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Biochemical Attributes of Efficient PGPR Bioinoculants and their Effect on Growth of *Dalbergia sissoo* (Roxb.).

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Biochemical characterization of *Bacillus* and *Pseudomonas* bioinoculants was done on the basis of phosphate solubilisation and indole-3-acetic acid production (IAA). It was observed that *Bacillus* isolate SD25 produced the highest solubilization zone (11mm) and solubilisation index (2.89) whileas; lowest solubilization zone was reported to be produced by *Bacillus* isolate SD4 and *Bacillus* isolate SD64 (2.5mm) but the lowest solubilisation index was reported in Bacillus isolate SD4 (1.26). Indole-3-acetic acid production (ìg mL^{-1}) by the *Pseudomonas* and *Bacillus* isolates was observed at two levels of tryptophan (0.05 and 0.1g tryptophan L^{-1} ìg mL^{-1}) and it was observed that maximum IAA was produced by *Pseudomonas* isolate SD2 (54.34 ìg mL^{-1} at 0.1 g tryptophan L^{-1}) while minimum by *Bacillus* isolate SD65 (7.17 ìg mL^{-1} at 0.1 g tryptophan L-1). In the same way the IAA production at 0.05 g tryptophan L^{-1} was highest and lowest levels produced by *Pseudomonas* isolate SD2 (32.368 ìg mL^{-1}) and by *Bacillus* isolate SD65 (3.981 ìg mL^{-1}) respectively. However it was observed that some isolates at both the levels of tryptophan did not produce IAA (SD64, SD66) and it was further observed that *Psudomonas* isolate SD47 produced IAA at 0.1 g tryptophan L^{-1} but not at 0.05 g tryptophan L^{-1}. The isolates were further mixed together to form a consortium named as "Rhizobacterial Consortium" or RC in a solution whose effect on plant growth was observed using different concentrations of the solution (0.2%, 0.5%, 1%, 2%, 5% 10% and 15%) in the form of seed treatment and inoculation into poly bags at fifteen days interval. Seed germination rate and growth parameters of seedlings were recorded, such as shoot and root length, the shoot and root dry and fresh weight. The effect of rhizobacterial consortia concentration on leaf pigments (Chlorophyll a, chlorophyll b and caeotenoid) was determined and it was found that contents of chlorophyll and carotenoids were highest (44.49, 14.54 and 36.29 mg mL^{-1} respectively) in 5% treatment and lowest 34.65, 10.32 and 24.34 mg mL^{-1} respectively) in control treatment.

Key words: Pseudomonas, Bacillus, *Dalbergia sissoo,* Isolate, RC.

Dalbergia sissoo (Roxb.) is an exceptionally significant tree of tropical Indian Himaliyas and is extensively eminent for its multiple uses from medicinal to timber. Being a forest tree its grown without knowing the soil fertility standing of the tropical soils so faces a discrepancy of multiple nutrients and in due course a reduced growth. *Dalbergia sissoo* (Roxb.) is considered to be originated in Bengal and is customary in countries like India, Nepal, Pakistan, Bhutan, Bangladesh, Myanmar, Malaysia, and Afghanistan. It has been reported to be used as aphrodisiac, abortifacient, expectorant, antihelminthic, antipyretic and other medicinal uses.

It is developed as monoculture or in agroforesty systems because of the quality of its wood and its sound effects on soil fertility all the way through N_2 fixation. Near to the ground soil fertility in tropical regions fallout in underprivileged plant growth. This is noteworthy in the case of forest trees, since they are by and

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large transplanted without making an allowance for the fertility status of soil (Bishti *et al.,* 2009). Plant growth is maintained by both biotic and abiotic factors (Sivasakthi *et al.,* 2014) and among the biotic factors microbes are recognized to interact with the plants and directly influence their growth (Palaniyandi *et al.,* 2013). Several plant linked bacteria have shown advantageous impression on the overall health of the plants; such bacteria residing in the proximity of a plant's root are termed as plant growth promoting rhizobacteria (PGPR) (Calvo *et al.,* 2014; Glick, 2014) and hence offer the suitable and sustainable solution. During the late 19th and early 20th centuries inorganic compounds containing nitrogen, potassium and phosphorus (NPK) were synthesized and used as fertilizers. Due to the intensification in human populations fertilizers were used to augment crop production and meet the mounting demands for food and fiber. Amplified production cost, and the perilous nature of chemical fertilizers for the environment has led to a renaissance of curiosity in the use of biofertilizers for enhanced environmental sustainability, lower cost production and excellent crop yields. Copious species of soil bacteria which prosper in the rhizosphere of plants, but which may nurture in, on, or around plant tissues, stimulate plant growth by a surfeit of mechanisms. These bacteria are as a group are known as PGPR (plant growth promoting rhizobacteria (Akhtar *et al.,* 2012). Plant-growth-promoting rhizobacteria (PGPR) help plants by increasing plant growth and defending them against pathogens (Fahimi *et al.,* 2014).

To accomplish the ceiling growth promoting interaction between PGPR and nursery seedlings it is imperative to discover how the rhizobacteria bring to bear their effects on plant and whether the effects are altered by various environmental factors, together with the occurrence of other microorganisms (Bent *et al.,* 2001). Thus PGPR can be isolated from plant rhizospheres and used as inoculants for improving the growth and yield of agricultural crops (Dasgupta *et al.* 2015).

The at hand study was conducted to divulge the plant growth promoting ability (IAA production and Phosphate solubilization) of *Bacillus* and *Pseudomonas* strains and to weigh up their effect on plant growth parameters of *Dalbergia sissoo* Roxb. At assorted concentrations under glasshouse conditions. The microorganisms have been combined into inoculant in the manufacturing process. The density of each culture in the RCranges from 1×10^{9} to 1×10^{10} cfu/mL and hence they can be used as inoculant to augment the microbial diversity in the soil. Such type of bacterial consortia have been used in a choice of integrated approaches of crop management besides organic farming systems. Therefore, the current exploration was carried out to study the effect of RC inoculants on seed germination and seedling growth of *Dalbergia sissoo* and also to determine the suitable concentration of RC solution for utmost seedling growth in the nursery.

MATERIALS AND METHODS

The present investigation was carried out in the college of Basic Sciences and Humanities CCS, HAU, Hisar Haryana India. The seeds of *D. sissoo* (Roxb.) were collected from Department of Forestry college of Agriculture CCS, HAU Hisar. The *Bacillus* and *Pseudomonas* isolates were selected among 100 isolates (isolated from the rhizospheric soil samples of Shisham rhizosphere) on the basis of their ability to produce a solubilisation zone on pikovskaya's mediam. Then All the selected rhizobacteria were inoculated in 25 mL respective broth. These flasks were incubated at 28+2°C in a BOD incubator for 3 days. Five ìL of each culture was taken and spotted on pikovskaya's plates. These plates were incubated at 28+2°C for 3-4 days. Zone of solubilization was measured and coloney size was also measured & these values were used to calculate solubilization index by the following formula (Edi-Premono *et al.,* 1996).

SI = colony diameter + halozone diameter/colony diameter

Thus indole-3-acetic acid production by selected isolates was determined by inoculating the isolates in 25 mL of their respective broth supplemented with 0.1 & 0.05 g L^{-1} DL-tryptophan. The flasks were incubated at 28+2°C in a shaking BOD incubator. After 4 days of incubation, 2 mL of culture broth was centrifuged at 7,000 rpm for two minutes and then IAA was determined in culture supernatant by adding an equal volume of

Salkowski's reagent to it. The contents were mixed by shaking and allowed to stand at room temperature for 30 minutes for development of pink colour which was estimated colorimetrically at 500 nm using spectrophotometer. Indole- 3- acetic acid was used as a standard method (Tang & Bonner, 1974).

The plant growth studies were carried out under glass house conditions using *Dalbergia sissoo* (Roxb.) as a host plant. The soil was collected from nursery of university campus and sieved well (Â 3mm) and mixed with decomposed cowdung in the ratio of 3:1. The poly bags of the dimensions of 20×10cm in size were filled with the prepared mixture. There were 8 treatments including the control and 5 replications for each treatment. Seeds in the poly bags of control were not inoculated with RC but water only and seeds in the treatments were inoculated with 0.2%, 0.5%, 1%, 2%, 5% 10% and 15% concentrations of RC solution. The 30 ml of solution (per kg of soil) was mixed with the soils before one week of sowing and 10 ml of solution was inoculated to each poly bag at weekly interval. To record the observations based on the impact of RC on seed germination, five seeds were sown in each poly bag. After completing the germination, only one seedling is maintained for observing the nodulation status and growth parameters of seedlings. Arrangements were made to avoid the direct sunlight and rain on the tender seedlings by covering the roof of glass house with polythene.

Germination rate was recorded daily from the date of seed sowing and continued up to the last germination. Then the seedlings were allowed to grow altogether for 2 months from the time of the last germination of seeds. After 2 months, seedlings from each treatment were selected for measuring their growth parameters. The recorded parameters included shoot and root length, collar diameter, leaf number, fresh shoot and root weight, dry shoot and root weight and nodulation status. For recording dry weight, shoots and roots were oven dried at 70°C until the constant weight was obtained. The pigment contents (chlorophyll a, chlorophyll b and caro- tenoid) were determined from the fresh leaves of seedlings in different treatments. Ten leaf discs were cut from leaves with a cork borer (inside diameter of 5 mm), weighted immediately after cutting and dipped in 100% acetone of 5 mL in test tube with stopper. After 24 h of incubation, the supernatant colored solution from the top was decanted carefully in 25-mL volumetric flask. The leaf discs were then crushed with a blunt glass rod gently and 5-mL fresh acetone was added to the test tube and left for 15 min. Then the supernatant colored solution from the top was again decanted to the same volumetric flask very carefully, avoiding the fragmented plant tissues. The process was repeated until the leaf fragments became colorless. Finally the volume was made up to 25 mL with fresh acetone and measurement was taken immediately after the preparation of solution. The measurement of chlorophyll a, chlorophyll b and carotenoid was made at 662 nm, 644 nm and 440.5 nm respectively, with a spectrophotometer. The pigment contents in the extract were calculated by following the formula of Wettstein (Wettstein *et al.*, 1957).

$$C_a = \frac{(9.784E662 - 0.99E644)\times V\times d}{1000F_w}$$

$$C_b = \frac{(21.426E644 - 4.650E662)\times V\times d}{1000F_W}$$

$$C_c = \frac{[4.695E440.5 - 0.268(C_a+C_b)\times V\times d}{1000F_w}$$

where, C_a is the chlorophyll a (mgÅ"L^{-1}), C_b the chlorophyll b (mgÅ"L^{-1}), C_C the carotenoid (mgÅ"L^{-1}), V the total volume (25 mL), L the litter of leaf extract solution, d the dilution factor, F_W the fresh weight of leaf disc (g), and E is the absorbance at a particular wavelength (440.5, 644 and 662 nm).

RESULTS AND DISCUSSION

All the isolates of *Bacillus* and *Pseudomonas* were found to solubilize phosphate (Table 1, Fig. 1) and further it was observed that *Bacillus* isolate SD25 produced the highest solubilisation zone (11mm) and highest solubilisation index (2.89) and lowest solubilization zone was reported to be produced by *Bacillus* isolate SD4 and *Bacillus* isolate SD64 (2.5mm each)

but the lowest solubilisation index was reported in *Bacillus* isolate SD4 (1.26). Similr to our findings numerous species of fluorescent pseudomonas such as *P. fluorescens* NJ101 (Bano & Masarrat 2004), *P. aeruginosa* (Jha *et al.*, 2009) and *Bacillus* sp. (Ahmad *et al.*, 2008) were reported as excellent phosphate solubilizers. Suresh *et al.* also reported the formation of halo zones by the various *Pseudomonas* species on sPikovskaya agar medium (Suresh *et al.*, 2010).

The capacity of bacteria to produce IAA in the rhizosphere depends on the accessibility of precursors and uptake of microbial IAA by plant. Growth encouragement may be ascribed to other mechanisms such as production of plant growth promoting hormones, siderophores, HCN and antibiotics in the rhizosphere and other PGP activities (Arshad & Frankenberger 1993; Glick *et al.*, 1995; Suresh *et al.*, 2010). Production of IAA by *Bacillus* and *Pseudomonas* is a general characteristic of our test isolates.

PGPRs are known to produce a class of phytohormones known as auxins. Auxins pose direct effect on the growth of the plant by controlling several stages of plant growth and development such as cell elongation, cell division,

Table1. Solubilisation zone and solubilisation index of different rhizobacterial isolates as observed on Pikoskaya's media

Isolate	Genus	Solibilisation zone (Radius in mm)	Solubilisation Index
SD2	*Pseudomonas*	8.0d	2.71b
SD4	*Bacillus*	2.5a	1.26a
SD5	*Pseudomonas*	4.5b	2.25a
SD21	*Pseudomonas*	3.5b	1.75a
SD25	*Bacillus*	11e	2.89b
SD31	*Bacillus*	6.5c	2.60b
SD47	*Pseudomonas*	4.5b	2.25a
SD59	*Pseudomonas*	5.0c	2.00a
SD64	*Bacillus*	2.5a	1.50a
SD65	*Bacillus*	3.0b	2.00a
SD66	*Pseudomonas*	4.5b	2.25a
C.D		2.25	0.98

*Figures superscripted are statistically significant at 0.5%

Fig. 1. Solubilization zone and solubilization index of various isolates

tissue differentiation, and aid apical dominance (Duca *et al.*, 2014). Thus indole-3-acetic acid producing ability (ìg mL^{-1}) of all the *Pseudomonas* and *Bacillus* isolates (Table 2, Fig. 2) was observed at two levels of tryptophan (0.05 and 0.1g tryptophan L^{-1}) and it was observed that 72.73% isolates produced IAA at 0.05g tryptophan L^{-1} concentration and 81.82% of isolates produced IAA at 0.1g tryptophan L^{-1} further it was observed that maximum IAA was produced by *Pseudomonas* isolate SD2 (54.34 ìg mL^{-1} at 0.1 g tryptophan L^{-1}) while minimum by *Bacillus* isolate SD65 (7.17 ìg mL^{-1} at 0.1 g tryptophan L^{-1}). In the same way the IAA production at 0.05 g tryptophan L^{-1} was highest and lowest quantity produced by *Pseudomonas* isolate SD2 (32.368 ìg mL^{-1}) and by *Bacillus* isolate SD65 (3.981 ìg mL^{-1}) respectively. However it was observed that some isolates at both the levels of tryptophan did not produce IAA (SD64, SD66) and further it was observed that *Psudomonas* isolate SD47 produced IAA at 0.1 g tryptophan L^{-1} but not at 0.05 g tryptophan L^{-1}. Tryptophan has been found to control the microbial biosynthesis of auxins under intense investigation by Duca *et al.*, 2014 which has deduced that different strains of bacteria produce auxins from tryptophan (Trp) by different routes similar to our findings.

Similarly higher level of IAA production by *Pseudomonas* species was recorded by other workers (Xie *et al.*, 1996). IAA production increases with the increase in tryptophan levels has also been reported (Yadav *et al.*, 2010).

The highest germination rate (71%) was observed in 5% RC treatment, followed by 68% and 66% in 2% and 0.5% respectively. The shoot

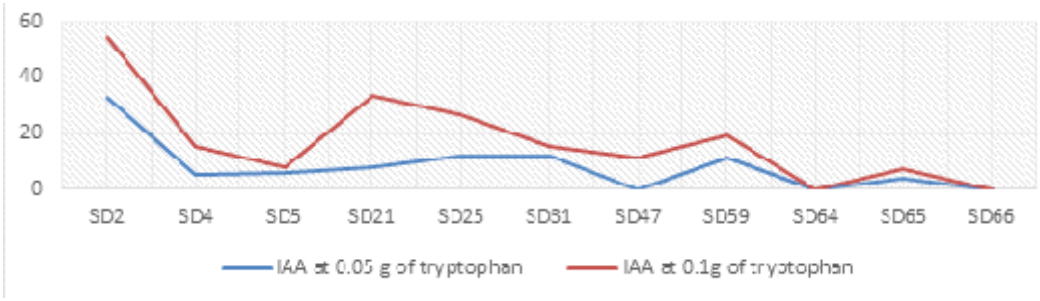

Fig. 2. IAA levels at different concentrations of tryptophan

Table 2. Indole-3-acetic acid production by various rhizobacterial isolates at two levels of tryptophan concentration

Isolate	Genus	IAA production at 0.05 g tryptophan L^{-1} (ìg mL^{-1})	IAA production at 0.1 g tryptophan L^{-1} (ìg mL^{-1})
SD2	*Pseudomonas*	32.368e	54.34
SD4	*Bacillus*	5.188b	15.43
SD5	*Pseudomonas*	5.882b	7.79a
SD21	*Pseudomonas*	8.144c	33.73
SD25	*Bacillus*	11.794d	26.98
SD31	*Bacillus*	11.855d	15.54
SD47	*Pseudomonas*	—	11.65b
SD59	*Pseudomonas*	11.071d	19.20
SD64	*Bacillus*	—	—
SD65	*Bacillus*	3.981a	07.17a
SD66	*Pseudomonas*	—	—
C.D		4.05	3.67

*Figures superscripted are statistically significant at 0.5%

length was highest (26.44 cm) observed in 5% RC treatment followed by 2% (25.33 cm) and 1% (24.29 cm) RC treatments while as highest root length (20.67 cm) was observed in 5% RC treatment. Number of leaves in seedlings were considerably higher in all the treatments than control (Table 3). Lugtenberg *et al*. also reported that there is increased seed germination, enhanced seedling emergence upon inoculation of bioinoculants (Lugtenberg *et al.,* 2002). Shaukat *et al.* (2006) reported that the germination percentage may be increased upto 100% upon inoculation. These findings could be attributed to increased production of plant growth promoting harmones that could trigger the activity of hydrolyzing enzymes in seeds and ultimately the germination.

Both fresh and dry weights from shoot and root, were maximum (3.09g and 1.559g; 1.075g and 0.508g respectively) in 5% RC treatment followed by 2% RC treatment. In all the RC treatments it was observed that both fresh and dry weight was higher than the control (Table 4). The increased biomass production in all the treatments could be as a result of the better root development and may be as a result of the production of biologically active substances produced by inoculated rhizobacteria. Yadav *et al*. also observed the enhanced biomass production, increased root/shoot length upon inoculation of rhizobacteria into the plant pots (Yadav *et al*., 2010). Tizzard *et al.*(2006) reported almost similar findings under pot culture

Table 3. Effect of different concentrations of rhizobacterial consortia on seed germination rate, shoot and root length, and leaf number of *D. sissoo* after two months from the last germination of the seeds in the nursery

Concentration of RC	Germination percentage (%)	Length (cm) Shoot	Root	Total	Compound leaf number
Control	58	19.1a	14.65a	33.75a	10.92
0.2%	63	22.23b	16.44a	38.67a	12.03
0.5%	66	23.34b	17.11a	40.45b	12.87
1%	64	24.29b	18.39a	42.68b	12.78
2%	68	25.33c	19.20b	44.53b	13.22
5%	71	26.44c	20.67b	47.11b	12.98
10%	62	23.54b	17.91a	41.45b	12.56
15%	62	22.65b	16.11a	38.76a	12.43
C.D	9	4.65	5.23	9.33	2.15

*Figures superscripted are statistically significant at 0.5%

Table 4. Effect of different concentrations of rhizobacterial consortia on shoot and root fresh and dry weight of *D. sissoo* after two months from the last germination of the seeds in the nursery

Concentration of RC	Fresh weight (g) Shoot	Root	Total	Dry weight (g) Shoot	Root	Total
Control	2.19a	0.723a	2.913a	1.12a	0.360a	1.486a
0.2%	2.59a	0.842 a	3.432a	1.311a	0.404a	1.715a
0.5%	2.71a	0.852a	3.562a	1.377a	0.420a	1.797a
1%	2.83a	0.949a	3.779a	1.433a	0.452a	1.885a
2%	2.94a	0.993b	3.933b	1.494a	0.472b	1.966b
5%	3.09b	1.075b	4.165b	1.559b	0.508b	2.067b
10%	2.71a	0.923a	3.633a	1.388a	0.440a	1.828a
15%	2.63a	0.834a	3.464a	1.336a	0.396a	1.732a
C.D	0.63	0.35	1.01	0.38	0.12	0.42

*Figures superscripted are statistically significant at 0.5%

Table 5. Effect of different concentrations of rhizobacterial consortia (RC) on nodule number and their fresh and dry weight of *D. sissoo*

Concentration of RC	Nodule number	Nodule weight (g)	
		Fresh	Dry
Control	19a	0.191a	0.063a
0.2%	28c	0.343c	0.113h
0.5%	27c	0.281b	0.095g
1%	25c	0.248a	0.085f
2%	24b	0.235a	0.079e
5%	23b	0.218a	0.076d
10%	21b	0.198a	0.071c
15%	20b	0.189a	0.067b
C.D	5.6	0.06	0.03

*Figures superscripted are statistically significant at 0.5%

conditions where he observed that rhizobacterial inoculation results into the enhanced growth in tree legumes. Lim *et al.* (1999) also reported that microbial consortia enhance the biomass production positively in plants due to biologically active substances in the solution.

Microbial consortia in various combinitions have been applied in USA, China, France, Japan, Brazil, Thailand and many other countries in the world. Application of microbial consortia can be an effective tool in enhancing seed germination, growth and yield of various agricultural crops and vegetables (Shin *et al*., 1995; Zacharia *et al.*, 1995; Iwaishi, 2000). The microbial consortium along with various organic fertilizers and other chemical substances has also been reported to enhance seed germination, seedling growth and yield of various agricultural crops

Table 6. Effect of different concentrations of rhizobacterial consortia on pigment contentents in fresh leaves of *Dalbergia sissoo*

Concentration of RC	Contents of pigments (mg L^{-1})			
	Chlorophyll a	Chlorophyll b	Caretenoids	Total pigment
Control	34.65a	10.32a	24.34a	69.31a
0.5%	36.65a	11.09a	27.65a	75.39b
1%	37.98a	12.32b	29.10b	79.40c
2%	40.06b	13.09c	31.22b	84.37d
5%	44.49b	14.54d	36.29c	95.32f
10%	41.88b	12.76b	32.43c	87.07e
15%	39.21a	11.18a	30.54b	80.93d
C.D	4.53	1.23	3.43	4.23

*Figures superscripted are statistically significant at 0.5%

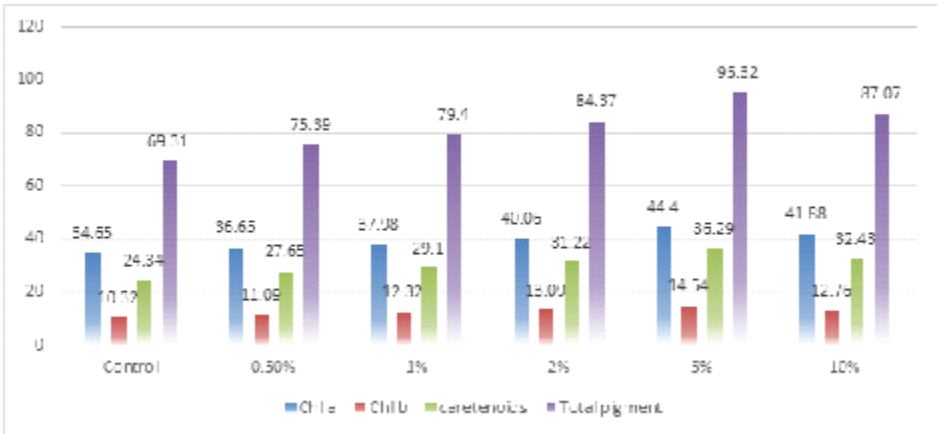

Fig. 3. Effect of different concentrations of rhizobacterial consortia on pigment contentents in fresh leaves of *Dalbergia sissoo*

(Anuar *et al.*, 1995 ; Xu, 2000). It has been reported that the effect of microbial consortia on growth of forest ccops has not been studied widely (Khan *et al.*, 2006). From this study on forest crop it is evident that soils amended with different concentrations of microbial consortium can improve seedling growth, seed germination, nodule number, chlorophyll and caretenoids. Khan *et al.* (2006) also reported that at lower concentrations of microbial consortia the growth of seedlings enhanced. However, as the concentration of microbial solution increased, seed germination was depressed, which may be due to toxicity of higher concentrations of microbial consortium. Nodule number was highest (28) reported in 0.2% RC treatment followed by 27 in 0.5% and lowest (20) in 15% RC. Both fresh and dry weights of nodule were maximum (0.343 g and 0.113 g respectively) in 0.2% treatment.and lowest in 15% RC treatment, the highest nodule number was reported at lower concentrations of microbial consortia as compared to its higher concentration.(Table 5). These results support the findings of Thach *et al.* (1999) that nodule number in soybean roots was not significantly increased due to higher concentrations of microbial consortium.

The effect of rhizobacterial consortia concentration on leaf pigments (Chlorophyll a, chlorophyll b and caeotenoid) was determined and it was found that contents of chlorophyll and carotenoids were highest (44.49, 14.54 and 36.29 mg mL^{-1} respectively) in 5% treatment and lowest 34.65, 10.32 and 24.34 mg mL^{-1} respectively) in control treatment (Table 6, Fig. 3). The total pigment content was highest (95.32 mg mL^{-1}) in 5% treatment. The present results are in agreement with the other findings (Xu, 2000 ; Wang *et al.*, 2000 ; Mridha *et al.*,2002).

Compliance with Ethical Standards

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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