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Response of Chickpea to Inoculation with Genetically Improved and Efficient *Rhizobium* Strains

Bontay

KEYWORDS :

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ABSTRACT

There is considerable interest in improving nitrogen fixation in legumes to increase soil fertility, particularly in the developing countries which help the plant to grow better and produce more. The effect of inoculation of chickpeaseed with rhizobium strain G-567 was examined under laboratory and field conditions.In laboratory, we have foundG-567 is a slow grower (generation time 10 h) which forms distinct colonies (white, opaque compact, dome shaped) on CR and BTB plates. The strain G-567 was screened in vitro for their symbiotic performance activities using assays multi drug test, rhizobiophages infection test,amplification and sequencing procedure as well as field condition to determine symbiotic effectiveness.

Derived phage resistant mutants (kanrvrtetrvr) showed symbiotic specificity with chickpea cultivars. Strain kanrvr revealed highest seed yield with chickpea cv. Radhe (14.8%), as compared to un-inoculated control. Yield increase recorded was approximately equivalent to 40 kg N-treatment (16.6%) above control.

It is, therefore, concluded that comparative functional analysis of chickpea rhizobia can be genetically improved through achieving resistance to various antibiotics and rhizobiophages, towards their sustained utilization in BNF-based pulse breeding programmes for improving symbiotic parameters in Cicer-Rhizobium mutualistic association, leading to significant grain yield enhancement.

Introduction

About a dozen of pulses are grown in India. The most important major pulse crops under cultivation in India are chickpea, pigeonpea, urdbean and lentil. India accounts for 72% of chickpea, and 90% of pigeonpea. These account together for 46% of cultivated area and 58% total production of pulses in India. Chickpea (Cicer arientinum) is a major source of protein for human consumption. It provides high quality crop residues for animal feed and helps to maintain soil fertility through biological nitrogen fixation (Herridge et al., 1995; Siddiqi and Mahmood, 2001; Kantar et al., 2007).

Rhizobium-legume symbiosis is the major source of biologically fixed nitrogen in agricultural system. Rhizobium is a gram negative, rod shaped, aerobic bacteria of family Rhizobiaceae and forms morphologically distinct nodules on the roots of leguminosae. Barring exception, this symbiosis is highly specific (Fred et al., 1932). Rhizobial ability to induce the formation of swollen structure, known as nodules on the roots of pulses, is followed by the reduction of atmospheric nitrogen into ammonia. This mutualistic symbiotic relationship between rhizobia and legumes is the most important biological mechanism for providing nitrogen to the plants as an alternative to the energy expensive ammonium fertilizer. Inoculation of leguminous seeds with the selected rhizobial strains is being widely practiced to ameliorate the plant yield by enhanced root nodulation and nitrogen uptake by the plant. Due to their paramount environmental and agriculture significance, these legume symbionts are being extensively characterized, and the assessment of diversity within rhizobial natural populations in various regions of the world has received increased attention (Rai et al., 2012).

It is now known that in Rhizobium-legume symbiosis there are at least eight distinct successive events which lead to the formation of nodules. These events are genetically controlled and have been recognized as root colonization root adhesion, root hair branching, root hair curling, formation of infected thread, nodule development, intercellular, release of bacteria into the plant cell bacteroid development (Vincent, 1980).

The rhizosphere is a habitat for a vast interactive community of rhizotrophic microorganisms whose activities largely determine the physiochemical properties of the rhizosphere soil (Zaidi et al., 2003). A continuous interaction exists between the plant root and the rhizotrophic microorganisms of different groups that exert an important influence on plant growth (Saxena and Tilak, 1994). Increased yield of various legumes after inoculation with nitrogen (N2) fixing microorganism have been reported earlier (Rajput et al., 1993; Wange and Patil, 1966). Nitrogen fixing microorganisms not only provide fixed N2 to the plants but also improve nitrogen (N) status of the soil. The increasing interest in nitrogen-fixing organisms is related to the fact that nitrogen is often the limiting factor in crop production (Alexandre et al., 2006). Rhizobia are taxonomically diverse and form a phylogenetic heterogeneous group.

Since the specific rhizobia is need to estabalish an effective association with plants, hence rhizobial inoculation is essential to ensure that a large and effective rhizobial population is available in rhizosphere of the plant. The success of the rhizobial inoculation is limited by several chemical, physical and biological factors of the soil as well as environmental factors. Among the many factors that affect soil population of rhizobia, parasitism and predation have been studied to a limited extent. Bacteriophage infecting rhizobia frequently occur in soils where legume is grown. Phages are ecologically important due to their profound effect on the relative proportions of different bacterial species or strains in a community. These are specific against different groups of soil bacteria that can form root nodules or both root & stem nodules on various legumes (Dhar and Ramakrishna, 1987; Dhar et al., 1993; Malek et al., 2005; Sharma et al., 2002). Existence of phages suggest that they can affect the outcome of legume-rhizobium symbiosis by reducing the population density of susceptible nodule bacterial strains in the soil and provide selective pressure for the evolution of less effective rhizobial variants that generally cause diminution of efficiency of symbiotic nitrogen fixation (Kleczkowska, 1971). In contrast, Kowalski et al. (2004) have indicated that the presence in rhizobial and phage mixture did not affect the symbiotic efficiency. Rhizobiophages are very specific to their host strain for which they have served as authentic genetic markers for differentiating rhizobial strains in chickpea (Dhar and Ramakrishna, 1987), pigeonpea (Dhar and Kumar, 1998), frenchbean (Dhar et al., 1993), cowpea (Singh et al., 1980), soybean (Hashem et al., 1996; Ali et al., 1998) and some other legumes.

Symbiotic genes are typically located in mega plasmids, known as pSyms (Brom et al., 2002; Garcia-de los Santos et al., 1996).

Non symbiotic plasmid may encode locally adaptive traits that confer phenotypic advantages, such as heavy metals or antibiotic resistance genes (Alexander et al., 2006). In evolutionary terms, plasmid encoded genes have the advantage of being more easily exchangeable within a certain population of the genes located in the chromosome (Downie, 1997). The study of rhizobial diversity has been achieved using several phenotypic and molecular approaches. Identification method that often lead to phylogeny inference include DNA-DNA hybridization, 16S rDNA sequencing (Stackebrandt et al., 2002), and 16S rDNA RFLP analysis (Laguerre et al., 1994).

MATERIALSAND METHODS

Isolation of Rhizobium and Rhizobiophage Strains:

A phage indicator *Rhizobium* strain G-567 and its phage RC-1 used in this study were obtained from Microbial Genetics Laboratory of the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. The rhizobial strain was grown on Yeast-Mannitol (YEM) medium (Vincent, 1970), the composition of which is presented in Table 1, at 28±2^0 C in a growth chamber. Bacterial cultures were maintained on the respective slants.

Media and growth condition

Strains were routinely maintained on yeast extract mannitol (YEM) agar slants (Vincent, 1970) and kept at 4°C. Purity was assured by routine plating on YEM agar supplemented with Congo red and selecting uniform colonies. The basal growth medium is that described by Vincent (1970). Primary cultures in 10 ml medium were started form agar slants and incubated at 28°C with shaking until the early logarithmic phase was reached. These cultures provided inocula for the experimental cultures that were also grown with at 28°C in 25 ml in 100 ml Erlenmeyer flasks, with 1 % inoculums.

Seed Inoculation

The Rhizobial strains were cultured in yeast extract mannitol broth until it reached the late logarithmic phase of growth. The inoculums were applied at an initial population level of 1.8×10^6 CFU seed^{-1}.

Reaction of rhizobial isolates to Congo red Bromothymol-blue:

Stock solutions of Congo red (CR) and Bromothymol blue (BTB) were prepared by dissolving 250 mg of the dye in 150 ml water and ethanol, respectively. Ten ml of stock was added to one liter of YMA in order to get the required concentration (25μg ml^{-1}). A loopful 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 3-4 days in growth chamber at 28°C (Fig 1.). Clear individual 20 colonies were taken for recording colony morphology data of each strain. In addition, change in color around individual colonies on YMA+BTB plates considered for acid (yellow) or alkali (blue) production by the rhizobial strain.

Growth under different carbohydrates and nitrogen sources:

The effect of five carbon sources (mannitol, glucose, arabinose, maltose and sucrose) on growth of rhizobial strain was studied in synthetic medium (SM) of O'gara and Shanmugam (1976), the composition of which is presented in Table 2, at a concentration of 1.0 g l^{-1}. Experiments were performed in 30 ml culture tubes containing 10 ml medium at 28±2^{0}C for 7 days. The data on optical density at 600 nm were harnessed with digital spectrophotometer (Electronic India 305). In the similar manner, the effect of different N-sources on rhizobial growth was studied in medium by replacing sodium glutamate (5mM) with suitable concentration of organic nitrogen sources, *i.e.*, potassium nitrate (1 mM), sodium nitrite (0.5 mM), ammonium sulphate (1mM), and urea (1 mM).

Antibiotic sensitivity and isolation of resistant mutants:

Sensitivity of the rhizobial strains to the antibiotics (kanamycin and tetracycline) was determined in YEM broth having graded concentrations of an antibiotic. For isolation of antibiotic resistant mutants, exponentially growing rhizobial cells were harvested by centrifugation (3000 g; 10 minutes) and washed twice with saline solution (1 m M NaCl). Cell suspension (0.2 ml) containing about $5x10^8$ cells ml^{-1} were suspended in 3 ml molten (45^{0}C) YEM –agar (1%), followed by addition of freshly prepared sterilized solutions of kanamycin (25, 50, 75, 100, 150, 200, 500, 600 and 700 μg ml^{-1}) / tetracycline (5, 10, 15, 20, 25 and 30 μg ml^{-1}). The entire content was poured over previously prepared YEM-agar plate having respective concentration of antibiotic. All plates were incubated in culture room at 28±2^{0}C for 15 days. Resistant colonies were picked up and purified by repeated streaking and maintained on agar slant containing antibiotic.

Isolation of phage resistant mutants:

The isolated kanamycin (200 μg ml^{-1}) and tetracycline (25 μg ml^{-1}) resistant clones of chickpea *Rhizobium* strain G-567 were used for isolation of phage (RC-1) resistant mutants. Exponentially growing cells were incubated with high phage titer (10^8 PFU ml^{-1}) in both with ratio of 1:10 for 1 h and contents were plated by double agar layer technique. Phage resistant colonies appeared on plates after 7-8 days of incubation. Colonies were randomly picked up and freed from phage by repeated sub cloning. Clones revealing resistance to both phage and kanamycin or tetracycline were selected for further studies on YEM broth.

DNA isolation, PCR amplification and purification of PCR products:

Total genomic DNA of each strain was extracted from bacterial cells grown in YEM broth until late exponential phase (10^9 cells ml^{-1}). Extraction of DNA was performed by a standard protocol (Appunu and Dhar, 2008). Purified DNA was dissolved in 10 mM Tris-HCl buffer containing 1 mM EDTA (pH 8.0).PCR reaction was carried out in final volume of 25 μl reaction mixtures containing 12.5 μl PCR master mix (Fermentas, USA), 1 μl of each primer (10 pM) of Operon USA, 1 μl (50 ng) genomic DNA and 9.5 μl sterilized water. The forward primer (5'GGAGAGTTAGATCTTGGCTC3') and reverse primer (5'CACCGCTACACCAGGAATTC3') of Operon, USA were used to amplify nearly 650 bp of 16S rRNA genes. Amplification was done by using a standard temperature profile, in a Thermo cycler, GeneAmp PCR System 2720 (Applied Biosystem, USA), including initial denaturation at 94^{0}C for 1 min, annealing at 57^{0}C for 1 min, and extension at 72^{0}C for 2 min, and a final extension at 7 min. The Amplified products were visualized on 1.5% of agarose gel.

Restriction digestion of 16S rDNA:

The amplified products of 16S rDNA regions of G-567 parent, tet^{r}v^r (tetracycline-resistant, virus-resistant) and kn^{r}v^r (kanamycin-resistant, virus-resistant) were digested completely with five restriction enzymes (Fermentas, USA) independently, *i.e.*, *Dde*I, *inf*I, *Hpa*I, *Nde*I and *Taq*I following the manufactur's instructions. The resulting DNA fragments were separated by horizontal gel electrophoresis on 2.5% agarose gel.

Field trail for evaluation of symbiotic effectiveness of mutant strains:

A trail was conducted at Agriculture farm, BHU main campus during Nov., 2012.One chickpea variety (Radhe) procured from the Department of Agronomy, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, was used in this study. Treatment consisted of five chickpea *Rhizobium* strains (G-567 parent, kn^r, tet^r, kn^{r}v^r and tet^{r}v^r), 60 kg N_2/ha and un-inoculated control. Field layout was in randomized block design having plot size (3**x**1.8 m^2 with 6 rows of 30 cm apart) with four replications. Data pertaining to nodule number, nodule dry weight, plant dry weight and nitrogenase activity (μ mol C_2H_2 production g^{-1} nod-

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ule h^{-1}) were recorded after 45 days and grain yield data was collected at the time of harvest. For plant dry weight each plant was kept separately in a paper bag and dried at 60^0C.

Nitrogenase Activity:
Nodulated plants were collected from each plot after 45 days of sowing. Nodules from each plant were freshly placed in 30 ml assay flask, incubated with 10% acetylene for 1 h. 0.5 ml gas sample of each flask was analyzed through Gas Chromatograph (Nucon, model 5765) equipped with hydrogen flame detector. The values were expressed as μ mol C_2H_2 production g^{-1} nodule h^{-1}, calculated as follows:

Stastistical Analysis:
Standard statistical procedure was followed for the analysis of data (Gomez and Gomez, 1984). Means were compared using critical difference.

RESULTS
The chickpea *Rhizobium* strains G-567, sensitive to rhizobiophage RC-1, formed distinct colonies reaching a diameter of 2.6 mm after 5 days of inoculation on YEM agar plate. In the presence of dye congo-red, it formed white opaque, compact, dome shaped colonies (Fig. 1). Rhizobial colonies gave alkaline reaction on YEM+BTB plates by the changing colour of agar medium from green to blue. Growth kinetics of the *Rhizobium* strain was studied in YEM medium. The generation time calculated was 10.0 h.

The rhizobiophage RC-1 produced clear plaques on *Rhizobium* strain G-567 when plated by double agar layer method. Homogenous plaques appeared in bacterial lawn within 48 h and had distinct morphology, showing central hallow and hazy margin (Fig. 2). Plaques attained 2.5 mm diameter after 3 days of incubation at 28^0C.

Antibiotic sensitivity of the rhizobial strain was determine in medium containing different concentration of kanamycin (25, 50, 75, 100, 150, 200, 500, 600 and 700 μg/ml) and tetracycline in (5, 10, 20, 25 and 30 μg/ml). Growth of the strain was inhibited with increasing concentrations of the antibiotics. No growth was observed at 50 μg/ml concentration of kanamycin (Fig. 3). The strain was found highly sensitive to tetracycline, since its 25 μg/ml concentration was found lethal (Fig. 4). Mutants resistant to kanamycin (200 μg/ml) and tetracycline (25 μg/ml) were isolated independently by plating high population of strain with lethal concentrations of the concerned antibiotics. Out of 2.16×10^{10} viable cells (CFU/ml) plated, only 82 and 70 colonies appeared on plates having 200 μg/ml kanamycin and 25 μg/ml tetracycline, respectively. The mutants were raised spontaneously with an approximate frequency calculated to be 3×10^{-9}. Both of the isolated antibiotic resistant mutants produced similar types of colonies as parent on agar medium. Both antibiotic resistant mutants showed sensitivity to phage RC-1 and formed plaques similar as to that of the parent strain. Mutant resistant to kanamycin (kn^r) and tetracycline (tet^r) were used for isolation of phage resistant clones. Phage resistant colonies were isolated randomly from antibiotic + phage containing plates, and colonies were purified by repeated cloning. Finally mutant clones showing resistance to both phage and kanamycin/tetracycline were used for further studies.

Growth of all the three strains of *Rhizobium* G-567 (Parent, kn^rv^r and tet^rv^r) were compared in YEM containing lethal concentrations of antibiotics and phage. Both mutants (kn^rv^r and tet^rv^r) showed growth only in the presence of respective antibiotics and phage (Fig.5). In the presence of phage, parent strain was unable to grow whereas the growth of kn^rv^r was better than tet^rv^r.

Effect of carbon and nitrogen sources on growth of parent and derived phage resistant mutants was studied in synthetic medium. Growth in different carbon sources (arabinose, glucose, sucrose, maltose) was compared with mannitol (1 gl^{-1}). Parent and tet^rv^r mutant showed almost parallel growth as in mannitol. Both mutants tet^rv^r and kn^rv^r grew better in arabinose compared to other carbon sources. Mutant tet^rv^r exhibited highest growth in arabinose and lowest in sucrose. (Fig. 6).

Growth rate of parent, kn^rv^r, and tet^rv^r was also compared in different nitrogen sources (Na-glutamate, $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ and Urea). Growth of these strains was distinctly different in inorganic nitrogen sources than Na-glutamate (Fig. 7). Parent and kn^rv^r strains were unable to grow in nitrite medium while tet^rv^r strain grew well. Strain tet^rv^r utilized all tested nitrogen sources for its growth. Mutant tet^rv^r exhibited highest growth rate in nitrate medium compared to other n-sources. Growth of parent and kn^rv^r mutant was comparatively less in presence of inorganic than organic (sodium glutamate) nitrogen.

The genomic DNA of parent and its derived mutants (kn^rv^r and tet^rv^r) was extracted and amplify by PCR using forward and reverse primers of 16S rDNA. The amplified products, as visualized in agarose gel, yielded single band of nearly 650 bp (Fig. 8). The resultant PCR product was digested separately with five restriction enzymes (*Dde*I, *Hinf*I, *Hpa*I, *Nde*I and *Taq*I). Only three enzymes (*Hinf*I, *Hpa*I and *Taq*I) were able to digest the amplified 16S rDNA of these rhizobial strains. *Hinf*I was unable to digest the 16s rDNA region of sm^rv^r whereas *Taq*I enzyme did not cut the 16S region of both parent and mutant kn^rv^r. Only *Hpa*I was able to digest the 16S rDNA amplified region of all the three strains. All digested bands were polymorphic in nature. Total 26 bands were observed by the 16S rDNA RFLP analysis which showed that Jaccard similarity coefficient between mutants (tet^rv^r and kn^rv^r) was 0.57, whereas it had 0.36 between parent and both mutants. (Fig. 9).

A field trail was conducted to determine symbiotic effectiveness of *Rhizobium* strain G-567 and its four derived mutants (kn^r, tet^r, kn^rv^r and tet^rv^r) along with an N-treatment (40 kg Nha^{-1}) on the chickpea cultivar, Radhe. (Table. 4). Nodulation data (45 days) revealed significant response of *Rhizobium* strain inoculation on both chickpea cultivars. Plant growth and close-up view of root system bearing nodule formed by parent and derived phage-resistant mutants are shown in (Fig 10 and 11). Nitrogenase activity in plant – nodules taken from different treatments was determined by gas chromatography. Data on acetylene reduction activity (ARA) by nodules has been expressed in (Table. 5). Similar to parent, both phage resistant mutants interacted specifically with the chickpea cultivar. The analysis of variance showed that effect due to strains was significant for all symbiotic parameters on chickpea *cv*. Radhe (Table 3). Both phage resistant mutant strains derived from antibiotic resistant parent produced significantly higher nodule dry weight, nodule nitrogenase activities $plant^{-1}$, plant dry weight and seed yield, as compared to the parent strain G-567 and un-inoculated control (Table 2). Phage resistant mutant tet^rv^r showed highest (110%) nodule nitrogenase activities followed by kn^rv^r (92%) and tet^r (83%), as compared to the control. In terms of seed yield, strain tet^rv^r (1287) was at par with 40 kg N ha^{-1} treatment (1278), which is approximately 14% higher than un-inoculated control (Table 4).

DISCUSSION
In the present investigation a phage indicator chickpea *Rhizobium* strain G-567, producing homogenous colonies on YEM plate and displaying a doubling time 10 h was used for isolation of genetically marked phage resistant mutants. This strain belongs to slow growing species of *Rhizobium* nodulating chickpea (Amarger, 2001), unlike fast growing chickpea rhizobia which were reported to produced their colonies every 2-5 days and have generation time less than 6 h. Strain G-567 gave alkali re-

action in YMA plate containing bromothymol blue which is in agreement with the report of Fred *et al.* (1932) and Noris (1965) that slow growing rhizobia produce alkali while the fast growing produced acid. Survival of applied rhizobia on seed and nodulation in the field is generally more effective when applied as solid based inoculants. When regulation exists, minimum standards at the time of inoculants expiry are 10^6 to 10^8 cells g^{-1} of inoculants. The most widely accepted standard for numbers of rhizobia per seed are 1000. Burton has recommended 1000 for fast-growing rhizobia and 100,000 for slow-growing rhizobia. The safest quality standards could be applied to inoculated seeds, where 10^5 rhizobia per large seed (*e.g.*, soybean, lupin), 10^4 rhizobia per medium seed (*e.g.*, Mungbean, pigeonpea) and 10^3 per small seed (*e.g.*, clover, siratro) are required.

As according to a report from Stowers (1985), rhizobia are chemo-organotrophs that have long been known as being able to utilize a variety of carbon and nitrogen compounds for their growth. Strain G-567 and its mutants exhibited the ability to utilize all five tested carbon sources but they were able to utilize monosaccharide more as compared to disaccharide which support the result reported by Chakrabarti *et al.*(1981) and Stowers and Eaglesham (1984).This suggests that isolation of slow growing rhizobial strain from *Cicer arietinum* utilizes disaccharide hydrolytic enzyme and uptake systems similar to those reported for the fast growers (Glenn and Dilworth, 1981) which metabolize a wider variety of carbohydrates than slow growers (Amarger, 2001). Besides sugars, all derived mutants of strain G-567 utilized nitrate, ammonium, urea and nitrite as a sole source of nitrogen for their growth which supported the result of Amarger (2001) that rhizobia can use nitrate, ammonium or amino acid as sole source of nitrogen, while rhizobia isolated from stem nodules of *Sesbania rostrata* utilized nitrogen for growth (Dreyfus *et al.*, 1998).

The RFLP analysis with five restriction enzymes of 16S rDNA regions of the genome of parent and antibiotic marked phage resistant mutants (kan^rv^r and tet^rv^r) was capable to diversify all the derived mutants and proved to be a useful method for grouping the isolates (Alexander *et al.*, 2006). This rather provides a rapid tool for identification of root nodule isolates and the detection of new taxa. It is, thus, a potent tool for determination of their known relatives (intra-species) (Laguerre *et al.*, 1994).

The symbiotic effectiveness of parent and all its derived mutant clones was tested with chickpea cultivar under field conditions. The study between chickpea cultivars and the different clones of a rhizobial strain indicates the existence of considerable host cultivar specificity. All six treatments (G-567 parent, kn^r, tet^r, kn^rv^r, tet^rv^r and 40 kg Nha^{-1}) showed significant symbiotic effectiveness with both chickpea cultivars (in relation to yield). The results clearly indicate that nodule number did not bear significant correlation with nodule dry weight, plant dry weight, Acetylene Reduction Activities (ARA) and yield, which support the report of Somasegaran and Hoben (1994) that nodule number is a less reliable indicator for clone effectiveness in legume host crop in general.

The effectiveness of the kanamycin and tetracycline resistant mutants was not as same as its parent with cultivars. It possesses significant symbiotic effectiveness with the chickpea cultivar Radhe with respect to yield and other used symbiotic characters. Antibiotic resistant mutants derived from both fast and slow growing rhizobia have been reported to exhibit variable symbiotic effectiveness and also competitive ability (Schwinghamer, 1967; Kowalska, 1971; Pankhurst, 1977; Pain, 1979; Bromfield and Jones, 1980; Sindhu and Dadarwal, 2001).

Symbiotic effectiveness of the antibiotic – marked phage resistant mutants (kn^rv^r and tet^rv^r) showed significant variations in their effects noted with the chickpea cultivar, Radhe, as compare to the parental strain inoculation. The kn^rv^r and tet^rv^r mutants were found superior to the parent strain in term of symbiotic parameters which in contrast to the results reported by Krassilnikov (1941) and Kleczkowska (1971) that phage resistant mutant was ineffective or partially effective (Patel, 1978). The results of the present investigation clearly indicates that the genetically marked phage resistant mutant (kan^rv^r and tet^rv^r) derived from phage sensitive chickpea *Rhizobium* strain G-567 had significantly higher symbiotic effectiveness compared to parent, indicating that acquisition of phage resistance character by a mutant may improve the symbiotic effectiveness of the strain with their host.

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REFERENCE

Alexander, A., Laranjo, M. & Oliveira, S. 2006. Natural population of chickpea rhizobia evaluated by antibiotic resistance profiles and molecularMethods.SpringerSciences+Business Media, Inc.,51: 128-136. | Ali et al. 1998.Polyvirulentrhizobiophages from a soybean rhizosphere soil, Soil BiolBiochem, 30:2171-2175. | Amarger, N. 2001. Rhizobia in the field.Adv.Agron.73: 109-168. | Brom, S., Girad, L., Gracia-de, los, Santos, A., Sanjuan-Pinilla, J.M., Olivares, J. &Sanjuan, J. 2002Conservation of plasmid-encoded traits among bean-nodulatingRhizobiumspecies.Appl. Environ.Microbiol.68:2555-2561. | Bromfield, E.S.P. and Jones, D.G.1980.Studies on double strain occupancy of nodules and the competitive ability of Rhizobium trifolii on red and white clover grown in soil and agar, Ann. Appl. Biol.94:51-59. | Chakrabarti, S., Lee, M.S. & Gibson, A.H. 1981. Diversity in the nutritional requirement of strains of various Rhizobiumspecies.Soil. Biol.Biochem.13: 349-354. | Dhar, B. & Kumar, B. 1998. Occurrence and characteristics of rhizobiophages in pigeonpeafields. Ind. J.Microbiol.38:141-144. | Dhar, B. &Ramkrishna, K. 1987. Morphology and general characteristics of phages of chickpea rhizobia.Arch.Virol.147:121-125. | Dhar, B., Ramkrishna, K., Singh, B.D. & Singh, R.M. 1987. Morphology and biological properties of phage RL4 of Rhizobium leguminosarumSU 391. Ind. J. Exp. Biol.25:38-41. | Dhar, B., Upadhyay, K.K. & Singh, R.M. 1993. Isolation and characterization of bacteriophages specific Rhizobium leguminosarumbiovarphaseoli.Can. J.Microbiol.39: 775-779. | Dreyfus, B.L.,Gracia, J.L. & Gillis, M.1998 Characterization of Azorhizobiumcaulinodansgen.nov.stemnodulating nitrogen fixing bacterium isolated from Sesbaniarostrara. Ins. J. Syst.Bacteriol.46:1016-1024. | Fred, E.B., Baldwin, I.L. & McCoy, E. 1932.Root nodule bacteria of leguminous plants, Uni. Wisconsin Press, Madison,USA. | Glenn, AR, and Dilworth, MJ, 1981. The uptake and hydrolysis of disaccharides by fast and slow growing species of Rhizobium, Arch. Microbiol.129, 233-239. | Gomez and Gomez 1984. Statistical Procedures for Agricultural Research Institute Book.A Wiley-Interscience Publication, John Wiley and Sons, New York. | Hashemet al. 1996. Isolation and characterization of rhizobiophage specific for Rhizobium japonicumUSDA 117, Canadian Journal of Microbiology32: 326-329. | Herridgeet al. 1995. Chickpea increases soil N fertility in cereals systems through nitrate sparing and N2 fixation. Soil Biol. Biochem. 27:545-551. | Kantar et al. 2007. Chickpea:Rhizobium management and nitrogen fixation. Chickpea Breeding Manage.pp.179-192. | Kleczkowaska, J. 1971.Genetical changes inRhizobium bacteria and their bacteriophages during co-existance.Plant&Soil(Suppl.) 47: 47-56. | Kowalski, M.G., Ham, G.E., Fredrick, L.R. & Anderson, I.E. 2004. Relationship between strains of Rhizobium japonicum and their bacteriophages from soil and nodules of field-grown soybeans.World J. Microbiol.Biotechnol.118:221-228. | Krassilnikov, N.A. 1941. Variability of nodule bacteria. I.The origin of strains under the influence of bacteriophages.Mikrobiologia. 10:396-398. | Laguerreet al. 1994. Rapid identification of Rhizobia by Restriction Fragment Length Polymorphism analysis of PCR – amplified 16S ribosomal RNA genes.Applied and Environmental Microbiology. 60: 56-63. | Malek, W., Sajnaga, E., Wdowiak-Wrobel, S., Studzinska, B., Swieicka, I., Nosalewicz, I., Slomka, M., Tatara, A. &Gawron, A. 2005. Characterization of phages virulent for Sarothamnusscopariousbradyrhizobia.Curr.Microbiol.51:1-7. | Norris, D.O. 1965. Acid production byRhizobium,a unifying concept.Plant& Soil.22:143-166. | Pain, A.N. 1979. Symbiotic properties of antibiotic resistant and auxotrophic mutants of Rhizobiumleguminosarum.J. Appl.Bacteriol.47:53-64. | Pankhurst 1977. Pankhurst CE, Symbiotic effectiveness of antibiotic-resistant mutants of fast and slow growing strains of RhizobiumnodulatingLotus species, Canadian Journal of Microbiology. 23: 1026-1033. | Patel, J.J. 1978. Symbiotic effectiveness of phage–resistants of two strains of Lotusrhizobia.Plant&Soil. 49: 251-257. | Rai,Rhitu, Dash, P.K., Mohapatra, T. & Singh, A. 2012. Phenotypic and molecular characterization of indigenous rhiozobianodulating chickpea in India. Ind.J. Exp. Biol. 50 : 340-350. | Rajput, R.P., Tilak, K.V.B.R. &Sastry, P.S.N. 1993. Effect of soil amendment on improvement of nodulation in soybean in problemsoils.Zentralblatt fur Mikrobilogie. 138: 217-221. | Saxena, A.K. &Tilak, K.V.B.R. 1994. Interaction among beneficial microorganism. Ind. J.Microbiol.34:91-106. | Schwinghamer, E.A. 1967. Effectiveness of Rhizobium as modified by mutation for resistance to antibiotics.J.Microbiol.Serol.33:221-136. | Sharma, R.S., Mohmmed, A. &Babu, C.R. 2002. Diversity among rhizobiophages from rhizospheres of legumes inhabiting three ecogeographical regions of India.Soil Biol. Biochem.34 : 965-973. | Siddiqi and Mahmood 2001. Effect of rhizobacteria and root symbionts on the reproduction of Meloidogynejavanica and growth of chickpea.Biores. Technol. 79:41-45. | Sindhu, S.S. &Dadarwal, K.R. 2001. Symbiotic effectiveness of spontaneous antibiotic-resistant mutants of Rhizobium sp. nodulating chickpea (Cicerarietinum) MicrobiolRes. 155 : 325-329. | Singh, R.B., Dhar, B. & Singh, B.D. 1980. Morphology and general characteristics of viruses active against cowpea Rhizobium CB 756 and 32 HI. Arch.Virol.64:17-24. | Somasegaran P and Hoben HJ, Handbook for Rhizobia: Methods in Legume-Rhizobium Technology, Springer-Verlag, Heidelberg, Germany, pp: 1-450, 1994. | | Stowers, M.D. 1985. Carbon metabolism in Rhizobium species. Ann. Rev.Microbiol.39:89-108. | Stowers and Eaglesham. 1984. Physiological and symbiotic characteristics of fast growing Rhizobium japonicumPlant and Soil. 77: 3-14. | Vincent. 1970.A Manual for the practical study of root nodule bacteria, IBP Handbook No. 15, Blackwell Scientific Publication, Oxford and Edinburgh. | Zaidiet al. 2003. Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (Cicerarietinum) Eur.JAgron. 19: 15-21.