RESEARCH PAPER

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Microbiolgical study of root nodule bacteria from wild legumes

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Nitrogen fixing microorganism of genus *Rhizobium* lives in root nodules of legumes and also in the soil. An attempt has been made to isolate and characterize this *Rhizobium* strains from different wild legumes like *Meliolotus indicus, Medicago denticulate, Desmodium triflorum* and *Alycicarpus hamosum*. Phosphorus is one of the major nutrients next to nitrogen which is present in the soil in insoluble form. Characterization of the isolated strains form wild legume plants was done by using certain other biochemical test, this included indole production, MR-VP, cirate utilization ketolactose production, nitrate reduection, phosphate solubilization, pH and Salt tolerance. Five isolates DT02, DT03, DT04, MI01, EH04 showed good phosphatase activity. Isolate DT02, DT03, DT04, MI01, EH04 grew well at concentration of 2%, 3% salt and grew well in alkaline and acid condition *i.e.* at pH10 and pH4.

Key words : Root nodule bacteria, Microbiological study, Wild legume *Rhizobium*

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INTRODUCTION

The legume *Rhizobium* interaction is a result of specific recognition of the host legume by *Rhizobuim*. Legumes play critical role in natural ecosystems, agriculture and agro-forestry. Their ability to fix nitrogen in symbiosis make them excellent colonizers of low environment and economics and environment friendly crop pasture and herb species.

Biological nitrogen fixation (BNF) is an efficient source of fixed nitrogen which plays an important role in land remediation. Interest in BNF has focused on the systems of leguminous plants and *Rhizobium* because associations have the greatest quantitative impact on the nitrogen cycle.

Wild legumes (herbs or tree) are widely distributed in arid regions and actively contributed to soil fertility in these environments. The nitrogen fixing activity and tolerance to drastic condition may be higher in wild legumes than in crop legumes. The wild legumes arid zone harbour diverse and promiscuous rhizobia in their root nodules. Specificity existed only in few rhizobia from wild legumes, however the majority of them are with the wide host range (Nagales *et al*., 2002).

Wild or crop legumes can be a source for genetic information to improve symbiotic character of other rhizobia. The significance of rhizobia of wild legumes are not restricted to their symbiotic nitrogen fixation activity or to several other activities in the soil, which eventually improved soil fertility and plant productivity, but some strains of rhizobia may be used for other biotechnological applications (Villegas, 2002). These biotechnologies include the production of polysaccharides enzyme and antibiotics. This field of research will be focus of future investigations for biotechnology purposes.

RESEARCH METHODOLOGY

Isolation of *Rhizobium* from wild legume plants:

Rooted plants of wild leguminous crops were collected from Botanical Garden, Dr. Hari Singh Gour Central University, Sagar (M.P.) and Patkui area of Sagar (M.P.) and were brought to microbiology laboratory of the Deptt. of Applied Microbiology and Biotechnology Dr. H.S. Gour V.V. Sagar for isolation of *Rhizobium* from root nodules of these plants.

Method of Isolation:

The details of methods followed for isolation of *Rhizobium* from root nodules of different samples of wild leguminous plants *viz.,Desmodium triflora, Elicicarpus hamosus, Medicago deniticulata, Meliotus indicus.* are as given below:

Surface sterilization of root nodules:

For the surface sterilization of nodules, roots of plants were thoroughly washed in running water to remove adhering soil particles (Aneja, 2003). The nodules were then immersed in 0.1% $HgCl_2$ for about 5 minutes to surface sterilize them. The nodule were placed in 70% of ethyl alcohol for 5 minutes and then again repeatedly washed in sterile distilled water. The nodules were then crushed with the help of glass rod in 1 ml of sterile distilled water to make uniform suspension.

Preparation of medium:

Isolation of *Rhizobium* was carried out by using a selective differential medium, Yeast Extract Mannitol Agar (YEMA) medium having congo red as differential reagent.

The composition of medium (YEMA) (Subba Rao and Tilak, 1977)

Isolation:

The isolation of *Rhizobium* was carried out by serial dilution of nodule extract followed by streak plate method (Waksman and Woodruff, 1942; Vincent, 1970).

Purification:

Purification (Waksman and Woodruff, 1942) was done by adopting streak plate technique in which a cell suspension of isolated colony was streaked across YEMA medium and incubated at 35ºC for 2-3 days.

Characterization was done by using following:

Characterization of the isolated strains was done using following:

Gram staining:

The stained slide was then examined under oil immersion objective to determine whether the bacteria is gram positive (violet) or gram negative (pink).

Carbol fuchsin staining:

The smear was heat fixed, cooled, and then flooded with dilute carbol fuchsin stain for 10-20 seconds. The slide was then washed in running water and allowed to dry. It is then examined under microscope for the presence or absence of poly-â-hydroxy butyrate granules.

Ketolactose agar test:

Agrobacterium utilize lactose by the action of enzyme ketolactase (Subba Rao, 1967), where as rhizobia can not utilize this sugar. Hence, this test was used to differentiate between *Rhizobium* and *Agrobacterium*.

Motility test:

The motility of isolated bacteria was tested using soft agar stab (having 1% agar in YEMA medium). Stabs showing fuzzy, diffuse growth of the bacteria at the edges indicate motile nature of the test bacteria.

IMViC test:

Indole, methyl red, vogues proskauer and citrate utilization test was performed on all the isolates biochemically.

Nitrate reduction test:

For this test organisms were grown in nitrate broth, the reduction of nitrate to nitrite was detected by adding 0.5 ml 1% sulphanilic acid in 5 N acetic acid followed by 0.5 ml of 0.6% dimethyl- á-naphthylamine in 5 N acetic acid. The development of a red colour indicated a positive reaction.

Phosphate solubilization activity:

For this test organism was grown in Pikovskaya's medium containing tricalcium phosphate (Ca_3PO_3) (Pikovaskaya *et al.,* 1948) and incubated at 35^0C for three to four days. The development of clear zone around the colony on the culture plates indicated that phosphate has been solubilized. (Halder *et al.,* 1991)

Test for salt tolerance:

Salinity has long been known to influence the distribution of plant nutrients in legumes (Greenway and Munns, 1980). For the test YEMA medium was prepared with varying salt concentration as 3% and 2% and were streaked with bacterial culture to observe growth.

Test of pH tolerance:

In current investigation bacteria was grown at varying pH of 4 and 10.

RESULTS AND ANALYSIS

For the isolation of *Rhizobium* four wild leguminous plants *Meliolotus indicus, Medicago denticulate, Desmodium triflorum* and *Elicicarpus hamosus* were collected from two different sites of Sagar (M.P.) as given in Table 1. A total of 16 isolates were obtained by using yeast mannitol agar medium with congo red as differential reagent. 13 isolates obtained formed gummy and sticky colonies due to exopolysaccharide secretion. The colonies were white in colour and elevation found to convex (pelvinate) while 3 of them caused dry colonies on

Table 1: Cultural characteristics of isolated bacteria on yeast extract mannitol agar

Place of collection	Name of the plant	No. of Isolates	Strain designation	Exopoly saccharide secretion	Colony texture	Density
University bare land	*Desmodium triflora*	5	DT01	+	Gummy	Translucent
			DT02	+	Gummy	Translucent
			DT03	+	Gummy	Translucent
			DT04	+	Gummy	Translucent
			DT05	-	Dry	-
Bare land near Patkoi, Sagar	*Elicicarpus hamosus*	5	EH01	+	Gummy	Translucent
			EH02	+	Gummy	Translucent
			EH03	+	Gummy	Translucent
			EH04	+	Gummy	Translucent
			EH05	-	Dry	-
University bare land	*Medicago denticulata*	2	MD01	+	Gummy	Translucent
			MD02	+	Gummy	Translucent
University bare land	*Melilotus indicus*	4	MI01	+	Gummy	Translucent
			MI02	+	Gummy	Translucent
			MI03	+	Gummy	Translucent
			MI04	-	Dry	-

aforesaid medium.

Characterization and identification of microorganism traditionally relies on the phenotypic and certain biochemical characteristics that are observed by growing them on selective and differential media. The pattern of physiological and morphological characteristics distinguishes one microbial species from another and thus forming basis for identification.

The isolates were characterized on the basis of Gram staining and carbol fuchin staining techniques (for the presence of poly b hydroxy butyrate granules), motility (was observed using YEMA medium with 1% soft agar) citrate utilization and lactose agar test. Out of 16 isolates only 13 were found to contain PHB granules and their typical characteristics of Rhizobium thus all the 13 isolates were confirmed to be as Rhizobium (Table 2).

For 3-ketolactose production isolates DT05, EH04, MI03 showed positive result while all other were found

Table 2: Biochemical characterization of the isolates isolated from the chick pea samples

Sr. No.	Strain No.	Motility	Presence of PHB	Citrate utilization test	3-Ketolactose production (lactose agar test)	Gram staining	Probable Identification
1.	DT01	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
2.	DT02	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
3.	DT03	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
4.	DT04	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
5.	DT05	-	-	+	Yellow zone	Gram-Ve	*Agrobacterium*
6.	EH01	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
7.	EHO2	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
8.	EHO3	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
9.	EHO4	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
10.	EHO5	+	-	-	Yellow zone	Gram-Ve	*Agrobacterium*
11.	MDO1	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
12.	MDO2	-	+	-	No Zone	Gram-Ve	*Rhizobiun*
13.	MI01	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
14.	MI02	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
15.	MI03	+	+	-	No Zone	Gram-Ve	*Rhizobiun*
16.	MI04	-	-	-	Yellow zone	Gram-Ve	*Agrobacterium*

Table 3: Biochemical characterization of isolated bacteria from wild legumes plants

Sr. No.	Isolate No.	Indole Test	MR	VP	Nitrate rweduction	Phosphate solubilization (Zone of phosphate solubilization)
1.	*Rhizobium* (DT01)	-	-	-	+	-
2.	*Rhizobium* (DT02)	-	+	-	+	+ (13)
3.	*Rhizobium* (DT03)	-	-	-	+	+ (12)
4.	*Rhizobium* (DT04)	-	+	-	+	+ (15)
5.	*Rhizobium* (EH01)	-	-	-	+	-
6.	*Rhizobium* (EHO2)	-	-	-	+	+ (5)
7.	*Rhizobium* (EHO3)	-	-	-	+	+ (8)
8.	*Rhizobium* (EHO4)	-	-	-	-	+ (12)
9.	*Rhizobium* (MDO1)	-	-	-	+	-
10.	*Rhizobium* (MDO2)	-	-	-	+	-
11.	*Rhizobium* (MI01)	-	-	-	+	+ (10)
12.	*Rhizobium* (MI02)	-	-	-	+	-
13.	*Rhizobium* (MI03)	-	-	-	+	-

to be negative. Work done by Bernaerts and Beley (1958) shows that Agrobacterium species produces 3-ketolactose. Present results indicate the typical characteristics of Rhizobium and Agrobacterium while studied for lactose agar test. All the 13 isolates which were identified as Rhizobium have been tested for IMViC test (indole, methyl red, vogues proskauer). Indole production, nitrate reduction and phosphate solubilization for indole production and VP all the isolate were found to be negative. Except two isolates DT02 and DT05 all the test isolates showed MR test negative.

All the 13 isolates were able to reduce nitrate to nitrite (Table 3). The ability of Rhizobium to solubilize inorganic phosphate was observed using Pikovskaya's media. Different isolates showed variability in their capability to solubilize tricalcium phosphate present in the media and the halo zones of different radius were obtained. Abd-Alla, (1994) and Antoun *et al.* (1998), showed that rhizobia are able to solubilize both organic and inorganic phosphate. Out of all test isolates, only 5 (DT02, DT03, DT04, MI01, EH04) showed good phosphatase activity. These were further studied for pH and salt tolerance.

The plant nodulating rhizobia have been found to show wide diversity in their pH tolerance. In the present study all the five selected isolates grew well in alkaline and acid condition *i.e.* at pH 10 and pH4. Thus showed their acid and alkali tolerant character. All the five bacterial/ isolates (*i.e.* DT02, DT03, DT04, M101 and EH04) grew well at a concentration of 2% and 3% salt. Nagales *et al.*, (2002) and Tharll *et al.,* (2008) suggested that saline soils naturally select strain more tolerant to salinity (Table 4).

Table 4: Physiological characterization of selected strain based on phosphate solubilizing efficiency

Sr. No.	Test strain no.	Growth at pH 4	Growth at pH 10	Growth at NaCl concentration 2%	Growth at NaCl concentration 3%
1.	*Rhizobium* (DT02)	+	+++	+++	++
2.	*Rhizobium* (DT03)	++	+++	+++	++
3.	*Rhizobium* (DT04)	++	+++	+++	++
4.	*Rhizobium* (MI01)	+	++	+++	++
5.	*Rhizobium* (EH04)	+	+++	+++	++

Growth Patterns, ++++ Excellent; +++ Good; ++ Fair; + Poor Growth

Conclusion:

In conclusion it can be said that in order to achieve better strains resistant to salt and alkali/acid, the enumeration and characterization of rhizobia from wild legumes should be encouraged. The typical characteristics of these bacteria may be found biotechnologically important and hence it is suggested that further investigations on these lines should be undertaken.

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