Survival and Efficiency of Temperature Tolerant and Temperature Sensitive Strains of *Rhizobium* sp. (*Acacia*) under Temperature Stress Conditions

N. Rustagi, J.S. Duhan and P.K. Sharma
Department of Microbiology, CCS Haryana Agricultural University
Hisar-125 004

ABSTRACT

Out of 100 isolates of *Rhizobium* spp. nodulating *A. nilotica* two temperature tolerant *Rhizobium* spp. (*Acacia*) strain AC-2 and AC-6 with ability to grow at 45° C and 40° C respectively, were compared with a temperature sensitive *Rhizobium* sp. (*Acacia*) strain AC-11 for their survival, nodulation and nitrogen fixation (nitrogenase activity) at elevated temperature. Survival of the temperature tolerant strains AC-2 and AC-6 was better at 40° C and 45° C than the temperature sensitive strain AC-11 in sterilized soil. Nitrogenase activity in nodules formed by AC-2 and AC-6 on *A. nilotica* was more at 45° C and 40° C in comparison to nitrogenase activity in the nodules formed by AC-11. Seedlings inoculated with strains AC-2 showed 7-13% reduction in shoot dry weight up to 45° C whereas 34-36% reduction in shoot dry weight was observed with AC-6. Inoculation with temperature sensitive strain AC-11 also showed the similar trend (33-40%) as that of AC-6 strain.

Key words : *Acacia nilotica, nitrogenase, nodulation, Rhizobium sp., temperature stress.*

1. INTRODUCTION

Nodulation of a legume under field conditions depend upon the presence of sufficient number of the appropriate rhizobia, their multiplication, colonization of root zones and establishment of effective symbiosis with the host (Danso et al., 1992). Temperature and moisture are important factors affecting rhizobial growth, survival in the soil and the symbiosis itself (Day et al., 1978, Munever and Wollum, 1981). In arid zones, temperature reaches up to 45° - 50° C in top soil for short duration in summer (Alexander, 1982; Chatel and Parker, 1973). Often inoculation with effective *Rhizobium* under such conditions is not sufficient to support plant growth (Bala et al., 1990). It is mainly due to poor survival of *Rhizobium* under stress conditions such as non-availability of nutrients, high temperature and low moisture (Gopal Krishanan and Dudeja, 1999; Kishnevsky and Weaver, 1992; Munever and Wollum, 1981).

Acacia nilotica, a deep rooted drought tolerant indigenous species has been widely planted for fodder, fuel and timber in arid and semi-arid zones of India. Being nitrogen-fixing tree (NFT), it is solely dependent for its nitrogen requirement on symbiosis (Aseefa and Kleiner, 1998; Bala and Giller, 2001). Attempts have been made to improve the nitrogen fixing ability of *Rhizobium* – *Acacia* symbiosis by selecting better nodulating hosts (Beniwal et al., 1995; Badhwar et al., 2002; Toky et al., 1995) and *Rhizobium* with wide host range and better survival under stress conditions (Bala et al., 1990; Hungria et al., 1993; Sharma et al., 2003). Temperature tolerant strains of *Rhizobium* have been isolated from *Leucaena, Lonchocarpus* and *Gliricidia* and have been found to improve biological nitrogen-fixation in cultivated legumes and tree legumes (Hungria et al., 1993; Zarhari et al., 2000; Rustagi et al., 1996).

In present investigation temperature tolerant strains *Rhizobium* sp. (*Acacia*) isolated from *Acacia* were compared with temperature sensitive strain of *Rhizobium* sp. (*Acacia*) for survival and nitrogen fixing efficiency.

2. MATERIALS AND METHODS

2.1 Isolation and Authentification of *Rhizobium* spp. (*Acacia*)

Nodules from *A. nilotica* seedlings (6-12 months old) were collected after rainy season. Rhizobial isolation was made from surface sterilized nodules as described by Vincent (1970). All the isolates were able to nodulate *A. nilotica* under sterilized conditions. Majority of *Rhizobium* sp. (*Acacia*) were fast growing and they were tested for their ability to grow at 40° C and 45° C in yeast extract mannitol (YEM) broth and YEM agar plates. Three strains of *Rhizobium* sp. (*Acacia*) AC-2, AC-6 and AC-11 with the ability to grow at 45° C, 40° C and 30° C, respectively were used in the present study (Rustagi et al., 1996). These strains were maintained on yeast extract mannitol agar (Vincent, 1970).

2.2 Survival of *Rhizobium* sp. (*Acacia*) in Soil at Elevated Temperature

Survival of rhizobia was studied in sterilized sandy loam soil having pH 7.6, organic C 0.42, total N 0.65%, P (Olsen) 10 ppm, electric conductivity 0.29 dSm^{-1}. *Rhizobium* sp. (*Acacia*) AC-2, AC-6 and AC-11 were grown in YEM broth at 30° C for 72 h under constant shaking conditions (100 revolutions min^{-1}). Cultures (109

Table 1. Survival of *Rhizobium* sp. (*Acacia*) strains AC-2, AC-6, AC-11 at elevated temperatures in sterilized soil

Strain	Temperature (°C)	Number of rhizobia g^{-1} soil						
		Time (days)						
		0	7	14	21	28	35	CD at 5%
AC-2	30	35 x106	43 x106	49 x106	55 x106	58 x106	62 x106	2.1
AC-2	40	32 x106	35 x106	40 x106	44 x106	46 x106	49 x106	1.8
AC-2	45	32 x106	29 x106	22 x106	14 x106	10 x106	6 x106	1.5
AC-6	30	30 x106	32 x106	35 x106	40 x106	43 x106	49 x106	2.2
AC-6	40	35 x106	40 x106	41 x106	39 x106	35 x106	35 x106	2.6
AC-6	45	32 x106	20 x106	3 x106	1 x106	59 x106	6 x104	1.5
AC-11	30	35 x106	35 x106	40 x106	42 x106	43 x106	47 x106	1.8
AC-11	40	48 x106	1 x106	3 x105	1 x105	2 x104	1 x104	2.2
AC-11	45	44 x106	1 x106	1 x105	1 x104	1 x103	1.6x102	1.5

cells ml^{-1}) were mixed to sterilized or unsterilized soil to give an initial population in the range of 32-48x106 rhizobia g^{-1} soil. The inoculated soil was dispensed into small disposable paper cups (100 g capacity) and incubated at 30° C, 40° C and 45° C for 7 to 35 days in BOD incubators. Soil moisture was maintained in these cups to field capacity throughout the study period. Three samples from each temperature treatment for each *Rhizobium* strain were collected after 7, 14, 21, 28 and 35 days and estimated for total rhizobial population by standard plate count method using YEMA medium.

2.3 Acetylene Reduction Activity in the Nodules

Nitrogenase activity of nodules of *A. nilotica* formed by different strains of *Rhizobium* sp. (*Acacia*) was determined by acetylene reduction assay (Hardy et al., 1968). Seeds of *A. nilotica* were surface sterilized using 0.2% mercuric chloride for 3 minutes, washed 6-7 times with sterilized distilled water and inoculated with *Rhizobium* sp. (*Acacia*) strain AC-2, AC-6 and AC-11 (one ml culture containing 108 cells ml^{-1} for 5 g seeds). Before surface sterilization, the seeds were given hot water treatment (Beniwal et al., 1995). Five seeds were sown into "Chillum jars" and after germination three plants were kept in each jar (Dahiya and Khurana, 1981). The jars were irrigated with Sloger's nitrogen free medium. After 45 days of sowing nodules were collected from each culture treatment. One gram of nodules were taken into tubes (150 x 50 mm) fitted with suba seal. One ml air in

Table 2. Effect of elevated temperature on acetylene reduction activity in nodules of *Acacia nilotica* formed by temperature tolerant and temperature sensitive strains of *Rhizobium* sp. (*Acacia*)

Strain	Temperature (°C)	Acetylene reduction activity* (n mols g^{-1} dry nodules)				
		Time (hr)				
		0.5	1.0	1.5	2.0	3.0
AC-2	30	2.09(100)	3.34(100)	4.56(100)	5.58(100)	8.1(100)
AC-2	40	1.68(80.5)	2.60(78.1)	3.98(87.2)	5.05(85.6)	6.21(76.0)
AC-2	45	1.57(75.1)	2.50(74.9)	3.55(77.7)	4.20(71.2)	4.80(58.7)
AC-6	30	2.14(100)	5.91(100)	8.80(100)	11.94(100)	15.80(100)
AC-6	40	2.11(98.5)	4.41(74.4)	6.00(68.1)	7.35(61.6)	9.33(59.0)
AC-6	45	1.87(87.6)	3.04(51.5)	3.42(38.8)	3.42(28.6)	3.42(21.6)
AC-11	30	2.63(100)	3.54(100)	3.96(100)	4.61(100)	5.09(100)
AC-11	40	1.15(43.7)	1.31(37.1)	1.64(41.0)	1.86(40.0)	1.86(36.5)
AC-11	45	0.99(37.9)	1.09(30.9)	1.14(28.8)	1.23(28.8)	1.23(24.3)
CD at 5%		0.16	0.16	0.28	0.30	0.23

* Figures in parenthesis indicate change in acetylene activity over control (ARA at 30° C).

Table 3. Effect of temperature stress on growth and nitrogen fixing ability of *Acacia nilotica* inoculated with temperature tolerant and temperature sensitive strain of *Rhizobium* sp. (*Acacia*)

Strain	Nodule dry weight mg/plant	Shoot dry weight mg/plant	Total N mg/plant	Nodule number per plant
Unstressed control				
AC-2	16.1	505	4.47	30
AC-6	15.6	604	5.13	38
AC-11	13.8	552	4.21	20
Control (uninoculated)	0	430	3.08	0
40° C/12h/day for 5 days				
AC -2	6.6	469	3.97	9
Ac-6	6.6	396	3.80	15
AC-11	5.2	369	3.02	12
45° C/12h/day for 5 days				
AC-2	4.4	439	4.06	10
AC-6	0.8	387	2.79	1
AC-11	1.5	329	2.47	2
CD at 5%	1.4	25.7	0.46	3.8

the tube was replaced by 1 ml acetylene and tubes were incubated at 30° C, 40° C and 45° C for 0.5, 1, 1.5, 2 and 3 h acetylene reduction was estimated by GLC (Nucon 5500) using flame ionization detector (Hardy et al., 1968).

2.4 Nodulation and Nitrogen Fixation by *A. nilotica*

Seedlings of *A. nilotica* inoculated with *Rhizobium* sp. (*Acacia*) strains AC-2, AC-6 and AC-11 were raised from surface sterilized seeds as described earlier. The "Chillum jar" containing 5 days old seedlings were shifted to growth chambers and incubated at 40° C/12 h/day or 45° C/12 h/day for 5 days and then shifted to normal growth conditions. Seedlings of *A. nilotica* inoculated with AC-2, AC-6 and AC-11 but grown at normal growth conditions served as control. After 45 days of sowing observations like nodule number, nodule dry weight, shoot dry weight of plant and total plant N were determined (Toky et al., 1995)

3. RESULTS AND DISCUSSION

3.1 Survival of *Rhizobium* sp. (*Acacia*) in Soil

Survival of *Rhizobium* sp. (*Acacia*) strain AC-2, AC-6 and AC-11 with ability to grow at 45° C, 40° C, 30° C respectively varied significantly in the sterilized soil at different temperature (Table 1). The populations of *Rhizobium* sp. (*Acacia*) strain AC-2, AC-6 and AC-11 remained unchanged (32x106 rhizobia g^{-1} soil) at 30° C. However at 40° C and 45° C, temperature tolerant strains AC-2 and AC-6 showed higher population in comparison to strain AC-11 sensitive to high temperature. The rhiozbial populations of the strains AC-2, AC-6 and AC-11 decreased from 32x106 to 6x106, 32x106 to 6x104 and 44x106 to 1x102 of rhizobia g^{-1} soil, respectively after incubation at 45° C for 35 days. At 40° C the population of AC-2 and AC-6 did not decrease. However, the population of AC-11 decreased from 48x106 to 1x104 log number of rhizobia g^{-1} soil after 35 days,

Thus, the temperature tolerant strains AC-2 and AC-6 showed better survival at elevated temperature than temperature sensitive strain AC-11. Total loss in rhizobial viability has been observed in chickpea, lentil and bean inoculants when they are exposed to ambient temperature of 44° C (Somasagaran et al., 1984). *Rhizobium* sp. (*Acacia*) strain AC-2 with the ability to grow at 45° C may have ecological advantage over temperature sensitive. *Rhizobium* sp. (*Acacia*) strain AC-11, which survive, and nodulates better at high temperature.

3.2 Nitrogenase Activity in Nodules

Nitrogenase activity (N_2ase) in nodules of *A. nilotica* formed by *Rhizobium* sp. (*Acacia*) strain AC-2, AC-6 and AC-11 decreased when nodules were incubated at high temperature (40° C or 45° C) for 0.5-3.0 h (Table 2). Considering N_2ase activity at 30° C as 100%, the decrease in N_2ase activity was 60% and 40% at 40° C and 45° C after 2 h of incubation in the nodules formed by temperature sensitive strain AC-11. The decrease in N_2ase activity was comparatively less in the nodules formed by temperature tolerant *Rhizobium* strains AC-2 and AC-6 when nodules were incubated at 40° C or 45° C for 0.5-2.0 h. Incubation of nodules formed by *Rhizobium* sp. (*Acacia*) strain AC-2, at 40° C or 45° C for 0.5 h decreased the N_2ase activity to 80.5 and 75.1%, respectively while incubation of nodules for 3 h at 40° C or 45° C N_2ase activity decreased by 76.0% and 58.7% respectively as compared to N_2ase activity at 30° C after 0.5 h. The N_2ase activity in nodule formed by strain AC-11 was moderately tolerant to high temperature and decreased to 59% and 26.6% after 3 h of incubation at 40° C and 45° C respectively.

High root temperature has been shown to strongly affect the nitrogen fixation. Critical temperature for nitrogen fixation has been reported, to be 30° C for clover and pea, 35°-40° C for guar (Arayangkoon et al., 1990) and, peanut (Kishinevsky and Weaver, 1992) and cowpea (Rainbard et al., 1990) and 28° C for soybean (Stoyanova, 1996). High temperature affects both synthesis and

functioning of nitrogenase system (Montanej et al., 1995). Temperature dependence of symbiotic N_2 fixation has also been shown to depend upon plant cultivar and nodulating strain (La Farve and Eaglesham, 1986; Munevar and Wollum, 1982 and Arayangkoon et al., 1990). Nitrogen activity was reduced to 58.7% and 24.3% in strain AC-2 and AC-11 after inoculation of nodules at 45° C for 3 h. This indicates that nodulation with temperature tolerant strain AC-2 not only improve nodulation at 45° C but also has the ability to fix more nitrogen at 45° C. Rhizobia from *Gliricidia, Leucaena* and *Lonchocarpus* have been reported to nodulate bean and fix nitrogen at 40° C/8 h/day (Hungria et al., 1993).

3.3 Nodulation and Nitrogen Fixation by *Rhizobium* sp. (*Acacia*)

Inoculation of *A. nilotica* with *Rhizobium* sp. (*Acacia*) strains AC-2, AC-6 and AC-11 improved the plant growth by accumulating more shoot dry weight and more total nitrogen content plant^{-1} (Table 3). Among the three strains, strain AC-6 performed better than other under unstressed conditions. However, when temperature stress was given the temperature tolerant *Rhizobium* strains AC-2 proved better than temperature sensitive *Rhizobium* strain AC-11. Temperature stress of 40° C/12 h/day for 5 days and 45° C/12 h/day for 5 days significantly reduced the number of nodules, dry weight of nodules, shoot dry weight and total nitrogen content of plant in comparison to unstressed plants. The plants inoculated with temperature tolerant *Rhizobium* sp. (*Acacia*) strain AC-2 performed better than other two *Rhizobium* strain AC-6 and AC-11 and accumulated more shoot dry weight and plant nitrogen under stress condition (45° C/12 h/day). However when temperature stress was continued for 10 days at 40° C or 45° C the host was affected adversely and showed stunted growth as resulted into significantly less shoot dry weight and total N in comparison to control plants.

Inoculation of *A. mlotica* with AC-2 showed 7-13% reduction in shoot dry weight and 9-11% reduction in plant N up to 45° C. With AC-6, 34-36% reduction in shoot dry weight was observed. Temperature sensitive strain AC-11 also showed similar trend (33-40%) as that of AC-6. Through drastic reduction in nodulation with temperature tolerant strain, AC-2 and AC-6 yet the performance AC-2 was better up to 45° C.

Root temperature in the range of 35-40° C is detrimental to nodule initiation, development and functioning (La Favre and Eaglesham, 1986; Munevar and Wollum, 1981, 1982; Rainbard et al., 1983; Arayangkoon et al., 1990). However, the detrimental effect of elevated temperatures on nodulation and nitrogen fixation can be alleviated by inoculating with temperature tolerant rhizobial strains (Day et al., 1978; Arayangkoon et al., 1990). Better nodulation was observed after inoculation of *A. nilotica* with temperature tolerant strain AC-2 and AC-6 at 40° C/12 h/day for 5 days and 45° C/12 h/day for 5 days in comparison to temperature sensitive strain AC-11.

CONCLUSION

In *Acacia-Rhizobium* symbiosis nodulation is inhibited at high temperature (45° C) but not completely blocked after stress for short-duration. Benefits of inoculation of *A. nilotica* with temperature tolerant *Rhizobium* strain were still evident in terms of nodulation, biomass accumulation and total plant N under 40° C temperature stress conditions only.

REFERENCES

Aseefa, F. and Kleiner, D. 1998. Nodulation pattern and acetylene reduction (nitrogen fixation) activity of some high land and low land *Acacia* species of Ethiopia. *Biol. Fert. Soil27: 60-64.*

Alexander, M. 1982. Ecology of *Rhizobium*. In: *Biological Nitrogen Fixation Ecology Technology and Physiology* (Ed. M. Alexander). Plenum Press, London.pp. 39-90.

Arayangkoon, T., Schomerg, M. and Weaver, R.W. 1990. Nodulation and nitrogen fixation of guar at high temperature. *Plant and Soil. 126: 209-213.*

Bala, A. and Giller, K.E. 2001. Symbiotic specificity of tropical tree rhizobia. *New Phytol. 149: 495-507.*

Bala, N., Sharma, P.K. and Lakshminarayana, K. 1990. Nodulation and nitrogen fixation by salinity tolerant rhizobia in symbiosis with tree legumes. *Agric. Ecosystem and Environ. 33: 33-46.*

Badhwar, S., Dogra, R.C. and Sharma, P.K. 2002. Symbiotic promiscuity among rhizobia isolated from tree legumes. *Ind. J. Agrofor. 4: 41-45.*

Beniwal, R.S., Toky, O.P. and Sharma, P.K. 1995. Genetic variability in symbiotic nitrogen fixation between provenances of *Acacia nilotica* (L.) Willd ex del. *Genetic Resources and Crop Evaluation 42: 7-13.*

Chatel, D.L. and Parker, C.A. 1973. The colonization of host root and soil by rhizobia. I. Species and strain differences in the field. *Soil Biol. Biochem. 5: 425-432.*

Dahiya, J.S. and Khurana, A.L. 1981. Chillum jar, a better technique fo screening of rhizobia under summer conditions. *Plant and Soil 63: 299-302.*

Danso, S.K.A., Bowen, G.D. and Sanginga, N. 1992. Biological nitrogen fixation in trees in agroforestry ecosystems. *Plant and Soil 141: 1771-96.*

Day, J.M., Roughley, R.J.I Eaglesham, A.R.J., Dye, M. and White, S.P. 1978. Effect of high temperature on nodulation of cowpea (*Vigna unguiculata*). *Annal Appl Biol. 88: 476-481.*

Gopala Krishnan, S. and Dudeja, S.S. 1999. Temperature tolerance and effectiveness of *Rhizobium* strains infecting pigeonpea (*Cajanus cajan*) host. *Indian J. Plant Physiol. 4: 6-9.*

Hardy, R.W.F., Holstein, R.D., Jackson, K. and Burns, R.C. 1968. The acetylene-ethylene assay for nitrogen fixation. Lab and field evaluation. *Plant Physiol. 43: 1185-1207.*

Hungria, M.M., Franco, A.A. and Sprent, J.I. 1993. New sources of high

temperature tolerant rhizobia for *Phaseolus vulgaris* L. *Plant and Soil 149: 103-109.*

Kishinevsky, B.D. and Weaver, R. 1992. Effect of high root temperature on *Bradyrhizobium* – pea nut symbiosis. *Plant and Soil 143: 275-282.*

La Farve, A.K. and Eaglesham, A.R. 1986. The effect of high temperature on soybean nodulation and growth with different strains of bradyrhizobia. *Can. J. Microbiol. 32: 22-27.*

Montanej, A., Danso, S.K.A. and Hardarson, G. 1995. The effect of temperature on nodulation and nitrogen fixation by five *Bradyrhizobium japonicum* strains. *Appl. Soil Ecol. 2: 165-174.*

Munevar, F. and Wollum, A.G. 1981. Growth of *Rhizobium japonicum* strains at temperature above 27°C. *Appl. Environ. Microbiol. 42: 255-258.*

Munevar, F. and Wollum, A.G. 1982. Response of soybean plants to high root temperature as affected by plant cultivar and *Rhizobium* strains. *Agron. J. 74: 138-142.*

Rainbard, R.M., Atkins, C.A. and Pate, J.S. 1983. Effects of temperature on nitrogenase functioning in cowpea nodules. *Plant Physiol. 73: 392-394.*

Rustagi, N., Sharma, P.K. and Dogra, R.C. 1996. Temperature tolerance and efficacy of *Rhizobium* sp. (*Acacia*) in symbiosis with *A. nilotica*. In: *Resource Management in Agriculture* (Eds.R.C. Dogra, R.K. Behl and A.L. Khurana, CCS HAU, Hisar & MMB, New Delhi.pp 107-111.

Sharma, P.K., Bala, N. and Kapoor, K.K. 2003. Moongbean – An inappropriate alternate host for rhizobia nodulating leguminous trees. *Environ. Ecol. 21(1): 128-130.*

Somasegaran, P., Reyer, V.G. and Hobon, H.J. 1984. Influence of high temperature on growth and survival of *Rhizobium* sp. in peat inoculant during prepartion, storage and distribution. *Can. J. Microbiol. 30: 23-30.*

Stoyanova, J. 1996. Growth, nodulation and nitrogen fixation in soybean as affected by air humidity and root temperature. *Biologia Plantarum 38(4): 537-544.*

Toky, O.P., Kaushik, N. and Sharma, P.K. 1995. Genetic variability in progenies of *Acacia nilotica* (L.) ex Deel. Sp. *Indica* (Benth.) brenan for nitrogen fixing ability. *Silvae-Genetica 44: 161-165.*

Vincent, J.M. 1970. *A Manual for Practical Study of the Root Nodule Bacteria.* IBP Handbook No. 15, Black Well, Oxford.

Zarhari, K., Aurag, J., Khabhya, B., Kharihar, D. and Filali-Maltouf, A. 2000. Phenotypic characteristics of rhizobia isolates nodulating *Acacia* species in arid and Saharan region of Morocco. *Lett. Appl. Microbiol. 30: 351-357.*