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Isolation, characterization and beneficial effects of rice associated plant growth promoting bacteria from Zanzibar soils

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This study was undertaken to isolate and characterize plant growth promoting bacteria (PGPB) occurring in four soils of Zanzibar, Tanzania as well as to evaluate their potential use as biofertilizers for rice. A total of 12 PGPB strains were isolated from rice and studied for growth characteristics, carbon/nitrogen source utilization patterns using QTS-24 kits, phosphate solubilization, indole acetic acid (IAA) production, antibiotic resistance patterns and growth at different pH, temperature and salt concentrations. All the isolates were motile and gram negative except Z3-4. Acetylene reduction activity was detected in all isolates ranging from 5.9–76.4 nmole C_2H_2 reduced/h × mg protein while 9 isolates produced IAA ranged from 20–90.8 mg/l. Most of the isolates showed resistance against different environmental stresses like 10–40 °C temperature, 0.2–1 M salt concentration and 4–8.5 pH range. Only one isolate Z2-7 formed clear zones on Pikovskaia's medium showing its ability to solubilize phosphates. Z3-2 was used to develop fluorescent antibodies to check the cross reactivity of the isolates. Inoculation of these bacterial isolates resulted in higher plant biomass, root area, and total N and P contents on Tanzanian rice variety *BKN PRAT3036B* under controlled conditions. *Bacillus* sp. Z3-4 and *Azospirillum* sp. Z3-1 are effective strains and, after further testing under field conditions, can be used for inoculum production of rice in Tanzania. The plant growth promoting effects of these PGPRs suggest that these can be exploited to improve crop productivity of rice in Tanzania.

Agriculture is the most important sector of Tanzanian economy. The government policy on food crops is food security and self-sufficiency through affordable domestic production. Production of rice is a major saving to the rural households in the sense that it obviates the need to buy rice. Use of nitrogen fertilizer is of great importance in rice production, as nitrogen is the major factor limiting growth under most conditions (DAWE 2000). Since agriculture is expected to move towards environmentally sustainable methods (STURZ *et al.* 2000), much attention has been paid to natural methods of biological nitrogen fixation. Rice species are likely to harbor unique populations of nitrogen fixing bacteria (HAFEEZ *et al.* 2002, ENGELHAND *et al.* 2000). Several diazotrophic bacteria have been isolated from the rhizosphere of rice (EGENER *et al.* 1998, FUJIE *et al.* 1987, BALDANI and DOEBEREINER 1980). Presently there is little documented information on the occurrence of PGPBs of potential to produce biofertilizers in Tanzania. PGPBs harbored by such unexplored environments may be looked upon as being wild types showing natural variability. Therefore, the study reported herein was undertaken to study PGPBs flora of some Tanzanian soils. The objectives were to isolate PGPBs occurring in some ecological location of Tanzania, to carry out a preliminary characterization and to evaluate the beneficial effects of these bacterial inoculants on rice.

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Table 1
Physico-chemical properties of four soil samples collected from Zanzibar, Tanzania

Soil	Source	pH	N	P	K^+	Ca^{2+}	Na^+
			%	µg/g	µg/g	µg/g	µg/g
Z1	Kizimbian, Zanzibar	7.5	1.9 ± 0.8	17 ± 0.3	08 ± 0.9	10.5 ± 1	13.8 ± 1.6
Z2	Maruhubi, Zanzibar	8.6	1.4 ± 0.8	38 ± 3.4	20 ± 2.1	12.5 ± 2.3	128.8 ± 4.5
Z3	Selem, Zanzibar	7.0	1.6 ± 0.6	160 ± 4.2	8 ± 1.1	04.5 ± 1.4	13.8 ± 1.7
Z4	Kizimbian, Zanzibar	7.5	2.9 ± 1.2	58.6 ± 4.8	21.5 ± 1.8	44.5 ± 3.8	60.7 ± 3.2

Results are an average of four replicates
± Standard deviation

Results

Physico-chemical properties of Zanzibar soils

The soil samples used in the present study had pH ranged from 7–8.6. Total N and P were ranged from 1.4–2.9% N and 17–169 µg P/g soil, respectively. Details of other ionic concentration are given in (Table 1).

Isolation and characterization of bacteria from rice

A total of thirty-two bacterial isolates were obtained initially from the rice roots and rhizosphere soils collected from Zanzibar. Only 12 isolates were selected for further studies on the basis of morphological and cultural characteristics, as others were just the re-isolation of the similar strains. All the isolates were observed to be gram negative except Z3-4 and motile rods. These isolates were found to be the members of different genera i.e. *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas* (Table 2).

Table 2
Acetylene reduction assay (ARA), indole acetic acid (IAA) production and P- solubilization of the PGPBs isolated from rice grown in the soils of Zanzibar, Tanzania

Strain	Source	[a]ARA	IAA*	IAA**	*P*	[b]Tentative
		nmol C_2H_2/mg protein × h		mg/l	µg/ml	Identification
Z1-1	soil	29.3 ± de	–	–	–	*Azotobacter* sp.
Z1-3	soil	20.6 ± 1.7 f	+	30.3 ± 4.5	–	*Pseudomonas* sp.
Z2-4	crushed root	58.4 ± 3.5 c	+	45 ± 4.2	–	*Azospirillum* sp.
Z2-5	soil	32.5 ± 2.7 d	+	38 ± 3.5	–	*Pseudomonas* sp.
Z2-7	crushed roots	21.9 ± 1.3 f	+	62 ± 8	310.6 ± 25	*Pseudomonas* sp.
Z2-10	soil	6.3 ± 0.9 g	+	53 ± 6.5	–	*Pseudomonas* sp.
Z3-1	crushed roots	76.4 ± 4.6 a	+	42 ± 4.8	–	*Azospirillum* sp.
Z3-2	crushed roots	68.6 ± 3.1 b	+	20 ± 3.7	–	*Azospirillum* sp.
Z3-3	crushed roots	21.2 ± 1.7 f	+	90.8 ± 10	–	*Azotobacter* sp.
Z3-4	crushed roots	27.2 ± 3.8 e	+	56 ± 8.2	–	*Bacillus* sp.
Z4-1	soil	8.4 ± 1.3 g	–	–	–	*Pseudomonas* sp.
Z4-2	soil	5.9 + 1 g	–	–	–	*Pseudomonas* sp

* Spot test ** HPLC
[a] The results of ARA, IAA and P are an average of four replicates. Means followed by the same letter differ non-significantly at $p = 0.05$ according to DMRT. ± Standard deviation
[b] Tentative identification based on morphological characteristics and biochemical tests using QTS-24 kit

Table 3
Nitrogen fixing activity of the plant parts from rice grown in different soils of Zanzibar, Tanzania

Soil	Plant part	[a]ARA nmoles C_2H_2 reduced/g dry wt × *h*
Z1	Shoot	16 ± 3.6
	Root	35 ± 5.7
Z2	Shoot	16 ± 4.9
	Root	39 ± 3.1
Z3	Shoot	20 ± 1.6
	Root	17 ± 2
Z4	Shoot	21 ± 1
	Root	27 ± 2.9

[a] The results of acetylene reduction assay (ARA) are an average of four replicates ± Standard deviation

Nitrogenase activity was detected in roots as well as in shoots of rice variety *BKN PRAT3036B*. Nitrogen fixing activity in shoot was ranged from 16–21 nmoles C_2H_2 reduced/g dry weight × *h* and from 17–39 nmoles C_2H_2 reduced/g dry weight × *h* in the roots (Table 3). In roots, relatively higher acetylene reduction was detected as compared to shoot. All the twelve PGPB isolates showed nitrogenase activity ranging from 5.6–76.4 nmoles C_2H_2 reduced/mg protein × *h*. An isolate Z3-1 showed maximum activity i.e. 76.4 nmoles C_2H_2 reduced/mg protein × *h* (Table 2).

Among all the isolates tested in colorimetric method, seven strains produced pink coloration showing their ability to produce IAA. Three isolates produced IAA ranged from 20.9–90.8 mg/l. Isolate Z3-3 produced the maximum IAA i.e. 90.8 mg/l (Table 2).

Only one isolate Z2-7 showed the ability of phosphate solubilization by forming the zone of P solubilization around the inoculated surface of PIKOVSKAIA's medium. Decrease in pH of Pikovskaia's broth from 7–5.4 showed the activity of phosphate solubilizing bacteria (PSB). The amount of phosphate solubilized by isolate Z2-7 was 310.6 µg/ml (Table 2).

All the isolates grew at the temperature ranging from 10–35 °C. Only one isolate Z1-1 was able to grow at all the temperatures tested even at 5 °C and 45 °C (Fig. 1). Most of the isolates tolerated salt concentrations ranged from 0.2–1 M (Fig. 2). All the isolates were

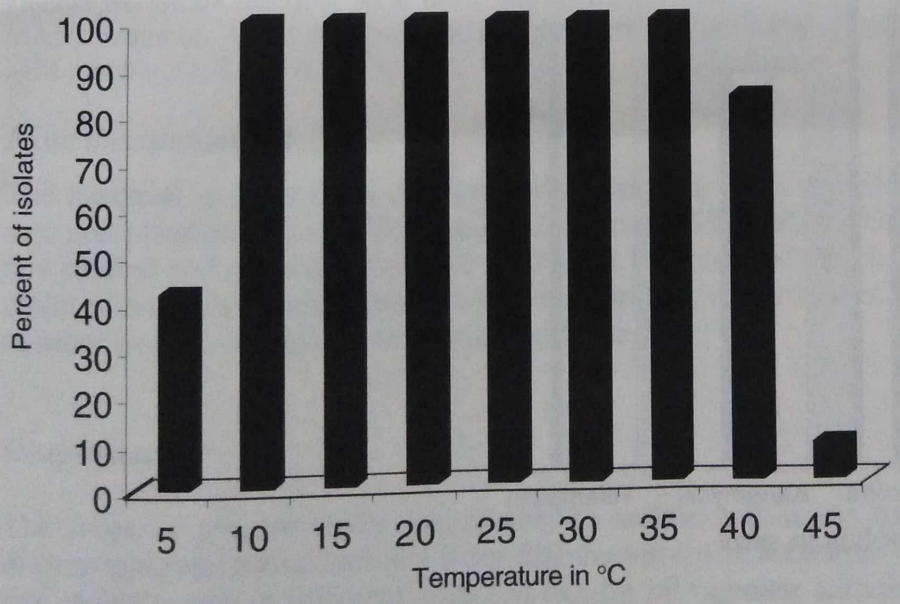

Fig. 1
Effect of temperature on growth of rice associated PGPBs isolated from soils of Zanzibar, Tanzania

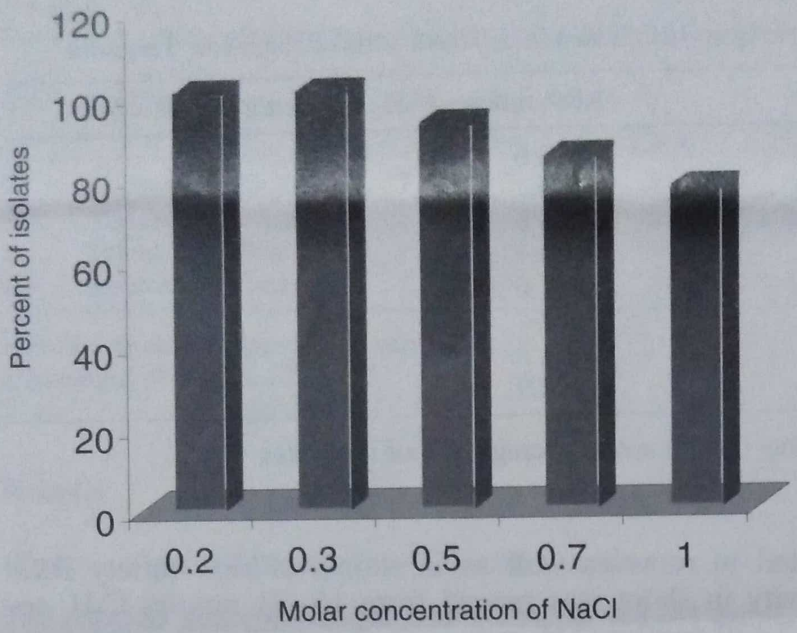

Fig. 2
Tolerance of rice associated PGPBs to different concentrations of NaCl isolated from soils of Zanzibar, Tanzania

able to grow at all the pH levels tested. Isolates Z1-3, Z2-10 and Z4-2 were resistant to all the four antibiotics used. Z1-1 and Z3-2 showed high susceptibility to rifampicin (Fig. 3). All the bacterial isolates were subjected to a group of tests and the observations yielded 26 recordable characters. Some of the distinct biochemical properties of these isolates have been presented in Table 4. The FA of an isolate Z3-2 showed antigenic uniqueness of Z3-2 and did not show any cross reactivity with other PGPB isolates.

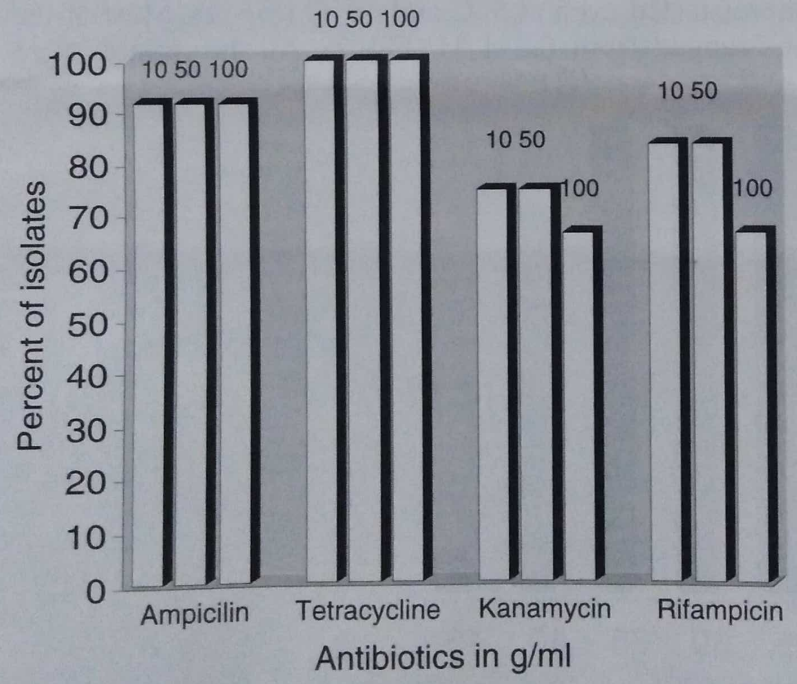

Fig. 3
Effect of different antibiotics on growth of rice associated PGPBs isolated from soils of Zanzibar, Tanzania

Table 4.
Physiological and biochemical tests using QTS-24 kit for characterization of PGPBs isolated from Tanzanian soils.

	Isolates											
Test	Z1-1	Z1-3	Z2-4	Z2-5	Z2-7	Z2-10	Z3-1	Z3-2	Z3-3	Z3-4	Z4-1	Z4-2
ONPG	+	+	+	+	+	+	+	+	+	+	+	+
CIT	–	–	–	–	–	+	–	–	–	–	+	+
MALO	–	–	–	–	+	+	–	–	–	–	–	–
LDC	–	–	–	–	–	–	–	–	–	–	+	–
ADH	–	–	–	–	–	+	–	–	–	–	+	–
ODC	–	–	–	–	–	–	–	–	–		+	–
H2S	–	–	–	–	–	–	–	–	–	–	–	–
UREA	–	–	–	+	–	–	+	+	–	+	+	–
TDA	–	–	–	+	–	–	–	–	–	–	+	–
IND	–	–	–	–	–	–	–	–	–	–	–	–
VP	–	–	–	–	–	+	–	+	–	–	+	–
GEL	–	–	–	+	+	–	–	–	–	+	+	–
GLU(a)	+	+	+	+	+	+	+	+	+	+	+	+
(b)	+	+	+	+	+	+	+	+	+	+	+	+
MAL	+	+	+	+	+	+	+	+	+	+	+	+
SUC	–	–	–	–	–	–	–	–	–	–	+	–
MAN	–	–	–	–	–	+	–	+	–	–	+	–
ARA	+	+	+	+	+	+	+	+	+	+	+	+
RHA	+	+	+	+	+	+	+	+	+	+	+	+
SOR	–	–	–	–	–	–	–	–			+	–
INOS	–	–	–	–	–	–	–	–	–	–	–	–
ADO	–	–	–	–	–	–	–	–	–	–	–	–
MEL	+	+	+	+	+	+	+	+	+	+	+	+
RAF	–	–	–	–	–	+	–	–	–	+	+	–
MOT	+	+	+	+	+	+	+	+	+	+	+	+
CO	–	+	+	+	+	+	–	+	+	–	+	+

CIT: Sodium Citrate, MALO: Sodium Malonate, LDC: Lysine decarboxylase, ADH: Arginine dihydrolase, ODC: Ornithine decarboxylase, H_2S: H_2S production, URE: Urea hydrolysis, TDA: Tryptophane deaminase, IND: indole, VP: Acetone, GEL: gelatin hydrolysis, GLU a: acid from glucose, b: nitrate reaction, Acid from (MAL: maltose, SUC: sucrose, MAL: maltose, SUC: sucrose, MAN: mannitol, ARA: arabinose, RHA: rhamnose, SOR: sorbitol, INO: inositol, ADON: adonitol, MEL: melibiose, RAF: raffinose

Plant inoculation test

The bacterial isolates from rice grown in Zanzibar soils showed beneficial effects on root area and plant biomass of Tanzanian rice variety *BKN PRAT3036B*. All the growth parameters studied and quantification of total N and P content of the plant samples showed that the effect of bacterial inoculation was more prominent with isolates Z3-4 and Z3-1 as compared to other isolates tested and the controls (Table 5).

Discussion

The scope of present study was mainly to isolate and assess the biochemical variation of diazotrophic microbial isolates from different soils of Zanzibar. Roots and shoots of all the rice plants grown in different soils did exhibit nitrogenase activity although with substantial variation. Isolation procedure led to the isolation of 32 microbial colonies, of which 12 morphotypic representatives were chosen and included in the present study. Morphological

Table 5
Effect of inoculated PGPBs on rice var. *BKN3036b* isolated from soils of Zanzibar, Tanzania

Treatment	Root area $(mm)^2$	Plant biomass mg	*N* µg/g	*P* µg/g
Z1-1	23 ± 1.4 ef	3.4 ± 0.4 gh	3.4 ± 1.2 g	4.2 ± 0.6 g
Z1-3	9.2 ± 0.4 h	3 ± 0.6 gh	4.8 ± 1.6 e	5 ± 1.3 efg
Z2-4	25 ± 1.6 def	21.3 ± 0.5 a	5.1 ± 1.3 e	6.4 ± 1.5 def
Z2-5	28 ± 2.2 d	5.2 ± 0.5 f	6.9 ± 0.9 cd	4.9 ± 2.2 g
Z2-7	24 ± 1.9 def	4.9 ± 0.8 f	4.4 ± 2.1 ef	6.9 ± 2.4 ab
Z2-10	22 ± 2.4 f	3.1 ± 0.9 gh	3.6 ± 0.7 fg	4.6 ± 0.9 fg
Z3-1	8 ± 3.4 b	37.2 ± 1.2 e	8.6 ± 0.8 a	6.3 ± 0.9 abcd
Z3-2	31 ± 3.5 c	10 ± 2.3 c	7.2 ± 0.4 bc	6.3 ± 0.9 abc
Z3-3	27 ± 2.6 de	18 ± 2.8 b	6.5 ± 0.7 cd	6 ± 0.8 bcd
Z3-4	46 ± 3.1 a	21 ± 3.1 a	7.8 ± 0.5 b	7.1 ± 1 a
Z4-1	31.5 ± 2.8 c	48 ± 3.8 f	6.2 ± 0.9 d	5.8 ± 0.9 cde
Z4-2	45 ± 3.3 a	4 ± 0.8 fg	6.3 ± 0.8 cd	5.9 ± 1.4 cde
Control *	16 ± 0.9 g	8.5 ± 0.7 d	4.4 ± 1 ef	4.9 ± 1.6 g
Control**	8.4 ± 0.8 h	2.6 ± 0.6 h	2.9 ± 1.2 h	3 ± 0.8 h

Control* uninoculated with N
Control** uninoculated without N
Means are the average of six replicates. Means followed by the same letter differ non-significantly at $p = 0.05$ according to DMRT. ± Standard deviation

characterization and biochemical tests (QTS-24 kit) of these bacteria showed that they belong to different bacterial genera including *Azospirillum, Azotobacter, Bacillus* and *Pseudomonas* as they showed maximum common characteristics with these genera (FARMER *et al.* 1992, GRIMONT and GRIMONT 1992, HOLT *et al.* 1994). The present data showed that all the three *Azospirillum* species had strong acetylene- reducing activity compared to the isolates of other genera. Nitrogen fixer *Azospirillum* isolated from the rhizosphere of rice collected from Kyonggi-do and Chungcheongnam-do in Korea had been reported to show the acetylene- reducing activity of 400 to 900 nmol C_2H_2 reduced/h × vial (KIM *et al.* 1994).

Nitrogen fixing activity was detected in roots as well as in shoots of rice var. *BKN PRAT3036B*. This indicates colonization of rice roots and shoots by diazotrophic bacteria. Higher acetylene reduction was determined in roots as compared to shoots. Acetylene reduction activity detected in shoots may be of practical importance, as the isolation and use of bacterial isolates of shoots along with root colonizing bacteria may enhance efficiency of such inocula. Earlier studies using ARA-based MPN (Most probable number) to estimate the population size of diazotrophic bacteria showed higher ARA activity in shoots as compared to roots of rice var. *NIAB IRRI-9* (MEHNAZ *et al.* 2001). In wetland rice, contribution of the basal portion of shoot to nitrogen fixation has been reported by WATANABE *et al.* (1981).

Most of these isolates produced the phytohormone IAA in growth medium except Z1-1, Z4-1 and Z4-2. Productions of phytohormones by different PGPR strains and their beneficial effects on plants have been reported (HAAHTELA *et al.* 1990).

Pseudomonas sp. Z2-7 was the only bacterial isolate that showed phosphate solubilization 310.6 µg/ml with an increase of 204.9% over control. Decrease in pH of PIKOVSKAIA's broth from 7-5.4 showed the activity of phosphate solubilizing bacteria. Earlier studies expressed that a number of phosphate solubilizing bacterial strains showed an effective role in P-uptake and growth promotion of plants by dissolution of inorganic insoluble phosphate (BELIMOV *et al.* 1995, DAS and MUKHERJEE 2000). The soil microorganism (PBS strain) can increase the availability of phosphorus to plant by mineralizing organic phosphorus compound and by converting inorganic phosphorus into more available form (MARSCHNER

1995, BARYOSEF *et al.* 1999). Numerous studies have shown a fall in pH by production of organic acids when PBS strains are grown in liquid cultures (ASEA *et al.* 1988, RODRIGUEZ and FRAGA 1999). The similar phenomenon was observed in this study too.

The survival of these PGPB isolates under different temperatures is of significance. Although more research work is needed to evaluate their efficiency under temperature stress conditions but it was well documented that the relative effectiveness of plant inoculation was higher under extreme conditions of soil temperature in different experiments. It was suggested that the antistress effect of diazotrophs on the plants is an important mechanism of their interaction and mutual resistance to unfavorable environmental conditions (BELIMOV 1994).

Salt tolerance of PGPBs is usually tested at 0.5–3% NaCl in LB growth medium. Favorable salt tolerance concentration for all isolates was found to be 0.2–1 M NaCl. It is often believed that saline soils naturally select the strains that are more tolerant to salinity. In earlier studies, it has been shown that bacterial strains isolated from saline soils survive inhibitory levels of salinity better than that from non-saline soil (HUA *et al.* 1982, DOUKA *et al.* 1978). In Zanzibar soils, salinity level is high and that is why most of these isolates could grow at high concentration. The bacterial strains that survive in saline environment can successfully colonize plants grown under the same condition (SALEENA *et al.* 2001). Our results also confirm that the natural habitat of the strains performs a selection pressure for tolerance to salinity.

The efficiency of the association between bacterial strain and the host plant is affected by various factors as soil moisture, available N and the presence of efficient and competitive bacterial strains (THIES *et al.* 1995, PALMER and YOUNG 2000, YUHASHI *et al.* 2000). Present study showed that tolerance to a wide range pH is a typical feature of these PGPB isolates.

The resistance of all bacterial isolates against different antibiotics was carried out to study their potential competitiveness. Isolates Z1-3, Z2-10 and Z4-2 having resistance against ampicilin, tetracycline, kanamycin and rifampicin may show better competitiveness under natural soil conditions but more field study will be carried out to confirm these findings. It was reported that the survival of *Azospirillum lipoferum* in soil increased if the activity of soil microflora was inhibited by rifampicin (BELIMOV *et al.* 1998) but in an other report it was found that the resistance of diazotrophs to antibiotics (streptomycin and rifampicin) significantly diminished the nitrogenase activity of the associations but not of the pure cultures of the components (ZLOTNIKOV *et al.* 1997). Very little is known about the competitiveness of the microorganisms and factors governing it. Therefore, more ecological studies are needed to select a competitive and effective strain.

Azospirillum sp. Z3-2 was characterized by serological technique. The development of a serological screening test for the detection of PGPB strains is used to study the taxonomic identity and ecology of diazotrophic bacteria associated with non-legumes (KIRCHHOF *et al.* 1997). The cross reactivity of twelve PGPB isolates with the FAs of isolate Z3-2 showed that all the tested isolates were different from Z3-2 and denied the possibility of re-isolation of the same strain. Strain-specific antibodies directed against *Azospirillum* sp. Z3-2 would enable us to localize and quantify inoculant bacteria in different parts of the rhizosphere.

The favorable impact of bacterial inoculation on Tanzanian rice variety *BKN PRAT3036B* showed the positive effects of PGPB application on plant growth. Bacterial isolates *Bacillus* sp. Z3-4 and *Azospirillum* sp. Z3-1 are effective strains and, after further testing under field conditions, can be used for inoculum production of rice in Tanzania. The beneficial effects of PGPB inoculants on plant growth can be explained on the basis of various mechanisms such as production of siderophores, antibiotics and extracellular metabolites but the production of plant growth regulars in the rhizosphere is considered the most plausible one (ARSHAD and FRANKENBERGER 1998).

Results of this study revealed the potential of PGPB to increase crop productivity. Therefore, these bacterial isolates could be good candidates for rice inoculation under Tanzanian edaphoclimatic conditions. These data are the basis for strain improvement and cross inocu-

lation experiments for different varieties when searching for well-adapted and compatible partners. However, intensive work should be done to isolate more promising PGPR strains capable of improving yields of specific crop.

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