STUDY OF VARIATION IN RESPONSE TO STRESS IN FAST GROWING RHIZOBIA

BY

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C E R T I F I C A T E

This is to certify that the thesis entitled "Study of variation in response to stress in fast growing rhizobia", submitted in partial fulfilment of the requirements for the award of the degree of DOCTOR OF PHILOSOPHY in Microbiology of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, is a bonafide research work carried out by Ms. Archna Suman under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma. The assistance and the help received during the course of present investigation have been duly acknowledged.

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Asuman

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C O N T E N T S

LIST OF TABLES

INTRODUCTION

The ecological and economic importance of biological nitrogen fixation has earned widespread attention of the agricultural researchers. This is due to the rising scarcity of various energy sources required in the production of nitrogenous fertilizers by chemical means. The nitrogen fixation ability is known among several prokaryotic groups of organisms, including Azotobacteriaceae, Enterobacteriaceae, Rhodospirillaceae, Baccilaceae, Rhizobiaceae and Actinomycetaceae and among Cyanobacteria (Burns and Hardy, 1975). The enzymatic apparatus involved in nitrogen fixation among all these organisms appears to be similar, although the optimum physiological conditions for expression of nitrogen fixation character may vary (Ruvkun and Ausbel, 1980). Gram negative bacteria of the genus *Rhizobium* are well known for their symbiotic ability to infect legumes and induce the formation of nitrogen fixing root nodules. Differentiated bacterial forms (bacterioids) within these nodules use photosynthetically generated carbon compounds provided by the plants to convert atmospheric nitrogen into ammonia which is directly assimilated by plant. The metabolic cooperation (symbiosis) makes this system more efficiently utilizable in agriculture than free living nitrogen fixing bacteria (Kondorosi and Johnston, 1981).

Rhizobia nodulate most members of the plant family Leguminosae, which is one of the largest family among angiosperms. This group of plants that vary from herbs to trees include grain and fodder crops, spreads over tropics to arctic region. Therefore, symbiotic nitrogen fixation process has attracted world wide biological and agricultural importance; it is the principal source of biologically fixed nitrogen in the upland agricultural soils.

The research on *Rhizobium* as biofertilizer is aimed at developing the inoculants which can promote higher levels of symbiotic nitrogen fixation in legumes under practical field conditions. The genetic constitution of both the partners (*Rhizobium* and plant) is known to affect the performance of a *Rhizobium* strain as a biofertilizer. An efficient and effective *Rhizobium* strain should possess several properties including the following: specificity for host plant, the high nitrogen fixation ability, competitive ability against other rhizosphere microorganisms and ability for survival and growth under stress conditions. The major factors affecting the competitiveness and survival of *Rhizobium* strains in nature are: host(s) (Franco and Vincent, 1976; Cregan and Keyser, 1988), O_2 (Ditta *et al.*, 1987), soil pH, moisture level, temperature (Kosslak and Bohlool, 1985). A good *Rhizobium* inoculant must be bestowed with genes to manage these factors favourably.

In the developing countries like India, pulses form

the principal source of protein in the cereal based diet. Majority of the pulse crops are grown under rainfed conditions in India. Chickpea (*Cicer arietinum* L.) is the extensively grown grain legume crop in the world (FAO, 1979) and in India. It accounts for more than one-third of area under grain legumes and 40% of total pulse production. Since all the legumes including chickpea are grown in marginal lands which might suffer from drought, high salinity and/or periods of high temperature, the need for *Rhizobium* strains to be effective under such stresses is obvious. In literature, some studies on the survival of rhizobia under environmental stresses like moisture and salt (Amara and Miller, 1986; Rudulier and Bernard, 1986; Zehran and Sprent, 1986), temperature (Kvien and Ham, 1985; Rawthorne *et al.*, 1985; Rennie and Kemp, 1986) and for acidity and metal ions (Amara *et al.*, 1986) are reported. There are also reports on the relationship between azide resistance and nitrogen fixing efficiency in rhizobia (Vashishat *et al.*, 1986; Singh and Kumar, 1989). The information on the genetic mechanism of stress response in rhizobia is meagre. In order to develop strains that maintain viability under different stress conditions, there is need to understand the genetic basis of these functions.

The present study was taken up (i) to contribute towards the understanding of genetics of stress tolerance

using *Rhizobium meliloti-Medicago sativum* system and (ii) to isolate relatively stress tolerant and effective strains from the pool of chickpea-*Rhizobium* strains already available. Thus the investigation had both applied and basic objectives. The study has led to (i) selection of effective strains of chickpea-*Rhizobium* that are tolerant to high temperature, salt and/or azide and (2) definition of a small number of genetic loci determining response of stress in *R.meliloti*.. It has indicated methodology for recovering strains varying in their response to stresses and provided some material for direct use in agriculture and for genetic investigations of response to stress in *Rhizobium*.

REVIEW OF LITERATURE

The agricultural importance of the gram negative bacteria of the genus *Rhizobium* is due to their ability to infect legumes and induce the formation of symbiotic nitrogen fixing nodules. The symbiotic nitrogen fixation is of immense importance because rhizobia in the root nodules are known to fix nitrogen at a higher rate than that of free living nitrogen fixing bacteria or azospirilla that form loose association with plants (Kondorosi and Johnston, 1981). Moreover, rhizobia form root nodules on the plants of family Leguminosae which is the third largest family among the angiosperms. This family includes the plant forms varying from herbs to trees and is spread throughout the world from the tropics to arctic region (Long, 1989). Biological nitrogen fixation in *Rhizobium*-legume association is the result of metabolic cooperation between the two organisms i.e. the plant and the bacterium. The bacteria reduce molecular nitrogen into ammonia and export to plant for assimilation. The plant reduces carbon dioxide into sugars during photosynthesis and translocates these to the root where the bacteria use them as fuel. This process, therefore, has attracted the attention of not only agricultural researchers but also the basic scientists. On one hand they are trying to identify the steps and genes involved

in the symbiotic interaction which involves a complex series of developmental steps. On the other hand, they are working with *Rhizobium* as a biofertilizer to develop an inoculant that can promote higher levels of nitrogen fixation in practical field conditions. The ultimate aim of the basic studies also is to provide information and material to carry out genetic manipulations for developing efficient *Rhizobium* strains. The qualities that an efficient *Rhizobium* strain should possess are: host specificity, effectivity in nitrogen fixation, competitive ability and higher amount of ability for survival and growth under stress conditions (Paau, 1989). Understanding of the genetics of these symbiotic and adaptive functions in *Rhizobium* is a pre-requisite for their directed improvement. The pertinent literature on these aspects has been reviewed under the following sections.

A. *Rhizobium*-legume symbiosis : The Process

1. Nodulation

Rhizobia stimulate leguminous plants to develop root nodules which the bacteria infect and inhabit. Nodules develop in a complex series of steps (Vincent, 1975; Newcomb, 1981). Specific plant attractants or exudates result into chemotactic movement of *Rhizobium* towards plant roots (Bergman *et al.*, 1988). The bacteria interact with the surface of root-hair such that hair grow deformed or get

curled (Yao and Vincent, 1969; Dazzo and Gardiol, 1984). This leads to the entry of *Rhizobium* into a root hair, formation of infection thread where bacteria proliferate. As this happens, the cells of root cortex begin dividing (Newcomb, 1981). As cell divisions in the plant root establish the body of nodule, infection threads ramify and penetrate individual cells within the nodules. Bacteria are released into plant cytoplasm itself, enveloped into a plant plasma membrane (Roberton *et al.*, 1978). The bacteria differentiate into bacterioids and begin symbiotic nitrogen fixation and metabolite exchange with plant cells.

2. Symbiotic genes

The genetic apparatus involved in the symbiotic nitrogen fixation is the primary focus of current *Rhizobium* research. *Rhizobium* genetics has been greatly advanced by the approaches of transposon mutagenesis, recombinant cloning and plasmid transfer techniques (Kondorosi and Johnston, 1981; Long, 1984; Denarie *et al.*, 1987). The fast growing *Rhizobium* species have been found to possess one to several large plasmids carrying symbiotic genes. These plasmids have been designated as pSym. Such plasmids have shown variation in size, i.e., from 200-300 kb plasmids of *R.leguminosarum* to the large megaplasmids of 1200-1500 kb of *R.meliloti*. In *Bradyrhizobium*, symbiotic genes do not seem to be located on plasmids (Nuti *et al.*, 1977; Casse

et al., 1979; Prakash *et al.*, 1980). Thus, the reports appearing indicate the location of symbiotic genes on chromosome (Forrai *et al.*, 1983) and/or on megaplasmids (Johnston *et al.*, 1978; Hirsch *et al.*, 1980; Banfalvi *et al.*, 1981).

Among fast and slow growing rhizobia, four kinds of symbiotic genes have been identified, i.e., *nif*, *fix*, *nod* and *hsn*.

(a) ***nif*** genes

nif genes are those which have structural homology to *Klebsiella pneumoniae* nitrogen fixation genes. The genes *nif H* , *nif D* and *nif K* have been reported to constitute single operon in *R.meliloti* (Ruvkun and Ausubel, 1980; Zimmerman *et al.*, 1983). These genes are nitrogenase structural genes. *nif A*, a regulatory gene for expression of *nif* and *fix* genes has also been reported (Szeto *et al.*, 1984).

(b) ***fix*** genes

fix genes are those which are required for nitrogen fixation by bacterioids in the nodules but lack homology to *nif* genes of *K. pnenmoniae*(Batut *et al.*, 1985). Four of these loci have been located as a *fix ABCX* cluster between *nif HDK* and *nif A* operons (Puhler *et al.*, 1984; Earl *et al.*, 1987).

(c) Nodule formation genes

These are required for the ability of rhizobia to nodulate host plants (Banfalvi *et al.*, 1981). In *R. meliloti*, they are organized into two clusters: one cluster which is of common nodulation (*nod*) genes and the second cluster is of host specific nodulation (*hsn*) genes (Kondorosi *et al.*, 1984). In addition to *nod* and *hsn* genes, nodule development is also governed by several genes concerned with development of surface components like exopolysaccharide synthesis (*exo*) genes (Banfalvi *et al.*, 1985; Finan *et al.*, 1985; 1986), nodule development (*ndv*) genes (Dylan *et al.*, 1986), lipopolysaccharide synthesis (*lps*) genes (Carlson *et al.*, 1987), bacteriophage receptor/ sensitivity (*sxf*) genes (Khanuja and Kumar, 1989).

B. *Rhizobium* as bio-inoculant

The *Rhizobium* strains, to be used as commercial inoculants, must be capable of efficient nitrogen fixation. But the strains showing good performance under laboratory conditions, generally, have not been successful under practical field conditions (Ham *et al.*, 1971; Nelson *et al.*, 1978). This it due to the reason that the inoculant strains often compete unsuccessfully for nodule occupancy with the indigenous strains. Maier and Brill (1978) observed that a mutant of *R.janonicum* having increased nitrogen

fixation ability was not competitive under most field conditions. The competitiveness of *Rhizobium* strains is, thus, a very significant property desired in an improved inoculant. Several factors are reported to affect the competitive ability of rhizobia. These factors include *Rhizobium* strains, legume host, soil type, moisture level, temperature, other stresses and microorganisms in the rhizosphere (Paau, 1989).

1. Growth, survival and competitiveness of rhizobia

The properties such as ability to survive and multiply in the soil and competitiveness in nodule formation in addition to nitrogen fixing ability have been singled out as essential for a strain of *Rhizobium* to be suitable for inoculant production (Franco and Vincent, 1976; Date and Roughley, 1977). It has been, therefore, argued that a *Rhizobium* strain, which has performed consistently well as inoculant under diverse soil conditions in presence of competing rhizobia, would possess these properties (vanRensburg and Strijdom, 1982a). The influence of environmental factors on inter-strain competition has been studied in terms of biotic as well abiotic components. These include soil parameters like soil pH (Ham *et al.*, 1971), organic nitrogen level (Semu *et al.*, 1979) and microflora in the rhizosphere (Kosslak and Bohlool, 1985). Although some studies show that inhibition of *Rhizobium* by soil microorganisms may occur under laboratory conditions, the presence

of inhibitory microorganisms in the legume rhizosphere has little effect on the nodulating ability of *Rhizobium* (Damirgi and Johnson, 1966; Habte and Barrion, 1984). Root surface association has been studied in relation to competitiveness (vanRensburg and Strijdom, 1982b). The competitive ability of a strain has been associated with its ability to infect rapidly in *R.meliloti* (Olivares *et al*., 1980).

Franco and Vincent (1976), while investigating inter-strain competition in rhizobia, observed dominance of ineffective over effective competitor. Inter- and intra-specific competition has also been studied in *R.japonicum* and *R.fredii* under field conditions. Indigenous *Rhizobium* populations have been observed to dominate over inoculant rhizobia (Dowdle and Bohlool, 1987; Cregan and Keyser, 1988). In *R.trifolii* and *R.leguminosarum* also similar observations have been made (Maartensson and Gustafsson, 1985; Ames-Gottfred and Christie, 1989). Between *R. leguminosarum* and *R.meliloti* the lateral transfer of symbiosis related genes has been reported to occur in rhizosphere indicating the existence of genetic transfer through plasmids under natural selection pressure (Broughton *et al.*, 1987). Markers for competitiveness assay have been developed for *R.japonicum* (Ayanaba *et al.*, 1986), cowpea *Rhizobium* (Rafique *et al.*, 1984) and chickpea *Rhizobium* (Garg, *et al.*,

1985).

The growth and survival of cowpea *Rhizobium*, *R.leguminosarum* , *R.meliloti* and *R.trifolii* have been studied under different soil types, environmental factors and inoculum production conditions (Mary *et al.*, 1985; Poi and Gosh, 1985; Aarons and Ahmad, 1987; Lawson *et al.*, 1987). Water stress has been reported to result in several fold increase in the activity of catalases in *R.trifolii* (Goyal *et al.*, 1986). Competitiveness in *R.trifolii* has been observed to be affected adversely by the low pH of plant root environment (Dughri and Bottomley, 1983).

2. Stress tolerance

Since majority of legumes are raised on problem soils in India which suffer from drought, high salinity and/or periods of high temperature, the researchers are trying to develop effective *Rhizobium* strains which can withstand such stresses. A survey of literature indicates that there is hardly any work done in the genetic analysis of mechanism of tolerance towards stress conditions in rhizobia. Such studies have been carried out in some other bacteria. But in *Rhizobium*, only the effect of such stresses has been studied in terms of their symbiotic properties.

(a) Salt tolerance

The osmotic strength of the environment is one of the physical parameters that determine the ability

of organism to proliferate in a given habitat. Although the ability to adapt to the fluctuations in external osmolarity is fundamental to the survival of organism, the mechanisms responsible for osmotic adaptation have been studied relatively recently. There seem to be close parallels between bacteria and plants for the functions they employ to regulate responses to osmotic stress (Csonka, 1989). The organisms for both kingdoms accumulate same set of cytoplasmic solutes upon exposure to conditions of hyperosmolarity. Osmoregulation has been studied much in details in *Escherichia coli* and *Salmonella typhimurium* compared to other bacteria. Mutations have been induced that confer increased sensitivity to osmotic stress although very little is known about their molecular mechanisms. In *Klebsiella pneumoniae* nitrogen fixation is affected more than overall cell growth under salt stress (LeRudulier *et al.*, 1982; 1984). Similarly, the conjugal transfer of F-plasmid in *E.coli* is more osmo-sensitive than the cell growth (Singleton, 1984). In *E.coli* potassium ions are supposed to have major role in osmoregulation (Epstein and Schultz, 1965). The cytoplasmic levels of glutamate and glutamine increase in procaryotes after exposure to high osmolarity. This has been observed in *R.melilloti* (Hua *et al.*, 1982; Botsford, 1984) and in *E.coli* (Richey *et al.*, 1987). Proline has been reported to be an osmoprotectant

in *Salmonella* (Measures, 1975; Csonka, 1981). The osmotic stress results in increase of intracellular levels of proline in large number of bacteria which may be due to enhanced retention of this metabolite by the cell as a result of increased activity of ProU and ProP systems under high osmolarity (Csonka, 1988). Glycine betaine is another important osmoprotectant compound accumulated by bacteria under conditions of hyperosmolarity. Cyanobacteria and some CO_2 fixing procaryotes carry out *de novo* synthesis of glycine betaine (Galinski and Truper, 1982; Imhoff, 1986) but most other bacteria are unable to do so. Thus these bacteria are dependent on the transport of this compound for its accumulation. Betaines have been reported to play some role in salt tolerance in *Rhizobium* also (LeRudulier and Bernard, 1986).

Growth inhibition by salts has been reported in *R.meliloti* (Botsford, 1984). Salt inhibition of nitrogenase activity has been observed in *Azotobacter vinelandii* by specific cationic effects of LiCl, NaCl and KCl. Dinegative anions (Na_2HPO_4 and Na_2SO_3) are the most inhibitory. This salt interaction is indicated with MoFe protein (Burns *et al .*, 1985). The comparative studies for induction of salinity stress proteins have been able to differentiate among *Anabaena* strains (Apte and Bhagwat, 1989). In cowpea *Rhizobium* response to salt stress for early and late

inoculated plants has been compared. Late inoculated plants were less sensitive to salt than the early inoculated ones for growth and nodulation. It has been suggested that ionic accumulation might have been restricted by nitrogen deficiency in late inoculated plants, thereby, limiting tissue injury (Wilson, 1985). The adverse effects of salt stress have been observed for nodulation and grain yield in Lentil *Rhizobium* (Rai *et al.*, 1985), survival and nitrogen fixation in *R.phaseoli* (Amara and Miller, 1986), root hair infection in *R.leguminosarum* (Zahran and Sprent, 1986). Douka and Xenoulis (1984) were able to isolate a strain of *R.meliloti* from salt affected soils. It was observed to be salt tolerant in broth as well as in saline soil. The nodulation, plant yield and elemental composition of the inoculated plants under salt stress was satisfactory.

(b) Temperature stress and heat shock

Effect of high temperature and heatshock has been studied in *Escherichia coli* in considerable details. In *Rhizobium*, effect of low and high temperature on growth, viability and symbiotic functions has been studied but molecular mechanism and genetic analysis of this response are least understood. In *E.coli*, progress has been made to reveal genetic basis of tolerance towards high temperature. It has been demonstrated that a set of 17 proteins is synthesized on exposure to high temperature in *E.coli* and

genes for several of them have been revealed (Neidhardt *et al.*, 1984; Grossman *et al.*, 1985). All these proteins are expressed under the control of a gene called htp^R (Neidhardt and vanBogelen, 1981) that specifies a σ factor of RNA polymerase (Grossman *et al.*, 1984).

Heat shock proteins have also been identified and studied in *Bacillus* (Streips and Polio, 1985; Todd *et al.*, 1985; Arnosti *et al.*, 1986), *Salmonella typhimurium* (Mackey and Derrick, 1986) and *Candida albicans* (Zeuthen and Howard, 1989). In *B.subtilis* heat shock proteins (*hsp*) have been classified in two main groups: specific *hsp* and general stress proteins. The latter group of proteins is induced in response to stress stimuli like heat, ethanol, hydrogen peroxide, aminoacid starvation, limitation of O_2 and osmotic pressure (Hecker *et al.*, 1988). Induction of heat shock proteins has also been reported by ethanol in *Zymomonas mobilis*(Michel and Starka, 1986) but has been ruled out in *Legionella pnenmophila* wherein these proteins could be stimulated by novobiocin, patulin or puromycin (Lema *et al.*, 1988).

Dispensability of htp^R gene in *E.coli* has been demonstrated at low temperatures (Yura *et al.*, 1984). The effect of this gene on proteolysis has been observed and marker rescue technique has been used to map this defect in htp^R

locus itself (Baker *et al.*, 1984). Other genes have also been implicated in response to heat (thermotolerance) in *E.coli* such as *rpo H*, *gro E* (Taglichet *et al .*, 1987), *dna K* (Paek and Walker, 1987), *grp E* (Ang *et al.*, 1986) and *xth A* (Paek and Walker, 1986). RNA polymerase subunit σ^{32} has been shown to be a regulatory protein for expression of heat shock genes in *E.coli* (Bloom *et al.*, 1986; Fujita *et al.*, 1987). Stringent response induction in *E.coli* by high temperature has been observed using *dna J* and *dna K* mutants (Itikawa *et al.*, 1986).

In rhizobia, studies have been carried out to evaluate their performance at higher and lower temperatures in laboratory and field studies. Effect on structure, functioning and number of nodules by temperature changes has been studied in *R.meliloti* (Jordan, 1981), *R.trifolii* (Roughley *et al.*, 1981) and *R.leguminosarum* (Rennie and Kemp, 1986). In general, adverse effects have been observed in all rhizobia studied under high temperature. Acetylene reduction by nitrogenase was found to decrease with increase in temperature in *R.leguminosarum* (Bertelsen, 1985). In chickpea, high temperature is reported to have delayed nodulation, decreased specific nitrogen fixation and hastened senescence of nodules compared to normal temperature (Rawthorne *et al.*, 1985). Toro and Olivares (1986) observed in *R.meliloti* that deletions in the pSym megaplasmid were produced upon exposure to heat treatments and thus affecting

the symbiotic functions directly. La Favre and Eaglesham (1986) could isolate thermotolerant bradyrhizobia on agar medium which behaved similarly in soil as well.

The impact of cold stress has also been studied in rhizobia. In *R.japonicum* it has been observed that low soil temperature (15-20°C) affected plant and nodule growth as well as the recovery of inoculant strain cells. The indigenous rhizobia were most competitive (Kvien and Ham, 1985). In *Galega-Rhizobium* survival and nitrogen fixation ability were found to decrease under freezing soil conditions (Lindstroem *et al.*, 1985). The storage temperature of 4°C was observed to be better than 30°C for cowpea- *Rhizobium* inoculant in terms of cell survival (Aarons and Ahmad, 1986).

In *Azospirillum brasilense*, biosynthesis of siderophore spirilobactin was strongly inhibited (20 folds) by growth at 42°C (Bachhawat and Ghosh, 1989). Haahtela (1985) studied 23 strains of diazotrophic root associated bacteria *Azospirillum*, *Enterobacter*, *Klebsiella* and *Pseudomonas* for nitrogenase activity during growth at different temperatures. Optimal temperatures were found to be 20-37°C for *Klebsiella*, 14-20°C for *Enterobacter* and 28-37°C for *Azospirillum*. Thermotolerance has been associated with stability of the respiratory chains of *Bacillus coagulans* at high temperature (Bell and Edwards, 1987) and with change in membrane composition (phospholipid deficiency) in *Zymomonas mobilis* (Benschoter and Ingram, 1986).

3. Azide resistance in *Rhizobium*

Genetic improvement of rhizobia depends on the existance of selective methods for identifying superior genotypes for nitrogen fixing ability. Ram *et al.* (1978) observed that a group of azide resistant mutants isolated in *R.leguminosarum* fixed significantly more nitrogen than the parent strain L4. Vashishat *et al.* (1986) tested 76 strains of slow growing mungbean *Rhizobium* for azide resistance and symbiotic effectiveness. The positive correlation for azide resistance and effectiveness was observed. In *R.trifolii* also the positive association was found with regard to azide resistance and symbiotic effectiveness. It was also seen that genetic variability exists among natural isolates for this property (Yadav and Vashishat, 1986).

The genetic linkage between *azi* marker and *nod* genes on the Sym plasmid has been demonstrated recently in *R.leguminosarum* by Singh and Kumar (1989). They have observed that a recombinant clone carrying Azi^R marker isolated from the genomic library of *R.leguminosarum* Rldl64 is able to confer Azi^R, Nod^+ phenotype to a mutant strain Rld7 (Azi^S Nod^- Leu^-). The size (11.5 kb) and restriction analysis of this insert carrying *azi* marker has also been done.

C. Interaction of *Rhizobium* with *Azospirillum* and *Azotobacter*

A study on the influence of *Azospirillum* cell contents on symbiosis by winged bean *Rhizobium* was carried out (Iruthayathas *et al.*, 1983a) using cell free extract (CFE) of three strains of *A.brasilense* and one strain of *A.lipoferum* . Inoculation of winged bean was done using *Rhizobium* strain KUL-BH and CFE from *Azospirillum* strains. CFE of all the strains except LUDHI drastically reduced the nodulation, nitrogen fixation and dry matter production and subsequently the symbiotic effectiveness. While in another study (Iruthayathas *et al.*, 1983b) combined inoculation of *Rhizobium* and *Azospirillum* in winged bean and soybean resulted into substantial increase in nodulation, nitrogen fixation, shoot dry matter production and nitrogen content. It was found that the response to combined inoculation was mainly dependent on genotypes (strains of these organisms).

Beneficial effect on yield and nitrogen assimilation in wheat was reported by mixed inoculation of *Azotobacter* and *Azopirillum* with cowpea *Rhisobium*. *Rhizobium* inoculation appeared to stimulate association of *Azotobacter* and *Azospirillum* in root zone of wheat (Kavimandan, 1985). Inhibition or stimulation both have been observed for the nodule formation on plant host by *R.trifolii* in combination with *Azospirillum*. Precise cell ratio (*R.trifolii*: *Azospirillum*) and time of inoculation affected the nodulation

process in either way. *Azospirillum* strains showed variation in their ability to inhibit or enhance nodulation by *R.trifolii* strains (Plazinski and Rolfe, 1985a). Inhibition of nodulation by *R.trifolii* when mixed with *Azospirillum* could be mimicked by the addition of auxins to the plant growth medium (Plazinski and Rolfe, 1985b). The possible relationship of the pectolytic activity of *Azospirillum* strains to the inhibition of clover nodulation was indicated using a pectin plate assay (Plazinski and Rolfe, 1985c). Transfer of Sym plasmid from *Rhizobium* to *Azosprillum* was carried out under laboratory conditions but it was unstable in *Azospirillum*. Tn5 showed a high frequency of transfer from *Rhizobium* to *Azospirillum* in the rhizosphere and in the root tissue upon mixed inoculation indicating possibility of genetic transfer between the two in natural conditions (Plazinski and Rolfe, 1985d). Milto and Karbanovich (1986) in another study used heat fixed liquid culture of *Azotobacter chrococcum* in liquid medium to explore effect on growth of *R.leguminosarum* , *R.meliloti*, *R.lupini* and *R. japonicum*. Addition of boiled suspension of *Azotobacter* (4% of the medium) facilitated the growth of rhizobia.

The survey of literature on the above aspects indicates that although there are investigations on the influence of stress conditions on the survival, growth, competitiveness and symbiotic functions of *Rhizobium*, there

is hardly any work on the genetic basis of these adaptive functions so that genetic manipulations can be carried out to develop competitive and efficient strains of rhizobia to be produced as bio-inoculants. In chickpea *Rhizobium* genetics of these functions is the least understood. Thus the present investigation was taken up to understand genetic basis of these adaptive functions in *R.meliloti* and use this information to isolate efficient and competitive strains in *Cicer-Rhizobium*.

MATERIAL AND METHODS

1. STRAINS

The *Rhizobium meliloti* strains used are listed in Table 1. The Table 2 lists the strains of *Escherichia coli* used. The plasmids and phages used are listed in the Tables 3 and 4, respectively. The *Rhizobium* strains of *Cicer arietinum* used are listed in Table 16. These strains have been called here as *Rhizobium ciceri* strains.

2. MEDIA

A. Complete medium for *Rhizobium meliloti*

The compositions of different media used for growing *R.meliloti* are given below. The chemicals used were obtained from Difco or BDH (India).

i) Tryptone-yeast extract agar medium (TY)

Bactotryptone, 5 g; Yeast Extract, 3 g; $CaCl_2.2H_2O$ (1 M), 1 ml; Agar, 16 g; and Water 1 L.

ii) TY broth

The composition was as that of TY agar medium except that agar was omitted.

iii) TY Soft agar

Soft agar used for plating phages was TY agar medium with 0.8% agar.

iv) Yeast extract mannitol agar medium (YEMA)

Mannitol, 10 g; Yeast extract, 1 g; K_2HPO_4 (2%),

Table 1. *Rhizobium meliloti* strains used

Serial no.	Designation	Relevant phenotype/genotype	Reference/ Source
1.	Rmd201	Spontaneous streptomycin resistant mutant of AK631, wild type	Khanuja and Kumar (1989)
2.	Rmd1004	Azide sensitive mutant of Rmd201 (Rmd201 *azt*::Tn5-156)	This work
3.	Rmd1005	Azide resistant mutant of Rmd201(Rmd201 *azs*::Tn5-1)	
4.	Rmd1006	Rmd201 *azs*::Tn5-2	
5.	Rmd1007	-4	
6.	Rmd1008	-8	
7.	Rmd1009	-11	
8.	Rmd1010	-13	
9.	Rmd1011	-16	
10.	Rmd1012	-17	
11.	Rmd1013	-18	
12.	Rmd1014	-19	
13.	Rmd1015	-20	
14.	Rmd1016	-22	
15.	Rmd1017	Heat shock sensitive mutant of Rmd201 (Rmd201 *hst*::Tn5-132)	
16.	Rmd1018	Rmd201 *hst*::Tn5-135	

Serial no.	Designation	Relevant phenotype/genotype	Reference/ Source
17.	Rmd1019	-136	This work
18.	Rmd1020	-139	
19.	Rmd1021	-142	
20.	Rmd1022	-143	
21.	Rmd1023	-144	
22.	Rmd1024	-145	
23.	Rmd1025	-146	
24.	Rmd1026	-147	
25	Rmd1027	-148	
26.	Rmd1028	-149	
27.	Rmd1029	-150	
28.	Rmd1030	-151	
29.	Rmd1031	-158	
30.	Rmd1032	-159	
31.	Rmd1033	-162	
32.	Rmd1034	-176	
33.	Rmd1035	-177	
34.	Rmd1036	-178	
35.	Rmd1037	-179	
36.	Rmd1038	-180	
37.	Rmd1039	-187	

Serial no.	Designation	Relevant phenotype/genotype	Reference/ Source
38.	Rmd1040	Heat shock resistant mutant Rmd201 (Rmd201 *hss*::Tn5-157)	This work
39.	Rmd1041	Rmd201 *hss*::Tn5-607	
40.	Rmd1042	-612	
41.	Rmd1043	-619	
42.	Rmd1044	-638	
43.	Rmd1045	-640	
44.	Rmd1046	Cotrimoxazole sensitive mutant of Rmd201 (Rmd201 *ost*::Tn5-152	
45.	Rmd1047	Rmd 201 *ost*::Tn-153	
46.	Rmd1048	-154	
47.	Rmd1049	-161	
48.	Rmd1050	-182	

Table 2. *Escherichia coli* strains used

Serial no.	Designation	Relevant phenotype/genotype	Reference/ Source
1.	CA8000	Hfr H, *thi, rel A, min, lam* B^+	Kumar(1976)
2.	HB101	*pro, leu, thi, lac Y, rec A, lam B,* str^R	Kumar and Srivastava (1983)

Table 3. Plasmids used

Serial no.	Designation	Relevant phenotype/genotype	Reference/ Source
1.	pGS9	Inc N, *rep 15-A*, Cml^R, carries Tn5 (Kan^R), suicidal in *Rhizobium*	Selvaraj and Iyer (1983)
2.	pJB3JI	Inc P-1, Kan^S derivative of plasmid R68.45, Cma^+ Amp^R, Tet^R	Brewin *et al.* (1980)
3.	pRK2013	Inc P-1, rep Col E1, carries Tra^+ function of plasmid pRK2, Neo^R, Kan^R	Figurski and Helinski (1979)
4.	pPHIJI	Inc P-1, Gen^R, Spc^R, Incompatible with pJB3JI and pRK290	Beringer *et al.* (1978)

Table 4. ***Rhizobium meliloti*** phages used

(Khanuja and Kumar, 1988)

Serial no.	Phage types	Relevant phenotype
1.	RMP26, 36, 46 and 50	Form semiturbid plaques on Rmd201 and the host range includes the strains Rm1021, 4013 and 102F34
2.	RMP38, 52, 79 and 145	Form turbid plaques on Rmd201 and the host range includes the strains Rm1021, 4013 and 102F34
3.	RMP86, 90 and M12	Form clear plaques on Rmd201 and host-range includes Rm1021, 4013 and 102F34
4.	RMP61, 64, 80 and 88	Form clear plaques on Rmd201 but do not plate on Rm1021, 4013 and 102F34
5.	RMP67 and 85	Form semiturbid plaques on Rmd201, but do not plate on Rm1021, 4013 and 102F34

10 ml; KH_2PO_4 (2%), 10 ml; $MgSO_4.7H_2O$ (1 M), 0.8 ml; $CaCl_2.2H_2O$ (1 M), 0.4 ml; Agar, 16 g; and Water, 1 L.

B. **Minimal medium for *R.melliloti* (RMM)**

First, stock solutions A and B were prepared. The solution A was 20% glucose and the solution B called 2xR salt solution (2xRSS) had the following composition (1 L): $Na_2HPO_4.12H_2O$ (4.5%), 20 ml; $(NH_4)_2SO_4$ (20%), 20 ml; $FeCl_3$ (1%), 0.4 ml; $MgSO_4.7H_2O$ (1 M), 0.4 ml; and $CaCl_2.2H_2O$ (1 M), 0.2 ml. To make 1 L of RMM, 16 g of agar and 10 ml of solution A were added to 500 ml of H_2O and autoclaved. Separately, 500 ml of 2xRSS was also autoclaved. Then the two 500 ml portions were mixed and cooled to 60°C.

To make liquid RMM medium, the above compositions were used except that agar was omitted.

C. **Complete medium for *R.ciceri***

Tryptone-yeast extract mannitol agar medium (TYM)

The compositon of TYM was as follows: Bactotryptone, 5 g; Yeast extract, 0.5 g; Mannitol, 10 g; $CaCl_2.2H_2O$ (1 M), 1 ml; Agar, 16 g; and Water, 1 L.

D. **Nitrogen free plant growth solution**

For carrying out nodulation tests two nitrogen free media were used

i) **McKnight's medium (MKS)**

This medium developed by McKnight (1949) was used. Five stock solutions called A to E with following compositions were prepared.

Solution A : H_3BO_3, 286 mg; $MnSO_4.4H_2O$, 154 mg; $ZnSO_4.7H_2O$, 22 mg; $CuSO_4.5H_2O$, 8 mg; H_2MoO_4, 9 mg; and Water, 100 ml.

Solution B : $FeCl_3$, 1.68 g; EDTA, 200 mg; and Water, 100 ml.

Solution C : $MgSO_4.7H_2O$, 20 g in 100 ml water.

Solution D : KH_2PO_4, 20 g in 100 ml water.

Solution E : KCl, 30 g in 100 ml water.

To make 10 L of MKS, 12.5 g of $CaSO_4$ was added to 2 L of water and boiled. To the resulting suspension, 10 ml of each of the solutions A, B, C, D and E were added. Water was mixed to make the final volume equal to 10 L. The pH of this medium was adjusted to 6.8 by using 0.1 N NaOH. The medium was then autoclaved and cooled.

ii) **Jensen's medium (JMS)**

This medium developed by Jensen (1942) was prepared by mixing the following: $CaHPO_4$, 1 g; K_2HPO_4, 0.2 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3$, 0.1 g; and Water 1 L. The pH of this medium was adjusted to 7.0 by using 0.1 N NaOH. JMS was mixed with MKS to make liquid medium for plant assays in tubes. The composition of the medium was JMS : MKS : H_2O::1:0.25:0.75.

E. Phage suspension medium (PSM)

PSM used for preparation of phage lysates had the following composition : K_2HPO_4 (2%), 10 ml; KH_2PO_4 (2%), 10 ml; $MgSO_4.7H_2O$ (1 M), 0.8 ml; $CaCl_2.2H_2O$ (1 M), 0.4 ml; and Water, 1 L.

F. Medium for *Escherichia coli*

The composition of different media used for growing cultures are given below:

i) Luria agar (LA)

It consisted of the following: Bactotryptone, 10 g; Yeast extract, 5 g; NaCl, 10 g; Agar, 16 g; and Water, 1 L.

ii) Tryptone agar (TA)

It consisted of the following : Bactotryptone, 10 g; NaCl, 5 g; Agar, 16 g; and Water, 1 L.

3. SUPPLEMENTS TO MEDIA

A. Antibiotics

Stock solutions of the antibiotics were prepared @ 100, 10 or 1 mg/ml in sterile water. Desired volume of an antibiotic was added to the autoclaved medium that had been allowed to cool to about 60°C. The antibiotics employed were : Kanamycin (Km) from Alembic (50 µg/ml), Streptomycin (Sm) from Sarabhai (100 µg/ml), Ampicillin (Ap) from Biochem

(500 µg/ml), Spectinomycin (Sp) from Sigma (50 µg/ml), Gentamycin (Gm) from Alembic (20 µg/ml) and Tetracycline (Tc) from Biochem (10 µg/ml).

B. Drug

Sodium azide (Azi) was used @ 5 to 100 µg/ml from the stock solution of 10 mg/ml azi in water.

C. Salt

Required amount of NaCl was added to the TY medium directly before autoclaving.

4. METHODS

A. Culture and incubation conditions

Single colonies were obtained by plating or streaking of cells of a strain on complete or minimal medium. When complete medium was used, incubation was done for 4 days at 30°C. On minimal medium, incubation was done for about one week at 30°C.

To raise a liquid culture, a colony was transferred with a loop to 5 ml of broth in a tube and tube was shaken in a water bath at 30°C. Cultures were, usually, harvested at about 36 h of incubation after which their titre was 2-5 x 10^8 cells/ml.

Often spot tests were employed to study growth responses of bacterial strains. For this purpose, cells from a colony were suspended in a drop of water (0.05 ml). With a loop, cells (10^5 to 10^6) were applied to the surface of agar.

B. Preparation of phage lysate

R.meliloti Rmd201 was used as the bacterial host for the preparation of phage lysates and the procedure of Khanuja and Kumar (1988) was followed.

C. Conjugational crosses between bacteria

Patch matings were employed. TY broth cultures of donor and recipient bacteria were grown for overnight to 36 h. They were mixed 1:1 and 0.05 ml of the mixture was spread on TY agar surface with the help of a loop. The plate was incubated for desired lengths of time from 8 to 36 h. The patch of the growth obtained was treated in two ways. (1) It was replica plated on selective media. (2) The growth was scrapped using a spatula and suspended in 1 ml of MSS and plated on the selective medium in required numbers. The plates were examined for exconjugants after 5 days of incubation at 30°C.

D. Transposon Tn5 mutagenesis

Cultures of donor *E.coli* strain WA803 (pGS9) and recipient *R.melliloti* strain Rmd201 were grown in TY broth by incubation for 12 h and 24 h respectively. The two cultures were mixed 1:1 and the mixture was patched on TY agar plates; several patches were made. The patched plates were incubated at 30°C for 8 h for allowing the mating.

The growth from a patch was transferred to 1 ml of MSS in a 5 ml tube using a spatula. The suspension of mated

cells was plated directly on one plate and after 10 fold dilution on another plate of TY agar medium containing Km (50 µg/ml) and Sm (100 µg/ml). The plates were incubated for 5 days at 30°C.

There were about 0 to 250 colonies on different plates when the suspension from a patch was plated directly. From a plate derived from a patch, one to five colonies were purified twice on YEMA medium containing Km (50 µg/ml) and Sm (100 µg/ml).

This way, a collection of about 1000 Tn5 mutants of Rmd201 was built up. The mutants were maintained on YEMA plates having Km and Sm.

E. Isolation of heat shock sensitive (Hst^-) and heat shock resistant (Hss^-) mutants

First, the conditions for screening of mutants were standardized. Rmd201 wild type cells were spotted on TY plates and were given heat shock at 50°C for 30 min to 6 h in an incubator and then shifted to 30°C incubator and retained there for 4 days. Control plate was kept at 30°C throughout. Since Rmd201 cells produced a spot of growth when given heat shock for 3 h, the mutants which did not form fully grown spots after heat shock for 3 h were treated as heat shock sensitive (Hst^-) and those that formed spots of growth even after heat shock for 4½ h were treated as heat shock tolerant mutants (Hss^-).

Like the Rmd201 cells, all the transposon mutants of Rmd201 were spotted on three TY plates. One plate was kept at 30°C as control and two were kept at 50°C for heat shock. One of these was removed after 3 h to 30°C and the other after 4½ h to 30°C. Growth on all the three sets of plates was examined after 4 days of incubation at 30°C.

F. Isolation of Osmo-sensitive (Ost^-) and Osmo-resistant (Oss^-) mutants

First, the conditions for screening of mutants were standardized. Rmd201 wild type cells were spotted on TY plates containing 0.05 to 0.8 M concentrations of NaCl and were incubated for 4 days at 30°C. Plain TY agar plate was used as control. Since Rmd201 cells produced a patch of growth when grown on TY medium containing 0.4 M NaCl, the mutants which did not grow on TY medium with 0.4 M NaCl were treated as Osmo-sensitive (Ost^-) and those that grew even at 0.8 M NaCl concentration in TY medium were treated as Osmo-resistant (Oss^-).

Like the Rmd201 cells, all the transposon mutants of Rmd201 were spotted on three TY plates, one plain TY, one TY having 0.4 M NaCl and one TY with 0.8 M NaCl and the plates were incubated at 30°C. Growth was examined after 4 days.

G. Isolation of sodium azide (NaN_3) sensitive (Azt^-) and NaN_3 resistant (Azs^-) mutants

To standardize the conditions for screening of mutants, Rmd201 wild type cells were spotted on TY plates containing 10 to 100 µg/ml of NaN_3. Plain TY agar medium was used as control. Since Rmd201 cells produced a spot of growth when grown on TY medium containing 30 µg/ml of NaN_3, the mutants which did not grow on TY medium with 25 µg/ml of azide were called as azide sensitive mutants (Azt^-) and those that grew on TY medium with 100 µg/ml of azide were called as azide resistant mutants (Azs^-).

For isolation of azide sensitive mutants, all transposon mutants of Rmd201 were spotted on plain TY and TY plate having 25 µg/ml of azide and incubated at 30°C. Growth was examined after 4 days.

For selecting azide resistant mutants, suspension of cells, obtained from the patch of mated cells of WA803 (pGS9) and Rmd201, was plated directly on TY plates containing azide (100 µg/ml) +Km (50 µg/ml) + Sm (100 µg/ml). Plates were incubated at 30°C for 7 days. Colonies that appeared on these plates were considered azide resistant (Azs^-) transposon mutants of Rmd201.

H. Phage growth test

To conduct these tests, 36 h liquid cultures (10^8 cells/ml) of mutants were used. About 0.2 ml portion of a culture in a 5 ml tube was added 3 ml of soft TY agar

and mixture was swirrled at 50°C and poured on agar surface of a TY plate. After soft agar had solidified, 16 different phage suspensions were spotted on the plate. Each spot was made with about 10^6 to 10^7 phage particles. The plates were examined for clearing of bacterial lawn at the sites of phage spots after 36 h of incubation at 30°C.

I. Nodulation tests

(i) On *R.meliloti* mutants using alfalfa as host

A test was carried out in a 20x2.5 cm glass tube. JMS-liquid growth medium was employed. A thick filter paper fashioned into a bridge provided support for the seeds in the tube. The growth solution was added in the tubes such that the middle fold of filter paper did not touch the liquid. The tubes were autoclaved after stopering with cotton plugs.

Medicago sativa cv T9 seeds of alfalfa were soaked in sterile water for ½ h. They were surface sterilized by soaking in 0.1% $HgCl_2$ for 1 minute, treatment with 70% alcohol for 1 min and several washings with water. Seeds were sown in the groove formed by the filter paper support in the growth tube.

To prepare the inoculum, a mutant was grown on YEMA slant. The growth that appeared was suspended in sterile water and inoculated on to 3 day old seedlings. The plants were kept under sufficient light and the root system was

prevented from direct exposure with the help of black paper. Growth was allowed for 45 days. Each plant was then characterized for number, colour and position of nodules and length of shoot and root.

(ii) On *R.ciceri* strains using *Cicer arietinum* Pusa256 as host

Leonard (1943) jar procedure was used. The assembly consisted of a top 'bottle' and a bottom 'jar' obtained by cutting the base and neck of beer bottles respectively. The narrow mouth of the top bottle was plugged with cotton tightly.

Yamuna sand was thoroughly washed in running tap water to get rid of soil. It was then dried in oven and $CaCO_3$ was mixed into it @ 1 g/kg sand. This mixture was filled in the bottles. The open ends were covered with petridishes and each bottle was kept in its jar. The whole assembly was covered with a paper envelope and autoclaved twice. Autoclaved MKS was poured into the jars before use which was absorbed by the sand.

Cultures of *R.ciceri* strains were grown as lawns on YEMA slants. The bacterial growth of a slant was suspended in 10 ml of water to serve as the inoculum.

Chickpea seeds were surface sterilized and immersed in bacterial suspensions (inoculum) for 1 h. For control, uninoculated seeds were used.

For planting of seeds in Leonard jars, 8-10 well spaced 1 cm deep holes were made in sand with a pencil. Each hole was sown with a seed and covered with sand. Jars were, then, covered with sterile petridishes. The assemblies were kept undisturbed till the germination of seeds. As the seedlings emerged, the petridishes were removed, sterilized cotton was placed around the plants on sand surface and assembly was covered with brown paper to avoid exposure of roots to light.

The sown assemblies were placed in growth chambers lighted for 12 h per day with fluorescent tubes. The plants were allowed to grow for 50 days. The plants were harvested by washing off the sand from the bottle in running tap water and studied to determine number and fresh weight of nodules, length of shoot and root, dry weight of shoot and acetylene reduction rate of root.

J. Estimation of reduced nitrogen

Reduced nitrogen in the dried plant samples was estimated using a N-auto analyser (Technicon Auto-analyser I) following the prescribed procedure (Technicon Monograph, 1971).

K. Construction of R-prime plasmids

Kanamycin sensitive derivative of plasmid R68.45 called pJB3JI was used to pick up Tn5 harbouring DNA from the genomes of the various insertion mutants of Rmd201.

E.coli HB101 (pJB3JI) and Rmd201::Tn5 mutants were patch mated for 24 h at 30°C. The growth of patch was replica plated on to the surface of RMM agar plate containing Km (50 µg/ml) and Tc (10 µg/ml). The replica was incubated at 30°C for 4-5 days. Colonies that appeared were taken as Rmd201::Tn5 (pJB3JI). These were purified and ascertained for markers.

Next, HB101 and Rmd201::Tn5 (pJB3JI) were patch mated for 24 h at 30°C. The patch of the growth that appeared was replica plated on TA plates containing Km (50 µg/ml), Ap (500 µg/ml) and Tc (10 µg/ml) and 0.6 M NaCl. The replica was incubated at 30°C for 2 days. Colonies produced were purified by streaking on the medium used for their selection. These were treated as HB101 carrying pJB3JI:Rm DNA::Tn5 R-prime plasmids i.e. HB101 (R'). Nature of each R-prime was confirmed by the test given below.

HB101 (R') and Rmd201 were patch mated and the resulting 24 h growth was replica plated on RMM agar surface containing Km (50 µg/ml) and Tc (10 µg/ml) and incubated for one week at 30°C. The colonies that appeared were of Rmd201 (R') and were purified by streaking on the same medium. Each Rmd201 (R') was then patch mated with HB101 (pPHIJI) for 24 h. The mating patch was replica plated on RMM containing Km (50 µg/ml) and Gm (20 µg/ml) and incubated for 4-5 days. The colonies produced were purified on the

same medium. The resulting colonies proved to be Gm^R at 20 µg/ml, Tc^S at 10 µg/ml and Km^R at 50 µg/ml. Since these were Gm^R but Tc^S, they must be harbouring pPHIJI in place of pJB3JI because of incompatibility between them and the gene in mutated form on chromosome because of their being Km^R.

Next, the phenotype of these derivatives was compared with the original Tn5 mutant from where insertion on R' had occurred. If the phenotypes were identical, then the right kind of R' had been constructed.

L. Complementation test

An R-prime was transferred to each of the Tn5 mutants having phenotype similar to the mutant used for the construction of the R-prime. These transfers were achieved by patch mating procedure. The merodiploids were selected and purified on RMM containing Km (50 µg/ml) and Tc (10 µg/ml). The derivatives were then checked for restoration back of wild type phenotype.

M. Screening for tolerance properties towards Heat, Osmotic and Azide shock in *Rhizobium ciceri* strains

Special TYM medium that is TY containing 0.5 g/L of yeast-extract and 10 g/L of mannitol was employed. It may be mentioned here that *R.ciceri* was found unable to grow on plain TY medium.

To screen for tolerance towards heat shock, all *R. ciceri* strains were spotted on five TYM plates. One of these was kept at 30°C throughout as control. Other four plates were kept at 50°C in an incubator. After 1 h one plate was shifted to 30°C and likewise other three plates were shifted to 30°C after 2, 3, and 4 h of heat shock at 50°C. Growth was examined after 5 days of incubation.

To screen for tolerance towards osmotic shock, all *R. ciceri* strains were spotted on TYM plates having 0 to 0.4 M NaCl. These plates were incubated at 30°C and growth was examined after 5 days.

To screen for tolerance towards azide, all *R. ciceri* strains were spotted on TYM plates containing 0 to 100 µg/ml of sodium azide. Growth was examined after 5 days of incubation at 30°C.

N. Procedure for studying effects of interactions between *Rhizobium*:*Azotobacter* and *Azospirillum* on chickpea plants grown in pots

Earthern pots were filled with sieved soil. The soil was obtained by digging 7 feet deep in a IARI field. Pots were watered one day before sowing to give proper moisture level for germination of seeds. Cultures were prepared by inoculating 250 ml of YEM broth in flasks and then incubating them on a shaker at 30°C for 2 days. Chickpea seeds were soaked in water for 1 h and then they were immersed in

different combinations of bacterial cultures for ½ h. Seeds of a treatment were sown in four pots. Holes were made in a pot with the help of a big spatula and each hole was sown with a chickpea seed. The leftover culture-mix was poured into the 4 pots of the concerned treatment. After germination of seedlings,pots were watered regularly with tap water and after 5 months yield of chickpea was estimated in terms of grain yield/pot. Completely Randomized block design (CRD) was employed.

RESULTS

A. CHARACTERISTICS OF Tn5 MUTANTS OF *Rhizobium meliloti* Rmd201, FOR THEIR RESPONSE TO STRESS

Rhizobium meliloti Rmd201 is a spontaneous streptomycin resistant derivative, of the strain AK631, isolated in this laboratory. The strain AK631 is compact colony morphology mutant of the wild type strain called Rm41. The derivatives of Rm41 have been extensively used in the genetical work for definition of genes determining symbiotic response of *R.meliloti* against alfalfa and related plants.

Since considerable amount of information is already available about the genetics of *R.meliloti* Rm41, the strain Rmd201 was chosen as the wild type strain for the isolation of mutants defective in their response towards different kinds of stresses, such as heat shock, hyperosmolarity and respiratory block.

In this study, first a total of 990 Tn5 mutants of Rmd201 were isolated by the procedure described in Material and Methods (4D). All of these insertion mutants were screened for their response to different durations of heat shock at 50°C and different concentrations of sodium chloride and sodium azide in the medium. This led to isolation of heat shock sensitive and resistant mutants, mutants sensitive to sodium chloride at 0.4 M and those that were sensitive to sodium azide at 25 µg/ml and mutants resistant to sodium

azide at 100 µg/ml. The characteristics of these mutants are presented here.

1. Mutants relatively more sensitive or resistant to heat shock than the wild-type

Nine hundred and ninety random Tn5 induced mutants of Rmd201 were studied for their response to heat shock at 50°C. The mutants, that could not withstand 50°C heat shock, for 3 h to as much extent as the wild type, were treated as heat shock sensitive mutants. They were given the name Hst^- mutants. In all, 23 mutants were Hst^-. Similarly the heat shock resistant mutants were isolated. The mutants, that tolerated 50°C heat shock for 4½ h relatively better than the wild type, were treated as heat shock resistant mutants. They were called as Hss^- mutants. In all there were 6 Hss^- mutants.

(a) Heat shock sensitive mutants defined two genes

The genomic fragment bearing the mutated site of the *hst*::Tn5-143 mutation was picked up on the plasmid pJB3J1 to construct an R-prime. This R-prime was then transferred to all the 23 Hst^- mutants. The merodiploids so obtained were compared with the wild type and the various Hst^- mutants for their response to heat shock at 50°C for 3 h. It was observed that 16 of the merodiploids had the mutant phenotype whereas the other 7 had the wild type phenotype. These

observations, given in the Table 5, were considered to have shown that the mutations *hst*::Tn5-132, -135, -139, -143, -144, -145, -146, -148, -149, -150, -176, -177, -178, -179, -180 and -187 defined a gene which has been called *hst A*. On the other hand it was considered that the mutations *hst* ::Tn5-136, -142, -147, -151, -158, -159 and -162 defined another gene which has been called as *hst B*.

(b) Heat shock resistant mutants defined three genes

The genomic segments harbouring Tn5 mutations *hss* ::Tn5-612 and -619 were individually incorporated into pJB3J1 plasmids to construct two R-primes. In order to study complementation between the 6 heat shock resistant mutants, the R-primes carrying *hss*::Tn5-612 and *hss*::Tn5-619 mutations were transferred to all the Hss^- mutants. The phenotypes of the 12 merodiploids, thus obtained, were compared with those of Hss^- mutants and the wild type using 50°C heat shock for 4½ h. The observations are given in the Table 6. It was found that the 6 Hss^- mutants defined 3 genes. The gene called *hss A* comprised of *hss*::Tn5-607, -612 and -640 mutations. The *hss B* gene was observed to carry *hss* ::Tn5-619 and -638 mutations. The *hss C* gene was defined by only one mutation, namely the *hss*::Tn5-157.

Table 5. Complementation tests among heat shock sensitive (*hst*) mutants of *R.meliloti*

pJB3JI-prime introduced to construct merodiploids	Mutants having heat shock sensitive phenotype by the lesions	
	hst::Tn5-132,-135,-139, -143,-144,-145, -146,-148,-149, -150,-176,-177, -178,-179,-180 and -187	*hst*::Tn5-136,-142, -147,-151, -158,-159 and -162
hst::Tn5-143	-	+
Complementation groups	*hst A*	*hst B*

Table 6. Complementation tests among heat shock resistant (*hss*) mutants of *R.meliloti*

pJB3JI-prime introduced to construct merodiploids	Mutants having heat shock resistant phenotype by the lesions		
	hss::Tn5-607, -612 and -640	*hss*::Tn5-619 and -638	*hss*::Tn5-157
hss::Tn5-612	-	+	+
hss::Tn5-619	+	-	+
Complementation groups	*hss A*	*hss B*	*hss C*

2. Osmotic mutants

The wild type strain Rmd201 is able to tolerate 0.4 M sodium chloride in TY medium. The available Tn5 mutants were screened for recovering any osmoresistant mutants by spotting on TY agar containing 0.5, 0.6, 0.7 and 0.8 M sodium chloride. No stable osmotolerant mutant was identified. Five mutants were identified which failed to grow on TY medium containing 0.4 M NaCl . These osmosensitive mutants have been given the name Ost$^-$. Complementation tests remain to be carried out with these mutants.

3.(a) Mutants differing from the wild type in their tolerance to azide

To detect azide sensitive and azide resistant mutants among the available Tn5 mutants, the entire collection was screened for colony forming ability on TY medium containing 25 µg/ml azide on one hand and 100 µg/ml azide on the other hand. It was found that none of the 990 Tn5 mutants was resistant to 100 µg/ml azide in the TY medium. Only one Tn5 mutant was found to be unable to grow on TY medium containing sodium azide at 25 µg/ml concentration. This mutant was called as azide sensitive mutant and was given the name Azt$^-$.

In order to further examine into the possibility of recovering azide resistant mutants, the transconjugants from the cross between Rmd201 recipient and WA803 donor

of Tn5 bearing suicide plasmid pGS9 were plated on TY medium containing 100 µg/ml sodium azide in addition to selective doses of Km and Sm. From 10 independent experiments, 12 stable azide resistant mutants were isolated. These mutants were called as Azs$^-$ mutants. The reasons for non-recovery of Azs$^-$ mutants from among the 990 randomly induced Tn5 mutants, without involvement of azide in the medium, has not been explored here.

(b) Azide tolerant mutants defined two genes

In order to carry out complementation tests between Azs$^-$ mutants, R-primes were constructed for *azs*::Tn5-11 and *azs*::Tn5-17 mutations. These two R-primes were transferred to all the 12 Azs$^-$ mutants. The 24 transconjugants were compared with the Azs$^-$ mutants and the wild type for their ability to tolerate azide at 100 µg/ml concentration in TY medium. The complementation data given in Table 7 showed that the 12 Azs$^-$ mutants defined 2 genes. One of these called *azs A* covers *azs*::Tn5-2, -4, -11, -18, -19 and -22 mutations. The other one called *azs B* covered *azs* ::Tn5-1, -8, -13, -16, -17 and -20 mutations.

4. Pleiotropy of azide sensitive mutants

The Azt$^-$ and Azs$^-$ mutants were studied for their toleration of sodium chloride at 0.4 M and heat shock for 3 and 4½ h (Table 8). They were also compared with the wild

Table 7. Complementation tests among azide resistant (*azs*) mutants of *R.meliloti*

pJB3JI-prime introduced to construct merodiploids	Mutants having azide resistant phenotype by the lesions	
	azs::Tn5-2, -4, -11, -18, -19 and -22	*azs*::Tn5-1, -8, -13, -16, -17 and -20
azs::Tn5-11	-	+
azs::Tn5-17	+	-
Complementation groups	*azs A*	*azs B*

Table 8. Response of azide sensitive (*azt*) and azide resistant (*azs*) mutantsof *R.meliloti* Rmd201 towards NaCl and heat shock

Serial no.	Strains	Genotype	Growth on TY medium containing				Survival after heat shock for	
			Azide (µg/ml)		(NaCl (M)			
			25	100	0.4	0.8	3 h	4½ h
1.	Rmd201	Wild type	+	-	+	-	+	-
2.	Rmd1004	*azt*	-	-	-	-	-	-
3.	Rmd1005-1016	*azs*	+	+	+	-	+	-

type for their response towards phages RMP26, RMP38 and RMP64 (Table 9). It was found from the observations that the Azt$^-$, Azs A$^-$ and Azs B$^-$ mutants had the same response to phages as the wild type. The Azs A$^-$ and Azs B$^-$ mutants were indistinguishable from the wild type for their response towards NaCl and heat shock. However, the Azt$^-$ mutant was sensitive to both NaCl and heat shock.

Representative mutants of the genes *azs A*, *azs B* and *azt* were also studied for their symbiotic response towards alfalfa. These observations are shown in the Table 10. It was found that the mutants Rmd1004 (*azt*), Rmd1005 (*azs B*) and Rmd1006 (*azs A*) were like the wild type in their symbiotic response.

The above observations demonstrated that the mutant of *azt* gene was osmo- and heat shock-sensitive besides being azide sensitive. The reasons for its pleiotropic behaviour require to be investigated.

5. Pleiotropic nature of osmosensitive mutants

The five osmosensitive mutants were compared with the wild type Rmd201 for their toleration towards azide and heat shock, abilities to propagate phages RMP26, RMP38, and RMP64 and for symbiotic response towards alfalfa. The results given in the Tables 11, 12 and 13 showed that the mutant Rmd1047 had the same kind of pleiotropic phenotype

Table 9. Plaque morphologies of phages on azide resistant (*azs*) and azide sensitive (*azt*) mutants of *R.meliloti* Rmd201

Serial no.	Strains	Growth properties of phages (+ = Growth ; - = No growth)	
		RMP26 and RMP38	RMP64
1.	Rmd201 (Azs^+ Azt^+)	+ (Turbid)	+ (Clear)
2.	Azs^-	+	+
3.	Azt^-	+	+

Table 10. Symbiotic response of azide sensitive (*azt*) and azide resistant (*azs*) mutants of *R.meliloti* Rmd201

Serial no.	Strains	Genotype	Nitrogen fixation ability of nodules
1.	Rmd201	Wild type	Fix^+
2.	Rmd1004	*azt*	Fix^+
3.	Rmd1006	*azs A*	Fix^+
4.	Rmd1005	*azs B*	Fix^+

Table 11. Response of osmosensitive (*ost*) mutants of *R.meliloti* Rmd201 towards Sodium Azide and heat shock

Serial no.	Strains	Genotype	Growth on TY medium containing				Survival after heat shock	
			NaCl (M)		Azide(µg/ml)		(50°C)	
			0.4	0.8	25	100	3 h	4½ h
1.	Rmd201	Wild type	+	-	+	-	+	-
2.	Rmd1046	*ost*	-	-	+	-	+	-
3.	Rmd1047	*ost*	-	-	-	-	-	-
4.	Rmd1048-1050	*ost*	-	-	+	-	-	-

Table 12. Plaque morphologies of phages on osmosensitive (*ost*) mutants of *R.meliloti* Rmd201

Serial no.	Strains	Genotype	Growth properties of phages (+ = Growth ; - = No growth)	
			RMP26 and RMP38	RMP64
1.	Rmd201	Wild type	+ (Turbid)	+ (Clear)
2.	Rmd1046 and Rmd1050	*ost*	+	+

Table 13. Symbiotic response of osmosensitive (*ost*) mutants of *R.meliloti* Rmd201

Serial no.	Strains	Genotype	Nitrogen fixation ability of nodules
1.	Rmd201	Wild type	Fix^+
2.	Rmd1046 to 1050	*ost*	Fix^+

as the Azt$^-$ mutant Rmd1004. A set of 3 mutants Rmd1048, 1049 and 1050 were heat shock sensitive besides being osmosensitive. All the Ost$^-$ mutants were like the wild type in their responses to phages and alfalfa.

6. Pleiotropy of heat shock mutants

In the Tables 14 and 15, the properties of *hst A*, *hst B*, *hss A*, *hss B* and *hss C* are compared with those of wild type. The *hss A* mutants such as Rmd1036 were like the wild type for their responses towards azide, NaCl and alfalfa. However, on their lawns the phages RMP26 and RMP38 formed hyperturbid plaques unlike the turbid plaques formed on Rmd201. The *hst B* mutants such as Rmd1030 were like the wild type in their responses to azide, NaCl, alfalfa and phages.

The *hss A* and *hss B* mutants did not differ from the wild type in their azide- and osmo- tolerance properties and in their response to phages RMP26 and RMP38. Significantly *hss A* mutant such as Rmd1041 induced nodules on alfalfa which were Fix$^-$. The *hss B* mutants like Rmd1043 induced normal nodulation response in alfalfa. The *hss C* mutant Rmd1040 differed from the wild type in two respects: on its lawn phages RMP26 and RMP38 formed hyperturbid plaques and it induced Fix$^-$ nodules on alfalfa.

Table 14. Pleiotropic phenotypes of heat shock sensitive (*hst*) mutants

Serial no.	Strain	Genotype *hst A*	Genotype *hst B*	Nodulation response (Fix)	Heat shock tolerance (W=Wild type) (S=Sensitive)	Azide tolerance (W=Wild type) (S=Sensitive)	Osmo-tolerance (W=Wild type) (S=Sensitive)	Plaques of phages RMP26 and RMP38 (T=Turbid) (HT=Hyper turbid)
1.	Rmd201	+	+	+	W	W	W	T
2.	Rmd1036	-	+	+	S	W	W	HT
3.	Rmd1030	+	-	+	S	W	W	T

Table 15. Pleiotropic phenotypes of heat shock resistant (*hss*) mutants

Serial no	Strain	Genotype			Nodulation Response (Fix)	Heat shock tolerance (W=Wild type) (T=Tolerant)	Azide tolerance (W=Wild type)	Osmotolerance (W=Wild type)	Plaques of phages RMP26 and 38 (T=Turbid) (HT=Hyper Turbid)
		hss A	*hss B*	*hss C*					
1.	Rmd201	+	+	+	+	W	W	W	T
2.	Rmd1041	–	+	+	–	T	W	W	T
3.	Rmd1043	+	–	+	+	T	W	W	T
4.	Rmd1040	+	+	–	–	T	W	W	HT

B. NATURALLY STRESS TOLERANT STRAINS OF *Rhizobium ciceri*

The experiments with *R.meliloti* described above demonstrated that through mutagenesis variability in tolerance to stresses such as heat shock and osmolar shock can be generated in both directions. This allowed the hypothesis that, in each of the species of *Rhizobium*, there should be strains in nature that are widely different in their response towards stresses. An experiment was carried out to use the procedures developed for *R.meliloti* for isolating, from the natural strains of *R.ciceri*, those resistant to heatshock and/or high osmolarity. The result of this set of experiments are presented below:

1. Variation for toleration towards heat shock, osmolarity and azide among *R.ciceri* strains

A collection of fifty eight strains of *R.ciceri* was screened for toleration to heat shock given at 50°C for 1, 2, 3 or 4 h and sodium chloride added in TYM agar at 0.05 to 0.4 M and for resistance to sodium azide present in TYM medium at concentrations ranging from 10 to 100 µg/ml. The results are presented in the Table 16, in terms of highest amount of stress tolerated; that is maximum period of heat shock, concentration of sodium chloride and sodium azide tolerated. The frequency distributions of the strains for toleration of heatshock, NaCl and azide are presented in Figures 1, 2 and 3 respectively. The curves were divided

Table 16 : Variation among *Rhizobium ciceri* strains for tolerance towards heat shock, NaCl and NaN_3 (Source:Dr.R.B.Rewari, Project Coordinator, BNF, I.A.R.I., New Delhi)

Strain			Maximum degree of tolerance* towards		
Designation	Origin @		NaCl(M)	Azide (µg/ml)	Heat shock 50°C (h)
1	2	3	4	5	6
Rcd1	Pant114	GP	0.15(LT)	30(LT)	1(LT)
Rcd2	BG256	ID	<0.05(S)	10(S)	<1(S)
Rcd3	BG576	ID	0.05(S)	10(S)	1(LT)
Rcd4	BG276	ID	<0.05(S)	30(LT)	4(T)
Rcd5	BG276-I	ID	0.2(T)	20(LT)	1(LT)
Rcd6	BG276-II	ID	0.05(S)	30(LT)	1(LT)
Rcd7	Tal 1148	NH	0.2(T)	10(S)	<1(S)
Rcd8	Tal 675	NH	<0.05(S)	20(LT)	1(LT)
Rcd9	IC59	IH	<0.05(S)	10(S)	1(LT)
Rcd10	IC76	IH	0.05(S)	20(LT)	1(LT)
Rcd11	IC94	IH	0.15(LT)	30(LT)	1(LT)
Rcd12	CHB-32	HH	0.1(LT)	<10(S)	1(LT)
Rcd13	KG-31	KP	0.15(LT)	20(LT)	1(LT)
Rcd14	KG-46	KP	0.15(LT)	80(T)	1(LT)
Rcd15	KG-61	KP	0.1(LT)	30(LT)	2(T)
Rcd16	NAG-1	ID	0.1(LT)	30(LT)	2(T)
Rcd17	NAG-1-88	ID	0.05(S)	20(LT)	<1(S)
Rcd18	MB-2-88	ID	0.15(LT)	30(LT)	1(LT)
Rcd19	MBG-13-12-88	ID	0.05(S)	20(LT)	<1(S)

1	2	3	4	5	6
Rcd20	DG-36	DP	<0.05(S)	<10(S)	<1(S)
Rcd21	DG-83-I	DP	<0.05(S)	30(LT)	<1(S)
Rcd22	GNA-586	ID	0.2(T)	10(S)	1(LT)
Rcd23	GNA-3	ID	0.05(S)	20(LT)	<1(S)
Rcd24	GNA-FG-787	ID	<0.05(S)	<10(S)	<1(S)
Rcd25	GM-1	ID	0.3(T)	30(LT)	<1(S)
Rcd26	GMB-5-FG	ID	<0.05(S)	20(LT)	1(LT)
Rcd27	GS-84-I	ID	<0.05(S)	10(S)	1(LT)
Rcd28	GSL-84	ID	0.4(T)	40(T)	4(T)
Rcd29	GSL-3-84	ID	<0.05(S)	30(LT)	<1(S)
Rcd30	GSL-5-84	ID	<0.05(S)	20(LT)	1(LT)
Rcd31	GSL-10-84	ID	<0.05(S)	<10(S)	<1(S)
Rcd32	GSL-24-84	ID	0.4(T)	40(T)	4(T)
Rcd33	F-75	ID	0.15(LT)	20(LT)	1(LT)
Rcd34	F-76	ID	0.15(LT)	30(LT)	1(LT)
Rcd35	G-IC-53	ID	0.1(LT)	20(LT)	1(LT)
Rcd36	G-B7	ID	<0.05(S)	20(LT)	1(LT)
Rcd37	G-301B	ID	<0.05(S)	<10(S)	4(T)
Rcd38	G-567	ID	0.2(T)	10(S)	1(LT)
Rcd39	G-1049	ID	0.2(T)	20(LT)	1(LT)
Rcd40	G-5-81	ID	0.1(LT)	10(S)	1(LT)
Rcd41	G-3-86	ID	0.2(T)	30(LT)	<1(S)
Rcd42	G-6-86	ID	0.1(LT)	20(LT)	1(LT)
Rcd43	G-7-87	ID	0.2(T)	40(T)	<1(S)
Rcd44	G-212-R	ID	<0.05(S)	<10(S)	<1(S)

1					
Rcd45	G-246-2	ID	<0.05(S)	20(LT)	1(LT)
Rcd46	G-246-26	ID	<0.05(S)	<10(S)	<1(S)
Rcd47	G-261-2	ID	<0.05(S)	30(LT)	<1(S)
Rcd48	G-261-4	ID	<0.05(S)	<10(S)	<1(S)
Rcd49	G-261-5	ID	<0.05(S)	<10(S)	<1(S)
Rcd50	G-261-6	ID	<0.05(S)	<10(S)	<1(S)
Rcd51	G-276-I	ID	0.15(LT)	30(LT)	2(T)
Rcd52	GTB-4	ID	0.05(S)	20(LT)	1(LT)
Rcd53	GA-88	ID	0.1(LT)	10(S)	2(T)
Rcd54	GA-27-86	ID	0.1(LT)	30(LT)	2(T)
Rcd55	GA-32-86	ID	0.1(LT)	10(S)	2(T)
Rcd56	GA-34-86	ID	0.2(T)	30(LT)	1(LT)
Rcd57	GA-37-87	ID	0.15(LT)	40(T)	1(LT)
Rcd58	H-45	JP	0.1(LT)	30(LT)	2(T)

*S = Sensitive; LT = Low tolerant; T = Tolerant

@GP = GBPUAT, Pantnagar

ID = IARI, Delhi

NH = Nif,Tal, Hawaii

IH = ICRISAT, Hyderabad

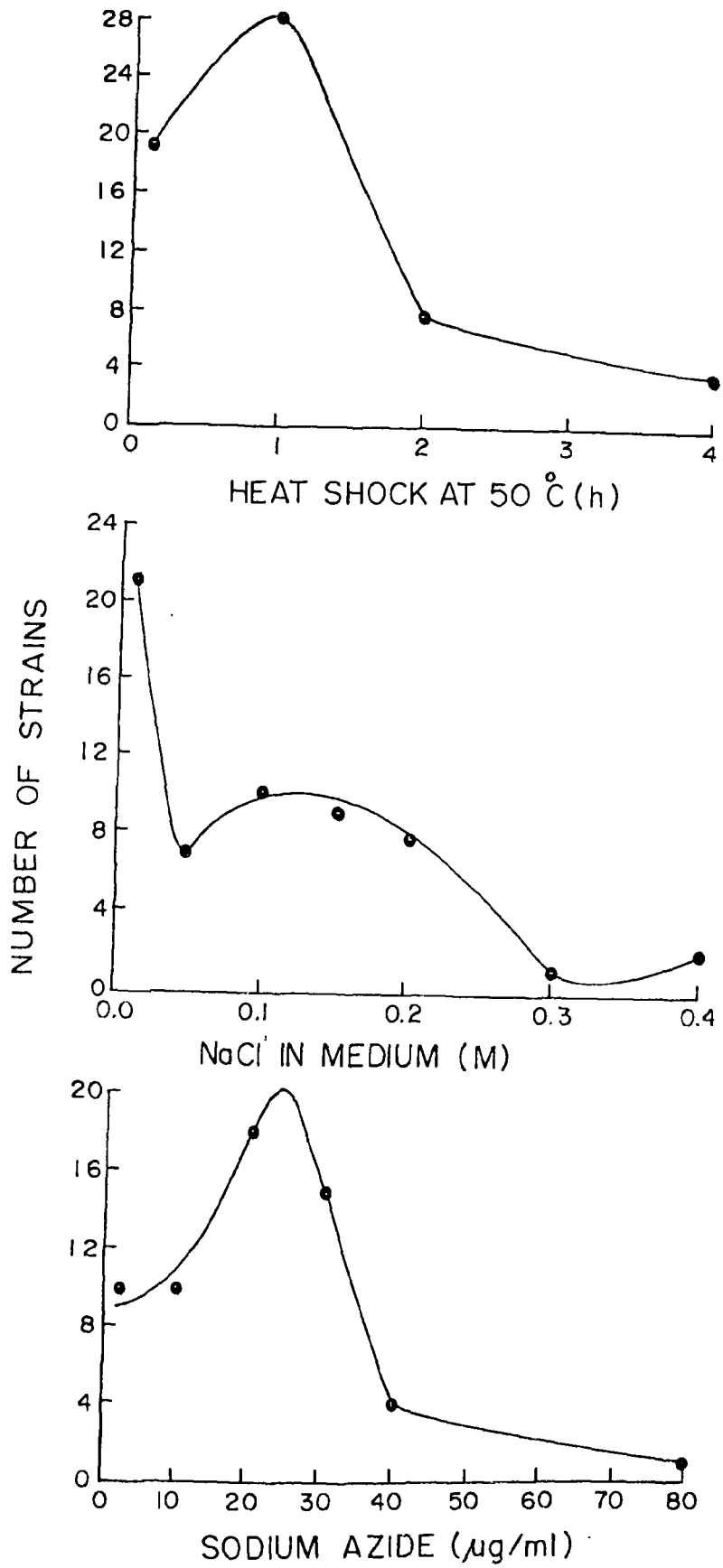
HH = HAU, Hissar

KP = Kanpur

DP = Durgapur

JP = Jabalpur

Figure 1-3 : Distribution of *Rhizobium ciceri* strains on the basis of their response to heat shock at 50°C (Figure 1), NaCl in the medium (Figure 2) and sodium azide in the medium (Figure 3).


28
24
20
16
12
8
4
0
0
1
2
3
4
HEAT SHOCK AT 50 °C (h)
NUMBER OF STRAINS
24
20
16
12
8
4
0
0.0
0.1
0.2
0.3
0.4
NaCl IN MEDIUM (M)
20
16
12
8
4
0
0
10
20
30
40
50
60
70
80
SODIUM AZIDE (µg/ml)

into three parts such that the strains falling in mode or central region were classified as having low tolerance. Those on the left of it were termed as sensitive and those falling on the right of central class were called as tolerant. Thus strains which tolerated heatshock of half an hour or less were called as heat shock sensitive strains (S). Those which tolerated maximum one hour of heat shock were called as low heat tolerant strains (LT). The remaining which tolerated heat shock up to 2 to 4 h were called as heat shock tolerant strains (T). Similarly the strains that tolerated 0.05 M or less salt concentrations were called as osmosensitive (S) and those which tolerated up to 0.15 M were treated as having low levels of osmotolerance (LT). The strains that tolerated 0.2 to 0.4 M NaCl were called as osmotolerant strains (T). Strains that tolerated 15 µg/ml azide or less were called as azide sensitive (S). Those which tolerated up to 30 µg/ml were treated as having low azide tolerance (LT). Strains having tolerance to 40 µg/ml or above concentrations of azide were called as azide tolerant strains (T).

There were nine heat shock tolerant strains, 3 of them tolerated up to 4 h of heat shock. There were 11 NaCl tolerant strains. Two of these tolerated 0.4 M NaCl. There were 5 azide tolerant strains, among these 4 were able to tolerate 40 µg/ml of azide and one up to 80 µg/ml of azide.

2. Isolation of two strains of *R.ciceri* that are simultaneously tolerant to heat shock, salt and azide

The Figure 4 represents covariation between maximum concentrations of NaCl tolerated and maximum duration of heat shock tolerated by the different strains. The data presented in Figure 4 showed that there were two strains namely Rcd28 and Rcd32 which were simultaneously resistant to both NaCl and heat shock. These were also observed to be tolerant to azide.

3. Symbiotic properties of *R.ciceri* strains varying in stress tolerance

Thirty one of *R.ciceri* strains were used for inoculation of chickpea cultivar Pusa 256 in Leonard jars and the nodulation response was examined.

The observations are presented in the Table 17. Each observation is a mean of 12 plants, that is 4 plants x 3 replications. For each plant, data was recorded on the lengths of shoot and root, number of root nodules, fresh weight of nodules, dry weight of shoot and total nitrogen content of shoot.

Figures 5, 6, 7 and 8 give the frequency distributions about dry weight of shoot, total nitrogen content, number of root nodules and fresh weight of nodules, respectively. Each distribution was divided into 3 parts. For dry weight values less than 0.5 g were called as low (L). Those between

Figure 4 : Co-distribution of *Rhizobium ciceri* strains on the basis of their tolerance towards NaCl in the medium and heat shock at 50°C. The number mentioned in each circle indicates the number of strains which fall on that position.

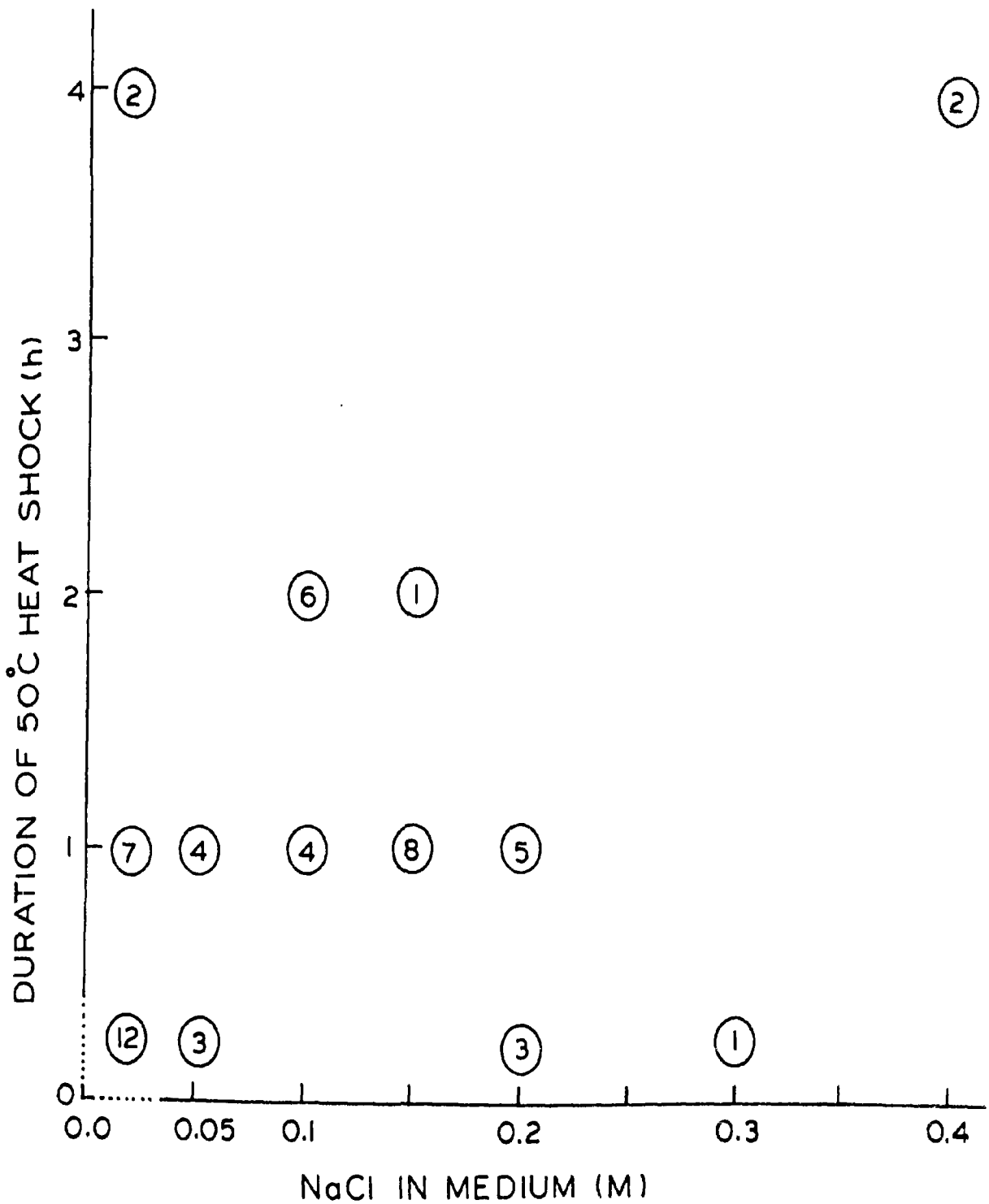

DURATION OF 50°C HEAT SHOCK (h)
4
3
2
1
0
2
2
6
1
7
4
4
8
5
12
3
3
1
0.0
0.05
0.1
0.2
0.3
0.4
NaCl IN MEDIUM (M)

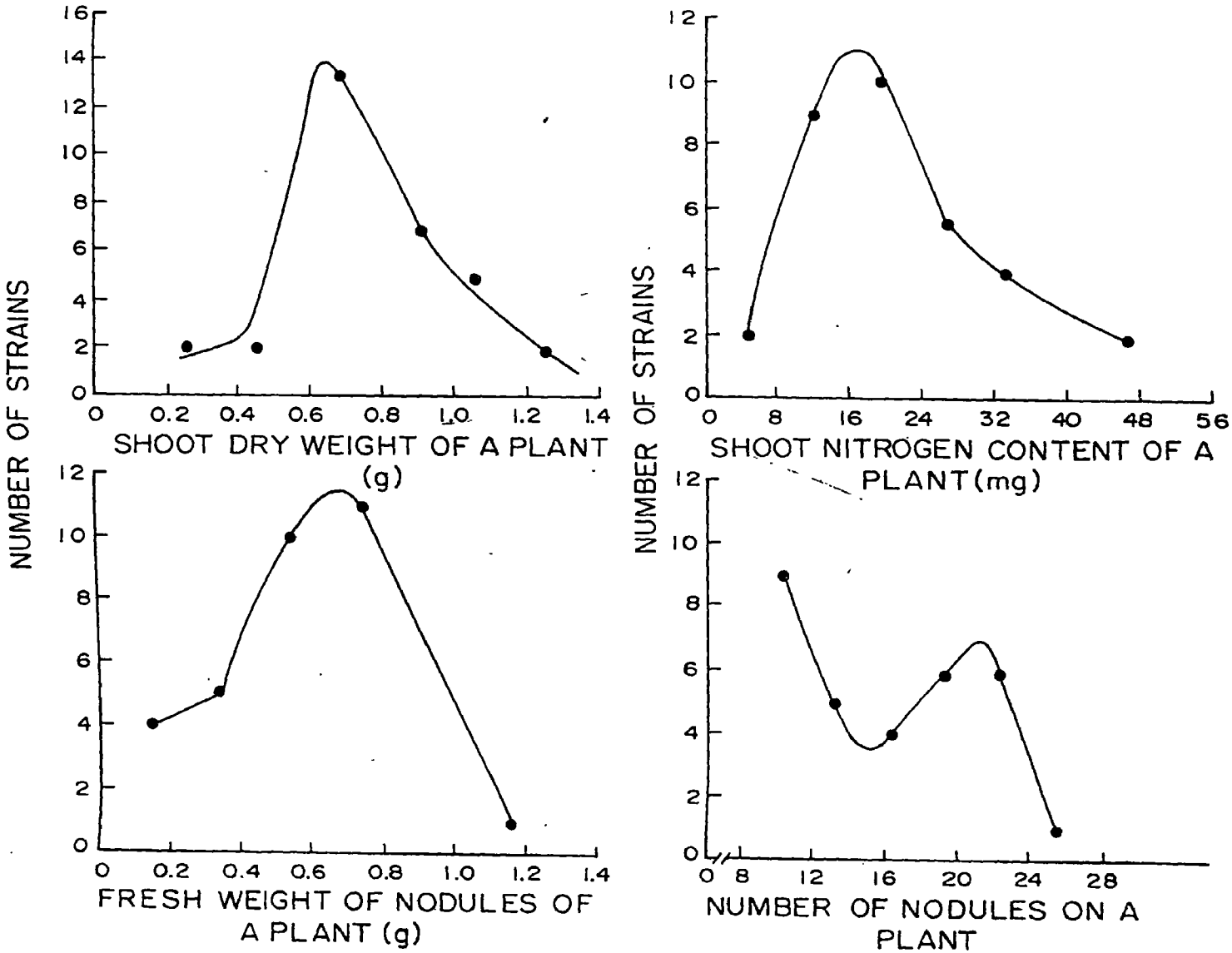
Table 17. Symbiotic response of *Rhizobium ciceri* strains towards *Cicer arietinum* cv. Pusa256

Serial no.	Strain	Plant length (cm)		Shoot		Root nodules	
		Shoot	Root	dry weight (g)	Nitrogen content (mg)	Number	Fresh weight (g)
	1	2	3	4	5	6	7
1.	Rcd1	32	22	0.6	16.2	11	0.4
2.	Rcd4	37	24	0.7	19.6	9	0.4
3.	Rcd5	37	22	0.5	14.0	16	0.7
4.	Rcd6	29	20	1.0	29.0	13	0.6
5.	Rcd9	37	23	1.1	34.1	11	1.2
6.	Rcd10	36	23	0.6	19.2	13	0.6
7.	Rcd11	35	26	0.8	23.2	10	0.7
8.	Rcd12	34	16	0.6	15.6	10	0.7
9.	Rcd13	27	25	0.8	21.6	16	0.5
10.	Rcd14	25	16	0.6	14.4	16	0.4

	1	2	3	4	5	6	7
11.	Rcd15	37	25	0.6	14.4	19	0.5
12.	Rcd16	36	22	1.3	46.8	14	0.7
13.	Rcd18	37	20	0.7	16.1	16.	0.7
14.	Rcd25	32	20	0.6	11.0	5	0.1
15.	Rcd28	40	21	0.8	23.2	8	0.7
16.	Rcd32	25	12	0.3	5.4	5	0.2
17.	Rcd33	37	22	0.9	30.6	17	0.6
18.	Rcd34	33	23	0.7	20.3	15	0.7
19.	Rcd35	33	20	1.2	45.6	23	0.8
20.	Rcd38	26	21	0.6	12.6	8	0.2
21.	Rcd39	32	18	0.7	16.8	12	0.7
22.	Rcd40	27	23	0.6	12.0	5	0.5
23.	Rcd41	32	22	0.8	18.4	25	0.5
24.	Rcd42	33	20	0.8	19.2	12	0.5

	1	2	3	4	5	6	7
25.	Rcd43	33	18	1.0	29.0	16	0.8
26.	Rcd51	25	24	0.5	10.0	10	0.1
27.	Rcd53	31	26	0.6	13.8	11	0.7
28.	Rcd54	35	22	0.8	28.8	26	0.7
29.	Rcd55	34	18	1.0	30.0	9	0.6
30.	Rcd56	34	24	0.6	15.0	10	0.5
31.	Rcd58	35	21	1.0	36.0	12	0.3
32.	Control	20	35	0.2	2.4	-	-

Figure 5-8 : Distribution of *Rhizobium cicori* strains on the basis of shoot dry weight of a plant (Figure 5), shoot nitrogen content of a plant (Figure 6), fresh weight of nodules of a plant (Figure 7) and number of nodules on a plant (Figure 8).


NUMBER OF STRAINS
SHOOT DRY WEIGHT OF A PLANT (g)
NUMBER OF STRAINS
SHOOT NITROGEN CONTENT OF A PLANT (mg)
FRESH WEIGHT OF NODULES OF A PLANT (g)
NUMBER OF NODULES ON A PLANT

0.5 to 0.85 g were called as medium (M). Those more than 0.85 g were called as high (H). Similarly, the limits for low (L), medium (M) and high (H) for nitrogen content were 8 mg, 26 mg and 46 mg respectively. When the number of nodules was less than 12, they were categorized as low (L). Medium category was for number 12 to 20 (M). More than 20 nodules was categorised as high (H). Fresh weight of nodules was treated medium (M) when it was between 0.4 and 0.9 g. The lower values were called as low (L) and the higher values as high (H).

Good correlation was noted between nitrogen content and dry weight (Figure 9). All the strains for which the nodulated plants had high dry weight, also had high nitrogen content. It was also noted that whenever dry weight of plant was low, nitrogen content was also low. The correlations between dry weight and nitrogen content of a plant on one hand and number of root nodules and fresh weight of root nodules on the other hand were not indicated to be tight.

4. Symbiotic properties of Rcd28 and Rcd32

The strains Rcd28 and Rcd32 of *R.ciceri* were shown to be tolerant towards heat shock, high osmolarity and sodium azide. The observations presented in Table 17 showed that while Rcd32 induced poor symbiotic response on chickpea plants, the strain Rcd28 induced a moderately good nodulation response. This point is graphically presented in Figure 10.

Figure 9 : Co-distribution of *Rhizobium ciceri* strains on the basis of nitrogen content and dry weight of a plant.

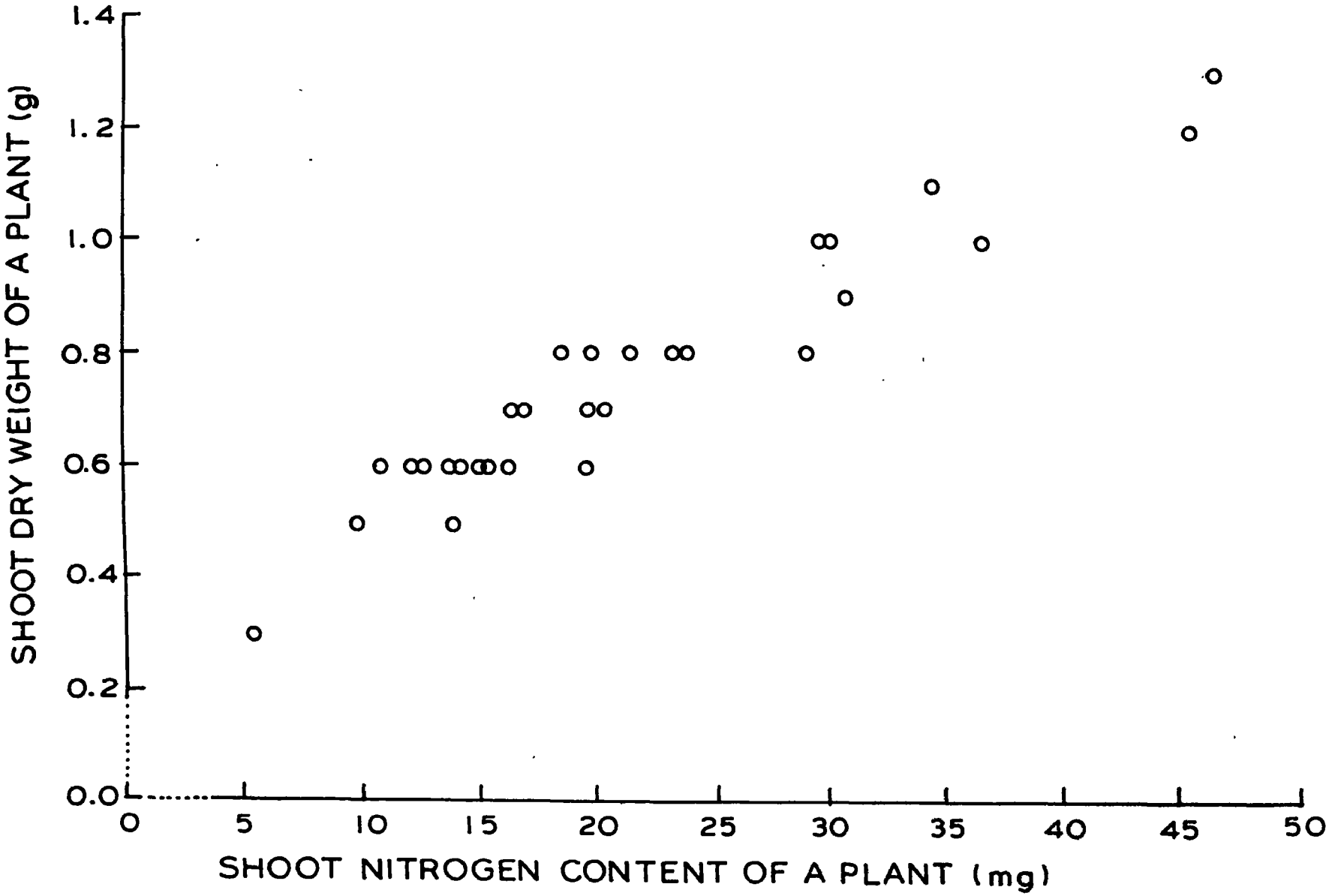
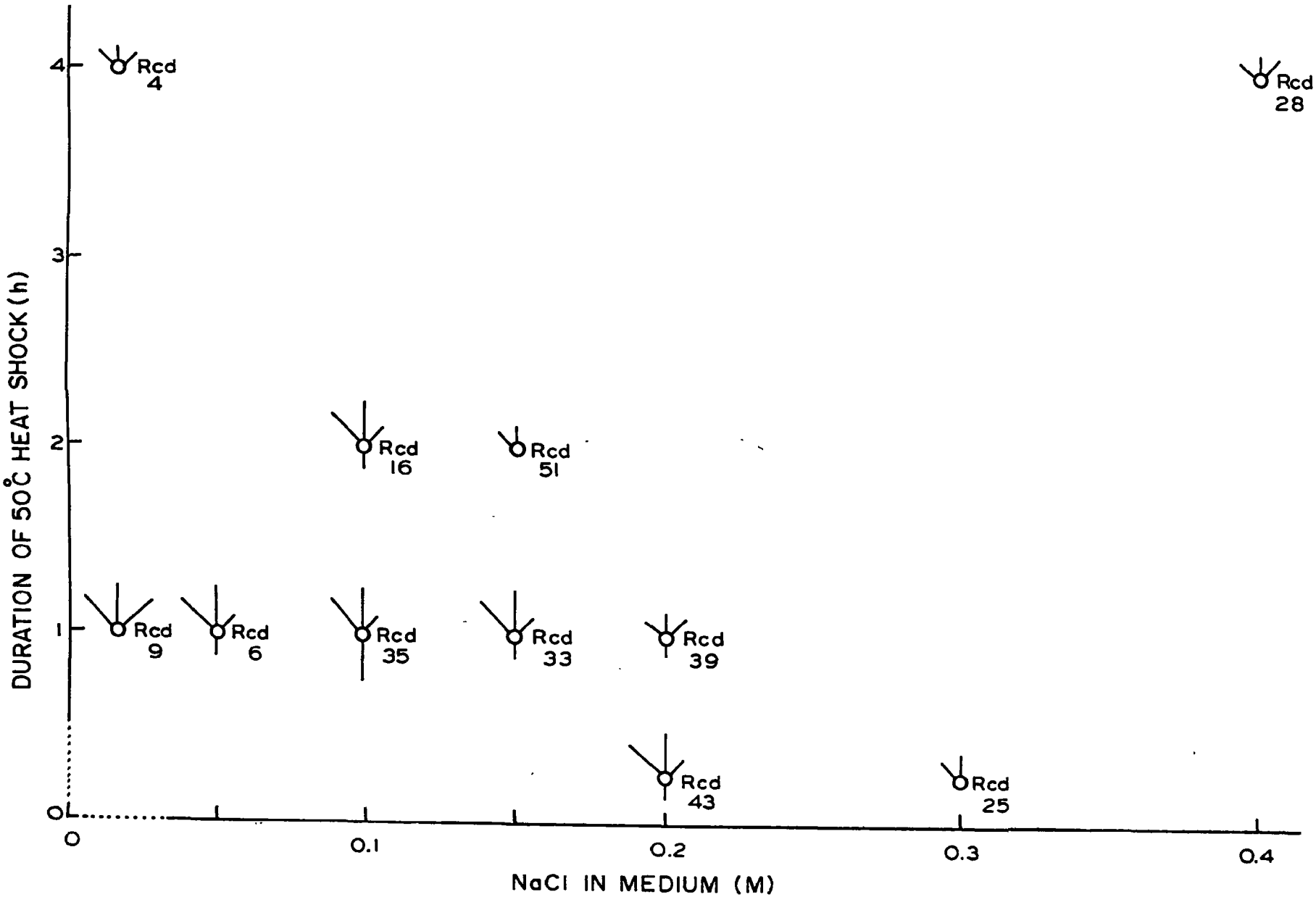

SHOOT DRY WEIGHT OF A PLANT (g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
45
50
SHOOT NITROGEN CONTENT OF A PLANT (mg)

Figure 10 : Co-distribution of twelve representative strains of *Rhizobium ciceri* for their response towards NaCl concentration and 50°C heat shock. Each strain, depicted here, has been picked up from each group shown in Figure 4 on the basis of maximum nitrogen content and dry weight of a plant (represented by a circle). The rods at 90°, 45°, 135° and 270° on the circle represent nitrogen content, nodule fresh weight, plant dry weight and number of nodules, respectively. Degree of expression of each character corresponds to the length of each rod.


DURATION OF 50°C HEAT SHOCK (h)
0
1
2
3
4
Rcd 4
Rcd 28
Rcd 16
Rcd 51
Rcd 9
Rcd 6
Rcd 35
Rcd 33
Rcd 39
Rcd 43
Rcd 25
0
0.1
0.2
0.3
0.4
NaCl IN MEDIUM (M)

C. INTERACTION OF *R.ciceri* STRAINS WITH THOSE OF *Azotobacter* AND *Azospirillum* WHEN INOCULATED TOGETHER ON CHICKPEA PLANTS

It has been reported that there is positive interaction between *R.ciceri* and *Azosprillum* for the promotion of growth of inoculated chickpea plants. Since it was shown above that the strain Rcd28 is symbiotically good and is tolerant towards stresses, such as heat shock, high osmolarity and sodium azide, it was desired to test its interaction with the strains of *Azospirillum* and *Azotobacter* which had been shown to elicit good growth response on a variety of plants including millets, rice and maize. This experiment was done using soil extracted from deep layers and which was not sterilized. Five strains of *R.ciceri* were used including Rcd28. These were used for inoculation individually and in combination with *Azotobacter* and *Azospirillum*. The experimental plants were grown to maturity. The response of treatments was measured in terms of mean grain yield/plant. The entire experiment was laid in complete randomized block design (CRD). The results are presented in Table 18. Considering critical difference at 5% level, the grain yields of 14 treatments could he classified into 4 non-overlapping groups called A, B, C and D. The range of the group A was between 13.9 to 16.6 g grain yield. Five treatments fell into this group. Only one

Table 18. Interaction of *Rhizobium ciceri* strains Rcd14, Rcd28, Rcd32, Rcd51 and Rcd56 with *Azotobacter chrococcum* strain A_{41}, *Azospirillum brasilense* strain CC, inoculated together on *Cicer arietinum* Pusa256 plants*

Serial no.	Bacterium and its strain used for inoculation			Mean grain yield of an inoculated plant (g)	Critically different grouping@ on the basis of grain yield data
	Rhizobium ciceri	*Azotobacter*	*Azospirillum*		
1.	Rcd14	Nil	Nil	13.9	A
2.	Rcd28	Nil	Nil	15.4	A
3.	Rcd32	Nil	Nil	11.5	B
4.	Rcd51	Nil	Nil	16.6	A
5.	Rcd56	Nil	Nil	14.5	A
6.	Rcd14	A_{41}	CC	9.1	C
7.	Rcd28	A_{41}	CC	9.4	C
8.	Rcd32	A_{41}	CC	9.4	C
9.	Rcd51	A_{41}	CC	8.2	C
10.	Rcd56	A_{41}	CC	8.6	C
11.	Nil	A_{41}	CC	8.1	C
12.	Nil	Nil	CC	15.7	A
13.	Nil	A_{41}	Nil	9.6	C
14.	Nil	Nil	Nil	5.8	D

* CD at 5% = 1.3; @Grain yields : A = 13.9-16.6 g; B = 11.5 g; C = 8.1-9.6 g ; D = 5.8 g

treatment giving a grain yield of 11.5 g fell into the group B. Seven treatments fell into group C having 8.1 to 9.6 g grain yield. The control uninoculated plant had the yield 5.8 g and fell into the group D. The average grain yield of group A was 15.2 g which is 2.6 times higher than that of the control. This showed that the control plants suffered in yield because of the deficiency of nitrogen arising from deficiency of N_2 fixing bacteria in the soil.

All the *R.ciceri* treatments produced group A or group B type of effects i.e they were symbiotically positive. It was interesting to note that the results corresponded with those elicited in the Leonard jars. The strain Rcd32 which was classified as having low symbiotic response in Leonard jars was categorised in this experiment as having group B type of positive symbiotic response.

Significantly, inoculation with *Azospirillum* benefitted the chickpea plants to about the same extent as the symbiotically good *R.ciceri* strains. However, the effect of *Azotobacter* treatment, although positive, was categorised as group C type of response.

Simultaneous inoculations with any of the *R.ciceri* strains, *Azotobacter* and *Azospirillum* produced group C type of response on grain yield. These observations showed that inclusion of *Azospirillum* along with *R.ciceri* in the inoculum suppressed the symbiotic response of *R.ciceri* strains on

chickpea plants.

These experiments also demonstrated that the strain Rcd28 which is resistant to azide and tolerant towards NaCl and heat shock is indeed symbiotically positive on chickpea plants grown in pots in the soil taken from a field.

DISCUSSION

A.1. *Rhizobium meliloti* has certain unique characteristics among rhizobia. It is considerably tolerant to both high temperatures and high osmolarity. For example, the strain Rmd201 is able to form colonies at 37°C and can grow in a medium containing up to 0.4 M NaCl. Thus *R.meliloti* offers opportunities to dissect the genetic basis of these adaptive characteristics. In the present study, random mutagenesis was carried out using the transposon Tn5. From among the mutants generated, those that were relatively more sensitive or tolerant than the wild type for heat shock at 50°C and sodium chloride at 0.4 M were isolated and characterized. Three particular kinds of mutants became available : heat shock tolerant, heat shock sensitive and osmosensitive. Osmotolerant mutants did not become available among nine hundred and ninety Tn5 mutants screened.

(a) Heat shock sensitive mutants (Hst^-)

A total of 23 Hst^- mutants were recovered among 990 Tn5 mutants, i.e. to an extent of 2.4%. By using complementation tests of one mutant with remaining twenty two, it was possible to group the mutants into 2 classes which have been named as genes *hst A* and *hst B*. Obviously the complementation analysis is preliminary only. The fact, that 2.4% mutants are heat shock sensitive, implies that a large number of genes must be determining the heat shock tolerance

involved in the strain Rmd201. It was found that on the Hst A^- mutant Rmd1036, the *R.meliloti* phages RMP 26 and 38 produced plaques which were more turbid than those produced by these phages on the wild type bacteria. This characteristic of heat shock sensitive *hst A* mutant is comparable to similar property of mutants of *E.coli* defective in certain heat shock responsive genes such as *dna J* and *dna K* (Ang *et al.*, 1986; Itikawa *et al.*, 1986). It has already been shown in *E.coli* that there are atleast 17 genes that are heat shock responsive. Some of them are involved in the toleration of heat by the wild type *E.coli* cells (Neidhardt *et al.*, 1984). The parallelism between the properties of *hst A* mutants of *R. meliloti* and *dna J* and *dna K* mutants of *E.coli* seem to indicate that similar type of functions must be involved in the determination of toleration to heat shock in the wild type bacteria.

The available mutants have provided tools for dissection of genes involved in heat shock tolerance as well as material for developing super heat shock tolerant strains of *R.meliloti*.

(b) Heat shock tolerant mutants (Hss$^-$)

Only 6 out of 990 Rmd201::Tn5 mutants were relatively more tolerant to 50°C heat shock than the wild type. Since this type of mutants were recovered to an extent of 0.6%, it can be concluded that only a small number of genes

regulate the sensitivity of *R.meliloti* towards the heat shock. Using two of the mutants in complementation against the available mutants, here, three genes called *hss A*, *hss B* and *hss C* could be defined. It was found that *hss A* and *hss C* mutants had pleiotropic phenotypes. These mutants were Fix$^-$ also. The *hss C* mutants were like the *hst A* mutants in their response to phages RMP26 and 38. The Fix$^-$ character of *hss A* and *hss C* mutants demonstrated that common functions or the functions originating from common operons might be involved in the determination of heat shock and symbiotic responses.

The present study has provided new material in the form of *hst* and *hss* mutants for analysing the mechanism in *R.meliloti* which promote toleration towards heat shock and also act in the opposite directions in determining sensitivity to heat shock. The *hss* kind of mutants are not reported in the literature but were previously isolated in our laboratory for *E.coli* (Raina, 1987).

(c) Osmosensitive mutants (Ost$^-$)

A total of 5 mutants became available from among 990 randomly induced Tn5 mutants. Four, out of five mutants, had pleiotropic phenotypes. Three mutants namely Rmd1048, Rmd1049 and Rmd1050 were heat shock sensitive besides being osmosensitive. Two of the mutants, out of the total collection of Rmd201::Tn5 mutants, were simultaneously

osmosensitive, heat shock sensitive and azide sensitive. The patterns of phenotypes of osmosensitive mutants and the low frequency of their recovery indicate that a small number of genes determine inborne osmotolerance in *Rhizobium meliloti* strain Rmd201.

Osmosensitive mutants are known in other bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Csonka, 1989). In these organisms, it has been demonstrated that the mutants defective in transport of certain aminoacids, such as proline (Measures, 1975; Csonka, 1981; 1988) and glycine betaines (Galinski and Truper, 1982; Imhoff, 1986), are osmosensitive. The correspondance of the osmosensitive mutants of *R.meliloti* isolated in this study with those of *E.coli* remains to be established.

A.2. Sodium azide is a respiratory inhibitor. The wild type strain Rmd201 is completely inhibited by about 40 µg/ml of sodium azide. Among the 990 randomly induced Tn5 mutants one mutant could be isolated which, unlike the wild type, was completely inhibited by 25 µg/ml of sodium azide. This was the Azt^- mutant which was simultaneously sensitive to heatshock and sodium chloride as well.

In an independent experiment, Tn5 mutants became available that were resistant to even 100 µg/ml of azide. These mutants, called Azs^-, were atleast of two kinds as

shown by preliminary complementation tests carried out on them. Apparently, there are genes in *R.melliloti* whose products make the wild type bacteria sensitive to azide. This is an interesting observation requiring further study.

In conclusion, the present study has demonstrated that it is possible to generate variability in both the directions in *R.melliloti* for adaptive characters such as response to heat shock, osmolarity and respiratory inhibitor azide. The material, that has been generated, will be useful in the genetic dissection of the concerned adaptive traits.

B. The realization from the experiments carried out with *R.melliloti* was that it would be possible to isolate mutants which differ from the wild type in both the directions in their response towards heat, osmolarity and respiratory inhibitor azide. This prompted screening of collection of *Rhizobium ciceri* strains for useful variation in such adaptive characteristics. The 58 strains studied revealed considerable variation among the strains for response towards 50°C heat shock, sodium chloride and sodium azide in the medium. Two strains could be identified which were simultaneously tolerant to heat shock, sodium chloride and sodium azide. One of these strains was symbiotically quite effective on chickpea variety Pusa256. The potential of this strain should now be evaluated at the field level. It is expected that the bacteria of this strain will survive

better in the field.

C. In Indian upland agriculture, certain crop rotations are practised. Often, a legume crop follows or precedes a cereal or a millet crop. Recently, strains of *Azospirillum* have been released as biofertilizers for millets, rice and wheat crop plants. On the other hand, several *Azotobacter* strains have been recommended for cotton crop. Thus, a situation has developed where the same field may be inoculated by *Rhizobium*, *Azospirillum* and *Azotobacter* strains in different cropping seasons. What effect this situation will have on the productivity of different crops is not known.

In this study, a pot experiment was carried out to examine the effect of interaction between a strain each of *Azotobacter* and *Azospirillum* and some strains of *R. ciceri* on the yield of inoculated chickpea plants of variety Pusa256. This experiment has led to several important observations: (1) The *R.ciceri* strain Rcd28 which was observed as highly tolerant to heat shock, osmolarity and azide. It is, indeed, symbiotically effective on chickpea plants under pot house conditions where natural non-sterlized soil had been used. (2) The *Azospirillum* strain was as much effective in promoting the growth of Pusa256 chickpea plants as was the *R.ciceri* strain Rcd28. (3) The *Azotobacter* strain, although effective, resulted only in poor growth of Pusa

256 chickpea plants. (4) In coinoculation treatments *Azospirillum* reduced the growth promoting effect of *R. ciceri*.

These observations imply that *Azospirillum* should be applied to the field judicially. Its indiscriminate application may prove counterproductive in agriculture, particularly for the productivity of legume crop plants such as chickpea.

SUMMARY

1. The overall objective of the present study was to understand genetics of stress tolerance in *Rhizobium meliloti* and to select relatively tolerant and effective strains of *Cicer-Rhizobium*.

2. In the *R.meliloti* wild type strain Rmd201, 990 Tn5 mutants were isolated and screened for their tolerance to 50°C heat shock and different concentrations of NaCl and NaN_3 in complete growth medium. This led to the isolation of 23 heat shock sensitive, 6 heat shock resistant, 5 osmosensitive and one azide sensitive mutants. Besides, a total of 12 azide resistant mutants were isolated by direct selection of Tn5 recipients.

3. With the use of complementation tests, heat shock resistant mutants have been classified into three complementation groups: *hss A* , *hss B* and *hss C* ; heat shock sensitive into two complementation groups: *hst A* and *hst B* and azide resistant mutants into two complementation groups: *azs A* and *azs B*.

4. Hst A^- and Hss C^- mutants allowed production of super-turbid plaques by rhizophages RMP26 and 38. Hss A^- and Hss C^- mutants were symbiotically defective in

being Fix^-. The osmosensitive Azt^- mutant Rmd1047 was azide sensitive and heat shock sensitive also.

5. A collection of 58 *R.ciceri* strains was screened for the response to 50°C heat shock, different concentrations of NaCl and NaN_3 in the complete medium. Considerable amount of variability was noted for each character. Two strains called Rcd28 and Rcd32 were identified to be highly tolerant to heat shock, hyperosmolarity and sodium azide. Among these, Rcd28 was found to be highly effective for symbiotic functions both in Leonard jars and pot experiments.

6. Interaction was investigated between selected *R. ciceri* strains and a strain each of *Azospirillum* and *Azotobacter*. *Azospirillum* was found to suppress the symbiotic effect of *R. ciceri* on Pusa256 chickpea plants.

BIBLIOGRAPHY

Aarons, S. and Ahmad, M.H. (1986). Examining growth and survival of cowpea rhizobia in Jamaican peat. Lett. Appl. Microbiol. 2: 115-118.

Aarons, S. and Ahmad, M.H. (1987). Growth and survival of cowpea rhizobia in bauxitic silt loam and sandy clay loam soils. FEMS Microbiol. Ecol. 45: 77-83.

Amara, D.S. and Miller, R.H. (1986). Effect of moisture and salt stress on selected *Rhizobium phaseoli* strains. Mircen J. Appl. Microbiol. Biotechnol. 2: 373-382.

Amara, D.S.; Mohjadi, W.A. and Miller, R.H. (1986). Tolerance of *Rhizobium phaseoli* to acidity, *aluminium and manganese. Mircen J. Appl. Microbiol.* Biotechnol. 2:289-299.

Ames-Gottfred, N.P. and Christie, B.R. (1989). Competition among strains of *Rhizobium leguminosarum* biovar trifolii and use of a diallel analysis in assessing competition. Appl. Environ. Microbiol. 55: 1599-1604.

Ang, D.; Chandrasekhar, G.N.; Zylicz, M. and Georgopoulos, C. (1986). *Escherichia coli grp E* gene codes for heat shock protein B 25.3, essential for both λ DNA replication at all temperatures and host growth at high temperatures. J. Bacteriol. 167: 25-29.

Apte, S.K. and Bhagwat, A.A. (1989). Salinity-stress-induced proteins in two nitrogen fixing *Anabaena* strains differentially tolerant to salt. J. Bacteriol. 171: 909-915.

Arnosti, D.N.; Singer, V.L. and Chamberlin, M.J. (1986). Characterization of heat shock in *Bacillus subtilis*. J. Bacteriol. 168: 1243-1249.

Ayanaba, A.; Haugland, R.A.; Sadowsky, M.J.; Upchurch, R.G.; Weiland, K.D. and Zablotowicz, R.M. (1986). Rapid colour nodule assay for assessing root exudate enhanced competitiveness of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 52: 847-851.

Bachhawat, A.K. and Ghosh, S. (1989). Temperature inhibition of siderophore production in *Azospirillum brasilense*. J. Bacteriol. 171: 4092-4094.

Baker, T.A.; Grossman, A.D. and Gross, C.A.(1984). A gene regulating the heat shock response in *Escherichia coli* also affects proteolysis. Proc. Natl. Acad. Sci. USA 81: 6779-6783.

Ball, A.S. and Edwards, C. (1987). Thermotolerance of the respiratory chains of *Bacillus coagulans*. J. Gen. Microbiol. 133: 1221-1226.

Banfalvi, Z.; Kondorosi, E. and Kondorosi, A. (1985). *Rhizobium meliloti* carries two megaplasmids. Plasmid 13: 129-138.

Banfalvi, Z.; Sakanyan, V.; Koncz, C.; Kiss, A.; Dusha, I. and Kondorosi, A. (1981). Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol. Gen. Genet. 184: 318-325.

Batut, J.; Terzaghi, B.; Gherardi, M.; Haquet, M.; Terzaghi, E.; Garnerone, A.M.; Boistard, P. and Haquet, T. (1985). Localization of a symbiotic *fix* region on *Rhizobium meliloti* pSym megaplasmid more than 200 kilobases from *nod-nif* region. Mol. Gen. Genet. **199**: 232-239.

Benschoter, A.S. and Ingram, L.D. (1986). Thermal tolerance of *Zymomonas mobilis:* temperature induced changes in membrane composition. Appl. Environ. Microbiol. **51**: 1278-1284.

Bergman, K.; Gulash-Hofee, M.; Hovestadt, R.E.; Larosiliere, R.C.; Ronco, P.G. and Su, L. (1988). Physiology of behevioural mutants of *Rhizobium meliloti:* evidence for a dual chemotaxis pathway. J. Bacteriol. **170**: 3249-3254.

Beringer, J.E.; Beynon, J.; Buchanan-Wollaston, A.V. and Johnston, A.W.B. (1978). Transfer of the drug-resistance transposon Tn5 to *Rhizobium*. Nature **276**: 633-634.

Bertelsen, H. (1985). Effect of temperature on H_2 evolution and acetylene reduction in pea nodules and in isolated bacterioids. Plant Physiol. **77**: 335-338.

Bloom, M.; Skelly, S. and vanBogelen, R. (1986). *In vitro* effect of the *Escherichia coli* heat shock regulatory protein on expression of heat shock genes. J. Bacteriol. **166**: 380-384.

Botsford, J.L. (1984). Osmoregulation in *Rhizobium meliloti* : inhibition of growth by salts. Arch. Microbiol. **137**: 124-127.

Brewin, N.J.; Beringer, J.E. and Johnston, A.W.B. (1980). Plasmid mediated transfer of host range specificity. J. Gen. Microbiol. **120**: 413-420.

Broughton, W.J.; Samrey, U. and Stanley, J. (1987). Ecological genetics of *Rhizobium meliloti*: symbiotic plasmid transfer in the *Medicago sativa* rhizosphere. FEMS Microbios Lett. **40**: 251-255.

Burns, A.; Watt, G.D. and Wang, Z.C. (1985). Salt inhibition of nitrogenase catalysis and salt effect on the separate protein component. Biochemistry **24**: 3932-3936.

Burns, R.C. and Hardy, R.W.F. (1975). Nitrogen fixation in bacteria and higher plants. Springer-Verlag, New York.

Carlson, R.W.; Kalembasa, S.; Turowski, D.; Pachori, P. and Noel, K.D. (1987). Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. J. Bacteriol. **169**: 4923-4928.

Casse, F.; Boucher, C.; Julliot, J.S.; Michel, H. and Denarie, J. (1979). Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**: 229-242.

Cregan, P.B. and Keyser, H.H. (1988). Influence of *Glycine* spp. on competitiveness of *Bradyrhizobium japonicum* and *Rhizobium fredii*. Appl. Environ. Microbiol. **54**: 803-808.

Csonka, L.N. (1981). Proline over production results in enhanced osmotolerance in *Salmonella typhimurium*. Mol. Gen. Genet. **182**: 82-86.

Csonka, L.N. (1988). Regulation of cytoplasmic proline levels in *Salmonella typhimurium* : effect of osmotic stress on synthesis, degradation, and cellular retention of proline. J. Bacteriol. **170**: 2374-2378.

Csonka, L.N. (1989). Physiological and genetic responses of bacteria to osmotic stress.Microbiol. Rev. **53**: 121-147.

Damirgi, S.M. and Johnson, H.W. (1966). Effect of soil actinomycetes on strains of *Rhizobium japonicum*. Agron. J. **58**: 223-224.

Date, R.A. and Roughley, R.J. (1977). Preparation of legume seed inoculants. *In*: A treatise on dinitrogen fixation, Section IV. Hardy, R.W.F. and Gibson, A.H. (eds.). John Wiley and Sons, New York. pp. 243-275.

Dazzo, F.B. and Gardiol, A. (1984). Host specificity in *Rhizobium* -legume interactions. *In*: Genes involved in microbe plant interctions. Springer-Verlag Publishers, New York pp. 3-31.

Denarie, J.; Boistard, P.; Casse, D.F.; Atherly, A.G.; Berry J.O. and Russell, P. (1981). Indigenous plasmids of *Rhizobium*. Int. Rev. Cytol. Suppl. 13. Bourne, G.H. and Danielli, J.F. (eds.) Academic Press, New York. pp. 225-246.

Ditta, G.; Stanfield, S.; Corbin, D. and Helinski, D.R. (1980). Broad host range DNA cloning system for Gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**: 7347-7351.

Ditta, G.; Virts, E.; Palomares, A. and Kim, C.H. (1987). The *nif A* gene of *Rhizobium meliloti* is oxygen regulated. J. Bacteriol. **169**: 3217-3223.

Douka, C.E. and Xenoulis, A.C. (1984). Salinity tolerance of a *Rhizobium meliloti* strain isolated from salt affected soils. Folia Microbiol. **29**: 316-324.

Dowdle, S.F. and Bohlool, B.B. (1987). Intra- and inter-specific competition in *Rhizobium fredii* and *Bradyrhizobium japonicum* as indigenous and introduced organisms. Can. J. Microbiol. 33: 990-995.

Dughri, M.H. and Bottomley, P.J. (1983). Effect of acidity on the composition of an indigenous soil population of *Rhizobium trifolii* found in nodules of *Trifolium subterraneum* cultivar Mt. Barker. Appl. Environ. Microbiol. **46**: 1207-1213.

Dylan, T.; Ilepi, L.;Stanfield, S.; Kashyap, L.; Douglas, C.;Yanofsky, M.; Nester, E.; Helinski,D.R. and Ditta, G. (1986). *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA **83**: 4403-4407.

Eaglesham, A.R.J.; Ahmad, M.H.; Hassouna, S. and Goldman, B.J. (1982). Cowpea rhizobia producing dark nodules: use in competition studies. Appl. Environ. Microbiol. **44**: 611-618.

Earl, C.D.; Ronson, C.W. and Ausubel, F.M. (1987). Genetic and structural analysis of the *Rhizobium meliloti fix A, fix B, fix C* and *fix X* genes. J. Bacteriol. **169**: 1127-1136.

Epstein, W. and Schultz, S.G. (1965). Cation transport in *Escherichia coli* : diverse systems with common controlling forces. Trends Biochem. Sci. 5 :21-23.

Figurski, D.H. and Helinski, D.R. (1979). Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76**: 1648-1652.

Finan, T.M.; Hirsch, A.M.; Leigh, J.A.; Johansen, E.; Kuldau, G.A.; Deegan, S.; Walker, G.C. and Signer, E.R. (1985). Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell **40**: 869-878.

Finan, T.M.; Kunkel, B.; Devos, G.F. and Signer, E.R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying expolysaccharide and thiamine synthesis genes. J. Bacteriol. **167**: 66-72.

Food and Agriculture Organization of UN (1979). 1978 FAO production year. Rome.

Forrai, T.; Vincze, E.; Banfalvi, Z.; Kiss, G.B.; Randhawa G.S. and Kondorosi, A. (1983). Localization of symbiotic mutation in *Rhizobium meliloti*.J. Bacteriol. **153**: 635-643.

Franco, A.A. and Vincent, J.M. (1976). Competition among rhizobial strains for the colonization and nodulation of two tropical legumes. Plant and Soil 45: 27-48.

Fujita, N.; Nomura, T. and Ishihama, A. (1987). Promoter selectivity of *E.coli* RNA polymerase: Purification and properties of holoenzyme containing the heat shock σ subunit. J. Biol. Chem. **262**: 1855-1859.

Galinski, E. and Truper, H.G. (1982). Betaine, a compatible solute in the extremely halophilic phototropic bacterium *Ectothiorhodospira halochloris*. FEMS Microbiol. Lett. **13**: 357-360.

Garg, F.C.; Beri, N and Tauro, P. (1985). Intrinsic antibiotic resistance in chickpea (*Cicer arietinum*) rhizobia. J. Agric. Sci. **105**: 85-89.

Goyal, V.; Chetal, S. and Nainawate (1986). Alteration in *Rhizobium trifolii* catalase under water stress. Folia Microbiol. **31**:164-166.

Grossman, A.D.; Cowing, D.; Erickson, J.; Baker, T.; Zhou, Y.N. and Gross, C.A. (1985). Analysis of the *Escherichia coli* heat shock response. *In* : Microbiology. Leive, L. and Schlessinger, D. (eds.) Am. Soc. Microbiol., Washington DC. pp. 327-331.

Grossman, A.D.; Erickson, J.W. and Gross, C.A. (1984). The htp^R gene product of *E.coli* is a sigma factor for heat shock promoters. Cell 165: 848-855.

Haahtela, K. (1985). Nitrogenase activity (acetylene reduction) in root associated, cold climate species of *Azospirillum*, *Enterobacter* , *Klebsiella* and *Pseudomonas* growing at various temperatures. FEMS Microbiol. Ecol. **31**: 211-214.

Habte, M. and Barrion, M. (1984). Interaction of *Rhizobium* sp. with toxin producing fungus in culture medium and in tropical soil. Appl. Environ. Microbiol. **47**: 1080-1083.

Ham, G.E.; Cladwell, V.B. and Johnson, H.W. (1971). Evaluation of *Rhizobium japonicum* inoculants in soil containing naturalized populations of rhizobia. Agron. J. 63: 301-303.

Hecker, M.; Heim, C. Volker, U. and Wolfel, L. (1988). Induction of stress proteins by sodium chloride treatment in *Bacillus subtilis*. Arch. Microbiol. **150**: 564-566.

Hirsch, P.R.; Van Montagu, M.; Johnston, A.W.B.; Brewin, N.J. and Schell, J.L. (1980). Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium legumionsarum*. J. Gen. Microbiol. **120**: 403-412.

Hua, S.S.; Tsai, Y.; Lichens, G.M. and Noma, A.T. (1982). Accumulation of amino acids in *Rhizobium* sp. strain WR 1001 in response to sodium chloride salinity. Appl. Environ. Microbiol. **44**: 135-140.

Imhoff, J.F. (1986). Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol. Rev. **39**: 57-66.

Iruthayathas, E.E.; Gunasekaran, S. and Vlassak, K. (1983b). Effect of combined inoculation of *Azospirillum* and *Rhizobium* on nodulation and N_2 fixation of winged bean and soybean. Sci. Hortic. **20**: 231-240.

Iruthayathas, E.E.; Vlassak, K. and Gunasekaran, S. (1983a). The influence of *Azospirillum* cell content and some amino acids on winged bean (*Psophocarpus tetragonolobus*) *Rhizobium* symbiosis. Z. Pflanzeneraehr Bodenkd. **146**: 405-408.

Itikawa, H.; Fujita, H. and Wada, M. (1986). High temperature induction of a stringent response in the *dna K* (Ts) and *dna J* (Ts) mutants of *E.coli*. J. Biochem. Tokyo **99**: 1719-1724.

Jensen, H.L. (1942). Nitrogen fixation in leguminous plants. Proc. Linn. Soc. N.S.W. **57**: 205-212.

Johnston, A.W.B.; Beyon, J.L.; Buchanan-Wollaston, A.V.; Setchell, S.M.; Hirsch, P.R. and Beringer, J.E. (1978). High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature **276**: 635-636.

Jordan, D.C. (1981). Reduction of the nodulation barrier in *Medicago laciniata* by alteration of the root temperature. Plant and Soil **61**: 93-111.

Kavimandan, S.K. (1985). Root nodule bacteria to improve yield of wheat (Triticum aestivum L.). Plant and Soil **86**: 141-144.

Khanuja, S.P.S. and Kumar, S. (1988). Isolation of phages for *Rhizobium meliloti* AK631. Indian J. Exp. Biol. **26**: 665-667.

Khanuja, S.P.S. and Kumar, S. (1989). Symbiotic and galactose utilization properties of phage RMP64-resistant mutants affecting three complementation groups in *Rhizobium meliloti*. J. Genet. **68**: 93-108.

Kondorosi, A. and Johnston, A.W.B. (1981). The genetics of *Rhizobium*. Int. Rev. Cytol. Suppl. **13**: 191-224.

Kondorosi, E.; Banfalvi, Z. and Kondorosi, A. (1984). Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*:identification of nodulation genes. Mol. Gen. Genet. **193**: 445-452.

Kosslak, R.M. and Bohlool, B.B. (1985). Influence of environmental factors on interstrain competition of *Rhizobium japonicum*. Appl. Environ. Microbiol. **49**: 1128-1133.

Kumar, S. (1976). Properties of adenyl cyclase and cyclic adenosine 3', 5' -monophosphate receptor protein-deficient mutants of *Escherichia coli*. J. Bacteriol. **125**: 545-555.

Kumar, S. and Srivastava, S. (1983). Cyclic AMP and its receptor protein are required for expression of transfer genes of conjugative plasmid F in *Escherichia coli*. Mol. Gen. Genet. 190: 27-34.

Kvien, C.S. and Ham, G.E. (1985). Effect of soil temperature and inoculum rate on the recovery of three introduced strains of *Rhizobium japonicum*. Agron. J. **77**: 484-489.

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

Lawson, K.A.; Barnet, Y.M. and McGilchrist, C.A. (1987). Environmental factors influencing numbers of *Rhizobium leguminosarum* biovar trifolii and its bacteriophages in two field soils. Appl. Environ. Microbiol. 53:1125-1131.

Lema, M.W.; Brown, A.; Butler, C.A. and Hoffman, P.S. (1988). Heat shock response in *Legionella pneumophila*. Can. J. Microbiol. 34: 1148-1153.

LeRudulier, D. and Bernard, T. (1986). Salt tolerance in *Rhizobium*: a possible role for betaines. FEMS Microbiol. Rev. **39**: 67-72.

LeRudulier, D.; Bernard,T.; Goas, G. and Hamelin, J. (1984). Osmoregulation in *Klebsiella pneumoniae* : enhancement of anaerobic growth and nitrogen fixation under stress by proline, betaine, γ-butyro betaines and other related compounds. Can. J. Microbiol. 30: 299-305.

LeRudulier, D.; Yang, S.S. and Csonka, L.N. (1982). Nitrogen fixation in *Klebsiella pneumoniae* during osmotic stress: effect of exogenous proline or a proline overproducing plasmid. Biochem. Biophys. Acta 719: 273-283.

Lindstroem, K.; Sarsa, M.L.; Polkunen, J. and Kansanen, P. (1985). Symbiotic nitrogen fixation of *Rhizobium* (*Galega*) in acid soils, and its survival in soil under acid and cold stress. Plant and soil 87: 293-302.

Long, S.R. (1984). Genetics of *Rhizobium* nodulation *In*: Plant microbe interactions. Kosuge, T. and Nester, E. (eds.). Macmillan Publishing Co., New York pp. 265-306.

Long, S.R.(1989). *Rhizobium*-legume nodulation: life together in the underground. Cell 56: 203-214.

Maartensson, A.M. and Gustafsson, J.G. (1985). Competition between *Rhizobium trifolii* strains for nodulation, during growth in a fermenter and in soil based inoculants studied by ELISA. J. Gen. Microbiol. 131: 3077-3082.

Mackey, B.M. and Derrick, C.M. (1986). Elevation of heat resistance of *Salmonella typhimurium* by sublethal heat shock. J. Appl. Bacteriol. 61: 389-393

Maier, R. and Brill, W.J. (1978). Mutant strains of *Rhizobium japonicum* with increased ability to fix nitrogen for soybean. Science 201: 448-450.

Mary, P.; Ochin, D. and Tailliez, R. (1985). Rates of drying and survival of *Rhizobium meliloti* strains during storage at different relative humidities. Appl. Environ. Microbiol. 50: 207-211.

McKnight, T. (1949). Efficiency of isolates of *Rhizobium* in cowpea group with proposed additions to this group. Queensland J. Agric. Sci. 6: 61-76.

Measures, J.C. (1975). Role of amino acids in osmoregulation of nonhalophilic bacteria. Nature 257: 398-400.

Michel, G.P.F. and Starka, J. (1986). Effect of ethanol and heat stresses on the protein pattern of *Zymomonas mobilis*. J. Bacteriol. 165: 1040-1042.

Milto, N.I. and Karbanovich, A.I. (1986). The use of *Azotobacter chrococcum* as a component of the nutrient medium for the cultivation of *Rhizobium* strains. Prikl. Biokhim. Mikrobiol. 22: 254-258.

Neidhardt,F.C. and vanBogelen, R.A. (1981). Positive regulatory gene for temperature controlled proteins in *Eschorichia coli*. Biochem. Biophys. Res. Commun. 100: 894-900

Neidhardt, F.C.; vanBogelen, R.A. and Vaughn, V. (1984). The genetics and regulation of heat shock proteins. Ann. Rev. Genet. 18: 295-329.

Nelson,D.W.; Swearingin, M.L. and Beckham, L.S. (1978). Response of soybeans to commercial soil applied inoculants. Agron. J. 70: 517-518.

Newcomb, W. (1981) Nodule morphogenesis and differentiation. *In* : Biology of the Rhizobiaceae, Int. Rev. Cytol. Suppl. 12. Giles, K.L. and Atherly, A.G. (eds.) Academic Press, New York. pp. 247-298.

Nuti, M.P.; Lederboer, A.M.; Lepidi; A.A. and Schilperoort, R.A. (1977). Large plasmids in different *Rhizobium* species. J. Gen. Microbiol. **100**: 241-248.

Olivares, J.; Casadesus, J. and Bedmar, E.J. (1980). Methods for testing degree of infectivity of *Rhizobium meliloti* strains. Appl.Environ.Microbiol.39: 967-970.

Paau, A.S. (1989). Improvement of *Rhizobium* inoculants. Appl. Environ. Microbiol. **55**: 862-865.

Paek, K.H. and Walker, G.C. (1986). Defect in expression of heat shock proteins at high temperature in *xth A* mutants. J. Bacteriol. **165**: 763-770

Paek, K.H. and Walker, G.C. (1987). *E.coli dna K* null mutants are inviable at high temperature. J. Bacteriol. **169**: 283-290.

Plazinski, J. and Rolfe, B.G. (1985a). Influence of *Azospirillum* strains on the nodulation of clovers by *Rhizobium* strains. Appl. Environ. Microbiol. **49**: 984-989

Plazinski, J. and Rolfe, B.G. (1985b). Interaction of *Azospirillum* and *Rhizobium* strains leading to inhibition of nodulation. Appl. Environ. Microbiol. **49**: 990-993.

Plazinski, J. and Rolfe, B.G. (1985c). Analysis of the pectolytic activity of *Rhizobium* and *Azospirillum* strains isolated from *Trifolium repens*. J. Plant Physiol. 120: 181-187.

Plazinski, J. and Rolfe, B.G. (1985d). *Azospirillum-Rhizobium* interaction leading to a plant growth stimulation without nodule formation. Can. J. Microbiol. 31: 1026-1030

Poi, S.C. and Ghosh, G. (1985). Carrier materials for *Rhizobium* and *Azotobacter*. Environ. Ecol. 3: 642-646.

Prakash, R.K.; Hooykaas, P.J.J.; Ledeboar, A.M.; Schilperoort, R.A.; Nuti, M.P.; Lepidi, A.A.; Casse, F.; Boucher, C.; Julliot, J.S. and Denarie, J. (1980). Detection,isolation and characterization of large plasmids in *Rhizobium*. *In* : Nitrogen fixation Vol. 2, Newton, W.E. and Orme-Johnson (eds.). University Park Press, Baltimore. pp. 139-163.

Puhler, A.; Aguilar, M.O.; Hynes, P.; Klipp, W.; Priefer, U.; Simon, R. and Weber, G. (1984). Advances in the genetics of free living and symbiotic nitrogen fixation bacteria. *In*: Advances in nitrogen fixation research. Veeger, V. and Newton, W.E. (eds.). Nijhoff-Junk/Pudoc. The Hague, pp. 609-619.

Rafique, M.; Mclaughlin, W. and Ahmad, M.H. (1984). Competition between inoculum and native rhizobia for nodulation of cowpea (*Vigna unguiculata* L. Walp): Use of a dark nodule strain. Plant and Soil **81**: 305-307.

Rai, R.; Nasar, S.K.T.; Singh, S.J. and Prasad, V. (1985). Interaction between *Rhizobium* strains and lentil (*Lens culinaris* Linn.) genotypes under salt stress. J. Agric. Sci. **104**: 199-205.

Raina, S.K. (1987). Ph.D. Thesis (Genetics). Submitted to Indian Agricultural Research Institute, New Delhi.

Ram, J.; Grover, R.P; Rewari, R.B. and Kumar, S. (1978). Improvement in the nitrogen fixing effectiveness of *Rhizobium leguminosarum* by incorporating genetic resistance to azide. Indian J. Exp. Biol. **16**: 1321-1322.

Rawthorne, S.; Hadley, P.; Summerfield, R.J. and Roberts, E.H. (1985). Effects of supplemental nitrate and thermal regime on the nitrogen nutrition of chickpea (*Cicer arietinum* L.) II. Symbiotic development and nitrogen assimilation. Plant and Soil **83**: 279-293.

Rennie, R.J. and Kemp, G.A. (1986). Temperature sensitive nodulation and N_2 fixation of *Rhizobium leguminosarum* biovar phaseoli strains. Can. J. Soil Sci. **66**: 217-224.

Richey, B.; Cayley, D.S.; Mossing, M.C.; Kolka, C.; Anderson, C.F.; Farrar, T.C. and Record Jr, M.T. (1987). Variability in the intracellular ionic environment of *Escherichia coli*: differences between *in vitro* and *in vivo* effects of ion concentrations on protein-DNA interactions and gene expression. J. Biol. Chem. **282**: 7157-7164.

Robertson, J.G.; Lyttleton, P.; Bullivant, S. and Grayston, G.F. (1978). Membranes in lupin root nodules. 1. The role of golgi bodies in the biogenesis of infection threads and peribacterioid membranes. J. Cell Sci. **30**: 129-149.

Roughley, R.J.; Nutman, P.S. and Chandler, M.R. (1981). Effects of host plant selection and temperature on the structure of the root nodules of red clover (*Trifolium pratense*). Plant and Soil **61**: 113-124.

Ruvkun, G.B. and Ausubel, F.M. (1980). Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. USA **77**: 191-195.

Selvaraj, G. and Iyer, V.N. (1983). Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. J. Bacteriol. **156**: 1292-1300.

Semu, E.; Hume, D.J. and Corke, C.T. (1979). Influence of soybean inoculation and nitrogen levels on populations and serogroups of *Rhizobium japonicum* in Ontario. Can. J. Microbiol. **25**: 739-745.

Singh, A. and Kumar, S. (1989). Evidence for linkage between the *azi* marker and nodulation determining (*nod*) genes in *Rhizobium leguminosarum* Rldl. Indian J. Exp. Biol. **27**: 201-206.

Singleton, P. (1984). Evidence for osmotic constraint in F-type bacterial mating. FEMS Microbiol. Lett. **25**: 37-39.

Streips, U.N. and Polio, F.W. (1985). Heat shock proteins in bacilli. J. Bacteriol. **162**: 434-437.

Szeto, W.W.; Zimmerman, J.L.; Sundaresan, V. and Ausubel, F.M. (1984). A *Rhizobium meliloti* symbiotic regulatory gene. Cell **36**: 1035-1043.

Taglichet, D.; Padan, E.; Oppenheim, A.B. and Schuldiner, S. (1987). An alkaline shift induces the heat shock response in *E. coli*. J. Bacteriol. **169**: 885-887.

Todd, J.A.; Hubbard, T.J.P.; Travers, A.A. and Ellar, D.J. (1985). Heat shock proteins during growth and sporulation of *Bacillus subtilis*. FEBS Lett. **188**: 209-214.

Toro, N. and Olivares, J. (1986). Analysis of *Rhizobium meliloti* Sym mutants obtained by heat treatments. Appl. Environ. Microbiol. **51**: 1148-1150.

vanRensburg, H.J. and Strijdom, B.W. (1982a). Competitive abilities of *Rhizobium meliloti* strains considered to have potential as inoculants. Appl. Environ. Microbiol. **44**: 98-106.

vanRensburg, H.J. and Strijdom, B.W. (1982b). Root surface association in relation to nodulation of *Medicago sativa*. Appl. Environ. Microbiol. **44**: 93-97.

Vashishat, R.K.; Yadav, A.S.; Sharma, P.K. and Khurana, A.L. (1986). Nitrogen fixing efficiency of sodium azide resistant strains of slow growing mungbean *Rhizobium*. Ann. Biol. **2**: 125-129.

Vincent, J. (1974). Root nodule symbiosis with *Rhizobium*. *In*: Biology of nitrogen fixation. Quispel, A. (ed.) North Holland Press, Amsterdam pp. 265-341.

Wilson, J.R. (1985). Comparative response to salinity of the growth and nodulation of *Macroptilium atropurpureum* cv. siratro and *Neonotonia wightic* cv. cooper seedlings. J. Agric. Res. **36**: 589-599.

Yadav, A.S. and Vashishat, R.K. (1986). Correlation between azide resistance and effectiveness of *Rhizobium trifolii*. Indian J. Microbiol. **26**: 288-289.

Yao, P.Y. and Vincent, J.M. (1969). Host specificity in the root hair "curling factor" of *Rhizobium* sp. Aust. J. Biol. Sci. **22**: 413-423.

Yura, T.; Tobe, T.; Ito, K. and Osawa, T. (1984). Heat shock regulatory gene (htp^R) of *Escherichia coli* is required for growth at high temperature but is dispensable at low temperature. Proc. Natl. Acad. Sci. USA **81**: 6803-6807.

Zahran, H.H. and Sprent, J.I. (1986). Effects of sodium chloride and polyethylene glycol on root hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. Planta **167**: 303-309.

Zeuthen, M.L. and Howard, D.H. (1989). Thermotolerance and the heat shock response in *Candida albicans*. J. Gen. Microbiol. 135: 2509-2518

Zimmerman, J.L.; Szeto, W.W. and Ausubel, F.M. (1983). Molecular characterization of Tn5-induced symbiotic (fix$^-$) mutants of *Rhizobium meliloti*. J. Bacteriol. **156**: 1025-1034.