JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1597-1609

Studies on Factors Affecting Root Colonization of Root and Nodule Endophytic Bacteria from Mungbean Rhizosphere

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(Received: 06 April 2015; accepted: 09 May 2015)

The present study was designed to study the factors affecting the root colonization by plant growth promoting endophytic bacteria. A total of 17 isolates (8 from root and 9 from nodule) of endophytic bacteria from mungbean rhizosphere were selected on the basis of their multifunctional traits and further studied for root colonization and growth parameters under *in-vitro* conditions. Highest biofilm formation was calculated at 12 hrs ranged between 0.180 to 0.411 (OD 595nm). Enhancement in autoaggregation was recorded after 24hrs (25.6 to 45.3%). Endophytic reactivity to antibiotics revealed that 58.8% of isolates were resistant to ampicillin (10 µg disc^{-1}). All the cultures exhibited significant increase in percent seed germination ranged from 80.8% to 90.0% in mungbean variety SML 668 and SML832 (except LREB1) as compared to nonbacterized control. Endophytic bacterization improved total length and fresh weight of seedlings in mungbean. Root endophytic *Klebsiella* sp. LREK25, nodule endophytic *Bacillus* sp. LNEB1 and *Pseudomonas* sp. LNEP16 were found to be most promising PGPR showing highest root colonization and seed vigor index. Phenotypic characteristics and genotypic distinctiveness of isolated strain from other phylogenetic neighbours of *Klebsiella*, *Bacillus* and *Pseudomonas* species, strain LREK25, LNEB1 and LNEP16 have been designated as *Klebsiella* sp. LREK25 (GenBank accession number KF424316), *Bacillus aryabhattai* LNEB1 (KF853102) and *Pseudomonas aeruginosa* LNEP16 (KF853103). These potential endophytes can be considered as highly competent microorganisms for better colonization, competitiveness and establishment in rhizosphere for improving mungbean productivity.

Key words: Biofilm, Endophytic bacteria, Mungbean, Vigor index.

The colonization of plant roots by soil-borne and introduced bacteria is a very important step in establishing an effective plant bacterial interaction. The success of inoculating seeds or seedlings with beneficial bacteria usually depends upon the colonization potential of introduced strains[1,2]. Beneficial endophytic rhizobacteria with plant growth promoting (PGP) traits are proposed as promising inocula for agriculture. Despite their potential as PGPR with low-input practical agents of plant growth promotion and application of Plant growth promoting rhizobacteria (PGPR) has been hampered by inconsistent performance in ûeld tests. This is usually attributed to their poor rhizosphere competence[3]. Rhizosphere competence of PGPR comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microûora[1]. Colonization of roots by inoculant strains seems to be a critical step in the interaction between beneficial bacteria and host plants. Endophytic PGPR include a broad spectrum of bacteria belonging to genera

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including *Acetobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Klebsiella, Pseudomonas* and *Serratia* etc. present in rhizosphere have beneficial effects on the host plants owing to their biological control traits[4], plant growth promotion[5], competition for nutrients and niches[6] and induction of systematic resistance in the host plant[7]. Bacterial endophytes and soil rhizospheric bacteria colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents[8]. This diverse group of bacteria not only out-compete with the others for rhizospheric establishments, but complement functionally for plant growth promotion. These bacteria live in consortia bound to surfaces such as in biofilms, flocs or granules at an early stage of development influenced enhancement of seedling germination and plant vigour even under adverse conditions[9]. Bacterial root colonization often starts with the recognition of specific compounds in the root exudates by the bacteria[10]. These compounds also play major roles in belowground community interactions[11]. Theoretically, plants simultaneously communicate with commensalistic, mutualistic, symbiotic and pathogenic microorganisms via compounds exuded by their roots[12]. However, it has been suggested that plants can communicate to specifically attract microorganisms for their own ecological and evolutionary benefit[1,13]. The ability of soil bacteria to approach plant roots via chemotaxis-induced motility and effectively colonize these via attachment, biofilm formation, autoaggregation and microcolony formation is probably among the strongest deterministic factors for successful endophytic colonization[1]. Besides organic and amino acids, plant secondary metabolites, especially flavonoids, have been proposed as important chemoattractants for endophytic colonization. The root surface and surrounding rhizosphere are signiûcant carbon sinks. Photosynthate allocation to this zone can be as high as 40%[14]. Competent bacterial endophytes in the vicinity of plant roots need to gear their metabolisms towards a physiological state that enables optimal nutrient acquisition, niche adaptation and competition[15]. Endophytic bacteria with high intrinsic antibiotic resiatance provide better competiton to other native bacteria and resulted into successful colonization of root/ nodule tissue. Several studies on gene expression in rhizobacteria have shown that the genes involved in nutrient acquisition and stress adaptation, next to activation of transcriptional regulators, are among the first responders when bacteria are exposed to root exudate compounds[16-18]. Hence, bacterial traits involved in the response to environmental stimuli, communication, niche adaptation and plant colonization are important for successful interactions with the plant, in a complex process. Thus, along root surfaces there are various suitable nutrient rich niches attracting a great diversity of microorganisms, including phytopathogens. Given the importance of rhizosphere competence as a prerequisite for selecting their effective PGP traits and better understanding of root-microbe communication will signiûcantly contribute in improving the efûcacy of endophytic PGPR. Therefore, the present study was devised to study the effect of biofilm formation, autoaggregation and Intrinsic Antibiotic Resistance (IAR) spectra of mungbean endophytic PGPR on seed germination and vigor index in mungbean (*Vigna radiata* L. Wilczek) *in vitro*.

MATERIALS AND METHODS

Selection of mungbean endophytic PGPR

A total of 8 isolates of root and 9 of nodule endophytic bacteria from mungbean rhizosphere were selected on the basis of their multifunctional traits as given in Table no.1 .

Biofilm formation assay

Each endophytic strain was grown in 10 ml of LB medium at 28ºC overnight. After vortexing, 100 ¼l volumes were transferred into Poly vinyl chloride (PVC) microtiter plate wells. After different incubation period (at 0,2,4,6 and 12 hrs), medium was removed from wells and microtiter plate wells was washed five times with sterile distilled water to remove loosely associated bacteria. Plates were air dried for 45 min and each well stained with 150 ¼l of 1% crystal violet solution in water for 45 min. Biofilm was visible as purple rings formed on the side of each well. The quantitative analysis of biofilm production was performed by adding 200

¼l of 95% ethanol to destain the wells. One hundred microliters from each well was transferred to a eppendorf tube and volume brought to 1 ml with distilled water. The level (OD) of the crystal violet present in the destaining solution was measured at 595nm[19].

Autoaggregation assay

Autoaggregation assays were performed according to Kos et al.[20], with minor modifications. Endophytic species were grown in Luria Bertanni broth (LB) at 28ºC to give cultures of 1×10^8 $CFUmL^{-1}$. The cells were harvested by centrifugation at 5000 g for 15 min, washed twice, and resuspended in phosphate buffered saline (PBS). Cell suspensions were mixed by vortexing, and autoaggregation was determined during 24 h of static incubation at room temperatures. After intervals of 6 h and 24 h , 0.1mL of the upper layer of the bacterial suspension was transferred to another tube with 3.9mL of PBS, and the absorbance (A) was measured at 600 nm. Autoaggregation percentage is expressed as $1-(A_t/A_0)/ 100$, where A_t represents the absorbance at different sampling times, and A_0 the absorbance at t=0.

Intrinsic Antibiotic Resistance (IAR) spectra

Intrinsic antibiotic spectra (IAR) test was carried out to identify the bacterial sensitivity or resistance to antibiotics. In order to check the sensitivity of the isolate towards different antibiotics, disc diffusion was done where culture was grown on NA in the presence of sterile filter paper discs (HiMedia, Mumbai) impregnated with different concentration of antibiotics viz. tetracycline (30 µg $disc^{-1}$), ampicillin (10 µg $disc^{-1}$), kanamycin (30 µg $disc^{-1}$), erythromycin (15 µg $disc^{-1}$), chloramphenicol (25 µg $disc^{-1}$), amoxycillin (10 µg $disc^{-1}$) and streptomycin (25 µg $disc^{-1}$) After 48 h incubation of plate at 28 °C, either development of zone of inhibition was observed and interpreted as sensitive (S) or resistant (R)[21].

Seed Germination and vigor index assay by roll towel method

Seed germination studies were conducted using paper towel method[22]. One hundred mungbean seeds of each variety; SML668 and SML832 were surface sterilized[23] and treated with different endophytic cultures as per treatment. Paper towel moistened with distilled water was stretched on a clean table and 10 seeds were arranged in a row on it. The paper towel was rolled and placed vertically in a seed germinator in upward position at 25-30ºC. After 5 days, percent seed germination , root-shoot length and fresh weight of were recorded. Seed vigour index was calculated by using formula:

Seed vigor index I= Per cent seed germination × (shoot length+ root length)

Seed vigor index II= Per cent seed germination × (fresh shoot weight+ fresh root weight)

Molecular identification of promising root/ nodule endophytic isolate

On the basis of PGP activities, root endophytic *Klebsiella* sp. LREK25 with GenBank accession number KF424316 (Data not shown), nodule endophytic *Bacillus* sp. LNEB1 (Data not shown) and *Pseudomonas* sp. LNEP16 were further identified by 16S rRNA based phylogenetic analysis. The 16S rRNA gene sequencing study was performed by Xcelris 178 Labs Limited, Ahmedabad (India). DNA was isolated from bacterial strain KG1 by using QIAamp DNA Purification Kit (Qiagen). The 16S rRNA gene was amplified by using PCR method with universal bacterial primers 8F (AGA GTT TGA TCC TGG CTC AG) and 1492R (ACG GCT ACC TTG TTA CGA CTT). Amplified PCR product was purified using Qiagen Mini elute Gel extraction kit according to the manufacturer's protocol. Sequencing of the purified 16S rRNA gene was performed using BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) as recommended by manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically using ABI 3730xl Genetic Analyzer (Applied Biosystem, USA). The 16S rRNA gene sequence of the strain LNEB1 was processed manually, analyzed at NCBI (National Centre for Biotechnology Information) server (http://www.ncbi.nlm.nih.gov) using BLAST tool and compared to the corresponding neighbour sequences from the GenBank-NCBI database. Multiple alignment of the strain KG1 was performed with related *Klebsiella, Bacillus* and *Pseudomonas* species (from GenBank-NCBI database) using Multalin program and phylogenetic tree was constructed by the neighbor joining method[24,25]. Evolutionary distance matrices for the neighbor joining method were

calculated using the algorithm of Kimuras two-parameter model[26]. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1000 replicates. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of the strain LNEP16 and related *Pseudomonas* species are shown in Figure 6.

Statistical Analysis

All data were analyzed statistically by analysis of variance (ANOVA). Significant difference among different treatment ($P<0.05$) was calculated by Tukey's Honestly significant test using SAS software.

RESULTS AND DISCUSSION

Biofilm formation is a dynamic process and different mechanisms are involved in their attachment and growth. The initiation of biofilm formation was assayed by the ability of 8 isolates of root endophytic and 9 isolates of nodule endophytic bacteria with attachment to the wells of microtiter plates formed of polyvinylchloride (PVC). The biofilm was detected by staining with crystal violet (CV) (Figure 1). Formation of colored ring stained with crystal violet at the interface between air and liquid indicated the biofilm formation (Figure 2). Further biofilm formation was quantitatively measured by level (OD) of the crystal violet present in the destaining solution at 595 nm. In the present study, increase in amount of crystal violet in destaining solution was observed with increase in time. At 0 hr, very low amount of CV was detected which increased with increase in time. Highest OD (595) was calculated at 12 hrs ranged between 0.180 to 0.411, being maximum for *Klebsiella* sp. LREK25 (OD= 0.411) followed by *Bacillus* sp. LNEB1 (0.370) and *Pseudomonas* sp. LNEP16 (0.321). Likewise, several researchers also reported successful colonization of roots and plant tissue by biofilm forming rhizobacteria[27-29]. Our results are in agreement with the earlier report where non-rhizobial mungbean bacterial isolates, M2, M4, M5 and M6 showed high efficiency in colonization on roots and biofilm formation on abiotic surface[27]. Our study in congurence with the earlier finding where *Pseudomonas putida* can respond rapidly to the present of root exudates in soil, converging at root colonization sites and establishing stable biofilm[30]. Previous studies on Plant-growth- promoting *Pseudomonas*, *Bacillus* and *Klebsiella* have been reported to discontinuously colonize the root surfaces[28,29]. *Azospirillum brasilense* and related species are motile heterotrophic proteobacteria that interact with roots of a variety of cereals such as wheat and maize, and often promote the growth of their host plant[31]. Bacterial biofilms established on plant roots could protect the colonization sites and act as a sink for the nutrients in the rhizosphere, hence reducing the availability of root exudate nutritional elements for pathogen stimulation or subsequent colonization on the root[32]. The ability of *nifH* gene containing endophytic PGPR to promote plant growth seems to be related to stimulation of root proliferation, rather than providing fixed nitrogen to the plant. Besides the Gram negative, Gram-positive microbes also effectively colonize the rhizophere and are well represented in plant rhizosphere[33]. Biofilm formation by different strains in present study could increase early root-microbe association and function as structures resistant against stress factors such as desiccation, UV radiation, predation, and antibiosis, which help in creating protective niches for mungbean rhizobia.

In order to elucidate whether some bacterial traits might be responsible for the low degree of rhizobial root attachment, we performed studies of autoaggregation as bacterial properties linked to surface adhesion. In the present study, the percentage of autoaggregation varied from 8.3% to 19.7% after 6 hrs whereas 25.6 to 45.3% of autoaggregation was observed after 24hrs (Figure 3). The highest percentage of autoaggregation was shown by *Klebsiella* sp. LREK25 (17.3-45.3%) followed by *Bacillus* sp. LNEB1 (15.6-44.0%) and *Pseudomonas* sp. LNEP16 (15.1-40.0%). Similarly, Albareda et al.[15] observed highest percentage of autoaggregation shown by *E. fredii* strains SMH12 (47–62%) and HH103 (65–88%) after 24 h of incubation. Results are well documented with earlier findings where bacterial ability for autoaggregation is well correlated with adhesion to different surfaces [20,34]. Our results are also in agreement with those obtained by Kos et al.[20] with

Lactobacillus strains. In present study, highest autoaggragation percentage was shown with endophytic bacteria which also record highest biofilm formation indicated the direct relation of biofilm formation with their autoaggregation ability. However, results are in contrast with the findings of Zaady and Okon[34] who reported that *Azospirillum* cell treatments leading to strong inhibition of aggregation culminate in the greatest adsorption to maize roots.

Antibiotics reactivity of 17 selected endophytes from mungbean rhizosphere revealed that 58.8 % of isolates were resistant to ampicillin whereas 41.2% showed resistance to tetracycline and chloramphenicol and 29.4% rhizobacterial isolates were resistant to kanamycin and streptomycin (Table 2). Resistance to erythromycin and amoxicillin was shown by 35.3% and 23.5% of isolates, respectively (Figure 4). Resistance of PGPR to several antibiotics might have an ecological advantage of survival in the rhizosphere when they introduced as inoculum . The data was supported by Siddiqui et a.l[35] where most of the isolates of *Pseudomonas* sp. were resistant to ampicillin. Kundu et al.[36] in their studies found that isolates belong to genera *Pseudomonas* from chickpea, wheat and mustard rhizosphere were resistant to ampicillin. They also reported that these isolates were resistant to tetracycline and kanamycin. Similarly, several workers revealed that rhizobacterial isolates were intrinsically resistant to antibiotics tested[35,37]. Inherent capacity of resistance to antibiotics might facilitate the endophytic bacteria in colonization through biofilm formation possibly by process of antibiosis against phytopathogens[38].

Increase in the seed germination, total length and weight of seedlings are considered as important PGP properties of endophytic rhizobacteria due to their effective root colonizing ability. The seed germination experiment was conducted by bacterization of mungbean seeds with different endophytic bacteria *in vitro* condition. Total length and fresh weight of seedling was measured for calculating the seed vigour index (Table 3). All the cultures except LREB1 exhibited significant increase in percent seed germination varied from 80.3 to

Table 1. Multifunctional traits of selected mungbean endophytic PGPR

Bacterial Endophytes	P Solubilization	IAA	OAP	*nifH* gene	Siderophore	HCN	Cellulase	Protease	ACC deaminase
LREB1	+	+	+	+	+	-	+	-	+
LREB4	+	+	-	-	-	-	+	-	+
LREP12	+	+	+	+	+	-	+	+	+
LREK19	+	+	-	+	+	-	+	+	+
LREK21	+	+	-	+	+	-	-	+	+
LREK22	+	-	-	+	-	+	-	+	+
LREK24	+	+	+	+	+	-	+	-	+
LREK25	+	+	+	+	+	+	+	+	+
LNEB1	+	+	+	+	+	+	+	+	+
LNEB4	+	+	-	-	+	+	-	-	+
LNEP10	+	+	-	+	-	-	+	+	+
LNEP11	+	+	-	-	-	-	+	+	+
LNEP12	+	+	+	+	+	-	+	+	+
LNEP13	+	+	-	-	-	-	+	+	+
LNEP16	+	+	+	+	+	+	+	+	+
LNEK32	+	+	+	+	+	+	+	+	+
LNEK37	+	+	+	+	-	-	+	+	+

P-solubilisation= Phosphate solubilisation, IAA=Indole acetic acid production, OAP- Organic acid Production, ACC deaminase= 1-amino-cyclopropane-carboxlic acid

LREB1 and LREB4= Root endophytic *Bacillus* spp. , LREP12= Root endophytic *Pseudomonas* sp., LREK19, LREK21, LREK22, LREK24, LREK25= Root endophytic *Klebsiella* spp.

LNEB1 and LNEB4= Nodule endophytic *Bacillus* spp., LNEP10, LNEP11, LNEP12, LNEP13, LNEP16= Nodule endophytic *Pseudomonas* spp. , LNEK32 and LNEK37= Nodule endophytic *Klebsiella* spp

Table 2. Intrinsic antibiotic spectra of mungbean endophytic bacteria

Bacterial Endophytes	Tetracycline	Amphicillin	Kanamycin	Erythromycin	Chloramphenicol	Amoxycillin	Streptomycin
LREB1	R	R	R	S	S	S	S
LREB4	S	R	S	R	S	S	S
LREP12	S	S	R	R	S	S	R
LREK19	R	S	R	S	S	S	R
LREK21	R	S	R	S	R	S	S
LREK22	S	R	S	S	S	R	R
LREK24	S	S	R	R	S	S	S
LREK25	R	R	S	R	S	R	S
LNEB1	R	R	S	R	R	S	R
LNEB4	R	R	S	R	S	S	R
LNEP10	S	R	S	S	R	S	S
LNEP11	R	S	S	S	S	S	S
LNEP12	S	S	S	S	S	R	S
LNEP13	S	R	S	S	R	S	S
LNEP16	S	R	S	S	R	R	S
LNEK32	S	R	S	S	R	S	S
LNEK37	S	S	S	S	R	S	S
% resistance of isolates	41.2	58.8	29.4	35.3	41.2	23.5	29.4

Table 3. Effect of endophytic bacteria on growth parameters in mungbean

Bacterial	Seed germination (%)			Plumule length (cm)			Radical length (cm)			Fresh weight of seedling (g)		
Endophytes	SML 668	SML 832	Mean	SML668	SML832	Mean	SML668	SML 832	Mean	SML668	SML 832	Mean
Control	79.3	78.4	78.8	5.8	6.7	6.3	1.9	2.3	2.1	0.141	0.164	0.152
LREB1	80.3	81.4	80.8	7.0	8.6	7.8	2.4	2.6	2.5	0.213	0.226	0.219
LREB4	86.9	86.5	86.7	11.5	12.9	12.2	3.2	3.7	3.4	0.298	0.319	0.308
LREP12	84.4	85.3	84.8	9.9	10.8	10.1	3.0	3.4	3.2	0.265	0.279	0.272
LREK19	85.9	86.7	86.3	10.6	11.7	11.1	3.1	3.5	3.3	0.286	0.304	0.295
LREK21	85.9	83.9	84.9	10.3	11.5	10.9	3.2	3.6	3.4	0.270	0.288	0.279
LREK22	87.6	88.4	88.0	11.9	13.6	12.7	3.6	4.0	3.8	0.323	0.340	0.331
LREK24	88.4	89.1	88.9	12.5	13.8	13.1	3.5	4.0	3.7	0.383	0.430	0.406
LREK25	89.7	90.3	90.0	14.6	15.3	14.9	4.4	5.0	4.6	0.460	0.487	0.474
LNEB1	88.9	89.9	89.4	13.5	14.9	14.2	4.3	4.6	4.4	0.427	0.452	0.439
LNEB4	87.0	87.2	87.1	11.7	13.5	12.6	3.3	3.9	3.6	0.308	0.327	0.317
LNEP10	82.4	84.5	83.4	8.4	9.8	9.0	2.9	3.2	3.0	0.225	0.247	0.235
LNEP11	83.9	85.1	84.5	9.6	10.8	10.2	3.1	3.3	3.2	0.258	0.275	0.266
LNEP12	81.2	83.2	82.2	8.0	9.5	8.7	2.6	3.0	2.8	0.220	0.237	0.236
LNEP13	82.5	83.8	83.2	9.1	10.4	9.75	3.1	3.3	3.2	0.235	0.256	0.245
LNEP16	88.6	89.6	87.7	13.0	14.7	13.8	3.9	4.2	4.0	0.356	0.382	0.369
LNEK32	83.6	84.2	83.9	9.4	10.7	9.9	3.3	3.5	3.4	0.248	0.269	0.258
LNEK37	86.6	87.4	87.0	10.9	11.8	11.3	3.4	3.7	3.5	0.290	0.314	0.302
Mean	85.1	85.7		10.4	11.6		3.2	3.5		0.289	0.311	
P<0.05	V=NS,T=1.73,V×T=NS			V=0.45,T=1.36,V×T=NS			V=0.13,T=0.41,V×T=NS			V= 0.031,T=0.091 ,V×T=NS		

Variety: V, Treatment:T

89.7% in SML 668 whereas 81.4 to 90.3% in SML 832 as compared to nonbacterized control. In both varieties, highest seed germination was obtained with *Klebsiella* sp. LREK25 (89.7% in SML 668 and 90.3% in SML 832) followed by *Bacillus* sp. LNEB1 (88.9% in SML 668 and 89.9% in SML 832) and *Pseudomonas* sp. LNEP16 (88.6% in SML 668 and 89.6% in SML 832).

All the cultures resulted into increase in total length and fresh weight of seedlings as compared to nonbacterized control (Figure 5). Of the selected root and nodule endophytes, significantly maximum plumule length was obtained by bacterization with root endophytic *Klebsiella* sp. LREK25 in both varieties i.e 14.6 cm in SML 668 and 15.3 cm in SML 832 followed by *Bacillus* sp. LNEB1 i.e 13.5 cm to 14.9 cm in SML 668 and in SML 832 as compared to control. Similar pattern was observed for radical length being significantly highest with root endophytic *Klebsiella* sp. LREK25 i.e 4.4 cm in SML 668 and 5.0 cm in SML 832. All the cultures resulted increase in total length and fresh weight of seedlings as compared to nonbacterized control. Total fresh weight of seedlings varied from 0.213 g to 0.460 g in SML 668 and 0.226 g to 0.487 g in SML 832 as compared to uninoculated control. Vigor index determines the state of the health of seedling and productivity of the plant. Among all the selected endophytes, root endophytic *Klebsiella* sp. LREK25 found to be most promising PGPR producing significantly highest seed vigor index I (1704.3 in SML 668 and 1833.1 in SML 832) and II (41.3 in SML 668 and 43.9 in SML 832) as compared to control (Table 4). However, difference among varieties was found to be nonsignificant. Ability of endophytic bacteria to autoaggregate and forming biofilm, on seed surface might have contributed in effective colonization and possibly improved seed germination and seed vigor index in the present study. Highest seed germination and growth parameters were also well correlated with highest biofilm, autoaggregation and IAR spectra with endophytic bacteria. Colonization success of PGP rhizobacteria reportedly increases the growth and fitness of many host plant species[39,40]. Several workers also reported better colonization, seed germination and plant growth by biofilm forming

Table 4. Effect of endophytic bacteria on vigor index in mungbean

Bacterial Endophytes	Vigor Index I			Vigor Index II		
	SML668	SML 832	Mean	SML668	SML832	Mean
Control	610.6	705.6	658.1	11.2	12.8	12.0
LREB1	754.8	911.6	833.2	17.1	18.4	17.7
LREB4	1277.4	1435.9	1356.6	25.9	27.6	26.4
LREP12	1088.7	1211.3	1150.0	22.4	23.8	23.1
LREK19	1176.3	1317.8	1247.0	24.6	26.6	25.6
LREK21	1159.6	1266.8	1213.2	23.2	24.2	23.7
LREK22	1357.8	1555.8	1456.8	28.3	30.1	29.2
LREK24	1419.2	1585.9	1502.5	31.3	33.5	32.4
LREK25	1704.3	1833.1	1768.7	41.3	43.9	42.6
LNEB1	1582.4	1753.0	1667.7	37.9	40.6	39.2
LNEB4	1305.0	1517.3	1411.1	26.8	28.5	27.6
LNEP10	931.1	1098.5	1014.8	18.5	20.9	19.7
LNEP11	1065.5	1199.9	1132.7	21.6	23.4	22.5
LNEP12	860.7	1040.0	950.3	17.8	19.7	18.7
LNEP13	1006.5	1148.1	1077.3	19.4	21.6	20.5
LNEP16	1485.5	1655.6	1570.5	33.9	38.3	35.8
LNEK32	1061.7	1195.6	1128.6	20.7	22.6	21.6
LNEK37	1238.3	1354.7	1296.5	25.1	27.4	26.2
Mean	1171.4	1321.4		24.7	26.9	
P<0.05	V=8.82,T=26.4,V×T=37.4			V=1.11,T=3.35,V×T=NS		

Variety:V, Treatment: T

and autoaggregating rhizobacteria[18,20,27] . Our results are in agreement with the results of Stajkovic et al.[9] who reported the positive effect of non-rhizobial nodule endophytic *Bacillus*, *Brevibacillus* and *Microbacterium* sp. on shoot length, shoot and root dry weight of alfalfa (*Medicago sativa* L.). Similarly, Tariq et al.[27] also reported biofilm forming non-rhizobial endophytic and showed very high root surface colonization in mungbean. Further, root colonization was directly related to their ability of forming highly efficient biofilm on abiotic surface. Significant reduction in the incidence of root rot and increased per cent seed germination and plant height in mungbean inoculated with endophytic *Burkholderia* sp. strain TNAU-1 well supported present study of using root and nodule endophytic bacteria with biocontrol activities (siderophore, HCN, cellulase, and protease production)[22]. This proves to be a promising feature since the endophytic movement from the point of application to the internal tissues provide a relatively uniform and protected environment and by colonizing the internal tissues, can exclude the entry of a pathogen and ineffective bacteria[22]. The results obtained in present study are in agreement with findings of Malleshwari and Bagyanarayana[41] who reported that all the isolates of PGPR (*Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas*) used in study, significantly enhanced seed germination and seedling vigor of sorghum, maize and green gram seeds.

Highest seed germination and growth parameters can also be contributed by PGP mechanisms of endophytic bacteria which are thought to be similar to those of PGP rhizobacteria; namely, they affect plant growth by producing phytohormones, such as cytokinins or auxins, by degrading hormone precursors, such as ACC by ACC deaminase, by suppression of

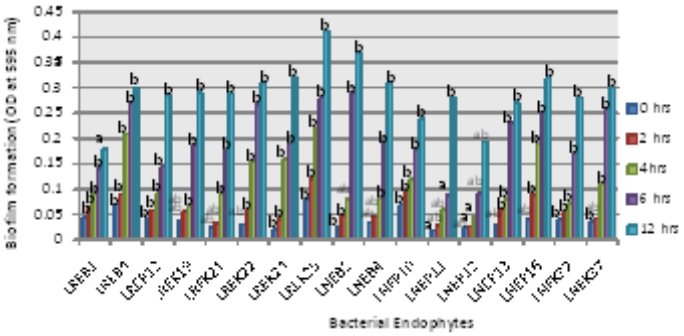

Fig. 1. Each bar represent the mean of triplicate values at different hours. Bars with different letters was statistically differed at 5% significant level. Letter 'a' represents the lowest value, 'b' represents the significant difference between treatments and 'ab' represents nonsignificant difference between treatments

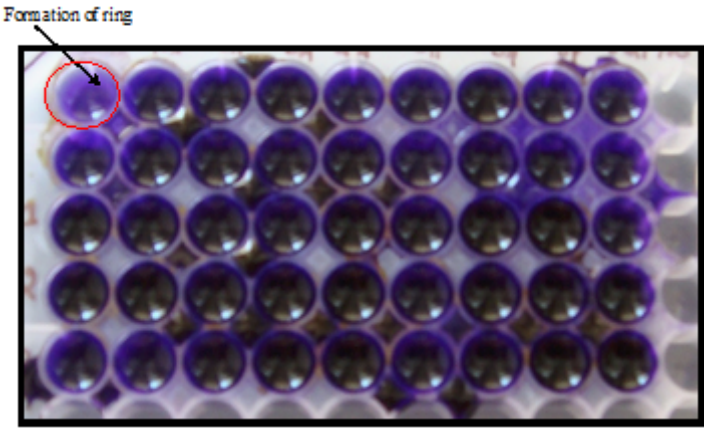

Fig. 2. Biofilm formation by endophytic bacteria on microtiter plate

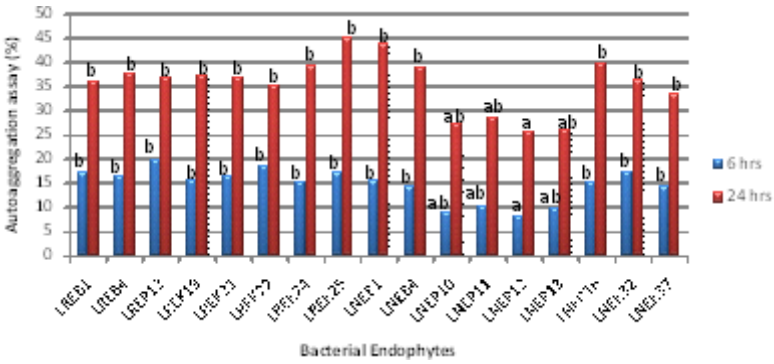

Fig. 3. Each bar represent the mean of triplicate values at different hours. Bars with different letters was statistically differed at 5% significant level. Letter 'a' represents the lowest value, 'b' represents the significant difference between treatments and 'ab' represents nonsignificant difference between treatments

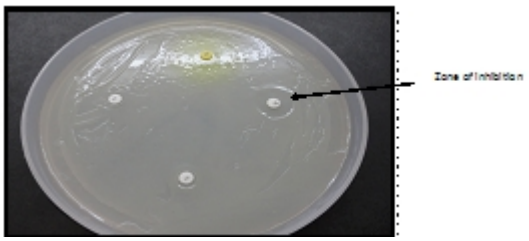

Fig. 4. Intrinsic antibiotic spectra of endophytic bacteria

Fig. 5. Effect of root and nodule endophytic bacteria on seedling length

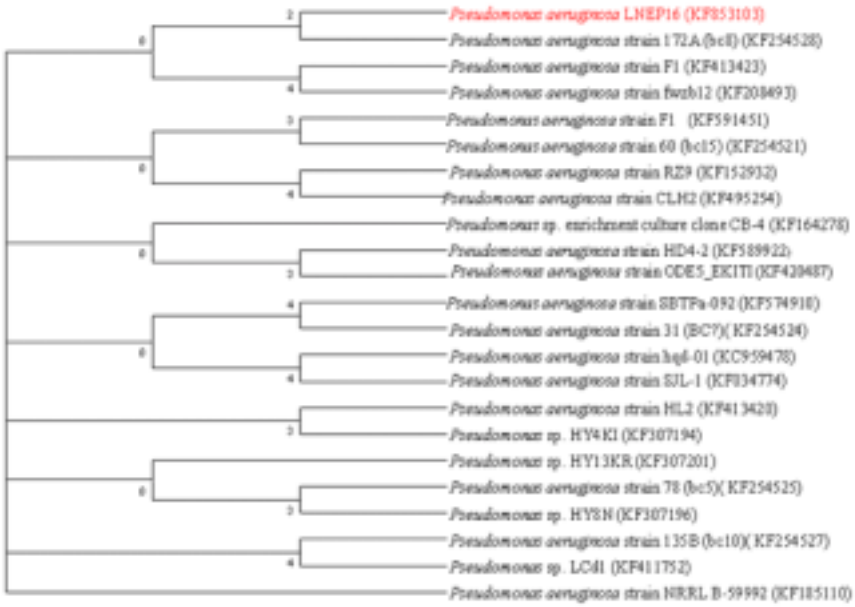

Fig. 6. Phylogenetic tree showing the relationships of the identified bacterial strain and the type strains of closely related *Pseudomonas* species, constructed using the neighbour-joining method based on 16S rRNA gene sequences. GenBank accession numbers are given in parentheses. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points.

deleterious microorganisms, phosphate solubilisation and promotion of mineral uptake[5,9]. This is largely supported in present study as selected root and nodule endophytic bacteria from mungbean rhizosphere solubilized phosphate, produced IAA and ACC deaminase. These selected endophytes showed multiple PGP traits and stress tolerant activities for better colonization and growth promotion of plant. Endophytes producing IAA are often found to promote root growth in an auxin dependant manner, although exact results on host roots depends on the amount of auxin produced, the presence of other interacting hormones, and plant sensitivity to IAA. Similarly, the present findings also agreed with results obtained by Patten and Glick[42] where *Pseudomonas putida* GR12-2 is able to stimulate up to 50% greater root elongation and adventitious root formation in mungbean, but this trait was reduced in ipdc insertion mutants deficient in auxin production. Another important mechanism of endophyte-dependent root growth promotion is through the reduction of the volatile plant hormone, ethylene by production of ACC deaminase enzyme which results into cleavage of ACC, immediate precursor of ethylene[43]. Ethylene affects roots by inhibiting elongation, promoting lateral root growth and stimulating root hair formation[44]. In this way, ethylene in roots antagonizes auxin function and reduces root surface area available for nutrient absorption. Similarly, Changes in root growth of *S. nigrum* are clearly correlated to the production of IAA and ACC deaminase by endophytic bacteria[45]. Other microbially produced phytohormones, including cytokinins and gibberellins (GA), can alter growth but have not been widely reported as root specific growth promoting mechanisms by endophytes[46].

Molecular characterization and phylogenetic analysis of endophytic bacteria LREK25 (root), LNEB1 (nodule) and LNEP16 (nodule) by 16s rRNA technique revealed 99% similarity to *Klebsiella* sp. (data not shown), 100% similarity to *Bacillus aryabhattai* (data not shown) and 100% to *Pseudomonas aeruginosa* (data given in figure 6) respectively. The morphological, biochemical and phylogenetic analysis suggested that strain LREK25, LNEB1 and LNEP16 are members of the genera *Klebsiella*, *Bacillus* and *Pseudomonas* respectively and designated as *Klebsiella* sp. LREK25 (GenBank accession number KF424316), *Bacillus aryabhattai* LNEB1 (KF853102) and *Pseudomonas aeruginosa* LNEP16 (KF853103).

Among all the selected endophytes, root endophytic *Klebsiella* sp. LREK25, nodule endophytic *Bacillus* sp. LNEB1 and *Pseudomonas* sp. LNEP16 can be considered as potential microorganisms producing large amount of biofilm, autoaggregation, IAR spectra and growth parameters (seed germination, seedling length, fresh weight of seedling and seed vigor index I and II). These endophytes can be promoted as highly robust microorganisms for better colonization and competitiveness in mungbean rhizosphere.

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