PLANT ROOT ASSOCIATED BACTERIA FOR ZINC MOBILIZATION IN RICE

MUHAMMAD TARIQ, SOHAIL HAMEED, KAUSER A. MALIK AND *FAUZIA Y. HAFEEZ

National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, 3800-Pakistan.

Abstract

The activity of Plant Growth Promoting Rhizobacteria (PGPR) to mobilize indigenous soil zinc (Zn) in rice (*Oryza sativa* L.) rhizosphere was observed in a net house micro plot experiment and compared with available form of chemical Zn source as Zn–EDTA. The PGPR application alleviated the deficiency symptoms of Zn and invariably increased the total biomass (23%), grain yield (65%) and harvest index as well as Zn concentration in the grain. The inoculation had a positive impact on root length (54%), root weight (74%), root volume (62%), root area (75%), shoot weight (23%), panicle emergence index (96%) and showed the highest Zn mobilization efficiency as compared with the un-inoculated control. The PGPR colonized rice plants were more efficient in acquiring Zn from either added or indigenous source, than non-colonized plants. Zinc mobilization by PGPR was also confirmed in liquid culture medium. It was concluded that, selected PGPR strains can serve as efficient solubilizer of Zn, allowing farmers to avoid the use of costly chemical Zn fertilizer in rice crop.

Introduction

Zinc is a micronutrient required in adequate concentration by living organisms (Di Simine *et al*., 1998; Martino *et al.,* 2003). In soil it undergoes a complex dynamic equilibrium of solubilization and precipitation that is greatly influenced by the soil pH and micro flora and that ultimately affects their accessibility to plant roots for absorption (Cunningham & Kuiack, 1992; Goldstein, 1995). Artificial chelates such as zinc ethylene diaminetetraacetate (Zn-EDTA) have been shown to be more plant available than inorganic forms (Brown, 1973).

Zinc deficiency is a serious constraint to rice production in many parts of the world (Anon., 1993) and this could only be compensated by the application of costly chemical fertilizers, either as foliar or soil applications (Reyes & Brinkman, 1989). Alternatively, numerous microorganisms, especially those associated with roots, have the ability to increase plant growth and productivity (Okon, 1985; Kloepper *et al*., 1998; Yanni *et al*., 2001; Rodriguiz *et al.,* 2004) by increasing the supply of mineral nutrients of low mobility in the soil like P, Zn and Cu (Cunningham & Kuiack, 1992; Tarafdar & Marschner, 1994; Goldstein, 1995; Thompson, 1996; Bashan *et al.,* 2004). Among these microorganisms, a group of bacteria referred to as plant growth promoting rhizobacteria (PGPR) are involved in nutrient cycling and therefore deserve particular attention for agriculture purposes (Weller & Thomashow, 1993; Glick, 1995).

The present study was undertaken to explore whether PGPR may increase the availability of native and added Zn to rice plants. This mechanism may account for their plant-growth promoting abilities and would provide new opportunities to study their interactions with plants, in detail.

*Corresponding Author: Head Biofertilizer Division,
Tel: 92-41-2651475-79, 92-41-2551395, Fax: 92-41-2651472,
E-mail: fauzia@nibge.org , fauzia_y@yahoo.com

Materials and Methods

The field experiments were conducted in net house micro-plots on rice (*Orzo sativa* L.) cv. Basmati–385. The 5 mm sieved sandy loam soil was filled in 1.5 x 1.5 m^2 cemented micro plots and submerged with water for one week before transplanting (Table 1). After one week, water was siphoned out and soil was stirred to a depth of 5 cm with a spatula to simulate puddle conditions. Recommended chemical N fertilizer corresponding to 120 kg ha^{-1} and P fertilizer corresponding to 50 kg ha^{-1} were applied as urea and DAP in two split doses, respectively. In PGPR inoculated treatments, N and P were applied as half than the recommended dose. First half of the N dose was applied at transplanting and the remaining half was top dressed as urea at the panicle initiation stage. Zinc was applied @ 20 kg ha^{-1} as Zn-EDTA as a basal dose. The experiment was laid out in randomized complete block design with four treatments and five replicates (Table 2).

The inoculum used for this study was carrier based biofertilizer (*BioPower*) containing a mixed consortia of five PGPR strains viz., *Azospirillum lipoferum* (JCM-1270, ER–20), *Pseudomonas* sp (K–1, 96-51) and *Agrobacterium* sp. (Ca–18) (Hafeez *et al.,* 2002). The *BioPower* material was dissolved in water to prepare inoculum suspension. Roots of one month old rice seedlings were dipped in the inoculum suspension for at least half an hour before transplanting seedlings in the micro plots (Hafeez *et al.,* 2002). Three seedlings per hill (36 hills plot^{-1}) were transplanted at 20x20 cm distance. Water was applied in each plot to maintain a standing water depth of 2.5 cm throughout the growing season.

In another set of experiment, conducted in the same way, the plants were grown for up to 45 d for the estimation of survival of PGPR and root and shoot parameters. For studying the bacterial survival in rice rhizosphere, viable cells were enumerated 45 d after transplantation (DAT) by direct plate count method and acetylene reduction assay (ARA)-based most probable number (MPN) using combined carbon medium (CCM) containing antibodies on vials and plates (Rennie, 1981). Five gram fresh roots were suspended in 45 mL saline and shaken for half an hour and ten fold serial dilutions of the suspension were prepared. From each dilution of roots, 100 μL of the suspension was either inoculated into vials or spread on plates of CCM medium. After 48 h, viable cell counts were taken by counting the bacterial colonies on CCM plates. For MPN counts, acetylene reduction assay was carried out with vials exhibiting visible growth. For ARA, 1mL acetylene was injected into each vial. The vials were incubated at 30°C in an incubator for 24 h. The vials were tested for ethylene production on a gas chromatograph (TRACE, Thermo-Quest, Italy). The MPN count of PGPR was calculated with the help of number of ARA vials and MPN table (Bilal *et al*., 1990). After 45 d of transplantation all the plants per hill were uprooted, shoot weight and total number of plants per hill were recorded. The root volume was determined by water displacement method (Pushpadas, 1979). Root area and root length were also recorded using root law software (Michigan State Univ., USA).

The number of panicles which emerged in each hill was recorded at 2 d intervals, beginning at 70–100 DAT and at maturity (125 DAT). The panicle emergence index (PEI) was calculated as the weighted mean of the number of emerged panicles (Srivastava *et al.,* 1999) as follows:

$$PEI = \Sigma_i W_i x_i / \Sigma w_i$$

where W_i represented the panicle weight (g), assigned to specific day of observation and x_i the number of emerged panicles hill^{-1}. The w_i were in decreasing order of magnitude

for each subsequent day of panicle emergence, beginning at 27 for 70 DAT, 25 for 72 DAT and so on, reaching 2 for 100 DAT and 1 for 125 DAT or maturity.

Biomass, grain weight, straw weight and harvest index were recorded as yield parameters at the time of plant maturity. After digestion with the acid mixture of H_2SO_4: HNO_3: $HClO_4$, (9:4:1), the total Zn content in grain and straw were determined by an atomic absorption spectrophotometer (SpectrAA.20, PerkinElmer) (Bhargava & Raghupti, 1993). Standard solutions for the analyses were prepared using $ZnCl_2$ (BDH). The available Zn concentration in the soil after the harvest of rice was determined by the diethylene triamine penta acetic acid (DTPA) method (Lindsay & Norvell, 1978). Zinc uptake in mg plot^{-1} was calculated as the product of yield, grain and / or straw (kg plot^{-1}) and tissue Zn concentration (mg kg^{-1}).

In vitro **Zn mobilization:** The cultures of five PGPR strains were grown in LB medium and maintained on agar plates at 28 ± 2 °C. The *in vitro* study in liquid culture was performed in a defined mineral salts medium (MSM) (Di Simine *et al*., 1998), containing 10 gL^{-1} glucose as the sole carbon source and Zn phosphate and Zn oxide (50 mg/100 mL MSM) were used as insoluble or sparingly soluble Zn compounds. Triplicate 250 mL samples of liquid MSM medium supplemented with Zn minerals were inoculated with an overnight grown pre-culture of PGPR (2.5% inoculums) and incubated in an orbital shaker (28 ± 2°C) for five days. Aliquots of the samples were analyzed for colony forming units (CFU) by inoculating plates of LB medium through serial dilutions of the culture. For Zn mobilization, samples were collected and filtered through a series of 0.5, 0.45 and 0.22 µm pore size filters. Liquid media without PGPR inoculation, processed in same way, was used as control. Samples were acidified with a few drops of concentrated HCl to prevent the loss of ions as well as any microbial growth until the analysis was completed. The acidified samples were analyzed for total Zn contents by atomic absorption spectrometry (AAS).

Results

The population of PGPR enumerated 45 d after rice transplantation on the roots was maximum in inoculated treatment T4, where half than recommended dose of N and P fertilizers were used, as determined by plate count method (3.8×10^7 viable cells mL^{-1}) and ARA based MPN counts (9.5×10^6 viable cells mL^{-1}) (Table 2).

Root weight, root volume, root length, root area, number of plants per hill and shoot weight at the panicle initiation stage (45 d after transplantation) increased significantly due to the PGPR inoculation (Table 2; Fig. 1). Moreover, all these variables were enhanced in T4 due to PGPR inoculation without the addition of Zn (Table 2).

PGPR inoculation resulted in a significantly higher number of emerged panicles hill^{-1} compared to the control as well as Zn-EDTA source from 70 - 100 DAT (Fig. 2). Prior to and after this period, the differences in the number of emerged panicles between control and PGPR inoculated plots were also significant. The PEI for the inoculated treatments with and without Zn source (T3, 14.6 and T4, 15.1) was significantly higher than the control without Zn (T1, 7.3) and with Zn (T2, 9.1), indicating a faster emergence of panicles (Table 3). These treatments differed with respect to PEI and total number of panicles at harvest and apparently were more effective than Zn-EDTA treatment (T2).

Table 1. Properties of dried soil.

Properties	Value	Reference
pH	8.2	Jackson (1967)
Organic C (g kg^{-1})	3.8	Walkley & Black (1934)
Total N (mg kg^{-1})	0.6	Bremner & Mulvaney (1982)
Available P (mg kg^{-1})	4.4	Olsen & Sommers (1982)
Available K (mg kg^{-1})	55.3	Knudsen *et al*. (1982)
Available Zn (mg kg^{-1})	0.6	Lindsay & Norvell (1978)
Total Zn (mg kg^{-1})	4.3	Bhargava & Raghupti (1993)
Sand (g kg^{-1})	661	Jackson (1967)
Silt (g kg^{-1})	240	Jackson (1967)
Clay (g kg^{-1})	99	Jackson (1967)
Texture	Sandy loam	

Table 2. Effect of PGPR on shoot and root parameters of rice at 45 d after seedling transplantation.

Treatments	Shoot wt (g hill^{-1})	Root wt (g hill^{-1})	Root length (m)	Root volume (m^3)	Root area (m^2)	Viable cell counts (×10^6)	MPN counts (×10^6)
T1	52.0 a	25.9 d	154.6 d	36.9 d	1.6 b	7.2 c	0.9 c
T2	56.0 a	34.5 c	206.0 c	39.9 c	2.2 ab	7.0 c	0.4 c
T3	66.0 a	40.4 b	241.2 b	50.3 b	2.5 ab	33.6 b	5.9 b
T4	64.4 a	45.1 a	269.2 a	59.8 a	2.8 a	38.0 a	9.5 a

T1 120 kg N ha^{-1}, 50 kg P ha^{-1}
T2 120 kg N ha^{-1}, 50 kg P ha^{-1}, Zn-EDTA
T3 60 kg N ha^{-1}, 25 kg ha^{-1}, Zn-EDTA, PGPR
T4 60 kg N ha^{-1}, 25 kg P ha^{-1}, PGPR
Values are average of five replicates
Values not sharing the same letter differ significantly at *P*=0.05

Table 3. Effect of PGPR on some yield attributes and harvest index of rice crop.

Treatments	Panicle emergence index	Total number of panicles hill^{-1}	Yield (g plot^{-1})			Harvest index (%)
			Total biomass	Grain	Straw	
T1	7.3 c	10.6 b	1.3 a	0.3 a	1.2 a	26.2 a
T2	9.1 b	12 b	1.6 a	0.3 a	1.2 a	22.0 a
T3	14.6 a	15.4 a	1.5 a	0.3 a	1.1 a	23.2 a
T4	15.1 a	15.4 a	1.6 a	0.5 a	1.0 a	27.1 a

Values are average of five replicates
For abbreviations see Table 2.
Values not sharing the same letter differ significantly at *P*=0.05

Table 4. Effect of treatments on Zn uptake and concentration in rice grain and straw and DTPA-Zn of soil after harvest of rice.

Treatments	Zn (mg kg^{-1})		Zn mobilization efficiency Index (Grain Zn / Straw Zn)	Zinc uptake (mg plot^{-1})			DTPA Zn (mg kg^{-1})
	Grain	Straw		Grain	Straw	Total	
T1	9.2 c	37.9 c	0.2 d	3.9 b	44.9 b	48.8 bc	0.3 b
T2	21.5b	49.4 a	0.4 c	7.3 a	61.5 a	69.6 a	1.3 a
T3	21.7b	43.8 b	0.5 b	7.1 a	49.8 ab	56.9 ab	1.8 a
T4	23.6a	28.9 d	0.8 a	7.7 a	28.3 c	36.0 c	1.7 a

DTPA-Zn = Diethylenetriaminepentaacetate-extractable zinc
Values are average of five replicates
For abbreviations see Table 2
Values not sharing the same letter differ significantly at *P*=0.05
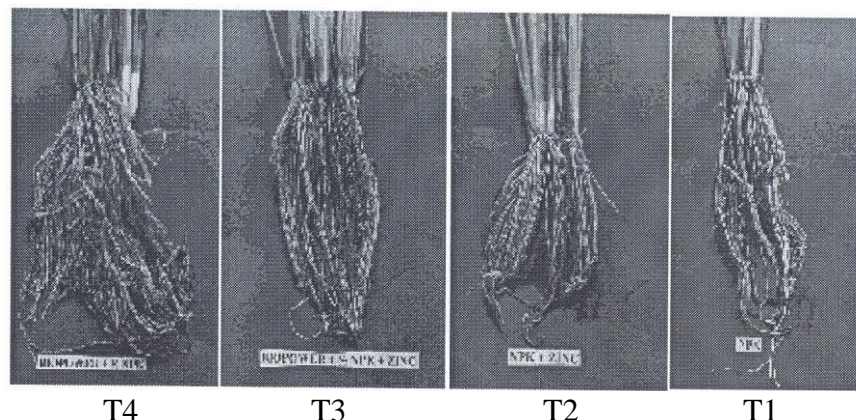
<center>T4   T3   T2   T1</center>

Fig. 1. Effect of PGPR on root area of rice plants at panicle initiation stage. For abbreviations see Table 2
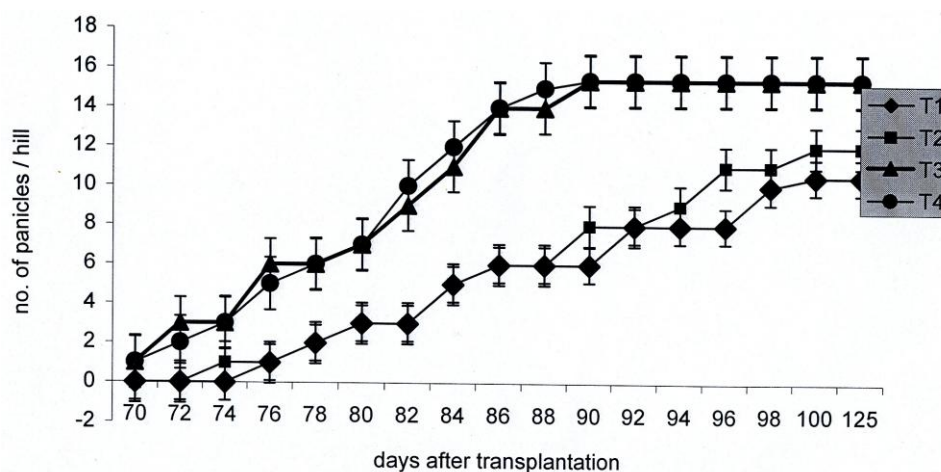

Fig. 2. Number of panicles emerged per hill in different treatments.

The effects of the microorganisms with respect to total biomass, grains and straw yield and harvest index did not differ statistically (Table 3). The application of PGPR inoculum significantly increased the concentration of Zn (23.6 mg kg^{-1}) in the rice grain over the control without Zn (T1, 9.2 mg kg^{-1}) and chelated Zn-EDTA treatment (T2, 21.5 mg kg^{-1}) and did not increase the Zn concentration in the rice straw (Table 4).

After the harvest of rice crop, a significantly higher content of DTPA-extractable Zn was found in the soil inoculated with PGPR (Table 4). The difference in the Zn content between the treatments T2 (1.3 mg kg^{-1}), T3 (1.8 mg kg^{-1}) and T4 (1.7 mg kg^{-1}) was non significant.

***In vitro* Zn mobilization:** Cultures were sampled daily for 5 days for the growth and the observations recorded during the growth of liquid cultures of PGPR are shown in Fig. 3. The PGPR were able to dissolve Zn minerals in liquid medium up to 5.4mM. Analysis of

AAS showed a 5.0mM increase in the concentration of mobilized Zn^{2+} by PGPR strains. The control filtrate consisting of un-inoculated MSM was not able to mobilize the same materials. During the time course of the experiment, bacterial proliferation occurred in both the control and the Zn minerals supplemented cultures (Fig. 3).
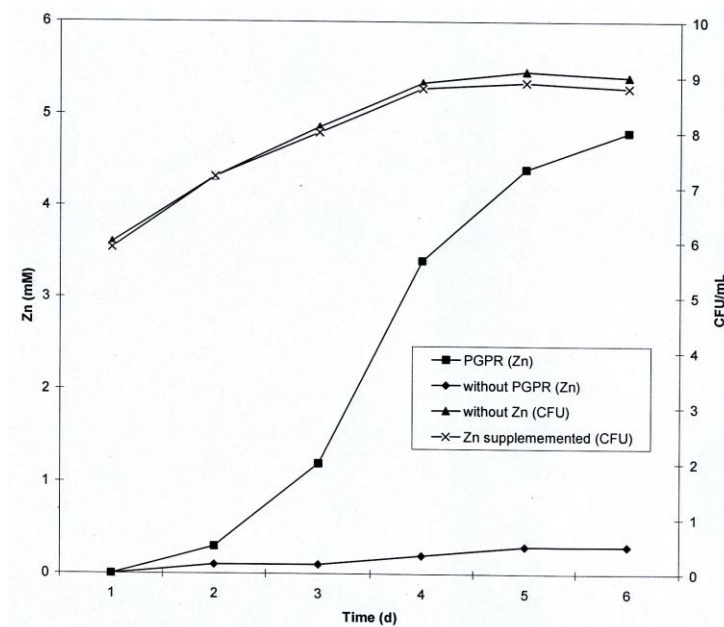

Fig. 3. Bacterial cell viability indicated as colony forming unit (CFU) and change in mobilized Zn^{+2} concentration due to PGPR inoculation. Values of CFU have been expressed in log.

Discussion

In control plots without Zn (T1), interveinal chlorosis appeared on older leaves. AT 5 DAT clear symptoms of Zn deficiency, showing elongated rusty spots on lower leaves, started to appear. The symptoms became more visible at 15 DAT, as also reported by Yoshida *et al.*, (1973), Forno *et al.*, (1975) and Anon., (1988). Considering 1.24 mg DTPA extractable Zn kg^{-1} soil as the critical level of Zn for rice crop (Srivastava & Gangwar, 1990), this soil which contained only 0.6 mg Zn kg^{-1} soil (Table 1) was graded as deficient in Zn. Although, total Zn in soil was 3.4 mg kg^{-1} (Table 1) but this was unavailable to plant because only DTPA-Zn is available to plant. So there was a need to mobilize this total Zn. But it was quite interesting that symptoms of Zn deficiency were not found during the whole growing period of rice crop in T4 where PGPR inoculum was used without additive Zn. This clearly shows that PGPR inoculation provided balanced Zn nutrition to rice plants during the whole growing period because of absence of Zn deficiency symptoms. Moreover, all the PGPR used possess the ability to produce some natural chelating agents like EDTA except the strain K-1 (unpublished data) that might have role in Zn mobilization as well as keeping Zn in available form for longer period of time.

The results of experiment showed that colonization of rice roots with PGPR persisted successfully. 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 & Chhonkar (2001). Root exudation also has direct correlation with number and survival of PGPR in the rhizosphere of cereals (Harris *et al*., 1989; Albrechet *et al*., 1983).

Increase in root and shoot parameters could be attributed to the efficiency of these bacteria to mobilize Zn from indigenous source as Zn is a micro-element required in conc. of 1.24 mg kg^{-1} soil (Srivastava & Gangwar, 1990), hence the Zn mobilized by these bacteria is sufficient for rice plant nutrition. Similarly, if this Zn mobilizing efficiency of PGPR is compared with treatment T2 where chelated Zn in the form of Zn–EDTA was added, there is a 26-30 % increase in all root parameters studied (Table 2). Increase in PEI could have been due to enhanced and sustained Zn availability and better Zn uptake efficiency in tiller production in response to PGPR inoculation.

The yield data indicated that contribution of the microorganisms to increase grain yield of paddy was due to the initiation of the early emergence of panicles, which might have allowed greater storage of assimilates in rice grains. However, the yield data should be interpreted in the light of rates of N and P fertilizer applications. As for the above variables, the lower rates of N and P fertilizer i.e., half than the recommended without Zn (T4) also gave comparable values to those treatments where Zn was applied as Zn-EDTA. This was again probably due to higher level and sustained supply of Zn, as well as better Zn uptake efficiency when PGPR were applied alone without Zn source as compared to the other treatments. The PGPR strains used have a defined role in N_2 fixation, P solubilization, and phytohormone production (like indole acetic acid) so these activities also attributed to saving of N and P fertilizer (Hafeez *et al*., 2002). A higher yield of rice crop due to mobilization of Zn by mycorrhizal fungi inoculation has been reported by Solaiman & Harita (1998) and Purakayastha & Chhonkar (2001) indicating a correlation between P and Zn. If we calculate the prevailing market cost of N, P and Zn fertilizer inputs ha^{-1} in each treatment it is US $ 41, 50, 32 and 23 for T1, T2, T3, and T4, respectively. So in this way there is a saving of US $ 27 in T4 over control (T1) and US $ 18 over treatment T2.

In comparison to the other treatments, the lowest Zn concentration in the paddy straw following PGPR application without chemical Zn (T4) could have been due to the mobilization of most of the Zn to the grains from vegetative tissue, as reflected by the "Zn mobilization efficiency index" (Table 4). Furthermore, this high concentration of Zn in rice grain by utilizing PGPR could improve the Zn deficiency in humans. The identical straw yields (Table 4) for all the treatments, indicated that the lower Zn concentration in rice straw in inoculated treatment (T_4) was not due to a dilution effect, but more Zn^{2+} translocation to the grain. Furthermore, it was interesting to note that the Zn concentration in the straw in T_4 was even lower than in the control. Whereas, the Zn concentration in the grain was higher in T_4 than in the control, again indicating the lower Zn concentration in the straw of T_4 was due to a higher Zn mobilization efficiency as the panicles (grain) emerge much earlier in inoculated treatment (T4) than the control making more translocation towards grain. Zinc uptake by grain and straw for different treatments followed a similar trend to that of the Zn concentration in these tissues respectively (Table 4). The highest total uptake of Zn was with Zn EDTA and the smallest with T_4. This was due to the fact that Zn taken up by plants in T4 was efficiently mobilized and utilized in grains and not wasted in straw. But in case of T2, the Zn content was highest in straw attributed to more total Zn uptake. The concentration of post harvest Zn in soil was related to the amount of Zn applied and the total Zn taken up by the crop.

Purakayastha & Chhonkar (2001) reported the same increase of Zn concentration in grains as compared to straw, showing Zn mobilization and more uptake of Zn by rice plant through mycorrhizal fungi inoculation.

Acknowledgements

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