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BRIEF COMMUNICATION

Enhancement of green gram nodulation and growth by *Bacillus* species

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Abstract

Rhizobacteria belonging to *Bacillus* sp. were isolated from the rhizosphere of green gram (*Vigna radiata*). Seed inoculation with the rhizobacteria showed stunting effect on root growth whereas four *Bacillus* strains caused stimulation of shoot growth at both 4 and 7 d of observations. Coinoculation of some *Bacillus* strains with effective *Bradyrhizobium* strain S24 resulted in enhanced nodulation and plant growth of green gram. The shoot dry mass (ratio to uninoculated control) varied from 1.32 to 6.33 at day 30 and from 1.28 to 3.55 at day 40 of plant growth. Nodule promoting effect after 40 d of plant growth was observed with majority of *Bacillus* strains except for MRS9 and MRS26. Maximum gains in nodulation, nitrogenase activity and plant growth were observed with *Bacillus* strains MRS12, MRS18, MRS22 and MRS27 after 40 d of plant growth, suggesting the usefulness of introduced rhizobacteria in improving crop productivity.

Additional key words: *Bradyrhizobium*, coinoculation, nitrogenase activity, rhizobacteria, seedling emergence, *Vigna radiata*.

A variety of rhizosphere microorganisms, including *Bacillus* and *Pseudomonas* species, are commonly found in the rhizosphere of leguminous and nonleguminous crops (Li and Alexander 1988, Mavingui *et al.* 1990, Sindhu *et al.* 1999). By virtue of their rapid colonization of the rhizosphere and stimulation of plant growth, there is currently considerable interest in exploiting these rhizosphere bacteria to improve crop production. 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 (Holl *et al.* 1988, Li and Alexander 1988, Podile 1995). Disease suppression of alfalfa by *B. cereus* UW85 (Handelsman *et al.* 1990), take-all reduction in wheat by *B. polymyxa* (Mavingui and Heulin 1994) and enhanced nodulation and seedling emergence (Halverson and Handelsman 1991, Podile 1995, Srinivasan *et al.* 1997) have been demonstrated as beneficial effects on plants.

Green gram (*Vigna radiata* L.) is a legume crop grown during summer in Central India. The present studies were undertaken to isolate *Bacillus* species from the rhizosphere of healthy green gram plants. The plant-growth promotion effects of *Bacillus* isolates were examined by inoculating the rhizobacteria in combination with a *Bradyrhizobium* strain on green gram.

Bacillus strains MRS9, MRS12, MRS18, MRS21, MRS22, MRS26, MRS27 and MRS31 were used in the present studies. They were maintained on Luria-Bertani medium (LB; Sambrook *et al.* 1989) and effective N_2-fixing *Bradyrhizobium* strain S24 was maintained on yeast extract-mannitol agar (YEMA; Vincent 1970) medium. The cultures were maintained by periodic transfers and stored at 4 °C. The *Bacillus* isolates were characterised with standard biochemical and physiological characteristics as described by Sneath (1986).

Seeds of green gram (*Vigna radiata* L.) cv. K851 were obtained from Pulses Section, Department of Plant Breeding, CCS Haryana Agricultural University, Hisar. Healthy seeds were surface-sterilized with H_2SO_4:ethanol (7:3, v/v) for 5 min followed by thorough washings with sterilized water six times (Sindhu *et al.* 1999) and inoculated by immersion in broth culture of *Bacillus*

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strains for 30 min. The inoculated seeds were germinated on 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 4 and 7 d after germination.

Surface sterilized seeds were dried in Petri plates in a laminar flow hood. For single inoculation, *Bradyrhizobium* strain S24 was used alone. In coinoculation experiment, seeds were treated with cultures of *Bradyrhizobium* and *Bacillus* by mixing broth of the inoculant strains in 1:1 ratio (v/v) as described by Goel *et al.* (2000). The inoculated seeds were sown in sterilized chillum jar assemblies containing washed river sand in upper jar and Sloger's nitrogen-free salt solution (Sloger 1969) 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 30 and 40 d after germination.

Nitrogenase activity was determined by measuring acetylene reduction activity (ARA) in 100-cm^3 flasks containing whole root system with intact nodules. Ten cm^3 freshly prepared C_2H_2 was added to the flasks to create a 10 % concentration in atmosphere. The flasks were incubated for 1 h at 28 ± 1 °C. A 0.5 cm^3 gas sample from each flask was injected to determine the amount of C_2H_4 formed using gas liquid chromatograph (*Nucon Aimil 5500*, New Delhi, India) according to Goel *et al.* (2000). The nodules were detached from the roots after measuring ARA and weighed. Calculation of ARA was based on nodule fresh mass. The root and shoot portions of the plants were dried at 90 °C in an oven for 24 h and the dry masses determined. All chemicals used in the present studies were of AR grade from *Hi-media*, Mumbai, India, *Sarabhai*, Glaxo, India, and *Sigma Chemical Company*, St. Louis, USA.

A total of 124 rhizobacterial isolates were obtained from the rhizosphere of field-grown healthy green gram plants, out of which 34 were Gram-positive, sporulating rods. Eight isolates were selected for the present studies based on typical cell shape, sporulation pattern and colony morphology which classified them as *Bacillus* spp. The classification was confirmed by biochemical tests with keys published by Sneath (1986). The chosen isolates were catalase positive, Voges-Proskauer (VP) and indole positive, methylated (MR) negative, utilized citrate, and were fermentative in Hough and Leifson's glucose medium. Two of these *Bacillus* isolates, MRS9 and MRS21, were further characterized as *Bacillus sphaericus* and *B. mycoides*, according to their fatty acid profiles by using MIS library search data at the Michigan Institute of Biotechnology (courtesy of Dr. M. K. Jain, Director, MIB Laboratory, East Lansing, Michigan).

Four *Bacillus* strains MRS9, MRS18, MRS21, and MRS31 stimulated seedling growth at both 4 and 7 d (Table 1). All the *Bacillus* strains, however, showed stunting effect on the root growth. Strains MRS21 and MRS26 showed maximum inhibition of root growth at both the stages. The inhibition of root growth may be due to synthesis or secretion of some toxic metabolites in the growth media as well as in rhizosphere of green gram. A similar inhibitory effect on growth of wheat root has been observed due to production of toxic metabolites by rhizosphere pseudomonad species (Astrom *et al.* 1993).

Table 1. Effect of inoculation with *Bacillus* strains on root and shoot growth of green gram determined in agar plates. Means ± SE, $n = 9$.

Bacillus strains	Root length [cm] 4 d	7 d	Shoot length [cm] 4 d	7 d
Control	8.63 ± 0.38	10.00 ± 0.97	7.59 ± 0.41	14.74 ± 1.18
MRS9	7.10 ± 0.33	9.08 ± 0.68	8.21 ± 0.47	15.06 ± 1.17
MRS12	7.64 ± 0.33	9.14 ± 0.59	7.69 ± 0.22	12.16 ± 1.45
MRS18	7.75 ± 0.59	9.35 ± 0.69	8.02 ± 0.96	15.15 ± 0.83
MRS21	5.11 ± 0.92	5.42 ± 0.33	7.65 ± 0.61	15.26 ± 0.73
MRS22	7.42 ± 0.45	7.46 ± 0.50	7.97 ± 0.36	13.83 ± 0.87
MRS26	4.26 ± 0.63	5.78 ± 0.55	8.41 ± 0.44	14.66 ± 0.97
MRS27	7.65 ± 0.56	9.43 ± 0.58	8.54 ± 0.39	14.70 ± 0.71
MRS31	7.61 ± 0.65	9.92 ± 0.91	8.73 ± 0.46	15.48 ± 0.57

Coinoculation with five *Bacillus* strains MRS12, MRS18, MRS22, MRS27 and MRS31 increased the shoot dry mass of green gram more (132 to 633 % of uninoculated control) than inoculation with *Bradyrhizobium* alone (103 to 167 %) (Table 2). Three *Bacillus* strains MRS9, MRS21 and MRS26 did not improve the symbiotic effectivity, however, strains MRS12, MRS22 and MRS27 also promoted nodulation. At 40 d of plant growth, the shoot dry mass was also increased (128 to 355 % of uninoculated control) (Table 3), but only five *Bacillus* strains showed enhanced plant growth in comparison to only *Bradyrhizobium*-inoculated treatments (shoot dry mass 112 to 179 %). Nodule promoting effect was observed with majority of strains at 40 d of plant growth except MRS9 and MRS26. Maximum gains in all the symbiotic parameters were observed with MRS12, MRS18, MRS22 and MRS27 at 40 d of plant growth.

Table 2. Effect of inoculation with *Bradyrhizobium* strain S24 and coinoculation with *Bacillus* strains on symbiotic parameters and dry matter accumulation in green gram at 30 d of plant growth. Means ± SE, $n = 3$.

Treatment	Nodule number	Nodule fresh mass [mg plant^{-1}]	ARA [μmol(C_2H_2 reduced) plant^{-1} h^{-1}]	Plant dry mass [mg plant^{-1}]
Control	-	-	-	184 ± 6.11
Bradyrhizobium S24	32 ± 2.64	170 ± 5.29	3.06 ± 0.11	696 ± 4.58
S24 + MRS9	14 ± 2.08	86 ± 6.11	2.26 ± 0.11	242 ± 8.72
S24 + MRS 12	55 ± 4.72	222 ± 9.86	4.88 ± 0.32	1164 ± 7.57
S24 + MRS18	24 ± 2.08	130 ± 9.16	3.12 ± 0.15	965 ± 13.65
S24 + MRS21	28 ± 3.21	145 ± 8.62	4.28 ± 0.15	387 ± 9.64
S24 + MRS22	36 ± 2.64	194 ± 6.93	2.64 ± 0.12	748 ± 9.07
S24 + MRS26	33 ± 2.51	162 ± 4.16	4.70 ± 0.16	524 ± 10.58
S24 + MRS27	40 ± 3.78	218 ± 8.72	2.32 ± 0.09	815 ± 6.66
S24 + MRS31	29 ± 2.45	150 ± 7.57	2.08 ± 0.12	718 ± 13.61
C.D.	8.43	21.54	0.67	27.92

Table 3. Effect of inoculation with *Bradyrhizobium* strain S24 and coinoculation with *Bacillus* strains on symbiotic parameters and dry matter accumulation in green gram at 40 d of plant growth. Means ± SE, $n = 3$.

Treatment	Nodule number	Nodule fresh mass [mg plant^{-1}]	ARA [μmol(C_2H_2 reduced) plant^{-1} h^{-1}]	Plant dry mass [mg plant^{-1}]
Control	-	-	-	460 ± 10.82
Bradyrhizobium S24	42 ± 3.78	246 ± 8.72	4.28 ± 0.14	912 ± 11.24
S24 + MRS9	22 ± 2.08	168 ± 5.29	3.80 ± 0.06	589 ± 8.96
S24 + MRS 12	57 ± 5.29	296 ± 8.62	5.16 ± 0.11	1546 ± 12.09
S24 + MRS18	54 ± 4.72	285 ± 11.56	4.68 ± 0.16	1634 ± 17.01
S24 + MRS21	47 ± 3.60	274 ± 9.86	4.52 ± 0.16	785 ± 14.93
S24 + MRS22	64 ± 4.16	348 ± 8.19	5.48 ± 0.18	1426 ± 11.53
S24 + MRS26	27 ± 3.05	192 ± 15.87	2.86 ± 0.08	628 ± 14.18
S24 + MRS27	56 ± 3.79	300 ± 8.74	5.64 ± 0.07	1495 ± 9.71
S24 + MRS31	44 ± 3.21	263 ± 9.29	4.32 ± 0.07	1024 ± 10.97
C.D.	10.77	31.38	0.36	36.50

Halverson and Handelsman (1991) also observed that seed treatment with *Bacillus cereus* UW85 increased nodulation of soybean in three field seasons as well as in three different sterilized soils in the growth chamber. In a similar study, Srinivasan *et al.* (1997) reported enhanced nodulation in *Phaseolus vulgaris* coinoculated with *Rhizobium etli* TAL182 and *Bacillus megaterium* S49. The coinoculation resulted in increased root hair proliferation and lateral root formation. Split root experiments revealed that coinoculation partially suppressed host-controlled regulation of nodulation, indicating a plant interaction with the two bacterial species. The potential of *Bacillus* sp. to enhance nodulation, plant dry matter and grain yield on coinoculation with rhizobia has also been reported for other legumes like peanut (Turner and Backman 1991), pigeonpea (Podile 1995) and white clover (Holl *et al.* 1988). These studies indicate consistently with our report that rhizobacteria from the local rhizosphere soil could be exploited for use as microbial inoculants to improve nodulation and crop productivity.

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