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Identification of Native Root Nodulating Bacteria from Mucuna (*Mucuna pruriens* L.)

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ABSTRACT

Background: Mucuna (*Mucuna pruriens* L.) is an annual herbaceous climber, grown as a medicinal, green manure, cover and smothering crop. The mucuna seeds contains L-DOPA (L-3, 4 dihydroxy phenylalanine), a non-protein amino acid, extensively used for Parkinson and hypertensive drug. Injudicious application of nitrogenous fertilizers leads to deterioration of soil quality which results into loss of crop yield and quality. The application of microbial inoculant containing efficient native rhizobia enhances the nodular properties, N_2-fixation and soil quality. Therefore, *Rhizobium* strain associated with mucuna was isolated, biochemically characterized and identified. The 16SrRNA sequencing revealed that *Sinnorhizobium mililoti,* a gram negative symbiotic nitrogen fixing bacteria is present in root nodules of mucuna.

Methods: Root nodules were extracted from mucuna grown at ICAR- Krishi Vigyan Kendra, Doddaballapura Taluk, Bengaluru Rural District, Karnataka, then cultured, screened and characterized in the laboratory. The 16SrRNA sequencing and phylogenetic analysis was done to identify the native rhizobial strain.

Result: Identification of native root nodulating bacteria through 16SrRNA sequencing concluded that *Sinnorhizobium mililoti* strain associated with the root nodules of Mucuna (*Mucuna pruriens* L.).

Key words: 16SrRNA, Mucuna, Rhizobium, *Sinnorhizobium mililoti.*

INTRODUCTION

Fabaceae plants have a special relationship with certain type of soil bacterium that gives them access to nitrogen in the atmosphere that would otherwise be inaccessible to them. The species of this family are greatly diversified as a result of their interaction and cognate bacterial symbionts, known as rhizobia (Sprent, 2007).

The positive effects of productive symbiosis between legumes and nitrogen-fixing bacteria in agricultural output are well known and these benefits result from the bacteria's ability to fix significant amounts of atmospheric nitrogen, which allows them to survive in soils depleted in nitrogen without the need for nitrogen fertilisers while also increasing the nitrogen content of the soil (Cheng *et al*., 2023; Alvarez-Aragón *et al*., 2023). Native legumes might not only be genetically predisposed to flourishing in the field, but also as a result of the development of advantageous bacterial interactions with soil microorganisms (Afzal *et al*., 2019). Since endophytic bacteria have the potential for biotechnological and agronomic uses, natural legumes are anticipated to offer a valuable reservoir of these organisms.

Rhizobia soil bacteria and legumes form a facultative symbiosis that is triggered by the host plant's starvation of nitrogen. The transformation of bacteria into nitrogen-fixing bacteroids takes place in root nodules, which are created when signal molecules are passed between the partners. In this mutualistic symbiosis, the bacteria exchange photosynthates from the host for nitrogen sources to support plant growth. Bacterial symbiosis can be irreversible or reversible depending on the host plant (MacLean *et al*., 2007).

Many modern medicines are created indirectly from plants, that are sources of novel pharmaceuticals. But analogus to

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the available traditional and scientific facts, legumes have a bright future in terms of nutrition, medicine and agricultural growth in developing nations, Mucuna botanically known as *Mucuna pruriens* is one such well-known legume.

Mucuna, commonly referred to as cowhage or velvet bean and a member of the fabaceae family, is also known as cowitch, Kawaanch, kapikachhu, or Alkushi. Tropical regions are where it originally evolved, mainly in Africa, India and the West Indies.It is an annual climber with 22 pairs of chromosomes in its diploid genome (2n = 2× = 22). Among other under-used legumes, the Mucuna is well adapted to tropical and subtropical regions of the world.It is used to treat hypertension, scorpion stings, leucorrhoea, spermatorrhoea, diabetes, Parkinson's disease, as an aphrodisiac, as a nervine tonic and other menstrual diseases. According to Janardhanan *et al*. (2005), it is regarded as a reliable source of dietary protein.

The Mucuna, like most legumes, has the ability to fix atmospheric nitrogen through a symbiotic connection with soil microorganisms. Global support has been given to ongoing study because of the significance of the rhizobium legume symbiosis for agriculture. The ability of many rhizobia to form nodules has not been studied, but as legumes are responsible for a large portion of the nitrogen flux from atmospheric N_2 to fixed forms such as ammonia, nitrate and organic nitrogen is crucial for both ecology and agriculture. The symbiotic fixation of atmospheric nitrogen by *Rhizobium* species and legumes is a sustainable source of nitrogen for agriculture (Peoples *et al*., 1995). Therefore, the aim of the present investigation is to isolate and identify native strains of *Rhizobium* in root nodules of Mucuna on the basis of molecular characteristics which could be used to inoculate Mucuna and enhance the yield and quality by reducing the application of nitrogenous fertilizers.

MATERIALS AND METHODS

An experiment was conducted at ICAR-Krishi Vigyan Kendra, Bengaluru Rural District and Department of Agricultural Microbiology, University of Agricultural Sciences, Bengaluru, Karnataka, India during the year 2021.

Collection and preservation of root nodules

The root nodules were collected from 10 mucuna plants at bloom stage during *kharif* season. The whole plant was dug up with its root system intact and partitioned in to herb and roots. The nodules were removed from the root was washed repeatedly with tap water and preserved in sterile water blanks under refrigeration in laboratory (Indu *et al*., 2018).

Isolation of native root nodulating rhizobial strain

The methodology developed by Sonali and Priya (2017) and Biswajit *et al*. (2015) was adopted for isolation of native root nodulating rhizobial strain. The root nodules were surface sterilized by immersing in 0.1% mercuric chloride and 4% hydrogen peroxide for 5 minutes and then washed repeatedly with sterile distilled water. Then nodules were washed in 70% ethyl-alcohol followed by washing with sterile distilled water. After being individually crushed with the help of a sterile glass rod, the root nodules were streaked on Yeast Extract Mannitol Agar media with Congo Red (YEMA) plates. The plates were incubated at 28°C for 48 hours, after which pure individual colonies were sub-cultured on the same medium and the isolates were further investigated for morphological and biochemical characteristics. To maintain purity, the partitioning procedure was executed (Fig 1).

16S rRNA sequencing and phylogenetic analysis

16S rRNA methodology was followed for molecular characterization of native root nodulating bacteria as procedure outlined by Patel, 2001. A single band of high-molecular weight DNA was detected after DNA was recovered from the prepared culture and its quality was assessed on a 1.0% agarose gel. 16S rRNA-F and 16SrRNA-R primers were employed to amplify a fragment of the 16SrRNA gene. When resolved on an agarose gel, a single discrete 801 bp PCR amplicon band was seen. To get rid of impurities, the PCR amplicon was cleaned. Using the BDT v3.1 Cycle sequencing kit and the ABI 3730xl Genetic Analyzer, a PCR amplicon was subjected to a forward and reverse DNA sequencing procedure. The 16SrRNA gene consensus sequence was created with aligner software using forward and reverse sequence data. BLAST searches with the NCBI Gene Bank's'nr' database were conducted using the 16S rRNA gene sequence. First 10 sequences were chosen and aligned using Clustal W's multiple alignment software based on maximum identity score. To determine the native rhizobium strain, a distance matrix and phylogenetic tree were built using MEGA 10 (Fig 2).

RESULTS AND DISCUSSION

Isolation of bacterial strains from root nodules of Mucuna grown under field conditions was carried out in Microbiology laboratory. In this context, 12 isolates were obtained from root nodules of Mucuna and congo red is often incorporated in culture media for isolating rhizobia and for testing the purity of rhizobia cultures. Rhizobia typically do not absorb congo red or absorb it weakly, while other bacteria absorb it strongly. All isolates were slow growers, showed little congo red absorption and had an alkaline reaction on BTB, indicated by a blue colour, which is usually produced by *Bradyrhizobium* spp. (Somasegaran and Hoben, 1994).

The assessment of genotypic diversity of rhizobium from Mucuna was achieved by biochemical characterization. Among various tests, catalase test, motility test and ONPG (O-nitro phenyl-b-D-Galacto-pyranoside) were found positive. Whereas, methyl red test, voges-proskauer (VP), indole test, citrate utilization test, hydrogen sulphide production, urea hydrolysis test and gel liquification test were found negative (Table 1). The prominent rhizobium species identified as *Sinorhizobium meliloti* and characterized as symbiotic, fast growing gram-negative bacterium (Marina *et al*., 2002). Morphologically, in contrast to *Bradyrhizobium japonicum* plain filaments, the *S. meliloti* filament is referred to as the complex filament and also when there is a low cell

density, each cell has peritrichous flagella and can swim in water. It converts atmospheric nitrogen into ammonia form that may be exercised by the host in which they reside and also it renounces excess nitrogen in the soil which may potentially reduce the need for fertilizers.

Table 1: Biochemical characteristics of rhizobial isolates from Macuna (*Mucuna pruriens* L.).

Biochemical tests performed	Results
Catalase test	Positive
Motility test	Positive
ONPG (O-nitro phenyl-b-D- Galacto-pyranoside)	Positive
Methyl red test	Negative
Voges-proskauer (VP)	Negative
Indole test	Negative
Citrate utilization test	Negative
Hydrogen sulphide production	Negative
Urea hydrolysis test	Negative
Gel liquification test	Negative

Genetic traits were more accurate and illuminating than the morphological and biochemical traits and same were utilised to characterise the native root nodulating rhizobial strain. In the present study, the isolate that resembled *Rhizobium* sp. was cultured on YEMA plates and incubated for 48 hours and the native organism was recognised by applying PCR with the aid of a universal primer based on 16S rRNA homology. This method assist to pick out poorly described, rarely isolated or phenotypically deviant strains. It can also help to identify novel pathogens and uncultured bacteria (Patel, 2001).

The purified PCR products of 801 bp were sequenced by using 27F and 1492R primers and the strain was identified as *Sinorhizobium meliloti*. The results of sequencing (BLAST queries of Gene Bank) showed that, the isolate have recorded the maximum score of 1696 bits, 91% query coverage, E value of 3e-91 and 100% identity were selected as the probable specifications of the isolate. The bacterial segregate shown 100% alikeness to the *Sinorhizobium meliloti* strain, according to the BLAST results, which were depicted in Table 2 and Fig 2.



Fig 1: Bacterial colonies *Sinorhizobium meliloti* strain isolated from root nodules of Mucuna (*Mucuna pruriens* L.).

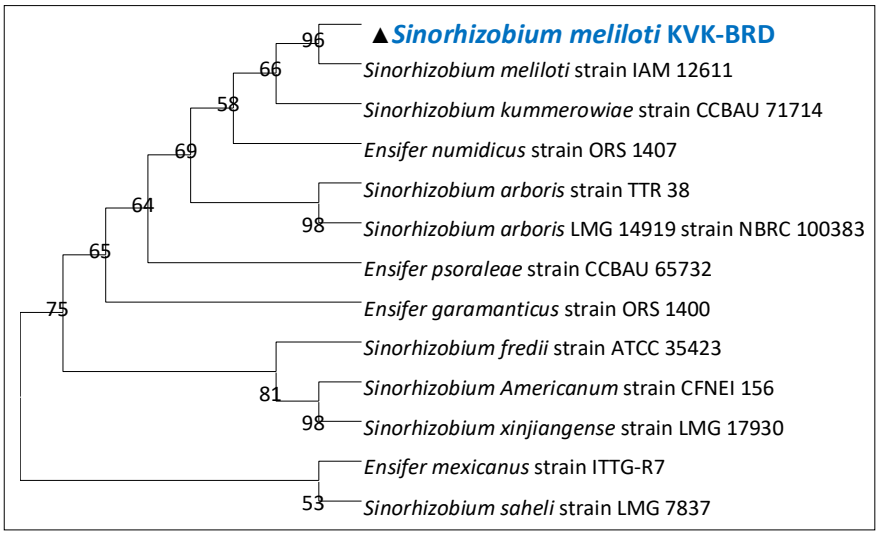


Fig 2: Phylogenetic tree of *Sinorhizobium meliloti.*

Table 2: BLAST result of 16S rRNA sequence showing similarity with *Sinorhizobium meliloti*.

Accession	Description	Maximum score	Total score	Query cover	E value	Per cent identity
CP021890.1	Complete sequence of *Sinorhizobium meliloti* strain Rm41 plasmid pSymA	350	1696	91%	3e-91	100.00%
HE995407.1	Complete sequence of*Sinorhizobium meliloti* Rm41 plasmid pSYMA	350	1696	91%	3e-91	100.00%
X01649.1	*Rhizobium meliloti* nodulation genes nodA, nodB and nodC	350	1379	95%	3e-91	100.00%
CP026526.1	Complete sequence of *Sinorhizobium meliloti* strain AK21 plasmid pSymA	344	1909	88%	1e-89	99.47%
CP019486.1	Complete sequence of *Sinorhizobium meliloti* strain B399 plasmid pSymA	344	1993	88%	1e-89	99.47%
CP019483.1	Complete sequence of *Sinorhizobium meliloti* strain B401 plasmid pSymA	344	1993	88%	1e-89	99.47%
CP021801.1	Complete sequence of *Sinorhizobium meliloti* strain USDA1021 plasmid pSymA	344	2003	91%	1e-89	99.47%
CP021798.1	Complete sequence of *Sinorhizobium meliloti* strain USDA1106 plasmid pSymA	344	2008	91%	1e-89	99.47%
CP003936.2	Complete sequence of *Sinorhizobium meliloti* GR4 plasmid pRmeGR4c	344	2008	91%	1e-89	99.47%
CP090104.1	Complete genome of *Sinorhizobium meliloti* strain Ak57 chromosome	344	2008	91%	1e-89	99.47%

Table 3: *Sinorhizobium meliloti* isolation from root nodules of mucuna (*Mucuna pruriens* L.).

Crop	Year of isolation	Organism identified	Accession no.	Closest type strain in the NCBI database	Sequence similarity
Cowhage	2020-2021	*Sinorhizobium meliloti* KVK-BRD	OP199029	*Sinorhizobium meliloti* strain IAM 12611	100%

The isolate of *Sinorhizobium meliloti* sequence strain of Mucuna is labelled as *Sinorhizobium meliloti* KVK-BRD and was deposited in the NCBI Gene Bank database with a unique accession number OP199029 (Table 3) as it is first of kind with respect to identification of native rhizobium starin of mucuna.

CONCLUSION

The rhizobium associated with root nodules of leguminous plants fixes the nitrogen from atmosphere. It is very essential to enhance the area under legumes to replenish nitrogen depleted soils and supply adequate nutrients particularly protein to the human beings and animals. The current study revealed that, a native root nodulating bacteria of mucuna as *Sinorhizobium meliloti* and same has been labelled as *Sinorhizobium meliloti* KVK-BRD and deposited in NCBI gene bank. The feeler research conducted to assess the efficacy of seed treatment of *Sinorhizobium meliloti* starin extracted from mucuna seeds confirmed the nitrogen fixation apart from enhancing growth, yield and L-DOPA content in mucuna.

Conflict of interest

All authors declared that there is no conflict of interest.

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