STUDIES ON THE ROLE OF PYRIMIDINE BIOSYNTHETIC PATHWAY OF *Rhizobium meliloti* IN SYMBIOSIS

A THESIS

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By

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CANDIDATE'S DECLARATION
(To be included in the thesis)

I hereby certify that the work which is being presented in the thesis entitled " STUDIES ON THE ROLE OF PYRIMIDINE BIOSYNTHETIC PATHWAY OF Rhizobium meliloti IN SYMBIOSIS", in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy and submitted in the Department of BIOSCIENCES AND BIOTECHNOLOGY of the University is an authentic record of my own work carried out during a period from Jan. 1999 to Oct. 2000 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.

Neeraj Vij

Date : Oct. 4 , 2000

Signature of the Candidate
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This is to certify that the above statement made by the candidate is correct to the best of my (our) knowledge.

G. S. Randhawa

Date : Oct. 4 , 2000

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The Ph.D. Viva-Voce examination of Neeraj Vij, Research Scholar, has been held on 3.10.2001

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ABSTRACT

The present work was undertaken to determine the symbiotic role of pyrimidine biosynthetic pathway in *Rhizobium meliloti* (presently called *Sinorhizobium meliloti*). The primary objectives of this work were isolation, characterization and symbiotic studies of pyrimidine auxotrophs of *S. meliloti*. Random transposon Tn5 mutagenesis was employed to generate pyrimidine auxotrophs of *S. meliloti* strain Rmd201 which is a Sm^r derivative of strain AK631. Conjugations between donor *E. coli* WA803 (pGS9) and recipient *S. meliloti* Rmd201 yielded 7,350 Km^r transconjugants from 147 crosses. Screening of these transconjugants resulted in isolation of 37 auxotrophs. Streaking of these auxotrophs on nutritional pools yielded twelve uracil, two uracil + arginine and two arginine auxotrophs. Six uracil and three uracil + arginine auxotrophs isolated by other researchers in this lab were also included in this study.

Intermediate feeding and intermediate accumulation studies were conducted for biochemical characterization of pyrimidine auxotrophs. On the basis of these studies. uracil + arginine and uracil auxotrophs, were divided into three groups:

(i) *car* mutants (NV1, NV15, RH33, RH37, RH47): Grew on minimal medium supplemented with uracil and arginine; also grew on carbamoyl phosphate supplemented minimal medium.

(ii) *pyrC* mutants (NV18, NV21, NV32, VK12, VK19, VK43, RH7, RH9): Grew on orotic acid or dihydroorotic acid supplemented minimal medium.

(iii) *pyrE/pyrF* mutants (NV6, NV9, NV12, NV19, NV23, NV26, NV33, NV34, NV37, RH36): Did not grow on minimal medium supplemented with any of the pyrimidine

biosynthetic intermediates (used in the study); accumulated orotic acid in liquid minimal medium.

Arginine auxotrophs grew on ornithine or citrulline supplemented minimal medium indicating that the positions of biochemical block in these auxotrophs were before ornithine.

The uracil, uracil + arginine and arginine auxotrophs were similar to the parental strain w.r.t. cell surface molecules (lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β-glucans), utilization of carbon sources, salt and acid tolerances, change in pH of the medium and growth patterns indicating that the symbiotic defects of these auxotrophs were not caused by a change in any of the above characteristics.

The linkage of Tn5 insertion to auxotrophy in each auxotroph (uracil/uracil + arginine/arginine) was determined by mobilizing the Tn5-containing genomic fragment from the auxotroph into the *S. meliloti* strain ZB555 (Cys^-, Phe^-, Rf^r, Sm^r) with the help of genome mobilizing plasmid pJB3JI and subsequently checking for donor's auxotrophy(ies) in Km^r transconjugants. All Km^r transconjugants thus obtained showed respective donor's auxotrophy confirming the linkage of Tn5 insertion to auxotrophy. This also proved that no other Tn5 insertion occurred in the genome of this auxotroph. Transconjugants of *S. meliloti* strain ZB555 carrying kanamycin resistance and respective auxotrophy when inoculated on alfalfa plants, showed the symbiotic defect like the donor auxotroph. The revertant of each auxotroph showed normal symbiosis, like the parental strain Rmd201, with alfalfa plants. These results showed that a single Tn5 insertion in each auxotroph was responsible for auxotrophy and symbiotic defect.

Genetic mapping of Tn5 insertion in each of the uracil and uracil + arginine auxotrophs was performed using plasmid pJB3JI mediated mapping method. These mutations were mapped in 41.7% region of chromosome between *cys*46 and *pur*15/168 loci. Precise mapping was not possible due to unavailability of mapping strains for complete chromosomal region.

Symbiotic properties of uracil, uracil + arginine and arginine auxotrophs were determined by inoculating them on alfalfa (*Medicago sativa* cv. T9) plants grown aseptically on nitrogen-free slants. All these auxotrophs induced white nodules, and mean plant heights and dry weights of these plants did not differ significantly from those of the uninoculated plants, indicating the inability of these auxotrophs to fix nitrogen. The *car*, *pyrC* and *arg* mutants induced spherical/irregular nodules whereas the nodules induced by the *pyrE/pyrF* mutants were cylindrical like the parental strain induced nodules. This showed that the extent of nodule development was related to the position of mutation in the pyrimidine biosynthetic pathway.

The symbiotic defects of uracil and uracil + arginine auxotrophs were not restored on addition of uracil or its intermediates (and arginine in case of uracil + arginine auxotrophs) to the plant nutrient medium. This may be due to the failure of the auxotrophs located in nodules to utilize these compounds from the medium. The symbiotic defects of arginine auxotrophs were restored on supplementation of arginine, citrulline or ornithine to plant nutrient medium.

The methylene blue stained root portions (1cm long) of alfalfa plants inoculated with the auxotrophs were observed for root hair curling and infection thread formation. All auxotrophs induced root hair curling and resulted in infection thread formation.

Six weeks old nodules induced by uracil and uracil + arginine auxotrophs were fixed and embedded in epoxy araldite resin. Semithin and ultrathin sections of these nodules were examined under light and electron microscopes.

In the nodules induced by *car* mutants lysis of rhizobial bacteria occurred immediately after their release into nodule cells from the infection threads. The defect in these nodules appears to be at the stage of bacterial release. The release of rhizobial bacteria into nodule cells occurred normally in the nodules induced by the *pyrC* mutants but the transformation of the released bacteria into bacteroids was not complete. Hence in these nodules the block occurred during the development of nitrogen fixation zone. In the nodules induced by the *pyrE/pyrF* mutants the rhizobial release into plant cells was normal and in most of the rhizobial cells transformation from bacterial to bacteroid stage was almost complete but the nitrogen fixation zones of these nodules were not fully developed like those of the parental strain induced nodules.

Since the nodules induced by *pyrC* mutants showed advanced structural features over those of the nodules induced by *car* mutants, carbamoyl phosphate/carbamoyl phosphate synthetase/carbamoyl aspartate may be involved in symbiosis. The nodules induced by *pyrE/pyrF* mutants were also structurally more advanced than the nodules formed by *pyrC* mutants, indicating that dihydroorotic acid/dihydroorotase/orotic acid may also have symbiotic function(s). The symbiotic defects of nodules induced by *pyrE/pyrF* mutants may be due to unavailability of orotidine monophosphate or lack of orotate phosphoribosyltransferase/orotidine monophosphate decarboxylase activity. Hence an undiminished metabolic flow through pyrimidine biosynthetic pathway in *S. meliloti* appears to be essential for the effective nodule development on alfalfa plants.

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Neeraj Vij

(NEERAJ VIJ)

LIST OF ABBREVIATIONS USED

A	=	Arginine
Am	=	Amyloplast
B	=	Bacteroid
BTB	=	Bromothymol blue
Cm	=	Chloramphenicol
Cma	=	Chromosomal mobilizing ability
ct	=	Central tissue
CW	=	Cell wall
DOC	=	Sodium deoxycholate
ed	=	Electron dense cytoplasm
EPS	=	Exopolysaccharide
ER	=	Endoplasmic reticulum
et	=	Electron transparent cytoplasm
exo	=	Exopolysaccharide
Fix	=	Nitrogen fixation
hc	=	Heterogeneous cytoplasm
hrs	=	Hours
hsn	=	Host specific nodulation
g	=	Gram
gc	=	Golgi complex
I	=	Infected nodule cell
If	=	Infection zone
it	=	Infection thread
Iz	=	Interzone
kb	=	Kilo base
Km	=	Kanamycin
LB	=	Luria Bertani
LPS	=	Lipopolysaccharide
M	=	Meristematic zone
Met	=	Methionine
mg	=	Milligram
min	=	Minute
ml	=	Millilitre
MSY	=	Mannitol salt yeast extract
n	=	Nucleus
Ndv	=	Nodule development
Nif	=	Structural genes for nitrogenase enzyme
Nm	=	Neomycin
nm	=	Nanometer
Nod	=	Nodulation
nol	=	Nodulation
Nf	=	Nitrogen fixation zone
Ori	=	Origin of transfer
pbm	=	Peribacteroid membrane

phb	=	Poly-β-hydroxybutyrate
pt	=	Peripheral tissue
rb	=	Rhizobial bacteria
rh	=	Root hair
Rf	=	Rifampicin
RMM	=	Rhizobial minimal medium
S	=	Senescence zone
sc	=	Shepherd's crook
sec	=	Second
sg	=	Starch granule
Tc	=	Tetracycline
Thi	=	Thiamine
Tra	=	Transfer
TY	=	Tryptone yeast extract
U	=	Uracil
UI	=	Uninfected nodule cell
v	=	Vacuole
VB	=	Vascular bundle
μg	=	Microgram
μm	=	Micrometer

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CONTENTS

Chapter 1

INTRODUCTION

Nitrogen (N), being a constituent of proteins, nucleic acids and other essential molecules, is a key component of nutrition for all organisms. It exists in gaseous form and constitutes about 78% (by volume) of the atmosphere but being chemically inert, is not available too most living organisms for use. Primary producers of food chain, plants, can utilize only combined forms of nitrogen (i.e. ammonia, nitrate, nitrite, etc.). Industrial production of nitrogenous fertilizers is done by Haber-Bosch, Berkland-Eyde and Cyanamide processes. It has been estimated that to produce and deliver 1Kg of nitrogen fertilizer to farm 1.5Kg of fuel oil is required. Moreover, 50% of this applied fertilizer gets leached which not only leads to pollution but also wastes energy and money. In this context alternative renewable source of nitrogen is biological nitrogen fixation. It involves the conversion of atmospheric nitrogen to ammonia by some prokaryotes called diazotrophs, which include bacteria and blue green algae. The bacteria on the basis of extent of their association with plants can be classified into three groups, (a) asymbiotic or free-living (*Azotobacter spp., Rhodospirillum spp., Pseudomonas spp., Klebsiella pneumoniae, Rhodopseudomonas spp.*, etc.), (b) symbiotic (*Rhizobium spp., Bradyrhizobium spp., Frankia spp.*, etc.) and (c) associative (*Azospirillum spp.*). The bacteria belonging to genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium* and *Mesorhizobium* are collectively termed as rhizobia. The symbiotically fixed nitrogen is the largest source of organic nitrogen in the global nitrogen cycle. The nitrogen fixed by legume-rhizobial symbiosis is an important promising candidate for practising sustainable agriculture as it accounts for more than 25% of the terrestrial nitrogen fixation (Burns and Hardy, 1975).

Rhizobia induce specialized structures called nodules on the roots of leguminous plants. The nodules provide bacteria the ambient environment for reduction of dinitrogen to ammonia. Plant assimilates this ammonia and in turn provides plant photosynthate to rhizobial bacteria to meet their cellular energy requirements. Rhizobia are classified as fast growing (*Rhizobium spp., Sinorhizobium spp., Azorhizobium spp.*, etc.) and slow growing (*Bradyrhizobium spp.*) rhizobia. Some strains of *Rhizobium meliloti* (presently called *Sinorhizobium meliloti*) are known to be fastest in growth among the fast growing rhizobia. *S. meliloti* forms symbiotic association with *Melilotus, Medicago and Trigonella* species. Due to small size of these plants, *S. meliloti-Medicago sativa* symbiotic system was selected for this study.

The nodules are the result of a multi-step process requiring the specific gene products of both the partners (Vincent, 1980). Among most critical steps of the nodule development are colonization, recognition and infection of the host by the bacterium (Newcomb, 1981). The rhizobial-legume symbiosis starts with the interaction between the bacteria and the root epidermis. First reaction of root system to presence of rhizobia is the curling and deformation of root hairs. The bacteria get entrapped in the curl; the host cell wall is hydrolyzed and a tubular structure called infection thread is formed. The bacteria invade root hair and root cortex with the help of infection thread. The nucleus at the tip of the curled root hair guides the path of the infection thread. Meanwhile, cells of root cortex are mitotically activated and form nodule primordium. Infection threads grow towards this primordium and rhizobia are released in plant cell cytoplasm. By endocytotic process bacteria are surrounded by membrane derived from host plasma

membrane. Rhizobia divide and transform into bacteroids inside nodules. Nitrogenase enzyme is synthesized in these bacteroids to convert dinitrogen to ammonia.

Developments in recombinant DNA technology has made it possible to characterize the rhizobial genes involved in complex symbiotic process. Several rhizobial genes involved in nodule formation (*nod, nol*) (Banfalvi *et al.*, 1981; Kondorosi *et al.*, 1984b; Fisher and Long, 1992; Freiberg *et al.*, 1997), nitrogen fixation (*nif, fix*) (Batut *et al.*, 1985; Putnoky *et al.*, 1988; Beringer *et al.*, 1990; Kundig *et al.*, 1993) and 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) have been characterized.

Bacteroidal development and nitrogen fixation in nodule have been reported to depend on nutritional conditions in the nodule. It has been reported that bacteria in nodule utilize C_4-dicarboxylates produced by sucrose metabolism of plants (Watson *et al.*, 1988; Watson, 1990; Boesten *et al.*, 1998). It has been established that bacteroids depend on their host plant for carbon and energy, but it is largely unclear which source(s) (such as carbon, nitrogen and sulfur) the bacteria require during bacteroidal development.

Auxotrophs of some amino acids (asparagine, leucine, tryptophan, etc.), nucleotide bases and vitamins have been reported to form ineffective nodules (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.*, 1999a; 1999b). This ineffectiveness may not pertain exclusively due to the role of the end products of the biosynthetic pathway; their intermediates/enzymes may have some specific role in symbiosis. Anthranilic acid, an intermediate of the tryptophan biosynthetic pathway, has

been shown to be involved in symbiosis in *S. meliloti* (Barsomian *et al.*, 1992; Prasad *et al.*, 2000). Recently it has been reported that tryptophan synthase enzyme is essential for effective symbiosis in *Rhizobium etli* (Taté *et al.*, 1999a). *S. meliloti ilvC* mutants and histidine auxotrophs of *Bradyrhizobium japonicum* did not induce nodule formation on their respective host plants (Sadowsky *et al.*, 1986; Aguilar and Grasso, 1991). Symbiotic defect in purine auxotrophs was restored on supplementation of the plant nutrient medium with purine intermediate 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Newman *et al.*, 1994). Relatively less study has been done on the role of pyrimidine biosynthetic pathway. Mutations in carbamoyl phosphate synthetase and in early steps of pyrimidine biosynthesis have been found to result in ineffective symbiosis in *S. meliloti* (Kerppola and Kahn, 1985, 1988a; 1988b). It has been reported that Ndv⁻ (nodule development defective) phenotype of pyrimidine auxotrophs of *R. leguminosarum* could not be suppressed by uridine addition (Noel *et al.*, 1988). In all these cases histological studies of nodules induced by pyrimidine auxotrophs were not performed and it is unclear if symbiotic ineffectiveness is due to unavailability of pyrimidine or a pyrimidine intermediate(s) is (are) responsible for defect in bacteroid development and function. There is also a possibility that some enzyme(s) of the pyrimidine biosynthetic pathway has (have) a dual role i.e. apart from its role in the biosynthesis of pyrimidine this enzyme also converts some unknown precursor into a product necessary for symbiosis. Vineetha (1998) on the basis of studies on nodules induced by pyrimidine auxotrophs indicated that some of the pyrimidine intermediates/enzymes might have some role in symbiosis. If the specific pyrimidine intermediate/enzyme has a role in symbiosis, it has to be worked out at which stage,

nodule development and function is arrested. Considering the above facts the present study on the pyrimidine biosynthetic pathway in *S. meliloti* was undertaken with following objectives:

(a) to generate pyrimidine auxotrophs of *S. meliloti* by random transposon Tn5 mutagenesis,

(b) to carry out genetic and biochemical characterization of pyrimidine auxotrophs,

(c) to study symbiotic properties of pyrimidine auxotrophs,

(d) to study histology of nodules induced by pyrimidine auxotrophs.

Chapter 2

Literature Review

The literature on the role of biosynthetic pathways in rhizobial-legume symbiosis and other related aspects was reviewed and has been presented under suitable headings.

2.1 Discovery and taxonomy of rhizobia

In 1679, Malpighi provided the first diagrams and descriptions of root nodules (as cited in Subha Rao, 1995). Two German chemists, Herman Hellriegel and Herman Wilfarth (1888) discovered the innate ability of legumes to fix atmospheric nitrogen. Beijerinck (1888) in Holland was first to isolate and cultivate a microorganism from the nodules of legumes. He named the organism as *Bacillus radicicola* which was later renamed as *Rhizobium* (rhizo = root and bios = living) by Frank (1889). Lohnis and Hansen (1921), on the basis of the growth rate, classified the root nodule bacteria into two groups: slow-growers with generation time more than 6 hours (which nodulate soybean and cowpea plants), and fast-growers with generation time less than 6 hours (which form nodules on alfalfa, clover, bean and pea). Further characterization of rhizobia was done on the basis of cross-inoculation groups assuming that one rhizobial species nodulate closely related legumes (Fred *et al.*, 1932); this concept was later disapproved on the basis of extensive genetic studies. A new genus *Bradyrhizobium* was proposed for slow growing rhizobia (Jordan, 1982; Jordan, 1984). Later, the root nodule bacteria, which can utilize gaseous nitrogen as a sole nitrogen source during growth in free living condition were placed under the genus *Azorhizobium* (Jordan, 1984; Dreyfus *et al.*, 1988). Existence of marked genetic diversity among rhizobia was shown by using modern methods of bacterial systematics like numerical taxonomy, 16s rRNA analysis and nucleic acid hybridization (Young *et al.*, 1991; Young, 1992). Subsequently two new

genera, *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994; Lindstrom *et al.*, 1995) and *Mesorhizobium* (Jarvis *et al.*, 1997) were recognized for rhizobial bacteria. Current taxonomic classification of rhizobia is presented in Table 1.

2.2 Development and metabolism of nodules

The development of an effective legume nodule is a result of complex rhizobial-legume interaction which involves several steps from initial recognition and infection to nodule formation and metabolism.

2.2.1 Recognition

Rhizobia can live saprophytically in soil. To colonize the plant roots, these bacteria reach near it by the processes called chemotaxis (Caetano-Anolles *et al.*, 1988; Malek, 1989; Maxwell and Phillips, 1990; Kape *et al.*, 1991) and electrotaxis (Miller *et al.*, 1986). Rhizobia are attracted towards the root surface by the presence of attractants in plant root exudates. Some of these attractants, such as sugars, amino acids, phenolic compounds and carboxylic acids are of nutritional value whereas others like flavonoids and chalcones are inducers or repressors of nodulation genes (Rolfe, 1988; Gottfert, 1993.).

Dazzo *et al.* (1984) and Smit *et al.* (1987) have proposed that rhizobial attachment to the root surface is a two step process. In the first step, rhizobial cells attach loosely to root surface in a non-specific manner. Smit *et al.* (1989; 1991) reported the role of rhizobial 14KD Ca^{2+} binding protein rhicadhesin in this step. A 32KD plant glycoprotein has been reported to be a possible receptor molecule for rhicadhesin. In the second step of

Table 1: Taxonomic classification of rhizobia (based on Scholla and Elkan, 1984; Eardley *et al.*, 1990; Nour *et al.*, 1995; Xu *et al.*, 1995; Chen *et al.*, 1995; 1997; Amarger *et al.*, 1997; de Lajudie *et al.*, 1994; 1998; Terefework *et al.*, 1998; Nick *et al.*, 1999)

Rhizobia	Host plants
Rhizobium	
Rhizobium spp. strain NGR234	Tropical legumes, *Parasponia spp.*(non-legume)
R. leguminosarum	
bv. *viciae*	*Pisum, Vicia, Lathyrus* and *Lens spp.*
bv. *trifolii*	*Trifolium spp.*
bv. *phaseoli*	*Phaseolus vulgaris*
R. tropici	*Leucaena spp., Macroptilium spp.,Phaseolus vulgaris*
R. etli	*Phaseolus vulgaris*
R. galegae	*Galega officinalis, G. orientalis* etc.
R. hainanense	Tropical legumes
R. gallicum	*Phaseolus vulgaris*
R. giardinii	*Phaseolus vulgaris*
Bradyrhizobium	
Bradyrhizobium spp. strain parasponia	*Parasponia spp.*
B. japonicum	*Glycine max, G. soja* and other legumes
B. elkanii	*Glycine max, G. soja* and other legumes
B. liaoningense	*Glycine spp.*
Sinorhizobium	
S. meliloti	*Medicago spp., Melilotus spp. and Trigonella spp.*
S. terangae	*Acacea laeta*
S. fredii	*Glycine max, G. soja* and other legumes
S. medicae	*Medicago spp.*
S. saheli	*Sesbania spp.*
S. arboris	*Acacia sengal, Prosopis chilenis,* etc.
S. kostiense	*Acacia sengal, Prosopis chilenis,* etc.
Azorhizobium	
A. caulinodans	*Sesbania spp.* (stem and root nodulating)
Mesorhizobium	
M. loti	*Lotus spp.*
M. huakuii	*Astragalus sinicus*
M. ciceri	*Cicer arietinum*
M. tianshanese	*Glycyrrhiza spp., Sophora spp.,* etc.
M. mediterraneum	*Cicer spp.*
M. plurifarium	*Acacia spp., Leucaena spp.,*etc.

rhizobial attachment, an effective contact is established by accumulation and anchoring of bacterial aggregates to root surface. In this step bacterial cellulose fibrils and plant lectins are involved. This step is called cap formation process (Vesper and Bauer, 1986; Smit *et al.*, 1987).

It has been shown that rhizobia respond to plant flavonoids. These flavonoids bind to rhizobial NodD protein which in turn binds to the promoters of other *nod* genes and induces expression of these genes (Fig.1) (Schlaman *et al.*, 1989; Goethals *et al.*, 1992). The *nod* gene products catalyze the biosynthesis of the Nod factors that induce deformation and curling of root hairs on the host plant (Schlaman *et al.*, 1989; Journet *et al.*, 1994). NodD proteins from different rhizobia require specific flavonoids from their respective host plants for optimal activation (Horvath *et al.*, 1987; Mc Iver *et al.*, 1989). *Rhizobium spp.* NGR234 that can nodulate various tropical legumes, synthesize 18 different Nod factors (Price *et al.*, 1992). *S. meliloti* have three variant NodD proteins, each having a different flavonoid and hence greater diversity for host plant (Györgypal *et al.*, 1988). Rhizobia secrete signal molecules i.e. Nod factors which are lipochito-oligosaccharides containing a backbone of 4-5 N-acetylglucosamine residues and a fatty acid at the non-reducing terminal sugar residue. The host specificity is determined by substitutions at terminal sugar residues. In *S. meliloti* the main host determinant is a sulphate group at the reducing sugar residue (Peters *et al.*, 1986; Lerouge *et al.*, 1990; Fisher and Long, 1992; Goethals *et al.*, 1992; Journet *et al.*, 1994; Pawlowski *et al.*, 1996).

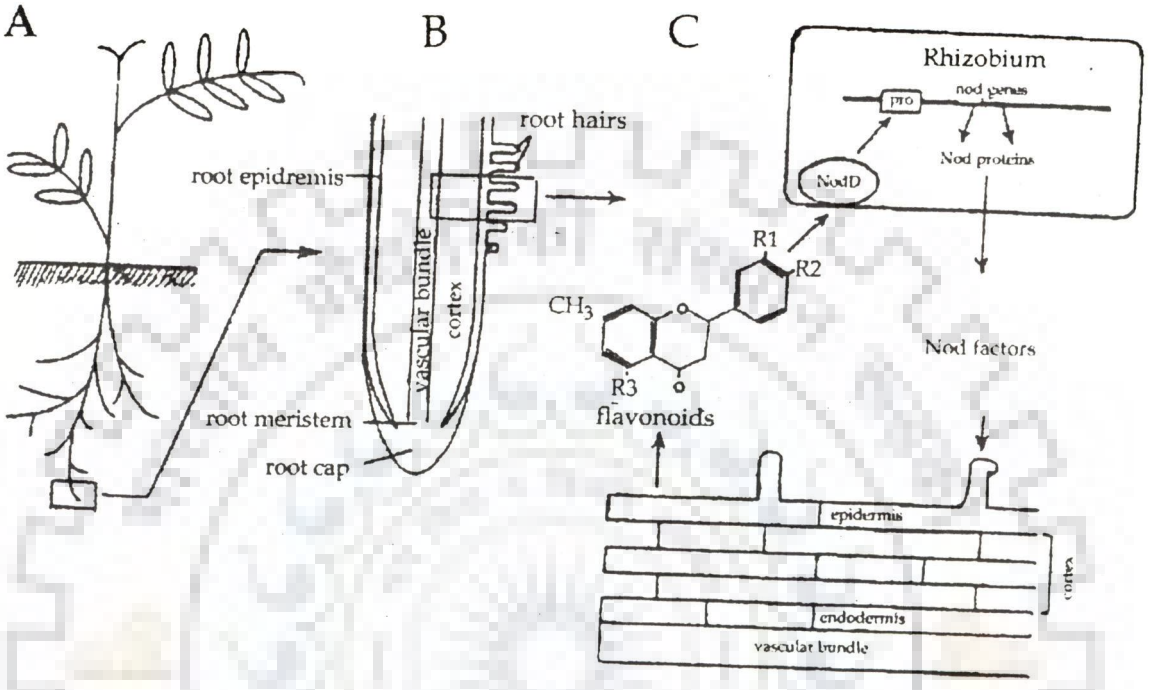

Fig. 1: Schematic representation of signal exchange during legume nodule induction, A. legume plant, B. root tip, C. interaction between legume roots and rhizobia (Pawlowski *et al.*, 1996).

2.2.2 Infection

The rhizobial bacteria attached to root hairs penetrate through plant cell wall derived "infection thread". These infection threads ramify and penetrate nodule cells (Callaham and Torrey, 1981; Newcomb, 1981; Vasse and Truchet, 1984).

2.2.2.1 Role of bacterial surface in infection

Bacterial exopolysaccharides are required for infection thread formation while lipopolysaccharides have a role in endocytosis and bacteroidal differentiation (Glazebrook and Walker, 1989; Brewin *et al.*, 1990). β-(1→2) glucans may be involved in infection thread development (Dylan *et al.*, 1990; Miller *et al.*, 1990).

2.2.2.2 Root hair deformation

The different types of rhizobial infection known are: (a) infection through infection thread and (b) infection via so-called crack entry, which takes place by the entry of bacteria into epidermal cells through gaps in epidermis (Chandler *et al.*, 1982; De Faria *et al.*, 1988). The infection through infection thread involves root hair deformation and formation of root hair curl called shepherd's crook. Microscopical studies have shown that root hair deformation is due to a new induction of root hair tip growth by Nod factors (Heidstra *et al.*, 1994). This process of root hair deformation involves host genes like *Mtrip1* encoding peroxidase (Cook *et al.*, 1995) and nodulin genes ENOD5 and ENOD12 that encode cell wall components (Scheres *et al.*, 1990). Purified Nod factors can induce expression of above mentioned plant genes and hence root hair deformation (Maxwell and Phillips, 1990; Journet *et al.*, 1994). Nod factors are also involved in infection by crack entry (Goethals *et al.*, 1994).

2.2.2.3 Infection thread formation

Rhizobia entrapped in root hair curl (shepherd's crook) induce infection thread formation by hydrolysis of plant cell wall (Callaham and Torrey, 1981). It has been

shown that invagination of plasma membrane at the site of hydrolysis results in formation of tubular structure called infection thread. The infection thread grows towards the root hair base and subsequently the nodule primordium (Turgeon and Bauer, 1985). In case of infection by crack entry rhizobial bacteria enter cortical cells of lateral roots and both membrane enveloped bacteria as well as plant cells divide repeatedly to form nodules (Chandler *et al.*, 1982).

2.2.3 Bacterial release

When infection thread has reached the nodule primordium, bacteria are released from infection thread into host cytoplasm. Bacteria become surrounded by plant-derived peribacteroidal membrane (pbm) and are then called symbiosomes (Hirsch *et al.*, 1983; Roth and Stacey, 1989; Brewin, 1991). The bacteria, after release, divide and undergo transformation to become bacteroids. Host plant cell increase in volume to enclose many bacteroids and supply them with nutrients. The plant cell organelles like mitochondria, vesicles, amyloplasts, etc. get arranged at periphery of the cell (Hennecke, 1990). It has been reported that Fix$^-$ (defective in nitrogen fixation) mutants of rhizobia show premature degradation of bacteroids (Hirsch and Smith, 1987).

2.2.4 Nodule development

Concomitant with infection of root hairs, root cortical cells are activated and start dividing to produce nodule primordium. Nod factors induce formation of nodule primordium (Vijn *et al.*, 1993; Journet *et al.*, 1994). Several nodulin genes like ENOD12, ENOD40 and GmN93 are expressed during nodule development (Kouchi and Hata, 1993). The location of nodule primordium in root cortex depends on the type of nodule formed by a particular plant (Newcomb, 1981). In temperate legumes like pea, vetch and alfalfa, inner cortical cells divide and form nodule primordium. Meristem, consisting of small cells with dense cytoplasm, is formed at the distal part of primordium (Libbenga

and Harkes, 1973; Dudley *et al.*, 1987). The nodule meristem differentiates during the complete nodule life into different cell types that build up the indeterminate cylindrical nodules (Newcomb, 1976; Vasse *et al.*, 1990). In case of tropical legumes such as Glycine, Phaseolus and Lotus, outer cortical cells of root divide to form nodule primordium (Rolfe and Gresshoff, 1988; Mathews *et al.*, 1989). Cells at the periphery of the primordium remain mitotically active and form a spherical meristem, which loses its activity at an early stage of development. The resulting nodules called the determinate nodules, are spherical and have a determinate growth pattern (Pawlowski *et al.*, 1996).

The temperate legumes form indeterminate nodules that export nitrogen as amides. These nodules have persistent meristem, open vasculature, pleomorphic bacteroids and vacuoles. The tropical legumes form determinate nodules, which export nitrogen as ureides. These nodules have non-persistent meristem, continuous vasculature and rod shaped bacteroids. Vacuoles are absent in determinate nodules (Dudley *et al.*, 1987). Determinate and indeterminate nodules have a similar tissue organization; there is central tissue surrounded by several peripheral tissues. The peripheral tissues comprise of cortex, endodermis, parenchyma and vascular bundles. The central tissue is infected by rhizobia and differentiates into distinct zones (Fig.2). The nitrogen fixed by a bacteroid in a nodule cell passes across peribacteroid membrane into cytoplasm of adjacent uninfected cells and reach vascular bundles at nodule periphery from where it is circulated to entire plant (Blevins, 1989).

2.3 Genetics of rhizobia

In the past three decades basic experimental conditions were established to study symbiotic genes of rhizobia. The exchange of genetic material by conjugation was worked out enabling construction of linkage maps of different rhizobial species. Many

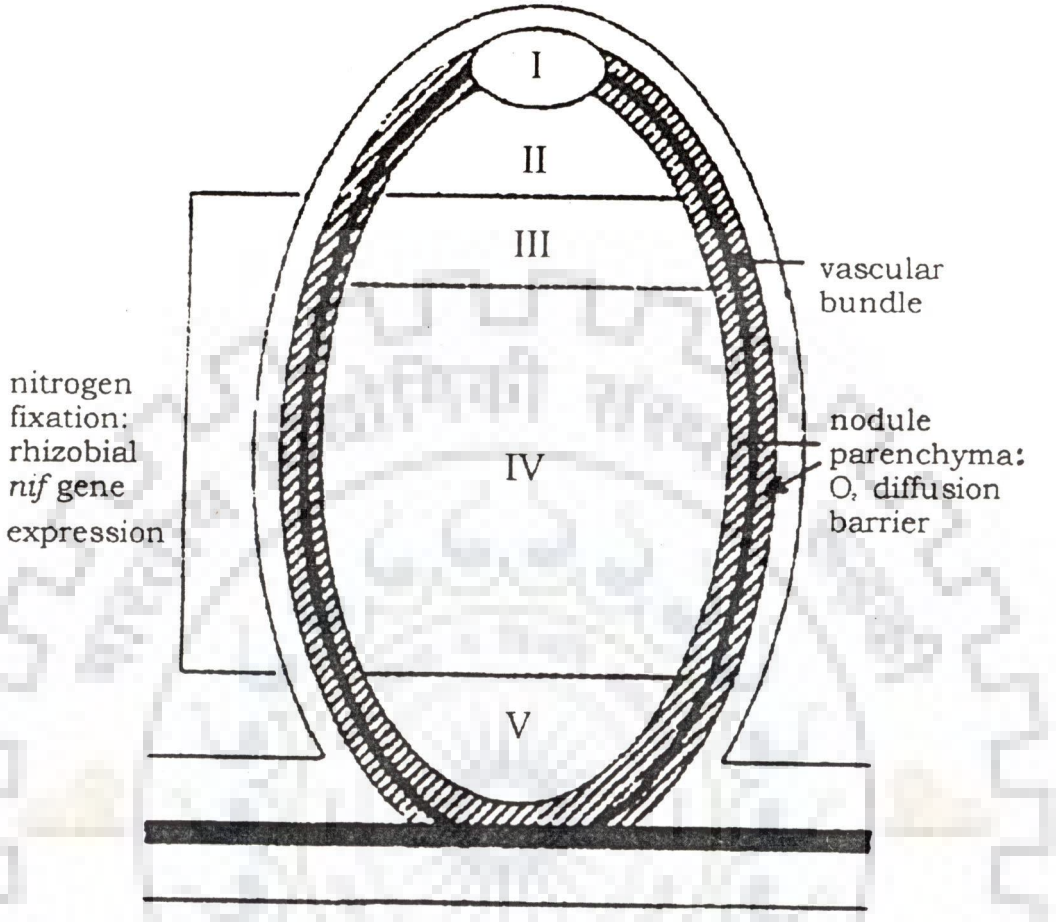

Fig. 2: Schematic representation of root nodule structure; Zonation: I, apical meristematic zone; II, infection zone; III, interzone; IV, nitrogen fixation zone; V, senescence zone (Pawlowski *et al.*, 1996).

mutants with altered symbiotic properties were generated by using various mutagenic techniques. Symbiotic genes from several rhizobial species were characterized using modern molecular biological techniques.

2.3.1 Mutagenesis

The primary focus of current rhizobial research is the identification of symbiotically essential genes and elucidation of their role in symbiotic process. To identify these genes of symbiotic importance both chemical and transposon mutagenesis techniques were employed. With the use of several mutagens like nitrous acid, N-methyl-N'-nitrosoguanidine (NTG), ethyl methane sulphonate (EMS), etc., mutants of rhizobial strains defective in metabolic pathways and symbiosis were generated (Scherrer and Dénarié, 1971; Federov and Zaretskaya, 1979; Pain, 1979; Singh *et al.*, 1984; Noel *et al.*, 1988; Mc Iver *et al.*, 1989; Agarwal, 1994). Transposon insertion can be used as an efficient technique to mutagenize gene(s) of symbiotic importance and metabolic pathways. Transposon induced mutation have following advantages over chemical mutagenesis: (a) insertion into a gene leads to complete loss of its function, (b) antibiotic resistance marker of the transposon is a positively selectable marker which makes genetic mapping of the affected gene simple, (c) molecular cloning of the transposon-marked gene becomes easier, (d) alteration of primary structure of DNA by transposon insertion is in a manner that it can easily be detected by restriction enzyme analysis, (e) mutations are relatively random due to little insertion specificity, (f) transposon insertions generally exert polar effects on downstream genes and hence used to determine limits of transcriptional unit, (g) transposon insertion mutants have relatively lower reversion frequency and (h) the mutated symbiotic gene, tagged by Tn5 can easily be identified by hybridization with Tn5 probe (Kleckner *et al.*,1977; de Bruijn and Lupinski, 1984; Kondorosi *et al.*,1984a). Transposon Tn5 conferring kanamycin/neomycin resistance has

been the transposon of choice for localization of important genes or gene clusters and their preliminary analysis (Selvaraj and Iyer, 1983). Transposon mutagenesis is of two types, random and site directed.

2.3.1.1 Random transposon mutagenesis

Random transposon Tn5 mutagenesis of a rhizobial strain involves insertion of Tn5 from a suicide plasmid vehicle into rhizobial genome. The suicide plasmid is introduced into rhizobia by conjugation from an *Escherichia coli* donor (Fig.3 & 4). As the suicide plasmid is not stable in rhizobia, selection of neomycin/kanamycin resistant transconjugants leads to isolation of Tn5 insertion mutants (Beringer *et al.*, 1978; Selvaraj and Iyer, 1983; Simon *et al.*, 1983). This technique has been used by several researchers to generate auxotrophs and symbiotically defective mutants of rhizobia (Meade *et al.*, 1982; Forrai *et al.*, 1983; Kerppolla and Kahn, 1988b; Khanuja and Kumar, 1989; Barsomian *et al.*, 1992). Mutated symbiotic gene can be identified and subsequently cloned into a suitable vector. Many genes of symbiotic importance have been identified and characterized by using this approach (Hirsch *et al.*, 1983; 1984; Hom *et al.*, 1984; Leigh *et al.*, 1987).

2.3.1.2 Site directed mutagenesis

Site directed mutagenesis is extremely useful for fine structural genetic analysis as it allows mutation of a specific gene. It involves the Tn5 mutagenesis of cloned DNA in *E. coli*, followed by transfer of selected insertion into rhizobia involving recombination. This procedure of recombination has been called by several names viz., homogenotization, gene replacement and marker exchange (Ditta, 1986). Site directed mutagenesis has been used for physical and genetic characterization of several rhizobial genes (Ruvkun and Ausubel, 1981; Ausubel, 1982; Ruvkun *et al.*, 1982; Corbin *et al.*, 1983; Watson and Rastogi, 1993; Kereszt *et al.*, 1998). Recently *in vitro* Tn5-

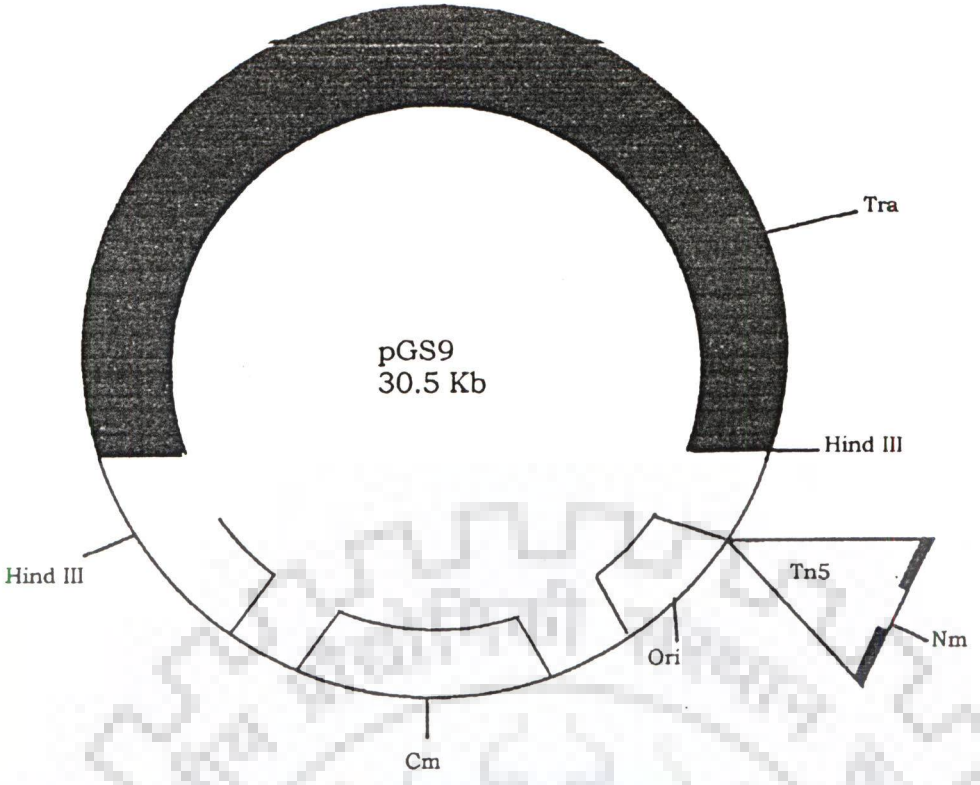

Fig. 3: Suicide vector pGS9 carrying Tn5 (based on Selvaraj and Iyer, 1983)

Abbreviations: Cm, chloramphenicol; Nm, neomycin; Ori, origin of replication

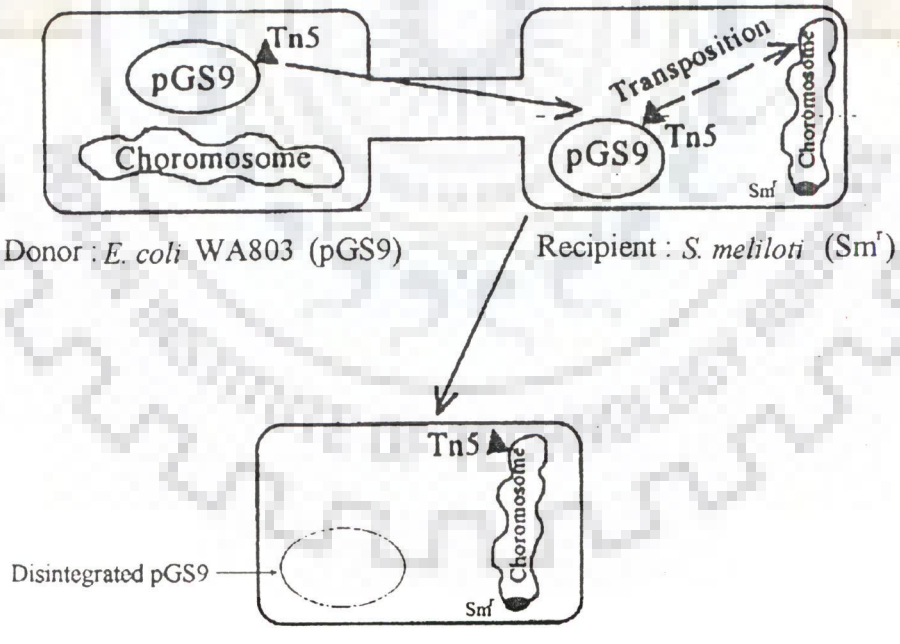

Fig. 4: Diagrammatic representation of transposon Tn5 insertion (based on Selvaraj and Iyer, 1983)

transposition system for directed mutagenesis has been developed (Goryshin and Reznikoff, 1998).

2.3.2 Symbiotic genes

The development of nodules and effective nitrogen fixation in symbiosis is a multistep process depending on the coordinated expression of specific genes acting at different stages in both partners. It has been reported that some symbiotic genes are present on chromosome (Meade and Signer, 1977; Casadeus and Olivares, 1979; Forrai *et al.*, 1983; Sobral *et al.*, 1991a; 1991b; Glazebrook *et al.*, 1992; Honeycut *et al.*, 1993; Kundig *et al.*, 1993) while most of them including host specificity of nodulation (*hsn*) are located on large plasmids (Hirsch *et al.*, 1980; Long *et al.*, 1982). Presence of large indigenous plasmids in different rhizobial species has been reported to be a common feature (Nuti *et al.*, 1977; Casse *et al.*, 1979; Dénarié *et al.*, 1981; Rosenberg *et al.*, 1981; 1982; Banfalvi *et al.*, 1981; 1985). Based on the function(s) symbiotic genes are classified as nodulation genes (*nod, nol*), nitrogen fixation genes (*nif, fix*), genes for cell surface components (*exo, pss, lps, ndv, rkp,* etc.) and genes for bacteroid metabolism (*dct, ntr*, etc.).

2.3.2.1 Genes involved in nodulation

Nodulation genes define the central functions required for plant invasion and host-recognition. Earlier these genes were designated as *nod* genes i.e. *nodA, nodB, nodC, ... nodZ* but after utilization of all the 26 alphabets, additional genes were designated as *nol* and *noe* genes. The nodulation genes have been divided into common *nod* genes and host-specific nodulation genes also called *hsn* genes. Functions of *nod* and *nol* genes are summarized in Table 2.

Table 2: Functions and properties of *nod* genes (based on Fisher and Long, 1992; Sharma *et al.*, 1993)

Genes	Function and properties
nod	
nodABC	Required for Nod factor production
nodC	Homology to chitin and cellulose synthase; proposed to form β-1,4-glycosyl bond
nodD	Transcriptional activator of inducible *nod* genes
nodE	Host -specific;homology to β-ketoacyl synthase(condensing enzyme) of fatty acyl synthase; proposed to synthesize Nod factor acyl chain
nodF	Host-specific; homology to acyl carrier protein; proposed to synthesize Nod factor fatty acyl chain
nodG	Host-specific; homology to reductases; proposed to modify Nod factor fatty acyl side chain
nodH	Host-specific; required for formation of sulphated Nod factor ;proposed to transfer activated sulphate to Nod factor
nodI	Homology to ATP-binding active transport proteins; proposed to form membrane transport complex with *nodJ*
nodJ	Homology to transmembrane proteins; proposed to form membrane transport complex with *nodI*
nodK	Unknown
nodL	Host-specific; homology to acetyl transferase; required for formation of 6-O-acetyl Nod factor; proposed to add O-acetyl group to Nod factor
nodM	Host-specific glucosamine synthase; proposed to synthesize Nod factor sugar subunits
nodN	Host-specific; involved in *Vicia hirsuta* nodulation
nodO	Exported Ca^{2+} -binding; homology to haemolysin; proposed to mediate in early stage in rhizobia-legume interaction
nodP	Host-specific ATP sulphurylase; proposed to provide activated sulphate for transfer to Nod factor
nodQ	Host-specific ATP sulphurylase and APS kinase; together with NodP makes activated sulphate; proposed to provide activated sulphate for transfer to Nod factor
nodR	Host-specificity
nodT	Host-specific; involved in *Trifolium subterranean* nodulation; proposed to be membrane protein
nodV	Homology to two-component regulatory system sensor proteins; proposed to regulate unknown target genes
nodW	Homology to two-component regulatory system activator proteins; proposed to regulate unknown target genes
nodX	Host-specific hydrophobic protein; extends host-range to Afghanistan peas
nodY Z	Host-specific nodulation
nol	
nolA	Extension of genotype-specific nodulation
nolB	Unknown
nolC	Extension of genotype-specific nodulation
nolE	Unknown
nolFGHI	Production of nod metabolites
nolP	Unknown
nolR	Repressor of *nodD*

2.3.2.1.1 Common *nod* genes

The common nodulation genes (*nodABC*), which are responsible for root hair curling and nodule induction are highly conserved among all rhizobial species (Long *et al.*, 1982; Horvath *et al.*, 1986; Schultze and Kondorosi, 1998). In *S. meliloti*, these genes are clustered in a 3kb segment which is located about 30kb away from *nifHDK* cluster (Long *et al.*, 1982; Kondorosi *et al.*, 1984b). Inactivation of *nodABC* genes abolishes the rhizobial ability to elicit any symbiotic reaction on the plant, like induction of root hair curling (*hac*), root hair infection (*hai*), thick and short root (*tsr*) and nodule formation (*nod*), regardless of the host (Long *et al.*, 1982; Long, 1989). The *nodABC* genes encode for enzymes required in the biosynthesis of core Nod factor (Fisher and Long, 1992). In most of the rhizobial species, *nodABC* genes exist as a single operon however there are some exceptions. In *Mesorhizobium loti, nodB* gene is absent from region between ORFs (open reading frames) of *nodA* and *nodC* genes (Scott *et al.*, 1996). In an arctic *Rhizobium spp.* strain N-33 the *nodA* gene has its own *nod* box, separated from *nodBCIJ* operon (Cloutier *et al.*, 1996). Expression of *nod* genes is activated by three allelic *nodD* genes in the presence of flavonoids from plant root exudates (Mulligan and Long, 1985; Peters *et al.*, 1986). The *nodD* gene thus define the host range of *Rhizobium* (Györgypal *et al.*, 1988; Mc Iver *et al.*, 1989; Schlaman *et al.*, 1992). *nodMN* have been identified as common *nod* genes; *nodM* code for signal production and bacteroid maturation (Baev *et al.*, 1992).

2.3.2.1.2 Host specific nodulation genes

Host specific nodulation (*hsn*) genes are involved in host specificity of nodulation. A mutation in a *hsn* gene leads to altered infection and nodulation which in turn lead to extension of host range. The *hsn* genes include *nodH, nodFEG* and *nodQP* genes. In *S. meliloti, hsn* genes cluster is 6kb away from common *nod* genes towards

nifHDK region (Long *et al.*, 1982; Kondorosi *et al.*, 1984b; Horvath *et al.*, 1986). *nodHPQ* genes are organized as an operon that is transcribed in a *nodD*-independent manner and is not regulated by flavonoids (Folch-Mallol *et al.*, 1998).

2.3.2.2 Genes involved in nitrogen fixation

Nitrogen fixation genes (*nif, fix*) are concerned with enzymology of nitrogen fixation. Biological nitrogen fixation involves conversion of nitrogen to ammonia and hydrogen in presence of nitrogenase enzyme.

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

Twenty adjacent *nif* genes are organized in eight operons in a 24kb region of DNA in *Klebsiella pneumoniae*. *Rhizobium fix* genes, shown to be equivalent to *K. pneumoniae nif* genes are assigned the same names (Beringer *et al.*, 1980; Arnold *et al.*, 1988). The *nif* and *fix* genes identified in *S. meliloti, B. japonicum* and *A. caulinodans* and their proposed functions are given in Table 3. In *S. meliloti, R. leguminosarum* and *Rhizobium spp.* NGR234 the *nod, nif* and *fix* genes are located on the symbiotic plasmids (Beringer *et al.*, 1990) while in *Rhizobium loti, Bradyrhizobium spp.* and *Azorhizobium spp.* these genes are chromosome borne. *S. meliloti* carries two megaplasmids pSyma (1400kb) and pSymb (1700kb), respectively (Banfalvi *et al.*, 1981; Burkhardt *et al.*, 1987). pSyma carries two clusters of symbiotic genes. The cluster I includes *nifHDKE, nifN, fixABCX, nifA* and *nifB* genes while the cluster II has *fixLJ, fixK, fixNOQP* and *fixGHIS* genes. The position of cluster II is 220kb downstream of *nifHDKE* operon and it is transcribed in direction opposite to *nifHDKE* (Batut *et al.*, 1985; David *et al.*, 1987; 1988).

2.3.2.2.1 *nif* genes

nif HDK genes are located in a single operon on a megaplasmid in *S. meliloti* and other *Rhizobium spp.* These genes code for nitrogenase polypeptides (Ruvkun and Ausubel, 1980; Rosenberg *et al.*, 1981). *nifA* is located upstream of *nifHDK* (Dixon, 1984) and is a regulatory gene for *nif* and *fix* genes (Ditta *et al.*, 1987). Proposed function(s) of various *nif* genes are given in Table 3.

2.3.2.2.2 *fix* genes

fixABCX genes required for nitrogenase activity are located between *nifHDK* and *nifA* operons (Ruvkun *et al.*, 1982). *fixGHIS* genes are predicted to be transmembrane proteins on the basis of amino acid sequences (Fisher and Long, 1992; Fischer, 1994). *fixR* is located upstream of regulatory *nifA* gene and is involved in oxidation-reduction process (Thony *et al.*, 1987). The predicted amino acid sequences of *fixNOQP* proteins imply that they encode membrane-bound cytochrome c - containing heme/copper cytochrome oxidase (Mandon *et al.*, 1993). Proposed function(s) of various *fix* genes are given in Table 3.

2.3.2.3 Genes for cell surface components

Normal expression of the genes for rhizobial cell surface components (exopolysaccharides, lipopolysaccharides, β-glucans and capsular polysaccharides) is essential for infection.

Table 3: Functions of *nif* and *fix* genes in *Sinorhizobium meliloti*, *Bradyrhizobium japonicum* or *Azorhizobium caulinodans* (Sharma *et al.*, 1993; Fischer, 1994)

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

2.3.2.3.1 Genes for synthesis of exopolysaccharides

Rhizobial exopolysaccharides have been shown to be essential for the successful invasion of the nodules by rhizobia (Gray and Rolfe, 1990; Leigh and Coplin, 1992; Rolfe *et al.*, 1996 and van Workum *et al.*, 1997). Exo$^-$ (defective in exopolysaccharide production) mutants of *R. leguminosarum* bvs. *trifolii* and *viciae* formed nodules but did not fix nitrogen (Chen *et al.*, 1985; Derylo *et al.*, 1986; Shorupska *et al.*, 1995; Rolfe *et al.*, 1996). Genetic control of succinoglycan (EPS I) and galactoglucan (EPS II) in *S. meliloti* is well known (Müller *et al.*, 1993; Reuber and Walker, 1993; Becker *et al.*, 1997). But relatively less is known about genetic control of synthesis and regulation of exopolysaccharide (EPS) in *R. leguminosarum* biovars. *pssA* and *pssCDE* genes, which are involved in EPS biosynthesis in *R. leguminosarum* bv. *trifolii*, have been recently characterized (van Workum *et al.*, 1997; Król *et al.*, 1998; Pollock *et al.*, 1998).

2.3.2.3.2 Genes for synthesis of lipopolysaccharides

Lipopolysaccharides (LPS) plays an essential role in rhizobial-legume symbiosis. Cava *et al.* (1989) cloned DNA segments containing two distinct sets of *R. leguminosarum* bv. *phaseoli* LPS genes. The mutants deficient in LPS biosynthesis have been reported to be impaired in symbiosis in *R. leguminosarum* biovars and *B. japonicum*. These mutants were arrested in the development of infection threads, leading to non-infected pseudonodules (Carlson *et al.*, 1987; Stacey *et al.*, 1991; Noel, 1992; Kannenberg and Brewin, 1994). *S. meliloti* LPS mutants were found to form nitrogen-fixing nodules on *Medicago sativa* (Clover *et al.*, 1989). The detailed analysis of *S. meliloti* LPS mutant Rm6963 showed reduced alfalfa nodulation competence as compared to *S. meliloti* wild type strain (Lagares *et al.*, 1992). Recently it has been

shown that the mutant Rm6963 form effective nodules with *Medicago sativa* but nodules formed with *Medicago truncatula* are ineffective (Niehaus *et al.*, 1998). Brzoska and Signer (1991) have identified a lipopolysaccharide gene (*lpsZ*) involved in symbiosis in *S. meliloti*.

2.3.2.3.3 Genes for synthesis of β-(1→2) glucans

β-(1→2) cyclic glucans are required for infection. The *ndvA* and *ndvB* genes involved in the synthesis of β-(1→2) glucans were identified in *S. meliloti* (Carlson, 1982; Dyaln *et al.*, 1986). A mutation in *ndvA* or *ndvB* gene of *S. meliloti* resulted in ineffective nodules. The *ndvB* gene product, a 235KD inner membrane protein, was reported to be involved in synthesis of glucan (Zorreguieta *et al.*, 1988). The *ndvA* gene product homologous to *E. coli* protein HlyB was postulated to be involved in export of glucan (Stanfield *et al.*, 1988).

2.3.2.3.4 Genes for synthesis of capsular polysaccharides

It has been reported that rhizobia possess surface polysaccharides analogous to groupII K antigens (KPS) of *E. coli* (Reuhs *et al.*, 1993); these *E. coli* polysaccharides can replace EPS of *S. meliloti* in infection process (Petrovics *et al.*, 1993; Reuhs *et al.*, 1995). KPS isolated from *S. meliloti* have shown banding patterns on PAGE that correspond to different degrees of polymerization controlled by *rkpZ* gene (Reuhs *et al.*, 1995). KPS of rhizobia have been shown to be composed of disaccharide repeating units of KDO (3-deoxy-D-mannose-hexouranic acid) (Reuhs, 1997). Two novel gene clusters, designated as *rkp-2* and *rkp-3* regions, involved in production of capsular polysaccharides have been identified in *S. meliloti*; genetic and biochemical analysis of *rkp-2* region was also done (Kereszt *et al.*, 1998).

2.3.3 Mapping studies

For many years in past, the major limitation for genetic analysis of rhizobia was the lack of indigenous chromosome mobilizing plasmids that could allow gene transfer studies. The difficulty was overcome by transferring broad host range plasmids with chromosome mobilizing ability (Cma) to rhizobia. The promiscuous P1 incompatibility group of R plasmids of *Pseudomonas aeruginosa* were used for linkage studies in rhizobia (Beringer and Hoopwood, 1976; Kondorosi *et al.*, 1977; Meade and Signer, 1977; Casadesus and Olivares, 1979). Due to inability of these plasmids to promote transfer from single distinct origins, co-inheritance frequencies have to be worked out (Hass and Holloway, 1976; Kondorosi *et al.*, 1977; Meade and Signer, 1977). P1 incompatibility group plasmids R68.45, R68.45, RP4 and R68.45 were used to construct circular linkage maps of rhizobial strains *R. leguminosarum* strain 300 (Beringer and Hoopwood, 1976), *S. meliloti* strain Rm41 (Kondorosi *et al.*, 1977), *S. meliloti* strain 2011 (Meade and Signer, 1977) and *S. meliloti* strain GR4 (Casadesus and Olivares, 1979), respectively. Using suppression tests of mutation and mapping function, circular linkage maps of *S. meliloti* 2011, *S. meliloti* Rm41 (Fig.5) and *R. leguminosarum* 300 were compared (Kondorosi *et al.*, 1980).

Many of the genes which are important in symbiosis are located on megaplasmids. Genetic and physical maps of megaplasmids of various rhizobial strains have been reported. In *S. meliloti*, genes required for nodule induction (*nod*), nitrogenase activity (*nif*) and other important functions of nodulation have been mapped on the megaplasmid pSyma (Fig.6), and the genes involved in exopolysaccharide synthesis (*exo, exp*), thiamine synthesis (*thi*) and dicarboxylic acid transport (*dct*), as well as two *fix*

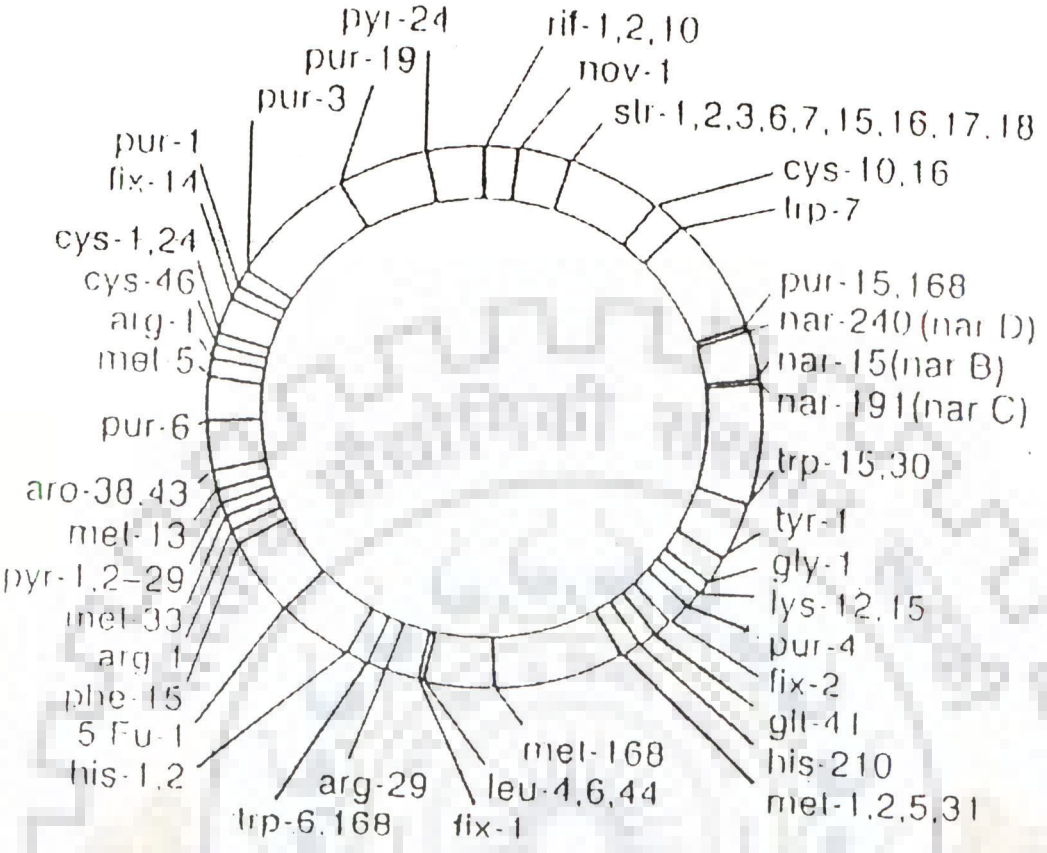

Fig. 5: Chromosomal map of *Sinorhizobium meliloti* Rm41 (based on Kondorsi *et al.*, 1980)

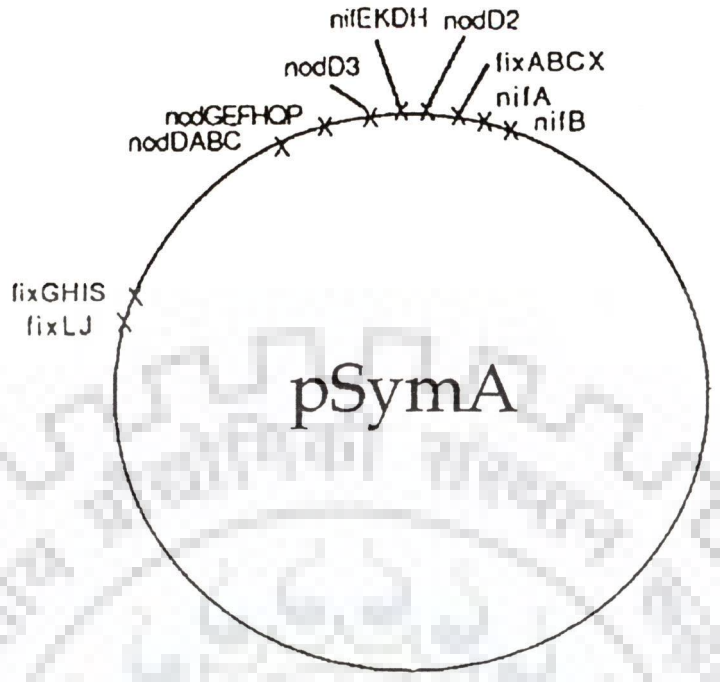

Fig. 6: Genetic map of plasmid pSymA of *Sinorhizobium meliloti* (Glazebrook and Walker, 1991)

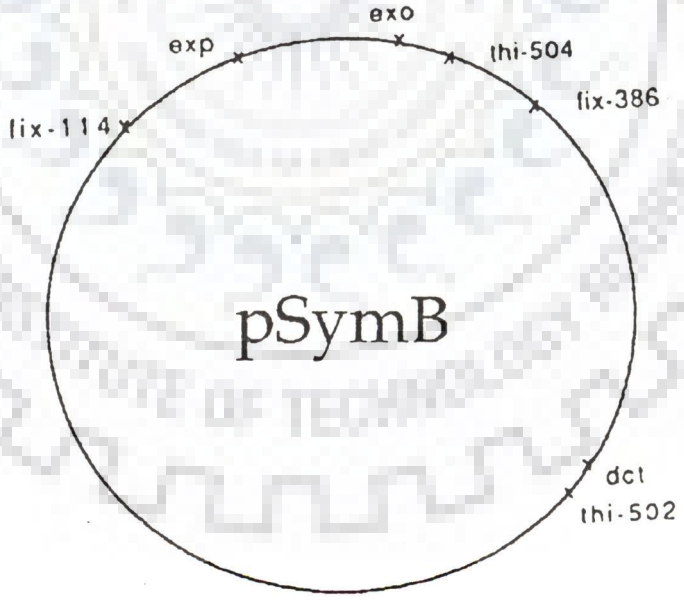

Fig. 7: Genetic map of plasmid pSymB of *Sinorhizobium meliloti* (Glazebrook and Walker, 1991)

genes have been mapped on pSymb (Fig.7) (Banfalvi *et al.*, 1981; Julliot *et al.*, 1984; Kondorosi *et al.*, 1984b; Batut *et al.*, 1985; Glazebrook and Walker, 1989; 1991; Charles and Finan, 1990). Restriction maps have been constructed for many rhizobial species with the use of restriction endonucleases and then separating resulting fragments by pulse field gel electrophoresis. Genetic and physical map of single circular chromosome of *B. japonicum* strain 110 was given by Kundig *et al.* (1993). The physical map of *S. meliloti* 1021 chromosome was reported by Honeycutt *et al.* (1993). The physical map of symbiotic plasmid of *Rhizobium spp.* NGR234 was established by Perret *et al.* (1991). Recently complete nucleotide sequence (5,36,165 base pairs with 416 ORFs) of megaplasmid pNGR234a of *Rhizobium spp.* NGR234 was given by Freiberg *et al.* (1997). These workers demonstrated the presence of new symbiotic loci and signaling mechanisms.

2.4 Role of bacteroidal metabolism in symbiosis

2.4.1 C_4-dicarboxylate transport

It has been shown that rhizobia utilize C_4-dicarboxylates from the plant host for their carbon and energy requirements. The dicarboxylic acid transport (*dct*) genes have been shown to be present on a megaplasmid in *S. meliloti* (Finan *et al.*, 1988; Watson *et al.*, 1988; Watson, 1990). The *dct* region has been characterized in *R. leguminosarum* and *S. meliloti.* It consists of divergently transcribed *dctA* and *dctBD* genes (Engelke *et al.*, 1989; Wang *et al.*, 1989). *dctBD* genes are controlled by *ntrA* gene that codes for sigma factor (Ronson *et al.*, 1987). Recently DctBD-dependent and independent *dctA* expression in *S. meliloti* has been established (Boesten *et al.*, 1998).

2.4.2 Symbiotic role of biosynthetic pathways of amino acids, nucleotide bases and vitamins

During colonization of root and nodule formation and function rhizobia require primary building blocks viz., amino acids, nucleotide bases and vitamins. Rhizobia have the ability to synthesize all these building blocks from nutrient materials; building blocks or their intermediates may also be available to rhizobia from plant source during symbiosis. A few genes involved in the biosynthesis of some amino acids, nucleotide bases and vitamins in rhizobia also appear to play a direct role in symbiosis (Schwinghamer, 1970; Pankhurst *et al.*, 1972; Kerppola and Kahn, 1988b; Noel *et al.*, 1988; Aguilar and Grasso, 1991; Barsomian *et al.*, 1992; Newman *et al.*, 1994; Taté *et al.*, 1999a; 1999b)

2.4.2.1 Amino acid biosynthesis and symbiosis

Arginine auxotrophs of *S. meliloti* strain 104A14 blocked either in ornithine transcarbamylase or arginosuccinate synthase were effective while mutants blocked at early steps were ineffective. It was proposed that ornithine is a precursor for polyamine synthesis and the absence of polyamines leads to a symbiotic defect (Kerppola and Kahn, 1988b).

Histidine auxotrophs of *B. japonicum* failed to induce nodules; histidine supplementation restored nodulating ability of these mutants indicating unavailability of histidine *in planta* (Sadowsky *et al.*, 1986; So *et al.*, 1987; Yadav *et al.*, 1998).

ilv mutants of *Rhizobium fredii* and *S. meliloti* were found to be Nod$^-$ (Kim *et al.*, 1988; Aguilar and Grasso, 1991). Aguilar and Grasso (1991) found that *nodABC* promoter was not activated by luteolin in *S. meliloti* 1021 *ilvC* mutant. They also

proposed the hypothesis that *ilvC* gene has a dual role in isoleucine-valine pathway and conversion of some unknown substrate into a product that is required for the expression of nodulation genes. Addition of isoleucine and valine or their intermediates to the plant nutrient medium could not restore the symbiotic defect. Leucine auxotrophs of *S. meliloti* were found to form ineffective nodules (Truchet *et al.*, 1980; Kerppola and Kahn, 1988b). Truchet *et al.* (1980) reported restoration of symbiotic defect in leucine auxotrophs of *S. meliloti* strain L5-30 by leucine supplementation whereas Kerppola and Kahn (1988b) did not find, such restoration in leucine auxotrophs of *S. meliloti* strain 104A14 by leucine addition.

Methionine auxotrophs have been found to be $Nod^{+}Fix^{+}$ in *R. leguminosarum* (Pain, 1979) and $Nod^{+}Fix^{-}$ in *S. meliloti* 104A14 (Kerppola and Kahn, 1988b). The difference in symbiotic behaviour of the methionine auxotrophs of *R. leguminosarum* and *S. meliloti* may be due to mutations in different genes of the methionine biosynthetic pathway. Recently, *Rhizobium etli metZ* gene has been reported to be essential in nodulation (Taté *et al.*, 1999b). It has been shown that cysteine or glutamate, but not methionine, is supplied by plant nodule cells to *R. etli* microsymbiont (Taté *et al.*, 1997; 1999b).

Kummer and Kuykendall (1989) found that except tryptophan synthase mutants all other tryptophan auxotrophs of *B. japonicum* were Nod^{-}. It seems that the synthesis of indole glycerol phosphate, an intermediate of tryptophan biosynthetic pathway, is essential for nodulation in *B. japonicum*. *trpE* mutants of *S. meliloti* have been found to be $Nod^{+}Fix^{+}$ but nodule histology revealed significant differences from nodule structure of parental strain, moreover these mutants fixed comparatively less nitrogen (Barsomian

et al., 1992; Prasad *et al.*, 2000). Barsomian *et al.* (1992) gave the hypothesis on the basis of the reports of Rioux *et al.* (1986a; 1986b) that anthranilic acid, synthesized by the expression of *trpE* gene, acts as an *in planta* siderophore helping in iron uptake for bacteroidal development. Normal metabolic flow through aromatic amino acid biosynthetic pathway has been found to be required for development of bacteroids in *S. meliloti* (Jelesko *et al.*, 1993). Recently, Taté *et al.* (1999a) have reported that the *trpB* gene of *R. etli* is essential for effective symbiotic interaction.

2.4.2.2 Purine biosynthesis and symbiosis

Purine auxotrophs of *S. meliloti* (Scherrer and Dénarié, 1971; Malek and Kowalski, 1983; Kerppola and Kahn, 1988b; Swamynathan and Singh, 1992; 1995; Gupta, 1996), *R. leguminosarum* (Pankhurst and Schwinghamer, 1974; Pain, 1979), *Rhizobium fredii* HH303 (Kim *et al.*, 1988), *Rhizobium* strain NGR234 (Chen *et al.*, 1985) and *Rhizobium etli* (Noel *et al.*, 1988) have been reported to be defective in symbiosis. The biochemical block in the purine auxotrophs (which were symbiotically defective) isolated by Pankhurst and Schwinghamer (1974) was before 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Chen *et al.* (1985) speculated that two adenine auxotrophs of *Rhizobium* strain NGR234 were symbiotically defective because of EPS overproduction. Purine auxotrophs of *R. etli* were found to elicit pseudonodules on bean plants. These auxotrophs lead to root hair curling and cortical cell divison but did not elicit infection thread formation, and as a result no bacteria could be isolated from resulting pseudonodules (Noel *et al.*, 1988). Supplementation of the root medium with adenosine had no effect on nodule phenotype while addition of AICAR significantly enhanced root nodule development (Noel *et al.*, 1988; Newman *et al.*,

1992). Purine auxotrophs of *R. fredii* HH303 and *R. leguminosarum* 128C56 (bv. *viciae*), each of which has the biochemical block before AICAR, elicited poorly developed and uninfected nodules on their respective hosts. Supplementation of the root environment with AICAR in case of these auxotrophs was effective in enhancing nodulation and promoting infection. The ability of AICAR to promote infection by purine auxotrophs, despite serving as a very poor purine source for these strains, supports the hypothesis that AICAR plays a role in infection other than merely promoting bacterial growth (Newman *et al.*, 1994).

2.4.2.3 Pyrimidine biosynthesis and symbiosis

Relatively less work has been done on the symbiotic role of the biosynthetic pathway of pyrimidines. Scherrer and Dénarié (1971) isolated a pyrimidine auxotroph of *S. meliloti* strain 2011. This auxotroph was found to be ineffective. Two out of fifteen pyrimidine auxotrophs of *R. leguminosarum* strain 3000 were also found to be defective in symbiosis (Pain, 1979). Ndv$^-$ (nodule development defective) phenotype of pyrimidine auxotroph of *R. leguminosarum* bv. *phaseoli* strain CFN42 could not be suppressed by uridine addition to the rooting medium (Noel *et al.*, 1988). Mutations in carbamoyl phosphate synthetase and steps of pyrimidine biosynthesis before orotic acid have been found to result in ineffective symbiosis in *S. meliloti* strain 104A14 (Kerppola and Kahn, 1985; 1988a; 1988b). In all these cases microscopic studies of nodules induced by pyrimidine auxotrophs were not performed. It was unclear whether symbiotic ineffectiveness of pyrimidine auxotrophs was due to unavailability of pyrimidine from plant source or due to lack of pyrimidine intermediate(s), which may be essential for efficient symbiosis. Recently it has been reported that there are some differences in the

internal structures of nodules induced by pyrimidine auxotrophs having biochemical block before and after orotic acid indicating that some of the intermediates/enzymes of pyrimidine biosynthetic pathway may have some role in symbiosis (Vineetha, 1998). However these conclusions were based on the histological studies of nodules induced by only two auxotrophs, one having a block before orotic acid and the other after it. Moreover in each of these mutants the exact position of the biochemical block was not specified.

2.4.2.4 Vitamin biosynthesis and symbiosis

Schwinghamer (1970) and Pankhurst *et al.* (1972) demonstrated the role of riboflavin biosynthesis in bacteroid transformation in *Rhizobium trifolii*. Streit *et al.* (1996) showed that biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *S. meliloti* 1021.

The above literature review shows that several genes having a role in rhizobial-legume symbiosis have been identified and characterized. However symbiotic role of some primary biosynthetic pathways, specifically pyrimidine biosynthetic pathway, of rhizobia has not been fully explored. There is a need to determine if any pyrimidine biosynthetic intermediate(s)/enzyme(s) is (are) involved in symbiotic process. Detailed histological studies of the nodules induced by pyrimidine auxotrophs, having mutations in different pyrimidine biosynthetic genes, of rhizobia are required to determine the role of the genes of pyrimidine biosynthesis in symbiosis.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial strains

The characteristics and sources of bacterial strains and plasmids used/constructed in this study are described in Table 4.

3.1.2 Host cultivar

Alfalfa (*Medicago sativa* cv. T9) seeds were used for plant inoculation test in this study. The seeds were obtained from National Seeds Corporation, New Delhi, India.

3.1.3 Composition of growth media used

3.1.3.1 Growth media for *Sinorhizobium meliloti*

3.1.3.1.1 Complete media

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

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

The pH of the medium was adjusted to 7.0 with 0.1N NaOH solution. To make solid medium 16g agar (HiMedia) was added before autoclaving.

3.1.3.1.1.2 Tryptone yeast extract (TY) swarm plates

Composition of medium was same as TY medium but agar was added @ 3g/litre. TY swarm plates were used to test the production of β-(1→2) glucans.

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

Strains/Plasmids	Relevant Characteristics	Source/Reference
Sinorhizobium meliloti		
AK631	Nod^+, Fix^+, compact colony variant of wild type strain Rm41	Adam Kondorosi
Rmd 201	Spontaneous Sm^r derivative of AK631 (Nod^+ Fix^+)	Khanuja and Kumar (1988)
PP631	AK631(pJB3JI)	Peter Putnoky
ZB178	Rm41 *ade15 rfl sm1*	Z. Banfalvi
ZB201	Rm41 *cys46 rfl 5fu^r sm1*	--- do---
ZB205	Rm41 *ade15 narB rfl 5fu^r*	--- do---
ZB555	Rm41 *cys46 phe15 rfl sm1*	--- do---
ZB556	Rm41 *gly1 met2 ade4 rfl sm1*	--- do---
ZB557	Rm41 *phe15 leu4 rfl sm1*	--- do---
NV1,NV15	Rmd201 *car*::Tn5	This Study
RH33,RH37,RH47	Rmd201 *car*::Tn5	This Lab
NV18,NV21,NV32	Rmd201 *pyrC*::Tn5	This Study
VK12,VK19,VK43,RH7, RH9	Rmd201 *pyrC*::Tn5	This Lab
NV6,NV9,NV12,NV19, NV23,NV26,NV33,NV34, NV37	Rmd201 *pyrE/pyrF*::Tn5	This Study
RH36	Rmd201 *pyrE/pyrF*::Tn5	This Lab
NV4,NV27	Rmd201 *arg*::Tn5	This Study
Escherichia coli		
WA803(pGS9)	Met^- Thi^- Cm^r Km^r	Selvaraj and Iyer (1983)
Plasmids		
pGS9	IncN repP15A Cm^r Km^r	Selvaraj and Iyer (1983)
pJB3JI	Km^r derivative of pR68.45 capable of mobilizing genomic segments of its host, Tc^r Cb^r Nal^r	Brewin *et al* (1980)

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

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

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

3.1.3.1.2 Minimal medium

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

Constituent	Amount used/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 1 litre

The pH of the medium was adjusted to 6.8 with 0.1N NaOH solution. Glucose solution (20% w/v) was prepared and filter sterilized, and 10ml of this solution was

added to 1 litre medium after autoclaving. To make solid medium 16g agar was added before autoclaving.

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

Constituent	Amount used/litre
Tryptone	10g
Yeast extract	5g
NaCl	10g
Distilled water	to make volume 1 litre

The pH of the medium was adjusted to 7.0 by using 0.1N NaOH solution. For solid medium 16g agar was added before autoclaving.

3.1.3.3 Nitrogen free plant nutrient medium for *Medicago sativa*

For plant assays, nitrogen free plant nutrient medium developed by Engelke *et al.* (1987) was used. Twelve stock solutions (A to L) of following composition were prepared.

Solution	Constituent	Amount of constituent used (g)	Distilled water used (ml)
A	K_2HPO_4	2.090	10
B	KH_2PO_4	0.544	10
C	$CaCl_2$	7.351	10
D	$C_6H_5O_7Fe.3H_2O$	0.335	10
E	$MgSO_4$	6.162	10
F	K_2SO_4	4.356	40
G	$MnSO_4$	0.034	20
H	H_3BO_3	0.026	20

I	$ZnSO_4$	0.030	20
J	$CuSO_4$	0.002	20
K	$CaSO_4$	0.006	20
L	Na_2MoO_4	0.006	20

The stock solutions (A to L) were autoclaved separately. To make one litre of plant nutrient medium, 10g of agar was added to 985ml of distilled water and the resulting water agar medium was autoclaved. Four ml of stock solution F (K_2SO_4) and one ml each of remaining stock solutions (A to E and G to L) were added to autoclaved agar solution and pH of the medium was 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.

Autoclaving was always done at 15psi for 20min.

3.1.4 Diluent used

3.1.4.1 Saline

Saline i.e. 0.9% (w/v) NaCl (HiMedia) was used for carrying out serial dilutions and to make cell suspensions.

3.1.4.2 Tween 80

0.02% Tween 80 was used to make cell suspension for cross feeding experiment.

3.1.5 Supplements to media

3.1.5.1 Antibiotics

All antibiotics used were purchased from HiMedia Laboratories. Streptomycin sulphate and kanamycin acid sulphate were dissolved in sterile distilled water while 50% ethanol was used as a solvent for tetracycline hydrochloride and chloramphenicol;

rifampicin was dissolved in dimethylsulphoxide. The final concentrations of different antibiotics used in different media were as follows: 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 before adding to autoclaved media.

3.1.5.2 Nitrogenous bases, amino acids and vitamins

The stock solutions of nitrogenous bases, amino acids and vitamins were made in distilled water and autoclaved separately. Required volumes from stock solutions were added to autoclaved media so as to make final concentrations of 50, 30 and 10μg/ml for amino acids, nitrogenous bases and vitamins, respectively. To determine the nature of auxotrophy of a strain, Holliday pools (Holliday, 1956) with few modifications were used. The composition of modified Holliday pools used in the study were as follows:

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

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

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

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

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

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

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

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

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

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

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

3.1.5.3 Intermediates

The pyrimidine intermediates, viz. carbamoyl phosphate, carbamoyl aspartate, dihydroorotic acid and orotic acid were purchased from Sigma Chemical Company, U.S.A. The arginine intermediates, ornithine monohydrochloride and citrulline, were obtained from s.d.fINE LTd, Mumbai, India. Stock solutions of all intermediates except carbamoyl phosphate, which was dissolved in dimethylsulphoxide, were made in sterile distilled water. All these stock solutions were filter sterilized and added to autoclaved media before pouring to make final concentrations of 30 and 50μg/ml for pyrimidine and arginine intermediates, respectively.

3.1.5.4 Sugars and dicarboxylic acids

All sugars and dicarboxylic acids were purchased from HiMedia Laboratories, Mumbai, India and each of these was added at a concentration of 2g/litre to the RMM (without glucose) medium as a sole carbon source before autoclaving. Sugars used in the study were lactose, galactose, maltose, arabinose, xylose, mannitol, sucrose and fructose; dicarboxylic acids used were malate, aspartate, glutamate, succinate and fumarate.

3.1.5.5 Sodium deoxycholate (DOC)

DOC (HiMedia) was added to MSY medium @ 1mg/litre before autoclaving.

3.1.5.6 Dyes

Aniline blue (HiMedia), calcofluor white (Sigma) and congo red (HiMedia) were added to MSY medium @ 0.02% (w/v), each before autoclaving. Methylene blue (0.01%) (w/v) and toluidine blue (1%) (w/v) from 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) from HiMedia was incorporated in the MSY medium @ 2.5mg/ml before autoclaving.

3.1.5.8 Salt

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

3.1.6 Reagents for orotic acid accumulation studies

3.1.6.1 Orotic acid

Stock solution of orotic acid (Sigma) (0.01mg/ml, pH 3.0) was prepared.

3.1.6.2 Monosodium citrate buffer (pH 2.5)

Citric acid (21.04g) was dissolved in 200ml of 1N NaOH and volume was made 1000ml with distilled water. 35.4ml of this solution was taken and 0.1N HCl was added to make the volume 100ml.

3.1.6.3 Saturated aqueous bromine solution

3.1.6.4 Sodium mercaptoacetate solution - 0.7% (v/v)

3.1.6.5 p-dimethyl amino benzaldehyde reagent

This reagent was made by dissolving p-dimethyl amino benzaldehyde in propanol so as to make final concentration of 2.5% (w/v).

3.1.7 Composition of solutions for light and electron microscopy of alfalfa root nodules

3.1.7.1 Requirements for preparation of blocks

(a) 0.2M phosphate buffer

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

(b) Fixative

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

12.5ml	8% (w/v) paraformaldehyde (Sigma) (Added 2g of paraformaldehyde in 25ml distilled water and heated the solution 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.) **Secondary fixative or post fixative i.e. 2% (w/v) OsO_4**

Stock solution of OsO_4 (2% w/v) was made by dissolving 1g of OsO_4 in 50ml of water. 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

Araldite CY212	10ml

Hardner (Dodecenyl succinic anhydride, DDSA; HY 964) 10ml

Accelerator (Tridimethyl aminomethyl phenol, DMP30; DYO64) 0.4ml

Plasticizer (Dibutylphthalate) 1.0ml

All ingredients were mixed and vigorously stirred.

3.1.7.2 Solutions for staining ultrathin sections for electron microscopy

3.1.7.2.1 Uranyl acetate solution

A saturated solution of uranyl acetate was prepared by adding excess of uranyl acetate to 10ml of 50% ethanol in a 15ml centrifuge tube. Centrifugation was done at 5000rpm for 2min and the supernatant transferred to another tube. This tube was tightly stoppered and stored at 4°C in a refrigerator.

3.1.7.2.2 Lead citrate solution

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

3.2 METHODS

3.2.1 General bacteriological procedures

3.2.1.1 Bacterial culture conditions

The strains of *Sinorhizobium meliloti* and *Escherichia coli* were maintained by culturing on slants of TY and LB agar media, respectively and storing these slants at 4°C. These strains were also maintained as stab cultures on respective media. Whenever these strains were required, a loopful of bacterial culture was inoculated into 10ml of TY (for

S. meliloti) or LB (for *E. coli*) liquid medium and kept on orbital shaker operating at speed 120rpm. The incubation temperatures for *S. meliloti* and *E. coli* were 28°C and 37°C, respectively. For re-culturing bacteria, 0.1ml of overnight grown culture was transferred to 10ml of respective liquid medium and incubated for 12-14hrs. The incubation times to obtain log phase cultures of *S. meliloti* and *E. coli* were 24-32hrs and 14-16hrs, respectively. For obtaining single colonies of *S. meliloti*, incubation time was 3 days on complete medium and one week on minimal medium.

3.2.1.2 Serial dilutions

Serial dilutions of bacterial cultures were done in saline i.e. 0.9% (w/v) NaCl. For obtaining 10^{-2} dilution, 0.1ml of neat culture was added to a screw cap glass tube containing 9.9ml saline. After proper mixing, 0.1ml of bacterial suspension from this tube was added to another tube containing 9.9ml saline so as to have 10^{-4} dilution. Further dilutions, as per the need of the experiment, were similarly done.

3.2.1.3 Bacterial conjugal matings

Bacterial matings were done according to Kondorosi *et al.* (1977). Log phase cultures (0.05ml of each) of both recipient and donor strains were mixed in 1:1 ratio (3:1 for Tn5 mutagenesis) and spread on TY agar plates. These plates were incubated at 28°C for 24hrs.

3.2.2 Isolation and screening of auxotrophs

3.2.2.1 Random transposon Tn5 mutagenesis

E. coli WA803 (pGS9) and *S. meliloti* Rmd201 strains were grown to log phase in TY liquid medium. *S. meliloti* (0.075ml) and *E. coli* (0.025ml) cultures were mixed and spread using sterile glass rod on half the surface of TY agar medium in a Petri dish.

Remaining half of the TY agar surface was divided into two halves; 0.05ml of Rmd201 culture was spread on one half and 0.05ml of WA803 culture on second half. These plates were incubated at 28°C for 24hrs. The growth obtained after incubation was scrapped and suspended in 2ml saline. The suspension was thoroughly mixed and 0.1ml of it was spread on TY agar plates containing streptomycin (100μg/ml) and kanamycin (400μg/ml). The donor (0.05ml) and recipient (0.05ml) suspensions were also plated on selective plates as controls. These plates were incubated at 28°C for five days. Fifty colonies were streak purified on TY+Km^{400}+Sm^{100} agar plates. All kanamycin resistant (Km^r) transconjugants were checked for the absence of vector encoded chloramphenicol resistance to confirm loss of suicidal plasmid pGS9. The above cross was repeated 147 times; 50 Km^r transconjugants were selected from each cross.

3.2.2.2 Screening for auxotrophs by replica plating method

The Tn5-induced Km^r transconjugants were streaked by sterile toothpicks on RMM and TY+Km^{400}+Sm^{100} agar plates. These plates were incubated at 28°C for 3 days and growth was observed after every 12hrs. The Tn5 derivatives showing growth on TY+Km^{400}+Sm^{100} but not on RMM agar plates were selected as auxotrophs. The selected Tn5 derivatives were streak purified on TY+Km^{400}+Sm^{100} agar plates and maintained for determining nature of auxotrophy.

3.2.3 Determination of the nature of auxotrophy

A loopful culture of each auxotroph was suspended in a drop of saline on a sterile glass slide and this suspension was patched on RMM agar medium + Holliday pools (one pool at a time). Incubation was done at 28°C for 5 days. The nature of auxotrophy was determined on the basis of growth patterns on Holliday pools. The auxotrophic

requirement(s) of each strain was further confirmed by streaking on RMM agar plates supplemented with suspected nutrient(s).

3.2.4 Location of biochemical block in each auxotroph

3.2.4.1 Intermediate feeding studies

Uracil, uracil + arginine and arginine auxotrophs were streaked on RMM agar plates supplemented with carbamoyl phosphate, carbamoyl aspartate, dihydroorotic acid, orotic acid and uracil (one at a time). These auxotrophs were also streaked on RMM agar + arginine + all the above mentioned intermediates (one at a time) of pyrimidine pathway. RMM + arginine/ornithine/citrulline or RMM + uracil + arginine/ornithine/citrulline agar plates were also streaked with these auxotrophs. The parental strain was streaked on each plate as a control. The growth was recorded after 3-5 days of incubation at 28°C.

3.2.4.2 Estimation of orotic acid accumulation

A sample of log phase culture (10ml) of each uracil and uracil + arginine auxotroph was centrifuged at 5000rpm for 10min. The pellet was washed twice with liquid RMM (2ml), re-suspended in 10ml of this medium and incubated at 28°C on an orbital shaker (speed 120rpm) for 48 hrs. The culture was centrifuged at 10,000rpm for 10min. To 1ml of the supernatant, 2ml of 5% monosodium citrate solution (as a buffer at pH 2.5) and 0.5ml of saturated bromine solution were added. After 10sec, 0.5ml of 0.7% sodium mercaptoacetate solution was added to decolourize excess bromine and the solution obtained was heated for 3min at 56°C. Finally, 2ml of 2.5% p-dimethyl aminobenzaldehyde solution in propanol was added and the resulting solution was read at 480nm against distilled water within 30min.

3.2.4.3 Cross feeding assays

Cell suspensions of three auxotrophs (uracil/uracil + arginine) were streaked adjacent to each other (like sides of three equilateral triangles facing each other) on RMM agar plate containing minimal (2μg/ml) amount of nutritional requirement(s); the cell suspension of parental strain was also streaked on one side as a control. Incubation was done at 28°C and growth pattern was recorded after 4-5 days of incubation. The other uracil and uracil + arginine auxotrophs, in combinations of three, were similarly streaked. Cells of uracil/uracil + arginine auxotrophs were also treated with 0.02% (v/v) Tween 80 and streaked, in combinations of three, as mentioned above.

3.2.5 Growth characteristics

The growth pattern of each auxotroph (uracil/uracil + arginine/arginine) used during this study was compared to that of the parental strain. A sample of the log phase culture (0.2ml) of each strain was inoculated into 20ml of liquid MSY medium. These cultures were incubated at 28°C on an orbital shaker (speed 120rpm). Samples of each culture were removed from zero to 24hrs at intervals of 4hrs; each sample was 1.5ml and time of inoculation was taken as zero. O.Ds. of these samples were recorded at 600nm; uninoculated liquid MSY medium was taken as a control. The generation time for each auxotroph was calculated using standard graphical method (O.D. vs. time plot).

3.2.6 Pleiotropic effects of Tn5 insertions in auxotrophs

3.2.6.1 Production of cell surface molecules

3.2.6.1.1 Test for production of lipopolysaccharides (LPS)

All auxotrophs (uracil, uracil + arginine and arginine) and the parental strain were streaked on MSY agar plates containing 1mg/ml of sodium deoxycholate (DOC). The

growth of the strains was recorded after incubation at 28°C for 3 days. Strains which showed growth on MSY + DOC agar plates were considered to be LPS producing (Swamynathan and Singh, 1995).

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

The binding of congo red dye and calcofluor white to rhizobial cells indicates the presence of cellulose fibrils (Kneen and LaRue, 1983) and succinylated exopolysaccharides (EPS I) (Leigh *et al.*, 1987), respectively, on the surface of these cells. The strains producing cellulose fibrils form red colonies on medium containing congo red while EPS I-producing strains show fluorescence under ultra violet (UV) light. MSY agar plates containing 0.02% (w/v) congo red and calcofluor (one at a time) were prepared. All auxotrophs (uracil, uracil + arginine and arginine) and the parental strain were streaked on these plates. These plates were incubated at 28°C for 3 days. Congo red plates were observed for red colonies in normal light whereas calcofluor plates were observed for fluorescence in UV light.

3.2.6.1.3 Test for production of β-glucans

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

Strains which produce β-(1→3) glucans form blue colonies on medium containing aniline blue (Nikanishi *et al.*, 1976). All uracil, uracil + arginine and arginine auxotrophs along with parental strain (as a control) were streaked on MSY agar plates containing 0.02% (w/v) aniline blue. These plates were incubated at 28°C for 3 days. After incubation, the plates were examined under normal light for blue colonies.

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

The production of β-(1→2) glucans can be assayed on swarm plates (Geremia *et al.*, 1987). TY swarm plates containing 0.3% (w/v) agar were spotted with a loopful of cell suspension of each auxotroph (uracil, uracil + arginine and arginine) and the parental strain. These plates were incubated at 28°C and the spotted strains were examined for swarming after 2-3 days of incubation.

3.2.6.2 Utilization of sugars and dicarboxylic acids by auxotrophs

Each auxotroph (uracil/ uracil + arginine/arginine) was streaked on RMM agar medium (without glucose) supplemented with the nutritional requirement(s) of the auxotroph and a sugar (lactose, galactose, maltose, arabinose, xylose, mannitol, sucrose or fructose)/dicarboxylic acid (malate, aspartate, glutamate, succinate or fumarate); the parental strain was also streaked, as a positive control, on this medium. Incubation was done at 28°C and growth was observed during 3-5 days of incubation.

3.2.6.3 Salt tolerance of auxotrophs

All auxotrophs (uracil, uracil + arginine and arginine) and the parental strain Rmd201 were streaked on MSY agar plates 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 media containing same NaCl concentrations. The flasks were incubated at 28°C on an orbital shaker (speed 120rpm) for 2-3 days and optical density (O.D.) of each culture was recorded using spectrophotometer; O.D. of the uninoculated control was also recorded.

3.2.6.4 Acid tolerance of auxotrophs

MSY agar and MSY liquid media of pH 4.0, 5.0, 6.0 and 7.0 were prepared using 1N HCl. Auxotrophs (uracil, uracil + arginine and arginine) and the parental strain were streaked on plates and inoculated in liquid media. Incubation was done at 28°C for 3 days; liquid cultures were incubated on an orbital shaker (speed 120rpm).

3.2.6.5 pH changes during growth of auxotrophs

The auxotrophs (uracil, uracil + arginine and arginine) and the parental strain were streaked on MSY agar plates containing bromothymol blue (25mg/litre). These plates were incubated at 28°C for 3 days. The production of blue, green and yellow colour of medium was used as an indicator of alkaline, neutral and acidic pH, respectively (Vincent, 1970). This test was also performed using liquid MSY medium containing bromothymol blue (25mg/litre).

3.2.7 Reversion analysis

Uracil, uracil + arginine and arginine auxotrophs were grown to late log phase in liquid TY medium at 28°C on an orbital shaker (speed 120rpm). A sample of each culture (10ml) was centrifuged at 5000rpm for 10min. After discarding the supernatant, the pellet was washed twice with liquid RMM medium (2ml each time) and re-suspended in liquid RMM medium to obtain about 10^9 cells/ml. A known number of cells of each strain were spread on RMM agar plates and these plates were incubated at 28°C. Prototrophic colonies were counted after 5 days of incubation and spontaneous reversion frequency was calculated. The excision of Tn5 in these prototrophs was determined by replica plating on TY plates containing kanamycin (400μg/ml).

3.2.8 Construction of donor strains of auxotrophs

Each auxotroph (uracil/uracil + arginine/arginine) of *S. meliloti* Rmd201 as a recipient was conjugated with the donor strain *S. meliloti* AK631 (pJB3JI) on a TY agar plate to transfer genome mobilizing plasmid pJB3JI (Tc^r). The transconjugants harbouring pJB3JI were selected on TY agar plate containing tetracycline (15μg/ml) and kanamycin (400μg/ml). Five transconjugants were purified for single colonies on the above selective medium. The purified transconjugants were used as donors in the subsequent experiments.

3.2.9 Linkage of Tn5 insertions to auxotrophy

The linkage of Tn5 insertion to auxotrophy was determined by mobilizing Tn5-containing genomic fragment of an auxotroph with the help of genome mobilizing plasmid pJB3JI and subsequently checking for donor's auxotrophy in Km^r transconjugants. The donor strain of each auxotroph (uracil/uracil + arginine/arginine) was conjugated with the recipient strain *S. meliloti* ZB555. Fifty Km^r transconjugants were selected on TY agar plates containing rifampicin (40μg/ml) and kanamycin (400μg/ml). The kanamycin resistant transconjugants were checked for donor's auxotrophy as described earlier.

3.2.10 Genetic mapping of Tn5 insertions in uracil and uracil + arginine auxotrophs

Transposon Tn5 insertion in each auxotroph (uracil/uracil + arginine) was mapped by using six mapping strains viz. ZB178, ZB201, ZB205, ZB555, ZB556 and ZB557 of *S. meliloti*. The donor strain of each auxotroph was mated with six mapping strains as recipients, separately. Fifty Km^r transconjugants from each mating were selected on TY agar plate containing rifampicin (40μg/ml) and kanamycin (400μg/ml). The co-transfer

of selected donor marker with each unselected recipient marker (Table 3) was determined by patching transconjugants from each cross on appropriate selective plates.

3.2.11 Characteristics of plants inoculated with auxotrophs

The plant inoculation tests were carried out in 20 x 2.5cm glass tubes. These tubes were plugged with cotton and autoclaved. The nitrogen free plant nutrient medium-containing agar was prepared as described earlier and 25ml of this medium at about 45°C was transferred to each tube under sterile conditions. These tubes were then plugged and immediately placed in slanting position. The supplements to the plant nutrient medium were filter sterilized and added to the autoclaved medium before transferring this medium to tubes. The nutritional supplements were the same as used in intermediate feeding studies.

The required quantity of clean and undamaged seeds of alfalfa (*Medicago sativa* cv. T9) was taken. These seeds were soaked in sterile distilled water for 20min and surface sterilized by treating with 0.1% $HgCl_2$ for 1min followed by 1min treatment with absolute alcohol. The seeds were then washed five times with sterile distilled water and spread on 1% agar plates for germination. These plates were wrapped in black paper and incubated at 25°C in dark. Two 2-days old seedlings were transferred to each tube.

The tubes containing seedlings were inoculated with the parental strain Rmd201 and auxotrophs (uracil, uracil + arginine and arginine) of *S. meliloti* Rmd201. Ten-ml of log phase culture (in liquid TY medium) of a rhizobial strain was centrifuged at 5000rpm for 10min. The pellet was washed twice with sterile distilled water and suspended in 5ml of sterile distilled water; 0.5ml of this suspension was inoculated in each tube containing seedlings. The lower portion of each tube was wrapped in black paper to protect the roots

from direct exposure to light. These tubes were placed in a plant growth chamber in which a temperature of 25°C was maintained and 2000 lux light was provided. The light and dark periods given to the plants were 16 and 8hrs, respectively. The plants were removed from tubes after 6 weeks and data on nodule characteristics (nodule appearance/shape/size/colour/location), mean plant height and mean dry plant weight were recorded. Dry plant weight was determined by drying plants in an oven at 85°C for 72hrs and then weighing these dry plants.

3.2.12 Nodule occupancy tests

All nodules from a plant were collected for re-isolating rhizobia. These nodules were surface sterilized (as described earlier) and crushed in a drop of saline. The resulting suspension was diluted in saline to obtain 10^{-1} and 10^{-2} dilutions. 0.1ml of neat suspension and 0.1ml of each dilution were spread on TY agar plates containing 100μg/ml of streptomycin; three replicates were used for neat suspension and each of the two dilutions. These plates were incubated at 28°C for 3 days. All colonies thus obtained were patched on TY + Km^{400} + Sm^{100}, RMM + Km^{400} + auxotrophic requirement(s) and RMM agar plates. All these plates were incubated at 28°C for 3 days. The colonies that showed growth on RMM but not on TY + Km^{400} + Sm^{100} and RMM + Km^{400} + auxotrophic requirement(s) plates, were considered to be prototrophic revertants.

3.2.13 Light microscopy (LM) for observing infection thread formation

Root hair deformation and infection thread formation were observed after 4 days of inoculation (Taté *et al.*, 1999b). Root portions of plants inoculated with auxotrophs (uracil, uracil + arginine and arginine) and the parental strain were removed, washed with sterile water and cut to obtain 1cm long pieces. These root pieces were stained with

methylene blue (0.01% w/v in distilled water) for 15min and washed with two changes of sterile water. These were then placed on a sterile glass slide and, after covering with a cover slip, examined at 40X magnification under light microscope (Leica *DM* LB).

3.2.14 Inoculation of plants with prototrophic revertants

Alfalfa plants (10 plants for each revertant) were inoculated (as described earlier) with five revertants of each auxotroph, obtained during nodule occupancy test, to establish that the symbiotic defect was indeed due to a particular auxotrophy. The data on the morphological features of each plant were recorded six weeks after inoculation.

3.2.15 Linkage of Tn5 insertion to symbiotic defect

Transposon Tn5 from each auxotroph (uracil/uracil + arginine/arginine) was transferred into *S. meliloti* recipient strain ZB555 (as described earlier) and five Km^r transconjugants thus obtained were inoculated on alfalfa plants (10 plants for each transconjugant) to confirm that the symbiotic defect was due to the said auxotrophy(ies). The data on the morphological parameters of each plant were recorded after six weeks of inoculation.

3.2.16 Microscopic studies of nodule sections

Nodules induced by the parental strain and pyrimidine auxotrophic strains, NV1, NV15, NV18, NV32, NV6 and NV9, were collected from plants after six weeks of inoculation; 10 nodules were taken for each strain. Each nodule was longitudinally cut into two halves. One half was used to determine nodule occupancy while another half was processed for histological studies. Nodule occupancy could not be checked for some very small nodules which were selected for histological studies; in such cases nodule occupancy of a similar nodule from the same plant was checked.

3.2.16.1 Primary fixation by immersion

The nodules, after washing with 2-3 changes of sterile distilled water, were transferred to Karnovsky fixative in 5ml glass vials. 0.1% (w/v) acrolein was added to the fixative to facilitate penetration of the fixative. The vials were placed in a vacuum chamber to immerse nodule specimens in fixative. The vials were kept at 4°C for 24hrs for penetration of fixative.

3.2.16.2 Preparation of specimen block

3.2.16.2.1 Washing of nodule samples

Samples were removed from the primary fixative, washed in 0.1M phosphate buffer and left overnight in the same buffer at 4°C.

3.2.16.2.2 Secondary fixation of nodule samples

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

3.2.16.2.3 Dehydration of nodule samples

The samples were passed through a graded acetone series, consisting of 30, 50, 70, 80, 90 and 95% acetone, to remove water. A sample was kept for 135min in each of the acetone concentrations followed by keeping for 15min in dry acetone. Finally two changes of 30min each in dry acetone were given. The process was carried out at room temperature.

3.2.16.2.4 Clearing acetone from nodule samples

The nodule samples were passed through two changes in toluene of 30min each at room temperature. This was done since clearing off acetone from the samples was necessary for the subsequent step.

3.2.16.2.5 Infiltration with liquid resin

Infiltration mixtures were prepared from embedding medium and toluene in following combinations (a) 1:3 parts, (b) 2:2 parts and (c) 3:1 parts. The nodule samples were removed from toluene and, after one change in mixture 'a', left overnight in the same mixture. Then the samples were kept in mixture 'b' for 1hr followed by keeping in mixture 'c' for also 1hr (under vacuum). Finally two changes of 1hr each in pure embedding medium at 50°C were given.

3.2.16.2.6 Embedding of nodule samples

Flat embedding of nodule samples was done in araldite embedding medium using gelatin blocks. Orientation of nodules, to facilitate subsequent longitudinal sectioning, was done before polymerization of the embedding medium.

3.2.16.2.7 Polymerization of araldite medium

Embedding blocks were kept at 50°C for 24hrs and then at 60°C for 48hrs in an oven. These blocks thus formed were used for sectioning.

3.2.16.3 Trimming of blocks

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

3.2.16.4 Preparation of semithin sections for optical microscopy

The block holder was attached to Ultracut E Microtome (C. Reichert, Austria OmU3) and semithin sections of 500-2000nm thickness were cut using a glass knife. The sections floating in distilled water in attached metallic trough were removed using a thin brush and placed on a clean glass slide. The slide was placed on a hot plate (at 80°C) for 30sec for drying. The sections were stained with 1% (w/v) toluidine blue for 1min,

washed with distilled water and observed under light microscope (Leica *DM* LB). The entire section or the area of interest of the section was photographed.

3.2.16.5 Preparation of ultrathin sections for electron microscopy

After light microscopic examination of a section, area of this section to be examined under transmission electron microscope was marked and blocks were trimmed with hand. Ultrathin sections (60-90nm thick) were cut using Ultracut E Microtome (C. Reichert, Austria OmU3). These sections were lifted from below on copper grids (200 mesh size) and coating of 3% formvar (polyvinyl formaldehyde) in ethylene dichloride and carbon was done by evaporation in vacuum.

3.2.16.5.1 Double staining (with uranyl acetate and lead citrate) of ultrathin sections

To obtain good contrast double staining with uranyl acetate and lead citrate was done. The grids carrying the sections were placed in 0.5ml of uranyl acetate solution on a clean watch glass for 15min. As staining is effective in dark, a cardboard cover was placed on the watch glass. The grids were washed with 50% ethanol and two lots of double distilled water, and carefully dried on a filter paper. These grids were placed in 0.5ml of lead citrate solution on clean watch glass for 10 min. After removing from this solution the grids were washed with 0.1N NaOH and two lots of double distilled water, and dried subsequently. These grids were stored in a grid holder at room temperature and viewed under transmission electron microscope (PHILIPS EM300). The areas of interest were photographed.

3.2.17 Statistical analysis

The data related to characteristics of plants inoculated with parental strain and auxotrophs were statistically analysed using standard statistical methods. The mean of ten

plants of a treatment was used. All values have been expressed as mean ± standard error of mean (SE). Significance between a control value and an experimental value was ascertained using standard 't' test.

Chapter 4

RESULTS

Random transposon Tn5 mutagenesis was carried out to generate pyrimidine auxotrophs of *Sinorhizobium meliloti* strain Rmd201 (spontaneous Sm^r derivative of strain AK631, which is a compact colony variant of wild type strain Rm41). Biochemical, genetic and symbiotic studies of these auxotrophs were done. Histology of the nodules induced by these auxotrophs was also studied. The results of these studies are presented here.

4.1 Isolation of auxotrophs by random transposon Tn5 mutagenesis

The suicide plasmid vector pGS9 from *E. coli* WA803 (pGS9), successfully delivered transposon Tn5 to *S. meliloti* Rmd201 genome by conjugation. Transposon Tn5-induced Km^r transconjugants were generated at a frequency of 3.67×10^{-5} per recipient whereas spontaneous Km^r frequency in Rmd201 strain was much lower i.e. $< 10^{-8}$. From 147 crosses a total of 7,350 Km^r transconjugants were obtained and streak-purified on TY + Km^{400} + Sm^{100} plates. All Km^r transconjugants were chloramphenicol sensitive indicating disintegration of delivery vector pGS9. Only 50 Km^r Tn5 derivatives were selected from each cross to avoid siblings. The Km^r transconjugants so obtained were patched on TY + Km^{400} + Sm^{100} and RMM agar plates. Thirty-seven transconjugants did not grew on minimal medium RMM but grew on TY + Km^{400} + Sm^{100} agar plates. These transconjugants, presumed to be auxotrophs, were streak purified and maintained.

4.2 Determination of nutritional requirements of auxotrophs

The growth patterns of 37 auxotrophs on modified Holliday pools revealed the nutritional requirements of these auxotrophs. The nutritional requirements of the auxotrophs were as follows (number of auxotrophs is given in brackets): cysteine/methionine (9), methionine (3), tryptophan + tyrosine + phenylalanine (1), tryptophan (2), isoleucine and valine (1), adenine (2), adenine + thiamine (3), arginine (2), uracil (12) and uracil + arginine (2). Uracil, uracil + arginine and arginine auxotrophs were used for further studies; such auxotrophs isolated by other researchers in the lab were also included for detailed investigations (Table5).

4.3 Location of biochemical block in uracil, uracil + arginine and arginine auxotrophs

4.3.1 Intermediate feeding and orotic acid accumulation studies

Based on intermediate feeding (Table 6) and orotic acid accumulation studies, uracil and uracil + arginine auxotrophs, were divided into three groups:

(i) *car* mutants (NV1, NV15, RH33, RH37, RH47): Required both uracil + arginine for their growth; grew on arginine/citrulline + any intermediate of the pyrimidine biosynthetic pathway; did not accumulate orotic acid in liquid RMM medium.

(ii) *pyrC* mutants (NV18, NV21, NV32, VK12, VK19, VK43, RH7, RH9): Grew on orotic acid/dihydroorotic acid but not carbamoyl aspartate/carbamoyl phosphate supplemented RMM agar plates; did not accumulate orotic acid in liquid RMM medium.

(iii) *pyrE/pyrF* mutants (NV6, NV9, NV12, NV19, NV23, NV26, NV33, NV34, NV37, RH36): Grew only on uracil supplemented RMM agar plate; accumulated orotic acid in liquid RMM medium.

Table 5: List of auxotrophic strains of *Sinorhizobium meliloti* used in this study

S.No.	Strain	Auxotrophy
1.	NV6	URACIL
2.	NV9	URACIL
3.	NV12	URACIL
4.	NV18	URACIL
5.	NV19	URACIL
6.	NV21	URACIL
7.	NV23	URACIL
8.	NV26	URACIL
9.	NV32	URACIL
10.	NV33	URACIL
11.	NV34	URACIL
12.	NV37	URACIL
13.	RH7	URACIL
14.	RH9	URACIL
15.	RH36	URACIL
16.	VK12	URACIL
17.	VK19	URACIL
18.	VK43	URACIL
19.	NV1	URACIL + ARGININE
20.	NV15	URACIL + ARGININE
21.	RH33	URACIL + ARGININE
22.	RH37	URACIL + ARGININE
23.	RH47	URACIL + ARGININE
24.	NV4	ARGININE
25.	NV27	ARGININE

Table 6: Growth responses of uracil, uracil + arginine and arginine auxotrophs of *Sinorhizobium meliloti* to different intermediates of pyrimidine/arginine biosynthetic pathway

STRAIN	Auxotrophy	RMM	RMM+													
			U	CP	CA	DHO	OA	A	ORNT	CITR	URA+ ARG	ORNT +URA	CITR+ URA	ARG +CA	ARG+ DHO	ARG +OA
Rmd201		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NV1	U+A	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+
NV15	U+A	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+
RH33	U+A	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+
RH37	U+A	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+
RH47	U+A	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+
NV18	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
NV21	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
NV32	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
VK12	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
VK19	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
VK43	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
RH7	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
RH9	U	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+
NV6	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV9	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV12	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV19	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV23	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV26	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV33	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV34	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV37	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
RH36	U	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-
NV4	A	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
NV27	A	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+

Abbreviations: U, uracil; CP, carbamoyl phosphate; CA, carbamoyl aspartate; DHO, dihydroorotic acid; OA, orotic acid; A, arginine; ORNT, ornithine; CITR, citrulline; RMM, Rhizobial minimal medium. ***Concentrations used*:** Uracil/uracil intermediate, 30μg/ml; arginine/arginine intermediate, 50 μg/ml.

Arginine auxotrophs grew on ornithine/citrulline supplemented minimal medium indicating that the positions of biochemical blocks in these auxotrophs were before ornithine. These auxotrophs were called *arg* mutants. The position of biochemical block in each *pyr/car/arg* mutant is shown in Fig.8.

4.3.2 Cross feeding assays

None of the uracil/uracil + arginine auxotrophs cross-fed any other uracil/uracil + arginine auxotroph. Cross feeding was also tried by treating cells, before streaking, with 0.02% (v/v) Tween 80. Even after this treatment no cross feeding was observed.

4.4 Growth characteristics

No significant differences between growth (optical density) of auxotrophs (uracil, uracil + arginine and arginine) and the parental strain Rmd201 was observed during entire logarithmic phase. The generation time of auxotrophs did not vary significantly from 2hrs 50min (i.e. generation time of parental strain).

4.5 Pleiotropic effects of Tn5 insertions in auxotrophs

4.5.1 Production of cell surface molecules

All auxotrophs (uracil, uracil + arginine and arginine) and the parental strain Rmd201 showed growth in presence of sodium deoxycholate, took up the congo red dye, fluoresced in presence of calcofluor white under UV light and showed swarming on swarm plates indicating the normal production of lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β-(1→2) glucans, respectively. All these auxotrophs and the parental strain Rmd201 did not show binding with aniline blue dye in medium

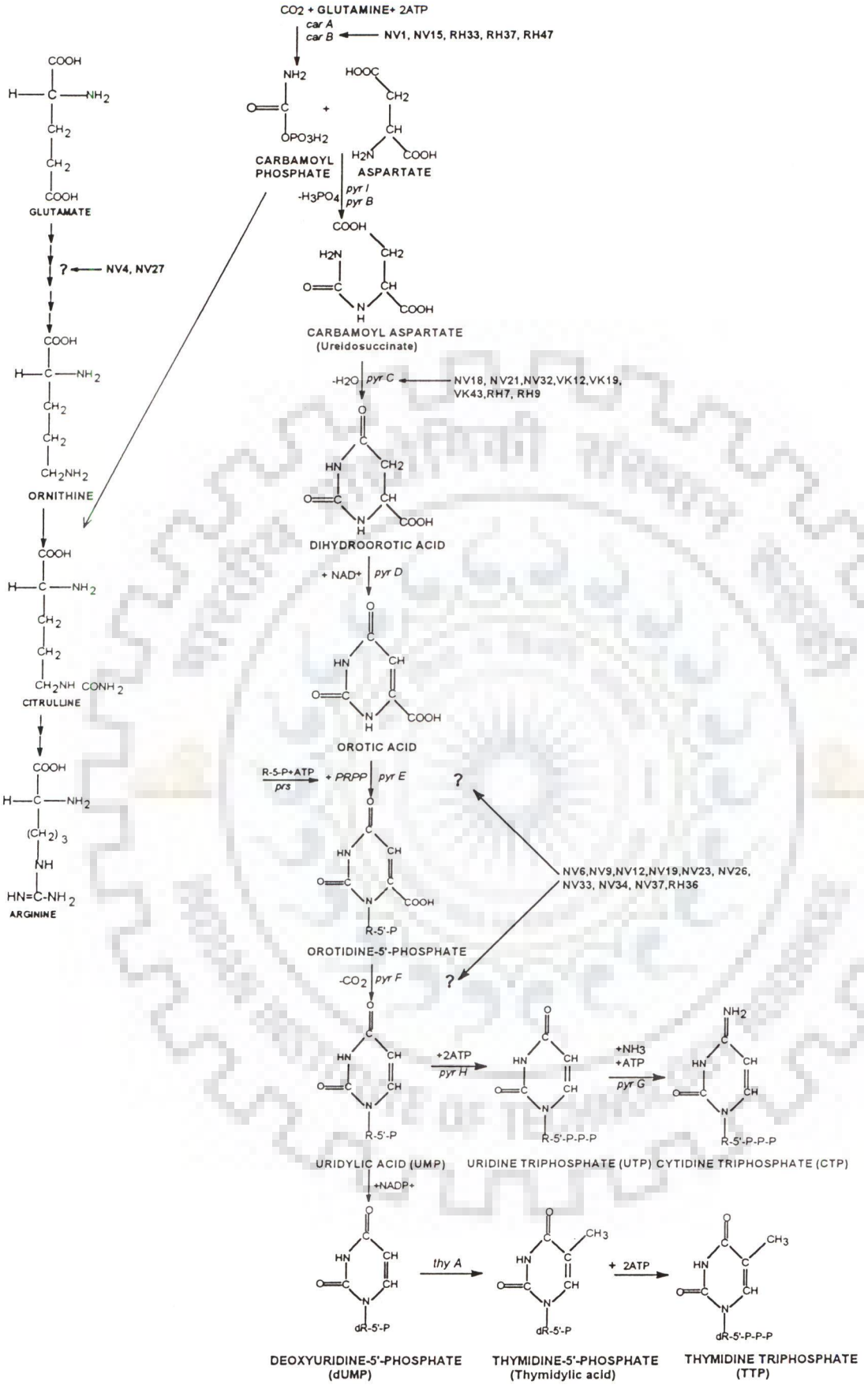

Fig. 8. The pathways of the synthesis of pyrimidines and arginine (based on Moat and Foster, 1995) showing the positions of mutation in the uracil, uracil + arginine and arginine auxotrophs of *Sinorhizobium meliloti* Rmd201 .

which indicated absence of β-(1→3) glucans production. These results showed normal production of cell surface molecules in auxotrophs like the parental strain.

4.5.2 Utilization of sugars and dicarboxylic acids

All uracil, uracil + arginine and arginine auxotrophs, like the parental strain Rmd201, showed normal growth on rhizobial minimal medium (RMM) in which glucose was replaced by any other sugar (lactose, galactose, maltose, arabinose, xylose, mannitol, sucrose or fructose) or dicarboxylic acid (malate, asparate, glutamate, succinate or fumarate). These results indicate that auxotrophs can utilize these sugars and dicarboxylic acids as a sole carbon source like the parental strain.

4.5.3 Salt and acid tolerance

All uracil, uracil + arginine and arginine auxotrophs showed growth, like the parental strain Rmd201, on MSY agar medium and in liquid MSY medium containing 1, 1.5, 2, 2.5 and 3% (w/v) NaCl; the parental strain and auxotrophs did not grow when 3.5 and 4.0% concentrations of NaCl were used. Similarly these auxotrophs like the parental strain grew at pH 6.0.

4.5.4 pH changes by auxotrophs

All auxotrophs (uracil, uracil + arginine and arginine) and the parental strain Rmd201, when grown on MSY agar medium or in liquid MSY medium containing bromothymol blue, changed the colour of the dye from blue to yellow indicating that auxotrophs are acid producers like the parental strain.

4.6 Reversion analysis

Protrophic revertants of uracil, uracil + arginine and arginine auxotrophs showed spontaneous Tn5 excision. Prototrophic colonies of each auxotroph were counted on

respective RMM agar plate and reversion frequency was calculated. The spontaneous reversion frequencies of auxotrophs are given in Table 7.

4.7 Linkage of Tn5 insertion to auxotrophy

The transposon Tn5-carrying genomic fragment of each auxotrophic mutant (uracil, uracil + arginine/arginine) was mobilized, with the help of genome mobilizing plasmid pJB3JI, to the recipient *S. meliloti* strain ZB555 (Cys^-, Phe^-, Rf^r, Sm^r). Fifty kanamycin resistance transconjugants were selected. In all crosses, all kanamycin resistant transconjugants showed respective donor's auxotrophy indicating 100% linkage of transposon Tn5 insertion to auxotrophy in all the auxotrophs.

4.8 Genetic mapping of Tn5 insertions in uracil and uracil + arginine auxotrophs

The donor strains of the uracil and uracil + arginine auxotrophs constructed by the introduction of plasmid pJB3JI were also used to locate the positions of transposon Tn5 in these auxotrophs. Each donor was conjugated with six *S. meliloti* strains ZB178, ZB201, ZB205, ZB555, ZB556 and ZB557. In all crosses selection was made for the transfer of kanamycin resistance marker, which is encoded by Tn5. The co-transfer of kanamycin resistance with any of the unselected markers of the available mapping strains was not observed for any of the uracil or uracil + arginine auxotrophs. The available mapping strains cover about 58.3% region of *S. meliloti* chromosome. The mapping results indicated that the Tn5 insertions in the uracil or uracil + arginine auxotrophs are not located in this region of the chromosome. These insertions are present in the remaining 41.7% region of chromosome between *cys*46 and *pur*15/168 loci.

Table 7: Spontaneous reversion frequencies of uracil, uracil + arginine and arginine auxotrophic strains of *Sinorhizobium meliloti* Rmd201

Strain	Auxotrophy	Spontaneous reversion frequency
NV6	Uracil	1.2×10^{-9}
NV9	-- do --	1.6×10^{-9}
NV12	-- do --	1.3×10^{-9}
NV18	-- do --	1.3×10^{-9}
NV19	-- do --	1.1×10^{-9}
NV21	-- do --	1.9×10^{-9}
NV23	-- do --	1.2×10^{-9}
NV26	-- do --	2.1×10^{-9}
NV32	-- do --	1.6×10^{-9}
NV33	-- do --	1.2×10^{-9}
NV34	-- do --	1.5×10^{-9}
NV37	-- do --	1.1×10^{-9}
RH7	-- do --	2.9×10^{-9}
RH9	-- do --	2.1×10^{-9}
RH36	-- do --	1.8×10^{-9}
VK12	-- do --	2.9×10^{-9}
VK19	-- do --	3.1×10^{-9}
VK43	-- do --	2.8×10^{-9}
NV1	Uracil + Arginine	1.2×10^{-9}
NV15	-- do --	1.5×10^{-9}
RH33	-- do --	1.8×10^{-9}
RH37	-- do --	1.7×10^{-9}
RH47	-- do --	1.9×10^{-9}
NV4	Arginine	1.3×10^{-9}
NV27	-- do --	1.2×10^{-9}

4.9 Characteristics of plants inoculated with auxotrophs

The morphological features of alfalfa plants inoculated with uracil, uracil + arginine and arginine auxotrophs, and the parental strain Rmd201 are given in Table 8. All auxotrophs induced white nodules on alfalfa plants; mean heights and dry weights of these plants did not differ significantly from those of the uninoculated plants (Table 8) indicating the absence of nitrogen fixation. The *car, pyrC* and *arg* mutants induced spherical/irregular nodules whereas the nodules induced by *pyrE/pyrF* mutants were cylindrical like the parental strain induced nodules (Plate 1). Some plants inoculated with auxotrophs showed morphological features like the plants inoculated with the parental strain. Nodule occupancy test revealed that the nodules of these plants were occupied by revertants; the data of these plants were not included.

4.10 Nodule occupancy tests

The bacteria were isolated from the nodules of each plant and checked for auxotrophic markers. The percentages of nodule occupancy by auxotrophs are given in Table 9.

4.11 Infection thread formation

Light microscopic studies of the roots of alfalfa plants inoculated with the parental strain Rmd201 showed root hair deformation and infection thread formation; similar results were obtained in inoculations with uracil, uracil + arginine or arginine auxotrophs. The root hairs of uninoculated plants were straight and devoid of infection threads (Plate 2).

Plate 1: Roots of representative alfalfa plants showing nodules induced by *Sinorhizobium meliloti* Rmd201 and its uracil, uracil arginine and arginine auxotrophs,

A. Rmd201 inoculated,

B. Uninoculated,

C. NV1 inoculated,

D. NV18 inoculated,

E. NV6 inoculated,

F. NV4 inoculated.


A
B
C
D
E
F

Plate 2: Infection thread formation in root hairs of alfalfa seedlings inoculated with *Sinorhizobium meliloti* Rmd201 and its uracil, uracil + arginine and arginine auxotrophs,

A. Rmd201 inoculated,

B. Uninoculated,

C. NV1 inoculated,

D. NV18 inoculated,

E. NV6 inoculated,

F. NV4 inoculated.

Abbreviations: it, infection thread; rh, root hair. Bar: 25μm (x400)

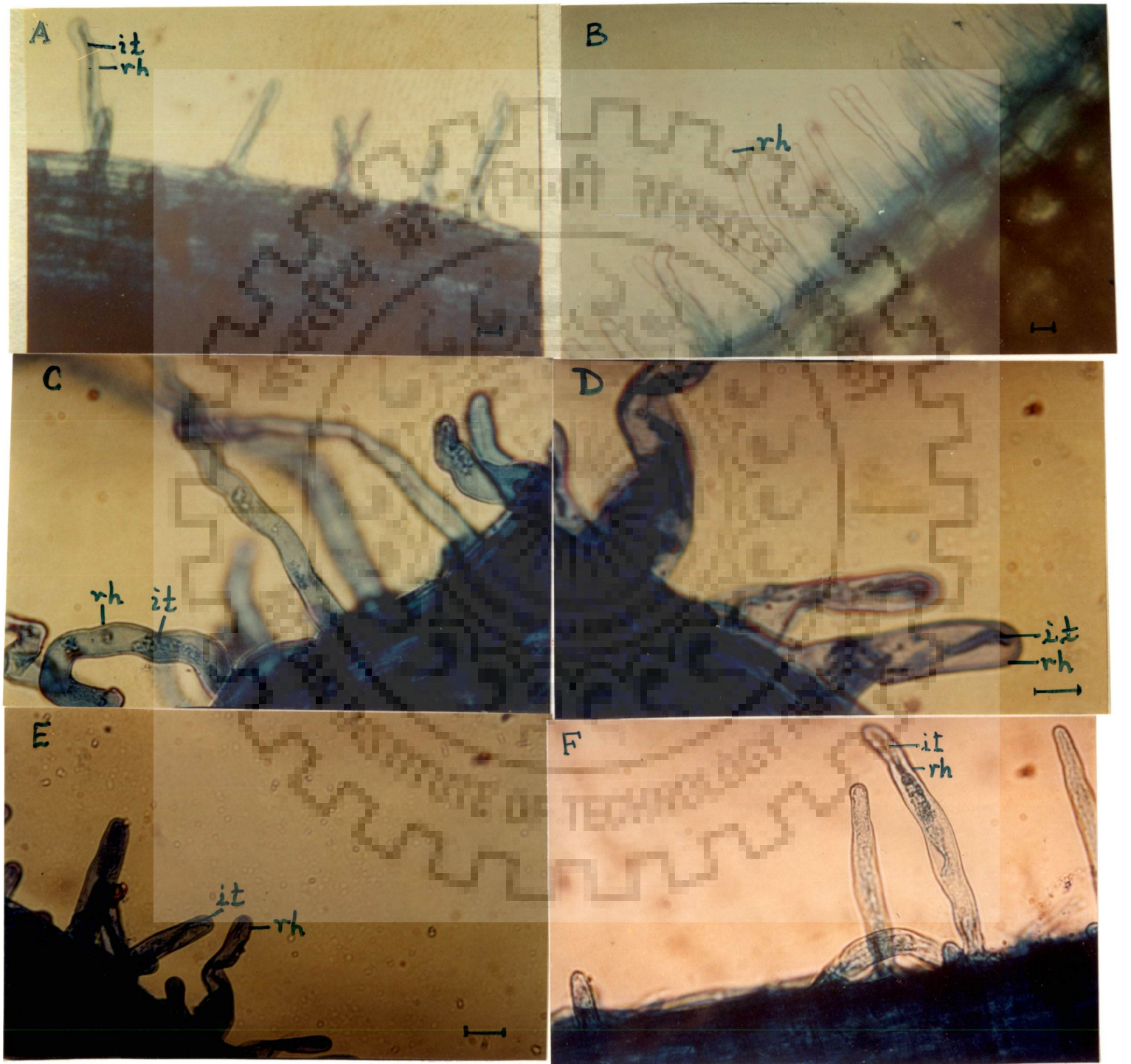
A
it
rh
B
rh
C
rh
it
D
it
rh
E
it
rh
F
it
rh

Table 8: Symbiotic properties of alfalfa plants inoculated with *Sinorhizobium meliloti* Rmd201 and its uracil, uracil + arginine and arginine auxotrophs

S. meliloti strain	Auxotrophic requirement(s)/ mutated gene	No. of days to appearance of first nodule	Nodule Characteristics				Mean plant height [#](cm)	Mean plant dry weight [#] (mg)
			Shape	Size(mm)	Colour	Location		
UNINOCULATED	-	-	-	-	-	-	5.7 ± 0.4	7.9 ± 0.9
Rmd201	-	9-10	Cylindrical	3-4	Pink	PR & LR	27.2 ± 1.2	37.8 ± 1.8
NV1	U + A/*car*	12-13	Spherical/Irregular	1-2	White	LR	6.7 ± 1.2*	8.1 ± 0.7*
NV15	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	6.3 ± 0.9*	7.9 ± 1.1*
RH33	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	5.8 ± 0.7*	7.5 ± 0.8*
RH37	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	6.1 ± 0.5*	7.7 ± 0.9*
RH47	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	6.9 ± 1.2*	8.3 ± 1.4*
NV18	U/*pyrC*	12-13	-- do --	-- do --	-- do --	-- do --	6.8 ± 0.4*	8.1 ± 0.7*
NV21	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	5.9 ± 0.8*	7.6 ± 2.2*
NV32	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	7.6 ± 0.7*	8.3 ± 1.7*
VK12	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	6.9 ± 0.9*	7.9 ± 2.1*
VK19	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	7.3 ± 1.1*	8.2 ± 1.3*
VK43	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	7.8 ± 0.9*	8.2 ± 0.9*
RH7	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	7.5 ± 0.7*	8.3 ± 0.7*
RH9	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.1 ± 1.2*	8.7 ± 1.8*
NV6	U/*pyrE*/*pyrF*	11-12	Cylindrical	2-3	-- do --	PR & LR	8.3 ± 1.8*	8.1 ± 1.1*
NV9	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.1 ± 0.9*	8.9 ± 0.7*
NV12	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	7.9 ± 1.1*	8.1 ± 0.5*
NV19	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.7 ± 2.2*	8.3 ± 1.1*
NV23	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.9 ± 2.7*	9.1 ± 2.1*
NV26	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.1 ± 2.1*	8.2 ± 1.1*
NV33	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.3 ± 1.9*	8.2 ± 0.9*
NV34	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.5 ± 2.1*	8.3 ± 0.7*
NV37	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	7.7 ± 1.2*	8.2 ± 1.3*
RH36	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	8.3 ± 0.9*	8.9 ± 0.8*
NV4	A/*arg*	12-13	Spherical/Irregular	1-2	-- do --	LR	6.9 ± 1.2*	8.1 ± 0.9*
NV27	-- do --	-- do --	-- do --	-- do --	-- do --	-- do --	7.3 ± 1.7*	8.5 ± 1.1*

[#] Each value is mean of ten plants; * Does not differ significantly from uninoculated plant value ($P<0.05$); ***Abbreviations:*** PR, Primary root; LR, Lateral root; U, Uracil; A, Arginine

Table 9: Nodule occupancies of alfalfa plants inoculated with uracil, uracil + arginine and arginine auxotrophs of *Sinorhizobium meliloti* Rmd201

S. meliloti strain	Auxotrophic requirement(s)	Mutated gene	No. of plants showing 100% occupancy of nodules by the auxotroph*	Percentage of plants showing 100% occupancy of nodules by the auxotroph
NV1	Uracil + Arginine	*car*	33	82.5
NV15	-- do --	-- do --	31	77.5
RH33	-- do --	-- do --	30	75.0
RH37	-- do --	-- do --	29	72.5
RH47	-- do --	-- do --	27	67.5
NV18	Uracil	*pyrC*	25	62.5
NV21	-- do --	-- do --	28	70.0
NV32	-- do --	-- do --	30	75.0
VK12	-- do --	-- do --	21	52.5
VK19	-- do --	-- do --	21	52.5
VK43	-- do --	-- do --	23	57.5
RH7	-- do --	-- do --	27	67.5
RH9	-- do --	-- do --	25	62.5
NV6	-- do --	*pyrE/pyrF*	32	80.0
NV9	-- do --	-- do --	28	70.0
NV12	-- do --	-- do --	31	77.5
NV19	-- do --	-- do --	29	72.5
NV23	-- do --	-- do --	26	65.0
NV26	-- do --	-- do --	30	75.0
NV33	-- do --	-- do --	32	80.0
NV34	-- do --	-- do --	26	65.0
NV37	-- do --	-- do --	28	70.0
RH36	-- do --	-- do --	30	75.0
NV4	Arginine	*arg*	32	80.0
NV27	-- do --	-- do --	28	70.0

*Forty alfalfa plants were inoculated with each auxotroph

4.12 Inoculation of plants with prototrophic revertants

The characteristics of the alfalfa plants inoculated with the prototrophic revertants of uracil, uracil + arginine and arginine auxotrophs were similar to those of the parental strain Rmd201 inoculated plants. The mean plant heights and dry weights of alfalfa plants inoculated with revertants of these auxotrophs are shown in Table 10.

4.13 Linkage of Tn5 insertion to symbiotic defect

The transposon Tn5-containing genomic fragment from each auxotroph was transferred to *S. meliloti* strain ZB555 with the help of Km^r marker of Tn5 and genome mobilizing plasmid pJB3JI. The resulting Km^r transconjugants showed respective donor's auxotrophy. Alfalfa plants inoculated with these transconjugants had characteristics like the plants inoculated with the donor auxotroph. These results indicated that a single Tn5 insertion was responsible for both auxotrophy and symbiotic defect in this auxotroph; similar results were obtained with other auxotrophs.

4.14 Feeding of nutritional requirements of auxotrophs to inoculated plants

No nutritional supplement to plants inoculated with uracil or uracil + arginine auxotrophs could restore the symbiotic defect of these auxotrophs. The symbiotic defect of arginine auxotrophs was restored on supplementation of the plant nutrient medium with ornithine, citrulline or arginine; restoration did not occur in uracil + arginine auxotrophs on uracil + arginine/ornithine/citrulline supplementations.

Table 10: Mean plant heights and dry weights of alfalfa plants inoculated with revertants of uracil, uracil + arginine and arginine auxotrophs of *Sinorhizobium meliloti* Rmd201

S. meliloti strain	Auxotrophic requirement(s)	Mutated gene	Mean plant height [#](cm)	Mean plant dry weight[#] (mg)
UNINOCULATED	-	-	5.6 ± 0.1	7.7 ± 0.7
Rmd201	-	-	27.3 ± 1.1	37.5 ± 2.3
NV1r	Uracil + Arginine	*car*	23.2 ± 0.9†	33.3 ± 2.1†
NV15r	-- do --	-- do --	24.6 ± 0.7†	35.2 ± 1.9†
RH33r	-- do --	-- do --	23.3 ± 0.1†	34.7 ± 1.2†
RH37r	-- do --	-- do --	23.2 ± 1.2†	36.1 ± 1.9†
RH47r	-- do --	-- do --	21.9 ± 1.9†	35.9 ± 1.8†
NV18r	Uracil	*pyrC*	26.2 ± 0.4†	36.2 ± 1.1†
NV21r	-- do --	-- do --	25.3 ± 0.6†	37.3 ± 0.5†
NV32r	-- do --	-- do --	27.2 ± 0.1†	37.4 ± 0.3†
VK12r	-- do --	-- do --	21.1 ± 2.1†	36.4 ± 0.9†
VK19r	-- do --	-- do --	20.9 ± 2.3†	35.6 ± 1.1†
VK43r	-- do --	-- do --	22.2 ± 1.8†	37.3 ± 0.2†
RH7r	-- do --	-- do --	23.2 ± 1.9†	33.7 ± 2.9†
RH9r	-- do --	-- do --	22.9 ± 1.5†	36.2 ± 1.2†
NV6r	-- do --	*pyrE/pyrF*	25.5 ± 0.8†	37.1 ± 0.9†
NV9r	-- do --	-- do --	26.2 ± 0.7†	37.4 ± 1.1†
NV12r	-- do --	-- do --	24.9 ± 1.1†	36.1 ± 1.8†
NV19r	-- do --	-- do --	27.1 ± 0.5†	37.4 ± 0.9†
NV23r	-- do --	-- do --	24.4 ± 0.9†	37.6 ± 0.6†
NV26r	-- do --	-- do --	23.7 ± 1.7†	33.9 ± 0.7†
NV33r	-- do --	-- do --	27.1 ± 0.5†	33.2 ± 2.9†
NV34r	-- do --	-- do --	26.5 ± 1.1†	35.5 ± 1.8†
NV37r	-- do --	-- do --	24.1 ± 2.1†	36.3 ± 1.9†
RH36r	-- do --	-- do --	28.3 ± 0.7†	35.5 ± 1.8†
NV4r	Arginine	*arg*	25.6 ± 1.2†	35.2 ± 0.7†
NV27r	-- do --	-- do --	27.8 ± 0.7†	37.5 ± 1.5†

[#] Each value is mean of ten plants; † Does not differ significantly from parental strain inoculated plant value ($P<0.05$)

4.15 Microscopic examinations of sections of the nodules induced by the parental strain Rmd201 and its pyrimidine auxotrophs

4.15.1 Parental strain Rmd201

4.15.1.1 Light microscopy

The nodule induced by the parental strain Rmd201 had a central tissue surrounded by several peripheral tissues which included vascular bundles (Plate 3A; 3B). The central tissue was differentiated into five zones viz., apical meristematic zone, infection zone, interzone between infection and nitrogen fixation zone, nitrogen fixation zone and senescence zone (Plate 3A). Apical meristematic zone contained uninfected and constantly dividing nodule cells (Plate 3B). Next to this zone was infection zone in which infection threads were seen in the intercellular spaces of the nodule cells. A few nodule cells of infection zone were found to be infected by rhizobia. Prominent nuclei were visible in some nodule cells of this zone. Many starch granules were also observed in the infection zone (Plate 3C). Interzone between infection and nitrogen fixation zones showed transition of nodule cells from uninfected to infected state; in this zone most of the cells towards the infection zone were without rhizobia whereas towards nitrogen fixation zone most of the cells contained rhizobial bacteroids (Plate 3D). A few starch granules were also seen in this zone (Plate 3D). Nodule cells containing bacteroids were seen in the nitrogen fixation zone; in each infected nodule cell a large number of bacteroids were arranged perpendicular to a centrally located large vacuole. Most of the nodule cells in this zone contained rhizobia (Plate 4A; 4B). The bacteroids in this zone were elongated and rod shaped (Plate 4C). Lysed nodule cells were seen in the senescence zone (Plate 4D).

4.15.1.2 Electron microscopy

The meristematic zone was devoid of infection threads (Plate 5A). The branching of infection threads was seen in intercellular spaces of the nodule cells in infection zone. (Plate 5B). In the interzone rhizobial bacteria in the infection threads were seen; the entry of infection threads into nodule cells was also visible (Plate 5C). Some of the nodule cells in the interzone were found to contain released rhizobial bacteria. (Plate 5D; 5E).

Poly-β-hydroxy butyrate (PHB) granules were seen in rhizobial bacteria freshly released into nodule cells. The freshly released bacterial cells were enclosed in a peribacteroidal membrane (pbm) (Plate 5C; 5F). PHB granules and a distinct pbm were not visible in the bacteroids of the nitrogen fixation zone. Bacteroids of several shapes were present in the nitrogen fixation zone; most of these were elongated (Plate 6A; 6B).

The cytoplasm of rhizobial bacteria in the infection zone was electron dense (Plate 6C; 6D) whereas the bacteroidal cytoplasm in the interzone and nitrogen fixation zone was heterogeneous having electron dense and electron transparent regions indicating the condensation of nuclear material (Plate 6A; 6B; 6E). Degenerated bacteroids, which had broken pbm and electron transparent cytoplasm, were seen in the senescence zone (Plate 6F).

Endoplasmic reticulum and amyloplasts were observed in nodule cells of infection zone (Plate 6C). These organelles were rarely seen in the nodule cells of the nitrogen fixation zone (Plate 6A; 6B).

Plate 3: 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 (M), infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S) are seen in central tissue, Bar: 100μm (x100), B. Nodule cells of meristematic zone (M), Bar: 25μm (x400), C. Nodule cells of infection zone showing starch granules (sg), prominent nuclei (n), rhizobial bacteria (rb) and infection threads (it), Bar: 25μm (x400), and D. Interzone showing infected nodule cells (I), uninfected nodule cells (UI) and starch granules (sg), Bar: 25μm (x400).

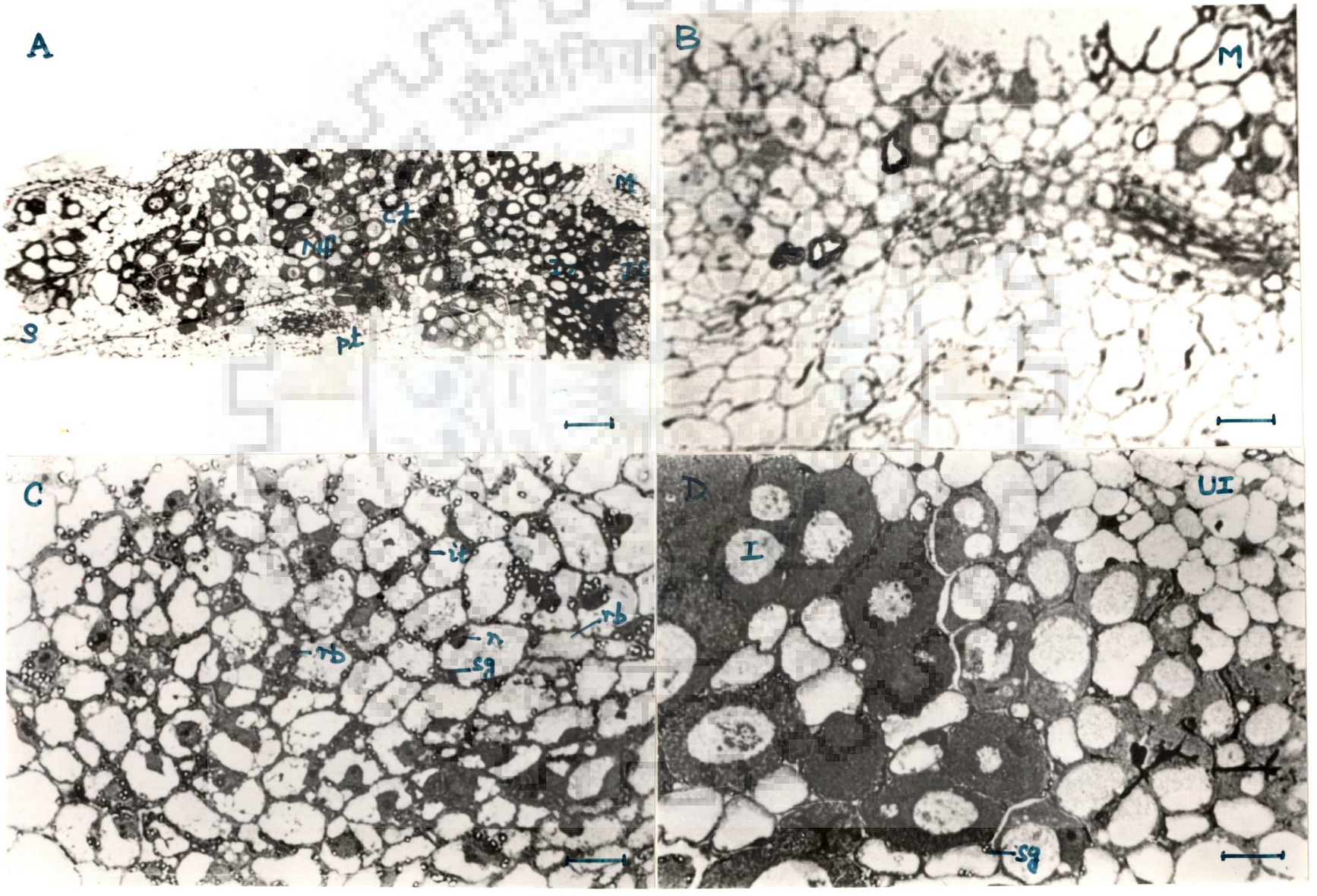
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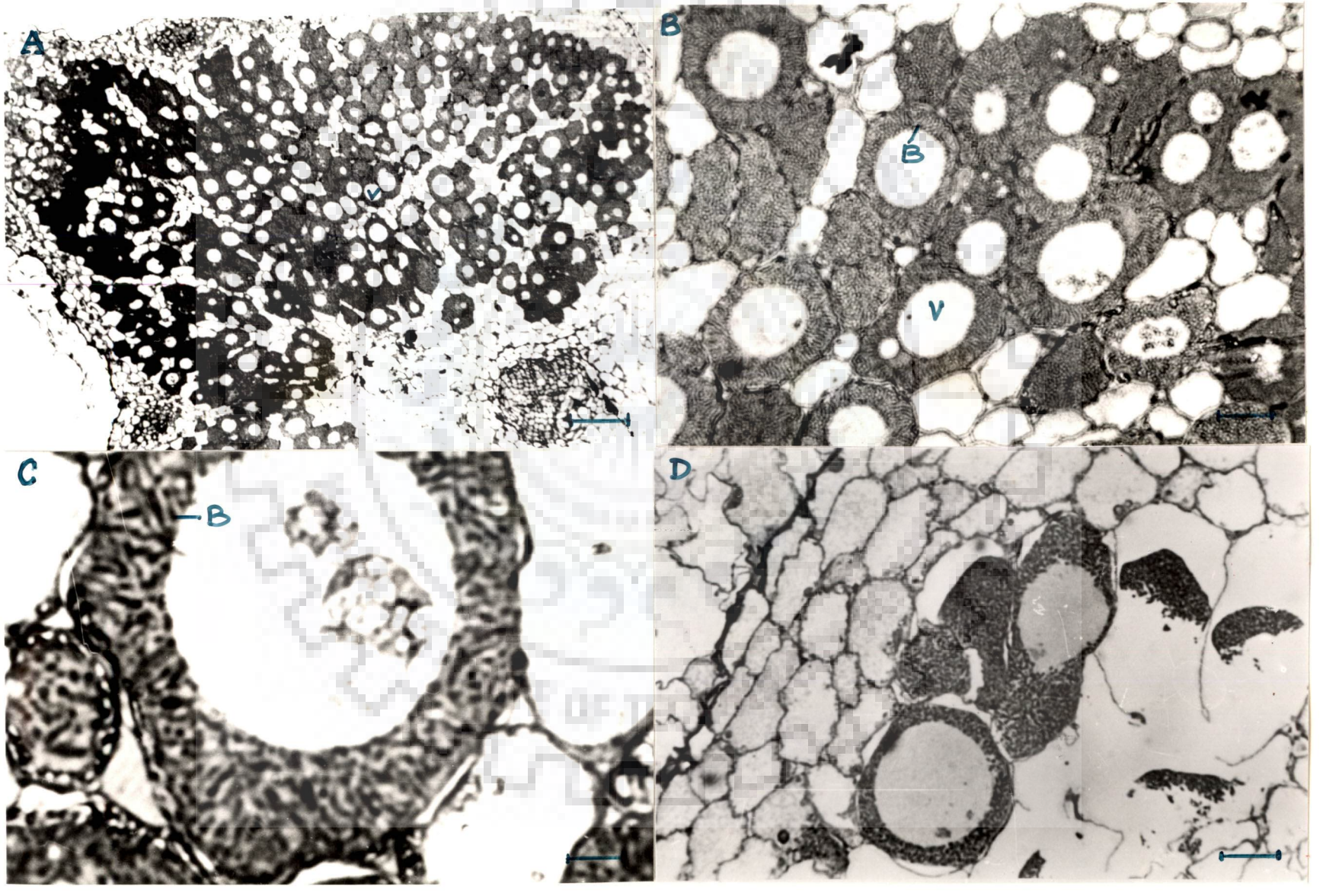
Plate 4: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A,** B. Nodule cells showing bacteroids (B) and large central vacuoles (v) in nitrogen fixation zone, Bar: 25μm (x400), **C.** Nodule cells showing elongated and rod shaped bacteroids (B) in nitrogen fixation zone, Bar: 25μm (x400), and **D.** Nodule cells showing lysis in senescence zone, Bar: 25μm (x400).


A
V
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B
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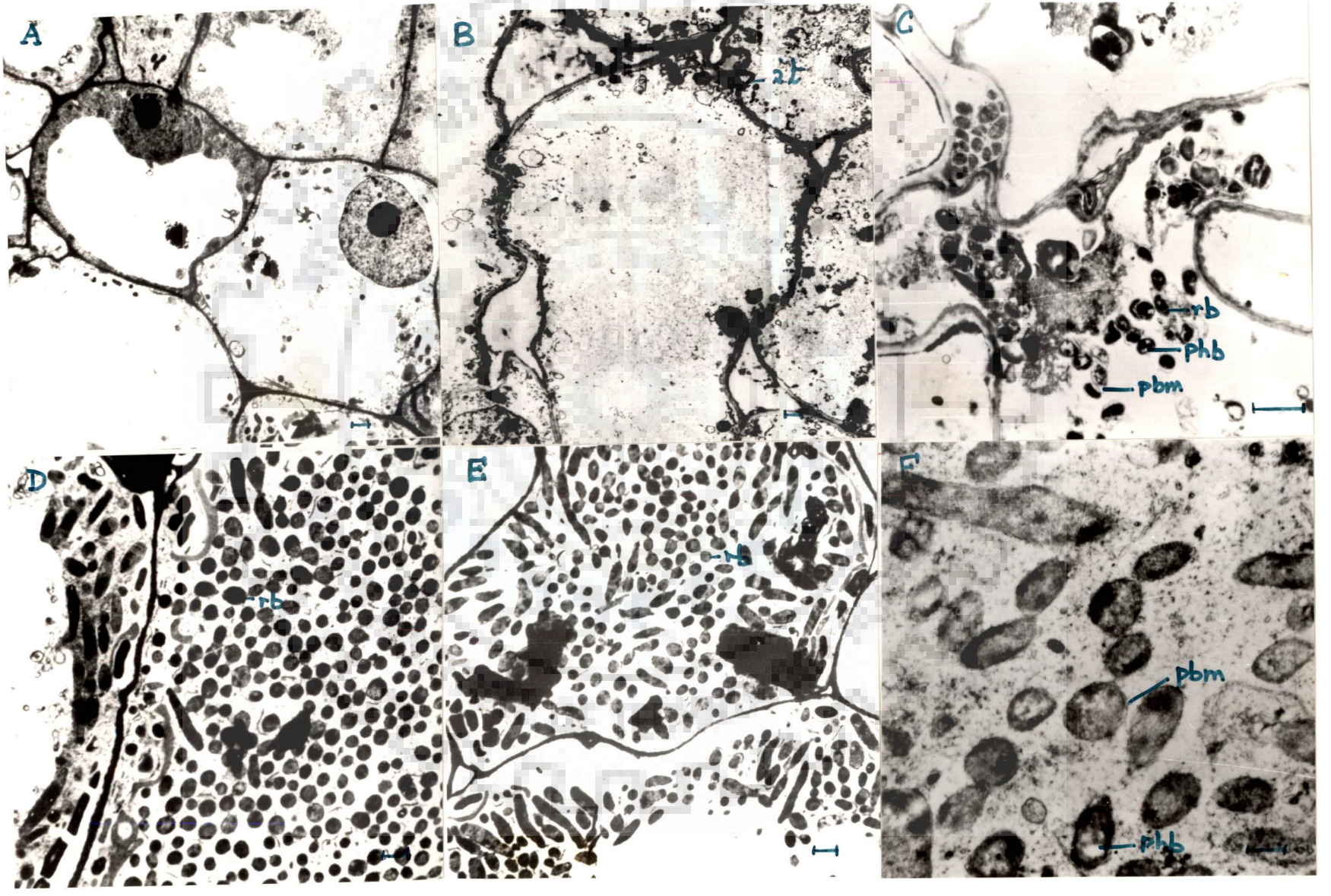
D

Plate 5: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A.** Nodule cells of meristematic zone, Bar: 1μm (x2900), **B.** Branched infection threads (it) in intercellular spaces of nodule cells of infection zone, Bar: 1μm (x2900), **C.** Poly-β-hydroxybutyrate (phb) granules and peribacteroidal membrane (pbm) of rhizobial bacteria (rb) in interzone, Bar: 1μm (x8400), **D, E.** A part of nodule cell showing released rhizobial bacteria (rb) in interzone, Bar: 1μm (x2900), and **F.** A part of nodule cell (of interzone) showing rhizobial bacteria containing phb granules and pbm, Bar: 1μm (x4100).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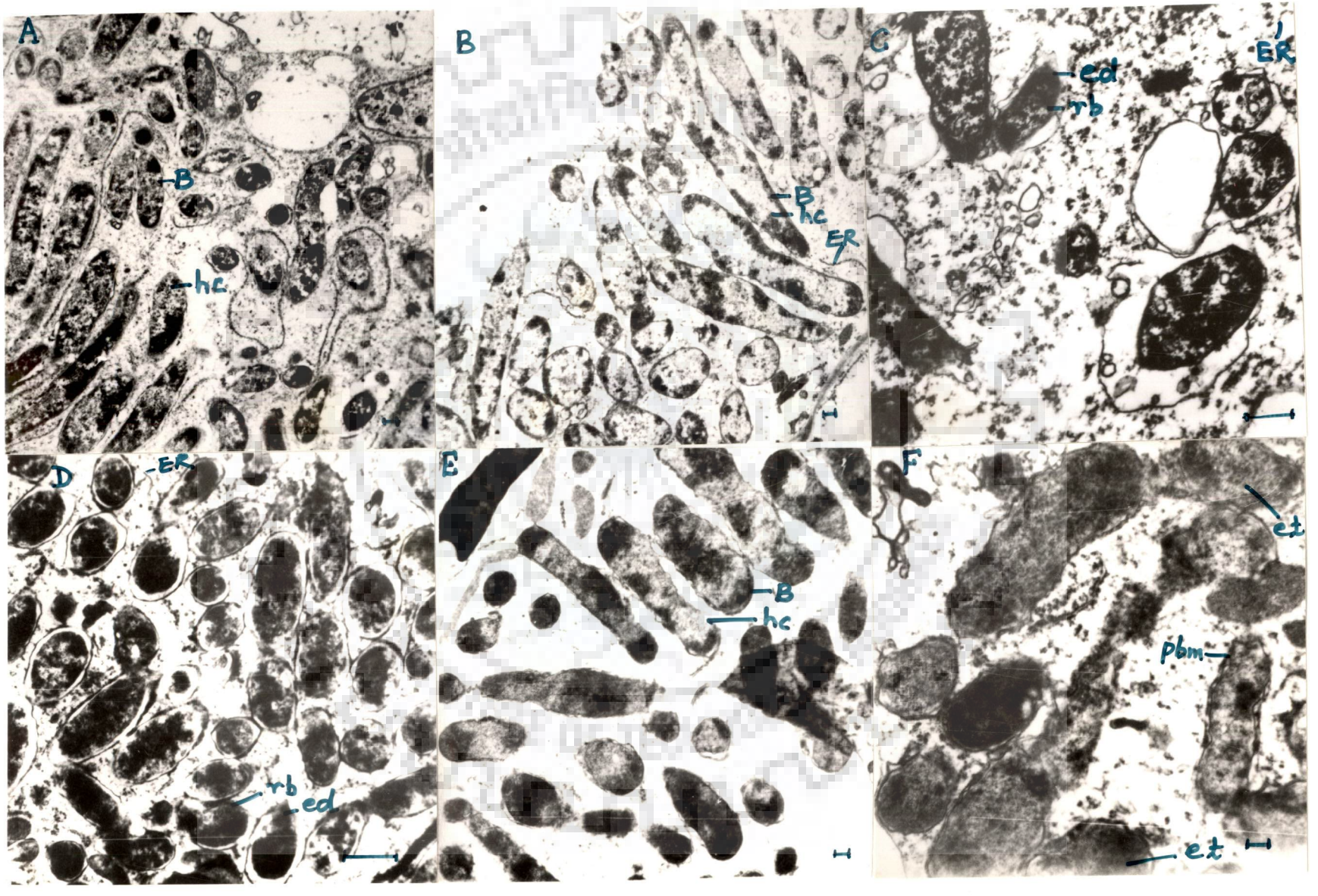
pbm
Phb

Plate 6: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A, B.** A part of nodule cell (of nitrogen fixation zone) showing rhizobial bacteroids (B) of different shapes containing heterogeneous cytoplasm (hc), Bar: 1μm (x2300), C, D. A part of nodule cell (of infection zone) showing endoplasmic reticuleae (ER) and rhizobial bacteria (rb) containing electron dense cytoplasm (ed), Bar; C: 1μm (x5800); D: 1μm (x4100),, E. A part of nodule cell showing rhizobial bacteroid (B) containing heterogeneous cytoplasm (hc) in interzone, Bar: 1μm (x2300), and F. A part of nodule cell showing broken peribacteroidal membrane (pbm) and electron transparent cytoplasm (et) of bacteroids in senescence zone, Bar: 1μm (x5800).


A
B
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E
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F
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pbm
et

4.15.2 *car* mutants

Out of 5 *car* mutants, two (NV1 and NV15) were selected for microscopic work. The nodules induced by these mutants had similar internal structure. The results of mutant NV1 are presented below.

4.15.2.1 Light microscopy

The nodule induced by the *car* mutant NV1 had underdeveloped central and peripheral tissues. The central tissue of this nodule was not differentiated into five zones like in the parental strain Rmd201 induced nodule; however it contained regions analogous to the infection zone and interzone of the Rmd201 induced nodule (Plate 7A). About three fourth of the nodule was represented by the so-called infection zone in which an extensive network of infection threads, transversing through the loosely packed nodule cells, was seen (Plate 7A; 7B). Nodule cells similar to the cells of the so-called interzone of the parental strain induced nodule were present in the basal part of the nodule (Plate 7C). Infection threads were also observed in the intercellular spaces of the nodule cells of interzone (Plate 7D). Centrally located large vacuoles were seen in some of the nodule cells of this zone (Plate 7D; 8A). Many starch granules were observed throughout the nodule section (Plate 7B; 8A). Peripheral vasculature was not visible (Plate 7A; 8B).

4.15.2.2 Electron microscopy

Poorly developed infection threads were observed in intercellular spaces of nodule cells; occasional release of bacteria from infection threads was seen (Plate 9A; 9B). The released bacteria were mostly in degenerating condition (Plate 9C). A few degenerating bacteria were also seen in infection threads (Plate 9A). Infection threads were occasionally branched and their contents were not electron dense (Plate 9A; 9B)

The rhizobial bacteria released into nodule cells had PHB granules and electron dense cytoplasm. In some nodule cells the freshly released bacteria had broken pbm and electron transparent cytoplasm indicating their senescence (Plate 9A; 9C; 9D; 9E). A few released bacteria were devoid of PHB granules indicating the tendency of these bacteria towards transformation (Plate 9F). The cytoplasm of bacterial cells (in nodule cells) was mostly electron dense indicating lack of nucleic material condensation in it (Plate 9F; 10A).

Many amyloplasts and endoplasmic reticuleae were seen in the nodule cells (Plate 10B; 10C). A few vesicles were observed in some nodule cells. Most of the nodule cells were without rhizobial bacteria (Plate 10D).

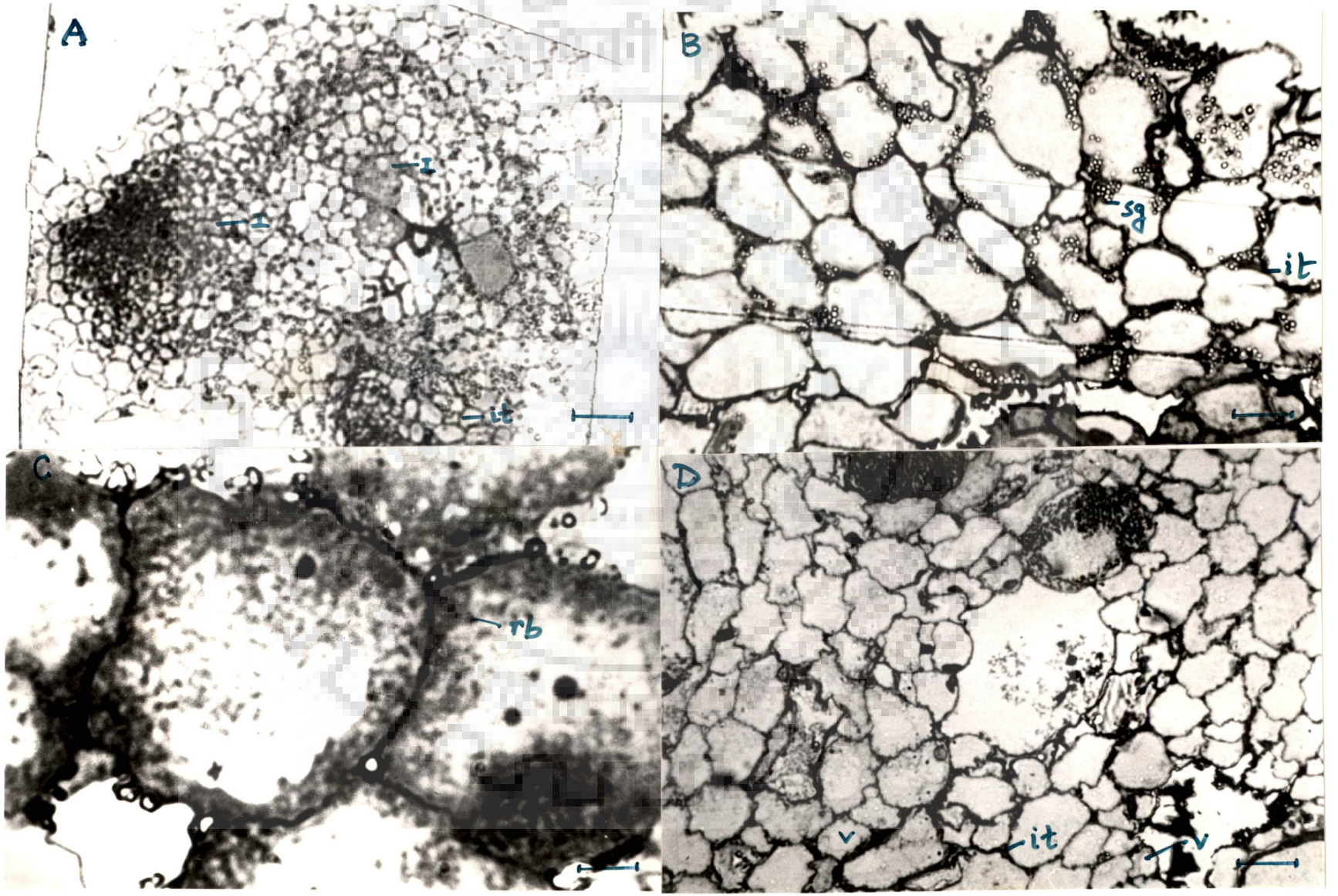
4.15.3 *pyrC* mutants

Out of 8 *pyrC* mutants, two (NV18 and NV32) were selected for microscopic work. The nodules induced by these mutants had similar internal structure. The results of mutant NV18 are presented below.

4.15.3.1 Light microscopy

The peripheral and central tissues of the nodule induced by the *pyrC* mutant NV18 were more developed than those of a *car* mutant induced nodule. The central tissue of this nodule was not differentiated into five distinct zones like in the parental strain Rmd201 induced nodule but some similarity to all the zones of Rmd201 induced nodule was observed (Plate 11A; 11B). Infection threads were visible in intercellular spaces of nodule cells in the so-called infection zone (Plate 11A; 11C). Bacterial release from infection threads occurred in several nodule cells of so-called interzone (Plate 11D; 12A). Nitrogen fixation zone was poorly developed; only a few nodule cells had peripheral

Plate 7: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain NV1, a *car* mutant of *Sinorhizobium meliloti* Rmd201, A. A nodule section showing infection threads (it) and infected nodule cells (I), Bar: 100μm (x100), B. Infection zone showing infection threads (it) and starch granules (sg), Bar: 25μm (x400), C. Nodule cells showing rhizobial bacteria (rb) in interzone, Bar: 25μm (x400), and D. Interzone showing infection threads (it), rhizobial bacteria (rb) and vacuoles (v) in this zone, Bar: 25μm (x400).


A
I
I
it
B
sg
it
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it
v

Plate 8: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain NV1, a *car* mutant of *Sinorhizobium meliloti* Rmd201, **A.** Nodule cells showing central vacuoles (v) and starch granules (sg) in interzone, Bar: 25μm (x400), and **B.** Nodule cells of peripheral tissue (pt), Bar: 25μm (x400).

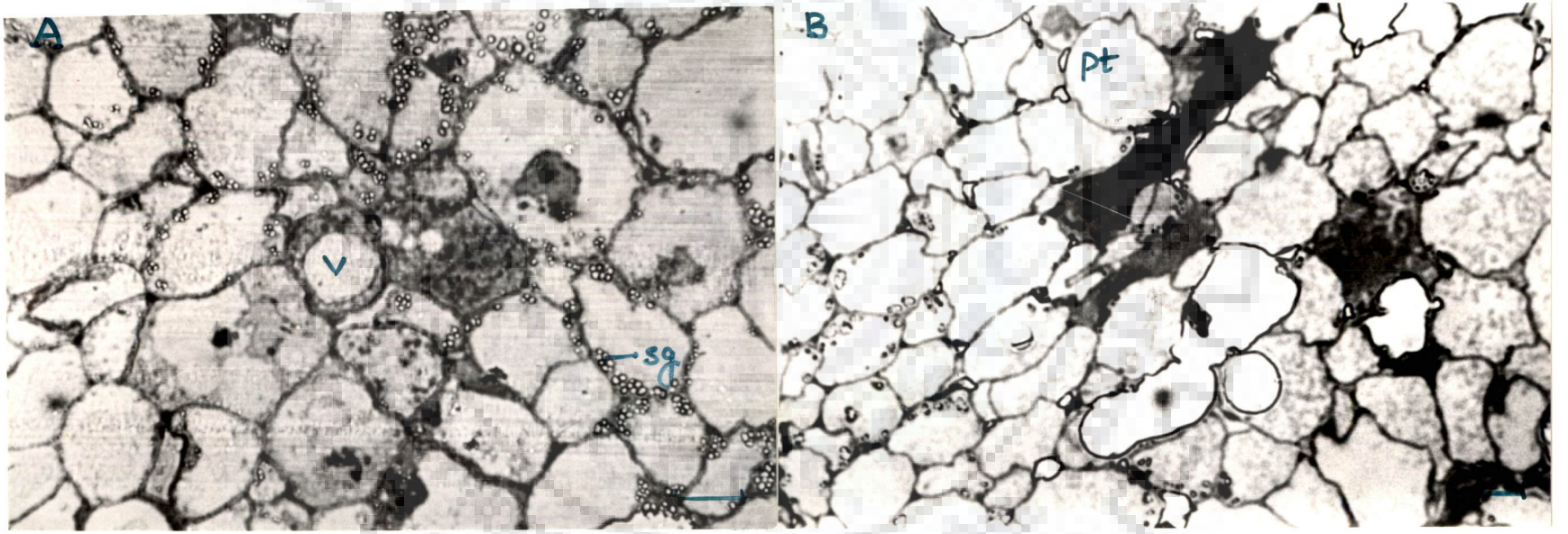
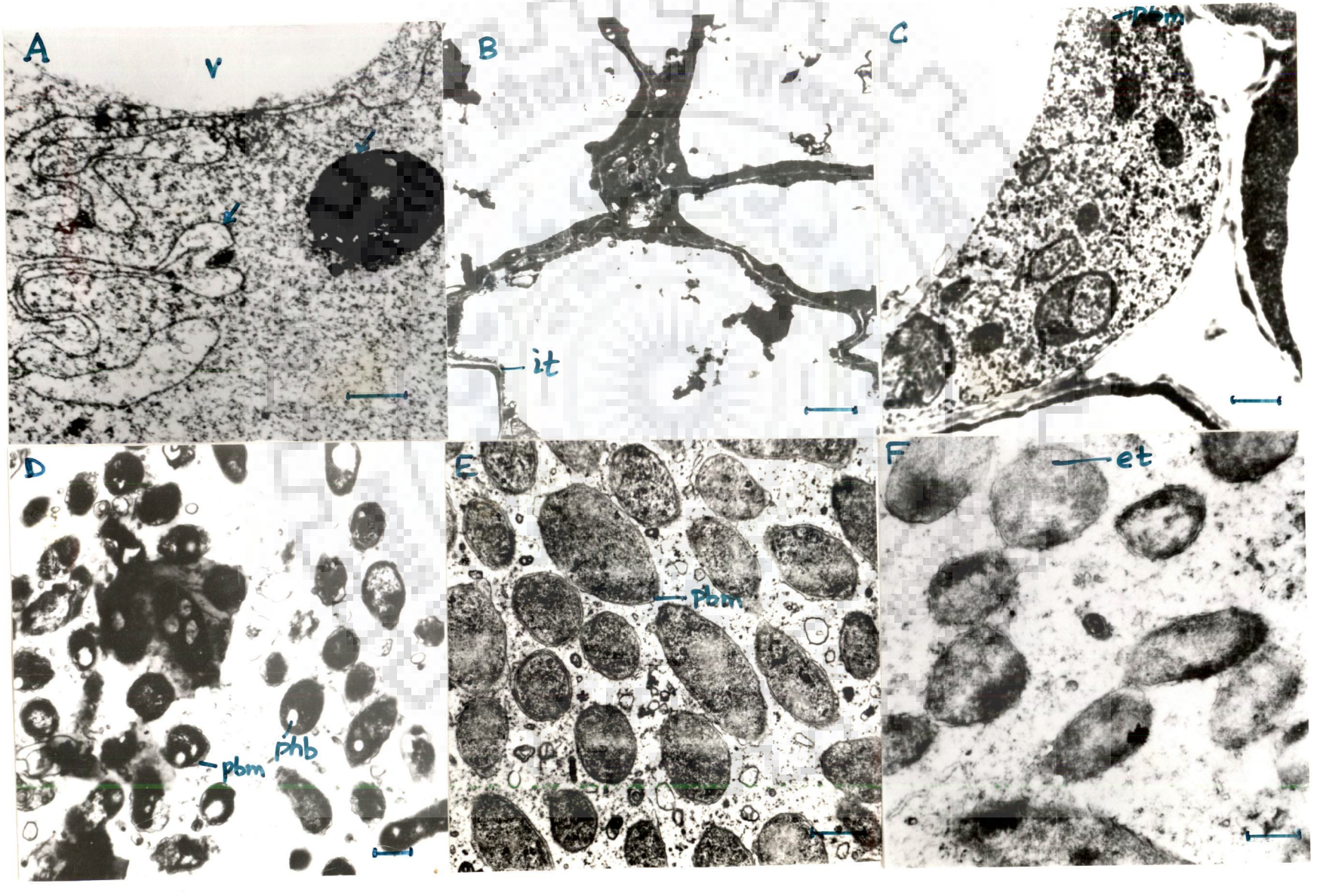

A
V
sg
B
pt

Plate 9: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain NV1, a *car* mutant of *Sinorhizobium meliloti* Rmd201, A, B. Infection threads (it) containing degenerating rhizobial bacteria (rb) in interzone, Bar: 1μm (x8600), C, D, E. A part of nodule cell showing broken peribacteroidal membrane (pbm) and electron transparent cytoplasm (et) of rhizobial bacteria in interzone, Bar; C: 1μm (x5800); D, E: 1μm (x8400), and F. A part of nodule cell showing poly-β-hydroxybutyrate (phb) granules and electron dense cytoplasm (ed) of rhizobial bacteria in interzone, Bar: 1μm (x4100).


A
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pbm
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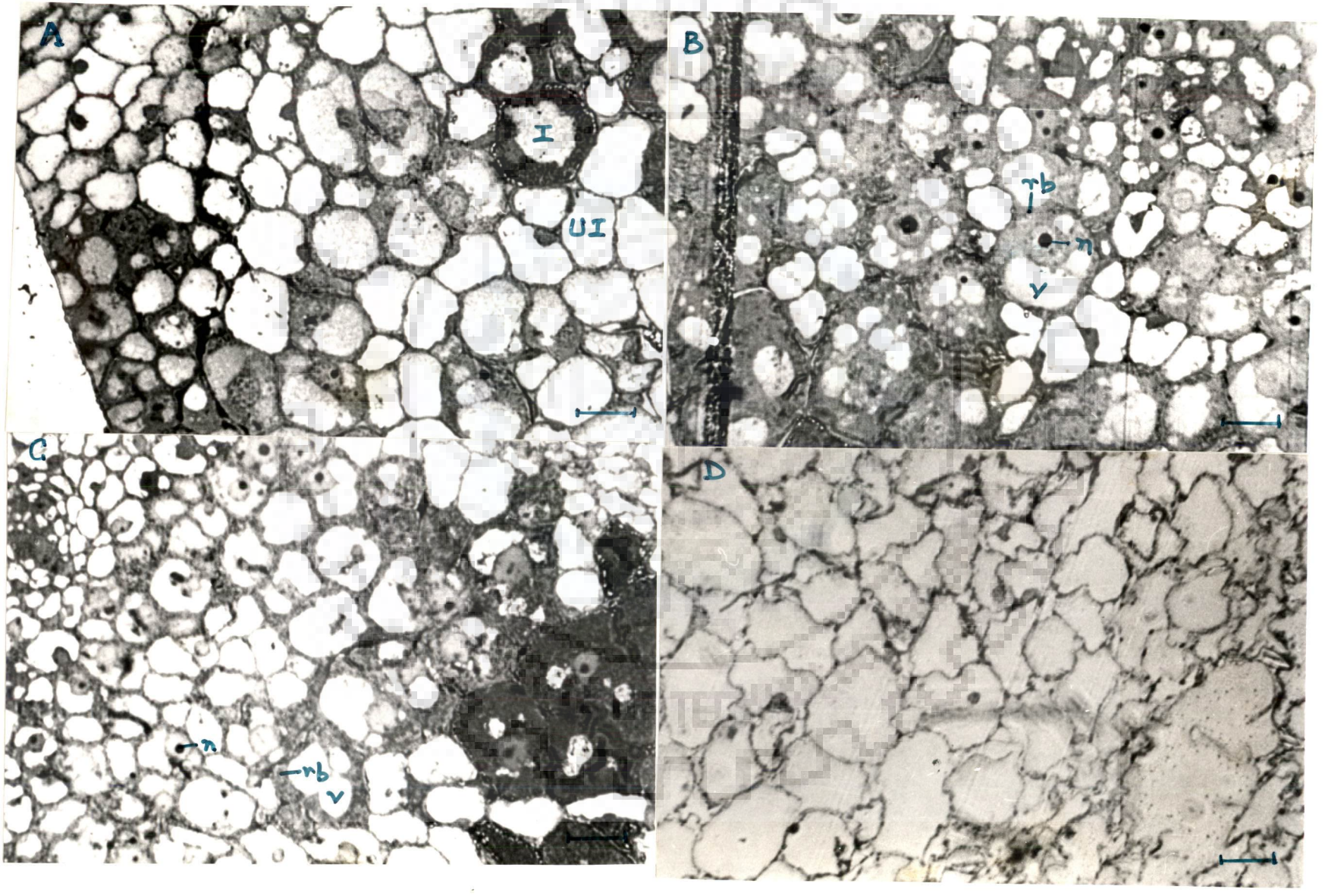
phb
E
pbm
F
et

Plate 13: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain NV18, a *pyrC* mutant of *Sinorhizobium meliloti* Rmd201, A. A part of nodule cell at the distal end (i.e. towards tip) of the nodule section showing a large central vacuole (v) and cell organelles (indicated by arrow), Bar: 1μm (x2900), B. Infection threads (it) in intercellular spaces of nodule cells of infection zone, Bar: 1μm (x5800), C, D, E. A part of nodule cell showing poly-β-hydroxybutyrate (phb) granules and distinct peribacteroidal membrane (pbm) of rhizobial bacteria in interzone, Bar; C: 1μm (x4100); D: 1μm (x5800); E: 1μm (x8400),, and F. A part of nodule cell showing broken pbm and electron transparent cytoplasm (et) of rhizobial bacteria in nitrogen fixation zone, Bar: 1μm (x8400).


A
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UI
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n
v
C
n
rb
v
D

Plate 12: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain NV18, a *pyrC* mutant of *Sinorhizobium meliloti* Rmd201, A. Nodule cells infected (I) with rhizobia and many uninfected nodule cells (UI) in interzone, Bar: 25μm (x400), **B, C.** Nodule cells of poorly developed nitrogen fixation zone showing rhizobial bacteria (rb), prominent nuclei (n) and vacuoles (v), Bar: 25μm (x400), and D. Lysed nodule cells in the basal part of the nodule section, Bar: 25μm (x400).

arrangement of rhizobia (Plate 11B; 12B; 12C). Starch granules were seen in most of the nodule cells throughout the nodule section (Plate 11A; 11D). Many nodule cells were without rhizobia (Plate 12A; 12C). Prominent nuclei and vacuoles were seen in several nodule cells (Plate 11A; 11D; 12B; 12C). Peripheral tissue of the nodule showed well-developed vasculature (Plate 11B). Lysed nodule cells were present in the basal part of the nodule (Plate 12C).

4.15.3.2 Electron microscopy

Uninfected nodule cells were seen in the distal end of the nodule i.e. towards the tip of the nodule. In each of these cells a large central vacuole was present; the remaining cytoplasmic material was arranged around this vacuole along the periphery of the cell (Plate 13A). In the so-called infection zone, infection threads were seen in the intercellular spaces of the nodule cells (Plate 13B). Normal bacterial release from the infection threads into nodule cells was observed in the so-called interzone (Plate 13C; 13D).

The rhizobial bacteria released from infection threads were electron dense. PHB granules and a distinct pbm were seen in these rhizobial cells (Plate 13D). Such rhizobial cells having distinct pbm were seen in most of the nodule cells indicating that the transformation of bacteria into bacteroids was not complete (Plate 13E). In some of the infected nodule cells, rhizobial cells had a broken pbm and electron transparent regions showing their lysis (Plate 13F; 14A). A few elongated bacteroids having electron transparent patches in the electron dense cytoplasm were also seen in some of the nodule cells (Plate 14B).

Amyloplasts and endoplasmic reticuleae were seen in several nodule cells (Plate 14C; 14D). In the basal end of the nodule degenerating rhizobial bacteria were seen in several nodule cells (Plate 14E).

4.15.4 *pyrE/pyrF* mutants

Out of 10 *pyrE/pyrF* mutants, two (NV6 and NV9) were selected for microscopic work. The nodules induced by these mutants had similar internal structure. The results of mutant NV6 are presented below.

4.15.4.1 Light microscopy

Distinct peripheral and central tissues were present in the nodule induced by the NV6 mutant. The central tissue comprised of five zones like those of the parental strain induced nodule (Plate 15A). The peripheral tissue contained vascular bundles. The meristematic zone was composed of uninfected, dividing nodule cells (Plate 15B). Infection threads were seen in intercellular spaces of the nodule cells of infection zone (Plate 15C). Starch granules and prominent nuclei were also observed in nodule cells of this zone (Plate 15C; 15D). A few nodule cells infected with rhizobia were seen in the interzone. Many nodule cells in this zone were without rhizobia; a few starch granules were also visible (Plate 16A). Nitrogen fixation zone was represented by nodule cells having peripheral arrangement of bacteroids; in each of these nodule cells the bacteroids were arranged around a central vacuole (Plate 16B). A few nodule cells lacked proper bacterial arrangement and many nodule cells were without bacteria in this zone; starch granules were also observed even in this zone (Plate 16C). Lysed nodule cells were seen in the senescence zone (Plate 16D).

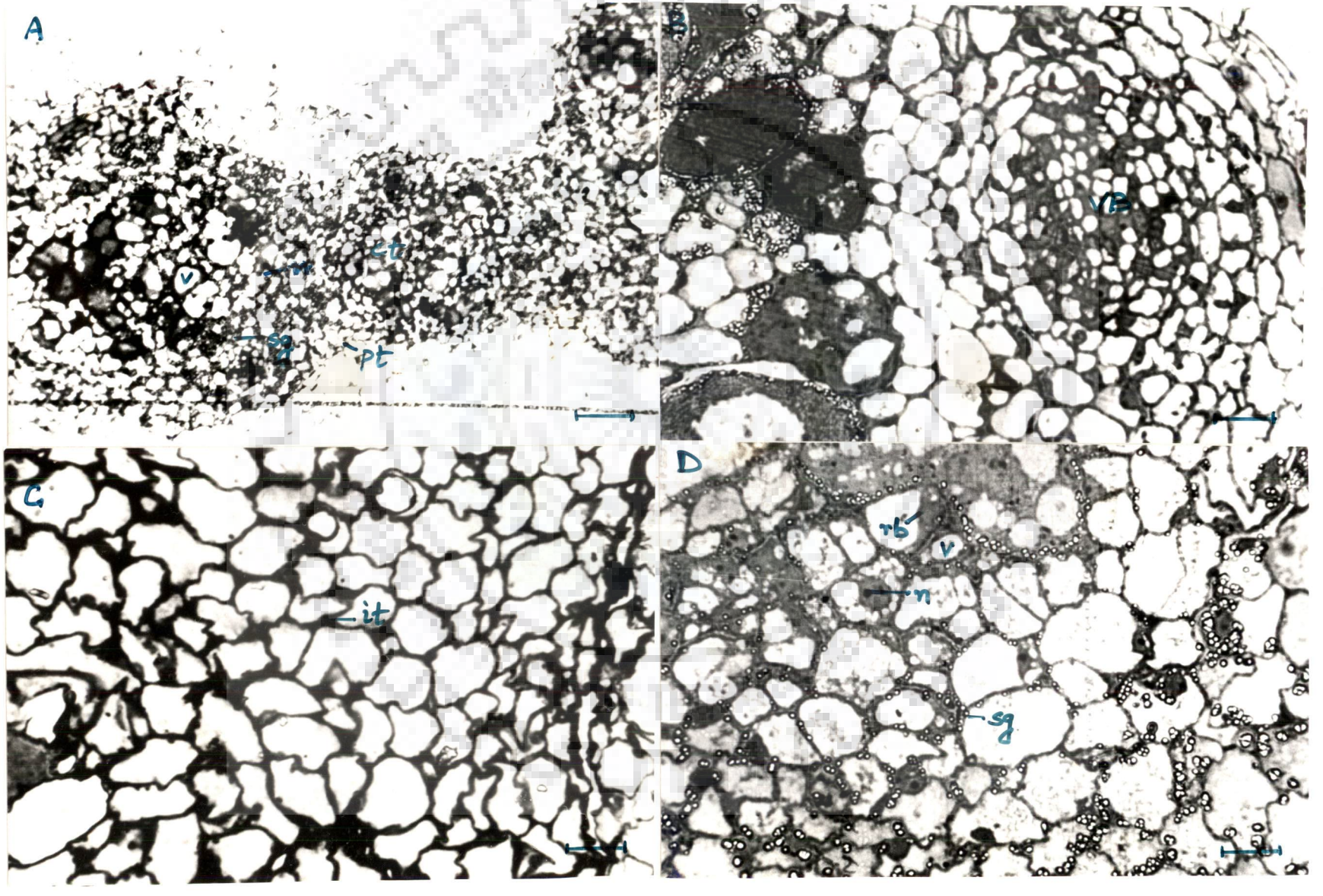
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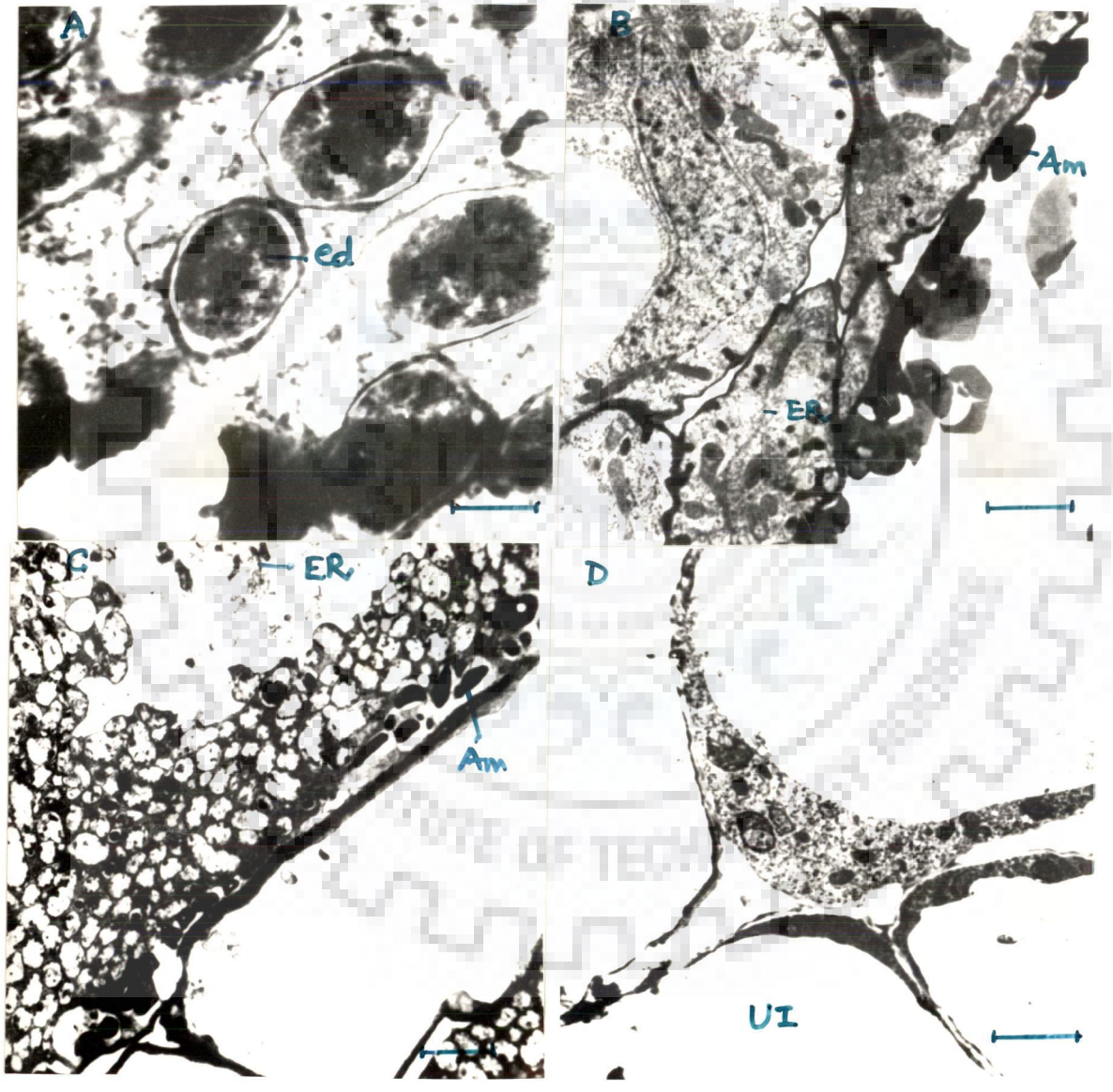
Plate 11: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain NV18, a *pyrC* mutant of *Sinorhizobium meliloti* Rmd201, A. A nodule section showing central tissue (ct) and peripheral tissue (pt); prominent nuclei (n), vacuoles (v) and starch granules (sg) are also seen, Bar: 100μm (x100), B. Nodule cells showing bacteroids (B) and central vacuoles (v) in nitrogen fixation zone; a vascular bundle (VB) is also seen, Bar: 25μm (x400), C. Infection threads (it) in intercellular spaces of nodule cells of infection zone, Bar: 25μm (x400), and D. Nodule cells showing released rhizobial bacteria (rb), starch granules (sg), prominent nuclei (n) and vacuoles (v) in interzone, Bar: 25μm (x400).


A
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B
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ER
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ER
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UI

Plate 10: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain NV1, a *car* mutant of *Sinorhizobium meliloti* Rmd201, A. A part of nodule cell showing electron dense cytoplasm (ed) of rhizobial bacteria in interzone, Bar: 1μm (x8400), B, C. A part of nodule cell showing endoplasmic reticuleae (ER) and amyloplasts (Am), Bar; B: 1μm (x2900); C: 1μm (x4100), and D. Uninfected nodule cells (UI), Bar: 1μm (x2900).

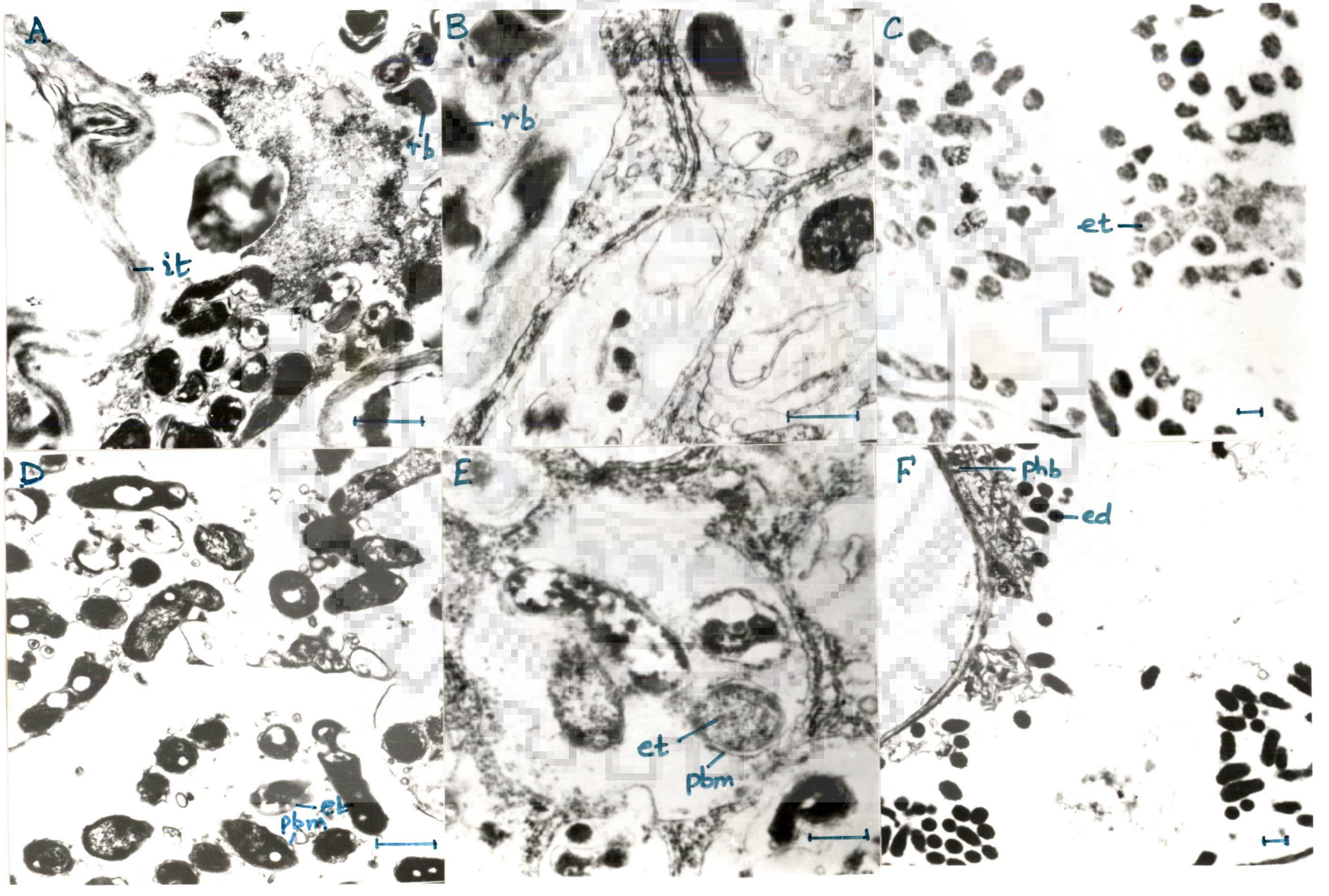
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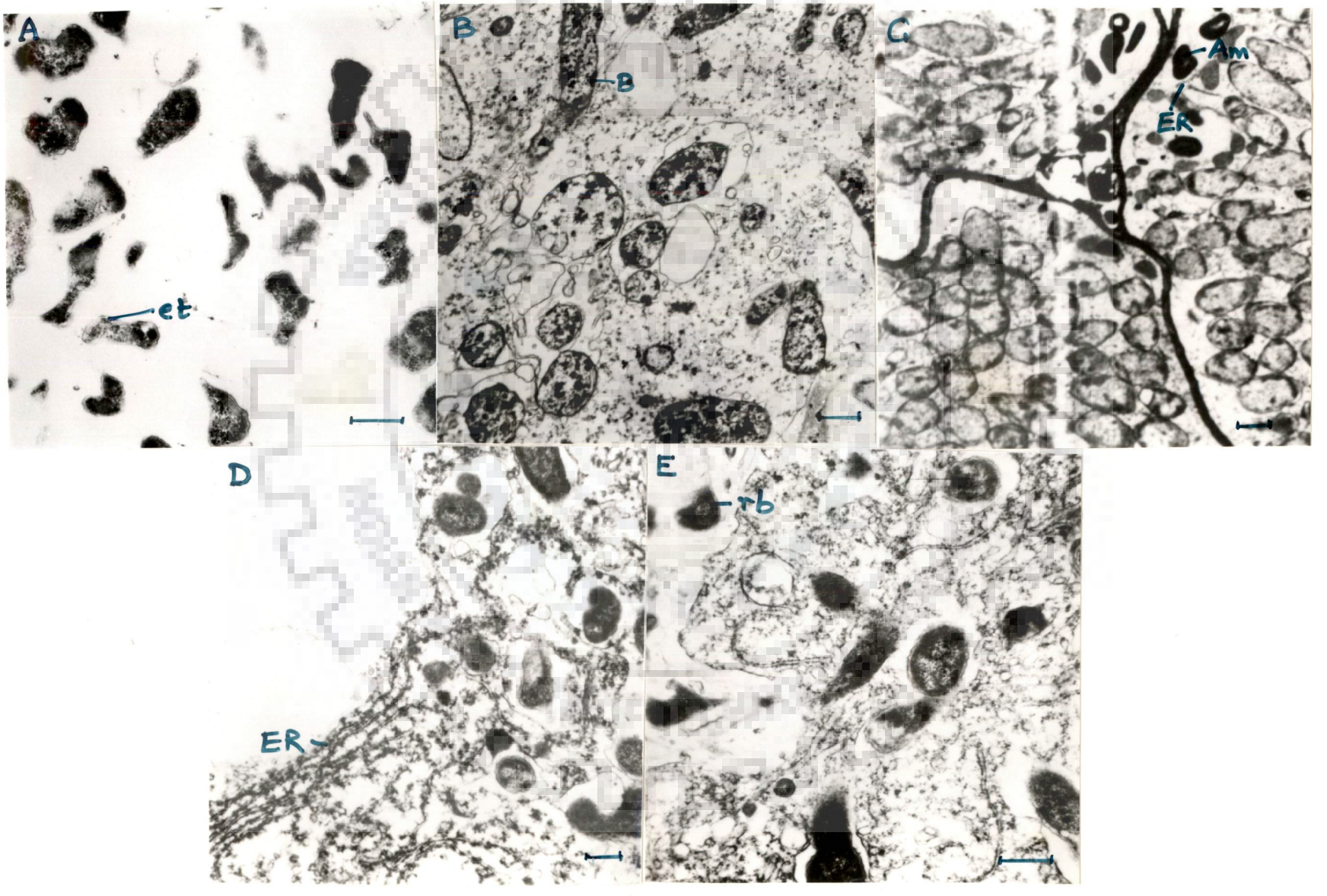
Plate 14: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain NV18, a *pyrC* mutant of *Sinorhizobium meliloti* Rmd201, A. A part of nodule cell showing broken pbm and electron transparent cytoplasm (et) of rhizobial bacteria in nitrogen fixation zone, Bar: 1μm (x8400), B. A part of nodule cell showing elongated bacteroids (B) in nitrogen fixation zone, Bar: 1μm (x5800), C, D. A part of nodule cell showing endoplasmic reticuleae (ER) and amyloplasts (Am) in nitrogen fixation zone, Bar: 1μm (x5800), and E. A part of nodule cell at the basal end of the nodule section showing degenerating rhizobial bacteria (rb), Bar: 1μm (x8400).


A
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B
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ER
D
ER
E
rb

Plate 15: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain NV6, a *pyrE/pyrF* mutant of *Sinorhizobium meliloti* Rmd201, **A.** A nodule section showing distinct peripheral tissue (pt) and central tissue (ct); meristematic zone (M), infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S) are seen in central tissue, Bar: 100μm (x100), **B.** Meristematic zone showing uninfected nodule cells (UI), Bar: 25μm (x400), **C & D.** Infection threads (it) in intercellular spaces of nodule cells of infection zone; starch granules (sg) and prominent nuclei (n) are seen in nodule cells, Bar: 25μm (x400).

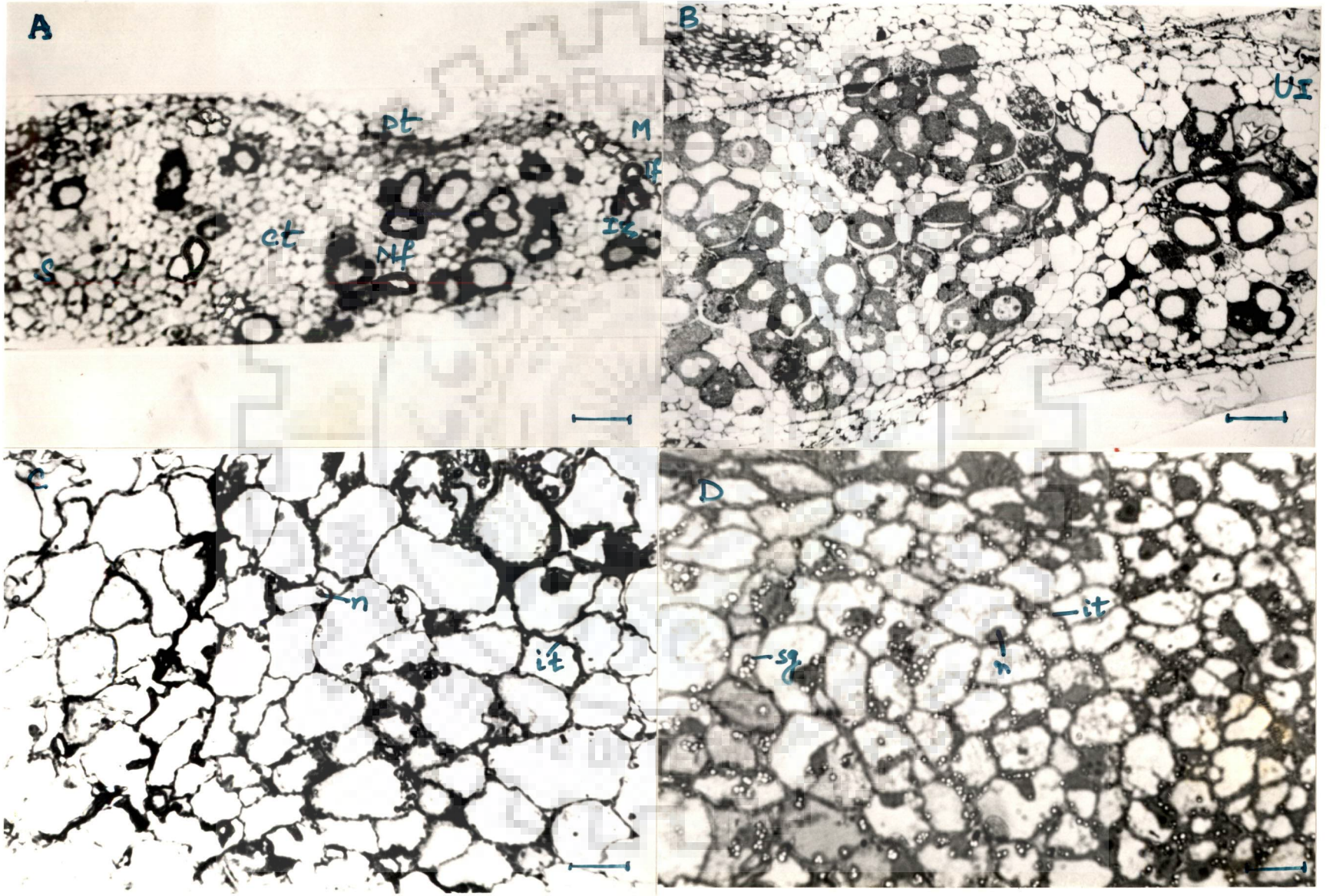
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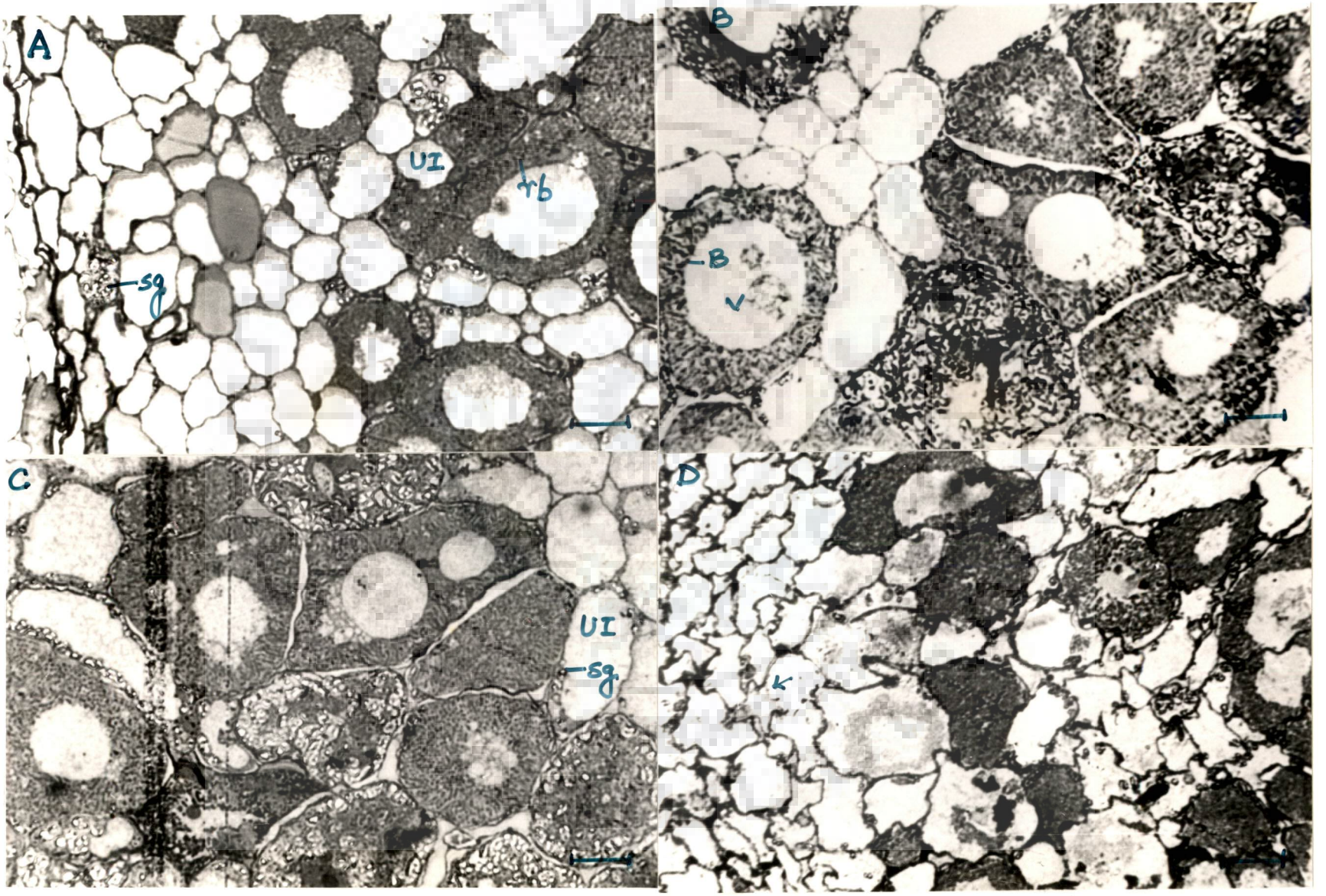
Plate 16: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain NV6, a *pyrE/pyrF* mutant of *Sinorhizobium meliloti* Rmd201, A. Nodule cells containing rhizobial bacteria (rb) and starch granules (sg) in interzone; uninfected nodule cells (UI) are also seen, Bar: 25μm (x400), B. Nodule cells showing peripheral arrangement of bacteroids (B) around central vacuoles (v) in nitrogen fixation zone, Bar: 25μm (x400), C. Uninfected nodule cells (UI) of nitrogen fixation zone; starch granules (sg) are seen in some nodule cells, Bar: 25μm (x400), and D. Lysed nodule cells of senescence zone, Bar: 25μm (x400).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K

4.15.4.2 Electron microscopy

The meristematic zone was represented by uninfected nodule cells having peripheral arrangement of cell organelles (Plate 17A). Branched infection threads were seen in the intercellular spaces of the nodule cells of infection zone (Plate 17B). Normal bacterial release in nodule cells was observed in interzone (Plate 17C).

Freshly released rhizobial bacteria contained PHB granules, pbm and electron dense cytoplasm (Plate 17C; 17D). The bacteroids were elongated and arranged perpendicular to a centrally located vacuole in each bacteroid containing nodule cell of nitrogen fixation zone (Plate 17E; 17F; 18A). Higher magnification of nitrogen fixation zone revealed bacteroids having electron dense and electron transparent regions. however, the heterogeneity of the bacteroidal cytoplasm was not to the extent observed in the parental strain induced nodule (Plate 18B). A few bacteria containing electron dense cytoplasm were also seen in this zone (Plate 18C). Rhizobial cells having a broken pbm and electron transparent cytoplasm were seen in the senescent zone (Plate 18D).

Unlike the parental strain induced nodules many endoplasmic reticuleae and amyloplasts were present in the nodule cells of the nitrogen fixation zone (Plate 18A; 18E).

Plate 17: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain NV6, a *pyrE/pyrF* mutant of *Sinorhizobium meliloti* Rmd201, A. A part of nodule cell showing peripheral arrangement of cell organelles in meristematic zone, Bar: 1μm (x2900), B. Infection threads (it) in intercellular spaces of nodule cells of infection zone, Bar: 1μm (x8400), C, D. A part of nodule cell (of interzone) showing poly-β-hydroxybutyrate (phb) granules, peribacteroidal membrane (pbm) and electron dense cytoplasm (ed) of rhizobial bacteria, Bar; C: 1μm (x4100), D: 1μm (x5800), E & F. A part of nodule cell showing elongated bacteroid (B) arranged perpendicular to a centrally located vacuole (v) in nitrogen fixation zone, Bar: 1μm (x2900).

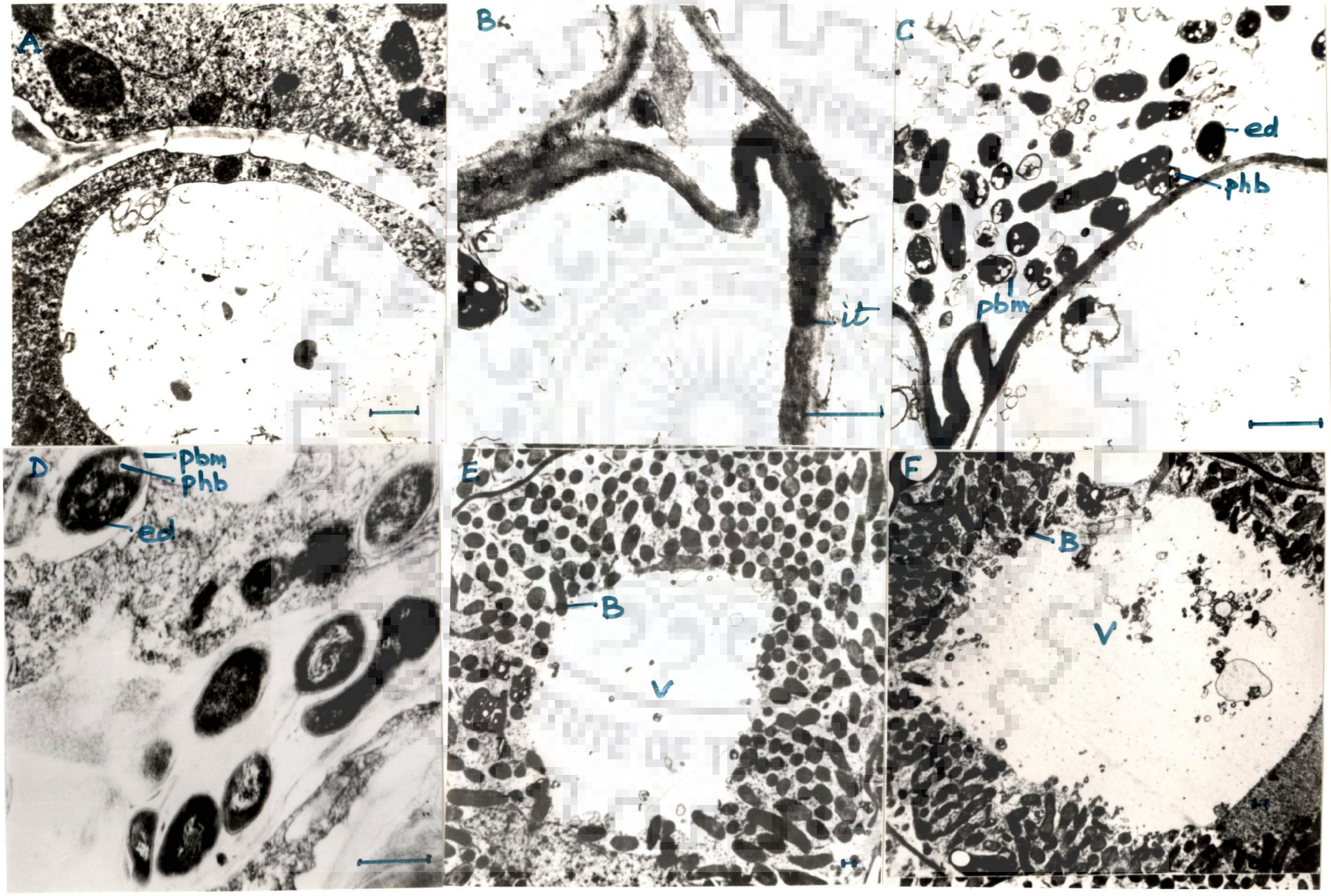
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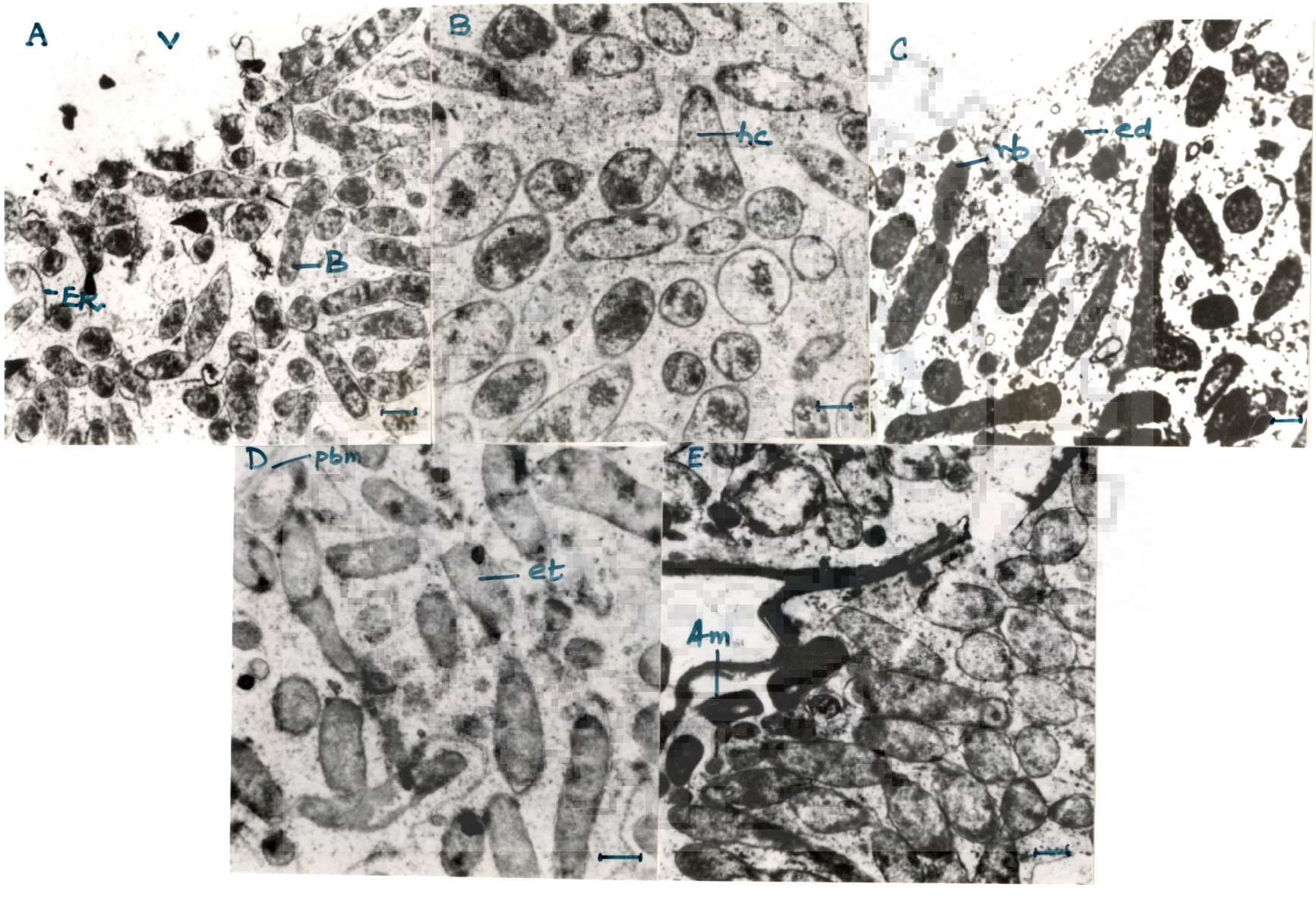
Plate 18: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain NV6, a *pyrE/pyrF* mutant of *Sinorhizobium meliloti* Rmd201, **A.** A part of nodule cell showing elongated bacteroids (B), centrally located vacuoles (v) and endoplasmic reticuleae (ER) in nitrogen fixation zone, Bar: 1μm (x2900), **B.** A part of nodule cell showing heterogeneous cytoplasm (hc) of rhizobial bacteroid in nitrogen fixation zone, Bar: 1μm (x2900), C. A part of nodule cell showing electron dense cytoplasm (ed) of rhizobial bacteria (rb) in nitrogen fixation zone, Bar: 1μm (x4100), D. A part of nodule cell (of senescent zone) showing broken peribacteroidal membrane (pbm) and electron transparent cytoplasm (et) of rhizobial bacteria, Bar: 1μm (x2300), and E. A part of nodule cell showing amyloplasts in nitrogen fixation zone, Bar: 1μm (x2300).


A
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Chapter 5

DISCUSSION

Random transposon Tn5 mutagenesis of *Sinorhizobium meliloti* strain Rmd201 strain, a Nod^+, Fix^+, Sm^r and compact colony variant of Rm41 strain, yielded 7,350 kanamycin resistant transconjugants. The frequency of Tn5 transposition ($3.67x10^{-5}$) was far greater than the spontaneous kanamycin (400µg/ml) resistance frequency (i.e. 10^{-8}) in *S. meliloti*. Thirty-seven auxotrophs were found among 7,350 Tn5-induced derivatives giving the frequency of occurrence of auxotrophs as 0.5%. The frequencies of Tn5 transposition and occurrence of auxotrophs were similar to those reported earlier in rhizobia (Meade *et al.*, 1982; Kim *et al.*, 1988; Vineetha, 1998; Prasad *et al.*, 2000). The auxotrophs isolated had diverse nutritional requirements indicating the randomness of Tn5 insertions as reported by the above researchers.

Twelve uracil, 2 uracil + arginine and 2 arginine auxotrophs, along with 6 uracil and 3 uracil + arginine auxotrophs were taken for further studies. Intermediate feeding, intermediate accumulation and cross-feeding studies were carried out to identify the block in each of these auxotrophs. No cross feeding was observed even where it was expected, based on results of intermediate feeding and intermediate accumulation studies, indicating that either small amounts of pyrimidine intermediates were accumulated or these intermediates could not be released to the surrounding medium. The uracil + arginine and arginine auxotrophs were classified into three groups on the basis of above studies.

Since carbamoyl phosphate synthetase enzyme is a common enzyme for both pyrimidine and arginine biosynthetic pathways, a mutation in the *carA* or *carB* gene (*carA* and *carB* genes code for carbamoyl phosphate synthetase) occurred in a uracil + arginine auxotroph. The growth of uracil + arginine auxotrophs on carbamoyl phosphate

supplemented minimal medium also indicated the occurrence of a mutation in one of the *car* genes in each of these auxotrophs. Thus these uracil + arginine auxotrophs (NV1, NV15, RH33, RH37, RH47) were designated as *car* mutants.

The uracil mutants, NV18, NV21, NV32, VK12, VK19, VK43, RH7, RH9, could grow on orotic acid or dihydroorotic acid but not on carbamoyl phosphate or carbamoyl aspartate supplemented minimal medium. Accumulation of orotic acid was also absent in these auxotrophs. Since dihydroorotase enzyme, coded by *pyrC* gene, is responsible for conversion of carbamoyl aspartate to dihydroorotic acid, each of these auxotrophs has a mutation in the *pyrC* gene. Hence these auxotrophic mutants were called as *pyrC* mutants.

The uracil auxotrophs, NV6, NV9, NV12, NV19, NV23, NV26, NV33, NV34, NV37, RH36, did not grow on minimal medium supplemented with any of the pyrimidine biosynthetic intermediates and accumulated orotic acid. Hence in these auxotrophs biochemical block was between orotic acid and uridine monophosphate (UMP). In each of these mutants a mutation occurred in either *pyrE* (coding for enzyme orotate phosphoribosyl transferase) or *pyrF* (coding for enzyme orotidine monophosphate decarboxylase) gene; therefore these mutants were called *pyrE/pyrF* mutants.

The uracil, uracil + arginine and arginine auxotrophs were similar to the parental strain w.r.t. cell surface molecules, utilization of carbon sources, salt and acid tolerances, change in pH of the medium and growth patterns indicating that the symbiotic defects of these auxotrophs were not caused by a change in any of the above characteristics.

When each *pyr/car/arg* auxotroph containing plasmid pJB3JI was mated to *S. meliloti* ZB555 recipient strain, 100% co-transfer of kanamycin resistance and

auxotrophy occurred; the transconjugants showed symbiotic defect similar to that of the *pyr/car/arg* donor strain. The revertant of each auxotroph had normal symbiosis with alfalfa plants. These results indicated that a single Tn5 insertion was responsible for auxotrophy and symbiotic defect in each auxotroph.

On the circular linkage map of *S. meliloti* Rm41 (Fig.5) pyrimidine mutations are present on two loci, *pyr1/2-29* and *pyr24* (Konodorosi *et al.*, 1977). During this study *pyr/car* mutations were mapped in the 41.7% region of the chromosome, between *cys*46 and *pur*15/168 loci, which includes already mapped *pyr24* locus. Precise mapping of these mutations could not be done due to unavailability of mapping strains from this chromosomal region. Since the present study was carried out on a derivative of *S. meliloti* strain Rm41 there is a possibility that *pyr* mutations are located at or near *pyr24* locus.

Occasional healthy plants were observed among the plants inoculated with *pyr, car* and *arg* mutants. The nodules of these plants were found to be occupied by prototrophic revertant cells. Revertant rhizobial cells formed as a result of spontaneous excision of transposon Tn5 apparently got selective advantage over auxotrophic cells in nodule formation since the frequency of nodule occupancy by revertants was much more than the frequency of spontaneous excision of Tn5.

Pyrimidine auxotrophs of rhizobia have been reported to induce white, ineffective nodules on their respective hosts (Scherrer and Dénarié, 1971; Pain, 1979; Noel *et al.*, 1988; Kerppola and Kahn, 1988b; Vineetha, 1998). Uracil and uracil + arginine auxotrophs in this study also induced white nodules, and mean plant heights and dry weights of these plants did not differ significantly from those of the uninoculated plants, indicating the inability of these auxotrophs to fix nitrogen. The *car* and *pyrC* mutants

induced spherical/irregular nodules whereas the nodules induced by the *pyrE/pyrF* mutants were cylindrical like the parental strain induced nodules. This showed that the extent of nodule development was related to the position of mutation in the pyrimidine biosynthetic pathway.

All pyrimidine auxotrophs of *S. meliloti* formed nodules which contained rhizobial bacteria. This result indicated that pyrimidines were provided by the plant to rhizobial bacteria in the nodule; multiplication of these auxotrophs in the nodules could not have occurred in the absence of pyrimidines. The symbiotic defect of uracil and uracil + arginine auxotrophs was not restored on supplementation of plant nutrient medium with uracil (for *pyr* mutants) or uracil + arginine (for *car* mutants). One possible reason for this could be the unavailability of these supplemented nutrients in sufficient amounts to the rhizobial auxotrophic cells inside the nodule cells. Another reason could be that some intermediate(s)/enzyme(s) of pyrimidine biosynthesis have a role in symbiosis.

None of the pyrimidine intermediates used in this study could restore the symbiotic defect of any of the *pyr/car* mutants. This result does not rule out the involvement of these intermediates in symbiosis since these supplemented intermediates may not be available in sufficient amounts to rhizobial cells inside nodule cells to restore symbiotic defect. Moreover an enzyme(s) involved in synthesis of these pyrimidine intermediates may also be responsible for conversion of some unknown precursor into a product(s) of symbiotic importance.

The rhizobial bacteria invading root cells induce them to differentiate into meristematic cells and different zones so as to form complete nodule structure. The bacteria themselves during this process transform into nitrogen fixing bacteroids

(Newcomb, 1981; Brewin, 1991; Pawlowski *et al.*, 1996). The rhizobial bacteria that induce non-nitrogen fixing nodules are Fix$^-$ mutants. Mostly the nodules induced by the Fix$^-$ mutants are blocked at the stage when bacteria are released from infection threads (Truchet *et al.*, 1980; Vincent, 1980) or leghaemoglobin and bacterial nitrogenase synthesis stage (Zimmerman *et al.*, 1983); in the second case almost normal organised nodular structure may be present. Light and electron microscopic examinations of the nodules induced by all the uracil and uracil + arginine auxotrophs in this study revealed blocks at different stages of nodule development.

In the nodules induced by *car* mutants lysis of rhizobial bacteria occurred immediately after their release into nodule cells from the infection threads. The defect in these nodules appears to be at the stage of bacterial release. The release of rhizobial bacteria into nodule cells occurred normally in the nodules induced by the *pyrC* mutants but the transformation of the released bacteria into bacteroids was not complete. Hence in these nodules the block occurred during the development of nitrogen fixation zone. In the nodules induced by the *pyrE/pyrF* mutants the rhizobial release into plant cells was normal and in most of the rhizobial cells transformation from bacterial to bacteroid stage was almost complete but the nitrogen fixation zones of these nodules were not fully developed like those of the parental strain induced nodules.

The numbers of endoplasmic reticuleae and starch granules were more in the nodules induced by *car* and *pyr* mutants in comparison to those in the parental strain induced nodules indicating nitrogen starvation of these ineffective nodules (Mackenzie and Jordan, 1974; Hirsch *et al.*, 1983).

A mutation in the carbamoyl phosphate synthetase genes leads to simultaneous arginine and pyrimidine auxotrophies. Arginine auxotrophs having blocks before and after ornithine have been reported to be symbiotically ineffective and effective, respectively (Kerppola and Kahn, 1988b). In the present study also arginine auxotrophs having blocks before ornithine have been found to be ineffective. It appears that the symbiotic defect of the first category of arginine auxotrophs is due to the lack of polyamines. Polyamines can be synthesized from ornithine or arginine (Tabor and Tabor, 1985). As both ornithine and arginine are not available in these auxotrophs, polyamines cannot be synthesized. This hypothesis is further strengthened by the findings of the present study that ornithine, citrulline or arginine restored symbiotic defect of arginine auxotrophs having a block before ornithine. Since carbamoyl phosphate synthetase enters the arginine biosynthetic pathway after ornithine, synthesis of polyamines is not a problem in *car* mutants as ornithine is available. Therefore symbiotic defects caused by *car* mutants may be entirely attributed to defective pyrimidine biosynthetic pathway.

Since the nodules induced by *pyrC* mutants showed advanced structural features over those of the nodules induced by *car* mutants, carbamoyl phosphate/carbamoyl phosphate synthetase/carbamoyl aspartate may be involved in symbiosis. The nodules induced by *pyrE/pyrF* mutants were also structurally more advanced than the nodules formed by *pyrC* mutants, indicating that dihydroorotic acid/dihydroorotase/orotic acid may also have symbiotic function(s). The symbiotic defects of nodules induced by *pyrE/pyrF* mutants may be due to unavailability of orotidine monophosphate or lack of orotate phosphoribosyltransferase/orotidine monophosphate decarboxylase activity. Hence an undiminished metabolic flow through pyrimidine biosynthetic pathway in *S.*

meliloti appears to be essential for bacteroidal transformation and effective nodule development (and function) on alfalfa plants.

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LIST OF PUBLICATIONS

*Vij, N., Randhawa, G. S and Chopra, A.K. 1998. Toxicity of pesticides 2, 4-D and cypermethrin against *Rhizobium meliloti*, *Chem. Environ. Res.*, 7: 123-128.

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