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Original Article

Nodule inhabiting non-rhizobial bacteria and their influence on growth of selected leguminous plants of Bangladesh

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ABSTRACT: The present study aimed to identify non-rhizobial bacteria present in the root nodules of four legume plants viz. *Arachis hypogaea* L., *Dolichos lablab* L., *Vigna mungo* L., *and Sesbania sesban* L. grown in Bangladesh. Pure isolates of the bacteria obtained from the surface sterilized root nodules were identified by sequencing of 16S rRNA gene. A total of 13 isolates belonging to 10 genera under 7 families were obtained. Effects of these non-rhizobial bacteria on nodulation, plant height, shoot biomass, and leaf chlorophyll contents were compared in between *A. hypogaeae* L. and *V. mungo* L. Of all the isolates identified, *Klebsiella* sp. and *Enterobacter* sp. produced nodules in both *V. mungo* L. and *A. hypogaea* L. and *Bacillus* sp. produced nodules only in *V. mungo* L. Results also demonstrated that most of the isolates enhanced shoot height, shoot biomass and leaf chlorophyll contents in *V. mungo*L. but not in *A. hypogaea* L. Results of the present study are thus relevant for enhancing our knowledge about the beneficial use of non-rhizobial bacterial in growth and development of leguminous crops in order to secure sustainable agriculture.

KEYWORDS: Legumes, Non-rhizobial nodule bacteria, DNA sequencing, 16S rRNA gene

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INTRODUCTION

Reports on the occurrence of non-rhizobial bacteria in root nodules of leguminous plants have created interests among the scientists and agriculturists for their role in survival, nodulation and grain yield of the crop plant[1,2]. Enhanced nodulation and grain yield by a number of activities such as indole acetic acid production, phosphate solubilization, fixing nitrogen and siderophore production has been reported by a number of studies [3,4]. Besides, a number of plant growth promoting bacteria have been reported to enable plants resist the environmental stress and exclude pathogens from rhizosphere [5,6]. Co-inoculation of growth promoting bacteria with crop specific rhizobia improves root infection which results in better nodulation and plant growth [1,7]. Therefore, use of non-rhizobial root-nodule bacteria has been increased over the years in order to increase the competitive survivability of rhizobial biofertilizer and thus achieve better plant growth under adverse environmental condition [8]. However, there has been little information on the identity of root nodule inhabiting bacteria present in the legume plants particularly in the context of Bangladesh soils. Black gram (*Vigna mungo* L.), hyacinth bean **(***Dolichos lablab* L.), groundnut (*Arachis hypogaea* L.) and Egyptian riverhemp *(Sesbania sesban* L.*)* are important legumes of those the first two are mostly used as pulses and the other three are respectively as oil seed, vegetable and soil quality improver in Bangladesh. Achieving enhanced growth in crops by using plant growth promoting bacteria is desired to secure sustainable agriculture of the country. Therefore, the objectives of this study were to identify non-rhizobial nodule inhabiting bacteria from selected leguminous plants *V. mungo* L., *D. lablab* L., *A. hypogaea* L. and *S. sesban* L. and also to examine the effects of these nodule occupying bacteria on the growth of *A. hypogaea* L. and *V. mungo* L.

MATERIALS AND METHODS

Collection of root nodules

Four legume plants viz. *A. hypogaea* L., *D. lablab* L., *V. mungo* L. and *S. sesban* L. were selected to isolate nodule inhabiting bacteria in the present study. Root-nodules of these four plants were collected from sites within 5 km area in the sub-district of Louhajong under the district of Munshiganj. Plant roots were collected carefully from the field with the help of a shovel and spade so that root nodules are not lost. Then, soils attached to roots were removed by gentle shaking and flow of water. After collection from the field in July 2010, the root systems along with nodules were brought to the Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

Extraction of bacteria from root nodule

Nodules were separated from roots and kept in a 1.5ml eppendorf tube. Five nodules per plant were randomly taken for the isolation of the nodule inhabiting bacteria. Surface sterilization of nodule was done by soaking into 5% Sodium hypochlorite for 10 min. Nodules were then rinsed five times with autoclaved Milli-Q water. Extraction of DNA from nodule bacteria started with crushing a nodule with homogenization solution (0.14 M NaCl, 0.003 M KCl, 0.04 M KH_2PO_4, 0.06 M K_2HPO_4, soluble PVP 10 g/100 ml and 10 mM DTT) in a flat-bottomed well-plate with the help of a sterile metallic rod. The homogenate was then passed through a 25 µm mesh filtration cup inside a 1.5 ml eppendorf tube. The homogenate was divided into

two parts: homogenate-1 for extraction of DNA for identification of dominant bacteria and homogenate-2 for pure isolation of non-rhizobial bacteria. Homogenate-1 was centrifuzed at 15,000 rpm and bacteria in the form of pellet was collected for extraction of DNA.

Isolation of nodule inhabiting non-rhizobial bacteria

Homogenate-2 was spread on YEM (Yeast-Extract Mannitol) agar media added with Congo Red. Colonies differing in color, morphology, and growth period were selected and sub-cultured into new plates to obtain pure isolate. Colonies purified in this way were cultured in liquid YEM medium at 28°C for 7 days. Liquid cultures of the isolates were centifuzed at 15,000 rpm and the pellet formed thus was used to extract DNA.

DNA extraction, PCR and sequencing

DNA was extracted by following heat-lysis method at 95°C for 10 min.16S rRNA gene was amplified using primers 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT). A PCR reaction mixture of 25 µl in volume contained 2.5 µl 10X PCR Buffer (Invitrogen), 1µl 50 mM $MgCl_2$ (Invitrogen), 0.5 µl 10mM dNTP (Fermentas), each 0.5 µl of forward and reverse primers, 0.1 µl of *Taq* DNA polymerase (Platinum® *Taq* DNA polymerase, Invitrogen), 1 µl template DNA and 18.9 µl autoclaved milli-Q water. A total of 35 cycles of reactions were done with the initial denaturation temperature of 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 68°C for 90 min, and final extension at 68°C for 4 min. The band size of the PCR product was confirmed by agarose gel (1% agar in 0.5X TBE solution) electrophoresis at 80V for 35 min.

PCR products obtained thus were sent to Macrogen Inc., Korea for DNA sequencing. After getting the sequencing results, sequences were checked for their quality. Most reliable parts of the consensus sequences were used for alignment. The 16S rRNA gene sequences were compared and identified with GenBank database using BLAST by selecting BLASTmethod[9].

Inoculation of legume plants

Selected isolates already identified were used to inoculate *A. hypogaea* L. and *V. mungo* L. After sterilization with 5% Sodium hypochlorite, seeds were kept for 48 h for germination. Inocula were prepared by letting isolates grow in liquid YEM medium for 4 days. Inocula with the similar concentration of bacterial cells of about $4.30X10^7$ (cfu/ml) were used for all isolates while applying to seedlings. After inoculation, seedlings were sown in the pre-wet and sterilized sand:vermiculate (4:1, v/v) mixture. The experiment was conducted in a growth room at controlled temperature with day length of 16 h at 24°C and night length of 8 h at 18°C.

Harvesting and measurements of plant growth parameters

Plants were harvested after three months of inoculation with the isolates. Shoot height and shoot biomass were measured. Shoots were cut and put into paper bags marked carefully and dried in oven at 65°C for 24h to weigh dry mass. Plant root systems were carefully separated from the sand-vermiculite mixture. Nodules were picked with the help of scalpel, counted and weighed to express biomass per nodule per plant.

Leaf chlorophyll content was determined using Chlorophyll Meter SPAD-502 (Minolta Co., Ltd. Japan). Ten fully expanded youngest leaves per plant were selected to determine the average SPAD value for plants treated with isolate.

RESULTS AND DISCUSSION

Dominant bacteria present in the root-nodule

Dominant bacteria present in the root nodules of the four host legumes are shown in Table 1. The similarity of the query sequences with the gene available on the GenBank data-base ranged from 97 to 99% and the number of nucleotides of the query sequences ranged from 807 to 933. *Bradyrhizobium* spp. were found in root nodules of *V. mungo* L., *D. lablab* L., and *A. hypogaea* L. and *Sinorhizobium* sp. were in *S. sesban* L. These results are in agreement with the results of other studies [10].

The family Enterobacteriaceae represented the highest number of species (38.5%) among all of the isolates which was followed by Bacillaceae and Xanthomonadaceae (15.40%) and each of the rest of the families contributed equally. These results indicate that root nodules of legumes are habitats for diverse non-rhizobial bacteria. Most of the non-rhizobial bacteria obtained along with the dominant rhizobia present in the root-nodule in the present study were also reported in root nodules of legume and non-legume plants in other studies [11-15].

As shown in Table 2, the present study revealed that the four selected host legumes differed in harboring bacterial species in their root nodules with the highest in *S. sesban* L. (6) followed by *A. hypogaea* L and *V. mungo* L. (3) and *D. lablab* (1). However, this result suggests further study to clarify whether the diversity in nodule associated bacteria is selected by the host plants or is determined by the local soil environments of the respective host plants.

Table 1. Dominant bacteria present in the root nodule of the selected four host legume plants. Identification was done by NCBI-BLAST using 16S rRNA gene obtained from PCR using template DNA extracted from the nodule bacteria.

Host plant	No. of query sequence	Organisms	% Similarity	Number of nucleotides
Vigna mungo L.	4	*Bradyrhizobium* sp.	98-99	888-928
Sesbania sesban L.	5	*Sinorhizobium* sp.	98-99	807-929
Dolichos lablab L.	2	*Bradyrhizobium* sp.	98	931-933
Arachis hypogaea L.	1	*Bradyrhizobium* sp.	99	933

Table 2. Systematic position of the bacteria isolated from the root nodules of selected host legume plants.

Isolates	Host	Organism	Family	Phylum
AH3.3	*Arachishypogaea* L.	*Enterobacter* sp.	Enterobacteriaceae	Proteobacteria
AH5	*A.hypogaea* L.	*Inquillinuslimosus*	Rhodospirillaceae	Proteobacteria
AH3	*A.hypogaea* L.	*Bacillus mageterium*	Bacillaceae	Firmicutes
SSB3.3	*S.sesban* L.	*Actinobacter* sp.	Microbacteriaceae	Actinobacteria
SSB3.1	*S.sesban* L.	*Stenotrophomonas* sp.	Xanthomonadaceae	Proteobacteria
SSL4.2	*S.sesban* L.	*Agrobacterium tumefaciens*	Rhizobiaceae	Proteobacteria
SSB1	*S.sesban* L.	*Pseudomonas geniculata*	Xanthomonadaceae	Proteobacteria
SSL1	*S.sesban* L.	*Pantoeaagglomerans*	Enterobacteriaceae	Proteobacteria
SSS2.1	*S.sesban* L.	*Labrys* sp.	'Beijerinckiaceae'	Proteobacteria
PM1.1	*Vigna mungo* L.	*Bacillus mycoides*	Bacillaceae	Firmicutes
PM2.1	*V. mungo* L.	*Enterobacter* sp.	Enterobacteriaceae	Proteobacteria
PM2.2	*V. mungo* L.	*Klebsiella pneumoniae*	Enterobacteriaceae	Proteobacteria
DL5	*Dolichos lablab* L.	*Enterobacter* sp.	Enterobacteriaceae	Proteobacteria

Effects of non-rhizobial bacteria on the growth of legumes

Effects of root-nodule inhabiting bacteria on nodulation, shoot height, shoot biomass and leaf chlorophyll contents on *A. hypogaea* L. and *V. mungo* L. are shown in Table 3. Of all the isolates identified, *Klebsiella* sp. and *Enterobacter* sp. produced nodules in both *V. mungo* L. and *A. hypogaea* L. indicating their infection and nodulation ability to these legume species. On the other hand, *Bacillus* sp. produced nodules only in *V. mungo* indicating the specificity of this genus to host species in infecting plants. Nodulation in legume plants by non-rhizobial bacteria has been reported by other studies [16]. *Enterobacter* spp. were reported to nodulate *Hedysarum* sp.[15]. Although *Bacillus* sp. has been reported to stimulate growth and nodulation in plants [17,18] there has been no data on nodulation by these bacteria in plants suggesting further study to confirm this result. *Klebsiella pneumonia* has been reported to contain nitrogen fixing gene [19]. Lateral transfer of genes responsible for nodulation in legumes from rhizobia to non-rhizobia might be the reason for such nodulation[16].

Results showed that most of the isolates enhanced shoot height, shoot biomass and leaf chlorophyll contents in *V. mungo* L. but not in *A. hypogaea* L. Results also showed that, except *Actinobacter* sp., *Labrys* sp. and *Enterobacter* sp., all other isolates stimulated leaf chlorophyll content in *V. mungo* L. Plant shoot height of *V. mungo* L. was increased by all isolates except that of *B. mageterium*, *Inquillinuslimosus*, *Pseudomonas geniculata*, *Pantoea agglomerans* and *Labrys* sp. Plant shoot mass was increased by all isolates except that of *Labrys* sp. where it remained unchanged. Results also demonstrated that *Labrys* sp. caused reduced and unchanged shoot height, mass and leaf chlorophyll content in both *V. mungo* L. and *A. hypogaea* L.

Results of the present study demonstrated that root nodules of the leguminous plants are habitats not only for the rhizobial bacteria but also for the non-rhizobial bacteria those enter the root nodules by the mixed infection with the rhizobial cell capable of nodulation in roots resulting mixed population within the nodule [16]. The result of the present study is in agreement with the results of the other studies those reported the presence of non-rhizobial bacteria in the root nodules of leguminous plants. Presence of *B. subtilis*, *B. simplex* and *Agrobacterium tumefaciens* were reported in the root nodules of *V. mungo*[1]. Formation of root nodules in the presence of *B. mycoides*, *K. pneumonia*, and *Enterobacter* sp. indicates their potentials in developing root nodules in the legume plants. Such infection to the legume plants might be due to the lateral transfer of nodule inducing gene to these bacteria. Nodulation in plants by the presence of *K. pneumonia* was reported by other study [20]. Indole acetic acid producing *Bacillus* sp. enhanced nodulation in *Phaseolus vulgaris* by *Rhizobium etli*[21].

The results that *Bacillus* spp. generally stimulated leaf chlorophyll content, shoot height and biomass in both *A. hypogaea* and *V. mungo* indicated that *Bacillus* spp. has the potential to be used in growth promotion of *A. hypogaea* L. and *V. mungo* L. Other study also reported growth stimulation in plants by *Bacillus* sp. [8]. Less responsiveness of *A. hypogaea* L. compared to *V. mungo* L. to isolates in nodulation, leaf chlorophyll contents, shoot height and shoot mass indicated the species specific potentials of isolates in the influence of growth of plants. Overall, the results of the present study reveal that root nodules of the legume plants grown in the soils of Bangladesh support diverse bacterial communities with varied range of growth promotion. Results of the present study are thus relevant for enhancing our knowledge about the potential use of non-rhizobial bacterial in growth and development of leguminous crops which is relevant for securing sustainable agriculture.

Table 3. Effects of isolates on nodulation, leaf chlorophyll content (SPAD value), shoot height (cm) and shoot biomass (g) of *Arachis hypogaea* L. and *Vigna mungo* L. (n = 3)

		Arachis hypogaea L.				*Vigna mungo* L.			
Isolates	Organism	Nodule number	SPAD value	Shoot height	Shoot mass	Nodule number	SPAD value	Shoot height	Shoot mass
AH3.3	*Enterobacter* sp.	0	39.55	12.25	0.36	0	24.0	13.5	0.06
AH3	*Bacillus mageterium*	0	51.9	20	0.77	0	29.45	11.25	0.06
AH5	*Inquillinus limosus*	0	36.1	15.5	0.53	0	26.57	8.33	0.08
SSB3.3	*Actinobacter* sp.	0	38.1	14.5	0.33	0	18.3	15.17	0.07
SSB3.1	*Stenotrophomonas* sp.	0	42.3	4	0.22	0	26.0	13.67	0.06
SSL4.2	*Agrobacterium tumefaciens*	0	37.3	14	0.45	0	36.5	13.0	0.06
SSB1	*Pseudomonas geniculata*	0	51.3	17.25	0.475	0	22.4	11.0	0.06
SSL1	*Pantoea agglomerans*	0	43.1	10.5	0.43	0	29.3	11.0	0.06
SSS2.1	*Labrys* sp.	0	41.5	14.75	0.33	0	19.6	11.0	0.05
PM1.1	*Bacillus mycoides*	0	39.4	12	0.39	41.67	30.23	11.83	0.06
PM2.1	*Enterobacter* sp.	0	40.8	15	0.52	0	22.3	13.67	0.07
PM2.2	*Klebsiella pneumoniae*	36	26.85	4.25	0.22	11	35.0	13.83	0.08
DL5	*Enterobacter sp*.	10	54.5	11	0.36	28.33	29.3	11.83	0.06
Control		0	44.05	15.5	0.605	0	22.46	11.79	0.05

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