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INFLUENCE OF INOCULATIONS OF RHIZOBIAL ISOLATES ON GROWTH OF PONGAMIA PINNATA

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Abstract: A nursery experiment was carried out at Department of Microbiology, Kakatiya University, Warangal to study the effect of *Rhizobium* inoculation on plant growth parameters of *Pongamia pinnata* under nursery conditions. 30 *Rhizobial* isolates were screened from the root nodules of *Pongamia pinnata* collected from different locations of Telangana. Eight isolates (RZETPP-1, RZBPPP-2, RZVVPP-4, RZIDPP-9, RZCPPP-19, RZKSPP-20, RZALPP-25 and RZMLPP-30) were positive for maximum number of plant growth promoting traits. Among the eight isolates three isolates, RZBPPP-2, RZVVPP-4, and RZCPPP-19 showed growth at temperatures of 45^0C. RZVVPP-4 isolate enhanced high percentage of seed germination (92 percent), seedling length (88 mm) and vigour index (8360) followed by isolate RZALPP-25 with seed germination (90 percent), seedling length (81 mm) and vigour index (7290). Maximum root length (15.61 cm), shoot length (33.67 cm) and dry weight (5.03 gm) was shown by isolate RZIDPP-9.
Keywords: *Rhizobium*, *Pongamia pinnata*, Indole Acetic Acid, Nodules, Vigour index.

INTRODUCTION

Rhizobia are a group of small, rod-shaped, aerobic, mostly motile, non-sporulating, gram-negative bacteria. However, the term *'Rhizobia'* has come to be used for all the bacteria that are capable of nodulation and nitrogen fixation in association with legumes and that belong to a genus that were at one time part of the genus *Rhizobium* or closely related to it (Denison and Kiers, 2011). However, the legume nodulating bacteria have been divided into two genera based on their speed of growth on yeast extract mannitol medium and various other characteristics under standard laboratory conditions (Singh *et al*., 2008). *Rhizobium*-legume associations are very specific and the legume will only form nodules when infected with a specific *Rhizobium*, while other legumes will form nodules with a range of rhizobia (Franche *et al*., 2009).

Pongamia pinnata (family: *Fabaceae*) is an indigenous multipurpose, biodiesel and medicinal beneficial tree species found throughout India. It is also used in agriculture and environmental management due to its N_2-fixing symbiotic associations with "rhizobia",

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insecticidal and nematicidal properties (Kesari *et al*.,2013; Kabir *et al*., 2001). It is considered as a valuable tree species in agroforestry (Chundawat and Gautam, 1993). In the present research, an attempt has been made to study the effect of inoculation *of Rhizobium* on growth and nodulation of *Pongamia pinnata.*

MATERIAL AND METHODS

Isolation of *Rhizobium*

Congo red-YEMA (Yeast Extract Mannitol Agar) medium (10.0 g mannitol, 0.5 g dipotassium hydrogen phosphate, 0.2 g magnesium sulphate, 15.0 g agar-agar, 15 ml congo red (0.47%), distilled water (DW) 1 L, pH 6.8 - 7.0) was used for isolation of *Rhizobium*. Pink multilobed nodules of *Pongamia pinnata* situated on the tap root were carefully collected and surface sterilized by $HgCl_2$ with 1% for 3 min and 70% C_2H_50H for 3 min. The sterilized root nodules were trampled and diluted suspensions 10^{-5} were chosen and 0.1 ml of suspension was spread and incubated at 30±2°C for 2-3 days. Small, round, colourless, translucent colonies with entire margin were picked out and streaked on YEMA medium. The rhizobial isolates were purified, subcultured and confirmed by congo red test, growth on Hoofer's alkaline medium, glucose peptone medium and ketolactose test (Vincent, 1970).

Nodulation Test: Healthy sterilized *Pongamia* seeds collected from University Campus at Kakatiya University, Warangal were mixed with bacterial (rhizobial) coating solution (100 ml 1% CMC + 10 ml log phase rhizobial broth culture) and allowed to dry for 30 min. Sets were placed in green house and strict aseptic and moist conditions were sustained. The nodulation was observed after 90 days of sowing and assessed the shoot length, root length and numbers of nodules were counted. Uninoculated seeds were maintained for comparison.

Germination Percent of Seeds: The percent germination of seeds was calculated by the following formula:

$$\text{Seed germination}\,(\%) = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sown}} \times 100$$

Root and Shoot Length: The length of roots and shoot was measured with a cm scale.

Determination of Dry Weight: The plant materials cut into bits were dried in an oven at 90°C for 3 days and dry weight was determined.

Nodule Number: Plants were removed carefully from the bags with their root system and nodules intact, after 90 days of growth. The nodules with roots were washed, separated and counted.

Seedling Vigour Index: The vigour index was calculated by using the formula:

Seedling Vigour Index = Seedling length × percent germination.

Statistical Analysis

All data were analyzed using Analysis of Variance (ANOVA). Significant differences between treatments were analyzed using SPSS 17 (Duncan's Multiple Range Test).

RESULTS AND DISCUSSIONS

Isolation of *Rhizobia*

A total 30 potential *Rhizobia* were successfully isolated from the nodules of *P. pinnata* was enumerated by an indirect method and the results are precised in table-1. Data presented in table reveals that sixty percent isolates were fast growers and acid producers. Earlier reports of rhizobia associated with woody legumes described them as either of slow-growing type or cowpea miscellany (Lange, 1961), but more recent reports have shown that this population includes very diverse type of rhizobia including fast, intermediate and slow-growing bacteria (Shetta *et al*.,2011). Appearance of yellow colonies on YEMA plates incorporated with indicator bromothymol blue would indicates acid producer is a fast growing strain (s) whereas a blue colony would indicate alkaline producer is a slow growing strain(s). In longer incubation *Rhizobia* isolated from nodules of Huzurabad (HBPP), Karimnagar (KNPP) Illandu (IDPP), Khammam (KMPP), Kesamudram (KSPP), Aleru (ALPP) and Mylaram (MLPP) were shown white but later observed to have red center. No correlation could be observed between the red color in the white slimy colony and slow grower or fast grower (Sharma *et al*., 2010).

Screening and Selection of Efficient Rhizobial Isolates

Besides N_2 fixation, *Rhizobia* can promote growth affecting, directly or indirectly by the production of various metabolites (Pavan Kumar Pindi, 2011; Vigya Kesari *et al.,* 2013). A total of 30 rhizobial isolates were screened for expression of plant growth promoting characters (PGP traits). Isolates with high relative efficacy of PGP characters were selected for further study. Details regarding the production of selected biochemical traits by the isolates of three host species were presented in the tables 2. A critical perusal of the table-2 reveals that out of thirty *P. pinnata* isolates, seventeen isolates showed significantly positive for protease (RZETPP-1, RZBPPP-2, RZHBPP-3, RZHPPP-6, RZKNPP-7, RZMGPP-8, RZSKPP-11, RZGKPP-14, RZNBPP-15, RZMKPP-16, RZSCPP-18, RZCPPP-19, RZAMPP-21, RZRPPP-24, RZALPP-25, RZMDPP-26 and RZBGPP-28). Six and five isolates were positive for chitinase (RZBPPP-2, RZVVPP-4, RZIDPP-9, RZCPPP-19, RZKSPP-20 and RZMLPP-30) and β-1,3-glucanase (RZBPPP-2, RZIDPP-9, RZNBPP-15,

RZALPP-25 and RZMLPP-30) production respectively. Gelatin, IAA production and starch hydrolysis were shown by 56.6, 50 and 53.3 percent of positive isolates. On subjecting inoculated plates to iodine test, clear zones around the colonies were seen. Thirteen, twelve, Nineteen and Seventeen isolates were found positive for HCN, gibberellic acid, ammonia production and phosphate solubilization respectively. Based on the maximum number of positive traits eight isolates (RZETPP-1, RZBPPP-2, RZVVPP-4, RZIDPP-9, RZCPPP-19, RZKSPP-20, RZALPP-25 and RZMLPP-30) were selected for further studies.

Evaluation of Tolerance of Selected Rhizobial Isolates Towards Stress Conditions

Many abiotic factors such as temperature, pH, salinity, alkalinity, antibiotics, heavy metals, osmotic pressure, moisture content, soil physico-chemical characters and its pollution due to organic and inorganic pollutants interact with the organisms affecting plant growth and yield production. (Ali *et al*., 2009). Hence a microorganism possessing tolerance traits along with PGP traits can be a potential bioinoculants (Dardanelli *et al*., 2009). Resistance of different isolates of rhizobia from *P. pinnata* towards pH, sodium chloride and temperature are presented in Table-3.

As indicated in Table-3, pH is an important parameters for the growth of the *Rhizobium*. In the present observation, pH 7.0 was found to be ideal for all rhizobial isolates. Similar findings were made by Singh *et al.* (2008). With regard to salt tolerance all isolates showed growth at 0.2 percent of $NaCl_2$. However, only 75 percent, 50 percent and 25 percent of the isolates were found to grow on media supplied with 0.4 percent, 0.8 percent and 1 percent $NaCl_2$ respectively. Isolates RZVVPP-4 could resist $NaCl_2$ of 2 percent and can be regarded as osmotolerant strains (Bouhmouch *et al*., 2005). Reports of previous workers (Keneni *et al.,* 2010) showed that fast growing *Rhizobium,* in general, grew well at $NaCl_2$ concentration between 3-5 percent. Temperature is known to influence survival, growth, and nitrogen fixation of *Rhizobium* (Graham *et al.,* 1991). Three isolates, RZBPPP-2, RZVVPP-4, and RZCPPP-19 showed growth at temperatures of 45^0C.

Influence of Inoculations of Rhizobial Isolates on Seed Germination and Seedling Growth

A critical perusal of the table-4 reveals that the percentage of seed germination and radical length varied from plant to plant and with also different rhizobial treatments. At 90 days (DAS) root length, shoot length and dry weight were higher in the *Rhizobium* inoculated plants when compared to control plants. In case of *P. pinnata* rhizobial isolates, RZVVPP-4 isolate enhanced high percentage of seed germination (92 percent), seedling length (88 mm)

and vigour index (8360) followed by isolate RZALPP-25 with seed germination (90 percent), seedling length (81 mm) and vigour index (7290). Maximum root length (15.61 cm), shoot length (33.67 cm) and dry weight (5.03 gm) was shown by isolate RZIDPP-9. Recently, several workers have attempted the role of rhizobial inoculation on improvement in growth and yield of agroforestry tree species (Uddin *et al.,* 2008; Kumudha, 2006). All isolates were also found to enhance root length and shoot length than control. Nodulation ability is the typical character of the genus *Rhizobium* and also essential for their symbiotic relationship with legumes. The nodulation ability of rhizobial isolates on *P. pinnata* when grown in pots varied with different *Rhizobium* isolates. Inoculation of isolate RZVVPP-4 significantly increased the nodule number (22) followed by RZALPP-25 (21). Nodules were assessed as effective based on their size and presence of legheamoglobin. Bigger nodules with legheamoglobin were considered as effective. Most of nodules were in spherical form and determinate type (Rasul *et al.,* 2012).

CONCLUSIONS

This research work concluded that eight isolates (RZETPP-1, RZBPPP-2, RZVVPP-4, RZIDPP-9, RZCPPP-19, RZKSPP-20, RZALPP-25 and RZMLPP-30) were positive for maximum number of plant growth promoting traits. Among eight isolates three isolates, RZBPPP-2, RZVVPP-4, and RZCPPP-19 showed growth at temperatures of 45^0C. RZVVPP-4 isolate enhanced high percentage of seed germination (92 percent), seedling length (88 mm) and vigour index (8360) followed by isolate RZALPP-25 with seed germination (90 percent), seedling length (81 mm) and vigour index (7290). Maximum root length (15.61 cm), shoot length (33.67 cm) and dry weight (5.03 gm) was shown by isolate RZIDPP-9. Production of plant growth promoting compounds indicated that these species have ability to increase the plant growth thereby these *Rhizobium* species can be used for the development of bioinoculants to generate potent biofertilizers.

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Table 1: Rhizobia isolated from nodules of *Pongamia pinnata* of different locations of Telangana

S.No	Location	Abbreviations	Morphological Characteristics	Growth
1	Eturnagaram	ETPP	2-3 mm diam; white; high convex; slimy	Slow grower
2	Bhupalpally	BPPP	1-2 mm diam; white; circular; slimy	Fast grower
3	Huzurabad	HBPP	3- 4 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy	Slow grower
4	Vemulavada	VVPP	1-2 mm diam; white; circular; slimy	Fast grower
5	Jammikunta	JKPP	1-2 mm diam; white; low convex; slimy	Slow grower
6	Hasanparthy	HPPP	1-2 mm diam; white; circular; slimy	Fast grower
7	Karimnagar	KNPP	2-3 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy	Slow grower

8	Mulugu	MGPP	2-3 mm diam; white; circular; excessively slimy	Fast grower
9	Illandu	IDPP	1-2 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy	Slow grower
10	Khammam	KMPP	1-2 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy	Fast grower
11	Sirpurkagaznagar	SKPP	3-4 mm diam; white; circular; slimy	Fast grower
12	Ramagundam	RGPP	1-2 mm diam; white; circular; slimy	Fast grower
13	Boinapally	BLPP	1-2 mm diam; white; circular; slimy	Slow grower
14	Gorrekunta	GKPP	2-3 mm diam; white; high convex; excessively slimy	Fast grower
15	Nizamabad	NBPP	White; circular; excessively slimy	Fast grower
16	Madikonda	MKPP	1-2 mm diam; white; circular; slimy	Slow grower
17	Bainsa	BSPP	1-2 mm diam; white; circular; slimy	Fast grower
18	Sircilla	SCPP	1-2 mm diam; white; circular; slimy	Fast grower
19	Chelpur	CPPP	1-2 mm diam; white; circular; slimy	Fast grower
20	Kesamudram	KSPP	3- 4 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy	Slow grower
21	Athmakur	AMPP	1-2 mm diam; white; high convex; excessively slimy	Fast grower
22	Paluvelpula	PPPP	1-2 mm diam; white; circular; slimy	Slow grower
23	Mucharlanagaram	MNPP	1-2 mm diam; white; circular; slimy	Fast grower
24	Ragunadpally	RPPP	3- 4 mm diam; high convex; excessively slimy	Slow grower
25	Aleru	ALPP	3- 4 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy	Slow grower
26	Miryalguda	MDPP	2-3 mm white; circular; excessively slimy	Fast grower
27	Pembarthy	PBPP	3- 4 mm diam; white; circular; excessively slimy	Fast grower
28	Bhuvanagiri	BGPP	2-3 mm white; circular; excessively slimy	Fast grower
29	Yadagiri Gutta	YGPP	2-3 mm white; circular; excessively slimy	Fast grower
30	Mylaram	MLPP	3- 4 mm diam; white but later observed to have red center in longer incubation; high convex; excessively slimy	Slow grower

Table-2: Screening of rhizobial isolates of *Pongamia pinnata* for production of growth promoting metabolites

S.No.	Isolates	Ammonia	HCN	Protease	Chitinase	β-1,3-glucanase	P' solubilization	Gelatin hydrolysis	Starch hydrolysis	GA	IAA	Siderophore production	Relative efficacy
1	RZETPP-1	+	+	+	-	-	+	-	+	+	+	+	0.72
2	RZBPPP-2	+	-	+	+	+	+	+	+	+	+	+	0.90
3	RZHBPP-3	-	-	+	-	-	-	+	-	-	-	-	0.18
4	RZVVPP-4	+	+	-	+	-	+	+	+	+	+	+	0.81
5	RZJKPP-5	+	+	-	-	-	+	-	-	-	-	-	0.27
6	RZHPPP-6	-	-	+	-	-	+	-	-	-	-	-	0.18
7	RZKNPP-7	+	+	+	-	-	-	+	+	-	-	-	0.45
8	RZMGPP-8	-	+	+	-	-	-	-	+	+	-	-	0.36
9	RZIDPP-9	+	-	-	+	+	+	-	+	+	+	+	0.72
10	RZKMPP-10	+	+	-	-	-	+	+	-	-	-	-	0.36
11	RZSKPP-11	-	-	+	-	-	-	-	+	+	-	-	0.27
12	RZRGPP-12	-	-	-	-	-	+	+	+	-	+	-	0.36
13	RZBLPP-13	+	-	-	-	-	-	-	+	-	-	-	0.18
14	RZGKPP-14	+	-	+	-	-	-	+	+	-	+	-	0.45
15	RZNBPP-15	+	-	+	-	+	-	+	-	-	-	-	0.36
16	RZMKPP-16	-	+	+	-	-	+	-	-	-	-	+	0.36
17	RZBSPP-17	+	-	-	-	-	+	-	-	+	-	-	0.27
18	RZSCPP-18	-	+	+	-	-	-	-	+	-	+	-	0.36
19	RZCPPP-19	+	+	+	+	-	+	+	+	+	+	+	0.90
20	RZKSPP-20	+	+	-	+	-	+	+	-	+	+	+	0.72
21	RZAMPP-21	-	-	+	-	-	+	+	-	-	-	-	0.27
22	RZPPPP-22	+	-	-	-	-	-	+	+	-	-	-	0.27
23	RZMNPP-23	-	+	-	-	-	-	+	-	+	+	-	0.36
24	RZRPPP-24	-	-	+	-	-	+	-	+	-	+	-	0.36
25	RZALPP-25	+	+	+	-	+	+	+	-	+	+	+	0.81
26	RZMDPP-26	-	-	+	-	-	-	-	+	-	-	+	0.27
27	RZPBPP-27	+	-	-	-	-	-	+	-	-	-	-	0.18
28	RZBGPP-28	+	-	+	-	-	-	-	-	-	+	-	0.27
29	RZYGPP-29	+	+	-	-	-	+	+	-	-	+	-	0.45
30	RZMLPP-30	+	-	-	+	+	+	+	+	+	+	+	0.81
	% of positive isolates	**63.3**	**43.3**	**56.6**	**20**	**16.6**	**56.6**	**56.6**	**53.3**	**40**	**50**	**33.3**	

+: Positive; - : Negative

Table-3: Evaluation of tolerance of selected rhizobial isolates of *Pongamia pinnata* towards stress conditions

S.No.	Isolates	Temperature^{0}C					NaCl					pH			
		4	10	15	35	45	0.20%	0.40%	0.80%	1%	2%	5	5.5	7	9
1	RZETPP-1	-	-	-	+	-	+	+	+	-	-	-	+	+	-
2	RZBPPP-2	-	-	+	+	+	+	+	-	-	-	-	+	+	+
3	RZVVPP-4	-	-	-	+	+	+	+	+	+	+	-	+	+	-
4	RZIDPP-9	+	+	+	+	-	+	-	-	-	-	-	+	+	+
5	RZCPPP-19	-	-	-	+	+	+	+	-	+	-	+	+	+	-
6	RZKSPP-20	-	-	-	+	-	+	+	+	-	-	-	-	+	+
7	RZALPP-25	-	+	+	+	-	+	-	-	-	-	-	+	+	+
8	RZMLPP-30	-	+	+	+	-	+	+	+	-	-	-	+	+	+
	% of positive isolates	**12.5**	**37.5**	**50**	**100**	**37.5**	**100**	**75**	**50**	**25**	**12.5**	**12.5**	**80.75**	**100**	**50**

+: Positive; - : Negative

Table 4: Influence of inoculations of rhizobial isolates on seed germination and seedling growth of *Pongamia pinnata*

S.No.	Isolates	% of germination	Seedling length (mm)	Vigour Index	Root length (cm)	Shoot length (cm)	Dry weight/plant	Nodule number/ plant
1	RZETPP-1	62	58±3.05^{a}	3596	11.12±0.17^{ab}	25.61±0.30^{b}	2.83±0.51^{ab}	12±1.52^{a}
2	RZBPPP-2	78	64±1.52^{a}	4992	12.23±0.21^{b}	29.32±0.45^{bc}	2.59±0.19^{a}	11±2.08^{a}
3	RZVVPP-4	92	88±3.72^{c}	8360	13.88±0.18^{bc}	26.84±0.25^{c}	4.33±0.46^{bc}	22±1.52^{c}
4	RZIDPP-9	85	74±1.85^{b}	6290	15.61±0.16^{b}	33.67±0.28^{b}	5.03±1.95^{c}	15±3.05^{b}
5	RZCPPP-19	83	76±2.64^{bc}	6308	10.94±0.33^{bc}	21.46±0.39^{a}	2.24±0.68^{a}	17±1.83^{bc}
6	RZKSPP-20	79	69±1.02^{ab}	5451	9.16±0.21^{a}	20.91±0.17^{a}	3.59±0.88^{bc}	18±0.68^{bc}
7	RZALPP-25	90	81±3.22^{bc}	7290	10.87±0.09^{a}	23.33±0.39^{ab}	2.55±0.13^{a}	21±2.43^{c}
8	RZMLPP-30	69	55±2.15^{a}	3795	11.43±0.12^{b}	27.25±0.14^{bc}	2.79±0.64^{ab}	16±0.45^{bc}
9	Control	55	47±1^{a}	2585	7.56±0.19^{a}	16.43±0.16^{a}	1.31±0.42^{a}	0

± SD; Means values followed by the same letter are significantly different based on Duncan's multiple range test ($p<0.05$) SPSS 17.