ROLE OF ARGININE BIOSYNTHETIC PATHWAY OF *Sinorhizobium meliloti* IN SYMBIOSIS

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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 **"ROLE OF ARGININE BIOSYNTHETIC PATHWAY OF *Sinorhizobium meliloti* IN SYMBIOSIS"**, in fulfillment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biotechnology** of the Institute is an authentic record of my own work carried out during a period from January 2001 to July 2003 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/ Institute.

Anvita Kumar

Dated: **July 8 , 2003** **(ANVITA KUMAR)**

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

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ABSTRACT

Biological nitrogen fixation, via the legume-rhizobium symbiosis, is a renewable and natural source of nitrogen for plants. Rhizobia enter into symbiotic relationship with legumes and induce the formation of root nodules where the atmospheric nitrogen is fixed in the form of ammonia by the changed form of rhizobial bacteria called bacteroids. A number of genes and nutritional conditions in the developing bacteroids are supposed to affect nitrogen fixation. Earlier reports have shown that some metabolites of the biosynthetic pathways of some amino acids, nucleotide bases and vitamins have a role in symbiosis. This work was taken up with the aim to study the role of arginine biosynthetic pathway of *Sinorhizobium meliloti* in symbiosis.

S. meliloti Rmd201, a streptomycin resistant derivative of the strain AK631, was conjugated with *Escherichia coli* WA803 (pGS9). Kanamycin resistant transconjugants of *S. meliloti* were selected by plating the mating mixture on complete medium (Tryptone yeast extract) agar plates containing kanamycin (400 μg/ml) and streptomycin (100 μg/ml). A total of 7,650 Tn5-induced kanamycin resistant transconjugants were obtained from 128 crosses. These transconjugants were screened for auxotrophs by streaking on *Rhizobium* minimal medium (RMM). The nutritional requirements of the auxotrophs were determined on RMM supplemented with Holliday pools. Following auxotrophs (the number of each type is given in brackets) were obtained: arginine (15), methionine (3), tryptophan (2), cysteine (7), uracil (3) and adenine (2). The 15

arginine-requiring auxotrophs (AK3, AK4, AK5, AK6, AK7, AK8, AK9, AK10, AK11, AK12, AK13, AK14, AK15, AK16, AK25) obtained were used for further studies. The frequency of spontaneous reversion to prototrophy was determined for each auxotroph by plating a known number of mutant cells on RMM. The reversion frequencies varied from 1.0 $x10^{-9}$ for AK5 to 3.0 x 10^{-9} for AK15. When transposon Tn5-encoded kanamycin resistance marker of each arginine auxotroph was transferred to *S. meliloti* ZB201 strain, all kanamycin resistant transconjugants (150 in each case) were found to be arginine auxotrophs. One hundred per cent co-transfer of Tn5 and auxotrophy indicated complete linkage of transposon Tn5 insertion to auxotrophy.

The biochemical block in each arginine-requiring auxotroph was determined by streaking it on the minimal medium (RMM) supplemented with different intermediates, viz. ornithine, citrulline and arginosuccinic acid, of the arginine biosynthetic pathway. On the basis of intermediate feeding arginine auxotrophic mutants were classified into following four categories: (i) *argA/argB/argC/argD/argE* mutants (AK3, AK4 and AK5) which grew on RMM supplemented with ornithine, citrulline or arginosuccinic acid; these mutants were also designated as ornithine auxotrophs, (ii) *argF/argI* mutants (AK6, AK7, AK8, AK10, AK11, AK13, AK14, AK15, AK16 and AK25) which did not grow on RMM supplemented with ornithine but grew on RMM supplemented with citrulline or arginosuccinic acid, (iii) *argG* mutant (AK12) which did not grow on RMM supplemented with ornithine or citrulline but grew on RMM supplemented with

arginosuccinic acid and (iv) *argH* mutant (AK9) which did not grow on RMM supplemented with ornithine, citrulline or arginosuccinic acid.

All the mutants, like the parental strain Rmd201, showed growth in presence of sodium deoxycholate (DOC), took up congo red dye, fluoresced in presence of calcoflour white under the UV light and showed motility on swarm plates, indicating the normal production of lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β(1→2) glucans, respectively. These auxotrophs did not show binding with aniline blue dye which indicated that β(1→3) glucans, like the parental strain, were not produced. These results showed that the Tn5 insertions had no pleiotropic effects on cell surface characteristics. No change in growth of arginine auxotrophs was observed when glucose in RMM medium was replaced by any one of the other sugars (sucrose, arabinose, xylose, maltose, lactose, galactose, mannitol or fructose) or dicarboxylic acids (malic acid, succinic acid, aspartic acid, glutamic acid or fumaric acid) as a sole carbon source. These findings showed that the uptake/utilization of sugars and dicarboxylic acids was not affected by Tn5 insertions in the arginine auxotrophs.

The symbiotic properties of arginine auxotrophs were determined by inoculating alfalfa (*Medicago sativa* cv. T9 and cv. A2) seedlings grown aseptically on nitrogen free agar media slants with these auxotrophs. The parental strain Rmd201 and the arginine mutants in the later part of the pathway (after ornithine) induced cylindrical, pink nodules on both primary and lateral roots of plants of both alfalfa cultivars, T9 and A2. These results indicated that

the nitrogen fixing efficiencies of these mutants were similar to that of the parental strain. The nodules induced by ornithine auxotrophs and AK10, an *argF/argI* mutant, were spherical/irregular and white; these nodules were located mostly on the lateral roots. These results indicated that these auxotrophs did not fix nitrogen.

Normal symbiosis, like that of the parental strain, was observed when alfalfa plants were inoculated with the spontaneous revertants of ornithine auxotrophs. The reisolation studies showed 100% occupancy of nodules by the arginine auxotrophs.

Light microscopic studies of the longitudinal section of the nodule induced by the parental strain Rmd201 showed a central tissue surrounded by several peripheral tissues. 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 internal structural features of the nodules induced by arginine auxotrophs *argF/argI* (AK11), *argG* (AK12) and *argH* (AK9) were similar to those of the parental strain induced nodules. Distinct peripheral and central tissues like those in the parental strain induced nodules were seen in the nodules of the ornithine auxotrophs AK3 and AK4. The central tissues of these nodules, like that of the parental strain induced nodules, were differentiated into five zones; however, the infection and the senescence zones were larger than those of Rmd201 nodules. The nitrogen fixation zone in the nodules of ornithine auxotrophs was identified on the basis of its visual resemblance to the nitrogen fixation zone of the Rmd201 induced

nodules. In comparison to the parental strain induced nodule, few nodule cells were infected with rhizobia in the interzone and the so-called nitrogen fixation zone.

TEM studies of the ultrathin section of the nodule induced by the parental strain Rmd201 showed the presence of poly-. -hydroxy butyrate (PHB) granules in the rhizobial bacteria in infection threads and in freshly released rhizobia in nodule cells. Peribacteroid membrane (pbm) surrounded each freshly released bacterial cell. Some bacteroids in the senescence zone had broken pbm. The cytoplasm of the rhizobial bacteria in the infection zone was electron dense whereas the cytoplasm of the bacteroids in the interzone and nitrogen fixation zone was heterogeneous in the sense that it contained electron dense and electron transparent regions. The cytoplasm of the bacteroids in the senescence zone was electron transparent. Most of the bacteroids in the nitrogen fixation zone were elongated. All stages of the infection of plant cells by rhizobial bacteria and the subsequent bacteroidal development in the nodules induced by the ornithine auxotrophs were similar to those seen in the parental strain induced nodule except that the bacteroids in the so-called nitrogen fixation zone of ornithine mutants were mostly spherical or oval. TEM studies revealed that the internal structures of the nodules induced by *argF/argI*, *argG* and *argH* mutants were similar to the internal structure of the nodule induced by the parental strain Rmd201.

The induction of fully effective nodules by arginine auxotrophs, each having a biochemical block in one of the last three steps (i.e. after ornithine) of

the arginine biosynthetic pathway (with the exception of one mutant AK10), on two cultivars of alfalfa plants showed that alfalfa host plant is able to provide the required quantity of arginine to *S. meliloti* bacteria during symbiosis. AK10 mutant, apart from having a Tn5 insertion in the *argF* gene, appears to have another mutation in one of its symbiotic genes. The studies on the ornithine requiring arginine auxotrophs indicate that ornithine or an ornithine-derived factor is required for the normal development of nitrogen fixation zone and complete transformation of rhizobial bacteria into bacteroids during the symbiosis of *S. meliloti* with alfalfa plants. Normal symbiotic activity of the prototrophs of ornithine auxotrophs indicated that a single Tn5 insertion in each of these auxotrophs was responsible for auxotrophy and the symbiotic defect.

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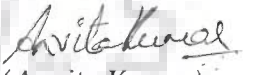

(Anvita Kumar)

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LIST OF ABBREVIATIONS

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

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

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1. INTRODUCTION

Chemical nitrogen fertilizers are an essential input in the modern agriculture. These fertilizers are expensive and cause pollution. The reliance on these fertilizers can be reduced by the use of certain bacteria which have the ability to carry out biological reduction of atmospheric N_2 to ammonia. This process of nitrogen reduction by these organisms is also known as biological nitrogen fixation. The nitrogen fixing bacteria are classified into three groups, viz., free-living (*Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Rhodospirillum rubrum*), associative (*Azospirillum* spp.) and symbiotic (*Rhizobium, Frankia*). Among these, the last group is very important to agriculture since about 25% of the terrestrial nitrogen is fixed by rhizobium-legume symbiosis.

The bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, collectively called rhizobia, enter into a symbiotic association with leguminous plants and a non-legume *Parasponia*. These bacteria induce cell proliferation in the cortical cells of a specific host plant and ultimately form a highly specialized organ called nodule on plant roots. Inside the nodule, the rhizobial bacteria invade the plant cells and get converted into a special form called bacteroids which fix nitrogen. The development and functioning of the symbiotic association is very complex and not fully understood at molecular level. The characterization of the genes and gene products involved in rhizobium-legume symbiotic process can help in constructing efficient rhizobium-legume combinations which may lead to increased agricultural productivity.

Several symbiotic genes of rhizobia and legumes have been identified and characterized. These include rhizobial genes involved in nodule formation (*nod*, *nol*) (Banfalvi *et al.*, 1981; Kondorosi *et al.*, 1984; Fisher and Long, 1992; Freiberg *et al.*, 1997), nodule invasion (*exo*, *pss*, *ndv*, *lps*) (Borthakur and Johnston, 1987; Priefer, 1989; Charles *et al.*, 1991; Noel, 1992; Leigh and Walker, 1994; Król *et al.*, 1998; Pollock *et al.*, 1998) and nitrogen fixation (*nif*, *fix*) (Batut *et al.*, 1985; Putnoky *et al.*, 1988; Beringer *et al.*, 1990; Kündig *et al.*, 1993), and plant genes involved in recognition of bacterial signals (Endre *et al.*, 2002; Stracke *et al.*, 2002) and control of nodule number (Nishimura *et al.*, 2002; Krusell *et al.*, 2002; Searle *et al.*, 2003).

Within the nodules, rhizobia obtain sources of carbon and energy from the plant in the form of dicarboxylic acids (Vance, 2000; Poole & Allaway, 2000) and in return provide the plant with ammonium. Apart from dicarboxylic acids and ammonium, information about the other small metabolites exchanged by symbiotic partners is very limited. The information on the identification and symbiotic role of such metabolites is expected to be very helpful in understanding the molecular biology of rhizobium-legume symbiosis.

The multiplication of rhizobial cells is required during the development of symbiosis. Hence, it is obvious that during the process of establishment of symbiosis, rhizobial bacteria/bacteroids require amino acids, nucleotide bases and vitamins. The detailed information about what metabolites are synthesized by rhizobia and what metabolites (or their intermediates) are provided by the host plant during the development and functioning of symbiosis is lacking. Such

information can be obtained by studying symbiotic properties of the mutants, which are auxotrophic or defective in the transport of these metabolites, of rhizobia. Auxotrophs of some amino acids, nucleotide bases and vitamins of rhizobium have been found to result in defective symbiosis (Schwinghamer, 1970; Pankhurst *et al*., 1972; Kerppola and Kahn, 1988b; Noel *et al*., 1988; Barsomian *et al*., 1992; Newman *et al*., 1994; Taté *et al*., 1999b; 1999c). In some cases it has been found that the reason for the defective symbiosis is not the unavailability of the end product; the defective symbiosis appears to be because of the lack of an intermediate(s) / enzyme(s) of the biosynthetic pathway (Taté *et al*., 1999a; López *et al*., 2001; Soberón *et al*., 2001; Vineetha *et al*., 2001). It has been recently shown, with the help of mutations in the amino acid transporters, *aap* and *bra*, of *Rhizobium leguminosarum* bv. *viciae* that amino acid cycling drives nitrogen fixation in the rhizobium-legume symbiosis (Lodwig *et al*., 2003).

Very little information is available on the role of arginine biosynthetic pathway in the rhizobium-legume symbiosis. Hence, the present work on *Sinorhizobium meliloti*, which makes symbiotic association with alfalfa (*Medicago sativa*) plant, was undertaken with the following objectives:

i) to generate arginine auxotrophs of *S. meliloti* Rmd201 by random transposon Tn5 mutagenesis,

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

iii) to study the symbiotic properties of these auxotrophs, and

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

2. REVIEW OF LITERATURE

The literature pertaining to this work has been reviewed under suitable headings.

2.1 Taxonomy of rhizobia

The bacterium isolated from the legume nodules was first named as *Bacillus radicicola* (Beijerinck, 1888) and later *Rhizobium* (Frank, 1889). Subsequently, slow growing rhizobia were placed under the genus *Bradyrhizobium* (Jordan, 1982; 1984). Later three more genera, viz., *Azorhizobium* (Downie and Brewin, 1992), *Sinorhizobium* (Chen *et al*., 1988; de Lajudie *et al*., 1994; Lindström *et al*., 1995) and *Mesorhizobium* (Jarvis *et al*., 1997) were recognized for rhizobia.

2.2 Signal exchange during nodule formation

The research work on the regulation of legume root nodule development has been recently reviewed by Stougaard (2000) and Long (2001). *Rhizobium*-legume relationship is specific in the sense that a particular species of rhizobia induces nodules on a particular legume or a group of legumes. The expression of several rhizobial genes, called as *nod*, *nol* and *noe* is required for host-recognition and plant invasion. Flavonoids secreted by the plant roots, in conjunction with the rhizobial NodD transcriptional activator, induce expression of the *nod* gene regulon. The *nod* gene products synthesize and transport Nod factors. Nod factors are β-1,4 linked N-acetyl glucosamine compounds typically carrying a fatty acid on the non-reducing sugar and sulfuryl, fucosyl, mannosyl or

arabinosyl groups at the reducing terminal sugar (Lerouge *et al.*, 1990). These lipochito oligosaccharides (LCOs) result in the deformation and curling of root hairs (Schlaman *et al.*, 1989; Journet *et al.*, 1994), and the division of cortical cells of the roots of the leguminous plants.

Recently Endre *et al.* (2002) have cloned a nodulation receptor kinase (NORK) gene from *Medicago sativa* that is essential for Nod - factor perception in alfalfa. Stracke *et al.* (2002) have also described the cloning of symbiosis receptor - like kinase (SYMRK) gene, from lotus and pea, which are required for both arbuscular mycorrhizal fungi and rhizobial recognition. The sequence analysis of NORK and SYMRK genes showed that these genes are analogous. The proteins encoded by these genes have been found to be related to receptors in animals and plants that function in the innate immune system and organ development (Spaink, 2002; Fig. 1).

The number of nodules in the wild type plants is regulated by a process referred to as autoregulation of nodulation. *Lotus japonicus* plants homozygous for a mutation in the hypernodulation aberrant root (*har1*) locus escapes this regulation and forms an excessive number of nodules (van Brusell *et al.*, 2002; Nishimura *et al.,* 2002; Shauser *et al.*, 1998). Recently, Krusell *et al.* (2002) have reported the molecular cloning and expression analysis of the *HAR1* gene of *Lotus japonicus* and the pea orthologue, *Pisum sativum SYM29*. *HAR1* has been found to encode a putative serine/threonine receptor kinase. Through the use of reciprocal and self-grafting studies using *Lotus japonicus* hypernodulating mutants, *har1* (also known as *sym78*), Nishimura *et al.* (2002) have shown that

the shoot genotype is responsible for the negative regulation of nodule development. Autoregulation of nodulation has been found to be controlled by the receptor like protein kinase GmNARK (*Glycine max* nodule autoregulation receptor kinase), similar to *Arabidopsis* CLAVATA1 (Searle *et al.*, 2003). CLAVATA1 and HAR1/NARK appear to have similar functions, because mutation of the corresponding genes results in increased meristematic activity, either in shoot apical meristems (*clavata1*) or in root and nodule meristems (*har1/nark*) (Downie and Parniske, 2002; Fig. 2).

2.3 Role of cell surface polysaccharide of rhizobia in symbiosis

Cell surface polysaccharides like cyclic β-(1→2)-glucans, lipopolysaccharides (LPS I, II), capsular polysaccharides (CPS) and extracellular exopolysaccharides (EPS I, II) of rhizobia are involved in early infection events. *S. meliloti* mutants that are unable to produce symbiotically active extracellular polysaccharides have been found to be defective in nodule invasion (Glazebrook & Walker, 1989; Leigh *et al.*,1985; Reuhs *et al.*, 1993). By using green fluorescent protein - expressing *S. meliloti* strains, it has been demonstrated that each of the three polysaccharides, viz., succinoglycan, EPS II, or K antigen, functions to mediate infection thread initiation and extension on alfalfa (Cheng & Walker, 1998; Pellock *et al.*, 2000). Recently, Pellock *et al.* (2002) have demonstrated that a LuxR homologue controls the production of symbiotically active extracellular polysaccharide II by *S. meliloti*. The wild type LPS layer in *Rhizobium leguminosarum* bv. *viciae* 3841 has been reported to be essential for

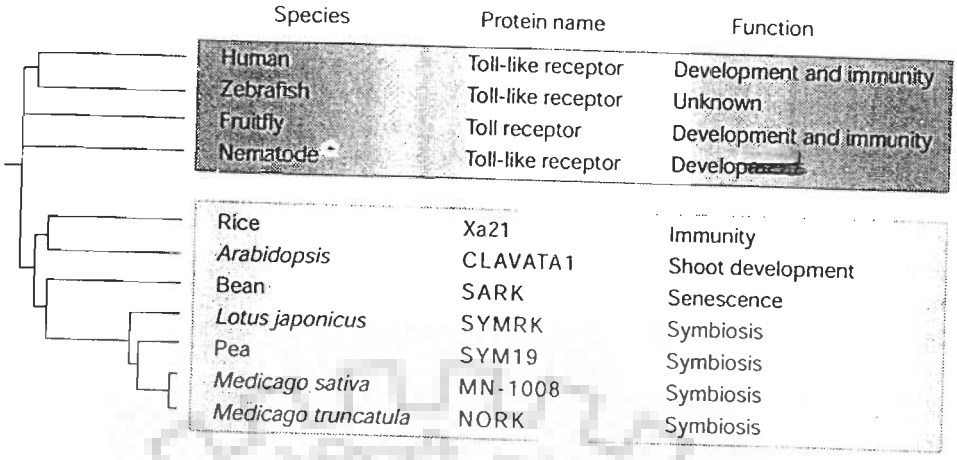

Fig. 1: Comparison of members of the protein family of receptors containing extracellular leucine-rich-repeats (LRR). This tree of protein relatedness compares examples from various subfamilies in plants and animals, and indicates the breadth of species in which the receptors are found, and the variety of functions that they have (Spaink, 2002).

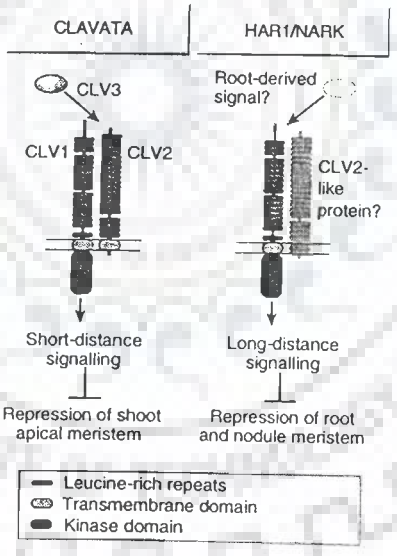

Fig. 2: Proposed functioning of HAR1/NARK proteins in signalling and root nodulation. HAR1 is most similar to CLAVATA (CLV) 1, a receptor kinase containing leucine-rich repeats which interacts with CLV2, forming a complex that recognizes the signalling peptide CLV3. CLV2 contains leucine-rich repeat and transmembrane domains, but lacks a kinase domain. HAR1/NARK might interact with a CLV2-like protein, which is yet to be identified, to recognize a signal produced by root and nodule meristems (Downie and Parniske, 2002).

nitrogen fixation (Poole *et al.*, 1994). Structurally conserved LPS of *Sinorhizobium fredii* and *S. meliloti* lack necessary information to influence host specificity (Reuhs *et al.*, 1998). Capsular polysaccharides (CPSs or K-antigens) of *S. meliloti* may be playing a role in the early recognition of rhizobia by alfalfa leaf cells (Kozak *et al.*, 1997). Delayed nodulation on soybean was observed by the EPS and CPS-deficient mutants of *B. japonicum* 2143 (Eggleston *et al.*, 1996). The *exoB* mutant of *R. leguminosarum* bv. *trifolii* was found to produce altered exopolysaccharide and induced abnormal nodules (Sanchez-Andujar *et al.*, 1997). Symbiotic function of EPS in *S. fredii* seems to differ fundamentally from that in *S. meliloti* (Krishnan & Pueppke, 1998). On the basis of symbiotic phenotype of EPS mutant *pssD133* of *R. leguminosarum* bv. *trifolii*, Krol *et al.* (1998) suggested a correlation between this mutation and defective endocytosis of rhizobial bacteria.

2.4 Early infection events and nodule development

In the most studied legumes, infection occurs via an infection thread that takes the bacteria through the root hair into the root cortex and distributes them to cells, which become the infected cells of the nitrogen-fixing nodule (Stougaard, 2000). The other mode of rhizobial infection is the so-called crack entry, where the bacteria enters the plant root through gaps in the epidermis (Chandler *et al.*, 1982) or enter between intact epidermal cells (De Faria *et al.*, 1988). During root hair infection, root hairs deform and curl, thus producing a pocket in each curl that provides a site for initiating the infection. Root hair deformation is due to a new induction of root hair tip growth by Nod factors (Heidstra *et al.*, 1994).

Simultaneously, the induction of several plant genes like *Mtrip1*, encoding a peroxidase (Cook *et al.*, 1995) and the early nodulin genes ENOD5 and ENOD12 that encode proline rich polypeptides (Scheres *et al.*, 1990a, 1990b) takes place.

Rhizobial bacteria entrapped in a curl carry out the local hydrolysis of the plant cell wall and hence induce the formation of an infection thread in the crook of the curled root hair (Callaham and Torrey, 1981). The infection thread, which is a plant - derived structure originating from plasma membrane invagination accompanied by external deposition of cell wall material, moves inwards in the root cortical cells (Stougaard, 2000). Even before the inward movement of the infection thread, root cortical cells starts dividing and form nodule primordium. Rhizobial bacteria are released into the cells of the nodule primordium by endocytosis and by undergoing several structural and biochemical changes get transformed into the symbiotic form called the bacteroids (Newcomb, 1981). The nodule primordium is formed by the divisions of inner and outer cortical cells in temperate (pea, vetch and alfalfa) and tropical (soybean) legumes, respectively (Newcomb, 1981). The nodules of the former kind are referred to as indeterminate type whereas those of the latter kind are called as determinate type. The rhizobial bacteria released into plant cells are surrounded by a plant - derived peribacteroid membrane (PBM). The functional structure formed by the bacteria and PBM is called a symbiosome. The development of the invaded host cells of the nodules induced by *R. etli* on the roots of *Phasoelus vulgaris* has been found to be synchronous (Cermola *et al.*, 2000). In the mature functional nodule, peripheral vascular bundles are connected to the root vasculature and

the main tissues of all types can be distinguished cytologically and to some extent with molecular markers (Stougaard, 2000).

In a typical legume nodule a central tissue containing rhizobial bacteria is surrounded by several peripheral tissues like nodule cortex, the endodermis and the nodule parenchyma (van de Wiel *et al.*, 1990). Both infected and uninfected cells are present in the central tissue. The infected cells are packed with a large number of rhizobia. Central tissue in the indeterminate nodules can be differentiated into meristem, infection, inter, nitrogen fixation and senescence zones. The meristem is at distal end of the nodule and consists of dividing cells. In the infection zone, infection threads are seen in the intercellular spaces and rhizobia are released into plant cells. Amyloplasts are present in the interzone. Nitrogen fixation zone contains fully differentiated nitrogen-fixing bacteroids. Degenerating rhizobia can be seen in senescence zone present in older nodules. Increased Cys protease activity has been shown in early senescing nodules of alfalfa, indicating a specific role for such proteases in the senescent phase of nodule development (Pladys and Vance, 1993).

2.5 *nif/fix* and other genes of rhizobia involved in symbiosis

Nitrogen fixation genes of rhizobia are designated as *nif/fix* genes. *nif* genes are those nitrogen fixation genes which were initially characterized in *Klebsiella pneumoniae*, a free-living nitrogen fixing bacterium. In this organism, 20 adjacent *nif* genes were found to be organized in 8 operons. Rhizobial nitrogen fixation genes, which are equivalent to *K. pneumoniae nif* genes, have

been given the same names (Beringer *et al.*, 1980, Arnold *et al.*, 1988). Rhizobial nitrogen fixation genes for which no equivalent is found in *K. pneumoniae* were designated as *fix* genes. Nitrogen fixation in all nitrogen fixing bacteria is carried out by enzyme nitrogenase. The structural genes which code for nitrogenase have been designated as *nifHDKE*.

The *nod*, *nif* and *fix* genes are located on chromosome in *R. loti*, *Bradyrhizobium spp.* and *Azorhizobium spp.* whereas these genes are present on symbiotic plasmids 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) have been found in *S. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Hynes *et al.*, 1986; Burkhardt *et al.*, 1987; Sobral *et al.*, 1991; Honeycutt *et al.*, 1993).

nifH, *nifD* and *nifK* genes which code for nitrogenase polypeptides are located in a single operon (*nifHDK*) in *S. meliloti* (Ruvkun *et al.*, 1982) and on two operons *nifH* and *nifDK* in *B. japonicum* (Kaluza *et al.*, 1983). In *S. meliloti*, nitrogen-fixing genes are present in two clusters, viz., cluster I (*nifHDKE*, *nifN*, *fixABCX*, *nifA*, *nifB*) and cluster II (*fixLJ*, *fixK*, *fixNOQP*, *fixGHIS*) on megaplasmid (Batut *et al.*, 1985; David *et al.*, 1987). The *fixA*, *fixB*, *fixC* and *fixX* have also been identified in *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). *fixF*, located near the common nodulation genes, was characterized in *S. meliloti* (Aguilar *et al.*, 1985).

Meloni *et al.* (2003) have cloned and characterized genetic determinants (*tat ABC* genes) of the Tat (twin-arginine translocation) system from *R. leguminosarum* bv. *viciae*, and constructed a *tatBC* deletion mutant. Analysis of the nodules induced by this mutant revealed that the Tat system is essential for *Rhizobium*-legume symbiosis. Krishnan *et al.* (2003) have demonstrated that a functional citrate synthetase gene of *S. fredii* USPA257 is essential for effective soybean nodulation and nitrogen fixation.

2.6 Characterization of genomes of rhizobia and legumes

The literature on the genes and genomics of the root symbiosis has been recently reviewed by Stougaard (2001). Leucine and isoleucine loci in *B. japonicum* D-211 were mapped by transformational analysis (Doctor and Modi, 1976). Circular chromosomal linkage maps of *S. meliloti* Rm41 (Kondorosi *et al.*, 1977), *R. leguminosarum* 300 (Beringer *et al.*, 1978) and *R. leguminosarum* bv. *trifolii* (Megias *et al.*, 1982) were constructed by using *Inc*P1 group plasmid R68.45 (Haas and Holloway, 1976). Chromosomal map of *S. meliloti* Rm2011 (Meade and Signer, 1976) was prepared by using plasmid RP4 (Datta *et al.*, 1971). Circular linkage maps of *S. meliloti* 2011, *S. meliloti* Rm41 and *R. leguminosarum* 300 were compared by suppression tests of mutations (Kondorosi *et al.*, 1980). Five *fix* alleles were localized on different chromosomal regions of *S. meliloti* 41 (Forrai *et al.*, 1983).

The complete nucleotide sequencing of the symbiotic plasmid of *Rhizobium* sp. NGR234 was reported by Freiberg *et al.* (1997). The sequencing

of *Mesorhizobium loti*, *S. meliloti* and *B. japonicum* USDA110 genomes has been completed in 2000, 2001 and 2002, respectively (Kaneko *et al.*, 2000a; Kaneko *et al.*, 2000b; Galibert *et al.*, 2001; Capela *et al.*, 2001; Barnett *et al.*, 2001; Kaneko *et al.*, 2002). This sequence information is available at http://www.kazusa.or.jp/rhizobase/Mesorhizobium/ (*Mesorhizobium*), http://sequence.toulouse.inra.fr/meliloti.html (*Sinorhizobium*) and http://gib.genes.nig.ac.jp/single/main.php?spid=Bjap_USDA110 (*Bradyrhizobium*). The sequencing of the *Medicago* genome was started in 2002 with the aim to sequence about 5Mbp per month. It is expected that about 20 - 50% of the genome sequence will become available by the end of the year 2003. The information on *Medicago* sequencing is available at http://www.genome.ou.edu/medicago_totals.html.

2.7 Transport and utilization of C_4-dicarboxylates by rhizobia

By studying symbiotic properties of Tn5-induced C_4-dicarboxylate transport mutants of *R. leguminosarum* strain GF160, Finan *et al.* (1983) concluded that a functional C_4 dicarboxylic acid transport system is essential for N_2 fixation in pea nodules. *S. meliloti* strain having a mutation in *ntrA* gene cannot transport dicarboxylates (Ronson *et al.*, 1987; Watson 1990). Aspartate has also been found to require dicarboxylate transport system for use as a carbon source (Watson *et al.*, 1988; Watson , 1990). *S. meliloti* contains an aspartate aminotransferase activity which is required for nitrogen fixation in the nodule (Rastogi and Watson, 1991). Boesten *et al.* (1998), on the basis of gene fusion studies, found that the DctBD - dependent *dctA* expression occurred

throughout the nodules induced by *S. meliloti*. The permease component of the Dct system, DctA, of *S. meliloti* Rm8002 has been found to transport orotate (Yurgel *et al.*, 2000).

2.8 Symbiotic role of biosynthesis and transport of amino acids, nucleotide bases and vitamins

Rhizobial bacteria have to multiply during colonization of plant roots and infection of plants. During multiplication, these bacteria require primary building blocks, viz., amino acids, nucleotide bases and vitamins. These metabolites or their intermediates are either synthesized by rhizobia or supplied by the host plant. The studies on the requirement and source of the supply of amino acids, nucleotide bases and vitamins (or their intermediates) in symbiosis have been carried out by isolating auxotrophic mutants of rhizobia and studying their symbiotic properties. Auxotrophic mutants of rhizobia have been isolated by using chemical mutagens and transposon Tn5. Suicide vectors containing Tn5 (Selvaraj and Iyer, 1983; Simon, 1984) were used in the transposon mutagenesis.

2.8.1 Biosynthesis of amino acids and symbiosis

2.8.1.1. Arginine

The auxotrophic mutant of *S. meliloti* blocked in the later part (after ornithine) of the arginine biosynthetic pathway have been reported to be effective in nitrogen fixation (Scherrer and Dénarié, 1971; Pain, 1979; Malek and Kowalski, 1977b) and the mutants of *S. meliloti* blocked in the early part of

arginine biosynthetic pathway that leads to ornithine were found to be effective (Scherrer and Dénarié, 1971), partly effective (Scherrer and Dénarié, 1971) or ineffective (Malek and Kowalski, 1977b). Different symbiotic phenotypes of the ornithine requiring auxotrophs isolated by Dénarié *et al.* (1976) and Kerppola and Kahn (1988b) emphasize the need for more research work to identify the intermediate(s), having a role in symbiosis, of arginine biosynthetic pathway of *S. meliloti*. Ornithine is also a precursor of polyamine synthesis (Tabor and Tabor, 1985). Considering this, Kerppola and Kahn have hypothesized that the lack of polyamines may be the cause of symbiotic defect of ornithine auxotrophs of *S. meliloti*. It seems that the alfalfa host plant is capable of providing sufficient quantity of arginine to *S. meliloti* bacteria during symbiosis.

2.8.1.2 Aspartic acid

Transposon Tn5 induced aspartic acid auxotrophs of *S. fredii* HH303 have been found to induce ineffective nodules on soybean. Symbiotic effectiveness of these auxotrophs was not restored on supplementation of aspartic acid to plant nutrient medium (Kim *et al.*, 1988). Aspartic acid from the soybean host plant does not appear to be available to *S. meliloti* bacteria inside the nodule.

2.8.1.3. Asparagine

Kerppola and Kahn (1988b) isolated 21 asparagine auxotrophs of *S. meliloti* 104A14. Seventeen auxotrophs whose symbiotic properties were studied were found to induce ineffective nodules. These workers concluded that

asparagine is probably not available to *S. meliloti* bacteria in the nodule from the alfalfa host plant.

2.8.1.4. Cysteine

Effective nodules on the respective plant hosts were induced by cysteine auxotrophs of *S. meliloti* 2011 (Scherrer and Dénarié, 1971), *S. meliloti* Rmd201 (Abbas *et al.*, 2002), *R. leguminosarum* 300 (Pain, 1979) and *S. fredii* HH303 (Malek and Kowalski, 1977b). The nodules induced by cysteine auxotrophs of *S. meliloti* L5-30 (Malek and Kowalski, 1977b) were ineffective. On the basis of their studies on the Tn5 induced *cysG* mutant of *R. etli*, Taté *et al.* (1997) concluded that sulfate (or sulfite) is the sulfur source of *R. etli* in the rhizosphere, while cysteine, methionine or glutathione is supplied by the root cells to bacteria growing inside the plant.

Twenty one Tn5 induced auxotrophs of *S. meliloti* Rmd201 isolated by Abbas *et al.* (2002) were sulfite reductase mutants and fully effective showing that the sulfite reductase activity of *S. meliloti* is not essential for effective symbiosis with alfalfa. This result showed that the alfalfa plant is able to supply cysteine and/or sulfide to *S. meliloti* bacteria during symbiosis. Cysteine auxotrophs of rhizobia have been found to grow on minimal medium supplemented with methionine, homocysteine or cystathionine (Kim *et al.*, 1988; Pain, 1979; Meade *et al.*, 1982; Schwedock and Long, 1992; Taté *et al.*, 1999c; Abbas *et al.*, 2002). Rhizobia appear to convert methionine or cystathionine to

cysteine as in *Saccharomyces cerevisiae* (Cherest and Surdin-Kerjan, 1992) and *Pseudomonas aeruginosa* (Foglino *et al.*, 1995).

2.8.1.5. Glutamate

Altered expression of nitrogenase activity was observed in the free - living cultures of 5 Tn5-induced glutamate auxotrophs of *B. japonicum* USDA110 (Hom *et al.*, 1984). On the basis of this result, the existence of a regulatory relationship between nitrogen metabolism and nitrogenase synthesis was suggested for *B. japonicum*.

2.8.1.6. Glutamine

None of the three *S. meliloti* loci involved in glutamine biosynthesis (*glnA, glnII* and *glnt*) are essential for symbiotic nitrogen fixation (de Bruijin *et al.*, 1989). Adenylylation of glutamine synthetase I is not essential for symbiotic nitrogen fixation in *S. meliloti* (Arcondéguy *et al.*, 1996).

2.8.1.7. Glycine

Three glycine auxotrophs of *S. meliloti* 2011 were more effective in nitrogen fixation than the parental strain (Scherrer and Dénarié, 1971). The reason for the more effectivity of these auxotrophs is not known.

2.8.1.8. Histidine

Histidine auxotrophs of *S. meliloti* strain L5-30 induced ineffective nodules (Malek and Kowalski, 1977a). The supplementation of histidine in the plant

nutrient medium restored the symbiotic effectiveness of these auxotrophs (Malek and Kowalski, 1977b). This result demonstrates that the alfalfa host plant is not able to provide the required quantity of histidine to the *S. meliloti* bacteria in nodules. Sadowski *et al.* (1986) isolated four histidine auxotrophs of *B. japonicum* USDA122; out of these, two were Nod$^-$ whereas the remaining two were symbiotically competent. Soybean plant appears to provide histidine to rhizobia; both Nod$^-$ histidine auxotrophs were somehow not able to utilize the histidine or its derivative provided by the host plant. The induction of symbiotically effective nodules by two histidine auxotrophs of cowpea *Rhizobium* strain IRC256 also shows that cowpea plant provides histidine to rhizobial bacteria.

2.8.1.9. Isoleucine and valine

Malek and Kowalski (1977a) reported that an isoleucine and valine *(ilv)* auxotroph of *S. meliloti* L5-30 was non-infective. Two Tn5 induced *ilv* auxotrophs of *S. fredii* HH303 were found to form ineffective nodules on soybean plants (Kim *et al.*, 1988). Symbiotic effectivity of *ilvD* mutants of *S. meliloti* suggested that alfalfa plant is able to provide both isoleucine and valine to *S. meliloti* bacteria during nodule formation and function (Aguilar and Grasso, 1991; Hassani *et al.*, 2001). The *ilvC* mutants of *S. meliloti* have been found to be Nod$^-$ (Aguilar and Grasso, 1991; Hassani *et al.*, 2002). In the *ilvC* mutant of *S. meliloti* 1021 *nodABC* gene were not activated by the inducer luteolin (Aguilar and Grasso, 1991). Variable activation of the common nodulation genes *nodABC* was

observed in the *ilvC* mutants obtained from different *S. meliloti* wild type strains (López *et al.*, 2001).

2.8.1.10. Leucine

Leucine auxotrophs of *S. meliloti* isolated by different workers have been found to induce ineffective nodules on alfalfa plants (Truchet *et al.*, 1980; Aronshtam *et al.*, 1993; Nichik *et al.*, 1995; Hassani *et al.*, 2001). All these workers, except Truchet *et al.* (1980), did not find restoration of symbiotic effectivity on supplementation of plant nutrient medium with leucine. Out of the three leucine auxotrophs of *S. meliloti* Rmd201, isolated by Hassani *et al.* (2001), one was *leuC/leuD* mutant and the two were *leuB* mutants. The nodules induced by *leuB* mutants were more advanced than the *leuC/leuD* mutant induced nodules; in the latter case, the bacteria remained in the infection threads. These findings indicate that the leucine biosynthetic pathway intermediate β-isopropylmalate of *S. meliloti* has a role in rhizobial release into alfalfa plant cells. Soybean plant host seems to supply the required quantity of leucine to rhizobia during symbiosis since the leucine auxotrophs of *B. japonicum* were found to induce effective nodules on soybean plants (Kummer and Kuykendall, 1989).

2.8.1.11. Lysine

The isolation of two lysine auxotrophs of *S. meliloti* Rm41 was done by Forrai *et al.* (1983). These workers did not report the symbiotic properties of these auxotrophs.

2.8.1.12. Methionine

Several workers have reported the isolation of methionine auxotrophs of rhizobia (Fedorov and Zaretskaya, 1977; Kerppola and Kahn, 1988; Scherrer and Dénarié, 1971; Pain, 1979; Meade *et al.*, 1982; Hom *et al.*, 1984; Forrai *et al.*, 1983; Ali *et al.*, 1984; Singh *et al.*, 1984). Methionine auxotrophs of *S. meliloti* have been found to induce effective (Scherrer and Dénarié, 1971) or ineffective (Kerppola and Kahn, 1988; Abbas *et al.*, 2002) nodules on alfalfa plants. Taté *et al.* (1997) characterized a Tn5 induced methionine auxotroph CTNUX23 of *R. etli* CE3 and concluded that *metZ* gene of *R. etli* is essential for nodulation of *Phaseolus vulgaris*. Supplementation of the plant nutrient medium with methionine was found to completely restore symbiotic effectiveness of the Tn5 induced methionine auxotrophs of *S. meliloti* Rmd201 (Abbas *et al.*, 2002). The lack of symbiotic effectivity of these auxotrophs appears to be due to the deficiency of methionine.

Methionine auxotrophs of *S. meliloti* Rmd201 did not grow in minimal medium (Khanuja and Kumar, 1989) supplemented with cysteine. It appears that the synthesis of methionine from *O*-succinylhomocysteine and cysteine through transulfurylation pathway is highly ineffective or inactive in *S. meliloti* like that reported in *Pseudomonas aeruginosa* (Foglino *et al.*, 1995). The *metE* mutants of *S. meliloti* Rmd201 grew in minimal medium (Khanuja and Kumar, 1989) supplied with vitamin B_{12} (Abbas *et al.*, 2002). The isolation of similar mutants in *R. leguminosarum* Rld1 has been reported (Singh *et al.*, 1984). These results

show that the final step of methionine biosynthesis in rhizobia is similar to that in *E. coli*.

2.8.1.13. Proline

Chien *et al.* (1991) isolated proline auxotrophic, catabolic and overlapping mutants of *R. leguminosarum* bv. *viciae* strain C1204b and studied the symbiotic properties of these auxotrophs with the pea host plants. The determination of cytosolic and bacteroid levels of proline in the nodular tissue did not support the role of proline as a key metabolite supplied by the host. On the basis of their studies on a *proC* mutant of *B. japonicum*, King *et al.* (2000) concluded that the *proC* gene of *B. japonicum* is essential for its symbiosis with soybean plants and the *proC* mutant is not able to get the supply of sufficient amount of proline from the host plant.

2.8.1.14. Phenylalanine

Prasad *et al.* (2000) isolated a Tn5 induced phenylalanine auxotrophic mutant of *S. meliloti* Rmd201. This mutant induced ineffective nodules on alfalfa plants and these nodules did not contain nitrogen fixation zone. It was concluded that the alfalfa plant is not able to provide the required quantity of phenylalanine to rhizobia in nodules.

2.8.1.15. Tryptophan

All tryptophan auxotrophs, except tryptophan synthase mutants, of *B. japonicum* strain USDA I-110 ARS were Nod^- (Wells and and Kuykendall, 1983;

Kummer and Kuykendall, 1989). These workers suggested that indole glycerol phosphate may be required by *B. japonicum* for nodulation of soybean plants. Kuykendall and Hunter (1997) found that the *trpCD* deletion mutants of *B. japonicum* were symbiotically ineffective. The *B. japonicum* operon consisting of *trpD*, *trpC* and a *moaC*-like gene was sequenced (Kuykendall and Hunter, 1997).

The tryptophan synthase mutants of *S. meliloti* showed normal symbiosis with alfalfa plants (Barsomian *et al.*, 1992; Prasad *et al.*, 2000). The *trpE* mutants of *S. meliloti* were found to form defective nodules on host plants; each of these nodules contained extended infection zone and fixed no or less nitrogen (Barsomian *et al.*, 1992; Prasad *et al.*, 2000). These results suggested that the expression of *trpE* gene of *S. meliloti* is required for normal symbiosis, and alfalfa host provides the sufficient quantity of tryptophan to *S. meliloti* bacteria in nodules.

The *trpE* locus of *S. meliloti* was subsequently found to be a fusion of *trpE* and *trpG* coding sequences and named as *trpE(G)* (Bae *et al.*, 1989). Barsomian *et al.* (1992) hypothesized that anthranilic acid, synthesized by the expression of *trpE* gene, acts as an *in planta* siderophore helping iron uptake for rhizobial development. Jelesko *et al.* (1993) observed that *S. meliloti* mutants with decreased DAHP synthase activity are sensitive to exogenous tryptophan and phenylalanine and form ineffective nodules. These workers concluded that the normal flow of metabolites through the biosynthetic pathways of aromatic amino acids in *S. meliloti* is essential for the development of bacteroids. Taté *et al.*

(1999b) found that *R. etli trpB* gene is essential for an effective symbiotic interaction with *Phaseolus vulgaris*.

2.8.1.16. Tyrosine

Kerppola and Kahn (1988b) isolated two tyrosine auxotrophs of *S. meliloti* 104A14. These auxotrophs were found to induce ineffective nodules on alfalfa plants. A Tn5 induced tyrosine auxotroph of *S. meliloti* Rmd201 was found to induce fully effective nodules on alfalfa plants (Prasad *et al.*, 2000). (Table 1).

2.8.2. Transport of amino acids and symbiosis

Lodwig *et al.* (2003) mutated *aap* and *bra*, both of which encode ABC-type broad specificity amino acid transporters, in *Rhizobium leguminosarum* bv. *viciae* (Hosie *et al.*, 2002; Hosie *et al.*, 2001; Walshaw and Poole, 1996). The single mutation in *aap* and *bra* reduced the uptake rates of all tested amino acids by 40-70% in free-living bacteria and the double mutant RU1357 was almost totally blocked for the uptake of a broad range of amino acids. Whereas the growth of peas nodulated by either *aap* or *bra* single mutants, or by the wild type (A34) was indistinguishable, peas nodulated by RU1357 progressively yellowed. It was concluded that the plant provides amino acids to the bacteroids, enabling them to shut down their ammonia assimilation, and in return bacteroids cycle back amino acids to the plant for asparagine synthesis (Fig. 3).

2.8.3. Biosynthesis of purines and symbiosis

Many researchers have found defective symbiosis of purine auxotrophs of *Rhizobium* strain NGR234 (Chen *et al.,* 1985), *R. leguminosarum* (Pain, 1979; Schwinghamer, 1969; Pankhurst and Schwinghamer, 1974; Noel *et al.,* 1988), *S. fredii* (Kim *et al.,* 1988) and *S. meliloti* (Kerppola and Kahn, 1988b; Scherrer and Dénarié, 1971; Malek and Kowalski, 1983b; Swamynathan and Singh, 1992; Al-Judi, 2001). Adenine auxotrophs of *R. leguminosarum* were Nod$^-$ (Pain, 1979; Schwinghamer, 1969; Pankhurst and Schwinghamer, 1974); these auxotrophs formed ineffective nodules on peas when adenine was supplemented in the plant nutrient medium (Schwinghamer, 1975; Pain, 1979). Pseudonodules were induced on bean plants by the purine auxotrophs of *R. leguminosarum* bv. *phaseoli*. Adenosine supplementation to the plant growth medium had no effect on nodule phenotype but the supplementation of 5 - aminoimidazole -4- carboxamide riboside (AICA-riboside) resulted in significant enhancement of nodule development (Noel *et al.,* 1988; Newman *et al.,* 1992). Newman *et al.* (1994) reported that purine auxotrophs, each having a biochemical block before 5 - aminoimidazole -4- carboxamide ribonucleotide (AICAR), induced poorly developed nodules. The expression of *purM* and *purN* genes of purine biosynthetic pathway was found to be essential for nodulation of peas by *R. leguminosarum* bv. *viciae* (Stevens *et al.,* 2000).

Purine auxotrophs of *S. meliloti* 2011 (Scherrer and Dénarié, 1971), *S. meliloti* L5-30 (Malek and Kowalski, 1983b) and *S. meliloti* Rmd201 (Swamynathan and Singh, 1992) were found to induce ineffective nodules on

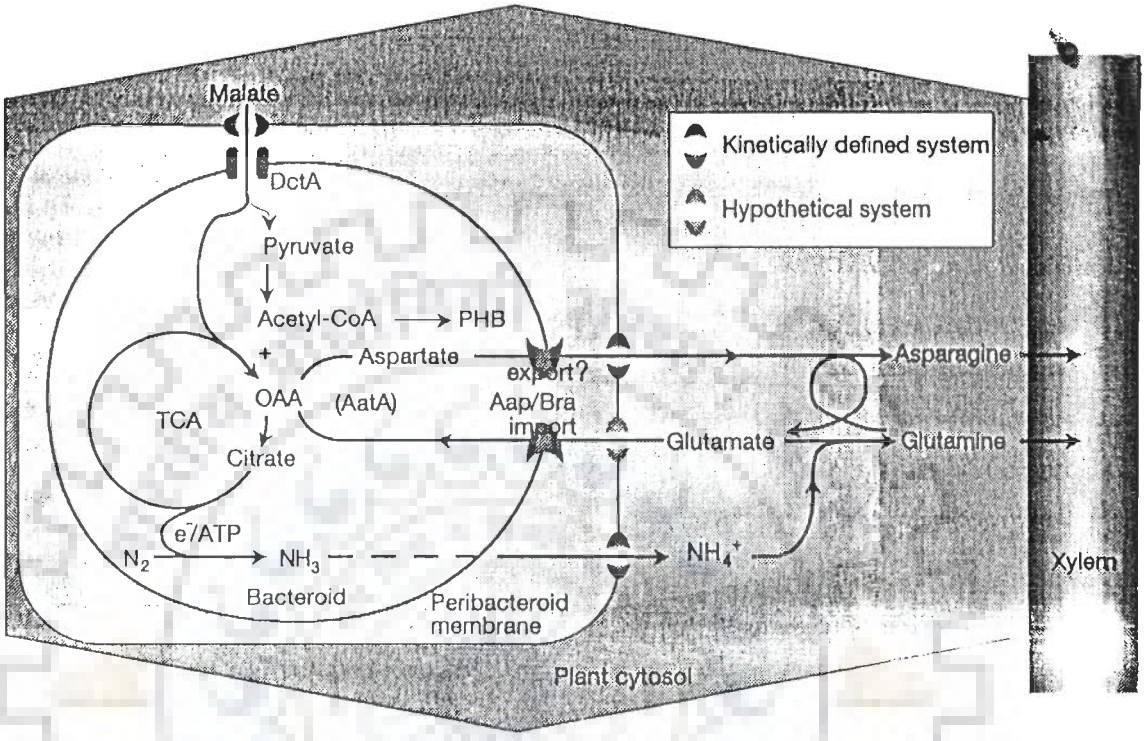

Fig. 3: The role of amino acid cycling (between the bateroid and the host plant) in nitrogen fixation in pea nodules. Two types of transport systems have been shown, kinetically defined but not genetically characterized system and hypothetical system. Glutamate and aspartate are shown to be the most likely cycled amino acids. The reaction catalyzed by AatA also forms 2-ketoglutarate, which may be either metabolized by the bacteroid or exported back to the plant. Export via Aap/Bra is shown with a question mark to indicate that it is hypothetical. ***Abbreviations:*** PHB, polyhydroxybutyrate; OAA, oxaloacetic acid; TCA, tricarboxylic acid cycle. (Lodwig *et al.*, 2003).

alfalfa plants. The symbiotic deficiency of the purine auxotrophs of *S. meliloti* Rmd201 appeared to be due to multiple changes in the cell surface molecules (Swamynathan and Singh, 1995). AICA - riboside has been found to negatively regulate the expression of *fixNOQP* in *S. meliloti* (Soberón *et al.*, 2001).

Two purine auxotrophs, ANU2861 and ANU2866, of *Rhizobium* strain NGR234 induced defective nodules on their host plants. In comparison to the parental strain, these auxotrophs produced less acidic exopolysaccharides and cyclic β-(1→2)-glucans (Chen *et al.*, 1985). The production of defective polysaccharides by the purine auxotroph ANU2861 was corrected by the addition of AICA - riboside in the growth medium (Djordjevic *et al.*, 1996).

The addition of guanine or guanosine to the minimal medium did not restore growth to the purine auxotrophs of *R. leguminosarum* bv. *phaseoli* (Noel *et al.*, 1988) and *S. meliloti* (Kim *et al.*, 1988; Swamynathan and Singh, 1992), showing that these bacteria cannot convert guanine to inosine monophosphate. Riccillo *et al.* (2000) have reported that inosine production of guanine via inosine monophosphate dehydrogenase in *R. tropici* is essential for growth at high temperatures and effective nodulation (Riccillo *et al.*, 2000).

2.8.4. Biosynthesis of pyrimidines and symbiosis

Scherrer and Dénarié (1971) reported that a pyrimidine auxotroph of *S. meliloti* 2011 induced ineffective nodules on alfalfa plants. Two out of 15 pyrimidine auxotrophs of *R. leguminosarum* 300 were found to be defective in symbiosis (Pain, 1979). The defective nodule development of a pyrimidine

auxotroph of *R. leguminosarum* bv. *phaseoli* strain CFN42 was not suppressed by the addition of uridine to the plant growth medium (Noel *et al.,* 1988). *S. meliloti* strain 104A14 auxotrophs having mutations in carbamoyl phosphate synthetase gene and the genes of the pyrimidine biosynthetic pathway before orotic acid induced ineffective nodules (Kerppola and Kahn, 1985; Kerppola and Kahn, 1988a; Kerppola and Kahn, 1988b). Kim *et al.* (1988) reported the induction of ineffective nodules by two Tn5 induced uracil auxotrophs of *S. fredii* HH303.

Transposon Tn5 induced five *car*, eight *pyrC* and ten *pyrE/pyrF* pyrimidine auxotrophs of *S. meliloti* Rmd201 induced ineffective nodules on alfalfa plants (Vineetha *et al.*, 2001). The structural features of the nodules induced by *pyrC* mutants were more advanced than those of the nodules induced by *car* mutants and the features of nodules induced by *pyrE/pyrF* mutants were more developed than those of *pyrC* mutants, indicating that carbamoyl phosphate/ cabamoyl aspartate and dihydroorotic acid/orotic acid may have a role in symbiosis of *S. meliloti* with alfalfa.

2.8.5. Biosynthesis of vitamins and symbiosis

2.8.5.1. Biotin

Streit *et al.* (1996) reported that both synthesis and uptake of plant derived biotin promoted colonization of alfalfa roots by *S. meliloti* 1021. The synthesis appeared to be the more important source of biotin for *S. meliloti* bacteria.

2.8.5.2. Nicotinic acid

Kim *et al.* (1988) found that a Tn5-induced nicotinic acid auxotroph of *S. fredii* HH303 induced ineffective nodules on soybean plants. This result showed that the *S. fredii* bacteria in the nodules are not able to receive the required supply of nicotinic acid from the plant host.

2.8.5.3. Riboflavin

Streit *et al.* (1996) found that riboflavin was required for the colonization of alfalfa roots by *S. meliloti* 1021 bacteria. Schwinghamer (1970) observed that a riboflavin auxotroph of *R. leguminosarum* bv. *trifolii* T-1, which formed ineffective or partially effective nodules on clover, was fully effective when riboflavin was added to the plant growth medium. Riboflavin appeared essential for the conversion of vegetative bacteria of *R. leguminosarum* bv. *trifolii* into functional, nitrogen-fixing bacteroids in the nodules (Pankhurst *et al.*, 1972).

2.8.5.4. Thiamine

Streit *et al.* (1996) demonstrated that thiamine is required for the colonization of alfalfa roots by *S. meliloti* 1021 bacteria.

2.8.5.5. Pantothenic acid

Kim *et al.* (1988) observed that a Tn5 induced pantothenic acid auxotroph of *S. fredii* HH303 induced fully effective nodules on soybean plants. This result

showed that soybean plant is able to provide the required quantity of pantothenic acid to *S. fredii* bacteria during symbiosis.

It is clear from the above literature review that rhizobia receive, apart from the dicarboxylic acids, several other metabolites (amino acids, nucleotide bases and vitamins, and/or their intermediates) from the plant host during symbiosis. The information about the source of supply of metabolites to rhizobia in different symbiotic systems can be useful for better understanding of the rhizobium-plant interactions. It also appears that not only the end products but also some of the enzymes/intermediates of the primary biosynthetic pathways are important in symbiosis. As very little information is available on the role of the arginine biosynthetic pathway in symbiosis, the present work on isolation and characterization of Tn5 induced arginine auxotrophs of *S. meliloti* Rmd201 was undertaken.

Table 1- Symbiotic characteristics of amino acid auxotrophs of rhizobia (reproduced from Randhawa & Hassani, 2001)

S. No.	Auxotrophy	Mutated gene/ Position of biochemical block	Name of rhizobium	Symbiotic phenotype	Symbiotic phenotype after supplementation	Reference(s)
1.	Arginine	after ornithine	*Sinorhizobium meliloti*	Nod^+Fix^+	-	Dénarié *et al.* (1976); Fedorov & Zaretskaya, 1977; Kerppola & Kahn, 1988
		before ornithine	-do-	Nod^+Fix^-	Not Determined	Kerppola & Kahn, 1988
2.	Aspartic acid	-	*S. fredii*	-do-	Nod^+Fix^-	Kim *et al.*, 1988
3.	Asparagine	-	*S. meliloti*	-do-	Not Determined	Kerppola & Kahn, 1988
4.	Cysteine	-	-do-	Nod^+Fix^+	-	Scherrer & Dénarié, 1971
		cysI/cysJ	-do-	-do-	-	Abbas *et al.*, 2002
		-	-do-	Nod^+Fix^-	Not Determined	Malek & Kowalski, 1977
		cysG	*Rhizobium etli*	Nod^+Fix^+	-	Taté *et al.*, 1997
		-	*R. leguminosarum*	-do-	-	Pain, 1979
		-	*S. fredii*	-do-	-	Kim *et al.*, 1988
5.	Glutamic acid	-	*Bradyrhizobium japonicum*	-do-	-	Hom *et al.*, 1984
6.	Glutamine	*glnA, glnII & glnT*	*S. meliloti*	-do-	-	de Bruijn *et al.*, 1989
7.	Glycine	-	-do-	-do-	-	Scherrer & Dénarié, 1971
8.	Histidine	-	*B. japonicum*	-do-	-	Sadowsky *et al.*, 1986
		-	-do-	Nod^-	Nod^+Fix^+	Sadowsky *et al.*, 1986
		-	-do-	-do-	Not Determined	So *et al.*, 1987
		-	*S. meliloti*	Nod^+Fix^-	Nod^+Fix^+	Malek & Kowalski *et al.*, 1977
9.	Isoleucine + Valine	*ilvD*	-do-	Nod^+Fix^+	-	Aguilar & Grasso, 1991; Hassani *et al.*, 2002
		ilvC	-do-	Nod^-	Nod^-	Aguilar & Grasso, 1991; Hassani *et al.*, 2002
		ilvC	-do-	-do-	Not Determined	López *et al.*, 2001
		ilvB/ilvG	-do-	-do-	Nod^-	Hassani *et al.*, 2002
		-	-do-	-do-	Not Determined	Malek & Kowalski, 1977
		-	*S. fredii*	Nod^+Fix^-	-do-	Kim *et al.*, 1988
10.	Leucine	-	*B. japonicum*	Nod^+Fix^+	-	Kummer & Kuykendall, 1989
		-	*S. meliloti*	Nod^+Fix^-	Nod^+Fix^+	Truchet *et al.*, 1980
		-	-do-	-do-	Nod^+Fix^-	Aronshtam *et al.*, 1993; Nichik *et al.*, 1995
		leuC/leuD & leuB	-do-	-do-	-do-	Hassani *et al.*, 2002
11.	Lysine	-	-do-	-	-	Truchet *et al.*, 1980
12.	Methionine	-	*S. meliloti*	Nod^+Fix^+	-	Scherrer & Dénarié, 1971
		-	-do-	Nod^+Fix^-	Not Detemined	Kerppola & Kahn, 1988
		metA/metZ, metE & metF	-do-	-do-	Nod^+Fix^+	Abbas *et al.*, 2002
		metZ	*R. etli*	Nod^-	Nod^+Fix^-	Taté *et al.*, 1999
13.	Proline	-	-do-	Nod^+Fix^+	-	Chien *et al.*, 1991
		proC	*B. japonicum*	Nod^+Fix^-	Not Determined	King *et al.*, 2000
14.	Phenylalanine	*pheA*	*S. meliloti*	-do-	-do-	Prasad *et al.*, 2000
15.	Tryptophan	*trpA & trpB*	-do-	Nod^+Fix^+	-	Barsomian *et al.*, 1992; Prasad *et al.*, 2000
		trpE(G)	-do-	Nod^+Fix^-/ Nod^+Fix^+	Not Determined	Barsomian *et al.*, 1992; Prasad *et al.*, 2000
		-	*B. japonicum*	Nod^-	-do-	Wells & Kuykendall, 1983; Kummer & Kuykendall, 1989
		trpB	*R. etli*	Nod^+Fix^-	-do-	Taté *et al.*, 1999b
16.	Tyrosine	*tyrA*	*S. meliloti*	Nod^+Fix^+	-	Prasad *et al.*, 2000
		-	-do-	Nod^+Fix^-	Not Determined	Kerppola & Kahn, 1988
17.	Phenylalanine + Tryptophan +Tyrosine	*aro*	-do-	-do-	-do-	Barsomian *et al.*, 1992; Prasad *et al.*, 2000

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Bacterial strains and plasmids

The description of the bacterial strains and plasmids used or constructed during this work has been given in Table 2.

3.1.2. Plant cultivars

Alfalfa (*Medicago sativa*) cv. T9 and cv. A2 plants were used in the plant inoculation studies. The seeds of the above cultivars were procured from National Seeds Corporation, Pusa Complex, New Delhi, India.

3.1.3. Composition of growth media

3.1.3.1. Growth media for *S. meliloti*

3.1.3.1.1. Complete media

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

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

The pH was adjusted to 7.0 with 0.1N NaOH solution. When required, the medium was solidified by adding 16g agar (M/s HiMedia Laboratories Pvt. Ltd., Mumbai, India) to the liquid medium before autoclaving.

Table 2— Bacterial strains and plasmids used/ constructed

Strains/ Plasmids	Relevant characteristics	Source /Reference
Sinorhizobium meliloti		
Rmd201	Spontaneous Sm^r derivative of AK631 (Nod^+ Fix^+)	Khanuja & Kumar (1988)
PP631	AK631(pJB3JI)	Peter Putnoky
ZB201	Rm41 *ade15 narB rf1 5fu*^{r}	---do---
AK3, AK4 & AK5	Rmd201*argA/argB/argC/argD/argE*::Tn5	This study
AK6, AK7, AK8, AK10, AK11, AK13, AK14, AK15, AK16 & AK25	Rmd201 *argF/argI*::Tn5	---do---
AK12	Rmd201 *argG*::Tn5	---do---
AK9	Rmd201 *argH*::Tn5	---do---
AK31, AK41 & AK51	AK3 (pJB3JI),AK4 (pJB3JI) & AK5 (pJB3JI)	---do---
AK61, AK71, AK81, AK101, AK111, AK131, AK141, AK151, AK161 & AK251	AK6 (pJB3JI),AK7 (pJB3JI),AK8 (pJB3JI), AK10 (pJB3JI),AK11 (pJB3JI), AK13 (pJB3JI), AK14 (pJB3JI),AK15 (pJB3JI),AK16 (pJB3JI) & AK25 (pJB3JI)	---do---
AK121	AK12 (pJB3JI)	---do---
AK91	AK9 (pJB3JI)	---do---
Escherichia coli		
WA803(pGS9)	Met$^-$ Thi$^-$ Cm^r Km^r	Selvaraj & Iyer (1983)
Plasmids		
pGS9	IncN rep15A Cm^r Km^r	Selvaraj & Iyer (1983)
pJB3JI	Km^r derevative of pR68.45 capable of mobilizing genomic segments of its host, Tc^r Cb^r Nal^r	Brewin *et al.* (1980)

To make TY swarm medium, 3g/litre agar was added to the liquid medium before autoclaving.

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

Constituents	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 was adjusted to 6.8 with 0.1N NaOH solution. When required, the medium was solidified by adding 16g agar (HiMedia) to the liquid medium before autoclaving.

3.1.3.1.2. Minimal medium

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

Constituents	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 was adjusted to 7.0 with 0.1N NaOH. When required, the medium was solidified by adding 16g agar (HiMedia) to the liquid medium before autoclaving. After autoclaving, the following glucose solution was added:

Glucose solution: 20% (w/v) D- Glucose solution in distilled water was prepared and filter - sterilized.

To prepare 1 litre of the medium, 10 ml from the glucose solution was added to 990 ml of the autoclaved RMM medium under sterile conditions.

3.1.3.2. Luria Bertani (LB) 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 was adjusted to 7.0 with 0.1N NaOH solution. When required, the medium was solidified by adding 16g agar (HiMedia) to the liquid medium before autoclaving.

3.1.3.3. Nitrogen free plant growth medium for *Medicago sativa* (Engelke *et al.*, 1987)

Twelve stock solutions (A to L) of following concentrations were prepared and used for plant inoculation studies:

Solution	Constituent	Amount of constituent (g)	Distilled water (ml)
A	$K_2HPO_4.3H_2O$	2.090	10
B	KH_2PO_4	0.544	10
C	$CaCl_2.2H2O$	7.351	10
D	$C_6H_5O_7Fe.3H_2O$	0.335	10
E	$MgSO_4.7H_2O$	6.162	10
F	K_2SO_4	4.356	40
G	$MnSO_4.H_2O$	0.034	20
H	H_3BO_3	0.026	20
I	$ZnSO_4.7H_2O$	0.030	20
J	$CuSO_4.5H_2O$	0.002	20
K	$CaSO_4.2H_2O$	0.006	20
L	Na_2MoO_4	0.006	20

The stock solutions were autoclaved separately. To make 1 litre of solidified plant growth medium, 10g agar was added to 985 ml distilled water and the resulting water agar medium was autoclaved. One ml from each of the stock solutions, except solution F, was added to the autoclaved agar solution; four ml of stock solution F was added and pH adjusted to 6.8 with 0.1N NaOH or 0.1N HCl. This medium was then poured into glass tubes to prepare slants for growing alfalfa plants. All autoclavings were done at 15psi for 20 min.

3.1.4. Diluent used

The diluent used for making serial dilutions and cell suspensions was normal saline, 0.85% (w/v) NaCl (HiMedia).

3.1.5. Supplements to media

3.1.5.1. Antibiotics

The antibiotics used were dissolved in different diluents to make stock solutions. The stock solutions of streptomycin sulphate (s.d. fine - chem Limited, Mumbai, India) and kanamycin acid sulphate (s.d. fine) were prepared in sterile distilled water; 50% ethanol was used as a solvent for tetracycline hydrochloride (HiMedia) and chloramphenicol (HiMedia), and rifampicin (HiMedia) was dissolved in dimethylsulphoxide. The antibiotics were used in the following concentrations: streptomycin sulphate, 100μg/ml; kanamycin acid sulphate, 40μg/ml for *E. coli* and 400μg/ml for *S. meliloti*; rifampicin, 40μg/ml; chloramphenicol, 40μg/ml and tetracycline hydrochloride, 15μg/ml. Stock solutions of antibiotics were filter-sterilized and added to the autoclaved media under sterile conditions.

3.1.5.2. Amino acids, nitrogenous bases and vitamins

The stock solutions of amino acids, nitrogenous bases and vitamins were prepared in distilled water and autoclaved. Measured volumes from stock solutions were added to the autoclaved media to make final concentrations of 50, 30 and 5μg/ml for amino acids, nitrogenous bases and vitamins, respectively. The auxotrophic requirement of each strain was determined on modified Holliday

pools (Holliday, 1956). Following are the compositions of modified Holliday pools used in the present study:

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

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

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

Pool4: Methionine, lysine, threonine, aspartic acid, alanine and cobalamine.

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

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

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

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

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

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

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

3.1.5.3. Intermediates of arginine biosynthetic pathway

L-ornithine, L-citrulline and L-arginine were purchased from s.d. fine. L-arginosuccinic acid was purchased from M/s Sigma Chemicals Co., St. Louis, USA. To prepare the stocks of the above intermediates, these were dissolved in sterile distilled water. The final concentration in which these intermediate(s) were added to the RMM medium was 50µg/ml.

3.1.5.4. Sugars and dicarboxylic acids

All sugars (glucose, galactose, fructose, arabinose, lactose, maltose, xylose, mannitol and sucrose) and dicarboxylic acids (malate, aspartate, glutamate, fumarate and succinate) used were purchased from HiMedia. A sugar/ dicarboxylic acid was added as a sole carbon source at the final concentration of 2g/ litre to the RMM medium before autoclaving, except glucose that was added as described earlier.

3.1.5.5. Sodium deoxycholate (DOC)

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

3.1.5.6. Dyes

Aniline blue (HiMedia), calcofluor white (Sigma) and congo red (HiMedia) were supplemented to the MSY medium at the concentrations of 0.02% (w/v) each before autoclaving. 0.01% (w/v) methylene blue (HiMedia) and 1% (w/v) toluidine blue (HiMedia) were used to stain infection threads and semithin sections of nodules, respectively. Toluidine blue was prepared in 1% (w/v) borax (Sigma).

3.1.5.7. pH indicator

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

3.1.5.8. Salt

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

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

3.1.6.1 Preparation of blocks

(a) 0.2M phosphate buffer

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

(b) Fixatives

(i.) **Karnovsky fixative (primary fixative)** (Karnovsky, 1965)

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

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

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

(c) Acetone series

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

(d) Araldite CY212 (resin) embedding medium

Constituent	Amount used
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 the ingredients were mixed by stirring.

3.1.6.2. Solutions for staining ultrathin sections for electron microscopy

3.1.6.2.1. Uranyl acetate solution

An excess of uranyl acetate was added to 10ml of 50% (v/v) ethanol in a 15ml centrifuge tube to prepare its saturated solution. This was centrifuged at 5000rpm for 2min and the supernatant 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 12ml of filtered 50% (v/v) ethanol in a 15ml centrifuge tube. After dissolution of NaOH, 50mg of lead citrate was added and the contents mixed by shaking. This was centrifuged at 5000rpm 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 cultures

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

3.2.1.2. Culturing of bacteria

Single colonies were obtained by dilution plating or streaking of cells of bacterial strains on complete or minimal medium. Incubation was done for 2-3 days at 28°C for *S. meliloti* and at 37°C for *E. coli* to obtain single colonies on complete medium. Incubation of about one week was required for single colonies of *S. meliloti* to appear on minimal medium.

Cell suspensions for studying growth responses of bacterial strains were prepared by suspending all the cells from a colony in 0.05ml of 0.85% NaCl. A loopful of the cell suspension (having 10^5 to10^6 cells) was applied to the surface of agar medium to mark a spot. Log phase *E. coli* culture was obtained by incubating a single colony into 10ml liquid LB medium containing the required

antibiotic for 14-16 hrs at 37°C. Similarly, log phase cultures of *S. meliloti* and its derivative strains were obtained by inoculating a colony of the required strain in 10ml liquid TY medium and incubating the inoculated medium for 24-32hrs at 28°C. For aeration, the tubes or flasks containing bacterial cultures were incubated at a suitable temperature in an orbital shaker (GALLENKAMP) operating at 120 rpm.

3.2.1.3. Serial dilutions

Serial dilutions were performed in normal saline i.e. 0.85% (w/v) NaCl. The serial dilutions were done to obtain single bacterial colonies according to the requirements of the experiment. 0.1 ml of neat culture was added to 0.9 ml saline to get 10^{-1} dilution; this was vortexed to get a uniform suspension. 0.1 of bacterial suspension from this tube was added to another tube containing 0.9 ml saline so as to obtain 10^{-2} dilution. Further dilutions were similarly made according to the experimental requirement.

3.2.1.4. Bacterial matings

All bacterial matings were done according to Kondorosi *et al*. (1977). Recipient (0.05 ml) and donor (0.05 ml) strains were grown to active log phase cultures, mixed in the ratio of 1:1 (3:1 for Tn5 mutagenesis as described in next section) and spread on TY agar plates. The plates were incubated at 28°C for 24 hrs.

3.2.2. Isolation and screening of auxotrophs

3.2.2.1. Random transposon Tn5 mutagenesis

Tn5 delivery suicide vector pGS9 (Selvaraj and Iyer, 1983) was used to perform random Tn5 mutagenesis of *S. meliloti* strain Rmd201. Log phase cultures of *E. coli* strain WA803(pGS9) as donor and *S. meliloti* strain Rmd201 as recipient, in liquid TY medium, were obtained. 0.07 ml of *S. meliloti* and 0.03 ml of *E. coli* cultures were mixed (in the ratio of 3:1) and spread on half of the surface of TY agar medium in a Petri dish with the help of a sterile glass rod. The remaining half of the TY agar surface was further divided into two halves. 0.05 ml of neat culture of *S. meliloti* on one half and 0.05 ml of neat culture of WA803(pGS9) on the other half were spread. Plates were incubated at 28°C for 24 hrs.

The bacterial growth from each area (donor / recipient / mating mixture) was scrap ed separately and each was suspended in 2 ml saline. 0.1 ml suspension of the mating mixture was spread on TY agar plates containing streptomycin (100 . g/ ml) and kanamycin (400 . g/ ml). The controls, donor (0.05 ml) and recipient (0.05 ml) suspensions, were also spread separately on these selective plates. The plates were incubated at 28°C for 5 days. The colonies obtained were purified on TY+Km^{400}+Sm^{100} agar plates. All kanamycin resistant (Km^r) transconjugants were checked for the loss of chloramphenicol marker to confirm the loss of suicide plasmid pGS9. The above cross was made for 128 times and all Km^r transconjugants were selected from each cross.

3.2.2.2. Screening for auxotrophs

Each Km^r transconjugant colony was streaked on RMM and TY+Km^{400}+Sm^{100} agar plates with the help of a sterile toothpick. Incubation was done at 28°C for 3 days; the bacterial growth on each streak was observed after every 12 hrs. Those Tn5 derivatives which showed growth on TY+Km^{400}+Sm^{100} but no growth on RMM agar plates were considered to be auxotrophs. Each of these auxotrophs was purified for a single colony on TY+Km^{400}+Sm^{100} agar plates and the cultures maintained as described earlier.

3.2.3. Determination of the nature of auxotrophy

To determine the nature of auxotrophy, a loopful of each auxotroph was suspended in a drop of saline. This suspension was streaked on RMM agar medium supplemented with Holliday pools (one pool at a time) with the help of a sterile toothpick. Incubation was done at 28°C for 5 days and the nature of auxotrophy established on the basis of growth patterns observed on various Holliday pools. The nutritional requirement(s) of each auxotroph was confirmed by streaking it on RMM agar medium supplemented with the suspected nutrient(s).

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

The biochemical blocks in each arginine auxotroph was determined by feeding the intermediates of the arginine biosynthetic pathway to the auxotroph and subsequently observing the growth. A cell suspension of each arginine auxotroph was made and streaked using a sterile toothpick on RMM medium

supplemented with L-ornithine, L-citrulline, L-arginosuccinic acid (one at a time). RMM medium supplemented with arginine was used as a positive control. The cell suspension of the parental strain was also streaked on the above media in each plate as a control. The growth pattern, with reference to the parental strain growth in each plate, was observed after incubation for 4-5 days at 28°C.

3.2.5. Growth characteristics

The pattern of growth of each arginine auxotroph used in this study was compared to that of the parental strain. Each auxotroph was grown to active log phase; 0.2ml of this culture was taken and inoculated into 20ml of liquid MSY medium. These cultures were incubated at 28°C on an orbital shaker operating at 120rpm. Considering the time of inoculation to be zero hour, a sample (1.5ml) from each culture was collected at an interval of 4 hrs, from zero to 24 hrs. Optical densities (O.D.s) of these samples were recorded at 600nm against uninoculated liquid MSY medium used as a control. The generation time for each auxotroph was calculated using standard graphical method. (O.D. v/s time graph).

3.2.6. Pleiotropic effects of Tn5 insertions in auxotrophs

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

3.2.6.1. Production of cell surface molecules

3.2.6.1.1. Test for the production of lipopolysaccharides (LPS)

All arginine 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 and growth of these strains was recorded. The strains which show growth on the DOC supplemented MSY agar medium are considered to be LPS producing (Swamynathan and Singh, 1995).

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

The binding of congo red dye and calcofluor white by the rhizobial strains indicates the normal production of cellulose fibrils and succinylated exopolysaccharides (EPS I), respectively. The strains producing cellulose fibrils form red colonies on medium containing congo red (Kneen and La Rue, 1983); the strains producing EPS I give fluorescence under ultraviolet light (Leigh *et al*., 1987). MSY agar plates containing 0.02% (w/v) congo red and 0.02% (w/v) calcofluor (one at a time) were prepared. All arginine auxotrophs were streaked on the above media. The parental strain was also streaked as a positive control. Incubation was done at 28°C for 3 days. After incubation, the congo red plates were observed for red colonies in normal light and the calcofluor plates were observed for fluorescence in ultraviolet light.

3.2.6.1.3. Test for the production of β-glucans

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

The rhizobial strains which produce β(1→3) glucans form blue colonies on medium containing aniline blue (Nikanishi *et al.*, 1976). All arginine auxotrophs were streaked on MSY agar medium containing 0.02% (w/v) aniline blue. The parental strain was also streaked as a positive control. The incubation was done at 28°C for 3 days. The plates were observed for the presence or absence of blue colonies of each strain.

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

The rhizobial strains producing normal β-(1→2) glucans showed swarming on TY swarm medium (Geremia *et al.*, 1987). A loopful of cell suspension of each arginine auxotroph was spotted on TY swarm medium containing 0.3% (w/v) agar. The cell suspension of the parental strain was also spotted as a positive control. The incubation was done at 28°C for 3 days and spots were observed for swarming after every 24 hrs.

3.2.6.2. Utilization of sugars and dicarboxylic acids by auxotrophs

Each arginine auxotroph was streaked on RMM agar medium (without glucose) supplemented with arginine and a sugar (galactose, fructose, arabinose, lactose, maltose, xylose, mannitol or sucrose) / dicarboxylic acid (malate, aspartate, glutamate, fumarate or succinate); the parental strain was also streaked as a positive control. All arginine auxotrophs and the parental strain were also streaked on RMM agar medium (with glucose). The incubation

was done at 28°C for 5 days and bacterial growth was observed after every 24 hrs.

3.2.6.3. Salt tolerance of auxotrophs

All arginine auxotrophs and the parental strain were streaked on MSY agar containing 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4% (w/v) NaCl and incubated at 28°C for 4-5 days. These strains were also inoculated in liquid MSY medium having the above NaCl concentrations. The incubation was done at 28°C for 2-3 days on an orbital shaker operating at 120rpm and O.D. of each culture was recorded against an uninoculated control.

3.2.6.4. Acid tolerance of auxotrophs

The arginine auxotrophs and the parental strain were streaked on MSY agar medium at pH 4.0, 5.0, 6.0 and 7.0 prepared using 1N HCl and incubated at 28°C for 4 - 5 days. These strains were also inoculated in liquid MSY media of above pH. The incubation was done at 28°C for 2-3 days on an orbital shaker operating at 120rpm.

3.2.6.5. pH changes during growth of auxotrophs

MSY agar plates and MSY liquid media containing bromothymol blue (25mg/litre) were prepared. Production/change in colour of medium to blue, green or yellow indicates the alkaline, neutral or acidic pH, respectively (Vincent, 1970). All arginine auxotrophs and the parental strain were streaked on MSY agar medium. The strains were also inoculated in liquid MSY medium. Incubation

was done at 28°C for 3 days; the inoculated liquid medium was incubated at 28°C for 2-3 days on an orbital shaker operating at 120rpm.

3.2.7. Reversion analysis of auxotrophs

All arginine auxotrophs were grown to late log phase culture in liquid TY medium at 28°C on an orbital shaker operating at 120rpm. A sample (10ml) of each culture was taken and centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet washed twice with liquid RMM medium (2 ml each time) and resuspended in a suitable volume of liquid RMM medium so as to obtain about 10^9 cells/ ml. A sample (0.1 ml) of this cell suspension was spread on RMM. Various dilutions (10^{-2}, 10^{-4} and 10^{-6}) of this cell suspension were made. A sample (0.1 ml) of this suspension as well as a sample (0.1 ml) of each of its dilutions were spread on TY agar medium. Incubation was done at 28°C for 5 days and observed for appearance of bacterial colonies. The number of colonies on RMM medium represented the number of prototrophic revertants whereas the number of colonies on TY agar medium multiplied by the dilution factor indicated the total number of cells in the volume (0.1 ml) of cell suspension spread. On the basis of these numbers, spontaneous reversion frequency for each auxotroph was calculated. Streaking cells from the prototrophic revertant colony on TY agar medium containing 400µg/ ml kanamycin determined the excision of transposon Tn5 from this colony.

3.2.8. Construction of donor strains of auxotrophs

The genome mobilizing plasmid pJB3JI (Tc^r) was introduced into all arginine auxotrophs. Each arginine auxotroph of *S. meliloti* strain Rmd201 and

the *S. meliloti* strain AK631 (pJB3JI) were mated, as a recipient and a donor, respectively, on TY agar medium. Incubation was done at 28°C for 18-24 hrs. The growth was scrapped and suspended in 1 ml of saline. Serial dilutions (10^{-1} & 10^{-2}) were made and 0.1 ml of each neat culture and its both dilutions were spread on TY agar medium containing tetracycline (15 µg/ ml) and kanamycin (400 µg/ ml). Incubation was done at 28°C for 4-5 days and the transconjugants containing plasmid pJB3JI selected. Five transconjugants were purified for single colonies on TY medium containing tetracycline (15 µg/ ml) and kanamycin (400 µg/ ml). These purified transconjugants were used as donor strains in the subsequent conjugation experiment.

3.2.9. Linkage of Tn5 insertion to arginine auxotrophy

The linkage of Tn5 insertion to arginine auxotrophy was determined by mobilizing the Tn5-containing fragment from an auxotroph with the help of genome mobilizing plasmid pJB3JI and subsequently checking for arginine auxotrophy in these Km^r transconjugants. The donor strain (containing plasmid pJB3JI) of each arginine auxotroph as obtained above, was mated with the recipient strain *S. meliloti* ZB201 (rifampicin resistant) on TY agar medium. The mating mixture was scrapped, suspended in 1ml saline and spread on TY agar medium containing rifampicin (40 µg/ ml) and kanamycin (400 µg/ ml). Incubation was done at 28°C for 3-5 days. All kanamycin resistant transconjugants were selected and checked for arginine auxotrophy.

3.2.10. Plant inoculation studies

3.2.10.1. Surface sterilization of seeds

Alfalfa (*Medicago sativa* cv. T9 and cv. A2) 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 treatment with absolute alcohol for 1 min. The seeds were washed five times with sterile distilled water and then spread on 1% agar plates. These plates were wrapped with black paper and incubated at 25°C in dark. After two days, two seedlings were transferred to each tube containing the plant growth medium.

3.2.10.2. Plant inoculation tests

Glass tubes (20 x 2.5 cm) were used to carry out the plant inoculation assays. Nitrogen free plant nutrient medium was prepared as described earlier. Twenty-five ml of this agar medium was transferred to each tube; the tubes were plugged with cotton and autoclaved. After autoclaving at 15psi for 15 min, the tubes were immediately placed in slanting position. Filter-sterilized supplements were added to the plant nutrient medium before transfer of this medium to tubes.

Two 2 - days old alfalfa seedlings were transferred to each of these tubes. *S. meliloti* Rmd201 and its arginine auxotrophs were grown to active log phase in 10ml of liquid TY medium. The cultures were centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet, after washing twice with 0.85% (w/v) NaCl, was suspended in 5 ml of 0.85% (w/v) NaCl. 0.5ml of the cell suspension was dispensed in each tube containing seedlings. The lower portions of tubes were wrapped with black paper to protect the plant roots from

direct exposure to light. These tubes were placed in a plant growth chamber maintained at 25°C and provided with 2000 lux light. Sixteen hrs of light and eight hrs of dark periods were maintained for the plants. The plants were pulled out from each tube after six weeks and data on nodule characteristics (shape, size, colour, location), plant height and dry plant weight were recorded. Dry plant weights were determined after drying the plants at 85°C for 72 hrs.

3.2.11. Nodule occupancy tests

The nodules were checked for per cent occupancy by the inoculated strains by reisolating the rhizobial cells from the nodules and testing their auxotrophic and antibiotic markers. All nodules from a plant were harvested, 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. 0.1ml of each of the dilutions and 0.1 ml of neat suspension were spread on a TY agar medium containing 100µg/ ml streptomycin. Three replications were used for the neat suspension and each of the two dilutions. Incubation was done at 28°C for 3 days. All the colonies obtained were streaked on RMM agar, RMM agar + Km^{400} + Sm^{100} + arginine and TY agar + Km^{400} + Sm^{100}. Incubation was done at 28°C for 3 days. The colonies which showed growth on RMM agar but not on RMM agar + Km^{400} + Sm^{100} + arginine and TY agar + Km^{400} + Sm^{100} were considered to be prototrophic revertants.

3.2.12. Plant inoculation tests with prototrophic revertants

The alfalfa plants were inoculated (as described earlier) with the prototrophic revertants of each arginine auxotroph to confirm that the symbiotic

defect was in fact due to arginine auxotrophy. Ten plants were inoculated for each revertant and the data on the plants was recorded six weeks after inoculation.

3.2.13. Microscopic studies of nodule sections

Microscopic examinations of the sections of the nodules induced by the parental strain *S. meliloti* Rmd201 and its arginine auxotrophs AK3, AK4, AK11, AK12 and AK9 were carried out. All the nodules from each of the representative plants of each strain were harvested six weeks after inoculation of these plants. The representative nodules from a plant were used for microscopic studies and the remaining nodules from this plant were used to determine nodule occupancy by the inoculated strain (as described earlier).

3.2.13.1. Primary fixation of nodules samples

The representative nodules to be used for microscopic studies were rinsed thrice with sterile distilled water and transferred to Karnovsky fixative in glass vials of 5 ml capacity. Acrolein (at 0.1% final concentration) was added to facilitate penetration of the fixative. For proper immersion of the fixative and to prevent the floating of the nodules on the surface, the vials were placed in a vacuum chamber at 4°C for 24 hrs.

3.2.13.2. Preparation of specimen block

3.2.13.2.1. Washing of nodule samples

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

3.2.13.2.2. Secondary fixation of nodules samples

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

3.2.13.2.3. 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. Subsequently, two changes of 30 min each in dry acetone were given at room temperature.

3.2.13.2.4. Removal of acetone

To clear off the acetone, the nodule samples were placed in toluene at room temperature for 60 min. Acetone was removed from the samples to facilitate infiltration in the next step.

3.2.13.2.5. Infiltration with resin

The following infiltration mixtures were prepared:

a. 1 part of embedding medium and 3 parts of toluene
b. 2 parts of embedding medium and 2 parts of toluene
c. 3 parts of embedding medium and 1 part of toluene

The nodule samples, after being removed from toluene, were placed in mixture 'a' and left overnight. These were then transferred to mixture 'b' for 1 hr and then to mixture 'c' for 1 hr under vacuum. Subsequently, two changes of 1 hr each in pure embedding medium at 50°C were given.

3.2.13.2.6 Embedding of nodule

Araldite embedding medium was used for embedding the nodule samples using gelatin blocks. Proper orientation of the nodules was done to facilitate subsequent longitudinal sectioning before polymerization of the embedding medium.

3.2.13.2.7. Polymerization of araldite medium

The embedding blocks were kept at 50°C for 24 hr and then the temperature of the oven was raised to 60°C. Complete polymerization of the embedding medium occurred in 48 hrs.

3.2.13.2.8. Trimming of blocks

The blocks containing the polymerized nodule samples were trimmed using pyramitome or trimmer and fitted in a specimen block holder.

3.2.13.3. Preparation of semithin sections

The block holder was attached to 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 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 then stained with 1% (w/v) toluidine blue for 1 min. The sections were then washed with distilled water and observed under light microscope (Leica *DM* LB). The areas of interest were photographed (Nikon Digital Camera).

3.2.13.4. Preparation of ultrathin sections

From the semithin sections, the area to be examined under TEM was selected and the blocks were further trimmed. Ultrathin sections (60-90 nm thick) were cut using Ultracut E Microtome (C. Reichert, Austria OmU3). These sections were lifted onto 200 mesh size copper grids. These grids were coated with 3% formvar (polyvinyl formaldehyde) in ethylene dichloride and carbon by the process of 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 then twice with double distilled water. After washing, the grids were dried on a filter paper and placed in 0.5 ml of lead citrate solution for 10 min on a clean watch glass. After removing the grids from the above solution, these were washed with 0.1N NaOH and then twice with double distilled water. The grids were dried after washing and viewed under transmission electron microscope (PHILIPS EM300). The areas of interest were photographed. The grids were stored in a grid holder at room temperature after viewing.

3.2.14. Statistical analysis

Statistical analysis of the data on characteristics of plants inoculated with the parental strain *S. meliloti* Rmd201 and its arginine auxotrophs was carried out. All values were expressed as mean ± standard error of mean (SE).

Significance between a control value and an experimental value was found out using the student ' t ' test.

4. RESULTS

Auxotrophs of *S. meliloti* strain Rmd201, a streptomycin resistant derivative of strain AK631 (a compact colony variant of the wild type strain Rm41) were generated through random transposon Tn5 mutagenesis. The genetic and biochemical characterizations of the arginine auxotrophs were done. *Medicago sativa* seedlings were used to study the symbiotic properties of these auxotrophs. Histological studies on the nodules induced by non-nitrogen fixing arginine auxotrophs were performed. The results of these experiments have been presented in this chapter.

4.1. Tn5 mutagenesis

A total of 7,650 Tn5-induced kanamycin resistant transconjugants were selected on TY agar medium containing kanamycin (400µg/ ml) and streptomycin (100µg/ ml). The transconjugants were obtained from 128 crosses of *E. coli* WA803(pGS9) donor and *S. meliloti* Rmd201 recipient. All the Tn5-induced transconjugants were found to be chloramphenicol-sensitive. This result indicated the absence of the Tn5 delivery plasmid pGS9 in the Tn5 derivatives. The Tn5 derivatives were streaked on TY agar medium containing kanamycin (400µg/ ml) and streptomycin (100µg/ ml) to get single colonies. The bacteria from one single colony of each Tn5 derivative were used in further experiment.

4.2. Screening of Tn5 derivatives for auxotrophs

The screening of Tn5 derivatives for auxotrophs was done by streaking all the Tn5 derivatives on RMM (*Rhizobium* minimal medium) agar medium. Thirty

two Tn5 derivatives did not show growth on RMM. These derivatives were considered as auxotrophs and used in the further work.

4.3. Determination of nutritional requirements of auxotrophs

The growth patterns of all the auxotrophs on modified Holliday pools showed that the nutritional requirements of the auxotrophs were as follows (the number of auxotrophs isolated have been given in brackets): arginine (15), methionine (3), tryptophan (2), cysteine (7), uracil (3) and adenine (2). The arginine auxotrophs were named as AK3, AK4, AK5, AK6, AK7, AK8, AK9, AK10, AK11, AK12, AK13, AK14, AK15, AK16 and AK25, and characterized further.

4.4. Reversion frequencies

All Tn5 induced arginine auxotrophs showed spontaneous reversion to prototrophy. The reversion frequencies of the arginine auxotrophs were found to vary from 1.0 $x10^{-9}$ for the strain AK5 to 3.0 x 10^{-9} for the strain AK15 (Table 3). All prototrophic revertants were kanamycin sensitive showing that the excision of Tn5 had occurred in these revertants.

4.5. Identification of positions of mutations

On the basis of intermediate feeding studies, arginine auxotrophic mutants were classified into following four categories:

(i) *argA/argB/argC/argD/argE* mutants (AK3, AK4 and AK5) which grew on RMM supplemented with ornithine, citrulline or arginosuccinic acid; these mutants were also designated as ornithine auxotrophs,

(ii) *argF/argI* mutants (AK6, AK7, AK8, AK10, AK11, AK13, AK14, AK15, AK16 and AK25) which did not grow on RMM supplemented with

Table 3: Spontaneous reversion frequencies of arginine auxotrophs of *Sinorhizobium meliloti* Rmd201

S. No.	Arginine auxotroph	Spontaneous reversion frequency
1.	AK3	1.8×10^{-9}
2.	AK4	1.5×10^{-9}
3.	AK5	1.0×10^{-9}
4.	AK6	1.8×10^{-9}
5.	AK7	1.7×10^{-9}
6.	AK8	2.0×10^{-9}
7.	AK9	1.4×10^{-9}
8.	AK10	1.9×10^{-9}
9.	AK11	1.6×10^{-9}
10.	AK12	1.3×10^{-9}
11.	AK13	2.1×10^{-9}
12.	AK14	1.7×10^{-9}
13.	AK15	3.0×10^{-9}
14.	AK16	2.2×10^{-9}
15.	AK25	1.4×10^{-9}

ornithine but grew on RMM supplemented with citrulline or arginosuccinic acid,

(iii) *argG* mutant (AK12) which did not grow on RMM supplemented with ornithine or citrulline but grew on RMM supplemented with arginosuccinic acid and,

(iv) *argH* mutant (AK9) which did not grow on RMM supplemented with ornithine, citrulline or arginosuccinic acid.

The position of biochemical block in each arginine auxotroph has been shown in Fig. 4.

4.6. Growth pattern

All arginine auxotrophs showed growth characteristics similar to that of the parental strain Rmd201. There was no significant difference between the generation time of each auxotroph and that of the parental strain (2hrs, 50 min).

4.7. Study of pleiotropic effects of Tn5 insertions

4.7.1. Production of cell surface molecules

The parental strain and all the arginine auxotrophs showed growth in presence of sodium deoxycholate, took up the congo red dye, fluoresced in presence of calcoflour white under UV light and showed swarming on swarm medium. These results showed that normal production of lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β-(1→2) glucans, respectively, occurred in all the auxotrophs like that observed in the parental Rmd201 strain. These auxotrophs, like the parental strain, did not show binding

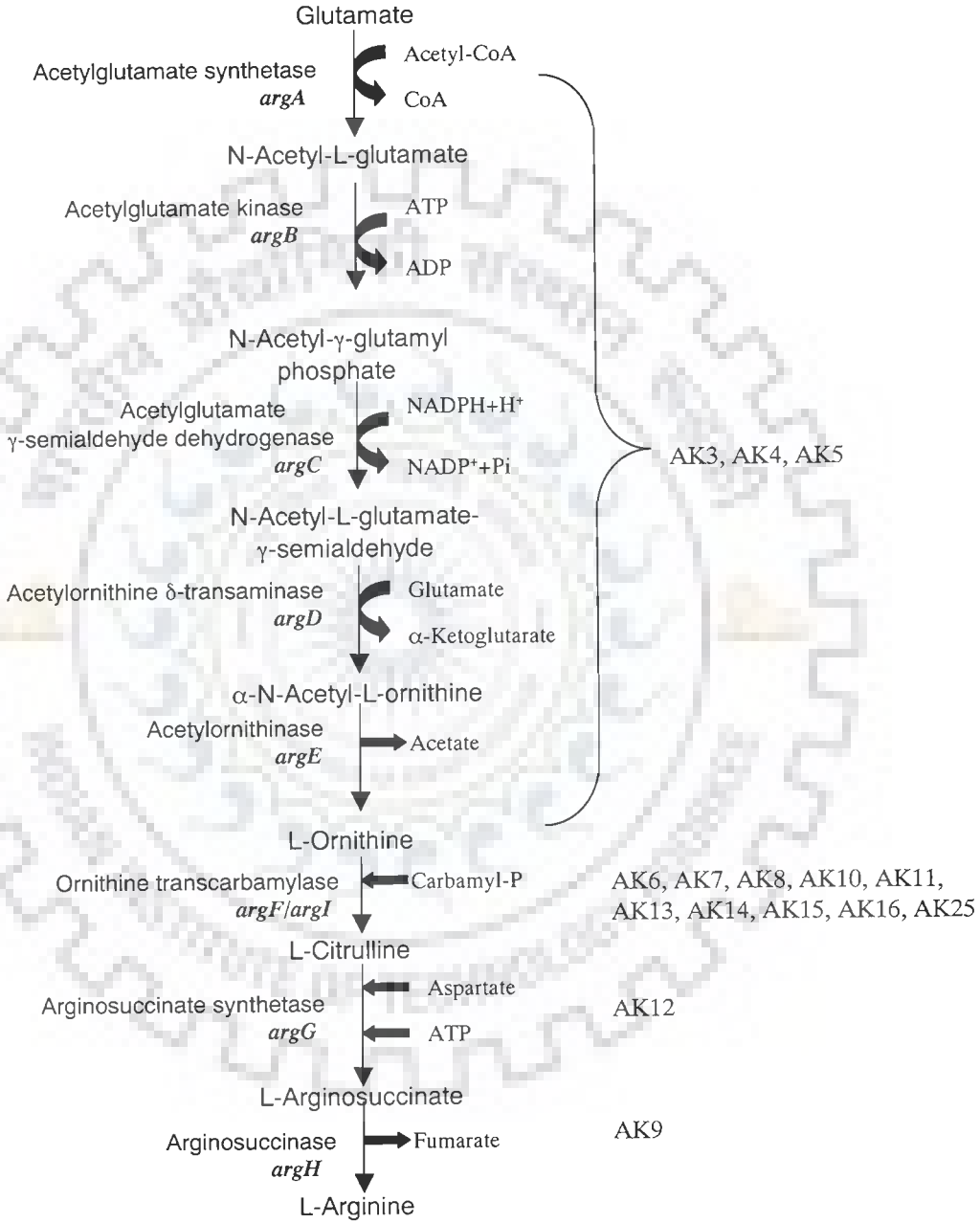

Fig. 4: The pathway for arginine biosynthesis in bacteria showing positions of mutations of arginine auxotrophs of *Sinorhizobium meliloti* Rmd201 (Moat and Foster, 1995).

with aniline blue dye. This result indicated that β-(1→3) glucans were not produced in these auxotrophs and the parental strain.

4.7.2. Utilization of sugars and dicarboxylic acids

The growth of all the arginine auxotrophs on RMM supplemented with arginine was similar to that of the parental strain Rmd201 on RMM. No change in the growth behaviour was noticed when glucose in RMM medium was substituted by any one of the other sugars (galactose, fructose, arabinose, lactose, maltose, xylose, mannitol or sucrose) or dicarboxylic acids (malate, aspartate, glutamate, fumarate or succinate) as a sole carbon source.

4.7.3. Salt tolerance

Like the parental strain Rmd201, all arginine auxotrophs grew on MSY medium (agar and liquid both) containing 1.0, 1.5, 2.0, 2.5 and 3% (w/v) NaCl but did not show any growth on MSY medium containing 3.5 and 4.0% NaCl (w/v) concentration.

4.7.4. Acid tolerance

All arginine auxotrophs and the parental strain showed normal growth on MSY medium (agar and liquid both) having pH 6.0; none of the strains grew on MSY medium at other pH values.

4.7.5. pH changes

When grown on MSY medium (agar and liquid both) containing bromothymol blue, all arginine auxotrophs and the parental strain Rmd201 changed the colour of dye from blue to yellow. This result indicated that arginine auxotrophs, like the parental strain, made the medium acidic.

4.8. Linkage of Tn5 insertion to auxotrophy

When transposon Tn5-encoded kanamycin resistance marker of each arginine auxotroph was transferred to *S. meliloti* ZB201 strain, all kanamycin resistant transconjugants (150 in each case) were found to be arginine auxotrophs. One hundred per cent co-transfer of Tn5 and auxotrophy indicated complete linkage of transposon Tn5 insertion to auxotrophy. In other words, each auxotrophic cell had only one Tn5 insertion and the auxotrophy was due to this insertion.

4.9. Symbiotic properties of auxotrophs

M. sativa plants of both cultivars T9 and A2 inoculated with the parental strain Rmd201 and the *argF/argI* (except AK10), *argG* and *argH* mutants were tall, healthy and showed no signs of nitrogen starvation; all these mutants and the parental strain induced cylindrical, pink nodules on both primary and lateral roots of plants of both alfalfa cultivars. The uninoculated plants were short, weak and showed chlorosis in all parts. The plants inoculated with ornithine auxotrophs and AK10 were short, weak and showed chlorosis, like the uninoculated plants, in the lower parts but the upper parts appeared greener suggesting that trace amounts of nitrogen was being fixed in the nodules induced by these auxotrophs. The nodules induced by these mutants were spherical/irregular and white, and were located mostly on the lateral roots. Representative plants inoculated with arginine auxotrophs, each having a biochemical block, either before or after ornithine, are shown in Plate 1. The mean dry weights of the plants of the alfalfa cultivars T9 and A2 inoculated with *argF/argI*, *argG* and *argH* mutants did not differ

significantly ($P<0.05$) from the mean dry weights of the plants of these cultivars inoculated with the parental strain Rmd201 (Tables 4 and 5). These results indicated that the nitrogen fixing efficiencies of these mutants were similar to that of the parental strain for both cultivars. The mean dry weights of the plants of alfalfa cultivars T9 and A2 inoculated with the *argA/argB/argC/argD/argE* mutants (ornithine auxotrophs) did not differ significantly ($P<0.05$) from the mean dry weights of the uninoculated plants of the above cultivars indicating that these auxotrophs did not fix nitrogen.

4.10. Inoculation of plants with prototrophic revertants

The revertants of arginine auxotrophs showed, like the parental strain Rmd201, normal symbiosis with alfalfa plants (Table 6). The plants inoculated with the revertants were tall and healthy, and induced pink coloured nodules on both primary and lateral roots. The mean dry plant weights of these plants did not differ significantly ($P<0.05$) from the mean dry plant weight of the parental strain. These results revealed that a single Tn5 insertion in each of these arginine auxotrophs resulted in auxotrophy and symbiotic defect.

4.11 Nodule occupancy tests

Nodule occupancy tests were conducted on nodules induced by the arginine auxotrophs on alfalfa plants. The rhizobial bacteria were isolated from the nodules of each plant and checked for arginine auxotrophy. Reisolation of bacteria from the nodules induced by the arginine auxotrophic mutants showed 100% occupancy of nodules by these mutants.

Plate 1: Morphological features of representative plants of alfalfa inoculated with *Sinorhizobium meliloti* Rmd201 and its arginine auxotrophs: 1. Uninoculated, 2. Rmd201 inoculated, 3. Ornithine auxotroph AK3 inoculated, 4. *argf/argI* mutant AK11 inoculated, 5. *argG* mutant AK12 inoculated, and 6. *argH* mutant AK9 inoculated.

Table 4: Characteristics of *Medicago sativa* cv. T9 plants inoculated with *Sinorhizobium meliloti* Rmd201 and its arginine auxotrophs

S. meliloti strain	Mutated gene	No. of days to appearance of 1st nodule	Nodule Characteristics				Mean plant height# (in cm)	Mean dry plant weight# (in mg)
			Shape	Size (in mm)	Colour	Location		
Uninoculated	-	-	-	-	-	-	5.5±0.5	7.9±0.2
Rmd201	-	10-12	Cylindrical	3-4.5	Pink	PR & LR	28.2±0.6	35.2±1.2
AK 3	*argA/argB/argC/argD/argE*	12-14	Spherical/Irregular	1-2.5	White	LR	5.8±0.7*	8.0±0.3*
AK 4	-do-	-do-	-do-	-do-	-do-	-do-	6.1±0.2*	8.2±0.5*
AK 5	-do-	-do-	-do-	-do-	-do-	-do-	6.0±0.5*	8.3±0.4*
AK 6	*argF/argI*	10-12	Cylindrical	3-4.5	Pink	PR & LR	26.5±0.3	34.3±0.5
AK 7	-do-	-do-	-do-	-do-	-do-	-do-	27.4±0.5	34.2±1.1
AK 8	-do-	-do-	-do-	-do-	-do-	-do-	28.0±0.2	33.6±1.4
AK 10	-do-	12-14	Spherical/ Irregular	1-2.5	White	LR	5.7±0.3*	8.5±1.0*
AK 11	-do-	10-12	Cylindrical	3-4.5	Pink	PR & LR	25.8±0.7	34.4±0.5
AK 13	-do-	-do-	-do-	-do-	-do-	-do-	26.3±0.5	33.8±1.0
AK 14	-do-	-do-	-do-	-do-	-do-	-do-	27.7±0.4	34.3±1.2
AK 15	-do-	-do-	-do-	-do-	-do-	-do-	27.2±0.8	35.0±2.2
AK 16	-do-	-do-	-do-	-do-	-do-	-do-	25.5±0.3	32.7±1.6
AK 25	-do-	-do-	-do-	-do-	-do-	-do-	28.0±0.9	32.5±0.8
AK 12	*argG*	-do-	-do-	-do-	-do-	-do-	28.1±0.1	32.3±0.3
AK 9	*argH*	-do-	-do-	-do-	-do-	-do-	27.7±0.4	32.1±1.5

#Each value is mean of 10 plants, *Does not differ significantly from uninoculated plant value ($P<0.05$); ***Abbreviations:*** PR, Primary root; LR, Lateral root.

Table 5: Characteristics of *Medicago sativa* cv. A2 plants inoculated with *Sinorhizobium meliloti* Rmd201 and its arginine auxotrophs

S. meliloti strain	Mutated gene	No. of days to appearance of 1st nodule	Nodule Characteristics				Mean plant height# (in cm)	Mean dry plant weight# (in mg)
			Shape	Size (in mm)	Colour	Location		
Uninoculated	-	-	-	-	-	-	5.8±0.6	7.8±0.4
Rmd201	-	10-12	Cylindrical	3-4.5	Pink	PR & LR	27.6±0.8	36.1±1.0
AK 3	*argA/argB/argC/argD/argE*	12-14	Spherical/Irregular	1-2.5	White	LR	5.9±1.0*	7.9±0.7*
AK 4	-do-	-do-	-do-	-do-	-do-	-do-	6.3±0.3*	8.1±0.5*
AK 5	-do-	-do-	-do-	-do-	-do-	-do-	6.1±0.4*	8.2±0.3*
AK 6	*argF/argI*	10-12	Cylindrical	3-4	Pink	PR & LR	28.0±0.7	35.5±0.8
AK 7	-do-	-do-	-do-	-do-	-do-	-do-	27.2±0.5	34.1±1.2
AK 8	-do-	-do-	-do-	-do-	-do-	-do-	26.0±0.5	34.8±1.5
AK 10	-do-	12-14	Spherical/Irregular	1-2.5	White	LR	25.4±0.3*	8.2±0.3*
AK 11	-do-	10-12	Cylindrical	3-4	Pink	PR & LR	26.5±0.8	35.3±1.8
AK 13	-do-	-do-	-do-	-do-	-do-	-do-	24.4±1.2	33.6±1.3
AK 14	-do-	-do-	-do-	-do-	-do-	-do-	27.4±0.5	34.5±2.1
AK 15	-do-	-do-	-do-	-do-	-do-	-do-	26.3±0.4	34.7±0.7
AK 16	-do-	-do-	-do-	-do-	-do-	-do-	27.4±0.7	33.3±0.9
AK 25	-do-	-do-	-do-	-do-	-do-	-do-	26.2±0.8	32.8±1.1
AK 12	*argG*	-do-	-do-	-do-	-do-	-do-	25.0±0.7	34.0±2.2
AK 9	*argH*	-do-	-do-	-do-	-do-	-do-	27.5±0.5	33.9±2.0

#Each value is mean of 10 plants; *Does not differ significantly from uninoculated plant value ($P<0.05$); ***Abbreviations:*** PR, Primary root; LR, Lateral root.

Table 6: Characteristics of *Medicago sativa* cv. T9 plants inoculated with *Sinorhizobium meliloti* Rmd201 and revertants of its arginine auxotrophs

S. meliloti strain	Mutated gene	No. of days to appearance of 1st nodule	Nodule Characteristics				Mean plant height# (in cm)	Mean dry plant weight# (in mg)
			Shape	Size (in mm)	Colour	Location		
Uninoculated	-	-	-	-	-	-	6.2±0.8	8.0±0.8
Rmd201	-	10-12	Cylindrical	3-4.5	Pink	PR & LR	30.1±1.1	32.4±1.5
AK 3	*argA/argB/argC/argD/argE*	-do-	-do-	-do-	-do-	-do-	27.2±1.2*	30.0±0.7*
AK 4	-do-	-do-	-do-	-do-	-do-	-do-	29.0±0.5*	31.4±0.5*
AK 5	-do-	-do-	-do-	-do-	-do-	-do-	27.6±0.7*	29.5±1.0*

#Each value is mean of 10 plants; *Does not differ significantly from parental strain inoculated plant value ($P<0.05$); ***Abbreviations:*** PR, Primary root; LR, Lateral root.

4.12. Light microscopy of nodules

The longitudinal semithin section of the nodule induced by the parental strain Rmd201 showed a central tissue surrounded by several peripheral tissues. Vascular bundles were seen in the peripheral tissue. 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 zone was made up of small, actively dividing and uninfected cells, each with a big nucleus and many mitochondria. Infection threads were seen in the intercellular spaces of the cells of infection zone; there was an occasional release of rhizobial bacteria from the infection threads in few cells. Some cells in the infection zone had large nuclei and vacuolar regions. Many amyloplasts were present in this zone. The interzone was the zone of the nodule showing transition of nodule cells from uninfected to infected state. The nitrogen fixation zone was quite large and most of the nodule cells in this zone were infected with rhizobia (Plate 2A). A large number of bacteroids were arranged perpendicularly around a large central vacuole in each infected cell in the nitrogen fixation zone (Plate 3B). The bacteroids seen in this zone were mostly elongated. Lysed nodule cells were observed in the senescence zone (Plate 3C).

Distinct peripheral and central tissues like those in the parental strain induced nodules were seen in the nodules of the ornithine auxotrophs AK3 and AK4. The central tissues of these nodules, like that of the parental strain induced nodules, were differentiated into five zones (Plates 6 and 7 for AK3; Plates 14

and 15 for AK11; Plates 10 and 11 for AK4). Unlike the parental strain, the infection zone in ornithine mutants was extensive and covered more than half of the nodule (Plates 6A and 10A). The meristematic zone, infection zone and interzone were filled with amyloplasts. The interzone was reduced to few cells (Plates 7A and 11A). The nitrogen fixation zone in the nodules of ornithine auxotrophs was identified on the basis of its visual resemblance to the nitrogen fixation zone of the Rmd201 induced nodules. Actually it is not a true nitrogen fixation zone since the nodules formed by these auxotrophs did not fix nitrogen. In comparison to the parental strain induced nodule, only a few nodule cells infected with rhizobia were seen in the so-called nitrogen fixation zone and uninfected cells were present amongst these infected cells (Plates 7B and 11B). Unlike the nitrogen fixation zone of parental and *argF/argI*, *argG* and *argH* mutants, many amyloplasts were present in this zone. Lysis of nodule cells was observed in the senescence zone which was made up of few cells. Several amyloplasts were seen in this zone (Plate 7A).

The internal structural features of the nodules induced by the arginine auxotrophs *argF/argI* (AK11), *argG* (AK12) and *argH* (AK9) were almost similar to those of the parental strain induced nodules. The meristematic, infection and the inter zones showed comparable characteristics as seen in the parental strain induced nodules. The nitrogen fixation zone in each of the nodules induced by these auxotrophs was like that seen in the nodules induced by Rmd201 (Plates 14 and 15 for AK11; Plates 18 and 19 for AK12; Plates 22 and 23 for AK9), except that the nitrogen fixation zone in AK11 mutant contained amyloplasts

which were not seen in the nitrogen fixation zone of other mutants. Some lysed nodule cells were seen in the senescence zone.

4.13. TEM studies of nodules

Ultrathin section of the nodule induced by the parental strain Rmd201 showed the presence of many mitochondria and endoplasmic reticulae in the meristematic zone (Plate 4A). Poly-. -hydroxy butyrate (PHB) granules were present in the rhizobial bacteria in infection threads and in freshly released rhizobia in nodule cells (Plate 4B). Peribacteroid membrane (pbm) loosely surrounded each freshly released bacterial cell (Plate 4C). The cytoplasm of the rhizobial bacteria in the infection zone was electron dense (Plate 4C) whereas the cytoplasm of the bacteroids in the interzone (Plate 5A) and nitrogen fixation zone (Plate 5B) was heterogeneous in the sense that it contained electron dense and electron transparent regions. Different shapes of rhizobial bacteria were seen in the infection and inter zone areas (Plates 5A and B). Most of the bacteroids in the nitrogen fixation zone were elongated (Plate 5B). Some bacteroids in the senescence zone had broken pbm (Plate 5C). The cytoplasm of the bacteroids in the senescence zone was electron transparent (Plate 5C).

All stages of the infection of plant cells by rhizobial bacteria and the subsequent bacteroidal development in the nodules induced by the ornithine auxotrophs AK3 and AK4 were similar to those seen in the parental strain induced nodule except that the bacteroids in the so-called nitrogen fixation zone of AK3 mutant (Plate 9A and 9B) and AK4 mutant (Plate 13A and 13B) induced nodule were mostly spherical or oval (Fig 4B). The bacteroids in the nitrogen

fixation zone of the ornithine auxotrophs AK3 and AK4 did not show heterogeneity in cytoplasm to the extent of the parental strain (Plate 13A and 13B). The peribacteroid membrane in the nitrogen fixation zones of ornithine mutants was loose and wavy (Plate 9B and 13B), unlike the peribacteroid membrane of bacteroids in the nitrogen fixation zones of Rmd201 (Plate 5B), *argF/*argI mutant (Plate 17A), *argG* mutant (Plates 20 C and 21A) and *argH* mutant (25B), showing reduced maturity and differentiation of bacteroids of ornithine auxotrophs. The bacteroids showing lysis in the senescence zone of ornithine mutants were electron dense (Plates 9C and 13C), unlike the parental strain Rmd201 (Plate 5C), *argF/*argI mutant (Plate 17C), *argG* mutant (Plate 21B) and *argH* mutant (25C).

The ultrastructure of each of the nodules induced by arginine auxotrophs AK11 (*argF/argI*), AK12 (*argG*) and AK9 (*argH*), each having a biochemical block after ornithine, was similar to that of the parental strain induced nodule (Plates 16 and 17 for AK11; Plates 20 and 21 for AK12; Plates 24 and 25 for AK9); however, the bacteroids in the nitrogen fixation zone of the nodule induced by AK11 mutant were not elongated to the extent observed in the nodules induced by Rmd201 (Plate 4B), AK12 (Plate 21A) and AK9 (Plate 25B). Intercellular spaces in the interzone of AK11 (an *argF/argI* mutant) induced nodule were seen filled with electron dense material (Plate 16C).

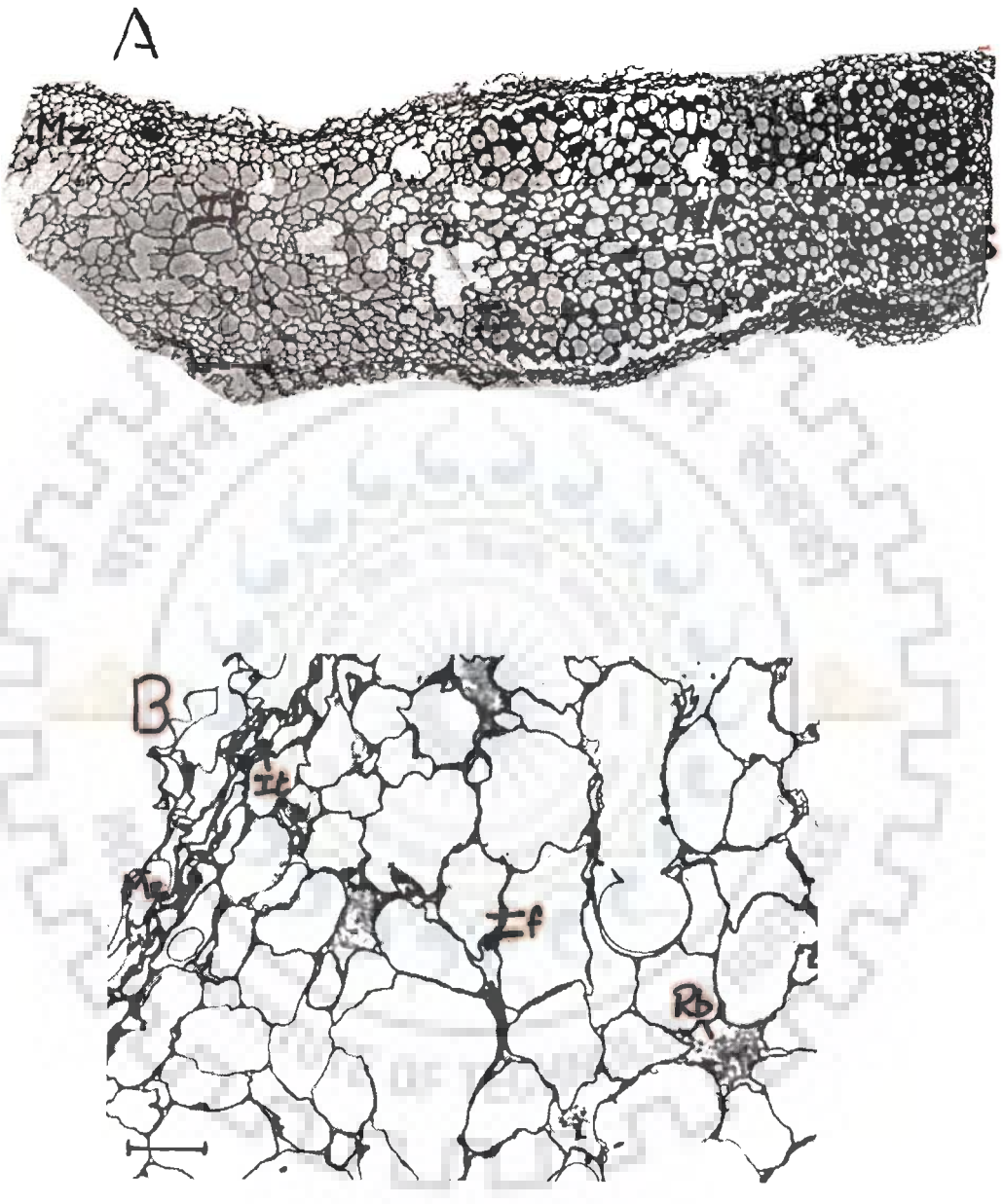

Plate 2: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A.** A nodule section showing distinct peripheral tissue (pt) and central tissue (ct); meristematic zone (Mz), infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S) are seen in the central tissue, Bar: 250μm (40X), and **B.** Nodule cells of meristematic (Mz) and infection zone (If) showing network of infection thread (It) and occasional release of rhizobial bacteria (Rb) into the nodule cells, Bar: 50μm (200X).

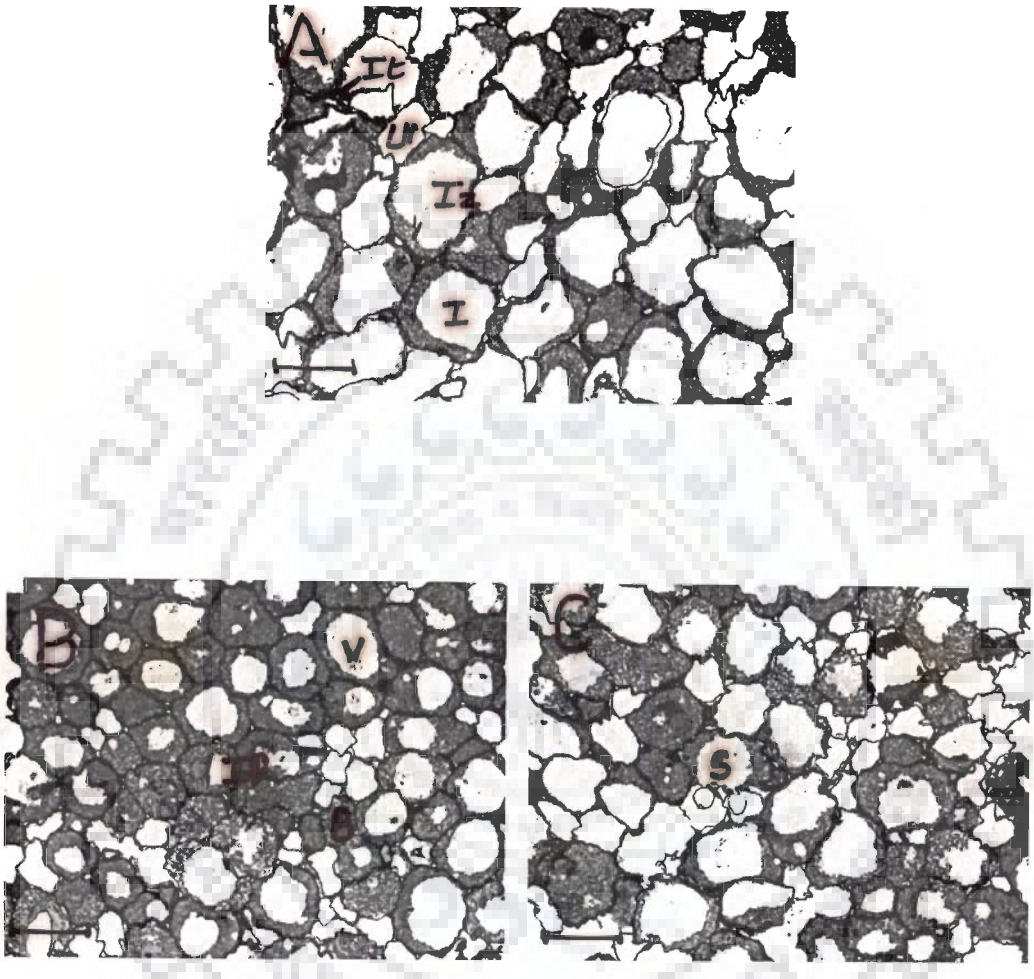

Plate 3: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A.** Nodule cells of interzone (Iz) showing infection thread (It) and infected (I), and uninfected (Ui) nodule cells, Bar: 50μm (200X), **B.** Nitrogen fixation zone showing cells packed with bacteroids (B), arranged perpendicularly to a central vacuole (V), Bar: 50μm (200X), and **C.** Few older cells showing bacterial lysis in senescence zone (S), Bar: 50μm (200X).

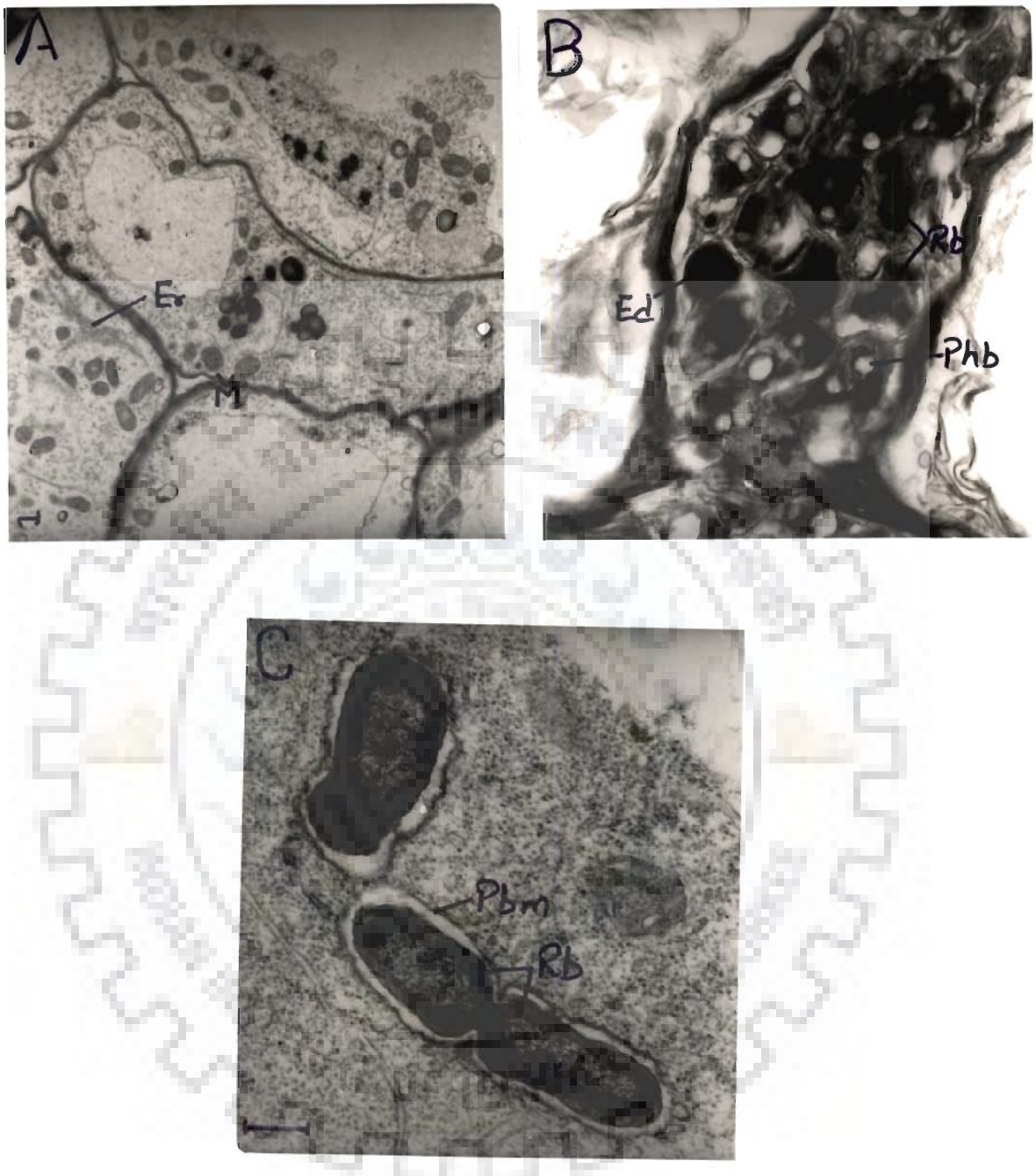

Plate 4: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by Sinorhizobium meliloti Rmd201, A. Nodule cells of meristematic zone having endoplasmic reticulum (Er) and many mitochondria (M), Bar: 1μm (1900X), B. Electron dense (Ed) rhizobial bacteria (Rb) containing poly-hydroxybutyrate granules (Phb) being released into the infection zone (If), Bar: 1μm (6200X), and C. A dividing freshly released rhizobial bacteria (Rb), in the infection zone (If), with wavy peribacteriod membrane (Pbm), Bar: 1μm (9700X).

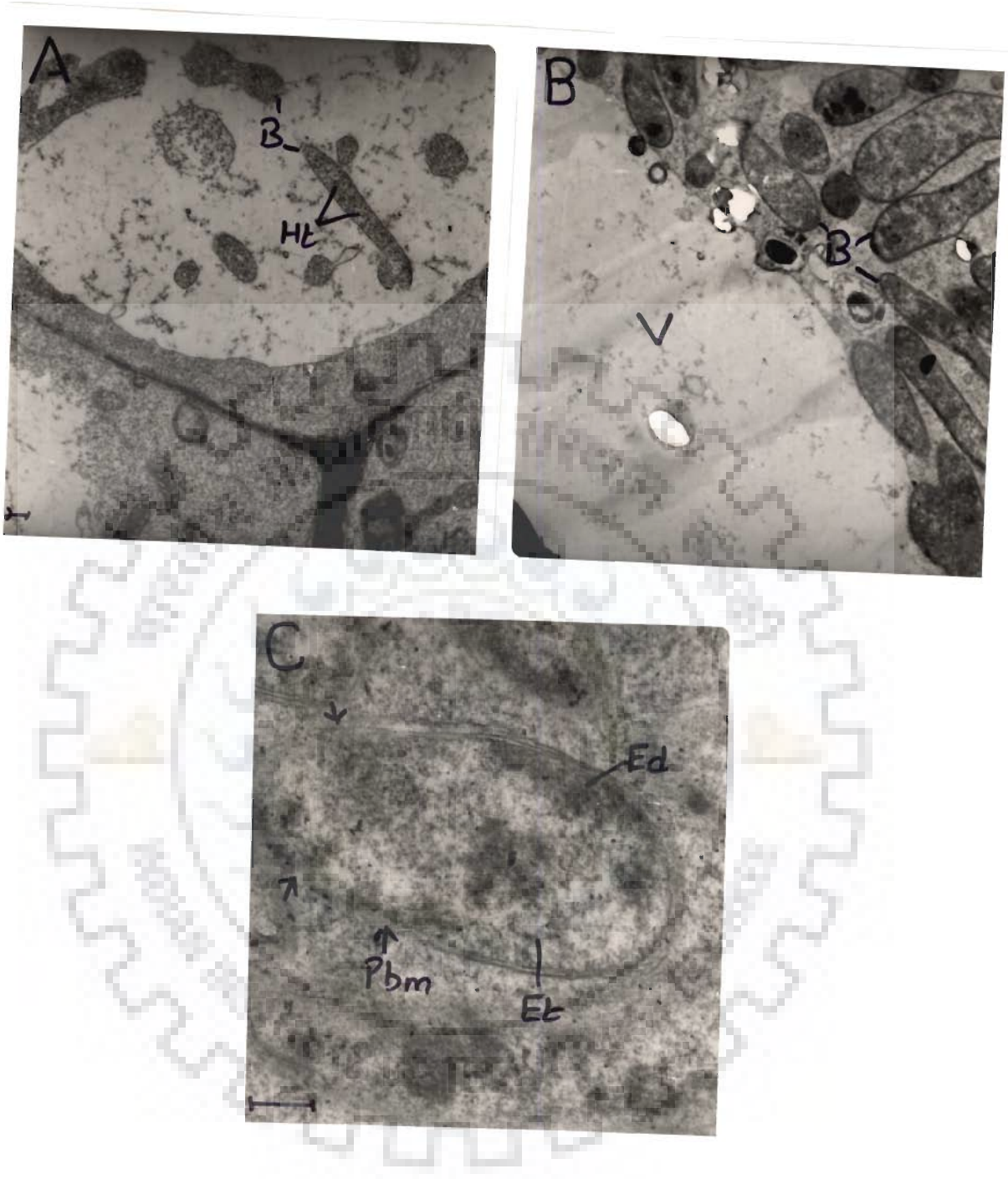

Plate 5: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by Sinorhizobium meliloti Rmd201, A. A part of a nodule cell of interzone (Iz) showing bacteroid (B) with heterogeneous cytoplasm (Ht), Bar: 1μm (3000X), B. A nodule cell of nitrogen fixation zone (Nf) with a large central vacuole (V) showing elongated bacteroid (B) with complete heterogeneity of cytoplasm, Bar: 1μm (2400X), and C. A degenerating electron transparent (Et) bacteroid (B) in the senescence zone (S), Bar: 1μm (9700X).

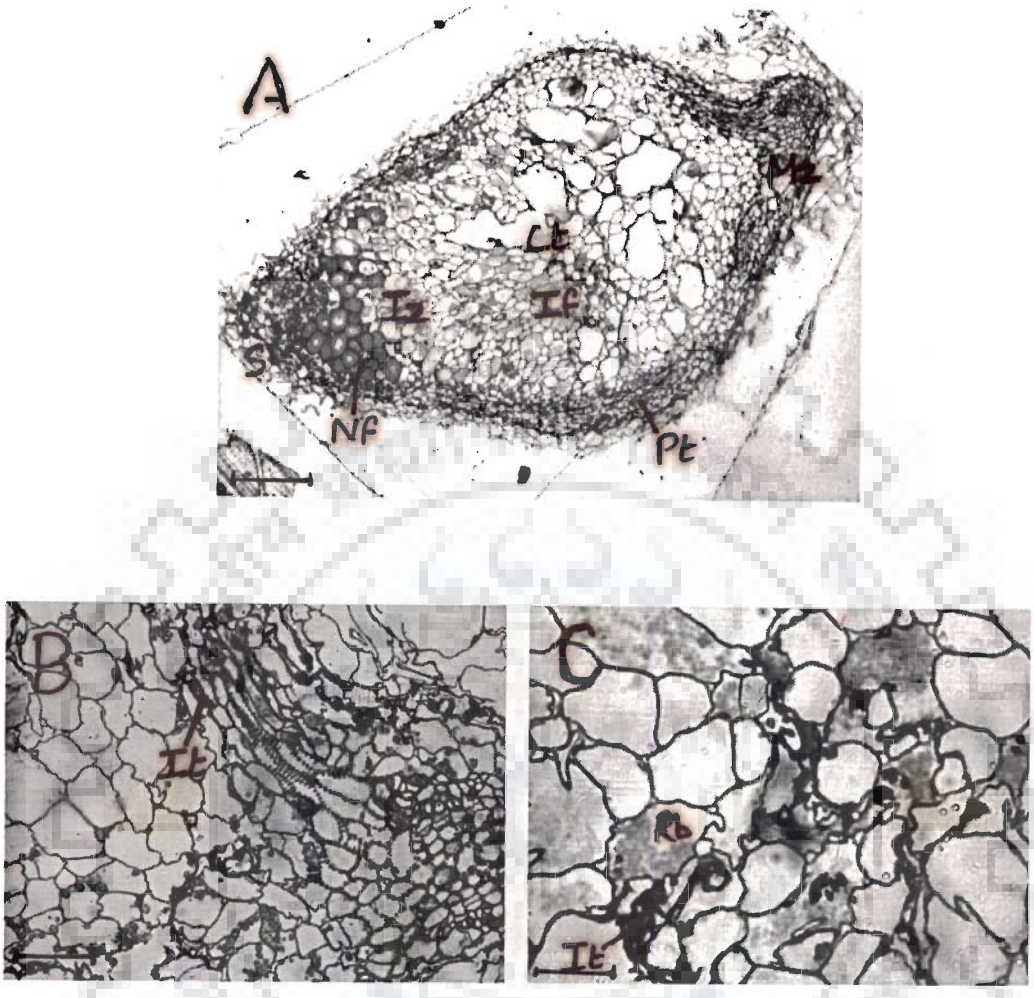

Plate 6: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK3, an *argA/argB/argC/argD/argE* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** A complete section showing distinct peripheral tissue (Pt) and central tissue (Ct); meristematic zone (Mz), extended infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S) are seen in the central tissue, Bar: 250μm (40X), **B.** A part of the meristematic zone (Mz) showing network of infection thread (It) and vascular bundle (Vb) in the peripheral tissue (Pt), Bar: 50μm (200X), and **C.** A part of the infection zone (If) showing thick infection threads (It) releasing rhizobial bacteria (Rb) into the nodule cells, Bar: 25μm (400X).

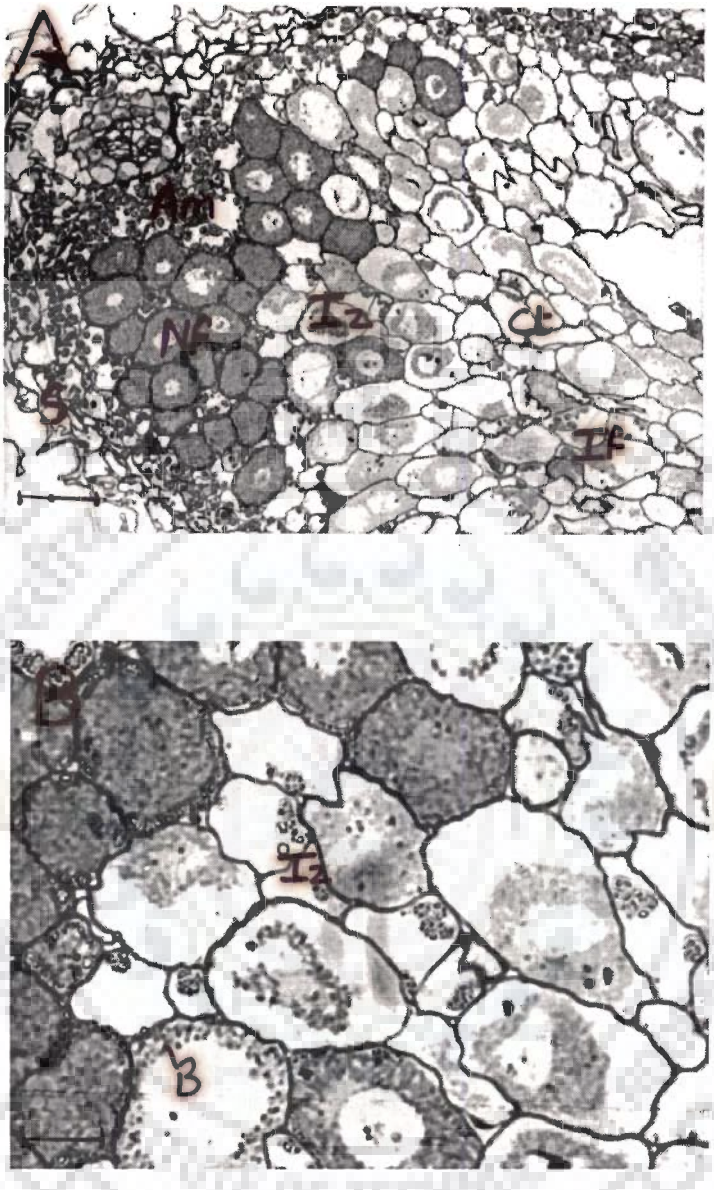

Plate 7: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK3, an *argA/argB/argC/argD/argE* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** Part of central tissue (Ct) showing infection zone (If), interzone (Iz), reduced nitrogen fixation zone (Nf) and senescence zone (S); senescence (S) and nitrogen fixation (Nf) zones are seen filled with amyloplasts (Am), Bar: 100μm (100X), **B.** A magnified part of the above section showing nitrogen fixation zone (Nf) with few cells of interzone (Iz), Bar: 25μm (400X).

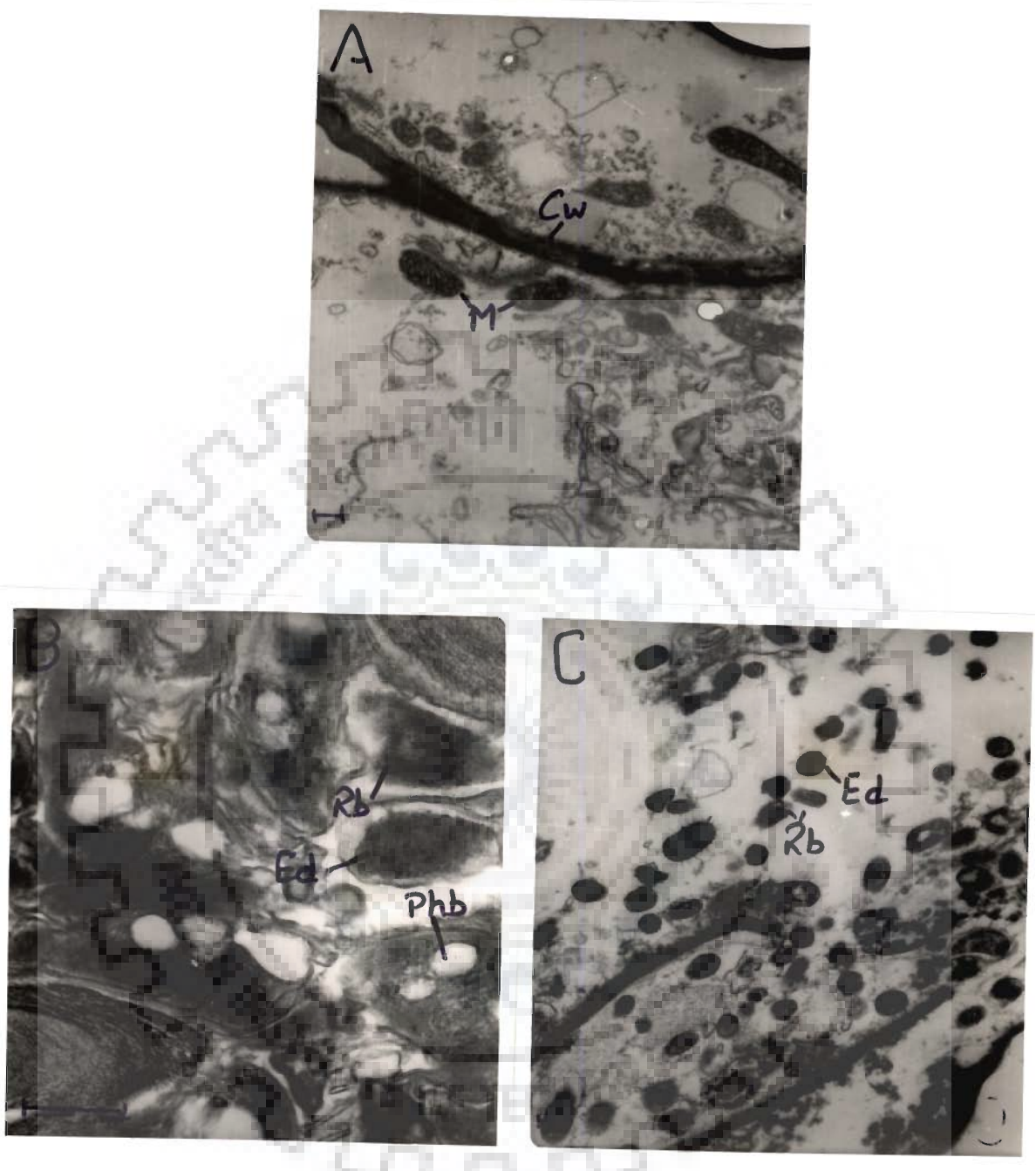

Plate 8: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK3, an argA/argB/argC/argD/argE auxotroph of Sinorhizobium meliloti Rmd201, A. A part of a cell of the meristematic zone (Mz) showing mitochondria (M); cell wall (Cw) between two cells is also seen, Bar: 1μm (3800X), B. Electron dense (Ed) rhizobial bacteria (Rb) containing poly-hydroxybutyrate granules (Phb) in the infection thread (It), Bar: 1μm (15,000X), and C. Freshly released electron dense (Ed) rhizobial bacteria (Rb) in a cell of the infection zone (If), Bar: 1μm (3000X).

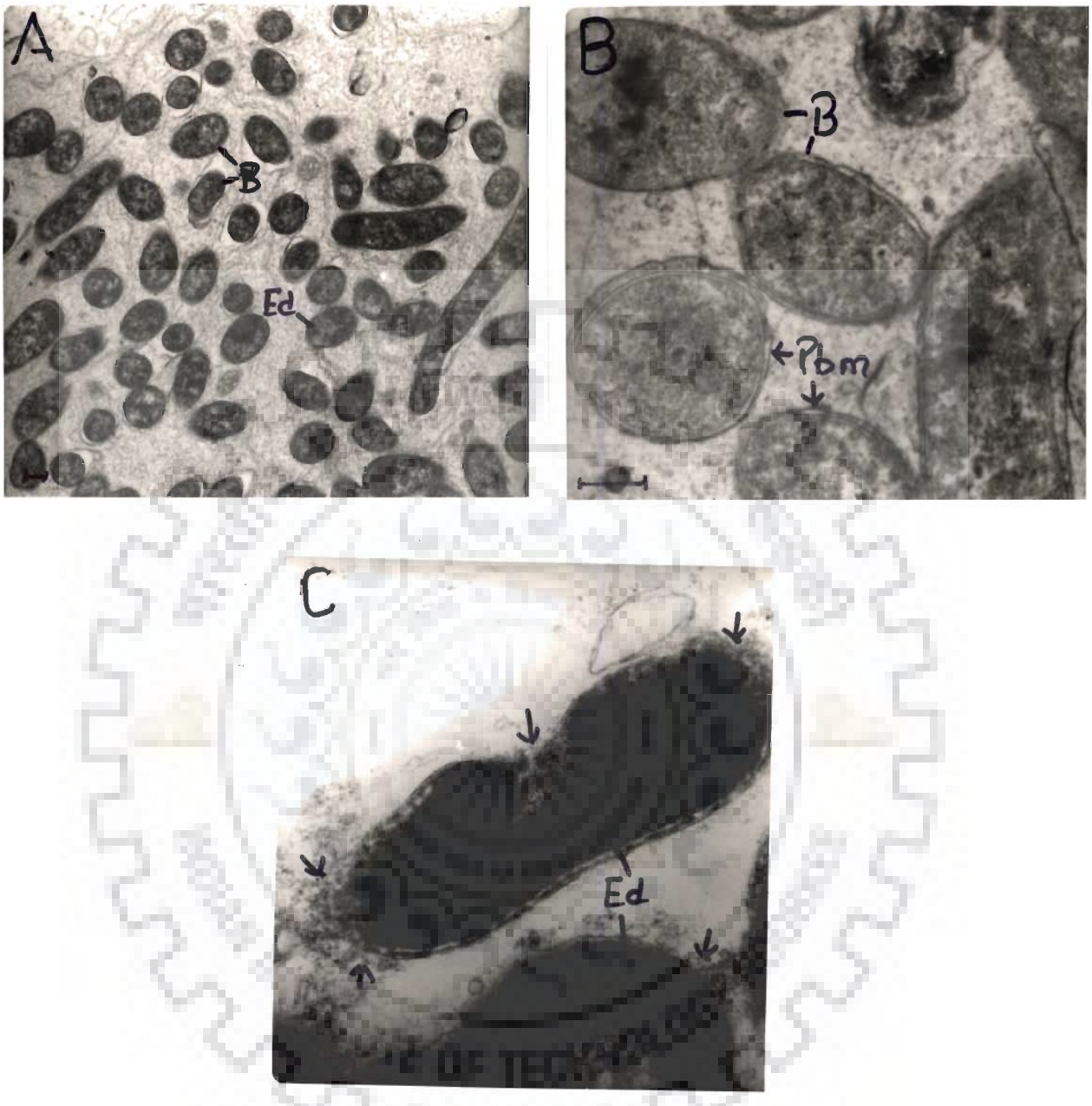

Plate 9: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK3, an argA/argB/argC/argD/argE auxotroph of Sinorhizobium meliloti Rmd201, A. A part of the cell of nitrogen fixation zone (Nf) showing electron dense (Ed) bacteriods (B), Bar: 1μm (3000X), B. A magnified portion of the above zone showing the wavy peibacteriod membrane (Pbm) surrounding the spherical bacteriods (B), Bar: 1μm (9700X), and C. A lysing electron dense (Ed) bacteroid (B) in the senescence zone (S), Bar: 1μm (9700X).

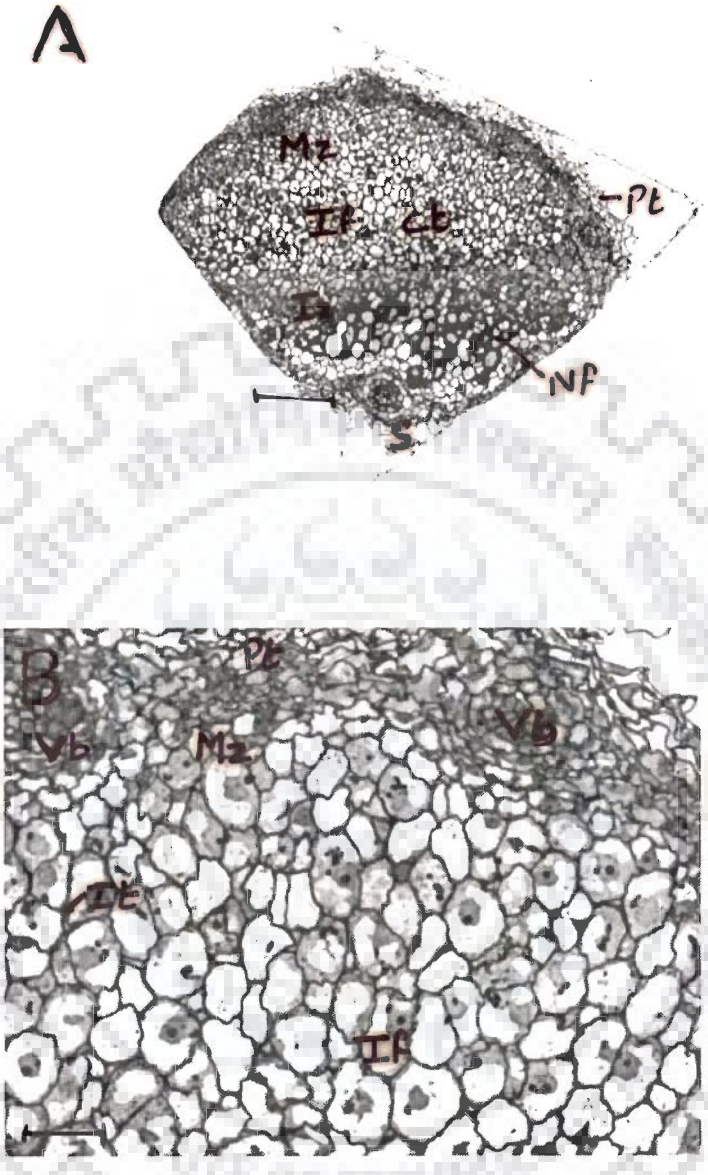

Plate 10: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK4, an *argA/argB/argC/argD/argE* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** A complete section showing peripheral tissue (Pt) and central tissue (Ct); central tissue (Ct) is divided into five zones, viz., meristematic zone (Mz), infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S), Bar: 250μm (40X), **B.** A part of the nodule cell showing vascular bundles (Vb) in the peripheral tissue (Pt) and infection threads (It) in the intercellular spaces of infection zone (If), Bar: 100μm (100X).

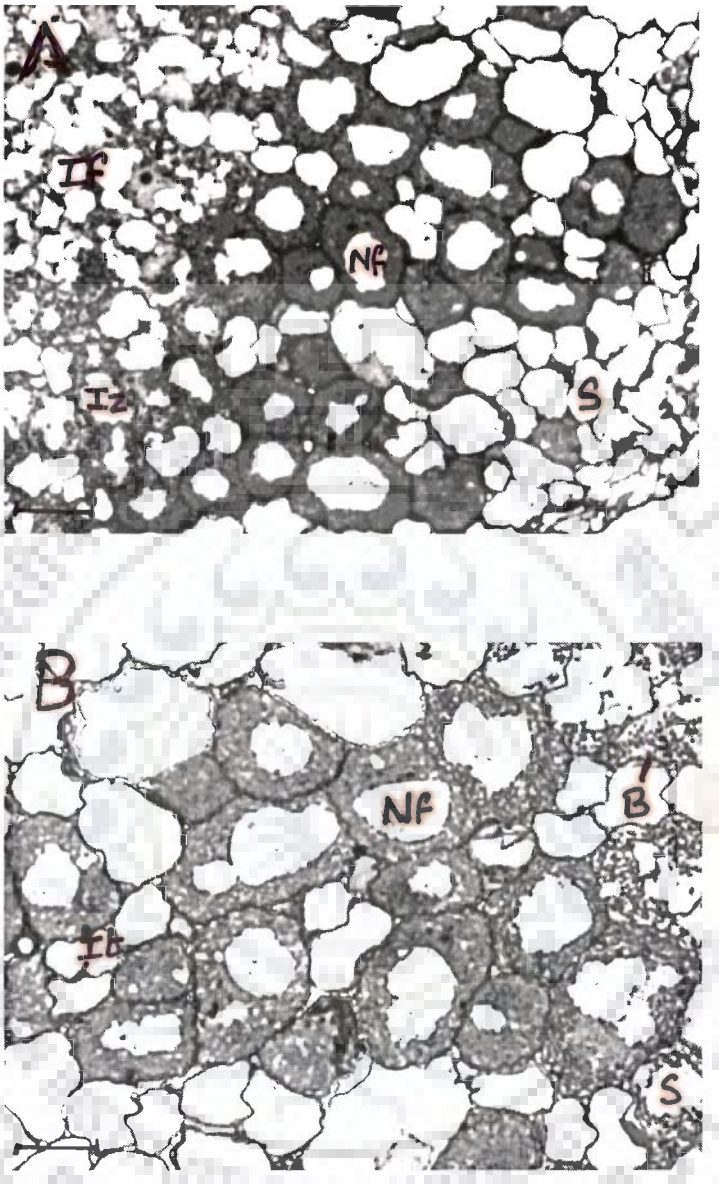

Plate 11: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK4, an *argA/argB/argC/argD/argE* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** A part of the nodule cell showing the extensive infection zone (If), few cells of interzone (Iz) and nitrogen fixation zone (Nf); cells of senescence zone (S) are also seen, Bar: 50μm (200X), **B.** A magnified portion of the above section showing infection threads (It) in the nitrogen fixation zone (Nf); lysing bacterial cells (B) are also seen in the nitrogen fixation (Nf) and senescence (S) zones, Bar: 25 μm (400X).

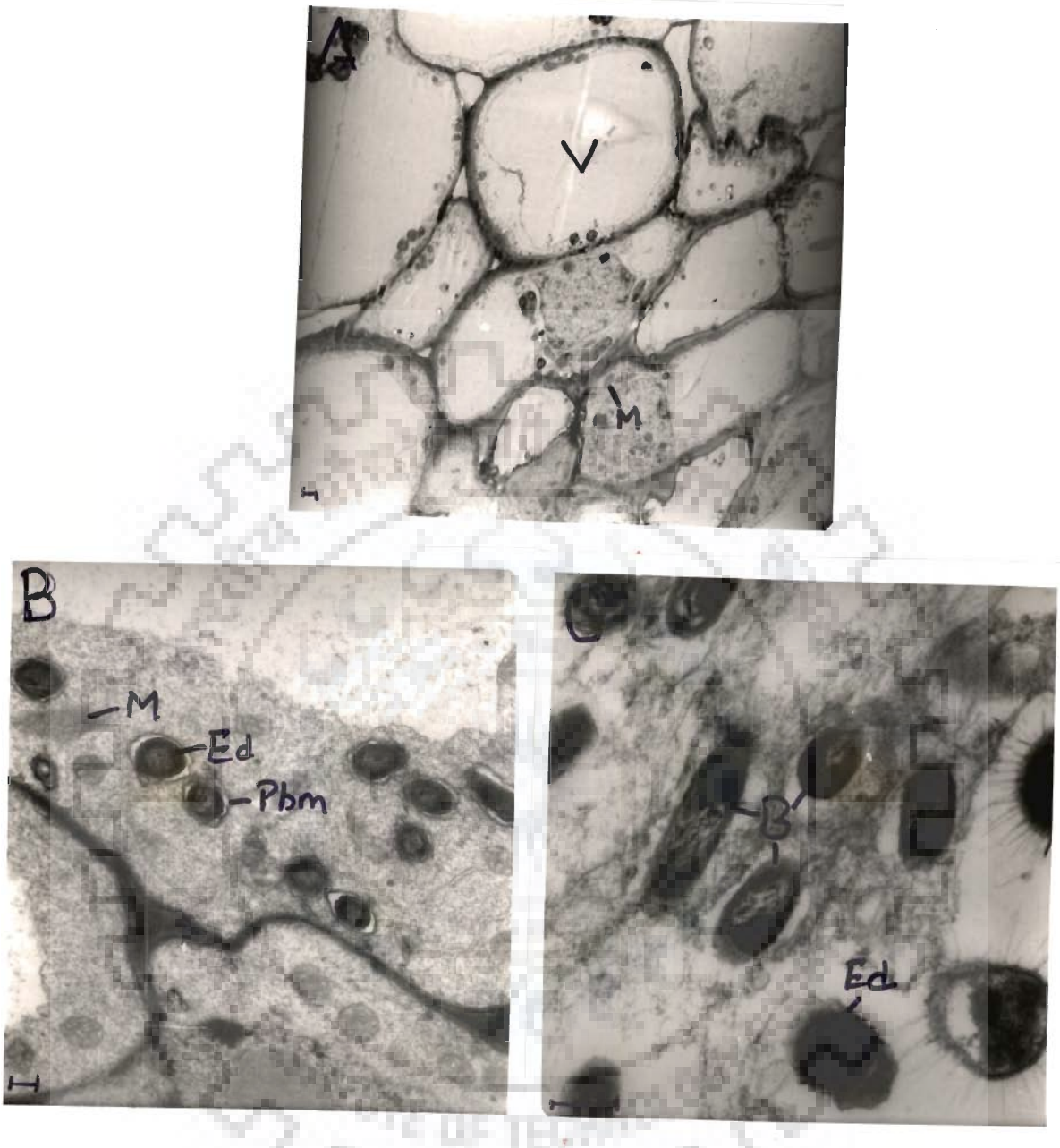

Plate 12: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK4, an argA/argB/argC/argD/argE auxotroph of Sinorhizobium meliloti Rmd201, A. A part of the meristematic zone (Mz) of the nodule having many mitochondria (M) arranged around a big central vacuole (V), Bar: 1μm (940X), B. Freshly released electron dense (Ed) rhizobial bacteria (Rb) in the infection zone (If); many mitochondria are also visible, Bar: 1μm (3800X), and C. A part of the cell of interzone (Iz) having electron dense (Ed) bacteroids (B) with wavy peribacteroid membrane (Pbm), Bar: 1μm (9700X).

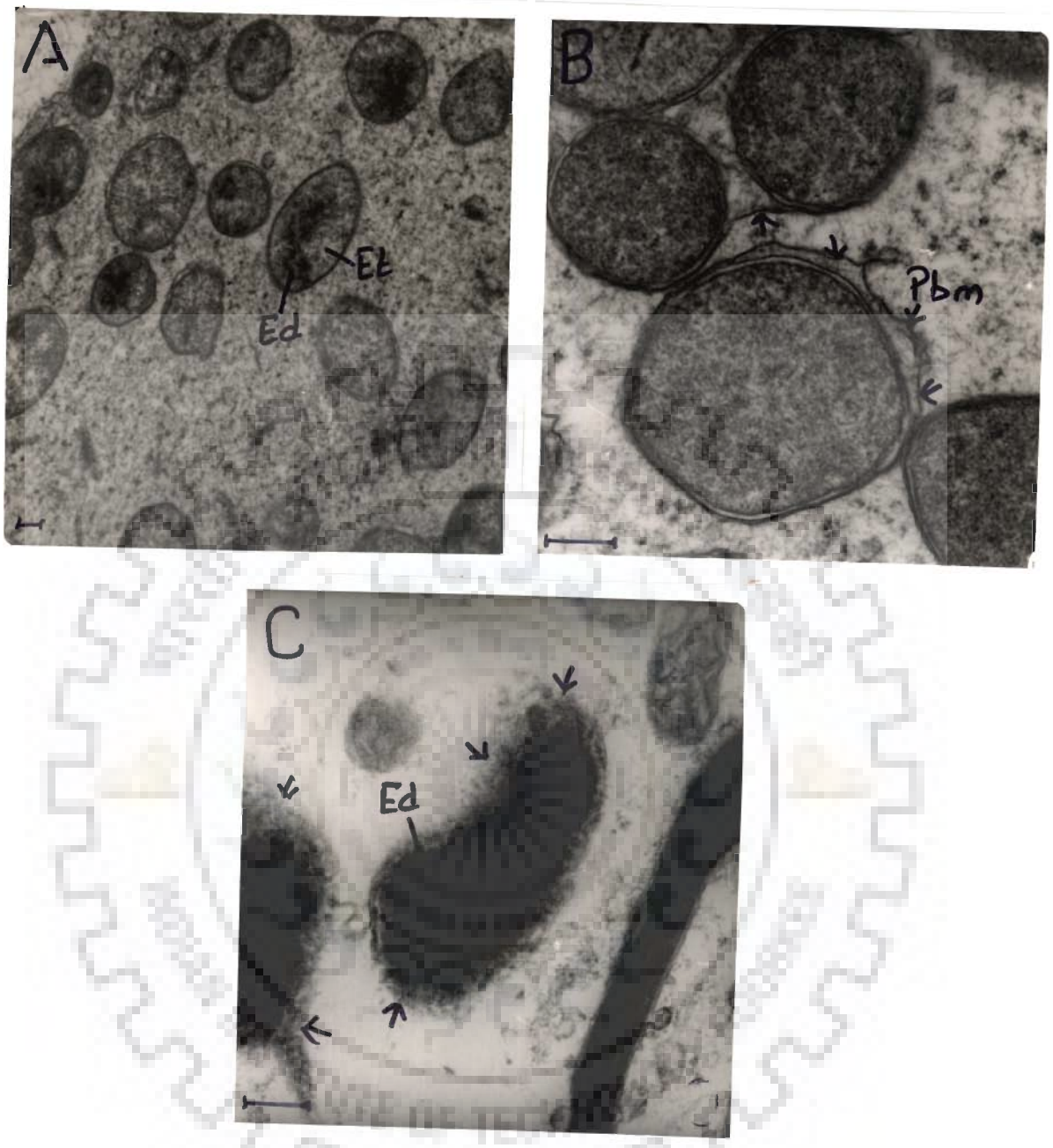

Plate 13: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK4, an argA/argB/argC/argD/argE auxotroph of Sinorhizobium meliloti Rmd201, A. Bacteroid (B) in the introgen fixation zone (Nf) having electron dense (Ed) and electron transparent (Et) regions, Bar: 1μm (3000X), B. An enlarged part of the above section showing bacteroids (B) in the nitrogen fixation zone (Nf) having loose peribacteroid membrane (Pbm), Bar: 1μm (9700X), and C. A degenerating electron dense (Ed) bacteroid (B) in the senescence zone (S), Bar: 1μm (9700X).

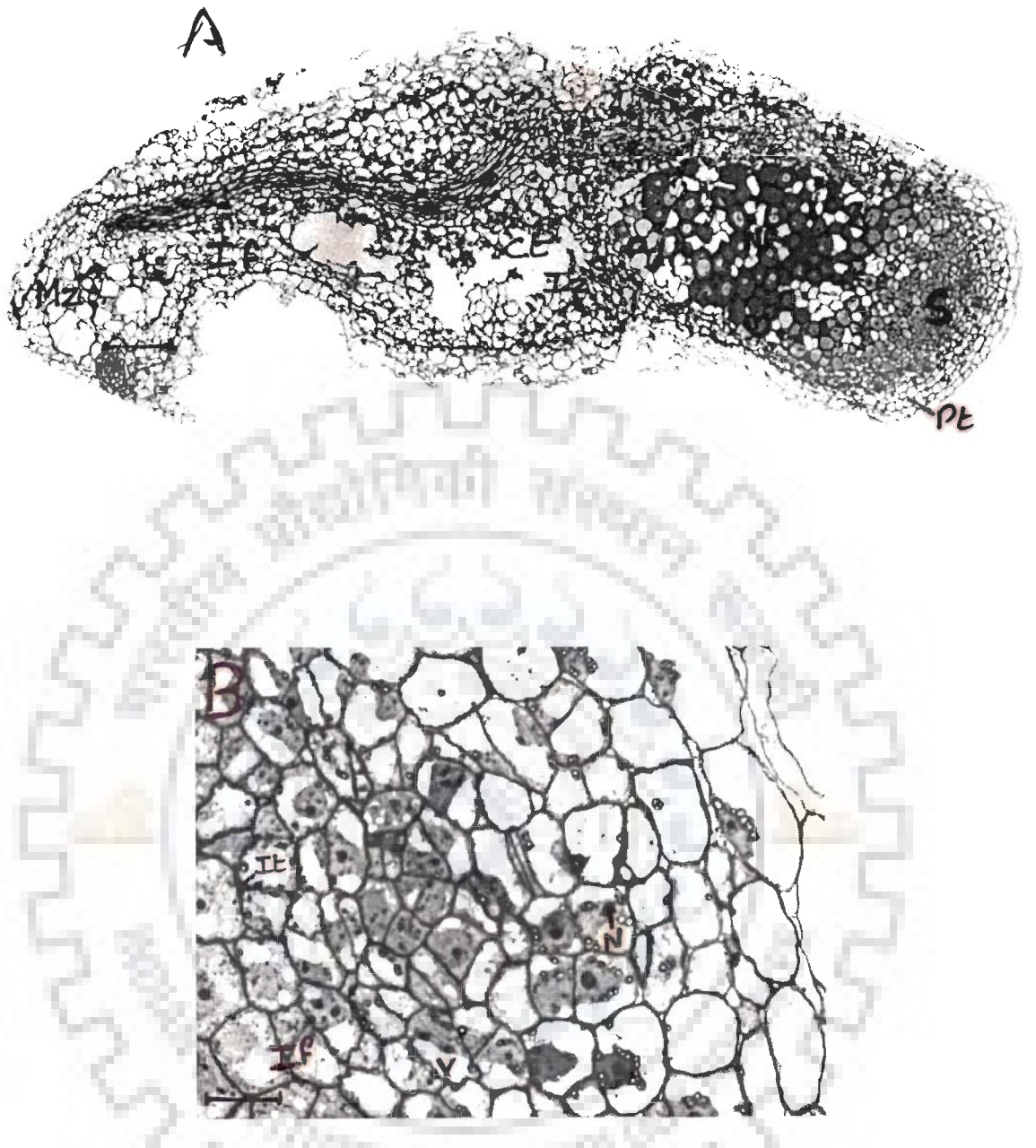

Plate 14: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK11, an *argF/argI* auxotroph of Sinorhizobium *meliloti* Rmd201, **A.** A nodule section showing distinct peripheral tissue (pt) and central tissue (ct); the central tissue (Ct) is divided into meristematic zone (Mz), infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S), Bar: 250μm (40X), and **B.** A part of the section showing infection threads (It) in cells of infection zone (If), each cell contains a big nucleus (N); vacuoles (V) are also seen, Bar: 50μm (200X).

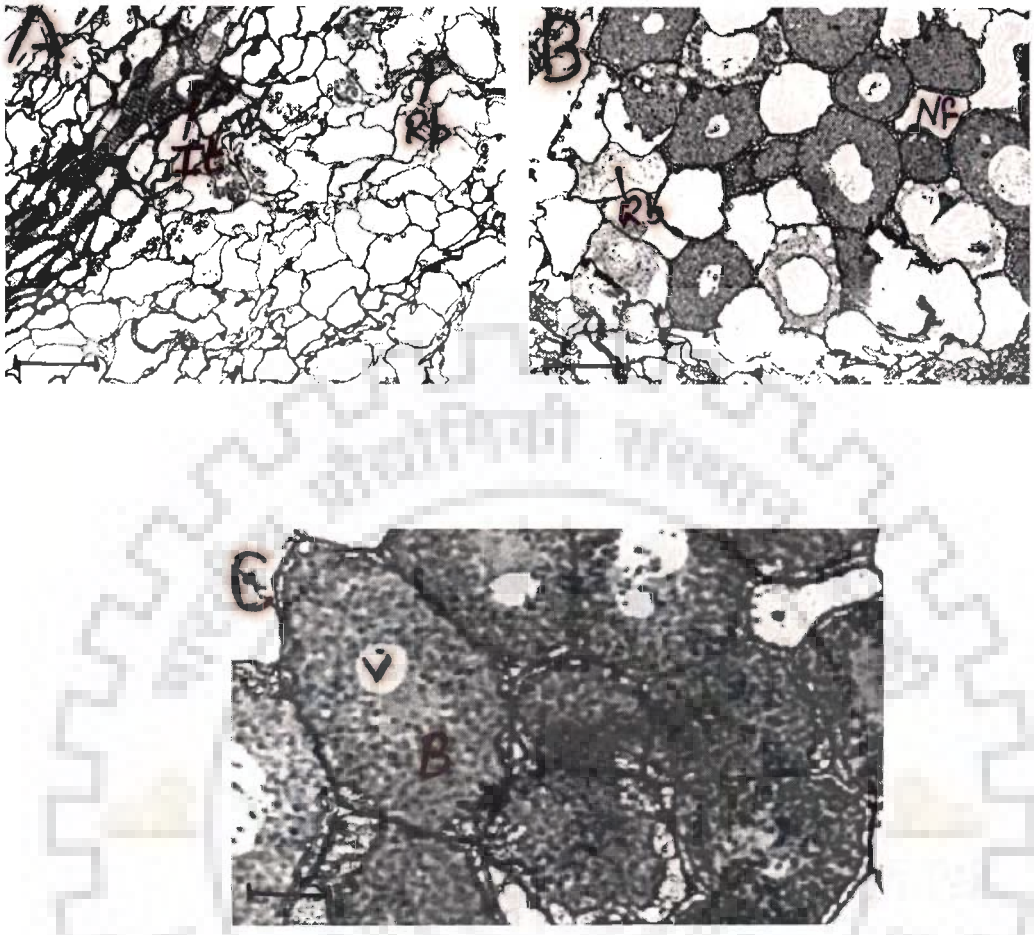

Plate 15: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK11, an *argF/argI* auxotroph of Sinorhizobium *meliloti* Rmd201, **A.** A part of the infection zone (If) showing network of infection threads (It) and release of rhizobial bacteria (Rb), Bar: 50μm (200X), **B.** A part of the section showing rhizobial bacteria (Rb) in the interzone (Iz); few cells of nitrogen fixation zone (Nf) are also seen, Bar: 50μm (200X), **C.** Magnified Cells of the nitrogen fixation zone (Nf) filled with bacteroids (B) with small vacuoles (V), Bar: 25μm (400X).

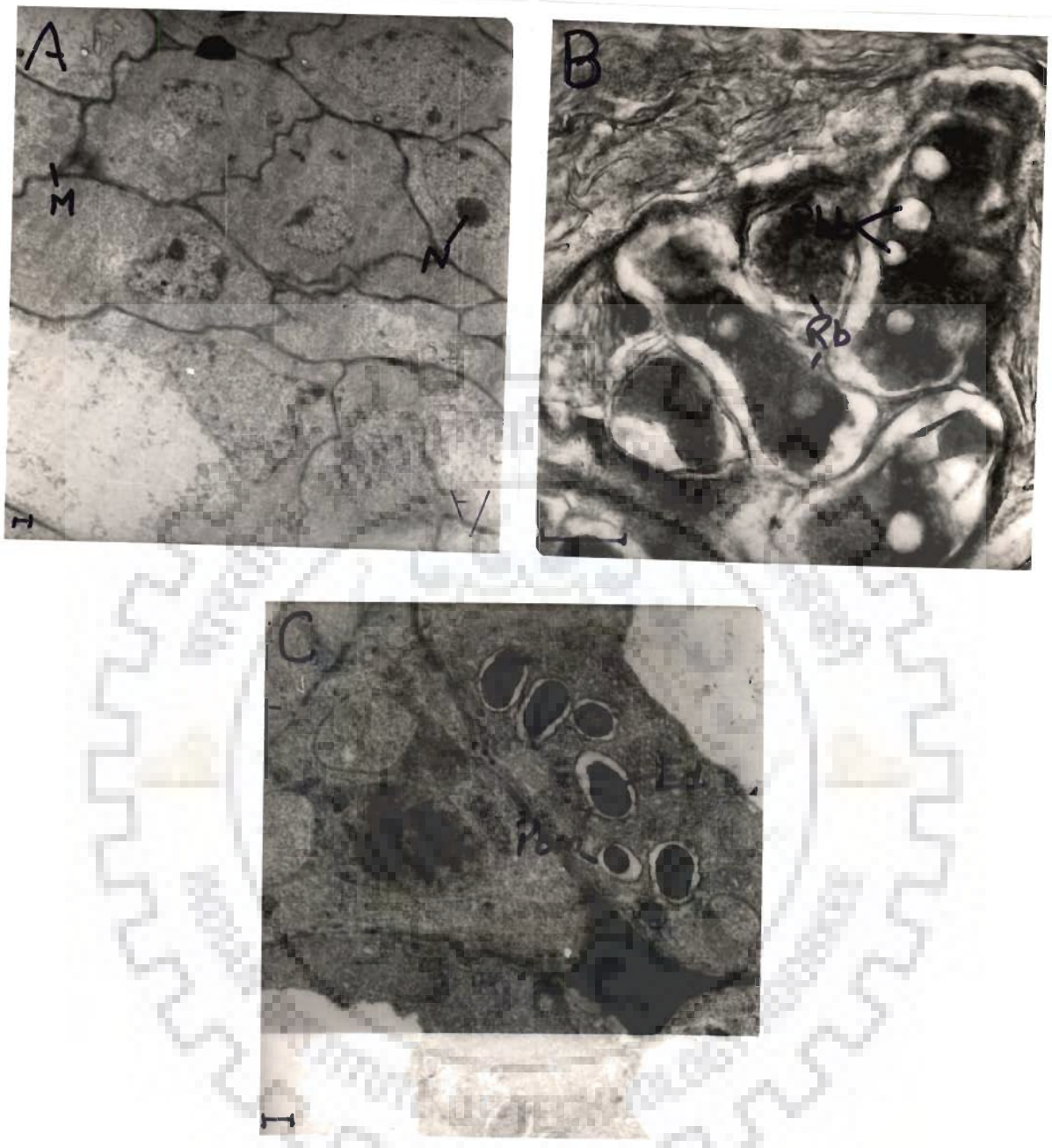

Plate 16: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK11, an argF/argI auxotroph of Sinorhizobium meliloti Rmd201, A. Cells of the meristematic zone (Mz) having many mitochondria (M) and big nuclei, Bar: 1μm (1900X), B. Rhizobial bacteria (Rb), having polyhydroxy butyrate granules (Phb) being released in the infection zone (If), Bar: 1μm (12,000X), and C. Freshly released rhizobial bacteria (Rb) with wavy peribacteroid membrane (Pbm) in the infection zone (If); intercellular space is filled with electron dense (Ed) material, Bar: 1μm (3800X).

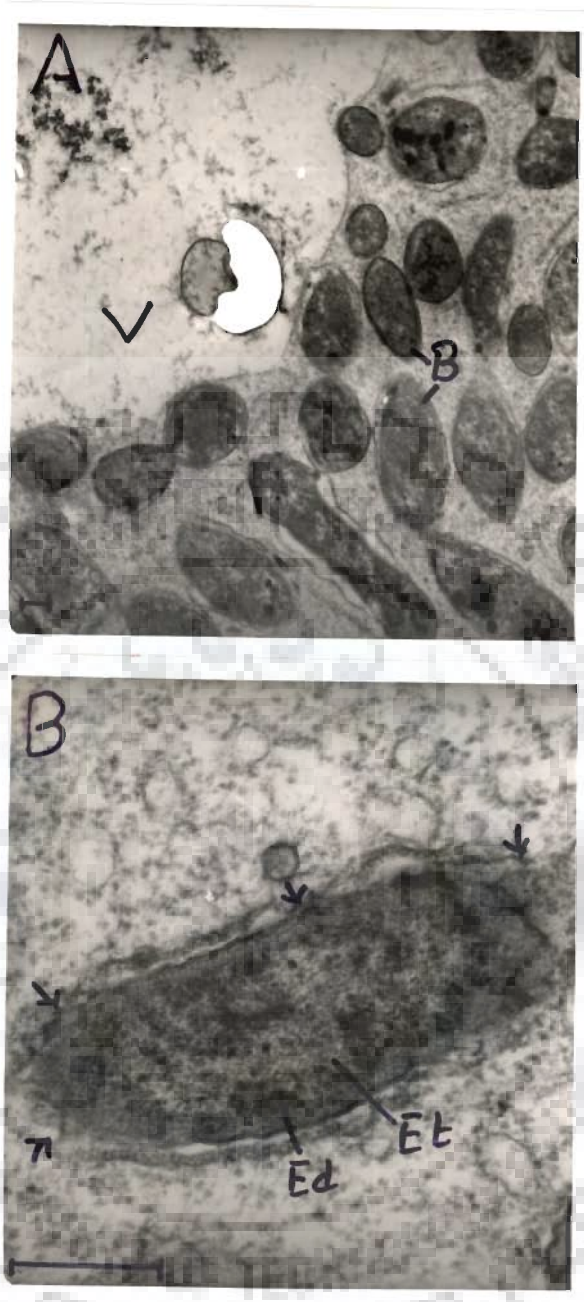

Plate 17: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK11, an argF/argI auxotroph of Sinorhizobium meliloti Rmd201, A. Bacteroids (B) arranged around a central vacuole (V) in the nitrogen fixation zone (Nf); please note the heterogeneity in the shapes of the bacteroids, Bar: 1μm (3000X), and B. A lysing electron transparent (Et) bacteroid (B) in the senescence zone (S), Bar: 1μm (19,000X).

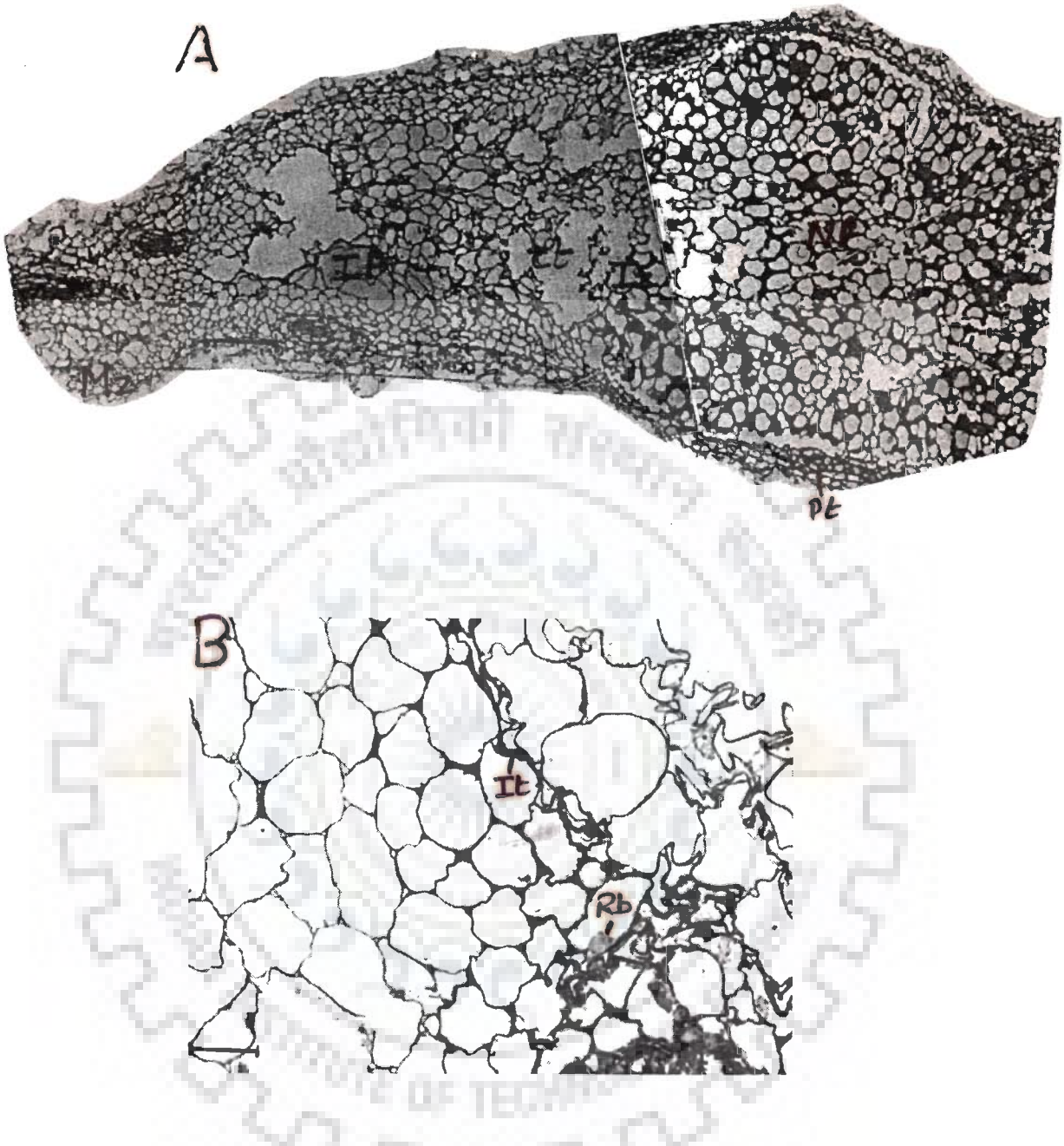

Plate 18: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK12, an *argG* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** A nodule section showing distinct peripheral tissue (pt) and central tissue (ct); meristematic zone (Mz), infection zone (If), interzone (Iz) and nitrogen fixation zone (Nf) are seen in the central tissue, Bar: 250μm (40X), and **B.** A part of the meristematic zone (Mz) showing entry and net work of infection threads (It) and the occasional release of rhizobial bacteria (Rb) in cells of infection zone (If), Bar: 25μm (400X).

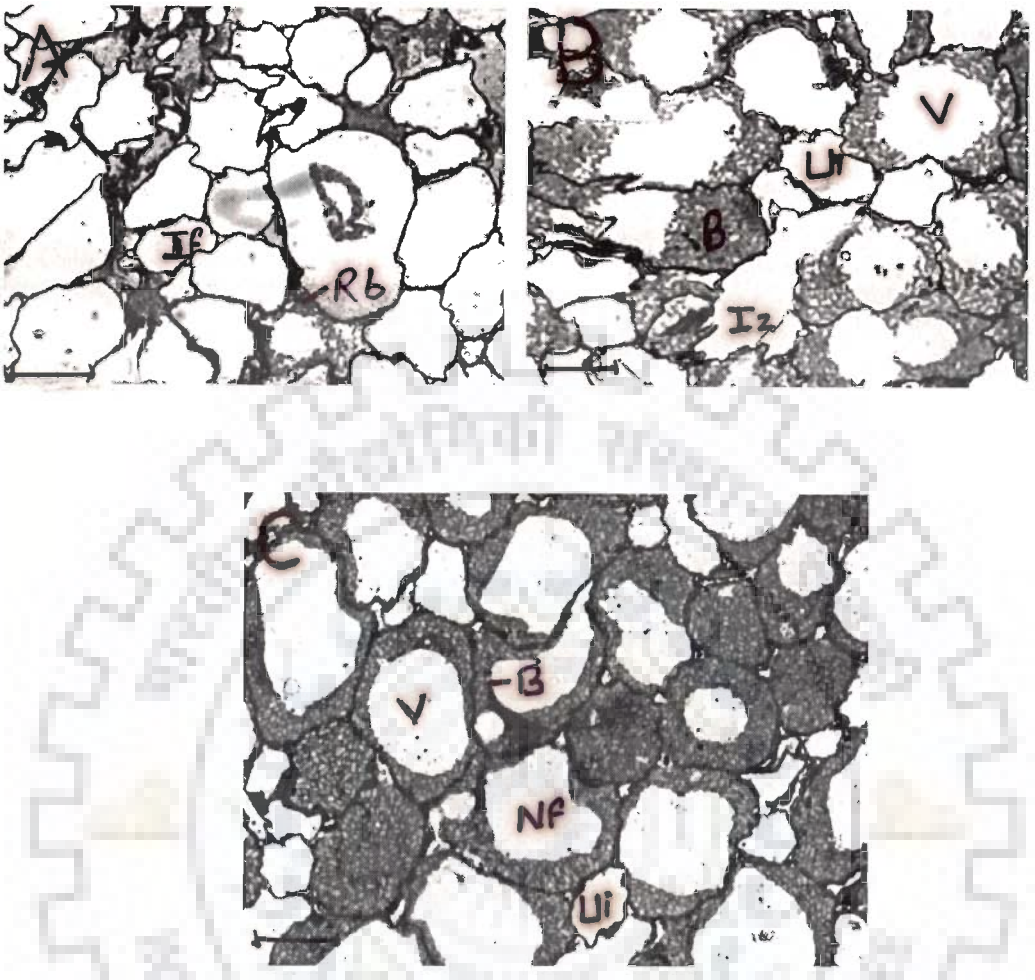

Plate 19: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK12, an *argG* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** Release of rhizobial bacteria (Rb) in cells of Infection zone (If), Bar: 25μm (400X), **B.** Freshly released bacteroids (B) getting arranged around a central vacuole (V) in the interzone (Iz), Bar: 25μm (400X), **C.** Bacteroids (B) arranged perpendicularly around a large central vacuole (V) in the nitrogen fixation zone (Nf); few uninfected cells (Ui) are also seen, Bar: 25μm (400X).

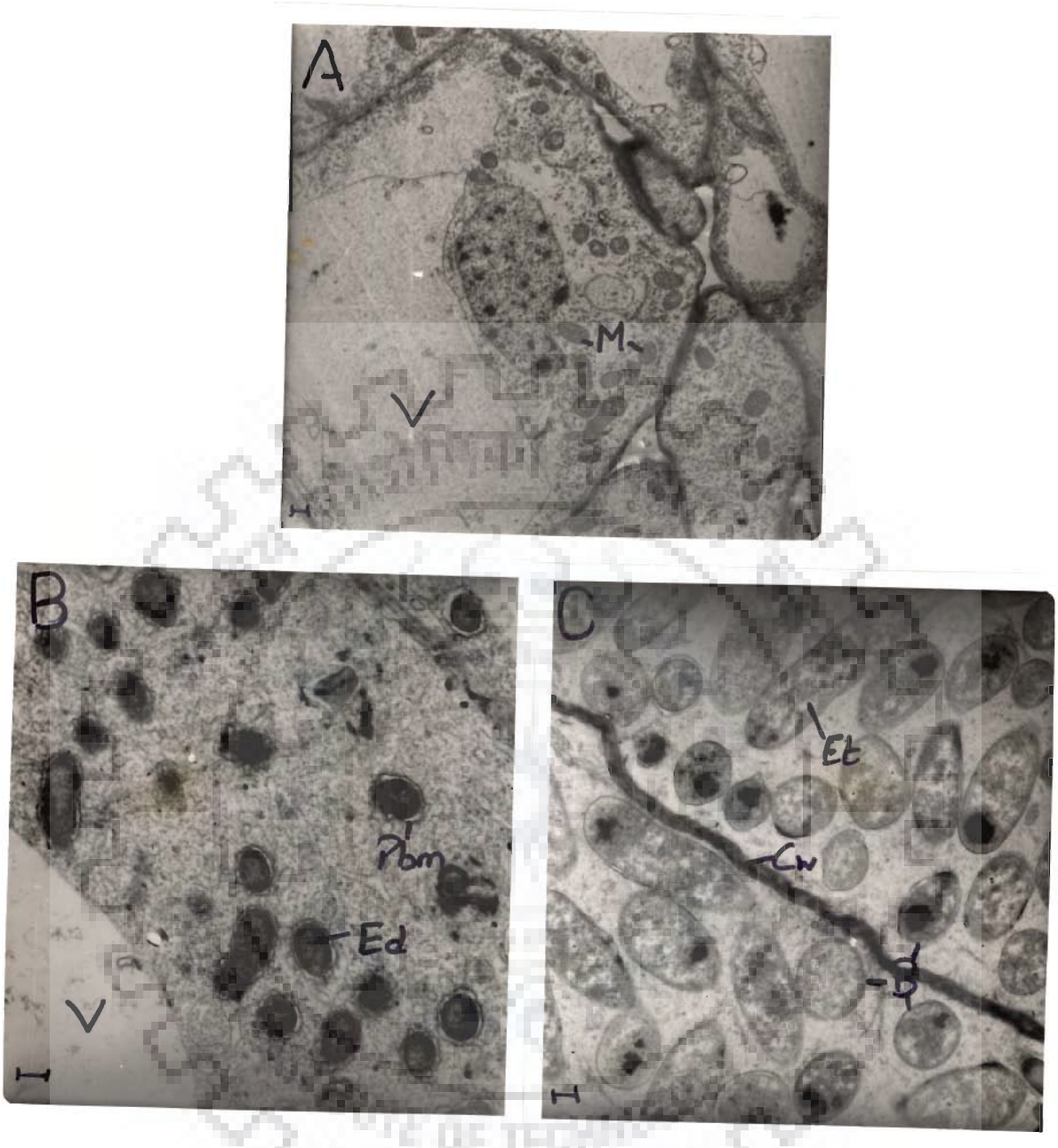

Plate 20: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK12, an argG auxotroph of Sinorhizobium meliloti Rmd201, A. Cells of the meristematic zone (Mz) showing mitochondria and central vacoule (V), Bar: 1μm (1900X), B. Electron dense (Ed) Rhizobial bacteria (Rb) in the infection zone (If) having wavy peribacteroid membrane (Pmb), Bar: 1μm (3800X), and C. Electron transparent (Et) bacteroids (B) of different shapes in the inter zone (Iz), Bar: 1μm (3000X).

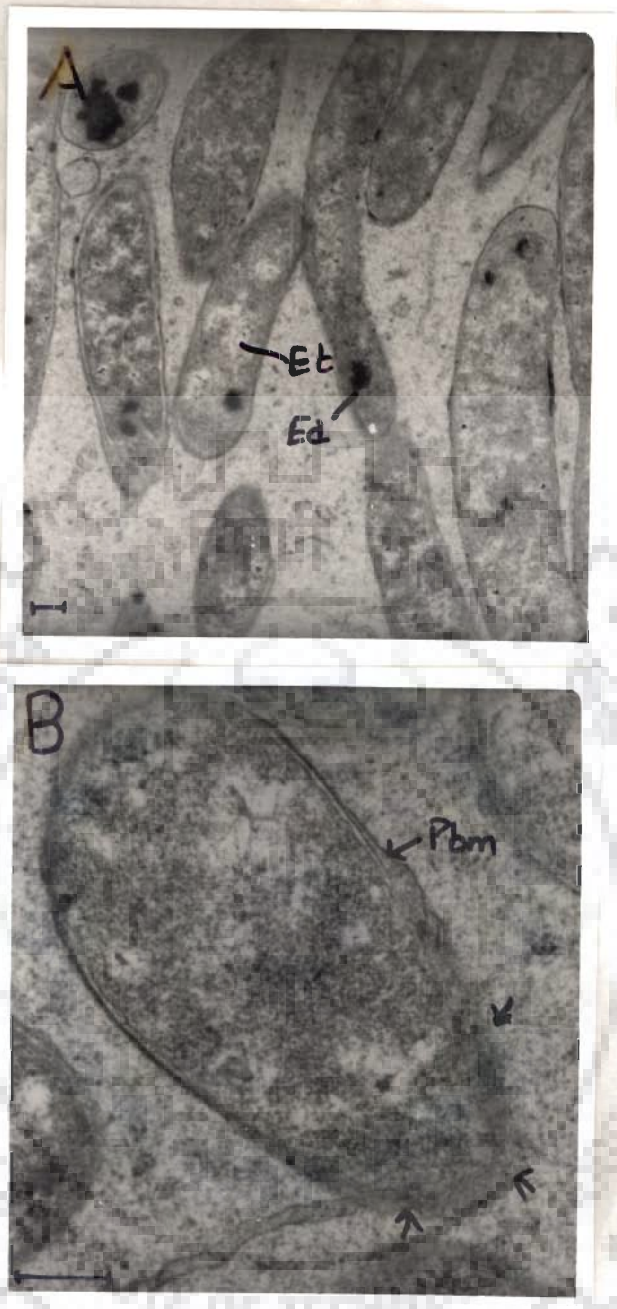

Plate 21: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK12, an argG auxotroph of Sinorhizobium meliloti Rmd201, A. Elongated bacteroids (B) having heterogeneous cytoplasm (Ht) seen in the nitrogen fixation zone (Nf), Bar: 1μm (3800X), B. A senescing bacteroid (B), with broken peribacteroid membrane (Pbm) in the senescence zone (S), Bar: 1μm (12,000X).

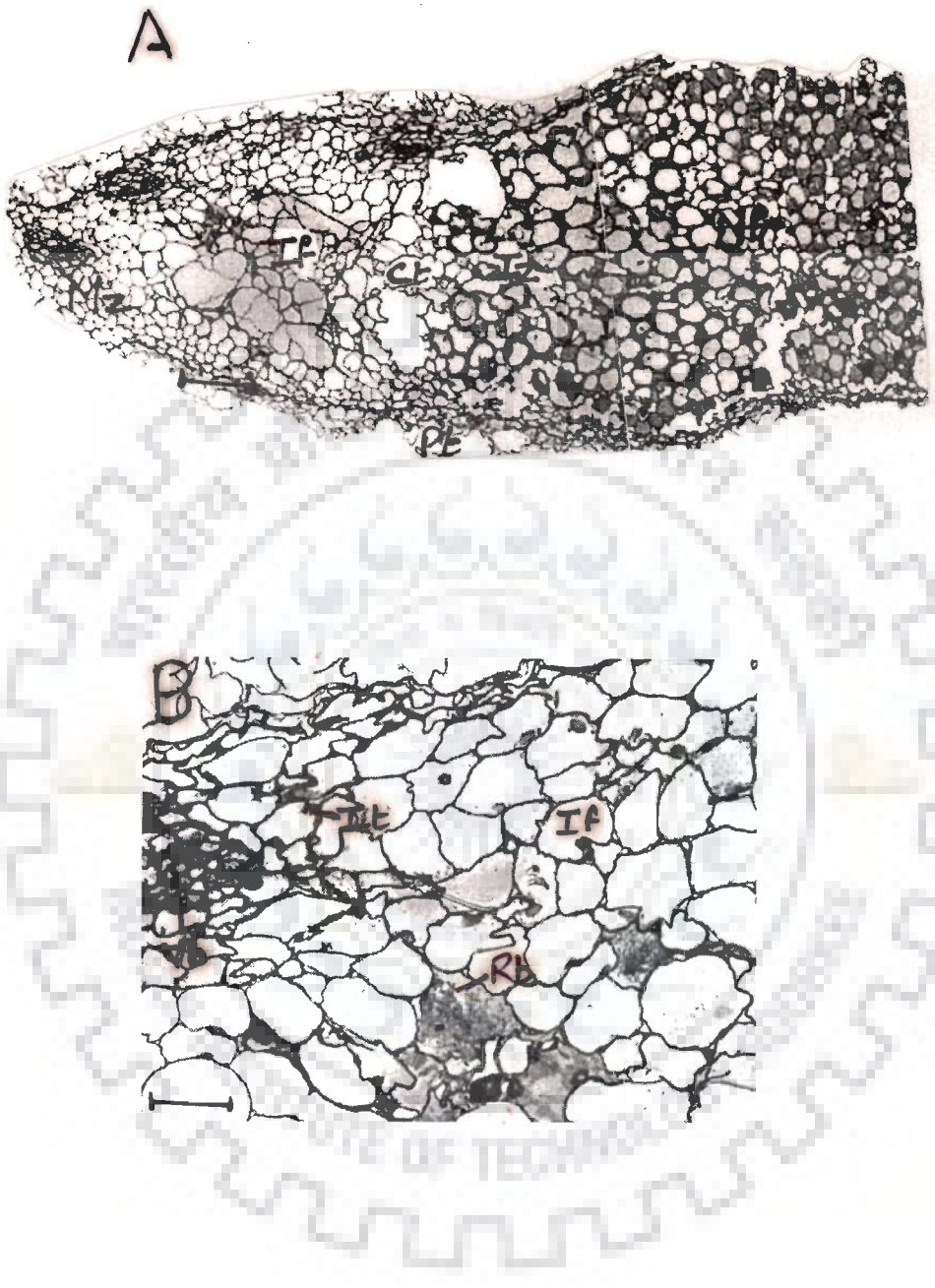

Plate 22: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK9, an *argH* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** A complete nodule section showing distinct peripheral tissue (pt) and central tissue (ct); meristematic zone (Mz), infection zone (If), interzone (Iz) and nitrogen fixation zone (Nf) are seen in the central tissue, Bar: 250μm (40X), **B.** Rhizobial bacteria (Rb) being released into the cells of Infection zone (If); vascular bundle (Vb) in the peripheral tissue (Pt) and infection threads (It) are seen, Bar: 50μm (200X).

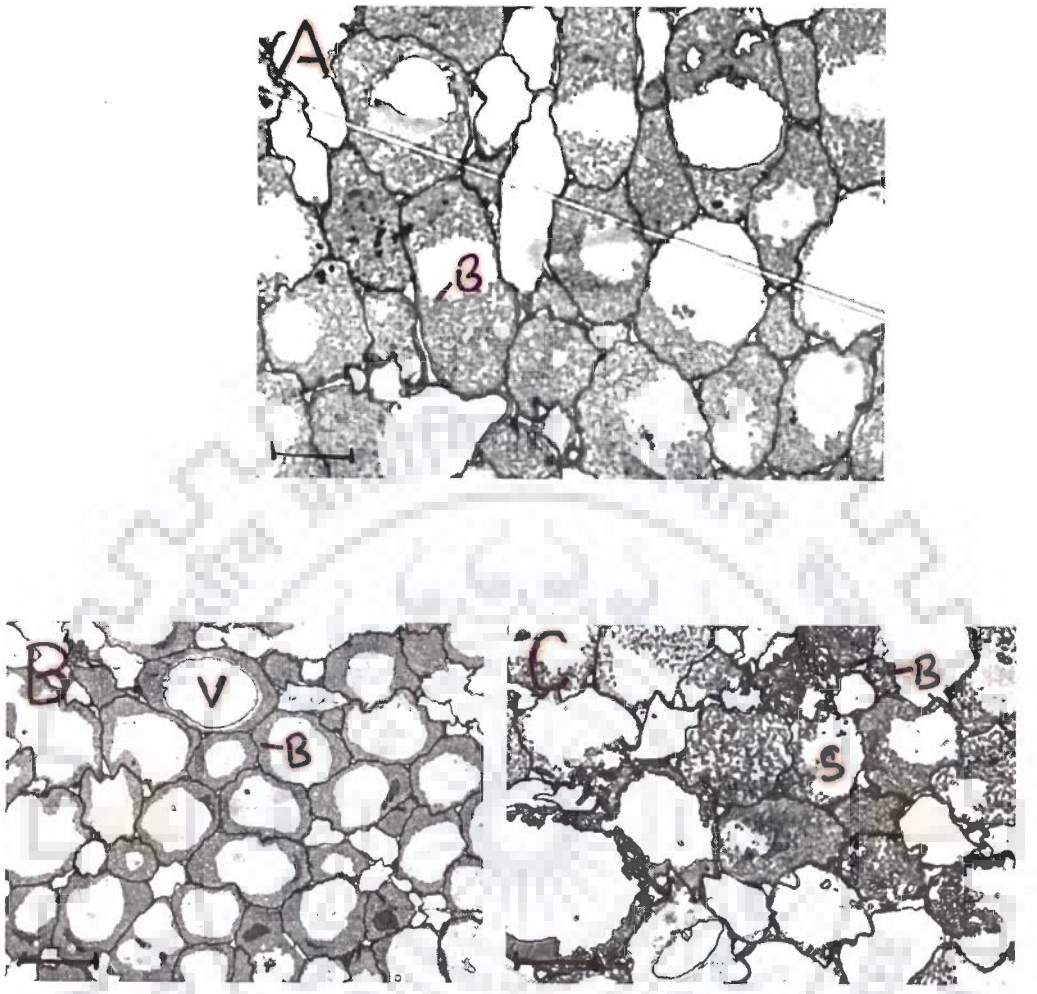

Plate 23: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by AK9, an *argH* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** Interzonal (Iz) cells filled with freshly released bacteroids (B), Bar: 25μm (400X), **B.** Cells of the nitrogen fixation zone (Nf) showing perpendicularly arranged mature bacteroids (B) around a large central vacuole (V) Bar: 25μm (400X), **C.** Lysing of older bacteroids (B) in the senescence zone (S), Bar: 25μm (400X).

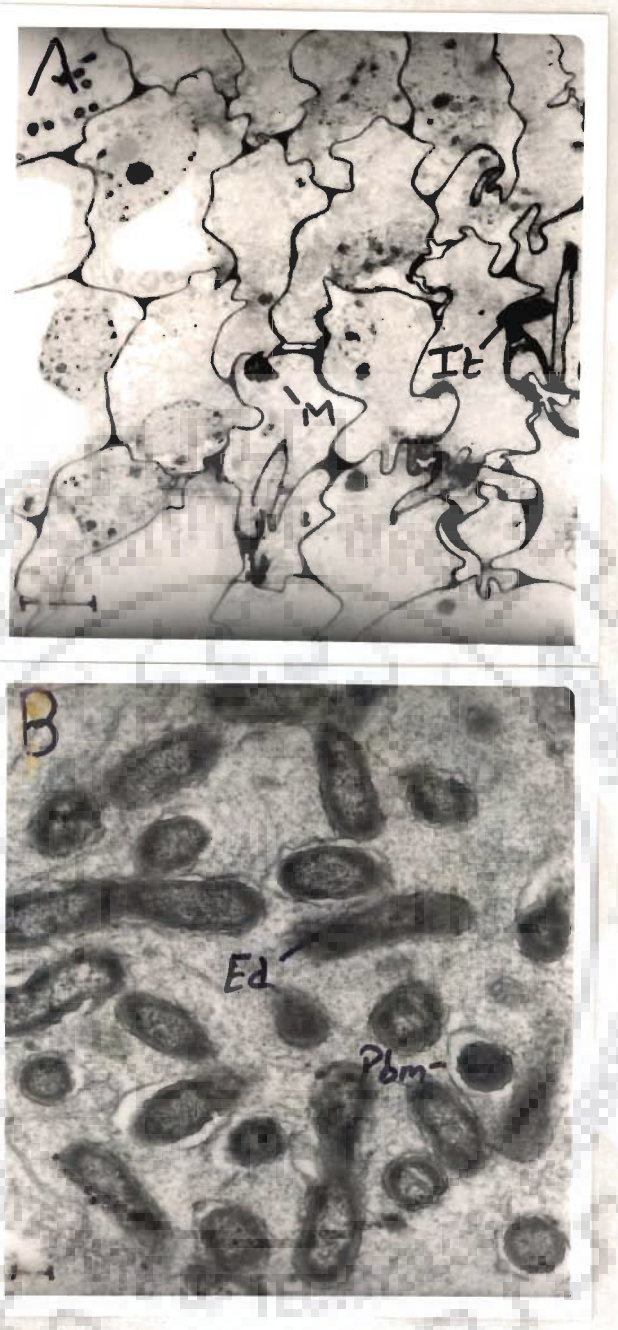

Plate 24: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK9, an argH auxotroph of Sinorhizobium meliloti Rmd201, A. Cells of the infection zone (If) showing infection threads (It); few mitochondria (M) are also seen, Bar: 1μm (9400X), B. Freshly released electron dense (Ed) rhizobial bacteria (Rb) having wavy peribacteroid membrane (Pbm), Bar: 1μm (4800X).

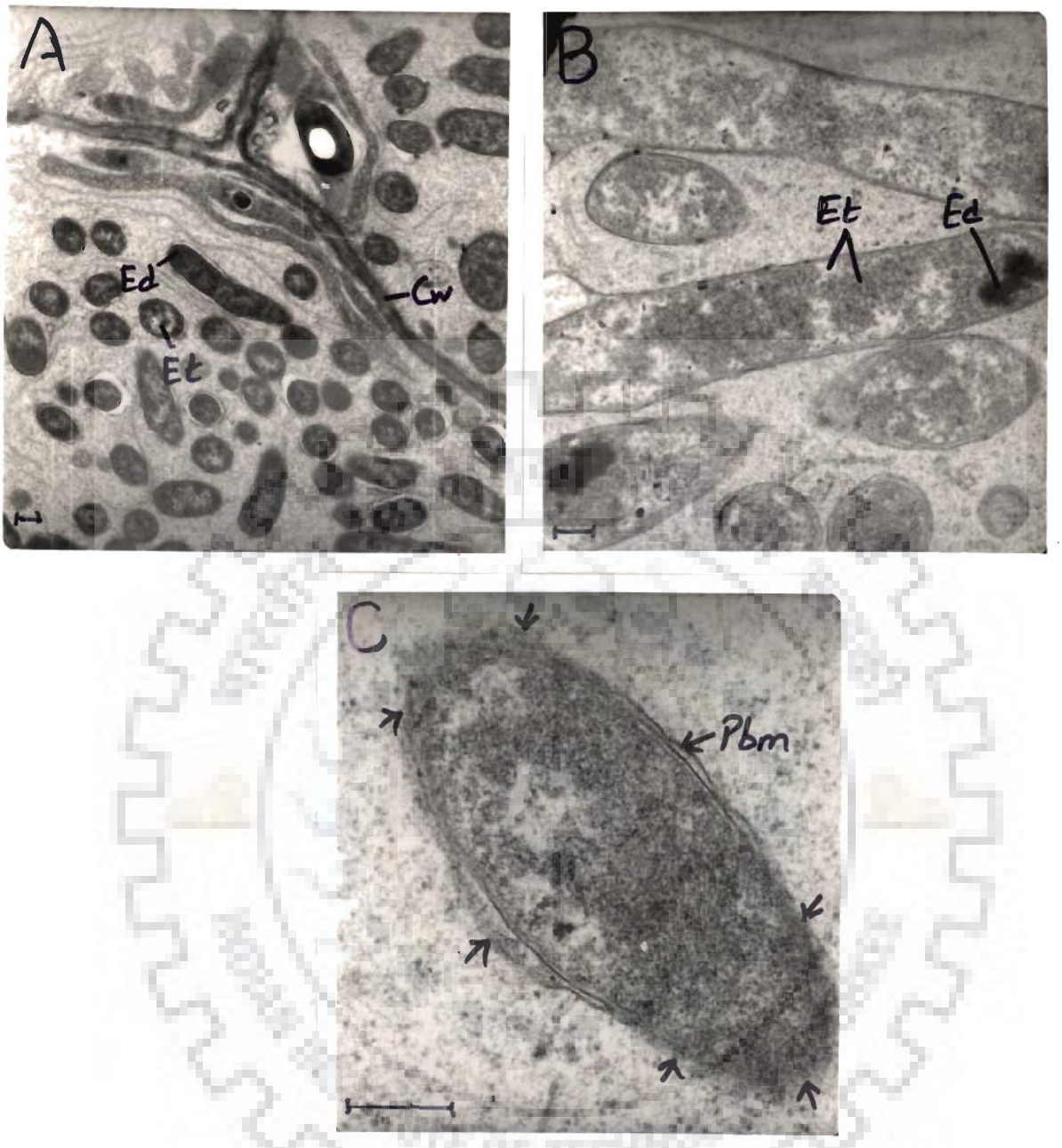

Plate 25: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by AK9, an argH auxotroph of Sinorhizobium meliloti Rmd201, A. Bacteroids (B) in the interzone (Iz) region showing decreased eletron density and variable shapes, Bar: 1μm (3000X), B. Elongated bacteroids (B) in the nitrogen fixation zone (Nf) having electron dense (Ed) and electron transparent (Et) regions, Bar: 1μm (4800X), and C. A lysing bacteroid (B) in the senescence zone (S) showing release of bacterial contents, Bar: 1μm (15,000X).

5. DISCUSSION

Sinorhizobium meliloti bacteria fix atmospheric dinitrogen in symbiotic association with alfalfa (*Medicago sativa*). The role of the arginine biosynthetic pathway of *S. meliloti* in symbiosis was studied by isolation and symbiotic characterization of transposon Tn5 induced arginine auxotrophs of *S. meliloti* strain Rmd201. *S. meliloti* Rmd201 strain was selected for the present study since this is a derivative of the genetically well-characterized *S. meliloti* strain Rm41.

Random mutagenesis of *S. meliloti* Rmd201 with transposon Tn5 was carried out using suicide delivery vector pGS9. A total of 7,650 Tn5 induced derivatives of Rmd201 were obtained from 128 crosses of *S. meliloti* Rmd201 strain with *E. coli* WA803(pGS9) strain. The screening of these derivatives yielded 32 auxotrophs. The frequency of occurrence of auxotrophs among Tn5 derivatives was 0.42%. This frequency was similar to the frequencies of occurrence of auxotrophs among Tn5 derivatives of rhizobia reported in previous studies (Meade *et al.,* 1982; Forrai *et al.*, 1983; Kim *et al.*, 1988; Prasad *et al.*, 2000; Vineetha *et al.*, 2001; Hassani *et al.*, 2001; Abbas *et al.*, 2002).

The nutritional requirements of the 32 isolated auxotrophs were arginine, methionine, tryptophan, cysteine, uracil and adenine. The diverse nutritional requirements of the isolated auxotrophs indicated randomness of Tn5 insertions in the *S. meliloti* genome as has been reported in different rhizobial species by

previous workers (Forrai *et al.*, 1983; Prasad *et al.*, 2000; Vineetha *et al.*, 2001; Hassani *et al.*, 2001; Abbas *et al.*, 2002).

Fifteen arginine auxotrophs were selected for further studies. On the basis of intermediate feeding studies, the arginine auxotrophs could be divided into two groups. The first group consisted of 3 arginine auxotrophs (AK3, AK4 and AK5). Each of these auxotrophs had a mutation in *argA, argB, argC, argD* or *argE* gene resulting in the biochemical block before ornithine in the arginine biosynthetic pathway. These auxotrophs were referred to as ornithine auxotrophs. The second group comprised of 12 arginine auxotrophs (AK6, AK7, AK8, AK10, AK11, AK13, AK14, AK15, AK16 and AK25). Each of these auxotrophs had a biochemical block after ornithine in the arginine biosynthetic pathway. The *argF/argI* gene was mutated in the arginine auxotrophs AK6, AK7, AK8, AK10, AK11, AK13, AK14, AK15, AK16 and AK25. The *argG* and *argH* genes were mutated in the arginine auxotrophs AK12 and AK9, respectively. The *argF/argI*, *argG* and *argH* auxotrophs covered all the genes involved in the arginine biosynthesis after ornithine. The mutated gene in each of the three arginine auxotrophs having metabolic blocks before ornithine could not be determined. As the intermediates for this part of the pathway are not commercially available, molecular studies are required to identify the mutated genes in the ornithine auxotrophs. In fact, more mutants having metabolic block before ornithine are needed for obtaining mutations in all the genes involved in ornithine biosynthesis.

Twelve arginine auxotrophs (each of which has a biochemical block in one of the last three steps, i.e. after ornithine, of the arginine biosynthetic pathway)

were, with the exception of one auxotroph AK10, found to induce fully effective nodules on two cultivars T9 and A2 of alfalfa plants. These results are consistent with those obtained for *S. meliloti* by earlier workers (Dénarié *et al.*, 1976; Fedorov & Zaretskaya, 1977; Kerppola & Kahn, 1988b). Light microscopic and TEM studies revealed that the internal structures of the nodules induced by *argF/argI*, *argG* and *argH* mutants were similar to the structure of a parental strain induced nodule. The elongation of the bacteroids in the *argF/argI* mutant induced nodule was, however, not to the extent observed in the parental strain induced nodule. It seems that the alfalfa host plant is able to provide the required quantity of arginine to *S. meliloti* bacteria during symbiosis. AK10 mutant showed plant growth characteristics similar to that of the ornithine auxotrophs. It appears that, apart from having a Tn5 insertion in the *argF/argI* gene, the AK10 auxotroph has another mutation in one of its symbiotic genes. One hundred per cent linkage of Tn5 insertion and arginine auxotrophy in AK10 mutant shows that the second mutation in this strain is not a Tn5 insertion; it could be any other type of mutation (point, deletion, addition, etc.).

Three arginine auxotrophs, each of which had a biochemical block before ornithine in the arginine biosynthetic pathway, induced ineffective nodules on alfalfa plants. Kerppola and Kahn (1988b) also reported that the mutants of *S. meliloti* 104A14 blocked in the early part of arginine biosynthetic pathway that leads to ornithine were ineffective. Light microscopic studies of the nodules induced by the ornithine auxotrophs showed that the infection zone in each of these nodules was enlarged, covering almost more than half of the nodule; the

nitrogen fixation zone was poorly developed. TEM studies showed that the bacteroids in these nodules were mostly spherical or oval and the heterogeneity in the bacteroids of the nitrogen fixation zone of ornithine auxotrophs was not to the extent observed in the bacteroids of the Rmd201. These results revealed that the transformation of the rhizobial bacteria into bacteroids was not complete in the nodules induced by the ornithine auxotrophs. It seems that ornithine or an intermediate(s) of ornithine biosynthesis, or a chemical factor derived from one of these compounds is required for the normal development of nitrogen fixation zone and transformation of rhizobial bacteria into bacteroids during the symbiosis of *S. meliloti* with alfalfa plants. The ornithine requiring mutants of *S. meliloti* isolated by Dénarié *et al.* (1976) had effective or partly effective symbiosis. The differences in the results obtained during the present work and by Kerppola and Kahn (1988b) on one hand and those obtained by Dénarié *et al.* (1976) on the other hand may be due to mutations in different genes.

Ornithine is also a precursor of polyamine synthesis (Tabor & Tabor, 1985). Kerppola and Kahn (1988b) hypothesized that the lack of polyamines may be the cause of symbiotic defect of ornithine auxotrophs, or rhizobium requires a spermidine-derived siderophore, similar to agrobactin (Ong *et al.*, 1979). Both these interpretations, according to Kerppola and Kahn (1988b), require that rhizobium be unable to make putrescine by degrading arginine via agmatine.

Though the dry weights of the alfalfa plants inoculated by the ornithine auxotrophs did not differ significantly from the dry weight of the uninoculated alfalfa plants, the former plants, however, were not as chlorotic as the latter ones.

The upper parts of the plants inoculated with ornithine auxotrophs were green. This observation indicates that trace amounts of nitrogen may have been fixed in the nodules induced by arginine auxotrophs having a metabolic block before ornithine.

All the arginine auxotrophs were similar to the parental *S. meliloti* Rmd201 strain with respect to cell surface molecules, utilization of carbon sources, salt and acid tolerance, changes in pH of the medium and growth patterns. These results showed that the symbiotic defect of these auxotrophs were not caused due to a change in any of the above mentioned characteristics.

One hundred per cent co-transfer of Tn5 and arginine auxotrophy in all the arginine auxotrophs indicated complete linkage between transposon Tn5 insertion and arginine auxotrophy. Normal symbiotic activity of the prototrophs of ornithine auxotrophs indicated that a single Tn5 insertion in each of these auxotrophs was responsible for auxotrophy and the symbiotic defect.

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