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Culturable total and beneficial microbial occurrences in long-term nutrient deficit wetland rice soil

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Abstract

A study was conducted to find the effect of long term (24 years) soil macro nutrient deficit condition on total soil microbial population and occurrences of free-living nitrogen fixing and phosphate solubilizing bacteria (PSB) population in wetland rice cultivation system. After the 48th crop cycle (two crops of rice grown per year) soil and plant samples were collected before transplanting and maximum tillering stages from plots with the following treatments: (i) Complete fertilizers (NPKSZn), (ii) NPSZn (-K), (iii) NKSZn (-P) (iv) PKSZn (-N), and (v) without fertilizer. The total bacteria, fungus, actinomycetes, free-living nitrogen fixing bacteria and phosphate solubilizing bacteria populations were determined from each treatment. Results showed that total bacteria, fungus and actinomycetes population were high in the "complete fertilizer" treatment and low in "without fertilizer" treatments, which proved that the absence of any single nutrient element (N or P or K) decreased total soil microbial populations. Significantly high free-living nitrogen fixing bacterial population and high biological nitrogen fixation was found in "without N" fertilizer treatment. The highest atmospheric nitrogen fixed in the nitrogen-free broth culture (3.7%) was by the endophytic strains isolated from "without N" treatment. The Population of PSB was high in the "complete fertilizer" treatment. The highest phosphate (P) solubilizing activity (80%) was observed with the isolated PSB in "complete fertilizer" treatments. The isolated strains produced indoleacetic acid at rates of 1-9 mg l^{-1}. The results of soil chemical analysis showed that the soil N reserve was not reduced as compared to P and K, although the soil received the same treatments over the 24 years. In general, total microbial population was significantly affected by the absence of nutrient elements, whereas the absence of nitrogen increased the free-living nitrogen fixing bacteria.

Keywords: Atmospheric nitrogen fixation, free-living nitrogen fixing bacteria, phosphate solubilizing bacteria, phosphate. solubilization, indoleacetic acid, nitrogen, phosphorus, potassium, yield.
Abbreviations: IAA_indoleacetic acid; MPN_most probable number; NA_nutrient agar; P_phosphate; PDA_potato dextrose agar; PSB_phosphate-solubilizing bacteria; SAS_statistical analysis system.

Introduction

The growth and colonization of soil microorganisms can be influenced by chemical, physical and biological properties of the soil. The availability of macro and micro nutrient elements can limit microbial population growth in a particular soil ecosystem. Essential soil elements for plant growth, such as, nitrogen, phosphorus, potassium, sulfur and micro nutrients influences the microbial population as these nutrient elements are also needed for microbial growth and activity. The presence or absence of plants also influences colonization of specific microbes as root exudates provide a suitable environment for microbial growth. The simple or low molecular weight carbon compounds released through root exuded are primary sources of energy for soil microbes (Naher et al., 2009a). However, soil types with variable chemical and biological properties affect the association between plants and bacteria. The long-term absence of a particular nutrient element in the soil-plant system influences microbial properties. Soil microbes play a vital role in providing soil nutrients specially N and P. Use of PSB as inoculants has concurrently increased phosphorous uptake in plants and improved yields in several crop species (Panhwar et al., 2011a). The long-term absence of nitrogen may favor the population of free-living nitrogen fixing bacteria. It was found that the presence of ammonium in the soil can inhibit the growth of diazotrophic bacteria. The growth of *Azospirillum lipoferum* was found to be restricted in the presence of 0.5 g NH_4Cl and 30 µM dissolved oxygen (Tsagou et al., 2003). Naturally, rice plants harbor a diverse group of microorganisms and beneficial soil microbes such as free-living nitrogen fixing bacteria (diazotrophs), PSB are associated with rice roots. In the lowland rice ecosystem, biological nitrogen fixation is a spontaneous process, where adequate carbon sources are available (Kennedy et al., 2004). Free living nitrogen fixing bacteria can supplement biological nitrogen directly (endophytes) or after cell death and incorporation within the soil. It has been proven that the rice-diazotroph association cannot supplement 100% of crop N requirements, but some of the diazotrophs can supplement about 40% of the rice crop N requirements (Naher et al., 2011). Besides biological nitrogen fixation, microbes have

made significant contributions in plant growth promotion. Production of growth hormones by certain beneficial microbes induces extensive root systems which enhance nutrient uptake and photosynthesis in rice (Naher et al., 2009b). Another beneficial microorganism is PSB, which have a crucial role in phosphorus mobilization in the soil-plant system. It was recorded that PSB has the ability to increase P availability in the soil solution to meet crop requirements (Panhwar et al., 2011b; Tao et al., 2008). A number of bacterial genera, including *Bacillus, Pseudomonas*, and *Rhizobium* found in the soil system have the capability to solubilize insoluble organic or inorganic soil phosphorus (Rodriguez and Fraga, 1999). The soil microbial community reacts to external stress even more than plants and animals (Panikov, 1999). Limited information is available on microbial diversity in long-term nutrient deficit rice environment. Information regarding long-term effect of absence or presence of nutrients on the microbial community is important for the regulation of the microbial populations in rice ecosystems. Hence, the present study was undertaken to determine the effect of long term (24 years) absence of major nutrient elements on total microbial population, free-living nitrogen fixing and phosphate solubilizing bacterial population in a wetland rice cultivation system.

Results and discussion

Microbial populations

The long term (24 years) presence or absence of major nutrient elements significantly reduced the total microbial populations in the wetland rice cultivation system. It has been proven that agriculture activities have significant implications on soil microbial communities (Hengeveld, 1996). Significantly high soil microbial populations were found in the complete fertilizer plots compared to the control treatments (Table 1). It is known that only culturable microbial populations can be determined by the total plate count method, which is only a portion of the total soil population. However, in the complete fertilizer treatment, higher bacterial populations were recorded compared to other treatments. The populations can change due to different fertilizer applications (Haynes, 1980). The results revealed that the absence of one particular major nutrient element, such as N, P or K affects the population of bacteria, while the population of fungus was not affected by the absence of nutrient elements. It was found that fertilizer directly stimulates microbial growth and may affect the composition of individual microbial communities (Khonje et al., 1989). An increased in actinomycetes population was found in the absence of P and N treatments. The soil nutrient contents are presented in Table 2. After the 45^{th} crop cycle there was no significant changes found for N, P, and K element in the full fertilizer and control treatment, but after the 47^{th} crop cycle the P nutrient content was drastically reduced. However, the microbial populations may be affected by the existing rice crop in the field as plant root exuded provide the required nutrients for microbes and every crop cycle adds on roots and straw stubble as organic matter in the missing treatment plots. Soil microbes have a role in nutrient cycling and soil fertility through biochemical processes which preserve the source and sink of soil mineral nutrients (Jenkinson and Ladd, 1981). However, a soil sample without a crop may generate different scenario of microbial populations in the missing elements experiment. Hence, further study is needed to find out the effect of missing nutrient in the absence of the crop plant on total microbial populations.

Diazotroph populations and biological nitrogen fixation (BNF)

A total of 45 diazotrophic strains were isolated from the five different treatments (Table 3). Irrespective of treatments, the population of diazotrophs was found to be higher in the rhizosphere compared to the soil and roots. Among the treatments, the higher rhizosphere, soil and endophytic populations were found in the complete fertilizer treatments and treatments without N. Comparatively, low populations were found in the control fertilizer treatment which proved that the nutrient requirements (other than nitrogen) of diazotrophs were like all living prokaryotes. In one study it was found that application of manure and fertilizer increased the populations of *Azotobacter* and *Azospirillum* (Mujiyati and Supriyadi, 2009). However, it was proven that application of high N fertilizers to lowland rice significantly inhibited the biological nitrogen fixation process (Henson et al., 1984; Sherestha and Ladha, 1996). Besides full fertilizer treatments, higher diazotrophic populations were found in fertilizer treatments without nitrogen. This may be due to the capability of nitrogen fixation of these bacteria in an nitrogen absent environment, which fulfills their life process as other nutrients were available for their growth. Isolated diazotrophs were able to fix nitrogen through biological process (Table 4). A total of 17 fast growing diazotrophic strains were selected and tested. Results showed that diazotrophs were able to fix 1.0 to 3.7% of atmospheric nitrogen during the incubation period (5 days). Root endophytic strain (strain 17) was able to fix the highest concentration of biological nitrogen. Diazotrophs that efficiently colonize the interior of rice roots might have higher potential to fix nitrogen. It has been reported that endosphere biological nitrogen fixation (BNF) of plant nitrogen are more extensive than rhizosphere contributions due to the lack of competition from other rhizosphere organisms and the availability of carbon sources with small fluctuations in pO_2 (Quispel, 1991). In terms of yield after 23 years of full fertilizer and without N treatments, there was only a slight decrease in yield (0.5- 0.6 t ha^{-1}) obtained in the absence of N compared to the full fertilizer treatment (Table 5). We know that nitrogen is the major nutrient element for rice production and that to produce 1 tonne of rough rice 15 kg nitrogen is required. The initial soil analysis report (in 1985) showed that total N was 0.08%, and in the year 2006 it was 0.12%. The addition of nitrogen in N deficit soil through the biological process might be the only possible source to obtain the high yields. Large amounts of nitrogen are derived in rice plants through biological nitrogen fixation (Sheno et al., 2001). In a ^{15}N study it was proven that biological nitrogen fixation can solely supplement 40% of the rice crop N requirement (Naher et al., 2011). Thus, the continuous twenty three (23) years of absence of the nitrogen element not only favored free living nitrogen fixing bacteria, but also benefitted from atmospheric nitrogen (N_2) through the biological nitrogen fixation process.

Phosphate solubilizing bacteria (PSB) population and phosphate solubilizing activity

A total of 12 phosphate solubilizing bacterial isolates were found in this study. Similar to nitrogen fixing bacteria, the PSB population was higher in the rhizosphere compared to the soil and endophytes (Table 6). Higher PSB populations have been reported in the aerobic rice rhizosphere compared to the soil (Panhwar et al., 2012). Significantly high populations were found in the full fertilizer treatments and the lowest population was recorded in the control treatment.

Table 1. Total microbial population with long term absence of major nutrient elements during Boro 2010 (Gazipur).

Treatments	Microbial population cfu g^{-1} soil		
	Total bacteria	Total fungus	Total actinomycetes
Complete fertilizers (NPKSZn)	7.2×10^7 a	1.5×10^4 a	7×10^3 c
NPSZn (-K)	6×10^6 b	1.2×10^4 a	7×10^3 c
NKSZn (-P)	6×10^6 b	5×10^4 a	5×10^5 a
PKSZn (-N)	5×10^6 b	1.3×10^4 a	2×10^5 a
Without fertilizers	1×10^6 b	9.8×10^4 a	1×10^4 b

Means within columns followed by the same letters are not significantly different according to Tukey's HSD at P≤0.05.

Table 2. Diazotroph populations of soil, rhizosphere and root endosphere of BRRIdhan29 grown in the Missing Element Trial experiment (year, 2010).

Treatments	Diazotroph population (cfu g^{-1})		
	Soil	Rhizosphere	Root endophyte
Complete fertilizers (NPKSZn)	2.3×10^5 a	3.2×10^7 a	5.7×10^5 b
NPSZn (-K)	2.2×10^4 b	1.9×10^6 b	2.1×10^4 c
NKSZn (-P)	1.2×10^4 b	3.1×10^6 b	1.3×10^4 c
PKSZn (-N)	3.9×10^5 a	5.4×10^7 a	2.2×10^6 a
Without fertilizers	2.2×10^2 c	4.2×10^5 c	6.3×10^4 c

Means within columns followed by the same letters are not significantly different according to Tukey's HSD at P≤0.05.

Table 3. Biological nitrogen fixation and indoleacetic acid production of isolated diazotrophs in the missing element trial experiment (year 2010).

Diazorophs	Treatment	Place of isolation	Nitrogen fixation (%) broth culture	Indoleacetic acid (mg l^{-1})
Strain 1	Complete fertilizer	Soil	1.0 c	4.21 c
Strain 2	Complete fertilizer	Rhizosphere	1.0 c	4.12 c
Strain 3	Complete fertilizer	Root endophyte	1.0 c	4.21 c
Strain 4	Complete fertilizer	Rhizosphere	1.0 c	4.29 c
Strain 5	Complete fertilizer	Soil	1.0 c	5.35 b
Strain 6	NPKZn (-P)	Rhizosphere	1.1 c	5.53 ab
Strain 7	NPSZn (-K)	Rhizosphere	1.1 c	4.38 bc
Strain 8	NPSZn (-K)	Root endophyte	1.3 c	4.56 b
Strain 9	Without fertilizers	Soil	1.1 c	5.56 ab
Strain 10	NPKZn (-P)	Rhizosphere	1.0 c	4.47 bc
Strain 11	PKSZn (-N)	Rhizosphere	2.3 b	4.35 c
Strain 13	NPKZn (-P)	Root endophyte	1.4 c	4.91 b
Strain 14	PKSZn (-N)	Root endophyte	2.3 b	5.06 b
Strain 15	PKSZn (-N)	Rhizosphere	2.3 b	4.41 c
Strain 16	Without fertilizers	Rhizosphere	1.4 c	4.24 c
Strain 17	PKSZn (-N)	Root endophyte	3.7a	6.91 a

Means within columns followed by the same letters are not significantly different according to Tukey's HSD at P≤0.05.

There was no significant difference in PSB populations between the P and K deficit treatments. This proved that a deficiency of either P or K reduced the PSB population. However, all the selected PSB isolates were able to solubilize P in NBRIP media plates, indicating clear halo zones for P-solubilizing activity (Plate 1). The highest P solubilizing activity was found in strain PSB6 (80%) followed by PSB7 (78.18%), while, the lowest activity (12%) was found in PSB12 (Table 7). Overall, higher P solubilizing activity was found in isolates obtained from treatments receiving complete fertilizer without P. Phosphorus (P) is the second most important nutrient element after nitrogen. The soil P cycle is a dynamic process involving the transformation of P by geochemical and biological processes. The PSB strains are able to solubilize certain amount of P from organic and inorganic soil P by mobilization (Khan et al., 2009). The inorganic P ranges between 25–42 μg P ml^{-1} (Tao et al., 2008) and the mineralizing organic P ranges between 8–18 μg P ml^{-1}. After 23 years of practicing the same treatment (without P), the yield was decreased by only 0.44-0.6 t ha^{-1} compared to the P fertilizer treatment. The nutrient content of the soil (without P treatment) analyzed after the 47th crop cycle (2006) showed that the soil P content was drastically reduced from initial (year, 1985) P content (9.7 mg kg^{-1}) compared to the full fertilizer treatment, but no similar trends in yield reductions were observed. This might be attributed to the PSB that solubilized soil organic P from the added crop residue and made it available for crop uptake.

Indoleacetic acid (IAA) production

The consequence of nitrogen fixation and phosphate solubilization by the isolated bacteria was plant growth enhancement activities with the production of phytohormones such as indoleacetic acid. Isolated diazotrophs were able to produce 4-9 mgl^{-1} of IAA. Beneficial microbes that are capable of producing indoleacetic acid (IAA) have pronounced effects on plant growth and development (Naher et al., 2011). The IAA improved amylase activity during rice seed germination, enhanced seedling vigor, speed of germination, and increased seedling length and dry weight. The extensive root growth influenced crop growth and development by increasing the plant's ability to absorb mineral nutrients from large volumes of soil (Biswas et al., 2000). The higher indoleacetic producing strains were isolated from complete fertilizer and without N treatments.

Table 4. Soil nutrient status after the 47th crop (Missing element trial experiment; Year, 2007).

Treatments	45th crop (2005)			47th crop (2006)		
	Total N (%)	Available P (ppm)	Exch K (ppm)	Total N (%)	Available P (ppm)	Exch K (ppm)
Complete fertilizers (NPKSZn)	0.13a	10.2a	54.6a	0.14a	20b	45c
NPSZn (-K)	0.11b	9.7a	39.0c	0.12c	20b	47b
NKSZn (-P)	0.13a	7.5b	46.8b	0.13b	2.0c	44d
PKSZn (-N)	0.08d	7.7b	23.4e	0.12c	27.5a	51a
Without fertilizers	0.09c	5.7c	31.2d	0.11d	2.0c	46c

Means within columns followed by the same letters are not significantly different according to Tukey's HSD at P≤0.05. Initial soil N= 0.08, P = 9.8, k = 70 ppm, Source, BRRI annual report, 2007.

Table 5. Effect of long-term absence of nutrient elements on grain yield (year, 2008-9).

Treatments	BR11 (t ha^{-1}) year, 2008	BRRIdhan31 (t ha^{-1}) year, 2009
Complete fertilizers (NPKSZn)	3.77a	3.53a
NPSZn (-K)	2.21b	2.58c
NKSZn (-P)	3.43a	3.09b
PKSZn (-N)	3.17a	3.02b
Without fertilizers	2.90b	2.57c
LSD (0.5)	0.11	0.11

Means within columns followed by the same letters are not significantly different according to Tukey's HSD at P≤0.05.
Source, BRRI annual report, 2008-9

Table 6. Phosphate solubilizing bacterial population of soil, rhizosphere and root endosphere of BRRIdhan29 (Missing Element trial experiment; Year, 2010).

Treatments	PSB population (cfu g^{-1})		
	Soil	Rhizosphere	Root endophyte
Complete fertilizers (NPKSZn)	1.1×10^5 a	1.3×10^7 a	2.3×10^5 a
NPSZn (-K)	2.6×10^4 b	1.2×10^6 b	1.1×10^3 b
NKSZn (-P)	3.4×10^4 b	2.1×10^6 b	1.3×10^3 b
PKSZn (-N)	1.9×10^4 b	5.4×10^6 b	2.0×10^3 b
Without fertilizers	1.1×10^2 c	1.8×10^5 c	7.3×10^2 c

Means in each column followed by the same letters are not significantly different according to Tukey's HSD at P≤0.05.

Table 7. Phosphate solubilizing bacterial activity and indoleacetic acid production by isolated diazotrophs (Missing Element trial experiment; Year, 2010)

Isolates	Treatment	Place of isolation	P solubilization (%)	IAA production (mg L^{-1})
PSB 1	NPKZn (-P)	Root endophyte	55.05d	3.0c
PSB 2	Complete fertilizer	Rhizosphere	63.0c	4.23b
PSB 3	Complete fertilizer	Rhizosphere	72.0b	4.34b
PSB 4	Complete fertilizer	Root endophyte	64.47c	5.23a
PSB 5	NPKZn (-P)	Soil	60.13c	3.0c
PSB 6	NPSZn (-K)	Soil	80.0a	2.1d
PSB 7	Complete fertilizer	Soil	78.18a	1.67e
PSB 8	Complete fertilizer	Soil	77.07a	1.05f
PSB 9	NPKZn (-P)	Rhizosphere	72.5b	2.7cd
PSB 10	Without fertilizers	Rhizosphere	13.59	0.82
PSB 11	Complete fertilizer	Rhizosphere	26.13f	3.17c
PSB 12	Complete fertilizer	Root endophyte	30.00ef	1.85e
PSB 13	NPSZn (-K)	Root endophyte	25.00f	2.42d
PSB 14	NPSZn (-K)	Soil	12.50g	1.5e
PSB 15	NPKZn (-P)	Rhizosphere	39.93e	1.0f
PSB 16	NPKZn (-P)	Rhizosphere	62.82c	2.79cd

Means within columns followed by the same letters are not significantly different according to Tukey's HSD at P≤0.05.

Materials and methods

Collection of soil samples

The microbial populations were determined from long-term Missing Elements Trail (-N, -P, -K, control and complete fertilizer treatments). The missing elements experiment was initiated in 1985 at the Bangladesh Rice Research Institute, Gazipur and has been continued to date to determine nutrient deficiency problems. The soil samples were collected from selected treatments (between four hills) and kept at 4 ^oC temperature until analyses. The *in vitro* studies were conducted at the BRRI soil microbiology laboratory, Gazipur, during the period of 2009. Total microbial population, rhizosphere and endophytic nitrogen fixing and phosphate solubilizing bacterial populations were determined in high-yielding rice variety BRRI dhan29.

Determination of total microbial population

Exactly 1 gm of soil sample was taken into 95 ml of sterile distilled water and shaken for 15 minutes. A series of 10 fold dilutions were prepared up to 10^{-8} and 0.1 ml of each dilution was spread on media plates. To enumerate total bacterial,

fungal and actinomycetes population nutrient agar (NA), potato dextrose agar (PDA) and actinomycetes media plates were used, respectively. After 3-5 days of incubation microbial population was counted following the spread plate technique.

Determination of free-living nitrogen fixing bacterial population in the rhizosphere

To determine the free-living nitrogen fixing bacterial population 2 g of rhizospheric soil including roots were used and a series of 10 fold dilutions were prepared. Nitrogen fixing bacterial population was determined using the most probable number (MPN) method in nitrogen-free semisolid media (Prasad et al., 2001).

Determination of nitrogen-fixing root endophyte bacterial population

About 2 g of fresh root was washed and surface sterilized with 70% ethanol for 5 min and then with 3% Clorox for 30 sec. After surface sterilization roots were washed several times with sterile distilled water. The root samples were checked for the efficacy of surface sterilization by rolling them on nutrient agar plates. The surface sterilized roots were meshed by using a sterilized mortar and pestle. A 10-fold series of dilutions were prepared and the diazotrophic populations were determined using the MPN method in Nfb semi-solid medium.

Determination of phosphate solubilizing bacterial population

Phosphate solubilizing bacterial (PSB) rhizosphere population was determined using the spread plate count method. After preparing a series of dilutions (10 fold), exactly 0.1 ml of samples were spread on NBRIP plates using hokey stick. The endosphere population was determined by meshing surface sterilized roots (2 g), and after serial dilution, 0.1 ml was spread on NBRIP media plates. After 5 days of incubation only halo zone producing colonies were counted.

Determination of P solubilizing activity

The phosphorus solubilizing activity of PSB isolates were determined by spotting 10 µl of 48 h cultures (exponential growth phase) on NBRIP agar media (Nautiyal, 1999) containing (g L^{-1}): $MgCl_2.6H_2O$ 5 g, $MgSO_4.H_2O$, 0.25 g, KCl 0.2 g, $(NH_4)_2SO_4$ 0.1 g, $Ca_3(PO_4)_2$ 5 g, and 20 g of bacterial agar, The plates were incubated at 30 ^oC for one week and observed for halo zone formation. The solubilizing activity was calculated using the following formula (Nguyen et al., 1992)

$$P\ solubilization\ efficiency = \frac{solubilization\ diameter\ (halo\ zone)}{growth\ diameter\ of\ colony} \times 100$$

Determination of total nitrogen from broth culture

Isolates that, grown only in nitrogen-free semi-solid media were selected for nitrogen fixing activity. The nitrogen fixation capability of the diazotrophs was determined from broth culture using the micro Kjeldahl method. Pure isolates were grown without adding bromothymol blue for 5 days in nitrogen-free broth (5 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$. 7 H_2O, 0.1 g NaCl, 0.02 g $CaCl_2$ and 4 ml of 1.64% Fe-EDTA solution, pH 7.2). One ml of broth was filtered through 0.2 µM pore syringe filter and total nitrogen was determined according to the kjeldahl digestion method.

Determination of indoleacetic acid (IAA) production

Isolates were inoculated in Jensen's broth and incubated at 29 ± 2 ^oC for 48 h. One ml of inoculated broth culture was transferred into new 50 ml fresh Jensen's broth containing 2 mg ml^{-1} of tryptophan and incubated at 29 ± 1 ^oC for 72 h. Approximately 2 ml of culture solution was centrifuged at 7000 rpm for 7 min and the supernatant was used to determine the IAA concentration. One ml of the supernatant was mixed with 2 ml of Salkowsky's reagent (2% of 0.5 M $FeCl_3$ in 35% perchloric acid) according to the method described in Gordon and Weber (1951). After 20-25 minutes, the absorbance was read using a spectrophotometer at 530 ηm. The IAA standard curve was prepared using pure IAA and the concentration of IAA was determined using the standard graph. Supernatants of un-inoculated test tubes were used as control. There was no visible colour was observed in the controls.

Statistical analysis

Data were analyzed using the analysis of variance procedure in the SAS statistical program version 9.2**.** The treatment means were compared using Tukeys' test at 5% level of confidence.

Conclusion

Long-term (24 years) absence in input of any one major nutrient element significantly reduced total microbial populations. The lack or absence of supplemental nitrogen favored free-living nitrogen fixing bacteria which contributed to the rice yield. The long term P deficit situation however did not enhance phosphate solubilizing bacterial populations although a number of efficient P solubilizers were isolated from the full fertilizer and P deficit treatments. Irrespective of fertilizer treatments, higher populations of bacteria were found at the rhizosphere. This proves that rice roots provide a suitable niche for bacterial growth.

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