

Isolation And Characterization Of Rhizobium Species From Pea Plant (*Pisum Sativum*) Root Nodules And Its Antibiotic And Antibacterial Activity Against Isolated Bacteria

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Abstract: The present study describes isolation and characterization of *Rhizobium* species from root nodules of pea plant (*Pisum sativum*) and its antibiotic and antibacterial activity against isolated bacteria. *Rhizobium* is a soil bacteria that fix nitrogen after becoming established inside root nodules of legumes. Selective media YEMA and CRYEMA were used to isolate *Rhizobium* and characterization was done by biochemical test. *Rhizobium* species were rod shaped, gram negative and found to be sensitive for pH, temperature and salt concentrations. The *Rhizobium* isolates showed antibiotic sensitivity against gentamycin, streptomycin and ampicillin whereas resistance against chloramphenicol. *Rhizobium* isolates showed antibacterial activity against *Enterobacter* whereas no antibacterial activity was observed against *Pseudomonas* and *Staphylococcus* species.

Keywords: *Rhizobium* species, antibiotics, antibacterial activity, *Pisum sativum*.

I. INTRODUCTION

Rhizobium plays a very important role in agriculture by inducing nitrogen fixing nodules on the roots of legumes such as peas, beans and clover. In the 19th century the scientific demonstration of this symbiosis was started and established that bacteria are present in legume root nodules which are responsible for fixing atmospheric nitrogen (Zsbrau, 1999). The *Rhizobium* species live inside the root nodules of host legumes so they are beneficial for the growth of the plants (Oblisami, 1995). They easily colonize in the plant root and promote solubilizing activity, nitrogen fixation and biocontrol activity (Deshwal *et al.*, 2011).

Root nodule bacteria generally grow under the following conditions 25-30°C (optimum) in the pH range of 6-7 (Vincent, 1970; Somasegaran and Hoben, 1994). *Rhizobium* growth normally occurs under aerobic conditions. However, when fixing nitrogen, low levels of oxygen are required to

protect the enzyme nitrogenase (Goldberg *et al.*, 1987) and hence, *Rhizobium* are able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994). Rhizobial nod genes are important in the determination of host specificity, infection and nodulation and are involved in the exchange of signals between the plant and bacteria (Denarie *et al.*, 1992).

The movement of rhizobia from the infection thread into the host cell results in the rhizobia being surrounded by a membrane known as the peribacteroid membrane (Prescott *et al.*, 1996). This is the formation of nodules and leads to the proliferation of the enlarged rhizobia and cortical cells. The enlarged rhizobia are often referred to as bacteroids in which the fixation of nitrogen by legumes occurs (Denarie *et al.*, 1992; Salisbury and Ross, 1992; Somasegaran and Hoben, 1994). The further differentiation of the bacteroid results in the nitrogen fixing structure known as a symbiosome (Prescott *et al.*, 1996). The cytosol of bacteroids is the site of synthesis of nitrogenase, the enzyme responsible for the reduction of

atmospheric nitrogen to ammonium (Somasegaran and Hoben, 1994).

Few endophytic bacteria isolated from young radishes can be used as biocontrol agent against human and plant pathogen (Seo *et al.*, 2010). Genetic variability in salt tolerance exists within *Rhizobium*, and may significantly affect crop performance. There is also a wide variation among chickpea *Rhizobium* strains in their ability to grow and survive under saline condition (Zurayk *et al.*, 1998). Symbiotic *Rhizobium* species of naturally growing legumes are more tolerant to some ecological condition (salt, severe drought, elevated temperature etc.) than *Rhizobium* from cultivated legumes (Zahran *et al.*, 2012). The Present study investigated isolation and characterization of *Rhizobium* species from pea plant (*Pisum sativum*) root nodules and its antibiotic resistance and antibacterial activity against isolated bacteria.

II. MATERIAL AND METHODS

COLLECTION OF SAMPLES

The pea plant (*Pisum sativum*) root nodules were collected from nearby fields. The root samples were collected in sterile containers and brought to laboratory. Then samples were processed immediately for analysis within one hour of collection.

ISOLATION OF RHIZOBIUM

Collected nodules were surface sterilized by using 70% ethanol and 0.1% HgCl₂ respectively and rinsed three times with sterilized distilled water. Isolation was done by serial dilution spread plate method on YEMA (Yeast Extract Mannitol Agar) media after crushing the root nodules. Plates were incubated at 30°C for 48 hrs (Aneja, 2003). Colony morphology was studied by observing various features (form, color, size, elevation, surface). Microscopic examination was done by using Gram staining.

BIOCHEMICAL TEST

All the collected samples were processed through different biochemical test such as catalase test, indole test, citrate utilization test, methyl red test, VP (Voges - Proskauer) test, glucose test, CRYEMA (Congo Red Yeast Extract Mannitol Agar) test, gelatin hydrolysis test.

PH VARIATION ASSAY

To analyze the effect of pH variation on the growth of microorganism, YEMA agar media plates were prepared with pH (4.0, 7.0 and 9.0). After inoculation, incubation was done and growth was determined after 48 hrs.

TEMPERATURE TOLERANCE

To analyze the effect of temperature variation on the growth of microorganism, YEMA agar media plates were prepared and inoculation was done. After inoculation,

incubation was done at different temperature (25°C, 30°C, 35°C and 40°C) and growth was determined after 48 hrs.

NACL VARIATION ASSAY

To analyze the effect of NaCl variation on the growth of *Rhizobium*, YEMA agar media plates were prepared and inoculation was done with NaCl variation i.e. 0.2, 0.4, 0.6, 0.8 (g/100ml). After inoculation, incubation was done and growth was determined after 48 hrs.

ANTIBIOTIC ACTIVITY TEST

The *Rhizobium* isolates were tested for antibiotic sensitivity by Kirby-Bauer disc diffusion method on YEMA agar media (Bauer *et al.*, 1966). Antibiotic activity of the *Rhizobium* isolates was tested against gentamycin, streptomycin, chloramphenicol and ampicillin.

ANTIBACTERIAL ACTIVITY TEST

Antibacterial activity of *Rhizobium* isolates was tested by disc diffusion method against bacteria such as *Enterobacter*, *Staphylococcus* and *Pseudomonas* isolated from different sources.

III. RESULTS

YEMA media was used for the isolation of *Rhizobium* bacteria. Colonies were observed on YEMA media after incubation at 30°C for 48 hours. The colonies were circular, creamy white, raised and smooth (Fig1.a). In biochemical testing, all the test except VP and gelatin hydrolysis were positive (Fig2). Medium containing gelatin gets solidified when kept at 4°C for 30 as well as 60 minutes as *Rhizobium* species was unable to hydrolyze the gelatin, therefore *Rhizobium* do not produce gelatinase enzyme (Fig2.d). *Rhizobium* cells were able to utilize glucose as a carbon source (Fig2.e). On CRYEMA media *Rhizobium* utilize congo red dye very slowly and formed white, circular and raised colonies (Fig1.b). Isolates were found to be highly sensitive for pH, temperature and salt concentration. Superior growth was observed at pH 7, temperature 30°C and salt concentration 0.6 (g/100ml).

Rhizobium isolates showed sensitivity against gentamycin, streptomycin and ampicillin antibiotics and resistance against chloramphenicol (Fig3). The diameter of zone of inhibition of *Rhizobium* isolates against gentamycin, ampicillin and streptomycin was found to be 2cm, 2cm and 1.5cm respectively. The *Rhizobium* isolates showed antibacterial activity against the *Enterobacter* species and diameter of zone of inhibition was found to be 1.5cm. Zone of inhibition was not observed for *Pseudomonas* and *Staphylococcus* species (Fig4).

IV. DISCUSSION

In present study, *Rhizobium* was isolated from root nodules of pea plant (*Pisum sativum*). YEMA media was used

for the isolation of *Rhizobium* bacteria. Colonies were observed on YEMA media after incubation at 30°C for 48 hours. The colonies were large (2-4 mm in diameter) mucilaginous, circular, convex with smooth edges, glistening translucent or white (Vincent, 1970; Holt *et al.*, 1994). Microscopic examination revealed that the isolates were rod shaped and gram negative in nature (Keyser, 1982; Anand and Dogra, 1991; Singh *et al.*, 2008). *Rhizobium* species in study were able to tolerate 2% NaCl, which is in accordance with the characteristics of fast growing *Rhizobium* (Holt *et al.*, 1994). In this study, *Rhizobium* isolates were catalase and glucose positive, which complements the finding of Lupwayi and Haque (1994).

Hashem *et al.*, (1998) have proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil. Superior growth of *Rhizobium* has been reported at neutral pH i.e. 7. Results showed that cells were able to grow only at pH 7.0 at 30°C temperature. No growth was observed in medium with pH 4.0 and 9.0. Similar observations were made by Gao *et al.*, 1994; Kucuk *et al.*, 2006; Baoling *et al.*, 2007. Glucose test for *Rhizobium* was positive showing the utilization of glucose as the carbon source by the *Rhizobium*. It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source (Kucuk and Kivnac, 2008). Negative gelatinase activity is also a feature of *Rhizobium* (Hunter *et al.*, 2007).

Rhizobium isolates were showed antibacterial activity against *Enterobacter* whereas it did not showed antibacterial activity against *Pseudomonas* and *Staphylococcus*. Sensitivity of *Rhizobium* against gentamycin, streptomycin, ampicillin and chloramphenicol were studied. *Rhizobium* isolates showed sensitivity against gentamycin, streptomycin and ampicillin antibiotics and resistance against chloramphenicol. There are three determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge, and amount of the antibiotic and the *Rhizobium* that showed a high level of resistance did not take up the antibiotics (Hungaria *et al.*, 2000).



Figure 1: *Rhizobium* colonies: (a) on YEMA media (b) on CRYEMA media



Figure 2: Biochemical characterization of *Rhizobium*: (a) Indole test (b) Citrate test (c) MRVP test (d) Gelatin hydrolysis test (e) Glucose test



Figure 3: (a) Antibiotic sensitivity of *Rhizobium* against streptomycin and antibiotic resistance against chloramphenicol (b) Antibiotic sensitivity of *Rhizobium* against gentamycin and ampicillin

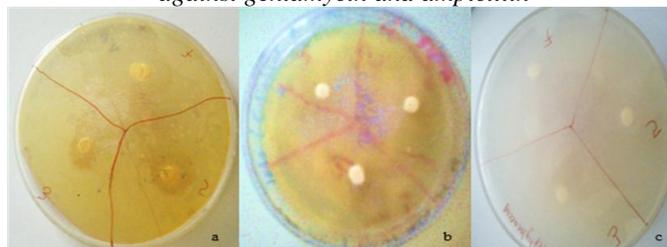


Figure 4: Antibacterial activity of *Rhizobium* isolates against (a) *Enterobacter* (b) *Pseudomonas* (c) *Staphylococcus*

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