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Isolation and Screening of Efficient Rhizobial Strains and Evaluation of Their Efficiency in Moth Bean (*Vigna aconitifolia* Jacq)

R. Brindavathy[1], R. Anandham[2], E. Jamuna[1], R. Anitha[3], Syed Abul Hassan Hussainy[5], M. Gnanachitra[2], U. Sivakumar[2], R. Krishnamoorthy[4], P. Sridhar[1]

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ABSTRACT

Background: Moth bean (*Vigna aconitifolia* Jacq.) is a legume possess a higher nutritional value with 22-24 per cent protein and also used as a pasture legume. Fertilizer applications to moth bean are uncommon in India and mostly grown with minimum inputs and care. The main objective is to increase the production and productivity in moth bean crop, using crop or location specific, native rhizobial strains.

Methods: *In vitro* studies on isolation and characterization of *Rhizobium* from moth bean root nodules was carried out and further its efficiency was tested under pot culture conditions.

Result: Among the thirty isolates obtained, 11 showed positive reactions for poor growth on glucose peptone agar medium, Keto-lactose and catalase activities, citrate utilization in *in vitro* studies and were tentatively identified as *Rhizobium*. By 16S rRNA sequencing and culture number MB-1 was identified as *Rhizobium skierniewicense*. Biometric observations revealed that MB-1 inoculated plants significantly recorded the highest plant height of 34 cm; root length of 14 cm; 24 numbers of leaves per plant having the maximum nodule number of 27 and maximum number of pods of 39 pods plant^{-1} and its corresponding single plant yield of seeds 18.6 g followed by COG15 inoculated plants which recorded 15.5 g of seeds in a plant. The native isolates *Rhizobium skierniewicense*, (MB-1) proven to be a effective strain resulted in higher and bigger nodules, increased biometrics and yield attributes.

Key words: Biochemical characterization, Biometrics, Moth bean, Nodulation, *Rhizobium.*

INTRODUCTION

Moth bean (*Vigna aconitifolia* L.) is a legume, belonging to the family *Fabaceae,* commonly known as kidney gram, aconite bean, or dew bean. It is exceptionally hardy legume and one of the most drought resistant pulses, mostly grown in arid and semiarid regions of India (Singh *et al.,* 2017). Moth bean is one of the *Vigna* species that possess a higher nutritional value where the seeds contain approximately 22-24% protein and also used as a pasture legume. It is a short-day crop and an annual plant with a spreading and prostate growth habit. The crop canopy spreads the low-lying soil and thus prevents soil erosion and also preserves moisture loss. This bean is thus a potential plant which may be used for intercropping with coconut, *Casuarina etc*. Fertilizer applications to moth bean are meagre or uncommon in India.

This arid legume is a tolerant to both drought and heat to some extent and in addition to its stress tolerance; they have adapted to grow with low inputs, light-textured soils and low rainfall (Kumar, 1996). The crop can survive with minimum to no irrigation and tolerates up to 40-50 days without water source and can withstand the temperatures up to 40°C. Its deep and fast penetrating root system favours its drought avoidance mechanism or capability. Grown in such condition this crop rhizosphere would have minimal microbial population and hence they often possess poor nodulation. If grown with proper management practices, nodulation can be increased and thus yield can be maximized.

[1]Oilseeds Research Station, Tamil Nadu Agricultural University, Tindivanam-604 102, Tamil Nadu, India.
[2]Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore- 641 003, Tamil Nadu, India
[3]Department of Crop Physiology, Sugarcane Research Station, Cuddalore, Velisemmandalam-607 001, Tamil Nadu, India.
[4]Department of Crop Management, Vanavarayar Institute of Agriculture, Pollachi-642 103, Tamil Nadu, India.
[5]Department of Agronomy, Agricultural College and Research Institute, Madurai-625 104, Tamil Nadu, India.

Corresponding Author: R. Brindavathy, Oilseeds Research Station, Tamil Nadu Agricultural University, Tindivanam-604 102, Tamil Nadu, India. Email: brindamuruga@yahoo.co.in

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Rhizobium, a saprophyte, exists as free-living bacteria in soil or as an endosymbiont within the root nodules of leguminous plant and accounts for a major share in biologically fixed nitrogen. In a symbiotic relationship, the bacteria fix a constant source of reduced nitrogen to legumes

and in turn, plant supplies nutrients and energy for its survivability (Singh *et al.,* 2008). In addition, this microsymbiont increases plant growth by the production of plant growth hormones, siderophores, induction of systemic resistance in plants (Hussain *et al.,* 2009). This association not only helps the existing crop but is known to benefit the succeeding crops (Thompson *et al*., 1994). These symbiotic interactions are so economical and reduce the inorganic fertilizers.

As per the survey conducted in many regions of Villupuram district, Tamil Nadu, India poor nodulations were found in most leguminous crops. Pertaining to poor irrigation and crop management, moth bean, when compared to other pulses crop had very lesser nodulation. To increase the production and productivity of moth bean crop, using crop or location specific, native, competitive and effective rhizobial strains is essential (Gilller, 2001). Hence this research is proposed to identify a best culture to trigger the nodulation potential and later to formulate a new combination of bacterial inoculants for integral approach for nutritional supplement in moth bean.

MATERIALS AND METHODS

Isolation of *rhizobium* from moth bean root nodules

The research was carried out at Oilseeds Research Station, Tindivanam, Villupuram district, Tamil Nadu, India during 2018 to 2020. Moth bean root samples with nodules were collected during *rabi* 2017 from five villages *viz*., Eraiyanur (12.2087°N, 79.6708°E), Konkarampattu (12.2210°N, 79.5287°E), Mannur (13.0217°N, 79.9574°E), Modaiyur (12.2344°N, 79.4915°E) and Rettanai (12.1958°N, 79.5519°E) of Villupuram district in Tamil Nadu. Unlike other legumes moth bean nodules are easily detachable and the plants were carefully uprooted by scooping it along with the soil in appropriate moisture. Fresh, healthy and larger sized nodules were carefully detached from each plant for study. The selected nodules were in ivory colour. The colour and freshness of the nodules indicated that the rhizobia are in active stage. The nodules were first washed thoroughly with sterile distilled water and were surface sterilized with 70% ethanol and 0.1% mercuric chloride and again washed thrice with sterile distilled water. The collected nodules were used for isolation and further morphological and physiological characterization.

Serial dilutions and plating technique

The root nodules were crushed in a test tube having sterile distilled water using glass rod. A turbid extraction was obtained at the end. Serial dilution was prepared from extraction of bacteroid solution. The extract was serially diluted up to 10^{-6} dilution and 10^{-4} to 10^{-6} dilutions were selected for plating. From the suspension 0.1 mL was spread onto the YEMA plates and incubated at room temperature. The same procedure was followed for the nodule samples collected from different locations and the isolates were named as MB (Table 1).

Morphological characterization

All the selected isolates were examined for the morphological, physiological and biochemical characterization as per the standard procedures given by Barthalomew and Mittewer (1950). Strains of rhizobia were identified by observing their growth on different solid and liquid media for their confirmation. Morphological and microscopic characteristics of all the isolates were investigated after an incubation period of 7 days at 28°C on yeast extract mannitol agar medium. Individual colonies were characterized for shape, size, colour, opacity, elevation, edge and microscopic features of the isolates were studied by Gram's staining technique.

Biochemical characterization

The most important rhizobial presumptive test *viz*., growth on YEMA and congo red medium, growth on glucose peptone agar, catalase activity test, citrate utilization test, Keto-lactose test and growth on YEMA containing BTB medium were performed (Singha *et al.,* 2015) as presented in Table 1.

Growth on YEMA with congo red

In general, rhizobia produced white colonies, whereas contaminants like *Agrobacterium* absorbs the dye strongly. Bacteria isolated from moth bean nodules were streaked on plates and incubated at 28°C for 48 hours.

Growth on glucose peptone agar (GPA) medium

Glucose peptone agar medium with Bromo-thymol blue (indicator) is widely used for isolating pure rhizobial colonies. The bacterial isolates were streaked on glucose peptone agar plates and incubated at 28°C for 48 hours. Rhizobia showed no growth or very poor growth on glucose peptone agar medium and caused very little change in pH Profuse growth in glucose peptone agar medium indicated contamination.

Keto-lactose test

Keto-lactose agar medium was prepared and bacterial isolates were streaked on plates. Inoculated plates were then incubated at 28°C for 48 hours. Plates were then flooded with Benedict's reagent, incubated at 25°C and results were observed after 1 hour.

Catalase activity test

The presence of the enzyme catalase in the rhizobial isolates was examined by suspending one loopful of the organism in a drop of 3 per cent H_2O_2 on a glass slide. Production of bubbles indicated a positive result or vice-versa.

Citrate utilization test

The ability of the isolates to utilize citrate was determined by the growth in Simmon's Citrate Agar (SCA). A distinct colour from green to blue referred to a positive utilization of citrate by the isolates.

Production of exopolysaccharide

The production of exopolysaccharide by each isolate was noted. The appearance of the colonies (*i.e*., gummy, watery,

translucent to a thick dense consistency, milky creamy appearance, opacity, presence of dark centres *etc*.) was observed (Grönemeyer *et al.,* 2014; Paudyal and Gupta, 2017).

Bromothymol blue test

Bromo-thymol blue test was done by inoculating the bacteria on YEM agar medium containing 0.025% bromothymol blue and the plates were incubated for 3 days (Be for acid or alkaline reaction. After incubation, positive sample showed yellow colour due to acid production.

16S rRNA sequencing

Genomic DNA from the efficient bacterium was isolated by using the HipurA® Bacterial Genomic DNA Purification Kit (M/s. Hi Media Laboratories, Mumbai, India). Bacterial genomic DNA was then amplified with 16S rRNA gene target universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) by a thermocycler (Mastercycler® nexus, Eppendorf India Private Limited). The PCR reaction mixture (25 µL) contained 20 ng of template DNA, 0.3 µL of 1 U *Taq*DNA polymerase (Sigma, India), 2.5 µL of 1x Taq DNA polymerase buffer (Sigma, India), 2 mM magnesium chloride (Sigma, India), 400 µM dNTPs (Sigma, India), 10 pmol of forward and reverse primer and the final volume was made up with sterilized deionized water. The thermoprofile condition of PCR consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 10 min and hold at 4°C. Genomic DNA was viewed and documented using a gel documentation and analysis system (Vilber E-box, Germany).

The fluorescent dye terminator method was used for obtaining the nucleotide gene sequences of 16S rRNA, which was then purified by the Millipore-Montage dye removal kit. Sanger sequencing was performed at the M/s. Barcode Biosciences, Bengaluru, India. The 16S rRNA sequences of the isolates obtained from the automatic sequencer were then aligned and identified using the e-server, EzTaxon (http://eztaxon-e.ezbiocloud.net/) (Yoon *et al.*, 2017) to determine their closest relative. The neighbour joining phylogenetic tree was constructed with the bootstrap values of 1000 replicates by using the MEGA version.

Nodulation study

Small sized pots were filled with 3 kg of sterile soil. Uninoculated plants served as negative controls and MB-1, MB-2 and MB-7 was the positive control. The seeds were surface sterilized with 1 per cent mercuric chloride. Seeds were inoculated with 1 ml of YEM broth containing approximately 10^9 cfu mL^{-1} and sterile water source was used. Twenty-five days after inoculation, plants were removed from the pots and the presence or absence of nodules assessed.

Pot culture studies

Pot culture experiment was conducted with six treatments and four replications and arranged in a completely randomized design. Twenty-four pots were filled with sterile soil, each having 7 kg of sterile soil. Surface sterilized seeds were used. Rhizobial isolates MB-1 and MB-2 was used for this study and these were compared with the standard rhizobial isolates *viz*., COG15 used for groundnut and BMBS14 culture used for green gram and black gram. Seeds were treated with the respective rhizobial isolate (2×10^9 cfu ml^{-1}) and shade dried seed were sown (5 seeds pot^{-1}) in the pots. The pots were labelled and watered regularly. Biometric observation *viz*., plant height, root length, number of nodules, nodules dry weight, pod yield per plant, haulms yield was recorded and statistically analysed (Fig 1).

RESULTS AND DISCUSSION

Cultural, morphological and biochemical characters

Rhizobial colonies were obtained on YEM agar plates, after incubation for 2 d at 36°C (Fig 2). The colonies were entire, with regular margin, translucent, circular in shape, shiny, raised (convex), sticky consistency of the colony with 2-4 mm in diameter. These characteristics were similar with the standard morphological characteristics of the *Rhizobium*. These cultures were aerobic, non-spore forming, Gram negative rods. Among the 28 isolates tested, 11 showed negative results to the chemical reaction namely indole, methyl red, Voges-Proskaur, hydrogen sulphide production, gelatin hydrolysis and citrate utilization which cleared the positivity for rhizobia. The results were concurrent with the findings of Sadowsky *et al.* (1983). Similarly, the cultural, morphological and biochemical characters performed in this study was parallel with the works done by Shahzad *et al*. (2012); Vishal and Abhishek, (2014). Nine isolates showed high production of exopolysaccharides. All these 11 isolates showed positive reaction for, keto-lactose, catalase activity, citrate utilization and exhibited poor growth on glucose peptone agar. Five isolates showed positive for growth on YEMA with Congo red. Among the five, MB-1, MB-2, MB-7 isolates showed positive results for Bromothymol blue test (Table 1). From the results of cultural, morphological, biochemical characters and presumptive tests, the three isolates MB-1, MB-2, MB-7 were tentatively identified as *Rhizobium*. Similar results were also observed by Singh *et al*. (2008) in his biochemical characterization test on rhizobia.

Nodulation study

Among the three isolates, MB-1, MB-2 and MB-7 tested for nodulation, MB-1 and MB-2 were able to nodulate the host plants and were found to be healthy. The uninoculated plants did not show nodule formation and the plants showed chlorosis symptoms. Similar results were presented by Beshah and Assefa (2019) in mung bean, where the native rhizobial cultures were tested for its nodulation potential. This finding was in conformity with the work done by Pawar *et al*. (2014) where, the nodulation and increased growth was observed in moth bean.

16S rRNA sequencing

The amplified PCR products sequences were then aligned with those of the closely related *Rhizobium* species. The similarities of the sequences were analyzed using the MegAlign program, version 7.1.0 (DNA Star, Madison, WI, USA). Distance Matrix was calculated based on Nucleotide Sequence Homology (Using Kimura-2 Parameter). Phylogenetic tree was constructed with the MEGA program, version 5.1 (Roohi *et al*., 2012). The accession number and per cent homology of full length 16S rDNA revealed that the isolate MB-1 belonged to *Rhizobium skierniewicense*. Phylogenic tree showed accession numbers and percentage homology of each strain (Fig 2). This is in agreement with the earlier report by Squartini *et al.* (2002).

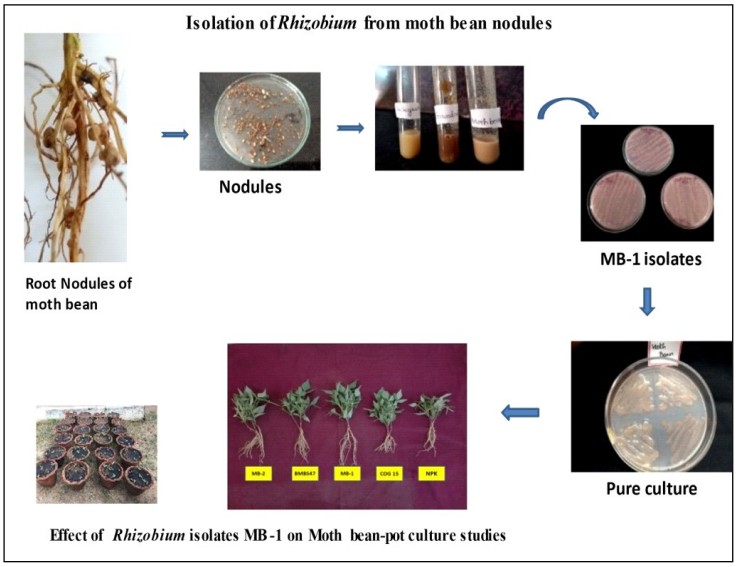


Fig 1: Images of biometric characters of moth bean.

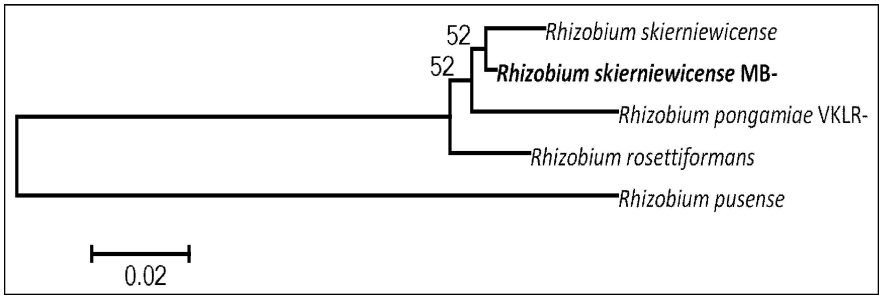


Fig 2: Phylogenetic relationship between known bacterial 16S rRNA sequences and MB-1. Bacterial sequence of MB-1 is in bold. Bootstrap values (from 1000 replicates). Bar 0.02 substations per nucleotide position.

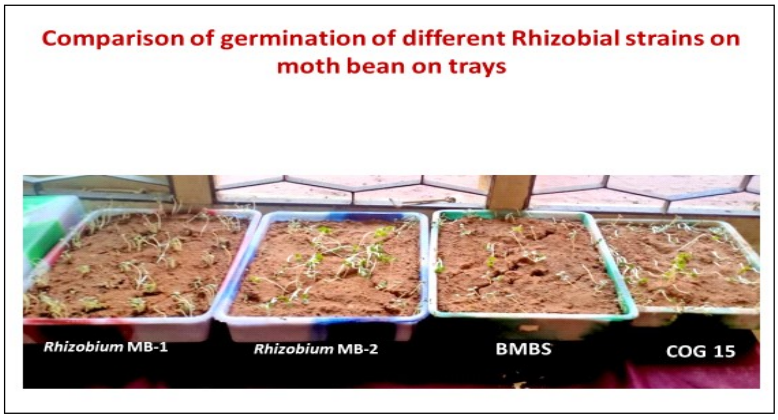


Fig 3: Germination studies in moth bean.

Table 1: Cultural, morphological and biochemical characters.

Cultural and morphological characters	Cultures positive	Tentative identification of rhizobia
Colony character	MB-1, MB-2, MB-6, MB-7, MB-11, MB-15,	
1. Translucent and Glistening	MB-20, MB-21, MB-22, MB-27, MB-28	11
2. Raised colonies	MB-1, MB-2, MB-7, MB-11, MB-15, MB-20, MB-22, MB-27	8
Production of EPS	MB-1, MB-2, MB-6, MB-7, MB-11, MB-15, MB-20, MB-21, MB-22	9
Gram reaction	MB-1, MB-2, MB-6, MB-7, MB-11, MB-15, MB-20, MB-21, MB-22, MB-27, MB-28	11
Presumptive test		
Growth on YEMA with Congo red	MB-1, MB-2, MB-7, MB-22, MB-27	5
Poor growth on Glucose Peptone Agar (GPA) medium	MB-1, MB-2, MB-7, MB-20, MB-22, MB-27	6
Keto-lactose test	MB-1, MB-2, MB-7, MB-20, MB-22, MB-27	6
Catalase activity test	MB-1, MB-2, MB-7, MB-22, MB-27	6
Citrate utilization test	MB-1, MB-2, MB-7, MB-22, MB-27	5
Bromothymol blue test	MB-1, MB-2, MB-7	3

Table 2: Effect of Rhizobial strain MB-1 on the growth promotion in moth bean.

Treatments	Plant height (cm)	Root length (cm)	No. of nodules plant^{-1}	Nodule dry wt. (mg) plant^{-1}	No. of leaves plant^{-1}	No. of pods plant^{-1}	Single plant yield (g)
T_1- MB-1	34.5	14	27.3	13.2	24.0	39 .0	18.6
T_2- MB-2	26.3	10.8	22.2	11.3	21.2	27.2	14.3
T_3- BMBS 47	29.0	12.5	20.6	11.1	20.5	31.0	14.8
T_4- COG 15	31.8	14.0	25.0	12.2	23.5	33.0	15.5
T_5- NKP	30.0	12.5	3.0	2.2	22.5	28.3	15.0
T_6- Control	22.5	10	4.0	2.4	21.5	17.0	13.8
SE(d)	1.39	0.51	1.00	0.77	0.42	0.81	0.47
CD @ 5%	2.93	1.08	2.10	1.61	0.88	1.70	0.99

Results of pot culture studies

Moth bean plants showed significant variation in the biometric and in yield attributes in the pot culture experiment (Table 2). MB-1 treated plant, significantly recorded the highest plant height (34 cm), root length (14 cm) and number of leaves per plant (24 numbers). The increase in plant growth might be due to the production of growth promoting substances and by the constant supply of nitrogen by the *Rhizobium*. These results are in conformity with the findings of Hussain *et al.* (2009). Production of nodules by the introduced rhizobial strain confirm the success of bacterization and in turn its symbiotic association.

In this present study, nodule number was higher in plants inoculated with MB-1 strain than the plants inoculated with standard rhizobial cultures. Nodule number (27 per plant) and nodule dry weight (13 mg) was significantly higher (Fig 1 and Fig 3). Nodulation and growth enhancement in moth bean was studied by *Pawar et al.* (2014). Their results and the present study proved that the inoculation of the efficient rhizobia by seed treatment positively alter the microbial population in the rhizosphere region. Maximum number of pod (39 pods plant^{-1}) was found in MB-1 culture treated plants and its corresponding single plant yield was 18.6 g followed by plants inoculated with the strain COG15 which recorded 15.5 g of seeds in a plant.

Nodule formation had a positive correlation with the production potential of the bacterized plants. Fatima *et al.* (2007) observed enhanced growth, yield, nitrogenous activity when inoculated with *Rhizobium* and phosphorus application in soybean. Their results support the findings of this research. In another study inoculation with rhizobia improved symbiotic nitrogen fixation and yield increase in common bean (Zaman-Allah *et al.,* 2007). These results supported this initial research work. Similar study on inoculation of *Azotobacter*, a nitrogen fixing bacteria, was carried out in alfalfa and found that crop rhizosphere had increased number of beneficial microbial population (Andjelkovic *et al*. 2020). Hence it was evidently clear that inoculation of crop specific, native bacterium will be more efficient, economical to the less cared crops grown in rainfed conditions.

CONCLUSION

Hence from this study, it can be inferred that the nodulation, growth parameters and yield are interrelated and it was

greatly influenced by the inoculation of the *Rhizobium* (MB-1) in moth bean. This basic research will be further tested in field condition at different locations. After productive results, this strain can be mass multiplied, commercialized and recommended specifically for moth bean, Parallelly, studies on compatibility and cross nodulation grouping are needed. The former research will lead to consortium development and the later will induce effective nodulation in other pulse crops.

Conflict of interest

Authors do not have any conflict of interests to declare.

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