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Need of Assessing Rhizobia for Their Plant Growth Promoting Activities Associated with Native Wild Legumes Inhabiting Aravalli Ranges of Rajasthan, India

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Abstract: The Aravalli region harbor germplasm of a number of species of legumes. Some of these are unique for several reasons as they are endemic. There is scope for introduction of certain legumes which can be cultivated for increasing productivity and income and for increasing productivity of other legumes through introduction of endophytic rhizobia associated with these plants. Nitrogen fixation studies have been largely ignored in native plants as compared to agricultural and industrial crops. There is need for characterization of indigenous microbes associated with economically or medicinally important native pants of Aravallies. The two important legumes namely *Mucuna pruriens* and *Pueraria tuberosa* are native to this region. These lianas are also medicinally important. There is very less literature available as per nitrogen fixation studies on these plant species. These plants are important for studies as these are not cultivated legumes. Therefore, researchers have not given much attention to these plants. It may be possible that these plants may harbour those rhizobia which can be important for increasing productivity in cultivated legumes and for large scale multiplication of these plants. Application of non-conventional approaches may further provide information regarding nodulation and plant microbe interactions which will be useful for upgrading the available information.

Key words: Aravalli · Rhizobia · *Mucuna pruriens* · *Pueraria tuberosa* · Nodules · Legumes

INTRODUCTION

The legumes are incredibly diverse in every way imaginable and defy generalization about almost any attribute. Even the characteristic fruit type that gives legumes their name is highly variable and ranges from tiny single seed forms to meter-long woody pods. Ecologically the family ranges from rain forests to deserts and from low land to Alpine habitats; there are even aquatic species. They include giant forest trees that are prominent sources of timber and expensive woods to tiny annual herbs. There is tremendous diversity in secondary compounds, particularly alkaloids; many of them are biologically active [1]. Despite their close phylogenetic affiliations, the genetic system represents within legumes are diverse ranging from simple autogamous diploids to complex out-crossing polyploidy [2]. Genome sizes also vary widely among the legumes [3]. Legumes are simultaneously one of the largest families of crop plants and a corner stone in biological nitrogen cycle [3]. Legumes broadly defined by their unusual flower structures, podded fruits and the ability of 88 % of species to form nodules with rhizobia, are second only to Gramineae in their importance to humans. The 670-750 genera and 18000-20000 species of legumes include important grain, pasture and agro forestry species.

Legumes are important in different agriculture and natural environment. Grain and forage are grown on 2-15% of the Earth's arable surface. They account for 27 % of the world's primary production, with grain legumes alone contributing 33 % of the dietary protein nitrogen needs of humans [4]. Legumes, through their symbiotic abilities can play an important role in colonizing disturbed ecosystems. In addition to traditional food forage uses, legumes can be milled into flour, used to make bread doughnuts, tortillas, chips, spreads and extruded snacks or used in liquid form to produce milks, yogurt and infant formula. Licorice (*Glycyrrhiza glabra*) and soybean candy provide novel uses for specific legumes. Legumes have been used industrially to prepare biodegradable plastics, oils, gums,

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dyes and inks. Galactomannan gums derived from *Cyamopsis* species and *Sesbania* species are used in sizing textiles and paper as a thickener and in pill formulation. Many legumes have been used in folk medicine [5]. Isoflavones from soybeans and other legumes have been more recently been suggested both to reduce the risks of cancer and to lower serum cholesterol. A few legumes are identified as good sources of biodiesel fuel. Some review legume natural products and stressed on need for understanding and manipulating complex pathways for human and animal health [1, 7]. A hallmark trait of legumes is their ability to develop root nodules and to fix nitrogen in symbiosis with compatible rhizobia. Carbon sequestration under *Prosopis* has also been reported.

The remarkable feature of Rajasthan is Aravallis range, the oldest folded mountain range in the world. This rocky and hilly area has rich biodiversity and occupies important position in the system as it probably affects movement of monsoon and influences the entire ecology (Fig.1A). The scenario is changing due to large scale deforestation of the area. The annual average rainfall in the region is less than 10 inches (250 mm), more than 90% of the precipitation occurs between July and September. Water is scarce and non availability of water is a major constraint. The vegetation is characterized by sandy plain, more or less barren of vegetation except in rainy season when large numbers of ephemerals come up and transform the land mass into a green carpet. These ephemerals complete lifecycle before the advent of summer heat. Plants with xerophytic adaptation are able to survive and evolve. Permanent vegetation of the entire area is therefore, xerophytic in character and shows various xenomorphic features like deep root, dry, hard, thick or fleshy stems; spines well developed; leaves either absent or much reduced and usually have a coating of wax or hair to prevent excessive evaporation. Plants of these arid regions have acquired through evolution physiological, biochemical and genetic traits which are of great importance and subject of curiosities. As a part of survival mechanisms the plant species produce numerous metabolites [8]. Some of these are used as pharmaceuticals, agrochemicals, flavors and fragrance ingredients, food additives etc. Increasing human population, mining, deforestation and developmental activities have caused irreversible damage to the natural habitats and reproduction cycle of numerous plant species in the Indian Thar Desert and the Aravallies. Besides these, the medicinal and herbal plants are being over exploited without any serious efforts to conserve and propagate them. Because of the genetic make up and environmental constrain, slow reproduction and poor regeneration the population of hese plant species is decreasing day by day. Efforts toward sustainable conservation and rational utilization of biodiversity therefore should get highest priority. Hence, knowledge of plant species became an important for plant prospecting [9, 10, 11].Despite the rapid development of new technologies, for example in the area of plant genomics, a crucial question that needs to be asked is whether the rate of increase in crop yield will be sufficient to feed the world population, which will increase from about 6 billion in 2000 to more than 10 billion in 2050 [12], given an ever decreasing area of arable land. Because drought and increased salinisation of arable land could result in a 50% land loss by the year 2050 [13], increased water-use efficiency and salt tolerance are important challenges for agricultural production [14, 15].

Several constraints that limit crop production or quality have been addressed by conventional breeding and enhanced management, but there are situations where the existing germplasm lacks required traits. Biotechnology techniques could help provide solution to certain constraints, thus improving food security in developing countries [16]. The emergence of modern biotechnology presents an important approach for a production of link between conservation and sustainable utilization of genetic diversity. Maintenance of wide genetic base, which is important dement of biodiversity, is essential to the biotechnology and the sustainable user of biological source. With the traditional breeding methods, the available gene pool is restricted by the sexual incompatibility of many interspecific and intergeneric crosses [17]. Genetic manipulation and *in vitro* culture provides a means for substantially broadened by allowing transfer of specific genes controlling well defined traits from one organism to another, thus improving crops.

The Indian Thar desert and the neighboring Aravallies provide unique environmental conditions for the growth, development and evolution of biological systems. Several plant species survive the harsh environmental conditions and produce biomass. These plant species provide ecosystems economic services and yield phytochemicals and nutraceuticals. Plants of these arid regions have acquired through evolution physiological, biochemical and genetic traits which are of great importance and subject of curiosities. As a part of survival mechanisms the plant species produce numerous metabolites. Some of these are used as pharmaceuticals,

Fig. 1: (A) Rich biodiversity of Aravalli ranges (B) Plant of *Mucuna pruriens* at Mount-Abu region (C) Flowering plant of *Pueraria tuberosa* at Aravalli region, Udaipur, India

agrochemicals, flavors and fragrance ingredients, food additives etc. Increasing human population and developmental activities have caused irreversible damage to the natural habitats and reproduction cycle of numerous plant species in the Indian Thar Desert and the Aravallies. Besides these, the medicinal and herbal plants are being over exploited without any serious efforts to conserve and propagate them. Because of the genetic make up and environmental constrain, slow reproduction and poor regeneration the population of these plant species is decreasing day by day. Efforts toward sustainable conservation and rational utilization of biodiversity therefore should get highest priority. Hence, knowledge of plant species became an important for plant prospecting.

Increasing human population and its consequences have threatened plant genetic resources and destroyed natural habitats of native plants. Several species of leguminous plants have found their habitats in these geographical regions. They provide not only ecosystem services by keeping the soils fertile but also produce biomass that sustain life in the otherwise hostile environment. Legumes are major contributors in economic welfare of this part of our country. Some of legumes of this are sources of herbal medicines.

In the Aravalli region, there are two important legumes namely *Mucuna pruriens* and *Pueraria tuberosa* which are native to this region (Fig.1 B and C). These lianas are also medicinally very important. There is very less writing available as per nitrogen fixation related research work on these plant species. These plants gain importance for research as these are not cultivated legumes. There is immense possibilities that these plants may harbor those rhizobia which can be important for increasing productivity in the cultivated legumes when cross inoculated. In this paper we provide an inside to preliminary work conducted to collect and isolate the edophytic rhizobia from root nodules associated with these plant species.

MATERIALS AND METHODS

Extensive Field Survey and Collection: Native legumes mentioned in "Flora of the Indian Desert [18] was targeted for collection of germplasm as well as for knowing the nodulation status in the field. The survey was restricted mostly around Aravalli ranges of Udaipur and Mount-Abu region of Rajasthan. The survey was conducted during monsoon and post monsoon period (July-September) in the year 2008. All necessary materials for field survey were used viz. Flora, Spade, field note book, paper tag, sponge, water cane, polythene bags, news paper etc. Plants species were noticed for occurrence of nodulation by digging the pant carefully with the help of spade or scoop etc. Five to ten plants from the population of same species from each site were examined for the presence of nodules. Plants with whole root system were carefully dug out along with root nodules. Plants with root nodules were bring to the laboratory wrapped in newspaper or in moist clothes. In laboratory they were spread in tray containing water and were examined for number and positions of nodules on the roots. Some plants were maintained and managed in earthen pots in a green house.

Collection of Soil Sample and Analysis: Soil were collected from the site of sampling (both rhizospheric and non rhizospheric) in small plastic bags and brings to laboratory to examine soil type, texture and pH. Soil texture was determined using 100 g of soil and sieved through brass sieve of different pores size and separated clay, silt, coarse and fine sand. For measuring pH, 5g of soil sample was dissolved in 100 ml of distilled water and left for one hour till soil dissolve and settle down. Supernatant was used to measured pH of the soil.

Isolation of Nodules from Secondary Roots: Nodules associated with secondary roots were isolated as per previously defined methods [19]. Legumes species were selected in field and with a spade, a circle of 15 cm radius and one fit depth were cut around the plant. The plants with soil were taken out and placed in polythene bags and brings to the laboratory without disturbing the plant root system. At laboratory they were placed in a sieve and flushed under the gentle stream of water to remove all the adherent soil particles. Similar method was used for pot grown plants by putting whole pot under the stream of water jet with slowly rotating them.

Preservation of Nodules for Long Term Studies: Fresh and healthy nodules were preserved in glutralaldehyde [19]. After rinsing, fresh and healthy nodules along-with small part of roots by water, these were surface sterilized with 0.1% $HgCl_2$. Toxic solution of $HgCl_2$ were removed by several washing (6 time) with sterile water. Then nodules were blotted between tissue paper and preserved in small vial containing dehydrated silica gel and cotton for long term preservations, transportation and microbiological studies. Nodules were also fixed in 1% glutareldyhide in 0.4 M phosphate buffer (pH 7.0) for anatomical studies. Vials were stored at low temperature.

Preparation for Morphological Studies of Nodules: Plants with nodules were removed from pots by above described method and then spread in a tray containing tap water. Roots were gently separated from one another and then position of nodule (attachment on roots), numbers of nodules, color, shape and size of nodules were recorded. Surface of nodules were observed under dissecting microscope. Presences of lenticels/scars/bark on nodules were recorded. Nodules of different developmental stage were collected from single root system and shape sizes were recorded accordingly.

Preparation for Anatomical Studies of Nodule: Fresh and healthy nodules of different developmental stages were excavated from roots of each legume species with a small adjoining root part. Radial, longitudinal and transverse hand sections were made using sharp razor blade. Middle half of nodules were observed under dissecting microscope for observing presence of leg-hemoglobin. Thin sections of approximately 0.2 to 0.5 mm thickness were selected and partially dehydrated with ethyl alcohol series (30% to 50%). These were stained with Safranin-Harris hematoxylin and 1% Toluedine Blue. Sections were then observed under compound microscope for the presence of bacteriods.

Smear Preparation and Staining of Bacteria: Nodules were washed with tap water; surface sterilized by 0.1% $HgCl_2$ and washed several times (6 times) with sterilized water. Surface sterilized nodules were punctured on glass slide containing one drop of sterilized water, white milky exudates were spread over the slide by the help of another slide and a thin smear were prepared. Smear was also prepared from emulsion of crushed sterile nodules. Smear was then stained by Gram's staining method. Slides were observed under phase contrast microscope.

Media Preparation and Sterilization: YEMA (Yeast Extract Mannitol Agar) medium was prepared by dissolving yeast extract 0.5 g, mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, Agar 15 g in one liter of distilled water adjust pH 6.8 [19]. YEM broth was prepared by above described contents excluding agar. In CR-YEMA (Congo red) medium Congo red final concentration was adjusted to 25.0 microgram per milliliter with pH 6.8. All cultural media were autoclaved at 121°C temperature and 15 psi pressure for 15 minute.

Isolation and Purification of Rhizobia from Excavated Nodules: Nodules were detached from root system along with small root part, washed with tap water then wrapped in cheese/muslin cloth for surface sterilization. Nodules were surface sterilized in 90% alcohol for 1 minute then by 0.1% $HgCl_2$ for 8-10 minutes. Surface sterilized nodules were subjected to several washing (at least 6 time) with sterilized water. The nodules were then detached from small root part and crushed in 0.5 ml of sterilized water in watch glasses by the help of sterilized glass rod and forceps. The CR-YEMA (Congo red-Yeast Extract Mannitol Agar) plates were marked into three zones (1, 2 and 3) on back side of plate for separate streaking within one plate. The three zones was streaked out by loop full of sterile water in zone 1st, streaking with water of sixth washing in zone 2nd and streaking of nodule exudates/emulsion in zone 3rd. This is for confirmation that nodule surface is properly sterilized and sterile water is free from any contaminations. After seven days plates were carefully observed and those plates on which colonies were also appeared on zone 1st or zone 2nd were discarded. The YEMA plates showing colonies formation only on zone third was used for further studies. From zone 3rd individual colonies were picked for further purification and for separating single colony of microsymbionts they were subjected to serial dilution method. More than one type of colonies from same culture was maintained separately on separate CR-YEMA plate and was treated as separate isolate. Rhizobia were also maintained in YEM-broth on shaker with 120 rpm.

***In vitro* Culture Conditions:** The inoculated YEMA, CR-YEMA plates were incubated at 28°C for 7 days in BOD incubator. Plates were regularly checked for growth of rhizobial/nodule endophyte colonies. Broth cultures were maintained on shaker with 120 rpm at 28°C.

Characterization of Isolated Rhizobia: Rhizobial cultures of were grown on above mentioned YEMA, CR-YEMA and BTB-YEMA medium at 28° C in incubator as described by [20]. They were purified on the basis of appearance on CR-YEMA, gram staining and growth parameters They were phenotypically characterized by growth rate, reaction on BTB and litmus milk, pH tolerance, NaCl tolerance and Antibiotic sensitivity test. The isolates were also characterized for their biochemical properties such as IAA production, Amylase, Hydrogen sulphide, Ammonia, Nitrate reduction, siderophore production, phosphate solubilization, utilization of citrate and starch, activity of pectinase and oxidase. catalase, indole group and chitinase.

RESULTS

Collection and *In vitro* Culture Initiation: The nodules were harvested during the months of July and August (Chart 1). A detailed survey was carried out in this region for nodule hunting. Plant was found associated with other native shrubs. Mature plants under flowering stage were taken out from the soil by digging a pit of diameter 3.0 foot with depth of 2.0 foot around the plant. Plant was immediately transferred to black polybags along with nodules on the roots (Fig. 2A). Rhizospheric soil was also taken along with the plant. Plants were then brought to laboratory for detailed analysis. Nodules were present on the lateral roots of *P. tuberosa* emerging out from the tuber. Nodules were harvested from the roots by immersing the roots along with nodules into a bath tub. Nodules harvested were then used for isolation of rhizobia. Some of the nodules were preserved in vials

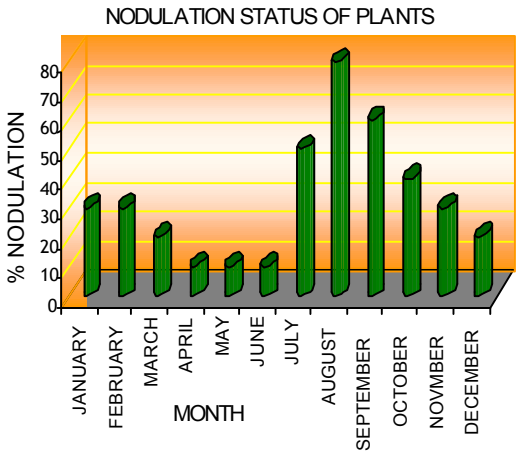

Chart 1: Nodulation status of wild legumes observed throughout the year

Fig. 2: (A) Roots of *Mucuna pruriens* showing intensive nodulation (B) After harvesting nodules plants were transferred to earthen pots in a greenhouse

containing 1% glutralaldehyde and the vials were kept in a cool dry place under room temperature. After harvesting nodules plants were shifted to earthen pots and were placed in a greenhouse (Fig. 2B). From each plant around 30 ± 5 nodules of diameter 0.5 ± 0.2 cm were harvested. Nodules were then analyzed under dissecting microscope for their phenotype. All nodules were found to be of desmoidial type. Lenticels were also present on the nodule surface. Cross-section of nodule showed presence of leghaemoglobin in the central part. It also revealed that these nodules were of indeterminate type. A total of ten rhizobial isolates were extracted from nodules of *Pueraria* in pure form and these were assigned as JNVU/PT1-JNVU/PT10. For *Mucuna pruriens* a total of 17 isolates were prepared and were designated as JNVU/M1-JNVU/M17.

Characterization of Rhizobial Isolates: The isolated rhizobia/root nodule endophytic bacteria were characterized both phenotypically and biochemically. The nodules of both plants were internally pink due to presence of leghaemoglobin. The isolated rhizobia exhibited various traits when grown on YEMA-CR and YEMA-BTB plates. The isolates were acid as well as alkali producing, slow as well fast growing, high to low EPS accumulating, tolerating 2-6% NaCl, growing at 3-11 pH and exhibited intrincsic antibiotic resistance. Majority of isolates were showing HCN production, NH_3 production and nitrate reductase. Few isolates were also showing siderophore production, IAA production, phosphate solubilization, utilization of citrate and starch and activity of pectinase and oxidase. All isolates from both the plants exhibited negative test for catalase, indole group and chitinase. The results of present investigation will be discussed with reference to making consortium of agriculturally important microorganisms.

DISCUSSION

Despite the successes of Green Revolution, the battle to ensure food security for hundreds of millions miserably poor people is far from won. Mushrooming populations, changing demographics and inadequate poverty intervention programs have eroded many gains of Green Revolution. However, for the genetic improvements of food crops to continue at a pace sufficient to meet the demands, both conventional technology and biotechnology are needed [21]. Legumes play a crucial role in the sustainability of agricultural systems and in food protein supply in developing countries. They can be improved by conventional and non-conventional methods of genetic manipulation. The origin of plant breeding can be traced back to the dawn of agriculture and the domestication of plant, when, a nomadic man first became a settler. The discovery of principles of inheritance or genetics provided scientific basis for modern plant breeding. The awareness of the particular nature of hereditary "factors" and the possibility to create novel combination of traits by making crosses contributed enormously to the more organized or professional plant breeding of the 20th century. However today with the emergence of the technology namely biotechnology, researchers can achieve the same kind of improvement in traits both with greater precision and accuracy and with much enhanced efficiency. New improved plants with desirable phenotypes and

genotypes can be rose which will offer traits not even possible through traditional breeding practices. Though legumes play critical role in natural ecosystems, agriculture and agro forestry. Legumes yield unfortunately continue to lag behind those of cereals. Some of these are: (a) yield has not kept pace with those of cereals, (b) there is need for developing drought and salinity tolerant leguminous crops, (c) diseases and pests are also the major constraints to legume production (d) uses of legumes in the human diet can also be problematic as many of the legumes contain low sulfur amino acids or anti nutritional factors and (e) some of the legumes have narrow genetic base and in the others there is need for selection and propagation of superior germplasm for increasing productivity and yield [22]. In the mid-twentieth century, overall food production in the developing countries could keep pace with population growth because higher-yielding crop varieties were introduced in the 'Green Revolution' [22]. To date, conventional breeding has led to a continuous increase in seed yield, which has generally resulted in an increased harvest index and improved yield stability [2]. Long-term selection experiments indicate that there is a sufficient potential for genetic improvement of quantitative traits over many generations [23]. However, there is evidence for declining crop yield increase [24], which will result in a gap between demand, caused by population growth and the amount of available food [25]. To meet the challenge of producing more crops from the suitable lands we need crop varieties with higher yield potential and greater yield stability [26]. Leguminous crops which are now days grown in salt prone areas or under arid climatic conditions are not very well adapted to situations existing here. So, to sustain their productivity and better survival, we need to have those bacteria colonizing which are well adapted to this harsh ecosystem. So, an attempt has been made to open a new aspect of research for arid wild legumes.

Relevance of Present Investigation to the Society: Plants growing in Indian Thar Desert are important bio-resources possessing many useful characteristics that make them to survive in extreme environmental conditions. Plants thriving in arid and semi-arid regions of non-agricultural lands are like supporting commodity for poor, laborious and innocent local peoples. These plants give them food, fodder, shelter as well as provide medical/health support in famine and uncongenial conditions. Therefore such plants should be given priorities to work. The current investigation will be useful in improving plant performance. Depending on the results of cross inoculation studies it might be possible to find out broad host range rhizobia applicable to agricultural/industrial crop and large scale inoculants preparation.

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