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Screening of Rhizobacteria Associated with Cassava for Plant Growth Promotion and Biocontrol Potential

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Abstract

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonise plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements. In the present study, rhizobacteria associated with five varieties of cassava, viz., Sree Padmanabha, Sree Vijaya, Sree Jaya, Sree Rekha and Sree Prakash were isolated and screened for their plant growth promotion traits and *in vitro* biocontrol potential. A total of 41 isolates were selected based on colony morphology and designated as RB1 to RB41. These isolates were screened *in vitro* for their plant growth promoting traits like production of indole acetic acid (IAA), ammonia (NH_3), phosphate solubilisation, hydrogen cyanide (HCN) and antifungal activity. Cowpea seeds were treated with rhizobacteria to assess seed germination and growth of seedlings. A pot experiment was conducted with cassava variety, Sree Vijaya, where stem cuttings were treated with rhizobacteria followed by a soil drench. About 88% of the isolates produced IAA, 83% produced ammonia and 50% solublized P. Only one isolate produced HCN and nine isolates showed antifungal activity against the plant pathogen, *Sclerotium rolfsii*. Rhizobacterial treatments promoted the germination in cowpea and growth in cassava.The present study suggests that PGPR isolates might have potential in future field applications as plant growth promoters or as biocontrol agents.

Key words: PGPR, plant growth promoting traits, seed germination, cassava

Introduction

The plant-microbe interaction in the rhizosphere can be beneficial, neutral, variable or deleterious for plant growth. Rhizobacteria that exert beneficial effects on plant development are termed plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). The term rhizobacteria is used for bacteria that aggressively colonize the rhizosphere (Subba Rao, 1999). The exact mechanism by which PGPR promote plant growth are not fully understood, but are thought to include the ability to produce or change the concentration of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene (Arshad and Frankenberger, 1993), asymbiotic nitrogen fixation (Boddey and Dobereiner, 1995), solubilizaton of mineral phosphates and other nutrients (De Freitas et al., 1997; Gaur, 1990), antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982), antibiotics (Shanahan et al., 1992) and cyanide (Flaishman et al., 1996). Most popular bacteria studied and exploited as biocontrol agents include the species of fluorescent *Pseudomonas* and *Bacillus*.

During the last couple of decades, the use of PGPR for sustainable agriculture has increased tremendously in various parts of the world. Moreover, PGPR mediate biological control indirectly by eliciting induced systemic resistance (ISR) against a number of plant diseases

(Jetiyanon and Kloepper, 2002). Application of some PGPR strains to seeds or seedlings has also been found to lead to a state of induced systemic resistance in the treated plant (Kloepper et al., 1999).

Cassava (*Manihot esculenta* Crantz)) is a woody shrub belonging to the family Euphorbiaceae (Spurge family) native to South America that is extensively cultivated as an annual crop in the tropical and subtropical regions for its edible starchy tuberous root, a major source of carbohydrate. It is the third most important food crop after cereals and grain legumes. Cassava is a high carbohydrate rich tropical root crop now grown in over 95 countries and provides a cheap food source for over 500 million people.

The objectives of the present study were to isolate the cassava rhizosphere bacteria and screen them for plant growth promotion and biocontrol potential.

Materials and Methods

Isolation and characterization

Soil samples were collected from the rhizosphere of apparently healthy plants of different varieties of cassava, namely, Sree Jaya, Sree Vijaya, Sree Padmanabha, Sree Rekha and Sree Prakash. Soils were collected from various cassava fields in Thiruvananthapuram and Kollam districts of Kerala and transferred to Laboratory, Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram. The samples were taken at the time of harvest. Ten soil samples were collected from each variety and composite sample was used for isolation. Bacterial isolation was done by serial dilution technique. Ten grams of rhizosphere soil was used to prepare 10 fold dilution series up to 10^{-6}.in sterile distilled water. 0.1 ml aliquots from dilutions (10^{-2} to 10^{-6}) were inoculated onto pre-poured nutrient agar and King's B agar plates and spread using a sterile glass spreader. After 48 h of incubation at 32°C, the plates were observed for number of bacterial colonies and their morphology. Forty one bacterial isolates showing prolific growth and having different morphological appearance on agar medium were selected for the present study.

In vitro screening of bacterial isolates for plant growth promoting activities

Indole acetic acid (IAA) production

The assay for IAA production by selected bacteria was carried out by the method proposed by Bric et al. (1991). Isolates were grown in 25 ml Luria Bertani medium containing 50 μg ml^{-1} tryptophan (LBT medium) and incubated at 30°C for 24 h in a rotary shaker (90 rpm). They were centrifuged at 5000 rpm for 25 min. Two volume of supernatant was mixed with one volume of Van urk Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) and the pink colour developed was measured after 2 h by spectroscopy at 530 nm. Concentration of IAA produced by cultures was calculated with the help of standard curve of IAA obtained in the range of 10 – 100 ìg ml^{-1}.

Phosphate solubilisation

All bacterial isolates were first screened on Pikovskaya's agar plates for phosphate solubilization as described by Gaur (1990). The isolates showing positive results in agar medium were assessed for quantification of P solublilization using vanadomolybdophosphoric yellow colour method (Clesceri et al., 1998). The cultures showing positive result in agar medium were inoculated in 50 ml of Pikovskaya's broth and incubated for 48 h. The broth was centrifuged at 8000 rpm for 10 min and 5 ml of the supernatant was taken to which 5 ml of the vanadomolybdate reagent was added. The volume was made up to 25 ml and incubated overnight. The yellow colour developed was read at 530 nm.

Ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 4 days at 30°C. Nessler's reagent (0.5 ml) was added in each tube. Development of a faint yellow colour indicated small amount of ammonia while deep yellow to brownish colour indicated increased production of ammonia (Dye, 1962).

HCN production

All the isolates were screened for the production of hydrogen cyanide (HCN) by adapting the method of Bakker and Schipper (1987). King's B medium was amended with 4.4 g l^{-1} glycine; sterilized in an autoclave and poured in sterilized petriplates under aseptic condition and the bacterial cultures were streaked on the agar plate. A Whatman filter paper No.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the upper lid of the petri plate. Plates were

closed and sealed with parafilm and incubated at 28±2°C for 4 days. Colour change of filter paper from deep yellow to orange and finally to orange brown to dark brown indicated HCN production.

In vitro screening of bacterial isolates for biocontrol potential

Antifungal activity

The bacterial isolates were tested *in vitro* on potato dextrose agar (PDA) for their antifungal activity against *Sclerotium rolfsii* by dual culture technique (Skidmore et al., 1976). An agar plug (5 mm in diameter) was cut from an actively growing (96 h) fungal culture and placed at the centre of the agar plate. Simultaneously, the bacterium to be tested was streaked 2 cm away from the agar plug. Plates with only fungus without bacterial culture were used as control and all plates were incubated at 30±1°C until fungal mycelia covered the agar surface of the control plate.

Plant bioassays for growth promotion

Seed germination test

The selected rhizobacterial isolates were bioassayed for their ability to promote or inhibit seedling growth using the method as described by Shende et al. (1997) and Elliot and Lynch (1984) with a few modifications. Cowpea seeds were surface sterilized with 0.1% mercuric chloride for 3 min followed by successive washing with sterile distilled water. The cultures were grown in their respective medium for 48 h containing at least 10^6 cells ml^{-1}. The seeds were kept in culture medium for 10 min and then kept on sterile soft agar plates (1%) and incubated at 30°C for 3 days. Seeds treated with sterilized medium alone served as control. Three replicates were maintained for each treatment. Seed germination and radicle length were recorded on the third day of incubation.

Influence of different PGPRs on agronomic characteristics of cassava

A pot culture experiment was conducted to evaluate the effects of selected rhizobacteria on cassava growth. Stem cuttings (setts of 15 cm) of cassava variety Sree Vijaya were surface disinfected by treatment with 95% ethanol for 2 min, followed by washing in sterile distilled water several times. Before planting, cassava stem cuttings were dipped thoroughly in bacterial culture broth for 10 min. Cell concentrations in the whole culture were adjusted with sterile distilled water to 1×10^8 cfu ml^{-1}, based on absorbance. Sterile distilled water was used as the control. Treated cuttings were planted in pots (30 cm diameter) containing soils sterilised at 121°C for 30 min for two consecutive days. There were three replicates per treatment with two cuttings per pot. The pots were watered daily and kept in a greenhouse with a 12 h photoperiod. After 30 days, agronomic characters like height of the plant, girth of the plant, number of leaves and total biomass were taken.

Biochemical characterisation of the isolated bacteria

A number of biochemical tests viz. gram reaction, indole test, methyl red test (MR), voges-proskauer test (VP), citrate utilization, nitrate reduction, triple sugar iron agar (TSI) test, starch, gelatin and casein hydrolysis, hydrogen sulphide production (H_2S), urease test, oxidase test, catalase test were performed for the characterization of isolates with the help of standard methods (Cappuccino and Sherman, 1992). Fermentation of carbohydrate was tested with sugars viz. glucose, lactose and sucrose. The bacterial isolates were identified by comparing the results with Bergey's Manual of Determinative Bacteriology.

Statistical analysis

Data from the experiment was subjected to analysis of variance using SAS version 9.3 (2010). Separation of treatment means was accomplished by Duncan's Multiple Range Test (DMRT) and all tests for significance were conducted at p d" 0.0001

Results and Discussion

Isolation of rhizosphere bacteria

The bacterial population (cfu g^{-1} of soil) recorded from the rhizosphere soil of five different cassava varieties are presented in Table 1. The population ranged from 2 x 10^6 to 6 x 10^6 cfu g^{-1} of soil. The bacterial colonization was maximum (6 x 10^6 cfu g^{-1} of soil) in the rhizosphere of cassava variety, Sree Padmanabha followed by Sree Prakash, Sree Vijaya, Sree Rekha and Sree Jaya.However, rhizobacterial population did not vary much among different cassava varieties.

The morphological characters of rhizosphere bacterial isolates varied widely. A total of 41 morphologically

Table 1. Population of rhizobacteria associated with different varieties of cassava

Variety	Bacterial population (x 10^6 cfu g^{-1} of soil)
Sree Padmanabha	6
Sree Prakash	4.5
Sree Vijaya	3.3
Sree Rekha	3
Sree Jaya	2

Values are the means of three replications

different isolates were selected based on their colony characteristics and pigment production. They were designated as RB1 to RB41.

Plant growth promoting traits of the isolates

IAA production

IAA, a member of the group of phytohormones, is generally considered to be the most important native auxin. IAA may function as important signal molecule in the regulation of plant development. It has been reported that IAA production by PGPR can vary among different species and strains and it is also influenced by culture conditions, growth stage and substrate availability (Mirza et al., 2001). Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil (Sarwar and Kremer, 1992). In the present study, results of colorimetric analysis indicated that 88% of the isolates produced IAA *in vitro*. Auxin production ranged from 1.33 μg ml^{-1} to 102 μg ml^{-1}. Five isolates (RB9, RB26, RB31, RB37 and RB41) were high producers of IAA (>63.33 μg ml^{-1}), 13 isolates were moderate producers (20.67 μg ml^{-1} to 63.33 μg ml^{-1}), while 18 isolates were weak producers (1.33 μg ml^{-1} to 17.33 μg ml^{-1}). Five isolates (RB22, RB23, RB24, RB25 and RB28) did not produce IAA.The results of major IAA producing rhizobacteria (16 isolates) are represented in Fig. 1.

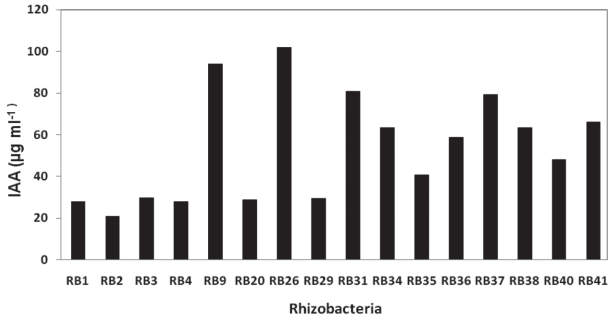

Fig. 1. *In vitro* IAA production capacity of rhizobacterial isolates

Phosphate solubilisation

Phosphorous is one of the major nutrients, second only to N in requirement for plants. Most of the P in soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Pradhan and Sukla, 2006). The ability of bacteria to solubilise mineral phosphates has been of interest to agricultural microbiologists as it can enhance the availability of phosphorous and iron for plant growth. In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate solubilising bacteria is commonly found in the rhizosphere (Raghu and Mac Rae, 1966). In the present study, a clearing zone around the colonies was shown by RB1, RB2, RB4, RB7, RB8, RB9, RB13, RB17, RB19, RB26, RB27, RB28, RB29, RB30, RB31, RB32, RB33, RB34, RB35, RB36, RB37, RB38, RB39, RB40 and RB41 in Pikovskaya's agar. Highest solubilisation of P was shown by RB17 (106.02 ppm) and the P solubilizing capacity of the major 25 rhizobacteria are presented in Fig. 2. Other isolates solubilised P in the range of 37 to 100 ppm.

Ammonia production

In the present study, 83% of the isolates produced ammonia indicating that it is a common trait in these bacteria which indirectly influence plant growth. Thirty three isolates showed high production of ammonia, while eight isolates did not produce ammonia.

HCN production

Out of the 41 isolates, only one isolate, RB1, produced HCN. It has been reported that overproduction of HCN may control fungal diseases in wheat seedlings (Flaishman et al., 1996). It has been reported that ammonia and HCN production by the isolates was positively related to N accumulation and elongation of the roots and P accumulation, biomass production and elongation of shoots in *Zea mays* (Marques et al., 2010)

Biocontrol potential of PGPR

Antifungal activity

Antifungal assay was done by dual culture technique. Inhibition of mycelial growth of the fungus, *Sclerotium rolfsii*, was shown by RB1, RB4, RB9, RB12, RB15, RB16, RB19, RB26 and RB37, which indicated their potential to resist the pathogen.

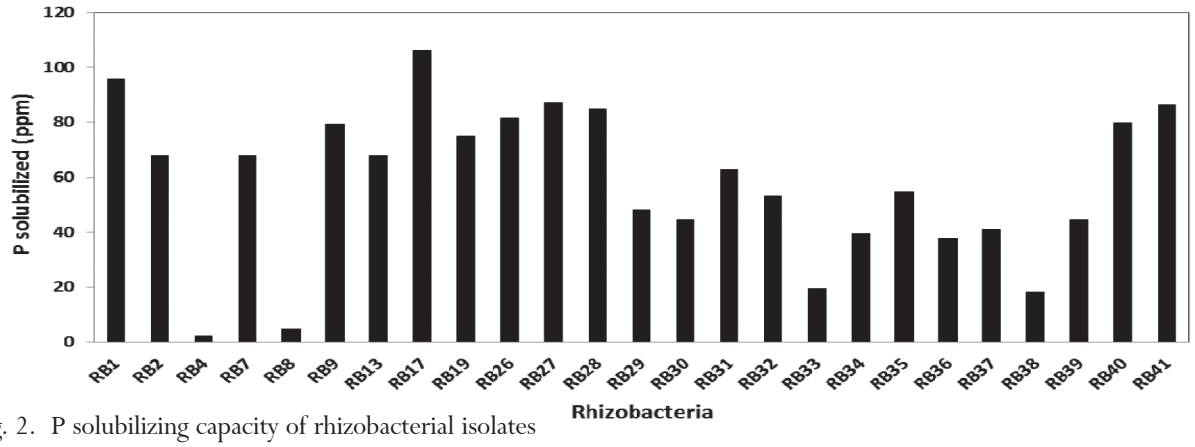

Fig. 2. P solubilizing capacity of rhizobacterial isolates

Plant bioassays

Seed germination test

The present study investigated the effectiveness of PGPR to increase the seed germination rate as well as growth of seedlings. Germination of seeds was found to be 80% in both rhizobacterial treatments and control. Twenty isolates showed a two fold increase in radicle length than control seedlings (2.4 cm). Thirteen isolates showed a neutral effect and 8 isolates produced a negative effect on radicle length of seedlings (Fig. 3). A large body of evidence suggests that PGPR enhance growth, seed emergence and crop yield and contribute to the protection of plants against certain pathogens and pests (Dey et al., 2004 ; Kloepper et al., 2004 ; Kokalis Burelle et al., 2006 ; Herman et al., 2008 ; Minorsky , 2008).

Influence of different PGPRs on agronomic characteristics of cassava

Significant increase in growth and yield of agronomically important crops in response to inoculation with PGPR have been reported (Chen et al., 1994; Amara and Dahdoh, 1997; Biswas et al., 2000a; 2000b; Hilali et al., 2001; Asghar et al., 2002). The agronomical characteristics of cassava were significantly influenced by different PGPR strains (Table 2.). One month after planting, RB9 and RB26 treatments resulted in significantly higher growth characters. Plant height was greater in RB9 (88 cm) followed by RB26 (85.33 cm) which was on par with RB9. The treatments viz., RB35 (81cm) and RB38 (80.33 cm) also resulted in higher plant height followed by RB25 (78 cm). Height of the plants ranged from 60 cm to 75 cm by the other PGPR treatments and that in the control plant was 52.67cm, which was relatively less. Girth of the plant was seen slightly higher due to RB26 (2.86 cm) followed by RB9 (2.84 cm) and less in control plants (2.11 cm). Significantly less increase in number of leaves was noted in bacteria treated cassava plants. Highest biomass (fresh weight) was shown by RB9 (112.33 g plant^{-1}) followed by RB26 (111.67g plant^{-1}), which were on par. The bacteria RB35 (107.33 g plant^{-1}) also resulted in higher biomass followed by RB25 (105.33 g plant^{-1}), which was on par with RB25. Other PGPRs also yielded a biomass in the range of 91 g plant^{-1} to 103 g plant^{-1} which was superior over the control. In general, all the bacterial treatments produced significantly higher agronomic characteristics viz., plant height, girth, number of leaves and biomass in cassava plant compared to the control treatment.

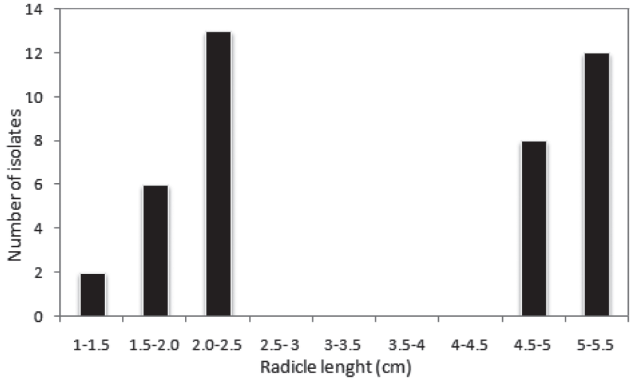

Fig.3. Effect of rhizobacterial isolates on radicle length of cowpea seedlings

Biochemical characterisation of PGPR isolates

The biochemical test results showed that most of the rhizobacteria belonged to *Bacillus* and *Pseudomonas* species (Table 3). The isolates, RB9 and RB26, which

Table 2. Effects of PGPR on agronomic characteristics of cassava

Treatment	Height (cm)	Girth (cm)	No. of functional leaves	Biomass (g plant^{-1})
RB1	62.33^{uvw}	2.34^{ijklmnopq}	12.00^{a}	92.33^{no}
RB2	65.33^{qrs}	2.32^{jklmnopq}	10.00^{c}	93.67^{mn}
RB3	62.67^{uvw}	2.17^{pqr}	10.00^{c}	91.00^{o}
RB4	69.33^{lmn}	2.48^{fghijkl}	11.00^{b}	93.67^{mn}
RB5	63.33^{tuvw}	2.25^{mnopqr}	11.00^{b}	94.00^{mn}
RB6	73.67^{fgh}	2.33^{ijklmnopq}	10.00^{c}	97.00^{ijk}
RB7	64.00^{rstu}	2.19^{opqr}	12.00^{a}	91.00^{o}
RB8	67.33^{op}	2.40^{ghijklmno}	10.00^{c}	93.67^{mn}
RB9	88.00^{a}	2.84^{ab}	12.00^{a}	112.33^{a}
RB10	70.00^{klm}	2.37^{hijklmnop}	11.00^{b}	100.00^{fgh}
RB11	64.67^{rst}	2.20^{opqr}	10.00^{c}	98.00^{ghi}
RB12	63.67^{stuv}	2.24^{mnopqr}	11.00^{b}	95.33^{jklm}
RB13	68.33^{mno}	2.14^{qr}	11.00^{b}	101.67^{ef}
RB14	71.67^{ijk}	2.27^{mnopqr}	11.00^{b}	96.67^{ijkl}
RB15	66.67^{opq}	2.29^{lmnopqr}	12.00^{a}	95.33^{jklm}
RB16	74.33^{efg}	2.53^{defghi}	12.00^{a}	102.00^{def}
RB17	70.67^{jkl}	2.26^{mnopqr}	10.00^{c}	100.00^{fgh}
RB18	72.00^{hij}	2.50^{efghijk}	11.00^{b}	93.67^{mn}
RB19	74.33^{efg}	2.60^{cdefg}	12.00^{a}	98.00^{ghi}
RB20	70.67^{jkl}	2.51^{efghij}	11.00^{b}	94.67^{klmn}
RB21	65.33^{qrs}	2.34^{ijklmnopq}	10.00^{c}	92.67^{no}
RB22	68.33^{mno}	2.19^{pqr}	11.00^{b}	98.00^{ghi}
RB23	63.67^{stuv}	2.30^{klmnopqr}	11.00^{b}	93.67^{mn}
RB24	74.33^{efg}	2.51^{efghij}	12.00^{a}	101.33^{ef}
RB25	78.00^{d}	2.69^{abcde}	12.00^{a}	105.33^{bc}
RB26	85.33^{b}	2.86^{a}	12.00^{a}	111.67^{a}
RB27	72.67^{ghi}	2.57^{defgh}	10.00^{c}	97.33^{ij}
RB28	74.67^{ef}	2.69^{abcde}	12.00^{a}	102.33^{def}
RB29	61.67^{w}	2.17^{pqr}	10.00^{c}	93.33^{mno}
RB30	68.00^{no}	2.49^{efghijkl}	11.00^{b}	95.33^{jklm}
RB31	61.67^{w}	2.43^{ghijklmn}	10.00^{c}	97.33^{ij}
RB32	76.00^{e}	2.52^{defghij}	12.00^{a}	103.33^{cde}
RB33	65.67^{pqr}	2.44^{ghijklm}	11.00^{b}	93.00^{mno}
RB34	72.33^{hij}	2.68^{abcdef}	12.00^{a}	101.33^{ef}
RB35	81.00^{c}	2.79^{abc}	12.00^{a}	107.33^{b}
RB36	73.67^{fgh}	2.65^{bcdef}	10.00^{c}	97.67^{hij}
RB37	62.00^{vw}	2.19^{opqr}	12.00^{a}	94.33^{lmn}
RB38	80.33^{c}	2.73^{abcd}	12.00^{a}	104.33^{cd}
RB39	75.67^{e}	2.68^{abcdef}	12.00^{a}	103.67^{cde}
RB40	63.33^{tuvw}	2.22^{nopqr}	10.00^{c}	97.67^{hij}
RB41	70.00^{klm}	2.55^{defgh}	12.00^{a}	100.33^{fg}
Control	52.67^{x}	2.11^{r}	10.00^{c}	83.67^{p}
Mean	69.75	2.43	11.1	97.83
p-Value	<.0001	<.0001	<.0001	<.0001
CV (%)	1.74	5.32	0	1.59

The data are the average of three replications (two plants per replication) for each treatment. Mean values with the same superscript within a column are not significantly different according to DMRT.

Table 3. Biochemical characterisation of PGPR isolates

Isolate	Gram reaction	Motility	I	MR	VP	C	URE	CAT	OXI	TSI	NR	SH	GH	CH	H_2S	GF	LF	SF	Probable identity
RB8,10,11,12,19, 21,22,23,26,29,32, 33,34,36, 37, 38	Gram +ve rod	+	-	-	-	+	-	+	+	K/A	+	+	+	+	-	+	+	+	*Bacillus* sp.
RB 24, 28, 31	Gram+ve cocci	-	-	-	+	-	+	+	+	K/NC	-	+	+	-	-	-	-	-	*Micrococcus* sp.
RB1,2,3,4,5,9,17, 8,20,25,30, 39, 40, 41	Gram -ve rod	+	-	-	-	+	-	+	+	K/A	-	+	+	+	-	+	+	+	*Pseudomonas* sp.
RB 15,30, 35	Gram -ve rod	+	-	-	+	+	-	+	-	A/A	+	-	-	+	-	+	+	+	*Enterobacter* sp.
RB 14, 26, 27	Gram -ve rod	-	-	-	-	+	+	+	-	A/A	+	+	-	-	-	+	+	+	*Klebsiella* sp.
RB 6,7, 13, 16	Gram -ve rod	+	-	-	+	+	-	+	-	K/A	+	-	+	+	-	+	-	+	*Serratia* sp.

I-Indole; MR-Methyl red; VP-Voges-proskauer; C-Citrate utilization; URE- Urease test; TSI-Triple sugar iron agar; CAT-Catalase; OXI-Oxidase; NR-Nitrate reduction; SH-Starch hydrolysis; GH-Gelatin hydrolysis; CH-Casein hydrolysis; H_2S-Hydrogen sulphide production; GF-Glucose fermentation; LF-Lactose fermentation; SF-Sucrose fermentation

produced significant growth promotion in cassava belonged to *Pseudomonas* and *Bacillus* sp. respectively. It should be further identified by techniques like 16s rDNA sequencing, biolog identification etc.

Conclusion

It can be concluded that PGPR are able to induce the production of IAA, solubilisation of P and resistance to pathogens thereby improving growth of plants. The use of PGPR as biofertilisers or as biocontrol agents may be tested in fields to find out their application in cassava cultivation.

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