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Full Length Research Paper

Synergistic Effects of Psychrotolerant Plant Growth-Promoting Rhizobacteria and Rhizobium on Lentil Plant Growth Parameters and Yield

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Cold-tolerant plant growth-promoting rhizobacteria (PGPR) are of immense agronomic importance due to the fact that the winter season crop growing cycle is subject to varying spells of low temperatures and such PGPRs are metabolically functional at cold conditions. Thirty-five (35) rhizobacterial isolates were isolated from lentil rhizospheric soil, six isolates showing maximum exponential growth at both 10 and 15°C after 24 h were selected and characterized belonging to genera *Bacillus* and *Pseudomonas*. Production of indole acetic acid (IAA) ranged from 12.7-17.5 µgml^{-1} at 10°C to 18.4-20.4 µg/ml at 15°C. At 15°C, an isolate J-17 was found to be strong HCN producer whereas at 10°C, only two isolates: J-26 and J-30 were moderate producers. P-solubilizion at 15°C, ranged from 62.5 - 77.2 µgml^{-1}. However, at 10°C none of the isolates solubilized P. At 15°C, catechol type siderophore production ranged from 523.4 - 606.1 µgml^{-1}. On the basis of the PGP traits, four isolates J-3, J-17, J-18 and J-30 were selected for evaluation under field conditions of lentil at research farm of PAU, Ludhiana, Punjab, India. Coinoculation exhibited a significant increase in nodule number, nodule dry weight, plant and root dry weight, chlorophyll and leghaemoglobin content over *Rhizobium* alone. Application of J-3, J-17, J-18 and J-30 along with *Rhizobium* further enhanced the grain yield (1.8, 4.4, 3 and 1.4%).

Key words: HCN production, indole acetic acid, siderophore, P-solubilization.

INTRODUCTION

Lentil is the world's fifth largest pulse crop with annual production of 3-4 Mt (Sharpe et al., 2013). The important lentil-growing countries of the world are India, Canada, Turkey, Bangladesh, Iran, China, Nepal and Syria (Ahlawat, 2012). In Punjab, lentil occupied an area of 1.0 thousand hectares with a production of 0.73 thousand

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tones during the year 2011-2012. The average yield was 725 kg/ha (Anonymous, 2013). Lentil seeds contain 1-2% fat, 24–32% proteins and minerals (iron, cobalt and iodine) and vitamins (lysine and arginine). Being a legume crop, it can fix its own nitrogen (N) from the atmosphere and help in restoring the soil fertility. Presently, agricultural research has shifted to sustainable agriculture by supplementing chemical fertilizers with organic amendments. Lentil is constrained by extremities of temperature which are detrimental to the survival and functioning of most introduced mesophilic microorga-nisms. Such climatic conditions warrant the use of highly adapted microbial strains that retain their biological functions (Mishra et al., 2008).

The seed inoculation with the appropriate rhizobia at sowing is a recommended practice, but in recent years, the potential of combined inoculation of N_2-fixers and plant growth-promoting rhizobacteria (PGPR) is more effective than single organism (Khanna and Sharma, 2011). Sindhu et al. (2002) reported that coinoculation of some *Bacillus* strains with effective *Bradyrhizobium* resulted in enhanced nodulation and plant growth of green gram (*Vigna radiata* L.).

The present investigation deals with the isolation, characterization, determination of the growth promotion of cold tolerant PGPR and their coinoculation effects with *Rhizobium leguminosarum* in lentil (*Lens culinaris* L. Medikus) under field conditions.

MATERIALS AND METHODS

Isolation of rhizobacteria

Rhizobacteria were isolated from ten different soil samples collected from different lentil growing fields in Punjab. Pour plating was done on nutrient agar (NA) for *Bacillus* and on King's B for *Pseudomonas*. The isolates were grown at 10 and 15°C in respective media. Growth in terms of optical density was recorded at 10 and 15°C. The cultures showing maximum growth were selected for further analysis.

Biochemical characterization of rhizobacteria

Biochemical characterization of bacterial isolates was done on the basis of Gram reaction, catalase production, nitrate reduction, starch hydrolysis and methyl red test were conducted as per the standard methods (Cappuccino and Sherman, 1992; Holt et al., 1994).

Indole acetic acid production

Characterization of isolates for the production of IAA was done by method of Gordon and Weber (1951).

HCN production

HCN production was inferred by the qualitative method of Bakker and Schipper (1987).

Phosphate solubilization

Phosphate solubilizing isolates were screened by spotting on Pikovskaya's medium (Pikovskaya, 1948). Inoculated plates were incubated at 10 and 15°C and the diameter of clear zone (halo) surrounding the bacterial growth as well as the diameter of colony was measured after four weeks and P-solubilization index was calculated.

$$\text{Phosphate solubilization index} = \frac{\text{Total diameter (colony + halo zone)}}{\text{Diameter of colony}}$$

The colonies forming clear halos were considered as phosphate solubilizers. Microbial solubilization of insoluble phosphates in liquid media was detected by the method of Jackson (1973). The total phosphorus was estimated with the help of standard curve using different concentrations of phosphorus.

Production of siderophore

Siderophore production by the isolates was estimated qualitatively at two different incubation temperatures, that is, 10 and 15°C. Siderophore production was detected by the Chrome Azurol-S (CAS) assay given by Schwyn and Neilands (1987) in 100 mm Petri dishes. Catechol-type siderophores were estimated using the method given by Arnow (1937).

Field study

Field experiments were conducted during Rabi season of 2013-2014 on lentil at the research farms of Punjab Agricultural University, Ludhiana, Punjab, India (30°54'5"N 75°47'53"E). Lentil seeds of variety LL 931 were inoculated with recommended *Rhizobium* strain and PGPR, as per treatments. In single inoculation of *Rhizobium* ($1x10^8$ cell/g of carrier), inoculant was applied to seeds.

In dual inoculation (*Rhizobium* + PGPR), 500 mg each of the inoculant was used. In uninoculated treatment (absolute control), seeds were treated with sterilized charcoal only. Before inoculation, equal volume of different organisms were mixed and allowed to stand for 30 min at room temperature. Inoculated seeds were dried at room temperature under shade before sowing. Crop was sown on 14th November, 2013 following the recommended agronomic practice and harvested on 11th April, 2014. The experimental design used was randomised block design and five treatments were used having three replications each. Each plot size was 12 m^2. The experiment was performed as per the recommended practices.

Symbiotic and plant growth parameters such as nodule number and nodule dry weight, root dry weight, shoot dry weight, chlorophyll and leghaemoglobin contents were recorded both at vegetative and flowering stages, and grain yield was recorded at harvest. Five randomly selected plants were carefully uprooted from each plot at 60 and 90 days after sowing (DAS) with root system intact. The roots were washed in running tap water and nodules were detached carefully with forceps, and number of nodules per plant was counted as average. The detached nodules were dried in oven at 60°C for two days and their dry weight per plant was recorded in mg.

Dry weights of shoots and roots of five randomly uprooted plants from each plot were taken after drying at 60°C for 2 days. Chlorophyll content was estimated by the method of Witham et al.

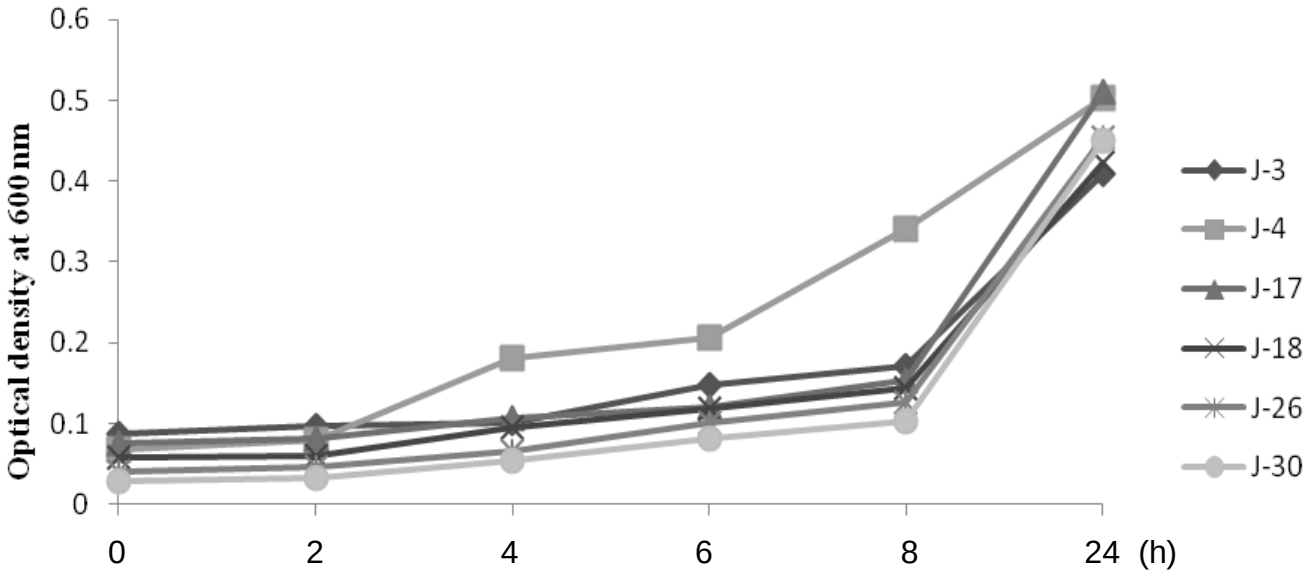

Figure 1. Growth curve of rhizobacterial isolates at 10°C.

(1971) and leghaemoglobin content by the method of Wilson and Reisenauer (1963). Grain yield from each plot (g/plot) was recorded at final harvest and was expressed in kg/ha.

Data was analysed using an analysis of variance appropriate for completely randomized block design (RBD). Statistical analysis was done using CPCS1 software developed by the Department of Mathematics, Statistics and Physics, PAU, Ludhiana.

RESULTS AND DISCUSSION

Isolation and characterization

Thirty-five rhizobacterial isolates were isolated from lentil rhizospheric soil sample collected from 10 different locations of Punjab and six isolates showing maximum growth in terms of optical density at both 10 and 15°C when incubated for 24 h were selected (Figures 1 and 2). Two isolates from Kings B medium showed the characteristic yellowish-green pigmentation, whereas the other 4 isolates from NA showed off-white to creamish colour. The isolates were evaluated for cultural, morphological and biochemical characteristics as per Bergey's manual of systematic bacteriology. On the basis of these tests, the isolates were tentatively assigned to genera *Bacillus* and *Pseudomonas* (Table 1).

Indole acetic acid production

The auxin IAA is an important phytohormone produced by PGPR, and reports have shown that treatment with IAA producing rhizobacteria increase the plant growth (Hayat et al., 2010). Indole acetic acid production ranged from 12.7 - 17.5 $\mu g ml^{-1}$ in the presence of tryptophan after 5 days of incubation at 10°C, whereas at 15°C, IAA production ranged from 18.4 - 20.4 $\mu g ml^{-1}$ in the presence of tryptophan after 5 days of incubation (Table 2). Isolate J-17 produced IAA at the same rate at 10 and 15°C. Similar results were reported by Selvakumar et al. (2008) in a cold tolerant *Serratia marcescens* strain SRM (MTCC 8708) which produced 11.1 $\mu g\ ml^{-1}$ IAA at 15°C.

HCN production

At 15°C, isolate J-17 was found to be strong producer of HCN (colour changed from yellow to reddish-brown), whereas J-18 was moderate producer (orange brown colour) as compared to yellow coloured control (Table 2). However, at 10°C, isolates J-26 and J-30 were found to be moderate producers. *Pseudomonas* sp. strain PGERs17 was reported to exhibit the HCN (cyanogenic compound) production at 15 and 4°C (Mishra et al., 2008).

Phosphate solubilization

At 15°C, P-solubilisation on solid media started on the 5th day and increased till the 15th day. P-solubilizing index ranged from 2.9 to 5.4, highest being recorded with isolate J-17 followed by J-18. The quantitative estimation showed that P-solubilization ranged from 62.5 - 77.2 $\mu g ml^{-1}$ (Table 3). The bacterial isolate J-17 was found to be the most potent P-solubilizer. Selvakumar et al. (2008) reported that at 15°C, the isolate *S. marcescens* strain

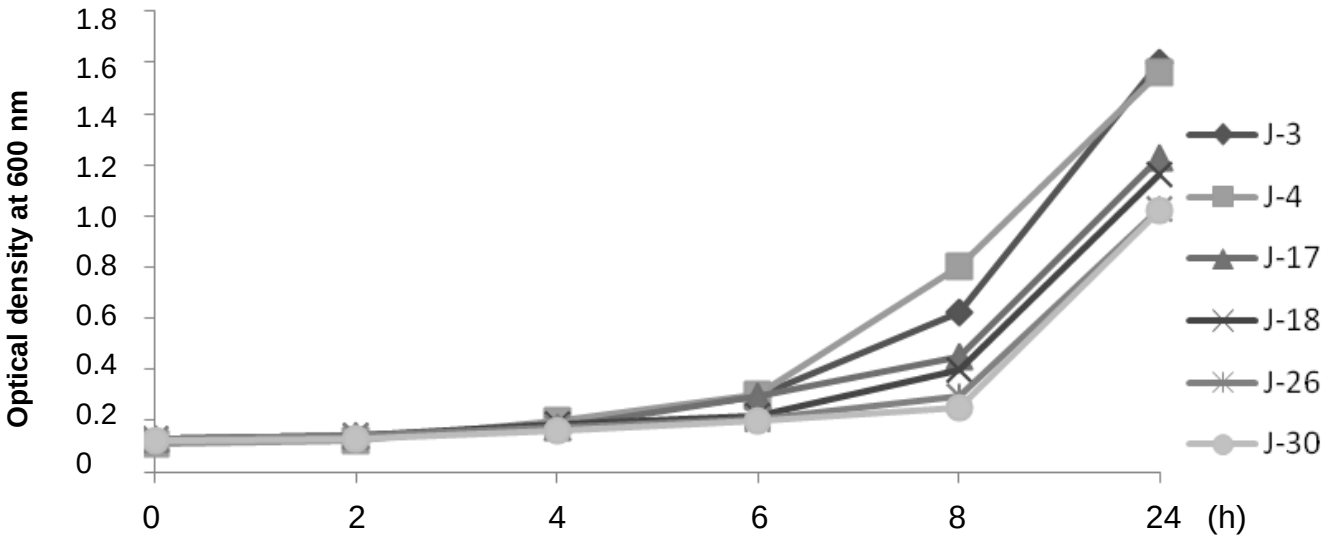

Figure 2. Growth curve of rhizobacterial isolates at 15°C.

Table 1. Cultural and biochemical characteristics of rhizobacterial isolates.

Characteristics of test organism	*Pseudomonas* spp.	*Bacillus* spp.
Gram's reaction	-ve	+ve
Shape	Rod	Rod
Pigment	+	-
Pigment colour	Yellow green	White
Starch hydrolysis	+	+
Catalase production	+	+
Methyl red test	+	+
Citrate	-	-
Nitrate production	+	+

SRM (MTCC 8708) was able to solubilize 76.6 µgml^{-1} of phosphorus. However, no P-solubilization activity was recorded at 10°C.

Siderophore production

The six isolates produced distinct halo (golden yellow colour) after three days of incubation, reaching a maximum after seven days. Diameter of halo varied from 1.5 - 3.0 cm on CAS agar plates, maximum being produced by fluoresecent *Pseudomonas* isolate J-18 (3 cm) (Table 3). Arnow's test gave positive reaction, indicating the presence of the catecholate group of siderophores.

At 15°C, isolate J-17 was the highest catechol producer. However, at 10°C no zone was formed. The isolates J-3, J-17, J-18 and J-30 were selected for coinoculation with *Rhizobium* in lentil under field conditions.

Effect of co-inoculation of *Rhizobium* and PGPR on symbiotic parameters and grain yield of lentil

Isolate J-17 showed highest nodule number (27.1 and 52.8 nn/plant) followed by J-18 (26.8 and 52.3 nn/plant) as compared to *Rhizobium* (23.2 and 30 nn/plant) at 60 and 90 DAS, respectively (Table 4). This may be credited to the abilities of PGPRs to produce IAA. An increase of 50 to 98% was reported in nodulation through dual inoculation of *Pseudomonas* and *Serratia* with *Rhizobium leguminosarum*, when compared with control in lentil (Zahir et al., 2011).

Table 2. IAA and HCN production by rhizobacterial isolates.

Rhizobacterial isolates	HCN production		IAA equivalents (μgml^{-1}) at 10°C		IAA equivalents (μgml^{-1}) at 15°C	
	10°C	15°C	L-TRP (-)	L-TRP (+)	L-TRP (-)	L-TRP (+)
J-3	-	-	5.4	13.8	11.8	18.7
J-4	-	-	6.7	12.7	12.7	17.5
J-17	-	++	6	19.3	13.5	20.4
J-18	-	+	5.2	17.5	12.8	19.8
J-26	+	-	5.9	15.5	11.8	18.4
J-30	+	-	5.6	14.9	12.3	18.6

Table 3. P-solubilisation and siderophore production by rhizobacterial isolates at 15°C.

Rhizobacterial isolates	P-solubilization index	P-solubilization on 9th day (μgml^{-1})	Siderophore production	
			Dia (cm)	Catechol type (μgml^{-1})
J-3	5.2	77	1.5	523.4
J-4	5.1	62.5	1.7	594.4
J-17	5.4	77.2	2.9	606.1
J-18	5.3	69.6	3.0	595.4
J-26	5.2	63.4	1.6	526.4
J-30	2.9	65.1	1.8	540.2

Table 4. Effect of dual-inoculation on symbiotic traits and plant biomass in lentil.

Treatments	No of nodules/ plant (nn/plant)		Nodule Dry weight (mg/plant)	Plant dry weight (g/plant)		Root dry weight (mg/plant)	
	60 DAS	90 DAS	90 DAS	60 DAS	90 DAS	60 DAS	90 DAS
Control	17.2	22.7	15.6	0.51	0.81	76.1	85.7
Rhizobium	23.2	30.0	21.6	0.71	1.37	82.88	99.4
Rhizobium+J-3	25.3	36.2	28.7	0.88	1.71	117.3	152.8
Rhizobium+J-17	27.1	52.8	34.6	1.20	2.23	122.2	162.2
Rhizobium+J-18	26.8	52.3	34.5	1.03	2.13	121.4	153.4
Rhizobium+J-30	25.4	36.0	26.6	0.89	1.73	89.62	124.3
CD at 5%	5.7	3.2	6.7	0.85	0.11	NS	9.18

Values represent mean of three replicates; * DAS– days after sowing.

Application of J-3, J-17, J-18 and J-30 alongwith *Rhizobium* further increased nodule dry weight (28.7, 34.6, 34.5 and 26.6 mg/plant) as compared to *Rhizobium* alone. Seed inoculation of lentil with effective strain of rhizobia ensures enhanced nodulation and N_2 fixation and combined use of *Rhizobium* with PGPR being more beneficial is well documented (Khanna et al., 2006; Siddiqui et al., 2007). Coinoculation of *R. leguminosarum*-PR1 with PGERs17 culture has also been reported to improve the symbiotic effectiveness in terms of nodule number (15.2%) and nodule dry weight (4.1%) in comparison with inoculation with *R. leguminosarum*-PR1 alone (Mishra et al., 2012).

At 60 DAS, maximum shoot dry weight was recorded (Table 4) in *Rhizobium* along with J-17(1.2 g/plant) followed by *Rhizobium* along with J-18 (1.03 g/plant) as compared to *Rhizobium* (0.71 g/plant). The findings are in agreement with the studies of Ogutcu et al. (2008) and Elkoca et al. (2008).

Coinoculation of non rhizobial *Bacillus thuringiensis*-KR1 with *Bradyrhizobium japonicum*-SB1 was reported to increase nodule number, root and shoot biomass, root length of soybean in comparison with *B. japonicum*-SB1 alone (Mishra et al., 2009). At 90 DAS, analogous results

Table 5. Effect of dual-inoculation on chlorophyll, leghaemoglobin content and yield in lentil.

Treatment	Chlorophyll content (mg/g fresh weight of leaves)		Leghaemoglobin content (mg/g fresh weight of nodules)	Yield (kg/ha)
	60 DAS	90 DAS	90 DAS	
Control	0.36	1.17	2.25	1297
Rhizobium	0.649	1.64	3.21	1442
Rhizobium + J-3	0.91	1.86	4.51	1468
Rhizobium + J-17	1.07	2.10	4.90	1506
Rhizobium + J-18	0.919	2.02	4.65	1488
Rhizobium + J-30	0.87	1.74	4.46	1463
CD (5%)	0.41	0.31	0.12	35.21

were obtained and maximum shoot dry weight was recorded in dual inoculation of *Rhizobium* with J-17 and J-18 (2.23 and 2.13 g/plant), respectively. A similar trend in root biomass was observed with coinoculation of *Rhizobium* along with J-17 (122.21 and 162.2 mg/plant) and J-18 (121.44 and 153.4 mg/plant) at 60 and 90 DAS, respectively (Table 4).

Highest chlorophyll content was recorded by *Rhizobium*+J-17 (1.07 and 2.10 mg/g) and *Rhizobium*+J-18 (0.919 and 2.02 mg/g) which was at par with *Rhizobium* (0.649 and 1.64 mg/g), respectively at 60 and 90 DAS (Table 5). A study showed that treatment with *Pseudomonas* sp. strain PGERs17 supernatant was also effective in reducing chlorosis and increased total chlorophyll (6.9%) over non-inoculated control plants in field pea as reported by Mishra et al. (2012). *Rhizobium* inoculation along with PGPR application showed significantly higher leghaemoglobin content, that is, 4.5, 4.9, 4.6 and 4.4 mg/g of nodule with J-3, J-17, J-18 and J-30, respectively, in comparison with *Rhizobium* (3.2 mg/g) alone (Table 5). Coinoculation of *Pseudomonas* sp. strain PGERs17 with *R. leguminosarum*- PR1 resulted in 17.4 and 4.76-fold increase in leghaemoglobin content over control and *R. leguminosarum*-PR1 treated plants, respectively (Mishra et al., 2012).

Grain yield is the most important economic trait. Improvement in grain yield due to *Rhizobium* inoculation has been reported in legume crops (Elkoca et al., 2008; Togay et al., 2008). Application of PGPRs J-3, J-17, J-18 and J-30 along with *Rhizobium* enhanced the grain yield (1.8, 4.4, 3.1 and 1.4%) over *Rhizobium* inoculation alone. The results are in corroboration with Saini and Khanna (2012) who reported that dual inoculation enhanced grain yield of lentil by about 13-15% over control. Kumar and Chandra (2008) reported that dual inoculation of *Rhizobium* sp. + PGPR showed significant increases in grain yields (20.8 and 23.5%) over *Rhizobium* sp. and PSB alone inoculation, respectively. The increase in yield can be attributed to comple-mentation of the functionality traits of the co-inoculants, resulting in improved symbiosis and nutrient uptake.

Conclusion

The present study reveals that a vast diversity of PGPRs possessing an array of functionality traits is available for exploiting as bio-inoculants for sustainable crop production. In the present study, native PGPR isolate *Pseudomonas* species (J-17) alongwith recommended *Rhizobium* inoculant improved plant growth, symbiotic parameters and enhanced grain yield by 4.43% over *Rhizobium* alone.

Conflict of interest

The authors did not declare any conflict of interest.

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