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Influence of Plant Growth Promoting *Rhizobium* on Growth of Pigeon Pea [*Cajanus cajan* (L.) Millsp.]

H.A. Harshitha, Geeta Goudar

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ABSTRACT

Background: Symbiotic nitrogen fixation plays a major role in the production of million tons of total biological nitrogen. The *Rhizobium* not only fixes the nitrogen but also produces plant growth promoting hormones. In this view, the present study was undertaken to characterize the native *Rhizobium* isolates for their functional properties and evaluate on pigeon pea for their plant growth promotional activities.

Methods: The present investigation was carried out during the year 2018-19 at UAS, Dharwad. Five of the native isolates obtained from Northern district of Karnataka were subjected for functional characterization using standard methods. The isolates were also tested for their plant growth promotional activities.

Result: The IAA production in the isolates was ranged from 20.50 to 22.85 µg IAA/ml of broth and GA production from 13.40 to 14.51 µg/25ml of broth. ACC deamination activity in the isolates was ranged from 57.50 to 75.5 nmoles of α-ketobutyrate/mg/h of broth. All the isolates exhibited their ability to solubilize P and Zn. Out of ten isolates, four isolates exhibited potential to inhibit mycelial growth of *Fusarium oxysporum* f. sp. *udum* and per cent inhibition was ranged from 48.75 to 73.75. All the isolates were found to be positive for siderophore and HCN production. The isolate AMVPR98 performed better with respect to all the growth parameters of pigeon pea. Molecular characterization of isolate AMVPR98 revealed 99% similarity with *Rhizobium pusense* strain AB3.

Key words: *Fusarium,* IAA production, PGPR, Pigeon pea, *Rhizobium*.

INTRODUCTION

Pigeon pea [*Cajanus cajan* (L.) Millsp.], is the second most important *Kharif* pulse crop grown in India after chickpea. In India, it is predominantly grown in rainfed conditions. It is used as food, fodder and fuel wood. It also maintains soil fertility through nitrogen fixation by its microsymbiont *Rhizobium,* as well as from the leaf fall and recycling of the nutrients. One of the major problems that limit economically successful agricultural production in yield of pigeon pea worldwide is poor soil fertility. Way to correct this problem is the addition of biofertilizers, which improves soil fertility by supplying nutrients needed for optimum crop growth by fixing an atmospheric nitrogen, mineral solubilisation *etc.,* (Osman *et al*. 2011).

Biological nitrogen fixation plays a major role in the production of million tons of nitrogen. The significance of these nitrogen fixing microbes is an evident from the fact that although a total of 100 million metric tons of synthetic nitrogen is produced per year, nitrogen fixing microorganisms yearly converts about 200 million tons of nitrogen to ammonia (NH_4) (Glazer and Nikaido, 2007).

All bacteria nodulating legumes described so far belongs to the Proteobacteria class. The majority of them belong to the genera of the α- Proteobacteria class, in the genera of *Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium* and Ensifer (formerly *Sinorhizobium*) (Berrada and Benbramhim, 2014). Rhizobia are gram-negative, rod shaped, motile, non sporulating bacteria that fix nitrogen from atmosphere after becoming established inside the root nodules of leguminous crops. Pigeon peas are often described as non-specific in their *Rhizobium* requirements and can be effectively inoculated by the indigenous soil populations. Inoculation of pigeon pea with *Bradyrhizobium* increased the number of primary branches, reduced the time to flowering and maturity. It also increased the nodulation and grain yield. Finally it was concluded that biological nitrogen fixation is one alternative to the inorganic N fertilizers (Ahmed *et al*. 2014). BNF values estimated for legume crops fall in the range of 200 to 300 kg of N $ha^{-1}year^{-1}$. Yield increases of crops planted after harvesting of legumes are often equivalent to those expected from application of 30 to 80 kg of fertilizers/N/ ha. Apart from N_2 fixation, *Rhizobium* also known to produce plant growth promoting substances thus stimulates plant growth.

Hence, the present study was carried out to characterize the *Rhizobium* isolates for their functional properties and plant growth promotional activities in pigeon pea.

Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad-580 005, Karnataka, India.

Corresponding Author: Geeta Goudar, Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad-580 005, Karnataka, India. Email: geetagoudar@gmail.com

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MATERIALS AND METHODS

The present investigation was carried out in the Department of Agricultural Microbiology, College of Agriculture, Vijayapur, UAS, Dharwad. Five of the isolates obtained from Northern district of Karnataka were subjected for functional characterization *viz.*, IAA (Gordon and Paleg, 1957), GA production (Paleg, 1965), ACC deaminase activity (Penrose and Glick, 2003), P (Vazquez *et al*. 2000), Zn (Di Simine *et al*. 1998), Si solubilization (Bunt and Rovira, 1955), HCN (Wei *et al*. 1991), siderophore production (Schwyn and Neilands, 1987) and antagonistic activity (Sakthivel and Gnanamanickam, 1987).

In vitro screening of *Rhizobia* isolates against fungal pathogen (*Fusarium oxysporum* f. sp. *udum*) of pigeon pea

The fungal pathogen was grown on potato dextrose agar plates until they completely cover the agar surface. With the help of a sterile cork borer (10 mm diameter), discs of fungal growth from the plates was taken and placed at the center of the fresh PDA plates. Each test isolate was then streaked parallel on either sides of the fungal disc leaving 1.5 cm distance from the edge of the plate. The PDA plates inoculated with only fungal pathogens were considered respective controls. The plates were incubated at 30°C for 96 h. The colony diameter of the fungus in control plate and the plates streaked with *Rhizobium* were recorded (Sakthivel and Gnanamanickam, 1987). The zone of inhibition (ZOI) of each fungal pathogen by different isolates were calculated by using the following formula:

ZOI = Colony diameter (control plate) - Colony diameter (in dual inoculated plates)

The per cent inhibition of pathogen was assessed by using the formula given below:

$$I = \frac{C-T}{C} \times 100$$

Where,
I = Per cent inhibition.
C = Radial growth of the pathogen in control.
T = Radial growth of the pathogen in treatment.

Evaluation of *Rhizobium* isolates for their growth promotional activities on pigeon pea under pot culture conditions

Rhizobium isolates were evaluated for their growth promotional activities under pot culture condition using pigeon pea as a test crop. The experiment was conducted during Kharif season 2018-19 at Department of Agricultural Microbiology, College of Agriculture, Vijayapur, University of Agricultural Sciences, Dharwad, Karnataka.

Inoculum preparation

The individual flask containing 20 ml of sterilized yeast extract mannitol broth was inoculated with overnight grown culture of each *Rhizobium* isolate separately and incubated on rotary shaker for 96 h at a temperature of 28±2°C to get a population of 10^8cfu/ml and mixed with sterilized talc powder in 1:3 proportions to prepare carrier based formulations. These formulations were used for seed treatment. Plastic pot of 35cm top diameter was filled with 8 kg sterilized soil and 30 grams of vermicompost. Pigeon pea seeds of variety TS-3R were surface sterilized in 0.1 per cent mercuric chloride for 3 minutes followed by repeated washing in sterile distilled water for 6 times to remove traces of mercuric chloride and seeds were treated with *Rhizobium* formulations. Treated pigeon pea seeds were sown in each pot by dibbling method at five equidistant spots of 4-5 cm depth where one seed was placed in each hole and immediately covered with soil. In control pots, untreated seeds were sown. After germination, thinning was done to retain only one plant in each pot. The pots were watered regularly to maintain optimum moisture.

Observations on plant growth parameters *viz.,* number of nodules, nodule dry weight (g/plant) and chlorophyll content (SPAD value) were taken after 60 DAS and the observation on shoot dry weight, root dry weight and total dry matter (g/plant) were recorded after 90 days of sowing. Plant nitrogen and phosphorous were estimated at 90 DAS.

Molecular characterization of selected isolates

The isolate AMVPR98 found to be positive for all functional traits and performed better under pot culture condition was subjected for molecular characterization through 16S rDNA analysis.

Statistical analysis of the data

The data obtained from the experiment were subjected to statistical analysis by Completely Randomized Design. Interpretation of the data was carried out in accordance with Pansey and Sukhatme (1985). The levels of significance used in the '*f*' and '*t*' test was P=0.01. The critical difference values were calculated wherever the '*f*' test values were significant.

RESULTS AND DISCUSSION

Functional characterization of *Rhizobium* isolates

All the isolates were positive for IAA and GA production. The IAA production was ranged from 20.50 to 22.85 µg IAA/ml of broth (Table 1) and GA production from 13.40 to 14.51µg/25ml of broth. Highest IAA production was observed in the isolate AMVPR 98 (22.85 µg/ml) followed by the reference strain PPM35B (22.45 µg/ml). With respect to GA production, the maximum of 14.51 µg/25ml of broth was observed in the reference strain which was on par with the isolates AMVPR98 (14.43 µg/25ml broth). ACC deamination activity in all the isolates was ranged from 57.50 to 75.5 nmoles of α-ketobutyrate/mg/h of broth (Table 1). The reference strain PPM35B reported 75.5 nmoles of α-ketobutyrate/mg/h ACC deamination activity which was followed by the isolates AMVPR98 and AMVPR79 with ACC deamination activity of 73.5 and 68.5 nmoles of α-ketobutyrate/mg/h respectively. All the isolates exhibited their

ability to solubilize P and Zn (Table 2). The dissolution and mobilization of soil minerals by microorganisms is well known (Calvaruso *et al*. 2006; Sheng and He, 2006; Uroz *et al*. 2007). The isolate AMVPR 98 showed highest diameter of zone of P-solubilization (10.3 mm) which was followed by reference strain (PPM35B), with zone of solubilization of 9.8 mm. The diameter of zone of zinc oxide solubilization was ranged from 9.5 to 20.5 mm. It was highest in reference strain PPM35B (20.5mm) followed by AMVPR98 (16.5 mm).Whereas only one isolate (AMVPR-98) and reference strain PPM35B exhibited their ability to solubilize silica.The results are in conformity with the work of Chandrakala *et al.* (2019) who reported that the *Rhizobium* isolate obtained from rice rhizosphere, besides possessing mineral (P, K, Si and Zn) solubilising capacities, also exhibited phytostimulatory behaviour by producing IAA (0.95 +0.14 µg IAA /µg bacterial cell protein) and ACC deaminase activity (2.52+0.73 µg α ketobutyrate /µg bacterial cell protein/24 h).

All the isolates were found to be positive for siderophore and HCN production (Table 2). With respect to siderophore production, the diameter of zone of clearance on CAS agar in different isolates ranged from 6.5 to 10.5 mm. Maximum diameter of zone of clearance was observed in the isolate AMVPR98 (10.5 mm). Among six isolates, 2 isolates *viz.,* AMVPR98 and AMVPR79 were strong (+++) HCN producers. The reference strain has also exhibited strong (+++) HCN production ability. The results of this study are in line with the findings of Manasa *et al*. (2017) they studied HCN production in fifteen *Rhizobium* isolates, among those 53% isolates showed positive for HCN and siderophore production. Siderophore production and utilization in rhizobia is of particular interest due to the dominant role of iron in the N_2 fixation and assimilation process (Schwyn and Neiland, 1987). Bacteria with the ability to produce siderophore can enhance plant growth by increasing the availability of iron near the roots for plant uptake (Alexander and Zuberer, 1991).

In vitro screening of *Rhizobium* isolates against fungal pathogen (*Fusarium oxysporum* f.sp. *udum*) of pigeon pea

With respect to *in vitro* screening of *Rhizobium* isolates against *Fusarium oxysporum* f.sp. *udum*, out of ten isolates, four isolates exhibited potential to inhibit mycelial growth of *Fusarium oxysporum* f. sp. *udum* (Table 3). Per cent inhibition was ranged from 48.75 to 73.75. The maximum per cent inhibition of 73.75 was observed in AMVPR-98. Several studies on the mode of action of *Rhizobium* spp. have shown that the growth inhibition of plant pathogens is ensured by the production of toxic compounds. Early work has allowed the characterization of antimicrobial activities related to extracellular compounds of *Rhizobium* spp. such as trifolitoxin (Malajczuk *et al.* 1984) that antibiosis may be part of their reported biocontrol efficacy.

Evaluation of *Rhizobium* isolates for their growth promotional activities on pigeon pea under pot culture conditions.

Plant height

All the treatments with *Rhizobium* isolates differed significantly over the absolute control at 90 DAS with respect to plant height of pigeon pea (Table 4). The highest plant

Table 1: Quantitative estimation of growth hormones produced by native *Rhizobium* isolates obtained from Northern Karnataka.

Isolates	IAA production (µg/ ml)	GA production (µg/ 25ml)	ACC deaminase production(nmoles of –α-ketobutyrate/mg/h)
AMVPR-32	20.50	13.40	60.5
AMVPR-53	22.25	13.97	63.5
AMVPR-79	21.45	14.23	68.5
AMVPR-98	22.85	14.43	73.5
AMVPR-128	21.65	13.40	57.5
Reference strain(PPM35B)	22.45	14.51	75.5
S. Em. ±	0.16	0.21	0.5
C.D @ 1%	0.503	0.64	1.57

Table 2: Functional characteristics of native *Rhizobium* isolates obtained from Northern Karnataka.

Isolates	Zone of P- solubilization (Dia in mm) and amount of Pi released(µg/ ml)	Zone of Zn-solubilization (Dia in mm)	Zone of Si-solubilization (Dia in mm)	Siderophore production (Dia in mm)	HCN production
AMVPR-32	7.5(1.76)	10.5	0.0	8.5	++
AMVPR-53	8.5(2.31)	11.5	0.0	9.0	++
AMVPR-79	8.8(2.45)	12.5	0.0	9.5	+++
AMVPR-98	10.3(3.14)	16.5	6.5	10.5	+++
AMVPR-128	5.3(1.28)	9.5	0.0	6.5	++
Reference strain (PPM35B)	9.8(2.89)	20.5	5.5	10.3	+++

Note: (++) indicates - Moderate HCN production. (+++) indicates - Strong HCN production.

Table 3: *In vitro* screening of *Rhizobium* isolates against *Fusarium oxysporum* f. sp. *Udum* fungal pathogen of pigeonpea.

Isolates	Per cent inhibition
AMVPR-32	48.75(43.84)
AMVPR-53	53.75(46.68)
AMVPR-79	68.75(55.45)
AMVPR-98	73.75(58.59)
AMVPR-128	0.00
Reference strain(PPM35B)	72.5(57.78)
S.Em. ±	0.893
C.D @ 1%	2.850

Note: Figures in parentheses indicate Arcsine transformed values
Inhibition description: (0) indicates no inhibition.

height of 114.58 cm was recorded in T_6 (PPM35B), which was on par with the treatment T_4 (AMVPR98) (114.36 cm)). Lowest plant height (93.72 cm) was recorded in control (T_7). Increase in plant height might be due to the production of phytohormones by the *Rhizobium* and increased mobilization of nutrients (Ahmad *et al.* 2014).

Nodule number

All the treatments with *Rhizobium* isolates differed significantly over control with respect to nodule number to at 60 DAS (Table 4). The treatments T_6 (PPM35B) and T_4 (AMVPR98) were on par with each other recording 12.80 and 12.60 number of nodules/plant. The next highest nodule number was recorded in T_3 (AMVPR79) (11.60) followed by T_2 (AMVPR53) (10.8). Lowest number of nodules (4.40/plant) was recorded in control T_7 which was not inoculated with *Rhizobium*.

Nodule dry weight

Significant differences were observed between the treatments with respect to nodule dry weight at 60 DAS (Table 4). At 60 DAS, maximum nodule dry weight (76.40 mg) was recorded in treatment T_6 (PPM35B) which was significantly superior over all other treatments. The next highest was observed in the treatment T_4 (AMVPR98) (70.25 mg) followed by T3 (AMVPR79) (62.32 mg). Lowest nodule dry weight per plant (23.10 mg) was recorded in treatment T_7. The results are in agreement with the work of Thakare and Rasal (2000) who reported significant increase in nodule number and dry weight of ground nut when inoculated with native isolates of *Rhizobium*.

Growth promotional ability of these isolates could be attributed to production of higher amounts of IAA, GA and also greater ability to solubilize P, Zn and Si. Nascimento *et al*. (2012) reported that selection and use of rhizobial strains with high ACC deaminase activity is a promising strategy to improve the performance of rhizobia-legumes symbiosis by enhancing the nodulation. Similarly, in the present study the isolate AMVPR98 and reference strain exhibited highest ACC deaminase activity resulted in more number of nodules. Increase in dry weight of nodules may be due to enhanced nodulation, higher nitrogen fixation rate and general improvement of root development (Dakora, 2003).

Chlorophyll content (SPAD value)

All the treatments with *Rhizobium* isolates differed significantly over the treatment with absolute control. At 60 DAS, treatment T_6 (PPM35B) has recorded significantly highest chlorophyll content of 62.56 SPAD value, which was followed by treatments T_4 (AMVPR98) (60.62 SPAD value) and T_3 (AMVPR79) (58.42 SPAD value). Lowest chlorophyll content of 42.58 SPAD values was recorded in T_7 (control). Results are well in accordance with Samavat *et al*. (2012) and Bejandi *et al*. (2012) who reported that *Rhizobium* treatment had significantly high chlorophyll content as compared to control. Iron uptake by siderophore production might also increase chlorophyll content of legumes (Kamble *et al.* 2006). In the present study, *Rhizobium* isolates were found to be siderophore producers.

Shoot dry weight, root dry weight and total dry matter production

All the treatments with *Rhizobium* isolates differed significantly over the treatment with absolute control (Table 5). At 90 DAS, highest shoot dry weight was recorded in the treatment T_6 (PPM35B) (8.38 g/plant) which was on par with the treatments T_4 (AMVPR98) (8.37 g/plant), T_3 (AMVPR79)

Table 4: Effect of native *Rhizobium* isolates on plant height of pigeon pea under pot culture condition.

Treatments	Plant height (cm)	Number of nodules /plant	Nodule dry weight (mg/ plant)	Chlorophyll content (SPAD value)
	90DAS	60 DAS	60 DAS	60 DAS
T_1	110.82	10.40	55.04	54.60
T_2	112.02	10.80	56.70	56.12
T_3	113.32	11.60	62.32	58.42
T_4	114.36	12.60	70.25	60.62
T_5	111.34	10.20	54.08	53.10
T_6	114.58	12.80	76.40	62.56
T_7	93.72	4.40	23.10	42.58
S. Em. ±	0.71	0.25	1.07	0.16
C.D @ 1%	0.24	0.75	3.12	0.49

Table 5: Effect of native *Rhizobium* isolates on shoot, root and total dry weight of pigeon pea under pot culture condition.

Treatments	Shoot dry weight (gm)	Root dry weight (gm)	Total dry weight (gm)	N uptake (g/plant)	P uptake (g/plant)
	90DAS	90DAS	90DAS	90 DAS	90 DAS
T_1	8.21	1.76	9.97	0.690	0.122
T_2	8.29	1.81	10.10	0.720	0.126
T_3	8.34	1.84	10.19	0.780	0.132
T_4	8.37	1.85	10.22	0.830	0.156
T_5	8.13	1.76	9.88	0.620	0.106
T_6	8.38	1.86	10.23	0.850	0.152
T_7	3.43	1.30	4.73	0.480	0.084
S. Em. ±	0.04	0.012	0.03	0.013	0.005
C.D @ 1%	0.11	0.053	0.09	0.039	0.013

(8.34 g/plant) and T_2 (AMVPR53) (8.29 g/plant). Lowest shoot dry weight was recorded in T_7 (3.43 g/plant). With respect to root dry weight at 90 DAS, highest root dry weight of 1.86 g/plant was recorded in the treatment T_6 (PPM35B) which was on par with the treatments T_4 (AMVPR98) (1.85 g/plant), T_3 (AMVPR79) (1.84 g/plant) and T_2 (AMVPR53) (1.81 g/plant). Lowest root dry weight was recorded in T_7 (1.30 g/plant). At 90 DAS, highest total dry matter content was recorded in the treatment T_6 (PPM35B) (10.23 g/plant) which was on par with the treatments T_4 (AMVPR98) (10.22 g/plant) and T_3 (AMVPR79) (10.19 g/plant). Lowest total dry matter production was recorded in T_7 (4.73 g/plant). The results are in line with the findings of Rufini *et al.* (2016) observed the symbiosis of *Bradyrhizobium* sp. with pigeon pea Cv. Fava-larga increased the shoot dry weight and N accumulation in the plant under field conditions. Samudin and Kuswantoro (2018) also observed significant increase in the number of nodules, nodule dry weight, root length and root dry weight of soybean when inoculated with *Rhizobium*.

There are evidences that *Rhizobium* can enhance plant growth through the changes in root physiology and morphology along with nitrogen fixation (Biswas *et al.* 2000), which increases root dry weight. Root development is influenced by the phytohormone auxin which is known to affect a number of plant functions including promotion of cell division, elongation, root initiation and ethylene biosynthesis (Chasan, 1993).

Nitrogen and Phosphorous uptake

All the treatments with different *Rhizobium* isolates differed significantly over the treatment with absolute control at 90 DAS. The highest nitrogen uptake was recorded in the treatment T_6 (PPM35B) (0.85g/plant) which was on par with the treatments T_4 (AMVPR98) (0.83g/plant) (Table 5). Lowest nitrogen uptake was recorded in T_7 (0.48g/plant). With respect to P uptake, highest phosphorous uptake was recorded in the treatment T_4 (AMVPR98) (0.156g/plant) which was on par with the treatment T_6 (PPM35B) (0.152g/plant). Lowest phosphorous uptake was recorded in T_7 (0.084g/plant). It has been confirmed that IAA producing bacteria are reported to produce high levels of ACC, which inhibit ethylene levels reported to promote plant growth, enhanced rhizobial nodulation and mineral uptake (Glick, 2012).

Molecular characterization and phylogenetic analysis of efficient *Rhizobium* isolate

The isolate AMVPR98 was subjected for molecular identification using 16S rDNA sequence analysis. The alignment and assessment of the gene sequences data were performed by comparing with the sequences available in Gene Bank database of NCBI, using the algorithm BLAST program. The phylogenetic tree was constituted using BLAST-Webpage (NCBI). The isolate AMVPR98 showed 99% similarity with *Rhizobium pusense* strain AB3 (gene bank accession number KY392993.1). Similarly, Girija *et al.* (2020) identified the isolates obtained from cowpea root nodules as *Rhizobium* sp. based on 16S rRNA gene sequence analysis.

CONCLUSION

PGPR are beneficial bacteria, which are able to establish a symbiotic or nonsymbiotic association with plants in the rhizosphere. *Rhizobium* is the most important PGPR, which is able to develop a symbiotic association with its specific host plant and increase its growth and yield by biologically fixing atmospheric N_2. In addition, they support plant growth via synthesis of phytohormones or other growth-promoting or protecting substances like siderophores and antibiotics. The use of PGPR strains in agricultural practices is strongly encouraged as they may constitute a sustainable solution that can improve the efficiency of chemical fertilizers.

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