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A Study on Optimization of Potential Plant Growth Promoting Rhizobial Strain from Root Nodules of *Albizia procera*

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ABSTRACT

Symbiotic systems for biological nitrogen fixation (BNF) in agriculture are most promising. Nitrogen fixation is the reduction of N_2 (atmospheric nitrogen) to NH3 (ammonia) which is made possible by the enzyme nitrogenase. Strains of root-nodulating bacteria were isolated from root a nodule of Albizia. After confirmation test with yeast mannitol agar (YMA), isolates were collected for morphological and biochemical characterization. Twenty six nodule isolates from Albizia procera grown in YEM broth were studied for their physiological and biochemical characteristics. Most isolates tolerated high salt concentration (5% NaCl), able to grow at a temperature of 45 °C, survive in a pH range from 5 to 8 upto incubation of 120hrs.

Key words: *Nutrient, BNF, Micro-organisms, Albizia procera*

INTRODUCTION

Biological nitrogen fixation (BNF) is the cheapest and environment-friendly procedure in which nitrogen fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into the soil. Nutrient enrichment of soils by nitrogen-fixing symbiotic bacteria present in legumes have been known for centuries. Scientific demonstration of this symbiosis was started in the 19th century and it established the facts that the bacteria present in nodules on legume roots are responsible for fixing atmospheric nitrogen (Zahran, 1999). Nitrogen fixing bacteria are able to fix atmospheric nitrogen under different conditions independently, in loose association with other organisms, or in strict symbioses with them, such as in the Rhizobium legume plant symbiosis which is being considered the most efficient type of association between diazotrophic microorganisms and plants.

Albizia procera, commonly known as 'Safed siris' is1 one of the important Nitrogen Fixing Tree (NFT) species belongs to family Fabaceae, sub-family Mimosoideae. It is a large, fast-growing medium to large sized deciduous tree that occurs on many different sites. It is distributed througout moist and dry deciduous forests of India. This species provides wood for a variety of purposes, nutritious fodder for livestock and shade for tea plantations.

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It is extensively planted in farm lands, wasteland, road side avenues and is an important reforestation and agroforestry species. It provides an excellent fuel, fodder and small timber for making agricultural implements. Besides, it is an important nitrogen fixing tree and helps in ameliorating the soil productivity. The timber is usually employed in making materials for carts, carriages, small handel tools and agricultural implements and also provide fodder during lean periods of summer, when there is scarcity of green fodder in the region.

Keeping view in mind, the present study was an attempt to optimize the cultural investigate the plant growth promoting traits of rhizobia isolated from *Albizia procera*.

MATERIALS AND METHODS

Isolation of *Rhizobium*

The *Rhizobium* isolates were obtained from root nodules of *Albizia procera* seedling grown under net house conditions. Nodules located on the roots were spherical (2-4 mm in diameter) and pink. Root nodules were sterilized in 95% (v/v) ethanol for 10 s and then washed 7 times with sterile distilled water. Individual nodules were crushed with sterile glass rods and streaked onto yeast extract mannitol (YEM) agar containing 0.0025% (w/v) Congo red. After incubation for 2-3 days at 30 °C, single colonies were selected and restreaked on YEM agar for purity.

Growth on Yeast Extract Broth

Growth in YEM broth incubated at 250 rev min-1 was determined by measuring the optical density at 600 nm every 2 h. The generation time was calculated from the logarithmic phase of growth.

Morphological Characters For morphological characteristics, isolated root-nodulating bacteria were streaked over YEM agar plates. Plates were kept at 28°C for 48 hours and their characteristics were observed.

Gram staining and Spore staining Two colonies were taken from 48 hours old culture plate to perform gram staining and spore staining.

Biochemical Characters Biochemical tests were conducted to observe biochemical characteristics of Rhizobium bacteria.

1) Catalase Activity Isolates of 48 hours old culture were flooded with hydrogen peroxide to observe the release of bubbles of oxygen around the bacterial colonies according to Graham and Parker (1964).

2) Oxidase Activity Few drops of p-aminodimethylaniline oxalate were added on the surface of isolates on YEM agar to observe the production of colour according to Kovaks.

3) Acid from Glucose Mannitol in the YEM agar was replaced by the equal amount of glucose and bromothymol blue (25 mg/l) were added to it, the modified media used to observe the Change in colour around the colonies.

4) Starch Hydrolysis Starch hydrolysis test was performed to determine the ability of microorganisms to use starch as a carbon source (de Oliveira, et al, 2007). The starch medium was inoculated with Rhizobium and analyzed for starch utilization. Iodine test was done to determine the capability of microorganisms to use starch. Drops of iodine solution (0.1 N) were added on 24 hours old cultures grown in petri plates and incubated for 48 hours. Formation of a clear zone around the colonies will indicate utilization of starch.

5) Growth on Glucose Peptone Agar Streaking with isolated strains was done on Glucose Peptone Agar (GPA) plates. The presence of growth was observed after 48 hours according to Vincent (1970).

6) Urea Hydrolysis YEM broth altered with 2% (w/v) urea and 0.012% phenol red to check the urea hydrolysis. The broth was inoculated with log phase cultures and incubated for 48 hours at 28˚C to observe for the production of colour according to Lindstrom and Lehtomaki.

7) Gelatin Hydrolysis Log phase cultures from YEM broth was swab on the surface of YEM agar plates containing 0.4% (w/v) gelatin to examine gelatinase activity. The plates were incubated at 28°C for 7 days.

8) Citrate Utilization By replacing mannitol from YEM agar with an equal amount of sodium citrate and bromothymol blue (25mg/l), citrate utilization ability was determined. The plates with modified media

were inoculated with bacteria and then incubated for 48 hours at 28°C.

9) Growth in Presence of 8% KNO3 Bacteria were allowed to test for the ability to grow in the presence of 8% KNO3 in YEM broth for a 7 days incubation period at 28°C.

Phenotypic Characterization

All tests were carried out in triplicate. Before inoculation, isolates were grown on YEM liquid medium to log phase (108 cell min^{-1}). When test plates were used, inoculation was performed with 30 µl of these cultures. The results were scored after 5 days of incubation at 30 °C.

Colony Morphology

The colony morphology of isolates was examined on both YEM agar plates. After an incubation of 2-3 days at 30 °C, individual colonies were characterized based on their color, shape, Gram stain reaction, and capacity to produce exopolysaccharide gum.

Sodium Chloride and pH Tolerance

The ability of the *Rhizobium* isolates to grow in basic or acidic media was tested by streaking them on YEM agar plates with pH adjusted to 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 with HCl or NaOH (2,10). The ability of the isolates to grow in different concentrations of salt was tested by streaking isolates on YEM medium containing 0%, 0.5%, 1%, 2%, 3%, 3.5%, 4%, and 5% (w/v) NaCl (11,12).

Temperature Tolerance

Tolerance to high temperatures was tested by typing on YEM broth and incubating at 37, 40, 42, and 45 °C (10,13).

2.2 Identification Identification of isolated strain was carried out based on morphological and biochemical characteristics.

RESULTS

Sampling

Study was conducted with *Albizia procera* root nodules. Samples were collected from different locations of Himachal Pradesh and Uttrakhand. *Rhizobium* isolation was done in microbiology laboratory of University. Bacteria were isolated and maintained on Yeast mannitol agar medium for regular laboratory works and stored at low-temperature refrigerator for long term storage. The growth on the YEMA medium was counted and reported as cfu/g root.

Table 1: Rhizobial population and number of isolates from root nodules of *Albizia procera* seedlings

	Isolate name	Sites	No. of isolate	Rhizobial Population (10^2 cfu/g soil)
Himachal Pradesh	N A (Nauni *Albizia*)	Nauni	8	76.3
	BA (Baddi *Albizia*)	Baddi	11	84.1
	JA (Jachh *Albizia*)	Jachh	6	80.1
	GA (Gaggal *Albizia*)	Gaggal	7	73.6
	DA (Dhaulakuan *Albizia*)	Dhaulakuan	6	71.8
Uttrakhand	FA (FRI *Albizia*)	FRI	5	85.8
	IA (ITBP *Albizia*)	ITBP	4	71.0
	RA (Ram Nagar *Albizia*)	Ram Nagar	6	83.4
	PA (Pant Nagar *Albizia*)	Pant Nagar	5	80.3
	LA (Lal Kuan *Albizia*)	Lal kuan		77.6

Root nodules were collected from the seedling of tree grown under net house conditions. A total of 66 rhizobial isolates were isolated from root nodules of *Albizia procera* from seedling of different sites.

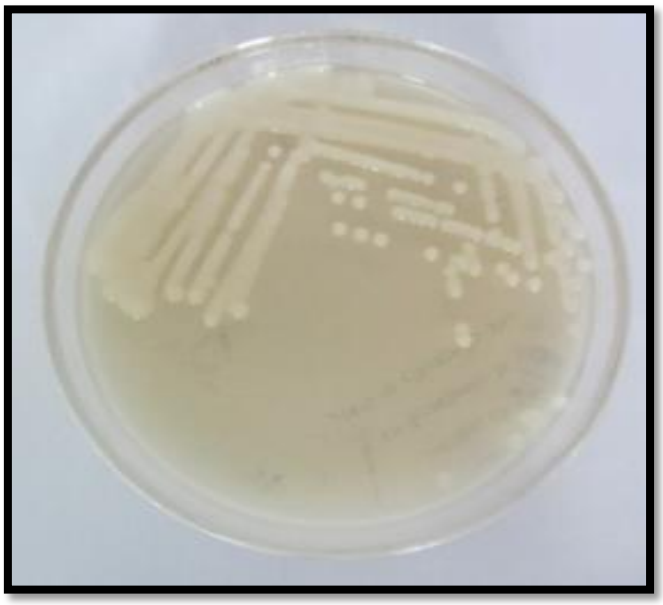

Plate 1: Isolation of rhizobial isolates of *Albizia procera*

Table 2: Characteristics of *Albizia procera- Rhizobium* isolate

	Properties	***Albizia procera- Rhizobium* isolate**
A. Behaviour		
1.	Gram staining	Gram –ve bacteria
2.	Shape	Short rod
B. Colony characters		
1.	Growth on	YEMA White translucent
2.	Shape/form of colony	Circular
3.	Size	1.5-2.00 mm
4.	Edge/margin	Nearly Entire
5.	Elevation	Convex (slightly flat)
6.	Surface	Smooth
7.	Color	Milky white

Morphological characteristics: Isolated bacteria were streaked over YEM agar plates. After incubation at 28˚-30˚ C for 48 hours, it was observed that isolates form milky white, circular, translucent, glistening, convex, elevated and raised colonies. The margin was regular and 2-4 mm in diameter. Figure 3.2 - Isolated bacterial colonies on Yeast extract mannitol medium.

Biochemical Tests:

Isolates were studied for their biochemical characteristics. Strains of 48 hours old were used to perform different biochemical tests.

Table 3: Results of different biochemical tests

Test	Results
Gram staining	Negative
Spore staining	Non spore forming
Catalase test	Positive
Oxidase test	Negative
Acid from glucose	Positive Colour changes from green to yellow
Starch hydrolysis	Positive Clear zone observed around colonies
Growth on Glucose peptone agar	Positive Utilize glucose as the sole carbon source
Urea hydrolysis	Positive Colour changes from red to orange
Gelatin hydrolysis	Negative No clear zone formed around colonies
Citrate utilization	Negative No colour change
Growth in presence of 8% KNO3	Positive
NaCl 2% tolerance	Positive

Salt, pH and Temperature Tolerance:

Isolates showed growth on 0.5%, 1%, 1.5% and 2% up to 4% (w/v) of NaCl concentration. No growth was observed on 4.5% and 5% NaCl concentration though temperature was 28˚C and pH was 7.

In the present study optimum, pH range for the growth of Rhizobia was between pH 6 to pH 7. No growth was observed in the medium with pH 3.5, pH 4, pH 8 and pH 9. Growth was observed at pH 6. pH 6.5 and pH 7.

No growth was seen on plates incubated at 34˚C, 37˚C, and 40˚C even at pH 7.

Optimization of Cultural Conditions for Selected Rhizobial Isolates

Effect of different incubation period will be studied on the growth of selected rhizobial isolates (0, 12, 24, 48, 72, 96 and 120 h). It was observed that rhizobial isolates showed best growth after 120 hours incubation.

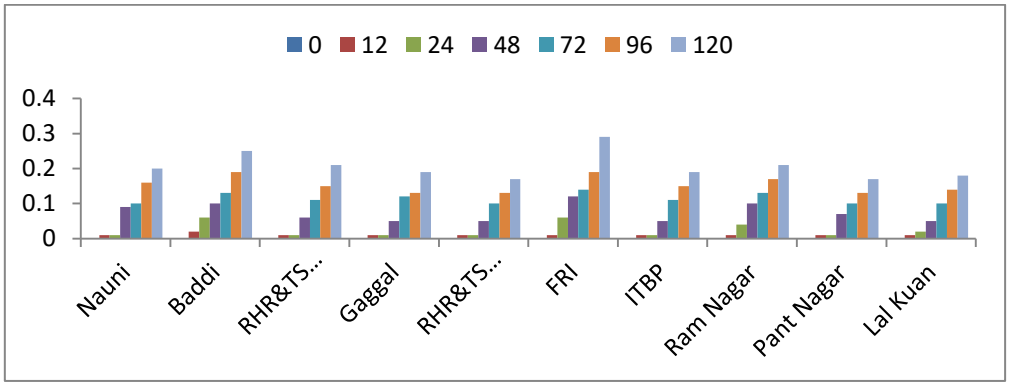

Fig. 2: Optimization of incubation period for rhizobial isolates of Himachal Pradesh and Uttrakhand

Growth of the rhizobial isolates at different pH (4, 5, 6, 7, 8 and 9) was carried out. Rhizobial isolates showed best growth at pH 7.

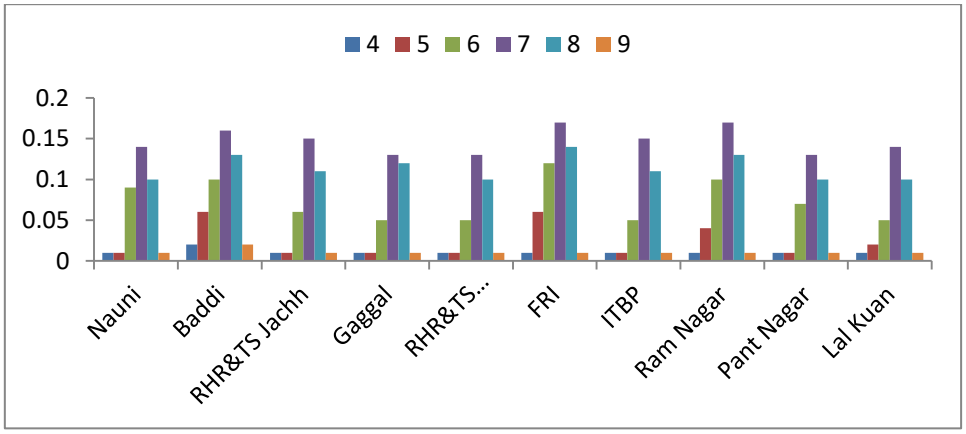

Fig. 3: Optimization of pH for rhizobial isolates of Himachal Pradesh and Uttrakhand

Effect of different temperature range (20, 25, 30, 35, 40 and 45 ºC) was studied for the optimum growth of selected rhizobial isolates. The rhizobial isolates showed maximum growth at temperature 30 ºC.

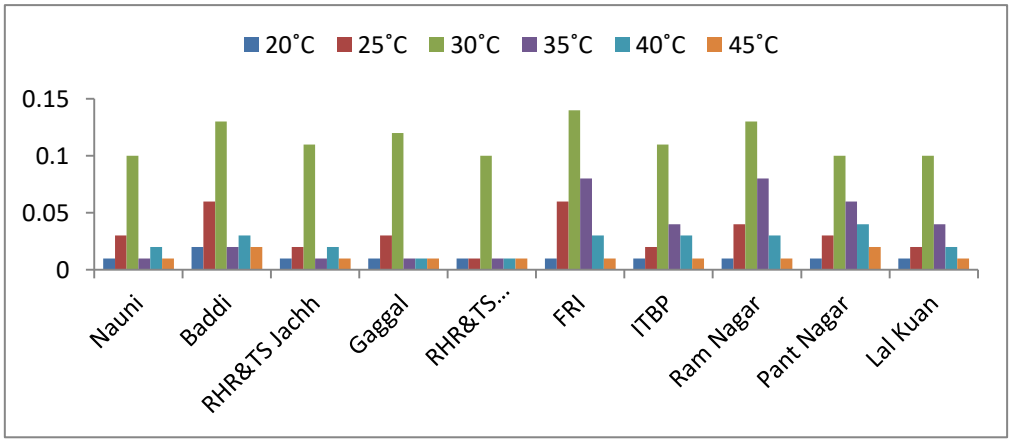

Fig. 4: Optimization of temperature for rhizobial isolates of Himachal Pradesh and Uttrakhand

DISCUSSION

In this study, strains of root-nodulating bacteria were isolated from the root nodules of Albizia procera growing in different areas of Himachal Pradesh and Uttrakhand. All the strains showed growth in three days and turned the yeast extract mannitol agar media containing bromothymol blue to yellow colour. It indicated that all were fast growers and acid producers (Alemayehu, 2009). The colonies were 2-4 mm in diameter, circular, translucent, glistening, elevated, convex with smooth edges, raised colonies. Microscopic examination revealed that the isolates were rod-shaped and gram negative in nature (Anand & Dogra, 1991, Singh et al., 2008). Strains were also nonspore forming. In the present study, all isolates were Oxidase negative as experiment showed no colour change in the region of the colonies after addition of p-aminodimethylaniline oxalate on the surface of isolates. The catalase test was positive. While isolates were flooded with hydrogen peroxide, the release of oxygen bubbles around bacterial colonies was observed. Urease test also showed positive results. In this experiment, YEM broth was altered with 2% w/v urea in company with 0.02% phenol red and inoculated with log phase culture. After 48 hours of incubation period, it was observed that colour of the broth changes from red to orange that is the indication of urea hydrolysis according to Lindstrom and Lehtomaki. It means that experimenting microbes are able to use urea as a source of carbon and energy for growth.

Strains were unable to utilize citrate. For this experiment, YEM media had to modify by replacing mannitol with an equal amount of sodium citrate and bromothymol blue (25 mg/l) and incubate. After finishing the incubation period colour remained blue. If strains were able to utilize citrate colour would be changed from blue to green. Our experimented Rhizobial cells did not produce gelatinase enzymes because clear zone formation was not seen around colonies. In the present study the isolate grown on modified YMA (contained 0.4% w/v) but did not show gelatinase activity. Negative gelatinase activity of *Rhizobium* was also observed by Hunter et al, (2007), Positive results were obtained from the starch hydrolysis assay as clear zone were present according to De Oliveria et al. (2007), which directed that investigational strains are able to use starch as a carbon source. Isolate in this study were able to tolerate 2% NaCl, which is in accordance with the characteristics of fast-growing *Rhizobium*.

Present study indicated that isolates showed growth on 0.5%, 1%, 1.5% and 2%, 2.5% (w/v) up to 4% of NaCl concentrations. No growth was observed at 5% NaCl concentration though the temperature was 28˚C and pH was 7. Growth rate decreased rapidly in high salt concentration. Some *Rhizobium* sp. are able to grow in the presence of 4.5% and 5% NaCl according to Kassem et al., & Kucuk et al. (2006). This study reported that the isolated strain is highly tolerant to high salt concentrations and have the potential to improve acquiesce of legumes in high salt concentration (El-Mokadem, et al., 1991). Results also showed that optimum pH for Rhizobia strain was between pH 6 to pH 7. No growth was observed in the medium with pH 3.5, pH 4 and pH 9. Growth was also observed at pH 7.5. At pH 8 very few amount of growth was observed. This result indicated that experimented strain are not acid adapted as they were not capable of surviving at pH lower than 6. Glenn and Dilworth, Correa and Barneix (1997), reported that different strains of the same species may vary extensively in their capability of pH tolerance. Some Rhizobia can show more sensitivity to low pH than their host and this may hamper the symbiotic relationship between them and may perhaps limit the survival of *Rhizobium* (Zahran, 1999). All isolates showed growth on temperature 26˚C, 27˚C, and 28˚C .No growth was seen on plates incubated at 34˚C, 37˚C, 38˚C according to Jordan (1984) and 40˚C though the pH was 7. This experiment indicated that strains are not heated tolerating. Moreover, temperature range depends highly on the strains of *Rhizobium* which are reported by Jordan (1984). Despite the fact that some strain may survive in high temperature but it does not represent that they are proficient in nitrogen fixation.

Indeed it is reported by Zahran (1999). that, some *Rhizobium* strains able to grow on higher temperature and formed nodules but the nodules became ineffective and the plant did not accumulate nitrogen.

The presence of the strains growing under stressed laboratory conditions in our study indicates their significance in contributing biologically fixed nitrogen to stressful ecosystems.

CONCLUSION

In this present study, *Rhizobium* sp. was isolated from the root nodules of leguminous tree *Albizia procera*, observed their morphological and biochemical characteristics by using different types of media and determined their nitrogen fixation ability and their effects on plant growth. Resulted strain is capable for nodulation and fixing the higher amount of nitrogen. Isolates also showed optimistic effects on plant growth. These findings may give us a prospect to explore extensive research and also using *Rhizobium* as a cheaper substitute for urea.

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