Isolation and characterization of plant growth promoting endophytic bacteria isolated from *Vigna radiata*

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ABSTRACT

A total of 22 endophytic bacteria were isolated from roots and nodules of *Vigna radiata* (mungbean) obtained from Jind district, Haryana. These were characterized on the basis of plant growth promoting traits. Almost all the endophytic bacteria produced IAA with maximum production of 81.63 µg/ml by isolate MJiR8. Among these, 100% root isolates and 84.6% nodule isolates resulted in *in vitro* root growth promotion of mungbean seedlings. All the isolates produced ammonia; eighteen (all root and nine nodule isolates) produced organic acid while only four root isolates were positive for siderophore production. The four isolates produced hydrogen cyanide and out of these only MJiR9 inhibited the growth of fungal pathogens *Fusarium oxysporium* and *Aspergillus niger*. All the endophytes were used to determine molecular diversity by ARDRA (Amplified Ribosomal DNA Restriction Analysis) Results revealed that the nodule isolates were more diverse, being present in separate clusters, in comparison to root isolates which were grouped together in cluster III.

Key words: Antifungal, Endophytic bacteria, IAA, Molecular diversity, Nodules, Roots, *Vigna radiata*.

INTRODUCTION

Endophytic bacteria are more bioactive than any other plant associated bacteria because they interact more closely with the host plant in internal microenvironment of the plant tissue (Dalal *et al*. 2013). They can benefit the host plants either directly by facilitating nutrient uptake, increasing plant hormone level or indirectly by biocontrol effect (Glick, 2012). Plant growth promoting endophytes also enable the associating plants to tolerate abiotic stresses like drought, salt, nutrient deficiency or excess of temperature and, presence of toxic metals (Jha *et al*. 2012). They interact with plants by producing plant growth regulators like indole acetic acid, asymbiotic N₂ fixation, antagonism against phytopathogens, production of siderophores, antibiotics and hydrogen cyanide, solubilization of mineral phosphates and other nutrients. There have been several reports of bacteria which elicit nodule formation and belong to rhizobial species (Gagne *et al*. 1987; Sturz *et al*. 1997; Suneja *et al*. 2016). Various non-rhizobial endophytes isolated from nodules and roots belonging to three major phyla actinobacteria, proteobacteria and firmicutes which include the members of *Bacillus, Paenibacillus, Pseudomonas*, and *Enterobacter* and have also been reported to perform plant growth promotion (Dudeja *et al*. 2014).

To fulfill the food demand of increasing population, plant growth and yield is generally enhanced by the input of chemicals which act as plant growth regulators and nutrients (Tilman *et al*. 2002). Excessive use of chemicals raises a number of concerns such as water contamination, soil degradation, loss of biodiversity and health risks for human as well as animals. Growing awareness about organic farming and use of biofertilizers instead of chemical fertilizers has led to the search for new promising bioinoculants. In order to better understand the plant microbe interaction and explore their functional potential as inoculants, it is important to study the diversity of endophytes within the same tissue as well as the different tissues of same plant.

*Vigna radiata*, is one of the important legume crop of *Kharif* season due to its short growing period, better storage ability and immense nutritional value (Tariq *et al*. 2012). Studies on the endophytic bacteria of *Vigna radiata* (mung bean) are very rare. Therefore, the functional and molecular diversity of bacterial endophytes in roots and nodules of mung bean was studied.

MATERIALS AND METHODS

Isolation of bacterial endophytes from leguminous plant: *Vigna radiata* plants were collected from the farmers fields of Jind district (29.45°N: 76.33°E) of Haryana, India. Healthy roots and nodules samples were surface sterilized by sequential immersion in 0.25% HgCl₂ for 1 minute, 95% ethanol for 30 seconds followed by 5-6 times washing with sterile distilled water. Macerated samples were used for isolation of endophytic bacteria on Tryptone soy agar (TSA) plates. Colonies were selected on the basis of variation in morphology, purified and maintained on TSA slants and preserved at 4°C for further investigations.

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**Determination of Plant growth promoting traits:**

**Indole acetic acid (IAA) production:** IAA production in the culture broth was determined by using standard colorimetric assay (Tang and Bonner 1947). IAA present in each sample was determined using standard curve of known concentration of IAA (10–200µg/ml) (Gordon and Weber, 1951). 2 ml of uninoculated YEM broth with equal volume of Salkowski reagent was taken as a negative control. All the experiments in this study were performed in triplicates and the average value was used.

**Siderophore production:** The isolates were spotted on Chrome azurol S (CAS) agar plates (Schwyn and Neilands 1987). After incubation at 28±2°C for 5 days, development of yellow-orange halo zone around the spot indicates siderophore production.

**Ammonia production:** Log phase cultures were inoculated in 10ml of peptone water in the tube and incubated at 28±2°C for 4-5 days. Development of color was assessed using Nessler’s reagent (Cappuccino and Sherman 1992).

**Organic acid production:** Organic acid producing ability of isolates was determined by methyl red test (Cappuccino and Sherman 1992).

**Phosphate solubilization:** The isolates were tested for its ability to solubilize calcium phosphate. For these isolates were spotted on Pikovskaya agar medium plates and incubated at 28±2°C for 4-5 days (Pikovskaya 1948). Development of clear halozone around bacterial isolates indicates phosphate solubilization.

**In vitro root growth promotion:** The seeds of mungbean (Var. Basanti) were surface sterilized using 0.25% HgCl₂ and placed on 1% agar media plate for germination. After 24 hours, germinated seeds were placed on 1.2% agar plates and 100µl of culture broth was inoculated on seeds. After 5 days of incubation, increase in root length was assessed (Narula et al, 2013).

**Hydrogen cyanide (HCN) production:** Production of hydrogen cyanide (HCN) was evaluated using method as described by Lorck (1948).

**Antifungal activity:** The bacterial isolates producing hydrogen cyanide were tested against Aspergillus niger and Fusarium oxysporium on potato dextrose agar media for fungistatic activity. The 24 hour old bacterial isolates were streaked 1 cm away from the 2 mm of fungal disc placed on media plate and uninoculated plate with fungal disc served as negative control (Kumar et al, 2015). Antifungal activity was evaluated by fungal growth inhibition after incubating the plates at 30°C for 7 days.

**Study of molecular diversity**

**DNA Isolation and 16s rDNA amplification:** The genomic DNA of freshly grown cultures was extracted by modified CTAB method (Ausubel et al. 1995). The universal primers 8F (5’AGAGTTTGATCCTGGTCAG 3’) and 1541R (5’AAGGAAGGTGATCCAGGCTCAG 3’) were used for 16S rDNA amplification (Weisburg et al. 1991). Reaction was performed in a volume of 30 µl and final amplified product was resolved on 1.2% agarose gel stained with ethidium bromide in 0.5X TBE buffer and observed under gel documentation system (Azure biosystems, Dublin, CA 94568 USA).

**ARDRA (Amplified Ribosomal DNA Restriction Analysis):** Restriction endonuclease (RE) MspI, Hinf I and HaeIII were used for restriction digestion of amplified 16S rDNA product.

| RE   | MspI   | Hinf I    | HaeIII  |
|------|--------|-----------|---------|
| RE   | 5’C\CGG3’ | 5’G\ANT\C3’ | 5’GG\CC 3’ |
| RE   | 3’GGC\C5’ | 3’CTN\AG 5’ | 3’CCA\GG 5’ |

**Source:** Moraxella sp. Haemophilus influenzae Haemophilus aegyptius

For this digestion, 5 µL of amplified 16S rDNA product was treated with 0.5 µL of restriction enzyme and held at constant temperature of 37°C for 4 hours in dry water bath. The digested product was resolved on 1.5% agarose gel. Two dimensional binary matrices were prepared from the polymorphic patterns of restriction digestion profiles and combined for cluster analysis. With the help of SimQual, similarity matrices were constructed and which were further analyzed by UPGMA cluster analysis using biostatistical analysis program NTSYS-PC programme 2.1 of Exeter Softwares USA (Rohlf, 1998).

**RESULTS AND DISCUSSION**

**Isolation and Morphological characterization of bacterial endophytes:** Roots and nodules of leguminous crops form a nutrient rich niche for occurrence of large number of endophytic bacteria which promote plant growth. These can be isolated by surface sterilization of plant roots and nodules. Effective sterilization was also checked by absence of any growth in the final rinsed water as well as uncrushed roots and nodules when placed on TSA plate. Twenty two morphologically distinct endophytic bacteria were isolated from roots and nodules of V. radiata collected from Jind districts of Haryana. All isolates were Gram positive except two while all the isolates were of rod shape of different sizes (Fig.1- 2. and Table1-2).

**Plant Growth promoting traits of endophytes:** Endophytic bacteria are well known to augment the growth and development of host plant by production of a number of metabolites like ammonia, organic acid, indole-3-acetic acid (IAA), siderophore, hydrogen cyanide etc (Table 3 and 4). The functional potentialities in relation to plant growth promoting activities of root and nodule isolates were assessed.

**Quantitative analysis of IAA production:** IAA is the most common physiologically active auxin synthesized by microorganisms. Most of the endophytic bacteria as well as...
rhizospheric bacteria are reported to have the ability to produce phytohormones (Aswathy et al. 2013). The data in Table 3 (root isolates) and Table 4 (nodule isolates) depict that all the isolates, except MJiR1 were producing IAA. The IAA production ranged from 9.5-81.63µg/ml. Highest amount of IAA produced by MJiR8 (81.63µg/ml) whereas lowest amount was produced by MJiN8 (9.5µg/ml). Pandya et al. (2015) reported highest IAA production of 10.80µg/ml by endophytic bacteria isolated from *V. radiata* nodules. Several genetic factors have been reported which result in variation in IAA production among the isolates (Passari et al. 2016).

**Siderophore production:** Bacterial endophytes are also known to liberate iron chelating molecules, which increase the accessibility of iron to the plants in iron-limiting conditions (Szilagyi-Zecchin et al. 2014). The only 4 root isolates MJiR 3, MJiR 5, MJiR 6, MJiR 8 were producing siderophore and MJiR 8 showed the formation of strong orange halo zone on CAS agar plate (Fig. 3a). Similarly, Rajendran et al. (2008) isolated three endophytic bacteria from root nodules of *Cajanus cajan* and two out of three isolates were reported to produce siderophore. Liaquat et al. (2016) also reported siderophore production by only 2 out of 7 endophytic bacteria isolated from peach rootstock.
Table 3: Plant growth promoting properties of endophytic bacteria from mungbean roots.

| Isolates | Ammonia production | Organic acid production | HCN production | Phosphate solubilization | IAA production (µg/ml) | Siderophore production | Root growth promotion (Control-3cm) |
|----------|---------------------|------------------------|----------------|--------------------------|-----------------------|------------------------|-------------------------------|
| MJiR 1   | ++                  | +                      | ++             | -                        | -                     | -                      | 7.5                           |
| MJiR 2   | +                   | +                      | ++             | +                        | 13.7±0.66             | -                      | 5.75                          |
| MJiR 3   | +                   | ++                     | ++             | ++                       | 20.4±1.7              | +                      | 6.25                          |
| MJiR 4   | ++                  | ++                     | +++            | ++                       | 11.6±0.8              | -                      | 5.75                          |
| MJiR 5   | ++                  | ++                     | ++             | +++                      | 22.7±0.6              | +                      | 6.75                          |
| MJiR 6   | +++                 | +++                    | ++             | +++                      | 27.6±1               | +                      | 4                             |
| MJiR 7   | ++                  | +++                    | +              | +++                      | 24±0.5               | -                      | 5                             |
| MJiR 8   | +++                 | +++                    | -              | +++                      | 81.6±2.7             | +++                    | 5.25                          |
| MJiR 9   | +++                 | +++                    | -              | -                        | 10±0.5               | -                      | 7.5                           |

IAA values are mean of three replicates ± SD
“–” means showed no production
“+” means showed low production
“++” means moderate production
“+++” means high production

Table 4: Plant growth promoting traits of endophytic bacteria from mungbean nodule.

| Isolates | Ammonia production | Organic acid production | HCN production | Phosphate solubilization | IAA production (µg/ml) | Siderophore production | Root growth promotion (Control-3cm) |
|----------|---------------------|------------------------|----------------|--------------------------|-----------------------|------------------------|-------------------------------|
| MJiN 1   | +++                 | -                      | +++            | +                        | 31.88±0.5             | -                      | 5.25                          |
| MJiN 3   | ++                  | +                      | +++            | -                        | 30.8±1.1              | -                      | 3.75                          |
| MJiN 4   | +++                 | +++                    | ++             | +                        | 51±3.6               | -                      | 5                             |
| MJiN 5   | ++                  | -                      | +++            | -                        | 18±3.2               | -                      | 2.5                           |
| MJiN 6   | +                   | ++                     | +              | -                        | 24.6±1.7             | -                      | 4.5                           |
| MJiN 7   | +++                 | -                      | +              | -                        | 14.4±0.6             | -                      | 2                             |
| MJiN 8   | ++                  | +                      | +              | -                        | 9.5±0.5              | -                      | 4.75                          |
| MJiN 9   | ++                  | ++                     | +              | -                        | 17.2±0.8             | -                      | 4.5                           |
| MJiN12   | ++                  | ++                     | +              | -                        | 48.86±54             | -                      | 4                             |
| MJiN13   | +++                 | +++                    | ++             | -                        | 36.2±2.2             | -                      | 5.25                          |
| MJiN14   | +                   | -                      | +++            | -                        | 18.8±2               | -                      | 9                             |
| MJiN15   | +                   | ++                     | +++            | -                        | 15.8±0.43            | -                      | 9                             |
| MJiN16   | +++                 | +++                    | -              | -                        | 22±0.6               | -                      | 4.5                           |

IAA values are mean of three replicates ± SD
“–” means showed no production
“+” means showed low production
“++” means moderate production
“+++” means high production

Ammonia production: All the isolated endophytic bacteria from roots and nodules produced ammonia (Fig. 3. b and c). Ammonia production is an important trait of plant growth promoting endophytes, which helps in plant growth and biomass accumulation indirectly (Marques et al. 2010).

Organic acid production: Plant growth promoting bacteria plays an important role in plant nutrition and physiology by producing organic acid. All the isolates from roots and nine isolates from nodules were producing organic acid (Fig. 3. d and e). These organic acids lower the soil pH, acts as stable metal chelating agent and helps in up taking important minerals (Barros et al. 2013; Yadav et al. 2013).

Phosphate solubilization: Phosphorus is the key macronutrient for plant growth and yield after nitrogen but present in insoluble form. Endophytic bacteria have the capability of solubilizing phosphate making it accessible to the plants (Duanpaenga et al. 2013; Young et al. 2013). In the present study, 5 out of 9 root isolates while 2 out of 13 nodule isolates were able to solubilize phosphate. Numerous studies have also reported the occurrence of phosphate solubilizing endophytes in leguminous plants and their role in soil improvement by increasing the phosphate availability (Palaniappan et al. 2010; Tariq et al. 2012).

In vitro root growth promotion: Plant growth promoting endophytes with multiple traits help in acquisition of nutrients and positively influence root architecture (Remans et al. 2010).
Therefore, in vitro root growth promotion was assessed by these isolates. All the root isolates and 84.6% from nodules resulted in increase in root length of Mungbean seedlings as compared to control (3cm). The increase in root length can be accounted for multiple PGP traits of isolates. Results indicated that most of the isolates with multiple PGP promote the root growth in vitro.

HCN Production: Hydrogen cyanide is a volatile compound which exhibit antifungal properties protecting plants against various soil borne pathogens and help in plant growth indirectly (Ramette et al. 2003). There are several reports on HCN production by endophytes (Ngoma et al. 2013; Etesami et al. 2014; Rodrigues et al. 2016). All the endophytic isolates were producing HCN and MJiN9, MJiR4, MJiR9, MJiR13 were the best among these (Fig. 3. f and g).

Antifungal activity: The antifungal activity of selected MJiN9, MJiR4, MJiR9 and MJiR13 was assessed against phytopathogenic species i.e. Fusarium oxysporium and Aspergillus niger. Isolate MJiR9 exhibited antagonism against both the fungal species (Fig 4. a and b) while rest did not show any antimicrobial activity. The efficacy of HCN for controlling black root rot by strain CHA0 has already been reported (Voisard et al. 1989).

Molecular diversity of endophytic bacteria: Diversity of endophytic bacteria isolated from roots and nodules of mungbean was studied by amplifying 16s rDNA sequence followed by restriction digestion using three restriction enzymes (Fig. 5-7). Banding pattern was scored for number of reproducible bands for each isolate. There were 29 polymorphic bands produced by restriction digestion where each isolates showed 2-5 reproducible bands ranging from 100-1000bp. Two predominant bands at 350bp and 470bp were observed in all the isolates. Based upon the presence or absence of bands, dendrogram was prepared (Fig. 8) and at 70% similarity, five clusters were observed. Cluster I included isolate MJiN3 and MJiN14 while in cluster II, MJiN4 and MJiN5 were 100% similar. A maximum of eight
isolates were present in cluster III which was further divided into two subclusters. In the first subcluster, isolates MJiN12, MJiR9 were 100% similar indicating that nodule endophytes get entry from primary and lateral roots in leguminous plants. These bacteria get entrapped into root hairs during curling and finally arrive at the nodules and multiply (Santoyo et al. 2016). In second subcluster isolates MJiR2, MJiR4, MJiR7 and isolates MJiR3 and MJiR5 also revealed 100% identity. The isolate MJiN9 formed separate cluster IV. Again root and nodule endophytes MJiN13, MJiN16 and MJiR8 were grouped together in cluster V. RFLP analysis based dendrogram reveals that all the isolates from nodules were present in separate clusters. These are in consistence with earlier findings that nodule endophytes are more diverse than that of root endophytes isolated from different legume and non-legume plants (Kumar et al. 2013).

CONCLUSION
Thirteen morphologically distinct endophytic bacteria isolated from roots and nodules of Vigna radiata were screened for multiple plant growth promoting traits. The isolates MJiR3, MJiR5 possessed all the PGP traits while other also had more than two traits. Almost all the isolates produced IAA. Therefore, they were used to study in vitro root growth promotion. Overall increase in root length of the mungbean seedlings by the root isolates was 100% while that of nodule isolates was 84.6% as compared to control. The isolates MJiN9, MJiR4, MJiR9 and MJiR13 produced high levels of HCN and checked for antifungal activity against Fusarium oxysporium and Aspergillus niger. The isolate MJiR9 only inhibited both the fungal pathogens. The dendrogram based on the similarity coefficients at 70% resulted in five clusters. The large variability was observed among the nodule isolates as all of them belonged to distinct clusters in comparison to root isolates, maximum of which grouped together in cluster III. This work provides the basis for selection of inoculum formulation to improve the respective plant growth.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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