CHARACTERIZATION OF MESORHIZOBIUM SP. ISOLATED FROM ROOT NODULES OF CICER ARIETINUM

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ABSTRACT

This study was carried out to investigate the effect of salt, pH, antibiotics, carbohydrate and fungicides on growth of *Mesorhizobium* isolates obtained from root nodules of chick pea plants from different sites of Dehradun. After morphological and biochemical test, 40 isolates were selected. Isolates were subjected to different (2%, 3%........up to 8%) NaCl concentrations and found that all isolates grew up to 4% NaCl concentration, variation starts from 5% NaCl and salt tolerance ability was reduced with increasing salt concentration. All isolates grew at pH 4.0 to pH 8.5 and none of the isolates at pH 9.0. Ten carbohydrates were used for describing carbohydrate utilization pattern. Most of the isolates catabolize nine carbohydrates while only 47.5% showed resistance to dextrose. All of the isolates were showed sensitivity to Meropenem, Netillin, Ceftriaxone and Amikacin antibiotics. All isolates were tested for their tolerance to four fungicides. The effect of the fungicides on the isolates was variable, depending on the fungicide and isolate. Further studies are needed to study the genes involved in salt, pH, carbohydrates, fungicides, antibiotic resistance and the relationship between these genes and the symbiotic genes.

KEYWORDS: Antibiotics, Carbohydrate, Chick pea, Fungicides and Mesorhizobium.

INTRODUCTION

The earth’s population grows annually by 1.4% and is expected to double in the next fifty years. This increase in population necessitates a simultaneous enhance in food production to maintain the dietary intake of the growing human population in an environmentally sustainable manner. This demand for higher crop production also implies a higher demand for fixed nitrogen (Graham & Vance,2000). Chemically produced nitrogen fertilizers can provide this nitrogen, but they are expensive to produce in addition to being harmful to the environment. This damage to the environment includes changes in the global nitrogen cycle, loss of nitrous oxides to the atmosphere, acid rain, nitrate pollution of ground water & induced leaching of soil nutrients. An inexpensive & environmentally friendly alternative to nitrogen fertilizer is biological nitrogen fixation (BNF), which is a process where by nitrogen gas in the atmosphere is converted into biologically useful & utilizable source of nitrogen for plants. The majority of the world’s land based biological nitrogen fixation can be accounted for by the symbiotic nitrogen fixation relationship between leguminous plants & rhizobia. The advantages of this type of BNF have led to numerous studies investigating the diversity & identity of the associated bacterial symbionts.
Although symbiotic nitrogen fixation by legumes is generally the dominant source of nitrogen input in soil for imparting fertility but soil stresses, pose a severe yield constraint in obtaining plant growth and development (Lawson et al., 1995). Environmental stresses adversely affect symbiotic nitrogen fixation in both tropical and temperate soils (Graham, 1981). Limiting rhizobial survival and persistence in soils and reduce nodulation. Typical environmental stress faced by the legume nodules & the symbiotic partners may include water stress, salinity, soil pH, temperature, heavy metals, fungicides etc (Kucuk & Kinvanc, 2008).

This study determines the diversity of the *Mesorhizobium* that nodulates Chick pea through their growth characteristics, salt and ph tolerance, intrinsic antibiotic resistance, carbon source utilization and fungicide resistance pattern. This could lead to an improvement in the selection of isolates adapted to the climatic conditions.

**MATERIALS AND METHODS**

**Collection of Samples**

Root nodulating bacterial strains were isolated from the root nodule of *Cicer arietinum* (chick pea) according to Vincent (1970), collected from different sites of Dehradun (U.K).

**Isolation and Characterization of Root Nodulating Bacteria**

The collected nodules were first surface-sterilized with 95% ethanol, then with 0.1% mercuric chloride and finally washed thoroughly with distilled water. strains were obtained by streaking the crushed root nodules on YEMA plates and incubated at 28±2°C. After 2 days of incubation, *Rhizobium* colonies were obtained. Further streaking, spreading and visual characterization of colony morphology helped in isolation of pure cultures. Biochemical characterization of recovered isolates were done according to Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994) All the test were carried out with 03 replicates.

**Salt and pH tolerance**

The ability of the isolated Rhizobial strain to grow in different concentration of salt was tested by streaking them on YEMA medium containing 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0% and 8.0% (wt/vol) NaCl. Differences in pH tolerance were tested in YEM agar. The pH was adjusted to 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, 8.5 and 9.0 with HCl or NaOH. All the plates were incubated at 28°C during 72 hours and plates containing basal YEMA medium were used as controls.

Strains were considered salt tolerant and resistant to acidity when growth was similar to the growth in the control plates.

**Antibiotic Resistance Pattern of Isolates**

All the isolates were tested for antibiotic sensitivity by Kirby-bauer disc diffusion method (Bauer et al.; 1966) on YEMA. Cultures were inoculated by swabbing with standard inoculums (corresponding 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 disc using sterile forceps. The plates were incubated at 30°C for 48 hours, sensitive to an antibiotic was detected by zone of inhibition around the disc. The following antibiotic discs were used, Cefalexin(CN30), Meropenem(MRP10), Polymixin-B(PB50), Netillin(NET30), Amikacin(AK30), Ceftriaxone(CTR30), Clindamycin(CD2), Streptomycin(S25), Amoxycillin(AMX25), Ampicillin(AMP25).

**Determination of Carbohydrate Utilization**

To determine the capability of isolates to use carbohydrates (Mannitol, Dextrose, Maltose, Galactose, Mannose, Sucrose, Raffinose, Arabinose, Sorbitol, Lactose) fresh culture was spread on YEMA plates. The agar surface was allowed to dry for 3-5 minutes before applying the carbohydrate disc using sterile forceps. The plates were incubated at 30°C for 48 hours. Carbohydrate resistance was detected by zone of inhibition around the disc.

**Determination of Fungicide Tolerance**

The effect of fungicides on the growth of strains on YEMA medium were tested as described by Bollich et al., (1985) using measurement of diameters of the growth inhibition zones. The fungicides used were:- Thiram (75%), Mancozeb (75%), Zineb (75%), Carbendazim (50%). The concentrations used were 25, 50, 75 and 100 g/l. The cultures were spread over the entire agar surface four disc of filter paper from different concentrations were applied using sterile forceps. The plates were incubated at 30°C. The diameters of the inhibited growth zones formed were measured after 24 hours. A plate with filter paper disc without any fungicide was used as control.

**RESULTS**

**Isolation and Characterization of Mesorhizobium Sp.**

Bacterial isolates were recovered from chick pea root nodules collected from different sites of Dehradun. All isolates showed colonies similar in appearance with large mucoid (LM) and large watery (LW) with diameter of 2-4.5 mm, round, white colored till 3-4 days of growth and turning yellowish in color after 4 days. All isolates were gram negative rods (Table 1) Selected isolates were catalase positive as confirmed by the liberation of oxygen around the bacterial colonies. Positive results for oxidase and urease were also observed by different isolates. None of the strain showed growth on medium containing methylene blue (0.1%), gentian violet (0.1%), glucose peptone agar and citrate agar. All isolates showed growth on lactose peptone agar. Positive results were obtained from starch hydrolysis assay, when the inoculated plates were subjected to iodine test, which was indicated by a clear zone around the colonies. All the isolates were gelatinase negative as there was no clearing zone formed around the colonies. On the basis of these tests forty isolates were designated as BCR1-BCR14, ECR1-ECR4, TCR1-TCR5, SCR1-SCR8, DCR1-DCR5, MCR1-MCR2 and HCR1-HCR2.(Table 2.0).
Effects of Salt and pH on Different *Mesorhizobium* Isolates

Tolerance of rhizobial isolates to NaCl concentration showed variation among strains. All isolates showed growth at YEMA containing 2%, 3% and 4% of NaCl isolate. At higher concentrations, number of tolerant strains decreased rapidly and only eight isolates i.e. BCR2, ECR2, SCR3, SCR5, SCR6, DCR3, DCR4, & DCR5 tolerated NaCl concentrations of 5%, 6%, 7%, & 8% (Figure 1)

Differences in pH tolerance are shown in Figure 2. All isolates except SCR5 grew at pH 4.0 to 8.5. All were found to resist at pH 9.0.

Antibiotic Resistance Pattern of Recovered Isolates

All isolates were sensitive to Meropenem, Netillin, Amikacin, and Ceftriaxone. Resistance to Ampicillin, Streptomycin, Amoxycillin, Clindamycin, Polymyxin-B, and Cefalexin was recorded for only 45%, 5%, 57.5%, 25%, 10% and 12.5% of the isolates, respectively. Isolates BCR13, BCR14, SCR4, SCR5, SCR6, SCR7, DCR3, DCR4, MCR1, and HCR1 were found to be tolerant to almost all antibiotics. Maximum resistance were observed with Amoxycillin and Ampicillin, the most potent antibiotics that allow the growth of only few isolates (Figure 3).

Carbohydrate Utilization Pattern of *Mesorhizobium* Isolates

Almost all of the isolates were able to catabolize a large variety of carbon substrates (Tables 4.7). All tested strains grew on Mannitol, Lactose, Sucrose, Sorbitol, Arabinose, Galactose, Mannose, Maltose, and Raffinose except Dextrose. Only 47.5% isolates utilized Dextrose. All isolates utilized Sorbitol except TCR2 & DCR7 and only DCR7 was also unable to utilized Mannose. 97.5% of the isolates utilized Raffinose and Mannitol (Figure 4).

Effects of Different Concentration of Fungicides on *Mesorhizobium* Isolates

The results presented in Figures 5-8 shown the effect of four fungicides namely Mancozeb, Zineb, Thiram & Carbendazim. All parameters depends on the tested fungicide and applied concentrations. Almost 98% isolates were found to be sensitive against Mancozeb, Thiram and Zineb. SCR4, SCR2, and MCR1 were found to be resistant at 25, 50, 75, concentrations, respectively against Mancozeb. DCR5 and MCR1 were resistant to all concentrations of Zineb. At lower concentration (25mg/l) BCR11, TCR5, SCR1, SCR6, SCR8 were showed no zones against Thiram. Most of the isolates showed resistance behavior while using Carbendazim. Twenty three isolates i.e. BCR1, BCR2, BCR7, BCR9, BCR12, BCR13, BCR14, ECR1, TCR1, TCR2, TCR3, TCR5, SCR1, SCR2, SCR3, SCR4, SCR6, SCR7, SCR8, DCR3, DCR4, HCR1 and HCR2 showed resistance to all tested concentrations used in present study.

DISCUSSIONS

The root nodule bacteria were isolated from *Cicer arietinum* (chick pea) plants growing in different regions of Dehradun. Microscopic examination showed that the isolates were gram negative rods. Biochemical characterization revealed that the selected isolates were oxidase, catalase and urease
positive. Isolates were able to utilize citrate, which complements with the findings of Lupwayi and Hague (1994). None of the isolate showed growth on medium containing dyes i.e methylene blue and gentian violet at 1% concentration, which correlates with the earlier studies by Wei et al. (2003), who indicated that *Rhizobial* cells were unable to grow in the presence of these two dyes. All the isolates were able to grow on lactose peptone agar and growth was absent on glucose peptone agar. The isolates were not producing gelatinase enzyme and it is shown by Hunter (2007) that negative gelatinase activity is a feature of *Rhizobium*. Positive results were obtained when the isolates were subjected to medium containing starch. De Oliveria et al (2007) also observed that *Rhizobium* strains obtained from different sources can utilize starch.

Growth of the rhizobial strains were not much affected as the concentration of the salt increases from 2%-4%. High concentration of NaCl solution can give high competitive value in the rhizosphere to survive and nodulate the host plants in harsh environmental conditions particularly at high concentrations of salt in the soil. This finding is in line with the report of Saraf and Dhandhukia (2005), who found that *Sinorhizobium meliloti* growth was not completely inhibited by 4% of NaCl concentration. Rabie and Alamadini (2005) also stated that the growth of *rhizobium* was not affected by low and moderate levels of salinity. *Rhizobium* has been reported to grow the best at neutral pH i.e. 7.0. All the isolates grown very well on pH 4.0-8.5. No growth was observed in pH 9.0. Our results were in agreement with previous studies (Kucuk et al, 2006; Baoling et al, 2007).

The results on the resistance patterns of the isolates to ten antibiotics reported high level of resistance against Ampicillin, Amoxycilline, Clindamycine and Streptomycin and least with Meropenem, Netillin, Amikacine, and Ceftriaxone. Several studies (Maâtallah et al., 2002; Küçük and Kıvanç, 2008) also observed great variation among chickpea rhizobia with respect to their intrinsic antibiotics resistance pattern (IAR). 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 differences in antibiotic resistance pattern, this technique could be successfully employed in ecological studies particularly in the recovery and enumeration of rhizobia introduced into soil.

It has been a well-established fact that *Rhizobium* can utilize a wide variety of carbon sources for growth, an effective tool to characterize the isolates. Results of the present study ion utilization of different C sources showed that the strains isolated from Chickpea nodules were able to utilize Mannitol, Lactose, Sucrose, Sorbitol, Arabinose, Galactose, Mannose, Maltose, and Raffinose as carbon sources. Similar results were also recorded by Sadowsky et al., 1983 and Stowers, 1985 in case of the *Rhizobium* strains. Fast-growing rhizobia were able to grow on a large variety of carbon substrates whereas slow-growing rhizobia were more limited in their ability to use diverse carbon sources. However, our result shows that the majority of tested slow-growing chickpea rhizobia were able to use a broad range of carbohydrates. This is in line with the result of other studies (Matalah et al., 2002; L’taief et al., 2007 Mulissa Jida and Fassil Assefa., 2012). It is very interesting to notice that the types of carbohydrates utilized also varied among chickpea rhizobia. Such characteristics are usually used as diagnostic features for root nodule bacteria (Küçük and Kıvanç, 2008; Hungria et al., 2001).
Nitrogen fixation by root nodulating isolate of *Rhizobium* contributes a significant input into the many types of farming systems. For instance, even 80% of total N in pasture legume plants can be supplied by root nodule bacteria (Goring and Laskowski, 1982). Fungicides applied as seed dressings protect seeds against fungal pathogens and pests (Martyniuk *et al*., 1999b). In the case of leguminous plants, treatment of seeds with *Rhizobium* inoculants is also very important (Martyniuk *et al*., 2002). When *Rhizobium* bacteria are inoculated on chemically treated seeds of crop, their survival and capacity to induce symbiosis can be markedly reduced due to possible toxic effects of fungicides on these bacteria (Rennie and Dubetz, 1984).

Therefore, the effect of fungicides on legume nitrogen fixation is of great importance. In present study, all the isolates were tested for their tolerance to Mancozeb, Carbendazim, Thiram and Zienb fungicides. Most of the isolates showed sensitivity to Thiram and resistance to Carbendazim. Many studies have indicated differential effect of fungicides on *Rhizobium*, nodulation and nitrogen fixation (Fox *et al*., 2007; Ramadoss and Sivaprakasam 1991).

The results obtained are a part of our successful efforts to contribute to screen bacteria from root nodules which can be future candidates for increasing productivity of agriculture crops which is at decline presently. The results on screening of *Mesorhizobium* strains resistant to various antibiotics and also to determine optimum level of tolerance for fungicides and other stress conditions may also help to grow crops in highly polluted soils.

REFERENCES


**APPENDICES**

**Table 1: Morphological and biochemical characteristics of all recovered isolates from Chickpea**

<table>
<thead>
<tr>
<th>CHARACTERS</th>
<th>RESULTS</th>
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<tr>
<td>Bacterium shape</td>
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<tr>
<td>Size of colony</td>
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<td>Color</td>
<td>Whitish pink and glistering</td>
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<td>Convex</td>
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<td>Margin</td>
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<td>Oxygen demand</td>
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<td>Spore formation</td>
<td>Non spore forming</td>
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<td>1% Gentian violet treatment</td>
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<td>Starch hydrolysis</td>
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<td>Unease test</td>
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Table 2 Mesorhizobium isolates obtained from different sites of Dehradun.

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<th>PLACE →</th>
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<th>SURVEY</th>
<th>DHARAPUR</th>
<th>MANDU</th>
<th>HATHIHATHI</th>
<th>No. Of</th>
</tr>
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<td>ESTATE</td>
<td>WALA</td>
<td>BARKAL</td>
<td>ISOLATES</td>
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<td>CHICK</td>
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<td>TCR 1-5</td>
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<td>8</td>
<td>5</td>
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Figure 1 Effects of salt concentrations on growth of Mesorhizobium sp.
Characterization of Mesorhizobium Sp. Isolated from Root Nodules of Cicer Arietinum

Figure 2 Effect of pH on growth on Mesorhizobium sp.

Figure 3 Effects of antibiotics on growth of Mesorhizobium sp.
Figure 4 Effects of carbohydrates on growth of Mesorhizobium sp.

Figure 5 Effects of mancozeb on growth of Mesorhizobium sp.
Characterization of Mesorhizobium Sp. Isolated from Root Nodules of Cicer Arietinum

Figure 6: Effects of zineb on growth of Mesorhizobium sp.

Figure 7: Effects of thiram on growth of Mesorhizobium sp.
Figure 8 Effects of carbendazim on growth of Mesorhizobium sp.