SYMBIOTIC DIVERSITY AMONG ACID-TOLERANT BRADYRHIZOBIAL ISOLATES WITH COWPEA

C. Appunu[1], L. M. L. Reddy[2], C. V. C. M. Reddy[2], D. Sen[2] and B. Dhar[2]

ABSTRACT

A total of eight acid tolerant strains of bradyrhizobia isolates from indigenous cowpea plants grown in acid soil in Varanasi, Uttar Pradesh, India, were examined for their ability to survive in soil and yeast extract mannitol broth at low pH levels. All these isolates survived in acidic (pH 3.5-6.5) conditions. Survival capacity of rhizobia was higher in soil than in nutrient medium at low pH 3.5-6.5 levels. Symbiotic effectiveness of these strains under polyhouse conditions in sterilized soil of pH 4.5 recorded the highest and lowest symbiotic characters for dry matter production and nitrogen improvement per plant in CR09 and CR20 inoculated plants, respectively. All the examined isolates showed variability in their symbiotic performances. The strain found to be more tolerant to stress were more effective N_2 *fixers in symbiosis with cowpea cv. Paiyur1 under acid-soil conditions. Symbiotic variation among different strains showed that there is potential to improve strain performance under stress conditions.*

Key words: *Acid tolerant, bradyrhizobia, cowpea, symbiotic effectiveness.*

INTRODUCTION

In India, cowpea (*Vigna unguiculata* L. Walp) is cultivated by commercial and subsistence farmers. They provide a valuable source of protein and thereby sustaining the nutritional balances of low income populations (Singh *et al*., 1997) and also help in maintaining soil health through biological nitrogen fixation by symbiotic rhizobia. Cowpea depends on their symbionts for a large part of their nitrogen requirements for growth and dry matter production. The cowpea symbionts are classified into slow-growers, *Bradyrhizobium* spp. and fast-growers, *Sinorhizobium* spp. (Zhang *et al*., 2007). Selected symbiotically efficient rhizobia were used as inoculants to increase biologically fixed N_2 under field conditions. The introduced strain must compete with highly adapted indigenous rhizobia for legume nodulation under specific physiological and biological soil conditions. Many biotic and abiotic factors affect the persistence of symbiotically effective introduced rhizobial strain in soil. Soil acidity is the one of the factors which restricts production of cowpea through its impact on nitrogen fixation. The failure of nodulation under acid soil conditions is common, especially in soils of pH less than 5. Soil acidity limits symbiotic nitrogen fixation by limiting *Rhizobium* survival and persistence in soils, as well as reducing nodulation (Ibekwe *et al*., 1997). High symbiotic effective rhizobial inoculation is a common practice in agricultural legume production (Catroux *et al*., 2001) which requires survival and establishment of inoculated rhizobia in the soil environment (Da and Deng, 2003). There is no history of inoculation of acid tolerant strains in acidic soil in India. A wide variation in the tolerance to acid-soil conditions have been reported among *Bradyrhizobium* strains of many agriculturally important legumes from

[1] *Section of Plant Breeding ,Division of Crop Improvement, Sugarcane Breeding Institute,Coimbatore- 641 007,Tamil Nadu, India.*
[2] *Microbial Genetics Laboratory, Department of Genetics and Plant Breeding, Institute of Agricultural Science, Banara Hindu University, Varanasi-221005, India.*

various countries (Ayanaba *et al*., 1983; Graham *et al*., 1994; van Rossum *et al*., 1994; Raza *et al*., 2001) and in India (Appunu *et al*., 2005). Keeping in view the role of acid-tolerant strain, we report here the survival of bradyrhizobial isolates in acidic conditions and their symbiotic effectiveness with cowpea.

MATERIALS AND METHODS

Bradyrhizobial Isolates

Eight acid tolerant bradyrhizobia strains, CR01, CR05, CR07, CR09, CR12, CR15, CR18 and CR20 were isolated from nodules of indigenous cowpea plants grown in acid soil by following the method of Vincent (1970) and were used in the experiments. All strains were maintained on yeast extract mannitol (YEM) medium and transferred to fresh slant every month.

Growth of bradyrhizobial isolates at low pH levels

Soil pH was estimated by suspending 40 g of air dried soil in 100 ml distilled water, after allowing the suspension to stand for 1 h at room temperature. Soil sample and yeast extract mannitol broth were sterilized in autoclave at 121°C for 15 min (JRIC-39E, Osworld India). The pH level of the soil sample (pH 4.5) and medium (pH 6.8) was modified to obtain the required pH values of 3.5, 4.5, 5.5 and 6.5 by adding 1 N HCl or NaOH and no changes in pH were observed after autoclaving. All the bradyrhizobia isolates were multiplied in YEM broth and 1 ml of multiplied rhizobial culture (about 10^8 rhizobial cells/ml) used as standard inoculum introduced into soil mixture (2.5 g of soil + 1 ml of distilled water) and to YEM broth of low pH levels and YEM broth with pH 6.8 as control for comparison. Standard volume of inoculum also introduced in the YEM Flasks containing treated soil and YEM broth were kept on a rotary incubator shaker (200 rev. min^{-1}) (ACM-22064-I, ACMAS Technology India) at 28 ± 2°C for 7 days. The rhizobial growth was determined (cfu ml^{-1}) by a plate count technique (Vincent, 1970) using YEM agar plates.

Symbiotic efficiency of rhizobial isolates

Genetically pure and healthy seeds of cowpea cv. Paiyur1 were surface sterilized as explained by Appunu *et al*. (2005). In brief, seeds were surface-sterilized with acidified mercuric chloride (0.2 % w/v) for 3-5 minutes and 70% ethyl alcohol for 1-2 minutes and then thoroughly rinsed with sterile distilled water for 4-5 times. Sterilized seeds were coated with bradyrhizobia as detailed by Vincent (1970). The untreated seeds were served as control. Seeds were grown in earthern pots containing sterilized soil [pH 4.5; organic carbon (0.78); CEC (11.8 cmol (+) Kg^{-1}); EC (<0.23 dSm^{-1}); exchangeable Al (cmol (+) Kg^{-1}); total N (0.10)] under polyhouse conditions. Each pot was maintained with one healthy seedling. Each treatment was replicated five times. Plants were supplied with water at appropriate times and were maintained to grow till 5 weeks in polyhouse having adequate temperature 30-35 °C, humidity 70-80% and light intensity 1,600-2000 lux. Plants were harvested after five weeks of sowing and data pertaining to symbiotic and vegetative characters were recorded as described previously (Appunu *et al*., 2005).

Data were subjected to analysis of variance, and means were classified using Duncan’s multiple-range test at the 0.05 probability level.

RESULTS AND DISCUSSION

The bradyrhizobia isolates were characterized to their growth response to low levels of pH 3.5 - 6.5. The results showed that these isolates were tolerant to extreme acidic conditions since they could survive and grew in the low pH, even at 3.5 (Figures 1 and 2).The rhizobia population showed higher level of survival capacity in acid soils than in nutrient broth at low levels of pH 3.5 - 5.5. It could be explained that attachment of rhizobial with cations/anions or organic molecules in the soil are one of the reasons for higher growth rate in soil than in nutrient broth. These results are in agreement with those reported earlier that *Rhizobium* strains which survived in the acid soil cannot grow on a nutrient medium with pH as low as that of the soil (Asanuma and Ayanaba, 1990). Rhizobial strains of a given species vary widely in their pH tolerance (Zahran *et al*., 1999; Appunu *et al*., 2005). The fast growing *Rhizobium* strains have generally been considered less tolerant to acid pH than slow growing strains of *Bradyrhizobium* (Graham *et al*., 1994). However, Mpepereki *et al*. (1997) reported that both fast- and slow-growing *Bradyrhizobium* strains of *Vigna unguiculata* are tolerant to pH values as low as 4.0. Rhizobia adopt various mechanisms to survive in the acid soil conditions (Zahran *et al*., 1999).

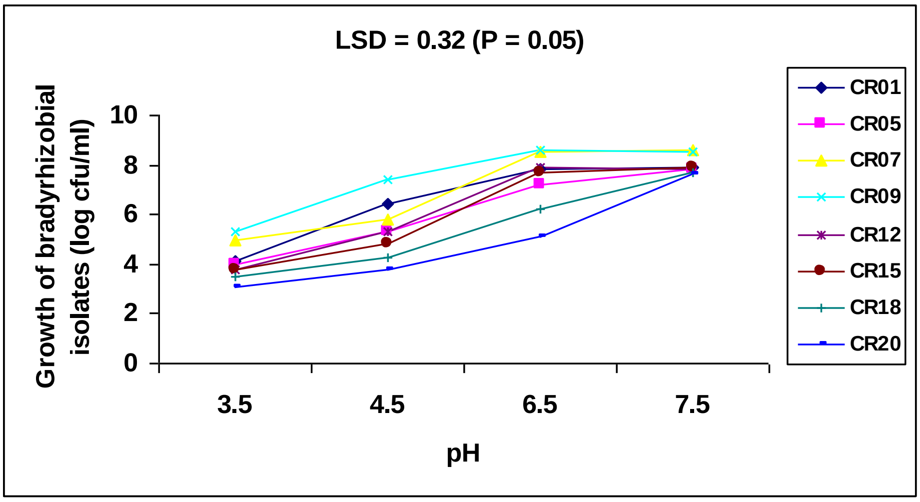

Figure 01: Effect of different level of acidic conditions (pH levels) on the growth of bradyrhizobial isolates of cowpea in soil.

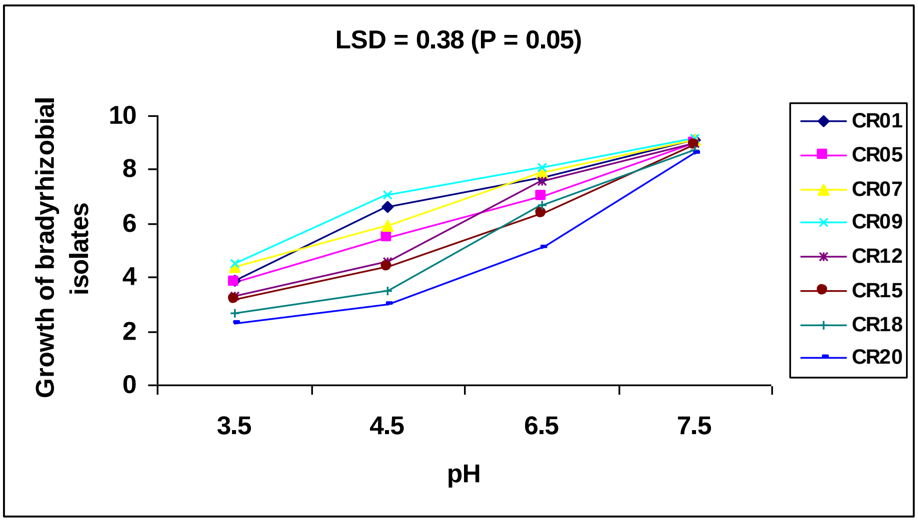

Figure 02: Effect of different level of acidic conditions (pH levels) on the growth of bradyrhizobial isolates of cowpea in yeast extract mannitol broth.

The effect of inoculation of eight acid tolerant bradyrhizobia isolates on cowpea cv. Paiyur1 is presented in Table 1. All strains formed nodules and a significant variation was noticed in nodule number, dry weight and nitrogenase activity, dry matter production and nitrogen accumulation per plant. Highest nodule frequency, dry weight and nitrogenase activity were found in the plant inoculated with the strain CR09. However, inoculation of strains CR01, CR05, CR07, CR09 and CR12 also resulted in significantly high dry weight. In comparison to control, an increase of 5.74-39.08% in total plant dry weight was observed with strain inoculated treatments. Maximum enhancement in total plant dry matter (39.08%) was noticed with the strain CR09, followed by CR07 (30.34%), CR12 (28.04%), CR05 (19.77%) and CR01 (14.71%). Percent increase of nitrogen content was also observed by the inoculation of strains. The highest nitrogen content (2.40%) per plant was estimated in strain CR09 inoculated plants, while the lowest (1.90%) was with CR18 and CR20 inoculated plants, which was 33.33 and 5.55% higher than that of control plants, respectively.

Table 01: Symbiotic effectiveness of acid-tolerant bradyrhizobial isolates on cowpea cv. Paiyur1

Strain	Total no. of nodules	Dry weight of nodules (g)	Nodules nitrogenase activity (μmol g^{-1} $plant^{-1}$)	Total plant dry matter production(g)	N content improvement of plant (%)
CR01	27.21c	0.19b	7.95b	4.99b	2.0
CR05	32.03b	0.17c	8.08b	5.21b	2.2
CR07	34.10b	0.17c	9.42a	5.67a	2.2
CR09	48.54a	0.22a	10.36a	6.05a	2.4
CR12	42.88a	0.20b	9.76a	5.57a	2.3
CR15	37.01b	0.23a	7.91b	4.85c	2.0
CR18	22.32d	0.14d	4.96c	4.73c	1.9
CR20	21.95d	0.11e	4.41c	4.55c	1.9
Control	0.00e	0.00f	0.00d	4.35c	1.8

Those not followed by common superscript letters differ significantly at the LSD probability of <0.05

This study showed that inoculation of acid-tolerant isolates CR01, CR05, CR07, CR09 and CR12 leads to good nodulation, dry matter accumulation and improvement of nitrogen content cowpea. These isolates exhibited great diversity in their symbiotic performance and a few of them accumulated considerably high total plant dry weight and percent nitrogen content. Differential symbiotic performance of *Bradyrhizobium* isolates has already been reported (Zhang *et al*., 2002; Meghvanshi *et al*., 2005; Appunu *et al*., 2008). In most cases, pH sensitive stage in nodulation occurs early in the infection process and that *Rhizobium* attachment to root hairs is one of the stages affected by acidic conditions in soils. Only one of the symbionts needed to be acid tolerant for good nodulation to be achieved at pH 4.5 (Vargas *et al*., 1988). van Rossum *et al*. (1994) reported that inoculation of acid tolerant *Bradyrhizobium* strains under acid-soil conditions improves the groundnut vegetative characters and yields. Selection of acid tolerant rhizobia to inoculate legume hosts under acid conditions will ensure the establishment of symbiosis and also successful performance (Correa and Barneix, 1997). However, the success or failure of inoculation depends on the competitive nodulation ability against indigenous bradyrhizobia under natural conditions. Graham (1992) and Carter *et al*. (1994) reported the existence of positive correlation between acid tolerance in laboratory and competitive nodulation on acidic soils.

CONCLUTIONS

In conclusion, four bradyrhizobia isolates CR07, CR09, CR01 and CR05 exhibited high growth at low pH levels and also showed better symbiotic performance in acid soils under laboratory conditions. These isolates could become useful inoculants in acid soils if they are superior in competitiveness under natural ecological conditions in the field.

REFERENCES

Appunu C, Sen D and Dhar B (2005). Acid and aluminium tolerance of *Bradyrhizobium* isolates from traditional soybean growing areas of India. Indian Journal of Agricultural Sciences 75(12),pp: 727-728.

Appunu C, Sen D, Singh MK and Dhar B (2008). Variation in symbiotic performance of *Bradyrhizobium japonicum* strains and soybean cultivars under field conditions. Journal of Central European Agriculture 9(1),pp: 185-190.

Asanuma S and Ayanaba A (1990). Variation in Acid-Al tolerance of Bradyrhizobium japonicum strains from African soils. Soil Science and Plant Nutrition 36(2),pp: 309-318

Ayanaba A, Asanuma S and Munns DN (1983). An agar plate method for rapid screening of *Rhizobium* for tolerance to acid-aluminium stress. Soil Science Society of American Journal 47,pp: 256-268.

Carter JM, Gardner WK and Gibson AH (1994). Improved growth and yield of faba bean (*Vicia faba* cv. Fiord) by inoculation with strains of *Rhizobium leguminosarum* biovar *viceae* in acid soils in South-west Victoria. Australian Journal of Agricultural Research 45,pp: 613-623.

Catroux G, Hartmann A and Revellin C (2001). Trends in rhizobial inoculant production and use. Plant Soil 230,pp: 21-30.

Chauhan GS and Joshi OP (2005). Soybean (*Glycine max*) - the 21st century crop. Indian Journal of Agricultural Sciences 75(8),pp: 461-469.

Correa OS and Barneix AJ (1997). Cellular mechanisms of pH tolerance in *Rhizobium loti*. World Journal of Microbiology and Biotechnology 13,pp: 153-157.

Da HN and Deng SP (2003). Survival and persistence of genetically modified *Sinorhizobium meliloti* in soil. Applied Soil Ecology 22,pp: 1-14.

Graham PH (1992). Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Canadian Journal of Microbiology 38,pp: 475-484.

Graham PH, Draeger K, Ferrey ML, Conroy MJ, Hammer BE, Martinez- Romero E, Naarons SR and Quinto C (1994). Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Canadian Journal of Microbiology 40,pp: 198-207.

Ibekwe AM, Angle JS, Chaney RL and van Berkum P (1997). Enumeration and nitrogen fixation potential of *Rhizobium leguminosarum* biovar *trifolii* grown in soils with varying pH values and heavy metal concentrations. Agriculture Ecosystem and Environment 61,pp: 103-111.

Meghvanshi MK, Prasad K and Mahna SK (2005). Identification of pH tolerant *Bradyrhizobium japonicum* strains and their symbiotic effectiveness in soybean (*Glycine max* (L.) Merr.) in low nutrient soil. African Journal of Biotechnology 4(7),pp: 663-666.

Mpepereki S, Makonese F and Wollum AG (1997). Physiological characterization of indigenous rhizobial nodulation *Vigna unguiculata* in Zimbabwean soils. Symbiosis 22,pp: 275-292.

Raza S, Jornsgard B, Abou-Taleb H and Christiansen JL (2001). Tolerance of *Bradyrhizobium* sp. (*Lupini*) strains to salinity, pH, $CaCO_3$ and antibiotics. Letters in Applied Microbiology 32,pp: 379-383.

Singh BB, Mohan Raj DR, Dashiell KE and Jackai Len (1997). Advances in cowpea research. IITA-JIRCAS, Ibadan, Nigeria

van Rossum D, Muyotcha A, De Hope BM, van Verseveld HW, Stouthamer AH and Boogerd FC (1994). Soil acidity in relation to groundnut-*Bradyrhizobium* symbiotic performance. Plant Soil 163,pp: 165-175.

Vargas AAT and Graham PH (1988). *Phaseolus vulgaris* cultivar and *Rhizobium* strain variation in acid-pH tolerance and nodulation under acid conditions. Field Crops Research 19,pp: 91-101.

Vincent JM (1970). A manual for the practical study of the root nodule bacteria. The Blackwell Scientific Publications, Oxford.

Zhang H, Daoust F, Charles TC, Driscoll BT, Prithiviraj B and Smith DL (2002). *Bradyrhizobium japonicum* mutants allowing improved nodulation and nitrogen fixation of field grown soybean in a short season area. Journal of Agriculture Science 138,pp: 293-300.

Zhang WT, Yang JK, Yuan TY and Zhou JC (2007). Genetic diversity and phylogeny of indigenous rhizobia from cowpea [*Vigna unguiculata* (L.) Walp.]. Biology and Fertility of Soils 44,pp: 201-210

Zahran HH (1999). Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews 63(4),pp: 968-989.