Screening of rhizobial diversity from the Wild medicinal legumes growing in Lucknow and adjoining areas

SUMMARY OF
Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY

LUCKNOW

प्रज्ञा शील करुणा

ESTABLISHED 1996

FOR THE DEGREE OF

Doctor of Philosophy

IN

ENVIRONMENTAL MICROBIOLOGY

Submitted By

Maya Verma

(Enrolment no. 638/12)

Under the Supervision of

Prof. Naveen Kumar Arora

DEPARTMENT OF ENVIRONMENTAL SCIENCE

SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A Central University, NAAC Accreditation 'A' Grade)

VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025

UTTAR PRADESH, INDIA

2018

Summary

Leguminosae is one of the largest plant family and leguminous plants are very important due to their outstanding activity of nitrogen fixation in association with their symbiotic root nodulating bacteria. Besides the BNF activity legumes are also source of various beneficial products for human welfare. Legumes produce diverse types of pharmaceutical and bioactive compounds and have a very big role in production of traditional herbal medicines and in this context wild legumes are very important. Besides having medicinal values wild legumes also show resistance against adverse environmental conditions and can not only survive in harsh conditions but can also maintain the fertility of neglected soils. Due to their over exploitation and habitat destruction these plants are now facing extinction. Most of such plants have not been explored for their root nodulating bacteria or commercial production of medicinal metabolites. Mining of rhizobial diversity from wild medicinal legumes growing in waste and uncultivated areas is very important to utilize them in pharmaceutics and reclaim degraded lands.

In this study 40 root nodulating bacteria were isolated from root nodules of five wild medicinal legumes (*Abrus, Clitoria, Crotalaria, Leucaena* and *Sesbania*) collected from different uncultivated sites of Lucknow and adjoining areas (Barabanki, Unnao, Kanpur). All the isolates formed convex, mucilaginous colonies with smooth margins on YEMA and CRYEMA media. All the isolates were identified as Gram negative, motile and rod shaped bacteria. Most of the isolates (75%) formed highly mucilaginous colonies and were fast growers. Of all, and 25% isolates were less mucilaginous and slow growers. Physiologically all the isolates were very diverse types and able to grow under various cultural conditions. Most of the isolates were able to grow at diverse range of pH (5-12), temperature (15-35 ºC) and salinity (1-8%) and were able to utilize various carbon and nitrogen sources. On the

basis of IAR activity large number of the isolates was sensitive to most of the antibiotics except penicillin. Biochemically all the isolates were very diverse types. All the forty isolates were positive for catalase, oxidase, nitrate reductase and ammonia production ability while none of the isolates showed gelatinase and cellulase activity. Most of the isolates, 62.5% and 72.5%, showed positive growth on GPA and potassium nitrate (8%) supplemented media, respectively. Among all the isolates 27.5% were able to grow on HAM media and showed positive lactose test. Most of the isolates, 80% and 77.5%, were positive for citrate utilization and PHB production respectively. Among all the isolates 57.5% were positive for urease activity, 62.5% for MR test and 37.5% for VP test. Some enzymatic activity such as amylase, protease and lipase were also showed by 67.5% 42.5% and 2.5% isolates respectively.

PGP nature of all the isolates was also determined by analysing activities, both qualitatively and quantitatively. Most of the isolates were positive for various PGP activities such as nitrogen fixation (97.5% positive), phosphate solubilisation (80% positive), zinc solubilzation (70% positive), gibberellin production (60% positive), IAA production (60% positive), HCN production (2.5% positive), siderophore production (50% positive) and EPS production (100% positive). However, only 2.5% isolates were positive for HCN production. On the basis of biocontrol activity it was observed that amongst all, 35% showed antagonism against *F. moniliforme*, 15% against *F. solani* and 10% against *F. oxysporum*. Of the forty isolates 11 were selected on the basis of PGP characteristics for further studies. Amongst these AB3 was identified as the most potent plant growth promoter with highest level of IAA and EPS production abilities.

IAA production is very important PGP trait of rhizobia. In this study AB3 was identified as efficient IAA producer with 120 µg/ml of IAA production under optimized cultural conditions which was quantitatively estimated by both traditional and microplate

method. It was found that AB3 produced maximum amount of IAA when growth medium was supplemented with 0.2% tryptophan, 0.5% salt concentration, mannitol (1%) as carbon source, potassium nitrate (0.1%) as nitrogen source at neutral pH for 72 hrs of incubation. At these optimized growth conditions IAA was produced and extracted. Extracted IAA was purified by thin layer chromatography (TLC) and chromatogram of culture showed a pink spot of purified IAA at the Rf value (0.65) almost same to standard IAA (0.67). Extracted IAA sample was further characterized at molecular level by Fourier transform infrared (FTIR) spectroscopy and characteristic indole group was observed. In the FTIR spectra peak observed at wavelength 3286.2 cm^{-1} showed the characteristic (N-H) stretching band of indole moiety. This is the identifying character of IAA.

EPS production is also a very important character of rhizobia and among all the isolates AB3 was identified as highest EPS producer (75 µg/ml). AB3 produced maximum amount of EPS when the media was supplemented with mannitol (1%) as a carbon source and yeast extract (0.1%) as a nitrogen source at 3% salt concentration and slightly alkaline. Under these conditions EPS was produced and extracted as water soluble solid white powder. Structural analysis of isolates was done by SEM, EDS and FTIR. On the basis of SEM analysis, EPS was observed as compact crystalline with irregular shape and rough surface and according to EDS analysis high concentration of calcium, phosphorus and potassium followed by oxygen and carbon was observed with trace amount of magnesium and sulphur. The recorded FTIR spectrum indicated the presence of water solubility with gucosamine, succinate and acetate groups in EPS. In this analysis EPS was observed as heteropolysaccharide with relative content of proteins and amino sugars having hydroxyl, carboxyl and amino as main functional groups.

Nodulation test was done to test the authenticity of isolate as root nodulating bacteria or rhizobia. Rhizobial isolates AB3, LM and VYS showed development of small

pink nodules on their respective host *Abrus*, *Leucaena* and *Sesbania* in culture tube under laboratory conditions. These three isolates (AB3, LM and VYS) were also able to nodulate their respective host in pot assay. Isolates were not able to nodulate legumes other than their host plant i.e. cross inoculation were not observed in this experiment. AB3, LM and VYS significantly enhanced the growth and dry weight of respective host in comparison to uninoculated control.

Phenotypic characterization was validated by genotypic analysis which is more authenticating for bacterial identification at species level. By the analysis of 16S rRNA gene sequencing and nucleotide homology, eleven best PGP isolates were characterized genotypically. On the basis of phylogenetic analysis these isolates were identified as strains from diverse genera. The sequence data of all the isolates were submitted to Gen Bank (NCBI) and assigned with respective accession numbers. Among all the eleven isolates five isolates (AB1, AB3, LM, LB2 and VYS) belonged to order rhizobiales, while six isolates (ClU1, ClU2, CV2, CV5, CGJ and LN) belonged to non-rhizobial order Enterobacteriales. Among five rhizobial isolates AB3, LM and VYS were identified as *Rhizobium pusense*, LB2 as *Rhizobium radiobacter* and AB1 as *Beijerinckia fluminensis.* Out of six non-rhizobial isolates two belonged to genus *Kosakonia* (ClU1 as *K. sacchari* and LN as K. *pseudosacchari*), two belonged to genus *Enterobacter* (ClU2 and CV5 as *E. cloaceae*), one (CGJ) to *Cronobacter sakazakii* and one (CV2) belonged to *Pantoea agglomerans.* Among all the five wild medicinal legumes rhizobia were isolated from only 3 legumes (*Abrus, Leucaena and Sesbania*) and no rhizobia was isolated from root nodules of *Clitoria* and *Crotalaria*. These isolates taxonomically have very important role because all of them were not much reported from root nodules of plants used in this study or other legumes also.

Among all the identified strains diversity of selected rhizobia was checked by RAPD. On the basis of dendrogram of RAPD analysis these rhizobial isolates were found to

be of three types. AB1, VYS and AB3 belonged to almost similar category and LB2 showed higher diversity among them. Diversity indices among isolates were also calculated in respect to their host plant (4.0) indicating very variability. This diversity index also showed the diversity of wild medicinal legumes collected from different sites. Diversity among eleven genetically identified isolates was high with diversity value 5.2 and diversity among only rhizobial isolates was moderate type true diversity value 2.6.

Eleven selected isolates with potent plant growth promoting abilities were applied (*in vitro* and *in vivo*) on their respective host to check their impact on plant growth. In case of *Abrus* AB1, and AB3, were applied and AB3 was observed as better plant growth promoter than AB1. It was observed that in comparison to control germination rate, root length, shoot length, leaves count, fresh weight and dry weight of AB3 treated plants were significantly increased by 26.42%, 38.05%, 18.70%, 99.88% 40.29% and 83.33% respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of AB3 treated plants were also significantly higher by 14.61%, 6.97%, 16.93%, 13.73%, 195.4% and 50.00% respectively, in comparison to uninoculated plants.

Isolates ClU1 and ClU2 were applied on their host plant *Clitoria* and observed that ClU2 was better plant growth promoter. ClU2 caused higher growth in plants than control and ClU1. It was observed that in comparison to control seed germination rate, root length, shoot length, leaves count, fresh weight and dry weight of ClU2 treated plants were significantly increased by 11.76%, 41.07%, 30.05%, 136.42%, 80.41% and 84.07% respectively. Various properties such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of ClU2 treated plants were also significantly higher by 18.66%, 6.28%, 16.24%, 15.84%, 136.00% and 45.16% respectively, in comparison to control.

Isolate CV2, CV5 and CGJ were applied on their host plant *Crotalaria* and among these three isolates CGJ was observed as best plant growth promoter in comparison to control and other isolates. It was observed that in comparison to control seed germination rate, root length, shoot length and leaves count of CGJ treated plants were significantly increased by 19.20%, 43.29%, 17.54%, 81.33%, 123.07% and 123.30% respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of CGJ treated plants were also significantly higher by 15.62%, 05.01%, 23.80%, 16.61%, 245.45% and 38.70% respectively in comparison to control.

Isolates LM, LN and LB2 were applied on their host plant *Leucaena* and among these three isolate LB2 observed as best plant growth promoter in comparison to control and other isolates. It was observed that in comparison to control seed germination rate, root length, shoot length and leaves count of LB2 treated plants were significantly increased by 15.25%, 69.59%, 31.64%, 52.68%, 90.00% and 70.32% with respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration flavonoid content, nitrate content and total protein of LB2 treated plants were also significantly higher by 25.47%, 05.25%, 16.53%, 14.91%, 179.16% and 53.33% with respectively in comparison to control.

Isolate VYS was applied on its host plant *Sesbania* and observed as an efficient plant growth promoter which caused significant enhancement in growth in comparison to control In comparison to control seed germination rate, root length, shoot length and leaves count of VYS treated plants were significantly increased by 4.18% , 55.86%, 16.75%, 32.25% 55.55% and 103.88% respectively. Various biochemical contents such as antioxidant activity, total carbohydrate, chlorophyll concentration, flavonoid content, nitrate

content and total protein of VYS treated plants were also significantly higher by 04.82%, 05.09%, 09.19%, 15.90%, 158.3% and 36.66% respectively in comparison to control.

Amongst all the isolates three isolates, AB3, LB2 and CV2 selected on the basis of their PGP activities were submitted in three Indian culture collection centres (MCC, Pune, MTCC, Chandigarh and NBAIM, Mau). Culture submission in ATCC is also in progress. Isolate **AB3** of ***Abrus*** was confirmed as type strain ***R. pusense*** and assigned with an accession number **MCC 3409** by MCC Pune, India. In the current study all the isolates from wild medicinal legumes were characterized by polyphasic taxonomy in which properties of isolates were determined phenotypically by morphological, physiological and biochemical characterization. After that nodulation test, functional diversity, genotypic analysis and diversity analysis were done. This work will be very useful for rhizobial taxonomy and with organic production of neglected wild medicinal legumes. This will also help in conservation of both the root nodule bacteria and their host plants by causing higher production at lower costs and less effort in sustainable manner.