Legume Research, 22(4): 227-232, 1999

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF FAST GROWING RHIZOBIA OF *SESBANIA BISPINOSA* IN THE *IN-VITRO* CONDITION

S. Gopalkrishnan* and A.G. Girish*
VHNSN College, Virudhunagar (T.N.)-626 001, India.

ABSTRACT

Five different strains of rhizobia were isolated from root nodules of *Sesbania bispinosa*. The native isolates varied in symbiotic performance in terms of nodule fresh weight and plant dry weight. The fast growing rhizobial isolates were found to be very efficient to give high nitrogenase activity, high nodulation count and biomass. Characterization of these different strains with regard to explanta nitrogenase, possession of intrinsic antibiotic resistance, ability to utilize various carbon sources, temperature tolerance and salinity tolerance were done after confirmed its nodulation by plant infection test. All the strains exhibited explanta nitrogenase activity and exhibited ampicillin, chloramphenicol, kanamycin, penicillin and nalidixic acid resistance and sensitive to gentamycin and tetracycline. The isolates could utilize wide range of carbon sources like mannitol, sucrose, dextrose, galactose, maltose, lactose, and cellobiose except citrate. These strains showed that it grows in wide range of temperature even up to 44° C and salinity levels of 600 mMolar. The biological nitrogen fixation can be symbiotically increased by doing further research on these efficient strains and could exploit its advantage characteristics.

INTRODUCTION

Selection of *Rhizobium* sp. strains adapted to specific environmental conditions has the potential to provide increased legume crop production through increased biological nitrogen fixation. (Mytton, 1984). The success of selected *Rhizobium* strains in increasing N_2 fixation in legumes has drawn attention because of the increasing need for long-term soil fertility and ecological sustainability. This interest culminated the identification of unknown weed legumes that has the potential as a green manure, is very much in need (Rinaudo *et al.*1983). Information about the effect of physiological and biochemical characteristics of different *Rhizobium* sp. strains is needed, in order to select superior strains. The present paper describes the effects of physiological and biochemical characteristics of five isolated strains of *Sesbania bispinosa*, a fast growing annual legume generally found in the semi arid areas of India.

MATERIAL AND METHODS

The host-*Sesbania bispinosa* : Six hundred and sixty one plants of the same legume were carefully uprooted from different locations of the Virudhunagar, Kamarajar District of Tamil Nadu with a crow bar to recover maximum roots and nodules. The legume was then identified with the help of Botanical Survey of India, Coimbatore, Tamil Nadu as *Sesbania bispinosa*. The temperature of the area where the samples were collected was around 42 - 45°C (in May-June), the soil was black and its pH was 8.1 to 9.2. Observations on nodule colour, size and

*Present address: Natural Resources Management Program, ICRISAT, Patancheru, 502 324, India

numbers were after washing the root system whenever required.

Rhizobial cultures : Among the plants screened, the dominated plants on the basis of nodule number, colour, size, nitrogenase assay, were selected for rhizobia isolation. Five cultures were selected for further studies. They were named DMB 001 - DMB 005 (DMB-Department of Microbiology). The cultures were maintained on Yeast Extract Mannitol Agar (YEMA) slants (Fred *et al.*,1932) at 4°C after 3-8 day's growth at 28 ± 2°C. The purity of the strains was tested by a plant infection test in 'Chillium Jar' assemblies under controlled environment conditions as per the procedure of Dahiya and Khurana, 1981. The cultures were defined as rapid and fast growers on the basis of their growth characteristics on the YEMA plates.

Growth studies : One ml of log phase cell suspension of rhizobia (ica 10^7 cells/ml) was inoculated to a 250 ml Erienmeyer flask containing 100 ml of Yeast Extract Mannitol broth (YEMB) which was then incubated on a rotary shaker (150 rev. min^{-1}) at 28 ± 2°C. Samples were drawn at intervals of 4th, as all were rapid growers. Viable counts were made and generation time of each strain was tabulated from the growth curves.

Intrinsic antibiotic resistance : Intrinsic antibiotic resistance (IAR) was determined by plating on medium containing different antibiotics and recorded the presence or absence of growth after incubation at 28±2°C for 6d.

Utilization of carbohydrates : Utilization of carbon was studied in an enriched medium of yeast extract with different carbon sources at 1% concentration. Carbon free controls were employed for evaluating the growth. Polyols and polysccharides were sterilized along with the medium. 250 ml of Erlenmeyer flasks containing 100 ml of yeast extract monnitol broth (YEM) which was then inoculated with 1ml of log phase cell suspension (*ica* 10^7 cells ml^{-1}) which were then incubated on a rotary shaker (150 rev. min^{-1}) at 28±2°C. Samples were drawn at intervals of 4h. O.D. was taken at 600 nm in spectronic 20 and viable count was made and generation time of each strain against each sugar was tabulated.

Screening of rhizobia for salinity tolerance : YEM broth (100ml) with different concentration of NaCl was sterilized in a 250 ml Erlenmeyer flasks fitted with side arm, permitting measurement of the turbidity of the cultures. These media were inoculated with 1ml of log phase cultures of the strain under test and incubated at 28°C in a rotary shaker (150-rev. $mins^{-1}$). Salinity tolerance was measured by reading OD_{600} after different time intervals.

Thermotolerance of rhizobia : The cultures were incubated at different temperatures i.e. 12°C, 28°C, 36°C, 42°C, 44°C and 50°C and the growth was evalutated. Erlenmeyer flasks of 250-ml capacity containing 100 ml of YEMB were Erlenmeyer flasks fitted with si permitting measurement of the of the cultures. It was inocula 1ml of log phase culture of the strains under test and incub respected temperatures in a rotar (150-rev. min^{-1}). Temperature was measured by reading OD different time intervals and viat was made and generation time strain against each temperat tabulated.

Table-1. Growth rate constant and generation time of different strains of *Rhizobium* spp of *Sesbania bispinosa*

Rhizobium strains	Mean growth rate constant	Mean generation time (h)
DMB 001	0.63	1.05
DMB 002	0.76	0.91
DMB 003	0.58	1.45
DMB 004	0.60	1.51
DMB 005	0.61	1.27

RESULTS AND DISCUSS

During the isolation of rhiz nodule yielded colonies that we slimy, transparent and non-spread appeared with in 2-3d and were as rapid growers. Colony sizes r 1mm d and appearance of colc in 7d are typical of fast growing (Graham and Parker 1966). Fi of rapid growers were selected generation time was determined growth curve (Table 1&2).

It was then observed that a lag of about 4h and the phase was reached with in generation time varied between 1.51h. The majority of the strai were rapid growers suggests t

Table-2. Efficiency of different str

Treatment	Nodule Number $plant^{-1}$
Uninocul.	0
DMB 001	38.4
DMB 002	49.6
DMB 003	29.9
DMB 004	30.7
DMB 005	19.3

Erlenmeyer flasks fitted with side arm, permitting measurement of the turbidity of the cultures. It was inoculated with 1ml of log phase culture of the different strains under test and incubated at respected temperatures in a rotary shaker (150-rev. min^{-1}). Temperature tolerance was measured by reading OD_{600} after different time intervals and viable count was made and generation time of each strain against each temperature was tabulated.

RESULTS AND DISCUSSION

During the isolation of rhizobia each nodule yielded colonies that were large, slimy, transparent and non-spreading. They appeared with in 2-3d and were designated as rapid growers. Colony sizes more than 1mm d and appearance of colonies with in 7d are typical of fast growing rhizobia. (Graham and Parker 1966). Five strains of rapid growers were selected and then generation time was determined from the growth curve (Table 1&2).

It was then observed that there was a lag of about 4h and the stationary phase was reached with in 36h. The generation time varied between 0.91 and 1.51h. The majority of the strains isolated were rapid growers suggests that native population of the rapid growing rhizobia were higher at time of root infection or that the strains were more competitive than the slow growers were. (Anand, R.C. and Dogra, R.C. 1991).

All the isolates were resistant to ampicillin, nalidixic acid, kanamycin, penicillin, streptomycin and chloramphenicol at various concentrations and were sensitive to tetracycline and gentamycin. The influence of various antibiotics on the isolates was given on the table. Similar type of antibiotic resistance pattern has been observed in other cowpea rhizobia infecting clusterbean (Mand, D.N, 1987), Pigeonpea (Anand, R.C. and Dogra, R.C. 1991). However, all the isolates studied were resistant to high concentration of nalidixic acid, so this resistant marker could be used as a selective trait or marker for the identification of the strain for the various ecological studies (Fig. 1).

Effect of different carbon sources on the growth of rhizobia showed that it utilized a variety of mono, di, polysaccharides and polyols. But rhizobia were unable to utilize citrate. The generation time varied between 1.9 to 3.2h depending on the carbon sources. The isolates utilizing glucose, galactose,

Table-2. Efficiency of different strains tested by the plant infection test in chillium jar assemblies under glass house conditions

Treatment	Nodule Number $plant^{-1}$	Nodule fresh wt (mg)	Shoot dry wt (mg)	Root dry wt (mg) $plant^{-1}$	Nitrogenase activity $MMC_2H_2h^{-1}g^{-1}$
Uninocul.	0	-	170.6	0.27	-
DMB 001	38.4	92.2	216.7	0.65	9.67
DMB 002	49.6	97.6	219.1	0.76	9.64
DMB 003	29.9	76.8	196.4	0.81	8.67
DMB 004	30.7	77.4	199.5	0.58	8.12
DMB 005	19.3	47.9	165.8	0.51	9.30

Fig.1. Intrinsic antibiotic resistance pattern of the isolates.

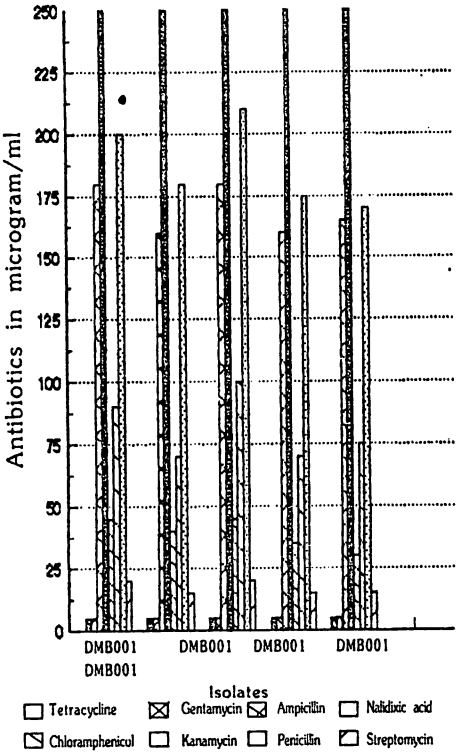

Fig.2. Growth of rhizobia of different isolates on various carbon sources.

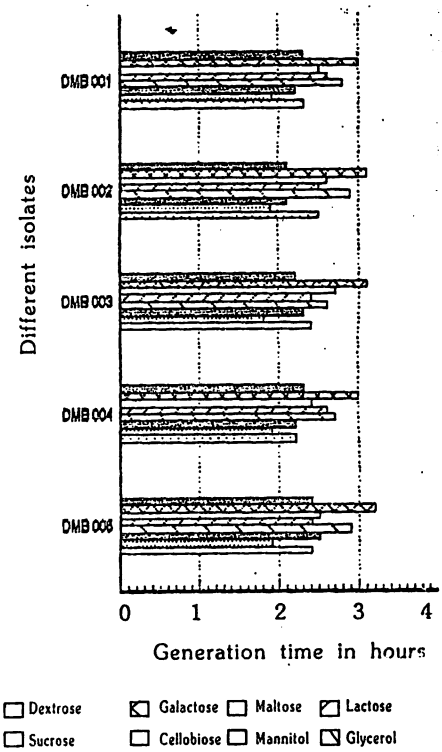

Fig.3. Growth of rhizobia of different isolates on various carbon sources at the end of the log phase.

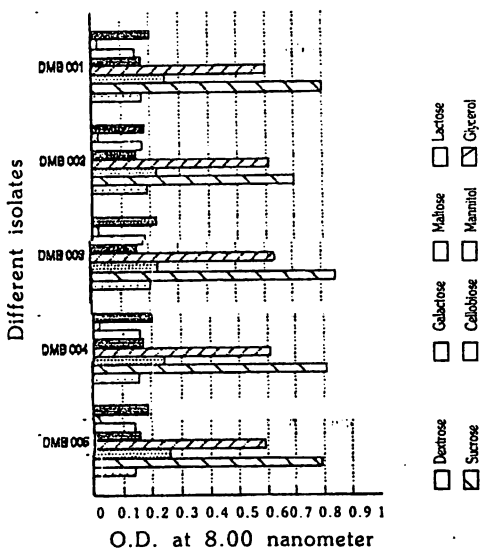

Fig.4. Relationship between temperature and generation time of different isolates.

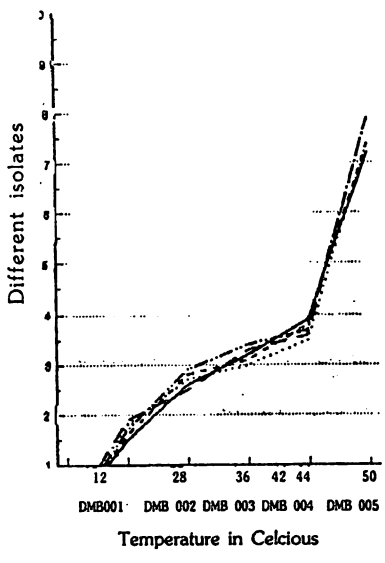

Table-3. Influence of sodium chloride on the growth of different rhizobial isolates of *S.bispinosa*

Isolates	NaCl in millimolar									
	100	200	300	400	500	600	700	800	900	1000
DMB 001	+	+	+	+	+	+	-+	-	-	-
DMB 002	+	+	+	+	+	+	-+	-	-	-
DMB 003	+	+	+	+	+	-+	-	-	-	-
DMB 004	+	+	+	+	+	+	-+	-	-	-
DMB 005	+	+	+	+	+	-+	-	-	-	-

Table-4. Growth pattern of different isolated rhizobia infecting Sesbania bispinosa on various temperatures.

Strain No.	Growth After 7 days ($O.D_{600}$)					
	12°C	28°C	36°C	42°C	44°C	50°C
DMB 001	0.05	1.0	0.7	0.45	0.35	0.15
DMB 002	0.05	0.95	0.75	0.4	0.3	0.1
DMB 003	0.05	1.1	0.65	0.5	0.3	0.1
DMB 004	0.05	0.85	0.75	0.4	0.35	0.15
DMB 005	0.05	1.06	0.65	0.45	0.3	0.1

The isolates utilizing glucose, galactose, sucrose, lactose, maltose, and mannitol were the typical character of fast growing rhizobia (Chakrabarti *et al*,1981; Stowers, M.D, 1985). Allen and Allen (1950) stated that the slow growing rhizobia were more specific in their carbohydrate requirement, for e.g. *R. trifoli* and *R. leuminosarum* can utilize 20 different carbohydrates, where as *R. japonicum* can utilize only 8 of the 20. Growth studies and ability to utilize different carbons strengthened the view that these rhizobia belonged to fast growing rhizobial group (Fig. 2 and 3).

The tolerance level of salinity to various isolates were up to 600m Molar indicating that all the isolates were highly salinity tolerant and it could grow well in saline conditions. (Table 3).

Under saline conditions, micro symbionts was not a limiting factor, however legumes have been recorded as more sensitive or only moderately resistant to slinity (Zahvan, H.H, 1991) because of the accumulation of toxic ions such as Na and Cl in plant tissues, where they disturb the enzyme activities.

The effect of different temperatures on the growth of rhizobia showed that all the isolates could well at 28°C, 36°C, delayed growth was found at 42°C and 44°C and no or very little was found at 50°C and at 12°C no growth was found. The generation time of the isolates at 28°C was the lowest among the others; this was true in all cultures. The generation time was increased with increase in temperature, because elevated temperature may delay nodule formation and development. Similar results were observed by Lie (1981). High temperature adversely affects the survival and persistence of rhizobia, competition, root hair formation, adsorption of rhizobia and nodulation. (Dudeja and Khurana (1989) (Table 4 & Fig. 4).

The isolates can grow well under extreme environmental conditions of salinity

and different physiological factors includes different carbon sources and antibiotics indicates that under natural ecosystem such strain could harvest a good amount of N_2 of legumes in soils. The results of this preliminary study indicate that the isolates can be used under field conditions and biological nitrogen fixation can be significantly increased.

REFERENCES

Allen, E.K. and Allen, O.N. (1950) *Bacteriol. Rev.*, **14**:273-330.

Anand, R.C. and Dogra, R.C. (1991). *J. Applied Bacteriol* **70**:197-202.

Chakrabarthi, S. *et al.* (1981). *Soil Biol. Biochem.*, **13**:349-354.

Dahiya, J.S. and Khurana, A.L (1981). *Pl. Soil,* **83**: 299-302.

Dudeja, S.S. and Khurana, A.L. (1989). *J. Expt. Bot.* **40**:460-472.

Fred, E.B. *et al.*(1932). Root nodule bacteria and leguminous plants. Wisconsin University Studies in Sciences, Masison: University Wisconsin press, 343P.

Graham,P.H. and Parker, C.A. (1996). *Pl. Soil,* **20**:383-386.

Lie, T.A. (1981). In : Nitrogen fixation, vol.1: Ecology (W.J. Broughton *ed.)* Clarendon Press, Oxford, 104-134.

Mand, S. (1987). Ph.D. thesis, Haryana Agricultural University, Hisar.

Mytton, L.R. (1984). *Pl. Soil,* **82**:329-335.

Rinaudo, G.*et al.* (1983). *Biol. Biochem. Soil,* **15**:111-113.

Stowers, M.D. (1985). *Ann. Rev. Microbiol.* **39**:89-108.

Zahvan, H.H. (1991). *Biol. Fertil. Soils,* **12**: 73-80.