STUDIES ON THE SYMBIOTIC ROLE OF ARGININE BIOSYNTHETIC PATHWAY OF TWO GENERA OF RHIZOBIA

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NAND KUMAR SINGH

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247 667 (INDIA)

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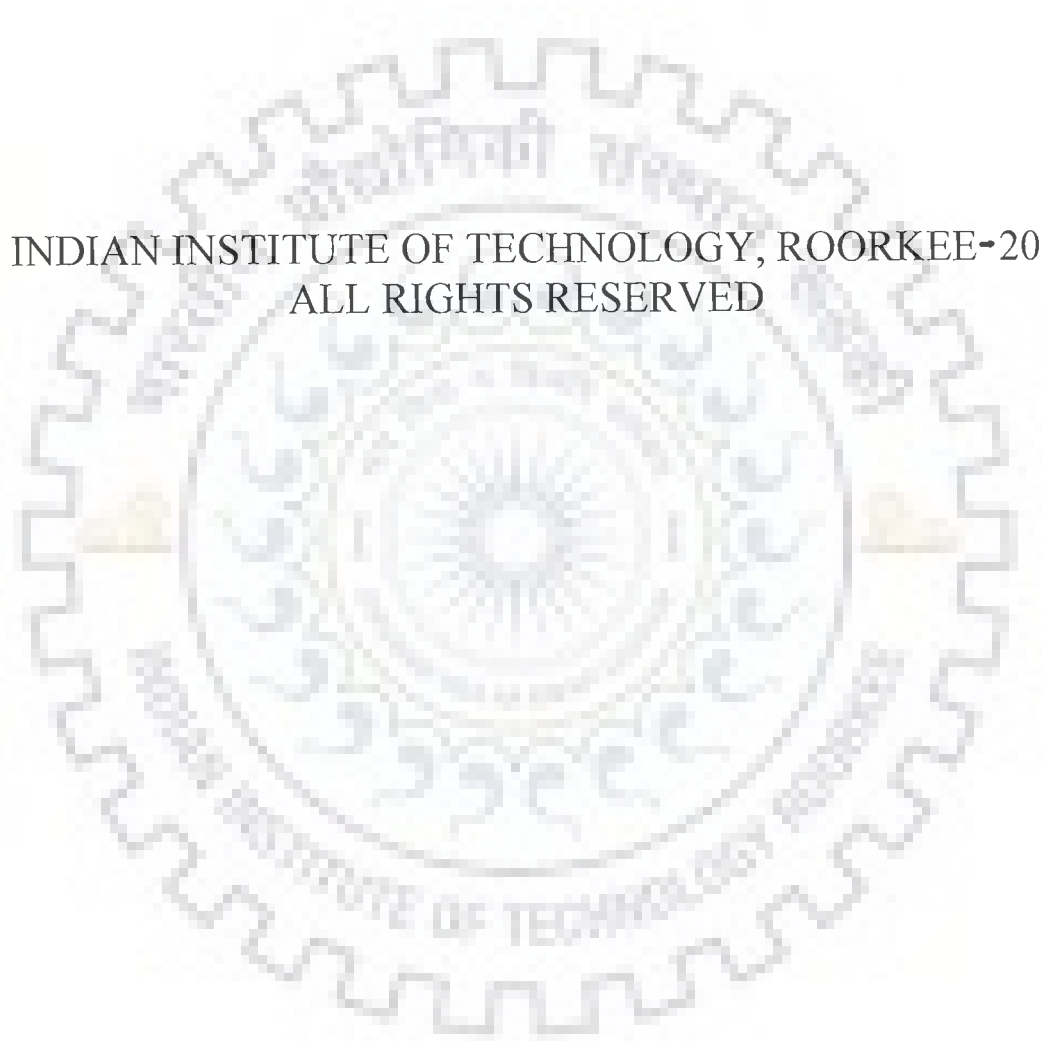

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I hereby certify that the work which is being presented in the thesis entitled **STUDIES ON THE SYMBIOTIC ROLE OF ARGININE BIOSYNTHETIC PATHWAY OF TWO GENERA OF RHIZOBIA** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2003 to December 2006 under the supervision of Dr. G. S. Randhawa, Professor & Head, Department of Biotechnology Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(NAND KUMAR SINGH)

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Dated: December 14, 2006

(G.S. RANDHAWA)
Professor & Head
Department of Biotechnology
Indian Institute of Technology Roorkee
Roorkee- 247667
India

The Ph.D. Viva-Voce Examination of **Nand Kumar Singh**, Research Scholar, has been held on .....

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ABSTRACT

Nitrogen is an essential nutrient for plants. Its deficiency results in reduced agricultural yields throughout the world. It is the most abundant gaseous element constituting about 78% of the earth's atmosphere but metabolically unavailable directly to higher plants or animals. It is available to some species of microorganisms through biological nitrogen fixation (BNF) in which atmospheric dinitrogen is converted to ammonia by the enzyme nitrogenase. Microorganisms that fix nitrogen are called diazotrophs. Symbiosis between legumes and rhizobia occurs within nodules which are induced mainly on the roots and, in a few cases on the stems. The plant supplies energy rich compounds to the diazotrophs, which in turn reduce atmospheric nitrogen to ammonia. This ammonia is transported from the bacteria to the plant to meet the plant's nutritional nitrogen needs for the synthesis of proteins, enzymes, nucleic acids, and chlorophyll.

A total of 415 crosses were performed between the donor strain *Escherichia coli* WA803 (pGS9) and each of the rhizobial recipient strains, viz., *Sinorhizobium meliloti* strain Rm1021, *Rhizobium leguminosarum* bv. *trifolii* strain MTCC905 and *R. leguminosarum* bv. *viciae* strain Rlv1. The transconjugants were selected on TY agar medium containing kanamycin (Km) (400 μg/ml) and streptomycin (Sm) (100 μg/ml). A total of 20,750 kanamycin resistant (Km^r) transconjugants were generated. The nutritional requirements of suspected auxotrophs were checked on the basis of their colony forming ability on modified Holliday pools. On the basis of their nutritional requirements all the auxotrophs were classified as follows (the number of auxotrophs

isolated have been given in brackets); *S. meliloti* strain Rm1021: arginine (7), methionine (10), leucine (2), tryptophan (1) and histidine (1); *R. leguminosarum* bv. *trifolii* MTCC905: arginine (4), glutamine (10), valine (15), phenylalanine (1), glycine (1) and histidine (1); *R. leguminosarum* bv. *viciae* Rlv1: arginine (4) and aspartic acid (8). The arginine auxotrophs of *S. meliloti* Rm1021 (IITR1, IITR2, IITR3, IITR4, IITR5, IITR6 and IITR7), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR8, IITR9, IITR10 and IITR11) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR12, IITR13, IITR14 and IITR15) were selected for further studies. The reversion frequencies of the arginine auxotrophs were found to vary from 2.0×10^{-9} in *S. meliloti* IITR 6 to 5.7×10^{-9} in *R. leguminosarum* bv. *viciae* IITR13.

The biochemical block in each arginine auxotroph was determined by feeding the intermediates of the arginine biosynthetic pathway to the auxotroph and subsequently observing the growth. On the basis of intermediate feeding studies, the arginine auxotrophs were placed in the following three categories: (i) *argA/argB/argC/argD/argE* mutants: IITR1 and IITR2 of *S. meliloti* Rm1021; IITR8 of *R. leguminosarum* bv. *trifolii* MTCC 905; IITR12 of *R. leguminosarum* bv. *viciae* Rlv1. These auxotrophs grew on RMM supplemented with ornithine or citrulline. These mutants were also designated as ornithine auxotrophs. (ii) *argF/argI* mutants: IITR3, IITR4, and IITR5 of *S. meliloti* Rm1021; IITR9 of *R. leguminosarum* bv. *trifolii* MTCC905; IITR13 and IITR14 of *R. leguminosarum* bv. *viciae* Rlv1. These auxotrophs grew on RMM supplemented with citrulline but did not grow on ornithine supplemented RMM. (iii) *argH* mutants: IITR6 and IITR7 of *S. meliloti* Rm1021; IITR10 and IITR11 of *R. leguminosarum* bv. *trifolii* MTCC905; IITR15 of *R.*

leguminosarum bv. *viciae* Rlv1. These auxotrophs grew on RMM supplemented with arginine but did not grow on citrulline or ornithine supplemented RMM. In order to find out the pleiotropic effects of Tn5 insertions on cell surface characteristics, all the auxotrophs were tested for production of cellulose fibrils, lipopolysaccharides, β(1→2) glucans, β (1→3) glucans and succinylated exopolysaccharides. All three parental strains and arginine auxotrophs took up the Congo red dye from the medium, grew in presence of sodium deoxycholate, showed motility on swarming media and fluoresced in presence of calcofluor white under UV light. This indicated the normal production of cellulose fibrils, lipopolysaccharides, β-(1→2) glucans and succinylated exopolysaccharides in the parental strains and auxotrophs. All the auxotrophs and the parental strains did not take up the aniline blue dye from the medium indicating that β-(1→ 3) glucans were not produced in these auxotrophs and parental strains. The growth of the auxotrophs on RMM supplemented with auxotrophic requirements was same as that of the parental strains on RMM. No change in growth was observed when the glucose in RMM medium was replaced by any one of the other sugars (sucrose, arabinose, xylose, maltose or mannitol) or dicaboxylic acids (malate, aspartate or glutamate) as a sole carbon source. These findings showed that the Tn5 insertions in these auxotrophs did not affect the cell surface characteristics or the uptake / utilization of sugars and dicarboxylic acids.

Symbiotic properties of all 15 arginine auxotrophs were determined by inoculating them, along with parental strains, onto their respective host plants, namely, alfalfa (*Medicago sativa* L.), clover (*Trifolium alexandrium* L.) and lentil (*Lens culinaris* Medik.). The *S. meliloti* Rm1021 strain and its *argF/argI* (IITR3, IITR4 and IITR5) and *argH* (IITR6 and IITR7) auxotrophs induced cylindrical and pink nodules on both

primary and lateral roots of plants of each of the 10 cultivars of alfalfa. The nodules induced by *argA/argB/argC/argD/argE* auxotrophs (IITR1 and IITR2) were of irregular shape and light pink colour and these were located on both primary and lateral roots of all the 10 cultivars of alfalfa. The data on mean dry plant weight, total nitrogen content per plant and acetylene reduction activity indicated that the nitrogen fixing efficiencies of the *argF/argI* and *argH* mutants were significantly less than nitrogen fixing efficiencies of the parental strain with 8 cultivars of alfalfa; with the remaining 2 cultivars the differences were nonsignificant. However, the nitrogen fixing efficiencies of *argF/argI* and *argH* mutants were significantly more than the nitrogen fixing efficiencies of *argA/argB/argC/argD/argE* mutants on all the cultivars of alfalfa. The dry plant weight and total nitrogen content per plant of *argA/argB/argC/argD/argE* mutants were very similar to those of the uninoculated plants showing that these strains fixed no or very little nitrogen. Similar results were obtained with the arginine auxotrophs of *R. leguminosarum* bv. *trifolii* MTCC905 (IITR10 and IITR11) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR15) on 4 and 6 cultivars of clover and lentil respectively. Normal symbiosis, like that of the parental strains, was observed when the host plants were inoculated with the spontaneous revertants of ornithine auxotrophs. Reisolation of bacteria from the nodules induced by the arginine auxotrophic mutants showed 100% occupancy of nodules by these mutants.

Light microscopic studies of the nodules induced by each of the parental strains *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905 and *R. leguminosarum* bv. *viciae* Rlv1 revealed that each nodule had a central tissue surrounded by several peripheral tissues. The central tissue consisted of five zones, viz., meristematic zone,

infection zone, interzone, nitrogen fixation zone and senescence zone. Small, uninfected cells and infection threads were seen in the meristematic zone. Nitrogen fixation zone was quite large and most of the cells in this zone contained differentiated rhizobia called bacteroids. Lysed nodule cells were present in the senescence zone. The internal structure of the nodules induced by the *argF/argI* and *argH* mutants of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC 905 and *R. leguminosarum* bv. *viciae* Rlv1 appeared almost similar to the internal structure of the nodules induced by the parental strains. The nodules induced by the ornithine auxotrophs of *S. meliloti* Rm1021 (IITR1 and IITR2), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR8) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR12) had distinct peripheral and central tissues. The central tissue in each of these nodules was differentiated into five zones as the nodules induced by the parental strains, however the nitrogen fixation zone was poorly developed and the senescence zone was quite extensive.

Transmission electron microscopic (TEM) studies of the ultrathin sections of the nodules induced by the parental strains *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905 and *R. leguminosarum* bv. *viciae* Rlv1 revealed the presence of the poly-β-hydroxybutyrate (Phb) granules in the rhizobial bacteria in the infection threads. The bacteroids were surrounded by a membrane called peribacteroid membrane (Pbm). In the senescence zone some bacteroids had broken Pbm. The cytoplasm of the rhizobial bacteria was electron dense where as the cytoplasm of the bacteroids in the interzone and nitrogen fixation zone contained both electron dense and electron transparent regions. The bacteroids in the nitrogen fixation zone were mostly elongated. The ultrastructure of the nodule induced by the *argF/argI* (IITR3, IITR4, IITR5, IITR9, IITR13 and IITR14)

and *argH* (IITR6, IITR7, IITR10, IITR11, and IITR15) auxotrophs was almost similar to that of the nodules induced by the parental strains. The TEM studies of the ultrathin sections induced by the ornithine auxotrophs showed the rupturing of Pbm in many bacteroids in the nitrogen fixation zone and bacteroidal cytoplasm had more electron transparent regions in comparison to that of the parental strains.

This work indicated that ornithine or an intermediate of ornithine biosynthesis may be required for the transformation of the rhizobial bacteria into bacterioids and normal development of the nitrogen fixation zone during the symbiosis of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC 905 and *R. leguminosarum* bv. *viciae* Rlv1 with alfalfa, clover and lentil plants, respectively. Some cultivars of alfalfa and lentil appear to provide the required amount of arginine to the rhizobia in the nodules.

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(Nand Kumar Singh)

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LIST OF ABBREVIATIONS

ade	Adenine
AICA	5-aminoimidazole-4-carboxamide
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
Am	Amyloplast
arg	Arginine
B	Bacteroid
Cm	Chloramphenicol
CPS	Capsular Polysaccharide
Ct	Central tissue
Cw	Cell wall
cys	Cysteine
DOC	Sodium deoxycholate
Ed	Electron dense cytoplasm
EPS	Exopolysaccharide
Er	Endoplasmic reticulum
Et	Electron transparent cytoplasm
exo	Exopolysaccharide
Fix	Nitrogen fixation
g	Gram
gly	Glycine
GmNARK	*Glycine max* nodule autoregulation receptor kinase
HAR/*har*	Hypernodulation aberrant root
Hc	Heterogeneous cytoplasm
hrs	Hours
I	Infected nodule cell
if	Infection zone
ilv	Isoleucine + Valine
It	Infection thread
Iz	Interzone
kb	Kilo base
Km	Kanamycin
LB	Luria Bertani
leu	Leucine
LPS	Lipopolysaccharide
M	Mitochondria
met	Methionine
mg	Milligram
min	Minute
ml	Mililitre
Mz	Meristematic zone
MSY	Manitol salt yeast extract
N	Normal
Nal	Nalidixic acid

Nf	Nitrogen fixation zone
Nif	Structural genes for Nitrogenase enzyme
Nm	Neomycin
nm	Nanometer
Nod/nol	Nodulation
NORK	Nodulation receptor kinase
Ori	Origin of transfer
Pbm	Peribacteroid membrane
Phb	Poly-β-hydroxybutarate
phe	Phenylalanine
Pt	Peripheral tissue
Rb	Rhizobial bacteria
Rf	Rifampicin
RMM	*Rhizobium* minimal medium
rpm	Rotations per minute
S	Senescence zone
sec	Second
Sm	Streptomycin
SYMRK	Symbiosis receptor- kinase
Tat	Twin-arginine translocation
Tc	Tetracycline
Thi	Thiamine
TY	Tryptone yeast extract
Ui	Uninfected nodule cell
V	Vacuole
Vb	Vascular bundle
µg	Microgram
µm	Micrometer

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1. INTRODUCTION

A major challenge for the 21st century is the production of sufficient food for the burgeoning population. Estimates of global human population are projected to reach ten billion by the year 2050. This will force us to employ new techniques to enhance the agricultural productivity at a very large scale. At the same time intensive application of chemical fertilizers to increase the yield will also create severe environmental problems. Thus processes like biological nitrogen fixation (BNF), which is defined as the conversion of the atmospheric nitrogen into ammonia by biological agents, will be of great importance in coming years. One of the well known BNF systems, the *Rhizobium*-legume symbiosis, is an attractive research field because of its importance in agriculture in an eco-friendly manner.

A relatively small number of bacterial species have the ability to reduce or fix atmospheric nitrogen to ammonia. Six bacterial genera, viz., *Rhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium* and *Mesorhizobium* fix gaseous dinitrogen to ammonia in symbiotic association with legumes. These bacteria invade roots of host plant and induce formation of nodules which they colonize (Okazaki, 2004). Nodule development and function are complex processes in which many genes of both the partners are involved. Some genes involved in the synthesis of amino acids, nitrogenous bases and vitamins in rhizobia have also been reported to play a vital role in symbiosis. The present work is focused on the role of biosynthetic pathways of arginine of rhizobia in symbiosis.

In spite of the large genetic complexity, *Rhizobium*–host symbiosis has been intensively investigated. Several symbiotic genes involved in nodulation and nitrogen fixation have been identified and characterized from both the partners. These include nodule formation (*nod*, *nol*) (Banfalvi *et al.*, 1981; Kondorosi *et al.*, 1984; Fisher and Long, 1992; Freiberg *et al.*, 1997), nodule invasion (*exo*, *pss*, *ndv*, *lps*) (Borthakur and Johnston, 1987; Priefer, 1989; Charles *et al.*, 1991; Noel, 1992; Leigh and Walker, 1994; Król *et al.*, 1998; Pollock *et al.*, 1998) and nitrogen fixation (*nif*, *fix*) (Batut *et al.*, 1985; Putnoky *et al.*, 1988; Beringer *et al.*, 1990; Kündig *et al.*,1993; Kumar, 2003) and plant genes involved in recognition of bacterial signals (Endre *et al.*, 2002; Stracke *et al.*, 2002) and control of nodule number (Nishimura *et al.*, 2002; Krussell *et al.*, 2002; Searle *et al.*, 2003). Rhizobia inside the nodules obtain carbon and energy sources from the plant as dicarboxylic acids (Vance, 2000; Poole and Allaway, 2000) and in return provide ammonia to the plant. Very little is known about the other small metabolites exchanged by the symbiotic partners. Identification of such metabolites and their symbiotic role will be very helpful in understanding of molecular aspects of rhizobia legume interaction.

The development of nodule needs multiplication of rhizobial cells which obviously requires amino acids, nucleotide bases and vitamins. These metabolites have to be synthesized by rhizobial bacteria and/or provided by the host plant. In a symbiotic system identification of the symbiotic partner providing a particular metabolite can be made by studying symbiotic properties of rhizobial mutant which is either auxotrophic or defective in the transport of these metabolites. Rhizobial auxotrophs of some amino acids, nucleotide bases and vitamins lead to defective symbiosis (Schwinghamer, 1970; Pankhurst *et al.*, 1972; Kerppola and Kahn, 1988b; Noel *et al.*, 1988; Barsomian *et al.*,

1992; Newmam *et al.*, 1994; Tate *et al.*, 1999b; 1999c). In case of certain amino acids and nucleotide bases the defective symbiosis does not appear to be due to lack of synthesis of the end products by *Rhizobium* rather it appears to be due to the unavailability of the intermediate(s)/enzyme(s) of the biosynthetic pathway which the plant is not able to provide (Tate e*t al.*, 1999a; Lopez *et al.*, 2001; Soberon *et a*l., 2001; Vineetha *et al.*, 2001; Kumar *et al.*, 2003). Amino acid cycling has been found to drive nitrogen fixation in the *Rhizobium*-legume symbiosis (Lodwig *et al.*, 2003).

Little is known about the role of arginine biosynthetic pathway in *Rhizobium*–legume symbiosis. Earlier work on this pathway has been done on one rhizobial species (*Sinorhizobium meliloti*). The mutants blocked in the early part of the arginine biosynthetic pathway that leads to ornithine were found to be defective in nitrogen fixation (Kerppola and Kahn, 1988; Kumar, 2003) whereas the other auxotrophs were effective (Dénarié *et al.*, 1976; Fedorov and Zaretskaya, 1977; Kerppola and Kahn, 1988; Kumar, 2003). This indicated that ornithine or a metabolite derived from it may have an important role in nitrogen fixation. There is a need to verify these findings and obtain detailed information in other rhizobia. It is also expected that the host plant may have genetic variability regarding the supply of metabolites provided to rhizobia. Hence there is a need to study the interaction of rhizobial auxotrophs with several cultivars of host plants.

The present work on arginine biosynthetic pathway has been carried out on *S. meliloti*, *Rhizobium leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae*; the host plants of the these rhizobia are alfalfa (*Medicago sativa* L.), clover *(Trifolium*

alexandrium L.) and lentil (*Lens culinaris* Medik.), respectively. Twenty cultivars of these three crop species were used in this study. This study was undertaken with the following objectives:

i) to generate arginine auxotrophs of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC 905 and *R. leguminosarum* bv. *viciae* Rlv1 by random transposon Tn5 mutagenesis,

ii) to carry out genetic and biochemical characterization of the arginine auxotrophs obtained,

iii) to study the symbiotic properties of the arginine auxotrophs with cultivars of *Medicago sativa* L., *Trifolium alexandrium* L. and *Lens culinaris* Medik. and

iv) to study the histology of the nodules induced by these arginine auxotrophs.

2. REVIEW OF LITERATURE

The literature on the role of amino acid biosynthetic pathways in *Rhizobium*–legume symbiosis and other related work was reviewed. The summary of the reviewed literature has been presented below.

2.1. Taxonomy of rhizobia

Beijerinck (1888) first isolated bacteria from the legume root nodule and named it as *Bacillus radicicola.* This bacterium was later named as *Rhizobium* by Frank (1889). Subsequently five more genera, *viz.*, *Bradyrhizobium* (Jordan, 1984; 1982), *Azorhizobium* (Dreyfus *et al.*, 1988), *Sinorhizobium* (de Lajudie *et al.*, 1994), *Mesorhizobium* (Jarvis *et al.*, 1997) and *Allorhizobium* (de Lajudie *et al.*, 1998) were recognized for rhizobia. All these genera belong exclusively to the α-subclass of Proteobacteria. Nodulation of legumes by *Buckholderia* spp., a member of the β-subclass of Proteobacteria, has also been reported (Moulin *et al.*, 2001).

2.2. Signal exchange during nodule formation

Establishment of *Rhizobium*–legume symbiosis is a complex process which involves a coordinated exchange of signals between the plant and bacteria. It leads to activation of a signal transduction cascade in the legume roots resulting in nodule formation.

Under nitrogen deficiency, legumes exude a variety of cyclic aromatic compounds called flavonoids and betains. Perret *et al.* (2000) have reported about 4000 flavonoids from vascular plants. Structurally the flavonoids contain two heterocyclic benzene rings linked through a heterocyclic pyran or pyrone ring (Brencic and Winans, 2005). Flavonoids include different classes such as chalcones, flavanones, flavones,

flavanols, isoflavonoids, coumestans and anthocyanidines (Harborne and Williams, 2000 and 2001).

The expression of several rhizobial genes, called as *nod, nol, noe* is necessary for host recognition and invasion of plant root. Flavonoids, secreted by plant roots, in combination with the rhizobial NodD transcriptional activator induce the expression of common *nod* genes, viz., *nodA, nodB* and *nodC*. The expression of common *nod* genes leads to the synthesis and transport of Nod factors.

The *nodABC* product is a lipo chito oligosaccharide, the core of which is structurally and functionally similar in all the rhizobia. Basic structure of the nod factor comprises of β-1,4 linked N acetyl D-glucosamine backbone of 2-5 residues, of which the nonreducing terminal residue is substituted at the C2 position with an acyl chain which can vary. The *nod*D gene encodes a transcriptional activator. In addition, each *Rhizobium* species possesses specific nod genes which are important in the production of species specific nod factors.

The nod factors are produced and secreted in pico molar concentrations. For their perception at the root cell surface a high affinity cell receptor is required. Nodule receptor kinase (NORK) gene from *Medicago sativa* (Endre *et al.*, 2002) and symbiosis receptor-like kinase (SYMRK) genes from lotus and pea (Stracke *et al.*, 2002) have been identified. The NORK and SYMRK genes, on the basis of sequence analysis, have been found to be analogous. In *Lotus japonicus* two receptor like kinases, *LjNFR1* and *LjNFR5,* jointly called NFR complex, is responsible for perceiving the nod factors (Radutoiu *et al.*, 2003; Madsen *et al.*, 2003).

The binding of Nod factor induces a signaling cascade in root cells. Upon binding of the Nod factor, a rapid ion flux and Ca^{2+} influx ares observed in root cells. Oldroyd and Downie (2004) have reported a gene *dmi2* which encodes trans membrane receptor–like kinase which appears to act downstream of the NFR complex and upstream of Ca^{2+} spiking. Ca^{2+} spiking is a periodic oscillation in calcium level in the cytosol of root cells. This plays a critical role in further signaling. Recently identified nucleoporin protein NUP 133 has been suggested to induce Ca^{2+} spiking. Ca^{2+} spiking in the cytosol induces a gene *dmi*3 which encodes a nuclear localized calcium/calmodulin dependent protein kinase (CCaMK) (Levy *et al.*, 2004). This kinase is supposed to activate two proteins NSP1 and NSP2 belonging to the GRAS family. These activators activate the early nodulin genes which induce nodule primordial formation. Recently an autoinhibitory domain of CCaMK has been identified which negatively regulates the kinase activity. Specific removal of this domain leads to the autoactivation of the signaling pathway in plant resulting in the induction of nodules in the absence of rhizobia (Gleason *et al.*, 2006).

The number of nodules is regulated by a shoot-root signaling process called autoregulation of nodules (AON). Searle *et al.* (2003) have demonstrated that AON in soybean is controlled by *Glycine max* nodule autoregulation receptor kinase (GMNRK) similar to *Arabidopsis* CLAVATA 1 (CLV1) which controls stem cell proliferation by short distance signaling. Nishimura *et al.* (2002) generated *Lotus japonicus* hypernodulating mutant, har1 and showed that shoot genotype is responsible for the negative regulation. They showed the similarity of the HAR-1 protein with the CLV1.

2.3. *nif/fix* genes of rhizobia

The symbiotic nitrogen fixation genes of rhizobia are known as *nif/fix* genes. Nitrogen fixation (*nif*) genes were first characterized in *Klebsiella pneumoniae*, in which 20 adjacent *nif* genes are organized in 8 operons. Some of the rhizobial nitrogen fixation genes are the same which are present in *K. pneumoniae*. These rhizobial *nif* genes have been given the same names as in *K. pneumoniae* (Beringer *et al.*, 1980; Arnold *et al.*, 1988). The other nitrogen fixation genes of the rhizobia for which no equivalent is present in *K. pneumoniae* are known as *fix* genes. The structural genes for the enzyme nitrogenase which converts nitrogen gas into ammonia are called *nifHDKE*. The rhizobial *nod*, *nif* and *fix* genes are located on chromosome (*Rhizobium loti*, *Bradrhizobium* spp. and *Azorhizobium* spp.) or plasmids (*S. meliloti*, *R. leguminosarum* and *Rhizobium* spp.). *S. meliloti* contains two megaplasmids, pSymA (about 1400kb) and pSymB (about 1700) (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Hynes *et al.*, 1986; Burckhardt *et al.*, 1987; Sobral *et al.*, 1991; Honeycutt *et al.*, 1993).

2.4. Infection and nodulation process

The most common mode of rhizobial infection of legume plants is through the infection thread that takes the bacteria through the root hair into the root cortex and delivers them to root cells (Stougaard, 2000). Second mode of rhizobial infection is the crack entry in which the rhizobial bacteria enter the plant root either through gaps in the epidermal layer or between the epidermal cells (Chandler *et al.*, 1982; De Faria *et al.*, 1988). As a result of rhizobial infection, the root hairs deform and curl, producing a pocket in each curl which provides a site for initiation of infection. The nod factors

induce root hair tip growth leading to root hair deformation (Heidstra, 1994). Simultaneously induction of plant genes like *Mtrip*1, *ENOD*5 and *ENOD*12 takes place (Cook *et al.*, 1995; Scheres *et al.*, 1990a, 1990b).

Rhizobial bacteria locally hydrolyze the plant cell wall and induce an infection thread formation (Callaham and Torrey, 1981). The infection thread moves towards the root cortical cells which start dividing to form nodule primordium (Stougaard, 2000). Rhizobial cells get released into nodule primordial cells by endocytosis and undergo several structural and biochemical changes to get converted into the symbiotic form, called bacteroids (Newcomb, 1981). In temperate and tropical legumes, the nodule primordium is formed by the division of the inner and outer cortical cells, respectively (Newcomb, 1981). The nodules of the temperate legumes are called indeterminate and those of the tropical legumes are known as determinate type. The rhizobial bacteria released in the plant cells are enclosed by a plant derived membrane, called peribacteroid membrane (Pbm). The mature nodule contains peripheral vascular bundles which are connected to the root vasculature (Stougaard, 2000).

A typical legume nodule has a central tissue surrounded by several peripheral tissues. The central tissue is made up of infected and uninfected plant cells whereas the peripheral tissue consists of nodule cortex, the endodermis and the nodule parenchyma (van de Wiel *et al.*, 1990).

2.5. Role of cell surface polysaccharides of rhizobia in symbiosis

Rhizobia produce different types of cell surface polysaccharides, such as lipopolysaccharides (LPS), capsular polysaccharides (CPS or K-antigens), neutral β (1→2) glucans and acidic extracellular polysaccharides (EPS). The EPS is supposed to play an important role in the invasion and nodule development, bacterial release from infection threads, bacteroid development, suppression of plant defense and protection against plant antimicrobial compounds (Skorupska *et al.*, 2006). EPS deficient *S. meliloti* mutants are defective in nodule invasion (Glazebrook and Walker, 1989; Leigh *et al.*, 1987; Reuhs *et al.*, 1993). It has been demonstrated by using green fluorescent protein-expressing *S. meliloti* strain that the cell surface polysaccharides mediate infection thread initiation and extension on alfalfa (Cheng and Walker, 1998; Pellock *et al.*, 2000). Zhan *et al.* (1991) had suggested that *S. meliloti* produce EPS II only under phosphate starvation. Pellock *et al.* (2002) have demonstrated that EPS II production is controlled by a Lux R homologue protein. It has been observed that under EPS deficiency bacterial agglutination is increased that inhibits elongation of infection threads and colonization of nodule primordium (Cheng *et al.*, 1998; Laus *et al.*, 2005; Pellock *et al.*, 2000). Poole *et al.* (1994) have suggested the important role of LPS layers of *R. leguminosarum* bv. *viciae* 3841 in nitrogen fixation. *S. meliloti* produce CPS or K-antigens which play an important role in the early recognition of rhizobia by alfalfa cell (Kozak *et al.*, 1997). In *R. leguminosarum,* K-antigen like polysaccharide has not been reported (Skorupska *et al.*, 2006). EPS and CPS deficient *B. japonicum* 2143 showed delayed nodulation on soybean (Eggleston *et al.*, 1996). EPS deficient *R. leguminosarum* bv. *trifolii* induced empty

nodules on the host *Trifolium* in which polyphenolic materials were deposited (Workum *et al.*, 1998; Rolfe *et al.*, 1996; Skorupska *et al.*, 1995; Pellock *et al.*, 2000).

2.6. Genomics of Rhizobia and Legumes

Genomics of the *Rhizobium*-legume symbiosis has been reviewed (Stougaard, 2001; Barnett *et al.*, 2001; Capela *et al.*, 2001; Galibert *et al.*, 2001; Kaneko *et al.*, 2000; González *et al.*, 2006). The sequencing of genomes of *Mesorhizobium loti* (Kaneko *et al.*, 2000), *S. meliloti* Rm1021 (Galibert *et al.*, 2001) and *B. japonicum* USDA 110 (Kaneko *et al.*, 2002) has been completed. In the genomics of legumes, focus has been on *L. japonicus, Medicago truncatula, G. max* and *Phaseolus vulgaris* (Cannon *et al.*, 2006).

2.6.1. Genomics of rhizobia

Bacterial genomes are usually partitioned into several replicons. The genomes of nitrogen fixing members of the Rhizobiaceae family have relatively large genome size (6-9 Mb) as compared to other prokaryotes, often including megaplasmids of 1.5 to 2 Mb and containing many repeated DNA sequences.

2.6.1.1. *Sinorhizobium meliloti*

Total genome size of *S. meliloti* is 6.69 Mb. Recent sequencing and annotation of *S. meliloti* genome revealed a high gene number with 6204 ORFs evenly distributed over three replicons (3.65 Mb chromosome, 1.35 Mb pSymA and 1.68 Mb pSymB megaplasmids) (Capela *et al.*, 2001; Finan *et al.*, 2001; Barnett *et al.*, 2001).

2.6.1.2. *Mesorhizobium loti*

The genome of *M. loti* strain MAFF 303099 consists of a single chromosome (7.036 Mb) and two plasmids, designated as pMLa (3.51 Mb) and pMLb (2.08 Mb). Chromosome comprises of 6752 potential protein coding genes, two sets of rRNA genes

and 50 tRNA genes representing 47 tRNA species (Kaneko *et al.*, 2002; VandenBosch and Stacy, 2003).

2.6.1.3. *Rhizobium etli*

Rhizobium etli CFN42 comprises a circular chromosome and six plasmids. The complete genome size is 6.530 Mb (chromosome 4.381 Mb, p42a 0.194 Mb, p42b 0.184 Mb, p42c 0.250 Mb, p42d 0.371 Mb, p42e 0.505 Mb and p42f 0.642 Mb). Six large plasmids comprise one third of the total genome size (González *et al.*, 2006).

2.6.1.4. *Rhizobium leguminosarum*

R. leguminosarum strain NGR234 genome consists of three replicons, viz., the chromosome (>3700 Kb), symbiotic plasmid pNGR 234a (536.165 Kb) and megaplasmid pNGR 234b (>2,000 Kb) (Mavingui *et al.*, 2002). *R. leguminosarum* bv. *viciae* strain 3841 has 7.75 Mb genome size, comprising a circular chromosome and six circular plasmids (chromosome 5.05 Mb, pRL12 0.870 Mb, pRL11 0.684 Mb, pRL10 0.488 Mb, pRL9 0.352 Mb, pRL8 0.147 Mb and pRL7 0.151 Mb) (Mavingui *et al.*, 2002).

2.6.1. 5. *Bradyrhizobium japonicum*

The genome of *B. japonicum* USDA110 is a single circular chromosome (9.105 Mb) with an average GC content of 64.1% and no plasmid. The chromosome comprises 8317 potential protein coding genes, one set of rRNA genes and 50 tRNA genes (Kaneko *et al.*, 2002).

2.6.2. Legume genomics

In order to understand the symbiotic interaction between legume and rhizobia, work is being done world over to know more about the genomics of legumes like *L. japonicus, Medicago truncatula, G. max* and *Phaseolus vulgaris*.

2.6.2.1. *Lotus japonicus*

The genome information of *L. japonicus* has been obtained from sequencing both cDNA and genome. A total of 70,137 3' ESTs have been clustered in 20,127 non redundant groups. The sequence data from ESTs are available at the website http://www.kazusa.or.jp/en/plantlotus.EST.

2.6.2.2. *Medicago truncatula*

DNA fingerprinting approach is being used to assemble the physical map of *M. truncatula* from BAC libraries. Total EST analysis for annotation and expression profiling has been adopted to map over ~480 Mb (95% of the genome). The ESTs database is available at http//medicago.Toulouse.innra.fr/Mt.EST (Cannon *et al.*, 2006).

2.6.2.3. *Glycine max*

The public soybean EST project began in 1998 and was funded by the North Central Soybean Research Program and the United Soybean Board, and by the USDA ARS. More than 80 cDNA libraries were generated through the project. More than 285,000 ESTs sequences have been deposited in dbEST and more are being processed (VandenBosch and Stacy, 2003).

2.7. Role of biosynthetic pathways and transport of amino acids, nucleotides bases and vitamins in symbiosis

Nodule formation and effective nitrogen fixation require multiplication of rhizobia. Rhizobial bacteria transform into bacteriods during which they increase in size. Multiplication and transformation processes require primary building blocks like amino

acids, nucleotide bases, vitamins, etc. Rhizobia synthesize all building blocks from simple nutrients; this synthesis depends upon genetic ability of the rhizobial bacteria. Rhizobia also depend on the host for supply of materials in symbiosis. Chemical and transposon mutagenesis studies have been useful to establish the sources of metabolite supply and their role in symbiosis (Noel *et al.,* 1988; Barsomian *et al.,* 1992 and Newman *et al.,* 1994; Taté *et al.,* 1999a; 1999b). The literature on the role of biosynthetic pathways of amino acids, nucleotide bases and vitamins has been reviewed by Randhawa and Hassani (2002).

2.7.1. Amino acids biosynthesis and symbiosis

2.7.1.1. Arginine

Arginine biosynthesis involves eight enzymatic steps from the precursor L-glutamate. First five steps involve N-acetylated intermediates leading to the synthesis of ornithine from which arginine is produced in last three steps (Fig.1) (Cunin *et al.*, 1986). Scherrer and Dénarié (1971), Pain (1979), Malek and Kowalski (1977b) and Kumar *et al.* (2003) have reported that the auxotrophic mutants of *S. meliloti* blocked after ornithine were effective in nitrogen fixation; the auxotrophs blocked before the ornithine were found to be effective (Scherrer and Dénarié, 1971) or partially effective or ineffective (Malek and Kowalski, 1977b; Kumar *et al.*, 2003). Diverse symbiotic phenotypes of ornithine auxotrophs isolated by Dénarié *et al.* (1976) and Kerppola and Kahn (1988b) show the necessity of doing further research work on this pathway. Ornithine is a partial precursor of polyamines (Tabor and Tabor, 1985); the absence of ornithine may be the cause of defective symbiosis in ornithine auxotrophs of *S. meliloti* (Kerppola and Kahn, 1985; 1988a; 1988b). Alfalfa host plant appears to provide

sufficient quantity of arginine to *S. meliloti* during symbiosis (Randhawa and Hassani, 2002).

2.7.1.2. Aspartic acid

Transposon Tn5 induced aspartic acid auxotrophs of *S. fredii* HH303 have been generated which induced ineffective nodules on host plant soybean. Symbiotic efficiency of aspartic acid auxotrophs was not restored, when aspartic acid was supplemented in the plant growth medium (Kim *et al.*, 1988). The host plant *G. max* may not be supplying aspartic acid to *S. fredii* inside the nodules or the supplemented aspartic acid may not be available to rhizobia inside the plant cell (Randhawa and Hassani, 2002).

2.7.1.3. Asparagine

Twenty one asparagine auxotrophs of *S. meliloti* 104A14 were generated by nitrous acid mutagenesis. Out of these, seventeen auxotrophs were unable to fix nitrogen in alfalfa. This indicated that the alfalfa host plant is unable to provide sufficient amount of asparagine to *S. meliloti* (Kerppola *et al.*, 1988 and Randhawa and Hassani, 2002).

2.7.1.4. Cysteine

The cysteine auxotrophs of rhizobia, viz., *S. meliloti* 2011 (Scherrer and Dénarié 1971), *S. meliloti* Rmd 201 (Abbas *et al.*, 2002), *R. leguminosarum* 300 (Pain, 1979) and *S. fredii* HH303 (Kim *et al.*, 1988) induced effective nodules on their respective host plants namely *M. sativa*, *Pisum sativum* and *G. max*. However, cysteine auxotrophs of *S. meliloti* L5–30 were unable to induce nodules (Malek and Kowalski, 1977b). The Tn5 induced *cysG* auxotroph of *R. etli* CTNUX8 required sulfate as sulfur source in rhizosphere, while cysteine, methionine or glutathione was supplied by the root cells to bacteria growing inside the plant (Taté *et al.*, 1997).

Twenty one Tn5 induced cysteine auxotrophs of *S. meliloti* Rmd201 isolated by Abbas *et al.* (2002) were sulfite reductase mutants. These auxotrophs induced fully effective nodules on host plant *M. sativa* (Abbas *et al.*, 2002) indicating that the sulfite reductase activity of *S. meliloti* is not essential for effective symbiosis with *M. sativa*. Cysteine auxotrophs of *S. meliloti* Rmd201 were found to grow on minimal medium supplemented with methionine, homocysteine or cystathionine (Khanuja and Kumar, 1989; Kim *et al.*, 1988; Pain *et al.*, 1979; Meade *et al.*, 1982; Schwedock and Long, 1992; Taté *et al.*, 1999; Abbas *et al.*, 2002; Randhawa and Hassani, 2002); *Rhizobium* seems capable to convert methionine, homocysteine or cystathionine to cysteine like *Saccharomyces cerevisiae* (Cherest and Surdin-Kerjan, 1992) and *Pseudomonas aeruginosa* (Foglino *et al.*, 1995).

2.7.1.5. Glutamate

Tn5 induced glutamate auxotrophs of *B. japonicum* USD110 had an altered expression of nitrogenase activity in free-living cultures. The regulatory relationship between nitrogen metabolism and nitrogenase synthesis found in *B. japonicum* seems similar to *K. pneumoniae* (Hom *et al.*, 1984).

2.7.1.6. Glutamine

Cloning and characterization of 3 genes (*glnA*, *glnII* and *glnt*) involved in glutamine biosynthesis in *S. meliloti* has been done. However, they are not essential for symbiotic nitrogen fixation (de Bruijn *et al.*, 1989). Adenylylation of glutamine synthetase I is not required for nitrogen fixation in *S. meliloti* (Arcondéguy *et al.*, 1996; Randhawa and Hassani, 2002).

2.7.1.7. Glycine

Scherrer and Dénarié (1971) have isolated three glycine auxotrophs of *S. meliloti* 2011 and found that these were more effective in nitrogen fixation then the wild type strain. The molecular basis of higher effectivity of these auxotrophs has not been understood.

2.7.1.8. Histidine

The L5-30 His^- mutant of *S. meliloti* induced ineffective nodules whereas the supplementation of histidine in plant nutrient medium restored the symbiotic efficiency (Malek and Kowalski, 1977a; 1977b). These results demonstrate that the alfalfa plant is unable to provide histidine to *S. meliloti*. Four histidine auxotrophs of *B. japonicum* USDA122 were isolated by Sadowsky *et al.* (1986), out of which two were defective in nodulation and the remaining two were symbiotically competent. These results suggested that soybean provides histidine to rhizobia but both Nod^- auxotrophs were not able to utilize it. The induction of effective nodules by histidine auxotrophs of cowpea *Rhizobium* strain IRC256 suggested that cowpea plant provides histidine to rhizobia (Mc Laughlin *et al.*, 1987).

2.7.1.9. Isoleucine and Valine

An isoleucine and valine (ilv) auxotroph of *S. meliloti* L5-30 was non-infective (Malek and Kowalski, 1977a). Kim *et al.* (1988) have reported two Tn5 induced isoleucine and valine auxotrophs of *S. fredii* HH303 which induced ineffective nodules on the host plant. These results showed the involvement of isoleucine and valine biosynthetic pathway in the symbiosis. Symbiotic efficiency of *ilvD* mutants of *S. meliloti* suggested that alfalfa plant is able to provide isoleucine and valine to rhizobia during

nodulation (Aguilar and Grasso, 1991; Hassani *et al.*, 2002). The *ilvC* Nod$^-$ mutants of *S. meliloti* have been found. In these mutants *nodABC* genes were not activated by inducer luteolin (Aguilar and Grasso, 1991). Variable activation of common nodulation genes *nodABC* was found in *ilvC* mutants of different wild type strains of *S. meliloti* (López *et al.*, 2001).

2.7.2.0. Leucine

Leucine auxotrophs of *S. meliloti* have been isolated by many workers and have been found to induce ineffective nodules on alfalfa plants (Truchet *et al.*, 1980; Aronshtam *et al.*, 1993; Nichik *et al.*, 1995; Hassani *et al.*, 2002). All these workers, except Truchet *et al.* (1980), did not find restoration of symbiotic effectivity on supplementation of plant nutrient medium with leucine. Hassani *et al.* (2002) isolated three leucine auxotrophs of *S. meliloti* Rmd 201 out of which one was *leuC/leuD* mutant and other two were *leuB* mutants. The nodules induced by *leuB* mutants were more effective than that of *leuC/leuD* mutants. In case of *leuC/leuD* mutants, bacteria remained in the infection thread showing that the leucine biosynthetic pathway intermediate β-isopropylmalate has a role in rhizobial release into alfalfa plant cells. Kummer and Kuykendall (1989) observed that the leucine auxotrophs of *B. japonicum* induced effective nodules on soybean plants. These results showed that soybean host plant is able to supply leucine to rhizobia.

2.7.2.1. Lysine

Two lysine auxotrophs of *S. meliloti* Rm41 were isolated by Forrai *et al.* (1983); however, they did not report symbiotic interactions of these auxotrophs.

2.7.2.2. Methionine

Methionine auxotrophs of rhizobia have been isolated by several workers (Fedorov and Zaretskaya, 1977; Kerppola and Kahn, 1988; Scherrer and Dénarié, 1971; Pain, 1979; Meade *et al.*, 1982; Forrai *et al.*, 1983; Ali *et al.*, 1984; Singh *et al.*, 1984; Hom *et al.*, 1984). Some of the auxotrophs have been found to induce effective (Scherrer and Dénarié, 1971) and some ineffective (Kerppola and Kahn, 1988; Abbas *et al.*, 2002) nodules on alfalfa plants. From Tn5 induced methionine auxotroph CTNUX23 of *R. etli* CE3, Taté *et al.* (1997) concluded that *metZ* gene is necessary for nodulation of *Phaseolus vulgaris*. Abbas *et al.* (2002) found that the symbiotic ineffectiveness of the Tn5 induced methionine auxotrophs of *S. meliloti* Rmd201 was restored by supplementing methionine in the plant nutrient medium.

Khanuja and Kumar (1989) observed that the methionine auxotrophs of *S. meliloti* Rmd201 did not grow in minimal medium supplemented with cysteine. It seems that the synthesis of methionine from cysteine is not taking place in *S. meliloti* as has been reported in *Pseudomonas aeruginosa* (Foglino *et al.*, 1995). The *metE* mutants of *S. meliloti* Rmd201 (Khanuja and Kumar, 1989) and *R. leguminosarum* Rld1 (Singh *et al.*, 1984) grew normally on the minimal media supplemented with vitamin B_{12}. These results indicate the similarity in the last steps of methionine biosynthesis in *Rhizobium* and *E. coli*.

2.7.2.3. Proline

Chien *et al.* (1991) reported the role of proline in symbiosis through generation of catabolic and overproducing proline mutants of *R. leguminosarum* bv. *viciae* strain C1204b. Their results oppose the role of proline as a key metabolite supplied by the host

plant. The *proC* mutants of *B. japonicum* produced underdeveloped nodules which lacked nitrogen fixation activity and plant leghaemoglobin. These results from King *et al.* (2000) suggest that the expression of *proC* is essential for normal symbiotic activity.

2.7.2.4. Serine

Serine auxotrophs of *M. ciceri* strain TL68, induced by Tn5 mutagenesis, unable to grow with ammonium as a sole source of nitrogen were isolated and characterized (Das *et al.*, 2006). Tn5 transposon has been inserted into the gene coding for 3-phosphoglycerate dehydrogenase which catalyses the first step in the serine biosynthetic pathway. These workers have concluded that serine biosynthetic pathway has an important role in symbiosis (Das *et al.*, 2006).

2.7.2.5. Phenylalanine

Prasad *et al.* (2000) isolated a Tn5 induced phenylalanine auxotrophic mutant RH38 of *S. meliloti* Rmd201 and found that the mutant bacteria degenerated as soon as they were released in the plant cells. Histological studies of nodules revealed the absence of nitrogen fixation zone. It seems that phenylalanine is not adequately supplied by the plant to its defective symbiotic partner (Randhawa and Hassani, 2002).

2.7.2. 6. Tryptophan

Wells and Kuykendall (1983) and Kummer and Kuykendall (1989) had isolated *trp* auxotrophs of *B. japonicum* strain USDA I-110 ARS and found that except tryptophan synthase mutants all other auxotrophs did not induce nodules on soybean. Kummer and Kuykendall (1989) suggested that indole glycerol phosphate, an

intermediate of tryptophan biosynthetic pathway, is necessary for nodulation in *B. japonicum*. The *trpC* and *trpD* deletion mutants of *B. japonicum* isolated by Kuykendall and Hunter (1995) were found to be symbiotically ineffective. Kuykendall and Hunter (1997) had sequenced *B. japonicum* operon consisting of *trpD*, *trpC* and a *moaC*-like gene. The mutation in *trpE* gene, which encode anthranilate synthase, induced defective nodules containing extended infection zone on alfalfa and fixed no or less nitrogen. The tryptophan synthase mutants of *S. meliloti* showed normal symbiosis with alfalfa plants (Barsomian *et al.*, 1992 and Prasad *et al.*, 2000). Thus it was concluded that anthranilic acid (product of *trpE* gene expression) of *S. meliloti* is essential for normal symbiosis and alfalfa plant is able to provide sufficient amount of tryptophan to *S. meliloti* in nodules. Taté *et al.* (1999b) found that the *trpB* mutant CTNUX4 of *R. etli* strain CE3 induced ineffective nodules on *P. vulgaris* plants. Under free living conditions this strain was unable to produce flavonoid-inducible nod factors, until the exogenous supply of tryptophan was provided.

2.7.1.17. Tyrosine

Using nitrous acid as a mutagen, two tyrosine mutants of *S. meliloti* 104A14 were generated by Kerppola and Kahn (1988b); these mutants induced ineffective nodules on alfalfa. Tn5 generated tyrosine auxotrophs of *S. meliloti* Rmd201 induced normal effective nodules (Prasad *et al.*, 2000)

The symbiotic characteristics of auxotrophs of amino acids of rhizobia have been summarized in Table 1.

2.4.2. Transport of amino acids and symbiosis

Amino acid transport was studied by mutating *aap* and *bra* genes by Lodwig *et al.* (2003). A single mutation in each of these genes which encode ABC-type broad specificity amino acid transporters in *R. leguminosarum* bv. *viciae*, reduced the uptake rates of all tested amino acids by 40- 70% in free living bacteria and the double mutant RU1357 was almost totally blocked for the uptake of a broad range of amino acids. The growth of the peas nodulated by either *aap* or *bra* single mutants, or by the wild type (A34) was indistinguishable; however peas nodulated by RU1357 progressively became yellow. These workers concluded that the plant provides amino acids to the bacteriods, in turn bacteriods cycle back amino acids to the plant for asparagine synthesis. Hence amino acid transport has been found to be playing a major role in *Rhizobium*-legume symbiosis.

2.4.3. Role of biosynthetic pathways of nucleotide bases

2.4.3.1. Purine biosynthesis and symbiosis

Purine auxotrophs of *Rhizobium* strain NGR234 (Chen *et al.*, 1985), *R. leguminosarum* (Schwinghamer, 1969; Pankhurst and Schwinghamer, 1974; Pain, 1979; Noel *et al.*, 1988), *S. fredii* (Kim *et al.*, 1988) and *S. meliloti* (Scherrer and Dénarié, 1971; Malek and Kowalski, 1983; Kerppola and Kahn, 1988b; Swamynathan and Singh, 1992; Al-Judi, 2001) have been reported to be defective in symbiotic ability. Nodulation was not induced by adenine auxotrophs (Pain, 1979; Schwinghamer, 1969; Pankhurst and Schwinghamer, 1974); however, these auxotrophs induced non-fixing nodules when the plant nutrient medium was supplemented with adenine (Schwinghamer, 1975; Pain, 1979). Purine auxotrophs of *R. leguminosarum* induced pseudonodules on bean plants;

supplementation of 5-aminoimidazole-4-carboxamide riboside (AICA-riboside) significantly enhanced nodule development (Noel *et al.*, 1988; Newman *et al.*, 1992). Purine auxotrophs, each having a biochemical block before 5-amino imidazole-4-carboxamide ribonucleotide (AICAR), of *R. leguminosarum* bv. *viciae* 128C56 and *S. fredii* HH303 induced poorly developed nodules which did not contain rhizobia. Stevens *et al.* (2000) observed that *pur*M and *pur*N genes of purine biosynthetic pathway were necessary for nodulation of peas by *R. leguminosarum* bv. *viciae*.

Ineffective nodules were formed on alfalfa plants by purine auxotrophs of *S. meliloti* 2011 (Scherrer and Dénarié, 1971), *S. meliloti* L5-30 (Malek and Kowalski, 1983) and *S. meliloti* Rmd201 (Swamynathan and Singh, 1992). Multiple changes in cell surface molecules appeared to be the cause of symbiotic defect of purine auxotrophs of *S. meliloti* Rmd201 (Swamynathan and Singh, 1995). Soberón *et al.* (2001) found that AICA- riboside negatively regulates the fix NOQP expression in *S. meliloti*.

Less acidic exopolysaccharides and cyclic β-(1→2) glucans were produced by two purine auxotrophs ANU2861 and ANU2866 of *Rhizobium* strain NGR234; these auxotrophs formed defective nodules on the host plants. Djordjevic *et al.* (1996) observed that normal polysaccharide production in the purine auxotroph ANU2861 occurred when AICA-riboside was added in the medium. Purine auxotrophs of *R. leguminosarum* bv. *phaseoli* and *S. meliloti* did not grow when the minimal medium was supplemented with guanine or guanosine; this demonstrated that these rhizobia are unable to convert guanine to inosine mono phosphate (Noel *et al.*, 1988; Kim *et al.*, 1988; Swamynathan and Singh, 1992). Production of guanine via inosine mono phosphate dehydrogenase in *R. tropici*

has been found essential for growth at high temperatures and effective nodulation (Riccillo *et al.*, 2000).

Pyrimidine biosynthesis and symbiosis

Only few reports are available on the study of the symbiotic role of pyrimidine biosynthetic pathway of rhizobia. A pyrimidine auxotroph of *S. meliloti* strain 2011 was reported to be ineffective (Scherrer and Dénarié, 1971). Pain (1979) found that out of fifteen pyrimidine auxotrophs of *R. leguminosarum* bv. *phaseoli* strain CFN42, two had defective symbiosis. Noel *et al.* (1988) did not succeed in suppressing the nodule development defective (Ndv^-) phenotype of pyrimidine auxotroph of *R. leguminosarum* bv. *phaseoli* strain CFN42 by addition of uridine to the plant nutrient medium. Ineffective symbiosis has been reported for the mutants having mutation in carbamoyl phosphate synthetase and steps of pyrimidine biosynthesis before orotic acid (Kerppola and Kahn, 1985; 1988a; 1988b). Two Tn5 induced uracil auxotrophs of *S. fredii* HH303 have been reported to induce ineffective nodules (Kim *et al.*, 1988).

Vineetha *et al.* (2001), by transposon Tn5 mutagenesis isolated, 5 *car*, 8 *pyrC* and 10 *pyrE/pyrF* pyrimidine auxotrophs which induced ineffective nodules on alfalfa plants. Microscopic studies showed that the structural features of the nodules induced by *pyr*C mutants were more advanced than those of the nodules induced by *car* mutants; similarly the features of the nodules induced by *pyrE /pyrF* mutants were more developed than those of *pyrC* mutants. These results indicated that carbamoyl phosphate/ carbamoyl aspartate and dihydroorotic acid/orotic acid may have role in *S. meliloti*-alfalfa symbiosis.

2.4.4. Vitamin biosynthesis and symbiosis

Symbiotic properties of auxotrophs of vitamins, viz., biotin, nicotinic acid, riboflavin, thiamine and pantothenic acid have been studied.

2.4.4.1. Biotin

Synthesis and uptake of plant derived biotin has been found to promote colonization of alfalfa roots by *S. meliloti* 1021 (Streit *et al.*, 1996). The results of these workers showed that synthesis was more important source for *S. meliloti* bacteria.

2.4.4.2. Nicotinic acid

A Tn5-induced nicotinic acid auxotroph of *S. fredii* HH303 has been reported to induce ineffective nodules on soybean plants (Kim *et al.*, 1988). It seems that the host plant is not able to provide the required amount of nicotinic acid to the *S. fredii* bacteria in the nodules.

2.4.4.3. Riboflavin

The availability of riboflavin is necessary for the colonization of alfalfa roots by *S. meliloti* 1021 bacteria (Streit *et al.*, 1996). A riboflavin auxotroph of *R. leguminosarum* bv. *trifolii* T-1 induced ineffective or partially effective nodules on clover; however fully effective nodules were induced by this auxotroph when the plant nutrient medium was supplemented with riboflavin (Schwinghamer, 1970). Pankhurst *et al.* (1972) observed that riboflavin was necessary for conversion of *R. leguminosarum* bv. *trifolii* bacteria into nitrogen fixing bacteroids in the nodules.

2.4.4.4. Thiamine

Thiamine has been found to be required for the colonization of alfalfa roots by *S. meliloti* 1021 bacteria (Streit *et al.*, 1996).

2.4.4.5. Pantothenic acid

A Tn5 induced pantothenic acid auxotroph of *S. fredii* HH303 has been reported to induce fully effective nodules on soybean plants (Kim *et al.*, 1988). It seems that during symbiosis soybean plant is able to provide the required amount of pantothenic acid to *S. fredii* bacteria.

The above literature review shows that the availability of small metabolites to rhizobia is very important for symbiotic relationship of rhizobia and legumes. Very little is known about the role of arginine biosynthetic pathway in symbiosis. Hence the present work on isolation and characterization of arginine auxotrophs of 3 strains of rhizobia was undertaken. Several cultivars of each host were included in the study to find out the cultivar differences in the supply of arginine and/or its intermediates.

Table 1: Symbiotic characteristics of amino acid auxotrophs of rhizobia (Modified from Randhawa and Hassani, 2002)

o.	Auxotrophy	Mutated gene/position of biochemical block	Name of *Rhizobium*	Symbiotic phenotype	Symbiotic phenotype after supplement-ation	Reference(s)
	Arginine	after ornithine	*S. meliloti*	Nod^+ Fix^+	–	Dénarié *et al.*(1976); Fedorc Zaretskaya (1977)
		before ornithine	-do-	Nod^+ Fix^+	ND	Kerppola & Kahn (1988)
	Aspartic acid	–	*S. fredii*	-do-	Nod^+ Fix^+	Kim *et al.* (1988)
	Asparagine	–	*S. meliloti*	-do-	ND	Kerppola & Kahn (1988)
	Cysteine	–	-do-	Nod^+ Fix^+	–	Scherrer & Dénarié (1971)
		cysI/cysJ	-do-	-do-	–	Abbas *et al.*(2002)
		–	-do-	Nod^+ Fix^-	ND	Malek & Kowalski (1977)
		cysG	*R. etli*	Nod^+ Fix^+	–	Taté *et al.*(1997)
		–	*R. leguminosarum*	-do-	–	Pain (1979)
		–	*S. fredii*	-do-	–	Kim *et al.* (1988)
	Glutamic acid	–	*B. japonicum*	-do-	–	Home *et al.* (1984)
	Glutamine	*glnA, glnII & glnT*	*S. meliloti*	-do-	–	de Bruijn *et al.* (1989)
	Glycine	–	-do-	-do-	–	Scherrer & Dénarié (1971)
	Histidine	–	*B. japonicum*	-do-	–	Sadowsky *et al.*(1986)
		–	-do-	Nod^-	Nod^+ Fix^+	Sadowsky *et al.* (1986)
		–	-do-	-do-	ND	Soet *et al.* (1987)
		–	*S. meliloti*	Nod^+ Fix^-	Nod^+ Fix^+	Malek & Kowalski (1977)
	Isoleucine+ Valine	*ilvD*	-do-	Nod^+ Fix^+	–	Aguilar & Grasso (1991); Hassani *et al.* (2002)
		ilvC	-do-	Nod^-	Nod^-	Aguilar & Grasso (1991); Hassani *et al.* (2002)
		ilvC	-do-	-do-	ND	López *et al.* (2001)
		ilvB/ ilvG	-do-	-do-	Nod^-	Hassani *et al.* (2002)
		–	-do-	-do-	ND	Malek & Kowalski (1977)
		–	*S. fredii*	Nod^+ Fix^-	-do-	Kim *et al.* (1988)
	Leucine	–	*B. japonicum*	Nod^+ Fix^+	–	Kummer & Kuykendall (1
		–	*S. meliloti*	Nod^+ Fix^-	Nod^+ Fix^+	Truchet *et al.* (1980)

		–	-do-	-do-	$Nod^+ Fix^-$	Aronshtam *et al.* (1993); Nichik *et al.* (1995)
		leuC/ leuD & leuB	-do-	-do-	-do-	Hassani *et al.* (2002)
11	Lysine	–	-do-	–	–	Truchet *et al.*(1980)
12	Methionine	–	*S. meliloti*	$Nod^+ Fix^+$	–	Scherrer & Dénarié (1971)
		–	-do-	$Nod^+ Fix^-$	ND	Kerppola & Kahn (1988)
		metA/ metZ/ metE & metF	-do-	-do-	$Nod^+ Fix^+$	Abbas *et al.* (2002)
		metZ	*R. etli*	Nod^-	$Nod^+ Fix^-$	Taté *et al.* (1997)
13	Proline	–	-do-	$Nod^+ Fix^+$	–	Chien *et al.* (1991)
		proC	*B. japonicum*	$Nod^+ Fix^-$	ND	King *et al.* (2000)
14	Serine	*serA*$^-$	*M. ciceri*	$Nod^+ Fix^-$	–	Das *et al.* (2006)
15	Phenylalanine	*pheA*	*S. meliloti*	-do-	-do-	Prasad *et al.* (2000)
16	Phenylalanine	*aro*	-do-	-do-	ND	Barsomian *et al.* (1992); Prasad *et al.* (2000)
17	Tryptophan	*trpA & trpB*	-do-	$Nod^+ Fix^+$	–	Barsomian *et al.* (1992); Prasad *et al.* (2000)
		trpE(G)	-do	$Nod^+ Fix^-$/ $Nod^+ Fix^{+*}$	ND	Barsomian *et al.* (1992); Prasad *et al.* (2000)
		–	*B. japonicum*	Nod^-	-do-	Wells & Kuykendall (1983); Kummer & Kuykendall (1989)
		trpB	*R. etli*	$Nod^+ Fix^-$	-do-	Taté *et al.* (1999b)
18	Tyrosine	*tyrA*	*S. meliloti*	$Nod^+ Fix^+$	–	Prasad *et al.* (2000)
		–	-do-	$Nod^+ Fix^-$	ND	Kerppola & Kahn (1988)

S. meliloti- Sinorhizobium meliloti; *S. fredii- Sinorhizobium fredii*; *R. leguminosarum- Rhizobium leguminosarum;*

R. etli-Rhizobium etli; *B. japonicum-Bradyrhizobium japonicum*; *M. ciceri- Mesorhizobium ciceri*

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Bacterial strains and plasmid

The description of the bacterial strains and plasmid used or constructed in this study is given in Table 2.

3.1.2. Plant cultivars

The study was conducted on different cultivars of three host plants, namely, alfalfa (*Medicago sativa* L.), clover (*Trifolium alexandrium* L.) and lentil (*Lens culinaris* Medik.). The sources of the accessions/cultivars of the respective host plants are listed in Table 3.

3.1.3. Composition of growth media

3.1.3.1. Growth media for rhizobia

3.1.3.1.1. Complete media

3.1.3.1.1.1. Tryptone yeast extract (TY) medium (Khanuja and Kumar, 1988)

Constituent	Amount (g)/litre
Tryptone	5.0
Yeast extract	3.0
$CaCl_2.2H_2O$	0.12
Distilled water	to make volume 1 litre

Table 2: Bacterial strains and plasmid used/constructed

Strain / plasmid	Relevant characteristics	Source / Reference
Sinorhizobium meliloti		
Rm 1021	Spontaneous Str^r (Nod^+ Fix^+)	T.M. Finan, Department of Biology, McMaster University, Hamilton, Canada
IITR1	Rm1021 *argA/argB/argC/argD/argE*::Tn5	This work
IITR2	-do-	-do-
IITR3	Rm1021 *argF/argI*::Tn5	-do-
IITR4	-do-	-do-
IITR5	-do-	-do-
IITR6	Rm1021 *argH*::Tn5	-do-
IITR7	-do-	-do-
Rhizobium leguminosarum bv. *trifolii*		
MTCC905	Spontaneous Str^r (Nod^+ Fix^+)	Institute of Microbial Technology, Chandigarh
IITR 8	MTCC905 *argA/argB/argC/argD/argE*::Tn5	This work
IITR 9	MTCC905 *argF/argI*::Tn5	-do-
IITR10	MTCC905 *argH*::Tn5	-do-
IITR11	-do-	-do-
R. leguminosarum bv. *viciae*		
Rlv1	Spontaneous Str^r (Nod^+ Fix^+)	This work
IITR12	Rlv1 *argA/argB/argC/argD/argE*::Tn5	-do-
IITR13	Rlv1 *argF/argI*::Tn5	-do-
IITR14	-do-	-do-
IITR15	Rlv1 *argH*::Tn5	-do-
Escherichia coli		
WA803(pGS9)	Met^- Thi^- Cm^r Km^r	Selvaraj and Iyer (1983)
plasmid		
pGS9	IncN rep15A Cm^r Km^r	-do-

Table 3: List of accessions/cultivars and their sources

Host plant	Accession/ Cultivar	Source
Alfalfa (*Medicago sativa* L.)	LCC3	Department of Plant Breeding, Genetics and Biotechnology, P.A.U. Ludhiana, India
	LCC5	-do-
	LCC8	-do-
	LCC10	-do-
	LCC12	-do-
	LCC13	-do-
	RL87	-do-
	Anand2	-do-
	A2	National Seeds Corporation, Pusa, New Delhi, India
	T9	-do-
Clover (*Trifolium alexandrium* L.)	EC 400733	Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi, India
	Pusa giant	National Seeds Corporation, Pusa complex, I.A.R.I., New Delhi, India
	Jawahar	-do-
	UPB110	-do-
Lentil (*Lens culinaris* Medik.)	PL639	Department of Genetics and Plant Breeding, C.C.S.University, Meerut, India
	P38	-do-
	Pusa1	-do-
	Pusa6	-do-
	Malika	National Seeds Corporation, Pusa Complex, I.A.R.I., New Delhi, India
	T36	-do-

3.1.3.1.1.2. Mannitol salt yeast extract (MSY) medium (Khanuja and Kumar, 1989)

Constituent	Amount (g)/litre
Mannitol	10.0
Yeast extract	1.0
$K_2HPO_4.3H_20$	0.2
KH_2PO_4	0.2
$MgSO_4.7H_2O$	0.1
$CaCl_2.2H_2O$	0.05
Distilled water	to make volume 1 litre

The pH of the medium was adjusted to 6.8 with the help of 0.1 N NaOH solution. To make solid medium 16 g/litre agar was added before autoclaving.

3.1.3.1.2. Minimal medium

3.1.3.1.2.1. *Rhizobium* minimal medium (RMM) (Singh *et al.*, 1984)

Solutions A and B were prepared as follows:

Solution A:

Constituent	Amount (g)/litre
$Na_2HPO_4.12H_2O$	0.45
$(NH_4)_2SO_4$	2.0
$FeCl_3$	0.002
$MgSO_4.7H_2O$	0.1
$CaCl_2.2H_2O$	0.04
Distilled water	to make volume 990 ml

This solution was autoclaved after adjusting its pH to 7.0 with 0.1 N NaOH.

Solution B:

Twenty percent (w/v) D-Glucose solution was prepared in double distilled water and filter sterilized.

To prepare 1 litre of RMM, 10 ml of solution B was added to 990 ml of the autoclaved solution A. To make solid RMM, 16 g/litre agar was added to solution A before autoclaving.

3.1.3.2 Luria Bertani (LB) medium for *Escherichia coli* (Sambrook *et al.*, 1989)

Constituent	Amount (g)/litre
Tryptone	10
Yeast extract	5
Sodium chloride	10
Distilled water	to make volume 1 litre

The pH of the medium was adjusted to 7.0 with 0.1 N NaOH solution. Solid LB medium was made by adding 16 g/litre agar before autoclaving.

3.1.3.3 Nitrogen free plant nutrient medium

Nitrogen free plant nutrient medium was prepared according to Engelke *et al.* (1987). Twelve stock solutions, A to L, were prepared as given below. All these stock solutions were autoclaved separately. To make 1 litre of plant nutrient medium, 10 g agar was added to 985 ml distilled water and resulting water agar medium was autoclaved. Four ml of stock solution F and one ml of each of the remaining stock solutions were added to autoclaved agar solution and pH of the medium was adjusted to 6.8 with 0.1 N NaOH or 0.1 N HCl. Twenty five ml of this medium was then poured into glass tubes (22 X 2.5 cm) to prepare slants to transfer the sprouting seeds of alfalfa, clover and lentil. All autoclavings were done at 15 psi for 20 min.

Solution	Constituent	Amount of constituent (g)	Distilled water (ml)
A	$K_2HPO_4.3H_2O$	2.090	10
B	KH_2PO_4	0.544	10
C	$CaCl_2.2H_2O$	7.351	10
D	$C_6H_5O_7Fe.3H_2O$	0.335	10
E	$MgSO_4.7H_2O$	6.162	10
F	K_2SO_4	4.356	40
G	$MnSO_4.H_2O$	0.034	20
H	H_3BO_3	0.026	20
I	$ZnSO_4.7H_2O$	0.030	20
J	$CuSO_4.5H_2O$	0.002	20
K	$CaSO_4.2H_2O$	0.006	20
L	Na_2MoO_4	0.006	20

3.1.4. Diluent used

Normal saline solution, 0.85 % (w/v) NaCl (HiMedia), was used to carry out serial dilutions and make cell suspensions.

3.1.5. Supplements to media

3.1.5.1. Antibiotics

Stock solutions of streptomycin sulphate (HiMedia) and kanamycin acid sulphate (s.d.fine-chem Limited) were prepared in sterile double distilled water. Stock solutions of tetracycline hydrochloride (HiMedia) and chloramphenicol (HiMedia) were prepared in 50 % ethanol; rifampicin (HiMedia) was dissolved in dimethylsulphoxide (DMSO).

Antibiotics were used in following concentrations: streptomycin sulphate, 100 μg/ml; kanamycin acid sulphate, 40 μg/ml for *E. coli* and 400 μg/ml for rhizobial kanamycin resistant transconjugants (Km^r): tetracycline hydrochloride, 15 μg/ml; rifampicin, 40 μg/ml. Stock solutions of antibiotics were filter sterilized and added to autoclaved media.

3.1.5.2. Amino acids, nitrogenous bases and vitamins

Stock solutions of amino acids, nitrogenous bases and vitamins were prepared in autoclaved double distilled water. Necessary volumes from stock solutions were added to autoclaved medium to make final concentrations of 50, 30 and 10 μg/ml for amino acids, nitrogenous bases and vitamins, respectively. The auxotrophy of each strain was determined by observing growth of the auxotroph on modified Holliday pools (Holliday, 1956). The composition of modified Holliday pools used in the study was as follows:

Pool 1: Adenine, histidine, phenylalanine, glutamine, thymine and pantothenic acid.

Pool 2: Guanine, leucine, tyrosine, asparagine, serine and riboflavin.

Pool 3: Cysteine, isoleucine, tryptophan, uracil, glutamate and biotin.

Pool 4: Methionine, lysine, threonine, aspartic acid, alanine and cobalamine.

Pool 5: Thiamine, valine, proline, arginine and glycine.

Pool 6: Adenine, guanine, cysteine, methionine and thiamine.

Pool 7: Histidine, leucine, isoleucine, lysine and valine.

Pool 8: Phenylalanine, tyrosine, tryptophan, threonine and proline.

Pool 9: Glutamine, asparagine, uracil, aspartic acid and arginine.

Pool 10: Thymine, serine, glutamate, alanine and glycine.

Pool 11: Pantothenic acid, riboflavin, biotin and cobalamine.

3.1.5.3. Intermediates of arginine biosynthetic pathway

Stock solutions of L-ornithine and L-citruline (s.d. fine-chem Limited), L-arginosuccinic acid (M/s Sigma Chemicals Co., St. Louis, USA) and N-Acetyl-L-glutamic acid (HiMedia) were prepared in sterile double distilled water. The intermediate(s) were added to RMM at a final concentration of 50μg/ml.

3.1.5.4. Sugars and dicarboxylic acids

Sugars (glucose, arabinose, lactose, galactose, maltose, xylose, mannose, mannitol and sucrose) and dicarboxylic acids (malate, aspartate and succinate) were purchased from HiMedia. A sugar/dicarboxylic acid was added at a final concentration of 2 g/litre to the RMM medium as a sole carbon source before autoclaving.

3.1.5.5. Sodium deoxycholate (DOC)

DOC (HiMedia) was supplemented to MSY medium at the concentration of 1mg/ml before autoclaving.

3.1.5.6. Dyes

Aniline blue (HiMedia), calcofluor white (Sigma) and Congo red (HiMedia) were supplemented separately to MSY medium at a concentration of 0.02 % (w/v) before autoclaving. To stain semithin sections of nodules 1.0 % (w/v) toluidine blue (HiMedia) in 1.0 % (w/v) borax (Rankem) was used.

3.1.5.7. pH indicator

Bromothymol blue (BTB) (Hi Media) was supplemented to the MSY medium at the concentration of 2.5 mg/ml.

3.1.5.8. Sodium chloride (NaCl)

Required amount of NaCl (HiMedia) was added to the MSY medium before autoclaving.

3.1.6. Composition of solutions for light and electron microscopy of root nodules

3.1.6.1. Preparation of blocks

(a) 0.2 M phosphate buffer (pH 7.4)

Constituent	Amount (g)/litre
$NaH_2PO_4.H_2O$	6.41
$Na_2HPO_4.7H_2O$	41.3
Double distilled water	1000 ml

(b) Fixatives

(i) Primary fixative (Karnovsky fixative) (Karnovsky, 1965)

12.5 ml	8 % (w/v) paraformaldehyde (Sigma) (added 2 g of paraformaldehyde in 25 ml double distilled water and heated the solution to 60-70°C followed by the addition of a few drops of 1 N NaOH)
5.0 ml	12.5 ml (v/v) glutaraldehyde (Sigma)
25.0 ml	phosphate buffer
7.5 ml	double distilled water

(ii) Post fixative (Secondary fixative), 2 % (w/v) OsO_4

Two percent (w/v) OsO_4 dissolved in double distilled water. This stock solution was stored at 4°C in a tightly packed brown bottle.

(c) Acetone series

Acetone solutions (30, 50, 70, 80, 90 and 95 %) (v/v) were prepared separately and stored at 4°C.

(d) Araldite CY212 (resin) embedding medium

Constituent	Amount used (ml)
Araldite CY212	10
Hardner (Dodecenyl succinic anhydride, DDSA; HY 964)	10
Accelerator (Tridimethyl aminomethyl phenol, DMP30; DYO64)	0.4
Plasticizer (Dibutylphthalate)	1.0

All ingredients were mixed and vigorously stirred.

3.1.6.2. Solutions for staining ultrathin sections for electron microscopy

3.1.6.2.1. Uranyl acetate solution

Uranyl acetate solution was prepared by adding excess of uranyl acetate (up to saturation point) to 10 ml of 50 % (v/v) ethanol in a 15 ml centrifuge tube. It was centrifuged at 5000 rpm for 2 min; the supernatant was transferred to another tightly packed tube and stored at 4°C.

3.1.6.2.2. Lead citrate solution

One and half pellets of sodium hydroxide were added to 12 ml of filtered 50 % (v/v) ethanol in a 15 ml centrifuge tube. After dissolution of NaOH, 50 mg of lead citrate was added and the contents were mixed by shaking. Centrifugation was done at 5000 rpm for 2 min and the supernatant was stored at 4°C in refrigerator.

3.1.7. Acetylene reduction assay

Acetylene reduction assay was performed by gas chromatographic method. Materials used in this study were: (i) GLC model HP 5890A (ii) column porapack Q (iii) hydrogen and nitrogen as carrier gases (iv) standard gas cylinders (Spantech Hi-Spech-Gas Technology) (v) gas collector 200 m (vi) injector syringe (vii) calcium carbide (CaC_2).

3.1.8. Nitrogen content

Nitrogen contents of plants were determined using CHNS (carbon, hydrogen, nitrogen and sulphur) method. Following materials were used for the nitrogen estimation (i) vario EL analyzer (ii) vario EL software (iii) electronic microbalance (0.001 mg) (iv) O_2 and He gas cylinder (v) sulfanilic acid (MERCK) (vi) benzoic acid (MERCK) (vii) tungsten trioxide (WO_3) powder and (viii) tin capsule.

3.2. METHODS

3.2.1. General bacteriological procedures

3.2.1.1. Maintenance of bacterial cultures

All bacterial strains were maintained in glycerol (15 % final concentration) at -20°C. These strains were also maintained at 4°C on TY and LB agar medium slants, respectively. These strains were also maintained as stab cultures on respective media.

3.2.1.2. Culturing of bacteria

Single colonies were obtained by plating or streaking the bacterial cells on complete or minimal medium. Rhizobial strains were incubated at 28°C for 2-3 days in complete and 3-4 days in minimal media to develop single colonies, whereas *E. coli* strain was incubated overnight at 37°C to get single colonies.

To study the growth response of rhizobia, single colony from each of these strains was suspended in 50 µl of 0.85 % NaCl. About 10^5 to 10^6 cells were applied to the surface of agar medium with the help of a loop to make a spot. Log phase culture of *E. coli* was obtained by incubating the inoculated liquid LB medium containing the antibiotic kanamycin sulphate (40µg/ml) for 14-16 hrs at 37°C. Log phase cultures of rhizobial strains were obtained by inoculating a single colony of the strain in liquid TY medium and containing streptomycin (100µg/ml) and incubating them upto 24-32 hrs at 28°C. The tubes and flasks containing cultures were incubated at the suitable temperature in an orbital shaker (GALLENKAMP) operating at 120 rpm.

3.2.1.3. Serial dilutions

Serial dilutions were done to obtain single bacterial colonies according to the requirements of the experiment. Serial dilutions were made in 0.85 % (w/v) NaCl. One hundred microlitre of neat culture was added to 900 µl saline to get 10^{-1} dilution; this was vortexed to get uniform suspension. One hundred microlitre of bacterial suspension from this tube was added to another tube containing 900 µl saline so as to obtain 10^{-2} dilution. Further dilutions were similarly made according to the experimental requirements.

3.2.1. 4. Bacterial matings

The matings between *Rhizobium* and *E. coli* strains were done according to Kondorosi *et al.* (1977). Log phase cultures of recipient and donor strains were mixed in 3:1 ratio and spread on TY agar medium. Incubation was done at 28°C for 24 hrs.

3.2.2. Isolation and screening of auxotrophs

3.2.2.1. Random transposon Tn5 mutagenesis

Random Tn5 mutagenesis of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905 and *R. leguminosarum* bv. *viciae* Rlv1 strains was done using Tn5 delivery suicide plasmid pGS9 (Selvaraj and Iyer, 1983). Log phase culture in liquid TY medium of *E. coli* strain WA803 (pGS9) was used as a donor and *S. meliloti* (Rm1021), *R. leguminosarum* bv. *trifolii* (MTCC 905) and *R. leguminosarum* bv. *viciae* (Rlv1) were used as recipients. Log phase cultures of donor and recipients were obtained and 750 µl of each rhizobial culture was mixed with 250 µl of *E. coli* culture separately. After thorough mixing 50 µl suspension was spread on half of the surface of TY agar medium in a Petri dish with the help of a sterile glass rod. The remaining half of the TY agar surface was further divided into two halves and 50 µl of *Rhizobium* culture was spread on one half and 50 µl of *E. coli* culture was spread on the other half. Incubation of these plates was done at 28°C for 24 hrs.

Each bacterial growth (donor/recipient/mating mixture) was scraped and suspended in 2 ml saline. One hundred µl suspension of the mating mixture was plated on TY agar medium containing streptomycin (100µg/ml) and kanamycin (400µg/ ml). The donor (50 µl) and recipient (50 µl) suspensions were also plated separately on selective media as controls. Incubation of these plates was done at 28°C for 4 days. Fifty colonies

were purified on TY+Km^{400}+Str^{100} agar plates. All kanamycin resistant (Km^r) transconjugants were checked for the loss of chloramphenicol marker to confirm the loss of suicide plasmid pGS9. The above cross was repeated 415 times and 50 Km^r transconjugants were selected from each cross.

3.2.2.2. Screening of auxotrophs

A colony of each Km^r transconjugant was streaked on RMM and TY+Km^{400}+Sm^{100} agar medium with the help of a sterile toothpick. Incubation was done at 28°C for 3 days. The bacterial growth was observed after every 12 hrs. The Tn5 derivatives which showed growth on TY+Km^{400}+Sm^{100} but not on RMM agar medium were considered as auxotrophs. Each of these auxotrophs was subcultured to get single colonies on TY+Km^{400}+Sm^{100} agar medium. The purified auxotrophs were maintained, as described earlier, for further studies.

3.2.3. Determination of the nature of auxotrophy

The nature of each auxotroph was determined as follows. A loopful culture of the auxotroph was suspended in a drop of saline. With the help of a sterile toothpick this suspension was streaked on RMM agar medium supplemented with Holliday pools (one pool at a time) and also on TY+Km^{400}+Sm^{100} and RMM medium, which served as positive and negative controls, respectively. Incubation was done at 28°C for 4 days. The nature of auxotrophy was found on the basis of growth patterns on various Holliday pools. Requirement(s) of each auxotroph was established by streaking it on RMM agar medium supplemented with the suspected nutrient(s).

3.2.4. Location of biochemical block in arginine auxotrophs by intermediate feeding studies

Biochemical block in each arginine auxotroph was determined by feeding the defined intermediates of the arginine biosynthetic pathway to the auxotroph and subsequently observing the growth. Arginine auxotrophs were suspended in normal saline and streaked using sterile toothpicks on RMM medium supplemented with L-ornithine and L-citrulline (one at a time). The cell suspension of the parental strain was also streaked on the above medium in each plate as a control. The growth pattern, with reference to the parental strain, was observed after incubation for 3-4 days at 28°C.

3.2.5. Growth characteristics

The pattern of growth of each arginine auxotroph used in this study was compared to that of the parental strain. Each auxptroph was grown to active log phase. Two hundred µl of this culture was taken and inoculated in 20 ml of liquid MSY medium. These cultures were incubated at 28°C on an orbital shaker at 120 rpm considering the time of inoculation and a sample of each culture was collected at an interval of 4 hrs upto 24 hrs. Optical densities (O.D.) of these samples were recorded at 600 nm against the uninoculated liquid MSY medium used as a control.

3.2.6. Pleiotropic effects of Tn5 insertions in auxotrophs

The pleiotropic effects of Tn5 insertions in all the arginine auxotrophs were studied by detecting changes, if any, in various characteristics like production of cell surface molecules and utilization of sugars and dicarboxylic acids.

3.2.7. Production of cell surface molecules

3.2.7.1. Test for the production of lipopolysaccharides (LPS)

All auxotrophs and the parental strains were streaked on MSY agar medium containing 1 mg/ml of sodium deoxycholate (DOC). The growth of these strains was recorded after incubation at 28°C for 3 days. The strains which showed growth on MSY + DOC agar medium were considered to be LPS producing (Swamynathan and Singh, 1995).

3.2.7.2. Test for the production of cellulose fibrils and succinylated exopolysaccharides (EPS I)

The presence of cellulose fibrils and succinylated exopolysaccharides (EPS I) on the surface of rhizobial cells was indicated by the binding of Congo red dye and calcofluor white, respectively. Red colonies on medium containing Congo red were produced by the strains producing cellulose fibrils (Kneen and La Rue, 1983). The strains producing EPS I gave fluorescence under ultraviolet light in presence of calcofluor (Leigh *et al.*, 1987). MSY agar medium containing 0.02 % (w/v) Congo red and 0.02 % (w/v) calcofluor (one at a time) were prepared separately. All arginine auxotrophs as well as the parental strains were streaked on the above media and incubated at 28°C for 3 days. After incubation the Congo red plates were observed for red colonies and calcofluor plates were observed for fluorescence in ultraviolet light.

3.2.7.3. Test for the production of β-glucans

3.2.7.3.1. Test for the production of β-(1→3) glucans

Blue colonies on medium containing aniline blue were formed by bacterial strains which produce β-(1→3) glucans (Nikanishi *et al.*, 1976). All auxotrophs and the parental strains were streaked on MSY agar medium containing 0.02 % (w/v) aniline blue. Incubation was done at 28°C for 3 days. The presence or absence of blue colonies was recorded for each strain.

3.2.7.3.2. Test for the production of β-(1→2) glucans

Production of β-(1→2) glucans was assayed on swarm plates (Geremia *et al.*, 1987). A loopful of cell suspension of the parental strain and that of each auxotroph were spotted on TY swarm medium containing 0.3 % (w/v) agar. Incubation was done at 28°C for 3 days and spots were examined for swarming after every 24 hrs.

3.2.7.4. Reversion analysis of auxotrophs

Ten ml of late log phase auxotroph culture in liquid TY medium was centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet, after washing twice with liquid RMM (2 ml each time), was resuspended in a suitable volume of liquid RMM so as to obtain about 10^9 cells/ml. One hundred µl of this cell suspension was spread on RMM and TY agar media. Incubation was done at 28°C for 4 days. The bacterial colonies on RMM and TY media were counted after incubation. The number of colonies on RMM medium represented the number of prototrophic revertants whereas the

number of colonies on TY medium indicated total number of cells spread on each medium. On the basis of these numbers spontaneous reversion frequency for each auxotroph was calculated. Excisions of transposon Tn5 in prototrophic revertant colonies were determined by streaking cells from these colonies on TY+Km^{400}+Sm^{100} agar medium.

3.2.8. Plant inoculation studies

3.2.8.1. Surface sterilization and germination of seeds

Seeds of 10, 4 and 6 cultivars of alfalfa, clover and lentil, respectively, were soaked in sterile distilled water for 20 min and surface sterilized by treating with 0.2 % $HgCl_2$ for 1 min followed by 1 min treatment with absolute alcohol. After five washings with sterile distilled water, seeds were spread on 1 % agar in a Petri plate. These plates were kept in inverted position at 25°C in dark. After two days, two seedlings of each cultivar were transferred to a culture tube containing nitrogen-free plant nutrient medium.

3.2.8. 2. Plant inoculation tests

Glass tubes were used to carry out plant inoculation assays. Nitrogen-free plant nutrient medium containing agar was prepared. The glass tubes were plugged with cotton and autoclaved. Twenty five ml of the autoclaved hot agar containing plant nutrient medium was poured into each tube. The tubes were plugged and placed in a slanting position.

Two 2-day old seedlings were transferred to each of these tubes. Parental strains of rhizobia and their arginine auxotrophs were grown to active log phase in 20 ml of liquid TY medium. The cultures were centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet, after washing twice with 0.85 % (w/v) NaCl,

was suspended in 5 ml of 0.85 % (w/v) NaCl. Five hundred µl of the cell suspension of a rhizobial strain was dispensed in each tube containing its host plant seedlings. The lower portions of the tubes were wrapped with black paper to protect the plant roots from direct exposure to light. These were placed in plant growth chamber maintained at 25°C and provided with 2000 lux light. Sixteen hours of light and eight hrs of dark periods were maintained in the growth chamber. The observations on nodule colour, nodule shape and position, mean plant height, mean dry weight per plant, nitrogen content (in percent), total nitrogen per plant and acetylene reduction for each plant were recorded.

3.2.8. 3. Nodule occupancy tests

Nodules were checked for percent occupancy by the inoculated strains by re-isolating the rhizobial cells from them and testing their auxotrophic and antibiotic markers. All nodules from a plant were surface-sterilized and crushed in 1 ml of saline. The resulting cell suspension was diluted in saline to get 10^{-1} and 10^{-2} dilutions. One hundred µl of neat suspension and each of the two dilutions were spread on a TY agar medium containing 100µg/ml streptomycin. Three replications were used for each plating. Incubation was done at 28°C for 3 days. The colonies obtained were streaked on agar medium containing RMM, RMM + Km^{400} + Sm^{100} + arginine and TY agar + Km^{400} + Sm^{100}. Incubation was done at 28°C for 3 days. The colonies which showed growth on RMM agar mediumbut not on RMM + Km^{400} + Sm^{100} + arginine agar medium and/or TY + Km^{400} + Sm^{100} agar were recorded to be prototrophic revertants.

3.2.9. Plant inoculation tests with prototrophic revertants

Alfalfa, clover and lentil were inoculated with the prototrophic revertants of arginine auxotrophs to confirm that symbiotic defect was in fact due to arginine auxotrophy. Ten plants were inoculated for each revertant and data on the plants was recorded after six weeks of inoculation.

3.2.10. Microscopic studies of nodule sections

Microscopic studies of the sections of nodules induced by parental strains *S. meliloti* Rm1021 and its arginine auxotrophs (IITR1, IITR2, IITR3, IITR4, IITR5, IITR6 and IITR7), *R. leguminosarum* bv. *trifolii* MTCC905 and its arginine auxotrophs (IITR8, IITR9, IITR10 and IITR11) and *R. leguminosarum* bv. *viciae* Rlv1 and its arginine auxotrophs (IITR12, IITR13, IITR14 and IITR15) were carried out. Only one cultivar of each host (LCC3 of alfalfa, Pusa giant of clover and PL639 of lentil) was chosen for these studies. The representative plants inoculated by the parental strains and the respective auxotrophs were harvested after six weeks of inoculation and their nodules were used for microscopic studies.

3.2.10. 1. Primary fixation of nodules

The representative nodules to be used for microscopic studies were rinsed thrice with sterile distilled water and transferred to Karnovsky fixative in glass vials of 5 ml capacity. Acrolein (0.1 % final concentration) was added to the fixative. The vials were placed in vacuum at 4°C for 24 hrs.

3.2.10. 2. Preparation of the specimen blocks

3.2.10. 3. Washing of nodule samples

The nodule samples were taken out of the primary fixative, washed in 0.1 M phosphate buffer and left overnight in the buffer at 4°C.

3.2.10. 3.1. Secondary fixation of nodules

The nodule samples were kept in secondary fixative (1 % w/v OsO_4) at 4°C for 2 hrs and subsequently washed with 0.1 M phosphate buffer.

3.2.10. 3.2. Dehydration of nodules

To remove water from the nodules, samples were passed through a graded acetone series (30, 50, 70, 80, 90, 95 % and dry acetone). A sample was kept in each of the acetone series at 4°C for 15 min. Subsequently, two changes of 30 min each in dry acetone were given at room temperature.

3.2.10. 3.3. Removal of acetone from nodules

Acetone from the samples was removed by placing them in toluene at room temperature for 60 min to facilitate infiltration in the next step.

3.2.10. 3. 4. Infiltration with resin

The following infiltration mixtures were prepared:

a. 1 part of embedding medium and 3 parts of toluene

b. 2 parts of embedding medium and 2 parts of toluene

c. 3 parts of embedding medium and 1 part of toluene

The nodule samples, which were taken out of toluene, were placed in mixture 'a'. After leaving overnight, these were transferred to mixture 'b' for 1 hr and then to mixture 'c' for 1 hr under vacuum. Thereafter two changes of 1 hr each in pure embedding medium at 50°C were given.

3.2.10. 3.5. Embedding of nodule

The nodule samples were embedded in araldite embedding medium using gelatin blocks. Proper orientation of the nodules was done to facilitate subsequent longitudinal sectioning. The embedding blocks were kept at 50°C for 24 hrs and then the temperature of the oven was raised to 60°C. In 48 hrs complete polymerization of the embedding medium occurred. These blocks were then trimmed using a trimmer and fitted in a specimen block holder.

3.2.10. 3.6. Preparation of semithin sections

The block holder was attached to ultracut E microtome (C. Reichert, Austria OmU3) and semithin sections (0.5 µm thick) were cut using a glass knife. The sections floating in water in the metallic trough were taken out with a thin brush and placed on a clean glass slide. The slide was dried by placing on a hot plate at 80°C for 30 seconds. The sections were stained with 1 % (w/v) toluidine blue for 1 min. After staining, the sections were washed with distilled water and observed under light microscope (Leica DM LB). The areas of interest were marked and photographed with Nikon Digital Camera.

3.2.10. 3.7. Preparation of ultrathin sections

The areas of the section to be examined under transmission electron microscope (TEM) ultrathin sections (60-90 nm thick) were cut using Ultracut E Microtome (C. Reichert, Austria OmU3). These sections were lifted onto 200 mesh size copper grids. These grids were coated with 3 % formvar (polyvinyl formaldehyde) in ethylene dichloride and carbon by evaporation under vacuum. After double coating, formvar was removed by dissolving in ethylene dichloride or chloroform. This left behind a carbon film. Double staining of sections with uranyl acetate and lead citrate was done to obtain a good contrast. The grids were placed for 15 min in 0.5 ml of uranyl acetate solution on a watch glass. A cardboard cover was placed over the watch glass to provide dark condition for staining. The grids were washed twice with 50 % ethanol and twice with double distilled water. Subsequently the grids were dried on a filter paper and placed for 10 min in 0.5 ml of lead citrate solution on a clean watch glass. The grids, after removing from the above solution, were washed with 0.1 N NaOH and then twice with double distilled water. After washing, the grids were dried and viewed under transmission electron microscope (PHILIPS EM 300). Areas of interest were photographed. After viewing, the grids were stored in a grid holder at room temperature.

3.11. Estimation of nitrogen content in dry plant matter

The plants were harvested after 6 weeks and dried in an oven at 65°C for 6 hrs and subsequently at 100°C overnight till all the moisture was removed. Oven dried material was ground with the help of a mortar and pestle and filtered through a sieve. The powdered material was weighed and packed in tin capsules with tungsten trioxide such

that air in the sample was minimized. This was then transferred to Vario EL for total nitrogen estimation. Sulfanilic acid was used as reference.

3.11.1. Measurement of Nitrogenase activity

Nitrogenase activity was measured as acetylene reduction assay (ARA) by GC analysis using HP 5890A chromatograph with Porapack Q column and ionization flame detector at 100°C. The roots containing the nodules were detached from the plant after six weeks of inoculation and incubated in 250 ml glass flasks containing 10 % (v/v) acetylene. After one hour 20 µl of gas from the flasks was removed with the help of a microsyringe and injected into preset GC system.

3.12. Statistical analysis

Data on various characteristics of plants inoculated with the parental strain *S. meliloti* (Rm1021), *R. leguminosarum* bv. *trifolii* (MTCC 905) and *R. leguminosarum* bv. *viciae* (Rlv1) and arginine auxotrophs were statistically analyzed. All values were expressed as mean ± standard deviation. Significant difference between control (uninoculated) value, and experimental value was calculated using Fisher't' test.

4. RESULTS

Auxotrophs of *Sinorhizobium meliloti* strain Rm1021, *Rhizobium leguminosarum* bv. *trifolii* strain MTCC905 and *R. leguminosarum* bv. *viciae* strain Rlv1 were generated and characterized. Symbiotic properties of these auxotrophs were studied by inoculating them onto seedlings of their respective host plants, viz., alfalfa, clover and lentil. Histological studies of the nodules induced by these auxotrophs were also done. This chapter presents the results of these studies.

4.1. Random transposon Tn5 mutagenesis

A total of 415 crosses were performed between the donor strain *E. coli* WA803 (pGS9) and each of the rhizobial recipient strains, viz., *S. meliloti* strain Rm1021, *R. leguminosarum* bv. *trifolii* strain MTCC905 and *R. leguminosarum* bv. *viciae* strain Rlv1. The transconjugants were selected on TY agar medium containing kanamycin (Km) (400 µg/ml) and streptomycin (Sm) (100 µg/ml). A total of 20,750 kanamycin resistant (Km^r) transconjugants (2,250 of Rm1021, 16,400 of MTCC 905 and 2,100 of Rlv1) were generated. All the Tn5-induced transconjugants were found to be chloramphenicol-sensitive indicating the absence of vector plasmid pGS9. Single colonies were obtained by subculturing the transconjugants on the TY agar medium containing Km (400 µg/ml) and Sm (100 µg/ml). The bacteria from a single colony of each Tn5 derivative were used in further experiments.

4.2. Screening of Tn5 derivatives for auxotrophs

The Tn5 derivatives were screened for auxotrophs by streaking on RMM (*Rhizobium* minimal medium) agar medium. Sixty five Tn5 derivatives did not grow on

RMM agar medium. These derivatives were assumed to be auxotrophs and used in further work.

4.3. Determination of nutritional requirements of auxotrophs

Nutritional requirements of suspected auxotrophs were checked on the basis of their colony forming ability on modified Holliday pools. On the basis of their nutritional requirements all the auxotrophs were classified as follows (the number of auxotrophs isolated have been given in brackets); *S. meliloti* strain Rm1021: arginine (7), methionine (10), leucine (2), tryptophan (1) and histidine (1); *R. leguminosarum* bv. *trifolii* MTCC905: arginine (4), glutamine (10), valine (15), phenylalanine (1), glycine (1) and histidine (1); *R. leguminosarum* bv. *viciae* Rlv1: arginine (4) and aspartic acid (8). Arginine auxotrophs of *S. meliloti* Rm1021 (IITR1, IITR2, IITR3, IITR4, IITR5, IITR6 and IITR7), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR8, IITR9, IITR10 and IITR11) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR12, IITR13, IITR14 and IITR15) were selected for further studies.

4.4. Analysis of reversion frequencies

All the Tn5 induced arginine auxotrophs showed spontaneous reversion to prototrophy. The reversion frequencies of the arginine auxotrophs were found to vary from 2.0×10^{-9} in *S. meliloti* IITR6 to 5.7×10^{-9} in *R. leguminosarum* bv. *viciae* IITR13. All prototrophic revertants were kanamycin-sensitive showing that the excision of Tn5 had occurred from these revertants (Table 4).

Table 4: Spontaneous reversion frequencies of arginine auxotrophic strains of *Sinorhizobium meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905 and *R. leguminosarum* bv. *viciae* Rlv1

S.No	Name of the auxotrophic Strain	Spontaneous reversion frequency
1	*S. meliloti* IITR1	$3.1x10^{-9}$
2	*S. meliloti* IITR2	$4.4x10^{-9}$
3	*S. meliloti* IITR3	$3.7x10^{-9}$
4	*S. meliloti* IITR4	$3.5x10^{-9}$
5	*S. meliloti* IITR5	$3.3x10^{-9}$
6	*S. meliloti* IITR6	$2.0x10^{-9}$
7	*S. meliloti* IITR7	$4.2x10^{-9}$
8	*R. leguminosarum* bv. *trifolii* IITR 8	$2.6x10^{-9}$
9	*R. leguminosarum* bv. *trifolii* IITR 9	$2.2x10^{-9}$
10	*R. leguminosarum* bv. *trifolii* IITR10	$3.7x10^{-9}$
11	*R. leguminosarum* bv. *trifolii* IITR11	$2.9x10^{-9}$
12	*R. leguminosarum* bv. *viciae* IITR12	$2.2x10^{-9}$
13	*R. leguminosarum* bv. *viciae* IITR13	$5.7x10^{-9}$
14	*R. leguminosarum* bv. *viciae* IITR14	$3.0x10^{-9}$
15	*R. leguminosarum* bv. *viciae* IITR15	$2.2x10^{-9}$

4.5. Identification of biochemical block in arginine auxotrophs

The position of the biochemical block in each arginine auxotroph was determined by feeding the intermediates of the arginine biosynthetic pathway to the auxotroph and subsequently observing the growth. On the basis of intermediate feeding studies, the arginine auxotrophs were placed in the following three categories:

I. *argA/argB/argC/argD/argE* mutants: *S. meliloti* IITR1 and *S. meliloti* IITR2; *R. leguminosarum* bv. *trifolii* IITR8; *R. leguminosarum* bv. *viciae* IITR12. These auxotrophs grew on RMM supplemented with ornithine or citrulline. These mutants were also designated as ornithine auxotrophs.

II. *argF/argI* mutants: *S. meliloti* IITR3, *S. meliloti* IITR4, and *S. meliloti* IITR5; *R. leguminosarum* bv. *trifolii* IITR9; *R leguminosarum* bv. *viciae* IITR13 and *R. leguminosarum* bv. *viciae* IITR14. These auxotrophs grew on RMM supplemented with citrulline but did not grow on ornithine supplemented RMM.

III. *argH* mutants: *S. meliloti* IITR6 and *S. meliloti* IITR7; *R. leguminosarum* bv. *trifolii* IITR10 and *R. leguminosarum* bv. *trifolii* IITR11; *R. leguminosarum* bv. *viciae* IITR15. These auxotrophs grew on RMM supplemented with arginine but did not grow on citrulline or ornithine supplemented RMM.

The position of biochemical block in each arginine auxotroph has been shown in Fig. 1.

4.6. Growth patterns of arginine auxotrophs

The growth characteristics of all the arginine auxotrophs appeared similar to their parental strains on TY medium.

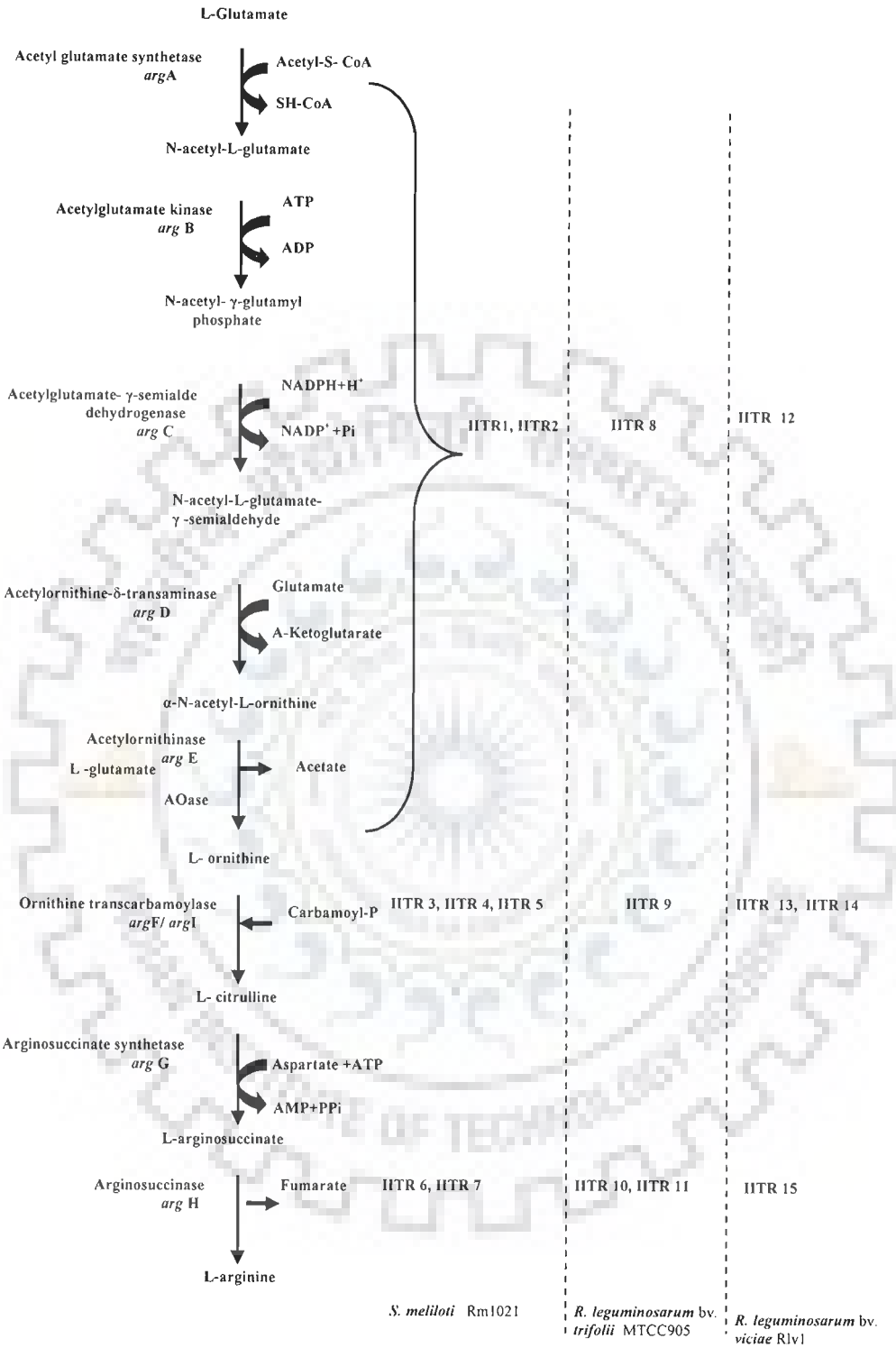

Fig. 1.The pathway for arginine biosynthesis in bacteria showing positions of mutations of arginine auxotrophs of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC 905 and *R. leguminosarum* bv. *viciae* Rlv1 (Cunin *et al.*, 1986)

4.7. Pleiotropic effects of Tn5 insertions in auxotrophs

4.7.1. Production of cell surface molecules

In order to find out the pleiotropic effects, if any, of the Tn5 insertions on cell surface characteristics the auxotrophs were tested for production of cellulose fibrils, lipopolysaccharides, β-(1→2) glucans, β-(1→3) glucans and succinylated exopolysaccharides. Three parental strains and all their arginine auxotrophs took up the Congo red dye from the medium, grew in presence of sodium deoxycholate, showed motility on swarming media and fluoresced in presence of calcofluor white under UV light. This indicated the normal production of cellulose fibrils, lipopolysaccharides, β-(1→2) glucans and succinylated exopolysaccharides in the parental strains and auxotrophs. All the auxotrophs and the parental strains did not take up the aniline blue dye from the medium indicating that β-(1→ 3) glucans were not produced in these auxotrophs and parental strains.

4.7.2. Utilization of sugars and dicarboxylic acids

The growth of the auxotrophs on RMM supplemented with auxotrophic requirements was same as that of the parental strains on RMM. No change in growth was observed when the glucose in RMM medium was replaced by any one of the other sugars (sucrose, arabinose, xylose, maltose or mannitol) or dicaboxylic acids (malate, aspartate or glutamate) as a sole carbon source. These findings showed that the Tn5 insertions in these auxotrophs did not affect the cell surface characteristics or the uptake/utilization of sugars and dicarboxylic acids.

4.8. Symbiotic studies

The results of the symbiotic interactions of 15 arginine auxotrophs of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905, *R. leguminosarum* bv. *viciae* Rlv1with their respective host plants have been presented in Plates 1-3 and Tables 5-24.

The *S. meliloti* Rm1021 strain and its *argF/argI* (IITR3, IITR4 and IITR5) and *argH* (IITR6 and IITR7) auxotrophs induced cylindrical and pink nodules on both primary and lateral roots of plants of each the 10 cultivars of alfalfa. The nodules induced by *argA/argB/argC/argD/argE* auxotrophs (IITR1 and IITR2) were of irregular shape and light pink colour and these were located on both primary and lateral roots of all the 10 cultivars of alfalfa. The data on mean dry plant weight, total nitrogen content per plant and acetylene reduction activity indicated that the nitrogen fixing efficiencies of the *argF/argI* and *argH* mutants were significantly less than nitrogen fixing efficiencies of parental strains with 8 cultivars of alfalfa; with the remaining 2 cultivars the differences were nonsignificant. However, the nitrogen fixing efficiencies of *argF/argI* and *argH* mutants were significantly more than the nitrogen fixing efficiencies of *argA/argB/argC/argD/argE* mutants on all the cultivars of alfalfa. The dry plant weight and total nitrogen content per plant of *argA/argB/argC/argD/argE* mutants were very similar to those of the uninoculated plants showing that these strains fixed no or very little nitrogen. Similar results were obtained with the arginine auxotrophs of *R. leguminosarum* bv. *trifolii* MTCC905 (IITR10 and IITR11) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR15) on 4 and 6 cultivars of *Trifolium alexandrium* L. and *Lens culinaris* Medik., respectively (Plates 1,2 and 3). Normal symbiosis, like that of the parental

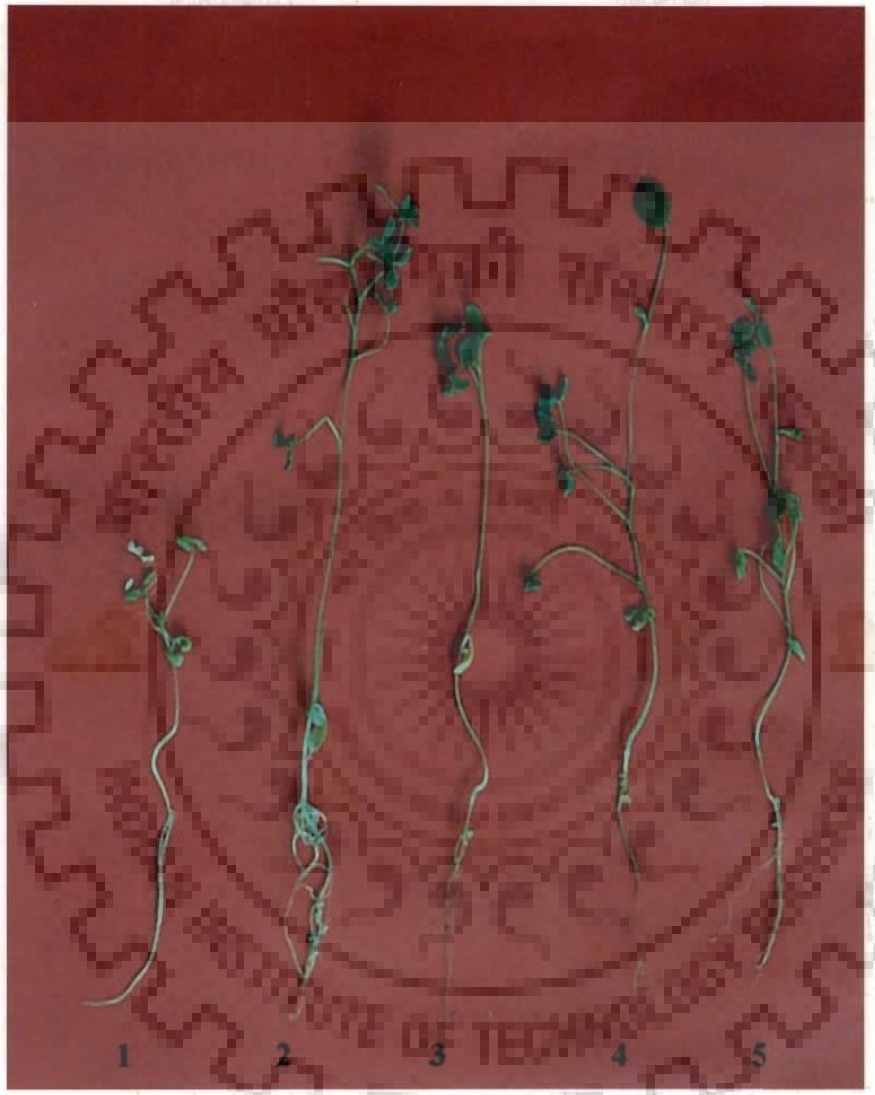

Plate 1: Morphological features of representative plants of alfalfa inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs: **1.** Uninoculated, **2.** Rm1021 inoculated, **3.** Ornithine auxotroph Rm1021 IITR1 (*argA/argB/argC/argD/argE* mutant) inoculated, **4.** Citruline auxotroph Rm1021 IITR3 (*argF/argI* mutant) inoculated and **5.** Arginine auxotroph Rm1021 IITR6(*argH* mutant) inoculated.

Table 5: Characteristics of *Medicago sativa* L. cv. LCC3 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)	Acetylene reduction (µM nitrogenase activity /mg nodule dry weight)
		Shape	Color	Nodule number/ plant					
Uninoculated	-	-	-	-	8.30±0.67	9.47±0.99	1.91	0.18	-
Rm 1021	-	Cylindrical	Pink	5.70±0.67	14.30±1.05	18.95±0.69	3.55	0.67	0.428
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.10±0.31	8.80±0.48*	9.59±0.69*	2.10 (40.8)	0.20 (70.1)	0.257 (40.0)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	3.30±0.67	8.50±0.07*	10.41±1.03*	2.30 (54.3)	0.22 (67.2)	0.295 (31.1)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	2.70±0.48	10.80±1.35	18.73±1.84†	3.08 (13.2)	0.57 (14.9)	0.382 (10.7)
IITR4	*argF/argI*::Tn5	-do-	-do-	3.60±0.84	9.20±0.63	18.44±1.45†	3.17 (10.7)	0.58 (13.4)	0.373 (12.9)
IITR5	*argF/argI*::Tn5	-do-	-do-	2.10±0.31	10.70±0.94	16.07±1.54	3.25 (8.5)	0.52 (22.4)	0.372 (13.1)
IITR 6	*argH*::Tn5	-do-	-do-	3.70±0.67	11.90±1.10	18.38±1.66†	3.02 (14.5)	0.55 (17.9)	0.352 (17.8)
IITR7	*argH*::Tn5	-do-	-do-	3.80±0.63	11.40±0.84	16.60±1.89	3.30 (7.0)	0.54 (19.4)	0.319 (25.5)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rm1021, figures in parenthesis represent Percent decrease in comparison to parental strain.

Table 6: Characteristics of *Medicago sativa* L. cv. LCC5 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg/plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	9.50±0.97	15.10±1.66	0.97	0.15
Rm1021	-	Cylindrical	Pink	6.10±0.66	15.40±1.34	25.9±1.85	3.17	0.82
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.7±0.67	10.00±0.94*	15.2±1.60*	1.94(38.8)	0.30 (63.4)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	3.1±0.56	9.70±0.67*	15.45±1.62*	2.01(36.6)	0.31(62.2)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	3.40±0.51	10.90±0.99	18.45±1.82	2.37(25.2)	0.44 (46.3)
IITR4	*argF/argI*::Tn5	-do-	-do-	4.3±0.94	12.10±0.87	19.82±1.32	2.77(12.6)	0.55 (32.9)
IITR5	*argF/argI*::Tn5	-do-	-do-	5.50±0.85†	13.00±1.15	21.76±1.46	2.97(6.3)	0.65 (20.7)
IITR6	*argH*::Tn5	-do-	-do-	5.10±0.56†	13.00±1.49	23.08±2.64	2.97(6.3)	0.69 (15.5)
IITR7	*argH*::Tn5	-do-	-do-	5.80±0.91†	12.00±0.96	22.31±1.70	2.67(15.8)	0.60 (26.8)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rm1021, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 7: Characteristics of *Medicago sativa* L. cv. LCC8 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg/plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	6.55±0.39	6.47±0.66	2.51	0.16
Rm1021	-	Cylindrical	Pink	5.7±0.48	10.40±0.96	17.52±1.51	3.92	0.68
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.30±0.48	6.60±0.51*	6.90±0.60*	2.70 (31.1)	0.19 (72.1)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	2.2±0.44	6.77±0.97*	7.00±0.62*	2.81 (28.3)	0.20 (70.6)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	2.7±0.48	8.60±0.69	8.66±1.26	3.50 (10.7)	0.31 (55.9)
IITR4	*argF/argI*::Tn5	-do-	-do-	2.20±0.42	8.40±0.97	8.06±1.17	3.70 (5.6)	0.30 (55.4)
IITR5	*argF/argI*::Tn5	-do-	-do-	3.80±0.63	9.10±0.73	9.78±0.76	3.60 (8.2)	0.35 (48.5)
IITR6	*argH*::Tn5	-do-	-do-	4.00±0.47	7.60±0.69	17.88±0.17†	3.42 (12.8)	0.61 (10.3)
IITR7	*argH*::Tn5	-do-	-do-	3.33±0.52	7.66±0.51	17.85±1.43†	3.52 (10.2)	0.62 (8.8)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rm1021, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 8: Characteristics of *Medicago sativa* L. cv. LCC10 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg/plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	5.80±0.84	6.35±0.52	2.76	0.18
Rm1021	-	Cylindrical	Pink	4.10±0.31	11.85±1.24	16.39±1.61	3.79	0.62
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.40±0.54	6.60±0.54*	6.60±0.50*	2.81(25.9)	0.19 (69.4)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	2.20±0.50	6.10±1.05*	6.80±0.67*	2.74 (27.7)	0.19 (69.4)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	2.20±0.44	7.20±0.44	10.23±0.85	3.70 (2.4)	0.38 (38.7)
IITR4	*argF/argI*::Tn5	-do-	-do-	1.90±0.33	8.20±0.97	7.66±0.97	3.66 (3.4)	0.28 (34.8)
IITR5	*argF/argI*::Tn5	-do-	-do-	2.30±0.50	9.00±1.45	6.86±0.55	3.66 (3.4)	0.25 (59.7)
IITR6	*argH*::Tn5	-do-	-do-	2.50±0.52	7.50±0.73	8.40±0.88	3.53 (6.9)	0.30 (51.6)
IITR7	*argH*::Tn5	-do-	-do-	2.20±0.44	8.00±0.70	8.80±1.30	3.63 (4.2)	0.32 (48.4)

*Does not differ significantly (P<0.05) from uninoculated plant and figures in parenthesis represent percent decrease in comparison to parental strain.

Table 9: Characteristics of *Medicago sativa* L. cv. LCC12 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg/plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	6.65±0.91	10.80±1.59	2.05	0.22
Rm1021	-	Cylindrical	Pink	4.12±0.64	12.12±0.99	17.56±2.04	4.14	0.72
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.50±0.92	6.90±0.64*	11.65±1.84*	3.11 (24.9)	0.36 (50.0)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	2.50±1.08	7.02±0.53*	10.98±1.23*	3.30 (20.3)	0.36 (50.0)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	2.50±0.83	9.83±0.98	16.15±1.23	3.19 (22.9)	0.52 (27.8)
IITR4	*argF/argI*::Tn5	-do-	-do-	2.50±0.70	10.90±1.29	14.75±2.06	3.51 (15.2)	0.52 (27.8)
IITR5	*argF/argI*::Tn5	-do-	-do-	2.80±0.70	10.50±1.17	13.88±1.21	3.46 (16.4)	0.48 (33.3)
IITR6	*argH*::Tn5	-do-	-do-	3.40±1.34†	11.16±1.78	14.57±1.15	3.42 (17.4)	0.50 (30.6)
IITR7	*argH*::Tn5	-do-	-do-	3.40±0.48†	10.21±1.69	15.02±2.13	3.51 (15.2)	0.53 (24.4)

*Does not differ significantly ($P<0.05$) from uninoculated plant and †does not differ significantly ($P<0.05$) from parental strain Rm1021, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 10: Characteristics of *Medicago sativa* L. cv. LCC13 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg/plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	6.00±0.71	8.92±1.17	2.94	0.26
Rm1021	-	Cylindrical	Pink	3.70±0.67	12.20±1.13	15.59±1.95	4.55	0.71
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	1.80±0.44	6.20±0.44*	9.08±1.35*	3.31(27.3)	0.30 (57.7)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	1.90±0.33	6.40±0.53*	9.57±1.30*	2.98 (34.5)	0.29 (59.2)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	2.50±0.52	7.70±0.82	10.01±049	3.80 (16.5)	0.38 (46.5)
IITR4	*argF/argI*::Tn5	-do-	-do-	2.70±0.48	9.20±0.91	11.27±1.13	3.75 (17.6)	0.42 (40.8)
IITR5	*argF/argI*::Tn5	-do-	-do-	2.40±0.69	7.70±1.05	10.46±1.08	4.01(11.9)	0.41(42.3)
IITR6	*argH*::Tn5	-do-	-do-	2.40±0.51	6.50±0.70	10.75±1.33	3.78 (16.9)	0.41(42.3)
IITR7	*argH*::Tn5	-do-	-do-	2.40±0.54	7.40±0.84	10.80±0.84	3.89 (14.5)	0.42 (40.8)

*Does not differ significantly ($P<0.05$) from uninoculated plant and figures in parenthesis represent percent decrease in comparison to parental strain.

Table 11: Characteristics of *Medicago sativa* L. cv. RL 87 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg/plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	8.66±0.60	9.37±0.75	2.73	0.25
Rm1021	-	Cylindrical	Pink	4.50±1.77	12.70±1.41	17.77±1.63	4.22	0.75
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.33±0.50	9.15±1.09*	10.19±0.98*	3.27(22.5)	0.33 (56.0)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	2.40±0.55	9.20±1.09*	9.62±0.65*	3.01(28.7)	0.29 (621.3)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	3.42±1.13†	10.87±0.84	11.00±1.16	3.92 (7.1)	0.43 (42.7)
IITR4	*argF/argI*::Tn5	-do-	-do-	3.60±0.55†	9.40±0.55	12.20±0.83	3.88 (8.1)	0.47 (37.3)
IITR5	*argF/argI*::Tn5	-do-	-do-	3.80±0.83†	10.20±1.09	11.22±0.92	3.87(8.3)	0.43 (42.7)
IITR6	*argH*::Tn5	-do-	-do-	2.60±0.89	9.20±1.30	13.21±1.12	4.12 (2.4)	0.54 (28.0)
IITR7	*argH*::Tn5	-do-	-do-	3.00±0.51	9.33±0.81	14.16±0.89	3.89 (7.8)	0.55 (26.7)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rm1021, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 12: Characteristics of *Medicago sativa* L. cv. Anand2 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content plant (mg)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	5.00±0.71	4.84±0.46	2.05	0.10
Rm 1021	-	Cylindrical	Pink	3.2±0.44	13.60±1.14	23.06±1.87	3.64	0.84
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.40±0.54	5.35±0.49*	5.20±0.39*	2.73 (25.0)	0.14 (83.3)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	2.80±0.44	5.09±0.69*	4.90±0.26*	2.63 (27.7)	0.13 (84.5)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	3.20±0.45†	12.04±0.77	13.12±1.10	2.88 (20.9)	0.38 (54.8)
IITR4	*argF/argI*::Tn5	-do-	-do-	3.20±0.44†	10.36±0.86	12.48±0.34	2.80 (23.1)	0.35 (58.3)
IITR5	*argF/argI*::Tn5	-do-	-do-	3.00±1.00†	10.68±0.95	15.64±1.73	3.12 (14.3)	0.49 (41.7)
IITR6	*argH*::Tn5	-do-	-do-	3.20±0.45†	11.60±0.89	11.65±0.65	2.95 (19.0)	0.34 (59.5)
IITR7	*argH*::Tn5	-do-	-do-	3.00±0.70†	10.60±0.54	12.52±0.52	3.15 (13.5)	0.39 (53.6)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rm1021, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 13: Characteristics of *Medicago sativa* L. cv. A2 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Uninoculated	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg/plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	6.10±0.74	2.95±0.37	1.66	0.05
Rm1021	-	Cylindrical	Pink	4.40±0.52	15.40±1.43	21.80±1.81	3.13	0.68
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.05±0.16	6.60±0.70*	3.15±0.34*	1.70 (45.7)	0.05 (60.6)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	1.89±0.33	6.33±0.71*	3.11±0.22*	1.69 (46.0)	0.05 (60.6)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	2.19±0.25	8.60±0.97	6.50±0.79	3.00 (4.2)	0.21 (37.1)
IITR4	*argF/argI*::Tn5	-do-	-do-	2.30±0.48	8.30±0.95	5.80±0.79	2.80 (10.5)	0.16 (44.5)
IITR5	*argF/argI*::Tn5	-do-	-do-	2.40±0.52	9.50±0.85	5.60±0.52	2.73 (12.8)	0.15 (45.9)
IITR6	*argH*::Tn5	-do-	-do-	2.05±016	8.30±0.67	9.20±0.79	2.80 (10.5)	0.26 (25.8)
IITR7	*argH*::Tn5	-do-	-do-	2.00±0.74	9.10±0.47	6.30±0.17	2.91 (7.0)	0.18 (41.5)

*Does not differ significantly (P<0.05) from uninoculated plant and figures in parenthesis represent percent decrease in comparison to parental strain.

Table 14: Characteristics of *Medicago sativa* L. cv. T9 plants inoculated with *Sinorhizobium meliloti* Rm1021 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content plant (mg)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	7.00±0.98	7.00±0.86	1.49	0.10
Rm 1021	-	Cylindrical	Pink	5.00±0.55	15.00±0.80	20.00±0.50	2.81	0.56
IITR1	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	3.00±0.70	7.68±1.10*	7.30±0.60*	1.44 (48.8)	0.10 (82.1)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	2.00±0.70	7.48±0.84*	7.35±0.46*	1.52 (45.9)	0.11 (80.4)
IITR3	*argF/argI*::Tn5	Cylindrical	Pink	3.12±0.74	13.20±0.07	11.00±1.02	2.88 (7.1)	0.32 (42.9)
IITR4	*argF/argI*::Tn5	-do-	-do-	3.00±0.03	11.30±0.89	10.00±0.63	2.52 (10.3)	0.25 (55.4)
IITR5	*argF/argI*::Tn5	-do-	-do-	3.00±0.89	12.12±0.88	10.20±0.96	2.46 (12.5)	0.25 (55.4)
IITR6	*argH*::Tn5	-do-	-do-	3.50±1.5	12.00±0.09	12.32±1.10	2.52 (10.3)	0.31(44.6)
IITR7	*argH*::Tn5	-do-	-do-	3.00±1.02	12.50±1.10	13.14±1.14	2.61 (7.1)	0.34 (39.3)

*Does not differ significantly (P<0.05) from uninoculated plant and figures in parenthesis represent percent decrease in comparison to parental strain.

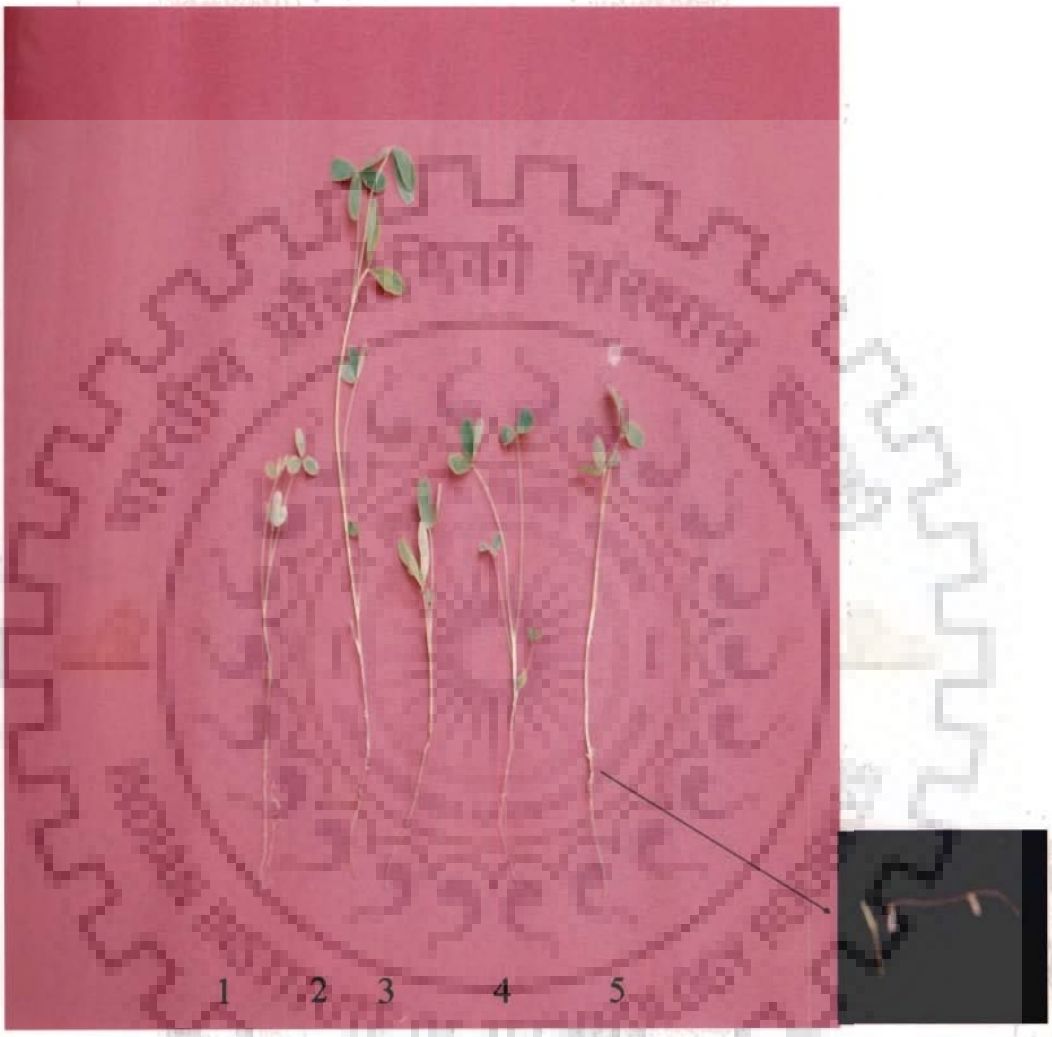

Plate 2: Morphological features of representative plants of clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* MTCC 905 and its arginine auxotrophs: 1. Uninoculated, 2. MTCC 905 inoculated, 3. Ornithine auxotroph IITR 8 (*argA/argB/argC/argD/argE* mutant) inoculated, 4. Citrulline auxotrophs IITR9 (*argF/argI* mutant) inoculated and arginine auxotroph IITR 10 (*argH* mutant) inoculated.

Table 15: Characteristics of *Trifolium alexandrium* L. cv. Pusa giant plants inoculated with *Rhizobium. leguminosarum* bv. *trifolii* MTCC905 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content/ plant (in mg)	Acetylene reduction(μ M /mg nodule dry weight)
		Shape	Color	Nodule Number/ plant					
Uninoculated	-	-	-	-	10.40±0.89	28.68±1.31	2.20	0.63	-
MTCC905	-	Cylindrical	Pink	8.80±0.71	19.30±1.40	83.79±6.94	3.70	3.10	0.237
IITR8	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	4.20±0.27	10.60±0.55*	30.18±2.88*	2.60(29.7)	0.78 (74.8)	0.016 (93.2)
IITR9	*argF/argI*::Tn5	Cylindrical	Pink	5.60±0.55	11.60±1.14	57.54±3.87	3.30 (10.8)	1.18 (61.9)	0.113 (52.3)
IITR10	*argH*::Tn5	-do-	-do-	6.80±0.71	14.10±0.89	66.94±7.21	3.10 (16.2)	2.07 (33.2)	0.151 (36.3)
IITR11	*argH*::Tn5	-	-	5.60±0.84	11.60±4.84	60.18±3.90	3.40 (8.1)	2.04 (34.2)	0.148 (37.6)

*Does not differ significantly (P<0.05) from uninoculated plant and figures in parenthesis represent percent decrease in comparison to parental strain.

Table 16: Characteristics of *Trifolium alexandrium* L cv. Jawahar plants inoculated with *Rhizobium. leguminosarum* bv. *trifolii* MTCC905 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content/ plant (in mg)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-		8.98±0.71	3.13±0.22	1.20	0.03
MTCC 905	-	Cylindrical	Pink	3.80±0.45	12.80±0.84	14.20±0.84	3.20	0.45
IITR8	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	3.40±0.55	9.00±0.71*	3.20±0.27*	2.10 (34.4)	0.06 (86.7)
IITR9	*argF/argI*::Tn5	Cylindrical	Pink	3.40±0.55	10.00±0.71	3.45±0.24	2.80 (12.5)	0.10 (77.8)
IITR10	*argH*::Tn5	-do-	-do-	2.80±0.45	10.00±0.71	4.06±0.55	2.10 (34.4)	0.08 (82.2)
IITR11	*argH*::Tn5	-	-	3.20±0.45	10.20±0.45	3.54±0.27	3.00 (6.3)	0.10 (77.8)

*Does not differ significantly (P<0.05) from uninoculated plant figures in parenthesis represent percent decrease in comparison to parental strain.

Table 17: Characteristics of *Trifolium alexandrium* L cv. USB 10 plants inoculated with *Rhizobium. leguminosarum* bv. *trifolii* MTCC905 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content/ plant (in mg)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-		8.42±0.56	44.15±3.20	1.30	0.57
MTCC905	-	Cylindrical	Pink	9.60±0.89	19.60±0.89	109.20±7.16	3.60	3.93
IITR8	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	6.00±1.00	9.30±0.84*	45.28±0.52	2.40 (33.3)	1.08 (72.5)
IITR9	*argF/argI*::Tn5	Cylindrical	Pink	4.80±0.84	11.40±0.55	55.98±6.31	3.10 (13.9)	1.73 (56.0)
IITR10	*argH*::Tn5	-do-	-do-	8.00±1.00	14.00±0.35	77.16±7.08	2.80 (22.2)	2.16 (45.0)
IITR11	*argH*::Tn5	-	-	4.60±0.55	11.60±0.89	56.84±4.86	3.10 (13.9)	1.76 (55.2)

*Does not differ significantly (P<0.05) from uninoculated plant and figures in parenthesis represent percent decrease in comparison to parental strain.

Table 18: Characteristics of *Trifolium alexandrium* L EC 400733 plants inoculated with *Rhizobium. leguminosarum* bv. *trifolii* MTCC905 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content/ plant (in mg)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	6.18±0.66	2.98±0.50	1.90	0.05
MTCC905	-	Cylindrical	Pink	3.750±0.50	11.40±0.55	8.50±1.02	3.10	0.26
IITR8	*argA/argB/argC/argD/argE*::Tn5	Irregular	Light pink	2.80±0.45	6.80±0.50*	3.00±0.80*	2.20 (29.0)	0.06 (76.9)
IITR9	*argF/argI*::Tn5	Cylindrical	Pink	3.5±0.45	8.00±0.58	4.02±0.05	2.80 (9.7)	0.11 (57.7)
IITR10	*argH*::Tn5	-do-	-do-	3.5±0.45	10.50±0.55	4.60±0.48	2.60 (16.1)	0.11 (57.7)
IITR11	*argH*::Tn5	-do-	-do-	3.5±0.55	9.00±0.71	4.45±0.36	2.90 (6.5)	0.12 (53.8)

*Does not differ significantly (P<0.05) from uninoculated plant figures in parenthesis represent percent decrease in comparison to parental strain.

Plate 3: Morphological features of representative plants of alfalfa inoculated with *Rhizobium leguminosarum* **bv.** *viciae* Rlv1 and its arginine auxotrophs: 1. Uninoculated, 2. Rlv1 inoculated, 3. Ornithine auxotroph IITR12 (*argA/argB/argC/argD/argE* mutant) inoculated, 4. *argF/argI* mutant IITR13 inoculated and 5. *argH* mutant IITR15 inoculated.

Table 19: Characteristics of *Lens culinaris* Medik. cv. PL639 plants inoculated with *Rhizobium leguminosarum* bv. viciae Rlv1 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)	Acetylene reduction (µM nitrogenase activity/mg nodule dry weight)
		Shape	Color	Nodule number/ plant					
Uninoculated	-	-	-	-					
Rlv1	-	Cylindrical	Pink	2.70±0.48	14.80±1.23	16.57±1.40	2.11	0.34	
IITR12	*argA/argB/argC/arg D/argE*::Tn5	Irregular	Light pink	1.95±0.16	25.90±1.37	29.37±1.43	3.67	1.08	0.285
IITR13	*argF/argI*::Tn5	Cylindrical	Pink	2.02±0.18	15.30±0.85*	16.97±2.97*	2.19 (40.3)	0.37 (65.7)	0.077 (73.0)
IITR14	*argF/argI*::Tn5	- do-	-do-	2.29±0.28	24.70±2.00†	21.36±1.94	3.20 (12.8)	0.68 (37.0)	0.267 (6.3)
IITR15	*argH*::Tn5	-do-	-do-	2.90±0.32†	21.20±2.25	24.09±1.94	3.55 (3.3)	0.85 (21.3)	0.212 (25.6)
					24.50±2.37†	21.97±2.13	3.24 (11.7)	0.71 (34.3)	0.235 (17.5)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rlv1, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 20: Characteristics of Lens *culinaris* Medik. P38 plants inoculated with *Rhizobium leguminosarum* bv. viciae Rlv1and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean Plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)
		Shape	Color	Nodule number/ plant				
Uninoculated	-	-	-	-	12.44±0.59	15.48±0.55	2.81	0.44
Rlv1	-	Cylindrical	Pink	2.70±0.48	27.70±1.89	25.87±2.23	4.06	1.05
IITR12	*argA/argB/argC/arg D/argE*::Tn5	Irregular	Light pink	2.30±0.48	13.20±0.92*	16.64±1.71*	3.30 (18.7)	0.55 (47.6)
IITR13	*argF/argI*::Tn5	Cylindrical	Pink	2.10±0.32	23.70±2.58	22.78±2.12	3.35 (17.5)	0.76 (27.6)
IITR14	*argF/argI*::Tn5	- do-	-do-	2.80±0.42†	24.70±2.58	28.52±2.48	3.39 (16.5)	0.97 (7.6)
IITR15	*argH*::Tn5	-do-	-do-	2.60±0.52†	23.40±2.22	26.76±1.54†	3.80 (6.4)	1.02 (2.9)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rlv1, figures in parenthesis represent percent decrease over parental strain.

Table 21: Characteristics of *Lens culinaris* Medik. cv. T36 plants inoculated with *Rhizobium leguminosarum* bv. viciae Rlv1and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)
		Shape	Color	Nodule Number/ plant				
Uninoculated	-	-	-	-	16.10±1.16	16.11±1.36	2.88	0.46
Rlv1	-	Cylindrical	Pink	3.00±0.71	28.70±2.89	28.95±2.24	4.50	1.30
IITR12	*argA/argB/argC/arg D/argE*::Tn5	Irregular.	Light pink	2.40±0.25	16.90±0.97*	16.50±1.43*	3.22 (28.4)	0.53 (59.2)
IITR13	*argF/argI*::Tn5	Cylindrical	Pink	2.40±0.52	24.10±2.33	22.73±2.13	3.89(13.6)	0.88 (32.3)
IITR14	*argF/argI*::Tn5	- do-	-do-	2.20±0.42	24.00±1.70	27.12±2.50†	3.55(21.1)	0..96 (26.2)
IITR15	*argH*::Tn5	-do-	-do-	2.40±0.52	23.80±2.21	23.86±1.97	4.16(7.6)	0.99 (23.8)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rlv1, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 22: Characteristics of *Lens culinaris* Medik. cv. Pusa1 plants inoculated with *Rhizobium leguminosarum* bv. viciae Rlv1and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean Plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)
		Shape	Color	Nodule Number/ plant				
Uninoculated	-	-	-	-	16.21±0.95	14.11±0.11	2.31	0.33
Rlv1	-	Cylindrical	Pink	4.30±0.60	28.01±0.66	25.23±0.21	4.19	1.06
IITR12	*argA/argB/argC/arg D/argE*::Tn5	Irregular	Light pink	2.40±0.55	16.90±0.87*	15.80±0.84*	2.43 (42.0)	0.38(64.2)
IITR13	*argF/argI*::Tn5	Cylindrical	Pink	2.80±0.84	24.40±1.82	19.40±1.34	3.05(27.2)	0.59(44.3)
IITR14	*argF/argI*::Tn5	- do-	-do-	2.50±16.80	24.00±1.70	16.40±1.34	3.10(26.0)	0.51(51.9)
IITR15	*argH*::Tn5	-do-	-do-	2.75±0.50	24.75±1.71	23.00±1.83	3.55(15.3)	0.82(22.6)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rlv1, figures in parenthesis represent percent decrease in comparison to r parental strain.

Table 23: Characteristics of *Lens culinaris* Medik. cv. Pusa 6 plants inoculated with *Rhizobium leguminosarum* bv. viciae Rlv1and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean Plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)
		Shape	Color	Nodule Number/ plant				
Uninoculated	-	-	-	-	15.56±1.17	14.10±1.13	3.55	0.50
Rlv1	-	Cylindrical	Pink	3.72±0.72	27.49±0.90	25.46±1.61	4.73	1.20
IITR12	*argA/argB/argC/arg D/argE*::Tn5	Irregular	Light pink	2.80±0.71	17.40±1.52*	15.00±1.41*	3.24 (31.5)	0.49 (59.2)
IITR13	*argF/argI*::Tn5	Cylindrical	Pink	3.20±0.53†	27.32±2.77†	23.92±2.29	4.19 (16.7)	1.00 (40.8)
IITR14	*argF/argI*::Tn5	- do-	-do-	3.32±0.69†	20.44±1.78	19.20±1.10	4.28 (10.7)	0.82 (38.0)
IITR15	*argH*::Tn5	-do-	-do-	3.00±0.50†	21.80±1.97	18.25±1.26	4.22 (11.9)	0.77 (52.4)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rlv1, figures in parenthesis represent percent decrease in comparison to parental strain.

Table 24: Characteristics of lentil (*Lens culinaris* Medik.) cv. Malika plants inoculated with *Rhizobium leguminosarum* bv. viciae Rlv1 and its arginine auxotrophs

Strain	Mutated gene	Nodule characteristics			Mean Plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)
		Shape	Color	Nodule Number/ plant				
Uninoculated	-	-	-	-	17.07±1.48	21.09±1.50	2.99	0.63
Rlv1	-	Cylindrical	Pink	5.00±0.69	43.28±2.95	40.72±3.77	4.28	1.74
IITR12	*argA/argB/argC/arg D/argE*::Tn5	Irregular	Light pink	3.18±0.62	18.50±1.79*	22.10±1.60*	3.12 (27.1)	0.69 (60.3)
IITR13	*argF/argI*::Tn5	Cylindrical	Pink	2.70±0.48	28.91±3.15	27.99±3.44	3.97 (9.9)	1.11 (90.3)
IITR14	*argF/argI*::Tn5	- do-	-do-	3.78±0.76	31.62±3.31	36.37±3.93	3.89 (9.8)	1.41 (29.3)
IITR15	*argH*::Tn5	-do-	-do-	3.22±0.78	27.28±2.45	31.81±3.29	4.02 (6.7)	1.28 (32.6)

*Does not differ significantly (P<0.05) from uninoculated plant and †does not differ significantly (P<0.05) from parental strain Rlv1, figures in parenthesis represent percent decrease in comparison to parental strain.

strains, was observed when the host plants were inoculated with the spontaneous revertants of ornithine auxotrophs (Table 25). Reisolation of bacteria from the nodules induced by the arginine auxotrophic mutants showed 100% occupancy of nodules by these mutants.

4.9. Light microscopy of nodules

The longitudinal semithin section of the nodules induced by the parental strains *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905, *R. leguminosarum* bv. *viciae* Rlv1, showed a central tissue surrounded by several peripheral tissues. The central tissue was differentiated into five zones: meristematic zone, infection zone, interzone between infection and nitrogen fixation zones, nitrogen fixation zone and senescence zone (Plates 4, 5 and 6). The meristematic zone was made up of small, non-vacuolated, actively dividing and uninfected cells, each with a big nucleus and many mitochondria (Plate 4B). Inner to the meristematic zone few layers of cells with visible infection threads in the intercellular spaces constitute the infection zone; there was an occasional release of rhizobial bacteria from the infection threads in few cells. Some cells in the infection zone had large nuclei and vacuolar regions. Many amyloplasts were present in this zone. The interzone was the zone of the nodule showing transition of nodule cells from uninfected to infected state (Plate 4C). The nitrogen fixation zone was quite large and contains packed nodule cells and most of the nodule cells in this zone were infected with rhizobia (Plates 4D, 5C and 6C). The bacteroids seen in this zone were mostly elongated. In the senescence zone lysed nodule cells were observed.

Table 25: Characteristics of *Medica sativa* L. cv. LCC3 with *Sinorhizobium meliloti* Rm1021, *Trifolium alexandrium* L cv. Puasa giant with *Rhizobium leguminosarum* bv. *trifolii* MTCC905 and *Lens culinaris* cv. PL639 with *Rhizobium. leguminosarum* bv. *viciae* Rlv1 and revertants its arginine auxotrophs.

Strain	Mutated gene	Nodule characteristics			Mean plant height (cm)	Mean plant dry weight (mg)	Nitrogen content (%)	Nitrogen content (mg /plant)
		Shape	Color	Nodule Number/ plant				
Uninoculated	-	-	-	-	7.5±0.55	10.11±0.81	1.97	0.20
Rm1021	-	Cylindrical	Pink	5.1±0.56	16.5±1.50	21.10± 2.10	3.60	0.75
IITR1	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	4.6±0.31†	14.78±0.50†	19.5±0.69†	3.55 (1.4)	0.69 (8.0)
IITR2	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	3.9±0.77†	13.90±1.11	18.28±2.0†	3.50 (2.7)	0.64 (14.6)
Uninoculated	-	-	-	-	10.40±0.89	28.68±1.31	2.00	0.57
MTCC905	-	Cylindrical	Pink	9.00±0.71	19.30±1.40	83.79±3.89	3.70	3.10
IITR8	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	7.70±0.84†	14.20±3.80†	72.11±4.17†	2.92 (21.0)	2.88 (7.0)
Uninoculated	-	-	-	-	14.80±1.23	16.57±1.40	2.11	0.34
Rlv1	-	Cylindrical	Pink	2.70±0.48	24.70±1.37	28.11±1.23	3.60	1.01
IITR12	*argA/argB/argC/argD/argE*::Tn5	-do-	-do-	2.29±0.28†	21.20±2.25†	24.09±1.94†	3.00 (16.6)	0.72 (28.7)

*Does not differ significantly ($P<0.05$) from uninoculated plant and †does not differ significantly ($P<0.05$) from parental strain Rm1021, MTCC905 and Rlv1, figures in parenthesis represent percent decrease in comparison to parental strain.

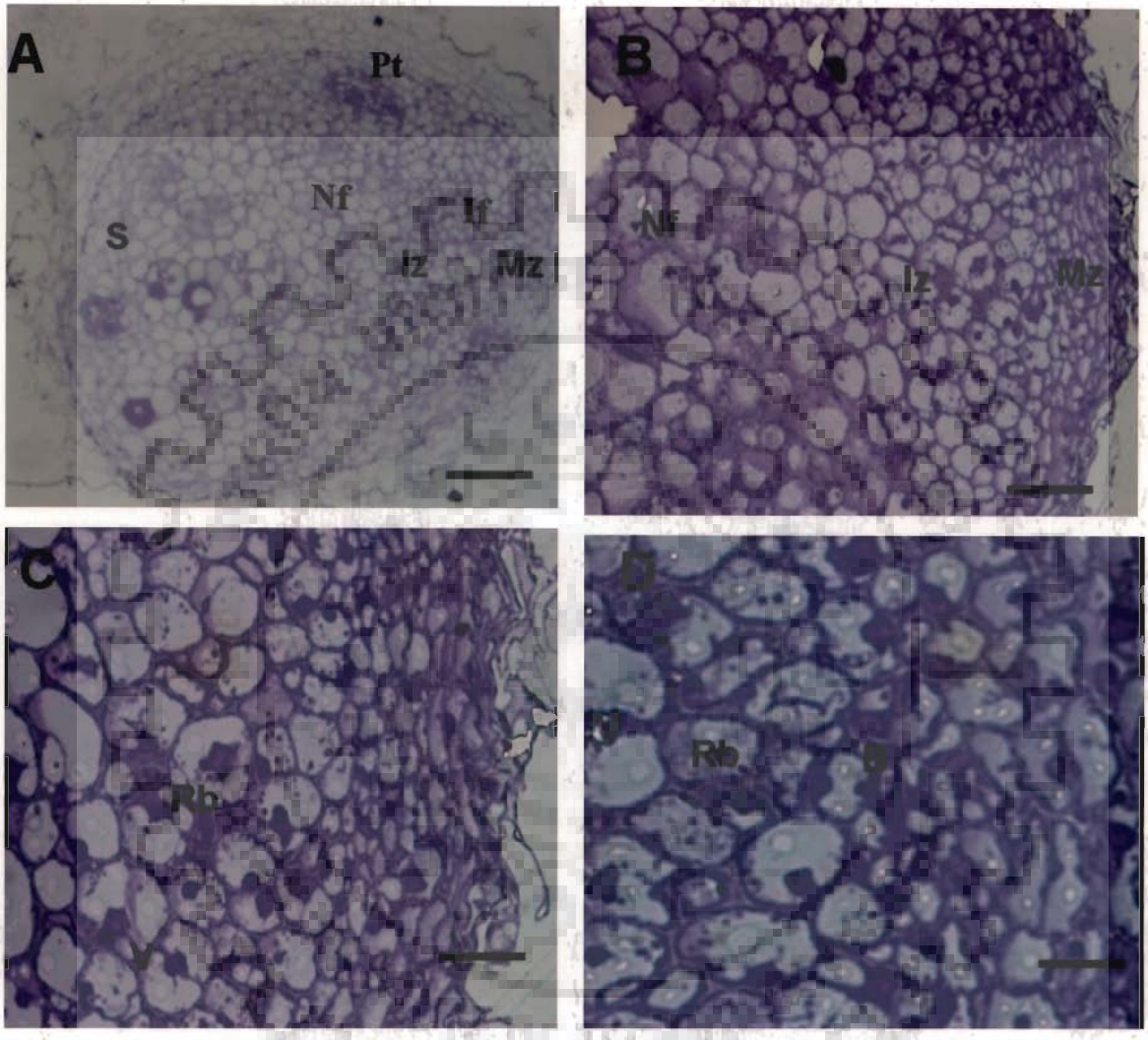

Plate 4: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rm1021, **A.** A nodule section showing distinct peripheral tissue (Pt), meristematic zone (Mz), nitrogen fixation zone (Nf), infection zone (If), interzone (Iz), and senescence zone (S), Bar: 250µm (40x), **B**. Nodule cells of meristematic zone (Mz), infection zone (If) and nitrogen fixation zone (Nf), Bar: 100 µm(100x), **C.** Cells of inter zone showing vacuoles (V) and rhizobial bacteria (Rb), Bar: 50 µm (200x), **D.** Nitrogen fixation zone showing cells packed with bacteroids (B), Bar : 25 µm (400x).

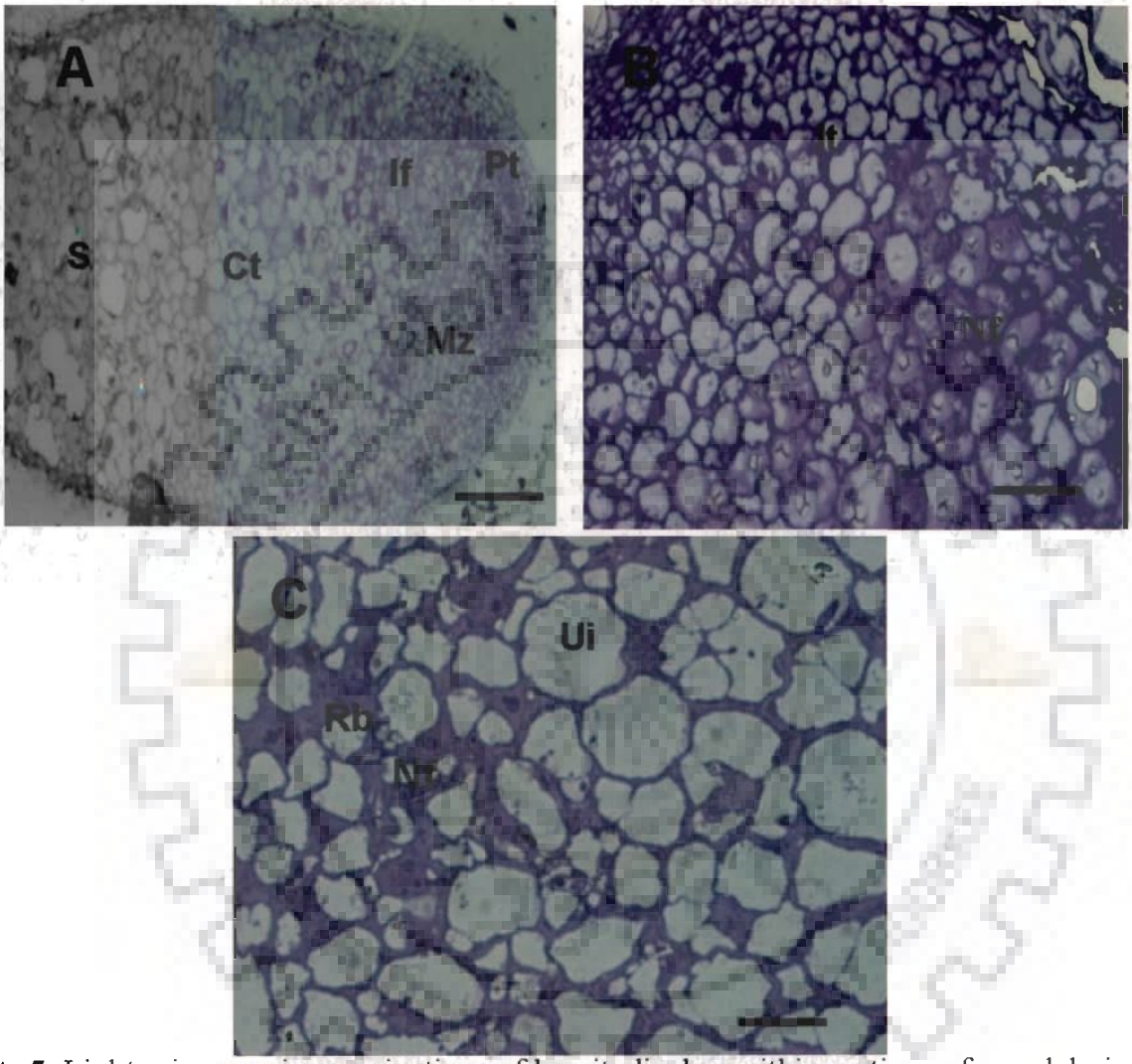

Plate 5: Light microscopic examinations of longitudinal–semithin sections of a nodule induced by *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A.** A complete section of nodule showing distinct peripheral tissue (Pt), meristematic zone (Mz), and central tissue (Ct) distinguished into an extended infection zone (If), nitrogen fixation zone (Nf) and senescence zone (S), Bar: 250 um (40x), **B.** A part of the nodule showing network of infection threads (It) in nitrogen fixation zone (Nf) , Bar: 100 um (100x), **C.** Portion of nodule showing nitrogen fixation zone (Nf) containing cells packed with rhizobial bacteria (Rb) and some uninfected cells (Ui), Bar: 50 um (200x).

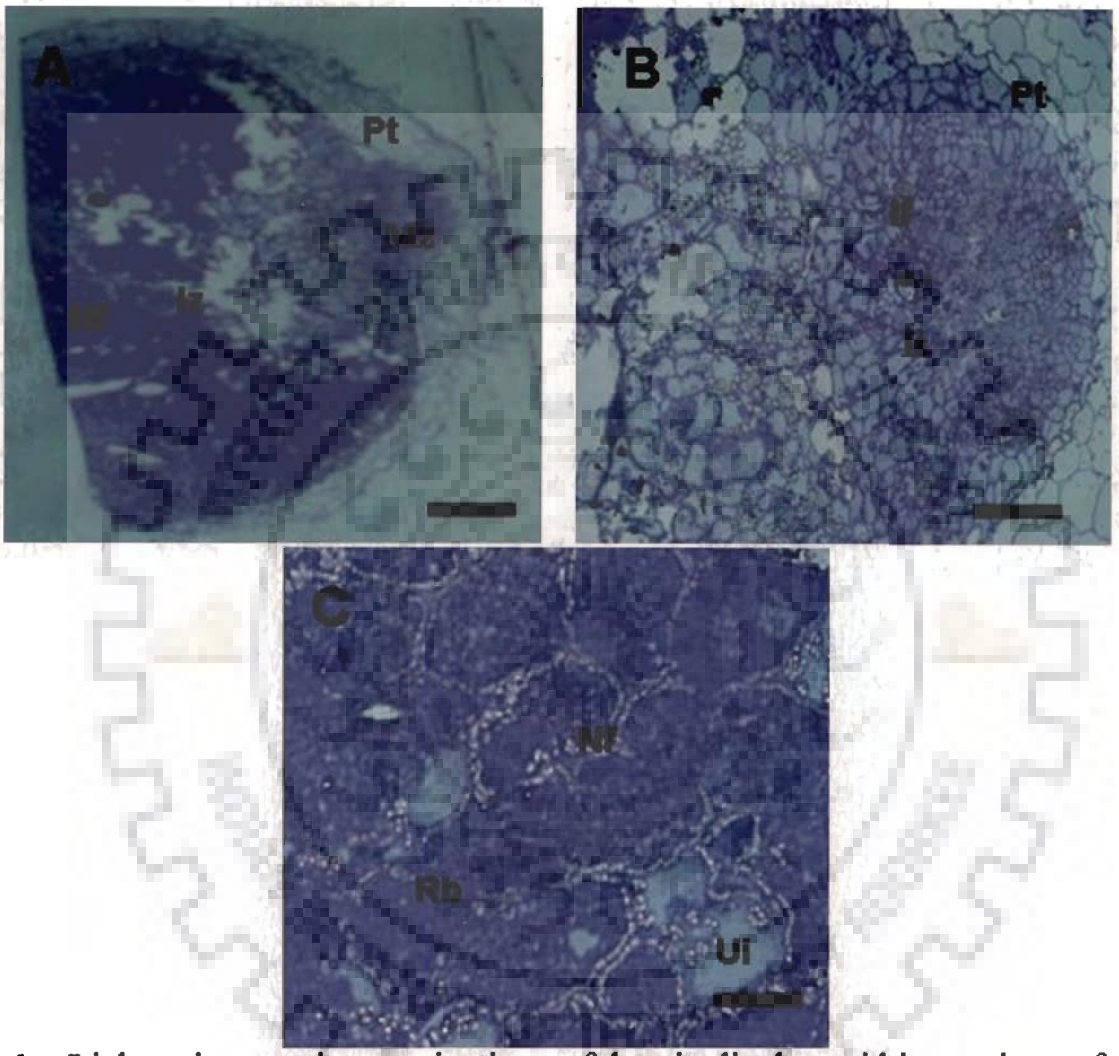

Plate 6: Light microscopic examinations of longitudinal–semithin sections of a nodule induced by *R. leguminosarum* bv. *viciae* Rlv1, **A.** A complete section of the nodule showing peripheral tissue (Pt), meristematic zone (Mz), inter zone (Iz), and nitrogen fixation zone (Nf), Bar: 250 µm (40x), **B.** A part of the nodule showing network of infection threads (It) in infection zone (If), Bar: 100 µm (100x), C. Nodule cells of nitrogen fixation zone (Nf) packed with rhizobia (Rb); few uninfected cells (Ui), Bar: 25 µm (400x).

The internal structure of the nodules induced by the *argF/argI* mutants, and *argH* mutants of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC 905 and *R. leguminosarum* bv. *viciae* Rlv1 appeared almost similar to the internal structure of the nodules induced by the parental strains (Plates 7,8,9,10,11 and 12). The nodules induced by the ornithine auxotrophs of *S. meliloti* Rm1021 (IITR1 and IITR2), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR8) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR12) had distinct peripheral and central tissues (Plates 13,14 and 15). The central tissue in each of these nodules was differentiated into five zones as in the nodules induced by the parental strains, however the nitrogen fixation zone was poorly developed and the senescence zone was quite extensive.

4.10. Transmission electron microscopy of nodules

Ultrathin sections of nodules induced by the parental strain *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905, *R. leguminosarum* bv. *viciae* Rlv1 (Plates 16,17 and 18) showed the presence of many mitochondria and endoplasmic reticulae in the nodule cells of interzone (Plate 16A). Polyhydroxy butyrate (Phb) granules were found in the rhizobial bacteria in infection threads and in freshly released rhizobia in nodule cells. Freshly released bacterial cells were loosely surrounded by peribacteriod membrane (Pbm) (Plate 17B). The cytoplasm of the rhizobial bacteria in the interzone was electron dense (Plate 17A) whereas the cytoplasm of the bacteroids in nitrogen fixation zone (Plates 16C and 18A) was heterogeneous in the sense that it contained electron dense and electron transparent regions. Different shapes of rhizobial bacteria were seen in the infection and interzone areas. Most of the bacteroids in the nitrogen fixation zone were elongated (Plate 16C and 18A).

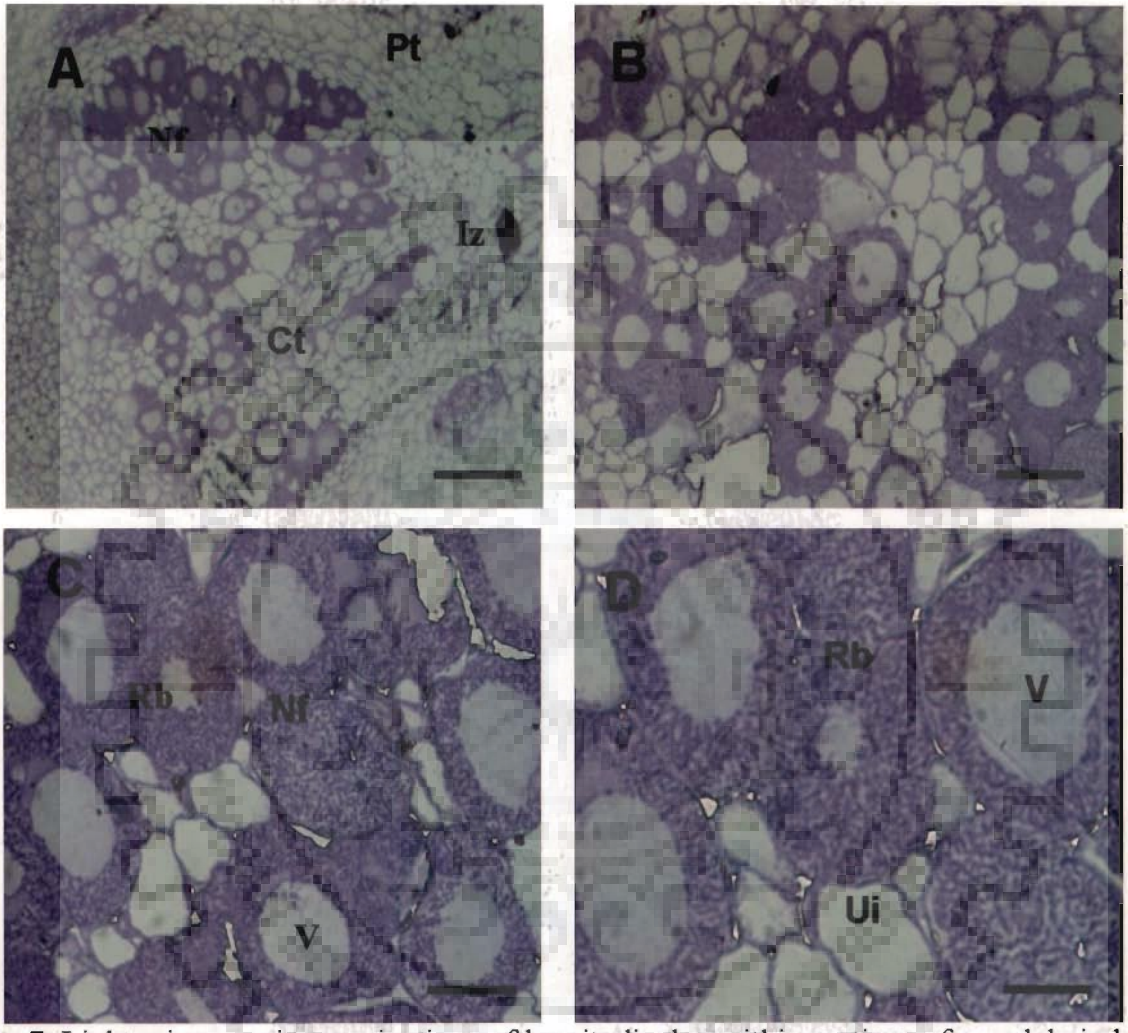

Plate 7: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by IITR3, an *argF/argI* mutant of *Sinorhizobium meliloti* Rm1021, **A.** A nodule section showing distinct peripheral tissue (Pt), inter zone and nitrogen fixation zone (Nf) in the central tissue, Bar: 250μm (40x), **B**. A part of nitrogen fixation zone showing heavily infected cells (I), Bar: 100 μm (100x), **C**. Nitrogen fixation zone showing cells packed with rhizobial bacteria (Rb) and vacuoles (V) in some cells, Bar:50 μm (200x), and **D.** Part of nitrogen fixation zone (Nf) showing Rhizobial bacteria (Rb) packed in many nodule cells and also showing few uninfected cells (Ui), Bar: 25 μm (400x).

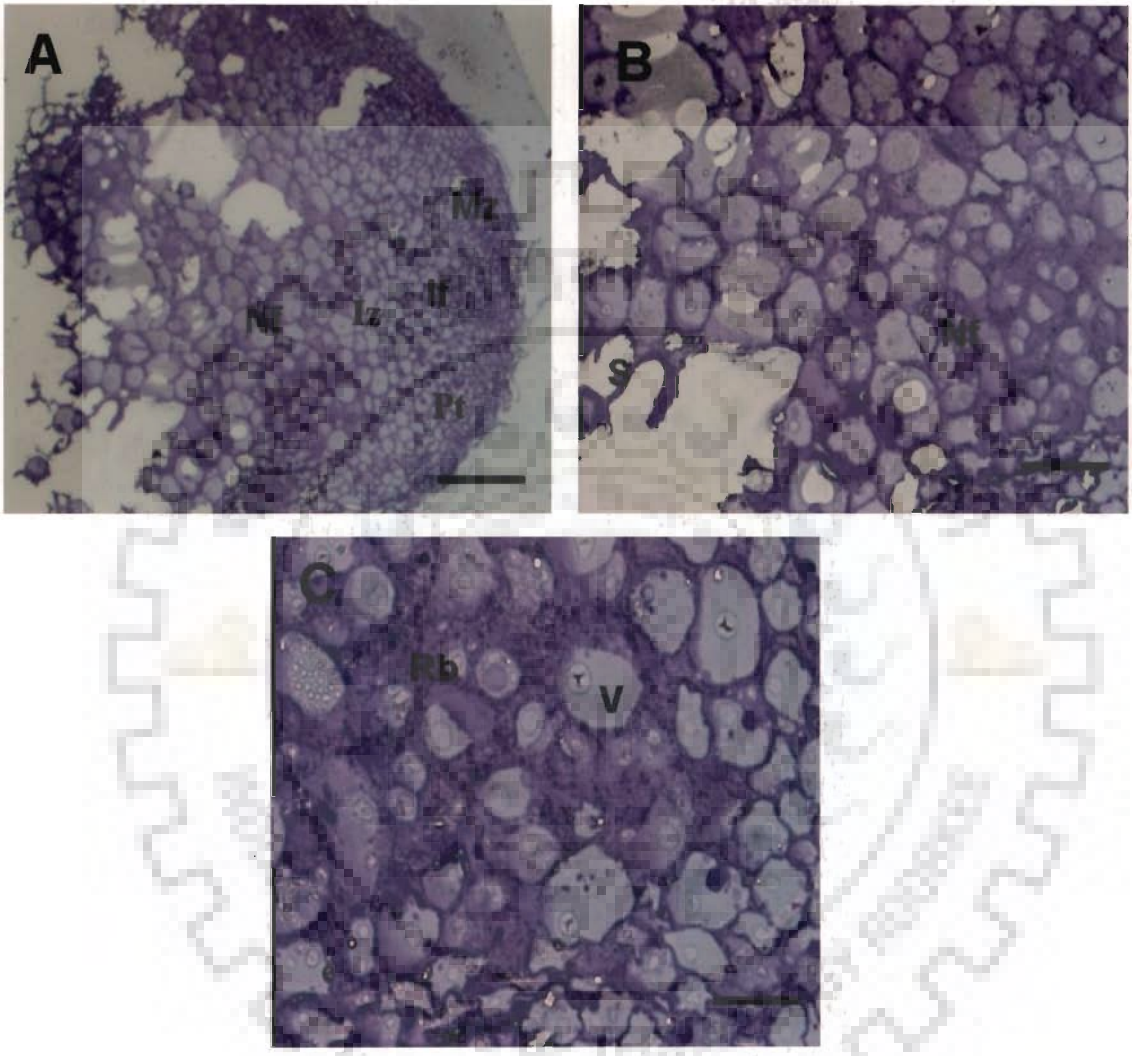

Plate 8: Light microscopic examinations of longitudinal- semithin sections of a nodule induced by IITR7, an *argH* mutant of *Sinorhizobium meliloti* Rm1021, **A**. A nodule section showing distinct peripheral tissue (Pt), meristematic zone (Mz), infection zone (If) and inter zone (Iz) in the central tissue, Bar: 250µm (40x), **B.** A part of the nodule showing nitrogen fixation zone (Nf) and senescence zone (S), Bar: 100µm (100x), **C.** A part of nitrogen fixation zone showing rhizobial bacteria (Rb), Bar: 50µm (200x).

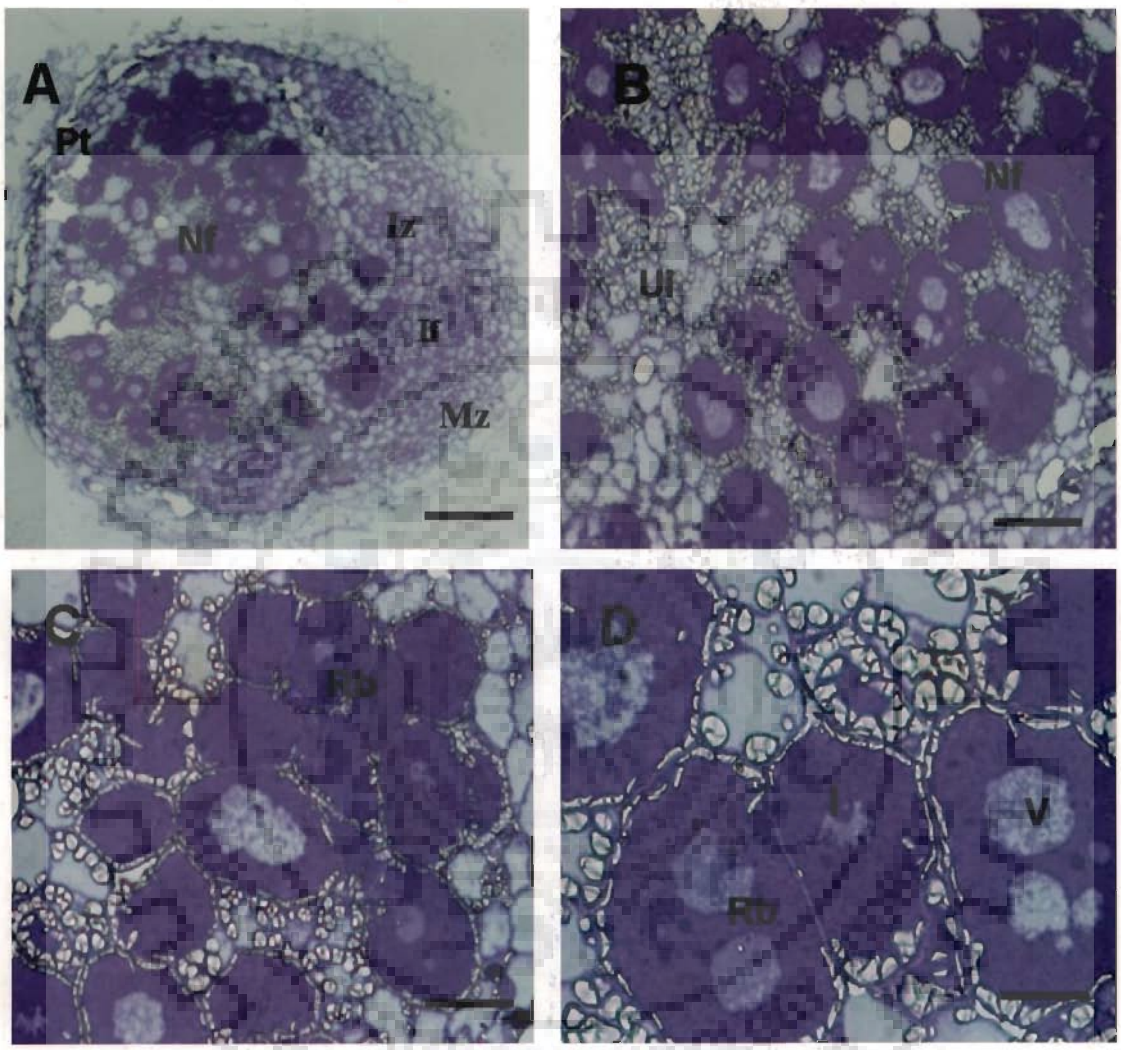

Plate 9: Light microscopic examinations of longitudinal–semithin sections of a nodule induced by IITR9, an *argF/argI* mutant of *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A.** A complete section of nodule showing distinct peripheral tissue (Pt), meristematic zone (Mz), infection zone (If), Inter zone (Iz) and nitrogen fixation zone (Nf), Bar: 250 um (40x), **B**. A part of the section showing nitrogen fixation zone (Nf) and unifected cells in central tissue, Bar: 100 um (100x), **C**. Nitrogen fixation zone showing cells packed with bacteriods (B), Bar:50 µm (200x) and **D.** Rhizobial bacteria (Rb) packed in many cells, arranged in perpendicular to a central vacuole (V), Bar: 25 µm (400x).

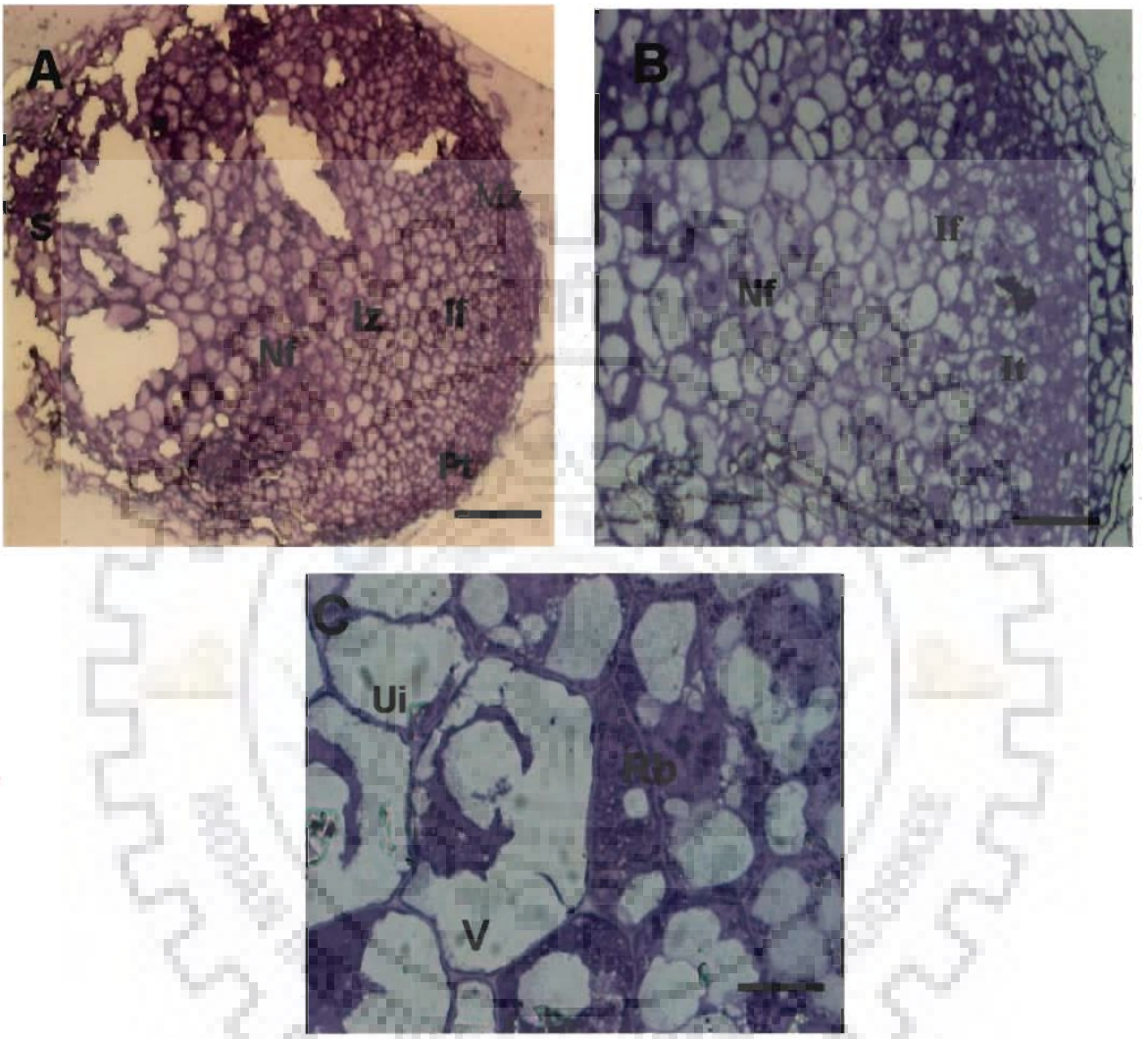

Plate 10: Light microscopic examinations of longitudinal- semithin sections of a nodule induced by IITR11, an *arg*H mutant of *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A**. A complete nodule section showing distinct peripheral tissue (Pt), meristematic zone (Mz), infection zone (If) , inter zone (Iz), nitrogen fixation zone (Nf) and senescence zone (S), Bar: 250µm (40x), **B.** A part of the nodule showing network of infection threads (It) in the nitrogen fixation zone (Nf), Bar: 100µm (100x), **C.** Portion of the nodule showing rhizobial bacteria (Rb) packed in many cells, arranged perpendicularly to the vacuole (V) and few uninfected cells (Ui), Bar: 25 µm (400x).

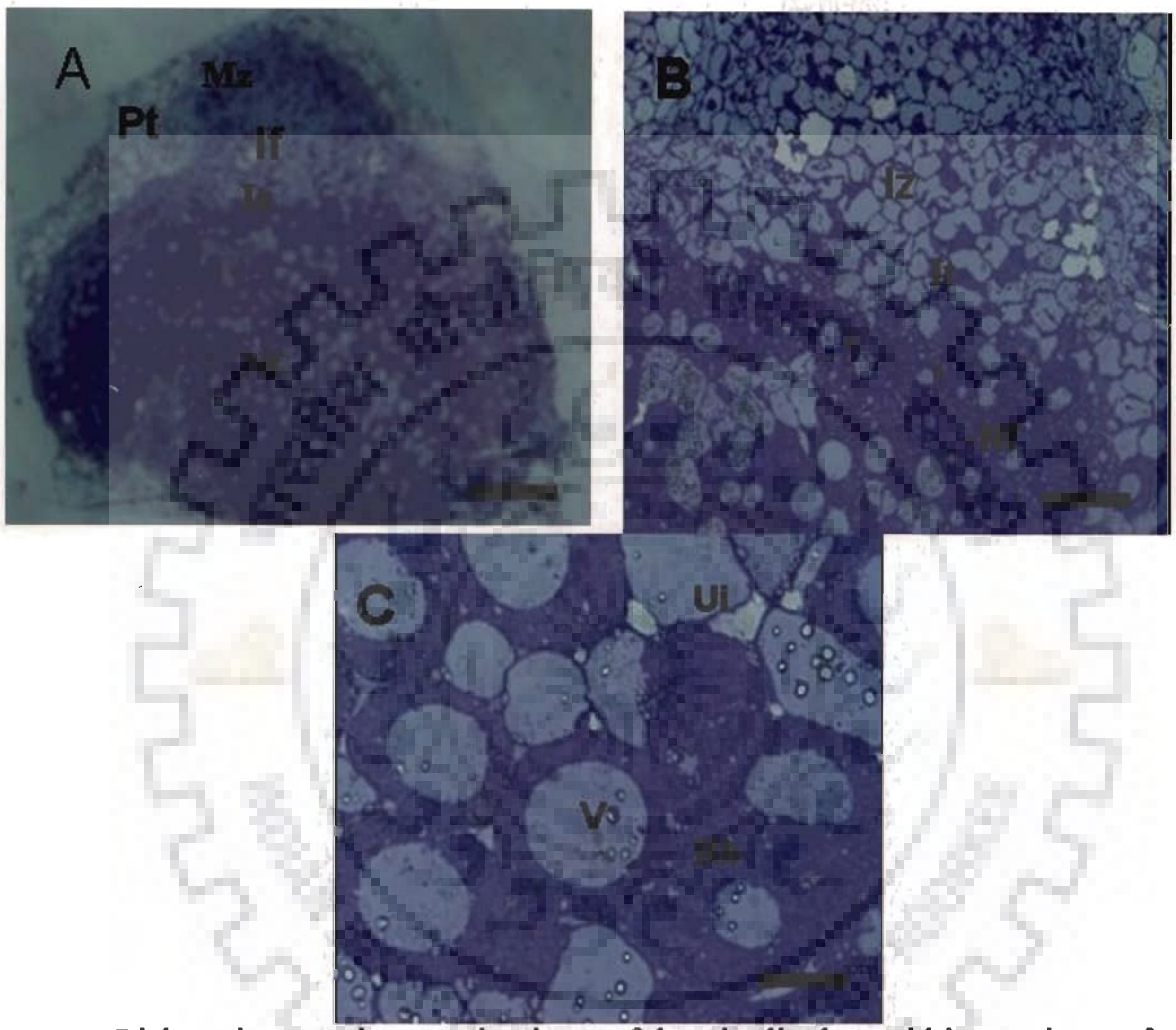

Plate 11: Light microscopic examinations of longitudinal–semithin sections of a nodule induced by IITR13, an *argF/argI* mutant of *R. leguminosarum* bv. *viciae* Rlv1, **A.** A complete section of the nodule showing distinct peripheral tissue (Pt), meristematic zone (Mz), infection zone (If), and nitrogen fixation zone (Nf), Bar: 250 µm (40x), **B.** A part of the nodule section showing network of infection threads (It), and nitrogen fixation zone (Nf), Bar: 100 µm (100x), and C. A part of nodule showing rhizobial bacteria (Rb) packed in cells of nitrogen fixation zone (Nf), vacuole (V) and some uninfected cells (Ui), Bar: 25 µm (400x).

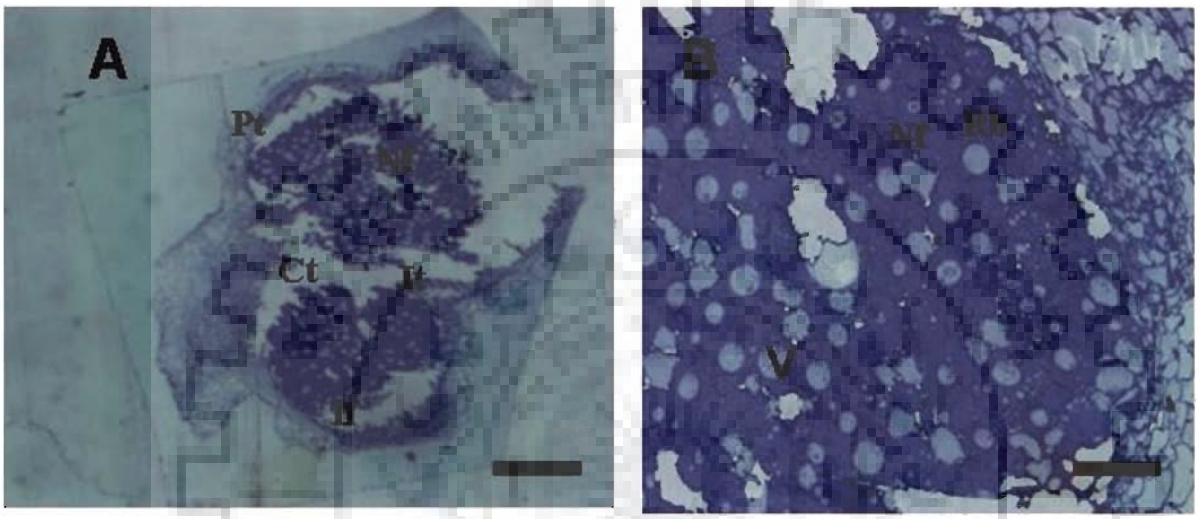

Plate 12: Light microscopic examinations of longitudinal–semithin sections of a nodule induced by IITR15, an *argH* mutant of *Rhizobium leguminosarum* bv. *viciae* Rlv1, **A.** A complete section of the nodule showing distinct peripheral tissue (Pt) and central tissue (Ct) containing infection zone (If), inter zone (Iz) and nitrogen fixation zone (Nz), Bar: 250 µm (40x), **B.** A part of the nodule section showing cells of nitrogen fixation zone (Nf) packed with rhizobia (Rb) around the vacuole (V); few uninfected cells, Bar: 50 µm (200x).

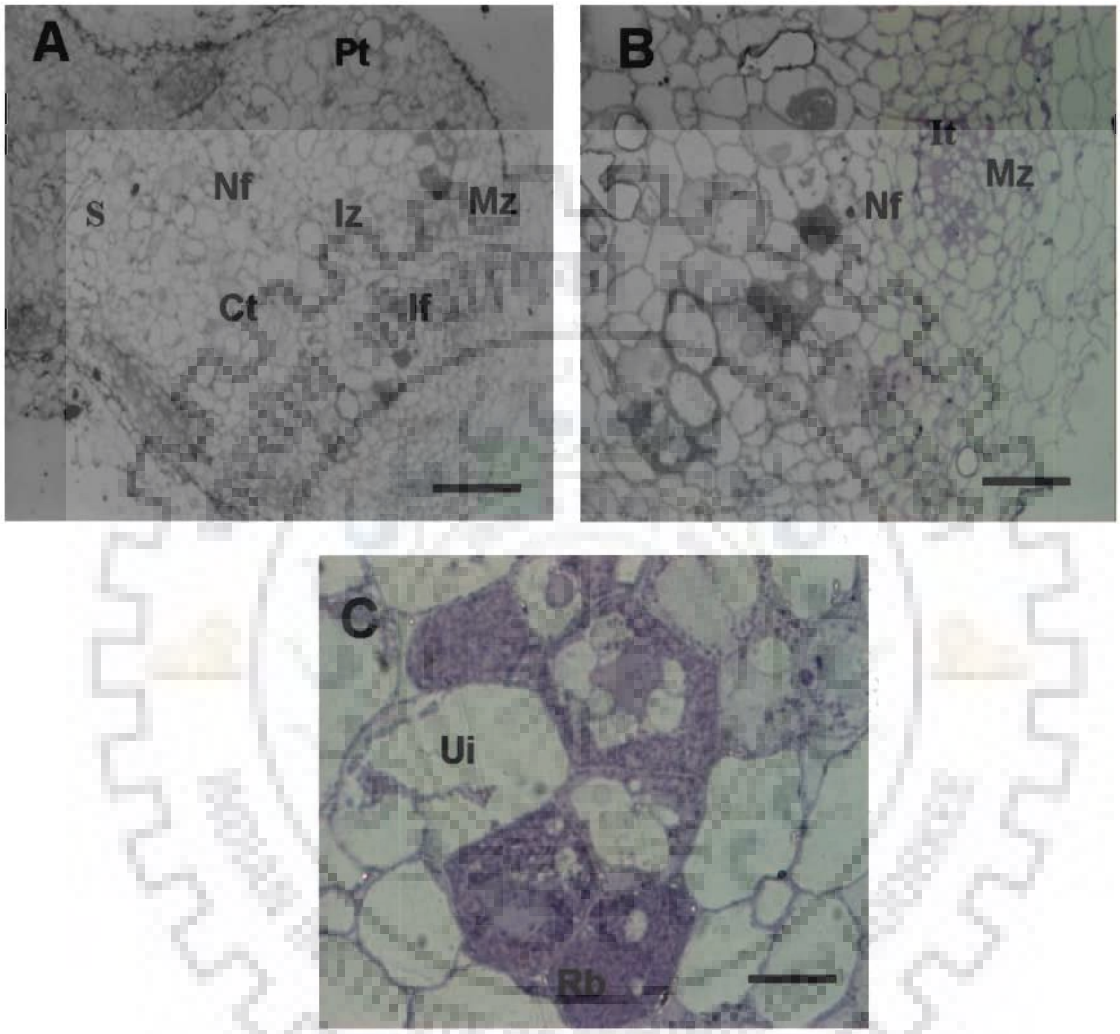

Plate 13: Light microscopic examinations of longitudinal- semithin sections of a nodule induced by IITR1, an *argA/argB/argC/argD/argE* mutant of *Sinorhizobium meliloti* Rm1021, **A**. A nodule section showing distinct peripheral tissue (Pt), inter zone (Iz) central tissue (Ct), infection zone (If) Bar: 250 µm (40x), **B**. A part of the meristematic zone (Mz) showing infection thread (It), Bar: 50 µm (200x), and **C**. Cells of infection zone showing rhizobial bacteria (Rb) and some vacant uninfected cells (Ui), Bar: 25 µm (400x)

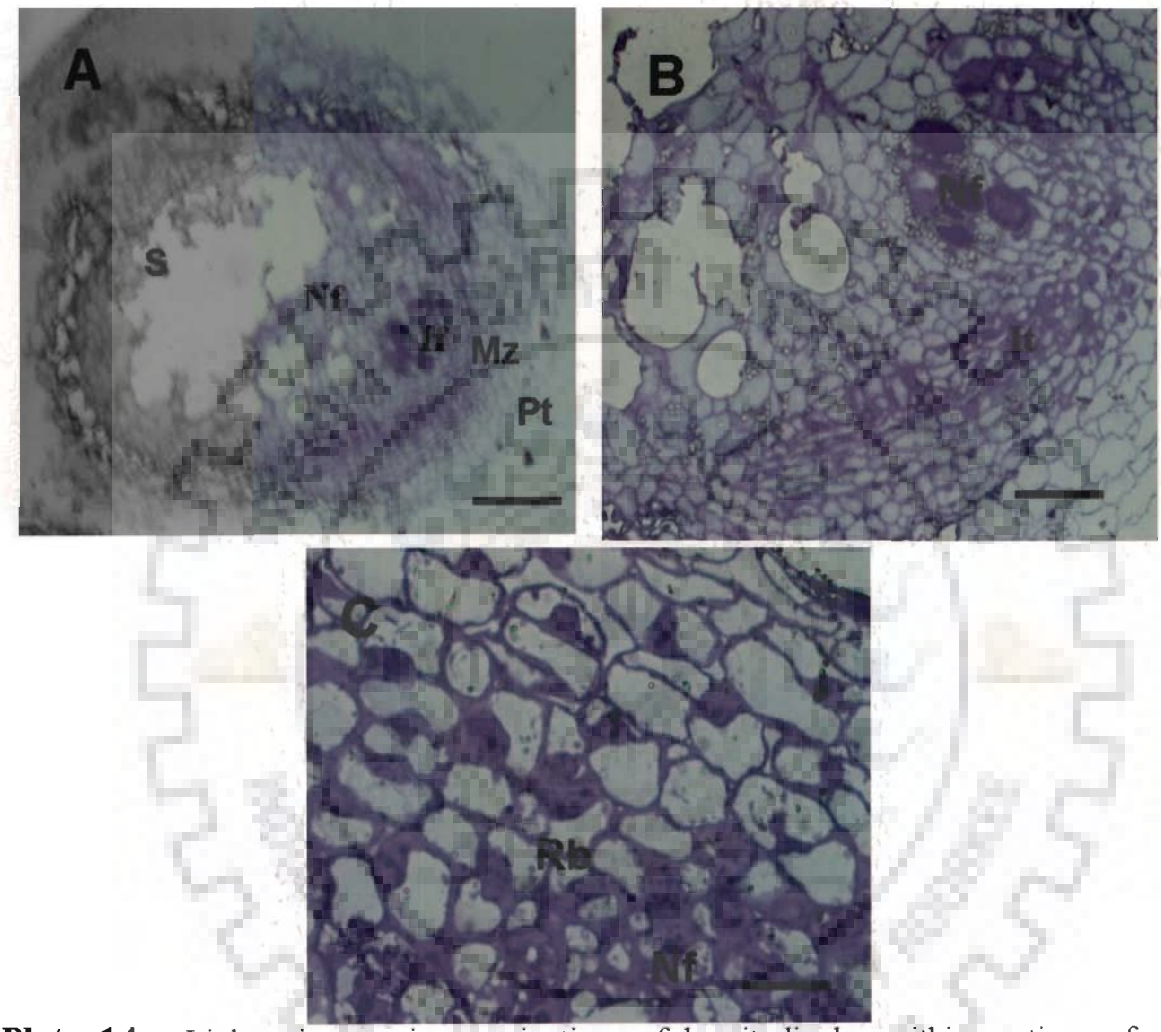

Plate 14 : Light microscopic examinations of longitudinal–semithin sections of a nodule induced by IITR8, an *argA/argB/argC/argD/argE* mutant of *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A.** A complete section of nodule showing distinct peripheral tissue (Pt), meristematic zone (Mz) and central tissue (Ct) containing infection zone (If), nitrogen fixation zone (Nf) and an extended senescence zone (S), Bar: 250 um (40x), **B.** A part of the nodule section showing network of infection threads (It), and nitrogen fixation zone (Nf) , Bar: 100 um (100x), **C.** A part of the nodule showing nitrogen fixation zone (Nf) containing cells packed with rhizobial bacteria (Rb) , Bar: 50 um (200x).

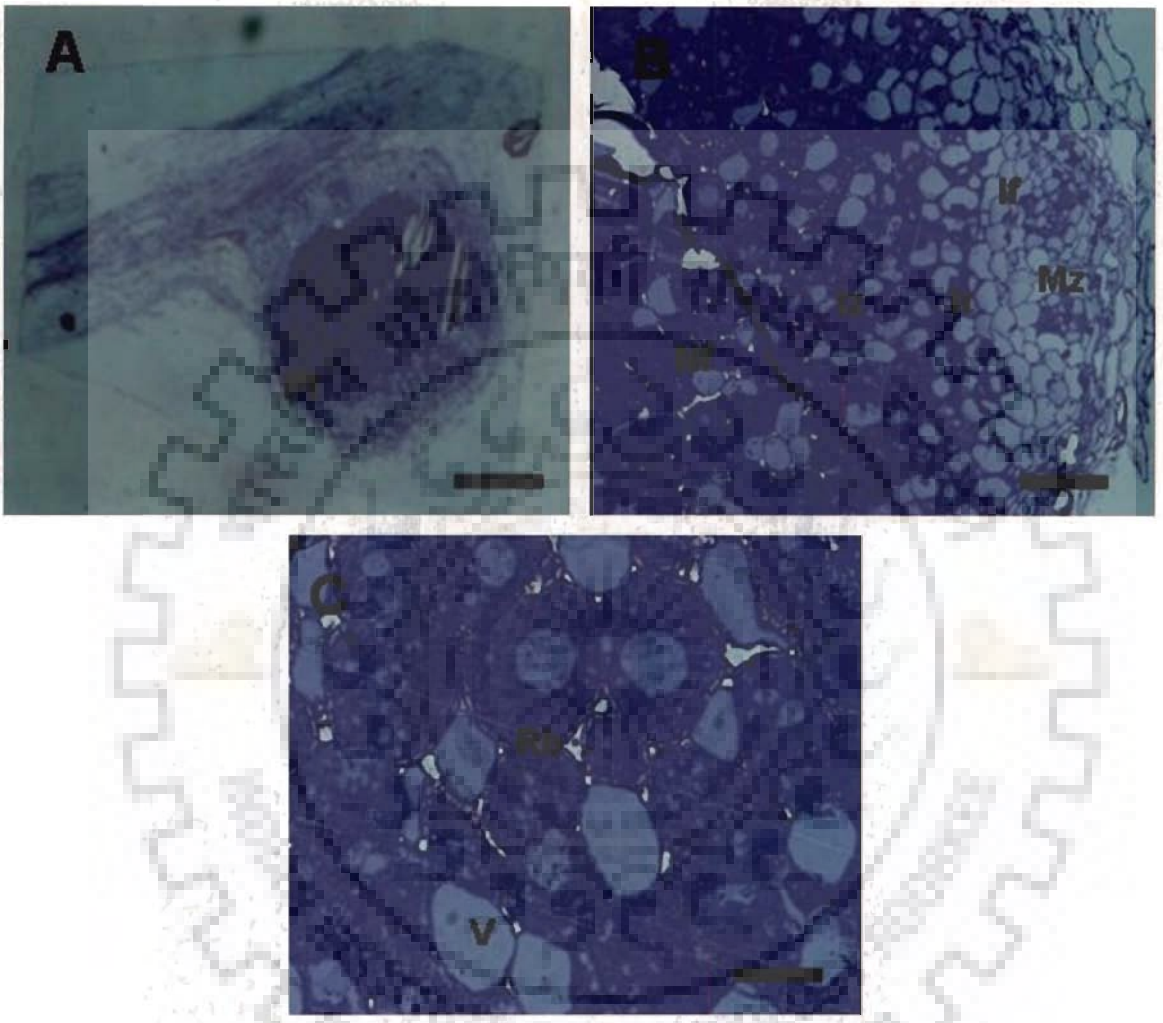

Plate 15: Light microscopic examinations of longitudinal–semithin sections of a nodule induced by IITR12, an *argA/argB/argC/argD/argE* mutant of *R. leguminosarum* bv. *viciae* Rlv1, **A.** A complete section of nodule showing distinct peripheral tissue (Pt) and central tissue (Ct), Bar: 250 µm (40x), **B.** A part of the nodule showing meristematic zone (Mz), infection zone (If), network of infection threads (It), inter zone (Iz) and nitrogen fixation zone (Nf), Bar: 100 µm (100x), and **C.** Enlarged section showing rhizobial bacteria (Rb) present in many cells around the centrally located vacuole (V), Bar: 25 µm (400x).

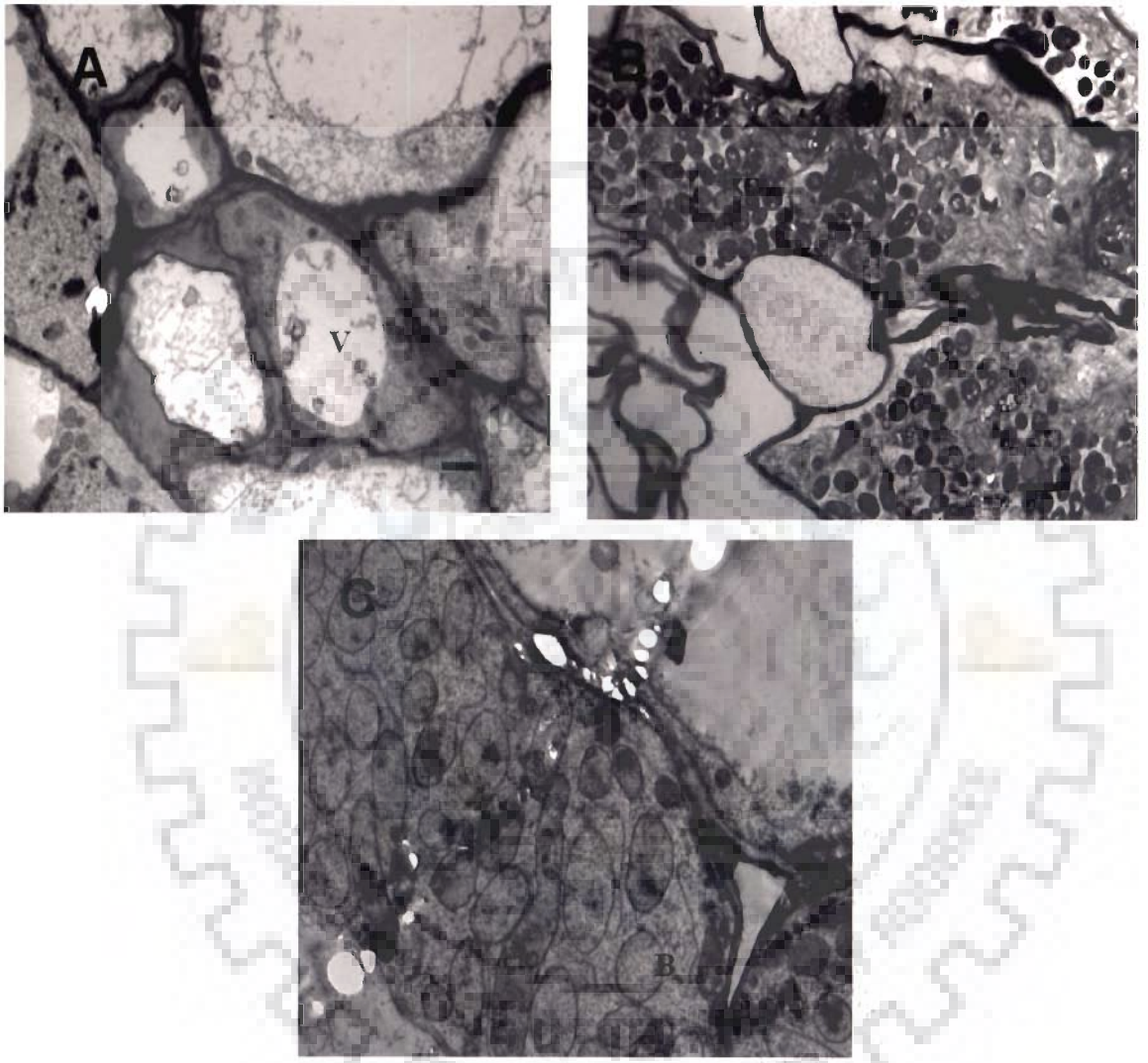

Plate 16 : Transmission electron microscopic examinations of longitudinal–ultrathin sections of a nodule induced by *Sinorhizobium meliloti* strain Rm1021, **A**. Nodule cells of nitrogen fixation zone showing peripheral arrangement of cell organelles around the vacuole (V), Bar : 1 µm (880X), **B.** Cells of inter zone showing freshly released rhizobial bacteria (Rb), Bar : 1 µm (880X) and **C.** Parts of nodule cells in the nitrogen fixation zone (Nf), showing some electron transparent bacteriods (B) and some bacteroids with heterogeneous cytoplasm (Hc), Bar : 1 µm (1100X).

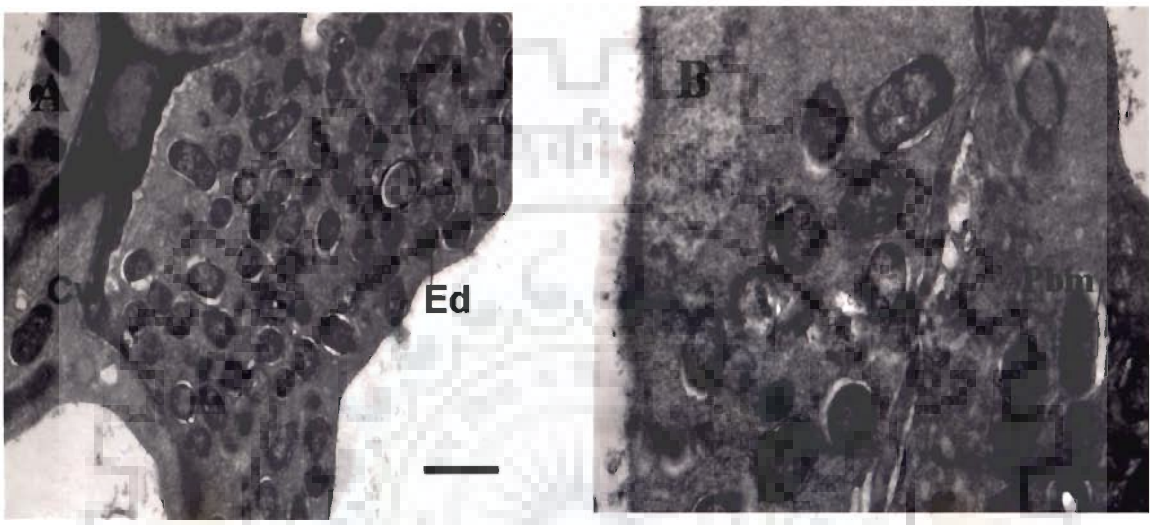

Plate 17: Transmission electron microscopic examinations of longitudinal–ultrathin sections of a nodule induced by *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A.** Parts of nodule cells showing freshly released, electron dense (Ed) rhizobia in inter zone (Iz), Bar: 1 μm (1400X) and **B.** Spherical bacteroids showing peribacteroid membrane (Pbm) in the inter zone, Bar: 1 μm (2800X).

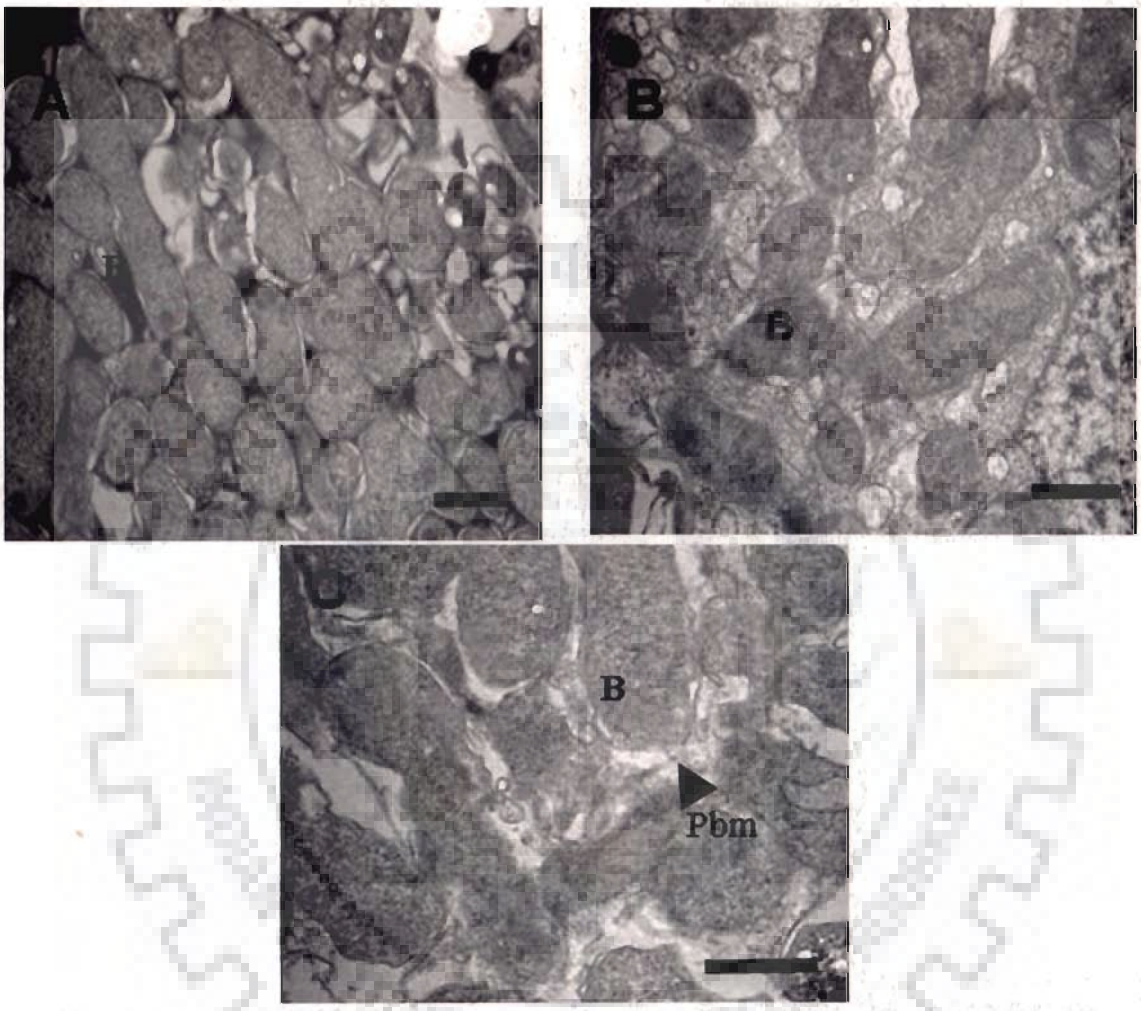

Plate 18: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by *Rhizobium leguminosarum* bv. *viciae* Rlv1, **A.** A part of nodule cell of nitrogen fixation zone (Nf) containing elongated and mature bacteroids (B) having electron dense and electron transparent cytoplasm Bar : 1 µm (1800X). **B.** A part of nodule cell showing bacteroids (B) having heterogeneous cytoplasm, 1 µm (2800X) and **C.** Elongated bacteroids (B) of nitrogen fixation zone bound within a peribacteroid membrane (Pbm), Bar: 1 µm (4400X).

The ultrastructures of the nodules induced by the *argF/argI* (*S. meliloti* IITR3, IITR4 and IITR5, *R. leguminosarum* bv. *trifolii* IITR9 and *R. leguminosarum* bv. *viciae* IITR13 and IITR14) and *argH* (*S. meliloti* IITR6 and IITR7, *R. leguminosarum* bv. *trifolii* IITR10 and IITR11 and *R. leguminosarum* bv. *viciae* IITR15) auxotrophs were almost similar to that of the nodules induced by the parental strains (Plates 19, 20,21,22,23 and 24). The TEM studies of the ultrathin sections induced by the ornithine auxotrophs (Plates 25, 26 and 27) showed the rupturing of Pbm (Plate 25C) in many bacteroids in the nitrogen fixation zone and bacteroidal cytoplasm had more electron transparent regions in comparison to that of the parental strains.

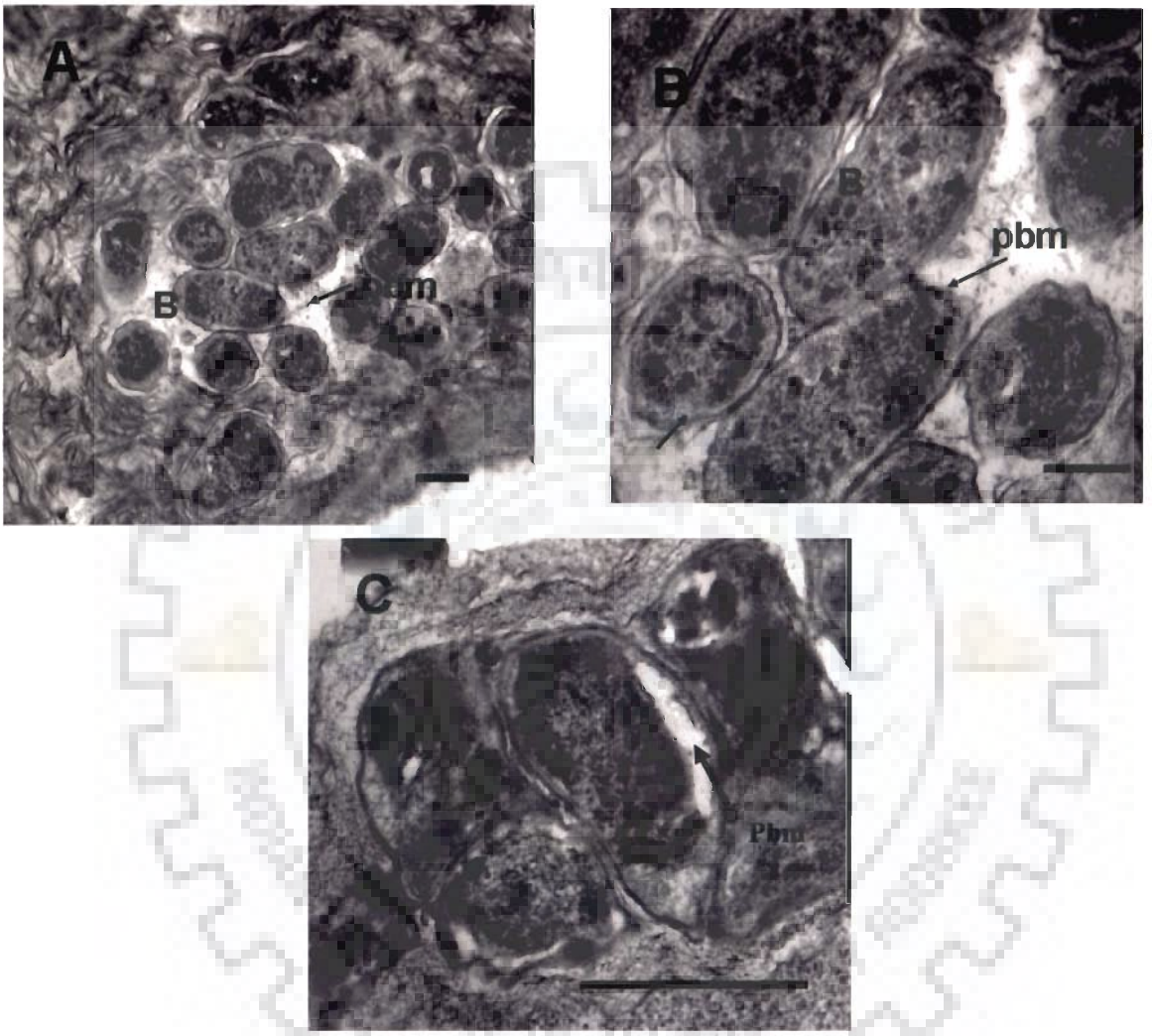

Plate 19: Transmission electron microscopic examinations of longitudinal–ultrathin sections of a nodule induced by IITR3, an *argF/argI* mutant of *Sinorhizobium meliloti* Rm1021, **A.** A part of nodule cell of the inter zone showing bacteroids (B), Bar: 1 µm (1400X), **B**.A part of nodule cell showing electron dense bacteroids (B), Bar: 1µm(3500X) and **C.** Mature bacteroids showing electron dense region and loosely bound peribacteroid membrane (Pbm), Bar: 1 µm (7100X).

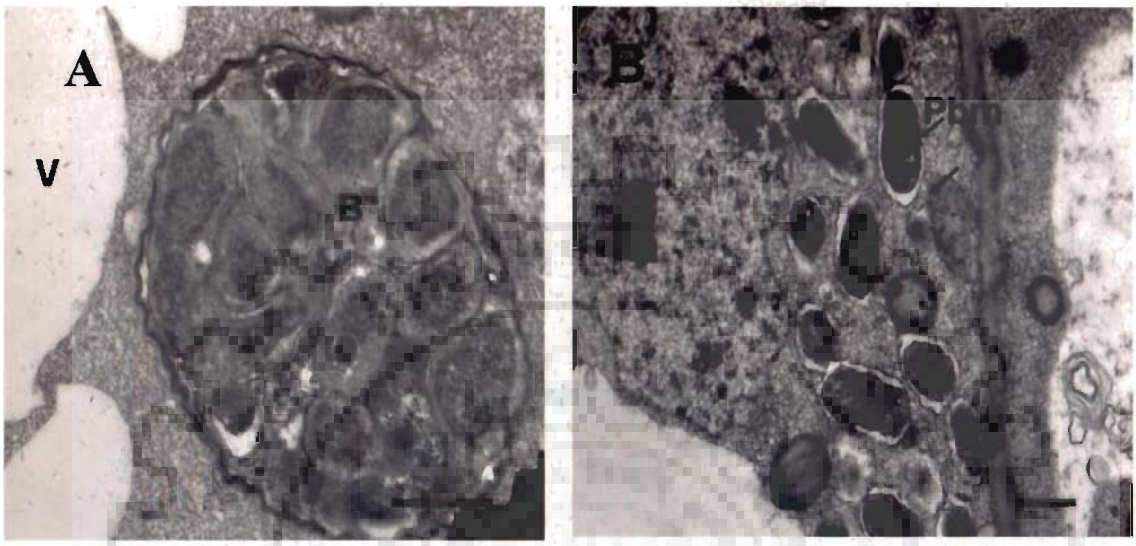

Plate 20: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by IITR7, an *argH* mutant of *Sinorhizobium meliloti* Rm1021, **A.** Rhizobial bacteria (Rb) in the infection thread, Bar: 1 μm (3500X), **B.** A part of the nodule cell of interzone showing spherical and elongated bacteroids, Bar:1 μm (2200X).

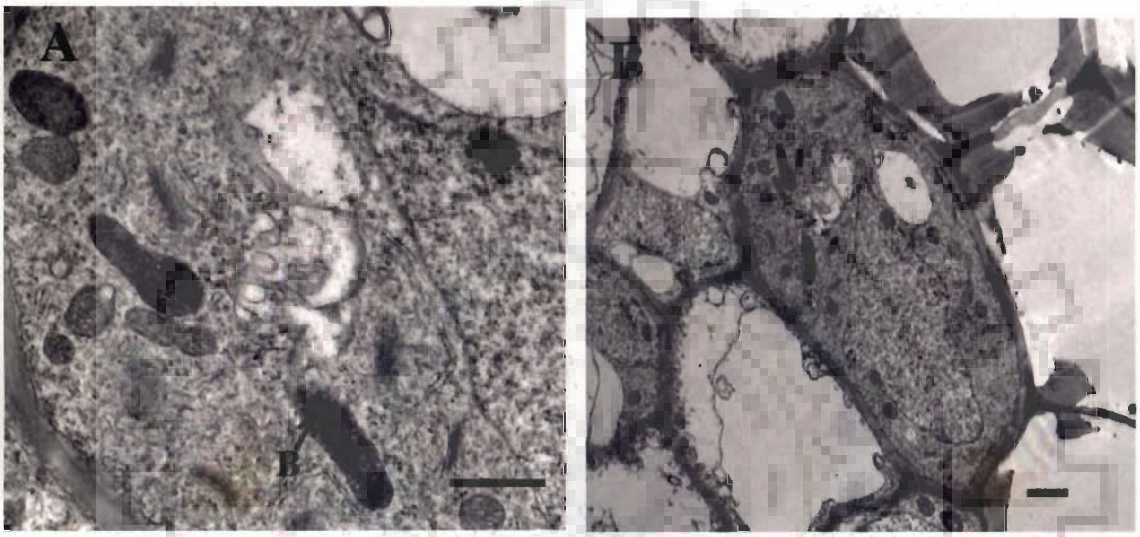

Plate 21: Transmission electron microscopic examinations of longitudinal–ultrathin sections of nodule induced by IITR9, an *argF/argI* mutant of *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A.** A part of nodule cell of nitrogen fixation zone showing polymorphic bacteroids (B), Bar : 1 μm (2800X)., **B.** A part of nodule showing uninfected cells in nitrogen fixation zone, Bar: 1 μm (880X).

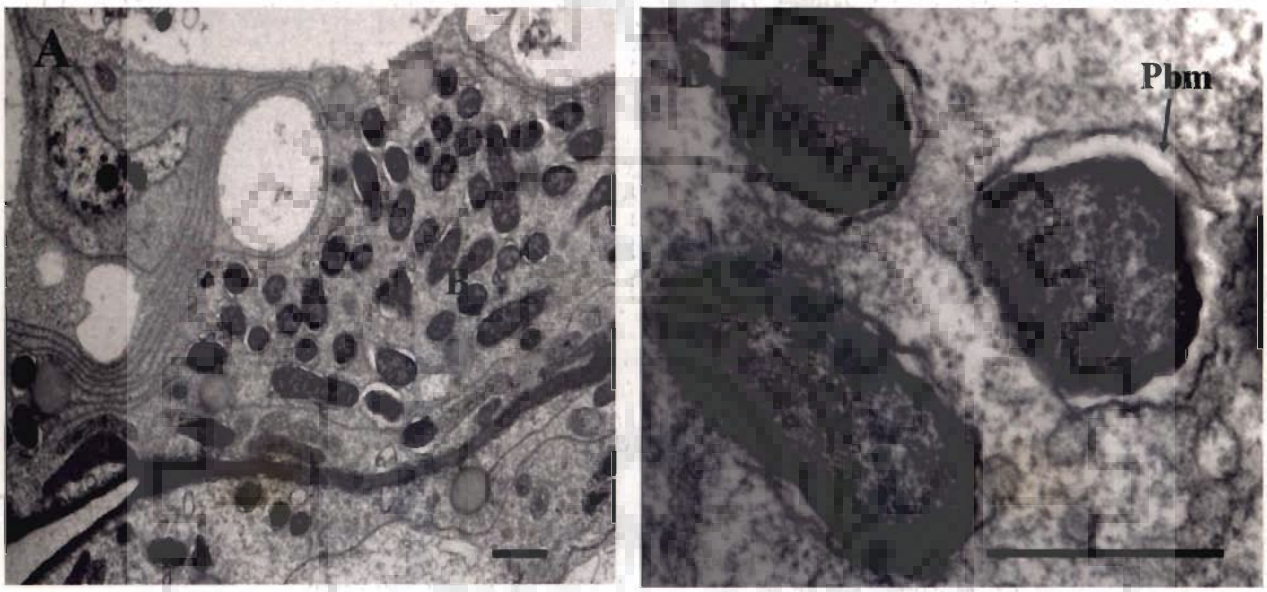

Plate 22: Transmission electron microscopic examinations of longitudinal–ultrathin sections of nodule induced by IITR11, an *argH* mutant of *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A**. Nodule cells showing electron dense and polymorphic bacteroids and cell organelles, Bar: 1 μm (1100X) and **B.** Nodule cells of the nitrogen fixation zone (Nf) showing electron dense and electron transparent bacteroids (B), and peribacteroid membrane, Bar: 1 μm (8900X) .

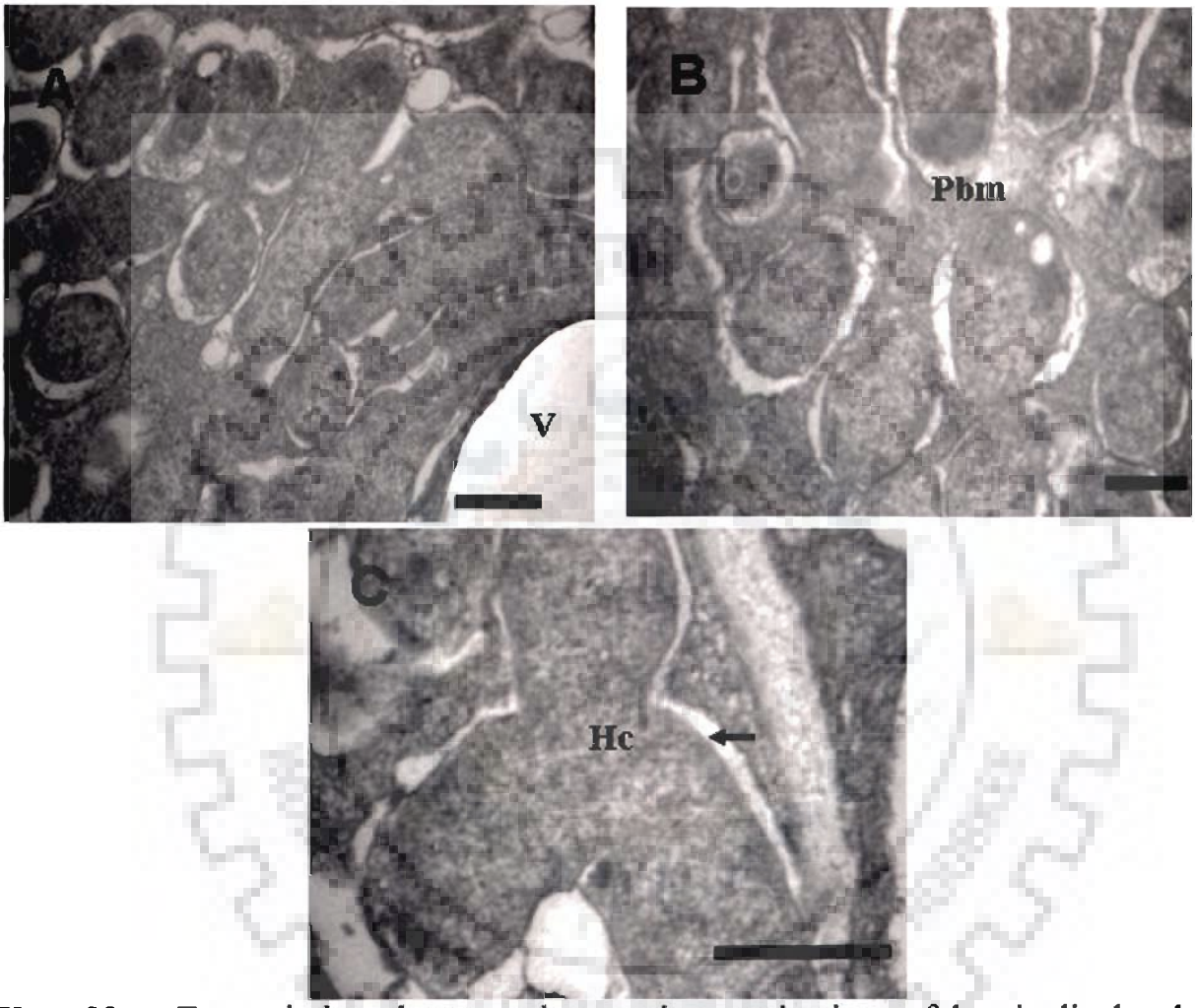

Plate 23 : Transmission electron microscopic examinations of longitudinal –ultrathin sections of a nodule induced by IITR13, an *argF/argI* mutant of *Rhizobium leguminosarum* bv. *viciae* Rlv1 **A.** A part of nodule cell of nitrogen fixation zone having bacteroids, Bar: 1 µm (2200X), **B.** Mature bacteroids within the peribacteroidal membrane (Pbm) , Bar : 1 µm (2800X) and C. A part of nodule cell showing a convoluted bacteroid with heterogeneous cytoplasm (Hc), Bar: 1 µm (5600X).

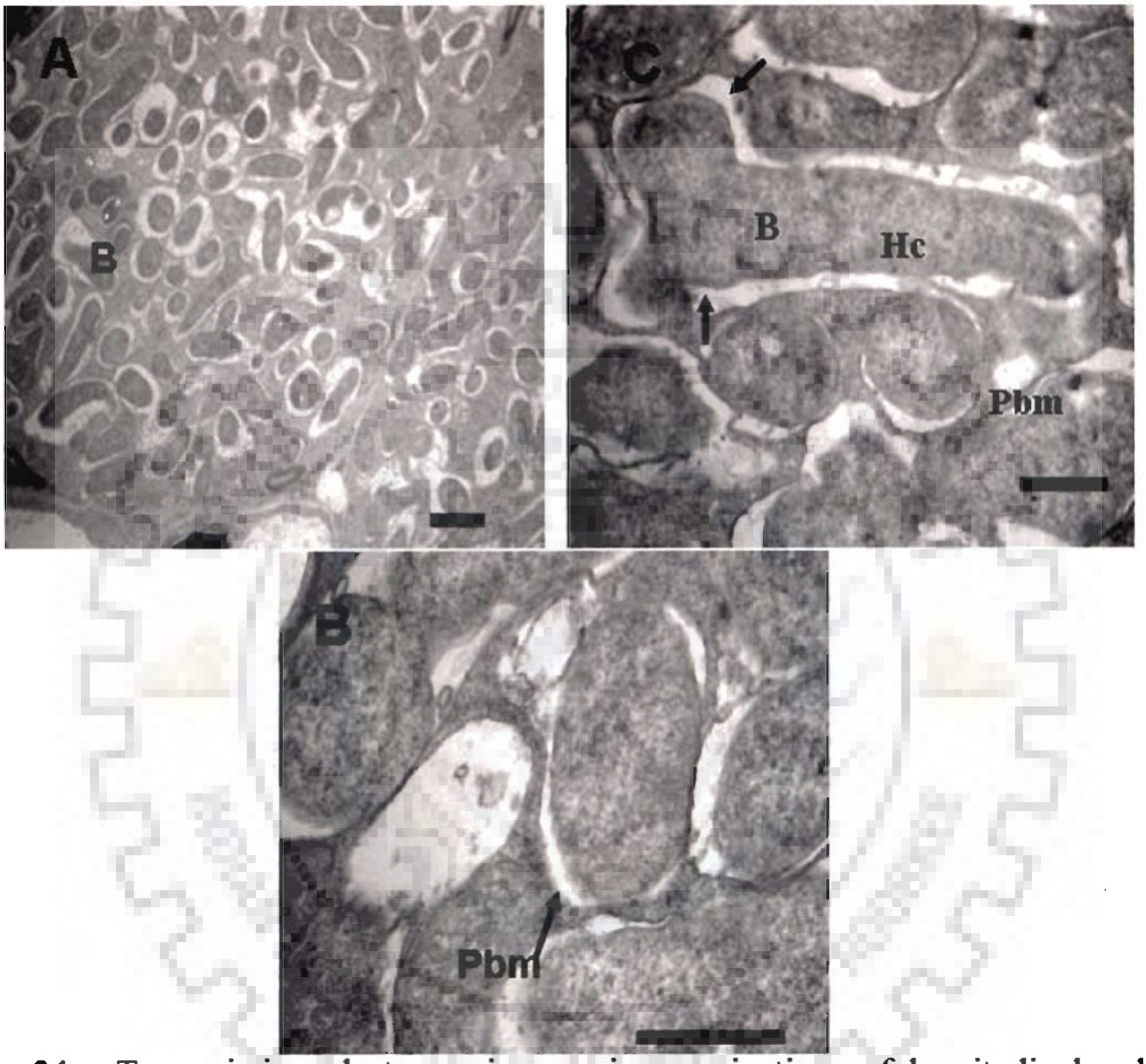

Plate 24: Transmission electron microscopic examinations of longitudinal –ultrathin sections of a nodule induced by IITR15, an *argH* auxotroph of *Rhizobium leguminosarum* bv. *viciae* Rlv1, **A.** nodule cell of the inter zone showing bacteroids (**B**), Bar :1 µm (1400X) **B.** A nodule cell showing polymorphic bacteroids having heterogeneous cytoplasm (Hc) and peribacteroid membrane (Pbm), 1µm (3500X) and **C.** A part of nodule cell of senescence zone showing degenerating bacteroids, Bar: 1 µm (5600X).

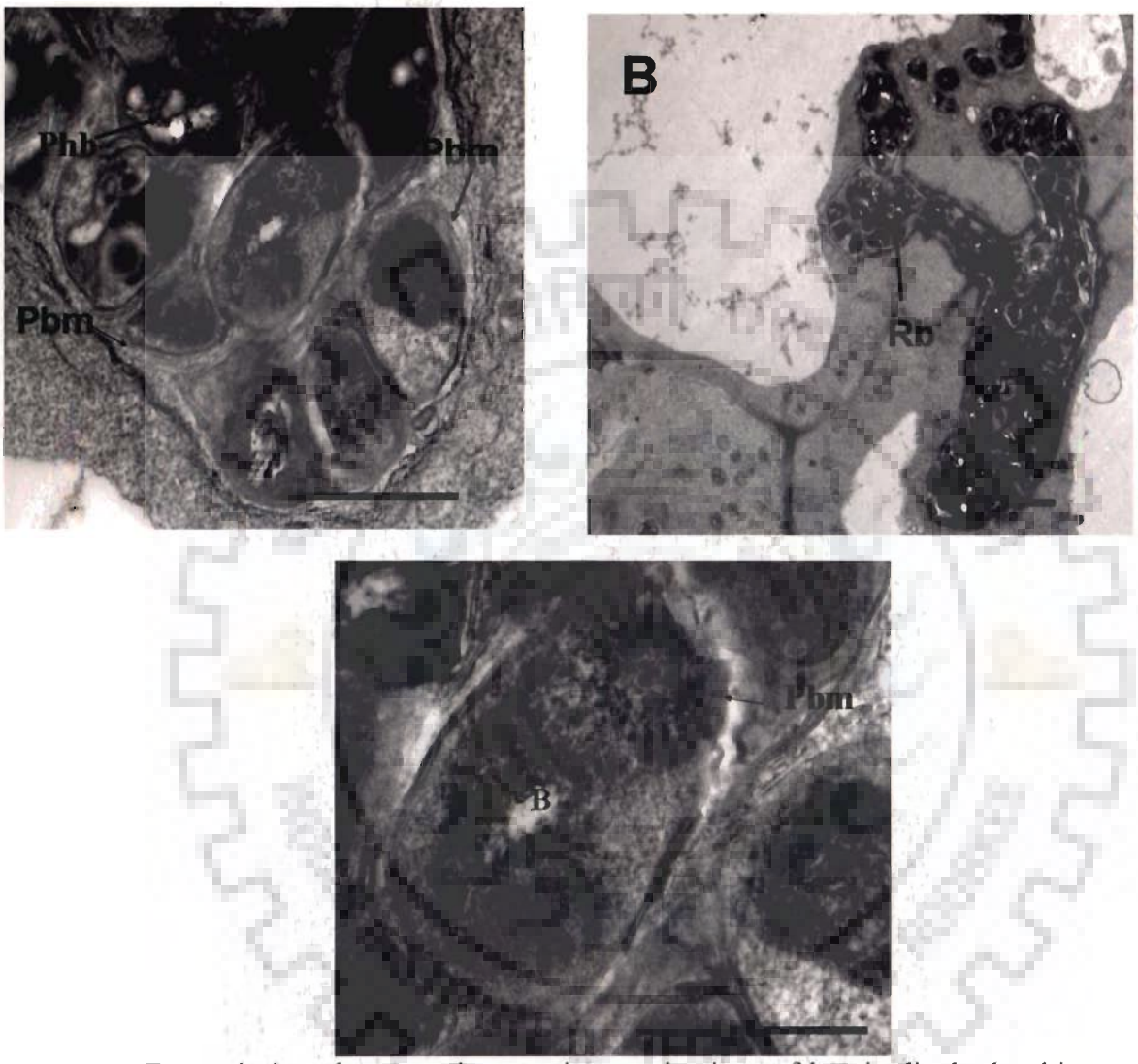

Plate 25 : Transmission electron microscopic examinations of longitudinal–ultrathin sections of nodule induced by IITR1, an *argA/argB/argC/argD/argE* mutant of *Sinorhizobium meliloti* Rm1021, **A.** A part of infection zone showing rhizobial bacteria (Rb) in the infection thread, Bar: 1 μm (5600X), **B.** A part of infection zone showing freshly released rhizobial bacteria (Rb), Bar : 1 μm (880X), and **C.** A part of nodule cell in the nitrogen fixation zone showing a bacteroid with broken peribacteroid membrane (Pbm), Bar: 1 μm (11000X).

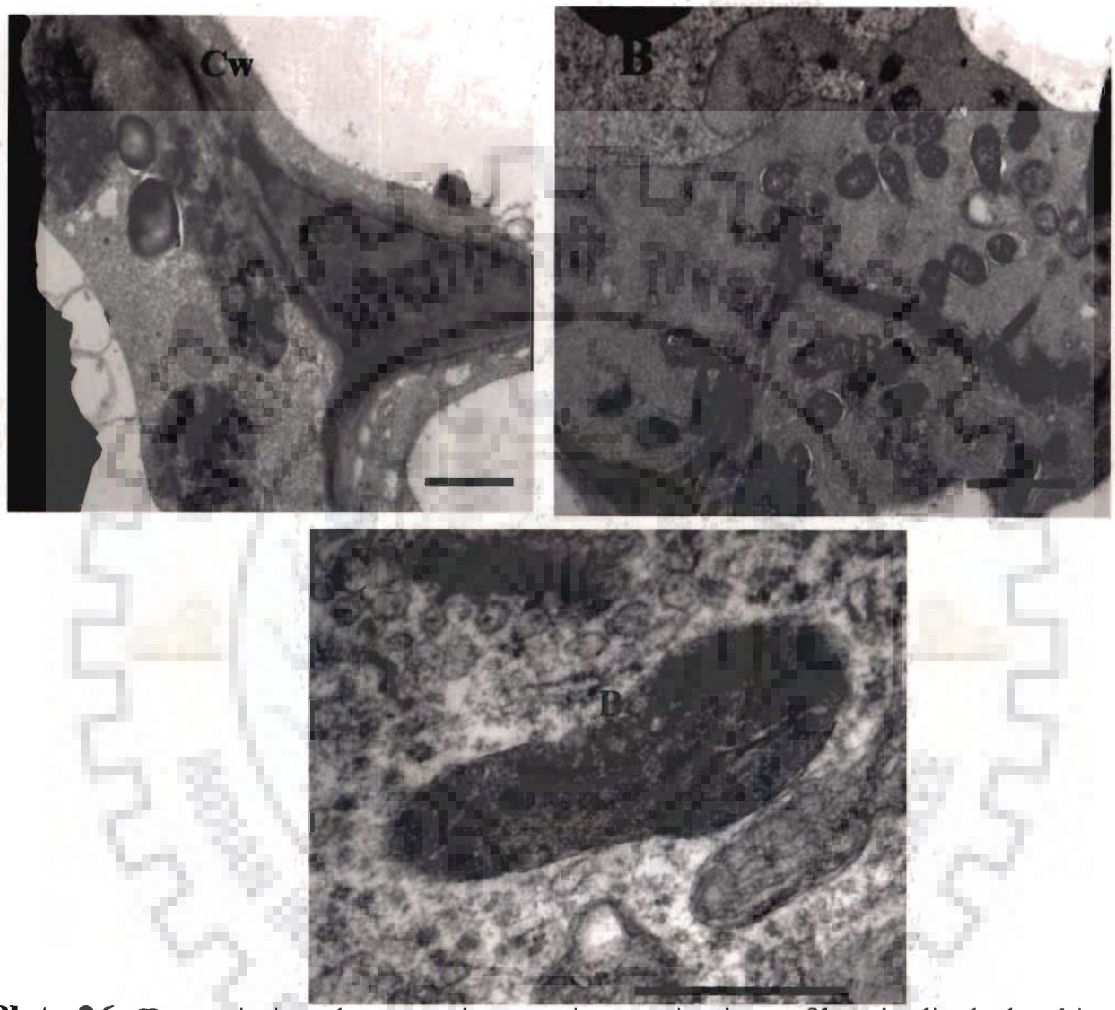

Plate 26: Transmission electron microscopic examinations of longitudinal-ultrathin sections of nodule induced by IITR8, an *argA/argB/argC/argD/argE* mutant of *Rhizobium leguminosarum* bv. *trifolii* MTCC905, **A.** Nodule cells of infection zone showing electron dense, rhizobial bacteria (Rb), Bar : 1 μm (2200X), **B.** Part of nodule cell of nitrogen fixation zone showing very less number of bacteroids, Bar: 1 μm (3500X) and **C.** Portion of the nodule cell of nitrogen fixation zone showing bacteroids having electron transparent and electron dense regions, Bar : 1 μm (8900X).

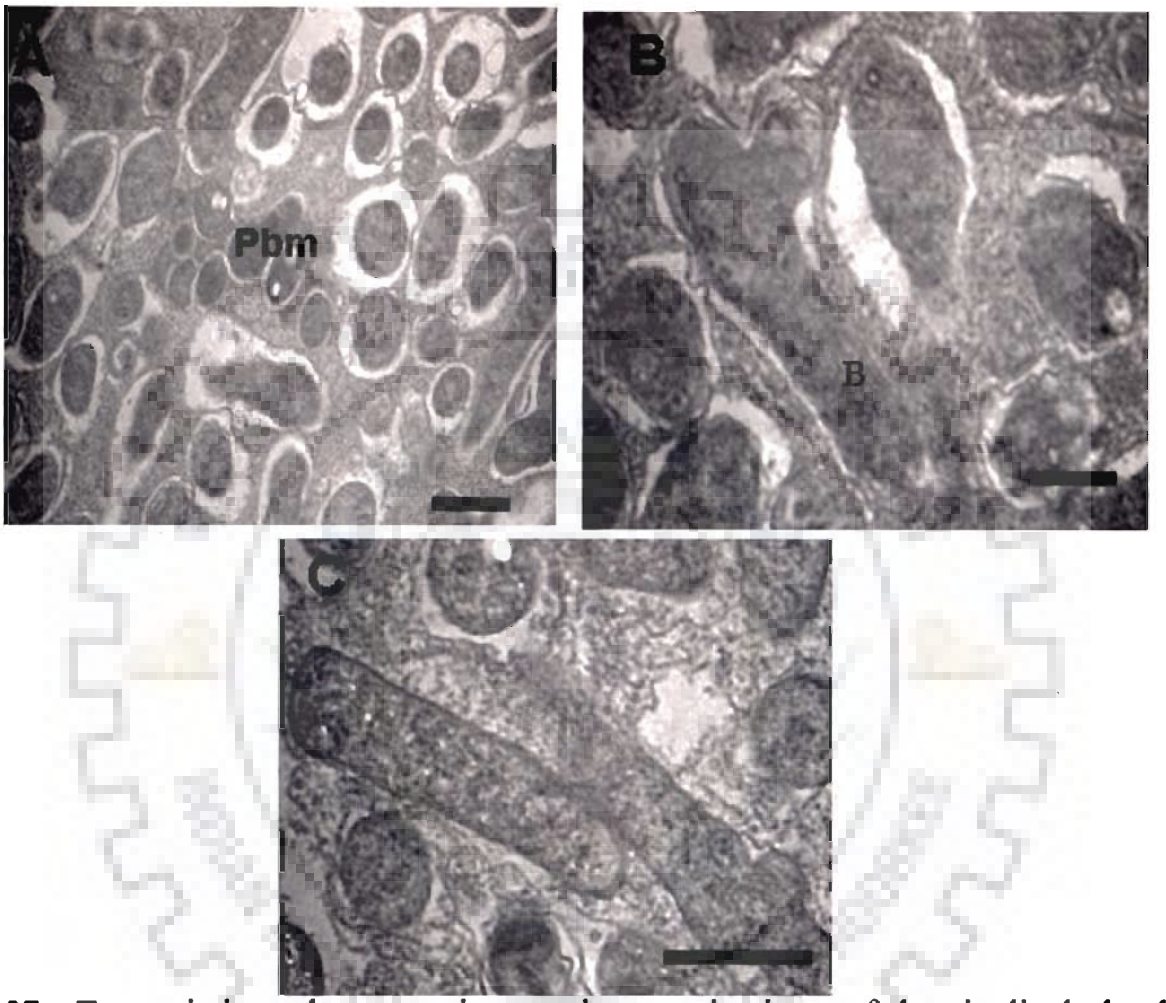

Plate 27: Transmission electron microscopic examinations of longitudinal-ultrathin sections of nodule induced by IITR12, an *argA/argB/argC/argD/argE* mutant of *Rhizobium leguminosarum* bv *viciae* Rlv1, **A.** A nodule cell of nitrogen fixation zone (Nf) showing bacteroids bound within the peribacteroid membrane (Pbm), Bar : 1 µm (1800), **B.** A part of nodule cell showing polymorphic bacteroids having heterogeneous cytoplasm (Hc) and loose peribacteroid membrane (Pbm) in the nitrogen fixation zone (Nf), 1 µm (3500X) and **C.** A nodule cell of senescence zone (S) containing degenerating bacteroids, Bar: 1 µm (4400X).

5. DISCUSSION

Rhizobial bacteria fix atmospheric nitrogen in symbiotic association with legumes by inducing specialized structures called nodules on the host plant roots. For the development and functioning of the symbiotic association rhizobia require, apart from sugars, many other metabolites like amino acids, nucleotide bases and vitamins. These are either synthesized by rhizobial bacteria and/or are supplied by the host plant. Very little information is available on the role of these metabolites in *Rhizobium*-legume symbiosis. Some of the intermediates of these metabolites also appear to be necessary in the symbiotic relationship. The research work on arginine biosynthetic pathway has been done in *Sinorhizobium meliloti* only (Dénarié *et al.*, 1976, Kerppola and Kahn, 1988b and Kumar *et al.*, 2003). On the basis of the interaction studies of the *S. meliloti* arginine auxotrophs with one or two varieties of the host plant alfalfa, it has been found that arginine auxotrophs blocked before ornithine are defective in nitrogen fixation. These results have indicated that ornithine may be important in symbiosis. It is obvious that further work on more strains of rhizobia belonging to different species needs to be done. The interaction of arginine auxotrophs should be studied with more cultivars of the host plant to find out the cultivar differences in providing the end product arginine and/or any of its intermediates.

The present work on arginine biosynthetic pathway has been carried out in *S. meliloti*, *Rhizobium leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*; the host plants of these rhizobia are alfalfa, clover and lentil, respectively. Twenty cultivars of the host plants were used in this study of the interaction of arginine auxotrophs of rhizobia with the host plants.

RandomTn5 mutagenesis of *S. meliloti, R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* was carried out using suicide delivery vector pGS9. A total of 20,750 Tn5 induced derivatives of these 3 rhizobial strains were obtained from 415 crosses of *S. meliloti, R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* with *E. coli* WA803 (pGS9) strain. From these Tn5 derivatives, 65 auxotrophs were obtained. The frequency of occurrence of auxotrophs among Tn5 derivatives was 0.31 %. This frequency of was similar to the frequencies of occurrence of auxotrophs among Tn5 derivatives of rhizobia reported by earlier workers (Meade *et al.,* 1982; Forrai *et al.,* 1983; Kim *et al.,* 1988; Prasad *et al.,* 2000; Vineetha *et al.,* 2001; Hassani *et al.,* 2002; Abbas *et al.,* 2002 and Kumar *et al.,* 2003).

The nutritional requirements of 65 isolated auxotrophs were: arginine, methionine, leucine, tryptophan, histidine, glutamine, valine, phenylalanine, glycine, histidine arginine and aspartic acid. The diverse nutritional requirements of the auxotrophs indicated randomness of Tn5 insertions in the genomes of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC905 and *R. leguminosarum* bv. *viciae* Rlv1. Similar randomness of Tn5 insertions has been reported in the previous Tn5 mutagenesis studies in rhizobia (Forrai *et al.,* 1983; Prasad *et al.,* 2000; Vineetha *et al.,* 2001; Hassani *et al.,* 2002; Abbas *et al.,* 2002 and Kumar *et al.,* 2003).

Fifteen arginine auxotrophs were selected for these studies. On the basis of intermediate feeding, the arginine auxotrophs could be divided into two groups. The first group consists of ornithine auxotrophs: *S. meliloti* Rm 1021(IITR1 & IITR2), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR8) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR12). Each of these auxotrophs had a mutation in *argA, argB, argC, argD* or *argE*

gene resulting in the biochemical block before ornithine in the arginine biosynthetic pathway. These auxotrophs were referred to as ornithine auxotrophs. The second group comprised of 11 arginine auxotrophs of *S. meliloti* Rm 1021 (IITR3, IITR4, IITR5, IITR6 & IITR7), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR9, IITR10 & IITR11) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR13, IITR14 & IITR15). Each of these had a biochemical block after ornithine in the arginine biosynthetic pathway. The *argF/argI* gene was mutated in the arginine auxotrophs of *S. meliloti* Rm 1021 (IITR3, IITR4, IITR5), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR9) and *R. leguminosarum* bv. *viciae* Rlv1 (IITR13, IITR14). The *argH* gene was mutated in the arginine auxotrophs of *S. meliloti* Rm 1021 (IITR6, IITR7), *R. leguminosarum* bv. *trifolii* MTCC905 (IITR10 & IITR11) and *R.. leguminosarum* bv. *viciae* (IITR15).

Arginine auxotrophs having a metabolic block after ornithine had reduced nitrogen fixation efficiency (upto about 23 %) in comparison to the parental type strains or were fully effective. These results are consistent with those obtained for *S. meliloti* by the earlier workers (Dénarié *et al.*, 1976; Fedorov and Zaretskaya, 1977; Kerppola and Kahn 1988b and Kumar *et al.*, 2003). The internal structure of the nodules induced by these auxotrophs was not very much different from those of the nodules induced by the parental strains. Arginine auxotrophs having a metabolic block before ornithine induced nodules in which no or very little nitrogen fixation occurred. The internal structure of these nodules showed that in these nodules nitrogen fixation zone was considerably reduced and the proper development of bacteriods did not take place. These results are in agreement with those of Kerppola and Kahn (1988b). However Dénarié *et al.* (1976) have reported effective or partially effective symbiosis of such auxotrophs.

Ornithine is also a precursor of polyamine synthesis (Tabor and Tabor, 1985). Kerppola and Kahn (1988b) have hypothesized that the lack of polyamines may be the cause of symbiotic defect of ornithine auxotrophs.

Some auxotrophs showed differential behavior in symbiosis with different cultivars of host plants. These results have shown that the genetic variability for the amounts of arginine and/or its intermediates provided to rhizobia exists among host cultivars. Thus the selection of host plants having better ability to provide amino acids, nucleotide bases, vitamins and/or their intermediates, may lead to better nitrogen fixation efficiencies.

The above results indicated that ornithine or an intermediate of ornithine biosynthesis may be required for the transformation of the rhizobial bacteria into bacteriods and the normal development of the nitrogen fixation zone during the symbiosis of *S. meliloti* Rm1021, *R. leguminosarum* bv. *trifolii* MTCC 905 and *R. leguminosarum* bv. *viciae* Rlv1 with alfalfa, clover and lentil plants, respectively. Some cultivars of alfalfa and lentil appear to provide the required amount of arginine to the rhizobia in the nodules.

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