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SCREENING OF CERTAIN RHIZOBACTERIA FROM *HEVEA BRASILIENSIS* FOR GROWTH PROMOTING ACTIVITIES

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Seventy nine IAA producing rhizobacteria, isolated and selected from rubber plants, were screened to identify efficient isolates under polybag conditions. The IAA production of the isolates in sucrose minimal salts medium was in the range of 0.48 - 62.84 μg mL^{-1}. Ten mL of each culture containing 10^8 cfu mL^{-1} was inoculated to the rubber seedlings in three splits at monthly intervals from transplanting of the germinated seedlings to the polybags along with uninoculated control plants. After six months growth, the plants were uprooted and measured various growth parameters. Out of the 79 isolates evaluated, 12 isolates showing more growth improvement of the seedlings were selected. They were the medium IAA producers (in the range of 4.08 - 25.46 μg mL^{-1}). All these isolates showed phosphate solubilising efficiency in Apatite agar medium. They solubilised $Ca_3(PO_4)_2$ and Al PO_4. $FePO_4$ solubilisation was shown by ten isolates and was less than the other two sources of phosphates. Three isolates *viz.* RB 88, Ri 25 and RH 104 showed the solubilisation of Raj phos, the sparingly soluble form of fertilizer. Eleven isolates showed medium to high levels of ammonia production and low to medium levels of phosphatase activity. Nine isolates showed low to high levels of siderophore production. All the isolates showed antagonistic activity against at least one of the five major rubber pathogens tested in dual culturing. The study showed that the top ten IAA producers were not included among the 12 selected isolates. The study suggests that a combination of different mechanisms, other than IAA production alone, may be involved in regulating and optimizing the plant promoting effects of root colonizing bacteria, the role of which under field conditions is to be further investigated.

Keywords: Growth promoting activity, Indole acetic acid, Rhizobacteria

INTRODUCTION

Plant growth in agriculture soil is influenced by myriads of abiotic and biotic factors. The roots support a large and active microbial population capable of exerting beneficial, neutral or detrimental effects on plant growth. The importance of root colonizing bacteria for maintenance of root health, nutrient uptake and tolerance of environmental stress is well recognised (Bowen and Rovira, 1999; Cook, 2002). The plant growth promoting rhizobacteria (PGPR) have been reported to enhance plant growth directly by a variety of mechanisms.

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Many are capable of producing auxins in addition to other plant hormones. They also fix atmospheric nitrogen that is transferred to the plant, produce siderophores that chelate iron and make it available to the plant roots and solubilize minerals such as phosphorus (Glick, 1995). Direct enhancement of mineral uptake due to increase in specific ion fluxes at the root surface in presence of PGPR has also been reported (Bertrand *et al.*, 2000). PGPR may use one or more of these mechanisms in the rhizosphere.

PGPR also indirectly enhance plant growth by the suppression of phytopathogens. The mechanisms include their ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesise antifungal metabolites such as antibiotics, fungal cell wall lysing enzymes or hydrogen cyanide which suppress the growth of fungal pathogens; the ability to successfully compete with pathogen for nutrients or specific niches on the roots and ability to induce systemic resistance (Glick, 1995; Bloemberg and Lugtenberg, 2001).

The ability of the microorganisms to produce and release various metabolites stimulating plant growth and health is considered to be one of the most important factors in soil fertility (Frankenberger and Arshad, 1995). Manipulating crop rhizosphere microbial population by introducing beneficial bacteria was found to enhance plant growth. It has been reported that PGPR isolated from native rhizosphere are more effective in growth enhancement and crop protection than other strains because of their better adaptability. *Hevea brasiliensis* is an important perennial cash crop in India. The regular use of chemical fertilisers and pesticides for better growth and yield of the crop may cause environment pollution problems and affect the useful microorganisms in soil. The selection of efficient naturally occurring rhizobacteria from rubber plantations could be an alternative for reducing the use of agrochemicals in rubber cultivation.

This study examined the ability of certain IAA (indole acetic acid) producing rhizobacteria isolated from rubber to promote seedling growth under polybog culture conditions. The efficient isolates were evaluated for other beneficial activities contributing plant growth under *in vitro* conditions and the isolates having more beneficial secondary characters were identified.

MATERIALS AND METHODS

Bacterial isolates

Seventy nine bacterial cultures screened and selected for any of the plant promoting activities from more than 500 isolates, including rhizosphere and root endophytic bacteria collected from different *H. brasiliensis* clones *viz.* RRII 105, RRII 414, RRII 417, RRII 422, RRII 429, RRII 430, PB 260, GT I and PB 260 were used in this study. They produced IAA (Sheng *et al.*, 2008) ranging from 0.48-62.84 μg mL^{-1} in sucrose minimal salt medium. Ten isolates recorded the production of IAA above 30 μg mL^{-1} and twenty isolates produced less than 5 μg mL^{-1} of IAA.

Plant growth promotion assay

Plant growth promotion assay was conducted as a screening procedure to select efficient isolates which were subsequently assessed for their other beneficial activities.

The ability of the isolates to promote growth of rubber seedlings was studied by transplanting the germinated rubber seeds to polythene bags containing unsterilised

field soil. Ten mL of each culture in nutrient broth containing 10^8 cfu mL^{-1} was applied to the rubber seedlings in three splits, at monthly intervals, from transplanting of the germinated seedlings to polythene bags. Since 79 isolates were used in the study, the experiment was divided in to seven separate batches, each consisting of 10-12 test isolates inoculated plants, including the controls applied with equal quantity of uninoculated culture media. The isolates used in every batch of the experiment were selected randomly. The experiment was performed in a completely randomised design. Ten replications were maintained for each treatment and seven were taken for biometric observations. After six-month growth, the plants were uprooted and recorded various parameters *viz.* shoot length, shoot girth, shoot fresh and dry weight, number of leaves, root length, root fresh and dry weight and root volume and the data were statistically analysed. Twelve efficient isolates were selected through this method and tested for the following beneficial activities.

Growth promoting activities of the selected isolates

The efficiency of the selected 12 isolates for various growth promoting activities *viz.* phosphate solubilisation, acid phosphatase activity, nitrogen fixation, ammonia production and siderophore production were studied under *in vitro* conditions.

Qualitative test for phosphate solubilisation was conducted by inoculating the bacteria in agar containing precipitated tricalcium phosphate (Apatite agar medium). Inoculated plates were incubated for four days and formation of clear zone around the colony was used to calculate the phosphate solubilising efficiency as per the method by Nguyen *et al.* (1992).

$$\text{Solubilising efficiency (SE\%)} = \frac{\text{Solubilisation diameter} \times 100}{\text{Growth diameter}}$$

The quantitative analysis of solubilisation of different forms of phosphate *viz.* tricalcium phosphate, aluminium phosphate, ferric phosphate and the sparingly soluble form of fertilizer, Rajphos was carried out in Pikovskaya's broth as described by Subba Rao (1982).

Acid phosphatase activity of the isolates was studied by colorimetric estimation of the *p*-nitro phenol released from *p*-nitro phenyl phosphate in the culture supernatants (Ilamurugu and Vigneswaran, 1998).

The ability of the isolates to fix atmospheric dinitrogen was checked by inoculating the isolates in N-free Dworkin-Foster (DF) salts minimal medium and observing growth (Husen *et al.*, 2009). The nitrogenase activity of the isolate showing growth in this media was estimated by measuring the ethylene produced by its acetylene reduction activity using a gas chromatograph.

The efficiency of the microorganisms to degrade organic nitrogen substrate with the resultant formation of ammonia was tested using Nessler's reagent (Cappuccino and Sherman, 1999). The relative amount of ammonia produced was determined by difference in the degree of yellow colouration.

Qualitative estimation of siderophore production by the isolates was carried out by ferric chloride test as described by Yeole *et al.* (2001). The isolates were graded based on the intensity of the orange or red brown colour developed.

Antagonistic activity against pathogens

The antagonistic activity of the selected isolates against the five major pathogens of rubber *viz. Phytophthora meadii, Corynespora cassiicola, Colletotrichum gloeosporioides,* (leaf pathogens); *Corticium salmonicolor* (stem pathogen) and *Phellinus noxius* (root pathogen) was studied by dual culturing in (potato dextrose agar). The zone of pathogen growth inhibition by the isolates was recorded after seven days growth.

RESULTS AND DISCUSSION

Plant growth promotion

Most of the isolates used in this study showed increase in at least one of the growth parameters of the plants upon inoculation. However 12 out of 79 isolates recorded significant increase in five or more number of variables used to measure the growth of the inoculated plants (Table 1) and were selected for studying other beneficial activities. These isolates were Ri 25, K 43, K 52, RB 88, K 24, A 1, RH 104, RH 34, 3 pt, Ps 20, Ri 10 and F 1. The study showed that the isolates in the groups of high and low levels of IAA production were not efficient growth promoters of *Hevea* seedlings. The IAA production of the selected isolates was in the medium range of 4.8 to 25.46 μg mL^{-1} (Table 2). Among the selected isolates Ri 10 and K 43 showed higher IAA production (25.46 and 25.06 μg mL^{-1}, respectively) while the isolates 3 pt, RH 104 and RB 88 were comparatively low producers of IAA.

Growth promoting activities of the selected isolates

The various beneficial activities of the isolates for promoting plant growth are shown in Table 2. All the isolates showed

Table 1. **Effect of 12 selected bacterial inoculants on growth of rubber seedlings**

Isolate	Shoot length (cm)	Shoot girth (cm)	Shoot fresh wt (g)	Shoot dry wt (g)	No. of leaves	Root length (cm)	Root fresh wt (g)	Root dry wt (g)	Root volume (mL)
Ri 25	59.58	1.30*	10.12	5.18*	7.2*	52.4*	13.12*	3.58*	20
K 43	63.80*	0.62	13.54*	5.28*	7.6*	58.0*	14.62*	4.28*	19
K 52	69.20*	0.74*	14.92*	6.52*	9.0*	57.2*	13.08*	2.54	18
RB 88	67.38*	0.84	14.62*	5.90*	5.6	58.2*	9.02	3.60*	17*
K 24	71.80*	1.34*	14.24*	8.46*	8.6	50.6	9.76*	3.24	10
A 1	66.40	1.54*	29.44*	9.78*	7.0	45.6	10.46*	2.06	25*
RH 104	71.60*	1.42*	15.56*	7.44*	11.0*	48.6	9.04*	3.96*	20
RH 34	71.40*	1.20*	18.90*	6.74*	12.8*	48.0	13.90*	3.74*	20
3 pt	68.80*	1.56*	19.52*	7.20*	6.6	42.0	10.28*	3.84*	20
Ps 20	78.00*	1.32*	18.18*	7.10*	7.4	51.8	7.42*	3.02*	18
R I 10	70.40*	1.38*	16.98*	5.84*	7.4	45.4	12.44*	2.54*	24*
F 1	86.00*	1.38*	19.32*	8.70*	9.2	46.4	5.48*	2.32*	10

* indicates values significantly higher than their corresponding controls at CD (P=0.05)

Table 2. **Beneficial activities of 12 selected isolates (Mean of three replications)**

Isolate	IAA production ($\mu g\ mL^{-1}$)	Phosphate solubilisation (P released in $\mu g\ mL^{-1}$)					Ammonia production	Phosphatase activity ($\mu gPNP\ mL^{-1}$)	Sidero-phore production
		SE %	$Ca_3(PO_4)_2$	$FePO_4$	$AlPO_4$	Rajphos			
Ri 25	23.10	220.00	509.0	131.60	252.30	95.5	Medium	0.78	High
K 43	25.06	127.27	380.0	84.45	117.00	-	Medium	0.08	-
K 52	17.40	112.50	407.5	117.10	219.85	-	High	0.08	-
RB 88	6.92	208.33	337.5	71.00	108.45	20.5	Low	1.16	High
K 24	13.40	106.67	418.5	-	147.55	-	nil	0.00	Low
A 1	9.04	162.50	420.0	-	107.90	-	High	0.16	Low
RH 104	5.36	200.00	479.0	36.60	101.70	47.5	High	3.10	Medium
RH 34	10.80	184.62	445.0	13.20	229.50	-	High	1.71	-
3 pt	4.68	121.43	246.5	15.15	125.30	-	High	0.78	Low
Ps 20	20.00	128.57	402.0	80.50	234.30	-	High	0.08	Medium
Ri 10	25.46	110.00	349.0	49.40	106.75	-	Medium	0.23	Low
F 1	15.90	118.19	420.0	73.70	104.85	-	Medium	0.08	Low

solubilisation of phosphate as indicated by zone of clearance around the colony. The solubilisation efficiency in Apatite agar medium ranged from 106.67 to 220 per cent. Higher phosphate solubilising efficiencies were showed by Ri 25, RH 104 and RB 88 while K 24 showed less zone of phosphate solubilisation. They solubilised $Ca_3(PO_4)_2$ and $AlPO_4$. Solubilisation of $FePO_4$ was recorded by 10 isolates and was less than the other two sources of phosphate. Only three isolates (RB 88, Ri 25 and RH 104) showed solubilisation of Rajphos, the fertilizer form of phosphate. Among the selected isolates Ri 25 showed highest solubilisation of all the four sources of phosphates.

Highest acid phosphatase activity was recorded by the isolate RH 104 followed by RH 34. The activity was less in other isolates. Among the 12 selected isolates only one isolate *viz.* F 1 grew in the nitrogen free medium. The nitrogenase activity of this isolate was 742.7 ppm ethylene produced per hour. Six isolates *viz.* K 52, 3 pt, Ps 20, RH 104, A 1 and RH 34 showed high level of ammonia production. K 24 did not show the production of ammonia. Except K 52, RH 34 and K 43 all the isolates showed siderophore production. RB 88 and Ri 25 showed high siderophore production.

Antagonistic activity against pathogens

The growth inhibition of the five major pathogens of rubber *viz. P. meadii, C. salmonicolor, C. cassiicola, C. gloeosporioides* and *P. noxius* by the isolates tested is shown in Table 3. The isolates showed different levels of growth inhibition of the pathogens in dual culturing. Highest antagonistic activity against *P. meadii* (2.7cm) and *C. gloeosporioides* (1.5 cm) was shown by the isolate A1, against *C. cassiicola* (2.3cm) by F1 and *C. salmonicolor* (2.4cm) by RB 88. Generally these isolates showed less growth inhibition of *P. noxius* the root pathogen.

Increased plant growth due to inoculation of selected rhizobacteria was

Table 3. **Antagonistic activity of selected isolates against five major rubber pathogens (Zone of inhibition in cm)***

Isolate	*P. meadii*	*C. cassiicola*	*C. gloeosporioides*	*C. salmonicolor*	*P. noxius*
Ri 25	0.2	0.9	0.6	0.1	0.1
K 43	0.3	0.8	0.7	1.3	0.2
K 52	1.4	1.0	0.9	0.7	0.2
RB 88	1.0	0.5	0.2	2.4	0.1
K 24	1.3	1.0	1.3	2.0	0.1
A 1	2.7	0.5	1.5	1.4	0.2
RH 104	0.4	0.6	0	0.2	0.1
RH 34	1.1	1.4	0.3	0.5	0.1
3 pt	0.6	0.2	0.1	0.5	0.2
Ps 20	1.2	0.6	0.7	0.3	0.2
Ri 10	0.7	0.8	1.0	0.3	0.1
F 1	0.6	2.3	0.9	0.1	0

* Mean of three replications

reported in many experiments and IAA production was considered as the major mechanism responsible for growth promotion (Kloepper *et al.*, 2004; Elena *et al.*, 2007). Microbially released auxins are not only economical but also provide a continuous supply which may prove to be better than a one time application of synthetic auxin (Frankenberger and Arshad,1995). In this study, out of the 79 isolates tested 12 isolates showed significant improvement in different plant growth parameters. The variable effects of the isolates in promoting the plant growth could be related to the IAA concentration in root tissues or the isolates failed to proliferate and grow well in the root zones (Husen *et al.*, 2009). The amount of IAA synthesised by the isolates from tryptophan or other small molecules present in root exudates may vary based on the ability of the isolates (Whipps, 1990). Previous studies have shown that IAA at low concentrations increased plant growth (Arshad and Frankenberger, 1993) and at high concentrations reduced plant growth (Husen and Saraswati, 2005). In general, exogenous IAA produced by bacteria stimulates plant cell proliferation and elongation, but it also activates the transcription of ACC (1- amino cyclopropane- 1- carboxylate) synthase to form ACC the immediate precursor of ethylene in higher plants. The IAA stimulates rooting, but rooting is opposed by ethylene generated by IAA, hence the promoting effects of IAA are offset by the inhibitory effects of ethylene (Arshad and Frankenberger, 1993). But the bacteria producing ACC deaminase facilitate plant growth by decreasing ethylene production and permitting IAA to stimulate plant cell without negative effect of increasing ACC (Husen *et al.*, 2009). In this study also it was found that 19 isolates with lower (<5mg mL^{-1}) and 10 isolates with higher (>30mg mL^{-1}) were not good growth promoters. Among the selected isolates also the ability to produce IAA varied from high to low levels (Table 2) indicating the involvement of other

mechanisms also in regulating the growth promoting effects of the bacterial isolates.

In addition to IAA production, the 12 selected isolates also recorded various beneficial activities. All the isolates were capable of solubilising insoluble phosphates, even though their ability to solubilize different forms of phosphate varied. In soils most of the P remains in insoluble form and is unavailable to plants. Many rhizobacteria have the ability to release phosphates and responsible for transforming immobilised soil P into plant available form. The traditional rubber growing soils are deficient in available P, due to high fixation of applied P by hydroxides of Fe and Al (Karthikakuttyamma, 1991).The isolate RB 88 and RH 104, the poor IAA producers among the selected were efficient solubilizers of all the four forms of phosphate. However, the isolate Ri 25 was both an efficient phosphate solubiliser and IAA producer.

A large proportion of the total P in rubber growing soils exists as organically bound form (Prasannakumari *et al.*, 2008). In order to become available to plants, organically bound P must be hydrolysed by the enzyme acid phosphatase which may be of plant or microbe origin (Tarafdar and Classen, 1998). Eleven isolates selected showed acid phosphatase activity. Highest acid phosphatase activity was shown by the isolate RH 104 followed by RH 34. Other isolates showed medium and low levels of acid phosphatase activity.

Only one isolate was categorized as diazotrophic bacteria that can fix atmospheric nitrogen. The sequential degradation of nitrogenous organic compounds in soil with the release of ammonia is initiated by excretion of extracellular proteolytic enzymes that are commonly produced by soil microorganisms. Except K 24, all the isolates showed positive response to this reaction. Siderophore producing bacteria promote plant growth by chelating the limited iron in the rhizosphere and making it available to the plant roots (Glick, 1995). It also reduces the availability of iron for growth of phytopathogens (Alexander and Zeeberi, 1991).

Out of the 12 isolates selected for this study, five isolates *viz.*, RH 104, K 43, K 52, RH 34 and Ri 10 gave significant increase in more growth parameters of the plants than others. They also showed their efficiency in the secondary characteristics studied. Even though RH 104 is poor IAA producer, phosphate solubilising efficiency and phosphatase activity by this isolate were higher and recorded high ammonia and medium siderophore production. Isolate RH 34 was also good phosphate solubiliser and showed comparatively high phosphatase activity and high ammonia production. K 52 was a good solubiliser of aluminium phosphate and ferric phosphate and showed high ammonia production. The isolates, K 43 and Ri 10 showed higher IAA production among the isolates. Hence, these isolates are promosing as efficient PGPR for further investigations on seedling growth of *H. brasiliensis*.

One of the challenges in developing PGPR for commercial application is ensuring that an effective screening and selection procedure is in place so that the most promising organisms are identified and brought forward. One approach is selection based on traits known to be associated with plant growth promotion (Nelson, 2004). The interaction between plants and rhizobacteria and the resultant plant growth response are variable according to the prevailing biotic and abiotic conditions. The use of multi-strain inocula of PGPR with known function is of interest as these formulations may increase

consistency in the field (Jetiyanon and Kloepper, 2002). They offer the potential to address multi-modes of action in variable biotic, edaphic and environmental conditions. In order to develop a consortium of inoculants with different activities, the compatibility among them is to be investigated. How much the plant is getting benefited from these different beneficial activities of the selected isolates under varying field conditions requires further study.

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