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Biochemical and Molecular Characterization of Endophytic Bacteria from Root Nodules of Mungbean (*Vigna radiata*)

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ABSTRACT

Bacterial endophytes are endosymbiotic in nature and employ advantageous effects on host plant as stimulation of plant growth, nitrogen fixation and induction of resistance to plant pathogen. In the present study, mungbean nodule samples were collected from farm area during summer and *kharif* season to isolate the endophytes. In total, 41 endophytes were isolated using Tryptone soya agar (TSA) and the majority of endophytes were found to be IAA producers, phosphorus solubilizer, potassium solubilizer and ACC utilizers and trove of many other plant growth traits. On the basis of morphological, biochemical and molecular characterization, four plant growth promoting endophytes found were *Bacillus flexus* strain PM1217, *Bacillus amyloliquefaciens* strain PM1218, *Beijerinckia fluminensis* strain PM1219 and *Pseudomonas* sp. strain PM1220. These non-rhizobial endophytes can significantly influence the mungbean growth and provide a strong basis for further development of these strains as efficient biofertilizers to attain the desired yield in fields.

Key words: Mungbean, non-rhizobial, endophytes, TSA, plant growth promotion (PGP)

INTRODUCTION

Mungbean belongs to the Papilionoideae family, order Leguminosae and botanically recognized as *Vigna radiata* (L.). The mungbean is one of the 13 food legumes grown in India and third most important pulse crop of India after chickpea and pigeonpea. Mungbean is considered as a substitute of animal protein and forms a balanced diet when used with cereals (Batra *et al.,* 2018). Seeds are more palatable, easily digestible and non-flatulent than other pulses allowing mungbean to consume in vegetarian and dietetic nourishment (Sansanwal *et al.*, 2017). Bacterial endophytes ubiquitously colonize the internal tissues of plants almost in all the plants and generally promote plant growth (Santoyo *et al.*, 2016). Presence of bacterial endophytes has been reported from many cultivated and flowering plants including rice, wheat, maize, sugarcane, potato, alfalfa, bean, chickpea, mungbean, clover, cowpea, pea, peanut, soybean, carrot, citrus plants, banana, *Acacia, Argyrolobium, Conzattia, Fenugreek, Hedysarum, Kennedia, Leucaena, Lotus, Mimosa, Medicago, Melilotus, Ornithopus, Onobrychis, Oxytropis, Psoralea, Scorpiurus, Sesbania, Tetragonolobus* and *Vicia* (Muresu *et al.*, 2019) . Endophytes have been isolated from almost all plant parts, including fruits, leaves, stems, seeds, nodules and roots (Lai *et al.*, 2015). Similarly, there is large variation in colony morphology of different isolates from different crops and the differences were observed in colony colour, shape and size. Batra *et al.* (2020a) found gram-negative and gram-positive endophytic bacteria in almost equal proportion in the roots of different crops. These endophytes from different environmental, geographical and physiological conditions may have resulted in allopatric polymorphism. The present study aims at characterizing morphological, biochemical and plant growth promotor (PGP) properties of the isolates.

The ribosomal RNA genes of bacteria, especially those for 16S and 23S-rRNA are excellent molecular markers for phylogenetic studies because of their functional constancy and their ubiquitous distribution (Harrison and

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Griffin, 2020). Molecular phylogeny of the diazotrophic spp. using ARDRA technique subjects the amplified 16S rDNA gene to restriction digestion and thus is termed as the 'amplified rDNA restriction analyses'. The most commonly found genera of bacterial endophytes are : *Pseudomonas*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, *Micrococcus*, *Pantoea* and *Microbacterium* (Naik and Rahi, 2022). Hence, this work involved the biochemical and molecular characterization of endophytic bacteria from mungbean root nodules.

MATERIALS AND METHODS

For the isolation of bacterial endophytes, the nodules were collected from the roots of mungbean (*Vigna radiata*) grown at the farm of CCSHAU, Hisar, Haryana during summer and *kharif* seasons. Nodules were surface-sterilized using 70% ethanol and 0.1% $HgCl_2$ and repeatedly washed with sterile water. Sterile nodules were crushed in a sterilized petri dish with the help of sterilized glass rod in one ml sterilized distilled water and the resulting suspension was streaked on Typtone soy agar (TSA) plates. These plates were incubated in BOD incubator at 30°C. After 24-36 h, the colonies were picked and purified by single colony streaking on the TSA plates and maintained at 4°C. These isolates had multiple plant growth promoting traits (Sansanwal, 2018; Sansanwal *et al.,* 2018; Batra *et al.*, 2020b).

The bacterial cultures were examined for various morphological, biochemical and physiological characteristics as per procedure described in Bergey's Manual for Determinative Bacteriology. The inter-relationship of the microorganisms in each section of Bergey's Manual is based on characteristics such as morphology, staining reactions, nutrition, cultural characteristics and biochemical test results for specific metabolic end products. Selected endophytes were subjected to different culture characterization (shape, texture, colour and size) and gram staining was performed. All the selected endophytes were processed for different biochemical tests i. e. indole production, methyl red test, Voges-Proskauer reaction, citrate utilization test, oxidase test, catalase production, starch hydrolysis, gelatin hydrolysis and motility test.

The nodule associated endophytic isolates were also characterized by 16S rRNA gene partial sequence analysis. The genomic DNA of the endophytic bacterial isolates was extracted by using CTAB method (Naik and Rahi, 2022). The extracted DNA was dissolved in 20 µl nuclease free water and used as a template for PCR reactions. PCR amplifications were performed in a total volume of 40 µl by mixing 20 ng of the template DNA with 10 mM concentration of each deoxynucleotide triphosphate (dNTPs), 2 µl of each primer (16S FORWARD 5'- AGG AGG TGA TCC AGC CGC- 3' =18 and 16S REVERSE 5'- AGA GTT TGA TCC TGG CTC AG-3'=20) and 2 µl of Taq DNA polymerase in 10X Taq buffer. These reactions were further subjected to initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, annealing of 50°C for 30 sec and a final extension step at 72°C for 5 min using thermo cycler. The PCR products were resolved using 1% agarose gel.

The amplified 16S rRNA gene partial sequences of four non-rhizobial bacterial isolates were obtained after sequencing from Xcelris lab limited, Ahmedabad, India. The nucleotide sequences were cross-checked using BLASTn tool available in National Center for Biotechnology Information (NCBI) database. The nucleotide sequence data were aligned, analyzed and compared using EditSeq, MegAline software (DNAStar Inc., Madison) and MEGA version 6.0. The phylogenetic trees were prepared by a neighbor-joining method Tamura-Nei statistical model using MEGA 6.0 (Molecular Evolutionary Genetic Analysis) software.

RESULTS AND DISCUSSION

A total of 41 endophytic isolates were obtained from healthy nodules of mungbean plant roots after sterilization with 0.1% $HgCl_2$. Bacterial colonies of different colours such as white, cream white and yellow were observed (Table 1). Based on the comparative analysis of various morphological and biochemical characteristics as per procedure described in Bergey's Manual of Determinative Bacteriology, two bacterial isolates E13 and E14 were found to belong to the genera of *Bacillus,* while E6 to the *Pseudomonas* and E17 to *Bejernickia* genus (Table 2). Huge number of plant growth promoting endophytes (PGPEs) were isolated from a large diversity of plants

Table 1. Morphological characters of selected endophytic bacterial isolates

Isolate	Colony morphology	Gram reaction	Morphology	Growth
E6	Raised, circular and gummy colony	-ve	Rods	Fast grower
E13	Creamish, small and rough colony	+ve	-do-	-do-
E14	Creamish, small, smooth, circular and gummy colony	+ve	-do-	-do-
E17	Creamish, small gummy and circular	-ve	-do-	Very fast-grower

Table 2. Biochemical characters of selected endophytic bacterial isolates

Biochemical test	E6	E13	E14	E17
Indole test	-	-	-	-
MR test	-	-	-	-
VP Test	-	+	+	-
Citrate utilization test	+	+	+	-
Oxidase test	+	+	+	+
Catalase test	+	+	+	+
Starch hydrolysis	-	+	+	-
Motility	+	+	+	-
Gelatin utilization	+	+	+	+
Genus	*Pseudomonas* sp.	*Bacillus* sp.	*Bacillus* sp.	*Beijerinckia* sp.

and found to be beneficial for plant growth, yield and crop quality, including strains in the bacterial genera of *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillium, Azotobacter, Azomonas, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Pseudomonas, Rhizobium* and *Serratia* (Mosqueira *et al.,* 2022). Etesami and Alikhani (2017) isolated endophytic bacterial isolates REN3 and REN4 and on the basis of morphological and biochemical tests on which these were found belonging to genera *Bacillus.* Sequencing of the 16S rRNA gene was aided as an important tool for identification, characterization and determination of phylogenetic relationship over a number of years. In the present study, approximately 1500 bp DNA fragment was amplified from 16S rRNA of all the four bacterial endophytes and was got sequenced from Xcelris lab limited, Ahmedabad, India (Fig. 1). The lengths of aligned sequences were 1517, 1539, 1437 and 1247 bp for *Bacillus flexus, Bacillus amyloliquefaciens, Beijerinckia fluminensis* and *Pseudomonas* sp., respectively. The 16S rRNA sequences were deposited in GenBank/EMBL/DDBJ under the accession numbers MG214343 (*Bacillus flexus* starin PM1217), MG214344 (*Bacillus amyloliquefaciens* strain PM1218), MG214345 (*Beijerinckia fluminensis* strain PM1219) and MG214346 (*Pseudomonas* sp. strain PM1220). The sequence identity matrices were generated with the nucleotide sequences of the present study with 40 reference strains obtained from Genbank (Fig. 2). The percentage of identity ranged from 98.2 to 99.6, 98.4 to 99.1, 86.3 to 99.1 and 98.2 to 99.1 of *Bacillus flexus* strain PM1217, *Bacillus*

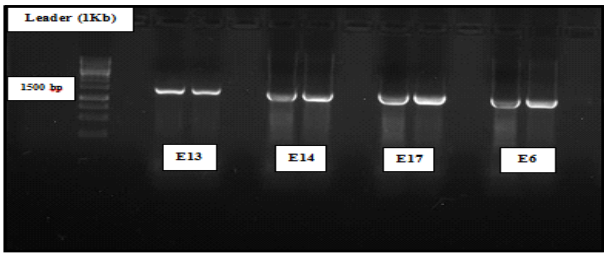

Fig. 1. Gel showing amplified 16S rRNA of promising endophytic bacteria E6, E13, E14 and E17.

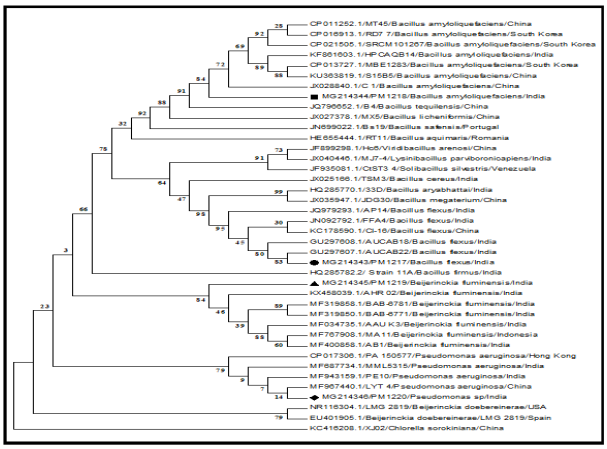

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of *Bacillus amyloliquifaciens* strain PM1218, *Bacillus flexus* strain PM1217, *Beijerinckia fluminensis* starin PM1219 and *Pseudomonas* sp. PM1220 between different taxa using Tamura-Nei statistical model (Bootstrap samples n=1000).

amyloliquefaciens strain PM1218, *Beijerinckia fluminensis* strain PM1219 and *Pseudomonas* sp. strain PM1220, respectively. The *B. flexus* strain PM1217 was clustered with other *B. flexus* Indian strains, *B. amyloliquefaciens* strain PM1218 with other *B. amyloliquefaciens* reference Indian strains. The *B. fluminensis* strain PM1219 was clustered with other *B. fluminensis* reference strains and was more similar with strain AHR02 in phylogenetic tree. The *Pseudomonas* sp. strain PM1220 was clustered with other *Pseudomonas aureginosa* reference strains and more similar with strain LYT 4 in phylogenetic tree. Korir *et al.* (2017) reported molecular characterization of endophytic isolates by 16S rRNA partial gene sequencing and the distributions were genetically diverse on several species of *Bacillus* sp., such as *B. megaterium, B. subtilis, B. aryabhattai* and *P. polymyxa.* In the present study, four endophytic isolates, namely, E6, E13, E14 and E17 possessed multiple plant growth promoting traits. In the light of present results, it may be concluded that these endophytic isolates can be used for growth promotion of mungbean under pothouse and field conditions.

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