ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 1, pp. 80–89. © Pleiades Publishing, Ltd., 2015.

EXPERIMENTAL ARTICLES

Exploring Plant Growth Promoting Potential of Non Rhizobial Root Nodules Endophytes of *Vigna radiata*[1]

M. Pandya, M. Rajput, and S. Rajkumar[2]

Microbiology Lab, Institute of Science, Nirma University, Ahmedabad, Gujarat, 382481 India

Received May 12, 2014

Abstract—Plant growth promoting (PGP) rhizobacteria exert beneficial effects and may establish as endophytes in their hosts. Here, plant growth promoting traits of 26 non rhizobial and one fungal endophyte previously isolated from *Vigna radiata* root-nodules were assessed for IAA and siderophore production, phosphate solubilization and hydrolytic enzymes production. Most bacterial endophytes improved seedling vigor index while fungal endophyte (*Macrophomina phaseolina*) lacked all PGP traits. Endophytes Ml, M10 and M15 were most influential in improving Seedling Vigor Index. Three endophytes having multiple PGP traits with maximum siderophore production: 46.77 μg mL^{-1} (*Bacillus anthracis*; Ml), IAA production: 10.81 μg mL^{-1} (*Paenibacillus taichungensis*; M10) and phosphate solubilization: 134.483 μg mL^{-1} (*Paenibacillus xylanilyticus*; M15) significantly increased root length (RL), shoot length (SL), number of lateral roots (NLR) and plant dry weight (DW) when inoculated/co inoculated with *E. adhaerens* (native rhizobia) to *V. radiata* in a small field trial. M10 inoculation produced longest RL while Ml when coinoculated with *E. adhaerens* produced highest SL and NLR. Ml inoculation or coinoculation was most effective in improving dry weight of mature plants. Most of the endophytes coinoculated with *E. adhaerens* improved growth parameters. We report that non rhizobial endophytes with PGP traits in combination with native rhizobia can be prospective candidates for use as biofertilizer.

Keywords: *Vigna radiata*, root nodule endophytes, plant growth promotion, Seedling vigor assay

DOI: 10.1134/S0026261715010105

In nitrogen limiting condition, association between legumes and symbiotic partners belonging to α- and β-*Proteobacteria* results in formation of specialized organs for N_2 fixation called root/stem nodules. Inspite of the benefits of symbiotic association to both partners, entry of rhizobia into host plant roots occurs by a series of well coordinated events from both symbionts.

An increasing number of α-, β- and γ-*Proteobacteria* have been isolated from root nodules of a wide range of legumes regardless of symbiosis specificity at multiple phases of the interaction between both the partners and are reported as nodule-associated bacteria or nodule endophytes [1, 2]. Such nodule-associated bacteria may be endophytic or free-living rhizobacteria and may establish neutral, detrimental or beneficial interactions with plants.

Some endophytic bacteria may promote plant growth [3], induce resistance to plant pathogens, fix nitrogen [4] and can be explored as plant growth promoting bacteria (PGPB). The numbers of plant growth promoting bacteria occurring in soil are not enough to compete with other bacterial strains commonly established in the rhizosphere. Therefore, for agronomic utility, inoculation of natural plant growth promoting endophytic bacteria may be taken advantage for plant yield enhancement [5].

The effect of inoculating PGPB with rhizobia on increased nodulation and growth in a wide variety of legumes has been reported [6] however, the effect of endophyte inoculation with native rhizobia are vivid *eg. Agrobacterium* strains reduced the nodulation of *Rhizobium gallicum* in the common bean [7], while it had no effect on nodulation of *Sinorhizobium meliloti* with alfalfa [8] signifying that a nodule endophyte may serve as PGPR or plant growth deleterious rhizobacteria (PGDR) based on its interaction with the rhizobial strain [9]. These reports highlight that biological significance and the agronomic implications of nodule endophytism are not well understood and hence remains to be explored.

V. radiata, is an important source of human food and animal feed which plays an important role in sustaining soil fertility by improving soil physical properties and fixing atmospheric nitrogen. In our previous study we reported isolation of non rhizobial endophytes and experimentally demonstrated that they invade root hair infection thread when coinoculated with host nodulating *Ensifer adhaerens* [10]. Here we aim to screen these endophytes for plant growth promoting traits and assess their impact on seed germina-

[1] The article is published in the original.

[2] Corresponding author; e-mail: shalinirjk@gmail.com

tion and plant growth when inoculated or coinoculated with *E. adhaerens* on *Vigna radiata*.

MATERIALS AND METHODS

Isolation and symbiotic test of nodule endophytes. One hundred undamaged, healthy root nodules of similar size sampled from the lateral roots of field grown *Vigna radiata* were surface sterilized and used for endophyte isolation on congo red yeast extract mannitol agar (CRYMA), Plate count agar (PCA) and Nutrient agar (NA) followed by 16S rDNA and ITS sequencing, as reported [10].

10^8 cells of each endophyte and native *E. adhaerens* (positive control) were inoculated to *V. radiata* seedlings grown in pots containing sterile soil [11] and checked for nodule formation 40 days post inoculation.

Plant growth promoting (PGP) traits of nodule endophytes. All the nodule endophytes and *E. adhaerens* were screened for the following PGP traits through qualitative and quantitative estimations.

Indole Acetic Acid (IAA) production, Siderophore production and Phosphate solubilization. The production of IAA by endophytes was determined as described previously [12] (Supplementary data, S1).

Siderophore production by endophytes was observed under Fe^{3+} limiting conditions as described previously [13]. Siderophore production was quantitated as described [14] (Supplementary data, S2).

The phosphate solubilization by individual endophytes was determined by plating onto Pikovskaya's agar as described [15]. Inorganic P concentration was estimated in culture supernatant every 24 h for 5 days at 820 nm and expressed as mg mL^{-1} [16] (Supplementary data, S3).

Production of pectinase, chitinase and antifungal activity. For pectinase assay, endophytes grown on 1% pectin agar [17] for 7 days at 28 ± 2°C were flooded with 0.1% aqueous red ruthenium solution for one hour, drained, rinsed with water and observed for red color halos around colonies which constituted positive test.

For chitinase assay, cells were seeded onto medium supplemented with 1.5 g L^{-1} colloidal chitin and incubated for 5 days at 28 ± 2°C to check chitinase production [18]. For detection of antifungal activity, a small block of PDA with growth of *Macrophomina phaseolina* (nodule endophyte) was cut using sterile blade and placed in the centre of a fresh PDA plate. Endophytic cultures were streaked at two ends of the plate and incubated at 28 ± 2°C for 48–96 h to record zone of inhibition.

Plant growth experiment. Seedling Vigor Assay, Seed germination—Root length (RL) and Hypocotyl length (HL). Surface sterilized *V. radiata* seeds were treated with endophytes and *E. adhaerens* (2% solution of 10^8 cfu mL^{-1}) for 45 min while control seeds were incubated in sterile distilled water. Germination tests were carried out by paper towel method with triplicated samples [19]. Seedling vigor was calculated on seventh day using formula as described [20].

$$\text{SVI} = (\text{mean root length} + \text{mean hypocotyl length}) \times \% \text{ germination}.$$

For seed germination assay, surface sterilized *V. radiata* seeds treated with endophytes and *E. adhaerens* for 45 min were sown in plastic pots (1 Kg soil holding capacity) filled with sterile soil. The seeds were inoculated with 10 mL of 2% diluted cultures and plants were grown under controlled environment (light intensity of 200 μE m^{-2} s^{-1}, 16-h day/8-h night cycle, constant temperature of 28 ± 2°C, relative humidity 50%) and watered regularly with sterilized tap water. The experimental design consisted of 3 treatments: no inoculation (control), inoculation with *E. adhaerens* (positive control) and inoculation of individual endophytes. Each plastic pot maintained 3 plants and each treatment were replicated thrice. Plants were harvested 10 DPI and scored for RL and HL.

Field trial. A small-scale field trial was performed by inoculating native *E. adhaerens* and selected nodule endophytes (M1, M10 and M15) with multiple PGP traits. The experimental design consisted of five treatments: no inoculation (control); inoculation with *E. adhaerens* (positive control), individual inoculation of M1, M10, M15, co-inoculation of M1, M10, M15 with *E. adhaerens* and triple coinoculation of M1, M10 and M15. Treatment plots of 5/5 m were separated by a margin of 2 m. The sowing density was 15 seeds m^{-2}.

Selected endophytes grown upto late exponential phase in 10 mL Luria Bertani broth (Himedia, India) were inoculated to 7 day old seedlings. The experiment was conducted without addition of fertilizers and weeding was carried out as required. Ten, 40 day old plants were randomly uprooted from each treatment for determination of RL, SL, NLR and DW.

Statistical analysis. The data were analyzed by Sigma Plot (Windows version 11.0, Systat Software Inc., California, United States). A one-way analysis of variance (ANOVA) was used to determine the statistical significance, which was assumed to be different when the comparison showed a significance level of $P \leq 0.05$. Results were reported as Mean ± SD.

RESULTS AND DISCUSSION

Isolation and symbiotic test of nodule endophytes. A total of 26 distinct non rhizobial colonies were obtained on PCA, NA and CRYMA from the sap of surface sterilized root nodule of *V. radiata*. *Macrophomina phaseolina* was the only fungal nodule endophyte isolated on PDA which was identified by ITS sequencing. All bacterial strains were further characterized by 16S rDNA sequencing to assess their taxo-

Molecular identification of *V. radiala* nodule endophytes by 16S rDNA sequencing

Endophyte	Species	Accession number of isolated endophytes	Accession No. of most closely related sequences, NCBI
M-l	*Bacillus anthracis*	JX280494.1	NR_074453.1
M-2	*Agrobacterium vitis*	JX280495.1	NR_036780.1
M-3	*Paenibacillus barcinonensis*	JX280496.1	NR_042272.1
M-4	*Paenibacillus pabuli*	JX280497.1	NR_040853.1
M-5	*Paenibacillus amylolyticus*	JX280498.1	NR_025882.1
M-6	*Paenibacillus validus*	JX280499.1	NR_116536.1
M-7	*Bacillus sonorensis*	JX280500.1	NR_113993.1
M-8	*Ensifer adhaerens*	JX280501.1	NR_113893.1
M-9	*Paenibacillus massiliensis*	JX280502.1	NR_115175.1
M-10	*Paenibacillus taichungensis*	JX280503.1	NR_044428.1
M-11	*Bacillus safensis*	JX280504.1	NR_1 13945.1
M-12	*Bacillus megaterium*	JX280505.1	NR_1 15953.1
M-13	*Klebsiella pneumoniae*	JX280506.1	NR_074913.1
M-14	*Bacillus circulans*	JX280507.1	NR_044546.1
M-15	*Paenibacillus xylanilyticus*	JX280508.1	NR_029109.1
M-16	*Paenibacillus kribbensis*	JX280509.1	NR_025169.1
M-17	*Bacillus pumilus*	JX280510.1	NR_074977.1
M-18	*Bacillus endophyticus*	JX280511.1	NR_025122.1
M-20	*Dyadobacter fermentans*	JX280512.1	NR_074368.1
M-21	*Paenibacillus xylanexedens*	JX280513.1	NR_044524.1
M-22	*Agrobacterium tumefaciens*	JX280514.1	NR_116874.1
M-23	*Bacillus mojavensis*	JX280515.1	NR_104873.1
M-24	*Paenibacillus panacisoli*	JX280516.1	NR_041381.1
M-25	*Chitinophaga filiformis*	JX280517.1	NR_040909.1
M-26	*Paenibacillus macquariensis*	JX280518.1	NR_041635.1
M-27	*Blastobacter aggregatus*	JX280519.1	NR_116445.1
M-28	*Macrophomina phaseolina*	KC513786	KJ609175.1

16S rRNA gene of isolated bacterial endophytes was amplified using universal primers Forward primer 27F Bacteria (5' AGA GTT TGA TC (A/C) TGG CTC AG 3') and reverse primer R1492 (5' TAC GG(C/T) TAG CTT GTT ACG ACT T 3') and ITS region of fungal endophyte Macrophomina phaseolina was amplified using forward primer (5' TCCGTAGGTGAACCTGCGG 3') and reverse primer (5' TCCTCCGCTTATTGATATGC 3'). The resulting amplicons were subjected to sequencing using automatic ABI 310 DNA sequencer (Big Dye Terminator cycle sequencing, ready reaction kit, Perkin-Elmer, USA. Accession numbers of the 16SrRNA sequences submitted to NCBI are enlisted and 16S rRNA sequences with highest similarity to submitted sequences are tabulated.

nomical positions [10]. The 16S rRNA genes from most isolates possessed 99–100% similarity with a species described in NCBI GanBank (table). When reinoculated, only *E. adhaerens* nodulated *V. radiata* among all nodule endophytes (data not shown).

Legume nodulating bacteria broadly referred to as rhizobia have intrigued researchers for many decades owing to intricate specificity and nitrogen fixing ability within specialized organs called nodules. The cooperative interaction between rhizobia and other plant root colonizing bacteria is of relevance in improvement of nodulation and growth in legumes [21]. The data presented here emphasize that root nodules of *V. radiata* are colonized by an array of culturable nonrhizobial endophytes. Probably all the microorganisms whose presence has a beneficial relation might get associated with the plant nodules [22]. The non rhizobial endophytes of *V. radiata* root nodules diverged to *Klebsiella, Agrobacterium, Dyadobacter*, and *Chitinophaga, Paenibacillus* and *Bacillus* and *Macrophomina* species (table). Our results paralleled the studies by Rajendran et al. [23] who reported presence of high proportion of gram positive endophytes within the root nodules of pigeon pea. Moreover, *Bacillus* species are commonly reported nodule endophytes [23, 24] as the spore-forming capability of many bacilli is advantageous for adaptation in field. The possibility of spore formers (*Bacillus*) being contaminants was ruled out following thorough validation of nodule sterility in this study.

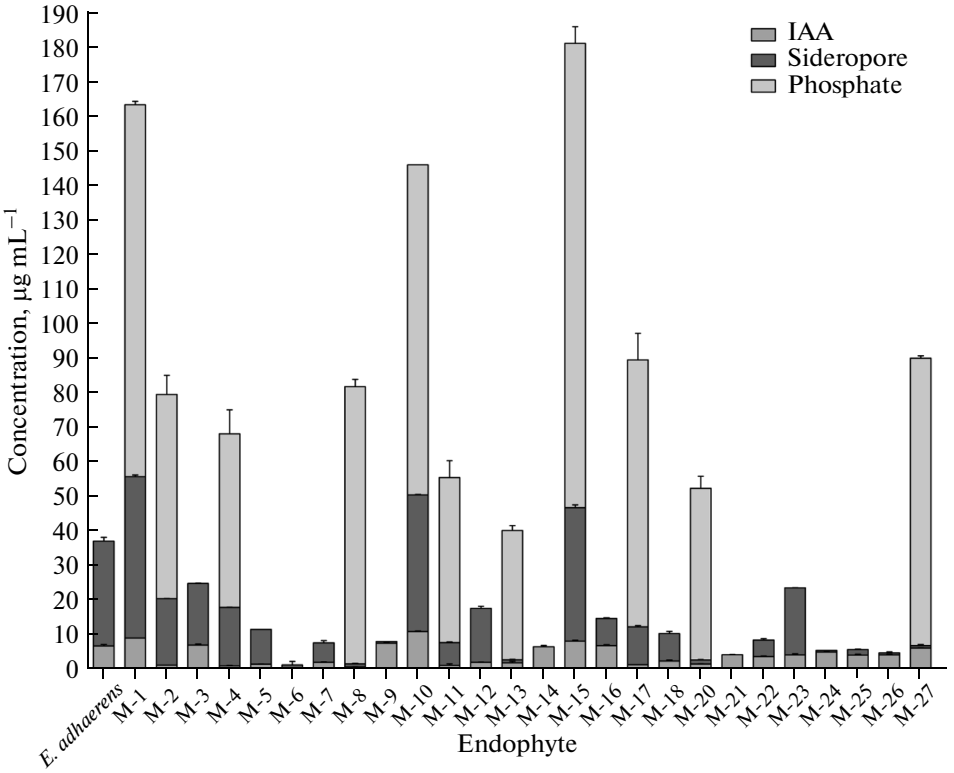

Fig. 1. IAA production, Siderophore production and Phosphate solubilization by *V. radiata* root nodule endophytes. Data are expressed as mean ± SD ($n = 3$), asterisk (*) represents significant difference between treatments and control, $P < 0.05$.

Plant growth promoting traits. Indole Acetic Acid (IAA) production, Siderophore production and Phosphate solubilization. A total of 25 endophytes produced IAA in a range of 0.52 to 10.81 μg mL^{-1}. M10 produced highest IAA (10.8 μg mL^{-1}) followed by M1 (8.9 μg mL^{-1}), M15 and M9 (~7.6 μg mL^{-1}). IAA produced by M3 and M16 was at par with *E. adhaerens* (6.61 μg mL^{-1}) (Fig. 1) while IAA production in other endophytes was significantly lower compared to *E. adhaerens* (positive control). IAA production varied greatly among endophytes with highest IAA producers being *Bacillus* and *Paenibacillus* spp. IAA concentration was lower in *V. radiata* endophytes compared to root endophytes and leaf epiphytes of peanut as previously reported [25]. It has been reported that IAA production is common in plant-associated bacteria as part of colonization strategy that involves phytostimulation and circumvention of plant defence mechanisms [26].

Twenty four (24) endophytes with orange halos around colonies on CAS agar produced Hydroxymate type siderophores in the range of 0.45 to 47.23 μg mL^{-1} with single exception of (Fig. 1), M18 that produced catechol type siderophore. Siderophore (hydroxymate) produced by M1 (46.7 μg mL^{-1}), M10 (39.5 μg mL^{-1}) and M15 (38.6 μg mL^{-1}) was significantly higher than *E. adhaerens* (30 μg mL^{-1}) but significantly lower than hydroxymate and catechol siderophores produced by *Trigonella foenum-graecum* root nodule endophytes reported previously [22]. Siderophore production is an essential PGP trait possessed by majority of rhizobacteria for iron uptake in the rhizosphere. Siderophores bind to the available form of Fe^{3+}, thus making it unavailable to the competing phytopathogens and protecting the plant. Some plants also have exceptional ability to bind, transport and exploit bacterial iron-siderophore complexes. As seen in IAA production, all endophytes producing significantly higher amount of siderophores belonged to gram positive *Bacillus* and *Paenibacillus* spp. which they are well known for siderophore production. *B. megaterium* produces schizokinen [27]. *B. subtilis* produces bacillibactin which is structurally similar to enterobactin produced by gram-negative bacteria such as *Escherichia coli* [28]. Interestingly, all the 11 siderophore producing endophytes were also phosphate solubilizers (Fig. 1) which indicate that the two traits may be correlated as organic acid secreted for phosphate solubilization might play siderophore like role and are released in iron depleted conditions [29].

Phosphate solubilization was not a common feature among the endophytes as only 11 endophytes solubilized phosphate and the trait was absent in *E. adhaerens*. The free phosphate release ranged from 37.4 μg mL^{-1} (M13) to 134.48 μg mL^{-1} (M15) (Fig. 1). Highest free phosphate released by M15 was significantly lower than free phosphate released by

endophyte P31 (*Bacillus cereus*) (354.3 μg mL^{-1}) isolated from potato roots as previously reported [30]. The most efficient phosphate solubilizing strains belonged to *Klebsiella* (M13), *Dyadobacter* (M-20), *Blastobacter* (M27), *Bacillus* (M1, M7, M11, M17) and *Paenibacillus* spp. (M4, M10 and M15). The phosphate solubilization by rhizospheric *Bacillus* [31], *Paenibacillus* [32] and *Klebsiella* [33] has already been reported. The phosphate solubilization and siderophore production seems to be rhizospheric trait of the endophytes. If they play a role in plant growth once bacteria become nodule occupant would be difficult to ascertain.

Production of pectinase, chitinase and antifungal activity. Three endophytes (M11, M15 and M17) that produce pectinase also produced chitinase and inhibited growth of fungal pathogen, *Macrophomina phaseolina*. M1, M2, M7, M9, M19, M25 produced chitinase and exhibited antifungal activity while M4, M10, M16 and M27 produced pectinase under in vitro condition. It is reported that microorganisms exhibit hyperparasitic activity and attack pathogens by secreting cell wall hydrolases like proteases and chitinase [34] which has been detected in several bacterial endophytes. Many endophytes produced pectinase which may be because pectic substances are predominantly located in the middle lamella and primary wall and presence or absence of pectinase might justify the ability of endophytes to rupture plant cell and enter within [35]. It is probable that endophytes positive for pectinase might facilitate entry of other rhizobacteria within plants.

Plant growth experiment. Effect of endophytes on Seedling Vigor (SV), Root length (RL) and Hypocotyl length (HL). Most of the endophytes (24) improved SVI of seedlings over controls. Increase in SVI by M10 was 2.5 folds followed by Ml and M15 where SVI increased by 2.25 folds over positive control. SVI increased by 2 folds with 3 endophytes (M17 and M24) and 1.5 folds with endophytes (M5, M7, M8, M14, M19, M23). M4, M6, M9, M11, M13, M18, M20 and M25 significantly improved SVI over positive control in all other endophytes treated 10 day old seedlings (Fig. 2a).

All inoculations significantly increased RL over uninoculated control (5.6 cm) while most endophytes improved RL over *E. adhaerens* treated 10 day old seedlings (8 cm) grown in pot. M-10 (14.9 cm) treated seedlings recorded longest RL followed by M3, M11, M16 and M17 (~11.9 cm). RL in seedlings treated with other endophytes were either at par with *E. adhaerens* treated seedlings or higher than controls (Fig. 2b). Fifteen endophytes achieved significant increase in HL over controls (6.9 cm). Longest HL of 10.9 cm was recorded with M-15 followed by M2, M12 (~10 cm) while other significant endophytes achieved HL of ~8.6 cm (Fig. 2b).

In pot experiment, endophytes bearing single or multiple PGP traits were equally competent in improving SVI, RL and HL in initial stages of germination. Similar improvement of seed germination parameters by endophytes has been reported in sorghum [36].

Field trials. Three multiple PGP traits bearing endophytes Ml (maximum siderophore); M10 (Maximum IAA and RL during seed germination) and M15(Maximum Phosphate solubilization, SVI and HL during seed germination) were selected for *V. radiata* inoculation in small field setup.

Longest RL was recorded in plants inoculated with M10 (26 cm) while M1/M15 were at par with *E. adhaerens* (13 cm) treated plants but was significantly higher than uninoculated control (11.5 cm). M10 maintained longest RL (21.3 cm) when coinoculated with *E. adhaerens* though it was lower compared to RL achieved with individual M10 inoculation. RL of plants inoculated with all three endophytes was similar to positive control (*E. adhaerens*) inoculated treatment (Fig. 3a). *E. adhaerens* inoculation significantly increased SL (26.7 cm) over uninoculated control (15.3 cm) which could not be achieved by simultaneous triple inoculation of the endophytes (21.9 cm) or coinoculation of endophytes except M10 which recorded longest SL of 35 cm with *E. adhaerens* (Fig. 3a).

All treatments improved NLR significantly higher over controls (18 cm). NLR was the only plant growth parameter which achieved significant increase over positive control in triple endophyte coinoculation treatment (22 cm). M1 (32 cm) was most suitable for coinoculation with *E. adhaerens* followed by M10 (26 cm) and M15 (24 cm) in this regard.

DW of 40 days old *V. radiata* plants did not improve with *E. adhaerens* inoculation. However, all endophytes significantly increased DW over controls (0.6 g) in individual, combined and *E. adhaerens* coinoculated treatments. M1 was most effective individually (1.4 g) and formed best combination with *E. adhaerens* (1.25 g). M10 and M15 were most effective singly (~1.1 g) a combined treatments with *E. adhaerens* (1 g) and triple endophyte inoculation was superior to *E. adhaerens* but at par with M-15 and combination of M10 and M15 with *E. adhaerens* (Fig. 3b). Similar increase in root nodule dry weight by inoculation of *Exiguobacterium* sp. M2N2c and B1N2b with *Sinorhizobium meliloti* has been reported [22].

Endophytes with multiple PGP traits (M1, M10 and M15) improved RL, SL, NLR (Fig. 3a) and DW (Fig. 3b) over control when applied singly or coinoculated with *E. adhaerens* in field. Significant increase in assessed growth parameters of *V. radiata* suggested that PGP traits improved plant growth directly or indirectly.

The results of endophyte inoculation on RL and HL in this study were in harmony with [37] who reported that individual inoculation of endophytes significantly increased root length while [38] reported

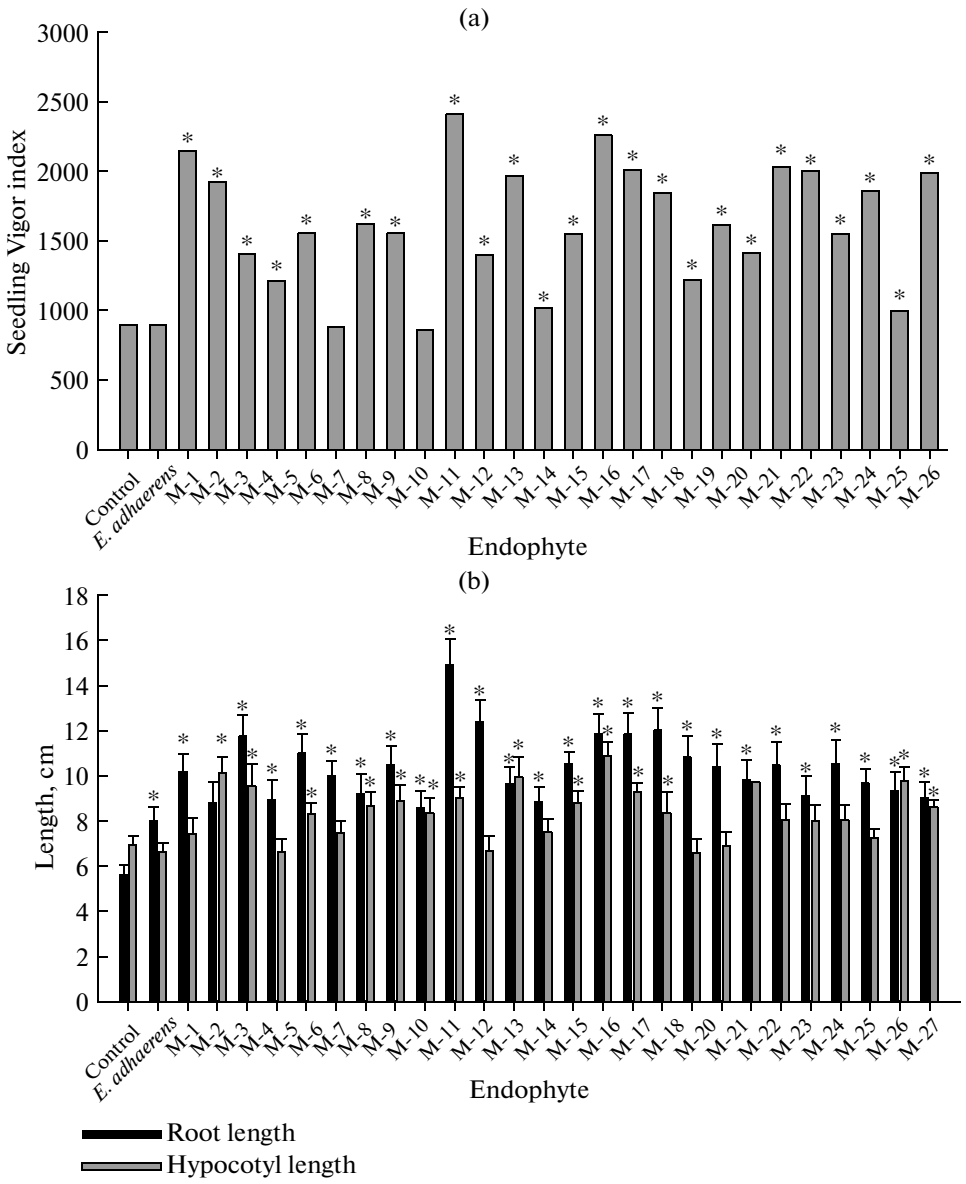

Fig. 2. (a)—Effect of endophytes on Seedling vigor. Data are expressed as mean ± SD ($n = 3$), *, $P < 0.05$. (b)—Effect of endophytes on root and hypocotyl length. Data are expressed as mean ± SD ($n = 3$), asterisk (*) represents significant difference between treatments and control, $P < 0.05$.

that some endophytes when inoculated increased root length but decreased shoot length of *Medicago sativa* [30] have reported increase in root and shoot length of potato upon endophyte inoculation.

Improvement in plant growth upon coinoculation of non rhizobial nodule endophytes with *Rhizobium* has been previously reported. In this study, coinoculation of *E. adhaerens* with nodule endophyte M1 significantly increased SL, NLR and DW (Figs. 3a, 3b) which accorded with [39] who reported coinoculation of *Mesorhizobium* sp. with nodule endophyte *Pseudomonas chlororaphis* in significantly improving RL and SL in *Sophora alopecuroides*. Similarly [22] have also reported coinoculation of nodule associated bacteria B1N2b with *Sinorhizobium meliloti* significantly increasing shoot and root length [40] reported improved soybean growth and nodulation by coinoculating *B. megaterium* B153-2-2 with *B. japonicum*.

The *V. radiata* nodule endophytes differed from each other with respect to PGP traits. Majority of the endophytes had one or the other PGP activity, few harboured multiple PGP traits while some lacked in all the tested PGP activities. Most endophytes that produced IAA with exception of M6 (*Paenibacillus validus*) and M22 (*Agrobacterium tumefaciens*) also inhibited fungal pathogen (*Macrophomina phaseolina*). *Bacillus anthracis* (M1) exhibited multiple PGP traits like antifungal activity, IAA and siderophore

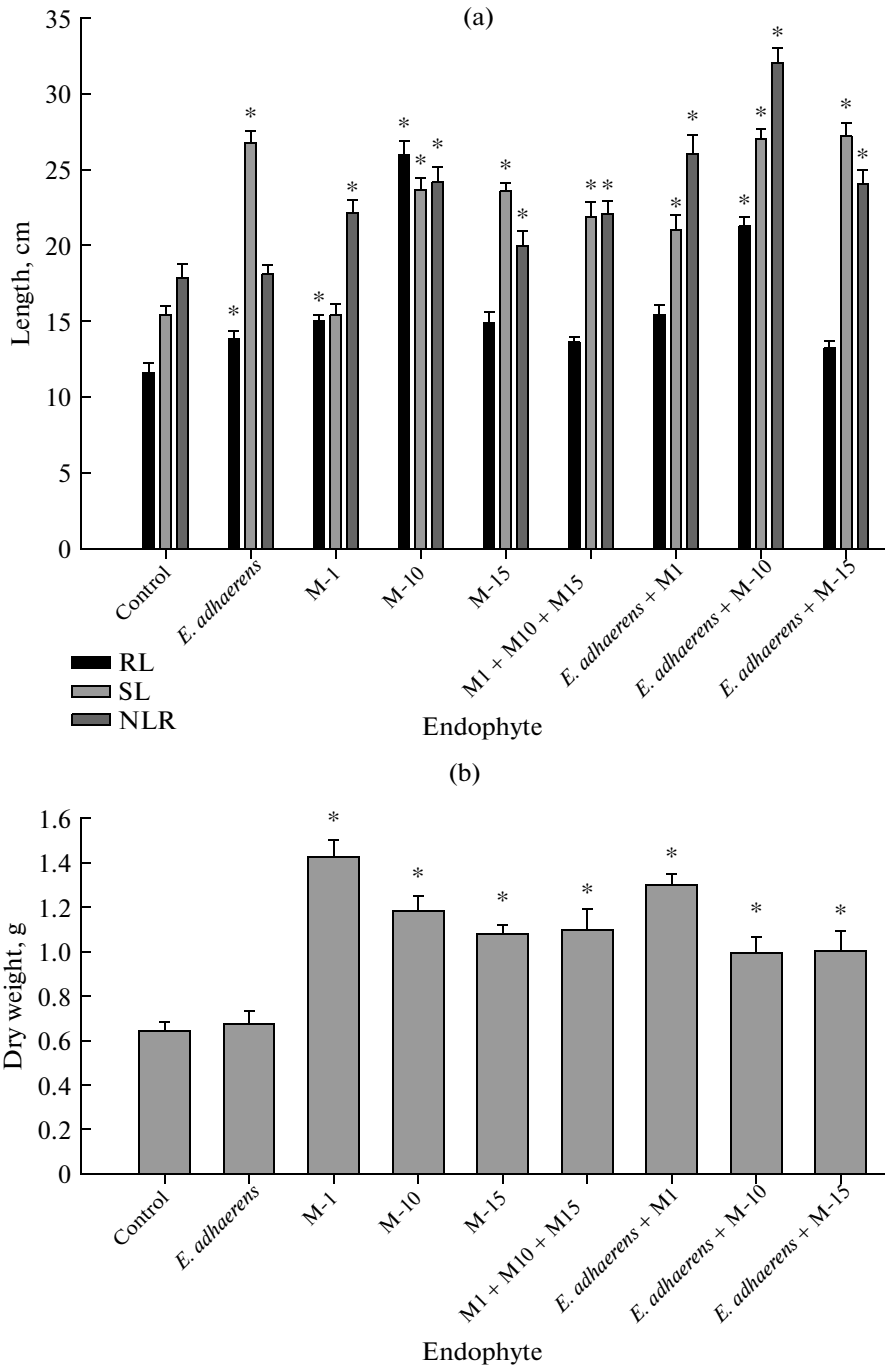

Fig. 3. (a)—Effect of endophytes on root length, shoot length and number of lateral roots in vivo. Data are expressed as mean ± SD ($n = 3$), asterisk (*) represents significant difference between treatments and control, $P < 0.05$. (b)—Effect of endophytes inoculation on dry weight of *V. radiata*. Data are expressed as mean ± SD ($n = 3$), asterisk (*) represents significant difference between treatments and control, $P < 0.05$.

production and phosphate solubilization. The antifungal activity of Ml might be attributed to the production of siderophores and chitinase or synergistic interaction of these two or with other metabolites. It has been reported that production of siderophores, secondary metabolites and lytic enzymes by rhizospheric bacteria like *Pseudomonas* strains were most effective in controlling plant root pathogens including *F. oxysporum* and *R. solani* [41]. The sampling site of *V. radiata* was infected with fungus which was also isolated from surface sterilized root nodules. ITS sequencing of the fungal endophyte revealed 99% similarity with *M. phaseolina*, the causal agent of chloral rot on many plant species. Therefore, *M. phaseolina*

was used as target to screen bacterial endophytes with antifungal activity. Despite of the fungal endophyte, the plants did not show any symptoms of infection and were robust enough to yield healthy pods which may be because of antifungal and PGP property of endophytes.

V. radiata root nodule endophytes were different from those isolated from root nodules of spontaneous legumes in Tunisia [1] and soybean [42]. The latter belonged to *Acinetobacter, Agrobacterium*, and *Burkholderia* genera. Probably the different genotypes, climate condition, soil type, and human activities are responsible for these differences [43]. Where rhizobacteria are known to influence plant growth, several studies have described selection pressure of host through root exudates in defining rhizospheric population [44] which in turn may influence endophytic population. Except for a few endophytes with neutral to negative effects, nodule endophytic bacteria were beneficial for the growth of *V. radiata*. Based on these observations it appears that nodules play vital role as a niche for their ability to harbor diverse bacterial taxa which together contribute to plant growth. These endophytic bacteria can be screened for their PGP traits and can be utilized to formulate a customized inoculum for betterment of respective host plant.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial grant received from Department of Biotechnology (DBT) (BT/PR4153/AGR/21/351/2011), Ministry of Science and Technology, Government of India, New Delhi and infrastructure provided by Nirma Education and Research Foundation (NERF), Ahmedabad Gujarat, India.

REFERENCES

1. Zakhia, F., Jeder, H., Willems, A., Gillis, M., Dreyfus, B., and de Lajudie, P., Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for *nifH*-lik gene within the genera *Microbacterium* and *Starkeya, Microb. Ecol.*, 2006, vol. 51, pp. 375–393.
2. Kan, F.L., Chen, Z.Y., Wang, E.T., Tian, C.F., Sui, X.H., and Chen, W.X., Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai-Tibet plateau and in other zones of China, *Arch. Microbiol.*, 2007, vol. 188, pp. 103–115.
3. Tariq, M., Hameed, S., Yasmeen, T., Zahid, M., and Zafar, M., Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (*Pisum sativum* L.), *World J. Microbiol. Biotechnol.*, 2014, vol. 30, pp. 719–725.
4. Kirchhof, G., Reis, V.M., Baldani, J.L., Eckert, B., Döbereiner, J., and Hartmann, A., Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants, *Plant Soil*, 1997, vol. 194, pp. 45–55.
5. Igual, J.M., Valverde, A., Cervantes, E., and Velazquez, E., Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study, *Agronomie*, 2001, vol. 21, pp. 561–568.
6. Tilak, K.V.B.R., Ranganayaki, N., and Manoharachari, C., Synergistic effects of plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and nitrogen fixation by pigeonpea (*Cajanus cajan*), *Eur. J. Soil Sci.*, 2006, vol. 57, pp. 67–71.
7. Mrabet, M., Mnasri, B., Romdhane, S.B., Laguerre, G., Aouani, M.E., and Mhamdi, R., *Agrobacterium* strains isolated from root nodules of common bean specifically reduce nodulation by *Rhizobium gallicum, FEMS Microbiol. Ecol.*, 2006, vol. 56, pp. 304–309.
8. Wang, L.L., Wang, E.T., Liu, J., Li, Y., and Chen, W.X., Endophytic occupation of root nodules and roots of *Melilotus dentatus* by *Agrobacterium tumefaciens, Microbial. Ecol.*, 2006, vol. 52, pp. 436–443.
9. Chihaoui, S.A., Mhadhbi, H., and Mhamdi, R., The antibiosis of nodule-endophytic agrobacteria and its potential effect on nodule functioning of *Phaseolus vulgaris, Arch. Microbiol.*, 2012, vol. 194, pp. 1013–1021.
10. Pandya, M., Kumar, G.N., and Rajkumar, S., Invasion of rhizobial infection thread by non-rhizobia for colonization of *Vigna radiata* root nodules, *FEMS Microbiol. Lett.*, 2013, vol. 348, pp. 58–65.
11. Vincent, J.M., *A Manual for Practical Study of the Root Nodule Bacteria* (IBP Handbook, no. 15, Int. Biol. Program, London): Oxford: Blackwell, 1970.
12. Gordon, S.A. and Weber, R.P., Colorimetric estimation of indoleacetic acid, *Plant Physiol.*, 1951, vol. 26, pp. 192–195.
13. Schwyn, B. and Neilands, J.B., Universal chemical assay for the detection and determination of siderophores, *Anal. Biochem.*, 1987, vol. 160, pp. 47–56.
14. Khan, A., Geetha, R., Akolkar, A., Pandya, A., Archana, G., and Desai, A.J., Differential cross-utilization of heterologous siderophores by nodule bacteria of *Cajanus cajan* and its possible role in growth under iron-limited conditions, *Appl. Soil Ecol.*, 2006, vol. 34, pp. 19–26.
15. Pikovskaya, R.I., Mobilization of phosphorus in soil connection with vital capacity of source microbial species, *Microbiologiya*, 1948, vol. 17, pp. 362–370.
16. Ames, B.N., Assay of inorganic phosphate, total phosphate and phosphatases, in *Methods Enzymology*, vol. 8: Complex Carbohydrates, Neufeld, E. and Ginsburg, V., Eds., New York: Academic, 1966, pp. 115–118.
17. Cotty, P.J., Cleveland, T.E., Brown, R.L., and Mellon, J.E., Variation in polygalacturonase production among *Aspergillus flavus* isolates, *Appl. Environ. Microbiol.*, 1990, vol. 56, pp. 3885–3887.
18. Tanaka, T., Fujiwara, S., Nishikori, S., Fukui, T., Takagi, M., and Imanaka, T., A unique chitinase with dual active sites and triple substrate binding sites from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 5338–5344.

19. Wold, A., International seed testing association history, 1974–1995, *Seed Sci. Technol.*, 1996, vol. 24, pp. 95–106.
20. Abdul-Baki, A.A. and Anderson, J.D., Vigor determination in soybean seed by multiple criteria, *Crop Sci.*, 1973, vol. 13, pp. 630–633.
21. Barea, J.M., Pozo, M.J., Azcon, R., and Azcon-Aguilar, C., Microbial cooperation in the rhizosphere, *J. Exp. Bot.*, 2005, vol. 56, pp. 1761–1778.
22. Rajendran, G., Patel, M.H., and Joshi, S.J., Isolation and characterization of nodule-associated *Exiguobacterium* sp. from the root nodules of Fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion, *Int. J. Microbiol.*, 2012, article ID 693982. doi: 10/1155/2012/693982
23. Rajendran, G., Sing, F., Desai, A.J., and Archana, G., Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp., *Biores. Technol.*, 2008, vol. 99, pp. 4544–4550.
24. Araujo, W.L., Maccheroni, W.Jr., Aguilar-Vildoso, C.I., Barroso, P.A., Saridakis, H.O., and Azevedo, J.L., Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks, *Can. J. Microbiol.*, 2001, vol. 47, pp. 229–236.
25. Taurian, T., Anzuay, M., Angelini, J., Tonelli, M., Luduena, L., Pena, D., Ibanez, F., and Fabra, A., Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities, *Plant Soil*, 2010, vol. 329, pp. 421–431.
26. Spaepen, S. and Vanderleyden, J., Auxin and plant-microbe interactions, *Cold Spring Harb. Perspect. Biol.,* 2011, vol. 3, a001438.
27. Byers, B.R., Powell, M.V., and Lankford, C.E., Iron-chelating hydroxamic acid (schizokinen) active in initiation of cell division in *Bacillus megaterium, J. Bacteriol.*, 1967, vol. 93, pp. 286–294.
28. Ollinger, J., Song, K.B., Antelmann, H., Hecker, M., and Hermann, J.D., Role of the Fur regulon in iron transport in *Bacillus subtilis, J. Bacteriol.*, 2006, vol. 188, pp. 3664–3673.
29. Lemanceau, P., Bauer, P., Kraemer, S., and Briat, J.F., Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes, *Plant Soil*, 2009, vol. 321, pp. 513–535.
30. Dawwam, G.E., Elbeltagy, A., Emara, H.M., Abbas, I.H., and Hassan, M.M., Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant, *Ann. Agric. Sci.*, 2013, vol. 58, pp. 195–201.
31. Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A., and Young, C.C., Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities, *Appl. Soil Ecol.*, 2006, vol. 34, pp. 33–41.
32. Lee, J.C., Kim, C.J., and Yoon, K.H., *Paenibacillus telluris* sp. nov., a novel phosphate-solubilizing bacterium isolated from soil, *J. Microbiol.*, 2011, vol. 49, pp. 617–621.
33. Ahemad, M. and Khan, M., Biotoxic impact of fungicides on plant growth promoting activities of phosphate-solubilizing *Klebsiella* sp. isolated from mustard (*Brassica campestris*) rhizosphere, *J. Pest. Sci.*, 2012, vol. 85, pp. 29–36.
34. Chernin, L. and Chet, I., Microbial enzymes in biocontrol of plant pathogens and pests, in *Enzymes in the Environment: Activity, Ecology and Applications,* Burns, R.G. and Dick, R.P., Eds., New York: Marcel Dekker, 2002, pp. 171–225.
35. Reinhold-Hurek, B. and Hurek, T., Life in grasses: diazotrophic endophytes, *Trends Microbiol.*, 1998, vol. 6, pp. 139–144.
36. Raju, N.S., Niranjana, S.R., Janardhana, G.R., Prakash, H.S., Shetty, H.S., and Mathur, S.B., Improvement of seed quality and field emergence of *Fusarium moniliforme* infected sorghum seeds using biological agents, *J. Sci. Food Agric.*, 1999, vol. 79, pp. 206–212.
37. Long, H.H., Schmidt, D.D., and Baldwin, I.T., Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses, *PLoS One*, 2008, vol. 3, e2702.
38. Stajkovic, O., Isolation and characterization of endophytic non-rhizobial bacteria from root nodules of alfalfa (*Medicago sativa* L.), *Bot. Serb.*, 2009, vol. 33, pp. 107–114.
39. Zhao, L.F., Xu, Y.J., Ma, Z.Q., Deng, Z.S., Shan, C.J., and Wei, G.H., Colonization and plant growth promoting characterization of endophytic *Pseudomonas chlororaphis* strain Zongl isolated from *Sophora alopecuroides* root nodules, *Braz. J. Microbiol.,* 2013, vol. 44, pp. 623–631.
40. Liu, Z.L. and Sinclair, J.B., Colonization of soybean roots by *Bacillus megaterium* B153-2-2, *Soil Biol. Biochem.*, 1993, vol. 25, pp. 849–855.
41. Nagarajkumar, M., Bhaskaran, R., and Velazhahan, R., Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen, *Microbiol. Res.*, 2004, vol. 159, pp. 73–81.
42. Li, J.H., Wang, E.T., Chen, W.F., and Chen, W.X., Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China, *Soil Biol. Biochem.*, 2008, vol. 40, pp. 238–246.
43. Seghers, D., Wittebolle, L., Top, E.M., Verstraete, W., and Siciliano, S.D., Impact of agricultural practices on the *Zea mays* L. endophytic community, *Environ. Microbiol.*, 2004, vol. 70, pp. 1475–1482.
44. Bremer, C., Braker, G., Matthies, D., Beierkuhnlein, C., and Conrad, R., Plant presence and species combination, but not diversity, influence denitrifier activity and the composition of nirK-type denitrifier communities in grassland soil, *FEMS Microbiol. Ecol.*, 2009, vol. 70, pp. 377–387.

SUPPLEMENTARY DATA

SI: IAA Estimation

The production of IAA by endophytes was determined as described (Gordon and Weber, 1951) by col-

orimetric measurement at 530 nm. The cultures grown in dark for 3 d were centrifuged at 13000 *g* for 10 min and 1 mL supernatant was mixed with 2 mL 0.01 M $FeCl_3$ in 35% $HClO_4$ and incubated in dark for 25 min (Gordon and Webber, 1951). The absorbance was read off a standard curve prepared using pure IAA (Sigma Chemical Co.) concentration (range of 10–100 μg mL^{-1}).

S2: Siderophore Production

Siderophore production by endophytes was observed under Fe^{3+} limiting conditions as described by Schwyn and Neilands (1987). The culture supernatant was obtained from bacteria grown in (deferrated MM9 minimal medium supplemented with CAS) with centrifiigation at 8800 *g* for 10 min. The culture supernatant was spotted (50 μL) on CAS (chrome azurol S) plates and incubated at 28 ± 2°C. The change in colour of the media (blue to yellow-orange) was considered positive siderophore activity. The composition of CAS medium for a litre of overlay was: Chrome azurol S (CAS) 60.5 mg, hexadecyltrimethylammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 mL. Siderophore production was quantitated according to Khan et al. (2006).

S3: Phosphate Solubilization

The phosphate solubilization by individual endophytes was determined by plating onto Pikovskaya's agar as described (Pikovskaya, 1948) and incubation at 28 ± 2°C for 72 h for formation of clear halo around colonies solubilizing phosphate. Log phase endophytic cultures that produced zone of clearance on Pikovasky's agar were inoculated in Pikovskaya's broth and incubated at 28 ± 2°C. Inorganic P concentration was estimated in culture supernatant every 24 h for 5 days at 820 nm and expressed as mg mL^{-1} (Ames 1966).