# STUDIES ON AUXOTROPHIC MUTANTS OF SULFUR-CONTAINING AMINO ACIDS OF *Sinorhizobium meliloti*

**A THESIS**

*Submitted in fulfilment of the requirements for the award of the degree of*

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*By*

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## CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**STUDIES ON AUXOTROPHIC MUTANTS OF SULFUR-CONTAINING AMINO ACIDS OF *Sinorhizobium meliloti***" in fulfilment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biosciences and Biotechnology** of the University is an authentic record of my own work carried out during a period from August 1997 to August 2001 under the supervision of **Dr. (Prof.) G.S. Randhawa.**

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

**Date: August 18 , 2001** **(Basil Abdul-Zahrah Abbas)**

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

**Date: August 18, 2001**

Signature of Supervisor
**(Dr. G. S. Randhawa)**

The Ph.D. Viva-Voce examination of Basil Abdul-Zahrah Abbas, Research Scholar, has been held on 24.11.2001

Signature of Supervisor Signature of H.O.D. Signature of External Examiner

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## ABSTRACT

The present work was taken up to focus on the influence of the biosynthetic pathways of sulfur-containing amino acids of *Sinorhizobium meliloti* on the symbiotic characteristics of this bacterium. *S. meliloti* strain Rmd201, a streptomycin resistant derivative of the strain AK631, was mutagenized with transposon Tn5 that codes for resistance to kanamycin. The suicide vector pGS9 carrying Tn5 was introduced into *S. meliloti* from *Escherichia coli* strain WA803(pGS9) by conjugation. Kanamycin resistant ($Km^r$) transconjugants were selected by plating the mating mixture on tryptone yeast extract (TY) agar medium containing kanamycin (400 µg/ml) and streptomycin (100 µg/ml). Three thousand Tn5 derivatives ($Km^r$) of *S. meliloti* strain Rmd201 were obtained and out of these derivatives 12 did not grow on *Rhizobium* minimal medium (RMM). These were considered to be auxotrophs and purified for single colonies on TY agar medium containing kanamycin and streptomycin.

On the basis of growth on RMM supplemented with modified Holliday pools, the auxotrophs were classified as follows (the number of each kind is given in brackets): adenine (2), adenine + thiamine (1), cysteine (2), methionine (1), uracil (2), tryptophan (1) and tryptophan + tyrosine + phenylalanine (1). The nutritional requirement(s) of two auxotrophs could not be determined. Three sulfur amino acid auxotrophs isolated during this study and 24 such auxotrophs generated previously in this lab were used for further studies.

Based on the results of cross feeding and intermediate feeding studies sulfur amino acid auxotrophs were placed in the following categories:

**Category I:** Cysteine auxotrophs (BA4, BA7, VK6, VK10, VK13, VK17, VK20, VK32, VK33, NV8, NV11, NV15, NV16, NV17, NV20, NV25, NV30 and NV55); grew on RMM supplemented with sodium sulfide but not sodium sulfite and hence these auxotrophs were sulfite reductase (*cysI/cysJ/cysG*) mutants. Further studies showed that these auxotrophic mutants were *cysI/cysJ* mutants.

**Category II:** *metA/metZ* methionine auxotrophs (BA8, VK21, VK31, NV22, NV35, and NV36); grew on RMM supplemented with cystathionine or homocysteine.

**Category III:** *metE* methionine auxotrophs (VK29 and VK36); did not grow on RMM supplemented with cystathionine or homocysteine but grew on RMM supplemented with cyanocobalamin (vitamin $B_{12}$).

**Category IV:** *metF* methionine auxotroph (VK39); did not grow on RMM supplemented with cystathionine, homocysteine or cyanocobalamin.

All cys*I*/cys*J* mutants were able to grow on RMM agar medium supplemented with methionine, homocysteine or cystathionine. The methionine auxotrophs did not show any growth in the liquid RMM medium supplemented with cysteine. For confirming the linkage of Tn5 insertion to auxotrophy in each auxotroph, the plasmid pJB3JI was used to mobilize the Tn5 containing chromosomal segment into *S. meliloti* recipient strain ZB557 ($Phe^-$, $Leu^-$, $Rf^r$, $Sm^r$). $Km^r$ transconjugants were selected and tested for the presence of the donor's auxotrophic marker. All $Km^r$ transconjugants obtained showed donor's auxotrophy. This result confirmed 100% linkage of auxotrophy to Tn5 insertion as well as ruled out the possibility of occurrence of other independent Tn5 insertions in each mutant.

The cysteine and methionine auxotrophs were similar to the parental strain with respect to the production of cell surface molecules (lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β-glucans) and utilization of dicarboxylic acids and sugars indicating that the Tn5 insertions in these mutants did not result in any change in the cell surface molecules or carbon source utilization properties and the symbiotic defects of methionine auxotrophs were not caused by a change in any of the above characteristics.

Symbiotic properties of sulfur-containing amino acid auxotrophs were determined by inoculating alfalfa (*Medicago sativa*) seedlings grown aseptically on nitrogen free agar slants with these auxotrophs. The parental strain Rmd201 and all its cysteine auxotrophs induced pink nodules. The dry weights of the plants inoculated with the cysteine auxotrophs did not differ significantly from that of the plants inoculated with the parental strain indicating that the nitrogen fixing abilities of the cysteine auxotrophs were similar to that of the parental strain. All methionine auxotrophs formed white nodules which did not fix nitrogen. Delayed nodulation and reduction in nodule number per plant were observed in case of methionine auxotrophs. Normal symbiosis was restored on addition of methionine to plant growth medium or when alfalfa plants were inoculated with methionine revertants.

Nodules from six weeks old alfalfa plants were fixed and embedded in araldite embedding medium. Semithin and ultrathin sections were obtained and observed under light and transmission electron microscopes, respectively. The parental strain Rmd201 induced nodule had the following distinct zones, viz., apical meristem, infection, inter, nitrogen fixation and senescence, in the central tissue. A large number of bacteroids were

arranged around a centrally located large vacuole in each infected nodule cell. All stages of bacteroidal development were visible under transmission electron microscope. Histological studies of the *metA/metZ* auxotrophs revealed that the nitrogen fixation zone in the nodule induced by each of these mutants was not fully developed like that of the nodule induced by the parental strain Rmd201. Some differences were found in the histological structures of the nodules induced by *metE* and *metF* mutants.

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*BASIL*

# LIST OF ABBREVIATIONS USED

| | |
|---|---|
| am | Amyloplast |
| ADP | Adenosine diphosphate |
| AICAR | 5-aminoimidazole-4-carboxamide ribonucleotide |
| AICAr | 5-aminoimidazole-4-carboxamide riboside |
| APS | Adenosine-5'- phosphosulfate |
| ATP | Adenosine triphosphate |
| b | Bacteroid |
| c | Cortex |
| Cm | Chloramphenicol |
| ct | Central tissue |
| cw | Cell wall |
| *cys* | Cysteine |
| d | Digestive vacuole |
| *dct* | dicarboxylic acid transport |
| ed | Endodermis |
| ep | Epidermis |
| EPS | Exopolysaccharide |
| er | Endoplasmic reticulum |
| *exo* | Exopolysaccharide |
| g | Golgi complex |
| *gln* | Glutamine |
| *hsn* | Host specific nodulation |
| ic | Infected cell |
| IF | Infection zone |
| it | Infection thread |
| IZ | Interzone |
| kb | kilobase |
| Km | Kanamycin |
| KPS | Capsular polysaccharide |
| LB | Luria Bertani |
| LPS | Lipopolysaccharide |
| m | Mitochondria |
| *met* | Methionine |
| mg | milligram |
| ml | milliliter |
| MZ | Meristematic zone |
| MSY | Mannitol salts yeast extract |
| n | Nucleus |
| Nal | Nalidixic acid |
| *ndv* | Nodule development |
| NF | Nitrogen fixation |
| nm | nanometer |
| Nm | Neomycin |
| Ori | Origin of replication |

| | |
|---|---|
| PAPS | 3'-phosphoadenosine-5'-phosphosulfate |
| pbm | Peribacteroid membrane |
| pbs | Peribacteroid space |
| phb | Poly-β-hydroxy butyrate |
| *phe* | Phenylalanine |
| *pro* | Proline |
| pt | Peripheral tissue |
| r | Root hair |
| rb | Rhizobial bacteria |
| rf | Rifampicin |
| RMM | *Rhizobium* minimal medium |
| s | Symbiosome |
| SZ | Senescence zone |
| SFMM | Sulfur free minimal medium |
| Sm | Streptomycin |
| Tc | Tetracycline |
| *thi* | Thiamine |
| Tra | Transfer |
| *trp* | Tryptophan |
| TY | Tryptone yeast extract |
| uc | Uninfected cell |
| v | Vacuole |
| µg | microgram |
| µm | micrometer |

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# Chapter 1

# INTRODUCTION

Gaseous nitrogen ($N_2$) is abundantly available in the atmosphere. Plants and animals cannot convert it into a biological useful form. Only a few prokaryotes have the ability to utilize the gaseous nitrogen. These organisms reduce nitrogen to ammonia. This process, known as biological nitrogen fixation, is carried out by the enzyme system nitrogenase. Some nitrogen fixing bacteria like *Klebsiella pneumoniae*, *Azotobacter* and *Rhodopseudomonas* are able to reduce nitrogen into ammonia as free living organisms. Other organisms fix nitrogen only in symbiotic relationship with an eukaryotic host plant, like rhizobia in legume symbiosis, *Frankia* in actinorhizal symbiosis and cyanobacteria in *Gunnera* symbiosis (Van Kammen, 1995). Symbiotic nitrogen fixation is of great importance to agriculture since it reduces the need of fertilizer nitrogen. It has been estimated that the rhizobia-legume symbiosis contributes as much as 25% of the total terrestrial nitrogen (Burns and Hardy, 1975).

The bacteria of the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium* and *Sinorhizobium,* collectively called rhizobia, form symbiotic association with leguminous plants. These bacteria induce the formation of specialized structures, called nodules, on the roots of legume plants. The reduction of atmospheric nitrogen takes place in these nodules. The establishment of symbiotic association takes place in several steps like recognition and infection of root hairs, development and differentiation of root nodules, multiplication of rhizobia and their conversion into bacteroids within plant cells and reduction of nitrogen to ammonia. Symbiosis also involves uptake and assimilation of energy rich carbon compounds by bacteroids and utilization of fixed nitrogen by plants.

The detailed knowledge of the genes and gene products involved in symbiosis will be of great help in obtaining symbiotic combinations having enhanced ability of nitrogen fixation. Such knowledge can also help in developing bacteria that can live on the plants and fix nitrogen. Development of a symbiotic nitrogen-fixing system for cereal crops, similar to the rhizobium-legume system, can greatly enhance the crop yield (Vance and Graham, 1995).

With the help of recombinant DNA techniques several symbiotic genes of rhizobia have been identified and characterized. Rhizobial genes involved in symbiosis and related functions include the genes involved in nodule formation (*nod*, *nol* and *noe*) (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; Król *et al.*, 1998) and nitrogen fixation (*nif*, *fix*) (Batut *et al.*, 1985; Putnoky *et al.*, 1988; Kündig *et al.*, 1993). Some genes of the primary biosynthetic pathways of rhizobia also appear to play a direct role in the symbiotic process. Several auxotrophs of rhizobia have been observed to be defective in symbiosis (Schwinghamer, 1970; Pankhurst *et al.*, 1972; Kerppola and Kahn, 1988a; Noel *et al.*, 1988; Barsomian *et al.*, 1992; Newman *et al.*, 1994; Taté *et al.*, 1997; 1999a; 1999b; Prasad *et al.*, 2000; Vineetha *et al.*, 2001). The defective symboisis with an auxotrophic mutant may be due to the unavailability of the primary metabolite (amino acid, nucleotide base or vitamin) or a specific intermediate(s)/enzyme(s) of its biosynthetic pathway. The specific intermediate/enzyme may have a direct role in symbiosis. The present work was undertaken to understand the symbiotic role of the pathways for the synthesis of cysteine

and methionine in *S. meliloti*, which forms symbiotic relationship with alfalfa (*Medicago sativa*) plants, with the following objectives:

- To generate auxotrophic mutants of *S. meliloti* by transposon mutagenesis.
- To do the biochemical characterization of sulfur-containing amino acid auxotrophs.
- To study the symbiotic relationship between alfalfa plants and these auxotrophic mutants.
- To study the internal morphology of nodules formed by these auxotrophs.

# Chapter 2

# Literature Review

## 2.1 TAXONOMY OF RHIZOBIA

The ability of legumes to fix atmospheric nitrogen was discovered by Hellriegel and Wilfarth (1888). Beijerinck (1888) in Holland did the first isolation and cultivation of microorganisms from the nodules of legumes. He named the organism as *Bacillus radicicola* which was later renamed as *Rhizobium* (rhizo = root and bios = living) by Frank (1889). Rhizobial taxonomy was initially based on the ability of a strain to nodulate a particular set of host plants. The rhizobial strains were placed into two categories, the slow-growers with generation time more than 6 hours and the fast-growers with generation time less than 6 hours. The taxonomy of rhizobia has been subjected to re-evaluation using features other than host range for the purpose of classifying bacterial strains. Numerous studies have analyzed properties such as metabolic requirements, serology, DNA homology and ribosomal gene sequences (Jarvis *et al.*, 1980; Segovia *et al.*, 1993; Khbaya *et. al.*, 1998). Based on DNA homology, Jarvis *et al.* (1980) merged *R. leguminosarum* and *R. trifolii* into one species. Slow-growing rhizobia were kept in a distinct genus, *Bradyrhizobium* (Jordan, 1982) and the fast-growing rhizobia were reclassified and two genera, *Sinorhizobium* and *Mesorhizobium,* were proposed (Chen *et al.*, 1988; Lindström *et al.*, 1995; Jarvis *et al.*, 1997). The latest taxonomic classification of rhizobia has been presented in Table 1.

## 2.2 GENETICS OF RHIZOBIA

### 2.2.1 Rhizobial plasmids

Strains of different rhizobial species possess indigenous plasmids. Megaplasmids have been found in *S. meliloti* strains. Many of the genes, which are important in

**Table 1: Taxonomic classification of rhizobia***

| Genus | Species | Reference | Host plant (s) |
|---|---|---|---|
| *Rhizobium* | | | |
| | *R. leguminosarum* | Jordan (1984) | *Vicia, Pisum, Trifolium, Phaseolus, Lens* spp. |
| | *R. galegae* | Lindström (1989) | *Galega officinalis* |
| | *R. tropici* | Martínez-Romero *et al.* (1991) | *Leucaena* spp., *Macroptilium* spp., *Phaseolus vulgaris* |
| | *R. etli* | Segovia *et al.* (1993) | *Phaseolus vulgaris* |
| | *R. gallicum* | Amarger *et al.* (1997) | *Phaseolus vulgaris* |
| | *R. giardinii* | Amarger *et al.* (1997) | *Phaseolus vulgaris* |
| | *R. hainanense* | Chen *et al.* (1997) | Tropical legumes |
| | *R. mongolense* | van Berkum *et al.* (1998) | *Medicago ruthenica* |
| | *R. huautlense* | Wang *et al.* (1998) | *Sesbania herbacea* |
| *Bradyrhizobium* | | | |
| | *B. japonicum* | Jordan (1984) | *Glycine max, G. soja,* etc. |
| | *B. elkanii* | Kuykendall *et al.* (1992) | *Glycine max, G. soja,* etc. |
| | *B. liaoningense* | Xu *et al.* (1995) | *Glycine soja, G. max* |
| *Azorhizobium* | | | |
| | *A. caulinodans* | Dreyfus *et al.* (1988) | *Sesbania rostrata* |
| *Sinorhizobium* | | | |
| | *S. meliloti* | Jarvis *et al.* (1982) | *Medicago* spp., *Melilotus* spp., *Trigonella* spp. |
| | *S. fredii* | Scholla and Elkan (1984) | *Glycine max, G. soja* etc. |
| | *S. medicae* | Eardly *et al.* (1990) | *Medicago* spp. |
| | *S. saheli* | de Lajudie *et al.* (1994) | *Sesbania* spp. |
| | *S. teranga* | de Lajudie *et al.* (1994) | *Sesbania* spp. |
| | *S. arboris* | Nick *et al.* (1999) | *Acacia senegal, Prosopis Chilensis,* etc. |
| | *S. kostiense* | Nick *et al.* (1999) | *Acacia senegal, Prosopis Chilensis,* etc. |
| *Mesorhizobium* | | | |
| | *M. loti* | Jarvis *et al.* (1982) | *Lotus* spp. |
| | *M. huakuii* | Chen *et al.* (1991) | *Astragalus sinicus* |
| | *M. ciceri* | Nour *et al.* (1994) | *Cicer arietinum* |
| | *M. tianshanense* | Chen *et al.* (1995) | *Glycyrrhiza, Sophora* spp. |
| | *M. mediterraneum* | Nour *et al.* (1995) | *Cicer arietinum* |
| | *M. plurifarium* | de Lajudie *et al.* (1998) | *Acacia* spp., *Leucaena* spp. |

* Reproduced from Hassani (2001).

symbiosis, are plasmid borne. In *S. meliloti,* genes required for nodulation and nitrogenase activity have been mapped on the megaplasmid pSyma (Banfalvi *et al.*, 1981; Hooykass *et al.*, 1981; Rosenberg *et al.*, 1981; Corbin *et al.*, 1982; Lamb *et al.*, 1982; Batut *et al.*, 1985). The second megaplasmid, pSymb, carries genes involved in exopolysaccharide synthesis, thiamine synthesis and dicarboxylate transport (Finan *et al.*, 1986; Watson *et al.*, 1988). The megaplasmid pRme2011a of *S. meliloti* strain Rm2011 is not required for viability but the cured derivative strain was unable to use a number of substrates as sole sources of carbon (one at a time) on defined media (Oresnik *et al.*, 2000). High resolution map of *S. meliloti* 1021 megaplasmid (pSyma) was constructed (Barloy-Hubler *et al.*, 2000). Recently, sequencing of the entire genome of *S. meliloti* 1021 has been reported (Galibert *et al.*, 2001).

Rhizobial plasmids also have been found to bear genes for bacteriocins (Hirsch, 1979) or for pigment production (Beynon *et al.*, 1980; Lamb *et al.*, 1982). In addition to their ability to restore symbiotic functions to mutants of their own species, rhizobial plasmids were shown to confer host range and nodulation properties on heterologous recipient strains through transfer studies (Beynon *et al.*, 1980). Some rhizobial plasmids, when transferred into *Agrobacterium*, conferred upon it ability to form (non-nitrogen fixing) nodules on legume hosts (Hooykaas *et al.*, 1981).

### 2.2.2 Symbiotic genes

#### 2.2.2.1 Nodulation genes

Nodulation genes (*nod*, *nol* and *noe*) of the rhizobia, have been divided into two classes. One class includes genes which, when mutated, completely abolish nodulation on all legumes. These genes can be complemented between different rhizobial genera or

species. They are often called common *nod* genes. The examples of common *nod* genes are *nodABCIJ, nodM and nodN*. *nodABC* genes are involved in the production of lipochito-oligosaccharides (LCOs) called Nod factors (Faucher *et al.*, 1988) which are required for root hair curling and infection, for eliciting mitosis in the cortex and for nodule formation (Kondorosi *et al.*, 1984; Debellé *et al.*, 1986; Horvath *et al.*, 1986; Dudley *et al.*, 1987; John *et al.*, 1993). *nodD* gene has been shown to be a determinant of host specific nodulation and it regulates the expression of nodulation genes in rhizobia (Spaink *et al.*, 1987; Schlaman *et al.*, 1992). *nodM* encodes functions for efficiency of nod signal production and bacterial maturation (Baev *et al.*, 1992).

The second class of *nod* genes is necessary for interaction with certain but not all, legumes. These genes have been termed host specific nodulation (*hsn*) genes. The examples of *hsn* genes are *nodH*, *nodFEG*, *nodQP* and *noeI*. Mutations in these genes cannot be fully complemented by cloned nodulation genes from other rhizobial species (Kondorosi *et al.*, 1984). *nodH* gene in *S. meliloti* determines production of an extracellular signal specific to alfalfa (Faucher *et al.*, 1988). Functions of nodulation genes are summarized in Table 2.

*nodH* gene catalyzes the transfer of sulfate from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the 6-*O* position of Nod factors (Ehrhardt *et al.*, 1995). *nodP* and *nodQ*, which exist in two copies in *S. meliloti*, encode both ATP sulfurylase and APS kinase activities (Schwedock and Long, 1990; 1992). *nolO* and *noeI* genes of *Rhizobium* sp. NGR234 are involved in 3-*O*-carbamoylation and 2-*O*-methylation of Nod factors (Jabbouri *et al.*, 1998), while *nolL* gene is required for *O*-acetyltransferase activity (Berck *et al.*, 1999). Sulfated compounds involved in sulfation and methylation of Nod factors are presented in Fig.1.

**Table 2: Functions and properties of nodulation genes**

| Genes | Functions and properties | References |
|---|---|---|
| *nodA* | Codes for acyltransferase which links the acyl chain to the acetyl free C-2 carbon of the nonreducing end of oligosaccharide | Debellé *et al.* (1996); Röhrig *et al.* (1994) |
| *nodB* | Removes the *N*-acetyl moiety from nonreducing terminus of the *N*-acetylglucosamine oligosaccharide | John *et al.* (1993); Spaink *et al.* (1991) |
| *nodC* | Involved in first step of Nod factors assembly by encoding for acetylglucosaminyltransferase | Geremia *et al.* (1994) |
| *nodD* | Mediates host-specific activation of *nodABC* | Honma *et al.* (1990); Horvath *et al.* (1987) |
| *nodE, nodF* | Involved in synthesis of lipid chain of Nod factors | Demont *et al.* (1993) |
| *nodG* | Involved in *nodH* production | Ogawa *et al.* (1991) |
| *nodH, nodP, nodQ* | Codes for the sulfation of Nod factors | Roche *et al.* (1991) |
| *nodI, nodJ* | Play a role in efficiency of secretion and export of Nod factors | Spaink *et al.* (1995); Cardenas *et al.* (1996) |
| *nodL* | Required for formation of 6-*O*-acetyl Nod factors by encoding O-acetyl transferase | Bloemberg *et al.* (1994) |
| *nodM* | Codes for glucosamine synthase | Marie *et al.* (1992) |
| *nodN* | Involved in host specific nodulation | Surin and Downie (1988) |
| *nodO* | Codes for $Ca^{2+}$- binding protein | Economou *et al.* (1990) |
| *nodS* | Involved in *N*-methylation of Nod factors | Jabbouri *et al.* (1995) |
| *nodT* | Production of outer-membrane transport protein | Rivilla *et al.* (1995) |
| *nodU* | Involved in 6-*O*-carbamoylation of Nod factors | Jabbouri *et al.* (1995) |
| *nodV, nodW* | Flavonoid recognition system regulating nod expression | Loh *et al.* (1997) |
| *nodX* | Involved in *O*-acetylation of Nod factors | Firmin *et al.* (1993) |
| *nodZ* | Adds fucosyl residue to Nod factors | Stacey *et al.* (1994); Mergaert *et al.* (1996) |
| *noeC* | Fucosylation and arabinosylation of Nod factors | Mergaert *et al.* (1996) |
| *noeE* | Host specificity, NoeE protein has sulfotransferase activity | Hanin *et al.* (1997); Quesada-Vincens *et al* (1998) |
| *noeI* | Required for 2-*O*-methylation of Nod factors | Jabbouri *et al.* (1998) |
| *nolK* | Fucosylation and arabinosylation of Nod factors | Mergaert *et al.* (1996) |
| *nolL* | Required for acetylation of fucosyl residue of Nod factors | Berck *et al.* (1999); Corvera *et al.* (1999) |
| *nolO* | Involved in 3-*O*-carbamoylation of Nod factors | Jabbouri *et al.* (1998) |
| *nolR* | NolR protein controls the expression of *nod* genes | Cren *et al.* (1995) |

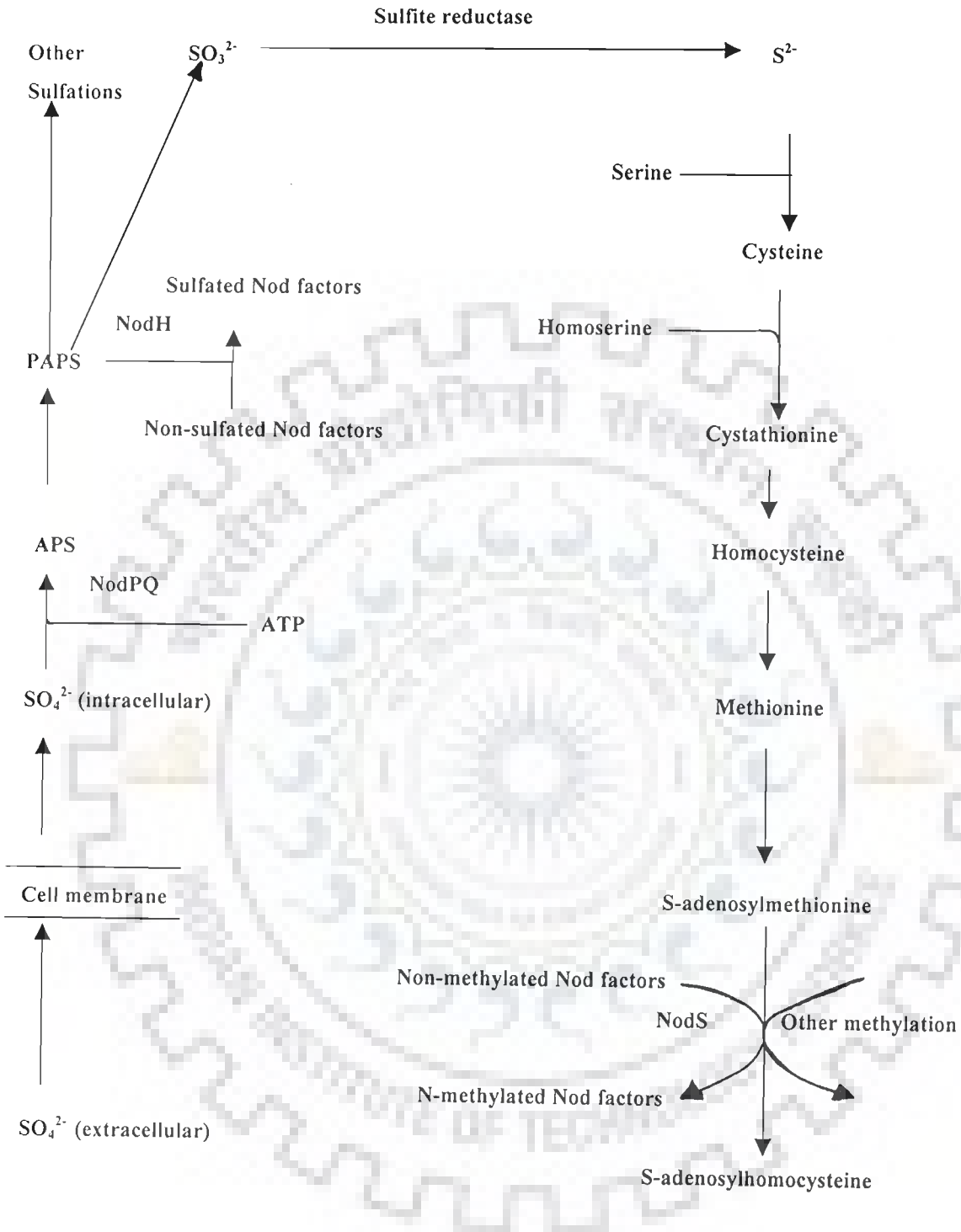



Fig.1: Sulfated compounds involved in sulfation and N-methylation pathways in Nod factors biosynthesis. (After Poupot *et al.*, 1995). APS=Adenosine-5'-phosphosulfate; PAPS=3'-Phosphoadenosine-5'-phosphosulfate.

### 2.2.2.2 Nitrogen fixation genes

Genes for nitrogen fixation in rhizobia are generally divided into two groups: Those with homologs in free-living nitrogen fixation systems, such as *Klebsiella pneumoniae*, are referred to as *nif* genes; those that are shown to be required for symbiotic nitrogen fixation, but whose function is not known to be analogous to a free-living function, are referred to as *fix* genes. Nitrogen fixation involves conversion of nitrogen to ammonia and hydrogen in presence of nitrogenase enzyme.

$$N_2 + 8H^+ + 8e^- \xrightarrow{16Mg\ A\ TP \longrightarrow 16Mg\ ADP + 16Pi\ /\ nitrogenase} 2NH_3 + H_2 \uparrow$$

*nif* and *fix* genes are present on chromosome in *R. loti*, *Bradyrhizobium* spp. and *Azorhizobium* spp. These genes are located on a symbiotic plasmid in *S. meliloti*, *R. leguminosarum* and *Rhizobium* spp. NGR234. Two megaplasmids of about 1400 kb (pSym-a or megaplasmid 1) and 1700 kb (pSym-b or megaplasmid 2) are present in *S. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Hynes *et al.*, 1986). The megaplasmid 1 carries the cluster I (*nifHDKE*, *nifN*, *fixABCX*, *nifA*, *nifB*) and the cluster II (*fixLJ*, *fixK*, *fixNOQP*, *fixGHIS*) of nitrogen fixation genes (Batut *et al.*, 1985; David *et al.*, 1987). *nifH*, *nifD* and *nifK* are structural genes for nitrogenase polypeptides (Ruvkun and Ausubel, 1980; Rosenberg *et al.*, 1981). These genes are present in a single operon (*nifHDK*) in *S. meliloti* (Ruvkun *et al.*, 1982). Two separate operons *nifH* and *nifDK* exist in *B. japonicum* (Kaluza *et al.*, 1983). *nifA* is a positively acting regulatory gene that controls the expression of nitrogenase and associated nitrogen fixation genes in *S. meliloti* and *B. japonicum* (Szeto *et al.*, 1984). The expression of *nifA* gene of *S. meliloti* has been found to be oxygen regulated; *nifA* gene is expressed when the external oxygen concentration is reduced to microaerobic levels (Ditta *et al.*, 1987). *S. meliloti fixL* gene is

involved in sensing oxygen (de Philip *et al.*, 1990). *fixA, fixB, fixC* and *fixX* genes have been identified in *S. meliloti* (Ruvkun *et al.*, 1982; Corbin *et al.*, 1983; Pühler *et al.*, 1984; Earl *et al.*, 1987), *B. japonicum* (Fuhrmann *et al.*, 1985; Gubler and Hennecke, 1986), *A. caulinodans* (Kaminski *et al.*, 1988; Arigoni *et al.*, 1991), *R. leguminosarum* bv. *viciae* (Grönger *et al.*, 1987) and *R. leguminosarum* bv. *trifolii* (Iismaa *et al.*, 1989). The functions of nitrogen fixation genes are summarized in Table 3.

**2.2.2.3 Genes for cell surface components**

Rhizobial cell surface, which plays an important role in infection of the legume host plant, is composed of exopolysaccharides (EPS), lipopolysaccharides (LPS), β-glucans and capsular polysaccharides (KPS). Normal expression of the genes for cell surface molecules is required for rhizobium-legume symbiosis.

**2.2.2.3.1 Genes for synthesis of exopolysaccharides**

Several workers have shown that rhizobial exopolysaccharides are essential for the successful invasion of nodules by rhizobia. *exo*$^-$ mutants, i.e. mutants having defects in exopolysaccharide production, of *S. meliloti* and *R. leguminosarum* bvs. *trifolii* and *viciae* formed ineffective nodules on the respective hosts (Chen *et al.*, 1985; Derylo *et al.*, 1986; Long *et al.*, 1988; Skorupska *et al.*, 1995; Rolfe *et al.*, 1996). *S. meliloti* synthesizes two exopolysaccharides, EPS I (calcofluor binding exopolysaccharide) and EPS II. Mutations affecting production of the EPS I were clustered in a 22-kb region and fell into 12 complementation groups (Long *et al.*, 1988). EPS II synthesis requires the products of at least seven loci on the second symbiotic megaplasmid of *S. meliloti* (Glazebrook and Walker, 1989). Becker *et al.* (1995) identified two genes, designated as *exsA* and *exsB*, having a function in EPS I biosynthesis in *S. meliloti*. *pssA* and *pssCDE*

**Table 3: Functions of nitrogen fixation genes (*nif* and *fix*)***

| Gene | Proposed function and/or product |
|---|---|
| ***nif* genes** | |
| *nifA* | Positive regulator of *nif, fix* and additional genes |
| *nifH* | Fe protein of nitrogenase |
| *nifD* | α subunit of MoFe protein of nitrogenase |
| *nifK* | α subunit of MoFe protein of nitrogenase |
| *nifB* | Involved in FeMo cofactor biosynthesis |
| *nifE* | Involved in FeMo cofactor biosynthesis |
| *nifN* | Involved in FeMo cofactor biosynthesis |
| *nifS* | Cysteine desulfurase |
| *nifW* | Required for full activity of FeMo protein |
| *nifX* | Unknown function |
| ***fix* genes** | |
| *fixABCX* | Required for nitrogenase activity |
| *fixNOQP* | Microaerobically-induced, membrane-bound cytochrome oxidase |
| *fixF* | Required for functioning of nitrogenase |
| *fixGHIS* | Redox process-coupled cation pump |
| *fixLJ* | Oxygen-responsive two component regulatory system involved in positive control of *fixK* (*S. meliloti, B. japonicum* and *A. caulinodans*) and *nifA* (*S. meliloti*) |
| *fixK*/*fixK*$_2$ | Positive regulator of *fixNOQP* (*S. meliloti, B. japonicum, A. caulinodans*), *nifA* (*A. caulinodans*), nitrate respiration (*B. japonicum*) and negative regulator of *nifA* and *fixK* (*S. meliloti*) |
| *fixR* | Similar to NAD-dependent hydrogenases |
| *fixU* | Unknown |
| *fixW* | May participate in a nitrogenase specific $e^-$ transport system |

*Reproduced from Vij (2000).

genes are involved in EPS biosynthesis in *R. leguminosarum* bv. *trifolii* (van Workum *et al.*, 1997; Król *et al.*, 1998; Pollock *et al.*, 1998).

**2.2.2.3.2 Genes for synthesis of lipopolysaccharides**

Cultured cells of *Sinorhizobium* spp. typically produce two forms of LPS: rough (R) LPS and smooth (S) LPS (Carrion *et al.*, 1990; Carlson *et al.*, 1992). The genes involved in LPS biosynthesis have been identified. *lpsZ* gene is involved in lipopolysaccharide biosynthesis and suppresses the symbiotic defect of *S. meliloti* exopolysaccharide mutants (Williams *et al.*, 1990b). This gene was located on the symbiotic megaplasmid pRm41c. Brzoska and Signer (1991) cloned and sequenced the *lpsZ* gene. The predicted LpsZ protein has a molecular weight of 48,589. Four loci, *lpsI*, *lpsN*, *lpsX* and *lpsY*, located on the chromosome are required for phenotype conferred by *lpsZ* (Williams *et al.*, 1990a). *lpsB* gene, located on the chromosome is responsible for both the LPS alteration and the symbiotic defect in *S. meliloti* (Lagares *et al.*, 1995). The genetic characterization of a 5.5 kb chromosomal region of *S. meliloti* 2011 that contain *lpsB* revealed the presence of six genes, *greA*, *lpsB*, *lpsE*, *lpsD*, *lpsC*, and *lrp*. Except for *lpsB*, none of the *lps* genes were relevant for nodulation and nitrogen fixation (Lagares *et al.*, 2001). So *et al.* (2000) reported the characterization of *rfaF* gene involved in lipopolysaccharide biosynthesis in *B. japonicum*.

**2.2.2.3.3 Genes for synthesis of capsular polysaccharides**

Rhizobia have surface polysaccharides analogous to group II K antigens (KPS) of *E. coli* (Reuhs *et al.*, 1993). These polysaccharides of *E. coli* can replace EPS of *S. meliloti* in infection process (Petrovics *et al.*, 1993; Reuhs *et al.*, 1995). KPS of rhizobia have been found to be composed of disaccharide repeating units of 3-deoxy-D-mannose-

hexouronic acid (Reuhs, 1997). Kereszt *et al.* (1998) identified novel *rkp* gene clusters of *S. meliloti* involved in capsular polysaccharide production and invasion of symbiotic nodule. The *rkpK* gene was found to encode a UDG-glucose dehydrogenase (Kereszt *et al.*, 1998).

**2.2.2.3.4 Genes for synthesis of beta glucans**

Two genes *ndvA* and *ndvB* involved in synthesis of β-(1→2)-glucans have been identified in *S. meliloti.* Ineffective nodules were formed when a mutation occurred in *ndvA* or *ndvB* gene (Dylan *et al.*, 1986; Geremia *et al.*, 1987). The *ndvA* gene probably codes for a single polypeptide of 616 amino acid residues which possesses striking homology to proteins involved in the export of hemolysin in *E. coli* and drugs in mammalian cells (Stanfield *et al.*, 1988). The *ndvB* locus of *S. meliloti* encodes a 319-kDa protein and is involved not only in invasion but also in bacteroidal development (Ielpi *et al.*, 1990). *B. japonicum* does not synthesize cyclic β-(1→2)-glucans. This organism produces cyclic β-(1→3), (1→6)-glucans composed of 11 to 13 glucosyl residues (Miller *et al.*, 1990; Cohen and Miller, 1991; Rolin *et al.*, 1992). Bhagwat *et al.* (1996) characterized a new locus (*ndvC*) influencing β-(1→6) linkages of β-glucans of *B. japonicum*. On the basis of their finding these workers suggested that the structure of the β-glucan molecule is important for a successful symbiotic interaction.

**2.2.3 Mutagenesis**

Mutagenesis of rhizobia has been done with physical and chemical mutagens, and transposons. Transposon mutagenesis has many advantages over the other methods of mutagenesis. Transposon insertion into a gene results into complete loss of the gene function and at the same time marks this gene with drug resistance locus which makes the

genetic analysis of the affected gene simple. Transposon mutagenesis is of two types, random and site-directed.

### 2.2.3.1 Random transposon mutagenesis

A suicide plasmid carrying transposon Tn5 is introduced into rhizobia by conjugation from an *Escherichia coli* donor (Fig. 2 and 3). This suicide plasmid is not stable in rhizobia and hence selection of neomycin/kanamycin resistant transconjugants results in the isolation of Tn5 insertion mutants (Beringer *et al.*, 1978; Selvaraj and Iyer, 1983; Simon *et al.*, 1983). Several workers have isolated auxotrophs and symbiotically defective mutants of rhizobia using suicide plasmid technique (Meade *et al.*, 1982; Forrai *et al.*, 1983; Kerppola and Kahn, 1988b; Khanuja and Kumar, 1989; Barsomian *et al.*, 1992).

### 2.2.3.2 Site-directed mutagenesis

A general method for site-directed Tn5 mutagenesis for Gram-negative bacteria was developed by Ruvkun and Ausubel (1981). In this method a cloned rhizobial DNA fragment is mutagenized in *E. coli* and then this mutagenized fragment is recombined in the cells of the rhizobial strain to be mutagenized. This method of mutagenesis has been used for physical and genetic characterization of several rhizobial genes (Ruvkun and Ausubel, 1981; Ausubel, 1982; Ruvkun *et al.*, 1982; Corbin *et al.*, 1983; Watson and Rastogi, 1993; Kereszt *et al.*, 1998). Goryshin and Reznikoff (1998) have recently developed an in vitro Tn5 transposition system for site-directed mutagenesis.

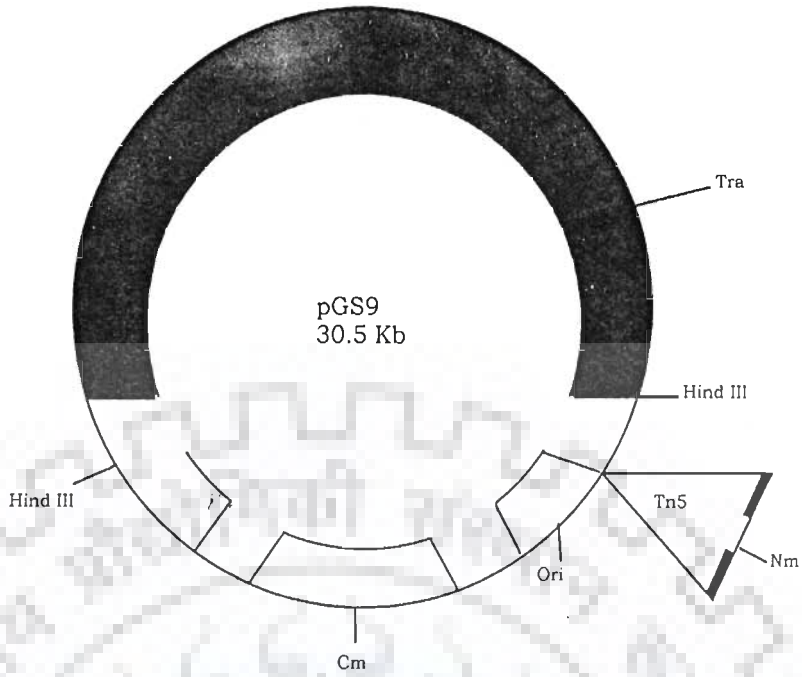



**Fig. 2: Suicide vector pGS9 carrying Tn5. *Abbreviations:* Cm, chloramphenicol; Nm, neomycin; Ori, origin of replication. (based on Selvaraj and Iyer, 1983)**

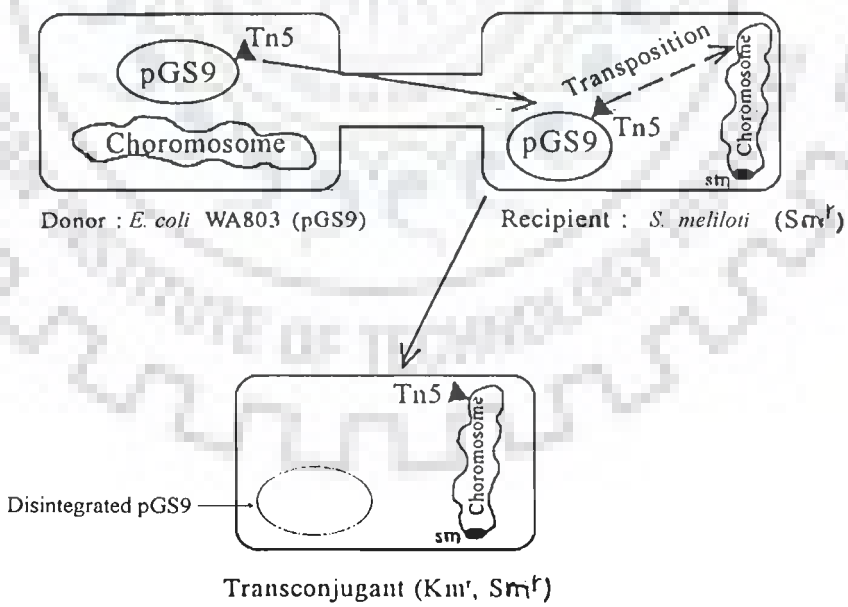



**Fig. 3: Diagrammatic representation of transposon Tn5 insertion. *Abbreviations:* Sm, streptomycin; Km, kanamycin.**

## 2.3 ROLE OF BACTEROIDAL METABOLISM IN SYMBIOSIS

### 2.3.1 Transport and utilization of $C_4$-dicarboxylates

The presence of a functional $C_4$-dicarboxylic acid transport system of *R. leguminosarum* was found to be essential for nitrogen fixation to occur in pea nodules (Finan *et al.*, 1983). Under free living conditions *B. japonicum* strain CJ1 containing a recombinant plasmid encoding *S. meliloti* sequences involved in dicarboxylic acid transport exhibited higher nitrogenase activity compared with the wild type strain (Birkenhead *et al.*, 1988). Aspartate has been shown to require the dicarboxylate transport system for transport into *S. meliloti* (Watson *et al.*, 1988; Watson, 1990). *S. meliloti* contains an aspartate aminotransferase activity required for symbiotic nitrogen fixation (Rastogi and Watson, 1991). Boesten *et al.* (1998) have found that the expression of *S. meliloti* $C_4$-dicarboxylate transport gene *dctA* is fully dependent on the regulatory genes *dctBD*. Permease component of DctA of *S. meliloti* Rm8002 has the ability to transport orotate, a monocarboxylic acid (Yurgel *et al.*, 2000).

### 2.3.2 Role of biosynthetic pathways of amino acids in symbiosis

The mutants of *S. meliloti* blocked in the steps after ornithine have been reported to be effective (Dénarié *et al.*, 1976; Fedorov and Zaretskaya, 1977; Kerppola and Kahn, 1988a) whereas the mutants of *S. meliloti* and *R. etli* blocked in the steps before ornithine were ineffective (Kerppola and Kahn, 1988a; Ferraioli *et al.*, 2001). Sequence analysis of a Tn5 induced mutant strain CTNUX5 of *R. etli* showed that Tn5 was inserted into an *argC*-homologous gene. This mutant strain was unable to produce flavonoid-inducible nodulation factors and did not induce nodules or nodule like structures on the roots of *Phaseolus vulgaris* (Ferraioli *et al.*, 2001).

Two Tn5 induced aspartate mutants of *S. fredii* HH303 were found to induce ineffective nodules on soybean. Symbiotic effectiveness was not restored in these auxotrophs after the addition of aspartic acid to the plant growth medium (Kim *et al.*, 1988). Seventeen out of 21 asparagine auxotrophs of *S. meliloti* 104A14 induced ineffective nodules on alfalfa (Kerppola and Kahn, 1988b).

The glutamate auxotrophs of *B. japonicum* showed an altered expression of nitrogenase activity in free living cultures (Hom *et al.*, 1984). de Bruijn *et al.* (1989) cloned and characterized three distinct *S. meliloti* loci involved in glutamine biosynthesis (*glnA, glnII* and *glnT*). None of these loci was essential for symbiotic nitrogen fixation. Arcondéguy *et al.* (1996) showed that symbiotic nitrogen fixation does not require adenylylation of glutamine synthase I in *S. meliloti*.

Histidine auxotrophs of *B. japonicum* and *R. leguminosarum* bv. *trifolii* were found to be symbiotically defective (Sadowsky *et al.*, 1986; Yadav *et al.*, 1998). However, on supplementation of histidine to the plant growth medium, normal symbiosis was shown by histidine auxotrophs (Sadowsky *et al.*, 1986; Yadav *et al.*, 1998).

Kim *et al.* (1988) obtained by transposon mutagenesis two isoleucine and valine (*ilv*) mutants of *S. fredii* HH303. These mutants formed ineffective nodules and the symbiotic effectiveness was not restored by addition of isoleucine and valine to the plant growth medium. Isoleucine and valine *ilvC* mutant of *S. meliloti* was $nod^-$. $Nod^+$ phenotype was not restored even after the supplementation of isoleucine and valine to the plant nutrient medium (Aguilar and Grasso, 1991). Lopéz *et al.* (2001) observed that *ilvC* mutants obtained from different *S. meliloti* wild-type strains are able to induce root hair deformation on alfalfa roots and show variable activation of the common nodulation

genes *nodABC*. Hassani (2001) reported that an *ilvB/ilvG* mutant of *S. meliloti* strain Rmd201 did not induce nodules on alfalfa plants and the nodulation ability was not restored by addition of isoleucine and valine to the plant growth medium. Nitrogen fixing nodules, like those of the parental strain, on alfalfa were induced by *ilvD* mutants of *S. meliloti* (Hassani, 2001).

Leucine auxotrophs of S. *meliloti* L5-30 formed ineffective nodules on alfalfa plants. However, normal symbiosis was restored when the plants were supplied with exogenous leucine or its precursors (Truchet *et al.*, 1980). Leucine auxotrophs of other strains of *S. meliloti* also induced ineffective nodules on alfalfa plants. But unlike in the above report, effectiveness was not restored on addition of leucine to the plant growth medium (Kerppola and Kahn, 1988a; Aronshtam *et al.*, 1993; Nichik *et al.*, 1995; Hassani, 2001).

Prasad *et al.* (2000) reported that a Tn5 induced phenylalanine auxotrophic mutant formed ineffective nodules on alfalfa plants. These results indicated that either the plant host is not able to provide phenylalanine to rhizobia in nodules or the gene involved in the synthesis of phenylalanine has a direct role in symbiosis.

Chien *et al.* (1991) determined cytosolic and bacteroid levels of proline in the nodules induced by the auxotrophic, catabolic and overproducing mutants of *R. leguminosarum* bv. *viciae* strain C1204b. The results did not support the role of proline as a key metabolite supplied by the host plant. King *et al.* (2000) found that a *proC* mutant of *B. japonicum* elicited underdeveloped nodules on soybeans that lacked nitrogen fixation activity and plant haemoglobin. It was concluded that *proC* gene is essential for

symbiosis and the mutant does not obtain an exogenous supply of proline in association with soybeans sufficient to satisfy its auxotrophy (King *et al.*, 2000).

Normal flow of metabolites through the aromatic biosynthetic pathway has been found to be essential for bacteroidal development in *S. meliloti* (Jelesko *et al.*, 1993). The nodules induced by the *trpE* tryptophan auxotrophs of *S. meliloti* showed defects in internal morphology. These nodules also fixed less amount of nitrogen as compared to the parental strain (Barsomian *et al.*, 1992; Prasad *et al.*, 2000). The tryptophan biosynthetic gene *trpB* of *R. etli* has been found to be essential for an effective symbiosis of this bacterium with *Phaseolus vulgaris* (Taté *et al.*, 1999a).

Sulfur-containing amino acid auxotrophs of rhizobia have been isolated by several workers (Pain, 1979; Meade *et al.*, 1982; Forrai *et al.*, 1983; Ali *et al.*, 1984; Hom *et al.*, 1984; Singh *et al.*, 1984). Methionine and methionine/cysteine mutants of *S. meliloti* strain 2011 were found to induce effective nodules on alfalfa plants (Scherrer and Dénarié, 1971). Cysteine-requiring mutants isolated from effective *S. meliloti* strain L5-30 showed loss of effectiveness (Malek and Kowalski, 1977). Methionine auxotrophs of *R. leguminosarum* have been reported to form effective nodules on pea plants (Pain, 1979). Kim *et al.* (1988) isolated two cysteine or methionine auxotrophic mutants of *S. fredii.* These two mutants formed effective nodules similar to those of the wild type on soybean plants. Kerppola and Kahn (1988a) observed that the methionine mutants of *S. meliloti* strain 104A14 formed nodules on alfalfa but these nodules were unable to fix nitrogen. Taté *et al.* (1997) found that a *cysG* mutant of *R. etli*, which was unable to grow on sodium sulfate as the sole sulfur source or nitrate as the sole nitrogen source, formed effective nodules on the roots of *Phaseolus vulgaris* plants. The *R. etli metZ* mutant was

unable to produce flavonoid-inducible lipochito-oligosaccharides (Nod factors) or to induce nodules or nodule-like structures on the roots of *Phaseolus vulgaris* unless methionine was added to the plant growth medium (Taté *et al.*, 1999b).

### 2.3.3 Role of biosynthetic pathways of purines in symbiosis

Defective symbiosis has been reported for the purine auxotrophs of *S. meliloti* (Scherrer and Dénarié, 1971; Malek and Kowalski, 1983; Kerppola and Kahn, 1988a; Swamynathan and Singh, 1992; 1995; Gupta, 1996), *R. leguminosarum* (Pankhurst and Schwinghamer, 1974; Pain, 1979), *R. fredii* HH303 (Kim *et al.*, 1988), *Rhizobium* strain NGR234 (Chen *et al.*, 1985) and *R. etli* (Noel *et al.*, 1988). Each of the purine auxotrophs isolated by Pankhurst and Schwinghamer (1974) had a biochemical block before 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Malek and Kowalski (1983) isolated two mutants of *S. meliloti* L5-30 requiring adenine and adenine with thiamine. Both mutants formed ineffective nodules on lucerne plants. EPS overproduction was observed in two adenine auxotrophs of *Rhizobium* strain NGR234 (Chen *et al.*, 1985). Pseudonodules on bean plants were induced by purine auxotrophs of *R. etli*; the supplementation of AICAR to the plant nutrient medium enhanced root nodule development (Noel *et al.*, 1988; Newman *et al.*, 1992). Newman *et al.* (1994) reported that purine auxotrophs of *R. fredii* HH303 and *R. leguminosarum* bv. *viciae* 128C56 induced poorly developed and uninfected nodules on their respective hosts. Each of these auxotrophs had a block before AICAR. The addition of AICAR to the plant growth medium enhanced nodulation by these auxotrophs. Soberón *et al.* (2001) found that 5-aminoimidazole-4-carboxamide riboside (AICAr), the precursor of AICAR, negatively regulates the expression of *fixNOQP* in *S. meliloti* by modulation of *fixK* expression.

Riccillo *et al.* (2000) reported that a *guaB* mutant of *R. tropici* had thermal sensitivity and was defective in symbiosis with beans. The study showed that, in *R. tropici*, the production of guanine via inosine monophosphate dehydrogenase is essential for growth at extreme temperature and for effective nodulation.

### 2.3.4 Role of biosynthetic pathways of pyrimidines in symbiosis

A pyrimidine auxotroph of *S. meliloti* strain 2011 was found to induce ineffective nodules on alfalfa (Scherrer and Dénarié, 1971). Pain (1979) isolated 15 pyrimidine auxotrophs of *R. leguminosarum* strain 3000. Two of these mutants showed defective symbiosis. Noel *et al.* (1988) obtained pyrimidine auxotrophs of *R. leguminosarum* bv. *phaseoli* strain CFN42 which induced ineffective nodules on *Phaseolus vulgaris*. Supplementation of the plant growth medium with uridine did not result in normal nodule development. Pyrimidine auxotrophs of *S. meliloti* strain 104A14 have been reported to induce ineffective nodules on alfalfa. Each of these mutants had a block before orotic acid (Kerppola and Kahn, 1985; 1988a; 1988b). Vineetha *et al.* (2001) obtained 23 pyrimidine auxotrophs of *S. meliloti* Rmd201. All these mutants induced ineffective nodules on alfalfa plants. On the basis of the internal structures of the nodules, these researchers concluded that some of the intermediates and/or enzymes of pyrimidine biosynthetic pathway of *S. meliloti* may have a role in bacteroidal transformation and nodule development.

### 2.3.5 Role of biosynthetic pathways of vitamins in symbiosis

Schwinghamer (1970) described a riboflavin-requiring auxotrophic mutant of *R. leguminosarum* bv. *trifolii*, which formed ineffective or partially effective nodules on some cultivars of clover in the absence of riboflavin, but was fully effective when

riboflavin was added externally to the roots of growing seedlings. The riboflavin requiring auxotrophs of *R. leguminosarum* bv. *trifolii* were unable to transform to bacteroids and resulted in the formation of ineffective nodules (Pankhurst *et al.*, 1972). A pantothenic acid auxotroph of *S. fredii* HH303 formed effective nodules on soybean plants, while nicotinic acid auxotroph of the same strain produced mature nodules like those of the wild type but these nodules lacked the characteristic pink color inside and were unable to fix nitrogen (Kim *et al.*, 1988). Streit *et al.* (1996) demonstrated that biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *S. meliloti* 1021.

## 2.4 RHIZOBIA-LEGUME INTERACTIONS

The formation of an effective legume nodule includes a series of events that involve both, bacteria and the host. These events include colonization of root by bacteria and signal exchange, root hair curling and infection, nodule initiation and bacterial release, and nodule development. Several reviews on rhizobium-legume interaction are available (Long and Cooper, 1988; Long, 1989; 2001; Pawlowski *et al.*, 1996; Perret *et al.*, 2000).

### 2.4.1 Plant genes involved in nodulation

Certain plant genes (*sym* genes) are required during all steps of nodulation. The products of these genes are involved in root hair deformation, infection thread formation and bacterial release (Nutman, 1984; Carroll *et al.*, 1986; Häser *et al.*, 1992). The plant genes which are not expressed in any plant organ other than nodules are termed nodulin genes (Van Kammen, 1984). Nodulin genes that are expressed before the onset of

nitrogen fixation are called early nodulin genes (*ENOD*). The examples of *ENOD* genes are *ENOD12* and *ENOD40.* Nodulin genes expressed at or after the onset of nitrogen fixation, are called late nodulin genes (*NOD*) (Nap and Bisseling, 1990).

### 2.4.2 Signal exchange

Certain chemicals present in root exudates attract rhizobia towards the root surface. These chemicals are called attractants. Some attractants like sugars, amino acids, phenolic compounds and carboxylic acids are of nutritional value. Others attractants like flavonoids and chalcones act as inducers or repressors of nodulation genes (Rolfe, 1988; Gottfert, 1993).

Rhizobia synthesize and secrete certain chemicals called Nod factors in response to appropriate inducers released by host plants. The Nod factors are lipochito-oligosaccharides containing a backbone of 4-5 *N*-acetylglucosamine residues and a fatty acid at the nonreducing terminal sugar residue (Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993). The enzymes encoded by the rhizobial *nod* genes mediate the biosynthesis of Nod factors (Okker *et al.*, 1993; Van Rhijn and Vanderleyden, 1995). Flavonoids secreted by the plant roots induce the expression of rhizobial *nod* genes (Peters *et al.*, 1986). One of the *nod* genes, *nodD*, is expressed constitutively. The NodD protein, upon binding the host flavonoids, activates the transcription of other *nod* genes (Goethals *et al.*, 1992). The lipochito-oligosaccharides secreted by different rhizobia are similar. The substitutions are in the terminal sugar residues that determine the host specificity of the lipochito-oligosaccharides. The major host determinant is a sulfate group at the reducing sugar residue of lipopolysaccharide (Roche *et al.*, 1991; Journet *et al.*, 1994).

### 2.4.3 Infection of plant by rhizobia

Rhizobia infect plants in two different ways: infection through deformed root hairs, and infection via so-called crack entry (Chandler *et al.*, 1982; Dreyfus *et al.*, 1986). The former way of infection occurs in only some tropical legumes whereas the latter way is found in most of legumes. Root hair deformation has been found to be due to a new induction of root hair tip growth by the Nod factors (Heidstra *et al.*, 1994). The deformation and curling of root hairs is accompanied by several host genes like *Mtrip1* which encodes a peroxidase and the early nodulin genes *ENOD5* and *ENOD12* which encode proline rich polypeptides (Scheres *et al.*,1990a,b; Cook *et al.*, 1995).

Rhizobia induce root hair curling and get entrapped in the curls. The rhizobial bacteria in a curl induce local hydrolysis of the plant cell wall (Callaham and Torrey, 1981). The plasma membrane grows inward and new cell wall material is deposited along the invaginating plasma membrane (Turgeon and Bauer, 1985). These events lead to the formation of a tubular structure, called infection thread (Dart, 1974). Rhizobial bacteria multiply in infection thread and are surrounded by a matrix which contains compounds of plant and bacterial origins (VandenBosch *et al.*, 1989). Infection thread wall is most likely of plant origin (Rae *et al.*, 1992).

Rhizobial bacteria are released from the infection thread into the cytoplasm of the plant cell. The released bacteria are surrounded by a plant-derived membrane, the peribacteroid membrane (pbm). The space within the pbm is called peribacteroid space (pbs). The bacteria, pbs and pbm form a functional structure called symbiosome (Roth and Stacey, 1989). The pbm controls metabolic exchanges between both symbiotic partners and is different from plasma membrane in phospholipid and protein composition

(Verma, 1992; Perotto *et al.*, 1995). Invasion of legume root hairs by rhizobia is illustrated in Fig. 4.

### 2.4.4 Nodule development and structure

During infection thread formation, root cortical cells start dividing and lead to the formation of a nodule primordium. The infection thread grows towards the nodule primordium. Several plant genes are expressed in the dividing cortical cells (Scheres *et al.*, 1990b; Kouchi and Hata, 1993).

There are two types of nodule development in legumes. In temperate legumes such as pea, vetch and alfalfa, inner cortical cells divide to form the nodule primordium (Libbenga and Harkes, 1973; Newcomb, 1981). After reaching the nodule primordium, the infection thread branches. The cells at the base of the primordium are infected whereas a meristem, consisting of small cells with dense cytoplasm, is formed at the distal part of the primordium (Libbenga and Harkes, 1973). The nodule meristem differentiates into different cell types (Fig 5). These nodules are called indeterminate nodules. In tropical legumes such as soybean, the nodule primordium is formed by outer cortical cells of the roots. The growing infection thread directly invades the cells of the primordium. Cells at the periphery of the primordium form a spherical primordium. These nodules are called determinate nodules.

A mature nodule consists of central tissue surrounded by several peripheral tissues. The peripheral tissues consist of nodule cortex, the endodermis and the parenchyma (Van de Wiel, *et al.* 1990). The central tissue contains infected and uninfected cells.

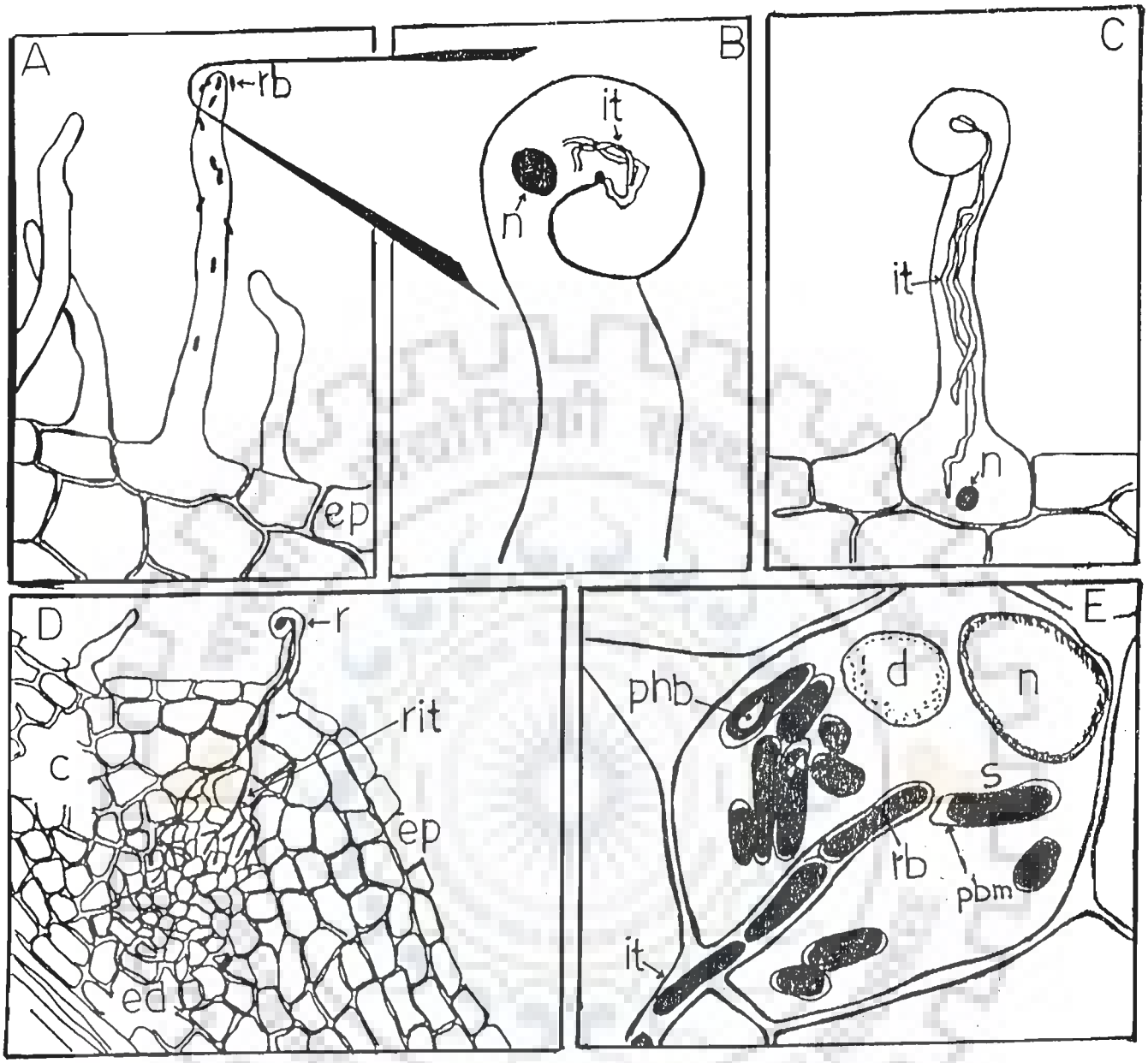



**Fig. 4.** Invasion of legume root hairs by rhizobia. **(A)** Rhizobial bacteria (rb) colonize the rhizophere and attach to the root hair (r). **(B)** Root hair curling and infection thread (it) formation. **(C)** Elongating infection thread (it) reaches the base of the root hair cell. **(D)** A developing infection thread ramifies (rit) near the nodule promordium formed by dividing cortical cells. **(E)** Rhizobial bacteria (rb) are released from the infection thread (it) and form symbiosomes (s) in nodule cells. Granules of polyhydroxybutyrate (phb) accumulate in rhizobial bacteria surrounded by the peribacteroid membrane (pbm). Other abbreviations: c, cortex; d, digestive vacuole; ep, epidermis; ed, endodermis; n, nucleus. (Perret *et al.*, 2000).

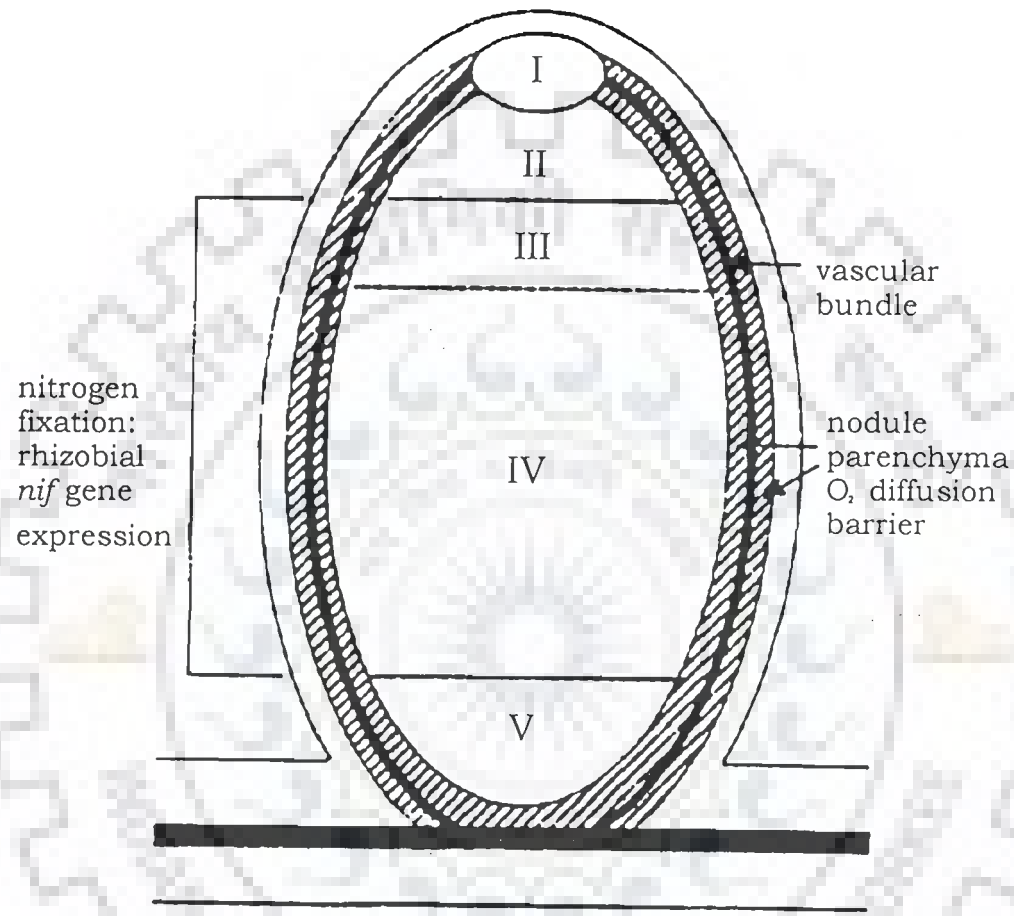



**Fig. 5: Schematic representation of root nodule structure; Zonation: I, apical meristematic zone; II, infection zone; III, interzone; IV, nitrogen fixation zone; V, senescence zone (Pawlowski *et al.*, 1996).**

The above review of literature reveals that several genes having a role in rhizobium-legume symbiosis have been identified and characterized in many species of rhizobia. The symbiotic role of genes involved in biosynthetic pathways of sulfur amino acids of rhizobia has not been fully explored. There is a need to determine if any intermediate(s)/enzyme(s) of these pathways is(are) involved in symbiotic process. Detailed histological studies of the nodules induced by sulfur amino acid auxotrophs, having mutations in different biosynthetic genes, of rhizobia are required to determine the role of the genes of sulfur amino acid biosynthesis in symbiosis.

# Chapter 3

# MATERIALS AND METHODS

## 3.1 MATERIALS

### 3.1.1 Bacterial strains and plasmids

The bacterial strains and plasmids used and constructed in this study are listed in Table 4.

### 3.1.2 Plant cultivar

Symbiotic properties of the rhizobial strains were tested with alfalfa (*Medicago sativa*) cultivar T9. The seeds of this cultivar were procured from National Seeds Corporation, New Delhi, India.

### 3.1.3 Growth media

The compositions of various growth media used for *S. meliloti* and *E. coli* are given below. Each of these media was solidified by adding 16g agar (HiMedia) to 1 litre liquid medium before autoclaving. All autoclavings were done at 15 psi for 20 min.

#### 3.1.3.1 Growth media for *Sinorhizobium meliloti*

**3.1.3.1.1 Tryptone yeast extract (TY) medium** (Khanuja and Kumar, 1988)

| Constituent | Amount/litre |
|---|---|
| Tryptone | 5g |
| Yeast extract | 3g |
| $CaCl_2.2H_2O$ | 0.12g |
| Distilled water | to make volume 1 litre |

The pH of the medium was adjusted to 7.0 with 0.1N NaOH solution.

**3.1.3.1.2 Tryptone yeast extract (TY) swarm plates**

These plates were prepared by adding 3 g/litre agar to the TY medium.

**Table 4: Bacterial strains and plasmids used/constructed in this study**

| Strains/Plasmids | Relevant characteristics | Source/Reference |
|---|---|---|
| *Sinorhizobium meliloti* | | |
| AK631 | $Nod^+$, $Fix^+$, compact colony variant of wild type strain Rm41 | Adam Kondorosi |
| Rmd201 | Spontaneous $Sm^r$ derivative of AK631 ($Nod^+$ $Fix^+$) | Khanuja and Kumar (1988) |
| PP631 | AK631(pJB3JI) | Peter Putnoky |
| ZB557 | Rm41 *phe15 leu4 rfl sm1* | --- do--- |
| BA4, BA7 | Rmd201 *cysI/cysJ*::Tn5 | This study |
| VK6,VK10,VK13 VK17,VK20,VK32 VK33, | Rmd201 *cysI/cysJ*::Tn5 | Vineetha, K.E. and Prasad, C.K. |
| NV8,NV11,NV15 NV16,NV17,NV20 NV25,NV30,NV55 | Rmd201 *cysI/cysJ*::Tn5 | Neeraj Vij |
| BA8 | Rmd201 *metA/metZ*::Tn5 | This study |
| VK21, VK31 | Rmd201 *metA/metZ*::Tn5 | Vineetha, K.E. and Prasad, C.K. |
| NV22,NV35,NV36 | Rmd201 *metA/metZ*::Tn5 | Neeraj Vij |
| VK29, VK36 | Rmd201 *metE*::Tn5 | Vineetha, K.E. and Prasad, C. K. |
| VK39 | Rmd201 *metF*::Tn5 | Vineetha, K.E. and Prasad, C. K. |
| *Escherichia coli* WA803(pGS9) | $Met^-$ $Thi^-$ $Cm^r$ $Km^r$ | Selvaraj and Iyer (1983) |
| Plasmids pGS9 | IncN repP15A $Cm^r$ $Km^r$ | Selvaraj and Iyer (1983) |
| pJB3JI | $Km^s$ derivative of pR68.45, capable of mobilizing genomic segments of its host, $Tc^r$ $Cb^r$ $Nal^r$ | Brewin *et al.* (1980) |

**3.1.3.1.3 Mannitol salt yeast extract (MSY) medium** (Khanuja and Kumar, 1989)

| Constituent | Amount/litre |
|---|---|
| Mannitol | 10g |
| Yeast extract | 1g |
| $K_2HPO_4.3H_20$ | 0.2g |
| $KH_2PO_4$ | 0.2g |
| $MgSO_4.7H_2O$ | 0.1g |
| $CaCl_2.2H_2O$ | 0.05g |
| Distilled water | to make volume 1 litre |

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

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

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

The pH of the medium was adjusted to 7.0 with 0.1N NaOH solution. 20% (w/v) glucose solution was prepared and filter-sterilized. Ten ml of glucose solution was added to 990 ml of the autoclaved medium.

### 3.1.3.1.5 Sulfur free minimal medium

| Constituent | Amount/litre |
|---|---|
| $Na_2HPO_4.12H_2O$ | 0.45g |
| $NH_4Cl$ | 2.0g |
| $FeCl_3$ | 2.0mg |
| $MgCl_2$ | 0.1g |
| $CaCl_2.2H_2O$ | 0.04g |
| Distilled water | to make volume 990 ml |

The pH of the medium was adjusted to 7.0 with 0.1N NaOH solution. 20% (w/v) glucose solution was prepared and filter-sterilized. Ten ml of glucose solution was added to 990 ml of the autoclaved medium.

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

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

The pH of the medium was adjusted to 7.0 with 0.1N NaOH solution.

### 3.1.3.3 Nitrogen free plant growth medium

The nitrogen free plant growth medium developed by Engelke *et al.* (1987) was used for plant inoculation experiments. The following 12 stock solutions were prepared.

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

The above stock solutions were autoclaved separately. To prepare 1 litre of plant growth medium, 10g agar was dissolved in 985 ml distilled water by heating. Four ml of stock solution F and one ml each of remaining stock solutions were added to the agar solution at about 50°C and pH of the medium was adjusted to 6.8 with 0.1N NaOH or 0.1N HCl. The resulting medium was autoclaved and poured at about 50°C into glass tubes to prepare slants for growing alfalfa plants.

### 3.1.4 Diluent used

Serial dilutions were made in 0.85% (w/v) NaCl (HiMedia). This solution was also used for preparing cell suspensions.

### 3.1.5 Supplements to media

#### 3.1.5.1 Antibiotics

Antibiotics used were purchased from HiMedia Laboratories. Streptomycin sulphate and kanamycin acid sulphate stock solutions were prepared in sterile distilled water. 50% ethanol was used as a solvent to prepare stock solutions of tetracycline hydrochloride and chloramphenicol. Rifampicin solution was prepared in dimethylsulphoxide. The final concentrations of antibiotics used in different media were as follows: streptomycin sulphate (100 μg/ml); kanamycin acid sulphate (100 μg/ml for *E. coli* and 400 μg/ml for *S. meliloti*), rifampicin (40 μg/ml), chloramphenicol (50 μg/ml) and tetracycline hydrochloride (15 μg/ml). Filter-sterilized stock solutions of antibiotics were added to the autoclaved media.

#### 3.1.5.2 Amino acids, nitrogenous bases and vitamins

Distilled water was used to prepare stock solutions of amino acids, nitrogenous bases and vitamins. All these solutions were autoclaved as described earlier. Stock solutions were added to the autoclaved media 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 on the following modified Holliday pools (Holliday, 1956):

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, glutamic acid and biotin.

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

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, glutamic acid, alanine and glycine.

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

**3.1.5.3 Intermediates**

Methionine intermediates were purchased from Sigma. The stock solutions of intermediates were prepared in sterile distilled water. Cystathionine and homocysteine were added to the medium at 50 µg/ml. Cyanocobalamin was added at 10 µg/ml concentration.

The final concentrations used of sulfur sources (HiMedia) were as follows: sodium sulfate (10 mM), sodium sulfite (0.85 mM), sodium sulfide (0.42 mM), and sodium thiosulfate (0.5 mM). These solutions were filter-sterilized and added to the sterile basal medium (Jones-Mortimer, 1968).

**3.1.5.4 Nirate source**

Sodium nitrate was used at 2 mg/ml final concentration as a sole nitrogen source and supplemented to the RMM (without ammonium sulfate).

### 3.1.5.5 Sugars and dicarboxylic acids

Sugars (glucose, arabinose, maltose, sorbitol, mannitol and sucrose) and dicarboxylic acids or their derivatives (malic acid, aspartic acid and sodium succinate) were purchased from HiMedia. Each carbon source was added at a final concentration of 2 g/litre to the RMM (without glucose) as a sole carbon source before autoclaving.

### 3.1.5.6 Sodium deoxycholate (DOC)

Sodium deoxycholate (DOC) (HiMedia) was added at the final concentration of 1 mg/ml to the TY medium before autoclaving.

### 3.1.5.7 Dyes

Calcofluor white (Sigma), aniline blue (HiMedia) and congo red (HiMedia) were added to MSY medium at the rate of 0.02% (w/v) each before autoclaving. Infection threads were stained with methylene blue (0.01%, w/v) (HiMedia). The staining of semithin sections of nodules was done with toluidine blue (1%, w/v) (HiMedia). The solution of toluidine blue was prepared in 1% (w/v) borax (Sigma).

### 3.1.6 Composition of solutions for light and electron microscopic studies of alfalfa root nodules

#### 3.1.6.1 Requirements for preparation of blocks

**(a) 0.2M phosphate buffer**

| | |
|---|---|
| $Na_2HPO_4$ | 6.41g |
| $Na_2HPO_4.7H_2O$ | 41.3g |
| Double distilled water | 1000 ml |

**(b) Fixatives**

**(i) Primary fixative i.e. Karnovsky fixative** (Karnovsky, 1965)

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

(ii) **Secondary fixative or post fixative i.e. 2% (w/v) $OsO_4$**

Stock solution of $OsO_4$ (2% w/v) was made by dissolving 1g of $OsO_4$ in 50 ml of distilled water. This stock solution was stored at 4°C in a tightly stoppered brown coloured bottle.

**(b) Acetone series**

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

**(c) Araldite CY212 (resin) embedding medium**

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

All ingredients were mixed and stirred vigorously.

**3.1.6.2 Solutions for staining ultrathin sections for electron microscopy**

**3.1.6.2.1 Uranyl acetate solution**

A saturated solution of uranyl acetate was prepared by adding excess of uranyl acetate to 10 ml of 50% (v/v) ethanol in a 15ml centrifuge tube. Centrifugation was done

at 5000 rpm for 2min and the supernatant was transferred to another tube. This tube was tightly stoppered 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 2min and the supernatant stored at 4°C.

## 3.2 METHODS

### 3.2.1 General bacteriological procedures

#### 3.2.1.1 Maintenance of bacterial strains

The bacterial strains used in this work were maintained in glycerol (15% final concentration) at -20°C. *S. meliloti* and *E. coli* strains were also maintained at 4°C on TY and LB agar medium slants, respectively.

#### 3.2.1.2 Culturing of bacteria

Single colonies of the bacterial strains were obtained by dilution plating or streaking methods. Single colonies on complete medium appeared after incubation for 2-3 days at 28°C for *S. meliloti* and 37°C for *E. coli*. Incubation of about one week at 28°C was required for the appearance of single colonies of *S. meliloti* on minimal medium. A single colony was suspended in 0.05 ml of 0.85% NaCl. This cell suspension was used for spot tests to study growth responses. To make a spot, $10^5$ to $10^6$ cells from this cell suspension were applied with a sterilized inoculation loop to the surface of agar medium.

A single colony of strain of *E. coli* was inoculated into 10 ml liquid LB medium containing the required antibiotic and incubation at 37°C was done for 14-16 hrs to obtain a log phase culture. Log phase culture of *S. meliloti* was obtained by the same method using liquid TY medium and incubating at 28°C for 24-32 hrs. Incubation was done in an orbital shaker (GALLENKAMP) operating at 120 rpm.

**3.2.1.3 Serial dilutions**

Serial dilutions of bacterial cultures were made in saline i.e. 0.85% (w/v) NaCl. 0.1 ml of a neat culture was transferred to a screw cap glass tube containing 9.9ml saline solution to obtain $10^{-2}$ dilution. After mixing, 0.1 of the bacterial suspension from this tube was transferred to another tube containing 9.9 ml saline to get $10^{-4}$ dilution. Further dilutions were similarly made.

**3.2.1.4 Bacterial conjugations**

Bacterial conjugations were done according to Kondorosi *et al.* (1977). 0.05ml log phase culture of recipient and 0.05ml of donor strain were mixed in 1:1 ratio (3:1 ratio for Tn5 mutagenesis) and spread on TY agar plates. Incubation was done at 28°C for 24 hrs.

**3.2.2 Isolation and screening of Tn5 derivatives**

**3.2.2.1 Transposon Tn5 mutagenesis**

Random mutagenesis of *S. meliloti* strain Rmd201 was carried out using Tn5 delivery suicide plasmid pGS9 (Selvaraj and Iyer, 1983). Log phase cultures in liquid TY medium of recipient *S. meliloti* strain Rmd201 (0.07 ml) and donor *E. coli* strain WA803 (pGS9) (0.03 ml) were mixed and spread with the help of a sterile glass rod on half of the surface of TY agar medium in a Petri dish. The remaining half of the TY agar medium

was further divided into two halves. In one half 0.05 ml of the donor culture was spread. In the other half 0.05 ml of the recipient culture was spread. These plates were incubated at 28°C for 24 hrs.

Each bacterial growth was scrapped with the help of a sterile spatula and suspended in 2 ml saline. The mating mixture suspension (0.1 ml) was spread on TY agar medium containing streptomycin (100 µg/ml) and kanamycin (400 µg/ml). The donor (0.05 ml) and recipient (0.05 ml) suspensions were also spread on the above selective medium separately as controls. Incubation was done at 28°C for 5 days. One hundred kanamycin and streptomycin resistant transconjugants were selected and each transconjugant was purified for a single colony on the same selective medium. All these transconjugants were streaked on TY agar medium containing chloramphenicol to confirm the loss of suicide plasmid pGS9. The above cross was repeated 30 times.

**3.2.2.2 Screening of Tn5 derivatives for auxotrophs**

The transconjugants obtained above were screened for auxotrophs by streaking them on RMM and TY+$Km^{400}$+$Sm^{100}$ agar medium with the help of sterile toothpicks. Incubation was done at 28°C for 3 days. The bacterial growth on each streak was observed 2-3 times each day. The Tn5 derivatives, which showed growth on TY+$Km^{400}$+$Sm^{100}$ but not on RMM agar medium, were assumed to be auxotrophs. Each of these auxotrophs was purified for a single colony on TY+$Km^{400}$+$Sm^{100}$ agar medium. The purified auxotrophs were used in further studies.

**3.2.3 Determination of the nature of auxotrophy**

A loopful of the growth of an auxotroph was suspended in a drop of saline. A sterile toothpick was dipped into the cell suspension and streaked on RMM agar medium

supplemented with modified Holliday pools (one at a time). Growth patterns were observed after incubation at 28°C for 5 days. The growth requirement(s) of the auxotroph was confirmed on the basis of its growth on RMM agar medium supplemented with the suspected nutrient.

### 3.2.4 Reversion analysis of auxotrophs

Spontaneous reversion frequencies of all auxotrophs were calculated. A sample (10 ml) of late log phase culture in liquid TY medium of an auxotroph was centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet washed twice with liquid RMM medium (2 ml each time). The washed pellet was resuspended in liquid RMM medium to obtain a cell suspension containing approximately $10^9$ cells/ml. 0.1 ml of this cell suspension was spread on RMM and the same volume was also spread on TY agar medium. The number of bacterial colonies were counted after incubation at $28^0$C for 5 days. Spontaneous reversion frequency for the auxotroph was calculated on the basis of the number of prototrophic revertants (number of colonies on RMM) and the total number of cells (number of colonies on TY medium). Each prototrophic revertant colony was also streaked on TY agar medium containing 400 µg/ml kanamycin to find out the excision of transposon Tn5.

### 3.2.5 Location of biochemical block in each auxotroph

#### 3.2.5.1 Intermediate feeding studies

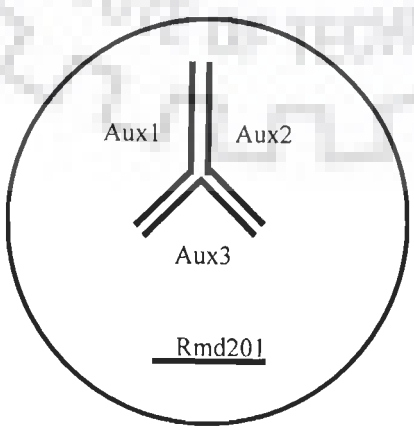
The cell suspensions of the methionine auxotrophs and parental strain as a control were streaked on minimal medium (RMM) supplemented with 3 intermediates (one at a time) of the methionine biosynthetic pathway.

For cysteine auxotrophs, sulfur free minimal medium was supplemented with 4 intermediates (one at a time) of the biosynthetic pathway of cysteine. The growth patterns were observed after incubation at 28°C for 4-5 days.

The growth of the strains in liquid cultures was also determined by measuring optical densities of these cultures at $590_{nm}$ ($O.D._{590}$). The parental strain and auxotrophic mutants were grown overnight in TY medium. Inocula of the strains was prepared by washing the cells from 10 ml late log phase culture in TY medium twice with minimal medium. The washed cells were suspended in 1 ml RMM. 0.1 ml of this cell suspension was added to 10 ml RMM supplemented with the particular intermediate at the concentration indicated previously. $O.D._{590}$ was recorded (three samples of each treatment) after incubation with shaking (120 rpm) at 28°C for 24 hrs.

**3.2.5.2 Cross feeding assays**

The cell suspensions (in 0.85% w/v saline) of cysteine/methionine auxotrophs, in combinations of three at a time, were streaked adjacent to each other on RMM agar medium containing 2 µg/ml cysteine. The streaking pattern of auxotrophs (Aux) was as follows:



The cell suspensions of methionine auxotrophs were also streaked as above on RMM medium containing 2 μg/ml methionine. The cell suspension of parental strain was also streaked on one side as a positive control.

### 3.2.6 Tests for the production of cell surface molecules

#### 3.2.6.1 Test for production of lipopolysaccharides (LPS)

All auxotrophs and the parental strain were streaked on MSY agar medium containing 1mg/ml of sodium deoxycholate (DOC). Incubation was done at 28°C for 3 days. The growth of a rhizobial strain on MSY + DOC agar medium indicated the ability of this strain to produce LPS (Swamynathan and Singh, 1995).

#### 3.2.6.2 Test for production of succinylated exopolysaccharides (EPS I)

The production of succinylated exopolysaccharides (EPS I) was detected by streaking all auxotrophic mutants along with the parental strain on MSY agar medium containing 0.02% (w/v) calcofluor white. Incubation was done at 28°C for 3 days. Fluorescence under long wavelength UV rays indicated EPS I production (Leigh *et al.*, 1987).

#### 3.2.6.3 Test for production of cellulose fibrils

All auxotrophic mutants and the parental strain were streaked on MSY agar medium containing 0.02% (w/v) congo red. The growth was observed after incubation at 28°C for 3 days. The presence of cellulose fibrils on the surface of rhizobial cells was indicated by production of red colonies (Kneen and La Rue, 1983).

#### 3.2.6.4 Test for production of β-(1→3)-glucans

The taking up of the aniline blue dye by the rhizobial cells of a given strain indicates the ability of this strain to produce β-(1→3)-glucans (Nikanishi *et al.*, 1976).

All auxotrophs and the parental strain were streaked on MSY agar medium containing 0.02% (w/v) aniline blue. The presence or absence of blue colonies was recorded after incubation at 28°C for 3 days.

### 3.2.6.5 Test for production of β-(1→2)-glucans

The production of β-(1→2)-glucans can be detected on swarm plates (Geremia *et al.*, 1987). A loopful of cell suspension of each auxotroph was spotted on a TY swarm plate containing 0.3% (w/v) agar. The cell suspension of the parental strain was also spotted on each of these plates. Incubation was done at 28°C for 3 days and spots were examined for swarming after every 24 hrs. Swarming indicated the production of β-(1→2)-glucans.

### 3.2.7 Utilization of sugars and dicarboxylic acids by auxotrophs

Utilization of several sugars (glucose, arabinose, maltose, sorbitol, mannitol and sucrose) and dicarboxylic acids (malic acid, aspartic acid and sodium succinate) by auxotrophs was studied. Each auxotroph was streaked on RMM agar medium (without glucose) supplemented with the nutritional requirement of the auxotroph and a sugar or dicarboxylic acid. The parental strain, as a positive control, was also streaked on this medium. All auxotrophs and the parental strain were also streaked on RMM agar medium. Incubation was done at 28°C for 5 days and after 48 hrs of incubation bacterial growth was observed daily.

### 3.2.8 Growth on nitrate containing minimal medium

To test the ability of a cysteine mutant to grow on minimal medium supplemented with nitrate as a sole nitrogen source, the mutant was streaked on RMM (without

ammonium sulfate) supplemented with sodium nitrate (2 mg/ml). The parental strain, as a positive control, was also streaked on this medium. Sodium thiosulfate was used as a sulfur source (Taté *et al.*, 1997).

### 3.2.9 Construction of donors of auxotrophs

The *S. meliloti* strain AK631(pJB3JI), as a donor of genome mobilizing ability plasmid pJB3JI ($Tc^r$), was mated with each auxotroph on TY agar medium. The transconjugants containing plasmid pJB3JI were selected on TY agar medium containing tetracycline (15 µg/ml) and kanamycin (400 µg/ ml). Five transconjugants were streaked for single colonies on the above selective medium. The purified transconjugants were used as donor strains in further experiments.

### 3.2.10 Linkage of Tn5 insertion to auxotrophy

The donor strain (containing plasmid pJB3JI) of each auxotroph was mated with the recipient strain *S. meliloti* ZB557 (rifampicin resistant) on TY agar plates. Fifty kanamycin resistant transconjugants were selected on TY agar medium containing rifampicin (40µg/ml) and kanamycin (400µg/ml). These transconjugants were checked for auxotrophy of the donor as described earlier.

### 3.2.11 Plant inoculation tests

Plant experiments were done in glass tubes (20 x 2.5 cm). The tubes were autoclaved after plugging with cotton. Twenty-five ml of nitrogen-free plant nutrient medium at about 45°C under sterile conditions was transferred to each tube. The tube was immediately placed in slanting position after plugging. Whenever required, filter-sterilized supplements were added to the plant nutrient medium before transfer of this medium to tubes.

Alfalfa (*Medicago sativa* cv. T9) seeds were soaked in sterile distilled water for 20 min and surface sterilized by treating with 0.1% $HgCl_2$ for 1 min followed by 1 min treatment with absolute alcohol. After giving five washing with sterile distilled water, the seeds were spread on 1% agar in Petri dishes. Incubation was done at 25°C in dark. After two days, two seedlings were transferred to each tube.

Alfalfa seedlings were inoculated with the parental strain *S. meliloti* Rmd201 and its auxotrophs. Ten ml of log phase culture of a rhizobial strain in liquid TY medium was centrifuged at 5000 rpm for 10 min. The pellet was washed two times with sterile distilled water. The washed pellet was suspended in 5 ml of sterile distilled water. 0.5 ml of this suspension was added to each tube containing seedlings. Black paper was wrapped on the lower portions of tubes to protect the plant roots from direct exposure to light. The tubes containing seedlings were placed in a plant growth chamber maintained at 25°C and provided with 2000 lux light. The plants were given 16 and 8 hrs light and dark periods, respectively. After six weeks the plants were taken out of the tubes and data on nodule characteristics and dry plant weights were recorded. Dry plant weights were recorded after drying plants at 60°C for 48 hrs (Sharypova *et al.*, 1994).

**3.2.12 Nodule occupancy test**

The nodules induced by rhizobial strains were checked for the occupancy of the inoculated strains. All nodules from a plant were surface-sterilized and crushed in a drop of saline. The resulting cell suspension was diluted in saline to get $10^{-1}$ and $10^{-2}$ dilutions. A sample (0.1 ml) of neat suspension was spread on TY agar medium containing 100µg/ml streptomycin. Both dilutions were also spread on the same medium. Three replications were used for the neat suspension and each of the two dilutions. Incubation

was done at 28°C for 3 days. The colonies obtained were streaked on agar medium containing RMM and RMM + $Km^{400}$ + $Sm^{100}$ + auxotrophic requirement. Incubation was done at 28°C for 3 days. The colonies which showed growth on RMM medium were recorded as prototrophic revertants.

### 3.2.13 Observation of infection thread formation

Alfalfa plants were taken out of tubes after 4 days of inoculation. The root portion of each plant was washed and cut into 1cm long pieces. These root pieces were placed in methylene blue solution (0.01% w/v in distilled water) for 15 min and washed twice with sterile water. Each stained root piece was placed on a glass slide, covered with a cover slip and examined at 10x and 40x magnifications under light microscope (Leica *DM* LB).

### 3.2.14 Microscopic examination of nodule sections

The sections of the nodules induced by the parental strain Rmd201 and its three methionine auxotrophs BA8, VK29 and VK39 were subjected to microscopic examination. Ten nodules for each strain were collected from plants after six weeks of inoculation. The methodology for these experiments consisted of specimen fixation, specimen block preparation and preparation of semithin and ultrathin sections of the specimens.

#### 3.2.14.1 Primary fixation of nodules

The nodules were washed thrice with sterile distilled water and transferred to glass vials (5 ml capacity) containing Karnovsky fixative. To facilitate penetration of the fixative, acrolein (at 0.1% w/v final concentration) was added. The vials were placed in a vacuum chamber to prevent the floating of the nodules on the surface. These vials were then kept at 4°C for 24 hrs.

### 3.2.14.2 Preparation of specimen blocks

#### 3.2.14.2.1 Washing of nodule samples

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

#### 3.2.14.2.2 Secondary fixation of nodules

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

#### 3.2.14.2.3 Dehydration of nodules

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

#### 3.2.14.2.4 Removal of acetone from nodules

The nodule samples were cleared off of acetone by placing in toluene at room temperature for 60 min. Acetone was removed from the samples to facilitate infiltration in the next step.

#### 3.2.14.2.5 Infiltration

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 in the previous step, were placed in mixture 'a'. After 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.

**3.2.14.2.6 Embedding of nodules**

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

**3.2.14.3 Preparation of semithin sections**

The block holder was attached to an ultramicrotome (Ultracut E Microtome, C. Reichert, Austria OmU3) and semithin sections (0.5 to 2.0 μm thick) were cut using a glass knife. The sections floating in water in the metallic trough fitted with the knife 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. The stained sections were washed with distilled water and observed under light microscope (Leica *DM* LB). The area of interest was photographed.

**3.2.14.4 Preparation of ultrathin sections**

The area of the section to be examined under TEM was selected and the blocks were further hand trimmed. Ultrathin sections (60-90 nm thick) were cut using an ultramicrotome (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 to leave behind the 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. After washing, the grids were dried on a filter paper and then 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 0.1N NaOH and then twice with double distilled water. After washing, the grids were dried and viewed under transmission electron microscope (PHILIPS EM300). The area of interest was photographed. After viewing, the grids were stored in a grid holder at room temperature.

### 3.2.15 Statistical analysis

The data on characteristics of plants inoculated with the parental strain Rmd201 and its cysteine and methionine auxotrophs were statistically analyzed. All values were expressed as mean ± standard error of mean (SEM). Significance between a control value and an experimental value was found out using student ' t ' test.

# Chapter 4

# RESULTS

The results of the experiments on the isolation and characterization of auxotrophic mutants of sulfur-containing amino acids of *S. meliloti* Rmd201 are presented below:

**4.1 Isolation of auxotrophs by random Tn5 mutagenesis**

Three thousand transposon Tn5 derivatives ($Km^r$) of *S. meliloti* strain Rmd201 were obtained and out of these derivatives 12 did not grow on *Rhizobium* minimal medium (RMM). These were considered to be auxotrophs and purified for single colonies on TY agar medium containing kanamycin and streptomycin.

**4.2 Nutritional requirements of auxotrophs**

On the basis of growth on RMM supplemented with modified Holliday pools, the auxotrophs were classified as follows (the number of each kind is given in brackets): adenine (2), adenine + thiamine (1), cysteine/methionine (2), methionine (1), uracil (2), tryptophan (1) and tryptophan + tyrosine + phenylalanine (1). Nutritional requirement(s) of two auxotrophs could not be determined. Three sulfur amino acid auxotrophs isolated during this study and 24 such auxotrophs generated previously in this lab were used in further studies.

**4.3 Intermediate feeding studies**

**4.3.1 Intermediate feeding studies on agar media**

The growth responses of cysteine/methionine and methionine auxotrophs to supplementation of minimal medium with different intermediates of cysteine and methionine biosynthetic pathways are given in Table 5. All cysteine/methionine auxotrophs grew on sulfur free minimal medium supplemented with sodium sulfide or sodium thiosulfate but were unable to grow on the above medium supplemented with sodium sulfate or sodium sulfite. Hence these mutants were sulfite reductase mutants.

Table 5: Growth responses of cysteine/methionine and methionine auxotrophic mutants of *Sinorhizobium meliloti* strain Rmd201 to different intermediates of the biosynthetic pathways of cysteine and methionine.

| Name of the strain | RMM | RMM supplied with | | | | | SFMM | SFMM supplied with | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Cysteine | Methionine | Cystathionine | Homocysteine | Vitamin $B_{12}$ | | Sodium sulfate | Sodium sulfite | Sodium sulfide | Sodium thio-sulfate |
| **Rmd201** | + | + | + | + | + | + | + | + | + | + | + |
| **Cysteine/methionine auxotrophs:** BA4, BA7, VK6,VK10, VK13,VK17, VK20, VK32, VK33, NV8, NV11, NV15, NV16, NV17, NV20, NV25, NV30, NV55 | - | + | + | + | + | - | - | - | - | + | + |
| **Methionine auxotrophs:** BA8, VK21, VK31, NV22, NV35, NV36 | - | - | + | + | + | - | - | - | - | - | - |
| VK29, VK36 | - | - | + | - | - | + | - | - | - | - | - |
| VK39 | - | - | + | - | - | - | - | - | - | - | - |

***Abbreviations***: RMM, *Rhizobium* minimal medium; SFMM, sulfur free minimal medium.

These auxotrophs were also able to grow on RMM supplemented with cystathionine, homocysteine or methionine. This result showed that *S. meliloti* is able to convert cystathionine, homocysteine or methionine into cysteine.

Six methionine auxotrophs, BA8, VK21, VK31, NV22, NV35 and NV36, grew on RMM supplemented with either cystathionine or homocysteine but not on cyanocobalamin (vitamin $B_{12}$) supplemented RMM. Two methionine auxotrophs, VK29 and VK36, showed growth on RMM supplemented with cyanocobalamin. The methionine auxotroph VK39 did not grow on RMM medium supplemented with any of the above methionine intermediates.

**4.3.2 Intermediate feeding studies in liquid culture**

The growth responses of the parental strain and its auxotrophic mutants in liquid RMM supplemented with different intermediates were studied by measuring optical densities of the liquid cultures. The growth of the parental strain Rmd201 in RMM supplemented with cysteine, methionine, cystathionine or homocysteine was more than its growth in RMM supplemented with cyanocobalamin or sodium sulfide (Fig. 6). The cysteine/methionine auxotroph BA4 showed less growth than Rmd201 in RMM supplemented with cysteine, methionine, cystathionine, homocysteine or sodium sulfide. The growth of this strain in sodium sulfide-supplemented RMM was more than its growth in RMM supplemented with other intermediates. The methionine auxotroph BA8 showed growth in RMM supplemented with methionine, cystathionine or homocysteine; the growth on cystathionine was more than the growth on homocysteine. The growth of the methionine auxotroph VK29 was less than the growth of the parental strain Rmd201 in RMM supplemented with methionine or cyanocobalamin. The methionine auxotroph

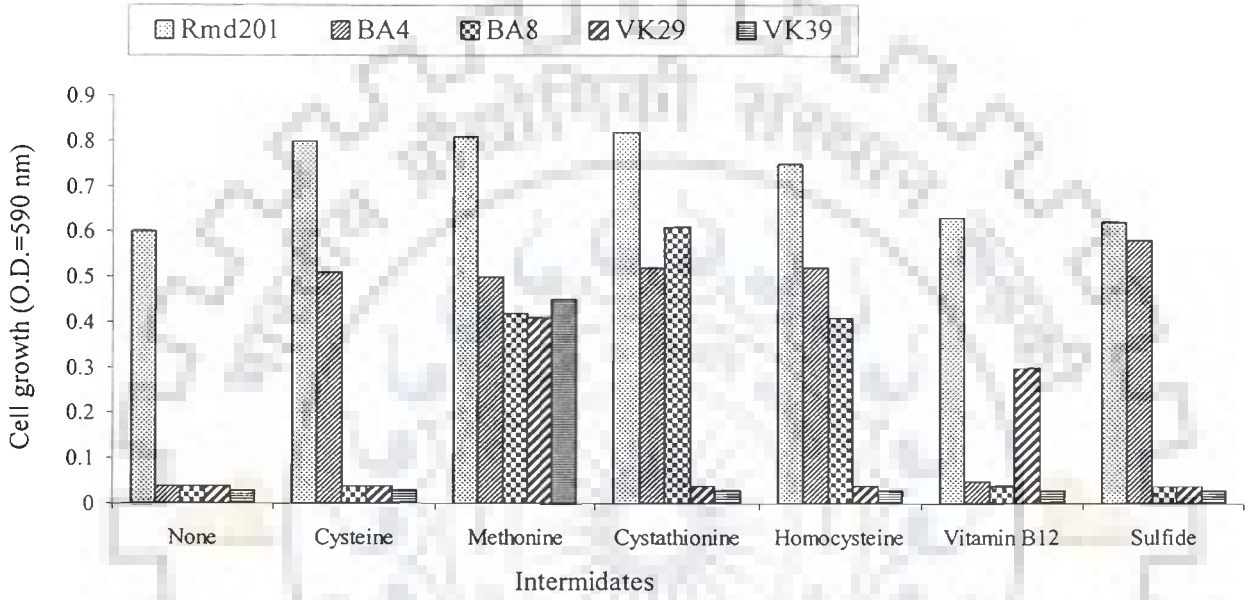



**Fig.6: Growth responses of *Sinorhizobium meliloti* strain Rmd201 and its cysteine (BA4) and methionine (BA8, VK29 and VK39) auxotrophs in liquid *Rhizobium* minimal medium supplemented with 4 intermediates of cysteine and methionine biosynthetic pathways.**

VK39 also showed less growth than the parental strain in RMM supplemented with methionine.

**4.4 Cross feeding studies**

None of the cysteine/methionine auxotrophs cross fed any other cysteine/methionine auxotroph. The methionine auxotrophs VK29, VK36 and VK39 cross fed the other methionine auxotrophs.

**4.5 Location of biochemical blocks in auxotrophs**

On the basis of intermediate and cross feeding studies, the following conclusions were drawn:

All cysteine/methionine auxotrophs were sulfite reductase mutants and hence, were cysteine auxotrophs. Since three genes (*cysI, cysJ* and *cysG*) control the reduction of sulfite to sulfide, these auxotrophs are *cysI/cysJ/cysG* mutants. All these mutants were able to grow on minimal medium supplemented with sodium nitrate; sodium thiosulfate was used as a sulfur source. This result showed that these mutants had normal nitrite reductase activity. It has been previously established that the *cysG* mutants of enteric bacteria are defective in sulfite reductase, nitrite reductase and cobalamin synthesis (Kredich, 1996) and *cysG* mutants of *R. etli* are defective in sulphate reduction and nitrite reduction (Taté *et. al.*, 1997). Therefore, normal nitrite reductase activity of the cysteine auxotrophs shows that each of these auxotrophic mutants has a normal *cysG* gene. Hence, these mutants were designated as *cysI/cysJ* mutants. Fig. 7 shows the position of block in each of the cysteine auxotrophs in the biosynthetic pathway of cysteine.

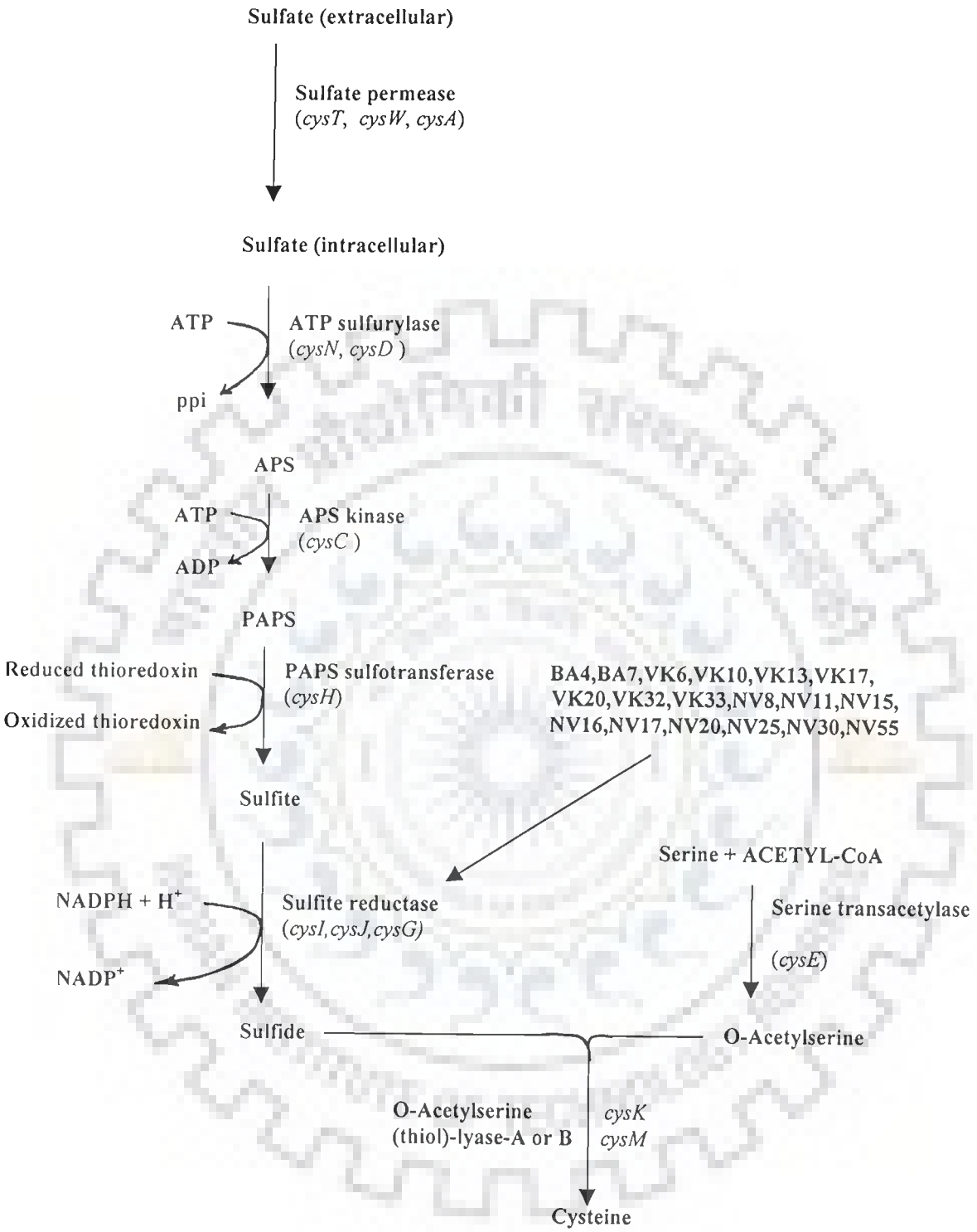



Fig. 7: The general biosynthetic pathway of cysteine in bacteria showing the positions of mutations in cysteine auxotrophs of *Sinorhizobium meliloti* Rmd201. *Abbreviations*: APS=Adenosine-5'-phosphosulfate;PAPS=3'-phosphoadenosine-5'-phosphosulfate (After Kredich, 1996).

The methionine auxotrophs were placed in the following categories:

**Category I:** *metA/metZ* mutants (BA8, VK21, VK31, NV22, NV35 and NV36).

**Category II:** *metE* mutants (VK29 and VK36).

**Category III:** *metF* mutant (VK39).

Fig. 8 shows the position of block in each of the methionine auxotrophs in the biosynthetic pathway of methionine.

**4.6 Linkage of Tn5 insertion to auxotrophy**

Transposon Tn5 encoded kanamycin resistance marker from each auxotroph was mobilized to *S. meliloti* ZB557 strain with the help of genome mobilizing plasmid pJB3JI. All kanamycin resistant transconjugants showed the respective donor's auxotrophic marker. Hence, there was 100% cotransfer of Tn5 and the auxotrophic marker. In other words Tn5 had complete linkage with auxotrophy in each auxotrophic mutant.

**4.7 Reversion analysis**

All auxotrophs showed spontaneous reversion to prototrophy.

**4.8 Production of cell surface molecules**

All auxotrophs, like the parental strain Rmd201, produced lipopolysaccharides, succinylated exopolysaccharides (EPSI), cellulose fibrils and β-(1→2)-glucans.

**4.9 Utilization of sugars and dicarboxylic acids**

Each auxotroph showed growth similar to that of the parental strain Rmd201 on minimal medium (RMM) supplemented with the respective auxotrophic requirement. No change in the growth behaviour of any auxotroph was detected when glucose in RMM was replaced by any one of the other sugars (arabinose, maltose, sorbitol, mannitol and

sucrose) or dicarboxylic acids (malic acid, aspartic acid and sodium succinate) as a carbon source.

**4.10 Characteristics of plants inoculated with the parental strain and its auxotrophic mutants**

Alfalfa plants were inoculated with the parental strain Rmd201 and its 18 cysteine and 9 methionine auxotrophs. The data on the characteristics of inoculated plants have been presented in Tables 6 and 7. The bacteria were isolated from the nodules of each plant and checked for their markers. The data on a plant were retained only if the nodules on this plant were found to be occupied by the inoculated strain.

The parental strain Rmd201 induced cylindrical nodules on both primary and lateral roots. The nodules induced by the cysteine auxotrophs were either cylindrical or irregular in shape and were located on both primary and lateral roots. The methionine auxotrophs induced nodules mostly on lateral roots and the shapes of the nodules were cylindrical, round or irregular. The nodules induced by the parental strain and the cystiene auxotrophs were pink in colour. The mean nodule number/plant for each cysteine auxotroph did not differ significantly from the mean nodule number/plant for the parental strain. All methionine auxotrophs induced white nodules. The mean heights and dry weights of the plants inoculated with cysteine auxotrophs did not differ significantly from those of the plants inoculated with the parental strain. On the other hand the mean heights and dry weights of the plants inoculated with the methionine auxotrophs did not differ significantly from those of the uninoculated plants. The above data showed that all cysteine auxotrophs were $Nod^+$ $Fix^+$ and their nitrogen fixing abilities were similar to that of the parental strain. The methionine auxotrophs were $Nod^+$ $Fix^-$.

**Table 6: Characteristics of alfalfa plants inoculated with *Sinorhizobium meliloti* strain Rmd201 and its cysteine auxotrophs**

| Name of the strain | Nodule shape | Nodule colour | Mean nodule number/plant | Mean plant height (cm)* | Mean dry plant weight (mg)* |
|---|---|---|---|---|---|
| Rmd201 | C | Pink | 8.7±0.7 | 24.0 ± 1.4 | 32.7 ± 1.0 |
| Uninoculated | ---- | ---- | ---- | 7.30 ± 0.5 | 10.6 ± 0.9 |
| BA4 | C or I | Pink | 7.8±1.0† | 21.3 ± 1.2 † | 28.5 ± 1.9 † |
| BA7 | -do- | -do- | 8.1±1.2† | 20.5 ± 1.2 † | 28.9 ± 1.5 † |
| VK6 | C | -do- | 8.2±1.3† | 19.8 ± 1.2 † | 28.2 ± 1.4 † |
| VK10 | -do- | -do- | 7.8±1.0† | 22.3 ± 1.0 † | 30.5 ± 1.8 † |
| VK13 | -do- | -do- | 7.8±1.8† | 23.4 ± 1.0 † | 31.8 ± 2.3 † |
| VK17 | C or I | -do- | 8.3±1.1† | 18.6 ± 1.0 † | 29.3 ± 1.4 † |
| VK20 | -do- | -do- | 8.6±0.9† | 19.4 ± 1.5 † | 29.7 ± 1.3 † |
| VK32 | C | -do- | 8.0±0.8† | 23.6 ± 0.8 † | 31.2 ± 1.1 † |
| VK33 | C or I | -do- | 8.1±1.3† | 20.4 ± 1.3 † | 29.3 ± 1.3 † |
| NV8 | C | -do- | 8.3±1.1† | 23.6 ± 0.5 † | 32.1 ± 2.3 † |
| NV11 | -do- | -do- | 8.5±0.9† | 23.2 ± 1.0 † | 31.6 ± 1.8 † |
| NV15 | C or I | -do- | 7.8±2.0† | 18.3 ± 1.3 † | 27.8 ± 1.7 † |
| NV16 | -do- | -do- | 7.7±2.2† | 19.6 ± 1.0 † | 29.5 ± 1.8 † |
| NV17 | -do- | -do- | 8.2±1.7† | 22.1 ± 1.6 † | 30.7 ± 1.1 † |
| NV20 | -do- | -do- | 8.1±1.4† | 23.0 ± 0.9 † | 31.6 ± 1.2 † |
| NV25 | -do- | -do- | 8.3±1.3† | 21.3 ± 1.2 † | 28.5 ± 1.7 † |
| NV30 | -do- | -do- | 8.6±1.0† | 22.6 ± 1.3 † | 30.4 ± 2.0 † |
| NV55 | -do- | -do- | 8.3±1.2† | 22.8 ± 1.2 † | 31.0 ± 1.6 † |

*Abbreviations*: C, cylindrical; I, irregular; * = Each value is a mean of values of eight plants; † = Does not differ significantly from the parental strain value ($P < 0.05$).

**Table 7: Characteristics of alfalfa plants inoculated with *Sinorhizobium meliloti* strain Rmd201 and its methionine auxotrophs**

| Name of the strain | Nodule shape | Nodule colour | Mean nodule number/ plant | Mean plant height (cm)# | Mean dry plant weight (mg)# |
|---|---|---|---|---|---|
| Rmd201 | C | Pink | 8.7±0.7 | 24.0 ± 1.4 | 32.7 ± 1.0 |
| Uninoculated | ---- | ---- | ---- | 7.30 ± 0.5 | 10.6 ± 0.9 |
| BA8 | C | White | 4.5±1.6† | 10.5 ± 1.5* | 13.5 ± 1.6 * |
| VK21 | C or R | -do- | 4.7±0.9† | 9.6 ± 1.1 * | 13.2 ± 1.5 * |
| VK29 | C | -do- | 5.0±1.1† | 10.3 ± 1.1* | 13.8 ± 2.1 * |
| VK31 | -do- | -do- | 4.3±0.8† | 11.6 ± 1.2* | 13.9 ± 2.1 * |
| VK36 | -do- | -do- | 4.6±1.2† | 9.8 ± 0.9 * | 12.1 ± 1.4 * |
| VK39 | R or I | -do- | 4.6±1.2† | 8.3 ± 0.8 * | 11.3 ± 1.8 * |
| NV22 | C | -do- | 4.0±2.0† | 9.8 ± 1.3 * | 12.7 ± 2.2 * |
| NV35 | -do- | -do- | 4.1±1.8† | 11.2 ± 1.6* | 13.6 ± 1.3 * |
| NV36 | -do- | -do- | 4.6±1.2† | 10.3 ± 1.2* | 12.8 ± 1.5 * |

***Abbreviations***: C, cylindrical; R, round; I, irregular; # = Each value is a mean of values of eight plants. * = Does not differ significantly from the uninoculated control value ($P < 0.05$). † = This value differ significantly from the parental strain value ($P < 0.05$).

Initiation of nodulation occurred 6 days after inoculation of plants with the parental strain Rmd201 (Fig. 9) and after 16 days of inoculation all plants were nodulated. In case of cysteine auxotrophs, nodulation started 8 days after inoculation and continued up to 22 days after inoculation. The methionine auxotrophs showed delayed nodulation (Fig 9) and reduction in nodule number (Table 7). In case of these mutants nodulation started 12 days after inoculation and continued to more than 30 days after inoculation.

**4.11 Characteristics of plants inoculated with revertants**

The revertants of methionine auxotrophs, like the parental strain Rmd201, induced pink and cylindrical nodules on both primary and lateral roots. The mean heights and dry weights of the plants inoculated with the revertants did not differ significantly from those of the plants inoculated with the parental strain (Table 8). These data indicated that the revertants formed normal nitrogen fixing nodules like the parental strain.

**4.12 Exogenous feeding of methionine to plants inoculated with methionine auxotrophs**

Symbiotic interactions of the methionine auxotrophs with alfalfa plants were studied using the plant nutrient medium supplemented with methionine (25 μM). The auxotrophs induced pink, cylindrical nodules. The mean heights and dry weights of the plants inoculated with the methionine auxotrophs did not differ significantly from those of the Rmd201 inoculated plants (Table 9). These results indicated that methionine supplementation to the plant nutrient medium completely restored the symbiotic effectiveness to the methionine auxotrophs.

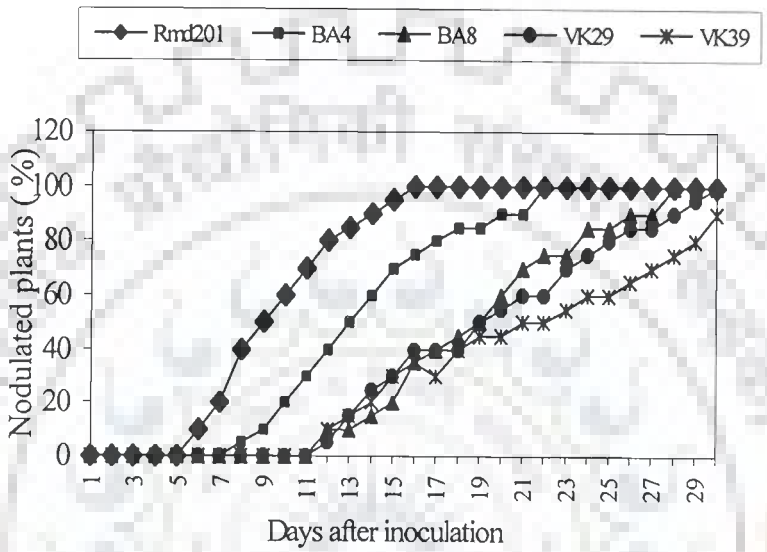



**Fig.9: Initiation of nodulation on alfalfa plants inoculated with *Sinorhizobium meliloti* Rmd201 and its cysteine (BA4) and methionine (BA8, VK29 and VK39) auxotrophs. Each value is a mean of values of 8 plants.**

**Table 8: Characteristics of alfalfa plants inoculated with revertants of methionine auxotrophs of *Sinorhizobium meliloti* Rmd201**

| Name of the strain | Mean plant height (cm)* | Mean dry plant weight (mg)* |
|---|---|---|
| Rmd201 | 25 ± 1.4 | 33.2 ± 1.0 |
| Uninoculated | 8.3 ± 0.5 | 10.9 ± 0.9 |
| BA8 | 22.1 ± 1.3† | 31.4 ± 1.4† |
| VK21 | 19.8 ± 2.3† | 29.7 ± 2.4† |
| VK29 | 23.2 ± 1.5† | 30.6 ± 1.7† |
| VK31 | 20.5 ± 1.2† | 32.4 ± 1.1† |
| VK36 | 18.9 ± 1.9† | 31.7 ± 1.3† |
| VK39 | 20.4 ± 1.6† | 29.6 ± 1.9† |
| NV35 | 21.7 ± 1.4† | 28.7 ± 2.4† |
| NV36 | 22.5 ± 1.7† | 30.6 ± 1.7† |

* = Each value is a mean of values of eight plants. † = This value does not differ significantly from the parental strain value ($P < 0.05$).

**Table 9: Characteristics of alfalfa plants inoculated with methionine auxotrophs of *Sinorhizobium meliloti* Rmd201 after supplementation of plant nutrient medium with methionine**

| Name of the strain | Mean plant height (cm)* | Mean dry plant weight (mg)* |
|---|---|---|
| Rmd201 | 26 ± 1.3 | 33.6 ± 1.1 |
| Uninoculated | 7.9 ± 0.5 | 9.8 ± 0.85 |
| BA8 | 23.2 ± 1.2† | 30.5 ± 1.3† |
| VK21 | 21.8 ± 1.5† | 29.6 ± 2.1† |
| VK29 | 24.2 ± 1.3† | 33.1 ± 1.6† |
| VK31 | 21.5 ± 1.3† | 30.1 ± 1.3† |
| VK36 | 22.9 ± 1.6† | 31.9 ± 1.2† |
| VK39 | 21.5 ± 1.7† | 30.5 ± 1.4† |
| NV22 | 22.6± 1.3† | 31.7 ± 1.3† |
| NV35 | 23.7 ± 1.3† | 30.7 ± 1.7† |
| NV36 | 21.4 ± 1.4† | 29.7 ± 1.4† |

* = Each value is a mean of values of eight plants. † = This value does not differ significantly from the parental strain value ($P < 0.05$).

### 4.13 Nodule occupancy tests

The nodule occupancy test was performed for each inoculation experiment. The bacteria were isolated from the nodules of each inoculated plant. The isolated bacteria were checked for the auxotrophic and antibiotic markers. The percentages of plants showing 100% occupancy of nodules by cysteine auxotrophs varied from 50 for auxotroph VK13 to 95 for auxotroph VK20 (Table 10). These percentages for methionine auxotrophs ranged from 55 for auxotroph VK39 to 92.5 for auxotroph NV22 (Table 11).

### 4.14 Microscopic studies

#### 4.14.1 Root hair curling and infection thread formation

The alfalfa plants inoculated with parental strain Rmd201 and all cysteine and methionine auxotrophs showed root hair curling and infection thread formation (Plate 1). The root hairs of the uninoculated plants were straight and devoid of infection threads.

#### 4.14.2 Histological studies of nodules

##### 4.14.2.1 Light and electron microscopic studies of nodules induced by the parental strain Rmd201

**Light microscopy:**

A section of the nodule induced by the parental strain Rmd201 showed a central tissue surrounded by several peripheral tissues (Plate 2A). The central tissue was differentiated into five zones, viz., meristematic zone, infection zone, interzone between infection and nitrogen fixation zones, nitrogen fixation zone and senescence zone. The meristematic and senescence zones were at the apex and base of the nodule, respectively. The meristematic zone consisted of small cells with dense cytoplasm and prominent nuclei (Plate 2B). These cells were not infected by rhizobia. In the infection zone, which was

**Table 10: Nodule occupancies of alfalfa plants inoculated with cysteine auxotrophs of *Sinorhizobium meliloti* Rmd201**

| Name of the strain | Number of plants showing 100% occupancy of nodules by the auxotroph* | Percentage of plants showing 100% occupancy of nodules by the auxotroph |
|---|---|---|
| BA4 | 32 | 80.0 |
| BA7 | 33 | 82.5 |
| VK6 | 30 | 75.0 |
| VK10 | 31 | 77.5 |
| VK13 | 20 | 50.0 |
| VK17 | 37 | 92.5 |
| VK20 | 38 | 95.0 |
| VK32 | 23 | 57.5 |
| VK33 | 21 | 52.5 |
| NV8 | 29 | 72.5 |
| NV11 | 27 | 67.5 |
| NV15 | 31 | 77.5 |
| NV16 | 33 | 82.5 |
| NV17 | 28 | 70.0 |
| NV20 | 32 | 80.0 |
| NV25 | 25 | 62.5 |
| NV30 | 36 | 90.0 |
| NV55 | 29 | 72.5 |

*Forty alfalfa plants were inoculated with each auxotroph

**Table 11: Nodule occupancies of alfalfa plants inoculated with methionine auxotrophs of *Sinorhizobium meliloti* Rmd201**

| Name of the strain | Number of plants showing 100% occupancy of nodules by the auxotroph* | Percentage of plants showing 100% occupancy of nodules by the auxotroph |
|---|---|---|
| BA8 | 31 | 77.5 |
| VK21 | 34 | 85.0 |
| VK29 | 35 | 87.5 |
| VK31 | 32 | 80.0 |
| VK36 | 29 | 72.5 |
| VK39 | 22 | 55.0 |
| NV22 | 37 | 92.5 |
| NV35 | 33 | 82.5 |
| NV36 | 30 | 75.0 |

*Forty alfalfa plants were inoculated with each auxotroph

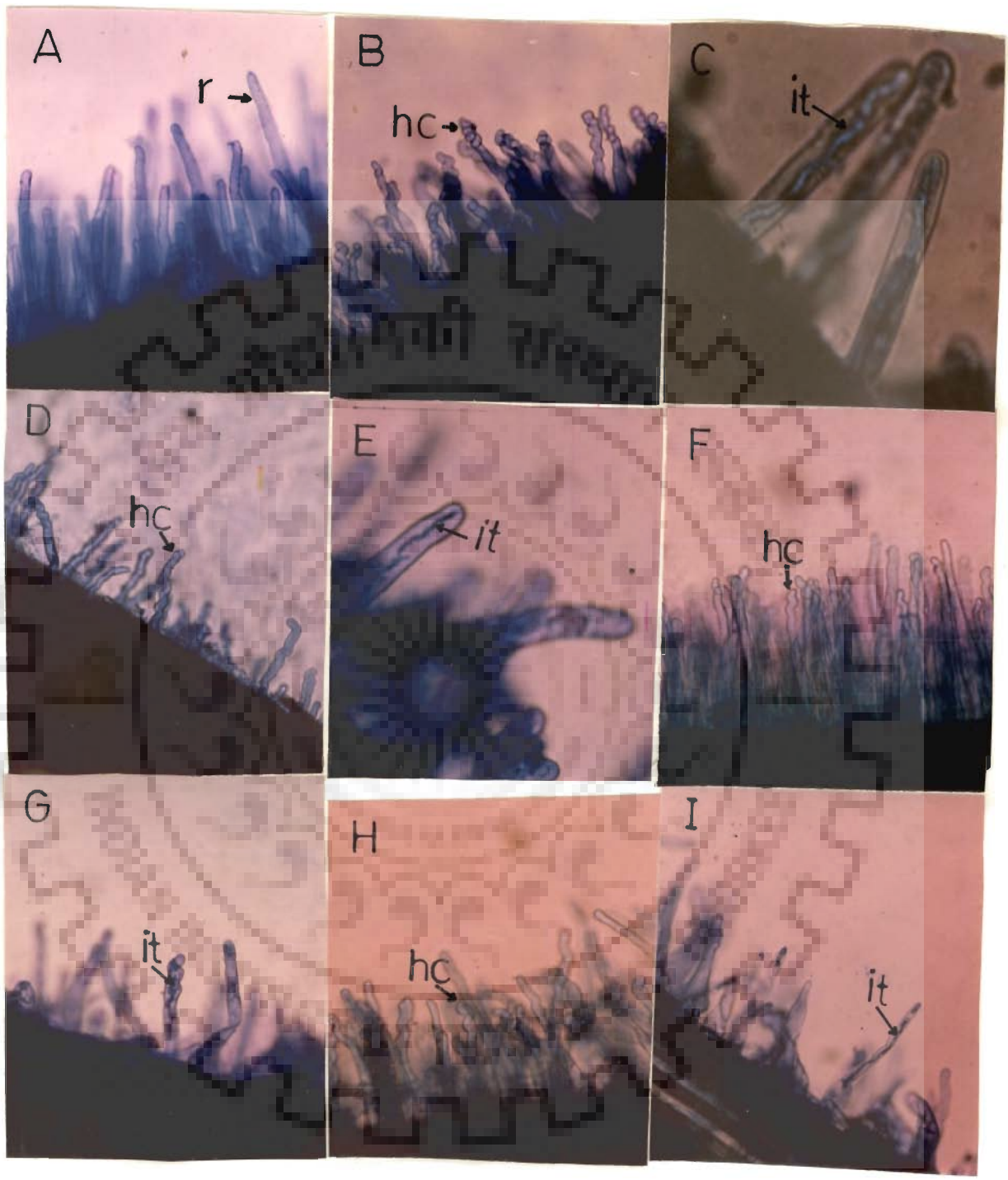



**Plate 1.** Root hair curling and infection thread formation in root hairs of alfalfa seedlings inoculated with *Sinorhizobium meliloti* Rmd201 and its methionine auxotrophic mutants. **A.** Uninoculated; **B.** and **C.** Rmd201 inoculated; **D.** and **E.** BA8 inoculated; **F.** and **G.** VK29 inoculated; **H.** and **I.** VK39 inoculated.

***Abbreviations:*** r, root hair; hc, root hair curling; it, infection thread. A, B, D, F, G & H (x100); C&E (x400).

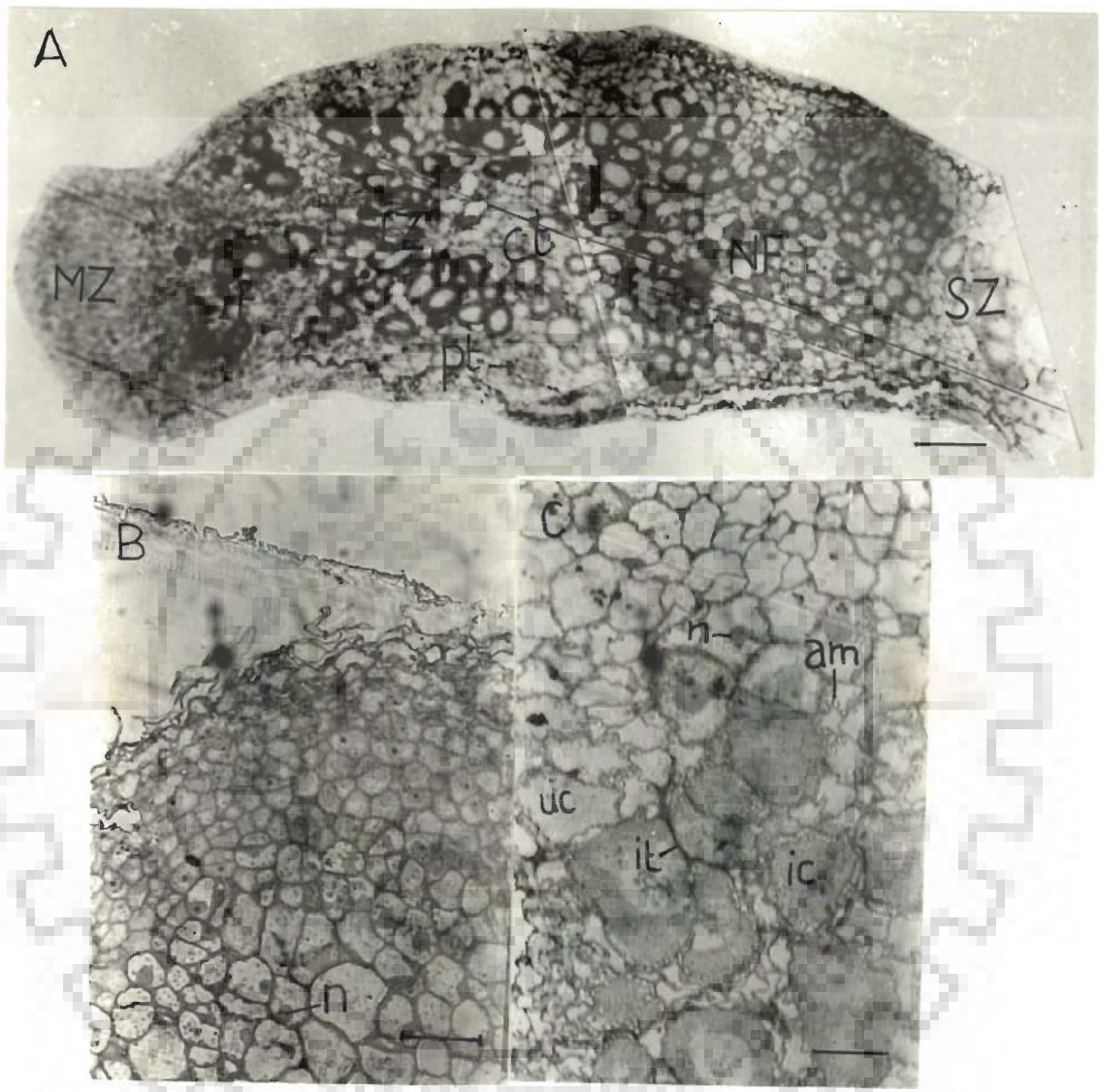



**Plate 2:** Light microscopic examination of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201. **A.** A nodule section showing distinct peripheral tissue (pt) and central tissue (ct); meristematic zone (MZ), infection zone (IF), interzone (IZ), nitrogen fixation zone (NF) and senescence zone (SZ) are seen in central tissue, Bar: 100μm (x 100). **B.** Nodule cells of meristematic zone showing prominent nuclei (n), Bar: 25μm (x 400). **C.** Nodule cells of infection zone showing infected nodule cells (ic), uninfected nodule cells (uc), prominent nuclei (n), infection thread (it) and amyloplast (am), Bar: 25μm (x 400).

located next to the meristematic zone, infection threads were seen in the intercellular spaces of the nodule cells (Plate 2C). Prominent nuclei and amyloplasts were also observed in the infection zone. In the interzone, most of the cells towards the infection zone were without rhizobia whereas most of the cells towards nitrogen fixation zone were infected (Plate 3A). Some amyloplasts were also seen in the interzone. Most of the nodule cells in the nitrogen fixation zone were infected. A large number of bacteroids were arranged around a centrally located large vacuole in each infected nodule cell (Plate 3B). Lysed nodule cells were observed in the senescence zone (Plate 3C).

**Electron microscopy**:

The meristematic zone was devoid of infection threads and rhizobial bacteria (Plate 4A). Infection threads were seen in intercellular spaces of nodule cells in the infection zone (Plate 4B). Rhizobial cells were present in the infection threads and in some of the nodule cells in the infection zone (Plate 4B; 4C). Poly-β-hydroxy butyrate (phb) granules were visible in rhizobial bacteria in infection threads (Plate 4B) and in freshly released rhizobia in nodule cells (Plate 4C; 4D). Each freshly released bacterial cell was enclosed in a peribacteroid membrane (pbm). phb granules and pbm were not seen in the bacteroids present in the nitrogen fixation zone (Plate 5B). The pbm of some bacteroids in the senescence zone was broken (Plate 5C).

The cytoplasm of the rhizobial bacteria in the infection zone was electron dense (Plate 4C; 4D). The cytoplasm of the bacteroids in the interzone and nitrogen fixation zone was heterogeneous having electron dense and electron transparent regions; the heterogeneity indicated the condensation of nuclear material (Plate 5A; 5B). The bacteroidal cytoplasm in the senescence zone was electron transparent (Plate 5C).

Amyloplasts and mitochondria were present in the nodule cells of the infection zone (Plate 4C). These organelles were rarely visible in the nodule cells of the nitrogen fixation zone.

**4.14.2.2 Light and electron microscopic studies of nodules induced by the *metA/metZ* mutant BA8**

**Light microscopy:**

Distinct peripheral and central tissues were present in the nodules induced by the *metA/metZ* mutant BA8. The central tissue of the nodule, like that of the parental strain induced nodule, was differentiated into five zones (Plate 6A; 6B). However, in comparison to the parental strain induced nodule, fewer cells infected with rhizobia were seen in the inter and nitrogen fixation zones (Plate 6C; 6D).

**Electron microscopy:**

The stages of the infection of plant cells by rhizobial bacteria and the stages of bacteroidal development in the nodule induced by the BA8 mutant were similar to those seen in the parental strain induced nodule (Plate 7A; 7B; 8A; 8B; 8C).

**4.14.2.3 Light and electron microscopic studies of nodules induced by *metF* mutant VK39**

**Light microscopy:**

A longitudinal section of the nodule induced by the *metF* mutant VK39 showed that the nodule consisted of peripheral and central tissues (Plate 9A). The meristematic, infection, inter and senescence zones were like those in the parental strain induced nodule (Plate 9B; 9C; 10B). A fully developed nitrogen fixation zone was not present. The arrangement of rhizobia around a large central vacuole in each infected nodule cell in

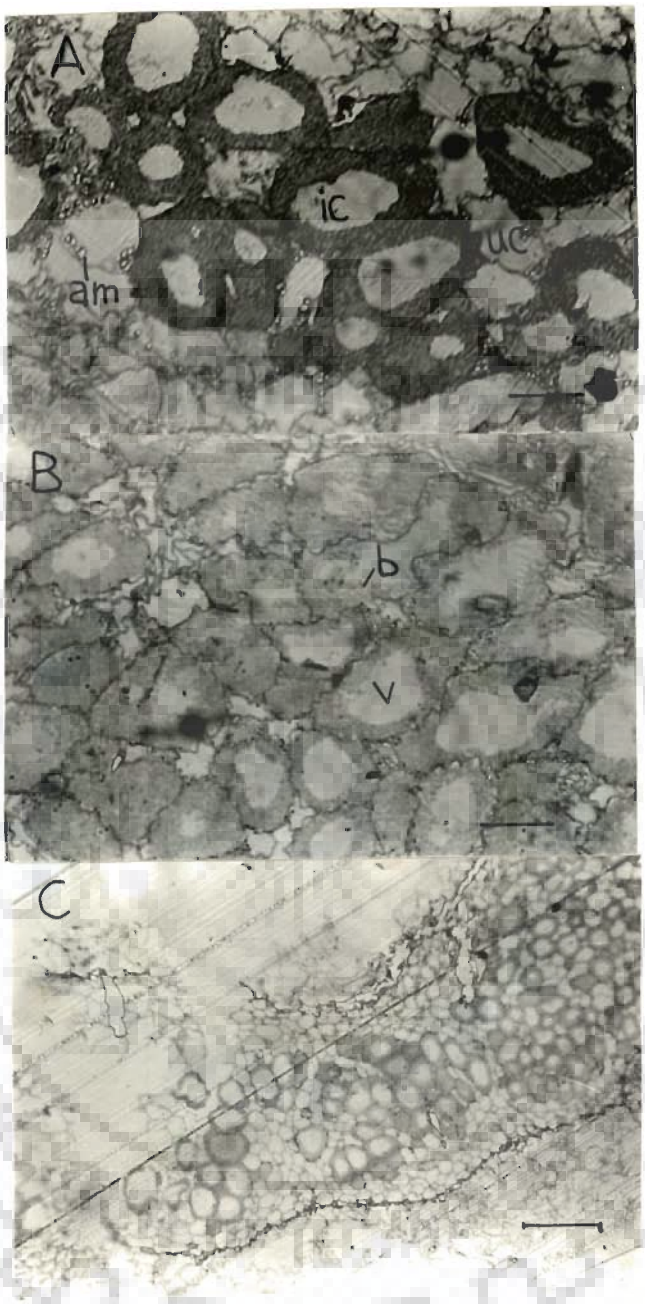



**Plate 3:** Light microscopic examination of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201. **A.** Nodule cells of interzone showing infected cells (ic), uninfected cells (uc) containing amyloplast (am), Bar: 25µm (x400). **B.** Nodule cells of nitrogen fixation zone showing infected cells (ic) containing central vacuole (v) and bacteroids (b), Bar 25µm (x400). **C.** Nodule cells showing lysis in senescence zone, Bar: 100µm (x100).

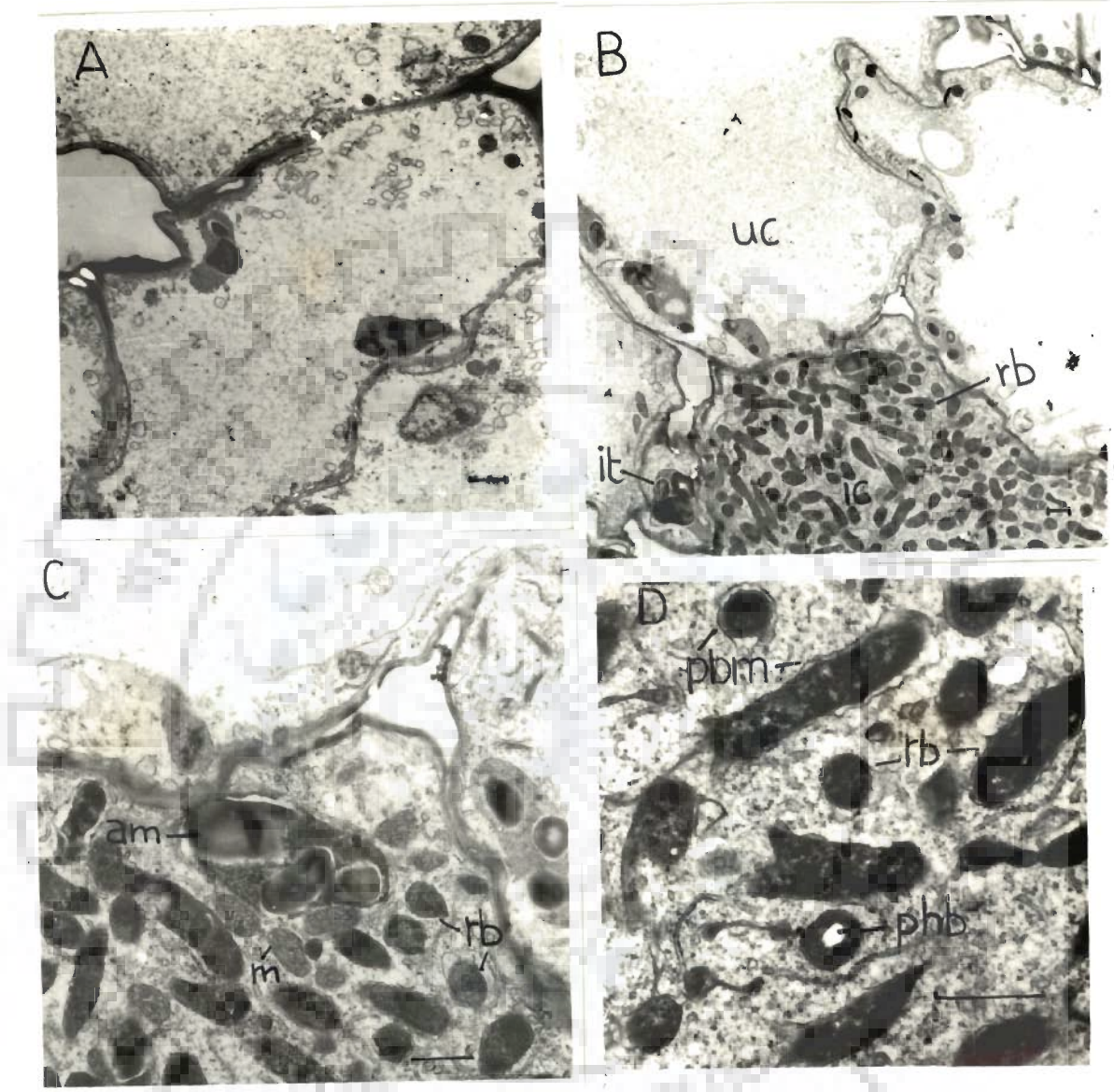



**Plate 4:** Transmission electron microscopic examination of longitudinal-ultrathin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201. **A.** Nodule cell of meristematic zone, Bar: 1µm (x4900). **B.** A part of infection zone showing an infection thread (it) in intercellular space of nodule cells, uninfected cells (uc) and infected cells (ic) containing released rhizobia (rb), Bar: 1µm (x2600). **C.** A part of an infected cell of infection zone containing released rhizobial bacteria (rb), mitochondria (m) and amyloplast (am), Bar: 1µm (x8500). **D.** A part of nodule cell of infection zone showing released rhizobial bacteria (rb) containing poly-β-hydroxybutyrate (phb) granules and peribacteroid membrane (pbm), Bar: 1µm (x15800).

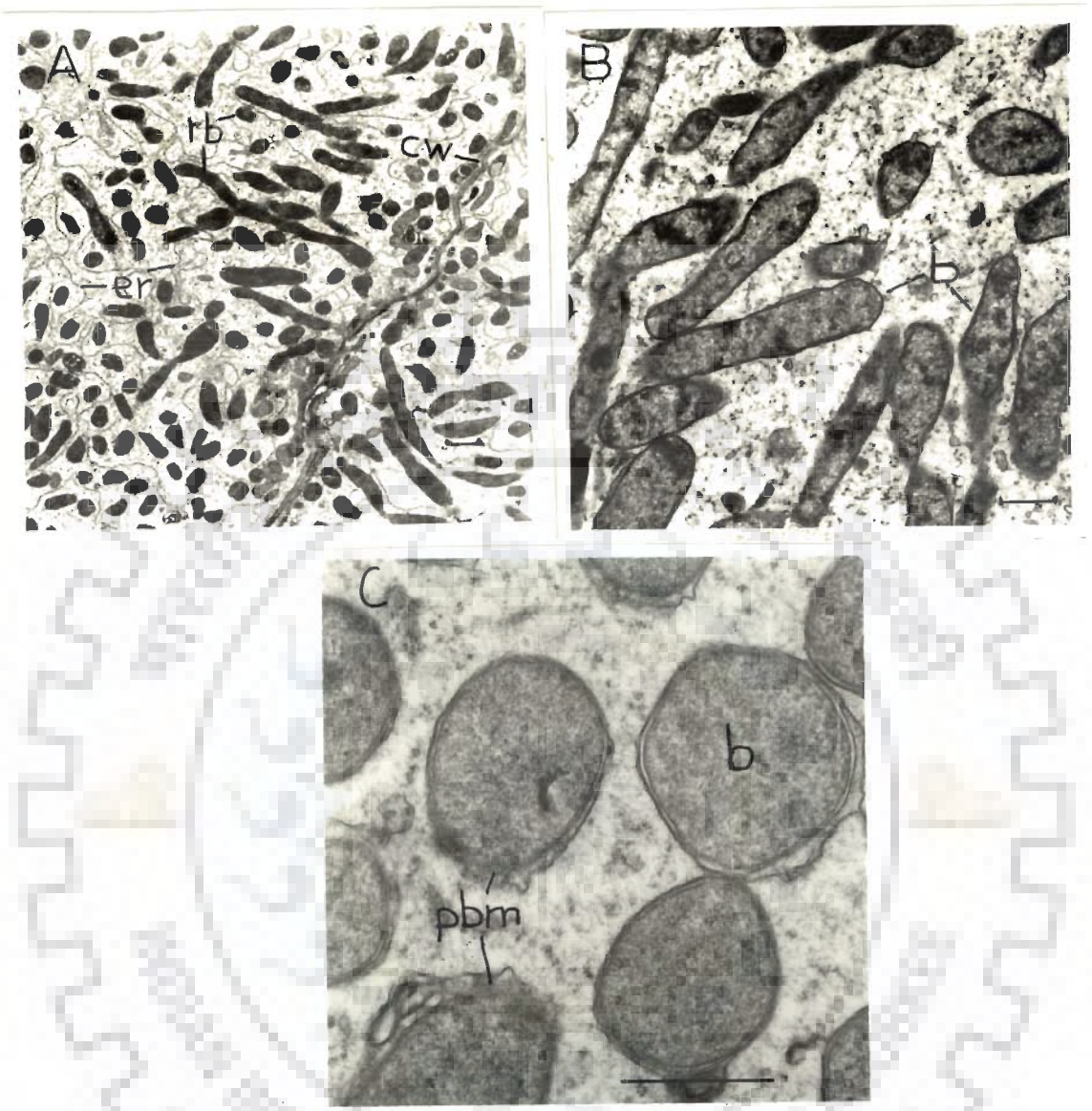



**Plate 5.** Transmission electron microscopic examination of longitudinal-ultrathin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201. **A.** A part of a nodule cell of interzone showing rhizobial bacteria (rb), endoplasmic reticulum (er) and cell wall (cw), Bar: 1μm (x3900). **B.** A part of a nodule cell of nitrogen fixation zone showing bacteroids (b) of different shapes containing heterogeneous cytoplasm, Bar: 1μm (x8500), **C.** A part of a nodule cell showing broken peribacteroid membrane (pbm) and electron transparent cytoplasm of bacteroids (b) in senescence zone, Bar: 1μm (x21000).

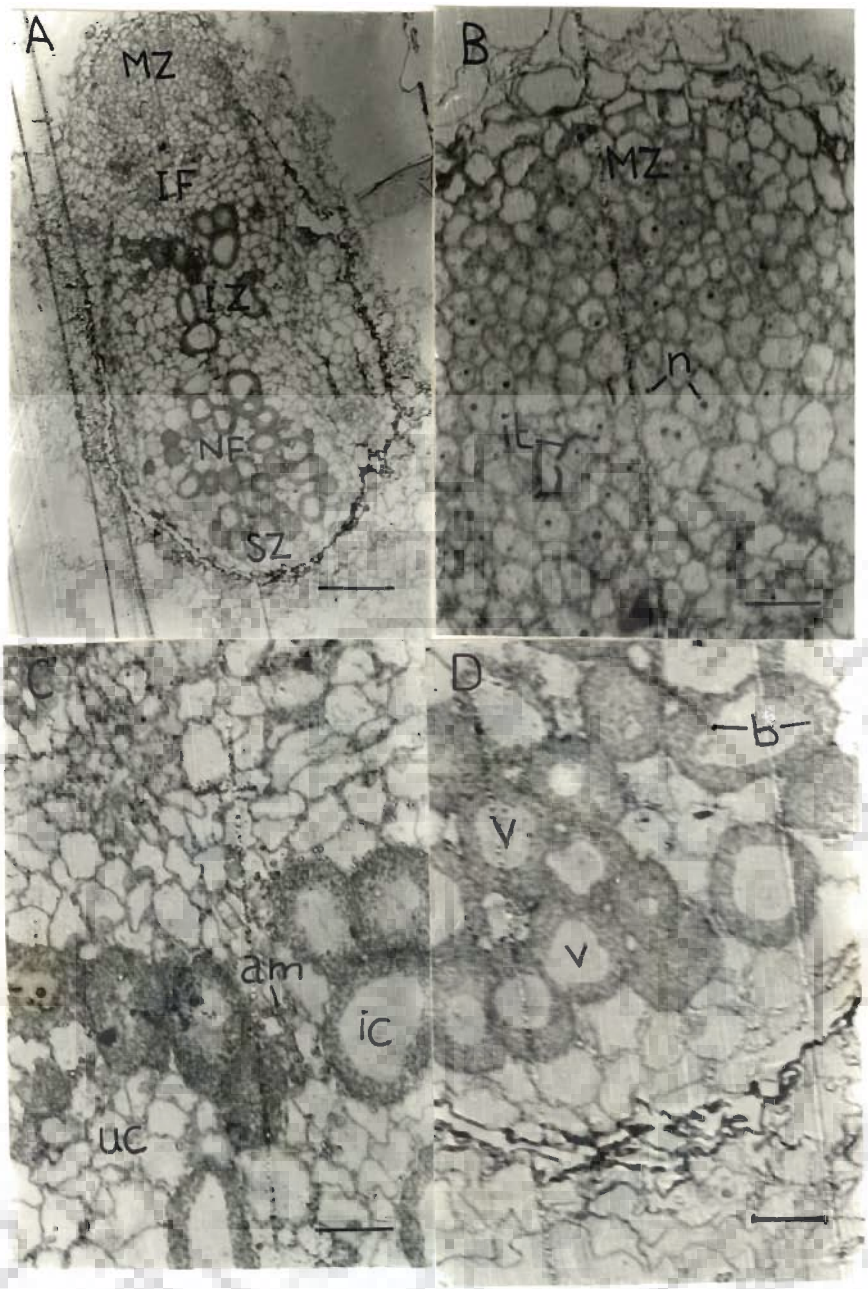



**Plate 6.** Light microscopic examination of longitudinal-semithin sections of a nodule induced by the strain BA8, a *metA/metZ* auxotrophic mutant of *Sinorhizobium meliloti* Rmd201. **A.** A nodule section showing meristematic zone (MZ), infection zone (IF), interzone (IZ), nitrogen fixation zone (NF) and senescence zone (SZ), Bar: 100µm (x100). **B.** Nodule cells of meristematic zone (MZ) and infection zone (IF); prominent nuclei (n) and infection threads (it) in the intercellular spaces are visible, Bar: 25µm (x400). **C.** Nodule cells of interzone showing uninfected (uc) and newly infected cells (ic). Amyloplast (am) visible in this section, Bar: 25µm (x400). **D.** Cells of nitrogen fixation zone; arrangement of bacteroid (b) around a large central vacoule can be seen in some infected nodule cells, Bar: 25µm (x400).

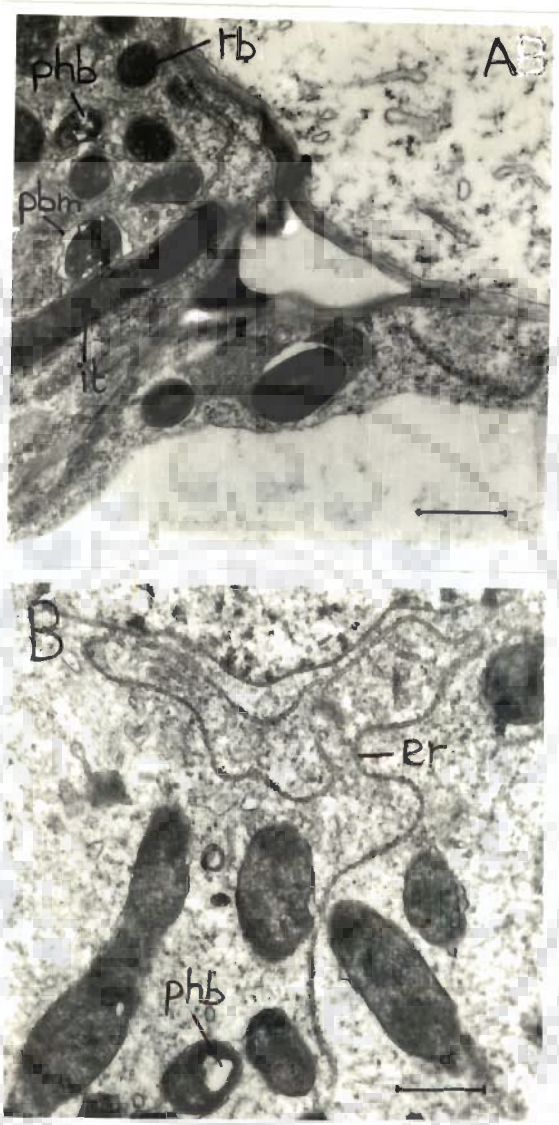



**Plate 7.** Transmission electron microscopic examination of longitudinal-ultrathin sections of a nodule induced by the strain BA8, a *metA/metZ* mutant of *Sinorhizobium meliloti* Rmd201. **A.** and **B.** Parts of nodule cells of infection zone showing released bacteria (rb) having poly-β-hydroxybutyrate (phb) granules and surrounded by peribacteroid membrane (pbm), Bar: 1μm (x11500).

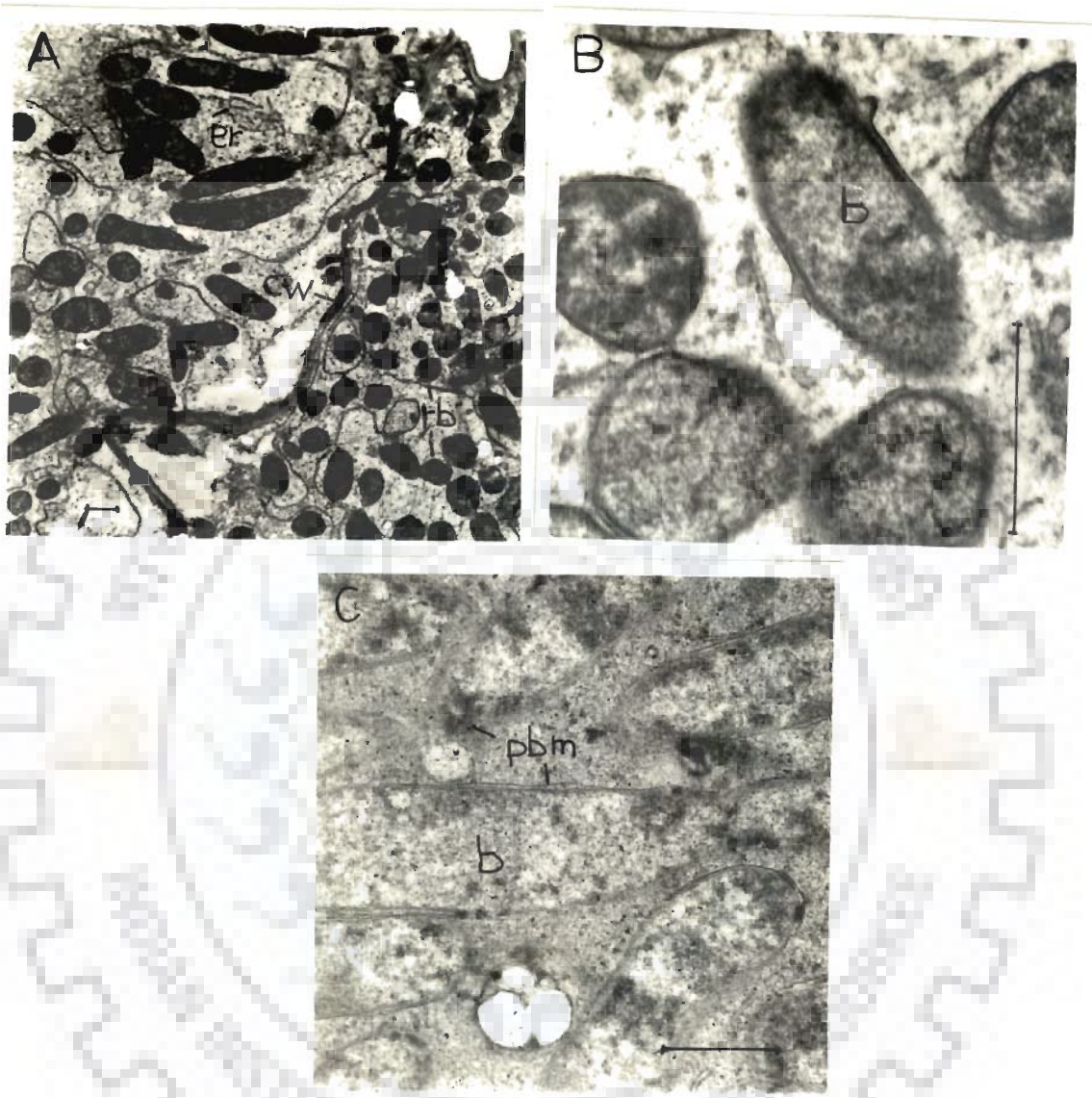



**Plate 8.** Transmission electron microscopic examination of longitudinal-ultrathin sections of a nodule induced by the strain BA8, a *metA/metZ* mutant of *Sinorhizobium meliloti* Rmd201. **A.** A part of nodule cells of interzone showing rhizobial bacteria (rb), endoplasmic reticulum (er), and cell wall (cw), Bar: 1μm (x4900). **B.** A part of nodule cell of nitrogen fixation zone containing mature bacteroids (b) having heterogeneous cytoplasm, Bar: 1μm (x2900). **C.** Degenerated bacteroids (b) from senescence zone having transparent cytoplasm and broken peribacteroid membrane (pbm), Bar: 1μm (x15800).

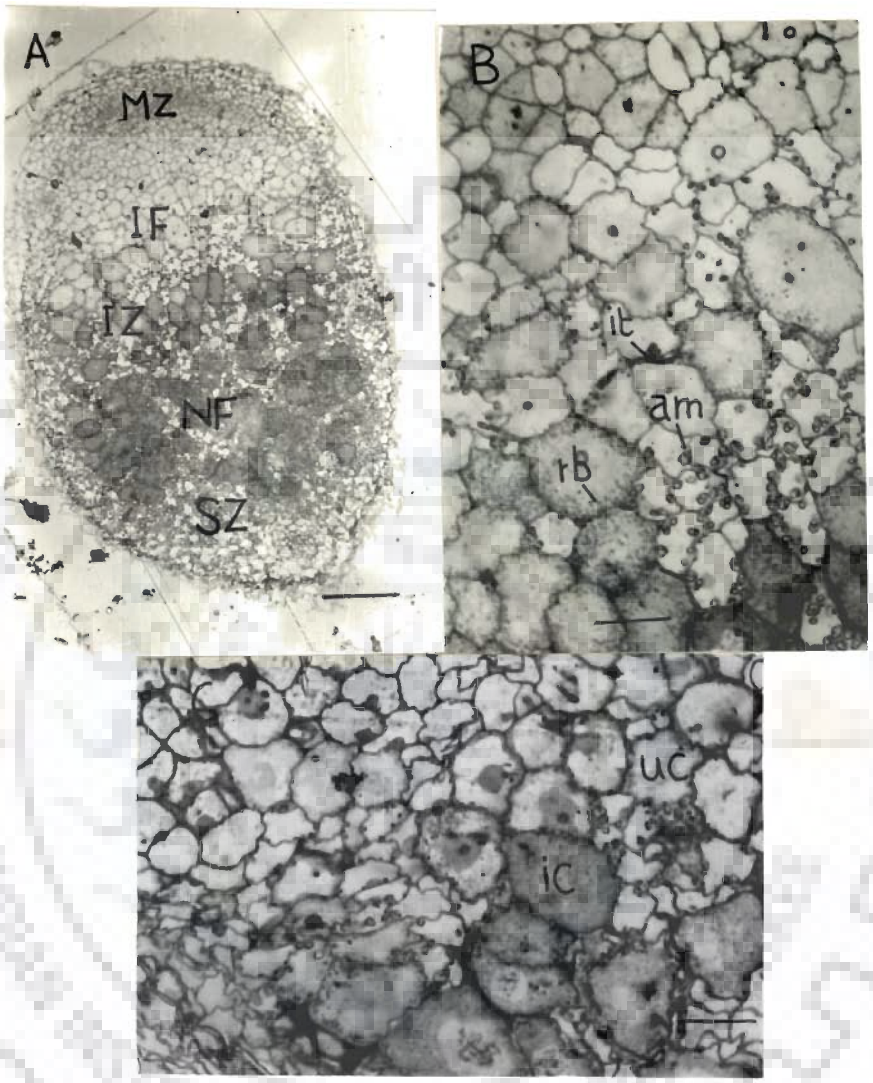



**Plate 9.** Light microscopic examination of longitudinal-semithin sections of a nodule induced by the strain VK39, a *metF* mutant of *Sinorhizobium meliloti* Rmd201. **A.** A nodule section showing meristematic zone (MZ), infection zone (IF), interzone (IZ), nitrogen fixation zone (NF) and senescence zone (SZ). Peripheral tissue (pt) is also seen in this section, Bar: 100μm (x100). **B.** Nodule cells of meristematic zone and infection zone showing infection thread (it), freshly released rhizobia (rb) and amyloplast (am), Bar: 25μm (x400). **C.** Cells of infection zone showing infected (ic) and uninfected (uc) cells, Bar: 25μm (x400).

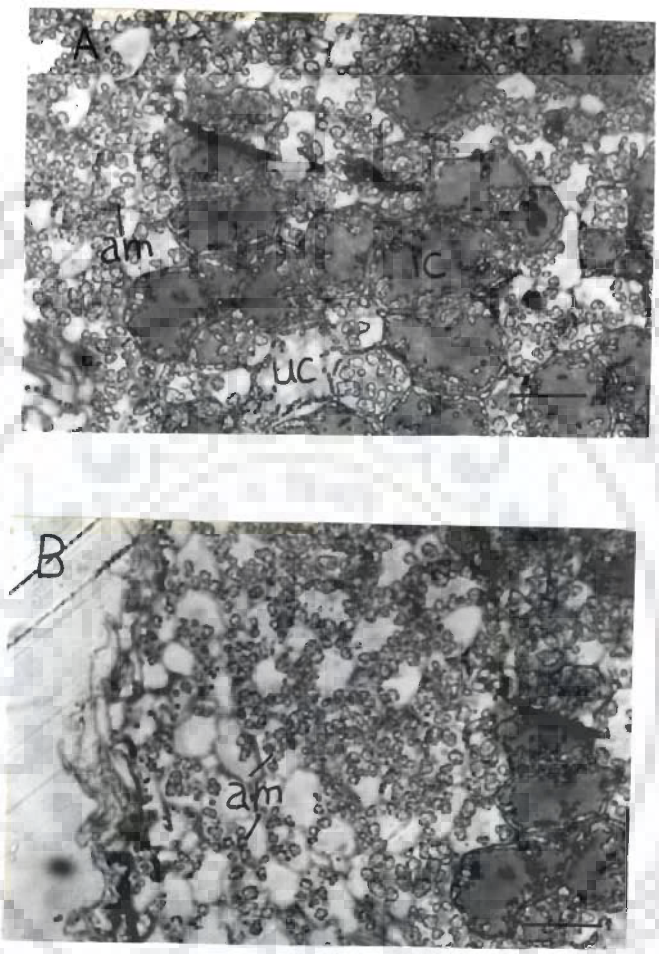



**Plate 10.** Light microscopic examination of longitudinal-semithin sections of a nodule induced by the strain VK39, a *metF* mutant of *Sinorhizobium meliloti* Rmd201. **A.** Nodule cells of nitrogen fixation zone showing infected (ic) and uninfected (uc) cells. Large number of amyloplasts (am) were seen in uninfected cells, Bar: 25μm (x400). **B.** Nodule cells of senescence zone accumulating large number of amyloplasts (am), Bar: 25μm (x400).

nitrogen fixation zone was not seen (Plate 10A). A large number of amyloplasts were observed throughout the nodule except the meristematic zone (Plate 9B; 9C; 10A; 10B).

**Electron microscopy:**

The cells in the meristematic zone of the nodule induced by *metF* mutant VK39 were without rhizobia (Plate 11A). The freshly released rhizobia in the nodule cells of the infection zone were small and had electron dense cytoplasm like that in the parental strain induced nodule (Plate 10B). In the so-called nitrogen fixation zone, some elongated rhizobial cells were seen. Most of the cytoplasm of each of the rhizobial cells in this zone was electron transparent (Plate 12A). Hence the heterogeneity of cytoplasm in these rhizobia was not to the extent seen in the nitrogen fixation zone of the parental strain induced nodule. The amyloplasts were found to contain 2-3 starch granules (Plate 12B).

**4.14.2.4 Light and electron microscopic studies of nodules induced by *metE* mutant VK29**

**Light microscopy:**

The longitudinal section of a nodule induced by *metE* mutant VK29 showed that the nodule was differentiated into peripheral and central tissues. Different zones like those in the parental strain induced nodules were visible in the central tissue but, unlike in the parental strain induced nodule, the infection zone was extended (Plate 13A; 13B). Hence uninfected cells occupied most of the nodule. A few nodule cells infected with rhizobia were present near the base of the nodule. The peripheral arrangement of rhizobia around a large central vacuole was observed in each of these cells. Some amyloplasts were also seen in the region of the nodule containing infected cells (Plate 13C).

**Electron microscopy:**

Rhizobial bacteria in the infected nodule cells were small in size and rarely elongated (Plate 14A). The cytoplasm of these bacteria was electron dense (Plate 14B). Lysed rhizobial cells were seen in the basal part of the nodule (Plate 14C).

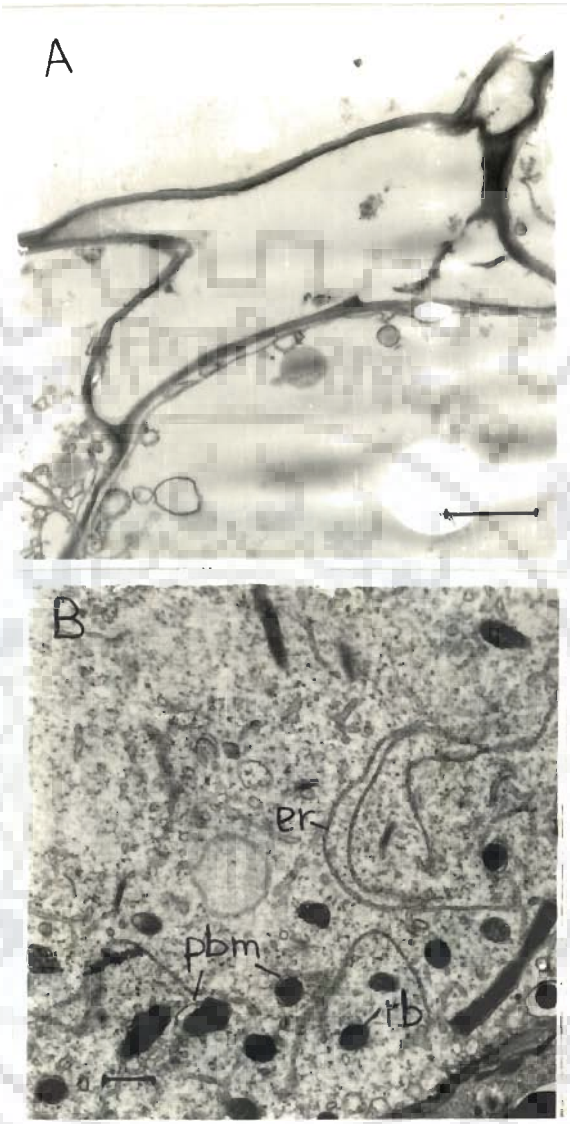



**Plate 11.** Transmission electron microscopic examination of longitudinal-ultrathin sections of a nodule induced by the strain VK39, a *metF* mutant of *Sinorhizobium meliloti* Rmd201. **A.** Nodule cells of meristematic zone devoid of rhizobia, Bar: 1μm (x11500). **B.** A part of nodule cell of infection zone showing freshly released rhizobia (rb) having peribacteroid membrane (pbm) and electron dense cytoplasm. Endoplasmic reticulum (er) also seen in this section, Bar: 1μm (x6650).

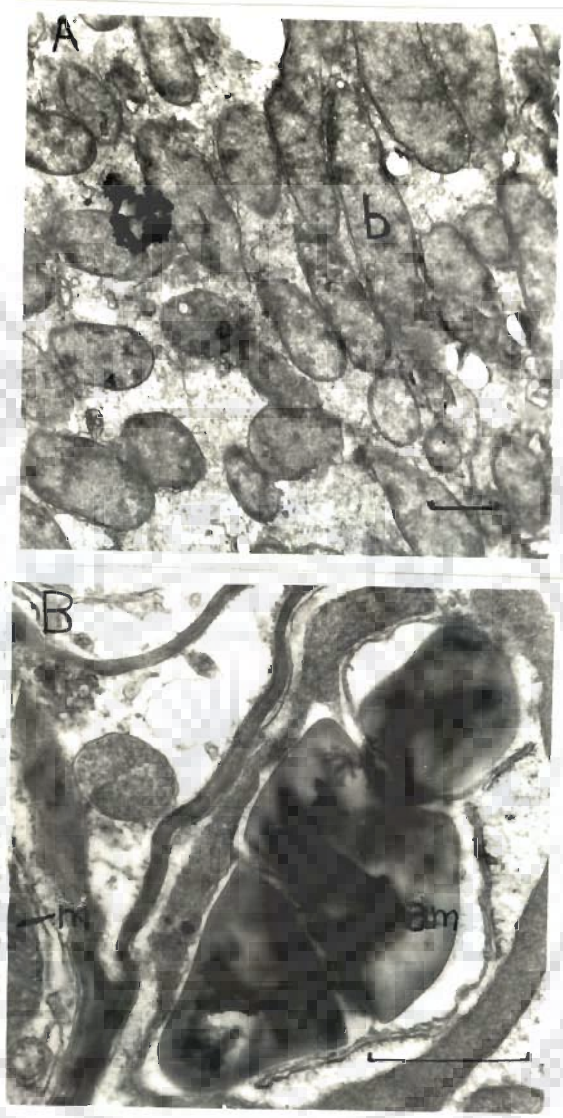



**Plate 12.** Transmission electron microscopic examination of longitudinal-ultrathin sections of a nodule induced by the strain VK39, a *metF* mutant of *Sinorhizobium meliloti* Rmd201. **A.** A part of nodule section of so called nitrogen fixation zone showing bacteroids (b) with less heterogeneity than those found in nitrogen fixation zone of nodule formed by the parental strain, Bar: 1μm (x8500). **B.** A part of nodule section of so called nitrogen fixation zone showing mitochondria (m) and amyloplast (am), Bar: 1μm (x21000).

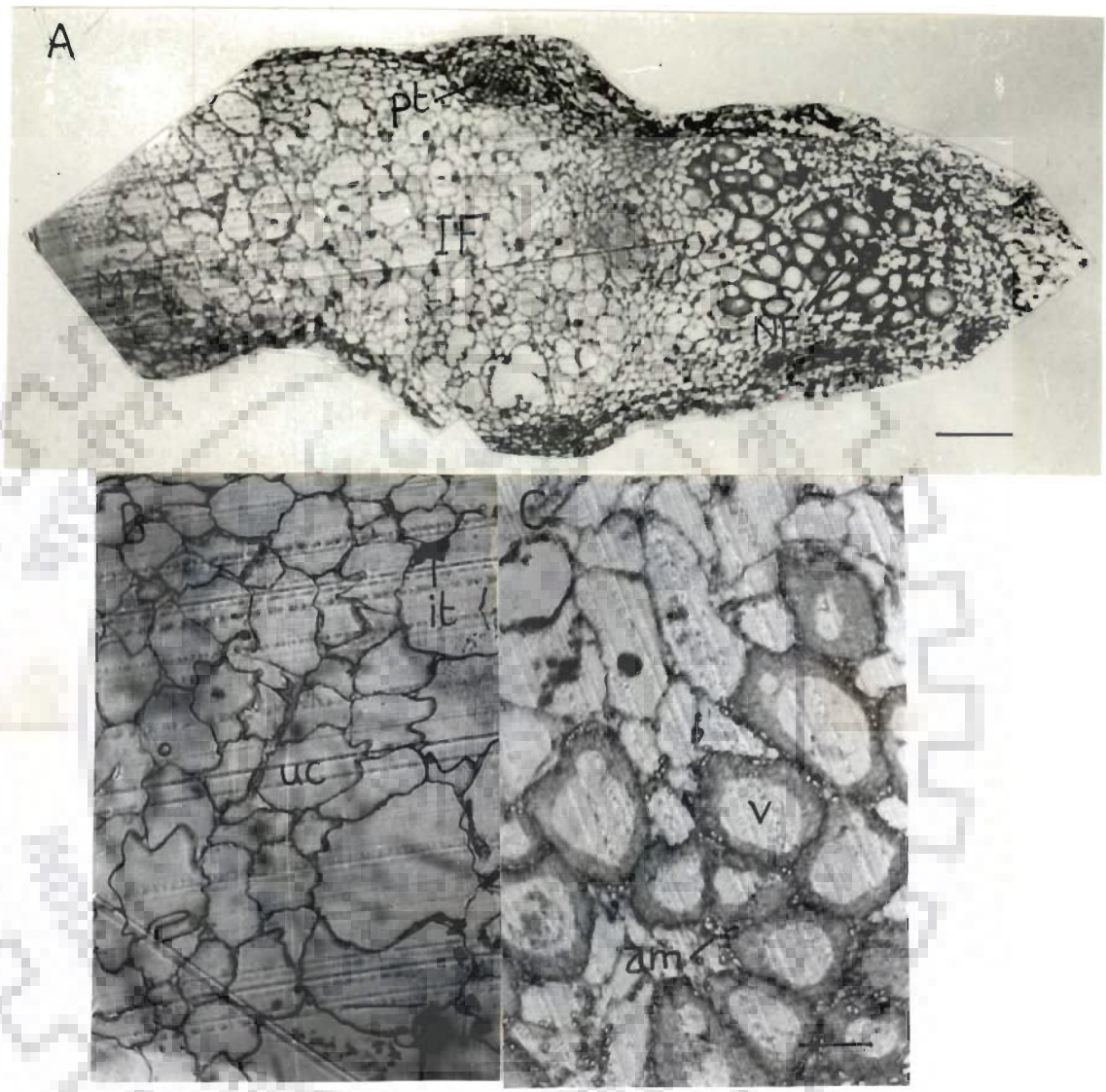



**Plate 13.** Light microscopic examination of longitudinal-semithin sections of a nodule induced by the strain VK29, a *metE* mutant of *Sinorhizobium meliloti* Rmd201. **A.** A nodule section showing meristematic zone (MZ), extended infection zone (IF) and nitrogen fixation zone (NF). Peripheral tissue (pt) also seen in this section, Bar: 100µm (x100). **B.** Nodule cells of infection zone showing infection thread (it) and uninfected cells (uc), Bar: 25µm (x400). **C.** Infected nodule cells having a large vacuole (v) and amyloplast (am).

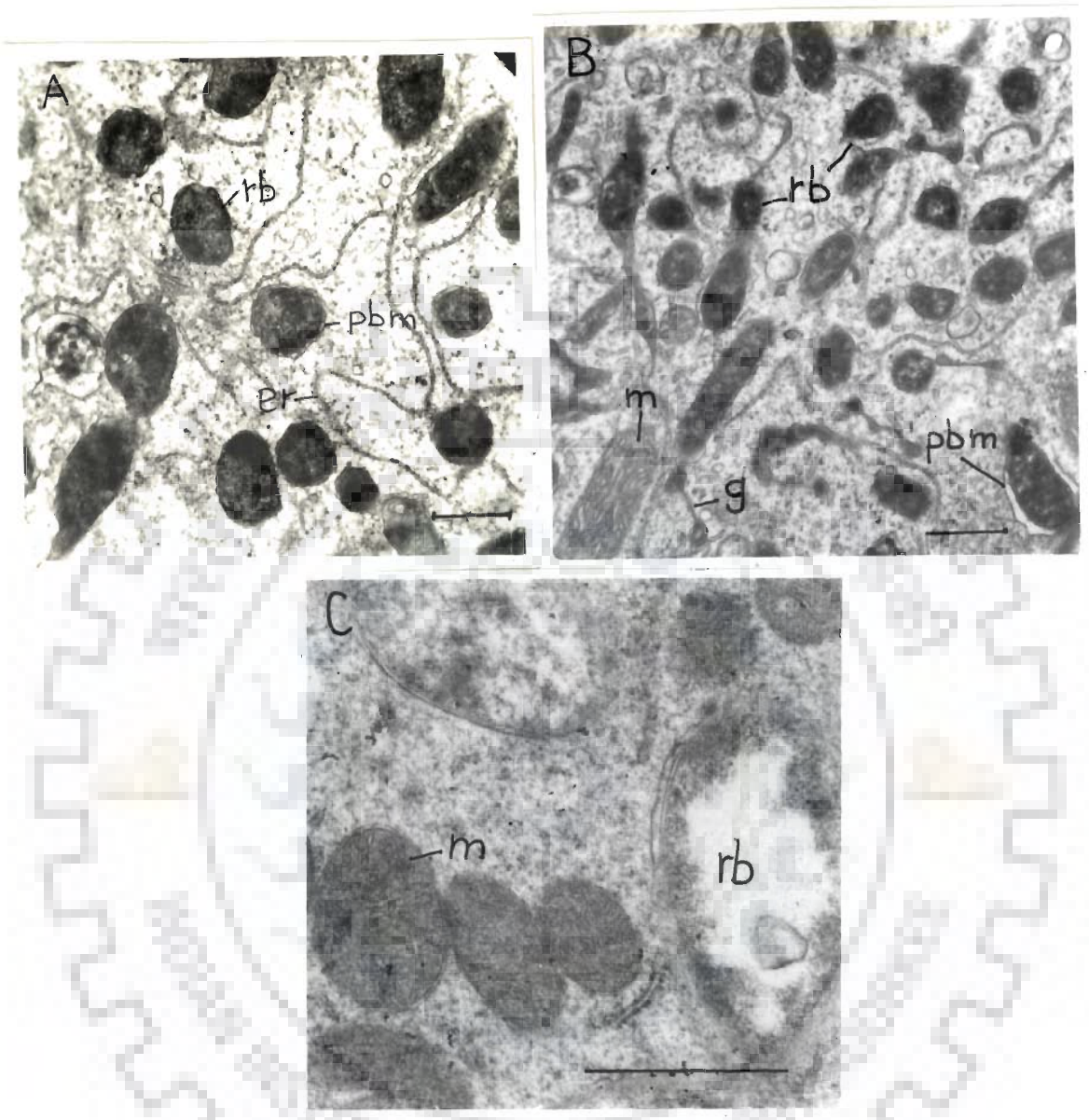



**Plate 14.** Transmission electron microscopic examination of longitudinal-ultrathin sections of a nodule induced by the strain VK29, a *metE* mutant of *Sinorhizobium meliloti* Rmd201. **A.** and **B.** Parts of infected nodule cells showing rhizobial bacteria (rb) with electron dense cytoplasm and peribacteroidal membrane (pbm). Cell organelles like endoplasmic reticulum (er), mitochondria (m), and Golgi complex (g) are visible in this section, Bar: 1µm (x11500) **C.** A part of nodule section of basal part of nodule showing rhizobial bacteria (rb) with electron transparent cytoplasm. Mitochondria (m) also visible in this section, Bar: 1µm (x28750).

# Chapter 5

# DISCUSSION

The role of the rhizobial genes involved in the biosynthesis of sulfur-containing amino acids in symbiosis was studied by isolation and characterization of cysteine and methionine auxotrophs of *S. meliloti* Rmd201. Twelve transposon Tn5 induced auxotrophic mutants of this strain were obtained with the help of Tn5 delivery vector pGS9. The auxotrophic mutants were identified by streaking the Tn5 derivatives on *Rhizobium* minimal medium (RMM). The frequency of occurrence of Tn5 derivatives was $3x10^{-5}$ per recipient. The frequency of occurrence of auxotrophs among Tn5 derivatives was 0.4%. Similar frequencies of occurrence of auxotrophs among Tn5 derivatives in rhizobia have been reported by earlier workers (Meade *et al.*, 1982; Kim *et al.*, 1988, Prasad *et al.*, 2000; Vineetha *et al.*, 2001). The isolation of auxotrophic mutants having different nutritional requirements indicated the randomness of Tn5 insertions as reported previously. The isolated auxotrophs included three sulfur-containing amino acid auxotrophs. High frequencies of occurrence of sulfur amino acid auxotrophs among auxotrophs of rhizobia were observed previously (Meade *et al.*, 1982; Hom *et al.*, 1984; Singh *et al.*, 1984). High frequencies of sulfur amino acid auxotrophs were considered to be due to the presence of a large number of genes involved in sulfur metabolism.

Three sulfur amino acid auxotrophs isolated during this work and 24 such auxotrophs isolated previously in this lab were used in further studies. Eighteen auxotrophs, which grew on RMM agar medium supplemented with cysteine or methionine were sulfite reductase mutants and hence cysteine auxotrophs. Further studies indicated that these auxotrophic mutants were *cysI/cysJ* mutants. It seems that there is a hot spot for transposon Tn5 insertion in *cysI or cysJ* gene of *S. meliloti* Rmd201. All the cys*I*/cys*J* mutants were able to grow on RMM agar medium supplemented with

methionine, homocysteine or cystathionine; the latter two are the intermediates in the biosynthesis of methionine. This result showed that *S. meliloti* is able to convert methionine, homocysteine or cystathionine to cysteine. The isolation of auxotrophs of rhizobia which grew on minimal medium supplemented with cysteine or methionine has also been reported by several workers (Meade *et al.*, 1982; Kim *et al.*, 1988; Schwedock and Long, 1992; Taté *et al.*, 1999b). It appears that the biosynthesis of sulfur-containing amino acids in rhizobia follows the pathway, in which methionine can be converted to cysteine, present in *Saccharomyces cerevisiae* (Cherest and Surdin-Kerjan, 1992) and *Pseudomonas aeruginosa* (Foglino *et al.*, 1995).

The methionine auxotrophs did not show any growth in the liquid minimal medium supplemented with cysteine. It seems that the synthesis of methionine from *O*-succinylhomocysteine and cysteine through transulfurylation pathway is highly inefficient or inactive in *S. meliloti* like that reported in *Pseudomonas aeruginosa* (Foglino *et al.*, 1995). Unlike this situation, methionine auxotrophs of yeast have been reported to grow in cysteine supplemented minimal medium (Kerjan *et al.*, 1986).

On the basis of intermediate and cross feeding studies methionine auxotrophs were designated as *metA/metZ* (BA8, VK21, VK31, NV22, NV35 and NV36), *metE* (VK29 and VK36) and *metF* (VK39) mutants. The *metE* methionine auxotrophs VK29 and VK36 grew on RMM agar medium supplemented with cyanocobalamin (vitamin $B_{12}$). The isolation of such a mutant has been reported in *R. leguminosarum* (Singh *et al.*, 1984). It appears that the final step of methionine biosynthetic pathway (methylation) in rhizobia resembles that present in *E. coli* which is catalyzed by two distinct enzymes; one

requiring only folic acid as a cofactor, and the other requiring both folic acid and vitamin $B_{12}$ as cofactors.

When each auxotroph containing plasmid pJB3JI was crossed with *S. meliloti* ZB557 recipient strain, 100% co-transfer of Tn5-induced kanamycin resistance and auxotrophy was observed. This result showed that each auxotrophic cell had a single Tn5 insertion which was responsible for auxotrophy. The revertants of methionine auxotrophs formed normal nitrogen fixing nodules like the parental strain. Hence, it can be concluded that a single Tn5 insertion was responsible for auxotrophy and symbiotic defect in each methionine auxotroph.

The cysteine and methionine auxotrophs were similar to the parental strain Rmd201 with respect to the production of cell surface molecules and the ability to utilize various carbon sources. These results indicated that a Tn5 insertion in each of these mutants did not result in any change in the cell surface molecules or carbon source utilization properties and the symbiotic defects of methionine auxotrophs were not due to any change in the above characteristics.

All *cysI/cysJ* auxotrophic mutants induced fully effective nodules on alfalfa plants. The reisolation of bacteria from these nodules confirmed the occupation of nodules by the *cysI/cysJ* auxotrophic mutants. This result lead to the conclusion that the mutated *cys* gene (*cysI/cysJ)* of *S. meliloti* has no direct role in symbiosis and the alfalfa plant is able to provide cysteine to rhizobia during symbiosis. The cysteine auxotrophs of *S. meliloti* (Scherrer and Dénarié, 1971) and *S. fredii* (Kim *et al.*, 1988) have been reported to form effective nodules. The cysteine requiring mutants of *S. meliloti* isolated by Malek and Kowalski (1977) showed loss of effectiveness. However, in all the above

reports, the mutated genes were not identified. Taté *et al.* (1997) found that a *cysG* mutant of *R. etli* formed effective nodules on the roots of *Phaseolus vulgaris* plants. Further work needs to be done to find out the role, if any, of the remaining genes of cysteine biosynthetic pathway of rhizobia in symbiosis.

The *metA/metZ* methionine auxotrophs isolated during this study formed nodules which were ineffective in nitrogen fixation. Histological studies revealed that the nitrogen fixation zone in the nodule induced by each of these mutants was not fully developed like that of the nodule induced by the parental strain Rmd201. The supplementation of the plant nutrient medium with methionine restored symbiotic effectiveness to these mutants. This result obviously rules out the direct involvement of homocysteine in symbiosis. But this conclusion may not be true since homocysteine appears to be formed in rhizobia from the externally supplied methionine. Hence, homocysteine may have a symbiotic role in *S. meliloti*. Taté *et al.* (1999b) have reported that a *metZ* mutant of *R. etli* did not induce nodules on the root of *Phaseolus vulgaris*. In the mutants isolated during the present work *metA*, not *metZ*, gene may be mutated. The different symbiotic behaviour of the *metA/metZ* auxotrophic mutants isolated during this work may also be explained on the basis of strain divergence.

The *metE* and *metF* methionine auxotrophic mutants, like the *metA/metZ* mutants, induced ineffective nodules. The exogenous supply of methionine restored symbiotic effectiveness to these mutants. This result indicates that the *metE* and *metF* genes of *S. meliloti* do not have a direct role in symbiosis. However, some differences were found in the histological structures of the nodules induced by *metE* and *metF* mutants. The significance of these differences is not clear at this stage. The methionine auxotrophs of

*S. meliloti* isolated by earlier workers have been found to form effective (Scherrer and Dénarié, 1971) or ineffective (Kerppola and Kahn, 1988a) nodules. However, these workers did not identify the mutated genes.

A few plants among the plants inoculated with the methionine auxotrophs had nodules occupied by prototrophic revertants. The frequencies of nodule occupancy by revertants were more than the frequency of Tn5 excision. This result shows that the prototrophic revertants produced as a result of spontaneous excision of Tn5 had a selective advantage over auxotrophs in nodule formation.

It is clear from the above account that further research work is required to find out the role of the enzymes and intermediates of the biosynthesis of sulfur-containing amino acids of rhizobia in the complex process of symbiosis.

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