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Bio-chemical characterization of rhizobia isolated from root nodules of Velvet bean (*Mucuna pruriens* L.)

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Abstract

Rhizobia are the symbiotic bacteria found in the soil which have potential ability to convert atmospheric di-nitrogen into usable form. A total of ten rhizobial strains were isolated from the root nodules of a medicinal legume *Mucuna pruriens* (L.) that commonly grow in the foothills of the Himalaya. All the ten strains isolated from different locations of same area were morphologically, biochemically and physiologically characterized based on the Bergey's Manual of systematic Bacteriology. They were tested for the antibiotics sensitivity. The isolates showed high sensitivity to amoxicillin and least to erythromycin. Authentication test was done in eleven legumes but shown nodulations only in *Trigonella foenum-graecum*, *Mucuna pruriens* and *Medicago sativa*. The morphology, physiology, biochemical and infection test studies carried out justifies that the bacteria isolated belonged to the species of *Rhizobium meliloti*.

Key words: Symbiotic bacteria, *Trigonella foenum-graecum*, *Medicago sativa*

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Introduction

Rhizobia are traditional soil bacteria because they possess the potential ability to fix atmospheric nitrogen by establishing symbiotic relationship with root nodule formation. The *Rhizobium* legume symbiosis because of its agricultural importance has got continuous research support worldwide. A great number of rhizobia being able to form nodules have not been examined. Most of those that have not been examined are from tropical areas (De Faria *et al*., 1989). Therefore, current taxonomy and phylogeny of rhizobia are based on only 15% of the 750 genera of legumes that has been explored so far. Rhizobia are specific to particular legume; therefore, it is essential to identify and characterize these organisms by morphological, physiological and biochemical basis to obtain their exact taxonomic position. The biochemical characterization of root nodulating bacteria still remains the only valid tests for establishing the identity of *Rhizobium*. Nevertheless, there have been reports from time to time (Graham and Parker, 1964; Kleczkowska *et al*., 1968; Skinner, 1977) on the usefulness of certain cultural and biochemical characteristics in the systematics of *Rhizobium*.

Legumes are very important both ecologically and agriculturally because they are responsible for the substantial part of the global flux of nitrogen from atmospheric N_2 to fixed forms such as ammonia, nitrate and organic nitrogen. Atmospheric nitrogen fixed symbiotically by the association between *Rhizobium*

species and legume represents a renewable source of N for agriculture (Peoples M.B., *et al*., 1995). Values estimated for various legume crops and Pasteur species are often impressive, commonly falling in the range 200-300 kg of $Nha^{-1}Yr^{-1}$ (Peoples M.B., *et al*., 1995). Yield increase of crops planted after harvesting of legumes is often equivalent to those expected from the application of 30-80Kg of fertilizer Nha^{-1} (Zahran, 1999).

At present time rhizobia are divided into 5 genera with 38 species including *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. Phylogenetically these bacteria belong to the α-sub-class of proteobacteria (Young and Haukka, 1996).

Materials and methods

Bacterial strains were isolated from the root nodules of *Mucuna pruriens* growing wildly on the foothills of the Himalaya according to Vincent (1970). Ten different strains were isolated and were characterized according to Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994). The cultures were incubated on Yeast Extract Mannitol Agar (YEMA) at 28 ± 1°C. The isolated strains were stored in YEMA slants at 4°C.

Morphology was determined by compound microscope through Gram staining. Generation time was calculated in YEM broth from the absorbance at 420nM recorded after every 2 hrs. at 28 ± 1°C. Catalase activity was determined according to Graham and Parker (1964). The ability to hydrolyze urea and gelatin were estimated according to Lindstrom and Lehtomaki (1988). Growth on Hofer's alkaline medium was done according to Hofer (1935), growth on Glucose Peptone Agar (GPA) was tested according to Kleczkowska *et al*. (1968). DNA base composition was studied according to Murmur and Doty (1962). Antibiotic resistance was detected using antibiotic discs. Carbon sources utilization by different carbohydrates were substituted for mannitol.

Isolates were tested for nodulation on their original hosts. Seedlings were grown on agar slants. Exponentially grown cultures (10^8 cells ml^{-1}) were inoculated during the seedling stage.

Results and discussion

All the strains isolated (MPR_1-MPR_{10}) were fast growing, motile, Gram negative, rod shaped with mean generation time 3.2 - 3.8 hrs (Table 1). The colonies on YEMA were circular, non-spreading, translucent, convex, smooth, entire and odourless with 2 - 4 mm in diameter after 48 hrs. of incubation at 28 ± 1°C. These morphological characteristics approaches closer to the genus *Rhizobium* as described by Jordan and Allen (1974).

No growth was observed on Glucose Peptone Agar (GPA) medium. Acid productions, ability to grow on Hofer's alkaline medium, ability of strains to precipitate calcium glycerophosphate were positive. Catalase activity, reduction of 2,3,5 triphenyl tetrazolium chloride (TTC), inability to utilize citrate with negative gelatinase activity were all positive for rhizobial strains as suggested by Kleczkowska *et al*. (1968), Deshwal and Chaubey (2014). Baoling *et al*. (2007) reported from the analysis of colony morphology that the pH of the medium (solid) and broth (liquid) during growth of the

Table 1. Biochemical characterization of the strains from *Mucuna*.

Tests	Strains of rhizobia									
	MPR_1	**MPR_2**	**MPR_3**	**MPR_4**	**MPR_5**	**MPR_6**	**MPR_7**	**MPR_8**	**MPR_9**	**MPR_{10}**
Gram stain	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Growth on GPA	-	-	-	-	-	-	-	-	-	-
Acid production	+	+	+	+	+	+	+	+	+	+
Growth on HAM	+	+	+	+	-	+	+	+	-	+
Urea hydrolysis	+	-	+	+	+	+	+	+	-	+
Growth on 8% KNO_3	+	+	+	-	+	+	+	+	+	+
Citrate utilization	-	-	-	-	-	-	-	-	-	-
Catalase activity	+	+	+	+	+	+	+	+	+	+
Ppt. with Calcium glycerophosphate	+	+	+	+	+	+	+	+	+	+
2% NaCl tolerance	+	+	+	+	-	+	+	+	+	+
Generation time (h)	3.46	3.6	3.8	3.5	3.45	3.3	3.6	3.54	3.2	3.7
Reduction of 2,3,5 TTC	+	+	+	+	+	+	+	+	+	+
Gelatinase activity	-	-	-	-	-	-	-	-	-	-

(-ve) Gram negative, (-) No growth observed, (+) Growth observed

isolates was changed from pH 7.0 to 6.0 thus showing the production of acid which is the characteristic of rhizobia. Different carbon sources used (Table 2) showed that no strains could grow on starch but other carbon sources used showed good growth. Vaishya and Senoria (1972) also observed great variations within the strains of *Cicer*-rhizobia of Indian origin in the utilization of carbohydrate substrates. Kucuk *et al*. (2006) also have reported that rhizobial strains were able to utilize glucose and sucrose more efficiently than YEM medium. Niste *et al*. (2015) also reports the use of wide range of carbohydrates for *Rhizobium leguminosarum* bv. *phaseoli* and *Sinorhizobium meliloti*. Rhizobia isolated to date differ significantly in carbohydrate metabolism and substrate utilization. Fast growing *Rhizobium* strains possesses NADP-linked 6-phosphogluconate-dehydrogenase activity and metabolite a wider range of carbohydrates (Zhang *et al*., 1991).

The average G + C (Guanine + Cytosine) content of DNA was 62.8 mol%. The rhizobial species usually have G + C values in the range of 59 - 64 mol% (Chen *et al*., 1988). Delay and Russel (1965) also studied the DNA base composition of *Rhizobium* which showed the range of 59-63 mol% corresponding to *Rhizobium leguminosarum* and *Rhizobium meliloti* group of Graham (1963). The strains of rhizobia based on generation time, flagellar arrangement, DNA base composition and many other biochemical characteristics (Jordan and Allen, 1974) have been classified into two broad groups: (a) fast growers - peritrichous strains with G + C mol% in the range of 59-63 mol% and (b) slow growers with sub polar flagellated strains with G + C mol% in the range of 62.8-65.5 mol%.

On the medium containing mannitol amended with brom thymol blue (BTB) dye, the strains found to produce acid by changing the blue colour of the media to yellow. Brockwell *et al*. (1966) have reported that acid or alkali production on mannitol was dependent of the soil from where rhizobia were isolated from *Trifolium* and *Lotus*. The rhizobial species associated with many of the tropical legumes

Table 2. Carbon source utilization by the strains from *Mucuna*.

Carbohydrates	Strains of rhizobia									
	MPR_1	**MPR_2**	**MPR_3**	**MPR_4**	**MPR_5**	**MPR_6**	**MPR_7**	**MPR_8**	**MPR_9**	**MPR_{10}**
Dextrose monohydrate	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+
D(+) Trehalose	+	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+

(+) Growth occurred, (-) No Growth occurred

Table 3. Antibiotic resistant studies on the strains from *Mucuna*.

Antibiotics	Strains of rhizobia										Diameter of inhibition zone (cm)
	MPR_1	**MPR_2**	**MPR_3**	**MPR_4**	**MPR_5**	**MPR_6**	**MPR_7**	**MPR_8**	**MPR_9**	**MPR_{10}**	
Neomycin	+	+	+	+	+	+	+	+	+	+	1.5
Erythromycin	+	+	+	R	+	+	+	+	R	+	1.0
Gentamycin	+	+	+	+	+	+	+	+	+	+	1.5
Ampicillin	R	R	R	R	R	R	R	R	R	R	-
Carbenicillin	R	R	R	R	R	R	R	R	R	R	-
Tetracycline	+	+	R	+	+	+	R	+	+	+	2.0
Chloramphanicol	+	+	+	+	+	+	+	+	+	+	1.2
Kanamycin	+	+	+	+	+	+	+	+	+	+	1.2
Bacitracin	R	R	R	R	R	R	R	R	R	R	-
Nalidixic acid	R	R	R	R	R	R	R	R	R	R	-
Furadolizone	R	R	R	R	R	R	R	R	R	R	-
Nistatine	R	R	R	R	R	R	R	R	R	R	-
Amoxycillin	+	+	+	+	+	+	+	+	+	+	3.5

(+) Sensitive, (R) Resistant

like *Cajanus*, *Sesbania* have also been reported to be acid producing in mannitol (Johnson and Allen, 1952). Paudyal and Gupta (1993) also reports the acid producing properties in fast growing *Rhizobium phaseoli* isolated from *Phaseolus vulgaris* in Kathmandu soils. The study on the antibiotic resistance showed that the strains were found resistant to ampicillin, carbanicillin, bacitracin, nalidixic furadolizone and nistatine (Table 3). Twenty percent of the isolates were resistant to erythromycin, tetracycline and chloramphanicol. The maximum area of inhibition was recorded 3.5 cm (diameter) by amoxycillin in the strain. It was reported by various workers that the fast-growing strains of rhizobia are less tolerant than do the slow growers (Gauri *et al*., 2011).

Bacterial growth was directly influenced by the change in temperature as it controls the cellular activities. During the present study, the optimum temperature for growth recorded was 28 ± 1°C. As the incubation temperature increased, a decreased growth was observed. The strain MPR_8 showed reduced CFU counts at 45°C, whereas the other strains could not survive at that temperature. Karanjan and Wood (1988) reported that rhizobia from hot dry areas are relatively more temperature and desiccation tolerant than the strains from the Polar Regions. Several temperature tolerant N_2 fixing rhizobial strains has been described that can grow up to 40°C (Hungria *et al*., 2000). Various workers have reported that temperature tolerance of *Rhizobium* from different habitats and hosts found that very few strains could grow above 40°C (Trotman and Weaver, 2000). Segovia *et al*. (1991) observed that high soil temperature can also contribute to the frequency of non-infective isolates in the 80% growth restriction on the strains of rhizobia at lower temperatures below 0°C either by slow or fast-growing strains (Graham, 1992).

The tolerances to pH from the isolated strains were also observed. The optimum pH for the strains *Rhizobium meliloti* (MPR_8) was observed at 7.0. The strains showed better growth at alkaline pH than in acidic ones. The reduction in CFU counts ml^{-1} of MPR_8 at pH 8.0, 10 and 11.0 were 12%, 13% and 19%, respectively after 24 hrs of growth compared to control (pH 7.0). The reduction at lower pH's 6.0, 5.0 and 2.0 were 13%, 46.7% and by 54%, respectively. The survival and growth of rhizobia were affected by soil acidity as well as the process of nitrogen fixation. The strains we have tested showed similarities in pH tolerance. The optimum pH for growth was 7.0. The fast-growing strains were reported to be less tolerant to the lower pH's (4.0 - 6.0) by Graham *et al*. (1994). In our study, it was found that the strains were highly affected by acidic pH rather than alkaline ones due to our strains were acid producing. Same types of results were reported by Brockwell *et al*. (1991). Elizabeth *et al*. (2000) screened the acid tolerant strains of *Rhizobium leguminosarum* for the improvement of clover plant and observed that the effectiveness of the strains showed on gradual loss. It is essential to develop acid tolerant strains of rhizobia to inoculate legumes under acidic soil conditions that will ensure the establishment of efficient symbiosis (Correa *et al*., 1999).

The strains isolated were subjected to cross inoculation study and found that the isolates showed nodulation on *Mucuna pruriens*, *Trigonella foenum-graecum* and *Medicago sativa* (Table 4).

Table 4. Cross inoculation studies using the strains from *Mucuna*.

Host Legumes	**Strains of rhizobia**									
	MPR_1	**MPR_2**	**MPR_3**	**MPR_4**	**MPR_5**	**MPR_6**	**MPR_7**	**MPR_8**	**MPR_9**	**MPR_{10}**
Pisum sativum	-	-	-	-	-	-	-	-	-	-
Vigna mungo	-	-	-	-	-	-	-	-	-	-
Vigna radiata	-	-	-	-	-	-	-	-	-	-
Phaseolus vulgaris	-	-	-	-	-	-	-	-	-	-
Lens culinaris	-	-	-	-	-	-	-	-	-	-
Cicer aerietinum	-	-	-	-	-	-	-	-	-	-
Medicago sativa	+	+	+	+	+	+	-	+	-	+
Mucuna pruriens	+	+	+	+	+	+	+	+	+	+
Trigonella foenumgraecum	+	-	+	+	-	-	+	+	+	+
Arachis hypogaea	-	-	-	-	-	-	-	-	-	-
Trifolium repens	-	-	-	-	-	-	-	-	-	-

(+) Nodulation occurred, (-) No nodulation occurred

It was concluded from the morphological, physiological, biochemical, molecular biological and in vitro infectivity tests were all similar as described by Holt *et al*. (1994) for rhizobial species. Further their generation time, carbon source utilization, DNA base composition, antibiotic resistance properties confirms to the species of *Rhizobium meliloti*.

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