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Full Length Research Paper

Physiological and phylogenetic analysis of rhizobia isolated from *Acacia nilotica* L.

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The legume-rhizobia relationship is known to be a beneficial symbiosis in the direction of agriculture. The present investigation was aimed to isolate and characterize five rhizobia isolates from woody legume tree; *Acacia nilotica* L. Physiological properties of all five isolated strains were similar. However, on the basis of nitrogen fixation test, only two isolates were symbiotic and the other three were non- symbiotic. Therefore, for phylogenetic analysis, only two isolates were subjected to 16S rRNA gene sequencing for strains. Hence, the DNA was isolated from the well grown bacterial cultures. Amplified DNA was sequenced for 16S rRNA. The phylogenetic tree was constructed and bacterial isolates were identified as rhizobia. Isolate first showed strong homology with *Mesorhizobium loti* and isolate second showed intermediate with *Rhizobium leguminosarum* and *Rhizobium hainanense.* For the confirmation of nitrogen fixing ability of isolates, *nif*H gene was amplified. Application of effective rhizobia strains as biofertilizers to improve legume production is an important approach in sustainable agriculture.

Key words: Legume, rhizobia, physiological, phylogenetic, biofertilizers.

INTRODUCTION

The fixation of N_2 by legumes plays a key role in agricultural sustainability. Moreover, the further assessment of rhizobial genetic diversity is contributing both to the worldwide knowledge of biodiversity of soil microorganisms and to the usefulness of rhizobial collections, and it is developing long-term strategies to increase contributions of legume-fixed to agricultural productivity. *Acacia nilotica* L. one of the legume tree, belongs to the family Leguminosae which fixes nitrogen via. a symbiosis with rhizobia. Rhizobia are soil bacteria that fix nitrogen after becoming established inside root nodules of legumes. The rhizobia cannot independently fix nitrogen and require a plant host.To improve nodulation and nitrogen fixation in nitrogen fixing legumes and legume species, the most practical method of obtaining a strain that may have effective nitrogen fixing ability and is suitable to local environment conditions. Nodulated wild legumes have potential for nitrogen fixation, reforestation and to control soil erosion (Lorite et al., 2010; Elsoni and Osman, 2011).

From last few years, wild legumes and their symbionts have drawn the attention of ecologist because of their tolerance to extreme environmental conditions such as severe drought, salinity and elevated temperatures. Addition, symbiotic rhizobia of naturally growing legumes successfully establish effective symbioses under these conditions (Zaharan, 2001).

The objective of this study was to isolate and characterize the rhizobial populations naturally associated wild legume *A. nilotica* L. originating from different ecological areas on the basis of chemical as well as molecular analysis.

MATERIALS AND METHODS

Isolation of Rhizobial and morpho-physiological, biochemical characterization of isolates

The root nodules were collected from *A. nilotica* L. (host plants are shown in Table 1) in arid and semi arid regions of Rajasthan and Haryana, India. From each plant sampled, three to four nodules were at random excised. Root material was washed in running tap

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Table 1. Isolates of Bacteria from root nodules of *Acacia nilotica* L. used in the present study.

S/N	Name of the Isolate	Origin
1	1	Jaipur
2	2	Hisar
3	3	Ajmer
4	4	Phulera
5	5	Jobner

water to remove adherent soil and surface sterilized with ethanol and hydrogen peroxide. Rhizobia were isolated on yeast-extract mannitol agar (YEMA) using standard procedures. Single colonies were marked and checked for purity by repeated streaking on YEMA medium (Vincent, 1970) and verifying a single type of colony morphology, absorption of Congo red (0.00125 mg kg-1) and were biochemically characterized by Gram's reaction, carbohydrate fermentation, oxidase test, H_2S production, IMViC tests, NO_2 reduction, starch and gelatin hydrolysis as per the standard methods (Cappuccino and Sherman, 1992; Raymond et al., 2004).

Purification and storage

Agar plates were incubated at 28°C for three to ten days. Individual colonies appearing over this period were re-streaked onto fresh Congo red YEMA plates to obtain pure Culture. For short term storage sub-cultured onto YMA+Ca slopes in test tubes. For long term storage broth culture suspended in 10% Glycerol and stored at -4°C.

DNA extraction from pure cultures

Total genomic DNA was extracted from bacteria samples using a modified method described by Petersen and Scheie (2000), modified.

Identification of the isolated bacteria by sequencing of the amplified 16S rRNA gene

The most powerful tool to identify the unknown bacteria is to sequence the gene (DNA) coding for 16S rRNA, which is present in the chromosome of the bacteria. The prokaryotic specific primers used for 16S rRNA gene amplification were 27F (5' AGAGTTTGA-TCCTGGCTCAG 3'), (Lane et al., 1991) and 519R (5-GWATTA-CCGCGGCKGCTG-3') (Turner et al., 1999). The gene coding for the 16S rRNA is amplified using the Polymerase Chain Reaction (Mullis, 1990). The amplified product has been subjected to sequencing with closely related sequences retrieved from EMBL by using CLUSTAL W (Felsenstein, 1993). Pair wise evolutionary distances were computed by using MEGA-4 software. Phylogenetic tree was constructed using NEIGHBOR, UPGMA, KITSCH, FITCH and DNAPARS of the PHYLIP package (Rump and Krist, 1992).

Amplification and sequencing of nif H gene

The *nif*H gene encodes dinitrogenase reductase which is essential for nitrogen fixation in the diazotropic organisms (Raymond et al., 2004). For amplification, a loop of bacterial culture from a yeast extract mannitol agar plate was suspended in water and boiled, and 25 µl was used to prepare a 50 µl PCR that also contained 1X reaction buffer, 2 mM $MgCl_2$, and 1 U of Taq polymerase (Imperial Life Science), each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 6.25 pmol of each primer (final concentration, 125 mM). The following temperature cycle was used: 2 min at 93°C, 35 cycles consisting of 45 s at 93°C, 45 s at 62°C, and 2 min at 72°C *nif*H was amplified by using primers *nif*H-1 and *nif*H-2 (Eardly et al., 1992), which amplify a 601-bp fragment.

RESULTS

Morpho-physiological, biochemical characterization

In the present study, 5 isolates (Table 1) were isolated from root nodules of *A. nilotica* L. collected from different locations in Rajasthan and Haryana. All strains tested were found to have circular colonies with regular borders, flat in elevation, creamy in color after 3 to 5 day of growth on YEMA at 28°C. On the basis of morphological and biochemical characterization, out of the five isolates, only two showed positive nitrogen fixation test (Table 2).

16S rRNA gene amplification and sequencing of amplified product

The almost complete 16S rRNA gene sequence (1565 bp) was aligned. Analysis of the 16S rRNA gene sequences on the two isolates was performed using NCBI BLAST (National Centre for Biotechnology Information). The complete sequences were aligned to the homologous sequence available for Rhizobia strains. The BLAST (NCBI) search using the sequences showed 99% homology of isolates to other GenBank as rhizobia with 16S rRNA gene sequences (Table 3).

Phylogenetic analysis

Phylogenetic tree analysis and sequence similarity calculations after neighbor joining analysis showed isolate 1 strong homology with *Mesorhizobium loti* and isolate 2 showed intermediate with *Rhizobium leguminosarum* and *Rhizobium hainanense* available in the database (Figure 1).

Table 2. Morphological and Biochemical tests of isolates.

Isolate number	1	2	3	4	5
Gram stain –reaction	-	-	-	-	-
Colony morphology	rod	rod	rod	rod	rod
Colony colour	Creamy	Creamy	Creamy	Creamy	Creamy
Bromo thymol blue with colony colour	+	+	+	+	+
Congo red with medium colony colour	White	White	White	White	White
Indole Acetic Acid Test	+	+	+	+	+
Nitrate reduction test	+	+	+	+	+
Siderophore production test	+	+	+	+	+
Nitrogen fixtion test	+	+	+	+	+
NNn	+	+	-	-	-

Table 3. Analysis of the 16S rRNA gene sequences for comparison of isolates with other rhizobia sp.using NCBI BLAST.

S/N	Name of Rhizobium	GenBank accession No.
1	*Rhizobium mediterraneum*	L38825
2	*Rhizobium leguminosarum*	U29386
3	*Mesorhizobium loti*	X67229
4	*Rhizobium giardinii*	U86344
5	*Rhizobium hainanense*	U71078
6	*Rhizobium tianshanense*	U71079
7	*Rhizobium multihospitium*	EF050765
8	*Bradyrhizobium betae*	AY372184

PCR amplification of *nif*H genes

After amplification of *nif*H gene by specific primers viz, *nif*H1 and *nif*H2, and comparison with low range DNA ruler, 601 bp long amplified products were obtained (Figure 2).

DISCUSSION

The ability of legumes to obtain the nitrogen required for their growth and reproduction from both soil and symbiosis sets them apart from other economically valuable crops. *A. nilotica* L. is one of the legume tree which fixes nitrogen through symbiosis with rhizobia. Earlier many research have been done on rhizobia in other species of Acacia (Manessila et al., 2007) and other plant species (Dubey et al., 2010; Stajkovic et al., 2011; Mehboob et al., 2011). Shetta et al. (2011) also isolated rhizobia from *Acacia nilotica* L., but they worked only up to physiological level.

Here, the present analysis specified isolation and characterization of rhizobia strains from both phy-siological and phylogenetic investigation. All five isolated strains were phenotypically similar except nitrogen fixation test; out of five only two showed positive test. Due to this, only two were symbiotic and the other three were non-symbiotic. Therefore, only two symbiotic rhizobia strains were selected for phyolgenetic analysis to 16S r-RNA sequencing. The 16S r-RNA gene is useful for characterization because it is slowly evolving and the gene product is both universally essential and functionally conserved. For this, the PCR product was bi-directionally sequenced using the forward, reversed and an internal primer. Sequence data was aligned and analyzed for finding the closest homologs for the microbes. Rhizobia are known to be nitrogen fixing ability so for the identification of nitrogenase activity, *nif*H gene were amplified in both the isolates. Recent study on *nif*H gene was identified in rhizobia isolated from *Cajanus cajan* L. (Dubey et al., 2010).

Conclusion

The isolates from the present study may be useful to increase the symbiotic nitrogen fixation in legume trees. Earlier reports depicted rhizobia as biofertilizers. Biofertilizers from microorganisms can replace chemical fertilizers to increase crop production. In principle, biofertilizers are less expensive and are more environmentally-friendly than chemical fertilizers.

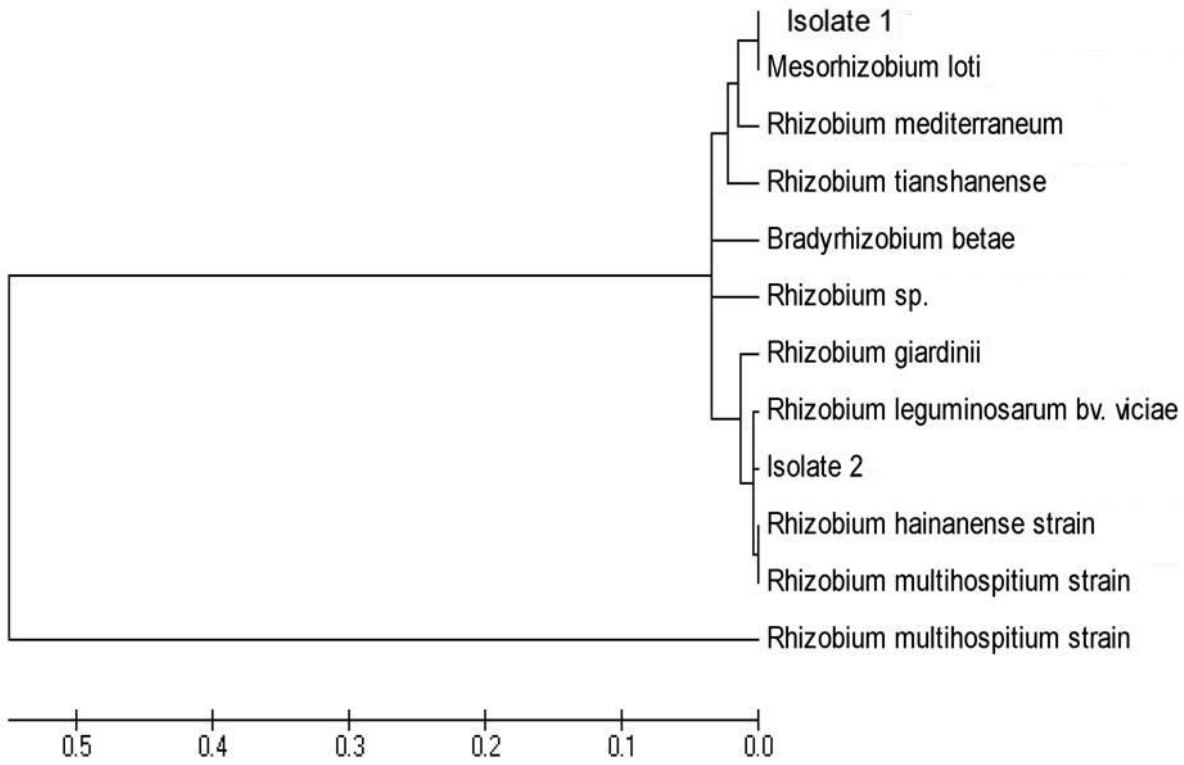

Figure 1. Phylogenetic tree based on the 16S rRNA sequencing of isolated bacteria.

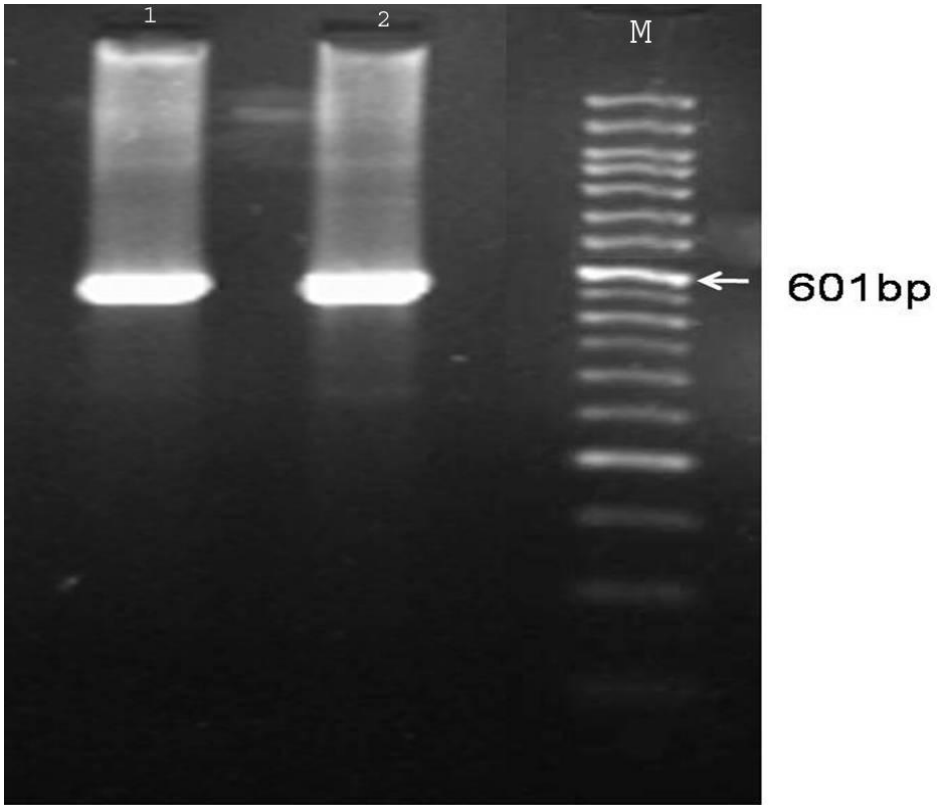

Figure 2. Amplification of *nif*H gene of Rhizobia Isolate 1 and Isolate 2.

Therefore, the study provides basis for further research on the phylogeny of rhizobia strains nodulating the legume trees, as well as their use as inoculants to improve growth and nitrogen fixation in arid and semi arid regions of India.

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