academicJournals

Vol. 7(19), pp. 2093-2102, 7 May, 2013
DOI: 10.5897/AJMR12.2263
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Physiological behavior and antibiotic response of soybean (*Glycine max* L.) nodulating rhizobia isolated from Indian soils

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Accepted 29 March, 2013

A total of 92 soybean nodulating rhizobia were isolated from different agro regions and screened for sensitivity against 8 rhizobiophages. Only 33 showed susceptibility with phage strains. Nineteen rhizobial isolates selected, including 9 isolates having high lytic behaviour and 10 resistant to all phages, were subjected to study on symbiotic effectiveness. Strains ALSR12, KNSR16, USDA123 and USDA94 showed highly significant interaction with host plant grown under axenic conditions. Strains USDA123 and ANSR12 had distinct lytic reaction with phage strain SR1 and SR2, respectively. Plaques formed by SR1 on USDA123 were quite clear with sharp margin, whereas central halo was surrounded by turbid concentric by SR2 on strain ALSR12. In view of generation time varying from 9.2 h (USDA94) to 12.6 h (ALSR12), they were marked as slow grower. Of the different carbon sources used, arabinose served as a potent carbon source for supporting highest growth of these strains. As compared to the other strain, ALSR12 was able to grow at 45°C and capable to tolerate a higher range of acidity and alkalinity. Strains ALSR12 and USDA123 showed highest resistance level with the antibiotics streptomycin and gentamicin, respectively. *Ex-planta* N_2-ase activity was determined only in USDA94 and ALSR12. This study provides means for conducting a systematic and scientific investigation towards obtaining a reliable understanding of the bradyrhizobial diversity in terms of their physiological behaviour.

Key words: Soybean, rhizobia, rhizobiophage, diversity.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is often called a miracle crop and is considered as one of the oldest crops of the world, with reports of its cultivation in China around 2500 B.C. (Morse, 1950). Soybeans improve the soil fertility through fixation of atmospheric nitrogen in its roots which is later utilized by the subsequent crop. Biological Nitrogen Fixation has emerged as an alternate means for quality grain production and restoration of declining soil fertility (Keyser et al., 1982). The world is now focused towards reducing the use of synthetic nitrogenous fertilizer because of its high cost of production and transportation, along with burden of increasing Govt. subsidy and detrimental effect on soil fertility. The beneficial effect of endosymbionts in agricultural legumes in terms of biological nitrogen fixation has been the main focus in the recent past (Deshwal et al., 2003; Herridge et al., 2008; Jaiswal and Dhar, 2011), as it is an important aspect of sustainable food production and long term crop-productivity. Various types of native rhizobia are present in cultivated soils of

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different geographical and ecological zones of the world, including India. Through inoculation of strains selected from indigenous population, improved symbiotic efficiency can be achieved leading to qualitative and quantitative yield enhancement of soybean. Rhizobia can show wide variations in numerous characteristics; rate of growth (fast or slow grower) (Fred, 1932), utilization of C and N sources (Stowers, 1985), tolerance to abiotic (elevated temperature and acidity) (Michiels et al., 1994; Surange et al., 1997) and biotic (Phage susceptibility) stress factors. Phage sensitivity pattern has been suggested as a potent method for differentiating bacteria according to their genotypic and phenotypic traits (Lindstrom and Lehtomaki, 1988; Sharma et al., 2002). In addition, it gives faster and reproducible results for discriminating other rhizobial strains and also proves useful enough for monitoring the purity of the rhizobial germplasm. Phages are believed to be responsible for the intensity and spatially varying selection pressure on their host and *vice versa* (Vos et al., 2009). The growth of strains of rhizobial species has been reported to vary with respect to various carbon and nitrogen sources, phage sensitivity and intrinsic antibiotic resistance level (Vincent, 1974; Yao et al., 2002). Intrinsic antibiotic resistance (IAR) properties of the strain has been successively used as a stable marker in ecological studies to determine heterogeneity (Eaglesham, 1987; Mueller et al., 1988). Though soybean is widely cultivated in some parts of India, their microbial symbionts have been poorly investigated for the fundamental and applied aspects. It was, therefore, found desirable to conduct a systematic and scientific investigation on native soybean rhizobia from various cultivated fields of Uttar Pradesh, India towards obtaining a reliable understanding of the existing bradyrhizobial diversity in this region.

MATERIALS AND METHODS

Collection and isolation of native rhizobia

Soil samples and fresh root nodules were collected from 40-45 day old soybean plants grown in the fields of Allahabad (25° 28' N; 81° 54' E) and Kanpur (26° 28' N; 80° 24' E) districts of Uttar Pradesh, India. The soil texture was loamy at both the locations, whereas pH 7.1 and 6.7 were observed at the collection fields in Allahabad and Kanpur, respectively. Rhizobia were isolated from individual root nodules using standard method as described by Somasegaran and Hoben (1994). Strains were maintained routinely on yeast extract mannitol agar (YEMA; Vincent, 1970). In addition, two standard soybean rhizobial strains (USDA 94 and USDA 123), very kindly provided by Dr. Patrick Elia, USDA/ARS, Beltsville, MD, USA, were also used in this study. The collected rhizobial isolates were screened for nodulation on soybean seed grown under axenic condition. Only those rhizobial isolates which produced nodules on soybean plants were used to stock on YM-agar slants and maintained in growth chamber at 28 ± 2°C for further study.

Reaction of rhizobial isolates to Congo red Bromothymol blue

Stock solution of Congo red (CR) and Bromothymol blue (BTB) were prepared by dissolving 250 mg of the dye in 100 ml water and ethanol, respectively. Ten milliliter of stock was added to one litre of YMA in order to get the required concentration (25 µg ml^{-1}). A loop full of exponentially growing culture of each strain was streaked on three different agar plates (YMA, YMA+CR and YMA+BTB). All plates were incubated for 6-7 days in growth chamber at 28°C. Clear white colony was cloned and used for recording colony morphology data. In addition, colour change around growing colonies on YMA+BTB plates was considered for acid (yellow) or alkali (blue) production by the rhizobial strain.

Growth under different carbohydrates sources

The effect of four carbon sources, that is, mannitol, glucose, arabinose, sucrose on growth of rhizobia was studied in synthetic medium (SM) at a concentration of 1.0 g l^{-1}. Experiment was performed in 30 ml culture tubes containing 10 ml medium, inoculated with 0.1 ml washed bacterial culture. Growth in treatment tubes was compared with respect to increase in optical density after 5 days at 28 ± 2°C.

Growth under different Temperature and pH

Rhizobial growth at varying temperature regimes (20, 30, 35, 40 and 45°C) was compared in bacterial incubator by keeping YM agar plates inoculated with 10 µl culture (~10^8 CFU ml^{-1}). Growth experiment was also performed in YM broth. Optical density was measured after eight days of incubation at 660 nm. Similarly, growth of rhizobial strains was compared at different pH (4.0, 4.5, 5.0, 7.0, 9.0 and 10.0) in YM medium. Hydrochloric acid (HCl) was used to adjust lower pH and NaOH was used to adjust higher pH in medium. Twenty five (25) µl of exponential phase cultures (10^9 CFU ml^{-1}) was inoculated in 10 ml YM broth tubes, adjusted to different pH. Optical density was measured after eight days of incubation.

Rhizobiophage bio-assay

Each rhizobiophage strain produced distinct types of lytic zone (plaque) after infection in bacterial lawn. This has been clearly observed by double agar layer technique (Adams, 1959) where 0.2 ml of suitably diluted phage lysate was mixed with 0.1 ml exponentially growing bacterial culture in 3 ml molten YM agar (0.7 % Bacto-Difco agar) medium at 40°C. The mixture was gently shaken and poured over the surface of solidified nutrient agar plate. After hardening of the top layer, plates were inverted and incubated in culture room at room temperature. Plaques in plates generally appeared on bacterial lawn as a result of phage-lytic reaction after 6-7 days of incubation.

Antibiotic sensitivity and isolation of resistant mutants

Two antibiotics, that is streptomycin and gentamicin procured from Sigma Chemical Co., USA were used. The stock solution of both antibiotics was prepared in distilled water and solution was used after filtration through Millipore membrane (0.2 µm porosity). Sensitivity pattern was studied on YM-agar plate containing graded concentration of antibiotics.

For isolation of antibiotic resistant mutants double agar layer technique was employed. Log phage rhizobial cells collected by centrifugation (5000 x *g* 10 min) and washed twice with YM broth. 0.2 ml cell suspension containing about 10^9 cells were taken in 3 ml molten (45°C) YM agar (1%) tubes, followed by mixing with lethal concentration of freshly prepared antibiotic solutions (200 µg ml^{-1} streptomycin) and (150 µg ml^{-1} gentamicin). The entire content was

poured over previously prepared agar plates having respective concentrations of antibiotics. Mutant colonies appearing on plates after 15 days of incubation were isolated and purified by successive growth and cloning from respective antibiotic plate.

Isolation of phage resistant mutants

An effective streptomycin resistant mutant of strain USDA 123 and gentamicin resistant mutant of strain ALSR 12 were used for further isolation of mutants, resistant to corresponding phages. Log-phase cells of the bradyrhizobial strain were incubated in broth with high phage titer (10^8 PFU ml^{-1}) in the ratio of 1:10 and plated by double agar layer technique. Plates were incubated in culture room for 8-10 days, when resistant colonies appeared. Colonies were randomly picked up, grown in medium and purified (free from phages) by repeated streaking. Isolated clones were tested for resistance to respective antibiotics and phage strains.

Ultraviolet sensitivity

A 15 watt general electric germicidal lamp emitting 7.3 ergs per mm^2 per second principally at 260 nm was used as a UV source. Exponentially growing bacterial cultures were diluted to 10^7 colony forming unit (CFU) per ml in YM medium. Ten milliliter samples were withdrawn in 80 mm Petri dishes and irradiated with UV-light by constant agitation of the suspension. Samples of 0.1 ml were withdrawn at intervals of 15 s, diluted and assayed for viable cell counts (CFU). The entire process was conducted in red light in order to prevent photo-reactivation.

Estimation of e*x- planta* nitrogenase activity

Expression of nitrogenase activity in cultured bacterial cells was estimated on agar slant prepared by using CS-7 medium, after Pagan et al. (1975). The agar slopes were prepared in 15 ml assay tube containing seven ml of the medium. YM grown culture of each strain was centrifuged, washed with saline solution and then transferred to the nitrogen free agar-slope and incubated for 8 days at 28°C. After bacterial growth, cotton plug of each culture tubes was replaced with subaseals and 10% air of each culture tube was replaced with acetylene gas. All tubes were incubated for 24 h and ethylene gas formed in tubes was determined by gas chromatograph using hydrogen flame detector. Cell protein content was measured after Lowery et al. (1951) using Bovine serum albumin as standard. The nitrogenase activity was expressed as µ mol C_2H_2 reduced per mg cell protein.

Statistical analysis

Standard statistical procedures were followed for the analysis of data (Gomez and Gomez, 1984). Means were compared using standard deviation.

RESULTS

Rhizobium Phage susceptibility and their symbiotic assessment under control condition

All native rhizobial isolates were screened for sensitivity to eight lytic rhizobiophages. A total of 92 native isolates along with 2 exotic *Bradyrhizobium* strains were tested for phage typing. Only 34 rhizobia including 33 native isolates and one exotic strain (USDA123) were found susceptible to these phages. Based on phage sensitivity pattern and place of rhizobia isolation, a total of 9 strains corresponding to phage sensitive group and 10 strains belonging to different phage resistant groups were selected for evaluation of nodulation efficiency on soybean plants. Results presented in Table 1 revealed that all were able to form distinct nodules on roots of host plant. Exotic strains USDA123, USDA94 and indigenous strains ALSR12 and KNSR16 showed highly significant interaction with host plant in terms of nodule number $plant^{-1}$, nodule dry weight and total plant dry weight. Strain KNSR16 produced maximum nodule dry weight and total plant dry weight as compared to other strains, whereas strain ALSR12 performed well in producing highest nodule number per plant. Strains USDA123 and ALSR12 had distinct lytic reaction with phage strains SR1 and SR2, respectively, whereas strain USDA94 and KNSR16 were completely resistant to all tested phage strains. These four strains were selected for further characterization.

Colony morphology and acid- alkali reaction

The plaques formed by SR1 on USDA123 were quite clear with sharp margin, whereas those formed by phage SR2 on strain ALSR12 were having central halo surrounded by turbid concentric. The colony diameter of the rhizobial strains varied between 1.2 ± 0.13 mm (USDA 94) to 2.3 ± 0.21 mm (USDA123) after 8 days (Table 2). The colonies produced by these strains were conspicuously different in size and shape. All selected strains showed alkaline reaction on BTB plates. Exotic strains USDA123 and USDA94 produced spherical round shaped colonies whereas both native strains formed opaque dome shape colonies (ALSR12) and opaque gummy colonies (KNSR16) on agar plate containing congo red. The generation time of the rhizobial strains varied from 9.2 (USDA94) to 12.6 h (ALSR12) in YM broth (Table 2).

Study under different carbon sources

Growth of the strains was studied in different carbon sources in synthetic medium by replacing mannitol (Figure 1). Results indicate that all strains grew under different carbon sources. Among all tested carbon sources, arabinose was found optimal for growth of these bradyrhizobial strains. Strain ALSR12 showed maximum growth in arabinose compared to other strains. The least growth of strains was observed in glucose medium.

Growth behaviour at different temperatures

The effect of different temperatures on growth of the strains was recorded after 10 days of incubation (Figure 2). Results show that growth of strains was least at 20°C and 45°C. All strains grew well in the temperature range of 30-40°C. At 45°C, strain ALSR12 showed maximum growth, as compared to other strains.

Table 1. Phage sensitivity and symbiotic efficiency of exotic and native soybean rhizobial strains.

Bradyrhizobium strain	Reaction with bacteriophages*	Symbiotic efficiency on soybean cv. JS335		
		Nodule number plant^{-1}	Nodule dry weight (mg) plant^{-1}	Total plant dry weight (g)
USDA123	Sensitive	37.3±3.1	42.1±9.3	1.87±0.34*
USDA94	Resistant	42.7±7.1	51.3±7.4	1.96±0.61*
ALSR1	Resistant	26.3±2.7	21.7±3.1	1.54±0.09
ALSR3	Resistant	19.7±3.3	21.6±2.1	1.67±0.31
ALSR5	Resistant	28.3±1.2	22.9±1.8	1.83±0.29
ALSR6	Sensitive	18.5±1.7	19.3±1.5	1.53±0.32
ALSR10	Sensitive	21.3±0.9	20.7±1.61	1.66±0.28
ALSR12	Sensitive	51.3±5.2	60.7±13.4	2.26±0.82*
ALSR15	Resistant	20.0±3.1	18.3±2.7	1.57±0.52
ALSR16	Sensitive	23.3±4.3	22.3±2.1	1.72±0.48
KNSR2	Resistant	17.3±1.8	16.5±1.9	1.64±0.67
KNSR6	Resistant	18.7±1.3	17.3±2.5	1.80±0.74
KNSR7	Resistant	21.0±3.2	19.7±4.2	1.53±0.21
KNSR8	Sensitive	23.6±3.9	20.5±4.9	1.65±0.47
KNSR11	Resistant	27.7±4.3	21.3±5.1	1.60±0.36
KNSR12	Sensitive	28.3±4.1	22.6±6.2	1.69±0.41
KNSR16	Resistant	48.6±5.2	61.9±12.1	2.31±0.73*
KNSR20	Sensitive	22.1±5.1	17.3±1.7	1.53±0.61
KNSR26	Sensitive	26.5±3.2	22.5±3.6	1.93±0.56
Uninoculated Control	-	0	0	1.30±0.14
CD (1%)	-	8.3	9.6	0.18

*Phage sensitivity tested against six strains (SR1, SR2, SR3, SR4, SR6 and SR8) available in our laboratory ± standard deviation.

Table 2. Characteristics of the selected bradyrhizobial strains.

Bradyrhizobial strain	Strain origin	Susceptibility towards rhizobiophages	Colony characteristics on growth medium (YMA)		Generation time in YM (h)
			YMA+CR	Diameter at 8d (mm)	
USDA123	Exotic	Sensitive to only phage strain SR1	White spherical, round shaped	2.3 ± 0.21	9.2
USDA94	Exotic	Resistant	White round shaped	1.2 ± 0.13	12.6
ALSR12	Native	Sensitive to only phage strain SR2	White opaque, dome shaped	1.8 ± 0.23	10.4
KNSR16	Native	Resistant	White opaque, gummy	1.5 ± 0.19	11.2

Growth behavior at different pH

All rhizobial isolates showed best growth at pH 7.0. Below and above the neutral pH, a gradual decrease in growth was recorded (Figure 3). Strain ALSR12 has shown its capability to tolerate a higher range of acidity and alkalinity, as compared to others. Strain KNSR16 exhibited high sensitivity in growth to acidic and alkaline pH.

Antibiotic drug sensitivity

Sensitivity of four bradyrhizobial (two exotic and two native) strains against two antibiotics (streptomycin and gentamicin) was determined and results are presented in Figures 4 and 5. Inactivation pattern was nearly exponential with respect to the concentration of antibiotics. Strains ALSR12 and USDA123 showed high resistance

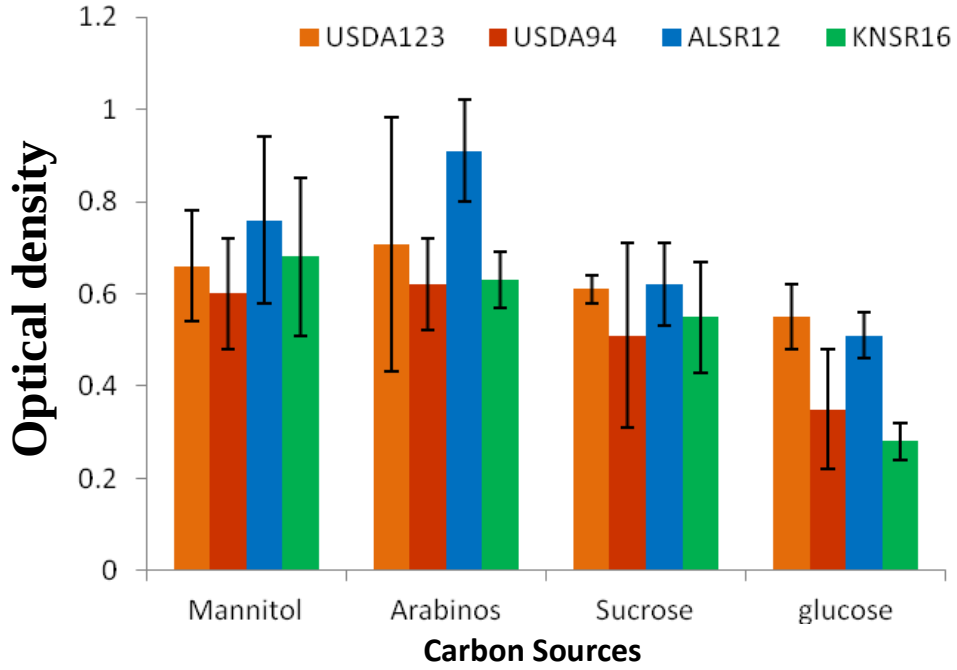

Figure 1. Effect of different carbon sources (1.0 g/l) on growth of the bradyrhizobial strains.

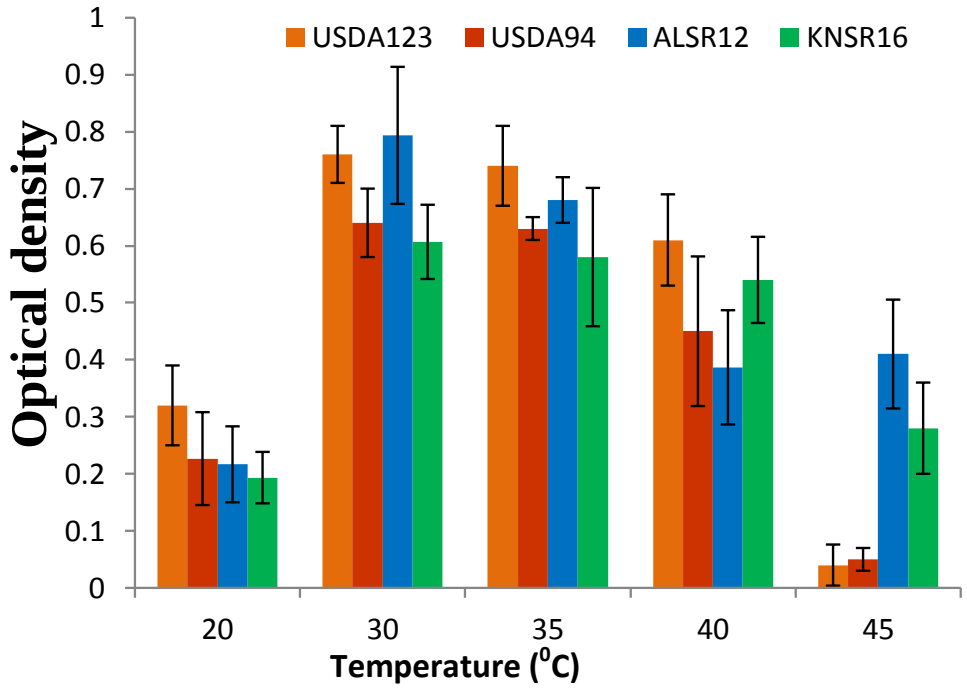

Figure 2. Effect of different temperature on growth of the bradyrhizobial strains.

level, whereas, strains USDA94 and KNSR16 displayed high sensitivity against streptomycin and gentamicin, respectively. Frequencies of spontaneously arising mutants for resistance to high level of streptomycin (200 µg ml^{-1}) and gentamicin (150 µg ml^{-1}) was determined in the two phage marked rhizobial strains (USDA123 and ALSR12), which varied significantly. Mutation frequency was low (3.0×10^{-8}) for gentamicin- resistance in strain ALSR12, whereas it was high (7.3×10^{-8}) for streptomycin-resistance in strain USDA123.

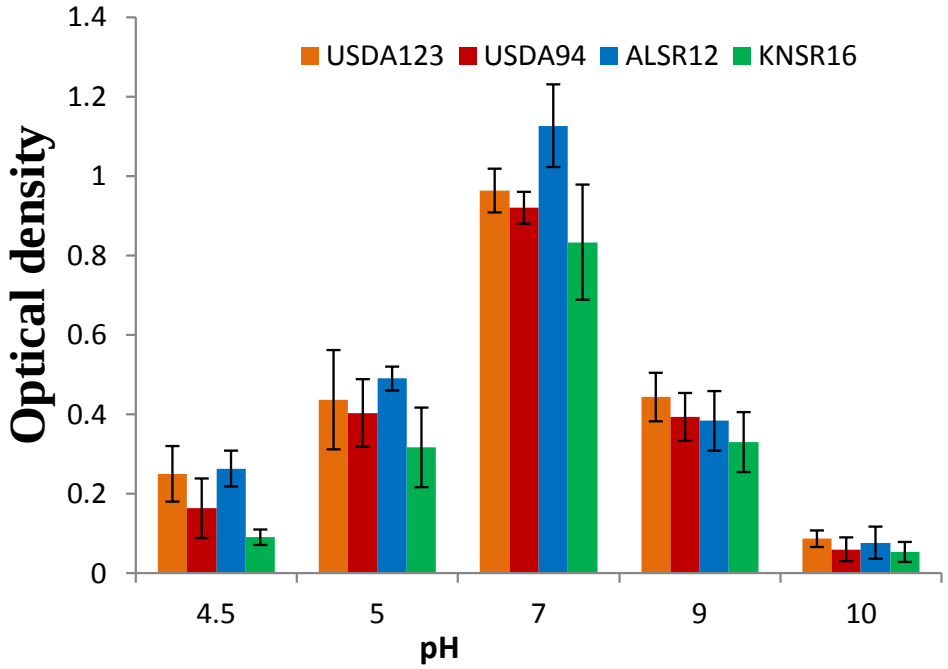

Figure 3. Effect of different pH on growth of the bradyrhizobial strains.

Ex-planta N_2-ase activity

Induction of nitrogenase activity under free living state in two exotic (USDA123 and USDA94) and native (ALSR12 and KNSR16) bradyrhizobial strains and their derived mutants was determined. Results presented in Table 3 indicates that nitrogenase activity was expressed only in two strains, that is USDA94 and ALSR12, and induction was more in strain USDA94 than that in ALSR12. The isolated gentamicin and phage resistant mutants of ALSR12 also showed expression of nitrogenase activity comparable to parent strain under cultured state.

Effect of UV-light

Effect of UV-light on survival and induction of antibiotic resistant mutants in two bradyrhizobial strains was studied. D_{37} value indicates that strain ALSR12 is nearly two times more resistant than USDA123. Survival pattern against UV-dose in strain USDA123 was exponential while it was sigmoidal in ALSR12. Mutation frequency increase towards streptomycin resistance in strain USDA123 was recorded high and exponential with UV-doses than gentamicin resistance in strain ALSR12.

DISCUSSION

In the present investigation a total of 34 out of 94 rhizobial strains showed susceptibility to isolated phages. The high degree of lytic specificity of phages suggests that they may impose very strong selection pressure among soil inhabiting rhizobia. Recently, Vos et al. (2009) have suggested that the biotic interaction, in addition to variation in physical environment plays a crucial role in the small scale spatial structuring of microbial diversity in soil.

Generally rhizobial strains having generation time below 6 h are called fast growers, while those doubling in more than 6 h are designated as slow growers (Amarger, 2001). Xu et al. (1995) reported the extra slow growers having generation time of 16 to 40 h, isolated from nodules of soybean collected in People's Republic of China. The generation time of the four rhizobial strains used in the study varied in between 8.8 to 12.2 h in YM. Thus, all the strains were categorized as slow growers so as to be safely grouped under *Bradyrhizobium* species. Colony size in rhizobial strains varied from small to large. These differences in colony morphology of *Bradyrhizobium* isolates have been reported by earlier workers (Boody and Hungria, 1997; Fremont et al., 1999). All strains produced large colonies on agar plate ranging from 1.2 ± 0.13 to 2.3 ± 0.21 which is contrary to earlier results that fast growers produce large colonies and slow growers produce small colonies (Upchurch and Elkan, 1977; Bromfield and Kumar Rao, 1983; Mullen and Wollum, 1989). Colony size variation has been utilized as the primary way for differentiating rhizobial isolates of soybean (Desa et al., 1997; Martins et al., 1997; Boddey and Hungria, 1997), cowpea (Eaglesham, 1987; Mpepereki et al., 1997), peanut (Yang et al., 2005) and other diverse legumes. The selected strains had ability to grow well when arabinose was used as the carbon source, which suggests that all strains possess disaccharide hydrolytic enzyme (Glenn and Dilworth, 1981).

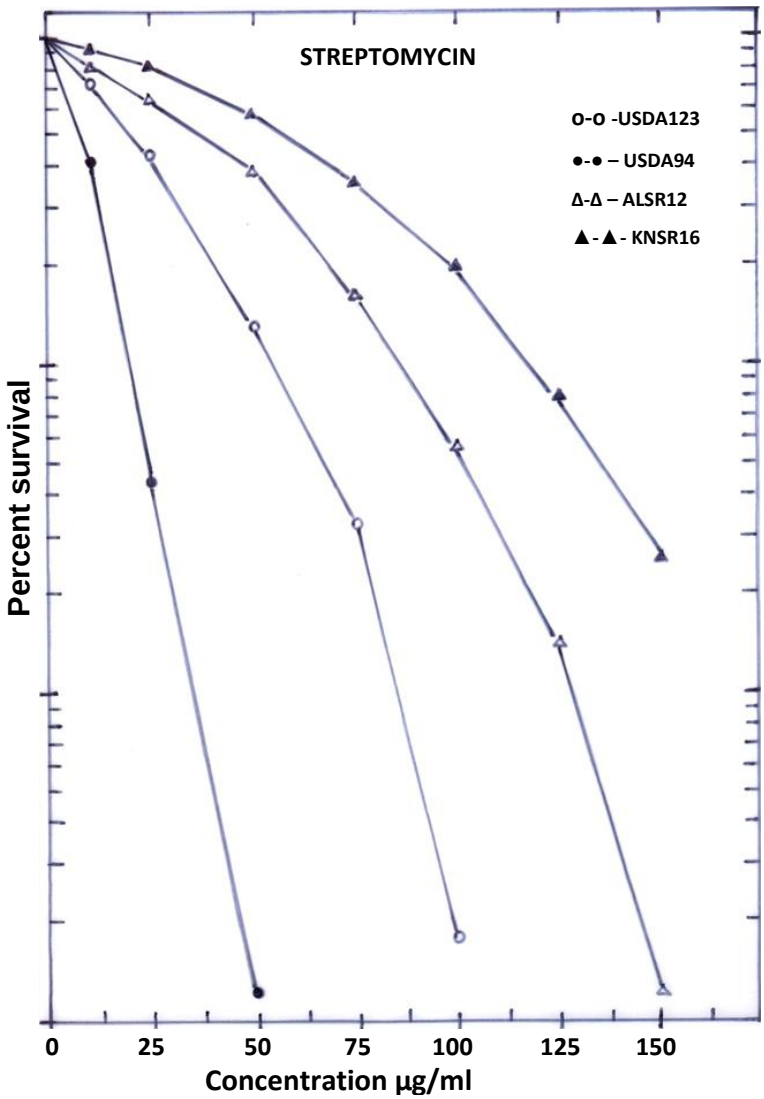

Figure 4. Inactivation pattern of selected bradyrhizobial strains.

The variable response of rhizobial strains towards tolerance to abiotic stresses like temperature and pH was observed. Such differences have been reported earlier with slow-growing rhizobial strains to higher temperature (Graham and Parker, 1964; Munevar and Wollum, 1989; Hartel and Alexander, 1984) and acidity (Graham et al., 1982; Hartel and Alexander, 1984; Graham et al., 1994; Elidrissi et al., 1996; Surange et al., 1997). Strain ALSR12 was able to survive and grow at higher temperature (45°C) which might be related to its greater energy requirements for maintenance of vital life processes (Dawes, 1985), and in turn might promote tolerance against heat stress (Bordeleau and Prevost, 1994). Heat tolerant strains have constitutive production of glyceraldehydes-3-phosphate dehydrogenase, alcohol dehydrogenase and phospho-fructokinase leading to emergence of enough disulphide bonds which strengthen the cellular membranes and organeller plasma membrane to stop heat permeation and save the vital cellular process (Fontana et al., 1976). Such strains have been found to have a high cross-resistance to protein denaturants (Fontana, 1984). There is, thus, a wide scope of testing the thermo tolerant bradyrhizobial strains with those biochemical parameters in order to raise the heat tolerance level through mutagenesis for resistance to protein denaturants. Studied strains varied widely in their pH tolerance from 4.5 to 10.0. The fast growing *Rhizobium* strains have generally been considered less tolerant to acid pH than slow growing strains of *Bradyrhizobium* strains (Graham et al., 1994). Two strains grew at 4.5 pH too, which aligned to the result of Mpepereki et al. (1997) that fast-and-slow growing *Bradyrhizobium* strains of *Vigna unguiculata* are tolerant to pH values as low as 4.0 and the rhizobia adapt various mechanisms to survive in the acid soil conditions (Zahran et al., 1999). Tolerance to high alkalinity has also been observed previously for rhizobial

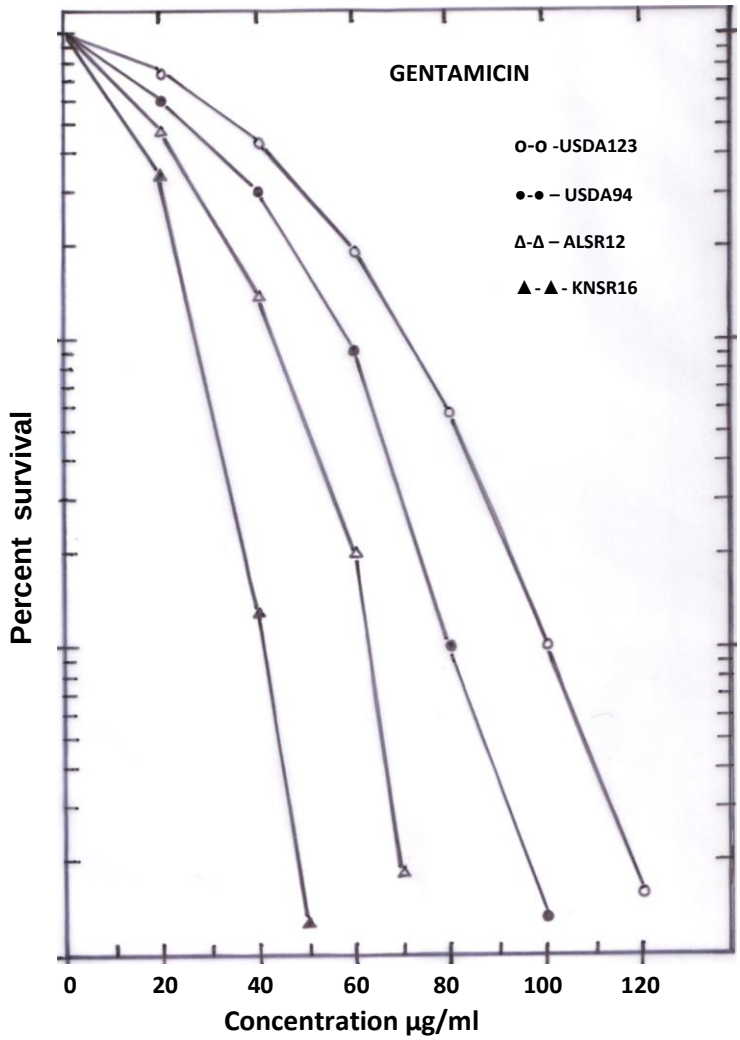

Figure 5. Gentamicin sensitivity pattern of selected bradyrhizobial strains.

Table 3. Expression of *ex-planta* nitrogenase activity in bradyrhizobial strains.

Bradyrhizobial strain	Nitrogenase activity*
USDA123	0
USDA94	102.5 ± 7.2
ALSR12	86.6 ± 3.6
KNSR16	0
USDA123-SMR	0
ALSR12-GMR	90.6 ± 4.4
USDA123-SMR-VR	0
ALSR12-GMR-VR	74.9 ± 5.4

*n mol C_2H_4 formed h^{-1} mg^{-1} protein

isolates.

Jordan (1984) reported tolerance of up to pH 9.0 for *Rhizobiaceae*. Apparently, therefore, the tolerant rhizobial strains can be further looked into and used as efficient inoculants in extreme environmental condition of India.

The intrinsic antibiotic resistance pattern towards streptomycin and gentamicin in two rhizobial strains differed significantly from each other. None of the strain showed a

high level (200 µg ml^{-1}) of resistance to the tested antibiotics. All rhizobial strains exhibited high sensitivity to gentamicin, as compared to streptomycin. Similar variations in IAR pattern among strains of fast growing rhizobia were reported earlier by several workers (Graham, 1963; Pankhurst, 1977; Hagedorn, 1979; McLaughlin and Ahmad, 1984; Sinclair and Eaglesham, 1984). The variation pattern in intrinsic antibiotic resistance (IAR) level has been used successfully to differentiate rhizobial strains of common bean (Josey et al*.,* 1979; Beynon and Josey, 1980).

Induction of nitrogenase activity in the parent and mutant rhizobial strains was estimated in agar grown cultures and recorded strain specificity. Expression of nitrogenase activity was noticed in one used native rhizobial strain (ALSR12) and all its derived antibiotic as well as phage resistant mutants. Induction of nitrogenase activity in *Rhizobium* cells in absence of plant (*ex-planta*) has mainly been demonstrated earlier with certain strains of slow growing *Rhizobium* groups (Pagan et al., 1975; McComb et al., 1975; Kaneshiro et al., 1978).

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