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BACTERIA WITH POTENITAL PLANT GROWTH-PROMOTING ABILITIES IN THE ROOT ENVIRONMENT OF SELECTED RICE VARIETIES GROWN IN SRI LANKA

W. S. Dandeniya* and R. M. C. P. Rajapaksha

Department of Soil Science, Faculty of Agriculture, University of Peradeniya.

ABSTRACT

Microorganisms with plant growth promoting traits can be isolated from plant root environment known as the rhizosphere. Culturable microorganisms with the ability to solubilize phosphrous (P), fix di-nitrogen (N_2) gas, secrete hormones like auxin are used in preparation of biofertilizers. The diversity of beneficial microorganisms in soils and their characters are poorly studied in Sri Lanka. The objective of the present study was to identify plant growth promoting bacteria inhabiting rice root environment. Rhizosphere soil samples were collected from 19 traditional varieties and one exotic variety, and two new improved varieties grown in irrigated paddy fields at Eppawala and Maha-Illuppallama at flowering stage. Total bacteria, cellulose decomposers, and *Azotobacter* in rhizosphere soils, rhizoplane and in endorhizosphere were enumerated using selective growth media and isolated and sub-cultured for characterization. Cultures were tested for P solubilization and auxin production and free-living N_2 fixation. Selected cultures positive for beneficial traits were identified using phospholipids fatty acid (PLFA) profiles.

The numbers of total counts of distinct colonies in selective media were different among varieties. Bacterial colonies with the ability to solubilize P, produce auxin, decompose cellulose and/or fix N_2 were found in rhizosphere soils in to varying degrees depending on the rice variety. Results indicated that despite the taxonomic similarity, differences exist in the beneficial traits among *Pseudomonas* sp., which is the dominant species among bacteria isolates. In addition, *Azospirillum, Enterobacter, Exigubacterium, Kluyvera, Aeromonas, Acinetobacter, Stenotrophomonas, Bacillus* and *Staphylococcus* were also isolated. The study confirms the presence of bacteria with possible plant growth promoting abilities in the rice rhizosphere. Their diversity and abundance may depend on the rice variety.

Key words: *Phosphorous solubilization, N_2 fixation, Auxin, Pseudomonas*

INTRODUCTION

High reliance on chemical fertilizers and pesticides is a characteristic feature of the widely practicing agronomic package for rice cultivation in Sri Lanka. An alternative option for nutrient management with high inputs of inorganic fertilizers is the inclusion of bio-fertilizers along with organic manures to the nutrient management package (Gunarto *et al.*, 1999; Garai *et al.*, 2013). Bio-fertilizers are widely formulated from bacteria belonging to genera such as *Rhizobia, Azotobacter, Azospirillum, Pseudomonas* and *Bacillus etc.* or mixtures aiming to fix nitrogen, solubilize phosphates or decompose fresh organic matter (Gunarto *et al.*, 1999; de Salamone *et al.*, 2010). In addition, mycorrhizae spores and infected roots are also used as biofertilizers particularly in nurseries. Development of a biofertilizer involves isolation and screening of microorganisms, testing under controlled condition and verifying under field condition.

Microorganisms having beneficial traits can be isolated from the rhizosphere and phyllosphere. The diversity of microorganisms in such habitats is affected by plant species, growth stage, microbial interactions, habitat characters, and agronomic practices leading to selective microbial communities more adapted towards specific environments (Berg and Smalla, 2009). A comprehensive study of rhizosphere microorganisms would provides an insight in to the diversity of microorganisms and also provides an opportunity to collect a large gene pool with beneficial traits.

Despite the fact Sri Lanka has a wide genetic pool of rice little information is available on the diversity of microorganisms associated with them. Thus, this study was undertaken to establish a culture collection of plant growth promoting rhizo-bacteria by screening the culturable bacteria inhabiting the root environment (rhizosphere, rhizoplane and endo-rhizosphere) showing beneficial traits such as fixing nitrogen, solubilizing Ca-phosphates, producing auxin and/or decomposing cellulose.

* Corresponding author: *warshisd@gmail.com*

MATERIALS AND METHODS

Rhizosphere soil and plant samples of 22 rice varieties were collected from paddy fields having Rhodustalf soils from Eppawala and Maha Iluppallam in the dry zone (DL1) of Sri Lanka.

Table 1. Rice varieties used in the study, their maturity period, sampling stage and cultivated location

Variety No.	Variety	Crop duration* (Months)	Growth stage at sampling	Location
V1	Buruma	6 ½	B	Eppawala
V2	Mahamariya	5 ½	B	Eppawala
V3	Kohu Ma Wee	5	B	Eppawala
V4	Raththal	9 ½	PI	Eppawala
V5	Rath Suwandal	3 ½	B	Eppawala
V6	Raththaran	7	B	Eppawala
V7	Muthu samba	7 ½	B	Maha Iluppallama
V8	Kalubala Wee	6 ½	B	Maha Iluppallama
V9	Sudu Heenaty	3 ½ to 4	B	Maha Iluppallama
V10	Suduru	7	B	Maha Iluppallama
V11	BG 450 [a]	4 ½	PI	Maha Iluppallama
V12	Bandara Haththawa	-	B	Maha Iluppallama
V13	Nona Bokra [b]	-	B	Maha Iluppallama
V14	BG 357 [a]	3 ½	B	Maha Iluppallama
V15	Rath Karal	3 ½	PI	Maha Iluppallama
V16	Al Wee	5 ½	PI	Maha Iluppallama
V17	Dik Wee	7 ½	B	Maha Iluppallama
V18	Goda Wee	-	B	Maha Iluppallama
V19	Heen Dik Wee	6	B	Maha Iluppallama
V20	Kalu Heenaty	6	M	Maha Iluppallama
V21	Weda Heenaty	5 ½	B	Maha Iluppallama
V22	Rathawalu	3 ½ to 4	B	Maha Iluppallama

a - new improved variety. b – exotic variety. B = Booting, PI = Panicle Initiation, M = Milky
***source**: Personal communication-Plant genetic resource center, Gannoruwa, Sri Lanka and The Movement of the Protection of Indigenous Seeds, Eppawala.

Field at Eppawala was owned by a private organic farm (The Movement of the Protection of Indigenous Seeds) and has been continuously cropped with traditional rice varieties in both *Yala* and *Maha* seasons for past 12 years.Field at Maha Iluppallama was located at the research farm of the University of Peradeniya hand has been cultivated in both seasons under conventional management for more than 20 years. Rhizosphere soils from one plant from each six traditional varieties being grown in *Maha* 2006/2007 season Eppawala and three plants from each 13 traditional, one exotic and two new improved rice varieties being grown in *Yala* 2007 season were collected (Table 1).

After transferring samples to the laboratory, shoots were removed and soil adhering to milky roots were separated as rhizosphere soil and stored at 4°C until commencing with analysis. Fresh milky roots were separated and used to stain for mycorrhizae fungi and enumeration of rhizoplane and endorhizosphere bacteria on the same day. Soil pH, available N forms (NO_3^- and NH_4^+), available P (Olsen and Sommers, 1982) and organic carbon content (Nelson and Sommers, 1996) in rhizosphere soils were determined. Sample preparation and the analyses were performed at Department of Soil Science of Faculty of Agriculture, University of Peradeniya.

Microbiological analyses

Selective growth media listed in SSSA special publication on microbiological methods were used in this study. Using plate count method with Tryptic Soy Broth (TSB) agar medium and a cellulose medium, population sizes of total culturable bacteria and cellulose decomposing bacteria in rhizosphere soils were estimated and expressed as number of colony forming units per unit of dry soil (CFU g^{-1}). Numbers of distinct colonies based on colony forming characters were used as a measure of diversity and selected colonies were isolated and sub-cultured for characterization and identification.

To isolate rhizoplane bacteria, a sample of milky roots of each rice variety was washed thoroughly with distilled water and then with sterilized distilled water to remove adhering soil particles. The washed roots were placed separately in flasks containing TSB and N free mineral medium selective for bacteria and *Azospirillum*, respectively, and incubated at room temperature in dark. Later TSB was sub cultured on Tryptic Soy Agar (TSA) medium, Kings' B medium and N free medium selective for generic bacteria, *Pseudomonas* sp. and *Azotobacter* type bacteria, respectively, and incubated at 28°C. *Azospirillum* broth was subcultured on to TSB agar medium and incubated until pigment production was observed. Colonies with distinct characteristics were isolated using TSA and stored at 4°C until further characterization.

To isolate endorhizosphere bacteria, a sample of young roots of each rice variety was washed thoroughly with distilled water to remove adhering soil particles and then surface sterilized (Lalande *et al.*, 1989). Roots were then crushed and aseptically transferred into bottles containing sterilized TSB and *Azospirillum* broth and incubated at room temperature in dark. The broth cultures were sub cultured onto agar plates as described previously and colonies were isolated for characterization. Fresh roots were examined for mycorhizal infections (Brundrett *et al.*, 1996).

Screening the isolates for beneficial traits

Rhizosphere, rhizoplane and endorhizosphere bacteria isolated on TSA medium and *Pseudomonas* selective media were tested for auxin production and phosphorous solubilizing activity. Qualitative determination of auxin production was performed following the procedure described by Gordon and Weber (1951). To test the P solubilizing ability isolates were streaked on plates containing yeast extract- glucose medium (Yeast extract 5 g L^{-1}, Glucose 10 g L^{-1}, Agar 20 g L^{-1}, Tri Calcium phosphate 3 g L^{-1}) amended with rock phosphate and incubated at 28 °C for a week. Colonies positive for phosphorous solubilizing activity result a clear zone or haloes on the medium surrounding them. Single colonies obtained on *Azotobacter* medium were subcultured on nitrogen free LG medium containing bromothymole blue as the pH indicator. A colour change from characteristic blue colour to yellow colour in the medium was used to screen nitrogen fixing organisms (Aquilanti *et al.*, 2004).

Identification of bacteria isolates

Bacteria were identified by analyzing whole-cell cellular fatty acid composition as described in the MIDI protocol (MIS Operating Manual, 2002). Fatty acids extracted were derivatized to methyl esters, *i.e.* FAMEs and identified using the gas chromatograph (Agilent 6890 GC). Identification of bacteria was performed using Sherlock Microbial Identification System version 4.5 (MIDI, Newark, DE). The extracted fatty acids from the unknown samples are automatically quantified and identified by the Sherlock software to generate a fatty acid profile. The generated profile is then compared to a digital library of profiles of reference strains and a list of best matches with associated similarity indices is produced by the software. The similarity index is a numerical value that expresses the closeness of the fatty acid profile of the unknown organism to the mean fatty acid compositions of the strains in the data base, thus used in deciding the identity of the organism or its most close relative. An exact match would result in a similarity index of 1.000. Similarity index of the first choice (>0.300) and the adequate separation from the second choice (at least 0.100 difference from the first) in the computer generated list were considered in determining the identity. The identified bacteria was given an accession number as **pdnVr X** (r = code number for variety ranging from 1 to 22; X = habitat of the isolate that denoted as R, RP or E for Rhizosphere, Rhizoplane and Endorhizosphere, respectiely). Isolates from cellulose medium and nitrogen free medium were indicated as Xc and Xn respectively.

Analysis of varience (ANOVA) was performed to assess statistical significance of microbial counts and soil data and mean separation was performed using LSD mean separation technique at $P < 0.05$ using statistical software SPSS 13.0 version.

RESULTS AND DISCUSSION

Rhizosphere soil properties varied across varieties (Table 2). Significant differences were observed among conventionally grown rice varieties with respect to the pH and nutrient contents, irrespective of the fact they were grown in the same soil type in close proximity (Table 2). Differences in plant uptake and microbial transformations lead to differences in nutrient availability in the root environment (Dandeniya and Thies, 2012).

Culturable bacteria population size varied from 5×10^8 to 8.57×10^{10} CFU g^{-1} Carbon in studied rhizosphere soils (equivalent to 3×10^7 to 1.14×10^9 CFU g^{-1} soil). Populations of aerobic bacteria in paddy soils are known to vary from 10^7 to 10^8 g^{-1} soil (Aragari and Tangcham, 1979). Cellulose decomposers are abundant in environment with high cellulose content. Aragari and Tangcham (1979) observed cellulose decomposing populations in rice rhizosphere increase with crop maturity and prolonged dry periods. They reported a population of cellulose decomposers of about 10^5 CFU g^{-1} Carbon for soils collected from Thailand. The populations observed in this study were several folds higher and varied from 1.4×10^7 CFU to 3.06×10^8 CFU g^{-1}Carbon. The differences in quality and quantity of organic inputs to root environment by plants could cause differences in microbial communities across varieties (Dandeniya and Thies 2011). The differences could not be explained by total organic carbon contents of the soils.

Table 2. Chemical properties of the rhizosphere soils

Variety	pH	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	Olsen P (mg kg^{-1})	Organic carbon (%)
		Organically managed rice*			
V1	6.15	13.04	4.35	72.84	0.7
V2	6.34	8.29	4.15	40.15	0.8
V3	6.44	26.08	17.38	71.70	1.1
V4	7.54	4.71	0.00	25.41	0.5
V5	6.48	4.03	4.03	73.39	0.8
V6	5.94	19.98	5.00	38.88	0.4
		Conventionally managed rice			
V7	6.33 f	10.67 abcd	4.93 bc	22.18 ab	3.9 ab
V8	5.65 ab	9.19 abcd	3.11 abc	26.68 ab	2.6 ab
V9	5.55 a	8.52 abcd	0.70 ab	33.41 ab	1.9 ab
V10	6.25 ef	8.05 abc	0.80 ab	41.08 b	2.3 ab
V11	5.88 bc	5.01 a	0.00 a	21.66 ab	1.7 a
V12	6.00 cdef	8.83 abcd	2.95 abc	21.74 ab	3.5 ab
V13	6.23 def	9.80 abcd	3.35 abc	26.85 ab	1.0 a
V14	5.74 abc	17.05 e	5.44 c	65.01c	3.5 ab
V15	6.04 cdef	7.10 ab	2.08 abc	15.59 a	1.7a
V16	6.06 cdef	9.04 abcd	2.27 abc	27.96 ab	3.0 ab
V17	5.89 bcd	9.63 abcd	4.09 abc	35.71 ab	3.6 ab
V18	5.92 bcde	11.09 bcd	1.58 abc	34.15 ab	1.9 ab
V19	5.91 bc	14.12 de	4.38 ab	36.70 ab	4.1 ab
V20	6.06 cdef	7.36 abc	0.76 ab	38.51 b	4.1 ab
V21	5.87 bc	7.55 abc	1.37 abc	26.19 ab	0.8 a
V22	6.05 cdef	13.11 cde	3.75 abc	31.16 ab	5.1 b

Means given in a column followed by different letters are significantly different (α= 0.05)
*Not included in statistical analysis as there was no field replicates at the farmer's field.

Rice rhizosphere is inhabited by a diverse group of microorganisms (Bai *et al*, 2000; Berg and Smalla, 2009; de Salamone *et al.*, 2010). The highest numbers of distinct colonies were identified for varieties V10 and V15; and the least for V1, V3 and V6. A number of varieties did not have culturable P solubilizers among tested isolates (*e.g.* V6, V7, V8 and V13).

Presence of *Pseudomonas, Clostridium, Choromatium, Thiospirillum, Azotobacter, Bacillus, Aeromonas, Staphylococcus* and *Azospirillum* species in the rice rhizosphere has been recorded previously (Guanrto *et al.*, 1999; Bai *et al.*, 2000; de Salamone *et al.*, 2010). Reche and Fuiza (2005) reported that *Enterobacter* sp. was present in irrigation water in channels of paddy fields in Brazil. *Pseudomonas putida* is known to colonize root surfaces aggressively (Anderson *et al.*, 1988). In line with the previous findings, predominant bacteria isolated in this study were genera *Acinetobacter, Pseudomonas, Bacillus* and *Staphylococcus* (Table 3). Some organisms promote plant growth by auxin production and/or solubilizing $Ca_3(PO_4)_2$. Number of phenotypically distinct colonies isolated from rhizoplane varied across varieties hinting difference in diversity (Figure 1).

Among tested colonies in this study, 52% of isolated bacteria inhabiting the rhizosphere and rhizoplane, was found to belong to genera *Pseudomonas*. *Bacillus, Pseudomonas, Herbaspirillum, Ideonella,* Enterobacter and *Azospirillum* are among the bacteria occupying the endorhizosphere of rice (Gunarto *et al.*, 1999; de Salamone *et al.*, 2010).

In the present study, bacteria belonging to genera *Pseudomonas* and *Bacillus* were isolated from endorhizosphere. According to the root staining study, tested rice roots were negative for mycorrhizal associations.

The culture collection made from 22 varieties comprised of 152, 80 and 77 isolates from rhizosphere, rhizoplane and endorhizosphere positions, respectively. The numbers of isolates that have the ability to decompose cellulose and fix nitrogen were 171 and 108, respectively. A total of 118 *Pseudomonas* sp were isolated from the rhizoplane of all varieties. Among the total collection, 48 and 26 were positive for P solubilizing and auxin production, respectively. Results indicated that despite the taxonomic similarity, differences exist in the beneficial traits among *Pseudomonas* sp. Therefore, this culture-based study suggests that the diversity of beneficial bacteria associated with the rhizosphere of different rice varieties is high and different.

Table 3. Taxonomic diversity of predominant bacteria in the rhizosphere and rhizoplane of selective varieties and their ability to solubilize P and produce auxin

Sample ID	Similarity Index*	Taxonomy	Auxin production	P solubilization
pdnV9R5	0.465	*Acinetobacter johnsonii*	+	+
pdnV10R5	0.327	*Paenibacillus polymyxa*(Bacillus)*	+	nd
pdnV10R15	0.805	*Pseudomonas syringae-syringae*	nd	+
pdnV10R17	0.878	*Enterobacter agglomerans*	+	+
pdnV11R4	0.647	*Staphylococcus cohnii-cohnii*	nd	nd
pdnV18Rc17	0.606	*Pseudomonas putida*-biotype B*	nd	nd
pdnV3RP4	0.848	*Pseudomonas putida*-biotype A	nd	+
pdnV4RP5	0.169	*Pseudomonas chlororaphis*	+	nd
pdnV5RP3-	0.348	*Pseudomonas putida*-biotype A	nd	+
pdnV6RP1	0.219	*Pseudomonas putida*-biotype B	nd	+
pdnV10RP2	0.356	*Exiguobacterium acetylicum*	nd	nd
pdnV12RP2	0.712	*Enterobacter cancerogenus*	nd	+
pdnV13RP1	0.677	*Kluyvera ascorbata*	nd	+
pdnV14RP1	0.823	*Pseudomonas putida*-biotype A	nd	nd
pdnV14RP2	0.758	*Pseudomonas syringae*-syringae	+	+
pdnV14RPN2	0.719	*Pseudomonas putida*-biotype A	nd	nd
pdnV17RP3	0.666	*Aeromonas caviae*	nd	+
pdnV18RP3	0.272	*Stenotrophomonas maltophilia*	nd	nd
pdnV20RPp1	0.480	*Pseudomonas putida*-biotype A	nd	nd
pdnV21RP1	0.922	*Enterobacter cloacae*	nd	+
pdnV21RP3	0.631	*Pseudomonas putida*-biotype A	nd	nd

* A value generated by Sherlock software by calculating the relative difference of the fatty acid profile of the sample (unknown organism) to the mean profile of the closest strains in the digital library. Values >0.500 – good comparison, 0.500 to 0.300 – a good match but atypical strain, <0.300 – do not have the species in the library but most closely related species is given. nd = Not detected

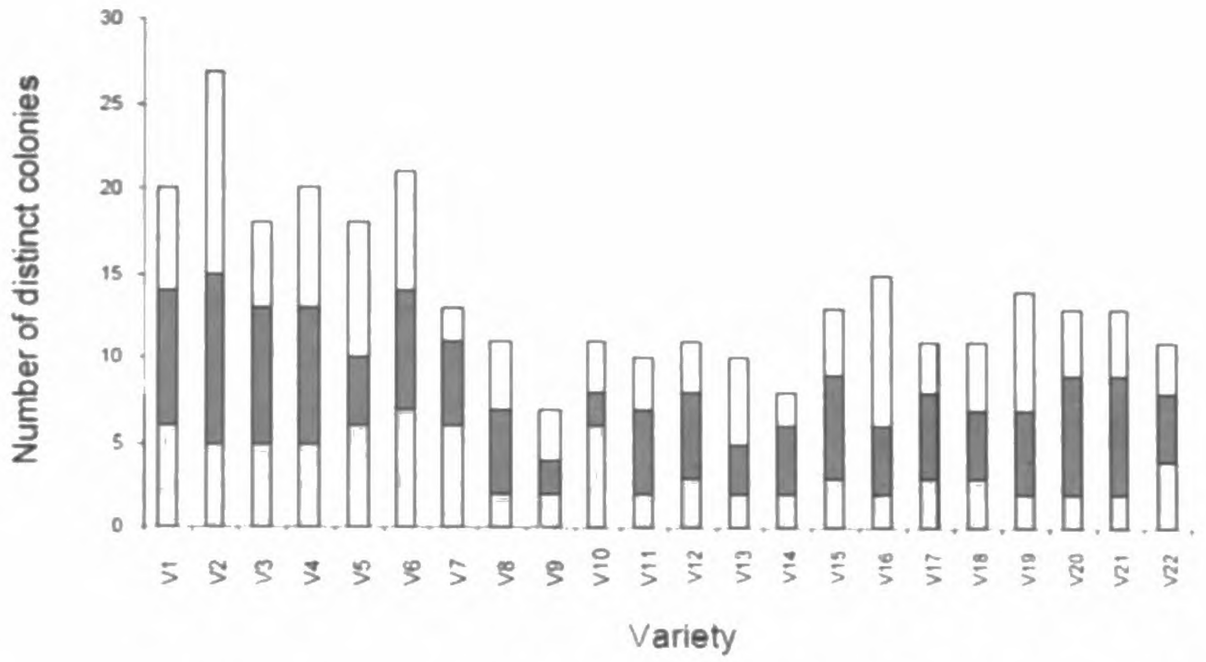

Figure 1. Number of distinct colonies isolated for bacteria with TSB medium (light grey), N_2 fixers with N free medium (dark grey), *Pseudomonas* sp. with King's B medium (white) from the rhizosplane of different rice varieties.

CONCLUSION

Diversity of microorganisms inhabiting rice root environment is influenced by the variety. *Azospirillum, Enterobacter, Exigubacterium, Kluyvera, Aeromonas, Acinetobacter, Stenotrophomonas, Bacillus* and *Staphylococcus* were among the isolates but *Pseudomonas* were found in high abundance among culturable bacteria isolated from rhizosphere, rhizoplane and endorhizosphere. There were microorganisms with potential plant growth promoting abilities such as producing auxin, fixing nitrogen, decomposing cellulose and solubilizing $Ca_3(PO_4)_2$ among the microbial occupants in the rice root environment. While there is possibility to screen beneficial microorganisms from the rice root environments findings suggest that different varieties harbor beneficial microorganisms to different extents.

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