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Siderophoregenic *Bradyrhizobia* Boost Yield of Soybean

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Abstract

After screening for siderophore (microbial iron chelator) production, of seven available cultures of soybean (*Glycine max* L.) root nodule bradyrhizobia, one strain, *Bradyrhizobium japonicum* NCIM 2746, was selected to confirm its phytopathogenic suppression and soybean growth promotion. Based on chromatographic and spectrophotometric studies, two different siderophores, a hydroxamate type (MW 734) and another catecholate type (MW 1000), were observed. Randomized block design (RBD) analysis of sick-pot studies (soil inoculated with phytopathogens) with an MACS 124 variety of soybean, bacterized with siderophoregenic *B. japonicum*, showed a marked increase in the percentage of germination, nodulation, chlorophyll, oil, protein content, and number of pods. Field trial study confirmed these pot results, which were evident from enhancement in shoot length, number of branches, chlorophyll content, number of nodules, root length, and number of pods. These results suggest the possibility of exploiting *B. japonicum* NCIM 2746 as a potential bioinoculant.

Index Entries: *Bradyrhizobium japonicum*; siderophores; soybean; growth promotion.

Introduction

Soybean (*Glycine max* L.) crop serves as a dominant supply of edible vegetable oil and high-protein feed supplements. Worldwide production of soybean is encouraging, with 108.6 MT/yr, with the United States, Brazil, China, Argentina, and India as the major producers (http://www.fas.usda.gov/WAP/circular/2000/00-08/avgoo.htm.). To meet the ever-increasing demand for soybeans, integrated plant disease management and integrated plant nutrition management strategies are being adopted.

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The plant's roots and its immediate environment constitute the rhizosphere *(1)*. Rhizospheric bacteria are ideal for use as a frontline defense for plant roots against pathogens. The soybean rhizosphere is a dynamic environment, which includes beneficial microorganisms, mainly *Rhizobia* and *Bradyrhizobia (2)*. These bacteria, being nitrogen fixers, have been used worldwide as legume inoculants for increasing crop yield *(3,4)*. All beneficial bacteria in the rhizosphere have been referred to as plant growth–promoting rhizobacteria *(5,6)*. They can synthesize siderophores, which can sequester and solubilize available insoluble iron from the soil to meet its iron requirement, which is also exploited by plant cells primarily based on iron stress mechanisms. These mechanisms include the production of specialized iron transfer cells in rhizodermal tissue and root hairs *(7,8)* and the release of protons and reductants into the rhizosphere to generate electrons for reduction of almost insoluble ferric to soluble ferrous form of iron *(9–11)*.

Irrespective of chemical nature, almost all siderophores exhibit a strong affinity for Fe^{3+} *(12)*. Hofte *(13)* classified siderophores as hydroxamates, catecholates, carboxylates, and mixed. *Bradyrhizobium japonicum*, the soybean symbiont, produces simple siderophores such as citrate as well as complex siderophores such as citrate and rhizobactin, respectively *(12,14,15)*. To date, very few strains of *B. japonicum* have been reported to produce both types of siderophores when tested for catecholates and hydroxamates *(15,16)*. Limited information is available regarding siderophore systems in *Bradyrhizobium* sp., and it may be strain specific with varying siderophore structures *(17–22)*. Hydroxamate siderophores can supply iron to a number of different plants, namely tomato and oat *(23)*. However, the ability of plants to acquire iron depends on plant species, relative concentration of iron, and type of siderophore produced by organisms in the rhizosphere *(24)*.

Materials and Methods

Preparation of Glassware

All glassware was cleaned in 16% HCl to remove residual iron and rinsed in deionized water. The deionized water (Millipore Q-Elix-3) was used in all growth media and in the preparation of reagents.

Bacterial Strains

Rhizobial strains were isolated from root nodules of soybean and maintained on yeast mannitol agar with Congo Red *(25)*. Standard culture of *B. japonicum* NCIM 2746 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, and Indian Agricultural Research Institute, IARI 102, New Delhi.

Media and Growth Conditions

Siderophore production was studied in maltose medium, which was prepared by replacing succinate with maltose (4 g/L) from the original succinate medium of Meyer and Abdallah *(26)*. Deferration of the medium was achieved by using 8-hydroxyquinoline *(14)*.

Preparation of Inoculum for Siderophore Production

Inoculum was prepared using the maltose medium just described, and the inoculum flask was incubated on a rotary incubator shaker at 140 rpm at 28°C for 24 h.

Siderophore Production

The inoculum (1% [v/v]) was added to the production medium, and the flasks were incubated on a rotary shaker for 36 h at 28°C. After 36 h of incubation, the flasks were harvested by centrifuging the culture at 165*g* in a cooling centrifuge (Remi, Mumbai) at 4°C for 10 min, and the supernatant was subjected to spectrophotometric analysis *(24)*, siderophore assays, and purification.

Siderophore Assays

Catechol-type siderophores were measured using the Arnow method *(27)*. Hydroxamate-type siderophores were determined by the modified Csaky method *(28)*. Quantitative analysis of siderophores was done using the Payne method *(29)*. In this method, siderophores present in the sample remove iron from dye complex resulting in a change in color of the dye from blue to orange. The absorbancy (A_{630}) is measured for loss of blue color. The percentage of siderophore units is defined as $(Ar - As/Ar) \times 100$, in which *Ar* and *As* are absorbance of reference and sample, respectively.

Extraction and Purification of Siderophores

The supernatant was acidified to pH 3.0 with 12 *M* HCl. The acidified supernatant was extracted with 1/5 volumes ethyl acetate (three times). The ethyl acetate layer was collected, combined, and dried by evaporation *(30)*. The extract was then resuspended in deionized water (1 mg/mL).

Similarly, the aqueous layer was concentrated (five times) and dried on a rotary evaporator (BUCHI R-124). Both the extracts were subjected to measurement of absorption maxima and assayed for Arnow and Csaky tests *(27,28)*.

Determination of Molecular Weight

Molecular weight was determined using 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver-staining technique. The standard markers used were 98, 67, 45, 31, 21.5, 14.4, 6.5, and 3.496 kDa, respectively. In the case of aqueous-phase sample, high-resolu-

tion fast-atom bombardment (FAB) mass spectra was recorded on an Autospec mass spectrophotometer for determination of molecular weight.

Thin-Layer Chromatography Detection

The dried aqueous fraction was analyzed with thin-layer chromatography (TLC) *(31)* using silica gel plates (E. Merck, Darmstadt, Germany) and butanol:acetic acid:distilled water (4:1:5) as the separation phase at 28°C for 30 min. The plate was air-dried at ambient temperature, and spots were identified using bromocresol green as a locating agent (100 mg of dye in 250 mL of absolute alcohol—green color), with the pH of the alcoholic solution adjusted to 7.0, giving a grass green color.

Sick-Pot Studies

Pots were filled with 5 kg of sterile soil. They were then infected with 10^9 spores/g of soil by *Rhizopus* sp. and, *Fusarium* sp. The pots were kept at 28 ± 2°C. The results of seven replicates were recorded after 30, 45, and 60 d, respectively.

Field Trial

Field trial was designed according to Prabhakaran and Ravi *(32)*. Soybean seeds were surface sterilized with acidified 0.1% $HgCl_2$ for 30 s and washed thoroughly three times with sterile distilled water. Seed bacterization was performed with *B. japonicum* (approx 10^8 cells/seed) and then the seeds were sown in the field. A 10% Jaggery solution and sterile soil were used for better adsorption of the cells to the seeds. No other soybean crop was grown on the site since introduction of the *Bradyrhizobium* strain *(22)*.

Seed Germination, Root Ramification, and Nodule Enumeration

The percentage germination of soybean seeds was recorded after 5 d of incubation. Root ramification was recorded by cleaning and measuring the length of soybean plantlet roots in centimeters. For nodule enumeration, isolates were tested for their ability to nodulate soybean MACS 124 *(33)*. The number of nodules was recorded after 30, 45, and 60 d of incubation. Plants were uprooted at each sampling time and after incubation, and root systems were harvested and rinsed to make plants free of adhering soil. Nodules were counted *(34)*, crushed, and observed for the presence of *Bradyrhizobia* by plating them on YMA agar and by using wet mount and Gram-staining techniques *(35)*.

Chlorophyll, Oil, and Protein Content

Chlorophyll content was determined according to Arnon *(36)*. Chlorophyll was extracted from 1 g of fresh soybean leaves in ice-cold 80% acetone under green light, and the amount of chlorophyll in the extract was estimated. For oil extraction, soybean oil content was determined on a weight/

weight basis by using the standard soxhlet extraction method. Protein content in soybean seeds was estimated by Folin and Lowry's method *(37)*.

Statistical Analysis

All data were subjected to statistical analysis (analysis of variance) using software developed by M/S Indostat Services, Hyderabad. The least significant difference (LSD) was compared at $p = 0.05$.

Results and Discussion

Five soybean-nodulating isolates (NMU isolate, Jalgaon; Agricultural college, Dhule isolate; Oil Research Station, Jalgaon isolate; Agricultural College, Pune isolate; and Agricultural University, Rahuri, Rahuri isolate) and two standard cultures (*B. japonicum* IARI 102 and *B. japonicum* NCIM 2746) were screened for siderophore production. As depicted in Table 1, on the basis of time course of siderophore production by all strains under study, maximum siderophore production (i.e., 89.10%) was observed in NCIM 2746 at a 36-h time interval. Therefore, further studies were focused on this strain.

Siderophore production was initially tried using standard synthetic succinate medium frequently used for obtaining siderophores of Pseudomonads *(26)*, but no sufficient growth was obtained probably because of the strain's inability to utilize succinate. Succinate was thus substituted with maltose, since the use of maltose has been reported to yield maximum biomass of *Rhizobium* and *Bradyrhizobium* sp. *(38)*. This medium with maltose as the carbon source gave maximum growth and siderophore (89.10% siderophore units) production by NCIM 2746.

Characterization of Siderophores

The supernatant was acidified (pH 3.0) and extracted with ethyl acetate. The solvent layer was collected, vacuum dried, and then dissolved in deionized water (1 mg/mL) and checked for Arnow's and Csaky's tests. A strong positive Arnow's assay indicated the presence of a catechol type of siderophores, whereas Csaky's test was negative. Spectroscopic studies of the sample revealed absorption maxima at 328 nm and a mol wt of about 1 kDa (Fig. 1) using semilog paper. Similarly, the aqueous layer was vacuum dried, resuspended (1 mg/mL) in deionized water, and checked for Arnow's and Csaky's tests. Arnow's test was observed to be negative, whereas Csaky's test was strongly positive. This indicates the presence of hydroxamate siderophores in the aqueous layer.

Spectroscopic studies of the sample revealed absorption maxima at 216 nm. The FAB mass spectrum for hydroxamate siderophore gave a mol wt of 734 Daltons (Fig. 2). The presence of a peak at m/e = 192 appearing as the base peak confirms the citrate moiety in the molecule, which is linked to the chain through ester bonds. The presence of a peak at m/e = 307 appears to be another major peak. The loss of the water molecule from this

Table 1
Screening of Siderophore-Producing *Bradyrhizobia*

Culture	Time (h)	Siderophore units (%)
Bradyrhizobium japonicum NMU isolate 1	24	32
	30	42
	36	65
	42	59
	48	56
Agriculture College, Dhule, isolate 2	24	43
	30	62
	36	73
	42	65
	48	62
Oil research station, Jalgaon, isolate 3	24	72.5
	30	78.6
	36	80
	42	54.2
	48	51.2
Agriculture College, Pune, isolate 4	24	38
	30	45
	36	53
	42	49
	48	40
Rahuri Krishi vidyapeeth, Rahuri isolate 5	24	58
	30	60
	36	64
	42	57
	48	49
Bradyrhizobium japonicum IARI 102	24	70
	30	76
	36	79
	42	59
	48	47
Bradyrhizobium japonicum NCIM 2746	24	73
	30	78
	36	89
	42	54
	48	47

gives a peak at m/e = 289. The loss of $NH^{+}_{2} = CH_2$ gave a peak at m/e = 259. The peak at m/e = 241 appears to be the result of the loss of another water molecule. This was followed by a peak at m/e = 213, which appeared as the result of the loss of $CH_2 = CH_2$.

O'Hara et al. *(39)* and Carson et al. *(16)* have reported catechol biogenesis by *Bradyrhizobium* sp. and hydroxamate biogenesis by *Rhizobium leguminosarum* separately. In contrast with these results *B. japonicum* 2746 seems to be an outstanding organism with the unique feature of producing

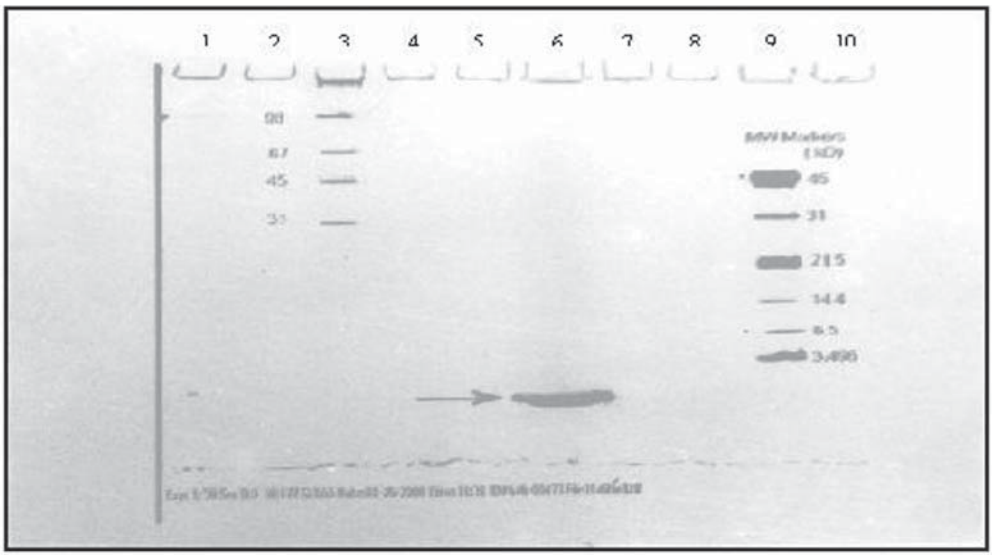

Fig. 1. SDS-PAGE of ethyl acetate fraction (catecholate siderophore) for molecular weight determination. Lanes 1, 2, 4, 5, 7, 8, and 10: empty; lanes 3 and 9: standard markers; lane 6: test siderophore sample.

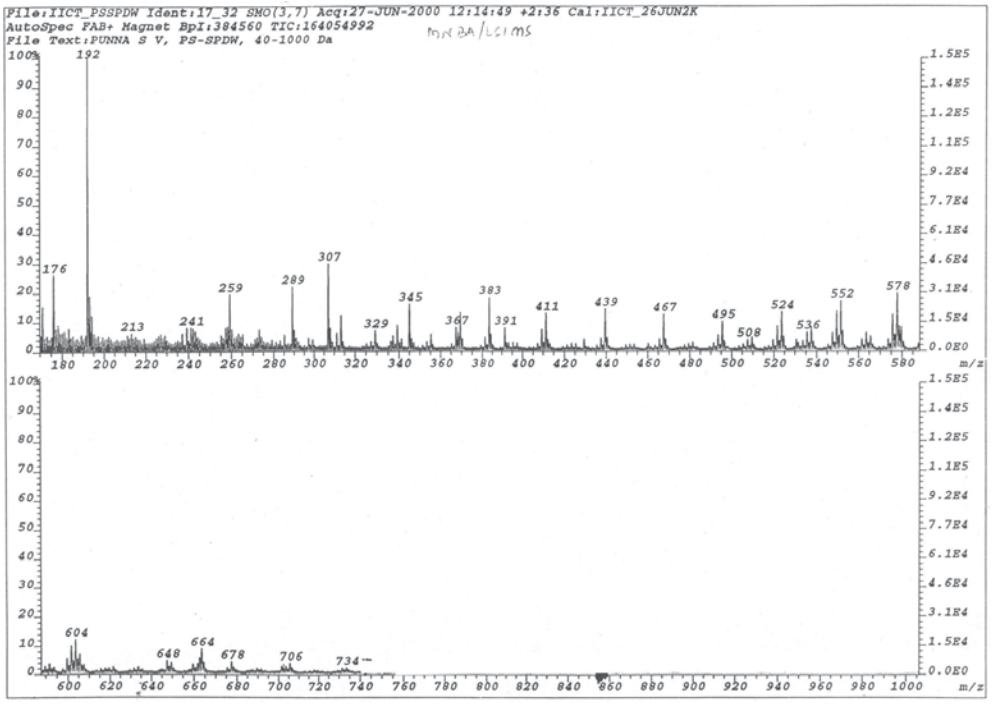

Fig. 2. FAB mass spectra of hydroxamate siderophore.

both, i.e., catechols and the hydroxamate-type of siderophores. Hydroxamates are a more useful type of siderophores in the field of medicine and agriculture because of their strong affinity toward Fe^{3+} *(40)*. In this regard, *B. japonicum* appears to be a more ecofriendly organism for sustainable iron nutrition of plants in an ecosystem.

The TLC plate showed a pale yellow spot with an R_f of 0.46 *(31)*, similar to that of the standard citrate. Other characterization studies of aqueous-phase sample are under way; however with the present data of partial identification, it can be nomenclatured as citrate-based siderophore.

In Vivo Growth Promotion by *Siderophoregenic* Bradyrhizobia

Sick-pot studies indicated a marked increase in the oil content and the protein content after harvesting of soybeans, whereas chlorophyll content was recorded after 60 d. The *Bradyrhizobium* bacterized seeds without fungal treatment showed a high oil content of 29.3% (SsBr; see Table 2 for definitions of abbreviations throughout) as compared with 27.2% in uninoculated seeds (control). The seeds infected with *Rhizopus* sp. and bacterized with *Bradyrhizobium* (SsRhBr) also surprisingly showed more oil content (28%) compared with that derived for *Rhizopus*-infected (SsRh) seeds (26.5%). In a similar way, soybean seeds infected with *Fusarium* sp. and bacterized with *Bradyrhizobia* (SsFuBr) showed more oil content (28.2%) compared with that derived from *Fusarium*-infected seeds (SsFu) (26%). Values of standard error of difference (SED) and critical difference (CD) at the 95% level obtained from control over NCIM and NMU strains were significant. This indicates that bacterization with *B. japonicum* NCIM was very effective against control, as indicated in Table 2. This treatment was more effective when the combination of *Bradyrhizobium* and *Pseudomonas* was used. Figure 3 shows that the number of pods after 90 d was too high (30) as compared to control (15), whereas seed weight per grams was increased in NCIM 2746–treated plantlets. In addition, enhanced root length was also found in NCIM 2746–treated plantlets.

The protein content was high (41.8 mg%) in siderophoregenic *Bradyrhizobium*–applied plants (SsBr) compared with uninoculated control (40.2 mg%). The *Rhizopus*-infected plantlets bacterized with *Bradyrhizobia* (SsRhBr) showed 41.7 mg% protein, which was greater compared to *Rhizopus* sp.–inoculated seeds (SsRh) 40 mg%. *Fusarium*-infected plantlets bacterized with bradyrhizobia (SsFuBr) showed 40.3 mg% protein, which was greater than that of only *Fusarium*-inoculated seeds (SsFu) 39.1 mg%. The chlorophyll content in SsRhBr plants was 1.4 mg/g as compared with 0.9 mg/g in the control. By contrast, SsFuBr plantlets showed more chlorophyll content (1.2 mg/g) as compared with SsFu (0.9 mg/g).

It was observed from sick-pot studies that siderophore-rich culture of *B. japonicum* NCIM 2746 suppresses the growth of *Fusarium* sp. and *Rhizopus* sp. The statistically analyzed data reveal that the number of pods, root ramification, plant dry weight, oil content, protein content, and chloro-

Table 2
Sick Pot Study[a]

Treatment	Pods	RL (cm)	Dry wt (g)	Size of seed (mm)	Oil content (%)	Protein (mg%)	Chlorophyll (mg/g)	Seed weight (g)
Control	15	13.0	15.51	5	27.2	40.2	0.9	2.05
SsBr	39	15.0	17.44	6	29.3	41.8	1.5	7.80
SsBrPs.	38	21.0	18.2	6	30.6	42.4	1.4	6.73
SsRh	30	12.5	12.16	5	26.5	40.0	1.0	5.11
SsRhBr	51	14.5	22.57	5.5	28.0	41.7	1.4	7.28
SsRhBrPs	49	18.0	21.0	6	28.0	41.2	1.8	5.06
SsFu	23	12.0	10.86	4.5	26.0	39.1	0.9	3.82
SsFuBr	28	14.0	11.65	5	28.2	40.3	1.2	4.0
SSFuBrPs	30	17.0	10.88	6	28.5	40.9	1.6	2.30
SED	0.773	0.366	0.154	0.163	0.425	0.669	0.054	0.447
CD 5%	1.556	0.736	0.309	0.328	0.855	1.345	0.109	0.899

[a]All values represent the mean of seven replicates each (one factorial randomized block design [RBD] was applied). Ss, Sick soil; Br, siderophoregenic *Bradyrhizobia*; Ps, siderophoregenic *Pseudomonas*; Rh, *Rhizopus*; Fu, *Fusarium*; RL, root length.

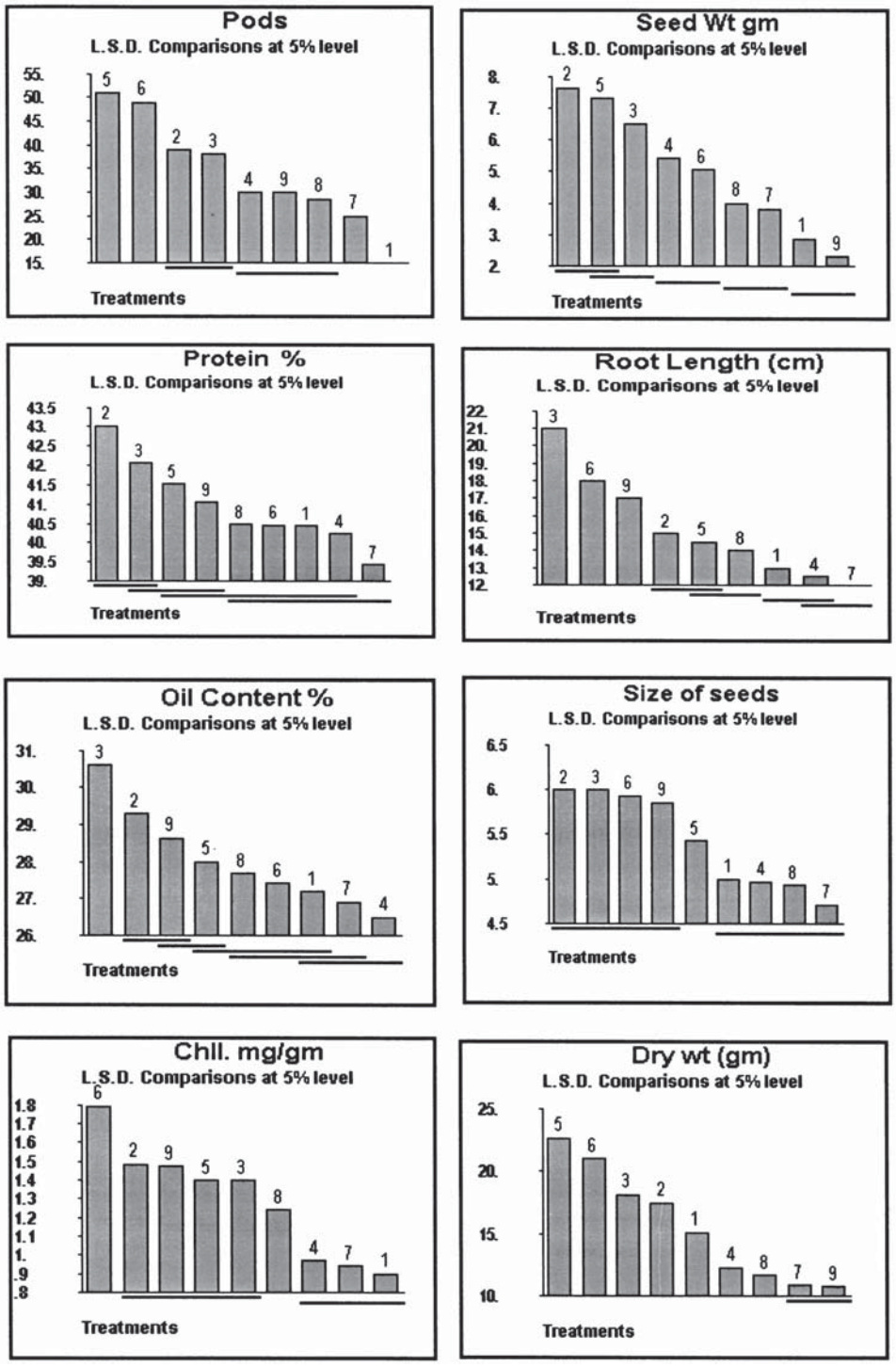

Fig. 3. LSD comparison at 5% level for sick pot studies. Treatments: 1 = control; 2 = SsBr; 3 = SsBrPs; 4 = SsRh; 5 = SsRhBr; 6 = SsRhBrPs; 7 = SsFu; 8 = SsFuBr; 9 = SsFuBrPs (see Table 2 for definitions).

phyll content were greater. This indicates that phytopathogenic fungi were suppressed with an increase in the parameters under study.

Pot studies were subsequently confirmed by field trial carried out with two *Bradyrhizobium* strains: NCIM 2746 and NMU 3 isolate. (NMU3 isolate was another high siderophore-producing bacterium compared with other NMU isolates.) It was performed at Krishi Vidnyan Kendra (KVK), Pal. During the field trial, various parameters such as root length, shoot length, number of nodules, number of pods, chlorophyll content, and number of branches were studied. The data obtained were statistically analyzed, as depicted in Table 3.

Field trial results were recorded after 30, 45, and 60 d, respectively. Root length after 60 d was greater (18 cm) in NCIM 2746–applied plants compared with control (14 cm). Shoot length was also greater (37 cm) after 60 d in NCIM 2746– and NMU-applied plants compared with control (27 cm). The number of nodules with respect to days was greater (14) in NCIM 2746–applied plants compared with control (7) after 60 d. A chlorophyll content of 0.31 mg/g was obtained in NCIM 2746–applied plants, whereas it was 0.20 and 0.09 mg/g in NMU-applied plants and control after 60 d, respectively. The number of pods was greater (18) in the case of NCIM 2746–applied plants compared with NMU-applied (16) and control (14), respectively. A similar pattern was observed for 30 and 45 d, respectively. SED and CD values at the 95% level obtained from control over NCIM and NMU strains were significant, as depicted in Fig. 4. This indicates that NCIM treatment is extremely effective against NMU and control.

Field trial results confirm the growth promotion abilities of *B. japonicum* NCIM 2746 on the basis of a rise in the various parameters under study as compared to NMU isolate and control.

Conclusion

B. japonicum NCIM 2746 is a dynamic, high-siderophore-producing strain on the basis of universal chemical assay for siderophores. It produces citrate-based siderophore as a major and catecholate as a minor. Sick-pot studies showed suppression of phytopathogenic fungi and an increase in growth parameters. Field trial results indicated enhancement in root length, pods, and chlorophyll content. Thus, *B. japonicum* NCIM 2746 has high potential of siderophore biogenesis, plant growth promotion, and phytopathogen suppression and, hence, could be exploited in agriculture for improvement in soybean yield.

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Table 3
Field Trial

Treatment (d)	Shoot length (cm)	No. of branches	Chlorophyll (mg/g)	No. of nodules	Root length (cm)	No. of pods
Control (30)	18	4	0.07	2	12	0.0
NCIM (30)	19	5	0.33	3	10	0.0
NMU (30)	19	5	0.20	2	14	0.0
Control (45)	20	8	0.11	5	13	4
NCIM (45)	27	11	0.35	8	16	8
NMU (45)	23	8	0.27	10	16	6
Control (60)	27	9	0.09	7	14	14
NCIM (60)	37	13	0.31	14	18	18
NMU (60)	37	11	0.20	14	18	16
SED	0.230	0.762	0.005	0.951	0.352	0.882
CD95%	0.462	1.532	0.011	1.913	0.708	1.774

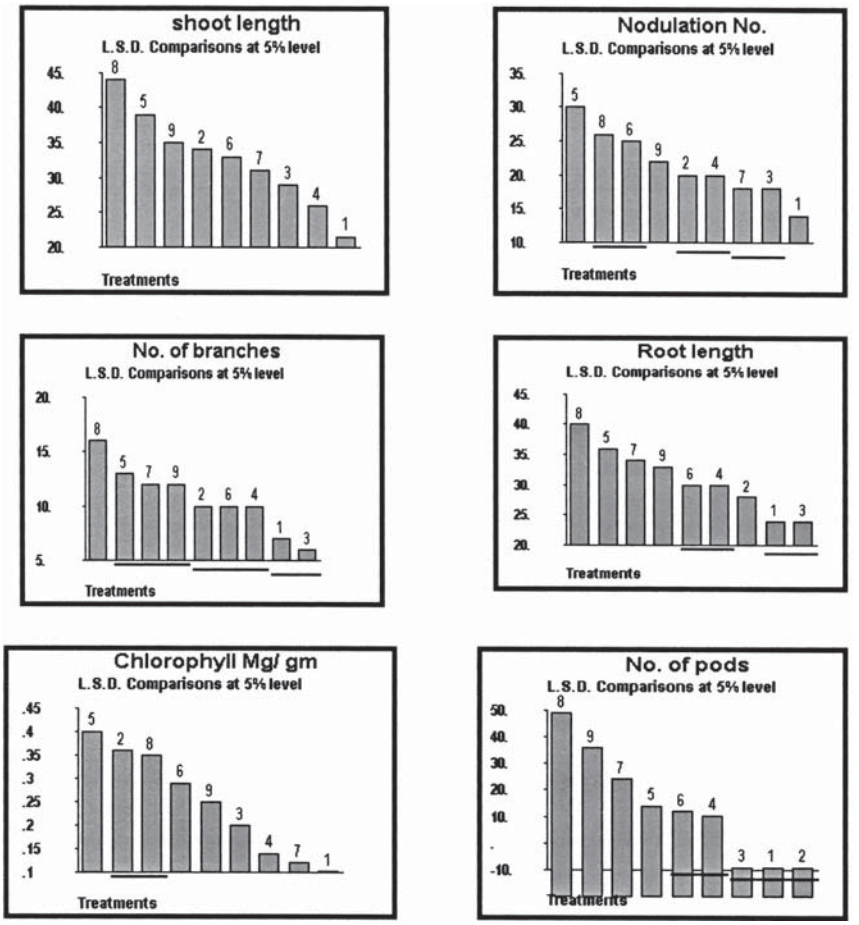

Fig. 4. LSD comparison at 5% level of field trial. Treatments: 1 = control (30 d); 2 = NCIM (30 d); 3 = NMU (30 d); 4 = control (45 d); 5 = NCIM (45 d); 6 = NMU (45 d); 7 = control (60 d); 8 = NCIM (60 d); 9 = NMU (60 d).

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