertilizer also can be helped by using microorganisms. The association of zinc-solubilizing bacteria (ZSB) and plant roots are involved in solubilization, biofortification, and also mineralization of the zinc pool, as ZSBs can solubilize zinc from inorganic and organic pools of total soil zinc and can be utilized to

* Corresponding author.
E-mail address: myzatul88@yahoo.com (N.M. Idayu Othman).

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increase zinc availability to plants (Fasim et al., 2002). ZSBs are known for their effectiveness to solubilize zinc through their association with plant roots due to the production of root exudates that act as a chemo attractant (Shakeel et al., 2015). However, this also involves competition with other bacteria even though ZSB have multiple beneficial characteristics that help plant growth. This can result in inconsistencies of colonization between the bacteria and plant roots. The application of ZSB on rice seedlings allows the acquisition of data related to its root colonization on rice at different zinc rates. Therefore, the present study was undertaken with the following objectives: 1) to determine the effects of different zinc rates on ZSB strain populations on rice plants; and 2) to observe the colonization ability of ZSB strains associated on rice roots using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Materials and methods

The experiment was conducted using *in vitro* (growth chamber) conditions located in the Soil Microbiology Laboratory, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The potential bacteria strains used were *Acinetobacte*r sp. (TM56) and *Serratia* sp. (TM9) that were selected during an isolation and characterization study of ZSB at the Tanjung Karang Irrigation Rice Field, Selangor, Malaysia.

Seed surface sterilization

Seed surface sterilization was conducted based on Amin et al. (2004). Rice seeds were dehusked and shaken in 70% ethanol for 5 min. The ethanol was discarded and the seeds were agitated in hypochlorite solution comprising 3% chlorox (2.6% NaOCl) and then were washed using sterilized distilled water.

Inoculation in vitro

Bacteria isolates were grown for 48–72 h in nutrient broth. The bacterial cells were harvested using centrifugation (3KC30; Sigma; St Louis, USA) at 3000 rpm for 10 min in an Eppendorf tube and were then washed using phosphate buffer saline (Somasegaran and Hoben, 1985). After washing, the bacterial cells were immediately suspended in phosphate buffer saline solution.

Gnotobiotic conditions

The experiment was conducted in a growth chamber under gnobiotic conditions to ensure the clear visualization of bacterial root colonization compared to the sand culture experiment. The surface of the seeds was sterilized and germinated, eight axenic, five-day-old rice plantlets were placed in a sterile 2 L glass tube on a stainless steel sieve. Prior to transplanting the rice seedlings, the sterilized glass tube was filled with 200 mL of sterilized Zn-free Hoagland nutrient solution. The zinc fertilizers used were zinc sulfate ($ZnSO_4$) and zinc oxide (ZnO) as soluble and insoluble sources, respectively. The zinc rates used were 0 mg/L, 0.2 mg/L and 0.4 mg/L. The seedlings were placed on a net so that only the roots were in touch with the solution. In the solution, there were three types of inoculation—non-inoculation with bacteria, inoculation with *Acinetobacter* sp. (TM56) and inoculation with *Serratia* sp. (TM9). For the inoculated treatments, about 1×10^8 colony forming units (CFU)/mL of bacteria were inoculated on rice plant roots.

Zinc-solubilizing bacteria population during rice plant growth using different types and rates of zinc

A sample of 1 mL of nutrient solution was taken from the respective glass tubes (gnotobiotic condition) containing inoculated plants at the sampling time for the determination of bacterial populations for the endosphere and rhizosphere. A series of 10-fold dilutions was prepared up to 1×10^{10}. Populations were determined using the drop plate method according to Somasegaran and Hoben (1985).

Visual observation of root colonization by ZSB using SEM and TEM

Inoculated five-day-old seedling roots were washed using sterile water and cut into 1 cm lengths for SEM analysis and into 1 mm pieces for TEM analysis. The root samples were pre-fixed using 4% glutalraldehyde and washed with 0.1 M sodium cacodylate buffer. Osmium tetraoxide buffer (1%) was used for post fixation. Then, the samples were dried in a critical point dryer and mounted on aluminum stubs, sputter coated in gold and viewed using SEM. Prior to TEM observation, the samples were infiltrated with an acetone and resin mixture and then coated with gold.

Sand culture experiment

The sand culture experiment was undertaken to determine the rice plant growth parameters using the modified method of Malik et al. (2011) and Naher et al. (2009). Sand was sieved through a 2.0 mm sieve and soaked overnight in 0.1 N (HCl) then the sands was washed using tap water until a pH of 6.0 was obtained. The sand was autoclaved at 15 psi and 121 °C for 15 min. Hoagland nutrient solution was used following the same formula as for the previous nutrient solution. The nutrient solution was watered once a week during the 40-day growing period. Each planting unit was kept in the growth chamber with a 12 h:12 h light:darkness cycle at constant room temperature. Treatments used were: T1 = without zinc; T2 = with 0.2 mg/L $ZnSO_4$; T3 = with 0.4 mg/L $ZnSO_4$; T4 = with 0.2 mg/L ZnO; and T5 = with 0.4 mg/L ZnO. In addition, three types of inoculation were tested: non-inoculation; inoculation with *Acinetobacter.* sp (TM56); and inoculation with *Serratia* sp. (TM9).

Determination of plant growth parameters

Different growth parameters were recorded after the 40 d of growth. The root morphology was studied at harvest using a root scanner, (Expression 1680; Epson; Nagano, Japan) and root scanning analysis software, (version Win-Rhizo, 2007d; Regent Instruments; Ville de Québec, QC, Canada). Fresh roots were washed using distilled water and placed on the root scanner in a Plexiglass tank. The total root length, total surface area and total volume were quantified using a scanner (Expression 1680; Epson; Nagano, Japan) equipped with a 2 cm depth Plexiglass tank (20 cm × 30 cm) filled with water After each harvest, plant samples were carefully washed to remove all soil particles and dried in an oven at 70 °C for 5 d until constant weight was achieved.

Data analysis

Plant growth parameters and root development were recorded. All data were subjected to analysis of variance and means comparison using the SAS statistical software package (version 9.4; SAS Institute Inc; Cary, NC, USA).

Results

Zinc-solubilizing bacteria population in different types and rate of zinc sources during rice plant growth

The mean population for the recovered ZSB population from the rhizosphere and endophytes was 4.13 $\log_{10}$ CFU/mL and 6.87 $\log_{10}$ CFU/mL, respectively. The highest ZSB population (6.87 $\log_{10}$ CFU/mL) for the rhizosphere was recorded for 0.2 mg/L $ZnSO_4$ added in the growth culture inoculated with *Serratia* sp. (TM9) at day 14 of growth (Table 1), while *Serratia* sp. (TM9) in 0.4 mg/L

Table 1
Bacterial population in rhizosphere of inoculated MR219 rice plant at different rates of zinc.

Rate of $ZnSO_4$	Bacterial population (log_{10} colony forming units per gram)					
	Acinetobacter sp. (TM56)			*Serratia* sp. (TM9)		
	day 7	day 14	day 21	day 7	day 14	day 21
0	6.53[aa]	6.29[b]	5.50[ab]	6.70[a]	6.37[b]	5.30[ab]
0.2	6.59[a]	6.27[ab]	5.51[ab]	6.31[a]	6.87[a]	5.08[b]
0.4	6.48[a]	6.55[ab]	5.91[a]	6.53[a]	6.39[b]	5.55[ab]

[a] Means in the same column followed by the same lowercase superscript letter are not significantly different at $p = 0.05$.

Table 2
Bacterial population in endosphere of inoculated MR219 rice plant at different rates of zinc.

Rate of $ZnSO_4$	Bacterial population (log_{10} colony forming units per gram)					
	Acinetobacter sp. (TM56)			*Serratia* sp. (TM9)		
	day 7	day 14	day 21	day 7	day 14	day 21
0	5.05[ba]	4.21[b]	4.13[a]	5.04[b]	4.46[b]	4.83[a]
0.2	5.56[a]	5.58[a]	4.52[a]	5.14[b]	5.47[ab]	4.33[a]
0.4	5.14[b]	4.91[ab]	4.38[a]	5.23[b]	5.88[a]	4.31[a]

[a] Means in the same column followed by the same lowercase superscript letter are not significantly different at $p = 0.05$.

$ZnSO_4$ had the highest population of the endosphere (5.88 log_{10} CFU/mL) in the same growing period (Table 2). Both isolates had decreased ZSB populations at day 21 of growth.

Visual observation of root colonization by zinc-solubilizing bacteria

The control treatment was bacteria-free. The applied ZSB levels were able to proliferate and colonize in the roots. These included the root tips, elongation zone, root hair zone and the primary and lateral roots. Cell aggregates were also found near the root tips and elongation zone (Fig. 1). Among the two ZSB isolates, *Acinetobacter* sp. (TM56) formed clusters on the root surfaces especially on new hair roots. The transverse sections of the root observed under TEM showed live ZSB in the epidermis, intercellular space and near vascular bundles (Fig. 2).

Plant growth parameters

The ZSB strains significantly increased the plant height, plant biomass and chlorophyll content (Table 3). The plants inoculated with *Acinetobacter* sp. (TM56) produced the significantly highest height (23.57 cm) at 0.2 mg/L $ZnSO_4$. The ZSB strains also affected the plant biomass with the highest (134.67 mg) produced by TM56 with 0.2 mg/L $ZnSO_4$ followed by TM9-inoculated plants with

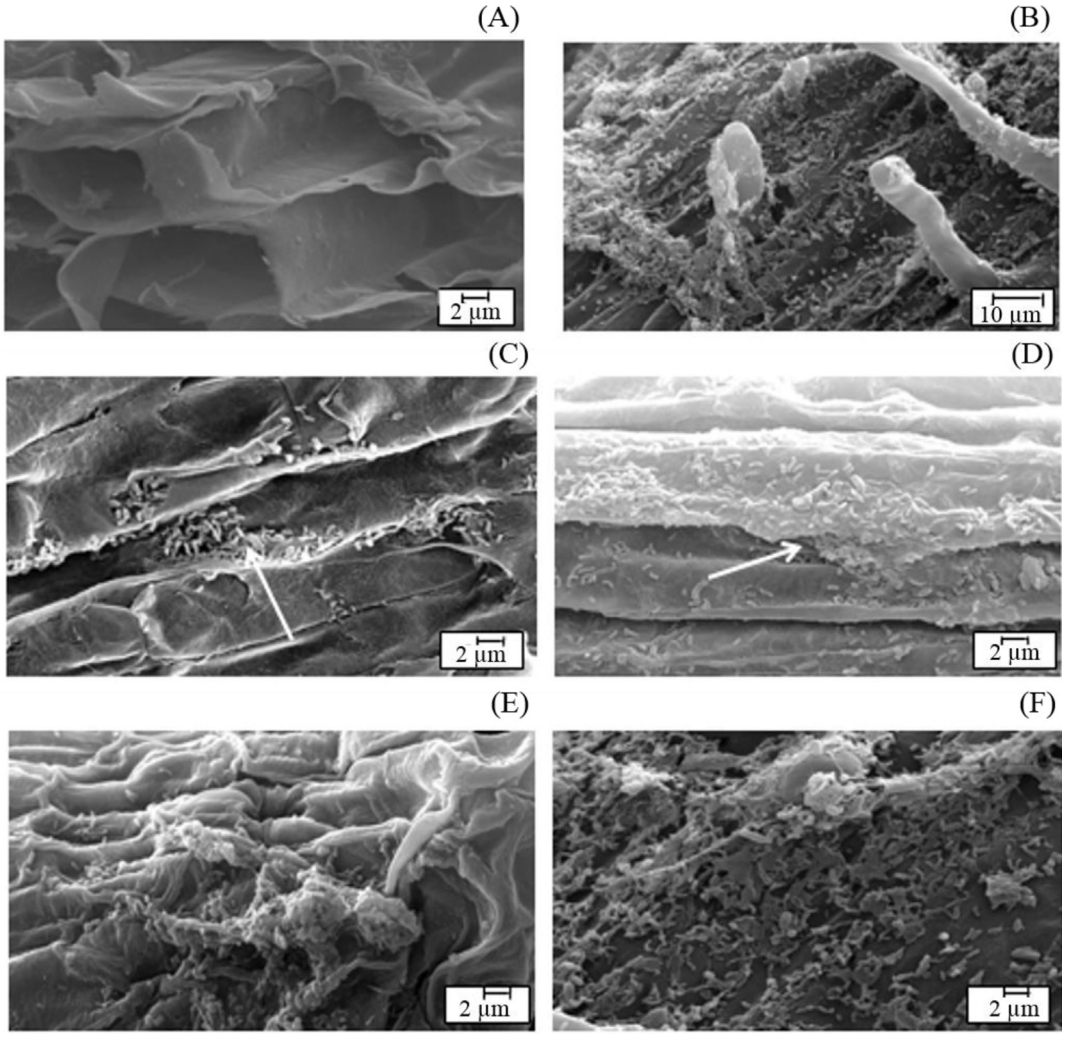

Fig. 1. Scanning electron microscopy micrographs showing the location of zinc-solubilizing bacteria colonization inoculated on MR219 rice roots: (A) control; (B) root hair zone; (C) in between cell wall root tips; (D) zone of elongation; (E) crevices and covered with mucillage material; (F) root tips, with white arrows showing the location of zinc-solubilizing isolates.

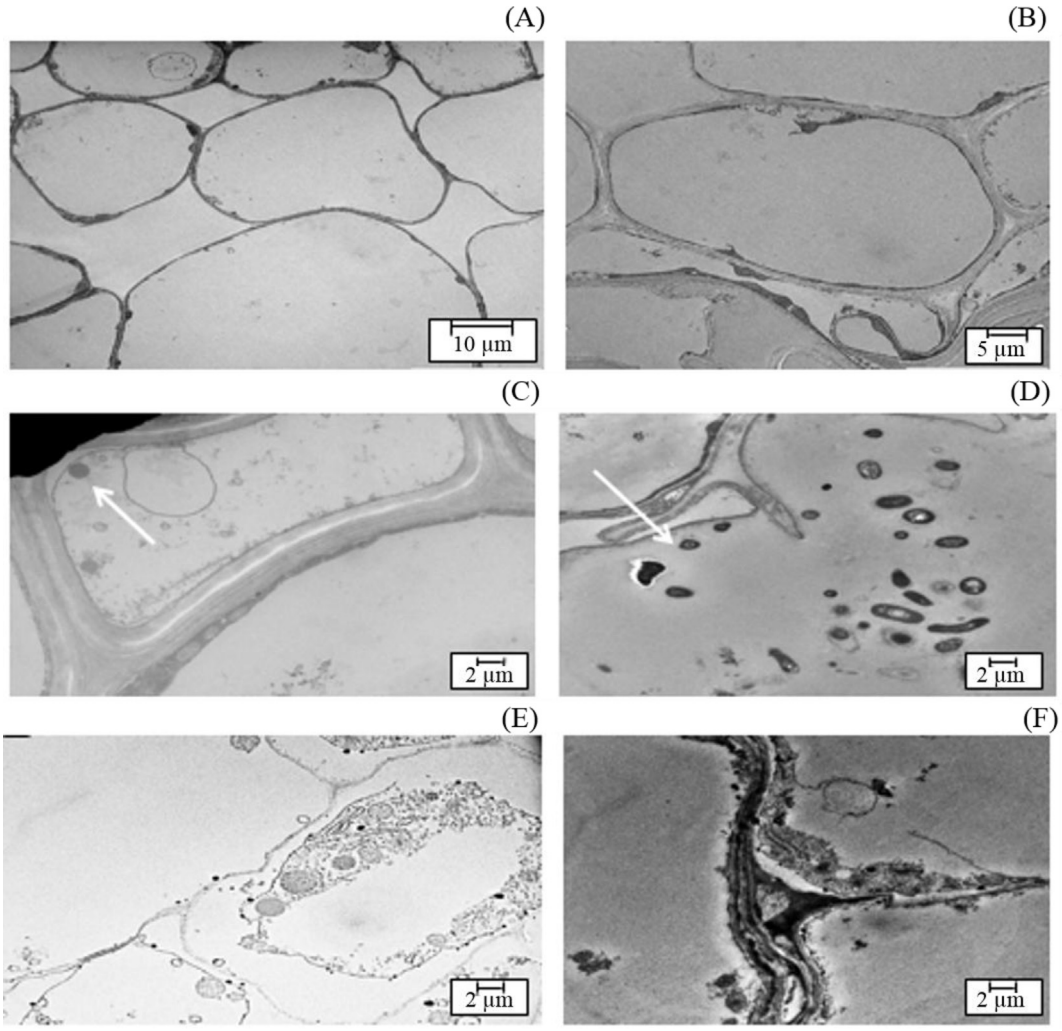

Fig. 2. Transmission electron microscopy micrographs showing the zinc-solubilizing bacteria inoculated on inner spaces of MR219 rice roots: (A) & (B) control; (C) intra cellular cell wall; (D) near cell wall; (E) near vascular bundles; (F) within epidermis, with white arrows showing the location of zinc-solubilizing isolates.

Table 3
Effect of different levels of zinc oxide and zinc sulfate with different types of inoculation on plant height, plant biomass, chlorophyll content, root volume and root length of rice plants.

Bacterial inoculation	Treatment	Plant height (cm)	Plant biomass (mg)	Chlorophyll content (mg cm^2)	Root volume (cm^3)	Root length (mm)
Non-inoculated	T1	5.97^{Aba}	48.33^{Ac}	0.86^{Cb}	0.07^{Bc}	77.91^{Cd}
	T2	6.77^{Cb}	64.67^{Cb}	1.62^{Cb}	0.14^{Aa}	99.79^{Bc}
	T3	14.70^{Aa}	63.67^{Cb}	6.53^{Ba}	0.07^{Bc}	114.65^{Bb}
	T4	15.30^{Ba}	85.33^{Ca}	6.65^{Ca}	0.10^{Bc}	145.17^{Ca}
	T5	15.03^{Ba}	81.67^{Ba}	5.51^{Ba}	0.13^{Ab}	155.66^{Ba}
Acinetobacter sp. (TM56)	T1	7.83^{Ad}	57.33^{Ac}	6.34^{Ac}	0.09^{Ad}	126.86^{Ae}
	T2	18.50^{Ab}	104.33^{Ab}	6.22^{Bd}	0.14^{Abc}	136.57^{Ad}
	T3	14.43^{Ac}	95.67^{Ab}	7.98^{Ac}	0.15^{Ab}	146.00^{Ac}
	T4	23.57^{Aa}	134.67^{Aa}	12.34^{Aa}	0.18^{Aa}	199.38^{Aa}
	T5	16.77^{Abc}	94.67^{Ab}	9.15^{Ab}	0.13^{Ac}	153.73^{Bb}
Serratia sp. (TM9)	T1	7.53^{Ac}	54.33^{Ad}	5.13^{Bc}	0.09^{Ab}	118.71^{Be}
	T2	9.90^{Bbc}	90.66^{Bb}	8.48^{Ab}	0.15^{Aa}	133.17^{Ac}
	T3	13.53^{Aab}	80.33^{Bc}	6.49^{Bc}	0.09^{Bb}	80.36^{Cd}
	T4	17.37^{Ba}	105.00^{Ba}	10.97^{Ba}	0.09^{Cb}	155.07^{Bb}
	T5	9.50^{Bbc}	94.33^{Ab}	8.32^{Ab}	0.15^{Aa}	176.66^{Aa}

T1 = without zinc; T2 = with 0.2 mg/L $ZnSO_4$; T3 = with 0.4 mg/L $ZnSO_4$; T4 = with 0.2 mg/L ZnO; T5 = with 0.4 mg/L ZnO.

[a] Means with the same letters are not significantly different at $p > 0.05$, where capital superscript letters indicate different types of inoculation and lowercase superscript letters indicate different types of zinc and rates.

0.2 mg/L ZnO (Table 2). TM56 plants also had the highest chlorophyll content (12.34 mg/cm^2) at 0.2 mg/L $ZnSO_4$ followed by *Serratia* sp. (TM9)-inoculated plants with a chlorophyll content (10.97 mg/cm^2) at 0.2 mg/L $ZnSO_4$ (Table 1). The ZSB strains showed better results with zinc fertilizer applications, especially with the zinc sulfate fertilizer.

Root development parameters

The rice plant root development was affected by the different treatments (Table 3). Greater root lengths and volumes were observed in the inoculated treatments. The greatest root length (199.38 mm) and root volume (0.190 cm^3) were recorded using the *Acinetobacter* sp. (TM56) inoculation at 0.2 mg/L $ZnSO_4$.

Discussion

The ZSB populations were affected by the zinc rates in the endosphere and rhizosphere. High populations were identified for the rhizosphere and endophere for *Serratia* sp inoculation because the micronutrient availability in the rhizosphere is controlled by individual plant properties, interactions of roots with microorganisms and the surrounding soil which can influence the bacterial population as well (Rengel, 2015). Similarly, a study done by Nunan et al. (1998) showed that the bacterial populations in different individual plants were a major driver for bacterial rhizoplane community composition. Statistical analysis of fingerprint patterns did not reveal a relationship between the bacterial community composition and plant species but did demonstrate an influence of plant community composition. In addition, nutrient deficiencies can influence rhizosphere microorganisms either directly by affecting their nutrition or indirectly via altering root morphology and exudation (Rengel and Marschner, 2005). In addition, the rhizosphere soil of different plant species and individuals shows differential composition and abundance of microbial populations (Ponmurugan and Gopi, 2006). The microbial community composition is influenced by soil properties as well as the macronutrient supply (Marschner et al., 2006) and increased agricultural activity resulting in decreased microbial diversity and reducing ecosystem function (Steenwerth et al., 2002). Naher et al. (2009) showed that the differential structure of microbial communities has also been noted among the different plant genotypes, different growth stages and root structure components which occur in rice roots.

In this study, the successful colonization of rice roots with ZSB persisted. These bacteria could survive in association with the rice roots probably because they get oxygen from the atmosphere through rice aerenchymatous tissue, as discussed for vesicular arbuscular fungi by Purakayastha and Chhonkar (2001). Another reason may be that root exudation also has a direct correlation with the number and survival of plant growth-promoting rhizobacteria in the rhizosphere of cereals (Harris et al., 1989; Albrechet et al., 1983). A similar finding was reported by Panhwar et al. (2012) where the phosphate-solubilizing bacterium (PSB16) *Bacillus* sp. was found to colonize aerobic rice. The bacterium has the ability to solubilize a substantial amount of insoluble phosphorus from the native soil. Rosas et al. (2009) also found *Pseudomonas aurantiaca* SR1 colonized the root system of maize, wheat and rice and it persisted in appropriate population densities.

The treatment inoculated with ZSB showed the highest plant growth parameters and root growth development. By promoting plant growth associated with rice plant roots, the inoculation had a positive impact on plant growth compared with the non-inoculated control (Hafeez et al., 2007). The association of colonization between roots and bacteria occurs because of glucose production in the root exudates of rice acting as a chemo attractant to ZSB to attach to the roots with glucose being preferred by the bacteria as a chemo attractant to attach to the rice plant roots (Naher et al., 2009). Microorganisms like fungi and bacteria are significant in zinc solubilization and plant-growth promoting activity especially when the microorganism has an association with its host, for example the plant roots (Shakeel et al., 2015). Rice plants inoculated with suitable zinc-solubilizing bacterial strains had higher plant growth and root development because of the higher zinc uptake that occurred during plant growth (Vaid et al., 2014). In the current study, ZSB helped in acquiring Zn from the Hoagland nutrient solution compared to the non-inoculated plants. Shakeel et al., (2015) proved that *Bacillus* sp. improved the rice grain yield and growth parameters in Indian rice as the strains enhanced Zn translocation toward grains and increased the yield of basmati-385 and super basmati rice varieties by 22–49% and 18–47%, respectively. This may have occurred because the zinc-solubilizing rhizobacteria also enhanced Zn translocation to the rice grains in similar way to that of chemical zinc supplied by farmers.

In conclusion, the applied ZSB inoculation was able to proliferate and colonize in the rice plant roots. Inoculation with *Acinetobacter* sp. (TM56) at 0.2 mg/L zinc sulfate produced the best results for plant growth parameters and root development.

Conflict of interest

The authors declare that there are no conflicts of interest.

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