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**DEVELOPMENT OF NITRATE TOLERANT  $Hup^+$   
STRAINS OF *CICER*-RHIZOBIA AND STUDIES ON  
THEIR NITRATE METABOLISM**

**DEBASIS CHATTOPADHYAY**

**DIVISION OF BIOCHEMISTRY  
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NEW DELHI - 110 012**

**1992**

# DEVELOPMENT OF NITRATE TOLERANT *Hup*<sup>+</sup> STRAINS OF *CICER-RHIZOBIA* AND STUDIES ON THEIR NITRATE METABOLISM

By  
**DEBASIS CHATTOPADHYAY**

A thesis submitted to the Post-Graduate School,  
Indian Agricultural Research Institute, New Delhi,  
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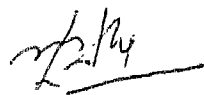
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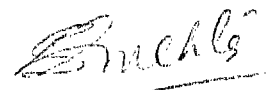
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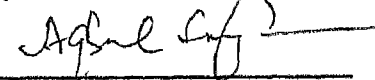
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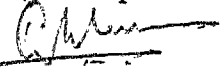
  
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## **CERTIFICATE**

This is to certify that the thesis entitled, **Development of nitrate tolerant Hup<sup>+</sup> strains of *Cicer-rhizobia* and studies on their nitrate metabolism** submitted in partial fulfilment of the requirement for the award of the the degree of **Doctor of Philosophy in Biochemistry** of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, is a faithful record of the *bona fide* research work carried out by **Shri Debasis Chattopadhyay** under my guidance and supervision and no part of this thesis has been submitted for any other degree or diploma. The assistance and help, received during the course of this investigation, has been duly acknowledged.

  
(M.L. Lodha)

Dated: July 27, 1992

Chairman  
Advisory Committee

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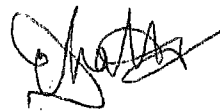
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## ABBREVIATIONS

|                  |   |                                           |
|------------------|---|-------------------------------------------|
| BSA              | - | Bovine serum albumin                      |
| CCC              | - | Covalently closed circular                |
| CRYMA            | - | YEMA with Congo Red                       |
| EDTA             | - | Ethylene diamine tetra-acetic acid        |
| EtBr             | - | Ethidium bromide                          |
| FID              | - | Flame ionisation detector                 |
| Fix <sup>+</sup> | - | Nitrogen fixation positive                |
| Fix <sup>-</sup> | - | Nitrogen fixation negative                |
| HUM              | - | Hydrogen uptake medium                    |
| Hup <sup>+</sup> | - | Hydrogen uptake positive                  |
| Hup <sup>-</sup> | - | Hydrogen uptake negative                  |
| <i>hup</i>       | - | Hydrogen uptake gene                      |
| IAA              | - | Indole acetic acid                        |
| LB               | - | Luria-bertani                             |
| Nod <sup>+</sup> | - | Nodulation positive                       |
| Nod <sup>-</sup> | - | Nodulation negative                       |
| NR               | - | Nitrate reductase                         |
| NiR              | - | Nitrite reductase                         |
| RNase            | - | Ribonuclease                              |
| SDS              | - | Sodium dodecyl sulphate                   |
| SSC              | - | Saline sodium citrate                     |
| TCA              | - | Trichloro acetic acid                     |
| TCD              | - | Thermal conductivity detector             |
| TE               | - | Tris-EDTA buffer                          |
| Tris             | - | 2-amino-2-hydroxymethyl propane-1, 3-diol |
| TY               | - | Tryptone yeast-extract                    |
| TYM              | - | Tryptone yeast-extract mannitol           |
| YEMA             | - | Yeast-extract mannitol agar               |

## **INTRODUCTION**

---

The whole process of symbiotic  $N_2$  fixation involving micro-symbiont *Rhizobium* and macro-symbiont legume, is very complex and developmentally regulated where both the micro- and macro-symbiont perform the pre-programmed role. The  $N_2$  fixation is not a single shot, one step event but consists of well defined steps *viz.*, pre-infection, infection and nodule formation, and nodule function. Moreover, being an entity of the biosphere,  $N_2$  fixation in general and enzyme nitrogenase in particular, are prone to different types of environmental controls.

In natural environment  $N_2$  fixation takes place against changing concentration of soil nitrate arising from mineralisation and/or fertilizer application. The metabolism of nitrate may occur by two pathways *viz.*, assimilatory under aerobic conditions or dissimilatory under microaerobic/ anaerobic conditions. However, in both the pathways, nitrite is the common intermediate. This nitrite, the product of bacterial nitrate reduction, is destructive to indole acetic acid (IAA) synthesized by the *Rhizobium* at the early stage of nodulation and is also inhibitory to nitrogenase, the enzyme responsible for the reduction of dinitrogen to ammonia.

Thus, among several other environmental stimuli, nitrate influences the  $N_2$  fixation process from pre-infection stage to nodule function, leading from time to time to the postulation of different theories of nitrate inhibition to  $N_2$  fixation *e.g.*, nitrate may block the recognition and adhesion of rhizobia to the root hairs or it may hamper the infection thread formation or leghaemoglobin and/or nitrogenase biosynthesis. At the infection stage nitrate through its reaction product nitrite may affect root hair curling by

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destroying IAA or by increasing its requirement. In its presence the photosynthate supply to nodule may be reduced or proper oxygen diffusion may not occur.

Even though lot of studies have been made, the mechanism of nitrate inhibition to nodule formation and function is not well understood. One way to understand the inhibition mechanism is to develop nitrate tolerant strains of *Rhizobium* and then study the levels of nitrate metabolizing enzymes and production of IAA in them. Besides nitrate, another important factor affecting the efficiency of  $N_2$  fixation is the evolution of  $H_2$  by nodules. Chickpea (*Cicer arietinum* L.) rhizobia are known to have no functional  $H_2$ -uptake (Hup) system. Therefore, the  $N_2$ -fixing efficiency of chickpea-*Rhizobium* symbiotic system can be increased by developing Hup<sup>+</sup> strains of *Cicer-Rhizobium*. Keeping this in view, in the present investigation first Tn5 mutants of *Cicer*-rhizobial strains were developed for nitrate tolerance under symbiotic conditions, and then studies were made to understand the biochemical basis of nitrate tolerance. Further Hup<sup>+</sup> transconjugants of the mutants so obtained, were developed. The main objectives of the investigation were:

- (i) to develop transposon Tn5 mutants of *Cicer*-rhizobial strains G36-84 and F-75, and screen them for nitrate tolerance under symbiotic conditions.
- (ii) to study nitrate metabolism and IAA production in the nitrate tolerant mutants so developed, under free-living conditions, and
- (iii) to develop Hup<sup>+</sup> strains of nitrate tolerant mutants in order to increase their symbiotic energy efficiency.

## **REVIEW OF LITERATURE**

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Biological nitrogen fixation, especially symbiotic nitrogen fixation is important to agriculture. A specific interaction between host legume and microsymbiont *Rhizobium* leads to the formation of nodules on legume roots. The process of nodule formation is complex and involves various steps. A number of factors are known to affect the establishment of an effective symbiosis, the most important being the presence of combined nitrogen in the soil medium.

Small amount of combined nitrogen may stimulate the symbiosis in soybean (Manhart and Wong, 1980a; Streeter, 1985), pea (Libbenga *et al.*, 1973; Chen and Phillips, 1977; Bisseling *et al.*, 1978) and white clover (Dazzo, 1980; Carroll and Gresshoff, 1983). However, combined nitrogen, specially nitrate, when present in higher concentration can affect the process of legume-*Rhizobium* symbiosis (Lodha *et al.*, 1991; Rawsthorne *et al.*, 1985; Singh and Lodha, 1989).

The whole process of symbiosis can be divided into two main steps: nodule formation and nodule function. The root hair infection process consists of several specific events which precede nodule formation in the legume-*Rhizobium* symbiosis. These include: mutual host-symbiont recognition of the *Rhizobium* species on the legume rhizo-plane, rhizobial adhesion to differentiated epidermal cells called root hairs, root hair curling, root hair infection, root nodulation, and transformation of vegetative bacteria into enlarged pleomorphic bacteroids which fix nitrogen. Nitrate has been shown to affect each step during the process of establishment of legume-*Rhizobium* symbiosis as reviewed below:

## 2.1 MECHANISMS OF INHIBITION OF NODULE FORMATION AND FUNCTION BY NITRATE

- a) Nitrate prevents the synthesis of lectin, thereby blocking the recognition and adhesion of *Rhizobium* to the root hairs, e.g., in white clover (Dazzo and Hubbell, 1975 a, b; Bishop *et al.*, 1977; Dazzo and Brill, 1977, 1978).
- b) Nitrate interferes with infection by preventing root hair curling and infection-thread formation, e.g., in soybean (Harper and Cooper 1971; Rabie and Kumazawa, 1979), white clover (Dazzo, 1980), and alfalfa (Munns, 1968a).
- c) Nitrate blocks or delays the onset of nodule appearance as observed in soybean (McNeil, 1982; Eaglesham *et al.*, 1983; Ralston and Imsande, 1983; Harper and Gibson, 1984; Streeter, 1985; Gibson *et al.*, 1986), and cowpea (Pate and Dart, 1961; Dart and Wildon, 1970).
- d) Nitrate lowers leghaemoglobin levels, e.g., in soybean (Bergerson, 1961; Harper and Cooper, 1971; Bergerson and Goodchild, 1976; Streeter, 1981), alfalfa (Vance *et al.*, 1979), and pea (Rojonen, 1970).
- e) Nitrate initiates senescence of the functioning of root nodule in soybean (Streeter, 1981), pea (*Pisum sativum*) (Chen and Phillips, 1977, Bisseling *et al.*, 1978), alfalfa (Becana *et al.*, 1986) and lupin (Lorenzo *et al.*, 1990).
- f) Nitrate affects hormone levels in the nodules of *Vicia sativa* L. (Pate, 1958), *Lupinus luteus* L. (Dullaart, 1970), pea (Libbenga *et al.*, 1973), alfalfa (Thornton, 1936; Valera and Alexander, 1965; Munns, 1968d), bengal gram (Yadav *et al.*, 1987) and mungbean (Singh and Lodha, 1989).

- g) Nitrate inhibits nitrogenase biosynthesis and activity within the nodule, e.g., in soybean (Hardy and Havelka, 1976; Latimore *et al.*, 1977; Manhart and Wong, 1980b; Ralston and Imsande, 1983), white clover (Carroll and Gresshoff, 1983), mungbean (Singh and Lodha, 1989) and chickpea (Lodha *et al.*, 1991).
- h) Presence of nitrate reduces the supply of photosynthates to nodules (Bethlanfalvy and Phillips, 1978; Wong, 1980).
- i) More recent evidence suggests that nitrate may inhibit  $N_2$  fixation by increasing the resistance of the nodules to oxygen diffusion (Carrol *et al.*, 1986; Minchin *et al.*, 1986; Sprent *et al.*, 1987; Vessey *et al.*, 1988) and not as a result of reduction to nitrite by nitrate reductase (NR) within the bacteroid zone (Sprent *et al.*, 1987).

### 2.1.1 Nitrate effect on legume-*Rhizobium* recognition

The recognition process in the legume-*Rhizobium* symbiosis includes a pre-infective event in which the root hairs preferentially absorb infective rhizobia (Dazzo *et al.*, 1976). This step occurred within 12 h after rhizobial inoculation in clover and may involve the specific binding of lectin, trifoliin, on root hair to unique carbohydrate structures of *Rhizobium trifolii* (Dazzo and Brill, 1977, 1978; Dazzo and Hubbell, 1975a, 1975b).

It is known for a long time that fixed nitrogen ( $NH_4^+$ ,  $NO_3^-$ ) is one of the many environmental factors which limits the development and success of legume-*Rhizobium* symbiosis in nature (Fred *et al.*, 1932). Dazzo and Brill (1978) observed that immunologically detectable levels of trifoliin and the specific attachment of *R. trifolii* to root hairs decreased in a parallel fashion as the concentration of either nitrate or ammonium was increased in the rooting medium. Either nitrate (16mM) or ammonium (1mM) completely

inhibited infection or nodulation of white clover seedlings inoculated with *R. trifolii*. This experiment supported the hypothesis that trifoliin is involved in binding of *R. trifolii* to clover root hairs. The levels of these ions which inhibited the attachment were well below the levels which stunted the seedling growth. Interestingly, the level of trifoliin and rhizobial attachment were increased at a relatively low concentration of nitrate. Thus, low levels of nitrate enhanced the recognition process, and high levels shut it off.

Immunochemical (Dazzo and Hubbell, 1975a, b) and genetic (Bishop *et al.*, 1977) studies suggested that the receptors on *R. trifolii* and clover root epidermis which bound trifoliin were similar. The result of this study indicated that fixed nitrogen ions in the rooting medium might play an important role in the regulation of this early recognition process. The inhibition by fixed nitrogen was expressed within a few hours after clover seed germination and it prevented *R. trifolii* from accumulating in high numbers on clover root surface. Since nodulation and nitrogen fixation occurred days to weeks after rhizobial absorption to root hairs, their inhibition by fixed nitrogen are of secondary ecological consequences.

Umali-Garcia *et al.* (1980) observed that adherence of *Azospirillum brasilense* to root hairs of the grass *Pennisetum americanum* (pearl millet) was suppressed when the roots were grown in critical concentrations of nitrate containing medium. Scanning electron microscopy showed a distinct degranulation in the surface topography of root hairs in the presence of nitrate. These observations explain that how fixed nitrogen regulates the developmental events of the legume-*Rhizobium* symbiosis.

### **2.1.2 Interference of nitrate in the infection process**

Nitrate retards the development of nodules on legumes nodulated with *Rhizobium* strain (Thornton, 1936; Gibson and Nutman 1960) an effect

ascribed to the initiation of root hair infection (Thornton, 1936; Munns, 1968a). The root hair infection process consists of several specific events which precede nodule formation in legume-*Rhizobium* symbiosis. Essentially, infection occurs at a root hair which then becomes curled (Lim, 1963). A channel called infection thread is formed through which the bacteria pass to the inner cortical cells and the pericycle of the roots, when multiplying, among the cells of root cortex (Fahraeus, 1957; Dart and Mercer, 1964; Nutman, 1962; Bauer, 1981). This, in turn, gives rise to nodules. These nodules have a very highly organised structure with vascular connections to the roots (Wilson, 1940; Dart and Mercer, 1965; Bauer, 1981).

Munns (1968a) observed that when nitrate was maintained in continuous supply in solution cultures at a concentration 0.02 mM to 2 mM, it reduced the number of curled root hairs and of nodules in *Medicago sativa*. But the addition of indole-3-acetate with the inoculum overcame the nitrate inhibition of curling without affecting nodulation, and there was no quantitative connection between the numbers of curled hairs and the number of nodules when nitrate treatments of limited duration were applied at different times in relation to inoculation. Thornton (1936) observed that if the growth medium contained nitrate, alfalfa plants had fewer root hairs, and fewer of the root hairs curled in response to inoculation with nodule bacteria. He considered curling to be the essential for infection by bacteria.

Thornton's observations were later confirmed and extended by Munns (1968a, b, c, d). He showed that nitrate reduced the nodule number to some extent even if present before inoculation, or when present only on the first, second or third day after inoculation and thus concluded that the reduction in nodule number could not be attributed solely or even chiefly to interference by nitrate with any one phase of nodulation process. However,

nitrate inhibited the formation of infection threads, to such an extent that this could be important in limiting nodule number (Munns, 1968c). Nitrate was also able to delay nodulation by causing a delay in the formation of infection threads (Nutman, 1959).

It has been noted that root hairs are eliminated on *M. sativa* roots at nitrate concentrations above 1 mM as would be found in fertile soils and conventional nutrient solution (Munns, 1968b). Rabie and Kumazawa (1979) observed reduction in number of nodules per plant in soybean which was explained to be due to inhibition of infection thread and nodule formation. Harper and Cooper (1971) found that in soybean nodule, fresh weight was decreased by nitrate application, while nodule number was not as markedly affected. This indicated an effect on nodule development rather than on root hair infection.

### **2.1.3 Block or delay in nodule appearance by nitrate**

Various soybean cultivar x *Rhizobium* strain combinations were taken, their pre-germinated seedlings were transplanted on day 7 to inoculated nutrient solutions containing nitrate levels 0, 0.5 and 1 mM (resupplied daily to initial levels) and 1, 2 and 4 mM (allowed to deplete with plant growth). It was observed by Harper and Gibson (1984) that rates of nitrate uptake were similar among all treatments inspite of an initial 8-folds range in nitrate level. Higher external concentration of nitrate was more inhibitory to nodule appearance even though when nitrate uptake rates were similar. The external concentration of nitrate in the solution rather than the rate of nitrate uptake appeared to have a major effect on the initial stages of nodulation.

It was observed by Pate and Dart (1961) that in the favourable summer sowing, primary root nodulation in cowpea was stimulated by low concentration of ammonium nitrate but higher levels progressively decreased nodule numbers. With moderate application of ammonium nitrate (0 to 125 mg N/pot), any reduction in nodule numbers was wholly or partly offset by increased nodule size. Nodule initiation (Gibson and Nutman, 1960; Gibson, 1974) and nodule development and function have also been found to be inhibited by increasing concentration of nitrate.

Lentils (*Lens esculenta*) grown in a nutrient solution containing 15 mM  $\text{NO}_3^-$  had 84% fewer nodules than lentils grown in  $\text{NO}_3^-$  free nutrient solution. Nodules from nitrate grown plants weighed 71% less than nodules from nitrate-free plants (Wong, 1980). Nodulation of the primary roots of cowpea was stimulated by low nitrogen doses and inhibited by larger ones, for purple vetch all doses were inhibitory (Dart and Wildon, 1970). The nodules formed at higher levels of urea, however, were much smaller. That the nitrogenous compounds applied differ in relative potency is consistent with the hypothesis that they influence the infection process in the rhizosphere in different ways (Tanner and Anderson, 1963). Urease activity in the rhizosphere of purple vetch may produce only low levels of ammonium ion during infection of the primary root. Induction of root tissue urease may result in levels of ammonium ions sufficient to restrict further nodule development.

A consistent observation during the experiment was made by Gibson *et al.* (1986) on soybean symbiosis with respect to the effect of combined N on it, and it was marked that the inhibition of nodule growth during the 2 day  $\text{NO}_3^-$  treatment, in one case, 1 mM  $\text{NO}_3^-$  caused a 50% reduction in the increase of nodule weight as compared to controls, whilst 2

mM caused a 75% inhibition. Therefore, mineral nitrogen has been attributed with delaying infection and nodule initiation. The delay to nodule establishment caused by  $\text{NO}_3^-$  is primarily determined by the host genotype, with little influence of strain of rhizobia (Gibson and Harper, 1985). Streeter (1985) reported that 4.3 and 6.4 mM nitrate treatments depressed nodule weight per plant in soybean. A nil or small positive effect of low concentrations of nitrate on nodule development has been reported by several workers (Eaglesham *et al.*, 1983; Ralston and Imsande, 1983; Streeter, 1981).

A study done by McNeil (1982) investigated differences in sensitivity to nitrate of soybean symbioses with 16 different *R. japonicum* strains, when nitrate (20 mM) was added to established symbiosis there were no significant differences in the degree of inhibition from any of the 16 strains. However, when nitrate was present during the establishment of nodules, high levels of nitrate (10 mM) were equally inhibitory on all symbioses.

A study carried out by Singh and Lodha (1989) have shown that continuous supply of nitrate for 40 days in the nutrient solution of mungbean plants even at a low concentration of 1 mM decreased nodule weight by 57% and 34% in plants inoculated with Hup<sup>-</sup> and Hup<sup>+</sup> rhizobial strains, respectively. At 10 mM  $\text{NO}_3^-$  concentration, however, the nodule formation was almost completely depressed.

#### **2.1.4 Lowering of leghaemoglobin content by nitrate**

An important constituent of nodule system is the occurrence of leghaemoglobin (Bergerson and Goodchild, 1976). It functions as an oxygen-transport system that support respiration in nodules (Smith, 1949a, b). Harper and Cooper (1971) found that nodule fresh weight and leghaemoglobin content was decreased by nitrate application to leguminous plants. Similar results were obtained by Streeter (1981).

Rigaud and Puppo (1977) investigated the ability of nitrite which is well known to interact with haemoglobin (Wallace and Caughey, 1975), to change the structure and properties of leghaemoglobin. The source of nitrite could be the nitrate reductase in nodules (Daniel and Appleby, 1972; Kennedy *et al.*, 1975; Manhart and Wong, 1978, 1979).

Earlier Cheniae and Evans (1957) and Bergerson (1961) have also suggested that inhibition of nitrogen fixation activity of nodules by nitrate could be caused by nitrite produced as a result of nitrate reductase activity in nodules.

Rigaud and Puppo (1977) showed that acetylene reduction by bacteroid preparation was significantly inhibited by nitrite addition and under low oxygen tensions (20 mm Hg) employed in those experiments, a three fold enhancement of  $C_2H_2$  reduction observed in the presence of 0.1 mM leghaemoglobin, is in good agreement with the results previously reported by Wittenberg *et al.* (1974). Spectrophotometric experiments showed that nitrite reacted directly with heme. Rapid deoxygenation of the chromoprotein occurred and simultaneously a typical ferric leghaemoglobin spectrum was obtained.

The small molecular size of nitrite is certainly responsible for its ability to penetrate to the bound oxygen as pointed out with haemoglobin by Wallace and Caughey (1975). However, Virtanen (1950), Kanayama and Yamamoto (1991), Kanayama and Yamamoto (1990) and Kanayama *et al.* (1990) had suggested the formation of a "NO-compound with leghaemoglobin", thus destroying its function as oxygen supplier to the bacteroids.

### **2.1.5 Nitrate effect on nodule senescence**

The effects of nitrate on root hair infection could be distinguished

from the effects on already nodulated plants (Thornton, 1936). The addition of 11mM  $\text{NH}_4\text{NO}_3$  to nodulated *M. tribuloides* and *T. subterreaneum* caused detectable structural changes within 2 or 3 days and massive disorganisation after 8 days (Dart and Mercer, 1965). In contrast to these results, there was 50% reduction in acetylene reduction activity when nodulated soybean plants were exposed to 7 mM  $\text{KNO}_3$  but no break down in nodule fine structure occurred (Gibson, 1976).

The proposed functions of leghaemoglobin, such as facilitating a high influx of oxygen (Wittenberg *et al.*, 1972) at a low concentration and an enhancement of the efficiency of the oxidative phosphorylation in the bacteroids (Appleby *et al.*, 1975; Bergerson and Turner, 1975) imply that reduction in the amount of leghaemoglobin in the nodule would decrease nitrogenase activity.

Bisseling *et al.* (1978) investigated the effect of ammonium nitrate on the *in vivo* synthesis of nitrogenase and the concentration of leghaemoglobin in the nodules of pea in symbiosis with *R. leguminosarum*. Plants treated with 20 mM  $\text{NH}_4\text{NO}_3$  lost their leghaemoglobin content and also nitrogenase activity in the nodules. They suggested that the decrease in nitrogen fixing capacity is caused by a decrease in leghaemoglobin content of the root nodules and not by the repression of the nitrogenase synthesis.

Becana *et al.* (1986) used nitrate to induce senescence in root nodules of alfalfa. All parameters of nodule activity, expressed on the basis of nodule dry weight, consistently decreased following treatment with nitrate. However, nitrate caused the selective decay of leghaemoglobin with respect to other nodular soluble proteins.

Senescence processes have been traditionally associated with variation in the enzyme levels which protect the tissues from toxic oxygen species ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ ,  $\text{OH}^-$ ). Catalase levels and nitrogen fixation have been shown to be positively correlated (Francis and Alexander, 1972). Oxygenated ferro-leghaemoglobin is highly sensitive to self-oxidation and this process results in the release of superoxide radical ( $\text{O}_2^{\cdot-}$ ). Becana *et al.* (1988) found that application of 20 mM  $\text{NO}_3^-$  to nodulated alfalfa plants led to a 20% decrease in catalase activity in the cytosol, whereas in the bacteroids, catalase activity remained practically constant. This result appears to be consistent with the findings of Lorenzo *et al.* (1990) who observed a catalase activity decrease in the peroxisomes of lupin treated with 20 mM nitrate.

#### 2.1.6 Influence of nitrate on hormonal levels

In the earlier work, Thimann (1936) and Link (1937) postulated that *Rhizobium* derived auxin may be the factor responsible for legume root nodule initiation and morphogenesis and specially for one of the earliest symptoms of nodule development-root hair curling. There is substantial evidence for the production of IAA by *Rhizobium* species (Bauer, 1981; Dart, 1977) though unequivocal evidence for its presence in culture filtrates has been obtained by Badenoch Jones *et al.* (1982) and Wang *et al.* (1981). Furthermore, it has been known for sometime that the production of IAA by rhizobia is stimulated by exogenous tryptophan (Dullaart, 1970). However, according to Badenoch Jones *et al.* (1982) IAA could be produced by nodulating and non nodulating tryptophan requiring strains of *R. leguminosarum*. There might be two pathways for auxin biosynthesis in *Rhizobium* sp. viz., chromosomal and plasmid borne.

Auxin and cytokinins have been found to be present in nodules of legumes and non-legumes and are believed to be involved in infection and

nodule development (Pate, 1958; Dullaart, 1970; Phillips and Torrey, 1970; Libbenga *et al.*, 1973; Henson and Wheeler, 1977a).

According to Kefford *et al.* (1960) and Libbenga and Torrey (1973) auxins, mainly, indole acetic acid (IAA) produced by rhizobia are involved in root hair infection, and nodule initiation in pea begins with cell division in the inner cortex at some distances from the advancing infection thread. Auxins have been reported to be acting as triggering agents for cell division in root nodule initiation. First evidence in favour of such a theory is the capacity of *Rhizobium* sp. to convert tryptophan into IAA (Allen and Allen, 1958; Hartmann and Glombitza, 1967; Dullaart, 1970). Second evidence supporting the theory is the presence of relatively higher content of IAA in root nodules (Allen and Allen, 1958; Pate, 1958; Dullaart, 1970). Thirdly, as reported by Kefford *et al.* (1960), release of IAA by *Rhizobium* at the surface of roots of inoculated plants, supports the theory.

Nitrate appears to have an influence on the action of IAA in the initiation and development of the symbiosis between legumes and rhizobia. For example, it is often noted that IAA is responsible for curling of root hairs that is taken to be a prelude to the bacterial invasion of the legume root. Thornton (1936) and Nutman (1956) noted that the percentage of alfalfa root hairs, that were deformed in the presence of *R. meliloti* cultures was reduced if the plants were simultaneously supplied with sodium nitrate. IAA is likewise proposed to be involved in the nodulation process itself (Kefford *et al.*, 1960), the nodule biogenesis likewise being repressed by nitrate. Tanner and Anderson (1964) reported that inhibition may be attributed to the destruction of auxin by the nitrite produced from bacterial reduction of nitrate. Added IAA could partly offset the inhibition of nodulation caused

by nitrate (Valera and Alexander, 1965; Munns, 1968d; Singh and Lodha, 1989). All these account along with evidence that nitrite is more inhibitory than nitrate. Vincent (1965) suggested that nitrite is prime inhibitor of nodulation operating by accelerating destruction of IAA. The source of nitrite could be the reduction of nitrate to nitrite by bacterial nitrate reductase.

Rigaud and Puppo (1975) showed that there was no relationship between oxidation of IAA by bacteroids and nitrogenase activity. However, they found that succinate, fumerate and pyruvate which stimulate nitrogen fixation, inhibited destruction of IAA. This indicated that there was a positive correlation between IAA content in the nodules and nitrogen fixing activity of the nodules.

It has been proposed by Valera and Alexander (1965) that nitrate in the rooting medium did not abolish nodule formation but rather increased the time for appearance of the first nodule and decreased nodule abundance. No enhancement in nodulation was noted in the presence of  $10^{-8}$  M IAA. Plants with both nitrate and  $10^{-8}$  M IAA nodulated to about the same rate as nitrate free alfalfa. At this concentration, the auxin likewise reversed the nitrogen-induced inhibition of nodule frequency.

It was reported by Yadav *et al.* (1987) that a 60-70% decrease in active leghaemoglobin content and 70% inhibition in *in vivo* nitrogenase activity was observed when nitrate (15 mM) was supplied in the growth medium of nodulated bengal gram plants. Application of IAA ( $10^{-7}$  M) alone resulted in about two-fold increase in leghaemoglobin content. Supplimentation of nitrate (15 mM) with IAA ( $10^{-7}$  M) removed the nitrate inhibitory effect.

The studies carried by Singh and Lodha (1989) indicated a role for IAA nodule biogenesis in mungbean. Continuous supply of 4 mM nitrate in a nutrient solution for 40 days inhibited nodule formation by 79% and 47% in Hup<sup>-</sup> *Rhizobium* strain M11 and Hup<sup>+</sup> strain S24 infected plants, respectively. Addition of IAA ( $10^{-6}$  M) before inoculation with *Rhizobium* alleviated nitrate inhibition on nodulation process by 31% on plants inoculated with Hup<sup>-</sup> strain. However, on plants inoculated with Hup<sup>+</sup> strain the same concentration of IAA not only reversed inhibitory effect of nitrate but further stimulated nodulation.

### 2.1.7 Inhibition of nitrogenase biosynthesis and activity by nitrate

One of the simplest explanation for the effect of nitrate on nodule growth and nitrogenase activity states that some product of nitrate metabolism is inhibitory. Streeter (1982) suggested that the involvement of nitrite in the effect of nitrate on legumes is more logical than the effect of ammonia, amino acids and ureides. Nitrite is a logical candidate for such an inhibitory role because: i) it is a potent inhibitor of nitrogenase activity when mixed with the purified enzyme (Trinchant and Rigand, 1980) or with bacteroids *in vitro* (Kennedy *et al.*, 1975; Rigaud and Puppo, 1977) or supplied with intact nodules, (Kamberger, 1977) and ii) it (i.e. nitrous acid) reacts spontaneously with primary and secondary amines.

After the discovery of nitrate reductase (NR) in *R. japonicum* bacteroids (Evans, 1954) it was generally assumed that the synthesis of nitrite in nodules occurs in bacteroids. The presence of NR and absence of NiR in *R. japonicum* nodule (Daniel and Appleby, 1972) could be a source of nitrite that could interfere with nitrogenase activity.

Nitrogen utilizes large amounts of metabolic energy and reducing power during the reduction of  $N_2$  to  $NH_4^+$  and as such is subject to several

types of cellular control (Postgate, 1971; Brill, 1975).

Nitrate plays a role in the regulation of nitrogenase biosynthesis through two separate processes - one through the nitrogen metabolism and the other mediated through the control system operating on the oxidation-reduction (redox) pathways (Hom *et al.*, 1980).

Hom *et al.* (1980) showed that addition of  $\text{NO}_3^-$  to an anaerobic culture of *Klebsiella pneumoniae* also repressed the biosynthesis of the nitrogenase polypeptides and this included not only nitrogenase but also nitrogenase reductase and the *nif-J* coded protein (120,000 daltons). Chlorate resistant mutant strains e.g. SK-563 and SK-564 did not respire in the presence of  $\text{NO}_3^-$  and nitrogenase biosynthesis was not repressed by  $\text{NO}_3^-$  in these strains. These results showed that the  $\text{NO}_3^-$  itself was not the repressor but that product(s) of  $\text{NO}_3^-$  metabolism play(s) an important role in the regulation of nitrogenase biosynthesis. Although  $\text{NO}_2^-$ , the product of nitrate respiration inhibited enzyme activity, it had no effect on the stability of preformed nitrogenase protein.

Hom *et al.* (1980) in *K. pneumoniae* observed that the  $t_{1/2}$  for nitrogenase repression by  $\text{NH}_4^+$  and  $\text{O}_2$  was 11 to 12 min. The  $t_{1/2}$  for repression by nitrate was about 1.5 h. This lag in the repression of nitrogenase by  $\text{NO}_3^-$  may be due to the requirement for the induction of NR.

Further, Hom *et al.* (1980) showed that addition of  $\text{NO}_2^-$  to the culture medium repressed nitrogenase biosynthesis without any time lag in *K. pneumoniae* strain SK-561. Although  $\text{NO}_3^-$  and  $\text{NO}_2^-$  could be converted to  $\text{NH}_4^+$  and nitrogenase biosynthesis may be repressed by  $\text{NH}_4^+$ , this was not the case with strains SK-25 and SK-561, since both these strains produce nitrogenase even in the presence of externally added  $\text{NH}_4^+$ . Differences may

exist between internally produced  $\text{NH}_4^+$  (from  $\text{NO}_3^-$  or  $\text{NO}_2^-$ ) and externally added  $\text{NH}_4^+$ . However, it has been observed with several strains including strain SK-25 and its chlorate resistant derivatives, that  $\text{NH}_4^+$  produced from  $\text{N}_2$  is excreted into the medium and nitrogenase was produced under conditions where  $\text{NH}_4^+$  was exported. (Andersen and Shanmugam, 1977).

Mahon (1977) reported that when 27-day old pea plants were exposed to 10 mM  $\text{KNO}_3$  for 6 days, there was 100% inhibition of  $\text{C}_2\text{H}_2$  reduction activity, but a similar exposure to 30 mM ammonium chloride caused only a 40% of inhibition. Streeter (1985) showed that the temporary treatment with high nitrate in soybean inhibited nitrogenase activity by 80% whether or not *R. japonicum* bacteroids had NR activity.

Lodha *et al.* (1991) while studying the effect of  $\text{NO}_3^-$  treatment on pre-established symbiosis, reported that when 43-day old chickpea plants were treated with 10 mM  $\text{NO}_3^-$  for 7 days, nitrogenase ( $\text{C}_2\text{H}_2$  reduction) activity was inhibited by 52.2 to 99.7% in nodules formed by four different *Cicer-Rhizobium* strains.

Trinchant and Rigand (1980) have shown that pre-incubation of nitrite with Mo-Fe component of nitrogenase results in a typical inhibition but pre-incubation with Fe-protein did not show any inhibition. This indicated that binding site of  $\text{NO}_2^-$  is Mo-Fe protein and this inhibition is competitive as nitrogen also binds to Mo-Fe protein completely after elimination of  $\text{NO}_2^-$  from the system, showing a total reversibility. Thus,  $\text{NO}_2^-$  is the actual inhibitor of  $\text{N}_2$  fixation though its mechanism of action is not very clear.

On the contrary, Gibson and Pagan (1977) and Manhart and Wong (1980b) examined the effect of nitrate on nitrogen fixing activity of nodules

produced by NR deficient rhizobia in cowpea (*V. unguiculata*) and lupine (*L. angustifolius*) and they found that the effect was inhibitory. There was neither assimilation or dissimilation effect of NR activity detected in nodules. Moreover, there was no traces of  $\text{NO}_2^-$  also. All these observations lead to the conclusion that depression in levels of nitrogenase activity in the nodules is due to some factors other than the  $\text{NO}_2^-$  effect.

### **2.1.8 Reduction in the supply of photosynthates to nodules by nitrate**

As early as in 1935, it was suggested that an adequate supply of carbohydrates to nodules is a limiting factor in biological nitrogen fixation by legumes (Allison, 1935). This idea is shared by a number of research workers (Gibson, 1966; Wheeler, 1971; Minchin and Pate, 1973; Laun and Brun 1974a, b; Lawrie and Wheeler, 1974; Brun, 1976; Hardy and Havelka, 1976). An adequate supply of carbohydrates is required to support the increased respiratory burden of nodulated roots and to provide carbon skeleton, necessary for the synthesis of organic forms of nitrogen which are transported from the nodules to the leaves and pods etc. (Schubert and Ryle, 1980). As the plant matures, the reproductive sinks compete with nodules for the available photosynthates (Lawrie and Wheeler, 1974). Similarly, due to assimilation of nitrate in the shoots and roots a diminished supply of photosynthates is available to the nodules (Oghoghorie and Pate, 1971). This has been termed as "Photosynthate deprivation hypothesis".

Similar type of competition between reproductive structure and nitrogenase for photosynthates has been observed in soybean (Streeter, 1974). Pod removal from every other node of soybean at the end of flowering increased nitrogen fixation, since it presumably made more photosynthates available to the nodules (Ham *et al.*, 1976).

Carroll and Gresshoff (1983) reported that the foliar application of nitrate caused temporal inhibition of nitrogenase activity. Data obtained from axenic plate method indicated that carbohydrate deprivation may be responsible for loss of nitrogenase activity in the early stages of nitrate-induced nodule senescence. Day *et al.* (1986) while studying the physiology of nitrate tolerant, supernodulating soybean mutant observed that nodule nitrogenase in the mutant was susceptible to inhibition by applied nitrate.

However, nodule initiation and growth, and consequently whole plant nitrogenase activity was much less affected by nitrate. These results suggested that at least part of the effect of nitrate on symbiotic development in soybean is via a shoot-regulated autoregulatory signal.

On the contrary, Oti-Boating and Silsbury (1986) while studying the effect of carbohydrate accumulation on dinitrogen fixation in faba bean (*Vicia faba* L.) suggested that photosynthate limitation was not responsible for the decline in nitrogenase activity, and the internal organic nitrogen pool of the plant exerted a regulatory influence over the symbiosis, termed as "feed back" control.

#### **2.1.9 Nitrate inhibition of $N_2$ fixation by increasing the resistance of the nodules to oxygen diffusion**

According to Minchin *et al.* (1986) exposure of mature nodulated plants of white clover (*Trifolium repens* cv. Blanca) to  $330 \text{ mg dm}^{-3} \text{ NO}_3^- \text{-N}$  for 8 days cause nitrogenase activity per plant to decrease by 80%. Total nodulated root respiration was not significantly affected but analysis of its components showed an 81% decrease in nitrogenase linked respiration and a 340% increase in growth and maintenance respiration. Carbon costs of nitrogenase activity (Mole  $\text{CO}_2$  respired per mole  $\text{C}_2\text{H}_4$  produced) increased by 45% over the exposure period. Sucrose content of the nodules decreased,

but the pattern of decrease did not correlate with that of nitrogenase activity. The oxygen diffusion resistance to the nodules was increased by a factor of five. Characterization of this resistance increase suggests an abnormal modification of the diffusion barrier and it is concluded that alteration in the oxygen supply to the bacteroids is involved in the effect of nitrate on nitrogenase activity. More recently, it has been suggested by Vessey *et al.* (1988) that nitrogenase activity in phloem-limited and nitrate-inhibited nodules is limited by O<sub>2</sub> diffusion.

## 2.2 NITRATE REDUCING SYSTEM

The enzymes of nitrate reducing system include: 1) nitrate reductase (NR), and 2) nitrite reductase (NiR).

### 2.2.1 Assimilatory and dissimilatory nitrate reductase:- localization, induction and regulation

In biological systems mainly two types of nitrate reductases exist, namely the assimilatory enzyme (nitrate reductase B) found in plants, fungi, bacteria and the dissimilatory enzyme (nitrate reductase A) found in denitrifying bacteria and facultative anaerobic bacteria (Beevers and Hageman, 1980). Dissimilatory NR is membrane bound and is involved in oxidative phosphorylation coupled to nitrate reduction. The other type namely assimilatory enzyme is usually found in the soluble fraction of the cell free extract. A particulate nature of the NR has been reported in many bacteria such as *Rhizobium* (Cheniae and Evans, 1959). Bhandari *et al.* (1984) reported the denitrification of nitrate in membrane fraction of *R. japonicum* CC-705. A soluble NR of assimilatory type was reported in *R. leguminosarum*, *R. trifolii* and *R. phaseoli* (Ishizawa, 1980). Almost all the NR activity (97.5%) of groundnut *Rhizobium* grown microaerobically with nitrate was localized in the membrane fraction (Reddy and Lodha, 1988).

Daniel and Appleby (1972) observed very high NR activity in nodule bacteroids in *R. japonicum* due to anaerobiosis. Kadam and Naik (1976) reported that nitrate is essential for the induced synthesis of NR in *Azotobacter vinelandii*. Kennedy *et al.* (1975) showed that NR is induced in *R. japonicum* only under partially anaerobic conditions indicating thereby a respiratory role for NR. Daniel and Gray (1976) reported that the activity of NR in *R. japonicum* is controlled by oxygen tension and not by nitrate. Manhart and Wong (1979) reported that all species of *Rhizobium* except *R. lupini* had NR activity. However, the conditions necessary for the induction of NR differed among various species of *Rhizobium*. *R. japonicum* and some *Rhizobium* sp. of the cowpea strains expressed NR activities both in the root nodules of appropriate leguminous hosts and in free-living cells when grown in the presence of nitrate. Zablotowicz and Focht (1979) reported denitrification and anaerobic, nitrate dependent acetylene reduction by nitrogenase in cowpea bacteroids. The denitrifying enzyme system, with the exception of nitrous oxide reductase, was shown to be functional in the bacteroids. Daniel *et al.* (1980) reported anaerobic growth and denitrification by *R. japonicum* and other rhizobia. According to Gollop and Avissar (1984) the expression of NR activity in free-living *Rhizobium* sp. 2206A appears to be regulated by the degree of aeration and by the physiological state of the culture and not by the presence of the substrate. In case of groundnut *Rhizobium* microaerobic condition could induce NR. However, addition of nitrate in the growth medium enhanced the synthesis of the enzyme by 30 per cent (Reddy and Lodha, 1988).

Kadam and Naik (1976) reported that ammonium sulphate (4 mM) added along with  $\text{KNO}_3$  (4mM) did not repress the BVH-NR in *A. vinelandii*. When 2 mM  $\text{KNO}_3$  was added along with 6 mM ammonium sulphate, it

stimulated synthesis of NR. Ammonium sulphate added alone did not induce the synthesis of NR. Thus, in general, the level of NR is high in organisms grown on nitrate and low if grown on ammonium. An organic nitrogenous compound whose generation from ammonium requires the participation of glutamine synthetase, rather than ammonium itself, seems to be the true repressor of NR synthesis (Dunn-Coleman *et al.*, 1979).

### **2.2.2 Assimilatory and dissimilatory nitrite reductase:- localization, induction, regulation**

Nitrite produced as a result of nitrate reduction can be metabolized by two pathways. In certain bacteria which possess dissimilatory pathway, nitrite is reduced to gaseous nitrogen or other oxides of nitrogen by a series of respiratory pathways (Payne, 1973). Most organisms with dissimilatory pathway are facultative bacteria which can grow anaerobically only when supplied with an inorganic substrate in place of oxygen. In assimilatory NiR the current evidence shows that nitrite is reduced to ammonia by the enzyme nitrite reductase (NiR). Assimilatory NiR has been reported in the nodule cytosol of soybean (Hunter, 1984), green gram and groundnut (Lodha *et al.*, 1984). NiR activity increased when increased amounts of nitrate were supplied to the soybean plants. The NiR which is associated with particulate fraction involves phosphorylation during nitrite reduction. In this case, nitrite acts as a terminal electron acceptor (Naik and Nicholas, 1966). The other is soluble NiR which requires NADH and flavin for its activity. There is no esterification of inorganic phosphate with the enzyme activity (Radcliffe and Nicholas, 1968). Anaerobic conditions could induce the enzymes of dissimilatory nitrite reduction (Payne, 1973). However, *E. coli* which can not reduce nitrite to nitrogen, reduces nitrite through an equivalent of an assimilatory pathway yielding ammonia (Losada *et al.*, 1981). A similar pathway namely, "nitrate-ammonification" has been shown to exist in

groundnut *Rhizobium* (Reddy and Lodha, 1986). Marwaha *et al.* (1972) reported that nitrite added to the growth medium was found to be effective in stimulating the synthesis of NiR in *A. vinelandii*. They further reported that ammonia is not a repressor of NiR in *Azotobacter*. Dissimilatory NiR has been induced under anaerobic conditions in *Spirillum lipoferum* grown with nitrate (Neyra and VanBerkum, 1977).

### 2.3 ASSIMILATORY AND DISSIMILATORY FUNCTIONS OF NR AND NIR:-THEIR RELEVANCE TO NITROGEN FIXATION

NR and NiR of free-living nitrogen fixing microorganisms fulfil both assimilatory and dissimilatory functions. Rigaud *et al.* (1973) reported that *Rhizobium* can use nitrate as an electron acceptor alternative to oxygen to generate ATP for nitrogenase activity, and nitrogenase and NR activities remain constant until nitrite accumulates and inhibits nitrogenase activity. Zablotowicz *et al.* (1978) reported denitrification in *Rhizobium*. According to them it is possible that dissimilatory NiR may obviate the problem of nitrite toxicity and the subsequent inhibition of nitrogenase activity that nitrite toxicity is known to bring about in legume nodules. Secondly, the ability of aerobic bacteria to utilize nitrogen oxides as terminal electron acceptors enables them to survive and grow during period of anoxia, which may be advantageous for the survival of *Rhizobium* in soil.

Daniel *et al.* (1980) reported that nitrate reduction was linked to ATP formation during anaerobic but not during aerobic growth in *R. japonicum* and other rhizobia. According to Lodha and Naik (1982) if the nodule NR is of assimilatory type, its end product ammonia would repress nitrogenase. Moreover, there will be a competition for reductants and photosynthates in the nodules between nitrogenase and NR which co-exist in the root nodules of legumes. On the other hand, if the nodule NR is of

dissimilatory type, nitrate can function as a terminal electron acceptor for the generation of ATP in the bacteroids under partially anaerobic conditions. This will facilitate nitrogen fixation in the bacteroids. Lodha *et al.* (1984) reported very high *in vivo* NR activity in the nodules of groundnut and green gram, while the activity of NR in the roots was extremely low. Based on the observation that the NR of bacteroids functioned efficiently with reduced MV, they suggested that it may be of dissimilatory type. Their results also suggested that  $\text{NO}_2^-$  produced in bacteroids may be metabolized in nodule cytosol and reduced to  $\text{NH}_3$  by the assimilatory system.

Kanayama and Yamamoto (1990) have reported that in soybean nodules, the ATP level and ATP/ADP ratio decreased significantly upon the supply of nitrate. Inhibition of nitrogen fixation by nitrate was caused by a decrease in oxidative phosphorylation attributable to the accumulation of nitrosylleghaemoglobin in the presence of nitrate.

#### **2.4 NITRATE REDUCTASE MUTANTS OF *RHIZOBIUM***

Singh *et al.* (1980) isolated forty mutants resistant to chlorate (10 mg/ml) from *R. japonicum* CB 1809. The mutants were unable to reduce nitrate used as sole source of nitrogen. Based on the results of the induction of NR activity in nitrate and nitrate plus glutamate containing minimal medium, these mutants were categorised into two classes. Mutants of class 1 showed a drastic increase in NR activity in the glutamate supplemented nitrate medium as compared to the medium containing nitrate only. Mutants of class 2 were unable to reduce nitrate in any one of the two media. Apparently class 1 type mutants were defective in the regulation of NR whereas class 2 type were probably affected at the structural genes, *nar*. Five mutants of each class were tested for nodulation and nitrogen fixation, they were infective and their efficiency was comparable to parental rhizobial

strain CB 1809. Thus NR deficient mutants do not have impaired nitrogenase activity.

## 2.5 HYDROGEN UPTAKE SYSTEM

Nitrogenase enzyme complex evolves hydrogen as a byproduct of dinitrogen reduction reaction. The energy loss in  $H_2$  evolution could be as high as 30-50% of energy flow through nitrogenase in nodules of different legumes (Schubert and Evans, 1976; Evans *et al.*, 1977). The evolution of  $H_2$  during  $N_2$ -fixation apparently is an inherent characteristic of the nitrogenase reaction (Rivera-Oriz and Burris, 1975). However, a few strains of rhizobia possess an oxygen-dependent hydrogen-oxidizing enzyme system, termed as 'uptake hydrogenase', which is capable of recycling the  $H_2$  released during nitrogen fixation (Schubert and Evans, 1976; Carter *et al.*, 1978).

In 1941, Phelps and Wilson reported the presence of a hydrogenase system in nodules of *Pisum sativum* formed by strain 311 of *R. leguminosarum*. More than 25 years later, Dixon (1967, 1968, 1972) confirmed this report and also concluded that nodules formed by *R. leguminosarum* with hydrogenase activity evolved little or no  $H_2$  during  $N_2$  fixation because the  $H_2$  produced as a by-product of  $N_2$  fixation was consumed internally by an  $H_2$ -oxidation system.

### 2.5.1 Occurrence of Hup system

Reports on survey of native rhizobia for distribution of  $H_2$  uptake (Hup) character are scanty. Uratsu *et al.* (1982) observed 75% *R. japonicum* isolates from major soybean growing areas of USA are  $H_2$ -uptake negative (Hup<sup>-</sup>). They also observed that 65% of 1400 commercial isolates of *R. japonicum* were Hup<sup>-</sup>. In a survey of native rhizobia nodulating various hosts of "cowpea miscellany" group, 56% of the *Rhizobium* isolates from

*V. radiata*, *V. mungo*, *V. unguiculata* and *Cyaneopsis tetragonoloba* were reported to be Hup<sup>+</sup>, whereas, all the isolates of *Cicer arietinum* were Hup<sup>-</sup> (Dadarwal *et al.*, 1985). Pahwa and Dogra (1981) reported 7 Hup<sup>+</sup> out of 30 native isolates of *Rhizobium* from *V. radiata*. Lim (1978) reported 3 out of 10 commercial strains of *R. japonicum* as Hup<sup>+</sup>.

### **2.5.2 Expression of uptake hydrogenase activity under free-living condition**

Maier *et al.* (1978) reproducibly demonstrated hydrogenase activity in free-living cultures of rhizobia when grown on H<sub>2</sub>-uptake medium and this helped in the genetic and physiological investigations of the hydrogenase system in agriculturally important rhizobia. They described a medium on which selected *R. japonicum* strains expressed hydrogenase activity under free-living conditions. They concluded that low concentrations of carbon substrate, decreased oxygen tension and the quantity of combined nitrogen in the medium are the major factors influencing hydrogenase expression.

### **2.5.3 Biochemistry and regulation of uptake hydrogenase**

Tilak *et al.* (1984) confirmed that *Rhizobium* hydrogenase is a membrane bound enzyme by immunological comparison of the crude extracts of *R. japonicum* strain SB16 with membrane bound and soluble hydrogenase of *Alcaligenes eutrophus* H 16. Arp (1985) purified *R. japonicum* hydrogenase to homogeneity and found the molecular weight to be 104,000 by sucrose density gradient centrifugation. SDS-PAGE revealed 2 sub-units with molecular weight 64,000 and 35,000 indicating an  $\alpha$ ,  $\beta$  sub-unit structure. Analysis of metal content indicated 0.59 mole Ni and 6.5 mole Fe/mol. Seefeldt and Arp (1986) purified *A. vinelandii* hydrogenase to homogeneity and observed that it was a nickel and iron containing  $\alpha$ ,  $\beta$  dimer.

The expression of the H<sub>2</sub> uptake system is regulated by a number of factors, including, H<sub>2</sub>, O<sub>2</sub> and C-substrates. The addition of H<sub>2</sub> was

required for the induction of  $H_2$  uptake, while high levels of  $O_2$  and C-substrates repressed the  $H_2$ -uptake system. All utilizable C-substrates repressed  $H_2$ -uptake, and nitrogenase expression was not required for the expression of hydrogenase (Maier *et al.*, 1978). Saini *et al.* (1987) observed derepression of uptake hydrogenase activity in poor as well as in rich carbon media.

Evidence of hydrogenase expression in nodules which is primarily controlled by the bacterial inoculant strain has been shown by Carter *et al.* (1978). However, Gibson *et al.* (1981) have reported that *Rhizobium* sp. CB 756 and 32 HI produced Hup<sup>-</sup> nodules on *V. radiata* and Hup<sup>+</sup> nodules on *V. unguiculata* and *V. mungo*. From reciprocal grafts of several *Vigna* species, they claim that root rather than the shoot genotype was the important factor in Hup expression.

#### 2.5.4 Advantages of uptake hydrogenase

Dixon (1972) pointed out several potential advantages of an efficient  $H_2$  recycling system to  $N_2$ -fixing organisms:

- a) The consumption of  $O_2$  by  $H_2$ -oxidation reaction may contribute towards the protection of the  $O_2$ -sensitive nitrogenase.
- b) The oxidation and thus removal of  $H_2$  produced via nitrogenase within the cell might prevent inhibition of nitrogenase by  $H_2$ .
- c) Oxidation of  $H_2$  may support the synthesis of ATP for use in  $N_2$ -fixation and other processes (Nelson and Salminen, 1982; Salminen and Nelson, 1984).
- d) Recycling of  $H_2$  may also generate reducing power (Lodha and Naik, 1984; Salminen and Nelson, 1984).

Specially, in the light of "Photosynthetic deprivation" theory uptake hydrogenase system has got special significance. Dixon (1968), Emerich

*et al.* (1980), Arima (1981), Drevon *et al.* (1982) and Rainbird *et al.* (1983) have shown that rates of CO<sub>2</sub> evolution were subsequently decreased in *Rhizobium* bacteroids or nodules formed by Hup<sup>+</sup> strains. This indicated that H<sub>2</sub>-oxidizing strains of *Rhizobium* can utilize H<sub>2</sub> as an energy source and as a consequence, conserve more of their carbohydrate supply than Hup<sup>-</sup> strains.

#### **2.5.5 Relationship between nitrate reductase and uptake hydrogenase system**

A previous study (Ligero *et al.*, 1987) on the relationship between the NR and hydrogenase activities in the pea *R. leguminosarum* symbiotic association indicated that nodules formed by strains with Hup<sup>+</sup> genotype showed lower *in vivo* NR activities than those formed by Hup<sup>-</sup> strains. Since *R. leguminosarum* bacteroids do not express NR activity (Chen and Phillips, 1977; Manhart and Wong, 1979; Streeter, 1985), it could not be tested whether differences between Hup<sup>+</sup> and Hup<sup>-</sup> strains at the whole nodules level may also occur in bacteroids.

All the seven strains examined by Delgado *et al.* (1989) expressed NR activity as free-living cells and as symbiotic forms, regardless of the Hup genotype of the strain used for inoculation. Although the presence of nitrate increased nitrate reduction by cultured cells and nodules, no differences in NR activity were obtained between bacteroids isolated from nodules of plants fed with nitrate or grown on N<sub>2</sub>-fixation exclusively. Cultured cells, nodules and bacteroids of strains with Hup<sup>-</sup> genotype (USDA 138, L 236, 3-15133 and PJ 17) had higher rates of NR activity than those with Hup<sup>+</sup> genotype (USDA 110, USDA 122, DES and CB 1003). These results suggest that NR activity is reduced in the presence of a genetic determinant associated with the *hup* region of *B. japonicum*.

If one were successful in obtaining an isogenic *R. leguminosarum* strain with a Hup<sup>+</sup>/NR<sup>-</sup> phenotype, it would be possible to elucidate whether the H<sub>2</sub>-uptake system confers any significant advantage upon the plant (Ligero *et al.*, 1987).

### 2.5.6 Organisation, transfer and stability of *hup* genes

Leyva and his co-workers (1990) have demonstrated that pAL 618 contains the entire Hup DNA region of *R. leguminosarum* and that *hup* genes are organised in six transcriptional units designated *hup* I to *hup* VI. Furthermore, they have reported that *hup* I region codes for the larger subunit of the hydrogenase. Similarly, Lambert *et al.* (1985) showed that all the genes which are necessary for hydrogenase are clustered in cosmid pHU52. In all the cases, one of the transcriptional units has been shown by sequence analysis to code for the structural genes of the hydrogenase (Leclerc *et al.*, 1988).

Acquisition of *hup*-gene cosmid pHU52 by 4 Hup<sup>-</sup> wild-type *R. japonicum* strains, USDA 16, USDA 117, USDA 138 and USDA 144 conferred upon these strains the ability to perform the oxy-hydrogen reaction after derepression in the free-living state (Lambert *et al.*, 1985). Likewise, three Hup<sup>-</sup> *R. meliloti* strains and *R. leguminosarum* 128 C 53 were converted to Hup<sup>+</sup> phenotype after acquiring pHU52 by conjugation. Hydrogen oxidation in these transconjugants was coupled to ATP formation as demonstrated by the ability of these strains to grow autotrophically using hydrogen gas as the sole energy source (Lambert *et al.*, 1985). Eight Hup<sup>-</sup> recipient strains and 17 transconjugant strains carrying pHU1, pHU52, pHU53 or the pLAFR1 vector, were screened for Hup activity and the presence of immunologically detectable hydrogenase subunits (Harker *et al.*, 1985). Only derepressed transconjugant strains carrying the cosmid pHU52 expressed Hup activity.

Concurrent screening of *E. coli* HB101 (pHU52) revealed immunological homology between the uptake hydrogenase of several strains of *E. coli* and *R. japonicum* hydrogenase.

Several reports indicate that the expression of Hup activity in nodules can depend upon the particular plant host (Bedmar *et al.*, 1983). Vasudev *et al.* (1990) reported symbiotic expression of *hup* gene in *Cicer-rhizobia* by transferring cosmid pHU52 and plasmid pIJ1008.

Several groups have observed instability of recombinant pLAFR1 cosmids in the extra-chromosomal state in *Rhizobium* during infection and nodule development. As for example, Long *et al.* (1982) showed that pLAFR1 recombinant cosmids containing *R. meliloti* DNA were unstable in bacteroids. Cantrell *et al.* (1983) and Haugland *et al.* (1984) observed *hup* cosmid instability, and Hom *et al.* (1985) reported *nif/hup* cosmid instability in *R. japonicum* bacteroids.

## 2.6 GENOMIC INTEGRATION OF FOREIGN DNA

Efforts to genetically engineer soil bacteria have been stimulated by the increasing importance of these organisms in modern agriculture (Lambert *et al.*, 1987). This engineering often entails adding foreign genes to the target organism and incorporating them into the chromosome rather than into plasmids, which tend to be unstable or transmissible to other bacterial genera. The genetic analysis and manipulation of bacteria and the molecular cloning of prokaryotic genes have been greatly facilitated by the use of antibiotic resistance transposons (Kleckner *et al.*, 1977). The *tox* gene, coding for delta-endotoxin protein, was integrated into chromosome of *Pseudomonas fluorescens* by using Tn5 (Obukowicz *et al.*, 1986a, b). Similarly, Stout and Iandolo (1990) have reported that the chromosomal gene transfer

during conjugation is mediated by transposon.

The addition of new DNA can be achieved by generalized recombination, if the donor molecule contains a segment homologous to part of the target chromosome (Acuna *et al.*, 1987). Integration of DNA into prokaryotic genomes has been achieved by using plasmids which lack the ability to replicate autonomously in the recipient cell (Renaud *et al.*, 1989).

Two plasmids utilizing the same replication system cannot co-exist stably and are called incompatible plasmid (Datta, 1979). When these two plasmids are introduced into the same cell, they compete with one another both during replication and subsequent processes. In some cells, one plasmid dominates whereas in others, its incompatible partner dominates. Over the course of a few generations of bacterial growth, the minority plasmid is completely eliminated. So far, more than 30 groups have been recognized (Datta, 1979). Friedman *et al.* (1982) constructed a cosmid pLAFR1 from the pRK290 by cloning 1.6kb *Bgl*III fragment containing the *Cos* site into the unique *Bgl*III site. This cosmid is mobilizable but not self-transmissible (*mob*<sup>+</sup>, *tra*<sup>-</sup>) and confers resistance to tetracycline. In 1978, Beringer *et al.* constructed a plasmid pPH1JI which is self transmissible and confers resistance to gentamycin, spectinomycin and streptomycin. Since the pPH1JI and pLAFR1 was derived from the same plasmid group (RK<sub>2</sub>) these two are incompatible to each other.

Recently Kunnimalaiyaan and Lodha (1992b) reported the expression of *hup* genes under symbiotic and free-living conditions in Hup<sup>+</sup> transconjugants of *Cicer-Rhizobium* G36-84 obtained by the integration of *hup* cosmid into the genomic DNA using Tn5 as an homologous sequence. The integrated cosmid was found to be maintained stably under both free-living and symbiotic conditions.

## **MATERIALS AND METHODS**

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## **MATERIALS**

### **3.1.1 Bacterial strains**

Pure cultures of *Cicer-Rhizobium* strains G36-84 and F-75 and *Escherichia coli* strains HB101 (pGS9), HB101 (pRK2073), HB101 (pPH1JI) and HB101 (pHU52) were obtained from the Division of Microbiology and Biotechnology Centre, IARI, New Delhi. The details of bacterial strains and plasmids are given in Table 1.

### **3.1.2 Plant seed material**

The seeds of chickpea (*Cicer arietinum* cv. Pusa 256) were obtained from the Pulse Research Laboratory, Division of Genetics, IARI, New Delhi.

### **3.1.3 Chemicals and Reagents**

L-arabinose, lysozyme, ethidium bromide, RNase-A, ficoll-400, salmon sperm DNA, nalidixic acid, kanamycin, tetracycline, chloramphenicol, gentamycin were obtained from Sigma Chemicals Co., USA. L-glutamic acid, mannitol, inositol, pyridoxal. HCl, thiamine. HCl were from Sisco Research Laboratory Pvt. Ltd., Bombay. Sodium gluconate was from Sarabhai M-chemicals, Baroda and nicotinic acid and methyl viologen from BDH, England. Bacto tryptone, bacto yeast extract were from Difco Laboratories, USA. Nick translation kit N-5500 was from Amersham and [ $\alpha^{32}\text{P}$ ] dCTP was obtained from BARC, Bombay. Absolute ethanol was obtained from the Bengal Chemicals Ltd. Ethylene standard was from Scott Research Laboratories, USA and  $\text{H}_2$  standard was from EDT Research, UK.

**Table 1. Bacterial strains and plasmids used**

| Strain/plasmid                                                                      | Relevant properties                                                                        | Source/Reference                                         |
|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|----------------------------------------------------------|
| <i>Cicer-Rhizobium</i>                                                              |                                                                                            |                                                          |
| G36-84                                                                              | Nd <sup>r</sup> , Km <sup>s</sup> , Tc <sup>s</sup> , Nod <sup>+</sup> Fix <sup>+</sup>    | Microbiology Division, IARI                              |
| F-75                                                                                | Nd <sup>r</sup> , Km <sup>s</sup> , Tc <sup>s</sup> , Nod <sup>+</sup> Fix <sup>+</sup>    | Microbiology Division, IARI                              |
| <i>Escherichia coli</i> HB101 plasmids                                              |                                                                                            |                                                          |
| pRK2073                                                                             | Sm <sup>r</sup> , Sp <sup>r</sup> , Mob <sup>+</sup> , Tra <sup>+</sup>                    | Figurski and Helinski (1979)                             |
| pGS9                                                                                | Suicidal to <i>Rhizobium</i> sp.<br>Km <sup>r</sup> , Cm <sup>r</sup>                      | Selvaraj and Iyer (1983)                                 |
| pHU52                                                                               | Tc <sup>r</sup> , Hup <sup>+</sup> , Mob <sup>+</sup> , Tra <sup>-</sup>                   | Cantrell <i>et al.</i> (1983)                            |
| pHU52::Tn5                                                                          | Tc <sup>r</sup> , Km <sup>r</sup> , Hup <sup>+</sup> , Mob <sup>+</sup> , Tra <sup>-</sup> | Kunnimalaiyaan and Lodha (1992a)                         |
| pPH1JI                                                                              | Self-transmissible, IncP-1<br>plasmid Sp <sup>r</sup> , Gm <sup>r</sup>                    | Beringer <i>et al.</i> (1978)                            |
| Gm, gentamycin;<br>Nd, nalidixic acid;<br>Sp, spectinomycin;<br>Cm, chloramphenicol |                                                                                            | Km, Kanamycin;<br>Sm, streptomycin;<br>Tc, tetracycline; |

All other chemicals used were of analytical grade from BDH, SRL and Merck. Deionized millipore water was used for the molecular biology work. Otherwise double glass distilled water was used throughout the study.

## METHODS

### 3.2.1 Incubation

For growth, cultures of *Rhizobium* were incubated at  $30 \pm 0.5^\circ\text{C}$  and *E. coli* cultures at  $37 \pm 0.5^\circ\text{C}$ .

### 3.2.2 Bacterial Growth Media

#### Medium for rhizobial growth

The medium used for the growth and maintenance of rhizobia was yeast extract mannitol (YEM) medium (Vincent, 1970) with the following composition:

|                                           |         |
|-------------------------------------------|---------|
| $\text{K}_2\text{HPO}_4$                  | 0.5 g.  |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.2 g.  |
| NaCl                                      | 0.1 g.  |
| Mannitol                                  | 10.0 g. |
| Yeast Extract                             | 1.0 g.  |
| Distilled water                           | 1 lit.  |

The pH of the medium was adjusted to 6.8 using 0.1 N NaOH.

**Medium for growth and maintenance of *Escherichia coli***

For growing *E. coli*, Luria-Bertani (LB) medium (Kahn *et al.*, 1979) was used having the following composition:

|                     |         |
|---------------------|---------|
| Bactotryptone       | 10.0 g. |
| Bacto Yeast extract | 5.0 g.  |
| NaCl                | 10.0 g. |
| Distilled water     | 1 lit.  |

pH was adjusted to 7.5. For maintenance of *E. coli* Bacto agar was added @ 16 g/lit.

**Medium for mating between *Rhizobium* and *E. coli***

Mating was carried out on tryptone yeast extract mannitol (TYM) medium (Khanuja, 1991).

|                                      |         |
|--------------------------------------|---------|
| Bactotryptone                        | 5.0 g.  |
| Bacto yeast extract                  | 2.0 g.  |
| Mannitol                             | 4.0 g.  |
| CaCl <sub>2</sub> .6H <sub>2</sub> O | 0.66 g. |
| Bacto agar                           | 16.0 g. |
| Distilled water                      | 1 lit.  |

pH was adjusted to 7.0. The details of different mating experiments have been given later.

Table 2. Mating experiments of bacteria

| Mating type | Donor                                                | Helper                            | Recipient                                                                  | Transconjugants                                                                                   |
|-------------|------------------------------------------------------|-----------------------------------|----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| 1.          | HB101 (pGS9)<br>Cm <sup>r</sup> Km <sup>r</sup>      | -                                 | G36-84<br>Cm <sup>s</sup> Km <sup>s</sup> Nd <sup>r</sup>                  | G36-84::Tn5<br>Nd <sup>r</sup> Km <sup>r</sup> Cm <sup>s</sup>                                    |
| 2.          | HB101 (pGS9)<br>Cm <sup>r</sup> Km <sup>r</sup>      | -                                 | F-75<br>Cm <sup>s</sup> Km <sup>s</sup> Nd <sup>r</sup>                    | F-75::Tn5<br>Nd <sup>r</sup> Km <sup>r</sup> Cm <sup>s</sup>                                      |
| 3.          | HB101(pHU52::Tn5)<br>Tc <sup>r</sup> Km <sup>r</sup> | HB101(pRK2073)<br>Sp <sup>r</sup> | G3684::Tn5<br>Nd <sup>r</sup> Km <sup>r</sup>                              | G3684::Tn5(pHU52::Tn5)<br>Nd <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup>                         |
| 4.          | HB101(pHU52::Tn5)<br>Tc <sup>r</sup> Km <sup>r</sup> | HB101(pRK2073)<br>Sp <sup>r</sup> | F-75::Tn5<br>Nd <sup>r</sup> Km <sup>r</sup>                               | F-75::Tn5 (pHU52::Tn5)<br>Nd <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup>                         |
| 5.          | HB101(pPH1JI)<br>Gm <sup>r</sup>                     | -                                 | 36-84::Tn5 (pHU52::Tn5)<br>Nd <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> | G36-84::Tn5::pHU52<br>(pPH1JI)<br>Nd <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> Gm <sup>r</sup> |
| 6.          | HB101 (pPH1JI)<br>Gm <sup>r</sup>                    | -                                 | F-75::Tn5 (pHU52::Tn5)<br>Nd <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup>  | F-75::Tn5::pHU52<br>(pPH1JI)<br>Nd <sup>r</sup> Km <sup>r</sup> Tc <sup>r</sup> Gm <sup>r</sup>   |

### **Maintenance of *Rhizobium***

Rhizobia were maintained on YEMA medium. The medium was prepared by adding 16.0 g agar and 0.5 g  $\text{CaCO}_3$  to one litre of YEM medium.

### **Testing of purity of *Rhizobium***

To the YEMA medium 1 ml of 2.5% aqueous solution of congo red per litre was added (CRYMA) to check the purity of the rhizobial culture.

### **3.2.3 Antibiotics**

Unless otherwise stated the concentration of antibiotics ( $\mu\text{g/ml}$ ) used in solid medium was as follows:

Kanamycin (Km), 25; tetracycline (Tc), 10; nalidixic acid (Nd), 25; spectinomycin (Sp), 25; gentamycin. (Gm), 25; streptomycin (Sm), 25.

Tetracycline was prepared in 50% ethanol, nalidixic acid in 1M NaOH and all other antibiotics were prepared in double distilled water.

### **3.2.4 Sterilization**

All media, solutions, cotton wool etc. unless mentioned otherwise were sterilized at 15 Psi for 15 min. The heat labile chemicals like vitamins were sterilized by passing through sterile millipore membrane filters (pore size 0.45  $\mu\text{m}$ ).

### **3.2.5 Inoculum**

The *Rhizobium* inoculum was prepared from fresh culture of 72 h growth. The size of the inoculum used in all experiments was kept more or less constant by inoculating 1 ml of 0.1 O.D. inoculum suspension to 100 ml of culture medium by measuring the turbidity at 420 nm with the help of

Spectronic-20 spectrophotometer (Bausch and Lomb, USA). For preparing *E. coli* inoculum 0.1 ml of overnight grown culture was inoculated to 100 ml of medium.

### 3.2.6 Growth of microorganisms

In the experiments where the effect of different concentrations of nitrate on the expression of nitrate reductase and nitrite reductase was studied, *Rhizobium* parent strains and their mutants were grown both aerobically and microaerobically.

Aerobically, the cultures were grown for 4 days at  $30 \pm 0.5^\circ\text{C}$  at 200 rpm in an environmental incubator shaker Model-G25 (New Brunswick and Co. USA). Always 100 ml of medium was taken in a 250 ml erlenmeyer flask.

For generating microaerobic conditions, milk shaking bottles (160 ml) (Borosil) were used. Both the bottles and the medium were autoclaved separately. The medium after inoculating it with *Rhizobium* culture, was added aseptically to sterilized milk shaking bottles using the laminar flow cabinet in such a way that no air space was left to ensure near anaerobic condition. They were incubated as stationary culture at  $30 \pm 1^\circ\text{C}$  for 10 days with occasional shaking.

After the required period of growth the cells were harvested by centrifuging the culture at  $1949 \times g$  for 10 min at  $4^\circ\text{C}$  in a RC-5B Sorvall refrigerated super speed centrifuge. The cell pellet was suspended in a minimum volume of (1 ml) of 200 mM sodium phosphate buffer, pH 7.5. *E. coli* was grown overnight at  $37 \pm 0.5^\circ\text{C}$  to get a count of  $10^8$  cells/ml.

### 3.2.7 Transposon Tn5 mutagenesis of *Cicer-Rhizobium* strains

*Cicer-rhizobial* strains G36-84 and F-75 were used for this experiment. *E. coli* strain HB101 carrying pGS9 was used as donor of Tn5 (Selvaraj and Iyer, 1983). This plasmid is suicidal in *Cicer-Rhizobium* species as it does not have replicon which function in *Rhizobium* sp. *E. coli* HB101 having pGS9 was mated with G36-84 and F-75 separately. Approximately  $2.3 \times 10^9$ ,  $8.7 \times 10^9$  and  $4.2 \times 10^9$  cells per ml of donor HB101 (pGS9) and recipients G36-84 and F-75, respectively were taken and biparental filter mating was done. In this case sterilized filtration assembly along with  $0.45 \mu\text{m}$  membrane filter was used. For a check, both the donor and recipients were streaked on the selective plates containing Nd Km. If any one of them showed growth on selective plates then the whole experiment was freshly conducted. The mutants were plated on the selective plates containing Nd Km and replica plated on the selective plates containing Nd Km Cm. Those transconjugants which were also growing on the plates containing Nd Km Cm were discarded. This may be due to the incorporation of both Cm Km genes into the *Cicer-Rhizobium*. It is the general phenomenon that the Tn5 may jump at any part of the DNA and therefore, it may or may not affect the expression of the neighbouring genes.

### 3.2.8 Screening of Tn5 mutants of *Cicer-Rhizobium* strains for nitrate tolerance under symbiotic conditions

Freshly harvested good seeds of chickpea (*Cicer arietinum* L. cv. Pusa 256) were rinsed with 95% ethanol and sterilized by immersing the seeds for 2 min in 0.1%  $\text{HgCl}_2$  acidified with 5 ml/lit of concentrated HCl and washed repeatedly with sterile distilled water until the washed water was negative to silver nitrate test. The sterilized seeds (three) were planted in plastic pots (4' diam.) containing sterilized river sand and inoculated with

mid log phase cultures of rhizobial strains (1 ml/seed), containing approximately  $1.5-2.5 \times 10^9$  cells. Plants were grown in *Rabi* season under natural environment. Hoagland nutrient solution (Hoagland and Arnon, 1950) containing 0, 1, 2 or 4 mM potassium nitrate was added to pots before sowing, and then as and when required for the growth of the plants.

The plants were harvested at 60 days after sowing. The roots were separated and washed free of sand. Intact nodules were used for the determination of nitrogenase (acetylene reduction) activity. The rhizobial mutants which did not produce effective nodules ( $\text{Nod}^+ \text{Fix}^-$ ) or did not form the nodules at all ( $\text{Nod}^-$ ) were discarded. Plants inoculated with parental strains were used as control. Two replications were used for each strain.

### **3.2.9 Assay of nodule nitrogenase (EC 1.18.2.1) activity**

Nitrogenase activity in intact fresh nodules was assayed by acetylene reduction method (Hardy *et al.*, 1968). The assay was performed in 25.5 ml vials provided with serum stoppers. In each vial 3.0 ml of air was replaced by 3.0 ml of  $\text{C}_2\text{H}_2$  and incubated at  $25 \pm 1^\circ\text{C}$  for 1 h. After incubation, gas samples (1 ml) were received from each vial and analyzed for  $\text{C}_2\text{H}_4$  using an AIMIL-NUCON gas chromatograph equipped with FID and porapak T (60-80 mesh) dual columns (182 X 0.32 cm) maintained at  $90^\circ\text{C}$  with  $\text{N}_2$  as carrier gas at a flow rate of 30 ml/min. Both injector and detector were maintained at  $110^\circ\text{C}$ , 1050 ppm  $\text{C}_2\text{H}_4$  in  $\text{N}_2$  (v/v) was used as standard. The ethylene values obtained for the samples were corrected by subtracting the value obtained for sample blank. Nodule specific nitrogenase activity has been expressed as n moles  $\text{C}_2\text{H}_4$  formed per gram nodule fresh weight per h. Those nitrate tolerant Tn5-mutants of *Cicer* rhizobial strains in which *nod* and *fix* genes were not affected, were taken for further studies for NR and

**Table 3. Composition of Hoagland solution  
(Hoagland and Arnon, 1950)**

| Salt                                          | Concentration | Qty./litre |
|-----------------------------------------------|---------------|------------|
| <b>Solution A (macro-nutrient)</b>            |               |            |
| $K_2SO_4$                                     | 0.500 M       | 5 ml       |
| $MgSO_4 \cdot 7H_2O$                          | 1.000 M       | 2 ml       |
| $Ca(H_2PO_4)_2$                               | 0.050 M       | 10 ml      |
| $CaSO_4$                                      | 0.010 M       | 200 ml     |
| <b>Solution B (micro-nutrient) (g/500 ml)</b> |               |            |
| Boric acid                                    | 1.430         |            |
| $MnCl_2 \cdot 4H_2O$                          | 0.905         |            |
| $ZnSO_4 \cdot 7H_2O$                          | 0.110         | 1 ml       |
| $CuSO_4 \cdot 5H_2O$                          | 0.040         |            |
| $Na_2MoO_4 \cdot H_2O$                        | 0.010         |            |
| <b>Solution C</b>                             |               |            |
| Iron citrate                                  | 0.500%        | 1 ml       |

**Note:** The pH was adjusted to 6.5 using 0.1 M NaOH. Required amount of potassium nitrate was added to get 1, 2 or 4 mM nitrate concentration. The nutrient solution was then autoclaved at 15 Psi for 30 min. The sterilized medium was stored, sometimes upto 3 to 4 days. Once the container was opened, the leftout medium was not used.

NiR activities, IAA production and integration of *hup* genes.

### **3.2.10 Estimation of nitrite**

Nitrite was estimated by Griss Ilosvay colorimetric method of diazotization of an aromatic amine compound by nitrite in acid solution and coupling with a suitable reagent to give an intense red purple colour as described by Snell and Snell (1949). Details of the method are given below:

#### **Reagents**

(a) 1% sulphanilamide in 1N HCl

(b) 0.02% N (1-naphthyl) ethylene diamine dihydrochloride in distilled water.

#### **Procedure**

To a suitable aliquot, 1 ml of reagent 'A' was added and mixed well. Then 1 ml of reagent 'B' was added and mixed immediately and final volume was made upto 8 ml by adding distilled water. The colour intensity was measured after 30 min at 540 nm using spectronic 20 spectrophotometer.

Standard curve for nitrite was prepared in the range of 10-100 n mol using  $\text{NaNO}_2$ .

### **3.2.11 Determination of nitrate reductase activity (anaerobic assay)**

*In vivo* nitrate reductase (NR) activity was measured by estimating the rate of formation of nitrite as described by Nicholas and Nason (1955) with suitable modifications.

The assay was carried out in small tubes (5 ml). The assay mixture in a final volume of 1 ml contained:

|                                                                   |        |
|-------------------------------------------------------------------|--------|
| 200 mM potassium phosphate buffer (pH 7.5)                        | 0.2 ml |
| 100 mM KNO <sub>3</sub>                                           | 0.2 ml |
| 1.5 mM methyl viologen                                            | 0.1 ml |
| Enzyme source (whole cells containing<br>30 to 160 µg/ml protein) | 0.2 ml |
| water                                                             | 0.1 ml |

Then about 2 mm film of liquid paraffin was layered carefully on the surface of reaction mixture. The reaction was started by the addition of 0.2 ml dithionite reagent (20 mg sodium dithionite and 20 mg sodium hydrogen carbonate dissolved in 5 ml of water just prior to use) to the reaction mixture through a fine syringe needle. The assay was carried out at 30°C for 1 h and then the reaction was stopped by agitating the tube on a vortex mixture until the dithionite was completely oxidized and the dye colour disappeared. The liquid paraffin layer was removed carefully by suction. The tubes containing boiled enzyme served as controls. By taking suitable aliquot (0.8 ml) the amount of nitrite formed was estimated as per the method described earlier. Suitable dilutions were made before taking the reading.

### **3.2.12 Determination of nitrite reductase activity (anaerobic assay)**

*In vivo* nitrite reductase (NiR) activity was measured by estimating the rate of nitrite disappearance as described by Miflin (1967) with slight

modification. The assay mixture in a final volume of 1.0 ml contained:

|                                                                              |        |
|------------------------------------------------------------------------------|--------|
| 200 mM potassium phosphate buffer (pH 7.5)                                   | 0.2 ml |
| 5 mM $\text{NaNO}_2$                                                         | 0.1 ml |
| 1.5 mM methyl viologen                                                       | 0.1 ml |
| Enzyme source (whole cells containing<br>30 to 160 $\mu\text{g/ml}$ protein) | 0.2 ml |
| Water                                                                        | 0.2 ml |

About 2 mm film of liquid paraffin was layered on the surface of the reaction mixture. The reaction was started by the addition of 0.2 ml of the dithionite reagent (as prepared in NR assay method) through a fine syringe needle. The reaction was carried out at 30°C for 1 h and then stopped by agitating the assay mixture on a vortex mixture until the dithionite was completely oxidized and the dye colour disappeared. The tubes containing boiled enzyme served as control. The liquid paraffin layer was removed by suction. By taking suitable aliquot (0.8 ml) the amount of nitrite disappeared was estimated as per the method described earlier. Suitable dilutions were made before taking the readings.

### **3.2.13 Estimation of indole acetic acid**

Indole acetic acid (IAA) was estimated according to the procedure given by Gordon and Weber (1950). To study IAA production rhizobia were grown in YEM broth containing tryptophan (200 mg/lit). The culture flasks were kept on a rotary shaker. Four day old cultures of slow growing rhizobia were centrifuged at 7796 X g for 5 min. The supernatant was filtered

through Whatman filter paper no. 1. IAA was extracted from the supernatant with diethyl ether (1:1) at pH 3 by adding few drops of ortho-phosphoric acid. The extraction procedure was repeated thrice to ensure complete extraction. The extracts were pooled together and evaporated to dryness. Residue was dissolved in 5 ml of 70% ethanol. To 2 ml of this alcoholic solution 4 ml of Salkowski's Reagent was added. The tubes were incubated at 28°C for 30 min. The intensity of light pink colour developed was measured at 520 nm wavelength using spectronic-20 spectrophotometer.

All the steps involved in extraction and determination of IAA were carried out in dark to avoid photo-oxidation of IAA. Colour development did not take place in case of control i.e., medium without inoculum.

#### **Salkowski's Reagent**

One ml of 0.5 M  $\text{FeCl}_3$  solution was mixed with 50 ml of 35%  $\text{HClO}_4$ .

#### **3.2.14 Soluble protein estimation from bacteria**

The soluble protein from the bacteria was extracted by the method of Stickland (1951). The cell suspension was centrifuged for 20 min at 17540 X g at 4°C, using a Sorvall RC-5B refrigerated centrifuge. The cell pellet was digested by suspending it in 3 ml of 0.75 M NaOH and the protein in the sample was precipitated by adding 0.6 ml of 25% TCA and 1.8 ml of ethanol. After thorough mixing, the sample was centrifuged as described earlier. The pellet obtained was again dissolved in water with the help of a minimal amount of NaOH solution (0.5 ml) and heated at 70°C for 5 min. The protein in the supernatant was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

### 3.2.15 Statistical analysis

In order to analyse the data generated from nitrate reductase, nitrite reductase assay and IAA estimation study randomised block design with the factorial scheme was followed. Finally two-way tables indicating bacterial strains and nitrate concentration in the growth medium were constructed. Interaction C.D. and bacterial strain C.D. were calculated at 5% and 1% level of significance (Panse and Sukhatme, 1954).

### 3.2.16 Transfer of pHU52::Tn5 to F-75::Tn5 and G36-84::Tn5

In order to transfer cosmid pHU52 containing *hup* genes to *Cicer*-rhizobial nitrate tolerant mutants by homologous recombination first Tn5 was inserted into the genomic DNA of *Cicer* strains G36-84 and F-75 as described earlier. pHU52::Tn5 was already available in our laboratory (Kunnimalaiyaan *et al.*, 1992a). The transfer was carried out as follows:

As cosmid pHU52::Tn5 is not self-transmissible, *E.coli* (pRK2073) was used as helper strain. Triparental mating (Ditta *et al.*, 1980) was done by mixing approximately  $2.7 \times 10^9$ ,  $7.1 \times 10^9$ ,  $2.5 \times 10^{10}$ ,  $1.8 \times 10^{10}$  and  $3.3 \times 10^{10}$  cells per ml of donor *E. Coli* HB101 (pHU52::Tn5), helper *E. Coli* HB101 (pRK2073), and recipient BC-35 (nitrate tolerant mutant of G36-84) or BC-46 (another nitrate tolerant mutant of G36-84) or AC-10 (nitrate tolerant mutant of F-75) respectively. The patch mixture was kept at  $30 \pm 1^\circ\text{C}$  for 48 h. To fish out spontaneous mutants, donor, helper and recipient were streaked on selective plates containing Nd Km Tc. Cosmid pHU52::Tn5 specifies resistance to Tc Km, and pRK2073 to Sp. After incubation for patch mating the different aliquots were plated on the selective plates containing Nd Km Tc. The resultant transconjugants were used for further studies. Since it was difficult to handle all the

transconjugants obtained, only some of these (eg. A<sub>1</sub>h<sub>22</sub> from AC10, B<sub>3</sub>h<sub>12</sub> from BC<sub>35</sub> and B<sub>4</sub>h<sub>16</sub> from BC<sub>46</sub>) were taken for further studies. The rhizobial transconjugants from the above experiment were tested for the presence of cosmid by isolating the cosmid and checking its presence by agarose gel electrophoresis.

### 3.2.17 Rapid plasmid DNA isolation

Plasmid DNA was isolated according to the method of Maniatis *et al.* (1989) with modification.

#### Solution I

|                             |          |
|-----------------------------|----------|
| 20% glucose                 | 2.25 ml  |
| 0.5 M EDTA pH8.0            | 1.00 ml  |
| 1 M Tris pH8.0              | 1.25 ml  |
| Sterilized H <sub>2</sub> O | 45.50 ml |

Store at 4°C. Add 2.0 mg/ml lysozyme immediately before use.

#### Solution II

|                             |         |
|-----------------------------|---------|
| 10N NaOH                    | 0.4 ml  |
| 20% SDS                     | 1.0 ml  |
| Sterilized H <sub>2</sub> O | 18.6 ml |

Do not autoclave. Shelf life 1 year at room temperature.

**Solution III**

3M sodium acetate (pH 4.8)

Dilute 17.25 ml glacial acetic acid to 80 ml in sterilized H<sub>2</sub>O. Add 12 ml 10N NaOH to get the pH 4.8. Make up the volume to 100 ml with sterilized distilled H<sub>2</sub>O.

**Solution IV**

|                           |          |
|---------------------------|----------|
| 1 M Tris (pH 8.0)         | 2.5 ml   |
| 3M Sodium acetate (pH4.8) | 1.65 ml  |
| Sterile H <sub>2</sub> O  | 45.85 ml |

**Tris-borate-EDTA (TBE) buffer (IOX) pH8.0**

|          |                    |
|----------|--------------------|
| 21.54 g  | Tris               |
| 11.004 g | Boric acid         |
| 8.0 ml   | 0.5 M EDTA (pH8.0) |

Finally the volume was made upto 200 ml by using distilled water.

**Bromophenol blue tracking dye**

|        |                       |
|--------|-----------------------|
| 0.5 ml | 50 X TBE              |
| 1 ml   | 0.5 M EDTA pH8.0      |
| 2.5 ml | glycerol              |
| 1 ml   | 0.5% bromophenol blue |
| 500 mg | Ficoll.               |

Mix well.

The cells of *E. Coli* HB101 (pHU52::Tn5) were grown overnight in 5 ml of LB medium, and the rhizobial transconjugants were grown for 3 days in 5 ml of YEM. About 1.5 ml of the culture was poured into an Eppendorf tube and centrifuged for a minute in an Eppendorf tabletop centrifuge at 8,800 X g. 100  $\mu$ l of ice cold solution-I (freshly prepared lysozyme in Tris-EDTA buffer) was added to the pellet, mixed immediately by vortexing, and kept on ice for 5 min. Then 200  $\mu$ l of freshly made solution-II was added to the Eppendorf tube. The contents were mixed gently by inverting the tubes twice or thrice and kept on ice for 5 min or until the SDS distinctly precipitated. Lysate used to be clear at first but got cloudy as SDS used got precipitated. (This alkaline pH denatures linear host DNA but not the CCC form). Sample should be viscous and stringy when cap is opened at the completion of this step.

Then 150  $\mu$ l of an ice cold solution-III was added at room temperature and mixed gently by inverting the tubes several times and kept on ice for 5 min. This high salt solution (3 mM sodium acetate) neutralizes the pH and precipitates protein, host DNA and RNA.

It was then centrifuged for 5 min at 15280 X g at 4°C in a Sorvall RC 5B refrigerated superspeed centrifuge without disturbing the pellet, 0.4 ml of supernatant was removed carefully and put into a new Eppendorf tube. The pellet was discarded. Two volumes of cold 100% ethanol (0.9 to 1.0 ml) was added to the supernatant, mixed gently and stored in a -70°C deep freezer for 15 min. Then it was centrifuged in a Eppendorf centrifuge for 2-3 min. The supernatant was discarded. Then 100  $\mu$ l of solution-IV was added to the pellet and dissolved gently with the help of a micropipette. Again, 200  $\mu$ l of cold 100% ethanol was added and frozen at -70°C for 15 min. It was

again centrifuged for 2-3 min in an Eppendorf centrifuge and the supernatant was removed. The pellet was washed with 70% ethanol to remove the salts. The pellet was dried for 5 min in vacuum. Finally the pellet was resuspended in 20  $\mu$ l of TE buffer (Tris 1 mM, EDTA 0.5 mM) and loaded on 0.7% agarose gel as described below:

### 3.2.18 Agarose gel electrophoresis of plasmid DNA

Agarose gel (0.7%) was prepared by boiling an appropriate amount of agarose in 1 X TBE buffer till the agarose dissolved completely. The gel solution was cooled to approximately 50°C, minimal quantity (4  $\mu$ l/40 ml of gel) of 1% ethidium bromide was added and the gel was poured into gel tray and allowed to set. The gel tray was placed on gel platform and buffer tank were filled with 1% TBE buffer just enough to cover the gel. The DNA samples were then loaded into the wells by adding a suitable tracking dye (bromophenol blue). The parental strains (G36-84 and F-75) served as controls. The electrophoresis was carried out for 3 h at 70 V using the power supply cross power 150 of the Atto Corporation. After completion of the electrophoresis the gel was viewed under a Fotodyne UV trans-illuminator and photographed by using a polaroid camera.

### 3.2.19 Integration cosmid pHU52:: Tn5 into the chromosome of *Cicer-Rhizobia*

The rhizobial transconjugants from the above experiment were tested for the presence of cosmid by agarose gel electrophoresis as mentioned earlier after its isolation. Since there was no force inside the cell for homologous recombination, the plasmid remained there as such in free form. Hence, forcing was done by introducing a new plasmid, which is incompatible to the cosmid, by biparental mating. *E. Coli* strain HB101 carrying

T-5422



incompatible plasmid pPH1JI ( $1.1 \times 10^{10}$  cells/ml) which specifies resistance to Gm, was mated with  $B_3h_{12}$  ( $1.36 \times 10^{10}$  cells/ml)  $B_4h_{16}$  ( $2.12 \times 10^{10}$  cells/ml) both G36-84::Tn5 (pHU52::Tn5) transconjugants, and with  $A_1h_{22}$  ( $1.28 \times 10^{10}$  cells/ml) a F-75:: Tn5 (pHU52::Tn5) transconjugant. Patch mating was done in TYM plates. At the same time to screen the spontaneous mutants both the donor and recipient were streaked on the selective plates containing Nd Km Tc Gm. The different aliquots of the cultures, after incubation, were plated on the selective plates containing Nd Km Tc Gm; both cosmid pHU52 and plasmid pPH1JI being incompatible to each other, will not grow unless selection pressure is given for both. To remove the incompatible plasmid (pPH1JI), the transconjugants were transferred to the plates containing only Nd Km Tc. The clones were subcultured six times for the subsequent generations to get rid off the plasmid. This was checked by growing on the plates containing Nd Km Tc Gm after each transfer. The clones which were not growing on the Nd Km Tc Gm plates but growing on the plates containing Nd Km Tc were taken for the further studies leading to the confirmation of integration of pHU52 into the chromosomal DNA of *Cicer-Rhizobium*.

### **3.2.20 Confirmation for the integration of pHU52 into the chromosomal DNA of *Cicer-Rhizobium***

If the cosmid pHU52 is not present in the free form in *Cicer-Rhizobium* strains then it shows indirectly that it has been integrated into the genomic DNA. This was confirmed by agarose gel electrophoresis of cosmid DNA isolated by alkaline lysis rapid plasmid DNA isolation method as described earlier. Plasmid DNA was isolated from  $B_3h_{12}$ ,  $B_4h_{16}$  and  $A_1h_{22}$  which served as controls and from the new *hup* integrated transconjugants eg.  $B_3H_9$ ,  $B_3H_{10}$  (from  $B_3h_{12}$ );  $B_4H_1$ ,  $B_4H_3$ ,  $B_4H_7$  and  $B_4H_{11}$  (from  $B_4h_{16}$ ) and

$A_1H_6$ ,  $A_1H_9$  (from  $A_1h_{22}$ ) and then, they were compared.

However, in order to directly confirm the presence of integrated form of pHU52 containing hydrogen uptake (*hup*) genes in the transconjugants, dot-blot hybridization was carried out using whole cosmid pHU52 as *hup* gene probe.

### 3.2.20.1 Preparation of probe by Nick translation

The pHU52 was isolated from *E. coli* HB101 by alkaline lysis method as described earlier. For the preparation of the probe, pHU52 was nick translated using Amersham nick translation kit N5500 according to the Amersham nick translation kit and Maniatis *et al.* (1989).

The nick translation reaction was set up as follows:

|      |   |            |
|------|---|------------|
| DNA  |   | 50 $\mu$ l |
| dATP | ] |            |
| dGTP |   |            |
| dTTP |   | 20 $\mu$ l |

(dATP, dGTP, dTTP are in equal proportion i.e. 6.8  $\mu$ l each, in a concentrated nick translation buffer containing Tris-Cl (pH 8.0),  $MgCl_2$  and 2-Mercaptoethanol)

|                                                   |             |
|---------------------------------------------------|-------------|
| ( $\alpha$ $^{32}P$ ) dCTP (sp act 3000 Ci/mmole) | 5 $\mu$ l   |
| Enzyme                                            | 10 $\mu$ l  |
| H <sub>2</sub> O                                  | 15 $\mu$ l  |
| Total volume                                      | 100 $\mu$ l |

The above mixture was incubated in a waterbath at 15°C for 90 min. Then 50  $\mu$ l of salmon sperm DNA (5 mg/ml) was added to the probe. In order to denature the probe, the mixture was kept in boiling water for 5 min and chilled immediately on ice. A brief spin was then given in an eppendorf centrifuge. Total probe had  $3 \times 10^6$  cpm.

### 3.2.20.2 Isolation of genomic DNA

Isolation of chromosomal DNA from Hup<sup>+</sup> final transconjugants of rhizobia was carried out by the following method.

The cells of *Cicer-Rhizobium* transconjugants were grown in 10 ml of TY medium for 3 days. The cells were collected by centrifugation at 2000 X g for 10 min. The cell pellet was resuspended in 5 ml of 50 mM Tris containing 50 mM EDTA (pH 8.0) and subsequently frozen at -20°C. Lysozyme was prepared @ 10 mg/ml in 0.25 M Tris, pH 8.0. 0.5 ml of lysozyme was added to frozen cell suspension and thawed with occasional mixing and incubating in water bath at room temperature. When just thawed, it was placed on ice for 45 min. Proteinase K solution was made @ 1 mg/ml in STE buffer (0.5% SDS, 50 mM Tris, 0.4 M EDTA, pH 7.5). One ml of this solution was added to the above ice cooled mixture and mixed well by inversion of tube. It was then heated at 50°C for 1 h.

Then 6 ml of buffered phenol (phenol was melted at 68°C and 8-Hydroxyquinoline was added to the final concentration of 0.1%. The melted phenol was then extracted several times with an equal volume of 1.0 M Tris (pH 8.0) first, followed by 0.1 M Tris (pH 8.0) and 0.2%  $\beta$ -mercaptoethanol until the pH of the aqueous phase was more than 7.6) was added to it. It was mixed by gently inverting the tube. This must not be

vortexed. Then it was centrifuged at 5000 X g for 15 min to separate the layers. DNA will be in the top aqueous layer. To the upper aqueous layer, 0.1 vol of 3 M sodium acetate was added and mixed gently by inversion. Then two volumes of ethanol were added and mixed well by inversion. Nucleic acid should precipitate as a stringy glob. The precipitate was spooled out and 5 ml of TE (10 mM Tris/1 mM EDTA pH 8.0) was added. The precipitate was allowed to dissolve overnight at 4°C. Next day an equal volume of chloroform was added to the DNA dissolved in T<sub>10</sub>E<sub>1</sub> buffer and inverted several times to mix. Then it was centrifuged for 5 min to separate the layers. The upper aqueous layer was transferred to a clear tube and 0.1 volume of 3 M sodium acetate followed by 2 volumes of ethanol was added to it. DNA will precipitate as long threads. The DNA was spooled out and dissolved completely in 0.1 ml T<sub>10</sub>E<sub>1</sub> for overnight. Finally DNA was stored at 4°C.

### 3.2.20.3 Dot-blot experiment

The total genomic DNA, isolated in the above mentioned manner was manually dotted on the bio-trans membrane. 5 µl of each sample was dotted (by taking 1 ul each time for individual sample) on the dry membrane along with the positive control i.e. pHU52 DNA. The membrane was then soaked for 5 min each in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and neutralizing solution (3 M sodium acetate pH 3.0). It was then dried at room temperature and baked between two fresh Whatman filter papers in vacuum oven at 80°C for 2 h.

The baked filter was then hybridized with labelled probe as mentioned below:

#### 3.2.20.4 Prehybridization

| Prehybridization solution          |               |
|------------------------------------|---------------|
| 20 X SSC                           | 16.05 ml      |
| 20% SDS                            | 1.07 ml       |
| 0.25 M EDTA                        | 0.418 ml      |
| 100 X Denhardt's solution          | 1.07 ml       |
| H <sub>2</sub> O                   | 80.785 ml     |
| Freshly denatured salmon Sperm DNA | 0.535 ml      |
| <b>Total</b>                       | <b>100 ml</b> |

The membrane was slipped in a heat-sealable bag and approximately 0.2 ml of prehybridization solution was added per square cm of the bio-trans membrane. The air was squeezed out from the bag as much as possible. The open end of the bag was sealed. Then the bag was incubated at 68°C for 3 h in a submerged condition in a shaking water bath.

After 3 h the bag containing the membrane was removed from the water bath and one of its corners was cut off and minimum amount of prehybridization solution just enough to saturate the membrane was retained and excess amount of prehybridization solution was drained off.

#### 3.2.20.5 Hybridization

The denatured probe was added to the bag containing membrane and prehybridization solution. Air was again squeezed out as much as

possible from the bag and the corner was re-sealed. The bag was then incubated at 68°C for 2 days in the submerged condition in the shaking water bath.

The next morning, after removing the bag from the water bath, the hybridization solution was poured out into a container suitable for disposal. The bag was then cut along its three sides. The filter was removed and immediately submerged in a tray containing sufficient amount of washing solution (2 X SSC and 0.1% SDS). Twice, the washing was carried out at room temperature. Then, the filter was washed again with the second washing solution (IX SSC, 0.1% SDS) at 50°C for 1 h.

#### **3.2.20.6 Autoradiography**

The bio-trans membrane containing the DNA blots was taken out from the washing solution, dried at room temperature, wrapped in a Saran wrap inside cassette and exposed using Indu-X-ray film for a period of 4 days at -70°C. The film was then developed by using Indu developer (for 4 min) and Indu fixer (for 4 min) at room temperature in a dark room followed by washing in running tap water. Finally, the film was dried.

#### **3.2.21 Studies on the expression of *hup* genes**

Once it was established that cosmid pHU52 had been integrated into the genome of final transconjugants the expression of the *hup* genes was studied by inducing the H<sub>2</sub>-uptake activity in them in the free-living conditions and then by measuring the uptake of H<sub>2</sub> gas chromatographically.

### 3.2.21.1 Hydrogen uptake assay

*Rhizobium* cells were cultured in 16 X 155 mm test tubes, each containing 6 ml of the H<sub>2</sub> uptake medium (HUM medium) as a 1.5% agar slant. 100 µl of suspension of early log phase cultures were distributed on the surface of the slants. The method of derepression and assay of hydrogenase as described by Maier *et al.* (1978) was used with some modifications. The cotton plugs in the slants were replaced by sterile serum stoppers and H<sub>2</sub> gas was injected aseptically into the slants after removing an equal volume of air to give an atmosphere of 5% H<sub>2</sub> in air. The inoculated slants were incubated at 30±1°C for growth and hydrogenase induction.

A series of incubations were carried out viz. 7, 6, 5 and 4 days. During incubation the slants were positioned in a more or less horizontal manner so that hydrogen gas comes in contact with rhizobial growth. After each incubation period, the serum stoppers were removed and the slants were left open in air for a minute and again closed with the stoppers. The whole operation was carried out in sterile conditions in a laminar flow cabinet. The slants were then provided with a new atmosphere of 1% H<sub>2</sub> in air after removing an equal volume of air with a syringe. The slants were incubated at 30±1°C and gas samples removed after 3 h and 24 h incubation, and analyzed for their H<sub>2</sub> content using an AIMIL-NUCON gas chromatograph equipped with a thermal conductivity detector (TCD) and dual column (0.32 X 200 cm) packed with molecular sieve 5A (80-100 mesh). The temperatures of the oven, injector and detector were 60°, 80° and 80°C respectively and the current flow was 100 mA. In order to account for the H<sub>2</sub> leakage from the tubes, uninoculated control slants were included in the experiments. For each strain/control, six replications were used. The hydrogenase activity was expressed as nmol of H<sub>2</sub> consumed per h per mg protein.

**Table 4. Composition of hydrogen uptake medium  
(Maier *et al.*, 1978)**

| Medium constituent                                   | Weight/l of medium | Stock solution                 | Quantity of stock/l |
|------------------------------------------------------|--------------------|--------------------------------|---------------------|
| <b>Part A</b>                                        |                    |                                |                     |
| Sodium gluconate                                     | 0.5 g              |                                |                     |
| Sodium glutamate                                     | 0.5 g              |                                |                     |
| Yeast-extract                                        | 0.1 g              |                                |                     |
| Distilled water                                      | 1.0 lit            |                                |                     |
| Agar                                                 | 14.0 g             |                                |                     |
| <b>Part B</b>                                        |                    |                                |                     |
| Iron EDTA                                            | 28.0 mg            | 14.000 g                       |                     |
| Boric Acid                                           | 3.0 mg             | 1.500 g                        |                     |
| ZnSO <sub>4</sub> · 7H <sub>2</sub> O                | 2.000 mg           | 1.000 g                        |                     |
| MnSO <sub>4</sub> · 7H <sub>2</sub> O                | 10.000 mg          | 5.000 g in 250 ml              | 0.5 ml              |
| CuSO <sub>4</sub> · 5H <sub>2</sub> O                | 0.040 mg           | 0.020 g H <sub>2</sub> O       |                     |
| CoCl <sub>2</sub> · 6H <sub>2</sub> O                | 0.025 mg           | 0.013 g                        |                     |
| KI                                                   | 0.780 mg           | 0.390 g                        |                     |
| Na <sub>2</sub> MoO <sub>4</sub> · 2H <sub>2</sub> O | 0.250 mg           | 1.125 g                        |                     |
| <b>Part C</b>                                        |                    |                                |                     |
| NaHPO <sub>4</sub> · 2H <sub>2</sub> O               | 150.000 mg         | 2.500 g/25 ml H <sub>2</sub> O | 1.5 ml              |
| <b>Part D</b>                                        |                    |                                |                     |
| CaCl <sub>2</sub> · 2H <sub>2</sub> O                | 150.000 mg         | 0.375 g In 25 ml               | 10.0 ml             |
| MgSO <sub>4</sub> · 7H <sub>2</sub> O                | 250.000 mg         | 0.625 g H <sub>2</sub> O       |                     |
| <b>Part E</b>                                        |                    |                                |                     |
| Sucrose                                              | 0.500 gm           | 5.000 g                        |                     |
| L-arabinose                                          | 1.000 gm           | 10.000 g In 50 ml              | 5.0 ml              |
| Inositol                                             | 100.000 mg         | 1.000 g H <sub>2</sub> O       |                     |
| Thiamine. HCl                                        | 0.010 g            | 0.100 g                        |                     |
| <b>Part F</b>                                        |                    |                                |                     |
| Nicotinic acid                                       | 1.000 mg           | 0.100 g In 10 ml               | 1 ml                |
| Pyridoxal. HCl                                       | 1.000 mg           | 0.010 g H <sub>2</sub> O       |                     |

**Note:** Part B, was added to part A, mixed thoroughly and the pH was adjusted to 6.8 using 0.1 N NaOH before adding agar. It was then autoclaved at 15 Psi for 15 min. Part C and D were autoclaved separately and added at the time of slant preparation. The stock solutions in Part E were prepared separately in 50 ml H<sub>2</sub>O, mixed with Part F proportionately by volume and filter sterilized using a bacteriological filter (grade 5 and pore size 0.45  $\mu$ m), it was then added at the time of slant preparation.

## **RESULTS**

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In natural environment  $N_2$  fixation takes place against changing concentration of soil nitrate. Now it is a well known fact that nitrite, an intermediate compound in nitrate assimilation and denitrification pathways, inhibits nitrogenase activity. Various research workers have found a significant negative correlation between nitrite in nodules and nodule weight per plant. At the same time, nitrite produced from bacterial reduction of soil nitrate appears to have some influence on the production of indole acetic acid (IAA) and/or in raising its requirement for the initiation and development of the symbiosis between legume and rhizobia. There are, however, evidences to suggest that if the legume roots are infected with rhizobia having a functional hydrogen uptake (Hup) system then they are not only more efficient in  $N_2$  fixation but also tolerant to nitrate inhibition (Singh and Lodha, 1989).

Considering all these relevant facts, attempts have been made to mutagenize the *Cicer*-rhizobial strains G36-84 and F-75 involving Tn5 mutagenesis to make them nitrate tolerant under symbiotic conditions so that the mechanism of tolerance could be understood. *In vivo* activities of nitrate reductase and nitrite reductase were then studied in the parental rhizobial strains and their Tn5 mutants by growing them both under aerobic and microaerobic conditions with and without nitrate. The production of IAA by aerobic cultures was also studied. Further, in order to increase the  $N_2$ -fixing efficiency of nitrate tolerant mutants so developed, their Hup<sup>+</sup> transconjugants were developed by incorporating pHU52 (containing *hup* genes) into the genome of the rhizobial mutants. The major findings are

reported under the following headings:

1. Development of Tn5 transposon mutants of *Cicer*-rhizobial strains G36-84 and F-75.
2. Screening the mutants of rhizobial strains for nitrate tolerance under symbiotic conditions.
3. Study on the activities of the enzymes nitrate reductase and nitrite reductase in nitrate tolerant rhizobial mutants and their respective parents grown under aerobic and microaerobic conditions in the presence of different levels of nitrate.
4. Study of IAA production by nitrate tolerant rhizobial mutants along with their respective parents grown under aerobic conditions at different levels of nitrate.
5. Transfer and integration of pHU52::Tn5 into genomic DNA::Tn5 of nitrate tolerant *Cicer*-rhizobial mutants using incompatible plasmid pPH1J1.
6. Confirmation of the integration of pHU52 into the genome of the nitrate tolerant *Cicer-Rhizobium* transconjugants, and
7. Expression of hydrogen uptake (Hup) activity in the transconjugants under free-living condition.

#### **4.1 DEVELOPMENT OF TRANSPOSON Tn5 MUTANTS OF *CICER*-RHIZOBIAL STRAINS**

In order to study the mechanism of nitrate tolerance, it was necessary first to develop nitrate tolerant mutants, and then to see how the

expression of nitrate reductase and nitrite reductase activities and production of IAA by them are affected.

The mutants of *Cicer-Rhizobium* strains G36-84 and F-75 were developed by Tn5 mutagenesis (Selvaraj and Iyer, 1983). *Escherichia coli* HB101 (pGS9) was used as Tn5 donor. Since this plasmid does not have a replicon which functions in *Rhizobium*, it will not replicate after its introduction into the *Rhizobium*. Hence, it is called a suicide plasmid vector.

Biparental mating between *E. coli* HB101 (pGS9) and *Cicer-rhizobial* strains G36-84 and F-75 was done as described in the Materials and Methods (Section 3.2.7). The mutants were selected by looking for colonies resistant to nalidixic acid (Nd) and Kanamycin (Km). The frequency of transfer of Km resistance to G36-84 was  $0.93 \times 10^{-6}$  per donor cell, and  $0.25 \times 10^{-6}$  per recipient cell (Table 5). Similarly, the frequency of transfer of Km resistance to F-75 was  $0.61 \times 10^{-6}$  per donor cell, and  $0.33 \times 10^{-6}$  per recipient cell. There is always a possibility that while jumping, Tn5 may carry a portion of DNA which may represent an altogether different gene. Since, the gene conferring resistance to chloramphenicol (Cm) is situated adjacent to Tn5, there is every possibility of carrying Cm gene along with Tn5 during its transposition (Selvaraj and Iyer, 1983). Therefore, in this mating experiment, out of a total 228 Tn5 mutants of G36-84 growing on Nd Km, 12 were discarded because they were showing growth on Nd Km Cm plates. Similarly, out of a total of 147 Tn5 mutants of F-75, 7 were discarded. G36-84 mutants were given code names BC-1 to BC-216 and F-75 mutants were coded as AC-1 to AC-140. However, for further screening, only 20 mutants of G36-84 (BC-30 to BC-49) and 21 mutants of F-75 (AC-6, AC-8 to AC-27) were taken because of management problem.

**Table 5. Transfer frequency of Tn5 (pGS9) into chromosomal DNA of *Cicer-Rhizobium* strains G36-84 and F-75 by conjugation**

| Donor       | Titer             | Recipient | Titer             | No. of mutants     | Frequency of transfer |                       |
|-------------|-------------------|-----------|-------------------|--------------------|-----------------------|-----------------------|
|             |                   |           |                   |                    | per donor             | per recipient         |
| HB101(pGS9) | $2.3 \times 10^9$ | G36-84    | $8.7 \times 10^9$ | $2.16 \times 10^3$ | $0.93 \times 10^{-6}$ | $0.25 \times 10^{-6}$ |
| HB101(pGS9) | $2.3 \times 10^9$ | F-75      | $4.2 \times 10^9$ | $1.4 \times 10^3$  | $0.61 \times 10^{-6}$ | $0.33 \times 10^{-6}$ |

## 4.2 SCREENING THE MUTANTS OF RHIZOBIAL STRAINS FOR NITRATE TOLERANCE UNDER SYMBIOTIC CONDITION

Random Tn5 mutagenesis technique as described above, was followed to create mutation in *Cicer-rhizobia*. And then, they were screened for nitrate tolerance under symbiotic condition. Only those mutants, which showed Nod<sup>+</sup> Fix<sup>+</sup> characters in presence of nitrate, were selected and their symbiotic efficiency was determined as described in the Materials and Methods (Section 3.2.8).

The seeds of chickpea, inoculated with 20 G36-84::Tn5 mutants and 21 F-75::Tn5 mutants were grown in pots containing sterilized river sand, in *Rabi* season. Nitrate at four levels (0, 1, 2 and 4mM) was added right from the sowing stage. Parental strains were tested at 0, 1, 2 and 4mM NO<sub>3</sub><sup>-</sup> level, whereas the mutants were tested only at 1, 2 and 4mM NO<sub>3</sub><sup>-</sup> level. The plants were harvested at 60 day stage, and nodule fresh weight and nitrogenase activity were determined (Table 6). Based on their nodulation and N<sub>2</sub> fixation ability, they have been classified as Nod<sup>+</sup> Fix<sup>+</sup>, Nod<sup>+</sup> Fix<sup>-</sup> and Nod<sup>-</sup> Fix<sup>-</sup> (Table 7). The results presented in Table 6 show that with the incorporation of Tn5 into the genomes of G36-84 and F-75, the nodule fresh weight as well as nitrogenase activity were affected. The results presented in Table 7 show that only 8 of G36-84 mutants and 5 of F-75 mutants were able to nodulate. Out of these, all the 8 mutants of G36-84 showed nitrogenase (acetylene reduction) activity at 1mM NO<sub>3</sub><sup>-</sup>, while only 5 mutants *viz.*, BC-33, BC-35, BC-44, BC-46 and BC-48 were able to show nitrogenase activity upto 2mM NO<sub>3</sub><sup>-</sup> (Table 6). However, only 5 mutants of F-75 *viz.*, AC-8, AC-9, AC-10, AC-24 and AC-25 expressed nitrogenase activity at 1mM NO<sub>3</sub><sup>-</sup>. All these mutants except AC-25 also showed the activity at 2mM NO<sub>3</sub><sup>-</sup>. However, none of the parents could form nodule even at 1mM NO<sub>3</sub><sup>-</sup> (Table 6). Besides, none of the 20 mutants of G36-84 and 21 F-75 mutants was able to nodulate at 4mM NO<sub>3</sub><sup>-</sup> (Table 7).

**Table 6. Tn5 mutants of *Cicer-rhizobial* strains G36-84 and F-75 showing their nitrate tolerance under symbiotic conditions, nodule fresh weight and nitrogenase activity**

(Pot culture, 60 day stage)

| Parent | Mutant | Nodule fresh wt.<br>per plant (mg)<br>at nitrate conc. |     |     | Sp. activity of nitrogenase<br>(n mol of C <sub>2</sub> H <sub>4</sub> formed/g nodule/h)<br>at nitrate conc. |      |      |
|--------|--------|--------------------------------------------------------|-----|-----|---------------------------------------------------------------------------------------------------------------|------|------|
|        |        | 0 mM                                                   | 1mM | 2mM | 0 mM                                                                                                          | 1mM  | 2 mM |
| G36-84 |        | 117                                                    | nil | nil | 2657                                                                                                          | -    | -    |
|        | BC-30  |                                                        | 92  | nil |                                                                                                               | 57   | -    |
|        | BC-33  |                                                        | 70  | 35  |                                                                                                               | 131  | 285  |
|        | BC-35  |                                                        | 128 | 63  |                                                                                                               | 2627 | 7247 |
|        | BC-36  |                                                        | 44  | nil |                                                                                                               | 156  | -    |
|        | BC-44  |                                                        | 224 | 133 |                                                                                                               | 1549 | 229  |
|        | BC-45  |                                                        | 92  | nil |                                                                                                               | 216  | -    |
|        | BC-46  |                                                        | 68  | 178 |                                                                                                               | 92   | 2918 |
|        | BC-48  |                                                        | 176 | 157 |                                                                                                               | 20   | 63   |
| F-75   |        | 126                                                    | nil | nil | 2538                                                                                                          | -    | -    |
|        | AC-8   |                                                        | 122 | 57  |                                                                                                               | 1760 | 506  |
|        | AC-9   |                                                        | 129 | 4   |                                                                                                               | 1992 | 50   |
|        | AC-10  |                                                        | 90  | 63  |                                                                                                               | 3875 | 948  |
|        | AC-24  |                                                        | 26  | 96  |                                                                                                               | 68   | 2968 |
|        | AC-25  |                                                        | 11  | nil |                                                                                                               | 150  | -    |

**Table 7. Characterization of Tn5 mutants of *Cicer--rhizobial* strains G36-84 and F-75 for their nodulation and nitrogen fixation ability in the presence of different levels of nitrate**

(Pot culture, 60 day stage)

| Phenotype                         | Tn5 mutants of G36-84                   |       |                                  | Tn5 mutants of F-75                     |       |                                       |
|-----------------------------------|-----------------------------------------|-------|----------------------------------|-----------------------------------------|-------|---------------------------------------|
|                                   | NO <sub>3</sub> <sup>-</sup> conc. (mM) |       |                                  | NO <sub>3</sub> <sup>-</sup> conc. (mM) |       |                                       |
|                                   | 1                                       | 2     | 4                                | 1                                       | 2     | 4                                     |
| Nod <sup>-</sup> Fix <sup>-</sup> | BC-31                                   | BC-30 | All Tn5 mutants<br>(BC-30 to 49) | AC-11                                   | AC-6  | All Tn5 mutants<br>(AC-6, AC-8 to 27) |
|                                   | BC-32                                   | BC-31 |                                  | AC-12                                   | AC-11 |                                       |
|                                   | BC-34                                   | BC-32 |                                  | AC-13                                   | AC-12 |                                       |
|                                   | BC-37                                   | BC-34 |                                  | AC-14                                   | AC-13 |                                       |
|                                   | BC-38                                   | BC-36 |                                  | AC-16                                   | AC-14 |                                       |
|                                   | BC-39                                   | BC-37 |                                  | AC-17                                   | AC-15 |                                       |
|                                   | BC-40                                   | BC-38 |                                  | AC-18                                   | AC-16 |                                       |
|                                   | BC-41                                   | BC-39 |                                  | AC-19                                   | AC-17 |                                       |
|                                   | BC-42                                   | BC-40 |                                  | AC-20                                   | AC-18 |                                       |
|                                   | BC-43                                   | BC-41 |                                  | AC-21                                   | AC-19 |                                       |
|                                   | BC-47                                   | BC-42 |                                  | AC-22                                   | AC-20 |                                       |
|                                   | BC-49                                   | BC-43 |                                  | AC-23                                   | AC-21 |                                       |
|                                   |                                         | BC-45 |                                  | AC-26                                   | AC-22 |                                       |
|                                   |                                         | BC-47 |                                  | AC-27                                   | AC-23 |                                       |
|                                   |                                         | BC-49 |                                  |                                         | AC-25 |                                       |
|                                   |                                         |       |                                  |                                         | AC-26 |                                       |
|                                   |                                         |       |                                  |                                         | AC-27 |                                       |
| Nod <sup>+</sup> Fix <sup>+</sup> | BC-30                                   | BC-33 |                                  | AC-8                                    | AC-8  |                                       |
|                                   | BC-33                                   | BC-35 |                                  | AC-9                                    | AC-9  |                                       |
|                                   | BC-35                                   | BC-44 |                                  | AC-10                                   | AC-10 |                                       |
|                                   | BC-36                                   | BC-46 |                                  | AC-24                                   | AC-24 |                                       |
|                                   | BC-44                                   | BC-48 |                                  | AC-25                                   |       |                                       |
|                                   | BC-45                                   |       |                                  |                                         |       |                                       |
|                                   | BC-46                                   |       |                                  |                                         |       |                                       |
| Nod <sup>+</sup> Fix <sup>-</sup> | BC-48                                   |       |                                  |                                         |       |                                       |
|                                   |                                         |       |                                  | AC-6                                    |       |                                       |
|                                   |                                         |       |                                  | AC-15                                   |       |                                       |

Nod, nodulation; Fix, nitrogen fixation.

Based on higher nodule nitrogenase activity, three Nod<sup>+</sup> Fix<sup>+</sup> Tn5 mutants (BC-35, BC-46 and AC-10) were taken for studying the mechanism of nitrate tolerance by determining the activities of NR and NiR and production of IAA after growing them under aerobic and microaerobic conditions.

### 4.3 STUDIES ON THE ACTIVITIES OF THE ENZYMES NITRATE REDUCTASE AND NITRITE REDUCTASE

*Cicer-Rhizobium* strain G36-84 and its nitrate tolerant Tn5 mutants BC-35 and BC-46, and strain F-75 and its mutant AC-10 were grown aerobically and microaerobically in YEM liquid medium with 0, 0.5, 1, 2 and 4 mM KNO<sub>3</sub> for 4 and 10 days, respectively, and the *in vivo* activities of nitrate reductase (NR) and nitrite reductase (NiR) were determined.

#### 4.3.1 Aerobic growth

The results on protein content (mg/ml culture) of parental strains G36-84 and F-75, and their mutants after 4 days of aerobic growth are presented in Table 8.

##### 4.3.1.1 *In vivo* NR activity

It is evident from the results presented in Table 9 that the overall NR activity as indicated by the treatment averages was significantly lower in the mutants *i.e.*, BC-35, BC-46 and AC-10 and was approximately 9%, 9% and 30% when compared to their respective parents *i.e.*, G36-84 and F-75. Specifically, the NR activity of mutants BC-35 and BC-46 was significantly lower than their parent G36-84 at all nitrate concentrations, and also in the case where no nitrate was added. However, between two mutants the differences observed in the activity of NR at different levels of NO<sub>3</sub><sup>-</sup> were non-significant. Likewise, when the nitrate tolerant mutant AC-10 was compared to its parent F-75, the *in vivo* NR activity of mutant was significantly

**Table 8. Cell protein content of *Rhizobium* strains and their nitrate tolerant mutants after 4 days of aerobic growth with different levels of nitrate**

| Strain/mutant | Protein content (mg/ml culture)         |      |      |      |      |
|---------------|-----------------------------------------|------|------|------|------|
|               | NO <sub>3</sub> <sup>-</sup> conc. (mM) |      |      |      |      |
|               | 0                                       | 0.5  | 1    | 2    | 4    |
| G36-84        | 0.14                                    | 0.14 | 0.15 | 0.16 | 0.19 |
| BC-35         | 0.04                                    | 0.12 | 0.09 | 0.08 | 0.08 |
| BC-46         | 0.03                                    | 0.13 | 0.10 | 0.09 | 0.07 |
| F-75          | 0.03                                    | 0.03 | 0.03 | 0.02 | 0.03 |
| AC-10         | 0.04                                    | 0.05 | 0.14 | 0.14 | 0.14 |

**Table 9. *In vivo* activity of nitrate reductase in *Cicer-Rhizobium* strains and their nitrate tolerant Tn5 mutants when grown aerobically with different levels of nitrate**

| Strain/<br>mutant | Nitrate reductase activity<br>(n mol NO <sub>2</sub> <sup>-</sup> formed/mg protein/h) |       |       |      |      |                      |
|-------------------|----------------------------------------------------------------------------------------|-------|-------|------|------|----------------------|
|                   | NO <sub>3</sub> <sup>-</sup> conc.(mM)                                                 |       |       |      |      | Treatment<br>average |
|                   | 0                                                                                      | 0.5   | 1     | 2    | 4    |                      |
| G36-84            | 9024                                                                                   | 10898 | 11936 | 9978 | 4265 | <b>9220</b>          |
| BC-35             | 1067                                                                                   | 750   | 1064  | 801  | 658  | <b>868</b>           |
| BC-46             | 1199                                                                                   | 754   | 921   | 764  | 516  | <b>831</b>           |
| F-75              | 3931                                                                                   | 4870  | 8351  | 4655 | 3615 | <b>5084</b>          |
| AC-10             | 2278                                                                                   | 1223  | 1312  | 1361 | 1340 | <b>1503</b>          |

|                      |      |          |
|----------------------|------|----------|
| C.D. for interaction | 713  | 937      |
| P =                  | 0.05 | P = 0.01 |
| C.D. for strains     | 319  | 419      |
| P =                  | 0.05 | P = 0.01 |

lower than F-75 at all nitrate concentrations and even in the case where no nitrate was added.

Both the parents, eg., G36-84 and F-75 showed a definite trend in NR activity *i.e.*, the activity increased upto 1mM  $\text{NO}_3^-$  level and thereafter it showed a decreasing trend at 2 and 4mM  $\text{NO}_3^-$ . At 4mM  $\text{NO}_3^-$  the activity was the lowest. However, for the mutants BC-35 and BC-46 the activity did not change much with the changing nitrate concentration. In case of mutant AC-10, the activity was significantly lower at all the nitrate concentrations studied compared to when no nitrate was added to the medium (Table 9) excepting the activity at 2mM  $\text{NO}_3^-$ .

#### 4.3.1.2 *In vivo* NiR activity

It is quite clear from the results presented in Table 10 that the overall NiR activity in the aerobically grown rhizobial cells as indicated by the treatment averages was also significantly lower in the mutants when compared to their respective parents. Mutants BC-35 and BC-46 had only 46% and 45% of the parental NiR activity respectively. However, mutant AC-10 had 64% of the parental NiR activity. To be more specific, the NiR activity of mutants BC-35 and BC-46 was significantly lower than their parent G36-84 at all  $\text{NO}_3^-$  concentrations including where no nitrate was added. The NiR activity of AC-10 was significantly lower than its parent F-75 at all concentrations of nitrate except where no nitrate was added.

For both the parental strains G36-84 and F-75 the NiR activity showed an increasing trend upto 1mM  $\text{NO}_3^-$  and thereafter it showed a gradual decreasing order at 2 and 4mM  $\text{NO}_3^-$ . A similar trend was also observed for all the mutants. The increase in the activity at 1mM  $\text{NO}_3^-$  was highly significant compared to when no nitrate was added. However,

**Table 10. *In vivo* activity of nitrite reductase in *Cicer-Rhizobium* strains and their nitrate tolerant Tn5 mutants when grown aerobically with different levels of nitrate**

| Strain/<br>mutant | Nitrite reductase activity<br>(n mol NO <sub>2</sub> <sup>-</sup> disappeared/mg protein/h) |      |      |      |      |                      |
|-------------------|---------------------------------------------------------------------------------------------|------|------|------|------|----------------------|
|                   | NO <sub>3</sub> <sup>-</sup> conc.(mM)                                                      |      |      |      |      | Treatment<br>average |
|                   | 0                                                                                           | 0.5  | 1    | 2    | 4    |                      |
| G36-84            | 2921                                                                                        | 3652 | 4845 | 3918 | 1934 | <b>3454</b>          |
| BC-35             | 968                                                                                         | 1727 | 2473 | 1804 | 948  | <b>1584</b>          |
| BC-46             | 1176                                                                                        | 1634 | 2515 | 1378 | 987  | <b>1538</b>          |
| F-75              | 1419                                                                                        | 2364 | 4710 | 4448 | 2936 | <b>3175</b>          |
| AC-10             | 976                                                                                         | 1400 | 3256 | 2382 | 2071 | <b>2017</b>          |

C.D. for interaction                      722                      949

P = 0.05                      P = 0.01

C.D. for strains                      323                      425

P = 0.05                      P = 0.01

between the two mutants *viz.*, BC-35 and BC-46 the differences observed in the activity of NiR at different levels of nitrate were non-significant.

#### **4.3.2 Microaerobic growth**

The results on protein content (mg/ml culture) of parental strains G36-84 and F-75 and their mutants after 10 days of microaerobic growth are presented in Table 11.

##### **4.3.2.1 *In vivo* NR activity**

The overall *in vivo* NR activity as indicated by the treatment averages was significantly lower in mutants BC-35 and BC-46 and was approximately 22% and 14%, respectively when compared to their parent G36-84. The mutant AC-10 also had only 13% of the parental NR activity (Table 12). In general, the decrease in the activity of NR at all the levels of added nitrate was highly significant in the mutants compared to their respective parents. Between the two mutants of G36-84, BC-46 showed significantly lower activity compared to BC-35 at nitrate concentrations of 0.5 and 1mM.

The *in vivo* NR activity of microaerobically grown culture of G36-84 and F-75 showed a very different trend. Their activities increased progressively and significantly upto 4 mM  $\text{NO}_3^-$  conc. For BC-35, the activity increased upto 0.5 mM  $\text{NO}_3^-$  then progressively decreased upto 4 mM  $\text{NO}_3^-$  level. However, for the mutants BC-46 and AC-10 the activity did not change significantly with the increasing nitrate concentration (Table 12).

##### **4.3.2.2 *In vivo* NiR activity**

The results presented in Table 13 show that the overall *in vivo* NiR activity of microaerobically grown mutants BC-35 and BC-46 as indicated by the treatment averages was significantly lower and was respectively 70%

**Table 11. Cell protein content of *Rhizobium* strains and their nitrate tolerant mutants after 10 days of microaerobic growth with different levels of nitrate**

| Strain/mutant | Protein content (mg/ml culture)         |      |      |      |      |
|---------------|-----------------------------------------|------|------|------|------|
|               | NO <sub>3</sub> <sup>-</sup> conc. (mM) |      |      |      |      |
|               | 0                                       | 0.5  | 1    | 2    | 4    |
| G36-84        | 0.19                                    | 0.35 | 0.23 | 0.04 | 0.04 |
| BC-35         | 0.14                                    | 0.15 | 0.14 | 0.15 | 0.13 |
| BC-46         | 0.15                                    | 0.14 | 0.13 | 0.13 | 0.11 |
| F-75          | 0.14                                    | 0.11 | 0.08 | 0.11 | 0.11 |
| AC-10         | 0.15                                    | 0.13 | 0.15 | 0.14 | 0.14 |

**Table 12. *In vivo* activity of nitrate reductase in *Cicer-Rhizobium* strains and their nitrate tolerant Tn5 mutants when grown microaerobically with different levels of nitrate**

| Strain/<br>mutant | Nitrate reductase activity<br>(n mol NO <sub>2</sub> <sup>-</sup> formed/mg protein/h) |      |      |      |      |                      |
|-------------------|----------------------------------------------------------------------------------------|------|------|------|------|----------------------|
|                   | NO <sub>3</sub> <sup>-</sup> conc.(mM)                                                 |      |      |      |      |                      |
|                   | 0                                                                                      | 0.5  | 1    | 2    | 4    | Treatment<br>average |
| G36-84            | 842                                                                                    | 1710 | 3536 | 5433 | 7500 | 3804                 |
| BC-35             | 432                                                                                    | 1264 | 962  | 783  | 733  | 835                  |
| BC-46             | 432                                                                                    | 768  | 517  | 460  | 441  | 524                  |
| F-75              | 1067                                                                                   | 1420 | 4760 | 6360 | 7227 | 4167                 |
| AC-10             | 764                                                                                    | 691  | 486  | 442  | 425  | 562                  |

|                      |      |          |
|----------------------|------|----------|
| C.D. for interaction | 349  | 459      |
| P =                  | 0.05 | P = 0.01 |
| C.D. for strains     | 156  | 205      |
| P =                  | 0.05 | P = 0.01 |

and 73% of the activity obtained in parental strain G36-84. Likewise, mutant AC-10 also had 63% of the activity shown in its parent F-75. To be specific, mutant BC-35 had significantly lower activity at 1, 2 and 4mM  $\text{NO}_3^-$  while mutant BC-46 had significantly lower activity only at 2 and 4mM  $\text{NO}_3^-$  in comparison to parent G36-84. AC-10 had significantly lower NiR activity at all levels of nitrate added. Surprisingly, in the absence of any added nitrate, the activity either did not change or increased non-significantly in the mutants compared to their respective parents. In mutants BC-35 and BC-46 the increase in the activity was highly significant compared to parent G36-84 (Table 13). The differences observed in the activities of NiR between the mutants BC-35 and BC-46 when grown with different levels of nitrate, were non-significant.

In strain G36-84 the *in vivo* activity of NiR gradually increased upto 2mM  $\text{NO}_3^-$  level and then decreased at 4mM  $\text{NO}_3^-$  level. Whereas, for F-75 the activity was highest at 0.5mM  $\text{NO}_3^-$  level, then it gradually decreased upto 4 mM  $\text{NO}_3^-$  level. For BC-35, BC-46 and AC-10 the activity was highest at 0.5mM  $\text{NO}_3^-$  level, and then it gradually decreased upto 4mM  $\text{NO}_3^-$  level.

#### 4.4 STUDY OF IAA PRODUCTION

In order to study the production of indole acetic acid (IAA) by the rhizobial strains and their mutants, they were grown aerobically for 4 days in YEM medium containing different levels of nitrate and tryptophan (200  $\mu\text{g/ml}$ ), and their growth data in terms of mg protein/ml culture are presented in Table 14. The IAA produced by the rhizobia and their mutants was estimated as described in the materials and methods (Section 3.2.13) and the results expressed as  $\mu\text{g}$  IAA/100 ml culture (Table 15) or  $\mu\text{g}$  IAA/mg protein (Table 16).

**Table 13. *In vivo* activity of nitrite reductase in *Cicer-Rhizobium* strains and their nitrate tolerant Tn5 mutants when grown microaerobically with different levels of nitrate**

| Strain/<br>mutant    | Nitrite reductase activity<br>(n mol NO <sub>2</sub> <sup>-</sup> disappeared/mg protein/h) |      |      |          |          |                      |
|----------------------|---------------------------------------------------------------------------------------------|------|------|----------|----------|----------------------|
|                      | NO <sub>3</sub> <sup>-</sup> conc.(mM)                                                      |      |      |          |          | Treatment<br>average |
|                      | 0                                                                                           | 0.5  | 1    | 2        | 4        |                      |
| G36-84               | 513                                                                                         | 1031 | 1650 | 1984     | 1417     | 1319                 |
| BC-35                | 565                                                                                         | 1371 | 1245 | 867      | 589      | 927                  |
| BC-46                | 520                                                                                         | 1536 | 1402 | 834      | 532      | 965                  |
| F-75                 | 836                                                                                         | 1596 | 1398 | 1330     | 943      | 1221                 |
| AC-10                | 1017                                                                                        | 1093 | 833  | 489      | 402      | 767                  |
| C.D. for interaction |                                                                                             |      |      | 250      | 329      |                      |
|                      |                                                                                             |      |      | P = 0.05 | P = 0.01 |                      |
| C.D. for strains     |                                                                                             |      |      | 112      | 147      |                      |
|                      |                                                                                             |      |      | P = 0.05 | P = 0.01 |                      |

**Table 14. Cell protein content of *Rhizobium* strains and their nitrate tolerant mutants after 4 days of aerobic growth with different levels of nitrate in the presence of tryptophan (200 µg/ml culture medium)**

| Strain/mutant | Protein content (mg/ml culture)         |      |      |      |      |
|---------------|-----------------------------------------|------|------|------|------|
|               | NO <sub>3</sub> <sup>-</sup> conc. (mM) |      |      |      |      |
|               | 0                                       | 0.5  | 1    | 2    | 4    |
| G36-84        | 0.17                                    | 0.19 | 0.18 | 0.22 | 0.20 |
| BC-35         | 0.09                                    | 0.12 | 0.09 | 0.08 | 0.08 |
| BC-46         | 0.10                                    | 0.13 | 0.10 | 0.09 | 0.09 |
| F-75          | 0.03                                    | 0.03 | 0.03 | 0.03 | 0.03 |
| AC-10         | 0.04                                    | 0.03 | 0.04 | 0.03 | 0.03 |

Table 15 shows that the overall IAA production ( $\mu\text{g}/100\text{ ml culture}$ ) as indicated by the treatment averages was lower in the mutants BC-35 and BC-46 compared to parent G36-84. However, mutant AC-10 showed an overall increase of about 100% in IAA production in comparison to the parent F-75. Specifically, BC-35 at 2mM and 4mM  $\text{NO}_3^-$  showed a significantly higher IAA production in comparison to its parent G36-84. Similarly, BC-46 at 4 mM  $\text{NO}_3^-$  conc. showed a significantly higher IAA production in comparison to G36-84. However, G36-84 showed a definite trend in IAA production *i.e.*, the IAA produced was highest at 0.5mM  $\text{NO}_3^-$  and thereafter it progressively decreased upto 4mM  $\text{NO}_3^-$  level. No such trend was observed with F-75. Even none of the three mutants showed any definite trend in IAA production.

A more clear picture on IAA production emerged when the results were expressed on protein basis (Table 16). The overall increase in IAA production (per mg protein) as indicated by the treatment averages was highly significant in all the three mutants when compared to respective parents, and this may be due to the difference in growth status. Once again, the parental strain G36-84 showed a definite trend in IAA production. The IAA produced by G36-84 was the highest when no nitrate was added and its production decreased gradually with the increase in nitrate concentration. In parental strain F-75 also IAA produced was the highest when no nitrate was added. However, least IAA was produced at 0.5mM  $\text{NO}_3^-$ . In case of mutants a different type of picture emerged. The IAA production either decreased or did not change at 0.5mM  $\text{NO}_3^-$  and thereafter its production increased upto 2mM or 4mM  $\text{NO}_3^-$  level compared to IAA produced in the absence of any added nitrate. Between two mutants, IAA production was significantly higher in BC-35 compared to BC-46 at 1mM and 2mM  $\text{NO}_3^-$  and also when no nitrate was added.

**Table 15. Production of IAA in the culture medium ( $\mu\text{g}/100\text{ml}$  culture) by *Cicer*-rhizobial strains and their nitrate tolerant Tn5 mutants when grown aerobically with different levels of nitrate in the presence of tryptophan**

| Strain/<br>mutant    | Nitrate concentration (mM) |      |     |          |          | Treatment<br>average |
|----------------------|----------------------------|------|-----|----------|----------|----------------------|
|                      | 0                          | 0.5  | 1   | 2        | 4        |                      |
| G36-84               | 1150                       | 1188 | 969 | 782      | 325      | 883                  |
| BC-35                | 888                        | 625  | 938 | 1000     | 775      | 845                  |
| BC-46                | 738                        | 725  | 813 | 850      | 963      | 818                  |
| F-75                 | 178                        | 91   | 137 | 131      | 94       | 126                  |
| AC-10                | 194                        | 141  | 222 | 347      | 347      | 250                  |
| C.D. for interaction |                            |      |     | 76       | 98       |                      |
|                      |                            |      |     | P = 0.05 | P = 0.01 |                      |
| C.D. for strains     |                            |      |     | 34       | 44       |                      |
|                      |                            |      |     | P = 0.05 | P = 0.01 |                      |

**Table 16. Production of IAA in the culture medium ( $\mu\text{g}/\text{mg}$  protein) by *Cicer*-rhizobial strains and their nitrate tolerant Tn5 mutants when grown aerobically with different levels of nitrate in the presence of tryptophan**

| Strain/<br>mutant | Nitrate concentration (mM) |     |     |     |     | Treatment<br>average |
|-------------------|----------------------------|-----|-----|-----|-----|----------------------|
|                   | 0                          | 0.5 | 1   | 2   | 4   |                      |
| G36-84            | 68                         | 63  | 54  | 36  | 16  | <b>47</b>            |
| BC-35             | 99                         | 52  | 104 | 125 | 97  | <b>95</b>            |
| BC-46             | 74                         | 56  | 81  | 94  | 107 | <b>82</b>            |
| F-75              | 59                         | 30  | 46  | 44  | 31  | <b>42</b>            |
| AC-10             | 49                         | 47  | 56  | 116 | 116 | <b>77</b>            |

C.D. for interaction

14

19

P = 0.05    P = 0.01

C.D. for strains

6

8

P = 0.05    P = 0.01

#### 4.5 TRANSFER AND INTEGRATION OF pHU52::Tn5 INTO GENOMIC DNA::Tn5

In order to develop  $H_2$  uptake positive ( $Hup^+$ ) transconjugants of nitrate tolerant Tn5 mutants of *Cicer*-rhizobial strains, cosmid pHU52::Tn5 was integrated into the genomic DNA::Tn5 of the mutants BC-35, BC-46 and AC-10 by homologous recombination utilizing Tn5 as an homologous sequence between them. The cosmid being  $Tra^-$  (not self transmissible), the helper strain *E. coli* HB101 (pRK2073) having the *tra* gene was used to transfer it into the mutant strains. Triparental mating was done as mentioned in the Materials and Methods (Section 3.2.16). The frequency of transfer of pHU52::Tn5 to three *Cicer*-rhizobial mutants ranged from  $1.6 \times 10^{-6}$  to  $3.6 \times 10^{-6}$  per donor and from  $0.17 \times 10^{-6}$  to  $0.5 \times 10^{-6}$  per recipient. In all 42 transconjugants of BC-35, 89 of BC-46 and 97 of AC-10 were obtained. However, for the purpose of integration of pHU52::Tn5, which is present in the free form, into the genomic DNA::Tn5 of these three mutants, only one transconjugant from each mutant was taken. The transconjugant selected have been coded as follows: transconjugant BC-35::Tn5 (pHU52::Tn5) has been coded as  $B_3h_{12}$ , transconjugant BC-46::Tn5 (pHU52::Tn5) as  $B_4h_{16}$  and transconjugants AC-10::Tn5 (pHU52::Tn5) as  $A_1h_{22}$ . To show the presence of cosmid pHU52::Tn5 as an episome in these transconjugants, agarose gel (0.7%) electrophoresis was carried out after isolating the cosmid. The cosmid DNA bands of all the three transconjugants tested were corresponding to the band of cosmid DNA obtained from donor strain *E. coli* HB101 (pHU52::Tn5) (Plate I).

Forcing was done in order to integrate the *hup* complementing cosmid pHU52::Tn5 DNA into the chromosome of transconjugants  $B_3h_{12}$ ,

### PLATE I.

Agarose gel (0.7%) electrophoresis of cosmid DNA isolated from *Cicer-Rhizobium* transconjugants by alkaline lysis method before ~~is~~ integration into chromosomal DNA

|               |                                                                                                                                                         |
|---------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|
| Lane 1,       | $\lambda$ DNA;                                                                                                                                          |
| Lane 2,       | pHU52::Tn5 <i>E. coli</i> HB101<br>(positive control);                                                                                                  |
| Lanes 3 to 5, | pHU52::Tn5 from B <sub>3</sub> h <sub>12</sub> ::Tn5,<br>B <sub>4</sub> h <sub>16</sub> ::Tn5 and A <sub>1</sub> h <sub>22</sub> ::Tn5,<br>respectively |

1

2

3

4

5



Table 17. Transfer frequency of *hup* cosmid pHU52::Tn5 to nitrate tolerant Tn5 mutants of *Cicer-rhizobial* strains by conjugation

| Donor             | Titer                 | Recipient                      | Titer                 | Helper         | Titer               | Number of transconjugants | Frequency of transfer per donor | Frequency of transfer per recipient |
|-------------------|-----------------------|--------------------------------|-----------------------|----------------|---------------------|---------------------------|---------------------------------|-------------------------------------|
| HB101(pHU52::Tn5) | 2.7X10 <sup>9</sup>   | BC-35                          | 2.48X10 <sup>10</sup> | HB101(pRK2073) | 7.1X10 <sup>9</sup> | 4.2X10 <sup>3</sup>       | 1.6X10 <sup>-6</sup>            | 0.17X10 <sup>-6</sup>               |
| HB101(pHU52::Tn5) | 2.7X10 <sup>9</sup>   | BC-46                          | 1.75X10 <sup>10</sup> | HB101(pRK2073) | 7.1X10 <sup>9</sup> | 8.9X10 <sup>3</sup>       | 3.3X10 <sup>-6</sup>            | 0.5X10 <sup>-6</sup>                |
| HB101(pHU52::Tn5) | 2.7X10 <sup>9</sup>   | AC-10                          | 3.28X10 <sup>10</sup> | HB101(pRK2073) | 7.1X10 <sup>9</sup> | 9.7X10 <sup>3</sup>       | 3.6X10 <sup>-6</sup>            | 0.3X10 <sup>-6</sup>                |
| HB101(pPH1JI)     | 1.11X10 <sup>10</sup> | B <sub>3</sub> h <sub>12</sub> | 1.36X10 <sup>10</sup> | —              | —                   | 3.14X10 <sup>4</sup>      | 2.8X10 <sup>-6</sup>            | 2.3X10 <sup>-6</sup>                |
| HB101(pPH1JI)     | 1.11X10 <sup>10</sup> | B <sub>4</sub> h <sub>16</sub> | 2.12X10 <sup>10</sup> | —              | —                   | 2.1X10 <sup>4</sup>       | 1.9X10 <sup>-6</sup>            | 1.0X10 <sup>-6</sup>                |
| HB101(pPH1JI)     | 1.11X10 <sup>10</sup> | A <sub>1</sub> h <sub>22</sub> | 1.28X10 <sup>10</sup> | —              | —                   | 2.88X10 <sup>4</sup>      | 2.6X10 <sup>-6</sup>            | 2.25X10 <sup>-6</sup>               |

$B_4h_{16}$  and  $A_1h_{22}$ . This was done by introducing an incompatible plasmid pPH1JI. The plasmid pPH1JI was transferred to the transconjugants by biparental mating. The selection was made by growing the transconjugants on the plates containing Nd Km Tc Gm. The frequency of transfer of the incompatible plasmid is given in Table 17. Since, the plasmid pPH1JI and the cosmid pHU52 (*hup* gene has been inserted into the pLAFR1 vector to get pHU52) are derived from the same plasmid group (RK2), these two are incompatible to each other. Therefore, during replication, one of them has to be eliminated. Since, there is a homology of Tn5 sequence between the genomic DNA::Tn5 and pHU52::Tn5, the integration of pHU52 into the genomic DNA should be favoured. The incompatible plasmid pPH1JI was not necessary after the integration of the *hup* cosmid pHU52 into the chromosome. Therefore, it was desirable to eliminate this. The elimination of pPH1JI was done by growing them on the plates without Gm but with Nd Km Tc and subculturing six times so that in the absence of selection pressure pPH1JI will be lost in the subsequent generations. Then the transconjugants were replica plated on the plates containing Nd Km Tc or Nd Km Tc Gm. Only those transconjugants which were growing on Nd Km Tc but not on Nd Km Tc Gm were retained (Plate II). In this way finally eight transconjugants were selected. They were coded in the following manner: Two final transconjugants from  $B_3h_{12}$  were coded as  $B_3H_9$  and  $B_3H_{10}$ , three final transconjugants from  $B_4h_{16}$  were coded as  $B_4H_1$ ,  $B_4H_3$ ,  $B_4H_7$  and  $B_4H_{11}$ , two final transconjugants from  $A_1h_{22}$  were coded as  $A_1H_6$  and  $A_1H_9$ . The genetic constitution of all the nitrate tolerant mutants and their transconjugants is given in Table 18.

In order to check whether the cosmid pHU52::Tn5 had been integrated into the chromosome of the final transconjugants, 0.7% agarose

**Table 18. Genetic constitution of nitrate tolerant mutants and their pHU52 transconjugants or presumptive transconjugants of *Cicer-rhizobia***

| Strain                                       | Genotype                 |
|----------------------------------------------|--------------------------|
| BC-35                                        | G36-84::Tn5              |
| BC-46                                        | G36-84::Tn5              |
| AC-10                                        | F-75::Tn5                |
| Transconjugant or presumptive transconjugant |                          |
| B <sub>3</sub> h <sub>12</sub>               | G36-84::Tn5 (pHU52::Tn5) |
| B <sub>4</sub> h <sub>16</sub>               | G36-84::Tn5 (pHU52::Tn5) |
| A <sub>1</sub> h <sub>22</sub>               | F75::Tn5 (pHU52::Tn5)    |
| B <sub>3</sub> H <sub>9</sub>                | G36-84::Tn5::pHU52       |
| B <sub>3</sub> H <sub>10</sub>               | G36-84::Tn5::pHU52       |
| B <sub>4</sub> H <sub>1</sub>                | G36-84::Tn5::pHU52       |
| B <sub>4</sub> H <sub>3</sub>                | G36-84::Tn5::pHU52       |
| B <sub>4</sub> H <sub>7</sub>                | G36-84::Tn5::pHU52       |
| B <sub>4</sub> H <sub>11</sub>               | G36-84::Tn5::pHU52       |
| A <sub>1</sub> H <sub>6</sub>                | F-75::Tn5::pHU52         |
| A <sub>1</sub> H <sub>9</sub>                | F-75::Tn5::pHU52         |

G36-84 and F-75 are the parental rhizobial strains.

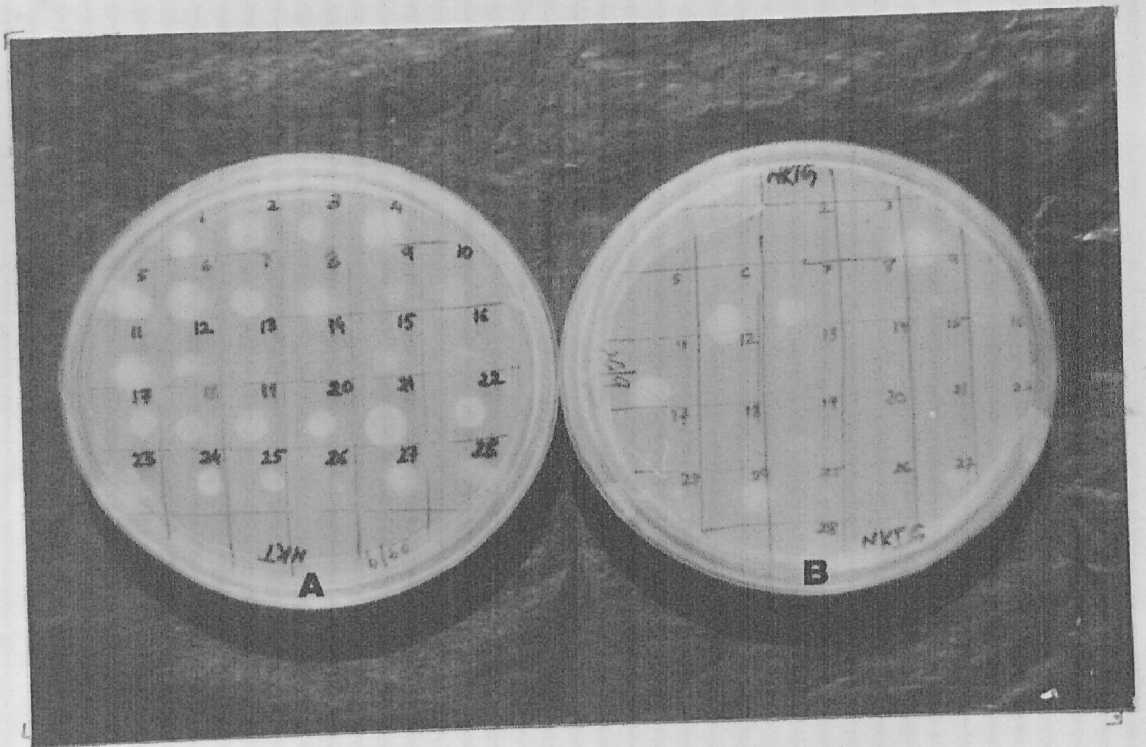
## PLATE II.

Petri plates showing transconjugant clones growing on the plates containing Nd Km Tc and Nd Km Tc Gm. The position of the transconjugant clones is as shown below:

| Parent | Colony number | Transconjugant |
|--------|---------------|----------------|
| F-75   | 1             | $A_1H_2$       |
|        | 2             | $A_1H_6$       |
|        | 3             | $A_1H_9$       |
|        | 4             | $A_1H_{12}$    |
|        | 5             | $A_1H_{15}$    |
|        | 6             | $A_1H_{10}$    |
|        | 7             | $A_1H_{13}$    |
|        | 8             | $A_1H_{11}$    |
| G36-84 | 9             | $B_3H_1$       |
|        | 10            | $B_3H_2$       |
|        | 11            | $B_3H_3$       |
|        | 12            | $B_3H_9$       |
|        | 13            | $B_3H_5$       |
|        | 14            | $B_3H_{11}$    |
|        | 15            | $B_3H_{10}$    |
|        | 16            | $B_3H_{15}$    |
|        | 17            | $B_3H_{16}$    |
|        | 18            | $B_4H_1$       |
|        | 19            | $B_4H_6$       |
|        | 20            | $B_4H_3$       |
|        | 21            | $B_4H_5$       |
|        | 22            | $B_4H_3$       |
|        | 23            | $B_4H_7$       |
|        | 24            | $B_4H_{14}$    |
|        | 25            | $B_4H_{19}$    |
|        | 26            | $B_4H_{11}$    |
|        | 27            | $B_4H_{12}$    |
|        | 28            | $B_4H_9$       |

A. The transconjugant clones growing with Nd Km Tc showing the presence of pHU52.

B. The transconjugant clones growing with Nd Km Tc Gm showing the presence of pHU52 as well as incompatible plasmid nPH1.II



gel electrophoresis was done after isolating the cosmid DNA from the transconjugants having pHU52 in free form as well as in the integrated form. The results are shown in Plate III. All the final transconjugants (with pHU52 integrated into the genome) did not show any band corresponding to the band of cosmid pHU52 DNA of the transconjugants having pHU52 in the episomal state. These results indicated that the cosmid pHU52::Tn5 was not present in the free form in the final transconjugants. However, this was only an indirect evidence to show the integration of cosmid into the chromosome.

#### **4.5.1 Confirmation of the integration of cosmid pHU52::Tn5 into the chromosome DNA**

In order to confirm the integration of pHU52 into the genomic DNA, dot-blot hybridization of the DNA isolated from the final transconjugants was done using ( $\alpha$ - $^{32}\text{P}$ ) labelled pHU52::Tn5 as a probe. The results presented in Plate IV show that all the 4 transconjugants tested gave positive signals.

#### **4.6 EXPRESSION OF HYDROGEN UPTAKE ACTIVITY IN THE FINAL TRANSCONJUGANTS UNDER FREE-LIVING CONDITIONS**

Once it is confirmed that the *hup* genes (cosmid pHU52) have been integrated into the genomic DNA, the next most important step is to know whether they are functional or not. Therefore, to study the expression of *hup* genes in the final transconjugants, they were grown on the HUM medium for 4 to 7 days for derepression of *hup* genes under free-living conditions in the presence of 5%  $\text{H}_2$ , and the  $\text{H}_2$  uptake (Hup) activity was measured. The activity has been expressed as n mol  $\text{H}_2$  taken up per mg protein per h.

The results presented in Table 19 show the expression of hydrogenase activity in all the transconjugants tested. However, both the parental strains G36-84 and F-75 did not show any expression. With respect

**Table 19. Hydrogen uptake activity of nitrate tolerant *Cicer-Rhizobium* transconjugants having chromosome integrated *hup* cosmid pHU52, when grown under free-living conditions**

| Parent            | Transconjugant                 | n mol H <sub>2</sub> /mg protein/h |          |          |          |
|-------------------|--------------------------------|------------------------------------|----------|----------|----------|
|                   |                                | Days incubation for derepression   |          |          |          |
|                   |                                | 4                                  | 5        | 6        | 7        |
| BC-35<br>(G36-84) | B <sub>3</sub> H <sub>9</sub>  | 316±44                             | 618±4    | 855±42   | 927±53   |
|                   | B <sub>3</sub> H <sub>10</sub> | nd                                 | 821±134  | 953±215  | 1061±273 |
| BC-46<br>(G36-84) | B <sub>4</sub> H <sub>1</sub>  | 2168±311                           | 1890±541 | 1584±253 | 1065±20  |
|                   | B <sub>4</sub> H <sub>3</sub>  | nd                                 | nd       | 377±64   | nd       |
|                   | B <sub>4</sub> H <sub>7</sub>  | 180±120                            | 684±36   | 470±239  | 489±154  |
|                   | B <sub>4</sub> H <sub>11</sub> | 299±66                             | 601±72   | 801±134  | 970±253  |
| AC-10<br>(F-75)   | A <sub>1</sub> H <sub>6</sub>  | nd                                 | 1081±427 | 55±220   | 284±119  |
|                   | A <sub>1</sub> H <sub>9</sub>  | 387±98                             | 578±138  | 810±65   | 892±187  |

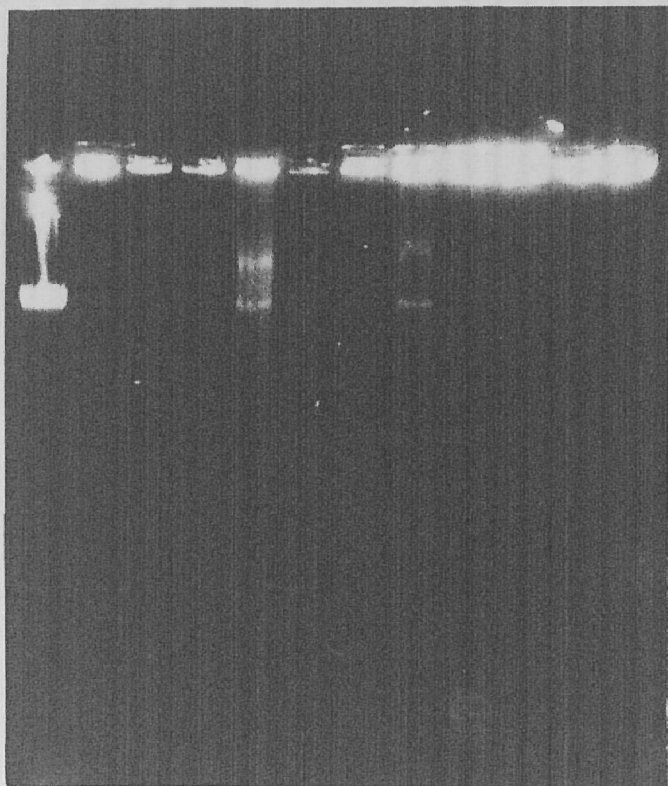
nd = not detectable

### PLATE III.

Plasmid analysis of final Hup<sup>+</sup> transconjugants of *Cicer-Rhizobium* strains by agarose gel (0.7%) electrophoresis

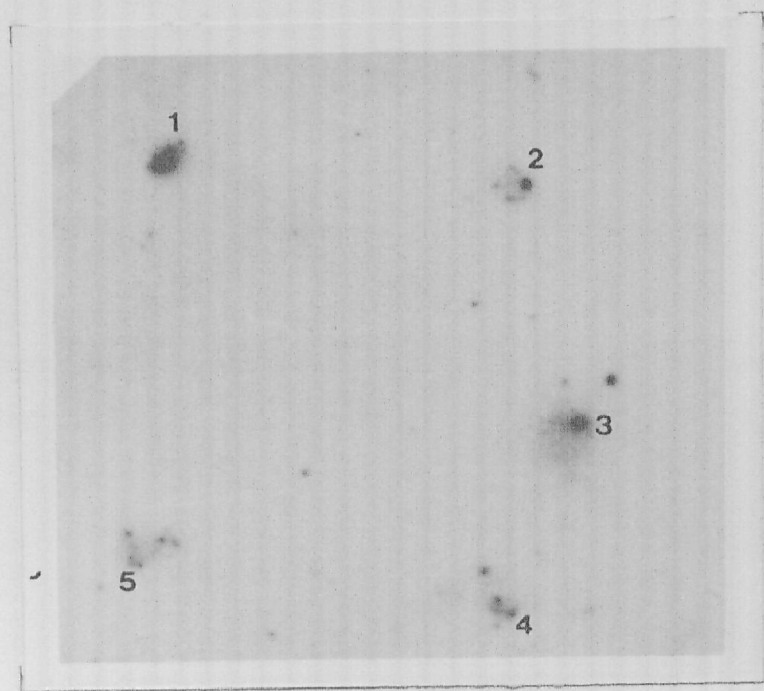
| Lane | Description                                       |
|------|---------------------------------------------------|
| 1    | $\lambda$ DNA                                     |
| 2    | B <sub>3</sub> h <sub>12</sub> ::Tn5 (pHU52::Tn5) |
| 3    | B <sub>3</sub> H <sub>9</sub> ::Tn5::pHU52        |
| 4    | B <sub>3</sub> H <sub>10</sub> ::Tn5::pHU52       |
| 5    | A <sub>1</sub> h <sub>22</sub> ::Tn5 (pHU52::Tn5) |
| 6    | A <sub>1</sub> H <sub>6</sub> ::Tn5::pHU52        |
| 7    | A <sub>1</sub> H <sub>9</sub> ::Tn5::pHU52        |
| 8    | B <sub>4</sub> h <sub>16</sub> ::Tn5 (pHU52::Tn5) |
| 9    | B <sub>4</sub> H <sub>1</sub> ::Tn5::pHU52        |
| 10   | B <sub>4</sub> H <sub>3</sub> ::Tn5::pHU52        |
| 11   | B <sub>4</sub> H <sub>7</sub> ::Tn5::pHU52        |
| 12   | B <sub>4</sub> H <sub>11</sub> ::Tn5::pHU52       |

1 2 3 4 5 6 7 8 9 10 11 12



#### PLATE IV.

Autoradiogram showing dot-blot hybridization of total DNA isolated from Hup<sup>+</sup> transconjugants of *Cicer-Rhizobium* strains G36-84 and F-75 to nick translated pHU52. Dot 1, pHU52 (positive control); dots 2 to 5, Hup<sup>+</sup> transconjugants B<sub>3</sub>H<sub>9</sub>, B<sub>4</sub>H<sub>1</sub>, B<sub>4</sub>H<sub>11</sub> and A<sub>1</sub>H<sub>9</sub>, respectively.



to derepression of *hup* genes different types of trends were observed. For one group of the final transconjugants eg.  $B_3H_9$ ,  $B_3H_{10}$ ,  $B_4H_{11}$  and  $A_1H_9$ , Hup activity increased progressively with the increase in days of incubation. Thus, for these transconjugants the optimum period for the maximum expression of Hup activity was 7 days. However, with the other group eg.  $B_4H_1$ ,  $A_1H_8$  Hup activity decreased gradually with the increase in days of incubation and for this group the optimum period of incubation for maximum expression of Hup activity was 4 days. Surprisingly, only one transconjugant  $B_4H_3$  showed Hup activity only at 6th day of incubation. In  $B_4H_7$ , however, no definite trend was observed. The maximum Hup activity of 2168 n mol  $H_2$ /mg protein/h was observed in  $B_4H_1$  after 4 days of incubation, while  $B_4H_3$  showed the lowest Hup activity of 377 after 6 days of an optimum period of incubation.

## **DISCUSSION**

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Combined nitrogen sources, more specifically soil nitrate, represent major inhibitors of nodulation and nitrogen fixation in legumes. Nitrate inhibition is interesting both at the physiological level, as a tool to understand the mechanisms controlling  $N_2$  fixation in the whole plant-rhizobia symbiosis, and at the applied level, since in the field  $N_2$  fixation occurs against a changing background of soil nitrate arising from mineralization and/or fertilizer application. The influence of nitrate on nodulation and  $N_2$  fixation by legumes is well documented in the literature. Nitrate or its metabolite has a deleterious effect on the entire process of nodulation starting from infection stage to nodule function, and accordingly several hypotheses have been put forward by different scientists. Two explanations that have received the most support are: (a) that a product(s) of nitrate reduction possibly nitrite, mediates degradation of indole-3-acetic acid (IAA) and inhibition of nitrogenase activity etc., and (b) that nitrate assimilation out-competes the symbiosis for available photosynthates (Carroll *et al.*, 1985). However, the most recent evidence suggests that oxygen supply limits the nitrogenase activity in nitrate inhibited plants (Vessey *et al.*, 1988).

Though the inhibitory effects of nitrate have been studied through nitrate reductase (NR) deficient mutants of *Rhizobium*, the mechanism of inhibition is not very well established. One school of thought says that mutants and wild types behave in the same way in relation to nitrate inhibition (Gibson and Pagan, 1977; Manhart and Wong, 1980a, b; Streeter, 1982, 1985), whereas the other school proposes that mutants are less inhibited by nitrate than the wild type (Stephens and Neyra, 1983).

In the present study efforts have been made to understand the mechanism of nitrate inhibition of nodulation and  $N_2$  fixation process by developing symbiotic nitrate tolerant mutants of *Cicer-Rhizobium* strains G36-84 and F-75 involving Tn5 mutagenesis and studying the activities of nitrate reductase and nitrite reductase and production of IAA under free-living conditions. The results obtained in this study are discussed under the following headings:-

### 5.1 DEVELOPMENT OF TRANSPONSON Tn5 MUTANTS OF CICER-RHIZOBIA

The development of nitrate tolerance could be achieved either by mutating the plant i.e. macro-symbiont (Gremaud and Harper, 1989) or *Rhizobium* i.e. micro-symbiont (Gibson and Pagan, 1977). However, the second method was followed in our study. Transposon Tn5 random mutagenesis was carried out. The source of Tn5 was pGS9 constructed by Selvaraj and Iyer (1983) and maintained in *E. coli* HB101. pGS9 contains p15A replicon which functions in *E. coli* but not in *Rhizobium* sp. Due to this reason, it acts as a suicide vector in *Rhizobium*. It also has a region encoding the N type of bacterial conjugation system which is very helpful in conjugation between *E. coli* and *Rhizobium* sp. In plasmid pGS9 chloramphenicol (Cm) resistance gene is situated by the side of Tn5. According to Selvaraj and Iyer (1983) during transposition of Tn5 into the recipient strain, there is every possibility that Cm gene might be carried away along with it. Such observations were also made in the present study and twelve G36-84::Tn5 and seven F-75::Tn5 mutants showing resistance to Cm in addition to resistance to Nd and Km were discarded. Ultimately twenty G36-84::Tn5 and twenty one F-75::Tn5 mutants showing resistance to only Nd Km were used for testing *nod* and *fix* gene expression under symbiotic condition.

## 5.2 SYMBIOTIC SCREENING OF RHIZOBIAL MUTANTS FOR NITRATE TOLERANCE

The interdependent series of steps responsible for the establishment of legume-*Rhizobium* symbiosis culminating in the formation of functional root nodules, consist of: (i) pre-infection events, (ii) infection and nodule initiation, (iii) nodule development, and (iv) nodule function. It is obvious that in Tn5 insertion mutants developed as above, any or all of the above events leading to the formation of effective nodules may be affected. These mutants are the result of a random mutagenesis process which means Tn5 could get inserted and disrupt the functioning of any gene and it is a chance process. For example had the Tn5 inserted itself into the *nod* and/or *fix* genes, the expression of these genes would have been affected, and no nodule formation or nitrogenase activity would have been observed. Therefore, the very purpose of screening Tn5 mutants was to select only those mutants which can form nodules (Nod<sup>+</sup>) in the presence of nitrate (1, 2 or 4 mM) and at the same time have nitrogenase activity (Fix<sup>+</sup>) under symbiotic conditions. Hence, the mutants were tested for their symbiotic efficiency. Out of a total of twenty G36-84 mutants, only 8 were able to form effective nodules at 1mM NO<sub>3</sub><sup>-</sup>, whereas only 5 of them were able to form nodules at 2mM NO<sub>3</sub><sup>-</sup>. The situation was not that good when F-75 mutants were taken for screening. Out of a total of twenty one mutants only 5 and 4 could form nodules at 1 and 2mM NO<sub>3</sub><sup>-</sup>, respectively (See Table 6). At a still higher NO<sub>3</sub><sup>-</sup> level of 4mM, none of the 41 mutants screened were able to form the nodules showing total nitrate inhibition (Table 6). Parental strains G36-84 and F-75 could not form even a single nodule at 1mM NO<sub>3</sub><sup>-</sup>. On the contrary Rawsthorne *et al.* (1985) reported that nitrate at 0.71 and 1.43 mM stimulated early nodulation and nodule growth but at 1.43 and 2.86 mM hastened nodule senescence in chickpea.

Carroll and Gresshoff (1983) also observed a significant inhibition of nodulation at 3-10 mM nitrate when white clover plants were cultured in an enclosed petriplate system. Similarly, in *Medicago sativa*, nitrate at 0.2 or 0.5 mM greatly reduced number of nodules (Munns, 1968b). In soybean also, plants grown at 2 mM and 4 mM nitrate had very few nodules, whereas plants grown at 0, 0.5, or 1.0 mM had large number of nodules (Ralston and Imsande, 1983). The addition of nitrate to the nutrient solution even at a low concentration of 1 mM substantially inhibited the nodule formation in Hup<sup>-</sup> strain M-11 (by 57 per cent) and in Hup<sup>+</sup> strain S-24 (by 34 per cent) infected mungbean plants (Singh and Lodha, 1989). As a result of this experiment rhizobial mutants were classified into three groups viz., Nod<sup>+</sup> Fix<sup>+</sup>, Nod<sup>+</sup> Fix<sup>-</sup> and Nod<sup>-</sup> Fix<sup>-</sup> (Table 7).

Only 40 and 25 per cent Tn5 mutants of G36-84 were found to be Nod<sup>+</sup> Fix<sup>+</sup> at 1 and 2mM NO<sub>3</sub><sup>-</sup>, respectively. The similar percentage for F-75 mutants was 24 and 19 at 1 and 2mM NO<sub>3</sub><sup>-</sup>, respectively. After taking into consideration nodule specific nitrogenase activity, two G36-84 mutants namely BC-35 and BC-46 and one F-75 mutant, AC-10 were taken for further studies on the mechanism of nitrate tolerance.

This study, thus, has shown a possibility of selecting Tn5 mutants of *Cicer*-rhizobial strains G36-84 and F-75 which can form nodules at a nitrate level of 1 and 2mM. Cen *et al.* (1982) reported that the successful use of transposon mutagenesis in a slow-growing *Rhizobium* permitted the isolation of auxotrophic and symbiotically defective mutants. In these mutants, it was shown that different single copy Tn5 insertions had occurred. Similarly, Djordjevic *et al.* (1985) showed that Tn5 mutagenesis of *R. trifolii* host-specific nodulation genes result in mutants with altered host-range ability.

Now, in order to elucidate the mechanism of nitrate tolerance in the Tn5 mutants BC-35, BC-46 and AC-10 the activities of enzymes nitrate reductase, nitrite reductase and the production of IAA were studied in these mutants *vis-a-vis* their parental strains.

### 5.3 STUDY ON THE ACTIVITIES OF THE ENZYMES NR AND NiR AND PRODUCTION OF IAA UNDER AEROBIC CONDITIONS

Rhizobia are known to be aerobic organisms. During the initial stage of symbiosis *i.e.*, from mutual recognition stage to nodule initiation stage rhizobia predominantly act in an aerobic environment. In order to have a better understanding of the response of *Cicer-Rhizobium* strains G36-84 and F-75 and their nitrate tolerant mutants BC-35, BC-46 and AC-10 to some of the growth conditions like nitrate utilization in aerobic (assimilatory) condition, the activities of nitrate reductase (NR) and nitrite reductase (NiR) and production of indole acetic acid (IAA) by rhizobia (both mutants and parents) were studied aerobically under free-living conditions with 0, 0.5, 1, 2 and 4 mM  $\text{NO}_3^-$ .

*In vivo* overall NR activities in mutants BC-35 and BC-46, as indicated by the treatment averages, were significantly lower than their parental strain G36-84. Similarly, mutant AC-10 also had a significantly low NR activity in comparison to its parent F-75 (See Table 9). Mutants BC-35, BC-46 and AC-10 had only 9, 9 and 30 per cent of the parental NR activities, respectively. Thus, in nitrate tolerant mutants less amount of nitrite was being produced in comparison to parents. Similarly, the overall *in vivo* NiR activity, as indicated by the treatment averages, was also significantly lower in mutants BC-35, BC-46 and AC-10 in comparison to respective parental strains. To be specific, BC-35 and BC-46 had only 46 and 45 per cent parental NiR activity respectively, whereas AC-10 had about 64 per cent parental NiR activity (See Table 10). Thus, in all the three nitrate

tolerant mutants, NR activity being very low, less amount of nitrite would be produced at a given point of time, and even whatever little nitrite produced would be further reduced to ammonia because of higher NiR activity compared to NR activity. Therefore, the chances of inhibition of lectin biosynthesis (Dazzo and Brill, 1978) and/or degradation of IAA (Tanner and Anderson, 1963) would be remote which means that the processes of recognition and adhesion of rhizobia to the root hairs and root hair curling would not be affected in nitrate tolerant mutants. This should facilitate onset of nodule appearance even in the presence of nitrate.

The simultaneous decrease in NR and NiR activity in the mutants could be due to Tn5 mutagenesis affecting some common regulatory gene(s) governing the biosynthesis of NR and NiR. Number of insertions of Tn5 and their exact locations are not known. But certainly Tn5 has not mutated the structural genes of NR and NiR because in that case no activity of these enzymes could have been traced. This hypothesis is indirectly supported by the findings of Marwaha *et al.* (1972) who reported that in *Azotobacter vinelandii* nitrate acted as an effective inducer of both NR and NiR. In this case nitrate might be acting by combining with a repressor product of a regulatory gene. A positive control was thus envisaged, wherein the combined product of co-inducer with the repressor acted as the inducer of both NR and NiR. Similarly in a *nit* mutant strain (2.1) of *Rhodopseudomonas capsulata* AD2 which was unable to grow with nitrate or nitrite as the sole N-source but was able to grow with other N compounds, it was thought not unlikely that a single mutation might have affected the expression of both assimilatory NR and NiR (Klemme *et al.*, 1981). It was suggested that the structural genes of these two enzymes either form a closely linked genetic group, or, alternatively, that one common regulatory element is required for the

expression of both the structural genes. A model also has been proposed by Cove (1979) for the mechanism of the induction of NR and NiR by nitrate in *Aspergillus nidulans*. According to this model, *nirA* gene product is required for the expression of the *niaD* (NR) and *niiA* (NiR) genes. In the absence of nitrate, NR converts the *nirA* gene product into a form which is no longer able to allow the *niaD* and *niiA* genes to be expressed. In the presence of nitrate, nitrate reductase does not inactivate the *nirA* gene product, thus allows the expression of *niaD* and *niiA* genes. Thus, based on all these observations it can be suggested that in the present case also Tn5 mutagenesis might have affected the regulatory gene common to structural genes of NR and NiR resulting in simultaneous decrease in the synthesis of NR and NiR.

As far as indole acetic acid (IAA) is concerned, it is known to play a role in infection-thread development and also in maintaining the organisation of infection-thread (Kefford *et al.*, 1960). However, when nitrate is present in the nutrient solution, the symbiotic production of IAA may become inadequate because nitrite, the rhizobial NR product, may destroy IAA (Tanner and Anderson, 1963). Rhizobia have the ability to reduce nitrate to nitrite and to convert tryptophan to IAA. Theoretically, it could be presumed that, when both nitrate and tryptophan are added to a rhizobial medium, nitrite and IAA production would occur and that there would be a concomitant destruction of IAA by the nitrite. Tonhazy and Pelczar (1954) reported that nitrite at 1 mM concentration greatly accelerated the oxidation of IAA enzymatically. However, nitrate could not produce this oxidation.

IAA must be helping in the establishment of symbiosis and once a functional symbiosis is established IAA may not have any role to play. Keeping all these points in view, in the present studies, the IAA production

status (per mg cell protein) of the three nitrate tolerant mutants *viz.*, BC-35, BC-46 and AC-10 was compared with their respective parents. In the absence of any added nitrate the difference in IAA production between the parents and their respective mutants except BC-35 were statistically non-significant. In other words they were producing more or less same amount of IAA (See Table 16). However, the overall quantity of IAA produced, as indicated by the treatment averages, was significantly higher in case of mutants in comparison to parents. At the same time, in case of both the parental strains G36-84 and F-75 the level of IAA production had gone down with increase in nitrate concentration in the culture medium which can be very well explained by Tanner-Anderson hypothesis of destruction of IAA by nitrite produced by rhizobial NR. Mutants BC-35 and BC-46 maintained, more or less, the same amount of IAA upto 1mM  $\text{NO}_3^-$ . But at 2 and 4 mM  $\text{NO}_3^-$  they produced either same amount of IAA or slightly higher amount of IAA in comparison to IAA produced when no nitrate was added in the medium (See Table 16). The maintenance of IAA level at different nitrate concentrations in the mutants BC-35 and BC-46 could be due to reduced production of nitrite as the activity of NR has gone down significantly in these mutants. However, it is difficult to explain increased production (more than double) of IAA in mutant AC-10 at 2 and 4 mM  $\text{NO}_3^-$  concentration in comparison to '0' level. There could be several possibilities. One such possibility is that Tn5 mutagenesis might have accelerated tryptophan biosynthesis which is the precursor of IAA (Tonhazy and Pelczar, 1954). A negative correlation, although non significant, exists in BC-35 and BC-46 in relation to per cent decrease in aerobic NR activities and per cent increase in IAA production over the parent G36-84 with the increasing nitrate concentration.

#### 5.4 STUDY ON THE ACTIVITIES OF THE ENZYMES NR AND NiR UNDER MICROAEROBIC CONDITIONS

Rhizobia are known to be aerobic organisms, nevertheless under *Rhizobium*-legume symbiosis, inside the nodule, the bacteria in the form of bacteroids grow and live under reduced oxygen tension or sometimes under anaerobic conditions. The biochemical characteristics of bacteroids which live in nodule cytosol can be partially mimicked by growing rhizobia under microaerobic condition (Daniel and Appleby, 1972). In order to have a better understanding of the behaviour of *Cicer-Rhizobium* strains G36-84 and F-75 and their nitrate tolerant mutants BC-35, BC-46 and AC-10 towards some of the growth conditions like nitrate utilization in microaerobic (dissimilatory) conditions, the activities of nitrate reductase (NR) and nitrite reductase (NiR) were studied. The parental strains and their nitrate tolerant mutants were grown in free-living conditions with (0.5, 1, 2 and 4 mM) and without nitrate under microaerobic conditions. The overall NR activity of the mutants, as indicated by the treatment averages, was significantly lower than their respective parents. To be specific, BC-35, BC-46 and AC-10 had only 22, 14 and 13 per cent parental NR activities, respectively (See Table 12). Whereas, their (mutant's) NiR activities were 70, 73 and 63 per cent in comparison to the respective parents (See Table 13). Under these conditions the NR may be of dissimilatory type as observed in groundnut *Rhizobium* grown microaerobically with nitrate (Reddy and Lodha, 1988). The whatever little nitrite produced by the activity of NR, will be used up by NiR. Thus, at a given point of time very little quantity of nitrite will be retained which means less inhibition of nitrogenase activity inside the nodule (Kemberger, 1977). The dissimilatory NR might also play some

positive role in  $N_2$ -fixation inside the nodule. According to Lodha and Naik (1982) if the nodule NR is of assimilatory type, its end product ammonia would repress nitrogenase. Moreover, there will be a competition for reductants and photosynthates in the nodules between nitrogenase and NR which co-exist in the root nodules of legumes. On the other hand, if the nodule NR is of dissimilatory type, nitrate can function as a terminal electron acceptor for the generation of ATP in the bacteroids under partially anaerobic conditions. This should facilitate nitrogen fixation in the bacteroids.

Now, as regards the regulatory mechanism for the expression of NR and NiR activities in microaerobically grown rhizobial mutants, it is quite likely that the mechanism might be the same as observed in the aerobically grown cultures. It means that Tn5 mutagenesis might have affected a regulatory gene common to both NR and NiR structural genes. It is possible that under both aerobic and microaerobic conditions, NR and NiR are synthesized by the same set of genes as observed in *E. coli* K12 (Showe and De Moss, 1968). And after the enzyme protein is synthesised, it could be present in the cell cytosol or it could go and bind the cell membrane depending on the prevailing oxygen status (*i.e.*, aerobic or anaerobic condition).

## 5.5 DEVELOPMENT OF HUP<sup>+</sup> TRANSCONJUGANTS OF NITRATE TOLERANT MUTANTS

In order to increase the symbiotic energy efficiency of *Cicer*-rhizobial strains, it is important that they should have  $H_2$  recycling ability. Since all the natural *Cicer*-rhizobial strains are known to be Hup<sup>-</sup>, they lose a significant amount of energy in  $H_2$  evolution. In the present study it was

thought worthwhile to develop Hup<sup>+</sup> transconjugants of nitrate tolerant Tn5 mutants of *Cicer-rhizobial* strains G36-84 and F-75 so that in addition to having nitrate tolerance they also have H<sub>2</sub>-uptake ability under symbiotic conditions. This has been achieved by integrating *hup* gene cosmid pHU52 into the chromosomal DNA of the mutants using Tn5 as an homologous sequence. The integrated *hup* genes have been found to express themselves under free-living conditions. It is, however, remains to be seen how they behave under symbiotic conditions and whether they improve symbiotic energy efficiency of nitrogen fixation. It also remains to be seen whether integration of cosmid pHU52 at the Tn5 site which has caused mutation affecting the expression of NR and NiR structural genes, has affected the nitrate tolerance of the mutants.

The study thus suggests that nitrate tolerant mutants of rhizobial strains could be obtained by transposon Tn5 mutagenesis and selecting the mutants which can form nodules and fix nitrogen (Nod<sup>+</sup> Fix<sup>+</sup>) in the presence of nitrate under symbiotic conditions. The biochemical basis of nitrate tolerance has been suggested to be a decrease in the production of nitrite, which destroys IAA produced by rhizobia at infection stage and inhibits nitrrogenase activity in nodules, due to a significant decrease in the activity of NR in both aerobically and microaerobically grown rhizobial mutants. The simultaneous decrease in the activities of both NR and NiR in nitrate tolerant mutants could be due to transposition of Tn5 on a regulatory gene common to both NR and NiR structural genes.

## **SUMMARY AND CONCLUSION**

Combined nitrogen, more specifically nitrate, is known to affect symbiotic nitrogen fixation in legumes. In the presence of nitrate both nodule formation as well as nodule function are affected. In chickpea (*Cicer arietinum* L.), which is an important pulse crop, even 1 mM  $\text{NO}_3^-$  is inhibitory to nodule formation. Therefore, it was thought worthwhile to develop nitrate tolerant symbiotic mutants of *Cicer*-rhizobial strains G36-84 and F-75, and establish the mechanism of nitrate tolerance. During the present investigation the activities of nitrate reductase (NR) and nitrite reductase (NiR) and the production of indole acetic acid (IAA) by mutants so developed were studied. To make these mutants symbiotically more efficient, cosmid pHU52 containing *hup* genes was integrated into their chromosomal DNA. The salient findings of the present investigations are summarized below:

- (i) Nitrate tolerant mutants of *Cicer*-rhizobial strains G36-84 and F-75 were developed involving random Tn5 mutagenesis. For this, biparental mating between *E. coli* HB101 (pGS9) and *Cicer-Rhizobium* strains G36-84 and F-75 was carried out and 216 G36-84::Tn5 mutants and 140 F-75::Tn5 mutants showing resistance to Nd and Km were selected. For G36-84, the frequency of transfer per donor and per recipient was  $0.93 \times 10^{-6}$  and  $0.25 \times 10^{-6}$ , respectively. Similarly for F-75, the frequency of transfer per donor and per recipient was  $0.61 \times 10^{-6}$  and  $0.33 \times 10^{-6}$ , respectively. However, for further screening under symbiotic conditions only 20 Tn5 mutants of G36-84 and 21 of F-75 were taken to avoid management problem.

- (ii) Symbiotic study involving all 41 *Cicer-Rhizobium* Tn5 mutants was carried out by infecting chickpea *cv.* Pusa 256 seeds with these mutants and growing them in pots containing sterilized river sand with three different levels (1, 2, 4 mM) of nitrate right from the sowing stage. The seeds infected with parental rhizobial strains were grown in the presence and absence of nitrate. The plants were harvested at 60 day stage and nodule fresh weight and nitrogenase activity determined. Based on their nodulation and nitrogen fixation ability, they had been classified into Nod<sup>+</sup> Fix<sup>+</sup>, Nod<sup>+</sup> Fix<sup>-</sup>, and Nod<sup>-</sup> Fix<sup>-</sup>. Eight and five G36-84::Tn5 mutants were found to be Nod<sup>+</sup> Fix<sup>+</sup> at 1 and 2 mM NO<sub>3</sub><sup>-</sup> concentration, respectively. Similarly, five and four F-75::Tn5 mutants were Nod<sup>+</sup> Fix<sup>+</sup> at 1 and 2 mM NO<sub>3</sub><sup>-</sup> concentration, respectively. However, the parental rhizobial strains G36-84 and F-75 could not form nodules even at 1 mM NO<sub>3</sub><sup>-</sup> concentration. None of the 41 mutants could form nodule at 4 mM NO<sub>3</sub><sup>-</sup> level. Based on higher nodule nitrogenase activity, two Nod<sup>+</sup> Fix<sup>+</sup> mutants of G36-84 namely BC-35 and BC-46 and one mutant of F-75 namely AC-10 were taken for studying the mechanism of nitrate tolerance.
- (iii) Rhizobial strains G36-84 and F-75 and their nitrate tolerant mutants (BC-35, BC-46 and AC-10) were grown aerobically (for 4 days) and microaerobically (for 10 days) in YEM liquid medium with (0.5, 1, 2 and 4 mM) and without nitrate and their *in vivo* NR and NiR activities were determined. In all the cases the overall activities of NR and NiR in the mutants, as indicated by the treatment averages, were significantly lower than their respective parents.

- (iv) Under aerobic condition, the overall decrease in the activities of NR of BC-35, BC-46 and AC-10 in comparison to their respective parents was 91, 91 and 70 per cent, respectively. Similarly, the overall decrease in the activities of NiR of BC-35, BC-46 and AC-10 in comparison to their respective parents was 54, 55 and 36 per cent, respectively.
- (v) Under microaerobic condition, the overall decrease in the activities of NR of BC-35, BC-46 and AC-10 in comparison to their respective parents was 78, 86 and 87 per cent respectively. Similarly, the overall decrease in the activities of NiR of BC-35, BC-46 and AC-10 in comparison to their respective parents was 30, 27 and 37 per cent, respectively.
- (vi). The transposon Tn5 symbiotic nitrate tolerant mutants BC-35 and BC-46 were found to accumulate in the culture medium less amount of IAA than their parent G36-84, whereas mutant AC-10 was found to accumulate double the amount of IAA than its parent F-75 when IAA produced was expressed as  $\mu\text{g}$  per 100 ml culture, and this may be due to the difference in growth status. However, when the IAA produced was expressed as  $\mu\text{g}$  per mg cell protein the trend was different. For all the three mutants the overall IAA produced as indicated by the treatment averages, was significantly higher than their respective parents. The overall increase in IAA produced by BC-35, BC-46 and AC-10 in comparison to their respective parents was 51, 43 and 45 per cent, respectively under aerobic condition.
- (vii) Hup<sup>+</sup> transconjugants of nitrate tolerant mutants were then developed. The three nitrate tolerant mutants had undergone

triparental mating involving *E. coli* HB101 (pHU52::Tn5) as donor and pRK2073 as helper. The free form of pHU52 was checked in the transconjugants B<sub>3</sub>h<sub>12</sub>, B<sub>4</sub>h<sub>16</sub>, A<sub>1</sub>h<sub>22</sub> by isolating cosmid and running the agarose gel. Then forcing of the cosmid was done by using incompatible plasmid pPH1JI in order to integrate pHU52 into the genome of the transconjugants involving Tn5 as an homologous sequence. Finally eight presumptive Hup<sup>+</sup> transconjugants were obtained *viz.*, B<sub>3</sub>H<sub>9</sub>, B<sub>3</sub>H<sub>10</sub>, B<sub>4</sub>H<sub>1</sub>, B<sub>4</sub>H<sub>3</sub>, B<sub>4</sub>H<sub>7</sub>, B<sub>4</sub>H<sub>11</sub>, A<sub>1</sub>H<sub>6</sub> and A<sub>1</sub>H<sub>9</sub>. In these transconjugants, agarose gel electrophoresis showed the absence of pHU52 as an episome.

- (viii) Dot-blot DNA hybridization study confirmed the integration of *hup* gene cosmid pHU52 into the chromosomal DNA of *Cicer-rhizobial* mutants.
- (ix) The chromosome integrated *hup* genes were found to express when the presumptive transconjugants were grown on H<sub>2</sub>-uptake medium for induction of Hup activity. The maximum Hup activity at an optimum period of incubation ranged from 377 to 2168 n mol H<sub>2</sub> taken up per mg protein per hour.

In conclusion, it can be said that nitrate tolerant mutants of *Cicer-Rhizobium* strains can be developed involving transposon Tn5 mutagenesis, and selecting the Tn5 mutants for nitrate tolerance under symbiotic conditions by looking for Nod<sup>+</sup> and Fix<sup>+</sup> phenotypes. The biochemical basis of nitrate tolerance has been suggested to be decreased activity of nitrate reductase

and at the same time increased levels of IAA because of low production of nitrite, which is destructive to IAA, at a given point of time. The study also suggests the possibility of development of Hup<sup>+</sup> strains of nitrate tolerant mutants by integrating *hup* gene cosmid using Tn5 as an homologous sequence.

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\* Original not seen

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