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RESEARCH PAPER

Isolation of root nodule bacteria from *Trigonella foenum graceium*

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Abstract : Wild legumes were collected from three different places of Patkoi village, district Sagar. A total of 27 bacteria were isolated from 3 different samples of wild leguminous plant *Trigonella foenum graceium.* The nodules of wild methi (*Trigonella*) yielded bacteria other then *Rhizobium* with different cultural characteristics. Only eight isolates were circular with exopolysaccharide secretion rest were showing different colony characteristics. The colonies were white in colour and the elevation were found to be convex also called as pelvinate colony. In citrate utilization test all isolates found to be negative except the isolates 13B and W07. Where as all isolates were able to grow at alkaline pH 11 but showed poor growth at acidic pH 4. W07 isolate were given positive result for indole production while remaining 7 isolates were negative. Isolates 13B and W07 shown positive result while tested for 3-ketolactose production.

Key Words : *Rhizobium*, Nodulation, Wild leguminous plant, Isolation, *Trigonella foenum graceium*

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INTRODUCTION

Soil micro-organism serves as biogeochemical agents for the conversion of complex organic compounds in to simple inorganic compounds or in to their constituent elements. This overall process is called as mineralization conversion of complex macromolecules in simpler usable forms (Pelczar *et al.,* 1996).

Rhizobia are genetically diverse and physiologically heterogeneous group of symbiotic nitrogen fixing bacteria that form nodules on the roots or rarely on stem of legume hosts within which bacteria fixes atmospheric nitrogen in to ammonia. A fully functional symbiosis recuries successful survival ability of bacteria even under adverse environmental conditions within the soil. Rhizobia frequently encounter various stress that effect their growth, their initial steps of symbiosis and the capability of nitrogen fixation (Zahran, 1999). The wild (naturally growing) leguminous plants living in arid or semi-arid regions are subject to severe environmental conditions. A competitive and persistant rhizobial strain is not expeted to expressits full capacity for nitrogen fixation as the limiting factor eg; salinity, unfavourable soil, temperature and nutrient deficiency impose limitation on the vigour of the host legumes (Brock *et al.,* 1995) According to Date (1974) the most satisfactory growth of *Rhizobium* can be obtained on the media containing yeast or other plant extract. According to Dubey *et al*. (2010) symbiotic nitrogen fixation by *Rhizobium* strains with legumes is important for agricultural productivity. Biological nitrogen fixation is a process that can only be performed by certain prokaryotes. In some cases, some bacteria are able to fix nitrogen in a symbiotic relationship with plants (Geurts and Bisseling, 2002).

The present investigation was aimed to study the *Rhizobium* associated with nodulation in certain wild leguminous plants. An attempt has also been made to characterize them on basis of certain biochemical properties.

MATERIALS AND METHODS

Collection of crop plants :

Rooted plants of wild leguminous crops have been

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collected from Patkoi area of Sagar M.P. and were brought to Microbiology laboratory of the Department 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 root nodule bacteria from of different samples of wild leguminous plant called *Trigonella foenum graceium* were as follows.

Surface sterilization of root nodules (Aneja, 2003) :

For the surface sterilization of nodules of roots of plants were thoroughly washed in running water to remove adhering soil particles. The healthy, pink, unbroken and firm root nodules were selected and detached from roots and washed in distilled water. The nodules were then immersed in 0.1 per cent $HgCl_2$ for about 5min to surface sterilize them. There after nodules were washed repeatedly in sterile distilled water for 3-4 times to get rid of sterilizing agents. The nodules were placed in 70 per cent ethyl alcohol for 5 minutes and then again repeatedly washed in sterile distilled water with the help of glass rod the nodules were then crushed 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. Composition of medium (YEMA) was as follows:

K_2HPO_4 - 0.5 g, $MgSO_4$. $7H_2O$- 0.2g, NaCl - 0.1 g, Mannitol - 10.0g, Yeast Extract -1.0 g, Agar - 20.0g, Distilled water - 1000ml (Note: 2.5 ml Congo red 1%).

The medium was autoclaved at 121^0C for 20 minutes and then congo red dye which was sterlized seperately was added in medium just before pouring and shaked gently.

Isolation :

The isolation of *Rhizobium* was carried out by serial dilution of nodule extract followed by streak plate method (Waksman, 1942; Vincent, 1970). Serial dilution of suspension of nodules extract were prepared by using sterile distilled water. 10^{-4} and 10 dilution was used and streak plate technique was performed for isolation of *Rhizobium*.

Purification :

Purification (Waksman,1942) was done by adopting streak plate technique in which a cell suspension of isolated colony was streaked across YEMA medium and incubated at 35^0C.

Morphological and biochemical characterization of isolated bacteria :

To study the morphological characterstics of isolated bacteria, each bacterial isolates strains was studied for different straining reaction.

Gram's staining :

The Gram stain procedure is the most widely used differential staining procedure in bacteriology. The stained slide was then examined under the oil immersion objective to determine whether the organism was Gram positive or Gram negative.

Carbol fuchsin staining :

The smear of bacteria was flooded with dilute carbol fuchsin stain for 10-20 seconds. The slide was then washed and then examined under microscope for the presence or absence of poly-β-hydroxy butyrate granules.

Biochemical characterization :

Lactose – Agar test :

This test was used to differentiate *Agrobacterium* from *Rhizobium*. *Agrobacterium* utilize lactose to form a reduced product ketolactose when Benedict's reagent was poured over agar medium containing lactose on which nodule bacteria were growing, the development of yellow colour due to Cu_2O around the colony indicates the presence of *Agrobacterium* (Subba Rao 1967).

IMViC test :

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

Nitrate reduction test :

For this test organisms were grown in nitrite broth. The reduction of nitrate to nitrite can be detected by adding 0.5 ml 1 per cent sulphanilic acid in 5 N acetic acid followed by 0.5 ml of 0.6 per cent dimethyl- α-naphthylamine in 5 N acetic acid. The development of a red colour indicates a positive reaction.

Phosphate solubilisation activity :

(Pikovskaya, 1948) - This activity shows the efficiency of bacteria to solubilize phosphate. For this test organism was grown in Pikovskaya's medium. The development of clear zone at inoculation site on the culture plates indicates that phosphate has been solubilized.

Test of pH requirement :

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

Test for salt tolerance :

For the test YEMA medium with varying salt concentration as 3 per cent, 4.5 per cent and 5 per cent were streaked with bacterial culture to observe the growth.

Amylase activity :

(Aneja, 2003) - For testing the amylase activity of the isolates, nutrient agar medium was prepared supplemented with 0.2 per cent starch and inoculated with bacteria and incubated. Gram's iodine solution was flooded over the plates and observed for the clear zone.

Catalase activity :

(Aneja, 2003) - For catalase activity a loopful of culture of bacteria was reacted with the 3 per cent H_2O_2 which was used as substrate. Formation of bubbles due to release of oxygen indicates positive catalase activity.

RESULTS AND DISCUSSION

The list of isolated bacteria from root nodules of wild legumes which were collected from three different places of patkoi village have given in Table 1 and 2. A total of 27 bacteria were isolated from 3 different samples of wild leguminous plant, *Trigonella foenum graceium.* The nodules of wild Methi (*Trigonella*) yielded bacteria other then *Rhizobium* with different cultural characteristics were not considered for further study. Only eight isolates were selected with circular and exopolysaccharide secretion. The colonies were white in colour and the elevation were found to be convex also called as pelvinate colony.

The results of Gram -staining are given in Table 1. All eight isolates were found to be Gram- negative and have properties of exopolysaccharide secretion. Work done by Singh and Sharma (1991) reported that when mannitol is used as carbon source in YEMA medium 4 out of 5 isolates showed maximum EPS production. In citrate utilization test all isolates were found to be negative except the isolates 13B and W07. Where as all isolates were able to grow at high pH 11 but showed poor growth at acidic pH 4. Zaharan (1999) showed that strains like ALL-1, ALL-4, ALL-5, BLL-1, BLL-2 isolated from wild legume *Leucaena leucocephala* and *Teprosia purpurea* were able to grow at high pH. W07 isolate gave positive result for indole production while remaining 7 isolates were negative.

For 3-ketolactose production, isolates 13B and W07 shown positive result. Work done by Bernaerts and Deley (1960) established that 24 out of 28 strains of *Agrobacterium tumefaciens* given positive result. All isolates except TFG1 were able to grow at high salt concentration (3%). Work done by Nagales *et al.* (2002) showed that *Rhizobium* strain ATP-2,

Table 1: Morphological characteristics of selected bacteria

Place	Wild legumes (No. of samples)	Number of isolates	Strain number	Exopolysaccharde	Colony texture	Density	Gram staining
Patkoie area	Seed Name – Trigonella foenum graecum						
Sagar	TFG Sample-1	Two	W09	+	Gummy	Translucent	Gram (-)
			W09A	+	Gummy	Translucent	Gram (-)
	TFG Sample-2	Three	TFG-1	+	Gummy	Translucent	Gram (-)
			TFG-2	+	Gummy	Translucent	Gram (-)
			TFGW09A	+	Gummy	Translucent	Gram (-)
	TFG Sample-3	Three	13B	+	Gummy	Translucent	Gram (-)
			W07	+	-		Gram (-)
			13BA	+	Gummy	Translucent	Gram (-)

Table 2: Biochemical characteristics of isolated bacteria

Sr. No.	Strain no.	Citrate utilization	3-letolactose production	Salt test (3%)	Indole production	Growth at different pH	
						pH 4.0	pH 11.0
1.	W09	-	-	+	-	-	+
2.	W09A	-	-	+	-	-	+
3.	TFG1	-	-	-	-	-	+
4.	TFG2	-	-	+	-	-	+
5.	TFGW09A	-	-	+	-	-	+
6.	13B	+	+	+	-	-	+
7.	W07	+	+	+	+	-	+
8.	13BA	-	-	+	-	-	+

ATP-3 from wild legume *Teprosia* were able to grow at 3 per cent salt.

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