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Characterization of *Rhizobium* isolated from root nodules of *Trifolium alexandrinum*

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Strains of root nodulating bacteria were isolated from root nodules of *Trifolium alexandrinum* growing widely in different agroclimatic regions of Dehradun. After authentication test, 85 isolates those nodulate host plant along with one standard strain *Rhizoboium trifolii* (MTCC 905) were selected. The isolates were further subjected to morphological, biochemical and physiological characterization to ascertain their taxonomic position. Antibiotic resistance pattern of all isolates was also evaluated against thirteen antibiotics. Most of the isolates exhibited highest resistance to streptomycin and least with tetracycline. On the basis of morphological, biochemical and physiological characters and their comparison with standard strain *R. trifolii* (MTCC 905) most of the characteristics were found similar to the standard strain.

Key words: Nodulation, *Rhizobium trifolii, Trifolium alexandrinum*

Introduction

Legumes have been used in agriculture since ancient time and legume seeds or pulses were among the first source of human food and their domestication. Legume plant posses a unique ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae. The bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Rinorhizobium* and *Mesorhizobium* (Martinez Romero, 2003; Willems, 2006) which are collectively referred to as rhizobia, are able to form nodules on their host plants

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inside of which they fix-nitrogen. This symbiotic relationship reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops and also enrich soil with nitrogen.

Leguminous plants are also of crucial importance as animal feed. Alfa-alfa and clovers are grown over extensive areas as forage crops for grazing or as dry hay, and they furnish not only high quality protein but also a variety of biologically active molecules such as vitamins, minerals and other nutrients (Burris and Roberts, 1993). Barseem (*Trifolium alexandrinum*), like other legume, is the most important winter season crop cultivated in an area of around two million hectares in India. The significance of this forage species lies in the development of milk industry. Its crude portion content i.e. up to 20%, coupled with 70% digestibility results in significant increase in milk production. Barseem is a most potent milk multiplier in the lactating buffaloes, sahiwal cows and cross breed cattles as compared to other forage crops. One of the most important limitations for enhancement of biological nitrogen fixation and productivity in symbiosis between *Trifolium alexandrinum* (barseem) and *Rhizobium trifolii* relates the lack of adequate ecological information about this symbiotic system. Although symbiotic nitrogen fixation by legumes is generally the dominant source of nitrogen input in soil for imparting fertility but also avoid soil stresses, such as temperature, acidity and salinity which pose a severe yield constraint in obtaining plant growth and development (Lawson *et al*, 1995). In this work, we characterized the diversity of 85 *Rhizobium* isolates by using morphological, biochemical and physiological tests, and to select isolates adapted to the climatic conditions.

Material and methods

Isolation of Root Nodulating Bacteria

Root nodules of *Trifolium alexandrinum* was used to isolate root nodulating bacterial strains were isolated (Vincent, 1970). Plants were collected from different agroclimatic regions of Dehradun. Plants were uprooted carefully and nodules were collected from the roots, washed with sterile water followed by surface treatment with 95% alcohol and again with sterile water. The washed nodules were surface sterilized with 0.1% mercuric chloride for 2-3 minutes and again washed for at least 10 times with sterile water as to remove the traces of mercuric chloride. The nodules were transferred in culture tube half filled with sterile water and crushed with a sterile glass rod to obtain a milky bacterial suspension. After serial dilution suspension was streaked on Yeast extract mannitol (YEM) agar plates and incubated for 2-3 days at 28°C. A single colony was taken from each nodule extract directly or after

purification through subsequent streaking. In order to compare the phenotypic traits of the field isolates with a commercial strain, a pure culture of *Rhizobium trifolii* (MTCC 905) obtained from Institute of Microbial Technology (IMTECH), Chandigarh was used and known to be effective in symbiotic nitrogen fixation with clover.

All the cultures obtained were tested for nodulation in the host plant before and after the series of experiments. The authentication test was performed using surface sterilized clover seeds planted on to nitrogen free agar slopes in 20×150mm test tubes as described by Pryor and Lowther, (2002). Uninoculated plants served as negative controls and *Rhizobium trifolii* strain (MTCC 905) was the positive control. Five days after planting, seedlings were inoculated with 1 ml of YEM broth containing approximately 10^7 cfu/ml and kept at 30°C in the incubator. Twenty five days after inoculation, plants were removed from the tubes and the presence or absence of nodules assessed. Then these nodules were crushed with sterile glass rod and streaked on yeast extract mannitol agar. After incubation for 2-3 days at 28°C., single colony was selected and re-streaked on YEM agar slant. After purity three replicates of per isolates were prepared for further study.

Cultural Characters

Log phase culture in 0.1ml quantity of the isolated root nodulating bacteria (RNB) strain was spread over YEM agar plates Strains were also streaked on YEM agar. The inoculated plates were incubated at 28°C for 48 hours and observed for colony shape, size, colour and texture. Fast growing rhizobia generally produce white, semi translucent, circular, mucilaginous colonies while slow growing strains produce white, opaque, circular, granular colonies, which do not exceed 1mm in diameter after prolonged incubation.

Biochemical and Physiological Characters

The biochemical tests were carried out in growth medium at 28°C for 48 hours incubation. All the tests were carried out with 03 replicates.

Catalase Activity

Different isolates which were 48 hours old were flooded with hydrogen peroxide and observed for liberation of effervescence of oxygen around the bacterial colonies according to Graham and Parker (1964).

Oxidase Activity

Few drops of p-aminodimethylaniline oxalate were added on the surface of isolates on YEM agar and observed for the production of color according to Kovaks (1956).

Acid from Glucose

Mannitol in the YEM agar was replaced by equal amount of glucose and bromothymol blue (25 mg/l) was added to it, this modified media was inoculated with the strains and incubated. Change in color around the colonies was observed.

Methylene Blue and Gentian Violet Treatment

Methylene blue dye was added to the growth medium at a concentration of 0.1%, then inoculated with *Rhizobium* and incubated at 28°C ± 2°C for 2-7 days. Similar experiment was done with gentian violet at the concentration of 0.1% and observations were made according to Gao *et al.* (1994).

Starch Hydrolysis

Starch hydrolysis test was performed to determine the ability of micro organisms to use starch as a carbon source (De Oliverira, 2007). This medium was inoculated with *Rhizobium* and analyzed for starch utilization. Iodine test was used to determine the capability of micro organisms to use starch. Drops of iodine solution (0.1 N) were spread on 24 hours old cultures grown in Petri plates. Formation of blue color indicated non utilization of starch and vice versa.

Growth on Glucose Peptone Agar

Glucose Peptone Agar (GPA) plates were streaked with isolated strains and incubated. Presence of growth was observed after 48 hours according to Vincent (1970).

Urea Hydrolysis

YEM broth was amended 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 and observed for the production of color according to Lindstrom and Lehtomaki (1988).

Growth on Hofer's Alkaline Broth

Autoclaved Hofer's Alkaline Broth (HAB) was inoculated with log phase cultures and observed for growth after 48 hours (Hofer, 1935).

Gelatin Hydrolysis

Log phase cultures from YEM broth were swabbed 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 (Sadowsky *et al*., 1983).

Citrate Utilization

Citrate utilization ability was determined, by replacing mannitol from YEM agar with equal amount of sodium citrate and bromothymol blue (25mg/l). The plates with modified media were inoculated and then incubated for 48 hours (Koser, 1923).

Growth in Presence of 8% kNO_3

Strains were tested for the ability to grow in the presence of 8% KNO_3 in YEM broth for a 7 days incubation period at 28°C (El Idrissi *et al*., 1996).

NaCl (2%) Tolerance

YEM agar media amended with 2% Nail (w/v) was inoculated and growth was observed after 48hours incubation.

Precipitation of Calcium Glycerophosphate

Precipitation of calcium glycerophosphate was carried out in YEM broth amended with calcium glycerophosphate. Formation of precipitate after the incubation indicates positive result (Hofer, 1941).

Antibiotic Resistance Test

The isolates were tested for antibiotic sensitivity by Kirby-Bauer disc diffusion method on YEM agar (Bauer *et al*., 1966). Cultures were inoculated

by swabbing with standard inoculums according to 0.5 Mc Farland tube over the entire agar surface. The agar surface was allowed to dry for 3-5 minutes before applying the antibiotic discs. Antibiotic discs were placed equidistantly on 90 mm Petri plate using sterile forcep. The plates were incubated aerobically at 28 ± 2°C for 48 hrs. Resistance to an antibiotic was detected by the inhibition zone formed around the discs. The antibiotics used were chloramphenicol (30 µg), kanamycin (30 µg), tetracycline (15 µg), rifampicin (5 µg), nalidixic acid (30 µg), streptomycin (10 µg), neomycin (10 µg), vancomycin (30 µg), erythromycin (15 µg), cephalothin (30 µg), ampicillin (10 µg), gentamycin (10 µg) and polyxifxin (300 units).

Utilization of Carbon and Nitrogen Sources

To determine the carbon and nitrogen utilization pattern, 80 µl of 10% w/v filter sterilized solutions of the carbohydrates (pentoses, hexoses disaccharides and trioses) and amino acids were added to 5 ml YEM broth in which yeast extract was reduced to 50 mg/l. The medium was then inoculated by the addition of exponentially (10^8 cells/ml) growing cultures of the isolates (Kumar *et al.,* 1999). The inoculated broth were incubated at 28°C and kept at 150 rpm in an incubator shaker. Optical density was taken at 610 nm after 7 days incubation for measuring the growth.

Salt, pH and Temperature Tolerance

The ability of the isolated Rhizobial strain to grow in different concentration of salt was tested by streaking them on YEM medium containing 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5% and 5.0% (wt/v) NaCl. Differences in pH tolerance were tested in YEM agar by adjusting the pH to 3.5, 4.0, 5.0, 6.0, 6.5, 7.5, 8.0 and 9.0. All the plates were incubated at 28°C for 72 hours and YEM medium plates were used as controls. Difference in the range of growth temperature were investigated by incubation of bacterial cultures in YEM agar at 32°C, 34°C, 36°C, 38°C and 40°C. Control plates were incubated at 28°C. Strains were considered salt tolerant, resistant to acidity and temperature resistant when growth was similar to the growth in the control plates.

Results

Rhizobium Isolation from Root Nodules

A total of 129 bacteria were recovered from root nodules of *Trifolium alexandrinum,* collected from different agro climatic regions of Dehradun. All isolates were gram negative, non-sporing rods. The authentication test confirmed that 85 isolates and *Rhizobium trifolii* (MTCC 905) were able to nodulate the host plant. Uninoculated plants were (negative control) and rest of the 44 isolates did not show nodule formation and the plants started to show chlorosis and wilting after the first two weeks of the experiment. After authentication test, 85 isolates that nodulate host plant confirms them as the isolates of *R trifolii*. These isolates were used for further cultural, biochemical and physiological characteristics.

Cultural Characters

All isolates showed the same colony characteristics, after 48 hours of incubation. The colonies were milky white, translucent, circular in shape, shiny, raised and 2-4 mm in diameter.

Biochemical and Physiological Characters

All isolates showed growth in two days and turned the yeast mannitol agar media containing bromothymol blue to yellow color showing that they were fast growers and acid producers. All the isolates were catalase and oxidase positive as confirmed by the liberation of effervescence of oxygen around the bacterial colonies and change in color of the oxidase strip, respectively. None of the strain showed growth on glucose peptone agar and medium containing 0.1% methylene blue and 0.1% gentian violet. Starch hydrolysis assay was positive for all isolates and a clear zones around the colonies were seen and colonies with yellow coloration of colonies. Most of the strains were positive for urease and were also able to grow in Hofer's alkaline broth. All the isolates were negative for gelatinizing ability and thus no clear zone was formed around the colonies on YEM gelatin agar. All isolates were unable to utilize citrate and were able to tolerate 8% KNO_3 and 2% NaCl and formed precipitate formation in calcium glycerophosphate medium (Table 1).

Table 1. Biochemical Characterization of the isolates recovered from *Trifolium alexandrium*

Isolate	Characteristics														
	Gram Reaction	Catalase	Oxidase	Acid From Glucose	Growth on GPA	Growth On 0.1 % Mthylene Blue	Growth on 0.10% Gentian Violet	Starch Hydrolysis	Urea Hydrolysis	Growth On HAB	Gelatin Hydrolysis	Citrate Utilization	8% KNO_3 Tolerance	2% NaCl Tolerance	Ppt. in Calcium Glycerophosphate
RGM 1	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 2	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 3	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 4	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 5	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 6	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 7	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 8	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 9	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 10	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 11	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 12	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 13	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 14	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 15	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 16	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM17	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 18	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 19	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 20	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 21	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 22	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM23	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 24	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 25	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 26	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 27	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 28	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 29	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 30	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 31	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 32	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 33	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 34	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 35	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 36	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 37	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 38	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 39	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 40	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 41	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 42	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 43	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 44	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 45	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 45	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 46	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 47	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 48	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 49	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 50	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+

RGM 51	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 52	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 53	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 54	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 55	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 56	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 56	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 57	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 58	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 59	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 60	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 61	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 62	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 63	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 64	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 65	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 66	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 67	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 68	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 69	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 70	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 71	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 72	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 73	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 74	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 75	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 76	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 77	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 78	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 79	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 80	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 81	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 82	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 83	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 84	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
RGM 85	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+
MTCC 905	−	+	+	+	−	−	−	+	+	+	−	−	+	+	+

+ Positive; − Negative; GPA = Glucose Peptone Agar; HAB = Hofer's alkaline broth

Antibiotic Resistance Pattern

On the basis of biochemical and physiological characters, 85 Rhizobium isolates were selected and along with one standard strain (*R trifolii*, MTCC – 905), were assessed for their IAR pattern against thirteen different antibiotics. The evaluation of intrinsic resistance to antibiotics of barseem rhizobia showed that most of the tested isolates exhibited highest resistance to streptomycin (96%), followed by chloramphenicol (94%), ampicillin (94%), erythromycin (93%), kanamycin (92%), polymyxin (92%), nalidixic acid (88%), gentamycin (86%) and neomycin (85%). On the other hand vancomycin, rifampicin, cephalothin and tetracycline had least antibacterial effect, the number of isolates showing resistance were 58, 44, 44 and 33, respectively. A positive IAR affected isolates in terms of percentage were 67%, 51%, 51% and 38% respectively (Figure 1). The overall pattern was shown by these antibiotics: streptomycin > chloramphenicol = ampicillin > erythromycin > kanamycin =

polymyxin > nalidixic acid > gentamycin > neomycin > vancomycin > rifampicin = cephalothin > tetracycline.

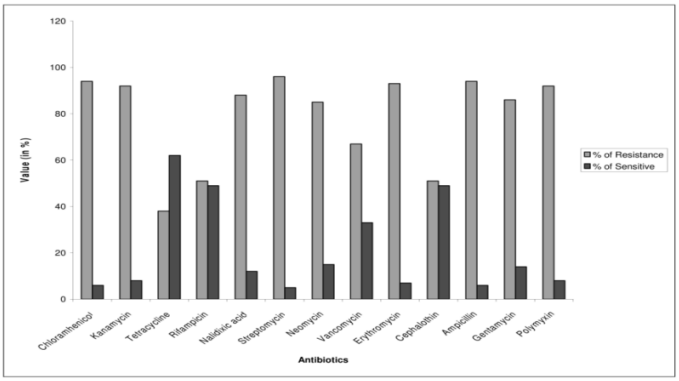

Figure 1: Intrinsic Antibiotic Resistance (IAR) for Rhizobial isolates

Utilization of Carbon and Nitrogen Sources

All strains were able to utilize a majority of carbon and nitrogen sources. Most of the strains were able to utilize hexoses (rhamnose and sorbitol), pentoses (arabinose and xylose), disaccharides (lactose and sucrose) and trioses (glycerol). Dextrin was assimilated by the isolate RGM 2, RGM 25, RGM 31 and RGM 39 (Table 2). All the amino acids, used in this study were utilized by isolates except valine and glycine (Table 2).

Table 2. Carbohydrates and amino acids utilization patterns of Rhizobial isolates recovered from *Trifolium alexandrium*

Isolates	Carbohydrates									Amino acids						
	Galactose	Dextrin	Rhamnose	Sorbitol	Arabinose	Xylose	Lactose	Sucrose	Glycerol	Alanine	Aspargin	Cystein	Glycine	Serine	Tyrosine	Valine
RGM 1	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 2	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 3	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 4	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 5	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 6	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 7	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 8	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 9	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM10	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 11	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 12	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–
RGM 13	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–

RGM 14	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 15	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 16	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 17	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 18	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 19	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 20	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 21	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM22	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 23	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 24	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 25	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 26	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 27	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 28	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 29	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 30	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 31	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 32	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 33	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 34	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 35	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 36	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 37	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 38	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 39	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 40	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 41	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 42	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 43	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 44	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 45	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 46	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 47	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 48	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 49	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 50	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 51	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 52	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 53	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 53	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 54	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 55	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 56	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 57	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 58	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 59	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 60	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 61	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 62	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 63	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 64	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 65	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 66	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 67	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 68	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 69	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 70	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−

RGM 71	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 72	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 73	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 74	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 75	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 76	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 77	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 78	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 79	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 80	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 81	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 82	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 83	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 84	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
RGM 85	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	−
MTCC 905	+	−	+	+	+	+	+	+	+	+	+	+	_	+	+	_

+ Positive; -Negative

Test for salt, pH and temperature tolerance

The physiological traits of 86 clover strains were summarized in Figure 2, 3 and 4. All strains were grown at controlled cultural conditions (0.01% NaCl, pH: 7.0 and temp: 28°C). All isolates showed growth up to 2.5% (w/v) of NaCl concentration and more than 85% of the isolates continued growth up to 4% (w/v) of NaCl concentration, after which salt tolerance character of strains reduced and only ten isolates (11.6%) showed growth at 5% NaCl concentration. In the above study optimum pH range for rhizobia was between 5.5 to 7.5. No growth was observed in the medium with pH 3.5. At pH 4, 50% of the isolates exhibited an acid tolerant character. The maximum temperature where more than 85% of the isolates grew was 36°C. The percentage of isolates which grew well at 32, 34 and 36 ^{0}C was 97.6, 94.18 and 88.3%, respectively. Differentiation according to temperature tolerance started at 38°C, of all the isolates 58% were survive at above temperature and none of the strains showed growth at temperature 40°C and 45°C.

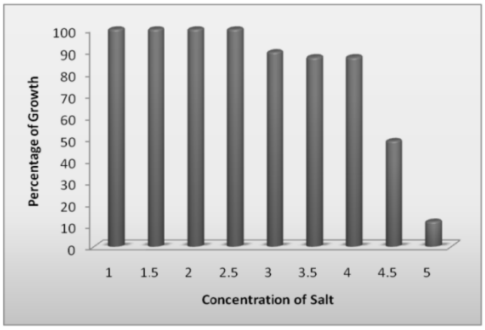

Figure 2: Tolerance of clover rhizobia to different concentrations of NaCl

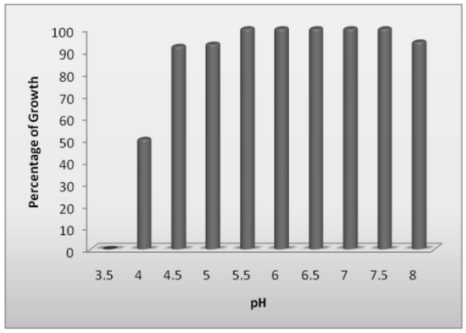

Figure 3: Effect of pH on the growth of clover rhizobia

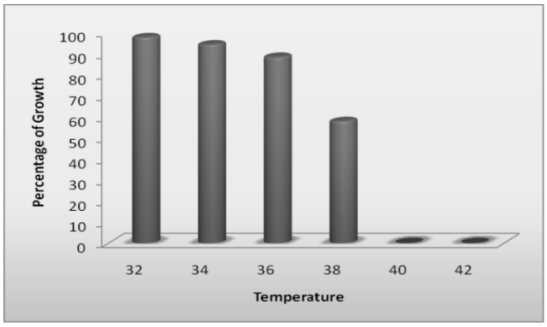

Figure 4: Effect of temperature on growth of rhizobia

Discussion

In the present study, strains of root nodulating bacteria were isolated from the root nodules of an important fodder legume i.e. *Trifolium alexandrinum* Barseem, growing in different regions of Dehradun. During this study eighty five strains were isolated from clover plants out of one hundred twenty nine strains and these were renodulated the host plant confirming them as the strains of *R. trifolii*. Similarly, Farrukh *et al*. (2004) isolated *Rhizobium leguminosarum* bv *trifolii* which were associated with clover. All the strains showed growth in three days and turned the yeast extract mannitol agar media containing bromothymol blue to yellow color showing that all were fast growers and acid producers (Alemayehu, 2009). The colonies were large (2-4 mm in diameter) mucilaginous, circular, convex with smooth edges, glistening translucent or white and precipitated calcium glycerophosphate present in YEM agar (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). In this study, all isolates were oxidase, catalase and urease positive and unable to utilize citrate, which complements the finding of Lupwayi and Hague, (1994). All strains in this study were able to tolerate 2% NaCl, which is in accordance with the characteristics of fast growing *Rhizobium* (Holt *et al*., 1994). Selected isolates were unable to grow on medium containing 0.1% methylene blue and gentian violet. Earlier studies also indicated that Rhizobial cells were unable to grow in the presence of these two dyes (Wei *et al*., 2003). It was observed that Rhizobial cells did not produce gelatinase enzymes. Negative gelatinase activity of *Rhizobium* was also observed by Hunter *et al*., (2007). Positive results were obtained from the starch hydrolysis assay. De Oliveria *et al.,* (2007) also observed that *Rhizobium* strains obtained from different sources can utilize starch. Further, resistance patterns of the isolates to

thirteen antibiotics were studied. Screening for antibiotic resistance in our study revealed that most of the strains were resistance to streptomycin, chloramphenicol, ampicillin, erthyromycin, kanamycin, polymyxin, nalidixic acid, gentamycin and neomycin and least to vancomycin, rifampicin, cephalothin and tetracycline. It is in accordance to Hungaria *et al*., (2000) that this character is due to three known determinants of bacterial permeability to antibiotics: hydrophobicity, electrical charge and amount of the antibiotic. Streptomycin resistance, when caused by a chromosomal mutation, has been shown to be due to an alteration of specific protein on the 30S ribosomal subunit to which streptomycin binds in the sensitive cell. Another class of streptomycin resistant bacteria owes their resistance to the presence of plasmid which mediates either the acetylation, adenylation or phosphorylation of the drug molecule, itself. According to Holt *et al.,* (1994) spontaneous mutants for resistance to most antibiotics are a component of all "wild type" strain. Resistance to streptomycin may be greater than 10 times that of "wild type", where as resistance to other antibiotics were generally of a lower order (2 or 3 times). Therefore, streptomycin resistant mutants, those are usually, effective are important in ecological field studies on strain competition.

Occurrence of higher resistance to antibiotics like penicillin, streptomycin and erythromycin was reported by Kahlon (1980). Similarly Kucuk and Kivnac, (2008) observed higher resistance of rhizobia against streptomycin, chloramphenicol, erthyromycin, kanamycin and penicillin. In this respect, Elbouthari *et al.,* (2010) reported that *Sinorhizobium*. *meliloti* and *S*. *medicae* were resistant to streptomycin, chlorompheni̇col, tetracycline and streptomycin. Josey *et al.,* (1979) studied the variation of IAR of eight antibiotics for identifying characteristics of twenty six *R*. *leguminasarum* strains and mentioned that the IAR of each strain was a stable property.

The markers obtained from the antibiotic resistance profiles can be helpful when selecting microorganisms. Eighty five percent of our isolates showed resistance to neomycin. Neomycin resistant strains had been often related to loss of effectiveness in nitrogen fixation (Amarger, 1981). Therefore, it is important to consider the use of these mutants when dealing with experiments for inoculant selection. Jarek (1989) reported that the most inhibiting effect on *R*. *leguminosarum* strains was observed with gentamycin. Eighty eight percent of the isolates used in this study were resistant to nalidixic acid which is DNA gyrase inhibitor. Similar studies was carried by Hagedorn (1979) who reported several strains of *R. leguminosarum* bv *trifolii* isolated from *Trifolium subterraneum* were resistant to nalidixic acid. Nalidixic acid has also been used to study the viability of many types of bacteria. Viable *R. leguminosarum* bv *trifolii*, bacteria undergoes cell elongation when exposed to

combination of substrate and nalidixic acid (Bottomley and Maggard, 1990). This feature is useful for direct viable counts of viable-non culturable bacteria and for determining the viability with in a population of soil bacteria.

The generalized sensitivity to tetracycline in our study agrees with the results of Jordan (1984) for the genus *Rhizobium* and Hagedorn (1979) for *R. leguminosarum* bv *trifolii*. Sensitivity of isolates to antibiotics may be due to the fact that these bacteria have not been exposed to these antibiotics in natural environments. Depending on the difference in antibiotic resistance pattern, this technique could be successfully employed for the field of ecological studies particularly in the recovery and enumeration of rhizobia introduced into soil with regard to carbon utilization, it has been established that *Rhizobium* is able to utilize a wide variety of carbon sources for growth and have several pathways available for carbon catabolism (Stowers, 1985). Utilization of different carbon sources is an effective tool to characterize the isolates (Mirza *et al*., 2007; Erum and Bono, 2008). All isolates obtained from barseem clover nodules were able to utilize glucose, galactose, rhamnose, sorbitol, arabinose, xylose, lactose, sucrose and glycerol as carbon sources. These carbon sources are generally utilized by bacteria of the genus *Rhizobium* (Stowers, 1985); Sadowsky *et al*. (1983) and Anand and Dogra (1991) also observed that fast growing Rhizobial strains utilized a wider variety of carbohydrates than the slow growing strains. Their ability to metabolize a broad range of carbon substrates may be advantageous for survival in soil. Only four isolates obtained in this study were able to use dextrin as a carbon source, which is in accordance with other works indicating that dextrin is rarely utilized by *Rhizobium* (Jordan, 1984).

In this study, most of the amino acids (alanine, aspargine, cysteine, serine, and tyrosine) were utilized by the strains except valine and glycine. Arora *et al*. (2000) also observed that fast growing Rhizobia can not utilize valine and glycine. These results further confirm the taxonomic position of *Rhizobium* isolates. Moreover morphological, biochemical, physiological and *in vivo* infectivity of the strains were found similar to that of *R. trifolii* (MTCC-905).

Soil temperature, physical and chemical composition, moisture content in soil varies within small areas and these variations affect the populations of the soil inhabitants. Therefore, differences in response towards salinity, pH and temperature are expected. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Hashem *et al*. (1998) 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 and rhizosphere or by inhibiting very early symbiotic events, such as colonization, thus directly

interfering with root nodule formation. Present study indicated that all isolates were able to grow in salt concentrations up to 2.5% (w/v) NaCl. The salt inhibitory concentrations varied among strains. Indeed, tolerance to sodium chloride was found since more than 85% of the tested rhizobia continued to grow with 4.0% NaCl (w/v). However, at higher concentrations, the percentage of tolerant strains decreased rapidly and only 10 strains showed moderate growth in 5% NaCl. The ability of some strains of *R. leguminosarum bv trifolii* to grow under NaCl concentrations up to 350 mM in broth culture has been reported previously (Zahran, 1999). Kassem *et al.,* (1985) observed that strains of *R. meliloti* are able to grow in the presence of 4.5% NaCl, similarly Kucuk *et al.* (2006) reported that some *Rhizobial* isolates grown under 4.5% NaCl. Our study, therefore reported the isolation of strains highly tolerant to high salt concentrations. Salt tolerant rhizobia may have the potential to improve yield of legumes under salinity stress (El-Mokadem *et al.*, 1991).

Results also showed that most of the isolates are acid adapted, capable of surviving at pH values lower than the pH range between 4.5 and 9.5, as reported for the genus *Rhizobium* by Jordan (1984). The fact that different strains of the same species may vary widely in their pH tolerance has been demonstrated previously (Glenn and Dilworth, 1994; Correa and Barneix, 1997). Some Rhizobial isolates can be shown more sensitive to low pH than their host and this affects the establishment of the symbiosis, limiting the survival and persistence of the rhizobia (Zahran, 1999).

The performance of some clover-*Rhizobium* symbiosis under acidic conditions is best when the Rhizobial strains were isolated from acidic soils (Zahran, 1999). Therefore, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic conditions may help the establishment of the symbiosis and also may improve the acid tolerance of legume. In our study 50% of the isolates grow at pH 4.0 which is lower than the pH tolerated by clover rhizobia. Such low tolerance has been previously reported for some strains of rhizobia that nodulates arrow leaf clover, which can survive and even increase in numbers at pH 4.2 (Weaver *et al*., 1985).

The ability of isolates to utilize a broad range of carbon substrates is also related to the survival of these isolates under acidic environments. Rhizobia are capable of metabolizing different carbon sources so that the products ameliorate the environmental pH (Glenn and Dilwarth, 1994). Under acidic conditions the catabolism of organic acids and amino acids leads to alkalinisation (Ibekew *et al*., 1997) and this buffering action may help in the establishment of the legume in acidic soils. This feature can make these strains more competent in acidic soils, thus it is important to couple the results of our investigation with the

selection of breeds or varieties of acid tolerant barseem clover, in order to establish and maintain symbiosis in soil with low pH.

Almost 90% of our isolates were able to grow at 36°C and 58% were survived at 38°C. None of the isolates showed growth above 38°C. According to Jordan, (1984), the maximum temperature reported for *R. leguminosarum* bv *trifolii* is 30°C, However, temperature range is highly strain dependent for genus *Rhizobium* (Jordan, 1984). Other studies with clover rhizobia have demonstrated that certain strains of *R. leguminosarum* bv *trifolii* are able to grow at various temperatures in artificial cultures, with growth response up to 41°C (Giddens *et al*., 1982). Nevertheless, survival under higher temperatures does not mean efficiency in nitrogen fixation. Rhizobial strains obtained from hot and dry environments that grew up to 45°C lost their ineffectiveness. Screening of *R. leguminosarum* bv *phaseoli* showed that some strains were able to nodulated *Phaseolus vulgaris* at high temperature (35°C and 38°C) but nodules formed at higher temperatures were ineffective and plants did not accumulate nitrogen in shoots (Zahran, 1999). Although critical temperatures for efficiency in nitrogen fixation for clovers have been reported to be around 30°C, this winter annual clover is exposed to temperatures in the range of 10°C up to 36°C. Despite the fact that high soil temperatures usually results in the formation of ineffective nodules, several strains of rhizobia have been reported to be heat tolerant and to form effective symbiosis with their host legumes. The selection of heat resistant isolates may be relevant for cultivation of ball clover, which is sown during late fall when temperatures can reach 36°C.

The presence of the strains growing under stressed laboratory conditions in our study indicates their significance in contributing biologically fixed nitrogen to stressful ecosystems. This shows the possibility of screening tolerant strains from the soil where they are naturally selected (O'Hara *et al*., 2002). The presence of tolerant strains becomes more interesting since the selected strains are good for nodulation and plant growth. This shows the possibility of getting effective stress tolerant strains through rigorous screening and characterization to exploit biological nitrogen fixation in low-input agricultural systems.

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