

Study of Stress Tolerant Forms of Rhizobia Isolated from *Trigonella foenumgraecum* in Semi Arid Region of Rajasthan



Microbiology

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ABSTRACT

*Rhizobia, a group of symbiotic nitrogen fixing bacteria plays a remarkable role in cycling of nitrogen. They are gram negative, motile rod shaped bacteria. In this study, attempts have been made to evaluate the effect of abiotic stresses (Salt, pH, Temperature, metal tolerance) on growth of Rhizobia. Ajmer being a semi arid zone of Rajasthan was selected for the study of stress tolerant forms of Rhizobia isolated from *Trigonella foenumgraecum*. Ten bacterial forms were isolated and screened for stress tolerant forms. Phenotypic and biochemical characterization of all the isolates were done followed by their plant assay test in growth pouches. Growth of isolates on yeast mannitol medium having variable range of pH (4-10) and different concentration of NaCl (0.05%-5%) were determined. Temperature tolerant isolates were also selected. 4 isolates (RTF-1, RTF-2, RTF-5, RTF-10) were found salt tolerant and 5 forms (RTF-1, RTF-2, RTF-3, RTF-9, RTF-10) were found pH tolerant and forms (RTF-1, RTF-2, RTF-3, RTF-5, RTF-7, RTF-8) were found temperature tolerant. Rhizobia with a high negative charge were found to be better protected in dry soils. Metal tolerant strains were also evaluated. It can be concluded from the study that these stress tolerant property of Rhizobia have enormous potential to be used as biofertilizers especially where the environmental constraints act as limiting factors and impose restrictions on growth of the host legumes and can also be used during forestation of degraded areas in adverse environmental conditions.*

INTRODUCTION

Rhizobia are gram negative, motile rod shaped bacteria, which belong to family Rhizobaceae. Role of Rhizobia in nitrogen fixation were first identified in root nodules of legumes in 1888 (Hirsch et al., 2001). Rhizobia are bacteria that establish symbiosis with legumes, forming root or stem nodules and fixing atmospheric nitrogen (Quatrini et al., 2002). The bacterium's enzyme system supplies a constant source of reduced nitrogen to the plant and this bacterial symbiosis with leguminous plant reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops. (Dilworth and Parker, 1969).

Fenugreek (*Trigonella foenumgraecum*), like other legumes, is a good source of dietary protein for consumption by man and animals. From ancient times Greeks used said plant as medicine, spice and cattle fodder and so it is still pronounced as Greek hay (Singh et al., 2008). Seeds of fenugreek are used in cosmetics and for medicinal purposes. Fenugreek is a good soil renovator and widely used as a green manure (Saeed and Elsheikh, 1995).

Leguminous plants growing in semi arid regions are subject to severe environmental constrain such as salinity, temperature extremes and pH. In addition, desertification causes disturbance of plant-microbe relationships, which are a key ecological factor in helping plant growth in adversely affected ecosystems (Requena et al., 2001). Surface properties of rhizobial cells are also important for adhesion to clay particles that has been considered to play important role in existence of bacterial cell in dry condition. (Gannon et al., 1991) As nutrient deficient soil of semi arid region is found to be contaminated by metal thus, heavy metal tolerant bacteria play a role in increasing nitrogen content of metal contaminated soil. Selection of effective, efficient and compatible stress tolerant rhizobial strains could help in ecological rehabilitation of degraded soils and an increase in soil fertility thereby improving the growth of associated plants of this region.

MATERIALS AND METHODS

Experimental site

Rajasthan state falls under arid and semiarid area, of which Ajmer and its adjoining area were selected to be the experimental site. Mean annual rainfall of the district is 453.2 mm. The annual rainfall is below 500mm, showing a semi-arid climate (Khan, 1999). The average maximum temperature of Ajmer district is 46.0 degrees Celsius. (Farook et al., 2009).

Collection of root nodules and isolation of Rhizobia

Isolation of rhizobia from root nodules of *Trigonella foenumgraecum* was done by the method of Somasegaran and Hoben (1985).

From each sample, two-three nodules were picked up and washed thoroughly with sterile distilled water. After washing, nodules were surface sterilized in 95% alcohol for 30-40 s to remove wax coating if any and subsequently immersed in 4% sodium hypochlorite for 3-4 min. Then nodules were immediately washed 5-6 times with sterile distilled water to remove traces of sodium hypochlorite. The surface-sterilized nodules were transferred to sterile tubes containing 100 µl sterile distilled water. Nodules were crushed with the help of sterile glass rod and then one loopful of each nodule suspension was streaked on to sterilized plates of yeast extract mannitol agar (YEMA). The pH of the medium was adjusted to 7.0 with 1N NaOH and HCl before autoclaving at 121°C for 15 minutes. Then, the streaked plates were incubated at 28°C in an inverted position for 4 to 10 days until colonies appeared along the lines of spreading. All the rhizobial isolates were subjected to their morphological, cultural and biochemical characterization ((Lupwayi and Haque, 1994). Furthermore, all the isolates were subjected to authentication test before performing any experiment

Stress tolerance studies

Tubes of YEM (Yeast Extract Mannitol) broth having either variable concentration (0.01-4.5%) of salt (sodium chloride) or variable range of pH (4.0-10.0) were used. These tubes were inoculated with pure rhizobial culture suspensions and incubated at 28±1°C. There after growth was measured as optical density (OD) at 540 nm using spectrophotometer. The pure bacterial isolates were also studied for temperature stress using YEMA agar using different temperatures (15°C - 60°C).

Heavy metal tolerance

The isolated bacterial strains were tested for their resistance to heavy metals by agar dilution method (Washington and Sutter, 1980). Freshly prepared agar plates were amended with various soluble heavy metal salts namely Co, Zn, Hg, Fe, Mo, Mn, Cd, Ni and Al at various concentrations ranging from 25 to 250 µg mL⁻¹ were inoculated with log phase cultures. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at room temperature for 24-72 hours.

Relative cell surface charge

Relative cell surface charge was determined in terms of the adsorption of positively charged methylene blue cations onto the negatively charged sites on rhizobium cell surface by using the calorimetric method using methylene blue solution described by Khokhar et al., 2001

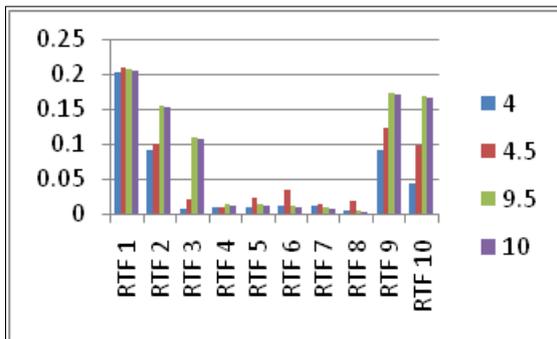
RESULTS AND DISCUSSIONS

Isolation and authentication of rhizobia

A good number of isolates were obtained from root nodules of *Trigonella foenumgraecum*. A total of 10 isolates were confirmed as Rhizobia after the authentication test in growth pouch and pot experiment using sterile sandy soil under controlled environmental conditions.

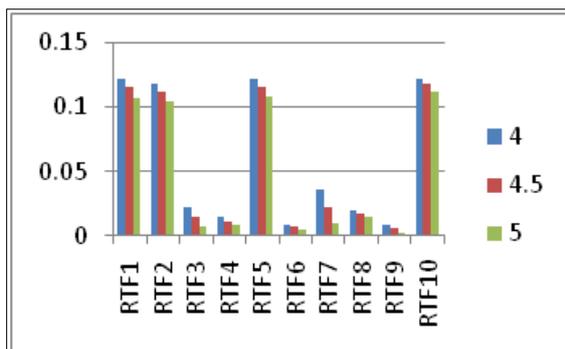
Salt tolerance

Tolerance to NaCl stress not only depicts bacterial ability to tolerate the stress but also the ability to respond and adapt to the environmental changes. Similar to previous studies of EAE, 1989 and Zerhari, 2000 four isolates (RTF-1,RTF-2,RTF-5,RTF-10) were found salt tolerant, showed growth even at 5% salt concentration as shown in Figure 1. Figure-1. Comparison of salt (NaCl) tolerance of rhizobial isolates In previous research work done by Tu 1981; Le Rudulier and Bouillard 1983; Bernard et al., 1986 reported that the different strains showed different level of tolerance towards salt stress as in the present study. Acid producing, fast growing rhizobium were more tolerant than the slow-growing, alkali-producing strains similar findings were also reported by Graham and Parker in 1964 . As concentration of salt increases growth of rhizobium decreases. Increasing salt concentrations cause detrimental effect on rhizobium strains due to osmotic stress(Nagaes et al., 2002 and Thrall et al., 2008)and salinity tolerant rhizobium from *Tephrosia purpurea* from Ajmer region were also screened by Ali et al.(2009)



PH tolerance

Rhizobia appear to be varying in their growth efficiency under acidic and alkaline conditions. In the current investigations, below pH 4.0 all the isolates showed growth less than the value of OD .500. Harwani (2006) showed that a few of the rhizobial isolates from Haroti region of Rajasthan were able to grow at pH 4.5. There was considerable increase in OD values with increasing pH upto 7.0. These findings are similar to study of Ali et al.(2009) and Rodrigues et al., (2006) concluded that the pH 6.5-7.0 is the most optimum pH for the growth of rhizobium bacteria. However, inhibitory effect of elevated pH(above 7.0) and lower pH(below 5) was clearly visible on the growth of rhizobia. The result of present show that the bacterial isolates resistant to both acid and alkaline conditions as shown in Figure 2 Figure-2. Comparison of pH tolerance of rhizobialisolates (RTF-1,RTF- 2,RTF-3,RTF-9,RTF-10)and maximam growth occur at pH 6.5-7.0. One of the most important factors that affects the efficiency of symbiosis between rhizobia and plants is the pH of the soil in which they interact (Glenn & Dilworth, 1994).So, these resistant forms can be used to increase growth of host plant.



Temperature tolerance

Majority of the rhizobium isolates exhibited luxuriant growth at the temperature ranging from 25-35°C. Some earlier workers also observed that optimum temperature for growth of root nodulating bacteria ranged from 25°C - 30°C(Graham,1992;Gaur, 1993; Harwani, 2006;Ali et al 2009). In this study RTF-1,RTF-2,RTF-3,RTF-5,RTF-7,RTF-8 were found temperature tolerant(Table 1). Strain adaptation to high temperature has also been reported by Hartel and Alexander , 1984 and Karanja and Wood, 1998 . Further increase in temperature led to noticeable decline in growth .Temperature range is highly strain dependent for the genus Rhizobium (Jordan, 1984). It is well stated that the growth and survival of rhizobia in soils are adversely affected by high soil temperatures (Meghvansi, 2006).

Table-1. Effect of Temperature on the growth of rhizobial isolates.

temperature	15	25	28	35	40	45	50	55	60
RTF1	+	+	++	++	++	++	++	++	++
RTF2	-	+	++	++	++	++	++	++	+
RTF3	++	++	++	++	++	++	++	++	++
RTF4	-	+	++	++	+	-	-	-	-
RTF5	+	+	++	++	+	+	+	+	+
RTF6	++	++	++	++	+	+	+	+	-
RTF7	+	+	++	++	++	++	++	++	+
RTF8	++	++	++	++	++	++	++	++	++
RTF9	+	+	+	+	-	-	-	-	-
RTF10	++	++	++	+	+	-	-	-	-

Metal tolerance

The samples of isolates showed good tolerance to heavy metals such as Mn, Zn Cd ,Co, Fe, Ni, Hg Mo ,Al , as shown in Table 2 Not a single isolate strain were grew well above the 25µg of cadmium and 25 µg of mercury. However only 2 isolate grew well on 200 µg of magnese. While only 3 strains were grew at 200 µg of molybedenum and 250µg of iron whereas 4 strains grew at 50 µg of nickel.5 isolate were grew on 200 µg of zinc and and no strains grew well above 100 µg of cobalt and 100 µg of aluminium. In a similar study, Paudyal et al. (2007) and Abdel-Salam et al 2010 determined the effect of heavy metals on the strains of Rhizobia. In the present study it has been observed that as concentration of metal increases growth of organism ceases, at higher concentration (250ug except 3 strains of iron) no organism shows growth. Similar to this numerous metals have been reported previously to inhibit the growth, morphology and activities of various groups of microorganisms (Khan and Scullion ,2002; Shi et al.,2002; ;Bondarenko et al.,2010)

Table 2: Heavy metals resistance patterns of rhizobial strains

metals	conc (in ug)	RTF1	RTF2	RTF3	RTF4	RTF5	RTF6	RTF7	RTF8	RTF9	RTF10
cadmium	25	1	1	0	1	0	1	1	1	1	1
	50	0	0	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0	0	0
	150	0	0	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0	0	0
magnese	25	1	1	1	1	1	1	1	1	1	1
	50	1	1	1	1	1	1	1	1	1	1
	100	1	1	1	1	1	1	1	1	1	1
	150	0	0	1	0	0	0	0	0	1	0
	200	0	0	1	0	0	0	0	0	1	0
mercury	25	1	0	0	1	0	1	0	1	1	0
	50	0	0	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0	0	0

	150	0	0	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0	0	0
	250	0	0	0	0	0	0	0	0	0	0
zinc	25	1	1	1	1	1	1	1	1	1	1
	50	1	1	1	1	1	1	1	1	1	1
	100	1	1	1	1	1	0	1	1	1	1
	150	1	1	1	1	1	0	1	1	1	1
	200	1	0	1	0	1	0	1	0	1	0
	250	0	0	0	0	0	0	0	0	0	0
molybede-num	25	1	1	1	1	1	1	1	1	1	1
	50	1	1	1	1	1	1	1	1	1	1
	100	1	1	1	1	1	1	1	1	1	1
	150	0	0	1	0	0	0	0	0	1	0
	200	0	0	1	0	0	1	0	0	1	0
	250	0	0	0	0	0	0	0	0	0	0
cobalt	25	1	1	1	1	1	1	1	1	1	1
	50	1	1	1	1	1	1	1	1	1	1
	100	0	1	0	1	1	1	0	1	0	1
	150	0	0	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0	0	0
	250	0	0	0	0	0	0	0	0	0	0
Iron	25	1	1	1	1	1	1	1	1	1	1
	50	1	1	1	1	1	1	1	1	1	1
	100	1	1	1	1	1	1	1	1	1	1
	150	1	1	1	1	1	1	1	1	1	1
	200	0	1	0	1	0	1	0	1	1	1
	250	0	1	0	0	0	0	0	1	0	1
nickel	25	1	1	1	1	1	1	1	1	1	1
	50	0	1	0	0	0	1	0	1	0	1
	100	0	0	0	0	0	0	0	0	0	0
	150	0	0	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0	0	0
	250	0	0	0	0	0	0	0	0	0	0
Aluminium	25	1	1	1	1	1	1	1	1	1	1

	50	1	0	1	0	1	1	1	1	1	1
	100	0	0	0	0	0	0	0	0	0	0
	150	0	0	0	0	0	0	0	0	0	0
	200	0	0	0	0	0	0	0	0	0	0
	250	0	0	0	0	0	0	0	0	0	0

0 SHOWS NO GROWTH 1 SHOWS GROWTH

Relative cell surface charge

Rhizobial cell have negative surface charge as stated by Bushby ,1990. cell surface negative charge was determined in terms of relative extent of methylene blue cations absorbed from methylene blue solution ,onto the cells. Relative absorbance was taken as inverse of cell surface negative charge (kokhar et al 2001) RTF 1 and RTF 2 contained maximam cell surface negative charge as shown in 6. As study of Kokhar and khan, 1994, cell which had negative charge were found to be more protected than cell carries positive charge in dry soils.

Conclusion

10 bacterial isolates were studied out of which different strains were found tolerant to environmental constrains like temperature, pH, salinity and metal tolerance and above discuss environmental conditions are regular feature of semi arid region .Thus, the present study can play role in improvement of agricultural fields as the symbioses between Rhizobium and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of N. Biological nitrogen fixation as a nonpolluting and more cost-effective way to improve soil fertility compared to chemical fertilizer. Thus, these stress tolerant property of Rhizobia have enormous potential to be used as biofertilizers especially where the environmental constrains act as limiting factors and impose restrictions on growth of the host legumes and can also be used during forestation of degraded areas in adverse environmental conditions.

Table 4 - Relative cell surface charge of Rhizobial isolates

strain	Relative absorbance in nm
RTF1	0.15
RTF2	0.16
RTF3	0.36
RTF4	0.42
RTF5	0.2
RTF6	0.35
RTF7	0.42
RTF8	0.19
RTF9	0.54
RTF10	0.45

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