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Evaluation of native rhizobacteria as promoters of plant growth for increased yield in lentil (*Lens culinaris*)

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Abstract

Six rhizobacterial isolates obtained from lentil rhizospheric soils were characterized and found belonging to genera *Bacillus* and *Pseudomonas*. The isolates were evaluated for their plant growth and antagonistic traits. Maximum catechol-type siderophore was produced by B-40 (97µg/ml) and hydroxamate-type siderophore by B-20 (129.5µg/ml). A wide variation in the phosphate-solubilizing efficiency (7.1-34.4 mg/100ml) was observed. Further, all these isolates were able to secrete phytohormone Indole-acetic-acid which ranged from 12.7-106.1µg/ml. On the basis of their PGP traits B-40 and P-1 were selected for field studies. Field experiments were carried out in order to test the effect of dual inoculations (selected PGPRs and *Rhizobium* sp. along with check strain KB-133) on lentil growth. The system productivity was highest in case of dual inoculations of PGPRs with *Rhizobium*. The grain yield with dual inoculations of *Rhizobium* with B-40 (1703 kg/ha) and P-1 (1679 kg/ha) was statistically on par to dual inoculation with KB-133 (1698 kg/ha). These data suggest that B-40 and P-1 can act synergistically with *R. leguminosarum* in promoting lentil growth.

Keywords: Co-inoculation; PGPR; *Rhizobium*; Siderophores and Symbiosis

INTRODUCTION

There is a mutualistic symbiotic association between legumes and rhizobia, which results in the formation of nodules (nitrogen fixing sites) on the roots of legumes. Seed inoculation of pulse crops with effective *Rhizobium* strains prior to sowing is a recommended practice, as it improves nodulation and N-fixation, which in turn is translated into enhanced growth and grain yield. Various free living soil bacteria that are capable of exerting beneficial effects on plants and can lead to increased yields of a wide variety of crops, are known as plant growth promoting rhizobacteria (PGPR). PGPR can promote growth by various mechanisms like production of phytohormones, asymbiotic nitrogen fixation, solubilization of mineral phosphates and other nutrients and antagonism against phytopathogens by production of siderophores, chitinases, antibiotics, and by lowering endogenous levels of plant hormone ethylene in roots. PGPR strains may use one or more of these mechanisms in the rhizosphere. These beneficial microorganisms can be a significant component of management practices to achieve the attainable yield, which has been defined as crop yield limited only by the natural physical environment of the crop and its innate genetic potential [1].

Lentil, also called *masoor dal* is a rich source of proteins, minerals and vitamins for human nutrition and the straw is a valuable animal feed. Moreover, being a legume crop, lentil can fix its own nitrogen (N) from the atmosphere and help in restoring the soil fertility. PGPR are known to stimulate the symbiotic relation between the host plant and *Rhizobium*. Keeping this in view, the present work was undertaken to isolate and evaluate PGPR from lentil rhizosphere and to study their effect on the symbiotic efficiency of *Rhizobium leguminosarum* and yield of lentil, under field conditions

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MATERIALS AND METHODS

Isolation and characterization

Rhizobacteria (6) isolated from lentil rhizosphere collected from different locations were tentatively assigned to genera *Bacillus* (3) and *Pseudomonas*(3) based on their morphological and biochemical characterization. These PGPRs were evaluated for plant growth-promoting traits viz. production of indole acetic acid (IAA) equivalents, siderophores and phosphorus solubilizing ability.

Evaluation of PGP traits

Production of siderophore

Catechol-type siderophores were estimated using the method of Arnow., 1937 [2]. The bacterial isolates were grown in succinate broth and incubated at 28°C for 72 hours. The culture supernatant was separated by centrifuging the cultures at 10,000 rpm for 15 minutes. Ethyl acetate extracts were prepared by extracting 20 ml of supernatant twice with an equal volume of solvent at pH 2. For the assay, one volume of sample was added to one volume of Hathway's reagent and absorbance was measured at 560 nm with sodium salicylate as standard.

Hydroxamate siderophores were assayed by Csaky., 1948 [3], adding 0.5 ml of 6 M H_2SO_4 to 0.5ml of culture supernatant; the mixture was autoclaved in a glass-stoppered tube at 121°C for 30 minutes and allowed to cool, and 1.0 ml of 1% (wt/vol) sodium arsenite solution was added. A solution of α-naphthylamine was added, and the total volume was increased to 10ml with distilled water. After 30min at room temperature, absorbance at 526 nm was

recorded.

Phosphate solubilization by test bacteria

Phosphate solubilizing isolates were screened on Pikovskaya's [4] medium. Plates containing Pikovskaya's medium were inoculated with 24 hours bacterial cultures and incubated at 28°C for 5 days. The colonies forming clarification halos were considered as phosphate solubilizers.

Microbial solubilization of insoluble phosphates in liquid media was detected by the method of Jackson., 1973 [5]. Culture broth was digested with 20 ml of triacid mixture. The volume was made to 50 ml with distilled water; specific aliquots were used to estimate the phosphorus by reacting with 5 ml of ammonium molybdate reagent in nitric acid. The volume was made upto 50 ml and yellow colour was estimated at 470 nm using spectronic-20. The total phosphorus was estimated with the help of standard curve using different concentrations of phosphorus.

Assay for Indole acetic acid (IAA) production

Characterization of isolates for the production of IAA was done by method given by Gordon and Weber., 1951 [6]. Bacterial cultures were grown in nutrient broth and 0.2 ml of overnight cultures were inoculated in Luria broth and incubated for 72 hours at 30 °C. 10 ml of grown culture was taken in eppendorfs and centrifuged at 10,000 rpm for 15 minutes. 2 drops of orthophosphoric acid were added to the supernatant (2 ml) if alkaline and then 4 ml of the Salkowski reagent and incubated for 25 minutes at room temperature for development of pink colour. Absorbance was taken at 530 nm.

Field study

Field experiments were conducted during *Rabi* season of 2010-2011 on lentil at the research farms of Punjab Agricultural University, Ludhiana. Eight treatments comprising of uninoculated control, recommended *Rhizobium leguminosarum* bv viciae, three PGPRs (native isolates P-1 and B-40, reference *Pseudomonas* culture KB-133 (Department of Microbiology, G.B. Pant University)) and *Rhizobium* co-inoculated with the PGPR were laid out in randomized block design (RBD) in three replications. The seeds were treated with the specific inoculants ($1x10^8$ cells/g of carrier) before sowing of lentil variety LL-931. Crop was sown on 15th November, 2010 following the recommended agronomic practice and harvested on 15th April, 2011. Symbiotic and plant growth parameters such as nodule number and nodule dry weight (NDW), root dry weight (RDW), shoot dry weight (SDW), 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 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 2 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 was taken after drying in at 60°C for 2 days. Chlorophyll content was estimated by the method of Witham *et al*., 1971 [7] and leghaemoglobin content by the method of Wilson and Reisenauer., 1963 [8]. Grain yield from each plot (g/plot) was recorded at final harvest and was expressed in kg/ha.

RESULTS AND DISCUSSION

Production of siderophore

The ability to sequester iron provides a competitive edge to microorganisms. In the present study, out of 6 isolates, 4 produced distinct yellow colored halo on Chrome-azurol S (CAS) plates indicating siderophore production. Conversion from blue to golden yellow colour after growth of rhizobacteria with CAS reagent confirmed production of siderophores, after 24 hours of incubation reaching a maximum after 72 h particularly in case of B-40. Culture supernatant of rhizobacteria subjected to Arnow's test gave positive reaction, indicating the presence of the catecholate group of siderophores, isolates B-40 and P-1 produced maximum catechol-type siderophore. Similar results have been reported by Singh *et al*., 2007 [9] in *Anabaena cylindrica*. However, maximum hydroxamate type siderophore production was exhibited by B-20 (129.5 µg/ml), followed by P-10 (108.9 µg/ml), followed by B-40 (94.9 µg/ml) and P-1 (69.5 µg/ml) (Table 1).

P-solubilization by rhizobacterial isolates

All of the isolates showed the ability to solubilize tricalcium phosphate, however, the P-solubilizing potential varied amongst these isolates as evidenced by the size of halo on Pikovskaya's agar plates. The relative efficacy of 6 isolates in solubilizing TCP ranged from 7.1 to 34.5 mg/100ml after 15 days of incubation. The phosphate solubilizing ability was observed upto 15 days after incubation thereafter it became stable. *Pseudomonas* isolate P-1 was a potent solubilizer and showed maximum P-solubilization after 72 hrs. of incubation. These results are in corroboration with Haque and Dave., 2005 [10] (Table 1).

Production of Indole-3-Acetic-Acid

The production of auxins, such as IAA, by rhizobacteria has been associated with plant growth promotion, especially root initiation and elongation. The rhizobacterial isolates assessed for their natural ability to produce IAA both in presence and absence of the precursor L-TRP showed considerable variation in the ability to produce IAA. IAA production ranged from 12.7-106.1 µg/ml in the presence of tryptophan after 5 days of incubation. This result is in accordance to Yasmin *et al*., 2009 [11]. They reported that higher IAA production was observed in the presence of precursor L-tryptophan and there was significant difference in the potential production of IAA amongst the isolates. *Pseudomonas* spp. were found to be stronger IAA producers than *Bacillus*. In the present study *Bacillus* spp. produced higher IAA equivalents as compared to *Pseudomonas* spp.

It was observed that B-20 and B-24 produced better IAA equivalents as compared to B-40 but B-20 was not found to be an efficient P-solubilizer and on the other hand, B-24 could not synthesize siderophore at all. Among the pseudomonads, P-15 isolate did not possess the ability to produce siderophores while P-10 produced sufficient quantities of siderophores but negligible amount of IAA equivalent. On the basis of above experiments, B-40 and P-1 were selected for evaluation under field studies, as these two were positive for all the functionality traits evaluated (Table 1).

Table 1. Evaluation of plant growth promoting traits of potential rhizobacterial isolates

Rhizobacterial isolates	IAA equivalents (µg/ml)	P-solubilization (mg/100ml)	Siderophore production (µg/ml) Catechol	Hydroxamate
B-20	74.5	11.6	37	129.5
B-24	106.1	7.1	-	-
B-40	43.0	13.1	97	94.9
P-1	35.3	34.5	80	69.5
P-10	12.7	20.8	32	108.9
P-15	17.9	24.5	-	-

Table 2. Effect of co-inoculation of *Rhizobium* and PGPR on symbiotic and growth parameters in lentil

Treatments	Number of nodules/ Plant		Nodule dry weight (mg/plant)		Root dry weight (mg/plant)		Shoot dry weight (g/plant)	
	60 DAS	90 DAS	60 DAS	90 DAS	60 DAS	90 DAS	60 DAS	90 DAS
Control	12.2	15.1	11.0	17.8	140	278	1.0	3.1
Rhizobium	20.1	24.3	16.9	44.3	236	313	1.5	6.7
P-1	15.2	16.3	12.5	33.0	206	251	1.1	3.4
B-40	16.1	17.4	13.5	37.3	214	224	1.3	3.7
KB-133	17.0	20.5	14.4	40.3	216	271	1.4	4.2
Rhizobium+ P-1	22.3	24.3	18.5	45.3	246	323	1.7	6.0
Rhizobium+ B-40	23.1	26.4	18.8	49.2	258	330	2.3	6.3
Rhizobium+ KB-133	25.1	26.6	22.7	50.6	282	338	2.3	6.7
CD (P=0.05)	1.8	2.9	2.7	3.3	NS	NS	NS	NS

Table 3. Effect of co-inoculation of *Rhizobium* and PGPR on chlorophyll, leghaemoglobin contents and yield in lentil

Treatments	Chlorophyll content (mg/g leaf fresh wt.)		Leghaemoglobin content (mg/g nodule fresh wt.)		Grain yield (kg/ha)
	60 DAS	90 DAS	60 DAS	90 DAS	
Control	1.02	1.34	0.85	1.42	1481
Rhizobium	1.09	1.63	1.09	2.15	1629
P-1	1.05	1.37	0.92	1.68	1530
B-40	1.07	1.35	0.96	1.82	1580
KB-133	1.11	1.41	1.06	2.03	1580
Rhizobium+ P-1	1.22	1.74	1.20	2.16	1679
Rhizobium+ B-40	1.27	1.73	1.20	2.19	1703
Rhizobium+ KB-133	1.29	1.77	1.29	2.29	1698
CD (P=0.05)	0.056	0.053	0.149	0.466	118

Field experiments

Data presented in Tables 2 and 3 revealed that inoculation with *Rhizobium* and PGPR individually enhanced the symbiotic parameters as compared to uninoculated control. Thus, inoculations with effective isolates of *Rhizobium* have significant effect on growth and nodulation [12]. Similar effects to *Rhizobium* sp. inoculation on nodulation in lentil have also been reported earlier [13,14] may be attributed to the presence of sufficient, but ineffective native *Rhizobium* sp. population in the soil. Dual inoculation with *Rhizobium* and PGPR showed significant improvement in nodulation over *Rhizobium* alone both in terms of number as well as dry weight of nodules. This suggests that P-1, B-40 and KB-133 acted synergistically with *R. leguminosarum* in promoting formation of

effective nodules. The involvement of plant hormones in nodule development is well documented, so it is attractive to speculate that the ability of P-1 and B-40 to produce IAA may have stimulated nodule growth. This suggests that PGPRs acted synergistically with *R. leguminosarum* in promoting growth of nodules. A number of reports clearly elucidate the effect of PGPR on stimulating *Rhizobium* efficiency and plant growth [15, 16, 14]. It is well documented the involvement of plant hormones in legume nodule development [17] and that PGPR produce phytohormones, and even are able to induce complex changes in the hormonal balance within the affected plant [18]. Therefore, it is attractive to speculate that some or the coordination of these mechanisms may act to stimulate nodule growth. Certainly, exhaustive studies will be needed to confirm this hypothesis.

A similar response to *Rhizobium* inoculation alone and *Rhizobium* in combination with PGPR was also noted in terms of plant biomass. The composite inoculation of *Rhizobium*+B-40 recorded the highest number and dry weight of nodules as well as plant biomass which was at par with *Rhizobium*+KB-133, indicating the potential of such consortia in enhancing crop productivity. Both these treatments were superior to *Rhizobium* sp. alone. Therefore, under the specific field conditions tested, B-40 and P-1 acted synergistically with *Rhizobium* in promoting growth of lentil. Increased shoot and root weight with dual inoculation have also been reported in chickpea [19] and urdbean [20].

Significantly higher chlorophyll, leghaemoglobin contents in dual inoculations of *Rhizobium*+B-40 and P-1 was recorded over rest of the treatments but was at par with the dual inoculation with *Rhizobium*+KB-133, during both the stages. Positive and significant correlation exists between photosynthesis and nitrogen fixation [21]. Increase in chlorophyll content due to PGPR inoculation may be due to delayed leaf senescence and improved photosynthesis. Deka and Azad., 2006 [22] have reported that the leghaemoglobin content has a positive correlation with N_2 fixation and nitrogenase activity in nodules. Higher leghaemoglobin content in nodules is characteristic of efficient symbiosis. Dual inoculation increased the grain yield of lentil by tune of 13.6% (*Rhizobium*+P-1), 14.6% (*Rhizobium*+KB-133) and 14.9% (*Rhizobium*+B-40) over the control. These results are in close conformity with the findings of Kumar and Chandra., 2008 [23].

The improvement in crop growth and yield attributes with dual inoculations could be due to their ability of good P-solubilization, siderophore production, IAA synthesizing abilities. Since the micro-organism involved in P-solubilization and scavenging of phosphorus can enhance plant growth by increasing the efficiency of biological nitrogen fixation, availability of trace elements such as Fe, Zn, etc. and by production of plant growth promoting substances.

In conclusion, all *Rhizobium* inoculated plants produced higher symbiotic as well as plant growth attributes than the uninoculated control plants, indicating the potential of inoculants for improving lentil productivity. Moreover, the results from the field experiment indicate benefits on lentil by combined inoculation of *Rhizobium* and PGPRs, and stress the suitability of using such mixed inoculants for the improvement of crop productivity.

The study revealed the availability of a vast diversity of PGPRs showing multifunctionality traits. These can be exploited to promote plant growth and improve the competitive ability and symbiotic effectiveness of inoculated *Rhizobium* sp. in lentil under field conditions, however, their benefits depend on the co-inoculated organisms. Compatibility of these organisms with *Rhizobium* sp. in consortia needs to be evaluated before being commercially exploited.

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