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SELECTION OF STRESS-TOLERANT RHIZOBIAL ISOLATES OF WILD LEGUMES GROWING IN DRY REGIONS OF RAJASTHAN, INDIA

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ABSTRACT

An attempt has been made to evaluate the effect of abiotic constraints (salt, pH and temperature) on the growth of rhizobia isolated from Leucaena leucocephala, Tephrosia purpurea and Crotalaria medicaginea grown in arid and semiarid regions of Rajasthan with a view to screen out stress tolerant isolates. A total of 27 isolates have been used for screening their stress tolerating ability with contrast to environmental abiotic soil conditions commonly prevailing in arid and semi-arid regions of Rajasthan. All the isolates were phenotypically and biochemically characterized followed by their plant assay test in growth pouches and pot experiment under controlled environmental conditions. Growth of pure rhizobial isolates on Yeast Extract Mannitol (YEM) medium having variable range of pH (4.0 to 10.0) and different concentrations of NaCl (0.01-4.5 %) were recorded at 540 nm using UV-VIS spectrophotometer after incubation at 28± 2^0C for two days. Survival of rhizobial isolates under variable stress of temperature was also selected using Thermal Death Point (TDP) process. On the basis of comparison of growth under varied stress conditions, five rhizobial isolates from each salt and pH stress study (salt tolerant: ALL-1, ALL-4, ALL-5, BLL-1 and BLL-2; pH tolerant: ATP-2, ATP-3, ATP-4, ATP-9 and ACM-2), where as seven root nodulating bacteria from temperature tolerant: ALL-1, ALL-2, BLL-1, BLL-7, ATP-2 ATP-3, and ATP- 9) were screened out. The stress tolerant traits of these rhizobia are of potential value from the point of view of biofertilization of legume seedlings during a forestation of degraded areas in arid and semi-arid tropics of Rajasthan.

Keywords: legume, rhizobia, temperature, pH, salt, stress tolerant.

INTRODUCTION

Rhizobia are genetically diverse and physiologically heterogeneous group of symbiotic nitrogen fixing bacteria that form nodules on the roots or rarely on the stem of legume hosts, within which the bacteria fix atmospheric nitrogen into ammonia. A fully functional symbiosis requires successful survival ability of bacteria even under adverse environmental conditions. Within the soil, rhizobia frequently encounter various stresses that affect their growth, their initial steps of symbiosis and the capability of nitrogen fixation (Zahran, 1999). The wild (naturally-growing) leguminous plants living in arid and semi arid regions are subject to severe environmental conditions. In addition, desertification causes disturbance of plant-microbe symbioses, which are a critical ecological factor in helping further plant growth in degraded ecosystems (Requena *et al.,* 2001). Among several environmental conditions, which are limiting factor such as salinity, temperature extremes and pH stress are probably the most problematic. A competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation as the limiting factors (e.g. salinity, unfavourable soil pH, temperature extremes, nutrient deficiency etc.) impose limitations on the vigour of the host legume (Brockwell *et al.,* 1995; Thies *et al.,* 1995). Inoculation of stress tolerant strains of rhizobia may enhance the nodulation and nitrogen fixation ability of plants under stress conditions. The ability of legume hosts to grow and survive in saline conditions is improved when they are inoculated with salt tolerant strains of rhizobia (Zou *et al.*, 1995; Hashem *et al* 1998; Shamseldin and Werner, 2005). Rhizobial populations vary in their tolerance to major environmental factors (Ulrich and Zaspel, 2000; Mahobia and Mahna, 2002; Sridhar *et al.*, 2005; Wei *et al.*, 2008; Biswas *et al.*, 2008).

About 60% of the Rajasthan state falls within the desert region (34°35'N to 30°10'N latitude and 69°31'E to76°55'E longitude) and covers the largest area (61%) of hot arid regions of the Indian Desert (DST, 1994). The natural rhizobia of wild legumes growing in arid zones exhibit higher tolerance to prevailing adverse conditions like salt stress, elevated temperature and drought. Selection of effective, efficient and compatible stress tolerant rhizobial strains could help in ecological rehabilitation of degraded soils and increases soil fertility thereby improving the growth of associated plants of this region. However, only few studies on this aspect have been carried out in dry regions of Rajasthan (Singh, 1998; Mahobia and Mahna, 2002; Mahobia, 2003). Considering these, in the present study, rhizobia from wild leguminous plants were isolated and their in-vitro physiological stress tolerance (salt, pH and temperature) ability was evaluated.

MATERIALS AND METHODS

Experimental site

Two regions (A-Ajmer and B-Bikaner) of Rajasthan were selected representing the typical semi-arid and arid zone, respectively with sparse vegetation.

Region A: Ajmer

Ajmer district is located in the centre of Rajasthan state between 25° 38' and 26°58' north latitudes and 73° 54' and 75° 22' east longitudes. The average maximum temperature recorded is 46.0 degrees Celsius. The normal annual rainfall is 60.18cm. Ajmer district

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occupies an area of 8480 km^2, and is located between 25°38': 26°58' north latitude and 73°54': 75°22' east longitude. The annual rainfall is below 500mm, showing a semi-arid climate (Khan, 1999). The northwestern part is covered with sand dunes and rest of the area is generally flat. Hydrogeologically, the major part of the region is occupied by crystalline rocks comprising of calc-schist, amphibolite/calc-gneiss and biotite schist (all Precambrian); sand and alluvium of younger age are other important formations (GSI, 1977; Srivastava, 2001).

Region B: Bikaner

Bikaner district lies in the north-west of Rajasthan in heart of 'Thar' desert between the latitude 27011'03'' to 29003' north and longitude 71054' to 74012' east comprising a total geographical area of 27,244 sq km (CAZRI, 1990). Climate of the district ranges from arid in the east to extremely arid in the west. The mean rainfall of the district is 247mm varying from 300mm in the east to 180mm in the west. The annual potential evapo-transpiration is 1770mm (Gheesa L., 1999). The mean maximum temperature ranges from 24.4 to 43.80°C and mean minimum for 7.3 to 31.0°C. Frequent droughts once in 2.5 years is a common phenomenon. Soils of this district are predominately light textured, weak structured and well drained. All the soils are calcareous, amount of calcium carbonate increases with depth merging at lower depths with lime concretionary zone particularly in the flat aggraded older alluvial plains and the flat interdunal plains (CAZRI, 1974).

Collection of root nodules and isolation of rhizobia

Root nodules of three commonly growing wild legumes namely Leucaena leucocephala, Tephrosia purpurea and Crotalaria medicaginea were collected from Ajmer and Bikaner regions and were transported to the laboratory in plastic bags along with seedlings, where bacterial strains were isolated. In the process, nodules were separated from the roots and washed in sterilized distilled water for several times. Following serial dilution agar plate technique as described by Somasegaran and Hoben (1993) using YEMA (Teast Extract Mannitol Agar) medium containing 0.0025 % Congo red dye (Vincent, 1970), bacterial isolation was carried out. After that these plates were incubated at 28±1ºC and observed daily. Bacterial colonies appeared after 2-3 days were picked up and streaked on YEMA plates. Pure cultures were obtained with one or more further sub-culturing steps. All the rhizobial isolates were subjected to their morphological, cultural and biochemical characterization (Vincent, 1970; Creager *et al.*, 1990; Cappuccino and Sherman, 1992). 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±1ºC for 48 h. Their after growth was measured as optical density (OD) at 540 nm using spectrophotometer (model- Systronics 118). The pure bacterial isolates were also studied for temperature stress by thermal death point precess using different temperatures (15ºC - 65ºC).

RESULTS AND DISCUSSIONS

Isolation and authentication of rhizobia

To begin with, a good number of isolates were obtained from root nodules of L. leucocephala, T. purpurea and C. medicaginea seedlings. A total of 27 isolates were confirmed as rhizobia after the authentication test in growth pouch and pot experiment using sterile sandy soil under controlled environmental conditions. Amongst these, 11 isolates (6 from Ajmer and 5 from Bikaner) belonged to L. leucocephala, while 10 and 6 isolates belonging to T. purpurea and C. medicaginea respectively were recovered from Ajmer. Nomenclature of the isolates was carried out representing their region of origin (first letter), parent plant (second two letters) and isolate number (numeric figure).

Salt tolerance

Tolerance to NaCl stress is a very complex phenotype that involves not only the bacterial ability to tolerate the stress but also the swiftness to respond and adapt to the environmental change. In the current study, decreased growth of rhizobial isolates with increasing salt concentration was registered. Similar to this, Nagales *et al.*, (2002) and Thrall *et al.*, (2008) were of the view that increasing salt concentrations may have a detrimental effect on rhizobial populations as a result of direct toxicity as well as through osmotic stress. At 3.5% NaCl concentration, the value of OD above 0.160 could be observed only for five isolates (ALL-1, ALL-4, ALL-5, BLL-1 and BLL-2). Somewhat similar to present findings, Hashem *et al.*, (1998) also reported three rhizobial isolates of Leucaena showing tolerance to >3% NaCl. However, at 4.5% salt concentration, at least six isolates (ALL-4, BLL-1, BLL-2, ALL-1, ALL-5 and ALL-3) could survive and OD>0.100 was observed for them (Figure-1). Isolates from herb legumes (T. purpurea and C. medicaginea) showed excellent growth on control as compared to those from tree legume (L. leucocephala). There was no distinct demarcation in salt tolerance level of rhizobial isolates belonging to two different agro-climatic conditions of Rajasthan.

PH tolerance

Rhizobia appear to be varying in their symbiotic efficiency under acidic and alkaline conditions. In the current investigation, at pH 4.0 all the isolates showed very poor growth except for six isolates (ATP-2, ATP-8, ATP-9, ACM-1, ACM-2 and ACM-6) showing value of OD above 0.050. Harwani (2006) reported that a few of the rhizobial isolates from Haroti region of Rajasthan were able to grow at pH 4.5. These findings are similar to what

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has been observed in the current study. There was considerable increase in OD values with increasing pH upto 7.0. Somewhat similar to this, Rodrigues *et al.*, (2006) quoted that the pH 6.5-7.0 is the most optimum pH for the growth of root nodulating bacteria. However, inhibitory effect of elevated pH (above 7.0) was clearly visible on the growth response of rhizobia since moderate growth was recorded for majority of the isolates (Figure-2). At pH 9.0, six isolates were able to survive as OD value recorded for them was >0.300. Maximum value (0.098) of OD at pH 10.0 was observed for ACM-2. However, OD values ≥ 0.080 could also be recorded for at least four other isolates (BLL-1, BLL-2, ATP-9, and ACM-6).

Temperature tolerance

In general, majority of the isolates exhibited luxuriant growth at the temperature ranging from 25-35^{0}C. Some previous workers also confirmed this finding by reporting that optimum temperature for growth of root nodulating bacteria ranged from 25°C - 30°C (Gaur, 1993; Harwani, 2006). However, at 15^{0}C only three isolates (ALL-1, ALL-4 and BLL-2) showed moderate growth while remaining showed scanty/no growth. Further increase in temperature led to noticeable decline in growth and at 45^{0}C, most of the isolates demonstrated moderate growth. It is well established that the growth and survival of rhizobia in soils are adversely affected by high soil temperatures (Meghvansi, 2006). Nevertheless, in the present study two isolates namely ALL-2 and BLL-1 could show moderate growth even at 55^{0}C while remaining showed scanty/no growth (Table-1).

CONCLUSIONS

This study showed that there was considerable variability in the level of stress tolerance of rhizobial isolates obtained from wild leguminous plants native to dry regions of Rajasthan. Based upon the comparative assessment, we have screened five isolates from each salt and pH tolerant study (salt tolerant: ALL-1, ALL-4, ALL-5, BLL-1 & BLL-2; pH tolerant: ATP-2, ATP-3, ATP-4, ATP-9 and ACM-2), whereas seven isolates recovered from temperature tolerant study (ALL-1, ALL-2, BLL-1, BLL-7, ATP-2 ATP-3, and ATP- 9) which could further be utilized for their symbiotic effectiveness determination under field conditions.

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

Identity of rhizobia	Range of temperature									
	15ºC	25ºC	28ºC	35ºC	45ºC	55ºC	65ºC	Cardinal temperature (ºC)		
								Min.	Max.	Opt.
ALL - 1	++	+++	+++	+++	++	+	+	15ºC	65ºC	28ºC
ALL - 2	+	+++	+++	+++	++	++	+	15ºC	65ºC	28ºC
ALL - 3	+	++	+++	+++	++	+	-	15ºC	55ºC	28ºC
ALL - 4	++	+++	+++	+++	+	-	-	15ºC	45ºC	28ºC
ALL - 5	-	++	+++	++	+	-	-	25ºC	45ºC	28ºC
ALL - 6	-	++	+++	+++	++	+	-	25ºC	55ºC	28ºC
BLL - 1	+	+++	+++	+++	++	++	+	15ºC	65ºC	28ºC
BLL - 2	++	+++	+++	++	+	-	-	15ºC	45ºC	28ºC
BLL - 5	+	++	+++	+++	++	+	-	15ºC	55ºC	28ºC
BLL - 6	+	++	+++	+++	++	+	-	15ºC	55ºC	28ºC
BLL - 7	+	+++	+++	+++	++	+	+	15ºC	65ºC	28ºC
ATP - 1	+	++	+++	+++	+	+	-	15ºC	55ºC	28ºC
ATP - 2	+	+++	+++	+++	++	+	+	15ºC	65ºC	28ºC
ATP - 3	+	+++	+++	+++	++	+	+	15ºC	65ºC	28ºC
ATP - 4	+	++	+++	++	++	+	-	15ºC	55ºC	28ºC
ATP – 5	-	+++	+++	+++	+	-	-	25ºC	45ºC	28ºC
ATP – 6	-	++	+++	++	+	-	-	25ºC	45ºC	28ºC
ATP – 7	-	++	+++	++	+	-	-	25ºC	45ºC	28ºC
ATP – 8	-	++	+++	++	+	-	-	25ºC	45ºC	28ºC
ATP – 9	+	+++	+++	+++	++	+	+	15ºC	65ºC	28ºC
ATP - 10	+	++	+++	+++	+	-	-	15ºC	45ºC	28ºC
ACM - 1	+	++	+++	+++	++	-	-	15ºC	45ºC	28ºC
ACM – 2	+	++	+++	++	++	+	+	15ºC	65ºC	28ºC
ACM – 3	-	+	++	++	+	+	-	25ºC	55ºC	28ºC
ACM – 4	-	++	+++	++	+	-	-	25ºC	45ºC	28ºC
ACM – 6	+	+++	+++	+++	++	+	-	15ºC	55ºC	28ºC
ACM – 7	+	++	+++	++	+	+	-	15ºC	55ºC	28ºC

(+++; luxuriant growth, ++; moderate growth, +; Scanty growth)
Growth recorded by Thermal Death Point (TDP) Procedure
* Data indicate mean value of three replicates.

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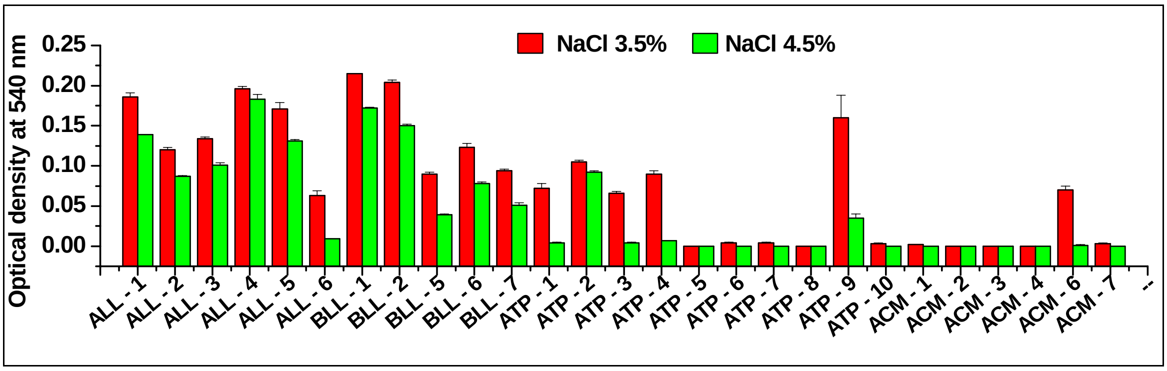

Figure-1. Comparison of salt (NaCl) tolerance of rhizobial isolates. Error bars are SEM.

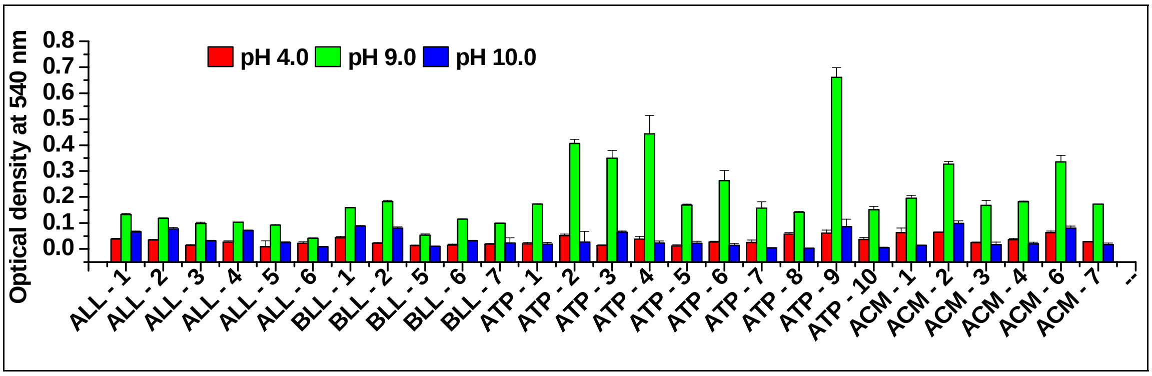

Figure-2. Comparison of pH tolerance of rhizobial isolates. Error bars are SEM.