Improvement in symbiotic efficiency of chickpea (*Cicer arietinum*) by coinoculation of *Bacillus* strains with *Mesorhizobium* sp. *Cicer*

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Abstract Rhizobacteria belonging to *Bacillus* sp. were isolated from the rhizosphere of chickpea (*Cicer arietinum*). Ten *Bacillus* strains were studied for their antifungal activity, effect on seedling emergence and plant growth promotion. Two *Bacillus* strains CBS127 and CBS155 inhibited the growth of all the four pathogenic fungi tested on nutrient agar medium plates *in vitro*. Seed inoculation with different *Bacillus* strains showed stimulatory effect on root and shoot growth at 10 d of observation in comparison to control whereas four *Bacillus* strains CBS24, CBS127, CBS129 and CBS155 caused retardation of shoot growth at 10 d. Maximum nodule-promoting effect was observed with *Bacillus* strains CBS106, CBS127 and CBS155. The symbiotic effectiveness of *Mesorhizobium* sp. *Cicer* strain Ca181 was further improved on coinoculation with six *Bacillus* strains i.e. CBS9, CBS17, CBS20, CBS106, CBS127 and CBS155 at 80 d of plant growth under sterile conditions and shoot dry weight ratios increased 1.62 to 1.74 times those of *Mesorhizobium*-inoculated treatments, suggesting the usefulness of introduced rhizobacteria in improving crop productivity.

Key words: *Bacillus* sp. · pathogenic fungi · *Mesorhizobium* sp. *Cicer* · symbiotic effectiveness · chickpea.

Introduction

Various bacteria including species of *Azospirillum*, *Bacillus*, *Enterobacter*, *Pseudomonas* and *Serratia* are commonly found in the rhizosphere of leguminous and nonleguminous crops^{1,2}. The early root colonizing microorganisms, in and around the growing roots, may interact with each other³ and with the plant in a way that may result in symbiotic, associative, neutralistic or detrimental effects depending upon the type of microorganisms involved, nutrient status in soil, abiotic and biotic soil environment and the plant defense system⁴. Usually, these rhizosphere bacteria have been identified to cause stimulation of plant growth, protection of plants from pathogens, or cause degradation of xenobiotics in the rhizosphere. For example, application of *Bacillus* species to seeds or roots has been shown to cause alteration in the composition of rhizosphere leading to increase in growth and yield of different crops^{2,5–7}. Disease suppression of alfalfa by *B. cereus*⁸, take-all of wheat by *B. polymyxa*^{9,10}, reduction in pigeon pea wilt by *B. subtilis* strain¹¹ has been reported. For their beneficial effects on plants, these bacteria have been termed as “plant growth-promoting rhizobacteria” (PGPR)¹².

Chickpea (*Cicer arietinum*) is a major legume crop and is cultivated in many parts of the world. Since rhizobacteria have been found to improve nodulation in other legumes, inoculation of PGPR could be one of the strategies to improve nodulation and nitrogen-fixing efficiency of this crop. The present studies were undertaken to characterize *Bacillus* species from the rhizosphere of healthy chickpea plants. The plant-growth promotion effects of *Bacillus* strains were examined by inoculating the rhizobacteria alone or in combination with *Mesorhizobium* strain on chickpea.

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Materials and Methods

Cultures, media, host seeds and growth conditions: Bacterial cultures, i.e. ten *Bacillus* strains and an effective N₂-fixing *Mesorhizobium* strain Ca181 strain, were used in the present studies. *Bacillus* strains were maintained on Luria Bertani (LB) medium¹³ and *Mesorhizobium* strain on yeast extract mannitol agar (YEMA) medium¹⁴. The *Bacillus* isolates were checked for standard biochemical and physiological characteristics¹⁵. The cultures were maintained by periodic transfers and stored in refrigerator for further studies. Seeds of chickpea (*Cicer arietinum*) cv. C235 were obtained from Pulses Section, Department of Plant Breeding, CCS Haryana Agricultural University, Hisar.

Antifungal activity test: Antifungal activity of *Bacillus* strains was tested against *Alternaria* sp., *Fusarium oxysporum*, *Pythium aphanidermatum* and *Rhizoctonia solani* in nutrient agar (NA) medium by the spot test method¹⁶. Spore suspensions of different fungi were prepared in 0.85% sterilized saline from 5 d old cultures and 0.1 ml of it was pre-seeded over the different media plates. The *Bacillus* strain was spot inoculated onto various pre-seeded medium plates and zones of fungal inhibition around the spotted growth of *Bacillus* were recorded after incubation for 5 d at 28 ± 1°C.

Seed treatment: Healthy seeds of chickpea cv. C235 were surface-sterilized with acid-alcohol (H₂SO₄ : ethanol, 7:3, v/v) for 5 min followed by thorough washings with sterilized water six times¹⁷. The surface sterilized seeds were inoculated with broth culture of *Bacillus* strains for 40 min. The inoculated seeds were germinated in 0.8% water agar at 28 ± 1°C under controlled conditions. Uninoculated seeds treated with LB broth were sown in agar plates as control. Effect of inoculation on root and shoot length was measured at 5 and 10 d of growth.

Coinoculation of *Bacillus* cultures with *Mesorhizobium* strain Ca181 on chickpea: Surface sterilized seeds were dried in petri plates in a laminar flow hood. Seed bacterization was carried out by the method as described by Sindhu and Dadarwal¹⁸. *Mesorhizobium* strain was cultured on YEMA slopes for 5 d at 28 ± 1°C and *Bacillus* strains were grown on LB slopes for 2 d at 30 ± 1°C. The bacterial growth was scrapped in sterile water and used for inoculation of surface sterilized seeds. In coinoculation experiment, seeds were treated with cultures of *Mesorhizobium* strain Ca181 and *Bacillus* by mixing broth of the inoculant strains in 1:1 ratio (v/v).

Ten grams of seeds were inoculated with 2 ml of the mixed growth suspension and populations of bacteria on inoculated seeds generally ranged from 10⁷ to 10⁸

colony-forming units per seed. The inoculated seeds were sown in sterilized chillum jar assemblies¹⁶ containing washed river sand in upper jar and Sloger's nitrogen free salt solution¹⁹ in the lower assembly. Uninoculated seeds were sown as control. Three healthy seedlings were retained in each chillum jar and three replicates were used for each treatment. The jars were kept in a net house under natural light conditions and watered with quarter strength Sloger's medium periodically. Observations for nodulation, nitrogenase activity and symbiotic effectiveness were taken at 40, 60 and 80 d of plant growth.

Measurement of nitrogenase activity: Nitrogenase activity was determined by measuring acetylene reduction activity (ARA) in 100-ml flasks containing whole root system with intact nodules. Ten ml freshly prepared C₂H₂ was added in the flasks to create a 10% atmosphere²⁰. The flasks were incubated for 1 h at 28 ± 1°C. Gas samples were assayed for C₂H₄ formed using gas liquid chromatograph (Nucon Aimil 5500, New Delhi, India) fitted with a dual flame ionization detector (FID) and Porapak N columns (2 m x 2 mm). The oven temperature was kept at 110°C. Nitrogen as carrier gas and H₂ as fuel gas, were used at 30–35 ml and 20–25 ml min⁻¹, respectively. A 0.5 ml gas sample from each flask was injected to determine the amount of C₂H₄ formed.

Measurement of nodule biomass and plant dry weight: The nodules were detached from the roots after measuring ARA and weighed. Calculation of ARA was based on nodule fresh weight. The root and shoot portions of the plants were dried at 90°C in an oven for 24 h and the dry masses measured.

Results and Discussion

A variety of rhizosphere microorganisms including *Bacillus* and *Pseudomonas* species have been found beneficial to both cereals as well as legumes. By virtue of their rapid colonization of the rhizosphere and stimulation of plant growth, there is currently considerable interest for exploiting these rhizosphere bacteria to improve crop production. A total of 124 rhizobacterial isolates were obtained in this study from the rhizosphere of field-grown healthy chickpea plants, out of which 45 were Gram positive, sporulating rods. Ten *Bacillus* strains were selected for the present studies based on typical cell shape, sporulation pattern, colony morphology and on biochemical tests with keys published by Palleroni¹⁵.

Antifungal activity of the selected *Bacillus* strains: Three *Bacillus* strains CBS3, CBS17 and CBS153 inhibited the growth of only two fungi, i.e. *F. oxysporum* and

Table 1 *in vitro* antifungal activity of selected *Bacillus* strains on nutrient agar medium.

<i>Bacillus</i> strains	Inhibition zone of fungal pathogens			
	<i>Fusarium oxysporum</i>	<i>Rhizoctonia solani</i>	<i>Pythium aphanidermatum</i>	<i>Alternaria</i> sp.
CBS3	+	+	–	–
CBS9	+	–	+	+
CBS17	+	+	–	–
CBS20	+	–	–	–
CBS24	–	–	–	–
CBS106	–	–	–	–
CBS127	++	+	+	++
CBS129	+	–	–	–
CBS153	+	+	–	–
CBS155	+	+	+	++

Data are diameters of inhibition zones across the spotted growth of *Bacillus* strains. The values represent: +, 1–3 mm inhibition zone; ++, 4–6 mm inhibition zone; –, no inhibition zone.

P. aphanidermatum; CBS9 inhibited the growth of three fungi whereas *Bacillus* strain CBS127 and CBS155 inhibited the growth of all the four fungi tested, i.e. *Alternaria* sp., *F. oxysporum*, *P. aphanidermatum* and *R. solani* in nutrient agar (NA) medium (Table 1). The growth inhibition zones of different fungi varied from 1 to 6 mm. Two *Bacillus* strains i.e. CBS24 and CBS106 did not cause inhibition to any of the four fungi tested. The production of siderophores was observed in two *Bacillus* strains CBS153 and CBS155 in low succinate medium and these strains formed orange halo zones by reduction of Chrome-azurol S dye (data not shown), suggesting the involvement of siderophore in conferring antifungal activity.

The inhibition of growth of a wide range of plant pathogenic fungi by the selected *Bacillus* isolates suggested their use as potential biocontrol agents to ensure the prevention of pathogenic forms establishing in the rhizosphere of certain plants. Recently, *Bacillus* strains isolated from plant tissues or soil have been reported to inhibit the growth of phytopathogenic fungi *Gaeumannomyces graminis* var. *tritici*⁹, *Rhizoctonia solani*, *Pythium ultimum* and *Sclerotium rolfsii*²¹.

Seed bacterization effect on chickpea seedlings: Five *Bacillus* strains i.e. CBS17, CBS106, CBS127, CBS153 and CBS155 caused inhibition of root growth of the seedlings at 5 d of observation (Table 2). All the *Bacillus* strains, however, showed stimulatory effect on the root growth in comparison to uninoculated seedlings at 10 d. Strains CBS3, CBS20, CBS106 and CBS153 showed maximum stimulation of shoot growth at 10 d of observation. *Bacillus* strain CBS20 showed significant stimulation of root and shoot growth at both 5 and 10 d of seedling growth. Inoculation with *Bacillus* strain CBS155 stimulated lateral root formation. The inhibition of seedling growth in some of the

treatments may be due to synthesis or secretion of some toxic metabolites in the growth media as well as in root exudates of chickpea. Similar inhibitory effects on growth of wheat root has been observed due to production of toxic metabolites by rhizosphere pseudomonad species²².

Effect of coinoculation of *Bacillus* strains with *Mesorhizobium* strain Ca181 on chickpea: Coinoculation of *Bacillus* strains with *Mesorhizobium* sp. *Cicer* strain Ca181 increased the shoot dry weight of chickpea in comparison to inoculation with *Mesorhizobium* strain alone or uninoculated controls (Table 3, 4). The shoot dry weight gains on coinoculation with different *Bacillus* strains varied from 1.10 to 1.35 times those of *Mesorhizobium* inoculated treatments and 1.53 to 2.30 times those of uninoculated controls at 40 d of plant growth except *Bacillus* strains CBS3 and CBS106. Similar beneficial effects of coinoculation were observed at 60 d of plant growth and more shoot dry weight enhancement was observed on coinoculation of *Mesorhizobium* with *Bacillus* strains CBS9, CBS17, CBS20, CBS129 and CBS155 (Table 3). Nodule-promoting effect was evident with *Bacillus* strains CBS9, CBS17, CBS20, CBS127, CBS129 and CBS155 at 60 d of observation.

The symbiotic effectiveness of *Mesorhizobium* sp. *Cicer* strain Ca181 was further improved at 80 d of plant growth on coinoculation with six *Bacillus* strains i.e. CBS9, CBS17, CBS20, CBS106, CBS127 and CBS155 (Table 4). The shoot dry weight ratios varied from 1.62 to 1.74 times those of *Mesorhizobium*-inoculated treatments. Maximum nodule-promoting effect was observed with *Bacillus* strains CBS106, CBS127 and CBS155. *Bacillus* strain-dependent variation was observed at different stages of plant growth (Table 3, 4).

In this study, coinoculation of *Bacillus* strains with *Mesorhizobium* sp. *Cicer* Ca181 resulted in increased

Table 2 Effect of *Bacillus* strains on seedling growth of chickpea cultivar C235 at 5 and 10 d of growth in agar plates.

Treatment	Root length (cm)		Shoot length (cm)	
	5 d	10 d	5 d	10 d
Control	3.14±1.4	4.25±1.1	1.07±0.7	6.08±2.8
CBS3	4.32±0.9	6.34±0.5	0.96±0.3	7.87±1.1
CBS9	5.2±2.2	6.8±1.8	4.6±0.4	6.5±0.6
CBS17	2.6±0.2	5.2±0.8	5.3±0.6	7.1±0.4
CBS20	7.34±0.5	9.1±2.2	7.1±0.2	8.6±0.8
CBS24	6.80±1.9	8.75±3.4	1.84±1.2	5.87±2.6
CBS106	1.6±0.2	4.68±3.1	1.30±0.6	8.83±3.7
CBS127	1.92±1.2	4.30±1.2	2.16±1.1	5.20±2.1
CBS129	3.40±2.8	7.28±2.6	2.25±0.3	3.82±1.3
CBS153	2.50±1.5	7.75±3.6	1.44±0.5	7.3±2.9
CBS155	2.85±1.6	9.00±4.2	1.21±0.6	5.25±0.8

Data are average values of five replications (Mean ± SE).

Table 3 Effect of coinoculation of *Bacillus* strains and *Mesorhizobium* sp. *Cicer* strain Ca181 on symbiotic parameters of chickpea at 40 and 60 (in bold) d of plant growth.

Treatments	Nodule number	Fresh nodule weight (mg plant ⁻¹)	Nitrogenase* activity	Plant dry weight (mg plant ⁻¹)
Control	-	-	-	183
	-	-	-	320
CBS20	-	-	-	210
	-	-	-	370
<i>Mesorhizobium</i> strain	27	550	5.2	310
Ca181	38	960	4.8	750
Ca181 + CBS3	23	460	3.4	290
	27	860	5.2	760
Ca181 + CBS9	20	410	8.9	380
	46	1450	7.5	960
Ca181 + CBS17	19	360	8.6	340
	44	1500	5.4	1030
Ca181 + CBS20	24	560	6.7	390
	42	1150	6.8	920
Ca181 + CBS24	29	610	7.0	360
	36	920	4.4	890
Ca181 + CBS106	18	370	5.2	280
	34	1080	5.3	750
Ca181 + CBS127	26	610	8.2	400
	45	1300	5.8	870
Ca181 + CBS129	18	450	9.6	420
	40	1430	6.2	1060
Ca181 + CBS153	30	480	7.4	410
	36	1120	4.8	740
Ca181 + CBS155	17	350	4.2	360
	52	1470	5.7	940

Figures are average values of five replications. *Nitrogenase activity is expressed as $\mu\text{M C}_2\text{H}_2$ reduced plant⁻¹ h⁻¹).

Table 4 Effect of coinoculation of *Bacillus* strains and *Mesorhizobium* sp. Cicer strain Ca181 on symbiotic parameters of chickpea at 80 d of plant growth.

Treatments	Nodule number	Fresh nodule weight (mg plant h ⁻¹)	Nitrogenase* activity	Plant dry weight (mg plant h ⁻¹)
Control	04	110	1.2	470
CBS20	-	-	-	490
<i>Mesorhizobium</i> strain Ca181	58	2280	6.1	1260
Ca181 + CBS3	60	2255	3.4	1200
Ca181 + CBS9	62	2300	4.8	2180
Ca181 + CBS17	64	2540	5.7	2110
Ca181 + CBS20	60	2240	4.5	2070
Ca181 + CBS24	50	2150	3.4	1410
Ca181 + CBS106	70	3420	5.2	2130
Ca181 + CBS127	74	2800	5.5	2140
Ca181 + CBS129	62	2450	6.8	1950
Ca181 + CBS153	42	1500	4.8	1230
Ca181 + CBS155	86	3450	5.4	2050

Figures are average values of five replications. *Nitrogenase activity is expressed as $\mu\text{M C}_2\text{H}_4$ reduced plant⁻¹ h⁻¹).

nodule number, nodule fresh weight and nitrogenase activity at 60 and 80 d of plant growth under chillum jar conditions (Table 3, 4). The flavonoids released by legume roots are the signal compounds that induce the transcription of rhizobial nodulation genes²³. Rhizobacteria have been found to enhance the production of flavonoid-like compounds or phytoalexins in roots of several crop plants^{24,25}. The localized plant hormone auxins have been shown to participate in the fundamental responses of nodule morphogenesis, biosynthesis of nodulation factors and induction of root cortical cell mitosis, early nodulin genes and/or nodule-like structures²⁶. Halverson and Handelsman⁶ also observed that seed treatment with *Bacillus cereus* UW85 increased nodulation of soybean in three field seasons and in three different sterilized soils in the growth chamber.

Inoculation of legumes and cereal plants with PGPR strains has been found to show a wide range of effects that varied among strain of PGPR²⁷. Some PGPR strains stimulated plant growth by affecting some plant physiological events such as photosynthesis, nodulation and nitrogen fixation^{28,29}. The production of biologically active metabolites particularly the plant growth regulators and antagonistic compounds³⁰ along with modification of the rhizosphere environment could also be the mechanism of plant growth promotion. Coinoculation with strains of *Bacillus* and *Mesorhizobium* under sterile chillum jar conditions showed a significant effect on plant growth and the shoot dry weight ratio varied from 1.53–2.30 at 40 d and 2.62–4.84 at 80 d of plant growth in comparison to uninoculated controls (Table 3, 4). The efficacy of *Bacillus* sp. to enhance nodulation, plant dry matter and grain yield

on coinoculation with rhizobia has also been reported for other legumes like white clover⁵, pigeon pea⁷ and peanut³¹. PGPR isolated from the maize rhizosphere including *Bacillus*, *Pseudomonas* and *Serratia* were reported to improve the yield by 9–14%³². Significant increase in the root dry mass of rapeseed was observed due to inoculation with *Proteus*, *Klebsiella* and *Bacillus*². The data from these studies along with earlier reported work of Sturz et al.²⁷ indicated that rhizobacteria from the local rhizosphere soil could be exploited for use as microbial inoculants to improve nodulation (in legumes) and crop productivity of both cereals as well as legumes.

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