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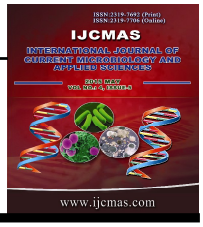

Original Research Article

Effect of plant growth promoting activity of rhizobacteria on Cluster bean (*Cyamopsis tetragonoloba* L.) plant growth and biochemical constituents

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Keywords

Rhizobacteria, PGPR, *Cyamopsis tetragonoloba*, Germination, Enzyme activity, Biochemical assays

A B S T R A C T

The present study aimed at assessing the effect of previously reported six rhizobacteria, *viz*. SJPB (*Acinetobacter baumannii*), SJPB-2a (*Aeromonas hydrophila*), SJPB-2b (*Acinetobacter sp.*), BJJ-4 (*Pseudomonas alcaliphila*), BJJ-5a (*Klebsiella pneumoniae*) and BJJ-7 (*Pseudomonas brassicacearum*), isolated from the semi arid regions of Southern India (Singh et al., 2015), on *Cyamopsis tetragonoloba* under pot culture experimental conditions. In comparison to control treated seeds, highest germination percentage, seedling length vigour index (SLVI), relative root elongation ratio (RER), lateral root density index; protein-, amino acids-, sugar-, and photosynthetic pigment-content; and catalase-, peroxidise-enzyme activity were observed with BJJ-5a (*Klebsiella pneumoniae*). The results of the present study, thus, indicate the potential of harnessing the benefit of plant growth promoting rhizobacteria to improve the germination and biochemical content of *Cyamopsis tetragonoloba*.

Introduction

An annual plant *Cyamopsis tetragonolobus*, also called cluster bean, of the pea family, cultivated chiefly in semiarid regions of South Asia as a forage crop, especially for its seeds from which guar gum is obtained. Guar as a plant has a multitude of different functions for human and animal nutrition but its gelling-agent-containing seeds is of most importance (Mudgil et al., 2014). Through the use of guar gum in the hydraulic fracturing (fracking) extraction of oil and shale gas, the demand for the plant has increased substantially (Mudgil et al., 2014). Furthermore, this legume is a very valuable plant within a crop rotation cycle, as it lives in symbiosis with nitrogen-fixing bacteria. In fact, agriculturists in semi-arid regions of Rajasthan follow crop-rotation and use guar as a source to replenish the soil with essential fertilizers and nitrogen fixation, before the next crop.

The crop production application of plant cultivation and farming practices need to be changed without adversely affecting the yield or quality of the crops with the advancement of new generation technologies. PGPR are known to improve

plant growth in many ways when compared to synthetic fertilizers, insecticides and pesticides (Glick, 1995; Kevin Vessey, 2003; Bhattacharyya and Jha, 2012). PGPRs have been demonstrated to enhance crop growth and help in sustainability of safe environment and crop productivity. The rhizospheric soil contains diverse types of PGPR communities, which exhibit beneficial effects on crop productivity.

In our recently published study (Singh et al., 2015), we had isolated six rhizobacteria from the rhizospheric soil of semi arid regions of Southern India, which demonstrated significant *in vitro* plant growth promoting activity, evident by increase in IAA-, siderophore-, EPS-, Ammonia- production and phosphate solubilisation. These six isolates were identified through molecular characterization by 16s rRNA sequencing and have been successfully submitted in National Centre for Biotechnology information (NCBI) repository. In the present study, to assess the *in vivo* capability of these six isolated rhizobacteria, effect on germination potential, biomass production of *Cyamopsis tetragonoloba* was investigated and further characterization of biochemical-, phytochemical- and enzymatic analysis were performed.

Material and Methods

Plant material

Certified seeds of *Cyamopsis tetragonoloba* plant were obtained from National seed Corporation, I.A.R.I, New Delhi**.**

Sterilization of Soil

Soil for studying plant growth promoting activity was collected from DRDO, Secunderabad. The soil was then sterilized three times for consecutive days 121ºC/15 lbs/in^2 for 1 hour.

Surface Sterilization of seeds

For the surface sterilization, the seeds of *Cyamopsis tetragonoloba* plant were sterilized in the 4% sodium hypochlorite solution for 2 minutes and washed thrice with sterilized distilled water for 10 minutes to remove the traces of sodium hypochlorite solution. *Cyamopsis tetragonoloba* seeds were air dried and sown into sterilized pots (3 seeds per pot)

Seeds Inoculation with Bacterial Culture

Bacterial strains were grown in nutrient broth for 48 hours at 100 rpm. After that seeds of selected plant were soaked in nutrient broth overnight. For the control, seeds were soaked in sterile water.

Preparation of Pots

The treated and control seeds were sown in sterilised soil in pots. They were maintained in Biological Hardening room at 28ºC, 16h light/8hrs dark with fluorescent light intensity1000 Lux and relative humidity 70%. The plants were irrigated with tap water on alternate days to maintain about 70% soil moisture.

Germination percentage

The number of seeds germinated in each treatment was counted on 7th day after sowing. Three replicates were maintained for each treatment. The Germination percentage was calculated by using the following formula.

Germination percentage =

(Total number of seeds germinated/Total number of seeds sown) x 100

Growth Parameters

The plants were harvested after 30th day and following parameters were recorded.

Root length

Underground parts were thoroughly washed under running tap water to remove the adhering soil particles. The root length was measured in centimetre using a thread along the root length and then the thread was measured with the help of a scale.

Shoot length

For measuring the shoot length same procedure for measuring root length was employed.

Number of leaves

The number of leaves per plant were counted and recorded**.**

Number of roots and lateral roots

The number of roots and lateral roots per plant were recorded.

The growth parameters were further analysed by seedling length vigour index (SLVI), relative elongation ratio (RER) of root, and lateral root density using the following formula.

Seedling Length Vigour index (SLVI) =

(Mean shoot length + Mean root length) x Mean germination percentage

Relative Elongation Ratio (RER) of root =

(Mean root length of test plant/ Mean root length of Control) x 100

Lateral Root Density =

Mean number of lateral roots/Primary root length

Fresh weight and Dry weight

Fresh weight of the plant was measured soon after harvesting with the help of weighing balance and data was recorded. For measurement of dry weight the plant was oven dried and then weight was measured with the help of weighing scale and noted.

Estimation of Chlorophyll (Arnon, 1949)

Five hundred mg of fresh leaf material was ground with a mortar and pestle with 10 ml of 80% acetone. The homogenate was centrifuged at 800 rpm for 15 minutes. The supernatant was saved and the residue was re-extracted with 10 ml of 80% acetone. The supernatant was saved and the absorbance values were read at 645 and 663 nm in a UV-Spectrophotometer (Shimadzu-1800). The chlorophyll-a, chlorophyll-b and total chlorophyll contents were estimated and expressed in mg/g of fresh weight.

Biochemical activity

Methanolic extract

For extraction of antioxidant compounds like phenol and flavonoids, leaves, were oven dried for overnight and then 1g of leaf sample homogenised in 10 ml of methanol (1:10 g/ml). The mixture was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and the pellet was centrifuged for 10 min. The supernatant was recollected, pooled and kept at 4°C for further use. Three extracts were prepared for each treatment.

Estimation of Protein (Lowry et al., 1951)

One ml of the extract was taken in a 10 ml test tube and 5 ml of Reagent C was added. The solution was mixed and kept in darkness for 10 minutes. Later, 0.5 ml of Folin phenol reagent was added and the mixture was kept in dark for 30 minutes. The sample was read at 660 nm in a UV Spectrophotometer (Shimadzu-1800).

Estimation of amino acids (Moore and Stein, 1948)

One ml of the extract was pipette out into a test tube. A drop of methyl red indicator was added. The sample was neutralized with one ml of 0.1N sodium hydroxide. To this, one ml of ninhydrin reagent was added and mixed thoroughly. The content of the test tube was heated for 20 minutes in a boiling water bath. Five ml of the dilute solution was added and heated in water bath for 10 minutes. The test tubes were cooled under the running water and the contents were mixed thoroughly. Blank was prepared with one ml of distilled water (or) ethanol. The absorbance was read at 570 nm in a UV Spectrophotometer (Shimadzu-1800).

Estimation of sugars (Nelson, 1944)

One ml of extract was taken in a 25 ml marked test tube 1 ml of reagent C was added. Then, the mixture was heated for 20 minutes at 100º C in boiling water bath, cooled and 1 ml of arsenomolybdate reagent was added. The solution was thoroughly mixed and diluted to 20 ml with distilled water. The sample was read at 520 nm in a UV Spectrophotometer (Shimadzu-1800).

Enzyme activity

Catalase (Machly and Chance, 1967)

One gram of leaf sample was homogenized in 10 ml of 0.1 N phosphate buffers (pH 7.0), and centrifuged at 4ºC for 10 minutes at 10,000 rpm. An aliquot of one ml of the supernatant of the enzyme extract was added to the reaction mixture containing one ml of 0.01M H_2O_2 and 3 ml of 0.01M phosphate buffer. The reaction was stopped after incubation of 5 minutes at 20° C by adding 10 ml of 1% H_2SO_4. The acidified medium without or with the enzyme extract was titrated against 0.005 N $KMNO_4$ and catalase enzyme activity expressed as n moles of H_2O_2 utilized (units /min/mg/protein).

Peroxidase (Kumar and Khan, 1982)

Assay mixture of peroxidase contained 2 ml of 0.1 M phosphate buffer (pH 6.8), 1 ml of 0.001 M pyragallol, 12 ml of hydrogen peroxidases and 0.5 ml of enzyme extract. The solution was incubated for 5 minutes at 25°C, after which the reaction was terminated by adding 1 ml of 2.5 N of sulphuric acid. The amount of purpurogallin formed was determined by reading the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N of sulphuric acid. The activity was expressed in unit = 0.1 absorbance mg/ protein / min.

Quantification of phytochemicals

Determination of total phenol content

Total phenolic concentration was estimated by Folin-Ciocalteu colorimetric method (McDonald et al., 2001). A methanolic plant extract (0.5 ml of 1:10 g/ml) was then oxidized with 5 ml Folin Ciocalteu reagent (1:10 diluted with distilled water) followed by neutralization with 4ml of 1M aqueous Na_2CO_3. The mixtures were then incubated for 15 min and absorbance was recorded at 765 nm. The standard curve was prepared by using different dilutions of gallic acid in

methanol: water (50:50, v/v). The results were expressed in terms of GAE (gallic acid equivalent) mg/g.

Determination of total flavonoid content

Flavonoid concentration was determined by Aluminum chloride colorimetric method (Chang et al., 2002). 0.5 ml of plant extract (1:10 g/ml) were separately mixed with 1.5 ml of methanol followed by treatment with 0.1 ml of 10% $AlCl_3$, mixed with 0.1 ml of 1M potassium acetate and the mixture was diluted with 2.8 ml of distilled water. The mixture was then incubated at room temperature for 30 min and the absorbance was measured at 415 nm. Total flavonoid content was determined by preparing standard curve of quercetin at different concentrations in methanol (g/ml).

Statistical analysis

All data are represented as mean ± SD for at least three replicates. Student's T-test was performed to determine significance of the results obtained in the experiments (** indicates $p<0.005$).

Results and Discussion

Germination studies

In earlier studies, a commercial soil amendment containing a mixture of four PGPR (*Azospirillu lipoferum*, *Azotobacter chroococcum*, *Pseudomonas fluorescence* and *Bacillus megaterium*) was evaluated and reported to increase germination rate and vigour index as compared with the control (Lenin and Jayanthi, 2012). Furthermore, effect of plant growth promoting rhizobacteria on seed germination, seedling growth and yield of field grown maize were demonstrated to be significantly enhanced (Gholami et al., 2009).

In the present study, *Cyamopsis tetragonoloba* seeds treated with BJJ-5a, SJPB-2a and SJPB-2b demonstrated significantly higher germination percentage 90%, 73.3% and 80%, respectively whereas uninoculated seeds (control) showed only 46.7% and 50% (Table 1). BJJ-5a treated seeds demonstrated better rooting, more number of leaves, increased root length, shoot length and dry weight as compared to control treated seeds (Fig. 1 and 2). Compared to control, increased number of roots was also observed for SJPB treated seeds (Fig. 1). Additionally, BJJ-7 treated seeds demonstrated more no. of lateral roots (Fig. 2). BJJ5-a showed highest seedling length vigour index (mean SLVI: 1669 ± 302), relative elongation ratio of root (mean RER: 157 ± 34%) and lateral root density (mean: 1.7 ± 0.4) compared to control (633 ± 236, 100 ± 0.0%, 1.0 ± 0.3, respectively) (Table 2). Our results suggest that the application of the rhizobacteria, in particular the BJJ-5a treatment, enhanced seed germination and plant growth.

Photosynthetic pigments

Next, we investigated the effect of Plant growth promoting rhizobacteria on the photosynthetic pigment contents of *Cyamopsis tetragonoloba* seedlings. BJJ-5a treated seeds demonstrated the maximal chlorophyll-a (12.15 mg/L), chlorophyll-b (1.40 mg/L) and total chlorophyll content (18.05 mg/L) (Fig. 3). The lowest chlorophyll-a, chlorophyll-b and total chlorophyll (4.14, 0.44, 6.12, mg/L) were observed in seedlings grown without PGPR treatment (Control). The seeds treated with SJPB, SJPB-2a, BJJ-4 and BJJ-7, but not SJPB-2b, demonstrated significantly higher chlorophyll-a, chlorophyll-b and total chlorophyll total chlorophyll content as compared to uninoculated control (Fig. 3).

Table.1 Germination percentage of rhizobacteria treated *Cymopsis tetragonoloba* seeds in comparison to untreated (Control) seeds (post 15 days of inoculation)

Replicate	Control I		Control II		SJPB		SJPB-2a		SJPB-2b		BJJ-4		BJJ-5a		BJJ-7	
	Germination		Germination		Germination		Germination		Germination		Germination		Germination		Germination	
	n/3	%	n/3	%	n/3	%	n/3	%	n/3	%	n/3	%	n/3	%	n/3	%
Pot 1	2	66.7	2	66.7	1	33.3	2	66.7	2	66.7	2	66.7	3	100.0	1	33.3
Pot 2	1	33.3	1	33.3	2	66.7	2	66.7	3	100.0	2	66.7	2	66.7	2	66.7
Pot 3	1	33.3	2	66.7	2	66.7	2	66.7	2	66.7	2	66.7	3	100.0	2	66.7
Pot 4	2	66.7	2	66.7	2	66.7	1	33.3	2	66.7	2	66.7	2	66.7	2	66.7
Pot 5	1	33.3	1	33.3	2	66.7	3	100.0	2	66.7	2	66.7	3	100.0	3	100.0
Pot 6	2	66.7	2	66.7	2	66.7	3	100.0	2	66.7	2	66.7	2	66.7	3	100.0
Pot 7	2	66.7	1	33.3	3	100.0	2	66.7	3	100.0	2	66.7	3	100.0	2	66.7
Pot 8	1	33.3	1	33.3	2	66.7	2	66.7	2	66.7	2	66.7	3	100.0	2	66.7
Pot 9	1	33.3	2	66.7	1	33.3	2	66.7	3	100.0	3	100.0	3	100.0	2	66.7
Pot 10	1	33.3	1	33.3	2	66.7	3	100.0	3	100.0	2	66.7	3	100.0	3	100.0
Mean %		**46.7 %**		**50.0 %**		**63.3 %**		**73.3 %**		**80.0 %**		**70.0 %**		**90.0 %**		**73.3 %**
Student's T-test		-		-		*p=0.0422*		*p*=0.0224		*p*=0.0038		*p*=0.0095		*p*=0.0007		*p*=0.0224

Table.2 Effect of rhizobacteria treatment on Seedling Length Vigour Index, Relative Elongation Ratio of root and Lateral Root Density of *Cyamopsis tetragonoloba*

	Control I	Control II	SJPB	SJPB-2a	SJPB-2b	BJJ-4	BJJ-5a	BJJ-7
Seedling Length Vigour index (SLVI)	633 ± 236	670 ± 250	962 ± 251	1171 ± 332	1239 ± 291	1159 ± 224	1669 ± 302	1202 ± 344
Relative Elongation Ratio (RER) of root	100 ± 0.0 %	98 ± 17.3%	133 ± 33.4 %	110 ±29.8%	114 ± 26.4%	130 ± 25.5 %	157 ± 33.4%	126 ± 24.6 %
Lateral Root Density (cm^{-1})	1.0 ± 0.3	1.2 ± 0.3	1.6 ± 0.6	1.6 ± 0.2	1.1 ± 0.4	2.0 ± 0.4	1.7 ± 0.4	2.2 ± 0.4

Mean ± Standard Deviation

Table.3 Effect of treatment of Plant growth promoting rhizobacteria on protein, amino acid and sugar (mg gm^{-1} of fresh wt.) content of *Cyamopsis tetragonoloba*

Isolates	Protein (mg/gm of fresh weight)	Amino Acid (mg/gm of fresh weight)	Sugar (mg/gm of fresh weight)
Control	13.43 ± 0.47	3.33 ± 0.46	3.34 ± 0.32
SJPB	17.02 ± 0.25	5.88 ± 0.34	3.95 ± 0.14
SJPB-2a	27.00 ± 1.51	7.84 ± 0.25	4.85 ± 0.31
SJPB-2b	27.78 ± 0.67	6.49 ± 0.29	3.92 ± 0.25
BJJ-4	20.93 ± 0.91	5.04 ± 0.07	4.34 ± 0.36
BJJ-5a	28.30 ± 0.54	7.80 ± 0.38	7.17 ± 0.72
BJJ-7	14.94 ± 0.50	5.52 ± 0.43	4.34 ± 0.40

Mean ± Standard Deviation

Table.4 Effect of treatment of Plant growth promoting rhizobacteria on enzyme (Catalase and Peroxidise) activity of *Cyamopsis tetragonoloba*

Isolates	Catalase (units/min/gm fresh weight)	Peroxidase (units/min/gm fresh weight)
Control	3.50 ± 0.48	4.94 ± 0.07
SJPB	7.74 ± 0.46	11.92 ± 0.10
SJPB-2a	9.70 ± 0.40	13.98 ± 0.03
SJPB-2b	8.58 ± 0.32	14.74 ± 0.38
BJJ-4	9.70 ± 0.25	14.58 ± 0.33
BJJ-5a	10.93 ± 0.16	15.29 ± 0.09
BJJ-7	7.88 ± 0.12	11.89 ± 0.13

Mean ± Standard Deviation

Table.5 Effect of treatment of Plant growth promoting rhizobacteria on Phenol and Flavonoids (mg gm^{-1} of fresh wt.) content of *Cyamopsis tetragonoloba*

Isolates	Phenol (mg/gm of fresh weight)	Flavonoids (mg/gm of fresh weight)
Control	216.85 ± 3.26	42.53 ± 0.51
SJPB	520.98 ± 0.52	51.68 ± 0.72
SJPB-2a	741.77 ± 2.52	77.38 ± 1.97
SJPB-2b	687.21 ± 2.95	63.22 ± 0.93
BJJ-4	345.72 ± 1.23	73.34 ± 0.68
BJJ-5a	784.94 ± 5.07	88.67 ± 1.06
BJJ-7	483.73 ± 1.67	74.76 ± 0.52

Mean ± Standard Deviation

Figure.1 Effect of rhizobacteria treatment on morphological parameters of *Cyamopsis tetragonoloba*

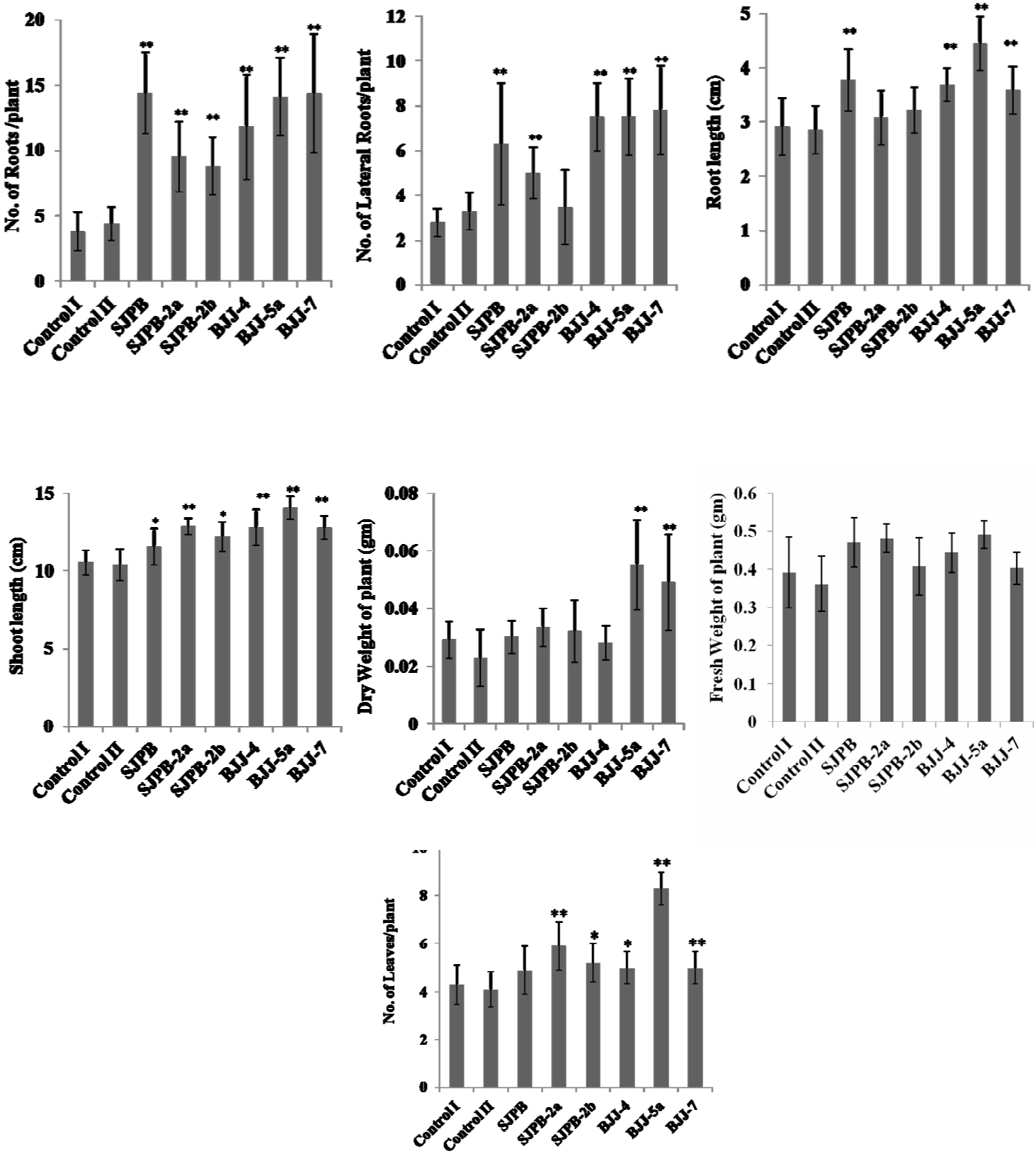
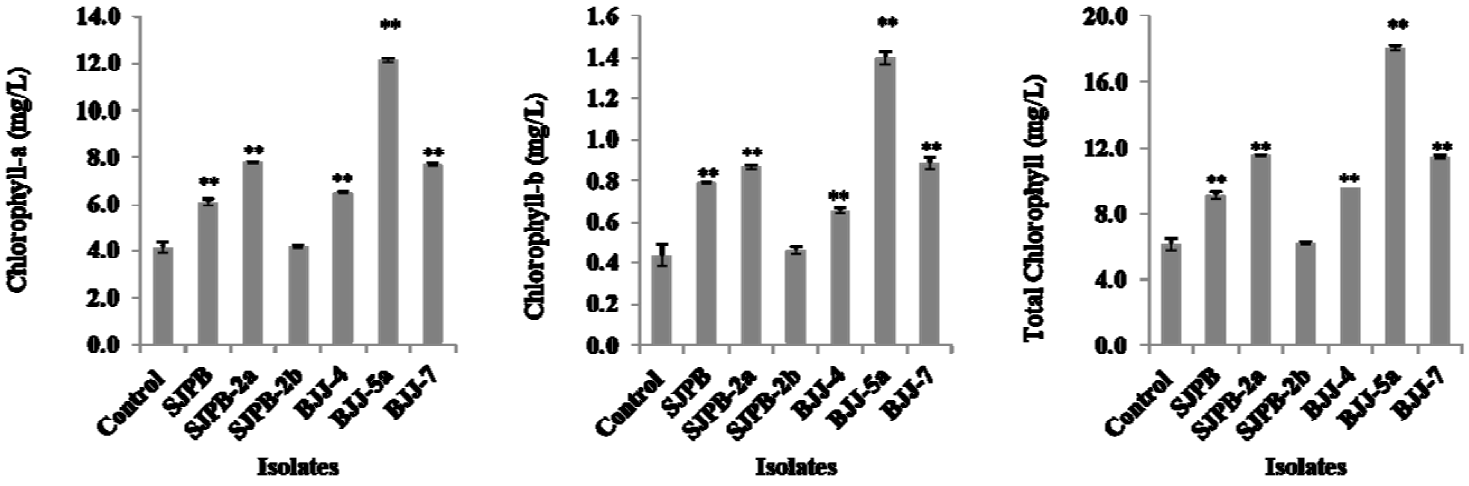


Figure.2 Representative photographs of Control and BJJ-5a treated *Cyamopsis tetragonoloba* (post 15 days of inoculation)

Control treatment

BJJ-5a treatment

Figure.3 Chlorophyll estimation (Chlorophyll-a, Chrolophyll-b and Total Chlorophyll) of rhizobacteria treated *Cyamopsis tetragonoloba*



Biochemical studies

Previous studies have reported that the inoculation of rhizobacteria increases protein content of the test plants (Akbari et al., 2011). We performed biochemical assays, to determine any augmentation of protein, amino acids and sugar content due to the treatment of *Cyamopsis tetragonoloba* seeds with isolated rhizobacteria. The highest protein, amino acid and sugar content (28.30, 7.8 and 7.17 mg/g fr. wt.) were observed in *Cyamopsis tetragonoloba* seedlings grown in BJJ-5a treatment (Table 3) while the lowest protein, amino acid and sugar contents (13.4, 3.33 and 3.34 mg/g fr.

wt.) were observed in *Cyamopsis tetragnoloba* seedlings grown without PGPR treatment (Control). In comparison to Control treatment, the seedlings treated with SJPB, SJPB-2a, SJPB-2b, and BJJ-4 demonstrated higher protein; SJPB, SJPB-2a, SJPB-2b, BJJ-4 and BJJ-7 demonstrated higher amino acid; and SJPB-2a, BJJ-4 and BJJ-7 demonstrated higher sugar content (Table 3).

Enzyme activity

PGPR application has been previously reported to induce plant defence enzyme (such as phehylamine ammonia lyase, peroxidise and polyphenoloxidase) activities in the leaf and root of *Piper betle* (Lavania et al., 2006) and *Antheraea assam* (Unni et al., 2008). To determine the effect of plant growth promoting rhizobacteria on catalase and peroxidise activity, we performed enzyme assays on PGPRs treated *Cyamopsis tetragonoloba*, results of which are presented in Table 4.

The highest catalase and peroxidase activity (10.93 and 15.29 units/min/g/fresh weight) were observed in *Cyamopsis tetragonoloba* seedling with BJJ-5a treatment while the lowest catalase and peroxidase activity (3.50 and 4.84 units/min/g/fresh weight) were recorded in *Cyamopsis tetragonoloba* seedling grown without plant growth promoting rhizobacteria treatment (Control). In comparison to Control treatment, the seedlings treated with SJPB, SJPB-2a, SJPB-2b, BJJ-4 and BJJ-7 demonstrated increased catalase and peroxidase activity (Table 4).

Phytochemical studies

Total phenolic and flavonoid content are one of the important secondary metabolites that present ubiquitously in plants and its products contains high amount of antioxidants (Razali et al., 2008). Previous studies have documented that phenolic compounds are the categories of antioxidant agents which help in the termination of free radicals (Shahidi and Wanasundara, 1992). Moreover, flavonoids have also demonstrated the antioxidant activity due to their scavenging and chelating process for free radicals (Prasad et al., 2013). Phenolic compounds play an important role in defence mechanism against microbial pathogens based on their toxicity and repellence to microbes and insects (Mazid et al., 2011).

We performed phytochemical assays on PGPR treated *Cyamopsis tetragonoloba* to determine the Phenol and Flavonoid content, results of which are presented in Table 5. The highest phenol and flavonoids content (784.94 and 88.67 mg/gm of fresh weight) were observed in *Cyamopsis tetragonoloba* seedling with BJJ-5a treatment while the lowest phenol and flavonoid content (216.85 and 42.53 mg/g of fresh weight) were recorded in *Cyamopsis tetragonoloba* seedling grown without plant growth promoting rhizobacteria treatment (Control). In comparison to Control treatment, the seedlings treated with SJPB, SJPB-2a, SJPB-2b, BJJ-4 and BJJ-7 demonstrated increased phenolic and flavonoid activity (Table 5).

Present study, thus, reflects the application of Plant Growth Promoting Rhizobacteria (PGPR), having significant effect on seed germination, plant growth, enzyme activity and contents of antioxidants in *Cyamopsis tetragonoloba*. Since these cardinal parameters are associated with plant yield, it can be suggested that application of isolated PGPRs may further enhance crop yield of *Cyamopsis tetragonoloba.*

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