GENETIC AND BIOCHEMICAL STUDIES ON BIOSYNTHESIS OF AMINO ACIDS, NUCLEOTIDE BASES AND VITAMINS IN *RHIZOBIUM*

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By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"GENETIC AND BIOCHEMICAL STUDIES ON BIOSYNTHESIS OF AMINO ACIDS, NUCLEOTIDE BASES AND VITAMINS IN *RHIZOBIUM*"**, 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 July 1995 to Dec. 1998 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.

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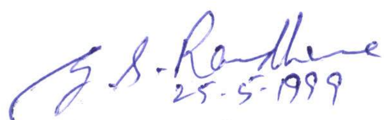

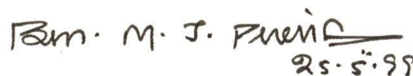

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ABSTRACT

The *Rhizobium meliloti* strain Rmd 201 (Nod^+ Fix^+ Sm^r), a derivative of wild type strain Rm 41, was subjected to random transposon Tn5 mutagenesis with the help of transposon introducing plasmid pGS9 carrying Tn5. Six thousand transconjugants obtained at a frequency of $3x10^{-5}$ from the crosses between the *E. coli* strain WA 803 (*met, thi*) harbouring suicide plasmid pGS9 and Rmd 201 were collected, purified and screened for auxotrophs based on their inability to grow on minimal medium. The screening of Tn5 derivatives yielded 29 auxotrophs, the frequency of isolation of auxotrophs being 0.48%. The nature of auxotrophy of each mutant was then determined on nutritional pools. Symbiotic properties of all auxotrophs were tested and are as follows : Nod^- (isoleucine + valine), Nod^+, Fix^+ (methionine and cysteine/methionine), Nod^+, Fix^- (uracil, uracil + arginine and adenine). When the revertants were similarly examined, they resembled the parental type strain Rmd 201, indicating that changes in the symbiotic behaviour of the above strains could be attributed to their auxotrophic mutations (Table 6). In all the above plant experiments, the bacteria were isolated from the nodules to confirm that nodules were colonized by the same strain with which the plant was inoculated. From a collection of 29 auxotrophs 9 pyrimidine auxotrophs were selected for further detailed studies.

The position of block in the pyrimidine biosynthetic pathway in these auxotrophs was determined by intermediate feeding and intermediate

accumulation studies. Based on the results the pyrimidine auxotrophs were divided into three groups as follows:

I Carbamoyl phosphate synthetase mutants which require carbamoyl phosphate for their growth (H33, H37 and H47).

II The mutants which have blocks in the pathway between carbamoyl phosphate and orotic acid (VE12, VE19, VE43, H7 and H9).

III The mutant that does not grow on orotic acid but grows on uracil supplementation, evidently having a block in the pathway after orotic acid (H36).

Symbiotic properties of pyrimidine auxotrophs were investigated by inoculating them on alfalfa *(Medicago sativa)* seedlings grown aseptically on nitrogen free agar slants. The symbiotic properties of auxotrophs in the second group except H7 (VE12, VE19, VE43 and H9) could not be determined as the nodules were always occupied by their prototrophic revertants. The carbamoyl phosphate mutants (H33, H37 and H47), the mutant H7 which has a block between carbamoyl phosphate and orotic acid and the mutant H36 that has a block in its pyrimidine biosynthetic pathway after orotic acid were nod^{+} and fix^{-}, i.e., they formed nodules which did not fix nitrogen.

In order to test the authenticity of the nodules formed by these auxotrophs, root hair curling and infection thread formation were observed under light microscope. All auxotrophs were found to induce root hair curling and infection thread formation.

For histological studies the nodules from six weeks old plants were fixed and embedded in araldite epoxy resin. Semithin and ultrathin sections

were obtained and observed under light microscope and transmission electron microscope, respectively.

Alfalfa nodules induced by the parental strain Rmd 201 were elongate, cylindrical and had distinct typical meristem, infection, nitrogen fixation and senescent zones. The bacteria in the infection thread and just after release into the host cell were approximately 1.0 to 1.5 μm long. At this stage poly-β-hydroxybutyrate inclusions were found in the bacterial cytoplasm. In the nitrogen fixation zone of the nodule the mature bacteroids increased significantly in size becoming 3 to 8 μm long. In addition, they proliferated to the extent that most of the host cytoplasm was occupied by elongated bacteroids. The younger bacteroids exhibited a relatively homogeneous cytoplasm and were found in the distal part of nitrogen fixation zone. In the mature bacteroids, which were present in more proximal part of the nitrogen fixation zone, the cytoplasm exhibited greater heterogeneity because of the condensation of nucleic acid material.

The nodules induced by strain H33 were ineffective and distinguishable in morphology from those induced by wild type. These nodules were usually white, spherical and found on secondary roots. The longitudinal section of the nodule did not show distinct zones. Most of the cells in the nodule were without bacteroids. Extensive branching of infection threads was found. At the ultrastructural level the occasional release of bacteria into plant cell cytoplasm was seen. The released bacteria showed homogeneous cytoplasm. Mature bacteroids with features as described above were not seen in the plant cells. It seems that transformation of young bacteroids to mature bacteroids did not occur in this case.

Nodules elicited by H7 were spherical or irregular in shape and white in colour. Light microscopic studies revealed absence of distinct zones. Ultrastructural studies showed extensive branching of infection threads and occasional release of bacteria into plant cell cytoplasm was observed. The released bacteria exhibited a relatively homogenous cytoplasm. The bacteroidal development did not occur beyond first stage.

The pyrimidine auxotroph H36 formed nodules that exhibited distinct zones viz. meristematic zone, infection zone, poorly developed nitrogen-fixing zone and senescent zone. Electron microscopic studies revealed that in the infection zone electron dense homogeneous young bacteroids with phb granules were seen. In the nitrogen-fixing zone bacteroids were elongated and properly organised. Bacteroids in this region showed partially heterogeneous cytoplasm which were lacking proper condensation of nucleic acid materials. It seems that transformation from young bacteroids to mature bacteroids did not occur fully. Moreover some cells showed this bacteroidal cytoplasm in electron transparent condition with ruptured peribacteroidal membrane. These bacteroids looks like lysing in this stage.

For confirming the linkage of auxotrophy to Tn5 insertion, the plasmid pJB3JI was used to mobilize the Tn5 containing chromosomal segments of pyrimidine auxotrophs into another *R.meliloti* strain ZB 555 (Cys^-, Phe^-, Rif^r, Sm^r). All Km^r transconjugants obtained showed donor's auxotrophy. This confirmed the linkage of auxotrophy to Tn5 insertion as well as ruled out the possibility of occurrence of other independent Tn5 insertions in these mutants.

Since the plasmid pJB3JI used in this study showed poor genome

mobilizing ability, precise mapping was not possible but the possibility of occurrence of *pyr* loci in 2/3 of the chromosome could be ruled out.

In order to find out the pleiotropic effects of Tn5 insertions all auxotrophs were tested for the production of exopolysaccharides, lipopolysaccharides, cellulose fibrils, β-1 $\rightarrow$ 2 glucans and utilization of dicarboxylic acids (malate, aspartate, succinate, etc.). Like the wild type all auxotrophs utilised dicarboxylic acids as carbon source and produced the above mentioned cell surface molecules.

From the above results it seems that intermediates of the pyrimidine biosynthetic pathway have a role in bacteroid development as blocks at different steps of the pathway resulted in bacteroids blocked at different stages of development.

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LIST OF ABBRIVATIONS USED

a	-	amyloplast
bc	-	bacteroid
CW	-	Cell wall
EC	-	Empty cell
edm	-	electron dense material
gc	-	golgi complex
I	-	Infection zone
it	-	infection thread
M	-	Meristematic zone
mg	-	milligram
ml	-	millilitre
n	-	nucleus
nm	-	nanometer
NZ	-	Nitrogen fixation zone
pbm	-	peribacteroidal membrane
phb	-	poly-β-hydroxybutyrate
r	-	rhizobia
RER	-	Rough endoplasmic reticulum
rh	-	root hair
S	-	Senescence zone
sc	-	shepherd's crook
V	-	Vacuoles
VB	-	Vascular bundles
µg	-	microgram
µm	-	micrometer

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Chapter 1

INTRODUCTION

Rapid increase in population and pollution pose a considerable burden on the economy of developing nations. Scientific utilization of the available fertile area can solve these problems to a certain extent. Nitrogen is a major component of earth's atmosphere and is an essential macronutrient of all living organisms. Plants, the primary producers in the food chain, cannot use the gaseous form of nitrogen from the atmosphere as such. They can take up only combined forms of nitrogen like ammonia, nitrate, nitrite, etc. The chemical fertilizers which are employed to compensate the deficiency of nitrogen in agricultural land are costly and polluting the ecosystem considerably. Biological nitrogen fixation is the most acceptable candidate to rectify these problems. This involves the conversion of atmospheric nitrogen to ammonia by a group of prokaryotes (bacteria and blue green algae) collectively called diazotrophs. These bacteria on the basis of the extent of their association with plants can be classified into three main groups which are as follows :

(i) free-living (*Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Rhodospirillum rubrum*, etc.),

(ii) associative (*Azospirillum* spp.), and

(iii) symbiotic (*Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Frankia*, etc.)

Rhizobial-legume symbiosis has a special significance because it contributes as much as 25% of the terrestrial nitrogen. Rhizobial bacteria interact with different legumes to produce root nodules. Establishment of the rhizobial-legume symbiosis involves infection of the host plant root and the subsequent formation of nodular growths containing modified rhizobial cells. Rhizobia are gram negative bacteria and well-adapted to survive in soil. The compatible rhizobia induce curling and branching of root hairs and subsequent formation of a tubular structure

called the infection thread in the host plant. The infection thread develops inward from its point of origin, near the most acutely curled region of the root hair. Rhizobia pass into the thread usually in a single file as the tip of the thread follows the movement of the nucleus towards the base of the root hair cell. The infection thread passes through the wall of root hair cell and the intercellular spaces of the adjacent cortical cells and branches into many newly dividing cortical cells. Rhizobia are released from the tip of the infection thread as vesicles into the host cell cytoplasm.

Though peribacteroidal membrane which surrounds each bacteroid is similar to plasma membrane, it does contain some characteristic proteins. Within the nodule the rhizobia differentiate into bacteroids which are the nitrogen fixing form of the rhizobia. The enzyme nitrogenase is synthesized in the bacteroids that converts dinitrogen to ammonia at the expense of host plant photosynthates (Bauer, 1981; Brewin, 1991). The plant produces globin (Sidloi-Lumbroso *et al.*, 1978) which combines with heme produced by the rhizobia (Cutting and Schulman, 1969) to form leghaemoglobin which appears to be a prerequisite for nitrogen fixation, Rhizobia can produce indole acetic acid (Dullart, 1970) and very small amounts of cytokinins (Phillips and Torrey, 1972) both of which are important plant hormones. There is also an indication that rhizobia can produce small amounts of polygalactouronases which may be important in obtaining an entry into the root and in the growth of the infection thread within the root. The significance and role of any of these substances during the differentiation remains to be established.

Rhizobia are further classified into two groups, viz., fast growing and slow growing. The fast growing rhizobia (*R. meliloti*, *R. leguminosarum*, etc.)

serve as a better genetic system because relatively lesser time is required for their colony formation. The growth of certain strains of *R. meliloti* is known to be fastest among the fast growing rhizobia. *R. meliloti* forms symbiotic association with three genera of Medicago group : *Medicago*, *Melilotus* and *Trigonella*. The small size of these plants facilitates assaying of bacterium-plant interactions under laboratory conditions. Because of the above advantages *R. meliloti* was chosen for the present study.

Recent advances in the recombinant DNA technology have made it easy to understand the complexity of symbiotic associations. The rhizobial genes responsible for nodule development and nitrogen fixation have been mapped and sequenced (Banfalvi *et al.*, 1981; Orenik *et al.*, 1994; Freiberg *et al.*, 1997). It has been reported that directly or indirectly some other functions of rhizobia can also influence symbiosis (Watson *et al.*, 1988; Finan *et al.*, 1991; Tombolini *et al.*, 1995; ϕsterås *et al.*, 1997). These include metabolic pathways for the synthesis of amino acids (Sadowsky *et al.*, 1986; Yadav *et al.*, 1998), nitrogenous bases (Swaminathan *et al.*, 1992, 1995) and vitamins (Graham, 1963). The biosynthetic study of amino acids, nucleotide bases and vitamins involve the use of auxotrophic mutants. Several mutagenesis procedures are used to induce a range of defined mutations including metabolic and symbiotic deficiencies.

Tyrosine, leucine and tryptophan auxotrophs have been reported to yield ineffective nodules (Kerppola and Kahn, 1988a). The role of some amino acids like serine, glycine, glutamine and glutamate in symbiosis is still not clear (de Brujin *et al.*, 1989). Except for few reports (Keppola and Kahn, 1985; 1988) only little information is available about the role of pyrimidines or their

intermediates in symbiosis. It has been reported that the defective symbiosis in purine auxotrophs was partially restored by supplementation of one of the purine intermediates, AICAR (Newman *et al.*, 1992; 1994). These studies indicate that either the end products or the intermediates in the amino acids/purines/ pyrimidines biosynthetic pathways are important for symbiosis. Thus analysing the symbiotic capabilities of auxotrophic mutants will give us an understanding of the contribution of different metabolites and various biosynthetic pathways in the nodulation and nitrogen fixation processes.

Keeping the above observations in view the present study was undertaken in *Rhizobium meliloti* with the following objectives :

(i) to generate auxotrophs of *R.meliloti* by random mutagenesis,

(ii) to study the biochemical and symbiotic characteristics of auxotrophs obtained,

(iii) to study the internal morphology of the nodules formed by pyrimidine auxotrophs and

(iv) to map the genes involved in pyrimidine biosynthetic pathway.

Chapter 2

LITERATURE REVIEW

For the present study the literature has been reviewed under the following headings :

1. Taxonomy of rhizobia.
2. Genetics of rhizobia.
3. Development of the legume root nodule.
4. Genes involved in nitrogen fixation
5. Symbiotic characteristics of auxotrophs of rhizobia.

2.1. TAXONOMY OF RHIZOBIA

The Rhizobiaceae group of bacteria which infect plants of the family Fabaceae (Leguminosae) were earlier exclusively placed in the genus *Rhizobium* (rhizo = root and bios = living). Subsequently these bacteria were re-grouped in the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. The bacteria of the genus *Rhizobium* grow fast and nodulate a narrow range of temperate legumes whereas *Bradyrhizobium* bacteria are slow growing and nodulate a broad range of tropical legumes. *Rhizobium* and *Bradyrhizobium* bacteria cannot grow in culture using gaseous nitrogen as a nitrogen source but *Azorhizobium* bacteria can do so. Recently fast growing rhizobia have been reclassified and a new genus called *Sinorhizobium* has been recognized. Phylogenetic relationships of fast growing rhizobia are shown in Figure 1.

2.2 GENETICS OF RHIZOBIA

2.2.1. MUTAGENESIS

Rhizobia were mutagenized by chemical mutagens or transposons to identify the genes involved in symbiosis.

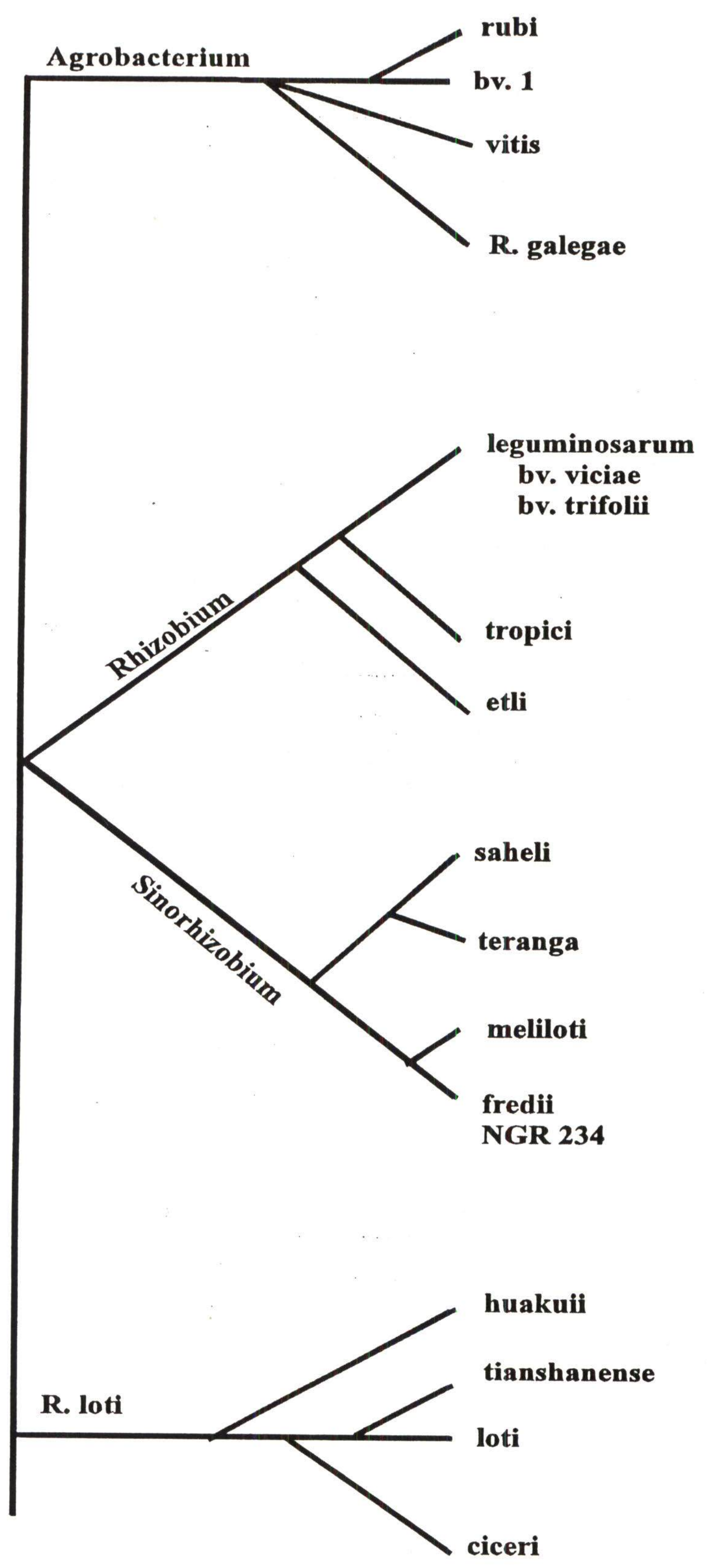


Fig. 1. A schematic phylogenetic tree of fast-growing rhizobia drawn after Lindstrom et al., 1995.

2.2.1.1 Chemical Mutagenesis

Several mutagens like ethyl methane sulphonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (NTG), nitrous acid, etc. have been employed to generate auxotrophs and symbiotically defective mutants of rhizobial strains (Kondorosi *et al.*, 1977b; Noel *et al.,* 1982; Forrai *et al.*, 1983; Singh *et al.,* 1984; Cava *et al.,* 1989; Kummer and Kuykendall, 1989; McIver *et al.,* 1989).

2.2.1.2. Transposon Mutagenesis

Several investigators have reported the use of transposon Tn5 mutagenesis to obtain various types of mutants of rhizobia. Suicidal plasmids carrying transposon Tn5 were constructed by Beringer *et al.* (1978a), Selvaraj and Iyer (1983) (Fig. 2) and Simon *et al.* (1983). These plasmids were used for the random transposon mutagenesis of *Rhizobium* to identify and characterize the genes of symbiotic importance (Meade *et al.,* 1982; Forrai *et al.*, 1983; Noel *et al.,* 1984; Hirsch *et al.,* 1984; Hom *et al.,* 1984; Sadowsky *et al.,* 1986; Leigh *et al.,* 1987 and Barsomian *et al.,* 1992). The mutagenesis was carried out by transferring the suicidal plasmid from *E. coli* to *Rhizobium* (Fig. 3). The symbiotic gene after mutation gets tagged by transposon Tn5 and can be located by the antibiotic resistant phenotype of the transposon. The tagged gene can also be detected by hybridization with a Tn5 probe.

Tn5 mutagenesis methodology was extended to a general method for site-directed Tn5 mutagenesis by Ruvkun and Ausubel (1981). This method involved the cloning of rhizobial *nif, nod* or *fix* genes into a suitable multicopy plasmid. The cloned genes were then subjected to Tn5 mutagenesis in *E.coli* and reintroduced into *Rhizobium*. Forced gene-replacement of the corresponding *Rhizobium* wild type sequences with

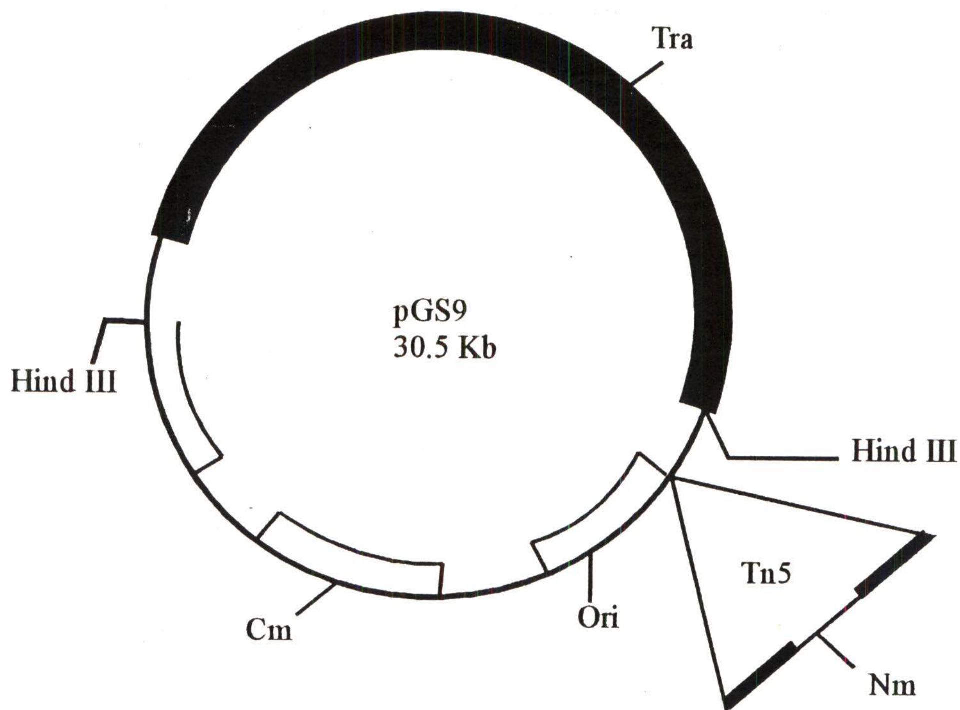


Fig. 2. Transposon Tn5-delivery vector pGS9. Abbreviations : Cm, Chloramphenicol; Nm, Neomycin; Ori, Origin of replication

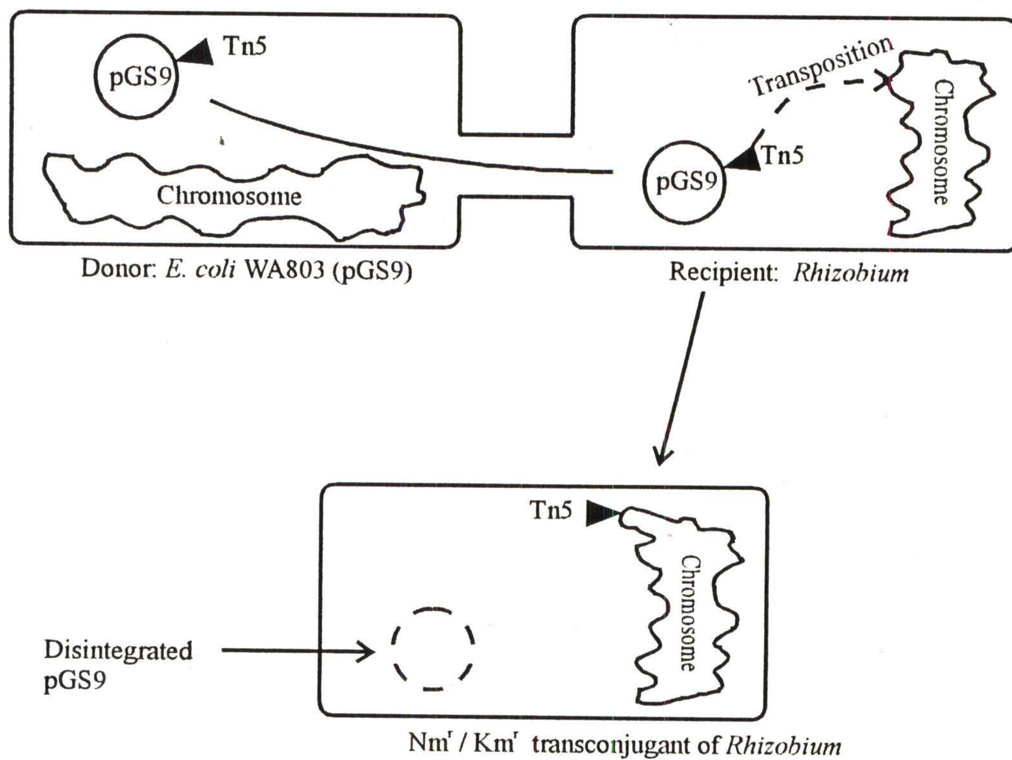


Fig.3. Diagramatic representation of transposon Tn5 mutagenesis

the Tn5-mutated counterparts was achieved. Through this method the physical and genetic characterizations of several *nod* and *nif* genes in a variety of *Rhizobium* species were done (Ausubel, 1982; Corbin *et al.*, 1983).

2.2.2 GENETIC AND PHYSICAL MAPPING STUDIES

Chromosomal transfer by conjugation allows rapid genetic mapping of bacterial chromosomes. But such studies were not possible in *Rhizobium* because of the lack of an indigenous conjugation system. The breakthrough came when P1 incompatibility group of R plasmids from *Pseudomonas* were shown to promote chromosome mobilization in a number of gram negative bacteria including rhizobia (Beringer and Hopwood, 1976; Kondorosi *et al.*, 1977a; Meade and Signer, 1977). These plasmids generally do not promote transfer from single, distinct origins (Hass and Holloway, 1976; Meade and Signer, 1977) and, therefore, coinheritance frequencies have to be worked out (Kondorosi *et al.*, 1977a; Beringer *et al.*, 1978a). The plasmid RP4 was employed to construct circular linkage map of *R. meliloti* 2011 (Meade and Signer, 1977). Linkage map of *R. meliloti* Rm41 (Kondorosi *et al.*, 1977a), *R. meliloti* GR4 (Casadesus and Olivares, 1979) and *R. leguminosarum 300* (Beringer and Hopwood, 1976; Beringer *et al.*, 1978b) were prepared by using plasmid R68.45. Circular linkage maps of *R.meliloti* 2011, *R.meliloti* Rm 41 (Fig.4) and *R. leguminosarum* 300 were compared by Kondorosi *et al.* (1980).

R. meliloti strain Rm41 has been found to harbour three plasmids, viz., pRme41a, pRme41b and pRme41c (Casse *et al.*, 1979 ; Banfalvi *et*

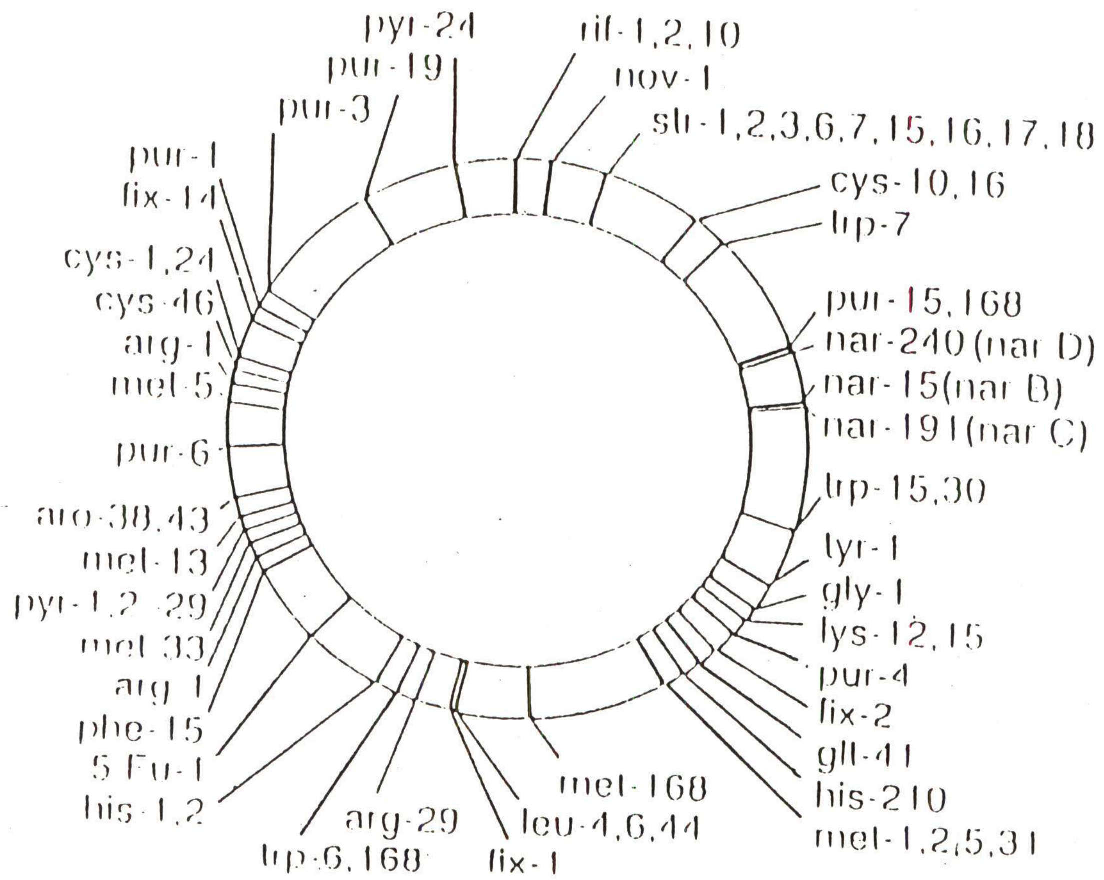


Fig. 4. Chromosomal map of *Rhizobium meliloti* strain Rm41(after Kondorosi et al., 1980)

al., 1981; Banfalvi *et al*., 1985). Nodulation (*nod*) and nitrogen fixation (*fix*) genes were localized on pRme41b, and detailed physical and genetic maps of the symbiotic region of this plasmid were established (Banfalvi *et al*., 1981; Kondorosi *et al*., 1984). In *R. meliloti* strain 2011 plasmid pRmeSU47a was found to carry the *nod* and *fix* genes (Batut *et al*., 1985). The genes for bacteroid development, exopolysaccharide biosynthesis and dicarboxylic acid transport in this strain were located on plasmid pRmeSU47b (Charles and Finan, 1990). Genetic and physical maps of plasmid pRmeSU47a have been published by Julliot *et al*. (1984), Batut *et al*. (1985), Swanson *et al*. (1987), Long *et al*. (1988) and Glazebrook and Walker (1989). The circular linkage map of pRmeSU47b has been constructed by Charles and Finan (1990).

The physical map of the symbiotic plasmid of *Rhizobium spp*. strain NGR234 has been established by Perret *et al*. (1991). A correlated physical and genetic map of the *B. japonicum* 110 chromosome was constructed by Kundig *et al*. (1993). Physical maps of the three replicons, viz., plasmid pRme1021a, plasmid pRme1021b and chromosome of *R. meliloti* 1021 were given by Honeycutt *et al*. (1993). Recently the complete nucleotide sequence of megaplasmid pNGR234a of *Rhizobium* sp. strain NGR234 was reported by Freiberg *et al*. (1997).

2.3. DEVELOPMENT OF THE LEGUME ROOT NODULE

The literature on the development of the legume root nodule has been reviewed by Brewin (1991). A lot of diversity in the morphology of legume root nodules has been reported. Inspite of this diversity the following four anatomical

features are generally common in all legume nodules (i) induction of a new plant meristem, (ii) tissue and cell invasion by rhizobial bacteria, (iii) development of a central tissue with reduced access to atmospheric oxygen and (iv) development of peripheral vascular tissue (Fig. 5).

The process of establishment of effective rhizobial legume symbiosis involves nodule initiation, nodule invasion and nodule maturation.

2.3.1. NODULE INITIATION

2.3.1.1. Diffusible Signals from Plant to Bacterium

Rhizobia are attracted by plant root exudates. These bacteria are chemotactic towards sugars, amino acids and some other nutrients (Bushby, 1982; Götz and Schmitt, 1987). Rhizobia also strong chemotaxic to specific flavonoids released by legume roots (Caetano-Anolles *et al.*, 1988; Aguilar, 1988; Maxwell and Phillips, 1990 ; Kape *et al.*, 1991). The flavonoids present in root exudates also induce the transcription of *nod* genes of *Rhizobium* (Peters *et al.*, 1986) and *Bradyrhizobium* (Kosslak *et al.*, 1987).

The *nodD* gene product, NodD protein, appears to interact with the flavonoids in root exudates (Recourt *et al.*, 1989; Schlaman *et al.*, 1989). These flavonoids are species specific and NodD proteins from different species of rhizobia interact with specific flavonoids preferentially. These interactions are an important determinant of host-*Rhizobium* specificity (Horvath *et al.*, 1987; Kondorosi *et al.*, 1989; McIver *et al.*, 1989; Spaink *et al.*, 1989). Some rhizobia like *R. meliloti* carry three variants of NodD, each of which may have a different flavonoid specificity resulting in greater diversity in host plant nodulation responses (Gyorgypal *et al.*, 1988).

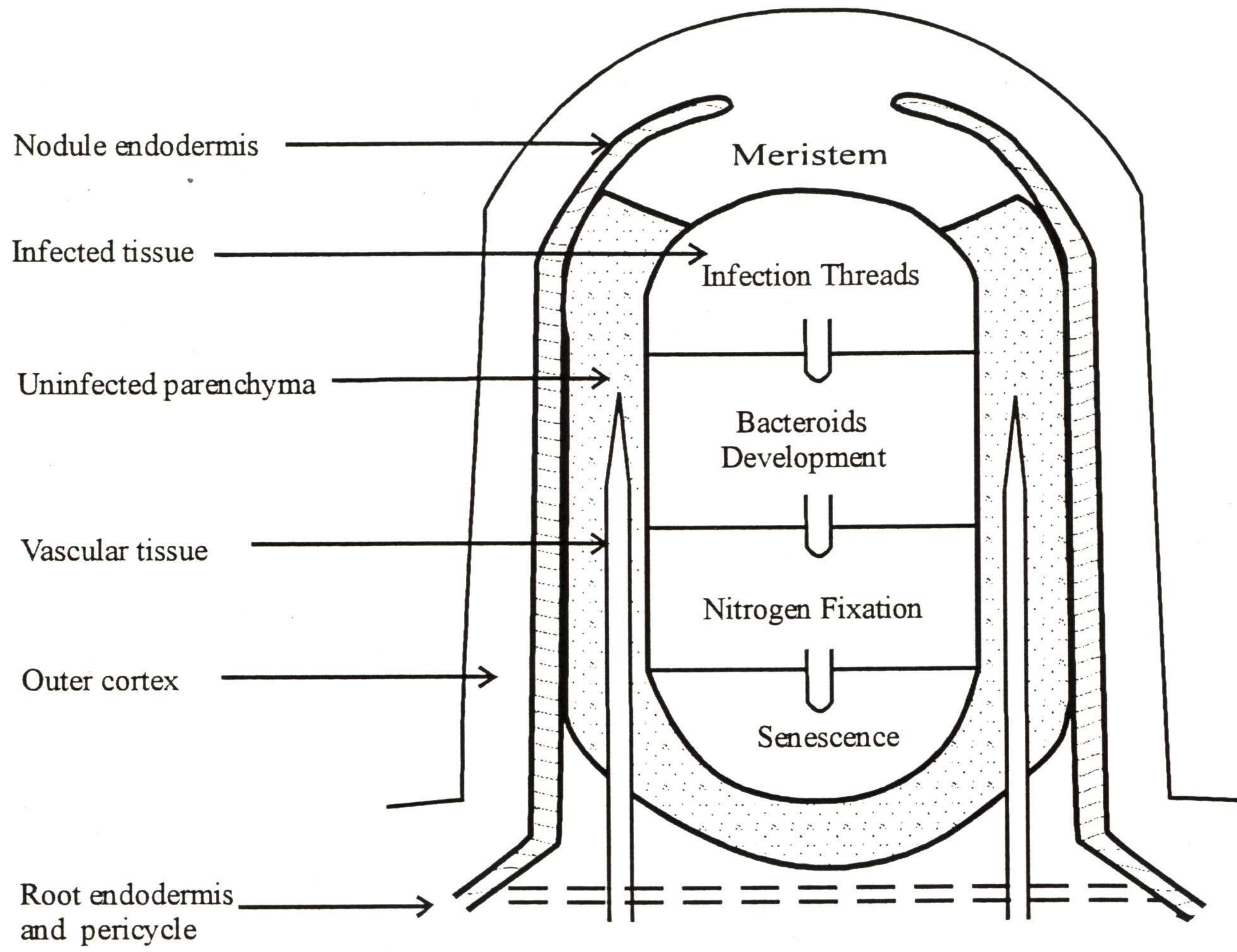


Fig. 5. Differentiation of cell types from the apical (uninfected) meristem of pea nodule. In the longitudinal axis of the nodule, the central mass of tissue shows successive stages of host cell invasion and differentiation by *Rhizobium* (after Brewin, 1991).

2.3.1.2. Diffusible Signals from Bacterium to Plant

Root hairs of a particular legume respond to inoculation with a specific rhizobial strain as well as the culture filtrate derived from such a strain. Bhuvaneswari and Solheim (1985) isolated a low molecular weight diffusible signalling substance from the culture filtrate of *R. trifolii*. A sulphated and acylated tetraglucosamine glycolipid called NodRm-1 was identified in the culture filtrate of *R. meliloti* by Lerouge *et al.* (1990). NodRm-1 was found to elicit root hair deformation and stimulate cortical cell divisions in alfalfa seedlings (Roche *et al.*, 1991). These studies revealed that the individual *nod* genes are involved in the biosynthesis and secretion of the glycolipid signalling molecule. Outline of ways in which nod genes in rhizobia may act is given in Figure 6. Mutations in *nodA*, *nodB* and *nodC* cause completely Nod$^-$ phenotype (Long *et al.*, 1989). Other nod genes such as *nodFE*, *nodG*, *nodH* and *nodL* are involved in specifying the type of host plant to be nodulated. Mutations in these genes lead to abnormalities in the root hair curling reactions elicited on their normal hosts and sometimes also result in the infection of host plants which are normally unresponsive (Lerouge *et al.*, 1990; Schwedock and Long, 1990). Downie and Surin (1990) have reported that *nodO* mutants have only minor effect on nodulation but the mutants lacking both *nodO* and *nodE* functions were severely affected in the nodulation process.

The region of the root just behind the apical meristem and the root region where the emergence of root hairs take place have been observed to be most susceptible to rhizobial infection (Bhuvaneswari *et al.*, 1981). Induction of mitotic activity in the root cortex has been noticed within 12-24 hrs of rhizobial inoculation (Calvert *et al.*, 1984). This mitotic activity has been found to be due to the expression of rhizobial *nod* gene (Dudley *et al.*, 1987). The location of

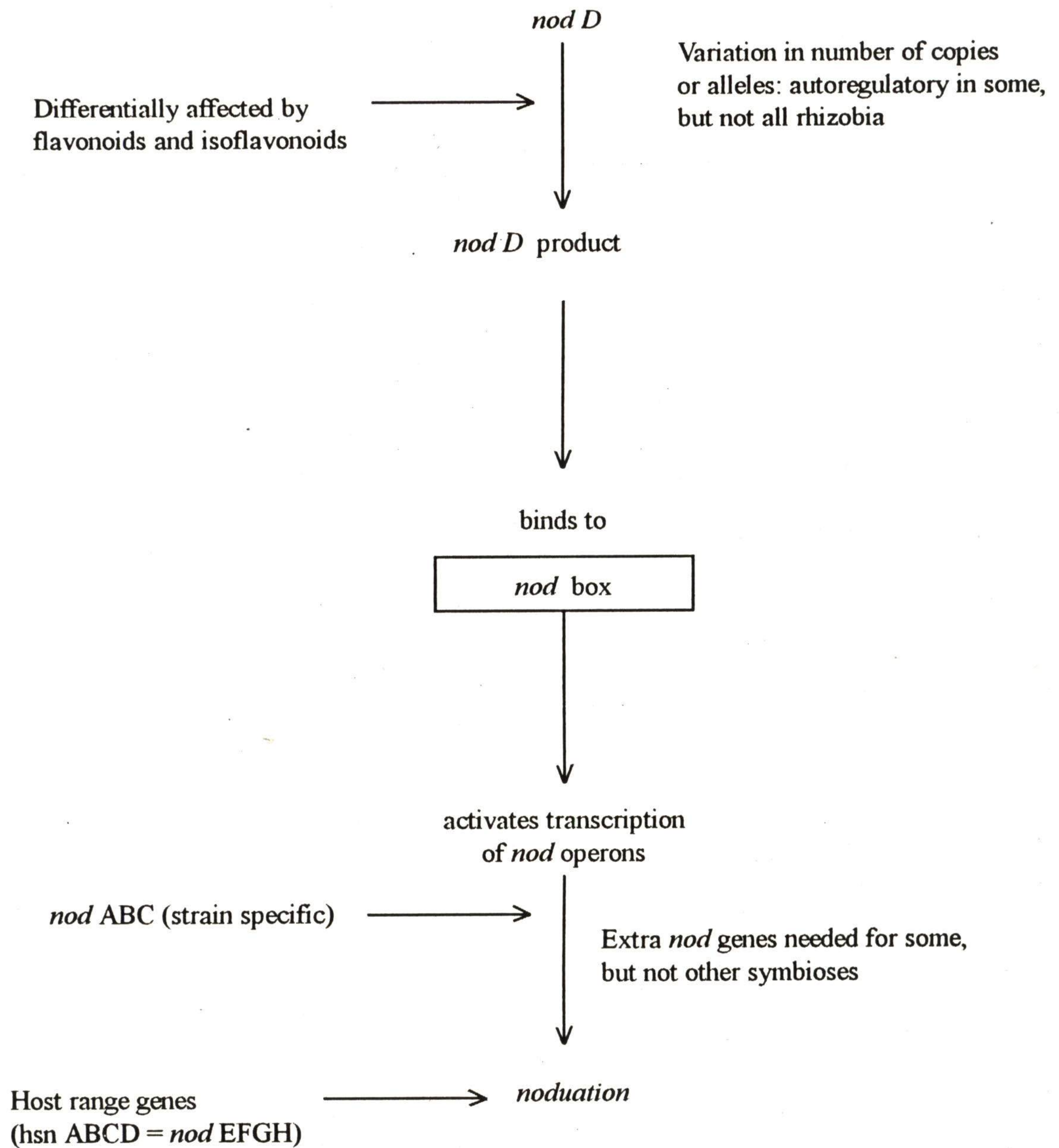


Fig. 6 : Outline of ways in which *nod* genes in rhizobia may act (after Sprent and Sprent 1990).

the nodule primordia in the root cortex depends on the type of nodule formed by a particular plant (Newcomb, 1981). The primordium is formed from the cells in the inner cortex in some plants like alfalfa and vetch (Libbenga and Harkes, 1973; Dudley *et al.*, 1987). This leads to the formation of indeterminate cylindrical nodules with a persistent apical meristem (Newcomb, 1976). This persistent activity of the meristem ensures nodule elongation since new cells are constantly added to the distal end of the nodule (Vasse *et al.*, 1990). The rhizobia are released from the infection threads to the cytoplasm of active meristematic cells. In indeterminate nodules, the nodule growth and functioning occur simultaneously and all developmental zones during differentiation can be observed in a single longitudinal section of nodule. In the plants like *Phaseolus* and *Lotus*, cortical cell divisions occur just beneath the epidermis (Mathews *et al.*, 1989) and these cells are invaded by rhizobia before they become meristematic (Rolfe and Gresshoff, 1988), thus the rhizobial cell divisions occur simultaneously with the plant cell divisions.

2.3.2 NODULE INVASION

2.3.2.1 Role of Bacterial Surface in Early Infection Events

Rhizobial attachment to the root surface is an important preliminary step before tissue and cell invasion. This attachment and other early infection events appear to be influenced by rhizobial cell surface molecules. These cell surface molecules include cellulose fibrils, lipopolysaccharides, $\beta(1\rightarrow2)$ glucans, $\beta(1\rightarrow3)$ glucans, $\beta(1\rightarrow6)$ glucans and extracellular polysaccharides.

Cellulose fibrils may be helping rhizobia to bind to the surface mucigel of

the plant root. This binding appears to be reinforced by calcium dependent protein (ricadhesins) produced by rhizobia (Smit *et al.,* 1989). The binding of the congo red dye with the cellulose fibrils of rhizobia results in the formation of red colonies.

Noel *et al.* (1986) reported that *R.phaseoli* mutants with defective production of lipopolysaccharides resulted in deformation of root hairs and stimulation of cell division but did not invade cortical cell. The lipopolysaccharides of *R.phaseoli* and *R.meliloti* were found to be actually involved in the interaction of these rhizobia with their host plants (Carlson *et al.,* 1987; Lagares *et al.,* 1992). Three genetic regions coding for the production of lipopolysaccharides in *R. leguminosarum* were identified. In *R. meliloti lpsZ*, a lipopolysaccharide gene, was found to be involved in symbiosis (Brzoska *et al.,* 1991).

Nikanishi *et al.* (1976) reported that a major type of curdlans $\beta(1\rightarrow3)$ glucans can be specifically stained with aniline blue. The genes *ndvA* and *ndvB* encoding functions required for $\beta(1\rightarrow2)$ glucans synthesis have been identified in *R. meliloti* (Dylan *et al.,* 1986). Geremia *et al.* (1987) observed that the absence of $\beta(1\rightarrow2)$ glucans correlated with absence of motility. The synthesis of $\beta(1\rightarrow2)$ glucans was found to be required for infection thread development in *rhizobia,* however, a $\beta(1\rightarrow3)$, $\beta(1\rightarrow6)$ glucan appears to replace $\beta(1\rightarrow2)$ glucan in *Bradyrhizobium* (Miller *et al.,* 1990). It has been suggested that these periplasmic oligosaccharides have a role in bacterial attachment or in osmotic adaptation (Dylan *et al.,* 1990).

Mutants defective in extracellular polysaccharides (EPS) induce little or no invasion in indeterminate nodules. In *R. meliloti eps* mutants, which were unable to produce normal acidic heteropolysaccharide (EPSI), were complemented by an entirely different acidic heteropolysaccharide EPSII

(Glazebrook and Walker, 1989). EPSI can be substituted by a particular form of lipopolysaccharide specified by the *lpsZ* gene to restore a symbiotic effectiveness in *R.meliloti* (Williams *et al.*, 1990).

It is interesting to note that the symbiotic phenotype of *Rhizobium* mutants defective in extracellular polysaccharides varies according to the nodule morphology of the host plant. *Rhizobium eps* mutants are *Nod⁻* or induce empty nodules (*Inf⁻*) on *Pisum*, *Medicago* and *Leucaena* which all produce indeterminate nodules. The same or similar mutations, however do not affect the development of determinate nodules on *Phaseolus*, *Glycine* and *Lotus* (Hotter and Scott, 1991). The lipopolysaccharide-defective mutants lacking O-antigen produce Inf⁻ phenotype on *Phaseolus* and *Glycine* (Puvanesarajah *et al.*, 1987). Such mutants show impaired bacteroid release in peas (Brewin *et al.*, 1990). It appears from these studies that extracellular polysaccharides have a role in infection thread development whereas lipopolysaccharides are probably involved in endocytosis and bacteroid differentiation.

2.3.2.2 Root Hair Deformation and Infection Thread Development

The root hair cells have been found to be most susceptible to deformation and infection thread development induced by rhizobia (Bhuvaneswari *et al.*, 1981). Root hair deformation occurs within a few hours of rhizobial exposure to root hairs but for most severe form of root hair curling physical attachment of rhizobia to root hair surface is required. It has been proposed that attached rhizobia induce a local stimulation in the rate of plant cell wall expansion (van Batenburg *et al.*, 1986). The normal process of cell wall growth in the root

hair appears to be turned inside out in such a way that the outwardly growing cell wall cylinder gets converted into an inwardly growing tunnel. This tunnel has been found to follow the nucleus towards the base of the root hair cell and cytoskeleton connection have been observed between the nucleus and the growing root hair tip (Lloyd *et al.,* 1987).

2.3.3 NODULE MATURATION

After approaching the region of newly dividing nodule meristematic cells, the infection thread branches and vesicles are blebbed off into the cytoplasm of plant cells resulting in the release of rhizobial bacteria into these cells (Fig.7). The membrane-enclosed bacteria released singly into the plant cytoplasm are known as bacteroids. The membrane which encloses bacteroids is known as peribacteroid membrane. This has been found to be antigenically similar to the plant cell membrane (Perotto *et al.*, 1991). The bacteroids continue to divide and eventually differentiate into nitrogen fixing endosymbionts. The volume of the host cells increase to accommodate many bacteroids. The plant cell organelles like mitochondria, amyloplasts are pushed to the periphery of these cells.

2.4 GENES INVOLVED IN NITROGEN FIXATION

Genes used in the final stages of symbiosis are generally identified by the observation that mutations in them make the *Rhizobium* unable to fix nitrogen; these genes are called *fix* genes (Beringer *et al.*, 1980). In *Klebsiella pneumoniae* 20 adjacent *nif* (nitrogen fixation) genes are organized in eight operons within a 24 kb region of DNA (Arnold *et al.*, 1988). The fix genes of *Rhizobium* which has been shown to be equivalent to *K.pneumoniae nif* genes are assigned the same name.

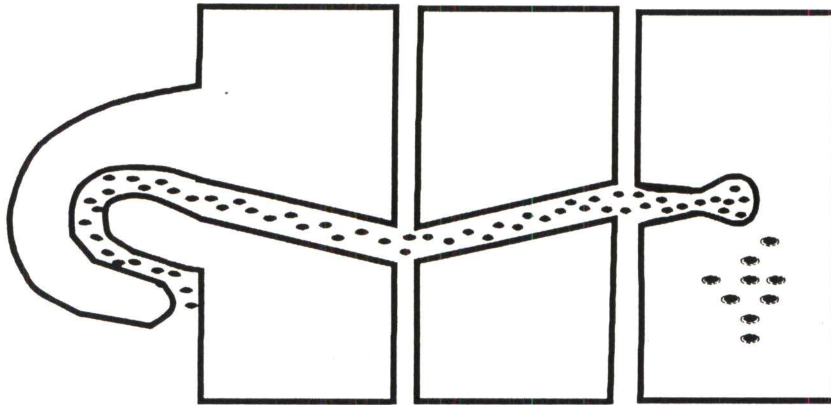


Fig. 7. Generalized diagram illustrating three stages (root hair curling and initiation of an infection thread, cell-to-cell spread through an infection thread and bacterial release) of plant cell and tissue invasion by *Rhizobium* (after Brewin, 1991).

The *nif* and *fix* genes location is species specific. Two cluster of these genes have been located on megaplasmid 1 (pSyma) of *R. meliloti*. The cluster 1 includes *nifHDKE, nifN, fixABCX, nifA, nifB* whereas the genes, viz., *fixLJ, fixF, fixNOQP, fixGHIS* are present in Cluster II (David *et al.*, 1987). The cluster II genes are located at about 220 kb down stream of the *nifHDKE* operon and are transcribed in opposite orientation to it. A cluster of *nod* genes including common *nod* genes is located in the 30 kb region between *nifE* and *nifN*. Some additional genes required for effective symbiosis are located on megaplasmid 2 or pSymb of *R. meliloti* (Watson *et al.,* 1988; Reuber *et al.,*1991; Honeycutt *et al.,* 1993; Reuber and Walker, 1993; Leigh and Walker, 1994).

2.5 SYMBIOTIC CHARACTERISTICS OF AUXOTROPHS OF RHIZOBIA

In several studies the role of some primary metabolic pathways in *Rhizobium*-legume symbiosis has been demonstrated by using auxotrophic mutants. In some cases specific metabolites affecting symbiotic interaction have been identified.

Kummer and Kuykendall (1989) and Barsomian *et al.* (1992) demonstrated the involvement of tryptophan biosynthetic pathway in symbiosis. It was found that the bacterial synthesis of indoleglycerol phosphate, an intermediate of tryptophan biosynthetic pathway was necessary for nodulation in *Bradyrhizobium japonicum* (Kummer and Kuykendall, 1989). In case of *R. meliloti trpE* mutants were found to be *nod*$^+$ but the nodules induced by them showed unusual defects in internal morphology and were able to *fix* only trace amounts of nitrogen (Barsomian *et al.* 1992; Prasad, 1998). The *trpE* mutants are characterized by their inability to synthesize anthranilic acid. During bacteroid transformation

there is an increasing demand of iron. On the basis of the earlier reports on secretion of anthranilic acid and iron uptake promoted by it in *R. leguminosarum* (Rioux *et al.*, 1986a; 1986b) Barsomian *et al.*, 1992 proposed that anthranilic acid may be acting as *in planta* siderophore, promoting the uptake required for the development of bacteroids and in the absence of this factor the bacteroids fail to differentiate. The expression of *trpE* gene in wild type nodules appeared to support this hypothesis. Hunter and Kuykendall (1990) reported another interesting feature of tryptophan biosynthetic pathway. They found that prototrophic revertants of a *nod*$^-$ tryptophan auxotroph of *B.japonicum* showed enhanced nodulation and nitrogen fixation.

ilv mutants of *R. meliloti* and *R. fredii* have shown nodulation defective phenotype in several studies (Dènariè *et al.*, 1976; Kim *et al.*, 1988; Aguilar and Grasso, 1991). It has been found that the promoter of the common nodulation genes (*nodABC*) in *R. leguminosarum* was not activated by luteolin in ilv mutants (Aguilar and Grasso, 1991). These mutants were unable to nodulate either on supplementation of isoleucine and valine or their intermediates. It has been hypothesised that the cellular concentrations of acetolactate in these *ilvC* mutants might have reached levels which inhibited the expression of nodulation genes.

B.japonicum histidine auxotrophs were also found to be *nod*$^-$ (Sadowsky *et al.*, 1986; Yadav *et al.*, 1998). However, external supplementation of histidine could restore nodulation in these mutants.

The linkage of leucine auxotrophy to symbiotic ineffectiveness in *R.meliloti* was reported by Truchet *et al.* (1980) and Kerppola and Kahn (1988a). External supplementation of leucine could restore symbiotic effectiveness in leucine auxotrophs isolated by Truchet *et al.* (1980) but not in leucine auxotrophs

isolated by Kerppola and Kahn (1988a).

Jelesko *et al.* (1993) observed that the normal flow of metabolites through the aromatic biosythetic pathway was essential for bacteroid development in *R. meliloti* . The mutants isolated by these workers showed decreased Deoxy arabino-heptonic acid-7-phosphate (DAHP) synthase activity and formed ineffective nodules.

R. meliloti auxotrophs blocked either in ornithine transcarbamylase or arginosuccinate synthase were effective but mutants which were blocked in early part of arginine biosythetic pathway, which leads to ornithine, were ineffective. It was proposed that since ornithine is a precursor for synthesis of polyamines, the lack of polyamines may be directly leading to the symbiotic defect (Kerppola and Kahn, 1988a).

Besides amino acids, vitamins also have been shown to play a role in symbiosis. Schwinghamer (1969) and Pankhurst *et al.* (1972) demonstrated the requirement of riboflavin for bacteroid development in *R. leguminosarum* and *R. trifolii*. The riboflavin requiring auxotrophs isolated by these workers were unable to transform to bacteroids and resulted in the formation of ineffective nodules. This defect was restored on external supplementation of riboflavin.

Several studies carried out on different species of *Rhizobium* have confirmed that purine auxotrophs were always defective in symbiosis (Newell and Tucker, 1968; Scherrer and Dénarié, 1971; Pankhurst and Schwinghamer, 1974; Federov and Zaretskaya, 1979; Pain, 1979; Kerppola and Kahn, 1988a; 1988b; Kim *et al.,* 1988; Swamynathan and Singh, 1992; Gupta, 1996). These auxotrophs were *nod*$^-$ except nod$^+$ purine auxotroph reported by Swamynathan and Singh, 1992. Defective symbiosis in purine auxotrophs could be partially restored on supplementation of one of the purine intermediates, i.e., 5-

aminoimidazole-4-carboxamide ribonucleotide (Newman *et al.* 1992; 1994). Except for the reports of Kerppola and Kahn (1985; 1988b) not much information is available about the role of pyrimidines or their intermediates in symbiosis.

The above review of literature shows that in *Rhizobium* several genes involved in symbiosis have been identified, mapped and characterized with respect to their role in symbiosis. But all physiological and biochemical aspects of nodule development are still not clear. The role of primary metabolic pathways in symbiosis has not been fully explored. Detailed studies in this direction will help in clarifying the remaining doubts in the *Rhizobium*-legume symbiosis.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 BACTERIAL STRAINS

The bacterial strains and plasmid constructs used in this study are listed in Table 1.

Table 1. Bacterial strains and plasmids used and constructed in this study

Strains/Plasmids	Relevant characteristics	Source / Reference
Rhizobium meliloti		
AK 631	Nod^+ Fix^+ compact colony variant of wild type strain Rm 41	Adam Kondorosi
Rmd 201	Spontaneous Sm^r derivative of AK 631 Nod^+ Fix^+	Khanuja and Kumar (1988)
PP 631	AK 631 (pJB3JI)	Peter Putnoky
ZB 201	Rm 41 *cys*46 Rif^r 5*fu*^{r} Sm^r	-do-
ZB 178	Rm 41 *ade*15 Rif^r Sm^r	-do-
ZB 555	Rm 41 *cys* 46 *phe*15 Rif^r Sm^r	-do-
ZB 556	Rm 41 *gly*1 *met*2 *ade*4 Sm^r Rif^r	-do-
ZB 557	Rm 41 *phe*15 *leu*4 Rif^r Sm^r	-do-
ZB 205	Rm 41 *ade*15 Nar B Rif^r 5*fu*^{r} Sm^r	-do-
VE 12	Rmd 201 Pyr^- (pyr :: Tn5)	This study
VE 19	-do-	-do-
VE 43	-do-	-do-
H7	-do-	Raad Hassani (This lab)
H9	-do-	-do-
H36	-do-	-do-
H33	Rmd 201 Car^- (*car AB* :: Tn5)	-do-
H37	-do-	-do-
H47	-do-	-do-
Escherichia coli		
WA803 (pGS9)	Met^-, Thi^-, Chl^r, Km^r	Selvaraj and Iyer (1983)
Plasmids		
pGS9	IncN rep P15A, Chl^r, Km^r	Selvaraj and Iyer (1983)
pJB3JI	Km^s derivative of pR68.45 capable of mobilising genomic segments of its host, Tet^r, Cb^r, Nal^r	Brewin *et al.* (1980)

3.1.2 PLANT CULTIVAR

Alfalfa (*Medicago sativa* cv T9) seeds obtained from National Seeds Corporation , New Delhi were used for plant tests.

3.1.3 GROWTH MEDIA

3.1.3.1 Media for *Rhizobium meliloti*

3.1.3.1.1 Tryptone Yeast Extract (TY) Medium (Khanuja and Kumar,1988)

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

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

3.1.3.1.2 Mannitol Salts Yeast Extract (MSY) Medium (Khanuja and Kumar, 1989)

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

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

3.1.3.1.3. Rhizobium Minimal Medium (RMM) (Singh *et al.*, 1984)

Constituent	Stock solution	Amount of stock solution added/litre	Amount/litre
$Na_2HPO_4 . 12H_2O$	0.45 g /10 ml	10 ml	0.45 g
$(NH_4)_2SO_4$	2.0 g /10 ml	10 ml	2.0 g
$FeCl_3$	2.0 mg/ml	1 ml	2.0 mg
$Mg SO_4 . 7H_2O$	0.1 g/ml	1 ml	0.1 g
$CaCl_2 .2 H_2O$	0.04 g/ml	1 ml	0.04 g
distilled water to make upto 1 litre volume			

pH of the medium was adjusted to 7.0 with 0.1 N NaOH solution. 20% glucose solution was prepared, filter sterilized and 10 ml from it was added to the autoclaved medium.

3.1.3.2 Medium for *Escherichia coli*

LB Medium (Luria Bertani Medium) (Sambrook *et al.*, 1989)

Costituent	Amount/litre
Bactotryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g
distilled water to make 1 litre volume	

pH of the medium was adjusted to 7.0 with 0.1 N NaOH solution. Wherever solid medium was needed agar @ 16 g/litre was used and all growth media were autoclaved at 15 psi for 20 min.

3.1.3.3 Nitrogen Free Plant Growth Medium

The nitrogen free plant growth medium developed by Engelke *et al.* (1987) was used to carryout plant assays. Twelve stocks of following compositions were prepared.

Solution	Salt	Amount of salt used (g)	Volume of water used (ml)
A	K_2HPO_4	2.0902	10
B	KH_2PO_4	0.544	10
C	$CaCl_2$	7.351	10
D	$C_6H_5O_7$ $Fe.3H_2O$	0.335	10
E	$MgSO_4$	6.162	10
F	K_2SO_4	4.3562	10
G	$MnSO_4$	0.034	20
H	H_3BO_3	0.026	20
I	$ZnSO_4$	0.03	20
J	$CuSO_4$	0.002	20
K	$CaSO_4$	0.006	20
L	$NaMoO_4$	0.006	20

These stock solutions were autoclaved separately. To make one litre of plant growth medium, 10 g agar was dissolved in 988 ml of water and autoclaved. One ml from each stock solution was added to this autoclaved agar solution and pH was adjusted to 6.8 with 0.1N NaOH solution or 0.1N HCl. The resulting medium was then used to prepare slants.

3.1.3.4 TY Swarm Plates

TY swarm plates were used for motility tests. The composition of the medium was the same as TY except for the reduction of agar concentration to 0.3%.

3.1.4 BACTERIAL GROWTH-CONDITIONS

The *E. coli* and *Rhizobium* strains were grown in their respective media as described above. The incubation temperatures for *E. coli* and *R. meliloti* were 37°C and 30°C, respectively.

3.1.5 SUPPLEMENTS TO MEDIA

3.1.5.1 Antibiotics

All antibiotics were purchased from Sigma Chemical Company. Distilled sterile water (for streptomycin sulphate and kanamycin acid sulphate), 50% ethanol (for tetracycline hydrochloride) and dimethyl sulphoxide (for rifampicin) were used as solvents for preparing stock solutions of antibiotics. The final concentrations of different antibiotics used in the media were as follows : Tetracycline hydrochloride (15 μg/ml), streptomycin sulphate (100 μg/ml), kanamycin acid sulphate (40 μg/ml for *E. coli* and 400 μg/ml for *R. meliloti*), rifampicin (30 μg/ml). Streptomycin and kanamycin were used after filter sterlization.

3.1.5.2 Amino Acids, Nitrogenous Bases and Vitamins

Stock solutions of amino acids, nitrogenous bases and vitamins were made in distilled water and filter sterilized. The final concentrations used for amino acids, nitrogenous bases and vitamins were 50 μg/ml, 20 μg/ml and 5 μg/ml, respectively. Required volumes from the sterile stocks were added to the

autoclaved media.

To identify the nature of auxotrophy, modified Holliday pools (Holliday, 1956) with modification were used. The compositions of different pools 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 Dicarboxylic Acids

All dicarboxylic acids (malic acid, sodium succinate, aspartate) were purchased from HiMedia and added to the RMM (without glucose) @ 2g per litre as a sole carbon source before autoclaving.

3.1.5.4 Intermediates

All pyrimidine intermediates were purchased from Sigma. Distilled sterile water (for carbamoyl aspartate, dihydroorotic acid), dimethyl sulphoxide (for

carbamoyl phosphate) were used as solvents for preparing stock solutions of intermediates. Intermediates were added to the medium at 50 μg/ml concentration.

3.1.5.5 Dyes

Calcofluor white (Sigma), congo red (HiMedia) and aniline blue (HiMedia) were added to the MSY medium at the rate of 0.02% before autoclaving.

0.01% methylene blue was used to stain infection threads in root hairs and 1% toluidine blue prepared in 1% borax was used for staining semithin sections for optical microscopy.

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

3.1.6 SERIAL DILUTION

0.9% NaCl solution was used for carrying out dilutions.

3.1.7 REAGENTS FOR ESTIMATION OF OROTIC ACID

1. Orotic acid (0.001 - 0.015 mg/ml pH 2-3)
2. Mono sodium citrate buffer at pH 2.5 (Solution A. 21.04 gm of citric acid was dissolved in 200 ml of NaOH (1N) and made it upto 1000 ml with water. 35.4 ml of the solution A was taken and made it upto 100 ml with HCl (0.1N).
3. Saturated aqueous bromine solution
4. 0.7% Sodium mercapto acetate solution
5. 2.5% p-Dimethyl amino benzaldehyde solution in propanol

3.1.8 COMPOSITION OF SOLUTIONS FOR LIGHT AND ELECTRON MICROSCOPIC STUDIES OF NODULES OF ALFALFA

3.1.8.1 Preparation of Blocks

(a) Karnovsky Fixative (Karnovsky, 1965)

12.5 ml	8% paraformaldehyde (Sigma)
5.0 ml	12.5% glutaraldehyde (Sigma)
25.0 ml	phosphate buffer
7.5 ml	distilled water

(b) 0.2 M Phosphate Buffer

NaH_2PO_4 - 6.41 g

$Na_2HPO_4.7\ H_2O$ - 41.3 g

or

$Na_2HPO_4.12H_2O$ - 55.2 g

Double distilled water to make 1 litre.

(c) Post Fixative (2% OsO_4)

A stock solution of 2% OsO_4 was prepared by dissolving 1 g in 50 ml of double distilled water. The solution was stored at 4^0C in a tightly stoppered brown coloured bottle.

(d) Araldite Resin

Araldite Cy 212 embedding medium of the following concentration was used for embedding.

Araldite Cy 212	-	10 ml
Hardener (Dodecenyl succinic anhydrite, DDSA; HY 964)	-	10 ml
Accelerator (Tridimethyl-amino-		

methyl phenol, DMP30 ; DYO64)- 0.4 ml

Plasticizer (Dibutylphthalate) - 1.0 ml

The ingredients were stirred vigorously to mix.

3.1.8.2 Solutions for Staining Ultrathin Sections

(a) Uranyl Acetate

A saturated solution of uranyl acetate was prepared by adding excess of uranyl acetate to 10 ml of filtered 50% ethanol in a 15 ml centrifuge tube.The solution was shaken vigorously for 2 min. spun down at 10,000 rpm to allow the excess of uranyl acetate to settle down, stoppered and stored at 4^0C.

(b) Lead Citrate

To 12 ml of double distilled water in a 15 ml centrifuge tube one and half pellet of NaOH was added. After proper dissolution of NaOH, 50 mg of lead citrate was added, shaken well to assist in dissolution and centrifuged at 10,000 rpm for 2 min. The solution was stored at 4^0C.

3.2 METHODS

METHODS OF BACTERIAL EXPERIMENTS

3.2.1 MAINTENANCE OF BACTERIAL CULTURE

The pure bacterial cultures were maintained in the final concentration of glycerol (15%). The strains were also maintained in slants of TY agar medium for *Rhizobium* and LB agar medium for *E. coli* strains and then stored at 4^0C in refrigerator.

Single colonies were obtained by plating or streaking cells of strain on complete or minimal medium. Incubation of 2-3 days was required at 30^0C for the colonies to appear on complete medium. In case of minimal medium incubation of about one week was required. A fully grown *R. meliloti* colony usually contained about 10^7 cells.

Spot tests for studying growth response of bacterial strains were done by suspending cells from a colony in 0.05 ml of 0.9% NaCl. About 10^5 to 10^6 cells were applied to the surface of agar with a loop to make a spot.

Log phase *E. coli* cultures were obtained by incubating the inoculum for 14-16 hours at 37^0C in LB broth with the required antibiotic. Log phase *Rhizobium* cultures were obtained by inoculating single colony of the culture in TY broth for 48 hours at 28^0C. The tubes or flasks containing the broth and required antibiotic were inoculated with single colony and incubated in shakers at suitable temperature (30/37°C) and 120 rpm speed of rotation for aeration.

3.2.2 BACTERIAL CONJUGATIONS

Patch matings were done according to Kondorosi *et al.* (1977a). TY broth cultures of *Rhizobium* were grown for 48 hrs and *E. coli* for 16 hrs. If the cross was between *E. coli* and *Rhizobium,* they were mixed in the ratio 3:7, i.e., 0.03 ml of *E. coli* culture and 0.07 ml of *Rhizobium* culture, on the agar TY plate with the help of sterile glass rod plated by spreading. Then the plate was incubated at 30^0C for the desired time interval. The growth obtained was scrapped using sterile spatula and suspended in 1 ml of 0.9% NaCl. The suspension was plated, directly and after dilution, on the selective medium. The plates were examined for transconjugants after five days of incubation at 30^0C.

3.2.3 SERIAL DILUTIONS

Serial dilutions were made in saline solution (0.9% NaCl) to obtain single bacterial colonies for many experiments. 0.1 ml of neat culture was added to the first tube containing 9.9 ml of saline solution to obtain 10^{-2} dilution. After mixing it properly 0.1 ml bacterial suspension from this tube was added to the next 9.9 ml saline solution tube to obtain 10^{-4} dilution. Similarly, further dilutions were made according to the requirements of the experiment.

3.2.4 ISOLATION OF MUTANTS AND SCREENING FOR AUXOTROPHS

3.2.4.1 Transposon Tn5 Mutagenesis

Cultures of *E. coli* strain WA 803 (pGS9) and *R. meliloti* strain Rmd 201 were grown to log phase in TY broth, 0.03 ml of *E. coli* and 0.07 ml of *Rhizobium* were delivered on the surface of TY medium in a Petriplate. The two cultures were mixed and spreading was done with the help of a sterile glass rod. Several patches were made, and incubated at 30^0C for 24 hrs.

After 24 hrs mating the growth from a patch was transferred to 1 ml 0.9% saline solution in a 5 ml tube. The suspension of mated cells was plated directly on TY medium supplemented with km^{400} and sm^{100}. In all matings, the donor and recipient cultures were also plated on TY medium supplemented with km^{400} and sm^{100} . The plates were incubated for 5 days.

There were 50-200 colonies on each plate when the neat suspension from a patch was plated on 2-3 plates. From a patch 40-50 colonies were purified twice on TY plates having km^{400} and sm^{100}.

This way a collection of 6000 mutants of *R. meliloti* from 120 patch matings was obtained. These mutants were maintained on TY plates having km^{400} and sm^{100}.

All Tn5 induced km^r mutants were then checked for the loss of the suicide plasmid. This was done by looking for the absence of vector encoded chloramphenicol resistance in these transconjugants.

3.2.4.2 Screening for Auxotrophs by Replica Plating Method

All the Tn5 derivatives obtained above were screened for auxotrophs. Each derivative was streaked on RMM (minimal) and TY (complete) media with sterile tooth picks. The derivative which grew on TY but not RMM medium was considered to be an auxotroph. This Tn5 mutant was streaked on TY medium to obtain single colonies. The cell population obtained from a single colony was again checked for auxotrophy. The nature of auxotrophy of this mutant was determined subsequently.

3.2.5 NATURE OF AUXOTROPHY

A loopful of cells of each mutant was suspended in a drop of 0.9% saline solution on a sterile glass slide and suspension was spotted on different Holliday pools (RMM medium supplemented with eleven Holliday pools as mentioned before) with the help of an inoculation loop. Incubation was done for three days at 30^{0}C. The nature of auxotrophy was estimated on the basis of the pattern of growth on these plates.

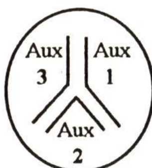
3.2.6 REVERSION ANALYSIS

The cells of an auxotrophic mutant were grown to late log phase in TY medium at 30^{0}C. These were washed twice with RMM medium and resuspended in the same medium to a concentration of about 10^9 cells per ml. Then the

frequency of spontaneous reversion was determined by plating a known number of mutant cells on RMM plates and counting the number of prototrophic colonies appearing after five days of incubation at 30^{0}C. These prototrophs were replica plated on RMM agar plates supplemented with kanamycin to determine whether Tn5 was retained or not.

3.2.7 CROSS FEEDING ASSAY

A loopful of cells of each auxotroph was suspended in 0.9% sodium chloride on a sterile glass slide and this suspension was streaked on RMM plates enriched with minimal (2μg/ml) nutritional supply. Three auxotrophic strains (having block at different steps in the pathway) were streaked on each plate in such a manner that each streak formed a side of an equilateral triangle as shown below.



The plates were then incubated for 2-3 days at 30^{0}C. At the end of the incubation period the results of the growth pattern were recorded.

3.2.8 INTERMEDIATE FEEDING TESTS

The block in the pyrimidine biosynthetic pathway in each pyrimidine auxotroph was determined by feeding of the intermediates of the pathway. RMM medium was supplemented with carbamoyl phosphate, carbamoyl aspartate, dihydroorotic acid or orotic acid. RMM medium supplemented with uracil was used as a positive control. A cell suspension of each auxotroph was

streaked on each supplemented medium. The parental cell suspension was also streacked on various plates as a control. Growth pattern was observed after incubation at 30^{0}C for 3-4 days.

3.2.9 OROTIC ACID ACCUMULATION STUDIES

Each pyrimidine auxotroph was grown in TY broth and the culture obtained was centrifuged. The cells were washed twice with RMM, suspended in 10 ml of RMM and incubated for 48 hrs at 30^{0}C. Then the culture was centrifuged for 10 min. at 10,000 rpm and the supernatant was tested for the presence of orotic acid as follows.

To 1 ml of sample added 2 ml of 5% solution of monosodium citrate as a buffer at pH 2.5 and 0.5 ml of saturated aqueous bromine solution. After 10 sec added 0.5 ml of 0.7% sodium mercapto acetate solution to decolourize excess bromine and heated at 56^{0}C for 3 min. To this 2 ml of 2.5% p-dimethyl aminobenzaldehyde solution in propanol was added and the resulting solution was read at 480 nm within 30 min. against water.

3.2.10 UPTAKE OF DICARBOXYLIC ACIDS

RMM (without glucose) supplemented with uracil/uracil + arginine (50 µg/ml) and malic acid, aspartate or sodium succinate as a sole carbon source was used for this test. All auxotrophic strains and parental strain (as a positive control) were streaked on the above media. Growth pattern of auxotrophs was compared with parental strain after incubation at 30^{0}C for 3-4 days.

3.2.11 CELL SURFACE CHARACTERISTICS

3.2.11.1 Tests For Production of β 1→3 glucans, Exopolysaccharides and Cellulose Fibrils

The production of β (1→3) glucans, exopolysaccharides, and cellulose fibrils in *Rhizobium* can be tested with dyes like aniline blue (Nikanishi *et al.*, 1976), calcofluor white (Leigh *et al.*, 1987) and congo red (Kneen and La Rue, 1983), respectively. Strains which produce β (1→3) glucans can form blue colonies on aniline blue containing medium. Same way strains which produce cellulose fibrils can form red colonies on congo red containing medium. Calcofluor staining was examined as the strains which produce EPS are fluorescent under long wave length UV light on calcofluor containing medium. MSY plates with aniline blue, calcofluor white and congo red were prepared and the pyrimidine auxotrophic strains were streaked on these plates. Rmd201 was also streaked on these plates and compared the growth pattern with these auxotrophic strains. These plates were then incubated at 30^0C for 4 days. Calcofluor plates were observed under long wave length ultra violet light whereas congo red and aniline blue plates were observed in normal light.

3.2.11.2 Test For Production Of Lipopolysaccharides (LPS)

Auxotrophic cell suspension of each pyrimidine auxotroph was streaked on TY plates containing sodium deoxycholate (1mg/ml) and then incubated at 30^0C for 2 to 3 days. Parental strain also was streaked on these plates as a positive control and growth pattern was compared after the incubation period. Strains which showed growth on these plates were considered to be LPS producing.

3.2.11.3 Test For Production of β 1→2 glucans

Motility of bacteria in swarm plates can be attributed to β(1→2) glucans production (Geremia *et al.*, 1987). A loopful of auxotrophic cell suspension was spotted on a TY swarm plate (containing 0.3% agar) and incubated at 30^{0}C for 2 days. Rmd201 cell suspension also was spotted as a positive control. Swarming of the bacterial strains was determined by the spread of the bacterial growth.

3.2.12 GENETIC EXPERIMENTS

3.2.12.1 Construction of Donors

The genome mobilizing plasmid pJB3JI (Tet^r) from *R. meliloti* AK631 (pJB3JI) was introduced into each pyrimidine auxotroph by conjugation. The transconjugants harbouring pJB3JI were selected on TY media supplemented with kanamycin and tetracycline. Five transconjugants from each cross were purified for single colony on the same selective medium. These were used as donors for testing linkage of Tn5 insertion to auxotrophy as described in the next section. The donor which showed highest transfer of Tn5 was used in mapping experiments.

3.2.12.2 Linkage of Tn5 Insertions to Auxotrophy

The linkage of transposon Tn5 insertion to auxotrophy in each auxotroph was determined by mobilizing the fragment containing Tn5 with the help of genome mobilizing plasmid pJB3JI to the recipient strain ZB555 followed by the testing for donor's auxotrophy in the km^r transconjugants. The donor strain of each auxotroph was conjugated on TY plate with the recipient *R.meliloti*

strain ZB555 and incubated at 30^0C for 24 hours. The km^r transconjugants were selected by plating the mating mixture on TY plates containing kanamycin (400 μg/ml) and rifampicin (50μg/ml) after 4-5 days of incubation at 30^0C. Sixty km^r transconjugants were checked for their donor's auxotrophy as described earlier. Percent cotransfer of km^r and pyrimidine auxotrophy was calculated.

3.2.12.3 Mapping Experiments

The donor strains, the construction of which has been described earlier were employed to map the pyr loci on the *R.meliloti* chromosome. Each auxotroph in which plasmid pJB3JI has been induced was crossed with six *R.meliloti* mapping strains (ZB178, ZB201, ZB205, ZB555, ZB556, ZB557). The description of the markers of these mapping strains has been given in Table 1. Fifty km^r transconjugants from each of the six crosses were selected by patching the mating mixture on TY medium containing kanamycin and rifampicin and the cotransfer of selected marker (km^r phenotype) with each of the unselected markers (markers of the donor) was determined by patching of these transconjugants on appropriate selective plates.

3.2.13 METHODOLOGY FOR PLANT EXPERIMENTS

Plant experiments were carried out in 20 cm x 2.5 cm glass tubes. These tubes were plugged with cotton and autoclaved. 988 ml of distilled water containing 0.8% agar and all the stock solutions of plant growth medium were also autoclaved separately. One ml of each solution was added under sterile conditions to the autoclaved distilled water containing agar at 50^0C with gentle

shaking. Whenever nutritional (uracil/orotic acid/uracil + arginine/carbamoyl phosphate / carbamoyl aspartate/ dihydroorotic acid) supplementations were required, the required amount of sterile nutrients were added to the medium. 25 ml of this plant growth medium was added to each autoclaved tube (20 cm x 2.5 cm) under sterile conditions and the tube containing the medium was placed in a slanting position.

Alfalfa (*Medicago sativa cv* T9) seeds were procured from the National Seeds Corporation, New Delhi. The seeds were soaked in distilled sterile water for 30 min. Then they were surface sterilized by treating with 0.1% $HgCl_2$ for one minute followed by treatment with absolute alcohol for one minute. These seeds were then quickly washed several times with distilled sterile water. Distilled water containing 1% agar was autoclaved and this agar solution was poured into sterile Petriplates. After the solidification of agar, surface sterilized seeds were spread on its surface. The plates were kept inverted at 25^0C in a dark place for germination. Two seedlings each 2-days old were then transferred to each tube.

For inoculation, fresh liquid cultures of *R. meliloti* Rmd 201 and pyrimidine auxotrophic strains in TY medium were prepared. Each culture was centrifuged (5000 rpm for 10 min), washed with sterile water and suspended in 4 ml of sterile water. 0.5 ml of this suspension was dispensed into each tube containing two-days old seedlings. The plants were kept under 2,000 lux light with roots protected from direct exposure to light. A 16 hrs light period and 8 hrs dark period were maintained at 25^0C temperature. Six weeks after inoculation with rhizobial strains, the data on each plant were recorded for number of days to first nodule appearance, nodule colour, nodule location, nodule shape,mean

plant height and mean shoot dry weight. Dry shoot weight was recorded after drying the plant top in an oven at 85^{0}C for 72 hours.

3.2.13.1 Nodule Occupancy Tests

Nodule occupancy by the inoculated strains was tested by reisolating rhizobia from the nodules and testing their auxotrophic and antibiotic markers. All the nodules from a plant were collected, surface sterilized and crushed in a drop of saline. The bacterial suspension thus obtained was diluted with saline to obtain 10^{-1} and 10^{-2} dilutions. 0.1 ml of each dilution and neat culture were spread on TY medium supplemented with streptomycin (100 µg/ml) and incubated for 2-3 days at 30^{0}C. The colonies obtained were patched on RMM, RMM supplemented with the nutrient requirement(s) of the auxotrophs and TY medium supplemented with kanamycin (400 µg/ml) and streptomycin (100µg/ml). Incubation was done at 30^{0}C for 2-3 days. The colonies which grew on RMM but not on TY + km + sm were considered to be prototrophic revertants and the colonies which did not grow on RMM but grew on TY + km + sm were considered to be auxotrophs.

3.2.13.2 Light Microscopy for Observing Infection Threads

The plant material needed for this study was prepared by the same method as used for nodulation assay. Six days after rhizobial inoculation the plants were taken for examination. The plant roots to be examined were dipped for 15 minutes in 0.01% methylene blue solution. The roots were then washed twice in sterile water and cut into 1 cm long pieces. Each root piece was placed on a clean and sterile slide, and covered with a coverslip. The slide was then

examined at 40x and 100x magnification under optical microscope (Leica DM LB).

3.2.13.3 Histology of Nodules

Histology of the nodules was studied through light and electron microscopies. For these experiments nodules were collected after 6 weeks of plant growth. The methodology for these experiments consisted of specimen fixation, specimen block preparation, and preparation of semithin and ultrathin sections of the specimen.

3.2.13.3.1 Fixation

Nodules from plants inoculated with the parental strain and auxotrophs were excised and washed quickly in 2 to 3 changes of sterile distilled water and dipped in Karnovsky fixative in 5 ml glass vials. Bigger nodules were cut longitudinally to 1 to 2 mm thickness and then placed in the fixative. To facilitate penetration additives like acrolein (at 0.1% final concentration) were added to the fixative. The vials were placed in vacuum chamber to prevent the material from floating on the surface. The fixation was carried at 4^0C for 2 to 24 hrs.

3.2.13.3.2 Specimen Block Preparation for Light and Transmission Electron Microscopic Studies

(a) Washing

Following fixation the nodules were washed twice in 0.1 M phosphate buffer and then left in the same buffer overnight at 4^0C. Final trimming of the nodules to appropriate size was done when these were in the buffer.

(b) Post Fixation

The nodules after fixation were taken out and placed in 1% OsO_4 for 2 hrs at 4^0C.

(c) Dehydration

Dry acetone was used as a dehydrating agent. The samples were passed through the following dehydration series at 4^0C:

30% acetone - 2 changes of 15 min. each

50% acetone - - do -

70% acetone - - do -

80% acetone - - do -

90% acetone - - do -

95% acetone - - do -

Dry acetone - 15 min.

The following step was carried at room temperature

Dry acetone - 2 changes of 30 min. each

(d) Clearing the Tissues Off Acetone

Though acetone is easily miscible with the resin it is advantageous to clear it off with a clearing agent to facilitate infiltration. The samples were placed in toluene at room temperature for 60 min. to clear off acetone.

(e) Infiltration and Embedding

The following infiltration mixtures were prepared from the embedding medium.

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

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

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

The samples were taken out of toluene, placed in the mixture `a'. and left overnight. These were then transferred to mixture `b' for 1 hr and then to mixture `c' for 1 hr under vacuum. Finally two changes of pure embedding medium for 1 hr each were given at 50^0C.

Embedding of the samples was carried out in araldite embedding medium using gelatin or beam capsules. Flat embedding was done where orientation of specimens for sectioning was required. The embedded blocks were kept at 50^0C in a special oven for 12 to 24 hrs. The temperature of the oven was then raised to 60^0C and the embedded tissues were kept for 24 to 48 hrs for complete polymerization of the embedding medium.

3.2.13.3.3 Preparation of Semithin Sections for Optical Microscopy

The resin blocks containing specimens were trimmed using a trimmer and were fitted in the specimen block holder. The block holder was attached to Ultracut E microtome (C. Reichert, Austria OmU3). Semithin sections (0.5 to 2.0 mm thick) were cut with glass knife. Thin sections floating in water filled in the metallic trough fitted closely around the knife were lifted with a glass-rod or a thin brush and placed on a clean glass slide. The slide was placed on a hot plate (at about 80^0C) for drying. The sections were then stained in 1% toluidine blue for 30 seconds. Later the sections were washed with distilled water. These slides were then observed under optical microscope (Leica *DM* LB) and areas of interest were photographed.

3.2.13.3.4 Preparation of Ultrathin Sections

After scanning the sections under the optical microscope, the area to be examined under TEM was selected and the blocks were further hand trimmed. Ultrathin sections (60-90 nm thick) were cut using Ultracut E microtome (C.Reichert, Austria). The silver coloured sections were lifted onto 200 mesh size copper grids. The grids were coated with 3% formvar (Polyvenyl formaldehyde) in ethylene dichloride and carbon by a process of evaporation under vacuum. After double coating, the formvar was dissolved either with ethylene dichloride or chloroform leaving behind the carbon film. To obtain a good contrast a double staining method using uranyl acetate and lead citrate was adopted. The grids carrying the sections were placed in uranyl acetate solution in a clean watch glass. A wooden or card-board cover was placed over the watch glass as the staining is effective when carried out in dark. The staining process was continued for 10 to 15 min. Then the grids were washed in 2 lots of 50% ethanol and 2 lots of double distilled water with continuous agitation. The grids were then dried carefully on a filter paper and then stained with lead citrate for 5 to 10 min. After this, these were washed briefly in 0.1 N NaOH and then in 2 lots of double distilled water and dried. The dried grids were stored in grid holder at room temperature and viewed under TEM (Philips EM 300) and the areas of interest were photographed.

3.2.14 STATISTICAL ANALYSIS

Significance between control vs experimental values was ascertained using student's `t' test. The data related to plant characteristics of parental strain and its auxotrophic mutants were statistically analysed. The means of eight

plants of a type were used.

Calculation of critical difference (C.D.) :

$C.D = t_{0.05} \times \overline{sd}$

Where

$t_{0.05}$ = value of t at 5% level of significance against error degree of freedom

$\overline{sd}$ = Standard error of difference, $\overline{sd} = \sqrt{2EMS/r}$

EMS = error mean square, r = no. of replications.

Chapter 4

Results

To determine the nature and extent of the involvement of the biosynthetic pathways of different amino acids, nitrogenous bases and vitamins of *Rhizobium meliloti* in symbiosis, auxotrophs of *R. meliloti* Rmd 201 strain were generated through random transposon mutagenesis and symbiotic properties of these auxotrophs were studied. Pyrimidine biosynthetic pathway was selected for detailed investigations. Biochemical and genetic characterization of pyrimidine auxotrophs obtained during the course of this work and by H. Raad in the lab were done and the nodules induced by these auxotrophs were subjected to microscopic studies. The results of these experiments are presented here.

4.1 TRANSPOSON MUTAGENESIS AND ISOLATION OF AUXOTROPHS

R. meliloti strain Rmd 201 was mutagenized with transposon Tn5 which codes for resistance to kanamycin. The suicide Tn5-delivery vector pGS9 was introduced into *R. meliloti* from *E. coli* WA803 (pGS9) strain by conjugation and Tn5 derivatives were selected by plating the mating mixture on TY medium containing kanamycin (400 μg/ml) and streptomycin (100 μg/ml). Km^r transconjugants were obtained at a frequency of 3×10^{-5} per recipient whereas the spontaneous resistance to 400 μg/ml concentration of kanamycin in Rmd201 strain was $<10^{-8}$. In order to avoid siblings not more than 100 Tn5 derivatives were selected from one cross. A total of 6000 km^r colonies from 120 crosses were collected and streak-purified on TY medium containing kanamycin and streptomycin. All Tn5 induced kanamycin resistant transconjugants were chloramphenicol sensitive indicating disintegration of Tn5 delivery vector pGS9 in these transconjugants.

All the Tn5 derivatives obtained above were streaked on minimal medium RMM and TY complete medium. Twenty nine derivatives did not grow on minimal medium. These were assumed to be auxotrophs and used in the further studies after purifying each derivative for single colony on TY medium containing kanamycin and streptomycin.

4.2. NUTRITIONAL REQUIREMENTS OF AUXOTROPHS

Nutritional requirements of isolated auxotrophic mutants were determined by patching these mutants on nutritional pools (Holliday, 1956) and observing growth after incubation. The results of these experiments in Table 2 show that out of 29 auxotrophs tested 25 had only single nutritional requirement for cysteine/methionine, methionine, uracil or adenine. Three auxotrophs, viz., VE4, VE5 and VE14, could grow when provided with both isoleucine and valine whereas the growth requirement of VE41 was both adenine and thiamine.

4.3 SYMBIOTIC PROPERTIES OF AUXOTROPHS

Plant inoculation tests were carried out with 29 auxotrophs isolated during this work and six uracil auxotrophs available in this lab. The data on nodule characteristics, plant height and dry plant weight of the alfalfa plants inoculated with the above strains have been presented in Table 3.

In order to find out the authenticity of nodulation by a particular strain the bacteria were reisolated from nodules and checked for their markers. Plant data were retained only if the nodules were found to be occupied by the inoculated strain.

Table 2. Nutrient requirements of the transposon Tn5 induced auxotrophic mutants of *Rhizobium meliloti* Rmd201 strain

S.no	Name of the auxotrophic mutant	Nature of auxotrophy
1	VE4	Isoleucine + Valine
2	VE5	"
3	VE14	"
4	VE2	Cysteine/methionine
5	VE3	"
6	VE6	"
7	VE10	"
8	VE13	"
9	VE17	"
10	VE20	"
11	VE32	"
12	VE33	"
13	VE9	Methionine
14	VE11	"
15	VE21	"
16	VE29	"
17	VE31	"
18	VE36	"
19	VE39	"
20	VE12	Uracil
21	VE19	"
22	VE43	"
23	VE8	Adenine
24	VE16	"
25	VE27	"
26	VE37	"
27	VE38	"
28	VE40	"
29	VE41	Adenine + Thiamine

All the isoleucine + valine auxotrophs did not induce nodules on alfalfa plants (Table 3). Initiation of nodulation occurred 6-7 days after inoculation with the parental strain Rmd201, all cysteine/methionine auxotrophs and all except one methionine auxotrophs. In case of methionine auxotroph VE39 nodules were formed 9-10 days after inoculation. In the remaining auxotrophs the appearance of the first nodule was slightly delayed as compared to that of the above mentioned strains.

Cylindrical nodules were formed by the parental strain Rmd201 as well as its uracil auxotroph H36. The nodules induced by all other auxotrophs were mostly round or irregular in shape.

Rmd201 induced pink nodules whereas the nodules formed by cysteine/ methionine and methionine auxotrophs were pinkish white; all other auxotrophs induced white nodules. The parental strain Rmd201 and all its cysteine/methionine and methionine auxotrophs resulted in nodulation on both primary and lateral roots whereas nodules appeared only on lateral roots when inoculation was done with any of the other auxotrophs.

The plants inoculated with cysteine/methionine auxotrophs did not differ significantly from the parental strain inoculated plant's mean plant height and dry shoot weight characteristics. On the other hand the plants inoculated with the remaining auxotrophs had mean plant heights and mean shoot dry weights similar to those of the uninoculated plants.

Table 3. The characteristics of alfalfa plants inoculated with *Rhizobium meliloti* strain Rmd201 and its auxotrophic derivatives.

S.no.	*R.meliloti* strain	Auxotrophic requirement	No. of days to first nodule appearance	Nodule shape	Nodule colour	Nodule location	Mean plant height* (cm)	Mean shoot dry weight* (mg)
1	Rmd 201		6-7	Cylindrical	Pink	PR & LR	25±1.8	34.6±2.5
2	Uninoculated control		-	-	-	-	4.1±0.9	7.2±0.9
3	VE4	Iso + Val	-	-	-	-	5±0.9⊗	8.2±1⊗
4	VE5	- do-	-	-	-	-	4.8±1.1⊗	7.8±1.1⊗
5	VE14	- do-	-	-	-	-	5.2±0.8⊗	7.5±1.7⊗
6	VE2	Cys/Met	6-7	Round/ Irregular	Pinkish white	PR & LR	16.8±6.3∅	26.7±6.6∅
7	VE3	- do-	- do-	-do-	-do-	-do-	22.9±3.7∅	32.3±4.5∅
8	VE6	- do-	- do-	-do-	-do-	-do-	18±5.1∅	28±5.1∅
9	VE10	- do-	- do-	-do-	-do-	-do-	21±1.1∅	31.2±1.5∅
10	VE13	- do-	- do-	-do-	-do-	-do-	23.2±2.9∅	32.9±5.9∅
11	VE17	- do-	- do-	-do-	-do-	-do-	19.2±4.1∅	27.2±6.5∅
12	VE20	- do-	- do-	-do-	-do-	-do-	19.5±4.1∅	30.5±2.3∅
13	VE32	- do-	- do-	-do-	-do-	-do-	18.2±6.9∅	26.5±7.2∅
14	VE33	- do-	- do-	-do-	-do-	-do-	15.9±7.9∅	32.9±1.8∅

S.no.	*R.meliloti* strain	Auxotrophic requirement	No. of days to first nodule appearance	Nodule shape	Nodule colour	Nodule location	Mean plant height* (cm)	Mean shoot dry weight* (mg)
15	VE9	Met	6-7	Round/ Irregular	Pinkish white	PR & LR.	18.1±5.8∅	29.1±2.9∅
16	VE11	-do-	-do-	-do-	-do-	-do-	21.8±3.9∅	23.1±1.2∅
17	VE21	-do-	-do-	-do-	-do-	-do-	23.9±1.2∅	34.1±0.9∅
18	VE29	-do-	-do-	-do-	-do-	-do-	22.8±0.2∅	30.8±2.8∅
19	VE31	-do-	-do-	-do-	-do-	-do-	22.2±0.7∅	31±1.2∅
20	VE36	-do-	-do-	-do-	-do-	-do-	21.8±1.1∅	30.2±2.7∅
21	VE39	-do-	9-10	-do-	-do-	-do-	19.3±5.1∅	29.3±3.9∅
22	VE8	Ade	-do-	Round/ Irregular	White	-do-	8.2±4.1⊗	8.9±1.1⊗
23	VE16	-do-	-do-	-do-	-do-	-do-	6.9±2.2⊗	7.1±2.3⊗
24	VE27	-do-	-do-	-do-	-do-	-do-	6.1±1.1⊗	8.0±0.9⊗
25	VE37	-do-	-do-	-do-	-do-	-do-	5.1±0.9⊗	7.9±1.1⊗
26	VE38	Ade	10-11	Round/ Irregular	White	LR	5.2±0.5⊗	8.1±1.2⊗
27	VE40	-do-	-do-	-do-	-do-	-do-	8.9±3.8⊗	7.5±1.3⊗

Contd...

S.no.	*R.meliloti* strain	Auxotrophic requirement	No. of days to first nodule appearance	Nodule shape	Nodule colour	Nodule location	Mean plant height* (cm)	Mean shoot dry weight* (mg)
28	VE41	Ade + Thia	-do-	-do-	-do-	-do-	7.2±4.1⊗	8.2±1.8⊗
29	VE12•	Ura	-	-	-	-	-	-
30	VE19•	-do-	-	-	-	-	-	-
31	VE43•	-do-	-	-	-	-	-	-
32	H7	-do-	10-11	Irregular	White	LR	4.8±0.8⊗	8.1±0.5⊗
33	H9•	-do-	-	-	-	-	-	-
34	H33	Ura + Arg	10-11	Round/ Irregular	White	LR	4.5±0.5⊗	7.9±0.9⊗
35	H36	Ura	-do-	Cylindrical	-do-	-do-	9.0±5.9⊗	10.2±2.5⊗
36	H37	Ura + Arg	-do-	Round/ Irregular	-do-	-do-	4.7±0.8⊗	8.8±1.2⊗
37	H47	-do	-do-	-do-	-do-	-do-	8.9±4.9⊗	7.1±1.6⊗

* Each value is a mean of eight plants

Abbreviations

PR. - Primary root

LR - Lateral root

⊗ - Does not differ significantly from uninoculated control ($P < 0.05$)

∅ - Does not differ significantly form the parental strain value ($P < 0.05$)

• - Nodules on all plants were occupied by revertants; hence no plant data of these strains are presented

4.4 CHARACTERIZATION OF PYRIMIDINE AUXOTROPHS

4.4.1 REVERSION ANALYSIS

Spontaneous excision of transposon Tn5 occurred in all pyrimidine auxotrophs resulting in the formation of km^s prototrophic revertants. The reversion frequencies of various mutants are presented in Table 4. Minimum reversion frequency was observed in mutant H7 (1.4×10^{-10}) whereas maximum was found in H9 auxotroph (9.2×10^{-9}). Reversion frequencies of VE12, VE19 and VE43 were very close to the reversion frequency of H9.

4.4.2 BIOCHEMICAL CHARACTERIZATION

Biochemical characterization to identify the metabolic block in each pyrimidine auxotroph included cross feeding assays, intermediate feeding tests and orotic acid accumulation studies (Fig.8).

4.4.2.1. Cross Feeding Assays

To detect cross-feeding among nine pyrimidine auxotrophs, all these strains were streaked near each other in all possible combinations on RMM supplemented with minimal quantity of their nutritional requirement (s) (uracil or uracil + arginine). None of the auxotrophs could cross-feed any other auxotrophs.

4.4.2.2 Intermediate Feeding Tests

Three arginine + uracil auxotrophs, viz., H33, H37 and H47 were able to grow on RMM supplemented with carbamoyl phosphate or arginine + orotic
248387
acid (a pyrimidine intermediate) but no growth occurred when orotic acid was

Table 4. Reversion frequency of pyrimidine auxotrophic mutants of *Rhizobium meliloti* strain Rmd201

S.No	Auxotrophic mutant	Spontaneous reversion frequency
1.	H33	3.1×10^{-9}
2.	H37	4.9×10^{-9}
3.	H47	3.3×10^{-9}
4.	VE12	7.2×10^{-9}
5.	VE19	8.3×10^{-9}
6.	VE43	8.1×10^{-9}
7.	H7	1.4×10^{-10}
8.	H9	9.2×10^{-9}
9.	H36	1.2×10^{-9}

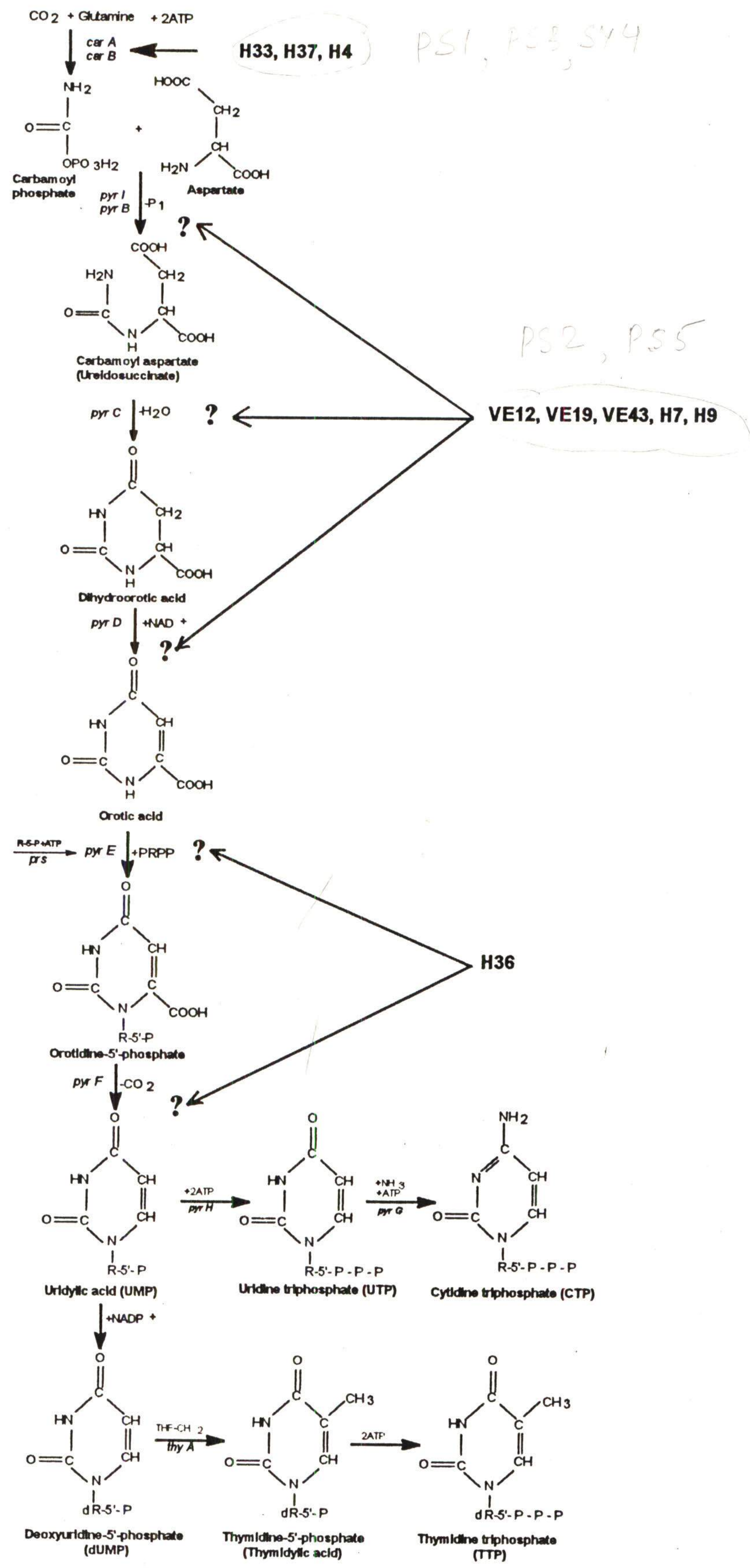


Fig. 8. The pathways of the synthesis of pyrimidines showing the positions of mutation in the pyrimidine auxotrophs of *Rhizobium meliloti* Rmd201 obtained.

replaced with carbamoyl aspartate or dihydroorotic acid which are also intermediates of pyrimidine biosynthesis (Table 5). Five auxotrophs, viz., VE12, VE19, VE43, H7 and H9 showed growth on RMM supplemented with orotic acid. These mutants did not grow on minimal medium supplied with carbamoyl aspartate or dihydroorotic acid. The growth of uracil auxotroph H36 did not occur on minimal medium supplemented with any of the above mentioned intermediates of pyrimidine biosynthesis.

4.4.2.3 Orotic Acid Accumulation Studies

Out of the nine pyrimidine auxotrophs tested for orotic acid accumulation in minimal medium only one (H36) showed positive result. Maximum orotic acid accumulation in this strain was found to be 2 μg/ml. On the basis of the above results the pyrimidine auxotrophs were divided into three groups as follows:

I Carbamoyl phosphate synthetase defective mutants which require carbamoyl phosphate for their growth (H33, H37 and H47).

II The mutants which have blocks in the pathway between carbamoyl phosphate and orotic acid (VE12, VE19, VE43, H7 and H9).

III The mutant that does not grow on orotic acid but grows on uracil supplementation, evidently having a block in the pathway after orotic acid (H36).

4.5 PLEIOTROPIC EFFECTS OF THE PYRIMIDINE BIOSYNTHETIC GENES

4.5.1 CELL SURFACE CHARACTERISTICS

The parental strain Rmd201 and all pyrimidine auxotrophs took up the dye in congo red medium grew in presence of sodium deoxycholate, showed

Table 5. **Growth responses of pyrimidine auxotrophs of *Rhizobium meliloti* strain Rmd 201 to different intermediates of pyrimidine biosynthetic pathway**

Strain	R MM supplied with 50µg/ml of									RMM
	CP	Arg+CA	Arg+Dho	Arg+Oro	CA	Dho	Oro	Arg+Ura	Ura	
Rmd 201	+	+	+	+	+	+	+	+	+	+
H33	+	-	-	+	-	-	-	+	-	-
H37	+	-	-	+	-	-	-	+	-	-
H47	+	-	-	+	-	-	-	+	-	-
VE12	-	-	-	+	-	-	+	+	+	-
VE19	-	-	-	+	-	-	+	+	+	-
VE43	-	-	-	+	-	-	+	+	+	-
H 9	-	-	-	+	-	-	+	+	+	-
H 7	-	-	-	+	-	-	+	+	+	-
H 36	-	-	-	-	-	-	-	+	+	-

Abbreviations

RMM - Rhizobial Minimal Medium

CP - Carbamoyl phosphate   Dho - Dihydroorotic acid   Arg - Arginine   '+' - growth

CA - Carbamoyl aspartate   Oro - Orotic acid   Ura - Uracil   '-' - No growth

motility in swarm medium and fluoresced in presence of calcofluor white under ultraviolet light indicating the normal production of cellulose fibrils, lipopolysaccharides, $\beta(1\rightarrow2)$ glucans and exopolysaccharides, respectively. All pyrimidine auxotrophs and Rmd201 strain did not take up the dye in aniline blue medium, indicating the absence of normal production of $\beta(1\rightarrow3)$ glucans. These results showed that the cell surface molecules in auxotophs were like those of the parental strain.

4.5.2 UPTAKE OF DICARBOXYLIC ACIDS

Like the parental strain Rmd201, all auxotrophs also showed growth on the minimal medium containing malic acid, sodium succinate or aspartate indicating that they were able to use any of these dicarboxylic acids as a sole carbon source.

4.6 GENETIC STUDIES OF PYRIMIDINE AUXOTROPHS

4.6.1 LINKAGE OF Tn5 INSERTION TO AUXOTROPHY

In all pyrimidine auxotrophs 100% cotransfer of kanamycin resistance and pyrimidine auxotrophy occurred indicating the complete linkage of transposon Tn5 insertion to auxotrophy in all these mutants.

4.6.2 MAPPING EXPERIMENTS

Since the markers of the available mapping strains cover only 2/3 of the chromosome the precise mapping of the pyrimidine mutations was not possible. However, the possibility of occurrence of pyrimidine loci in the 2/3 of the chromosome could be ruled out. The region of the chromosome in which pyrimidine loci are present is shown in Figure 9.

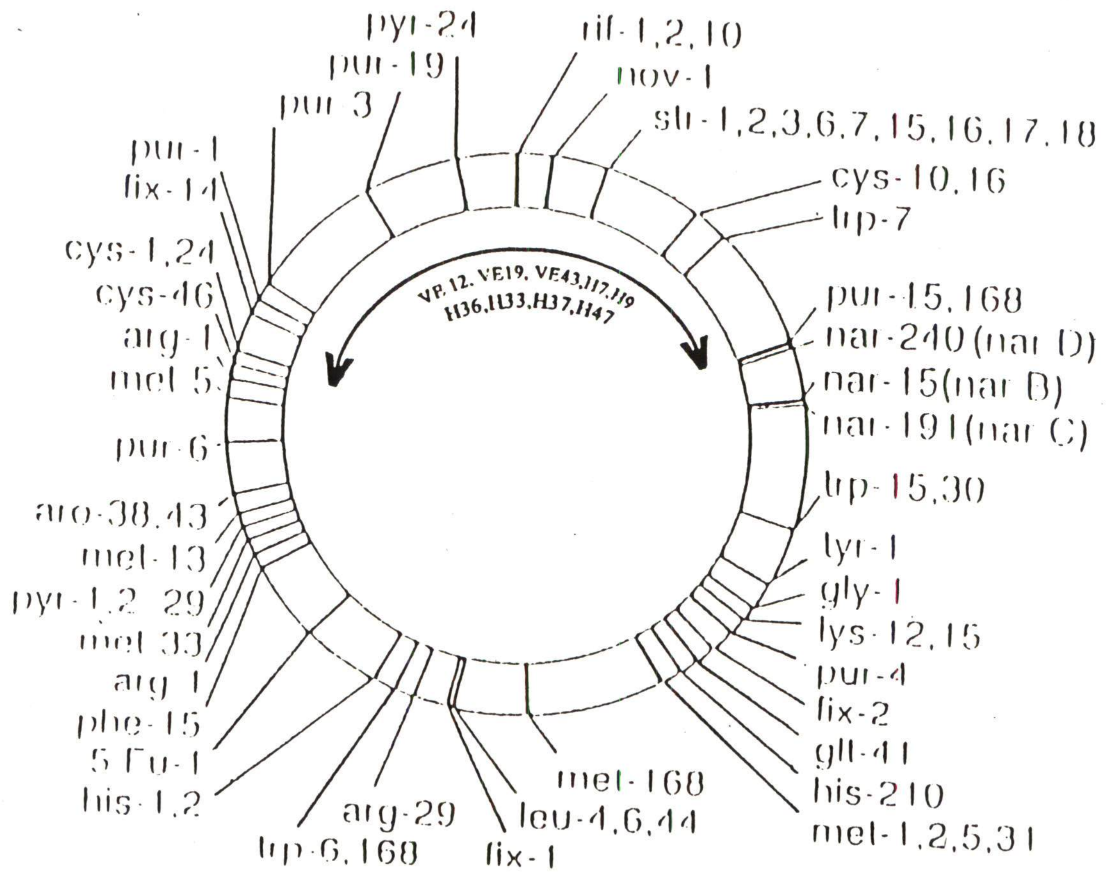


Fig. 9. Chromosomal map of *Rhizobium meliloti* strain Rm 41 showing the possible region of Tn5 insertion in pyrimidine auxotrophs

Plate 1. Morphological features of the representative plants of alfalfa inoculated with *Rhizobium meliloti* Rmd201 and its pyrimidine auxotrophic mutants.

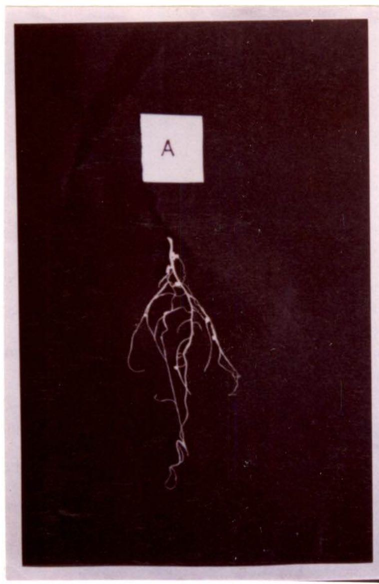
A. Rmd201 inoculated plant

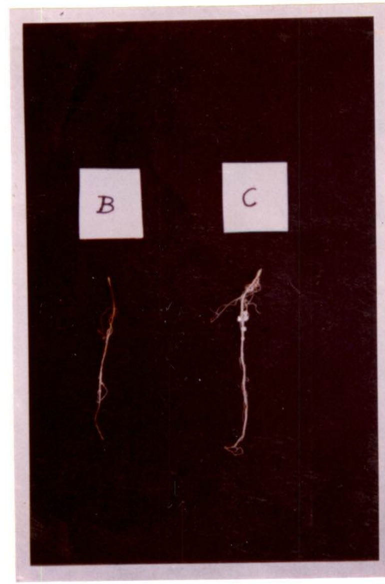
B. Uninoculated plant

C. H33 inoculated plant

D. H7 inoculated plant

E. H36 inoculated plant





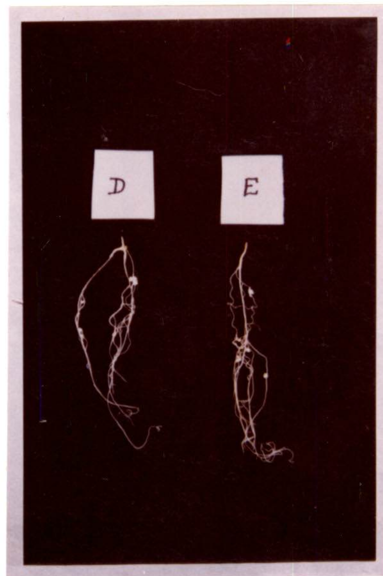


Plate 2. Plant roots showing the morphology of nodules induced by *Rhizobium meliloti* Rmd201 and its pyrimidine auxotrophic mutants. **A.** Rmd201 inoculated **B.** Uninoculated **C.** H33 inoculated **D.** H7 inoculated **E.** H36 inoculated

4.7 SYMBIOTIC PROPERTIES OF PYRIMIDINE AUXOTROPHS

4.7.1 PLANT INOCULATION TESTS WITH PYRIMIDINE AUXOTROPHS

The data on the characteristics of the alfalfa plants inoculated with the pyrimidine auxotrophs have been presented, alongwith the data of the other auxotrophs, in Table 3 and representative plants and their root portions are shown in plates 1 and 2. It has already been mentioned that the pyrimidine auxotrophs H33, H37, H47, H7 and H36 induced white nodules on alfalfa plants; mean plant height and mean shoot dry weights of these plants did not differ significantly from those of the uninoculated plants indicating the absence of nitrogen fixation. All the nodules induced by the mutants VE12, VE19, VE43 and H9 were found to be occupied by their prototrophic revertants.

4.7.2 PLANT INOCULATION TESTS WITH PYRIMIDINE AUXOTROPHIC REVERTANTS

The data on the characteristics of alfalfa plants inoculated with the revertants of pyrimidine auxotrophs in Table 6 shows that all revertants, like the parental strain Rmd201, induced nodules pink in colour and cylindrical in shape on both primary and lateral roots. The plants inoculated with revertants did not differ significantly from the parental strain inoculated plants w.r.t. mean plant height and shoot dry weight characteristics. The above results indicated that the revertants formed normal nitrogen fixing nodules like the parental strain Rmd201.

Table 6. The characteristics of alfalfa plants inoculated with the revertants of pyrimidine auxotrophs of *Rhizobium meliloti* Rmd201

S.no.	*R.meliloti* strain	No. of days to first nodule appearance	Nodule shape	Nodule colour	Nodule location	Mean plant height* (cm)	Mean shoot dry weight* (mg)
1	Rmd 201	6-7	Cylindrical	Pink	PR & LR	25±1.8	34.6±2.5
2	Uninoculat-ed control	-	-	-	-	4.1±0.9	7.2±0.9
3	VE112	7-8	Cylindrical	Pink	PR & LR	22±1.2Ø	33.1±2.5Ø
4	VE119	7-8	-do-	-do-	-do-	18.8±6.1Ø	29±3.5Ø
5	VE143	7-8	-do-	-do-	-do-	21.9±3.5Ø	34±2.2Ø
6	H17	7-8	-do-	-do-	-do-	18.8±4.8Ø	29.5±4.6Ø
7	H19	7-8	-do-	-do-	-do-	23.7±4.2Ø	33.1±0.9Ø
8	H133	7-8	-do-	-do-	-do-	16.9±7.3Ø	24.8±7.5Ø
9	H136	7-8	-do-	-do-	-do-	20.7±3.1Ø	30.2±4.7Ø
10	H137	7-8	-do-	-do-	-do-	19.1±5.8Ø	26.3±7.2Ø
11	H147	7-8	-do-	-do-	-do-	18.0±6.9Ø	28.8±5.3Ø

* Each value is a mean of eight plants
PR. - Primary root
LR - Lateral root

Ø – No Significant difference with parental Rmd201 strain inoculated plant ($P < 0.05$)
The strains VE112, VE119, VE143, H17, H19, H133, H136, H137 and H147 are spontaneous revertants of pyrimidine auxotrophs VE12, VE19, VE43, H7, H9, H33, H36, H37 and H47, respectively.

4.7.3 EXOGENOUS FEEDING OF URACIL OR ITS INTERMEDIATES TO PLANTS INOCULATED WITH PYRIMIDINE AUXOTROPHS

Plant nutrient medium supplemented with uracil or its intermediates was used to study the symbiotic interaction of the pyrimidine auxotrophs with alfalfa plants. The supplementation of uracil + arginine or carbamoyl phosphate did not restore the symbiotic defects of the auxotrophs H33, H37 and H47 (carbamoyl phosphate synthetase defective mutants). Similarly restoration did not occur in case of H7 auxotroph on supplementation of uracil, carbamoyl aspartate, dihydroorotic acid or orotic acid. The symbiotic defect of auxotroph H36 was also not restored by addition of uracil to the plant nutrient medium.

4.7.4 NODULE OCCUPANCY TESTS

Rhizobial bacteria were isolated from the nodules of each plant inoculated with a particular pyrimidine auxotroph. These bacteria were checked for the auxotrophic markers. From none of the plants a mixed population of auxotroph and prototroph was obtained. Nearly two third of the plants inoculated with five auxotrophs, viz., H33, H37, H47, H7 or H36 showed 100% occupancy of nodules by the inoculated auxotrophs. Only prototrophic revertants were obtained from the nodules of plants inoculated with four auxotrophs, viz., H9, VE12, VE19 and VE43 (Table 7).

4.7.5 INFECTION THREAD FORMATION

Root hair curling, shepherd's crooks and infection thread formation were observed in root hairs of alfalfa plants inoculated with parental strain Rmd201

Table 7. **Nodule occupancy characteristics of plants inoculated with pyrimidine auxotrophic derivatives of *Rhizobium meliloti* Rmd201.**

S. no.	Pyrimidine auxotroph	No. of plants inoculated	No. of plants showing 100% occupancy of nodules by the auxotroph	No. of plants showing 100% occupancy of nodules by the revertant	Percent plants showing 100% occupancy of nodule by the auxotroph
1	H33	38	28	12	70
2	H37	40	29	09	72
3	H47	35	24	72	68
4	H7	40	25	15	62
5	H9	36	0	36	0
6	VE12	40	0	40	0
7	VE19	38	0	38	0
8	VE43	34	0	34	0
9	H36	40	27	13	67

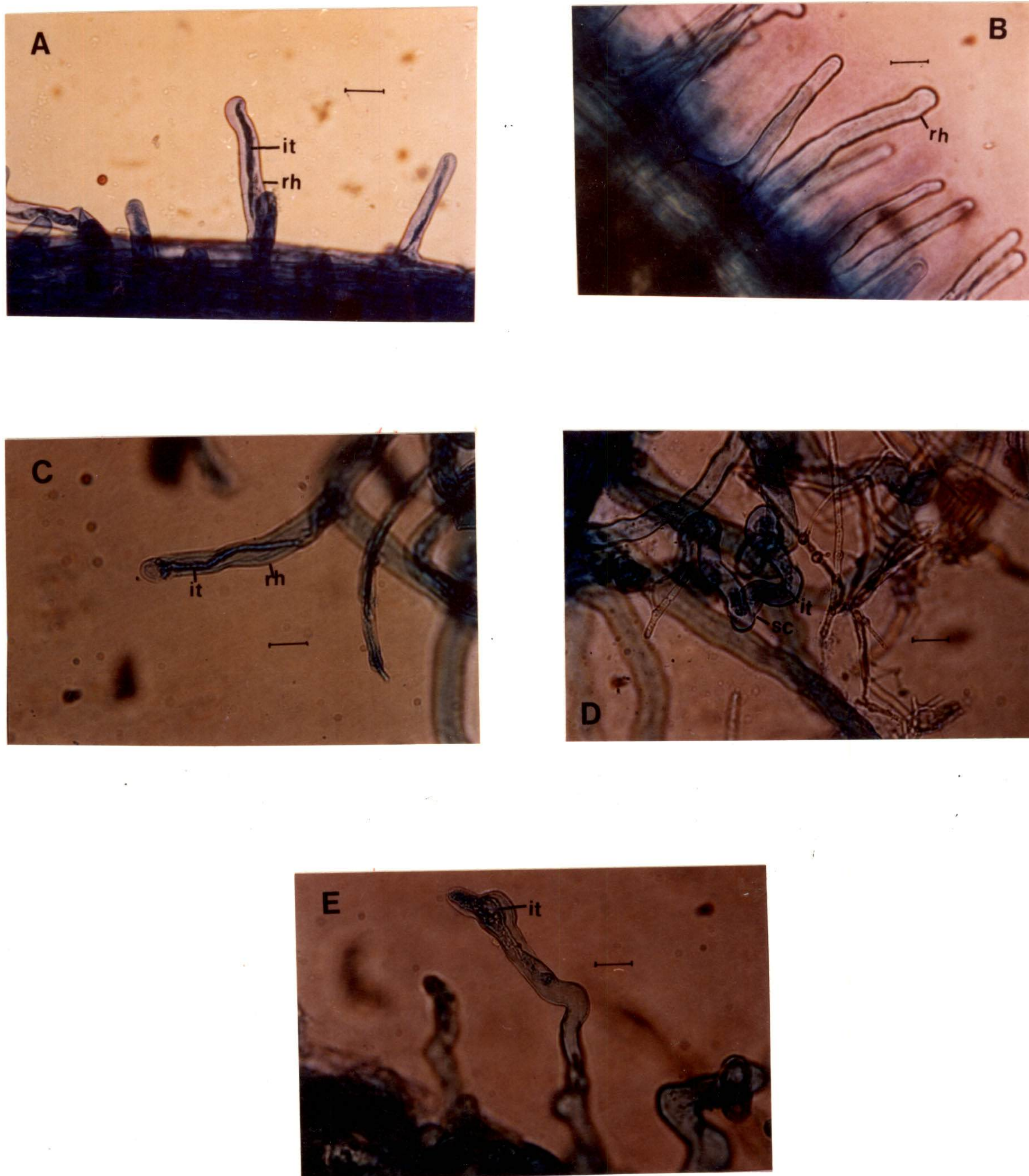
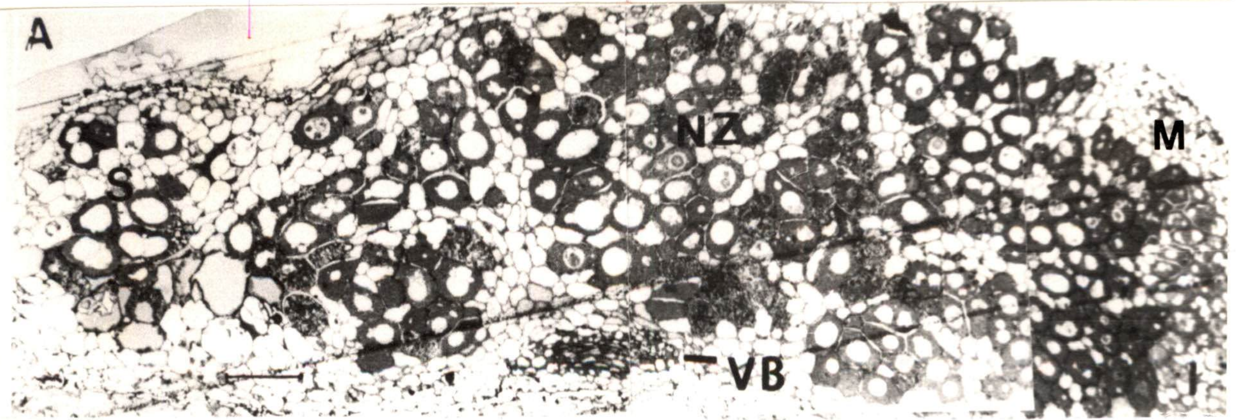
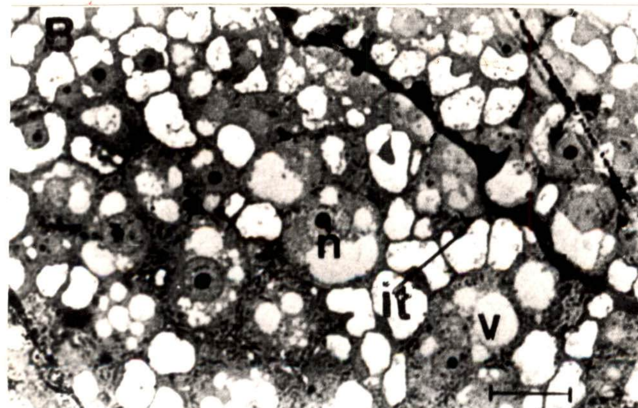


Plate 3. Root hair curling and infection thread formation in root hairs of alfalfa seedlings inoculated with *Rhizobium meliloti* Rmd201 and its pyrimidine auxotrophic mutants. **A.** Rmd201 inoculated **B.** Uninoculated **C.** H33 inoculated **D.** H7 inoculated **E.** H36 inoculated **Abbreviations :** it - infection thread, rh - root hair and sc - shepherd's crook. Bar, 25 μm (x 400).





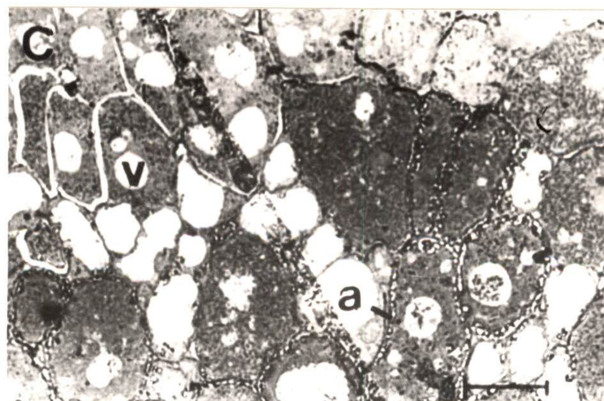
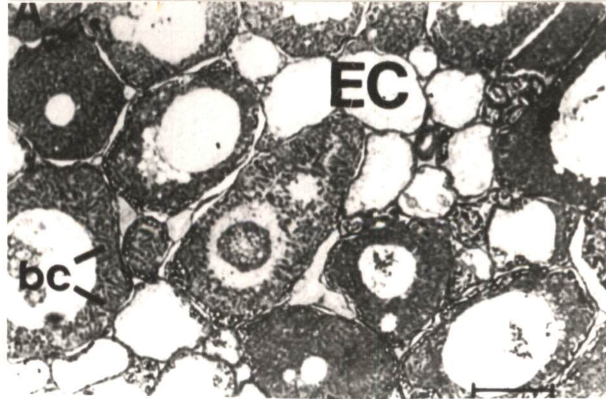


Plate 4. Light microscopic studies of longitudinal and semithin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** The nodule section showing infection zone (I), nitrogen fixation zone (NZ), senescence zone (S) and peripheral vascular bundles (VB). Bar, 100 µm (x100). **B.** Infection zone showing cells containing infection threads (it), vacuoles (V) and prominent nuclei (n). Bar, 25 µm (x400). **C.** Zone between infection and nitrogen fixation zones. The cells in this zone are packed with amyloplasts (a) Bar, 25 µm (x400).



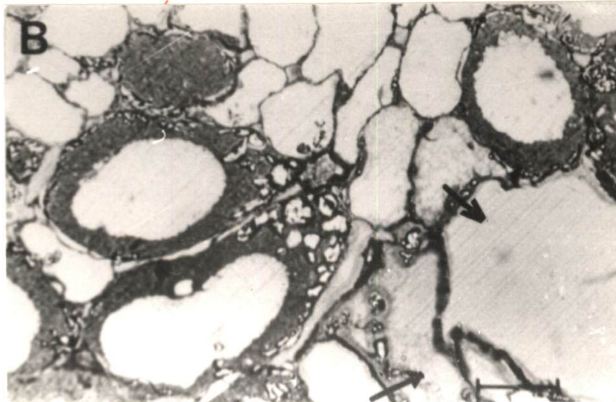
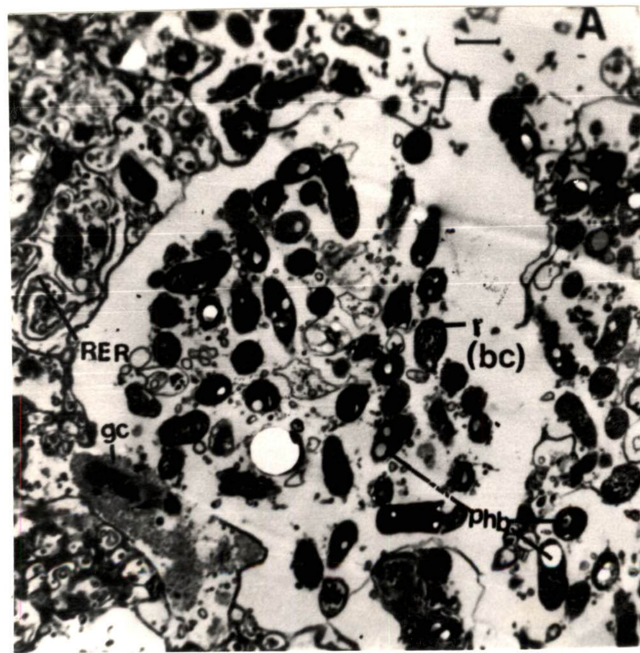


Plate 5. Light microscopic studies of longitudinal and semithin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** Nitrogen fixation zone showing nodule cells filled with mature bacteroids (bc) and nodule cells without bacteroids, i.e., empty cells (EC). Bar, 25 μm (x400). **B.** Senescence zone showing nodule cells in which bacteroids are degenerated (indicated by arrows). Bar, 25 μm (x400).



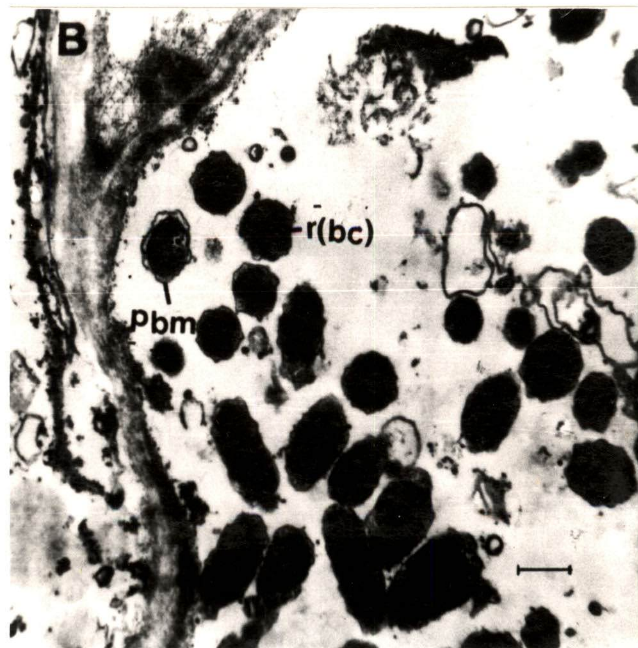


Plate 6. TEM studies of longitudinal and ultrathin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** Depicts the part of the nodule cell containing freshly released rhizobia (r), i.e., bacteroids (bc) which are electron dense and contain poly-β-hydroxybutyrate (phb) granules. Nodule cell cytoplasm also contains numerous rough endoplasmic reticulum (RER) and golgi complex (gc) Bar, 1 μm (x5,900). **B.** A part of nodule cell from early infection zone showing released bacteroids (bc) enclosed by a wavy peribacteroidal membrane (pbm) but without phb granules. Bar, 1 μm (x6,800).

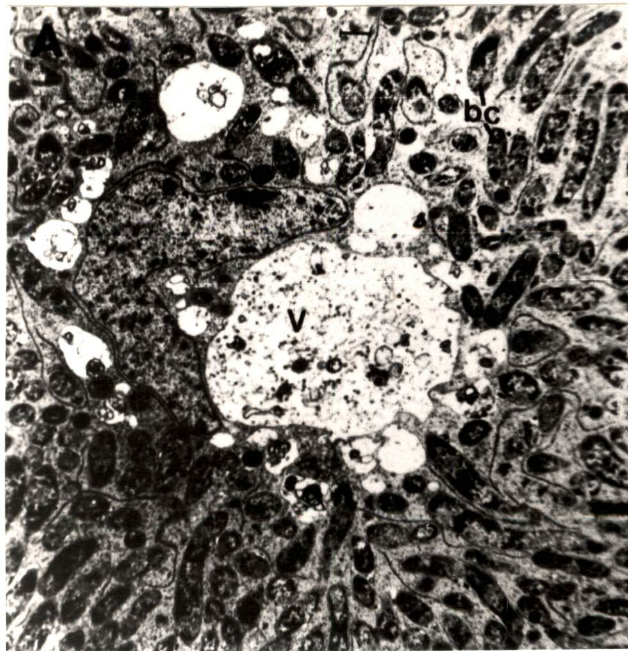
and all pyrimidine auxotrophs. In uninoculated alfalfa plants root hairs were straight and devoid of infection threads (Plate 3).

4.7.6 HISTOLOGICAL STUDIES OF NODULES

4.7.6.1 Light and Electron Microscopic Studies of Nodules Induced by the Parental Strain Rmd201

The longitudinal cross section of a nodule induced by the parental strain Rmd 201 revealed four main zones, viz., apical meristematic zone, infection zone, nitrogen fixation zone and senescence zone (Plate 4:A). The meristematic zone contained constantly dividing uninfected nodule cells. The infection zone that lied next to the meristematic zone comprised of cells with large, prominent nuclei, amyloplasts and many vacuoles (Plate 4:B). Few layers next to this infection zone constituted the interzone. Here the nodule cells were studded with amyloplasts but the number of amyloplasts in each cell was less as compared to that in infection zone (Plate 4:C). The nitrogen fixation zone formed the major part of the nodule. The nodule cells in this region were filled with mature bacteroids which were organised around the central vacuole in each cell (Plate 5:A). The senescence zone which occupied the basal portion of the nodule contained oldest nodule cells (Plate 5:B).

Electron microscopic studies showed that the bacteroids in the infection zone were electron dense. Most of the bacteroids were found to contain clear poly-β-hydroxybutyrate (phb) granules indicating that these were the freshly released bacteroids. Peribacteroidal membrane (pbm) was also visible in bacteroids (Plate 6). In the late infection zone, the nodule cells were studded with numerous amyloplasts, rough endoplasmic reticulum (RER), golgi



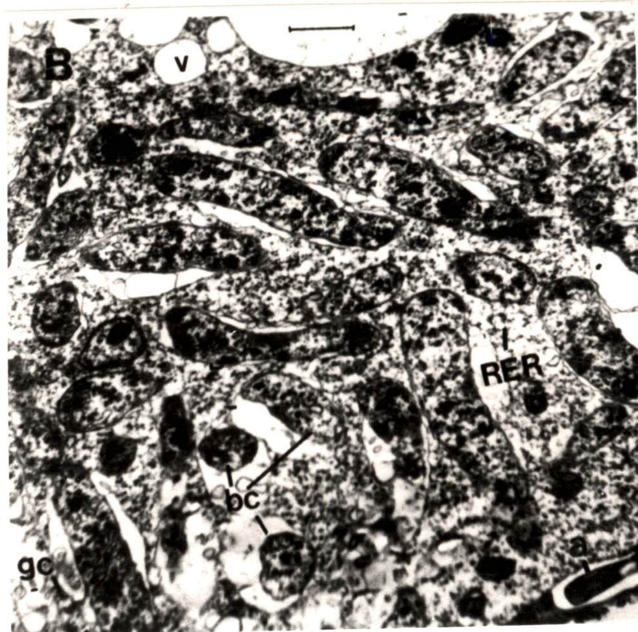


Plate 7. TEM studies of longitudinal and ultrathin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** A part of nodule cell from late infection zone containing partially heterogeneous bacteroids (bc) and numerous vacuoles (V), Bar, 1 μm (x3,900). **B.** Higher magnification of a part of the same field showing amyloplasts (a), golgi complex (gc), rough endoplasmic reticulum (RER) and vacuoles (V). Bar, 1 μm (x8,900).

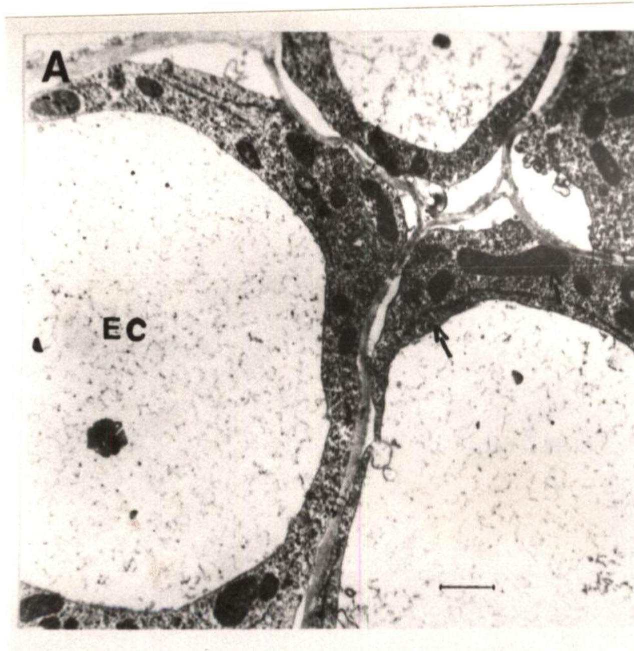
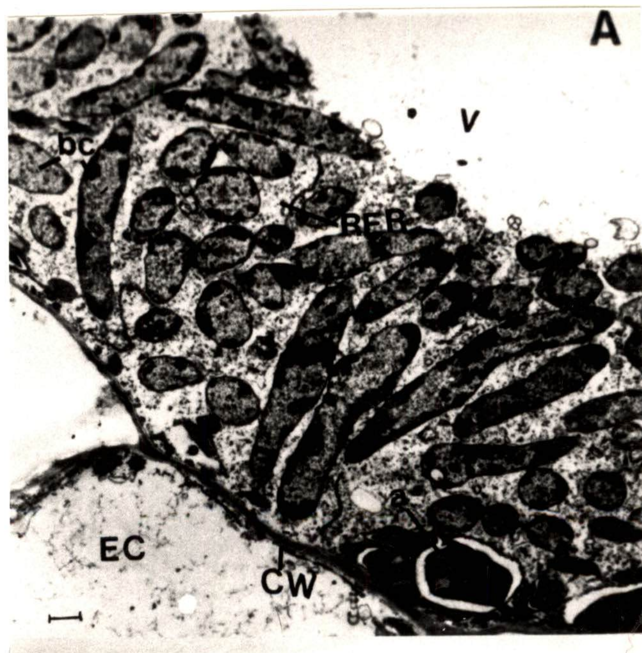


Plate 8. TEM studies of longitudinal and ultrathin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** The nodule cells without bacteroids, i.e., empty cells (EC) showing the cytoplasm restricted to the periphery. All cell organelles (indicated by arrows) are displaced near to the cell wall. Bar, 1 μm (x7,600). **B.** A part of the nodule cell from the inter zone between infection zone and nitrogen fixation zone showing vacuoles (V) and partially heterogeneous bacteroids (bc). Bar, 1 μm (x7,600).



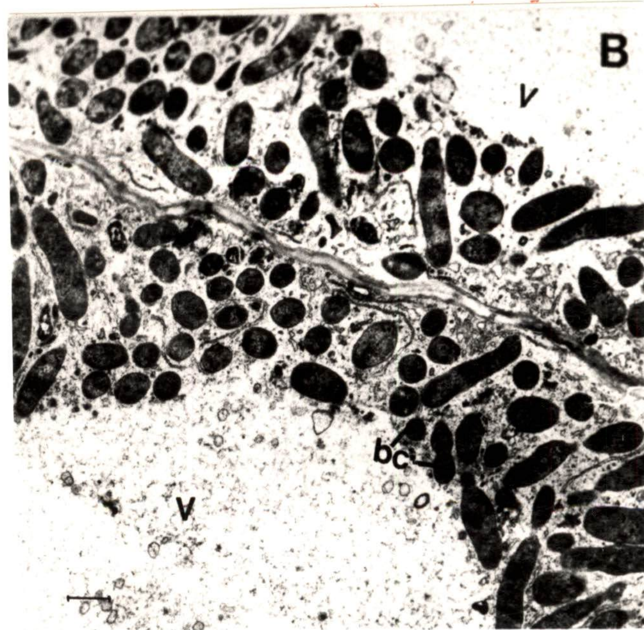


Plate 9. TEM studies of longitudinal and ultrathin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell without bacteroids, i.e., empty cell (EC) and a part of bacteroids filled nodule cell from the nitrogen fixation zone of the nodule. The filled nodule cell contains mature bacteroids (bc) with heterogeneous cytoplasm. Amyloplast (a) and rough endoplasmic reticulum (RER) are displaced to the periphery near to the cell wall (CW). Bar, 1 µm (x4,100). **B.** The part of the two bacteroids filled nodule cells from nitrogen fixation zone. The heterogeneous mature bacteroids (bc) are polymorphic and organised around the central vacuole (V) of the nodule cell. Bar, 1 µm (x5,800).

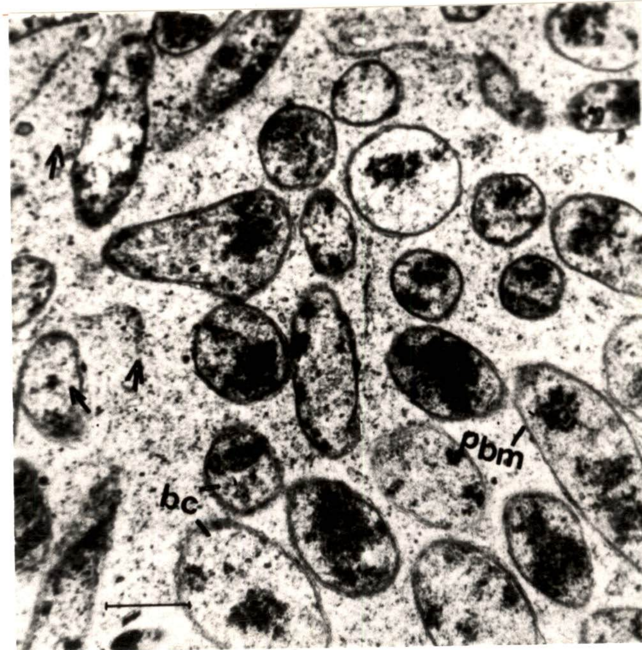
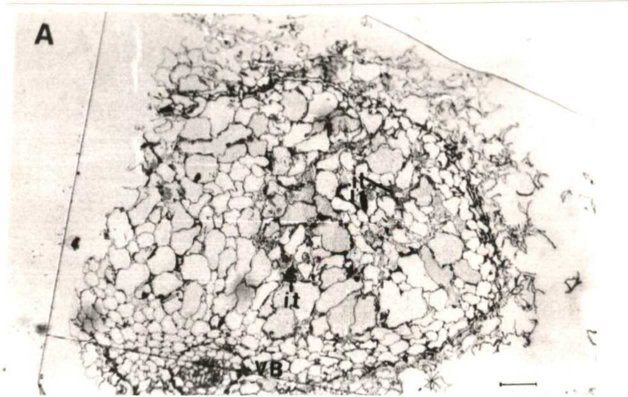


Plate 10. TEM studies of longitudinal and ultrathin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell from senescence zone showing degenerating bacteroids (indicated by arrows). The cytoplasm of each bacteroid is electron transparent and broken peribacteroidal membrane (indicated by arrows) is also visible in some bacteroids. Bar, 1 μm (x11,800).



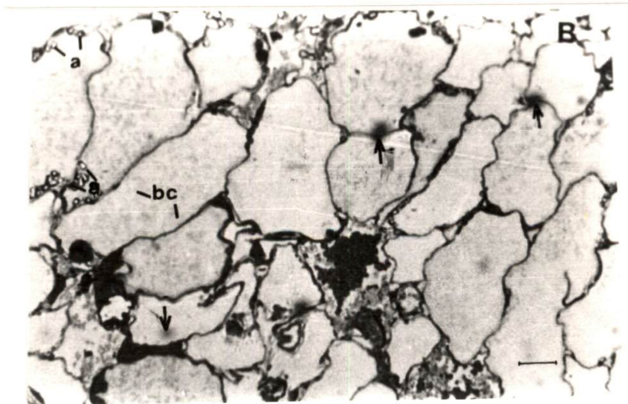
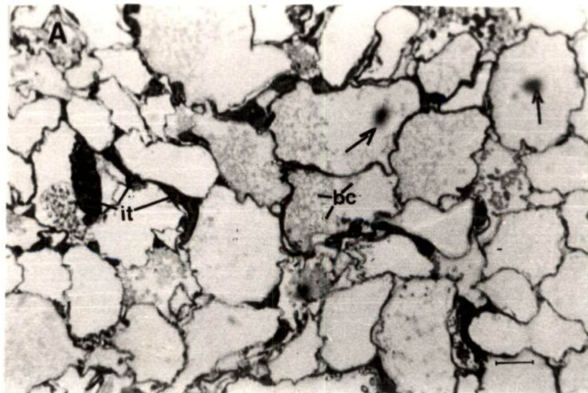


Plate 11. Light microscopic studies of longitudinal and semithin sections of a nodule induced by the strain H33, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** Nodule cells and extensively branched infection threads (it); different zones are absent in this nodule. Bar, 100 µm (x100). **B.** Nodule cells showing released bacteroids (bc), partially active nuclei (indicated by arrows) and amyloplasts (a). Bar, 25 µm (x400).

complex, vacuoles and bacteroids. These bacteroids were elongated and exhibited partial heterogeneous cytoplasm (Plate 7). The plant cell organelles in the empty cells as well as in the cells filled with bacteroids took the peripheral position near to the cell wall (Plates 8:A & 9:A). The mature bacteroids in the zone between infection zone and the nitrogen fixation zone exhibited more heterogeneous cytoplasm with electron dense and electron transparent regions (Plate 8:B). The bacteroids in the nitrogen fixation zone were mostly heterogeneous, elongated and properly organised around the central vacuole of the cell. RER and golgi complex contents were less in nitrogen fixation region in comparison to those of the infection zone (Plate 9). The senescence zone was found to contain degenerated bacteroids which had transparent cytoplasm. Broken pbm was also visible in these bacteroids (Plate 10).

4.7.6.2 Light and Electron Microscopic Studies of Nodules Induced by H33 Mutant

The longitudinal section of a nodule induced by the H33 mutant lacking the carbamoyl phosphate synthetase activity (Car^-) revealed poorly developed nature of the nodule in comparison to the parental strain induced nodule. The nodule lacked distinct zones and peripheral vascular bundles. The nodule cells were loosely packed and the infection threads were seen traversing all through the nodule (Plate 11). The bacteria were mostly confined to the infection threads but occasional release of bacteria was found in some cells. Unlike in the parental strain induced nodules where the nodule cells of infection zone contained prominent nuclei, many vacuoles and amyloplasts, the H33 mutant



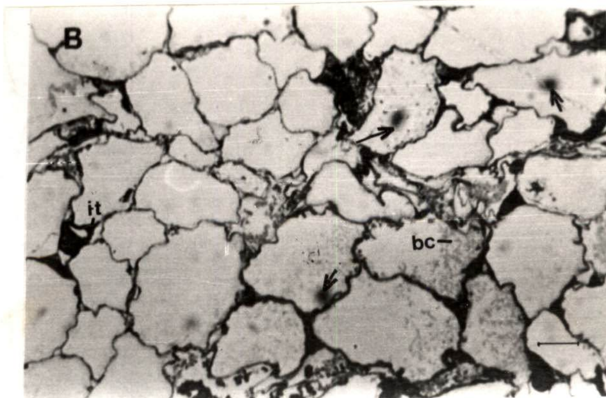
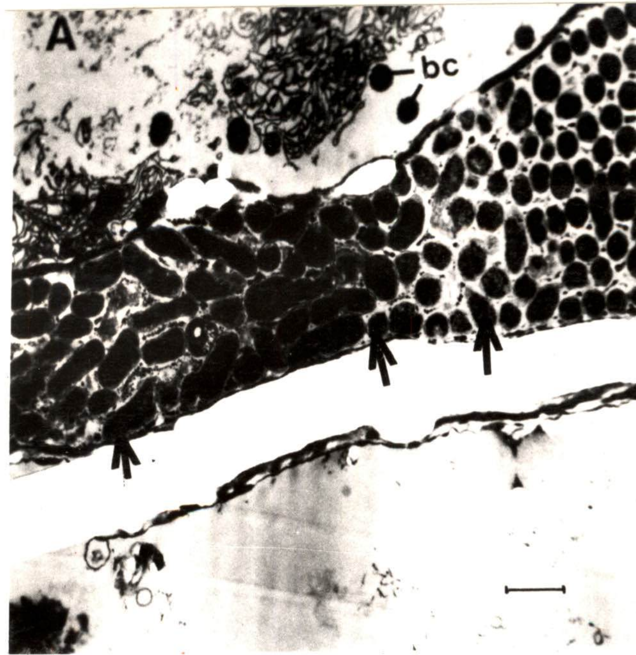


Plate 12. Light microscopic studies of longitudinal and semithin sections of a nodule induced by the strain H33, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** Nodule section showing aggregations of infection threads (it), released bacteroids (bc) and partially active nuclei (indicated by arrows). Bar, 25 μm (x400). **B.** Nodule section showing aggregated infection threads (it) and some nodule cells with electron dense materials (edm). Bar, 25 μm (x400).



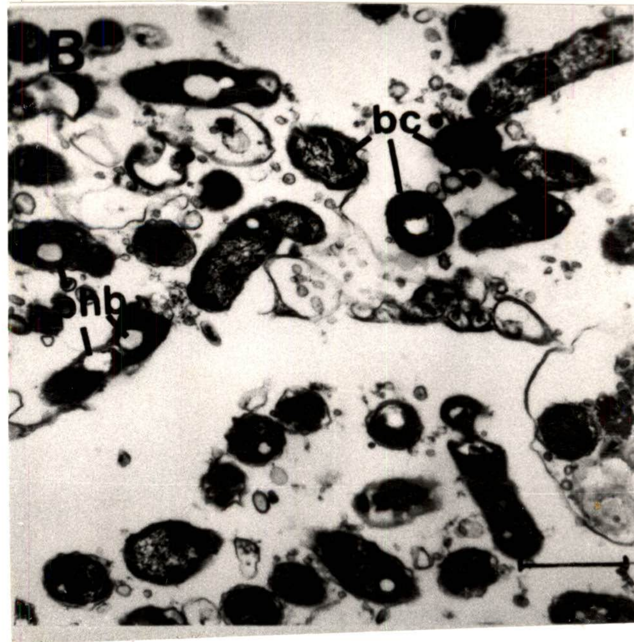
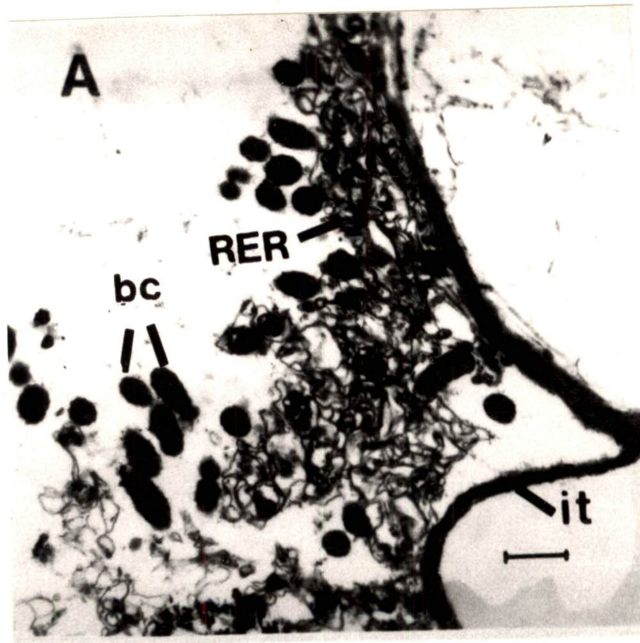


Plate 13. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H33, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of an infection thread containing rhizobial bacteria (indicated by arrows). A few bacteroids (bc) in the adjoining nodule cell are also visible. Bar, 1µm (x8,000). **B.** Depicts freshly released bacteroids (bc) which are electron dense and contain poly-β-hydroxybutyrate (phb) granules. Bar, 1µm (x14,800).



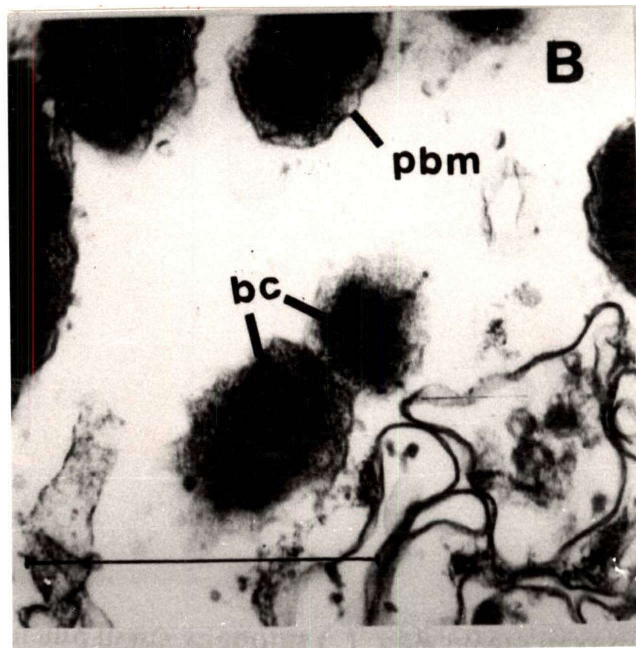
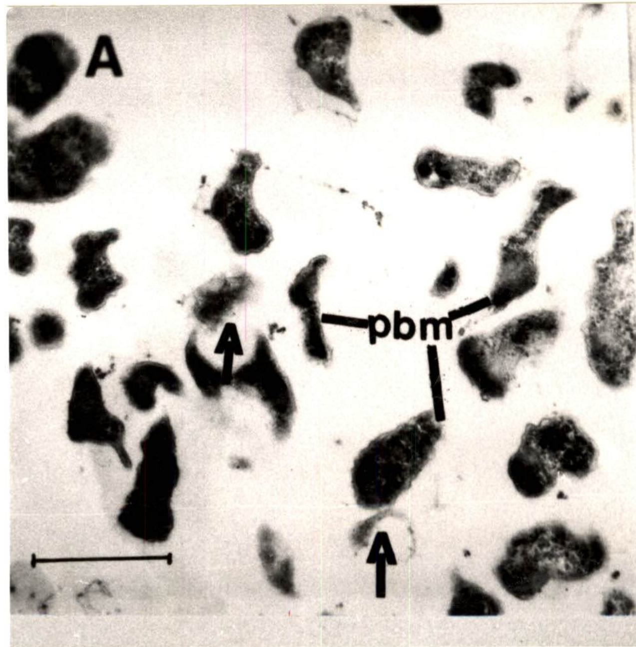


Plate 14. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H33, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell showing rough endoplasmic reticulum (RER) and released bacteroids (bc) without (phb) granules. Infection thread is also visible. Bar, 1μm (x8,600). **B.** Same field higher magnification showing young bacteroids (bc) with homogeneous electron dense cytoplasm and peribacteroidal membrane (pbm). Bar, 1μm (x49,800).



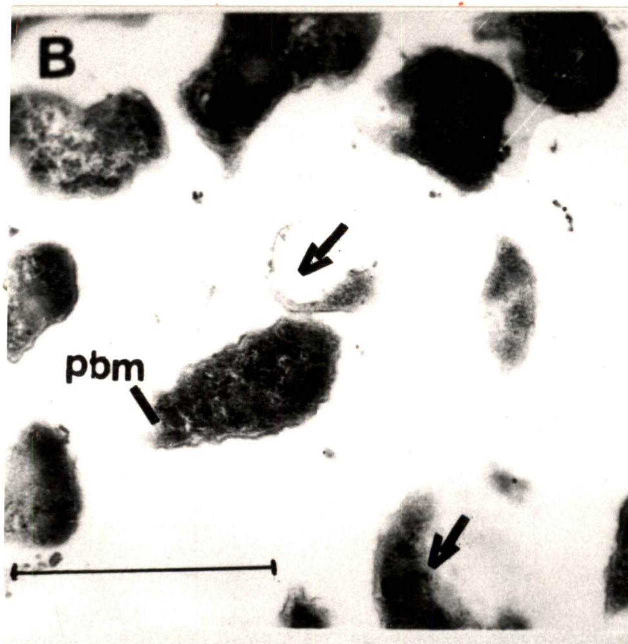


Plate 15. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H33, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** Degenerating bacteroids with ruptured peribacteroidal membrane (pbm) and electron transparent inner content (indicated by arrows). Bar, 1μm (x19,100). **B.** Same field higher magnification showing ruptured peribacteroidal membrane and electron transparent inner content (indicated by arrows). Bar, 1μm (x36,900).

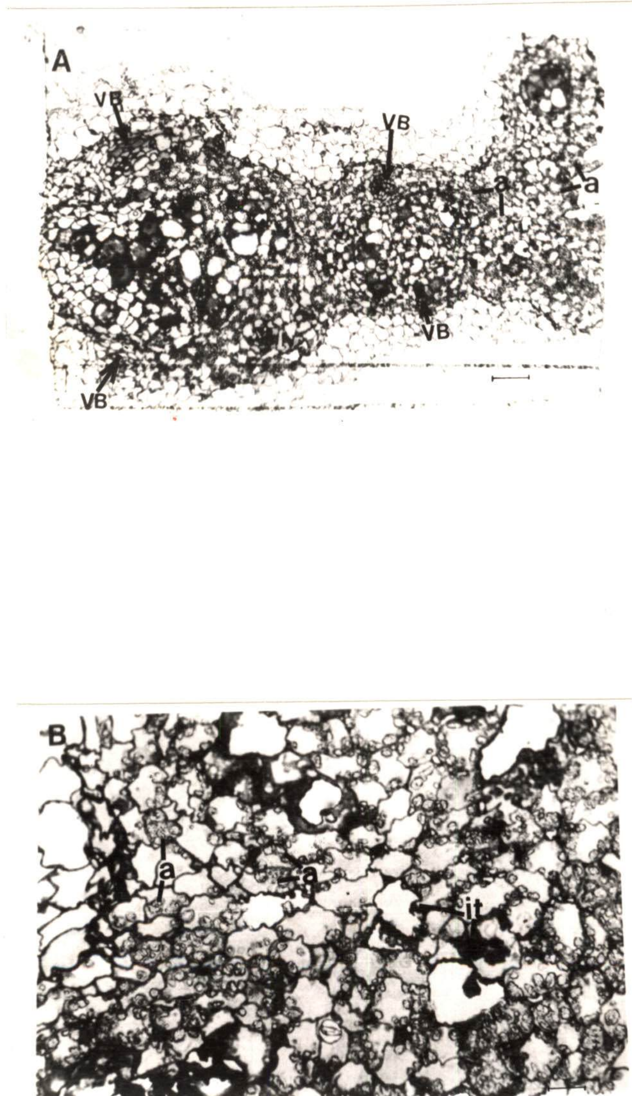


Plate 16. Light microscopic studies of longitudinal and semithin sections of a nodule induced by the strain H7, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** The nodule section showing peripheral vascular bundles (VB) and nodule cells containing amyloplasts (a) and bacteroids. Bar, 100µm (x100). **B.** Nodule section showing aggregated infection threads (it) and nodule cells containing amyloplasts (a). Bar, 25µm (x400).

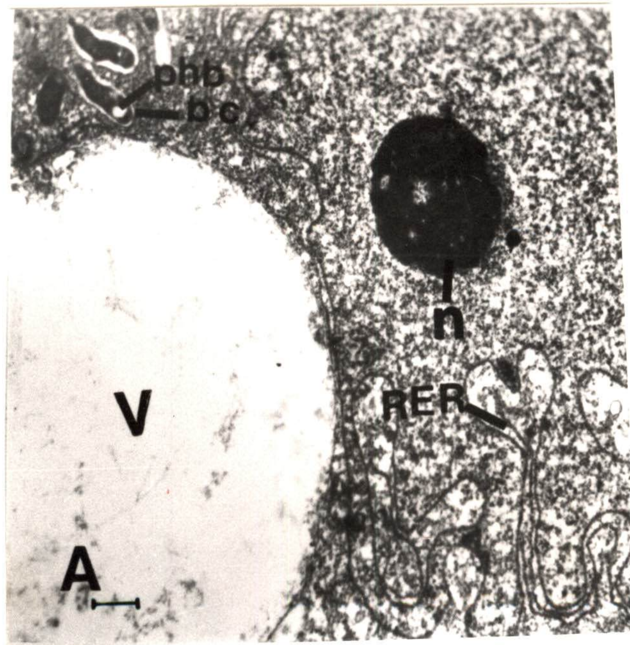
induced nodule cells were devoid of prominent nuclei, many vacuoles and amyloplasts (Plate 12). The initiation of nuclear activity was observed in these cells; some cells were studded with few amyloplasts (Plate 11:B).

At electron microscopic level the bacteria were seen in healthy condition inside the infection threads (Plate 13:A). Occasional release of bacteria into the nodule cells was observed. The freshly released bacteroids were electron dense and contained phb granules (Plate 13:B) but during maturation they were devoid of phb granules. Peribacteroidal membranes were clear in some bacteroids (Plate 14). Unlike in the parental strain induced nodules the bacteroidal transformation to mature bacteroids did not occur and in some cell these young bacteroids were seen in lysed condition; as their pbm were seen broken and the bacteroidal cytoplasm became electron transparent (Plate 15). In infected cells accumulation of RER was seen similar to that of infection zone cells of parental strain induced nodules (Plate 14:B).

4.7.6.3 Light and Electron Microscopic Studies of Nodules Induced by Mutant H7

The longitudinal section of a nodule induced by mutant H7 showed a few advanced features over the nodule induced by the Car$^-$ mutant. Peripheral vascular bundles were seen but out of the four distinct zones only infection zone was present (Plate 16:A). Many vacuoles, amyloplasts and immature bacteroids were seen in all infected cells (Plate 16:B).

Electron microscopic studies showed that the bacterial release was normal. The bacteroids were electron dense but contained clear phb granules (Plate 17:A). A clear pbm was visible in some bacteroids, some young



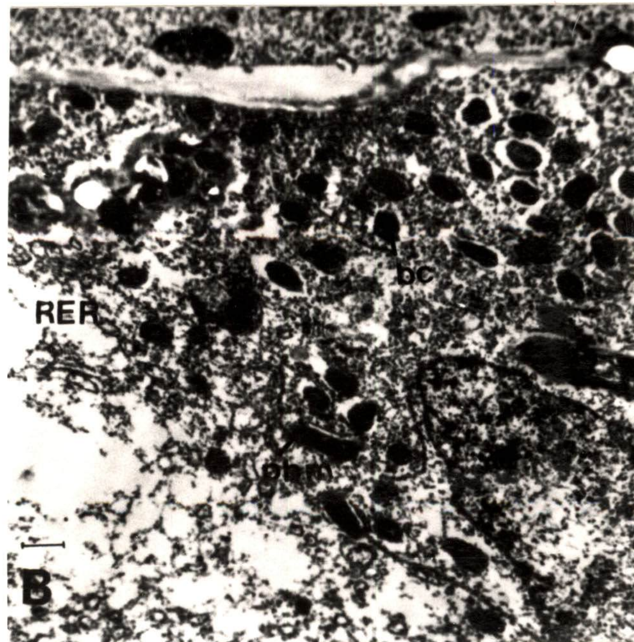


Plate 17. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H7, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell showing freshly released bacteroids (bc) containing phb granules, distinct nucleus (n), rough endoplasmic reticulum (RER) and large vacuole (V). Bar, 1μm (x6,000). **B.** A part of the nodule cell from early infection zone. The bacteroids (bc) are devoid of phb granules. A part of the peribacteroidal membrane (pbm) is also visible in some bacteroids. The nodule cell also contains high RER content. Bar, 1μm (x5,900).

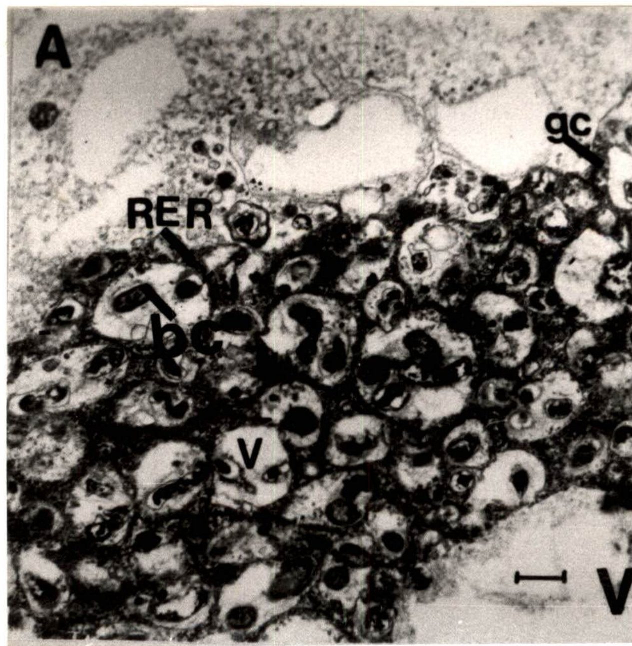
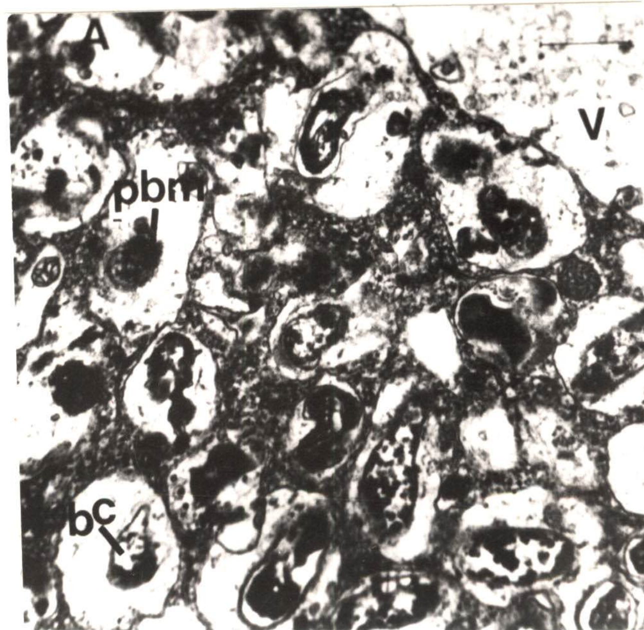


Plate 18. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H7, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell from late infection zone showing released bacteroids (bc), many vacuoles (V), rough endoplasmic reticulum (RER) and golgi complex (gc). Bar, 1μm (x6,000). **B.** Same field higher magnification. Bar, 1μm (x13,800).



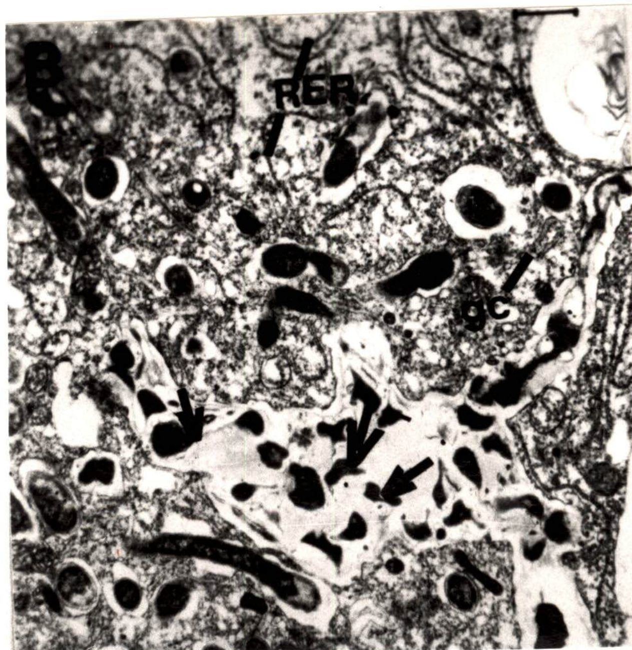
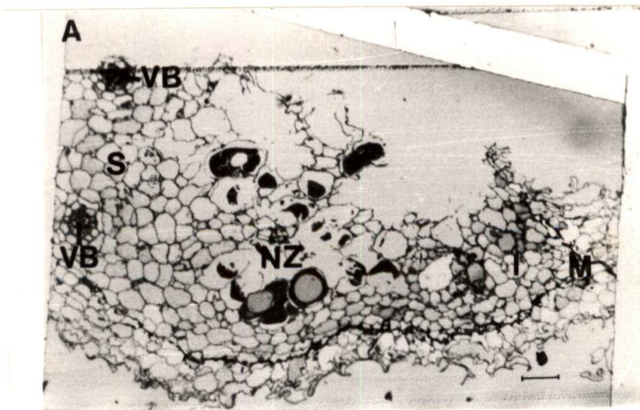


Plate 19. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H7, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell from late infection zone showing many vacuoles (V) and bacteroids (bc). A part of the cytoplasm of some bacteroids is electron transparent and peribacteroidal membrane of many bacteroids is ruptured. Bar, 1μm (x11,000). **B.** A part of the nodule cell showing released bacteroids in lysing stage (indicated by arrows), increased amount of rough endoplasmic reticulum (RER) and golgi complex (gc). Bar, 1μm (x8,000).



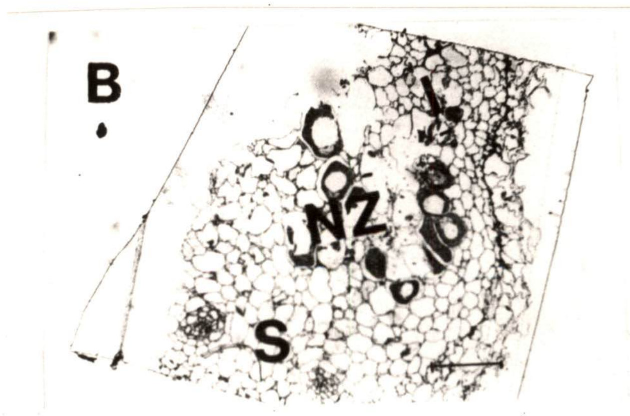
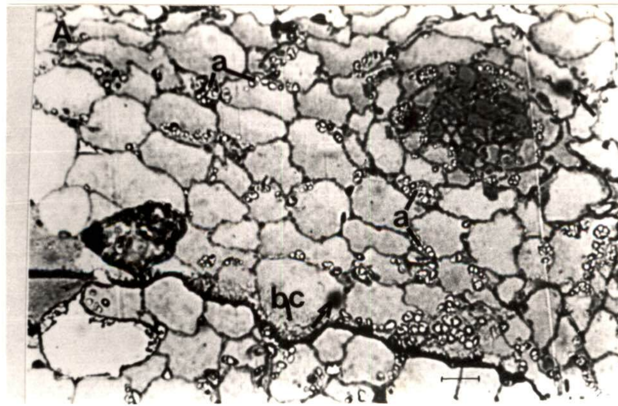


Plate 20. Light microscopic studies of longitudinal and semithin sections of the nodules induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** L.S. of the whole nodule showing four distinct zones, viz., meristematic zone (M), infection zone (I), nitrogen fixation zone (NZ) and senescence zone (S), and peripheral vascular bundles (VB). Bar, 100µm (x100). **B.** L.S. of another nodule showing all above mentioned features. Nitrogen fixation zone is more clear in this section. Bar, 100µm (x100).



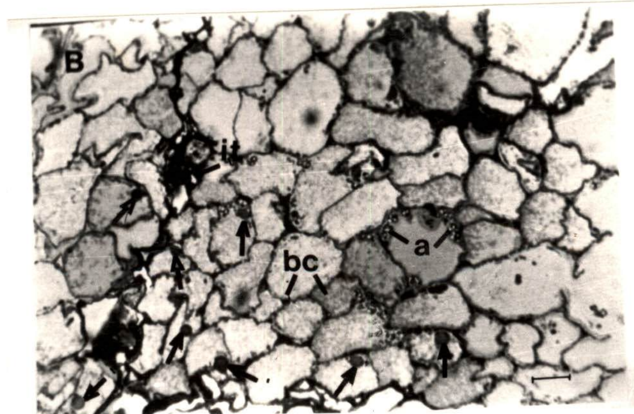
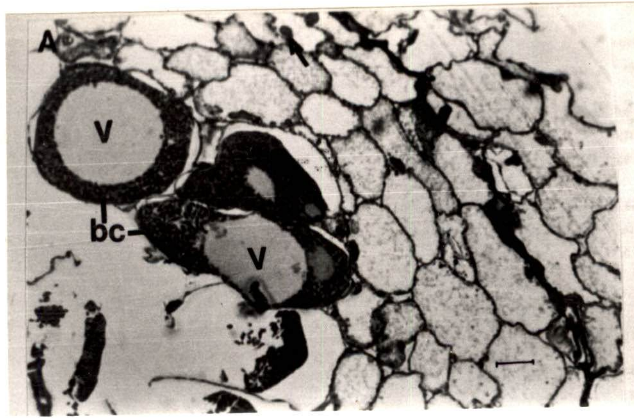
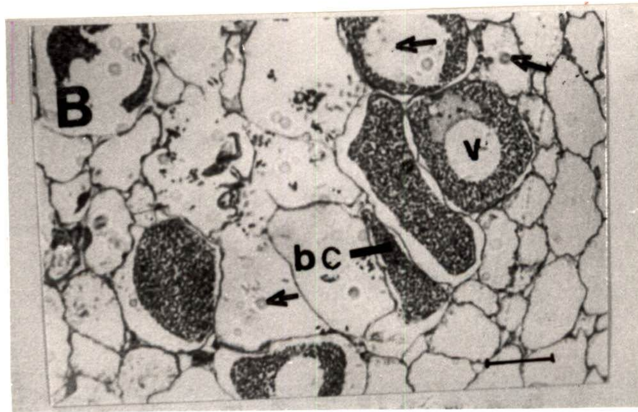


Plate 21. Light microscopic studies of longitudinal and semithin sections of a nodule induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** Infection zone of the nodule showing initiated nuclear activity (indicated by arrow). The nodule cells contain amyloplasts (a) and released bacteroids (bc). Bar, 25 μm (x400). **B.** Inter zone between infection and nitrogen fixation zone showing nodule cells containing prominent nuclei (indicated by arrows), released bacteroids (bc) and few amyloplasts (a). Branched infection threads (it) are visible in the intercellular spaces of nodule cells. Bar, 25μm (x400).





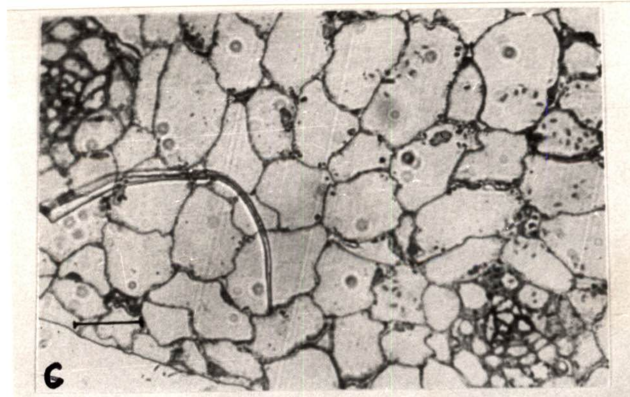


Plate 22. Light microscopic studies of longitudinal and semithin sections of a nodule induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** Poorly developed nitrogen-fixation zone. A few nodule cells contain bacteroids (bc) organized around the central vacuoles (V). Prominent nuclei (indicated by arrows) are also visible in some nodule cells. Bar, 25μm (x400). **B.** Nitrogen fixation zone from another nodule showing the above mentioned features. Bar, 25μm (x400). **C.** Senescence zone. Bar, 25μm (x400).

bacteroids were devoid of phb granules (Plate 17:B). The nodule cells in which bacterial release were found exhibited numerous RER, golgi bodies and vacuoles than in the infection zone of a nodule induced by the parental strain (Plate 18). Transformation of young bacteroids to mature bacteroids was revealed by their tendency to elongate but most of the bacteroids were lysed at this stage as were evident from the broken pbm and electrontransparent cytoplasm (Plate 19).

4.7.6.4 Light and Electron Microscopic Studies of Nodules Induced by the Mutant H36

The histology of nodules induced by the mutant H36 matched with that of the nodule induced by parental strain in having meristematic zone, infection zone, nitrogen fixation zone and senescence zone (Plate 20). The initiation of nuclear activity and amyloplasts accumulation was observed in the nodule cells of infection zone (Plate 21:A). The nodule cells of inter zone contained prominent nuclei and many vacuoles. The amyloplast content of these cells was less than that of the nodule cells in the infection zone; this pattern of amyloplast distribution was similar to that of the parental strain induced nodule (Plate 21:B). In the nitrogen fixation zone unlike the Rmd201 induced nodules only a few nodule cells were filled with bacteroids which were organized around the central vacuole (Plate 22: A;B). The senescence zone was quite prominent in this case (Plate 22:C).

Electron microscopic studies showed clear phb granules in the young bacteroids which were electron dense; a wavy pbm was also visible in some bacteroids (Plate 23). In the infection zone the nodule cells showed RER and

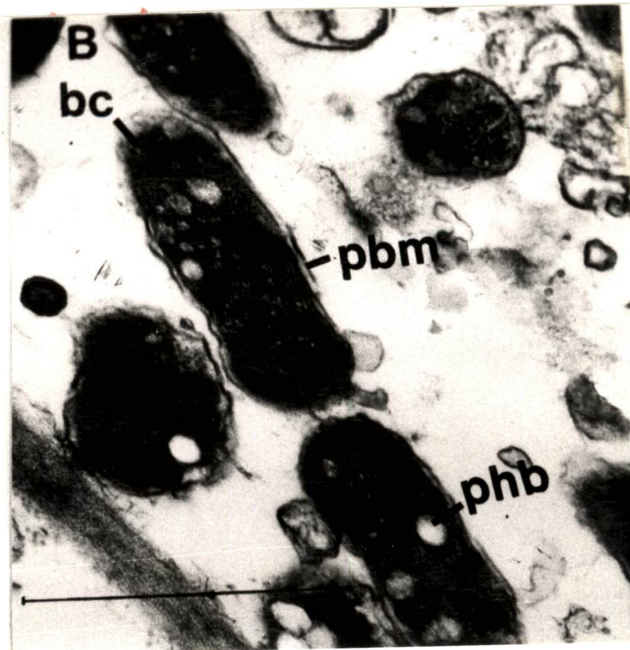
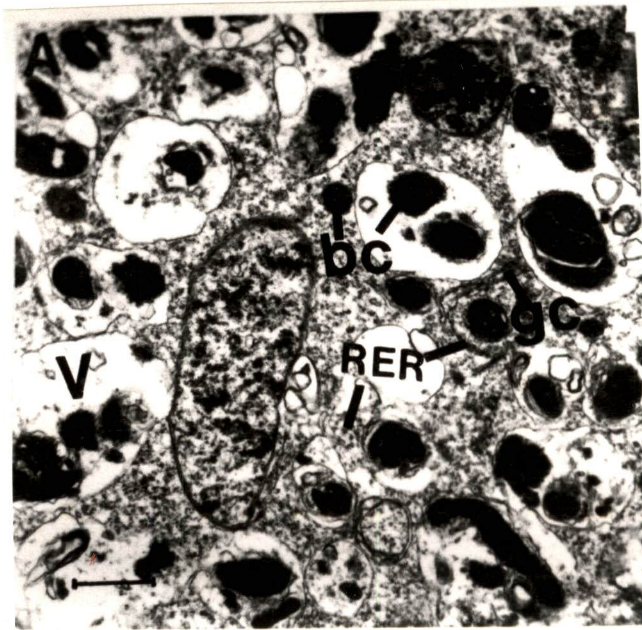


Plate 23. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell from early infection zone showing freshly released bacteroids (bc) containing phb granules. Bar, 1μm (x14,000). **B.** Same field higher magnification showing wavy peribacteroidal membrane (pbm) of some bacteroids (bc). Bar, 1μm (x44,900).



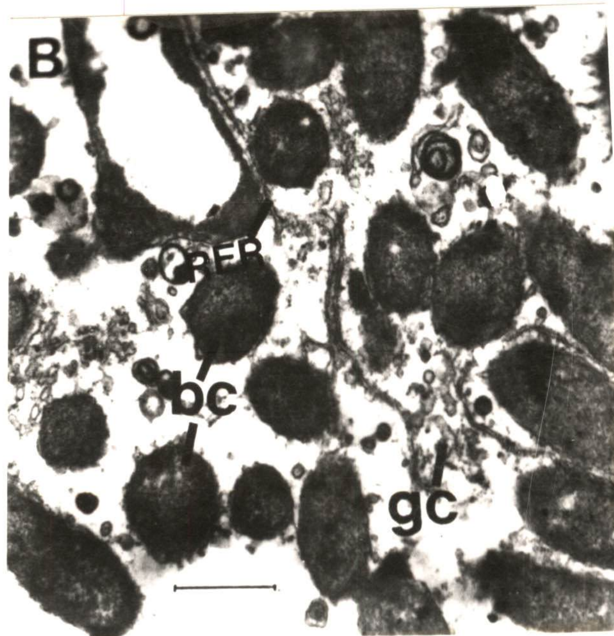
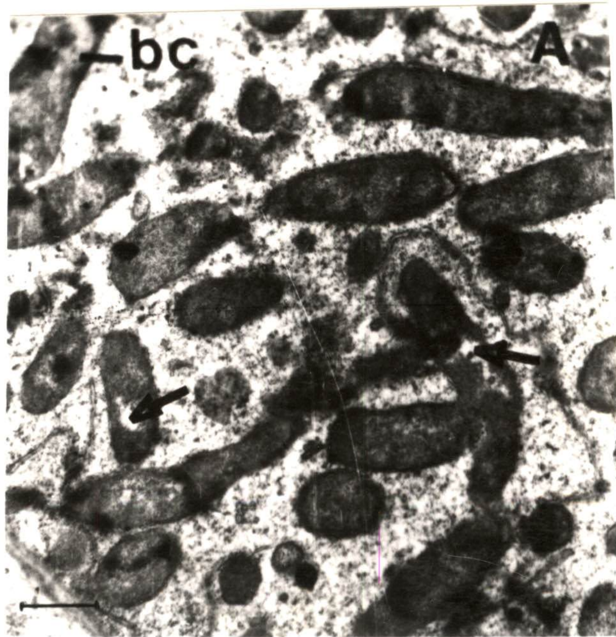


Plate 24. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of nodule cell from early infection zone containing numerous vacuoles (V), rough endoplasmic reticulum (RER), golgi complex (gc) and bacteroids (bc) without phb granules. Bar, 1μm (x10,900). **B.** A part of nodule cell from late infection zone showing bacteroids (bc), RER, and gc. The nuclear condensation in bacteroids is not prominent. Bar, 1μm (x14,000).



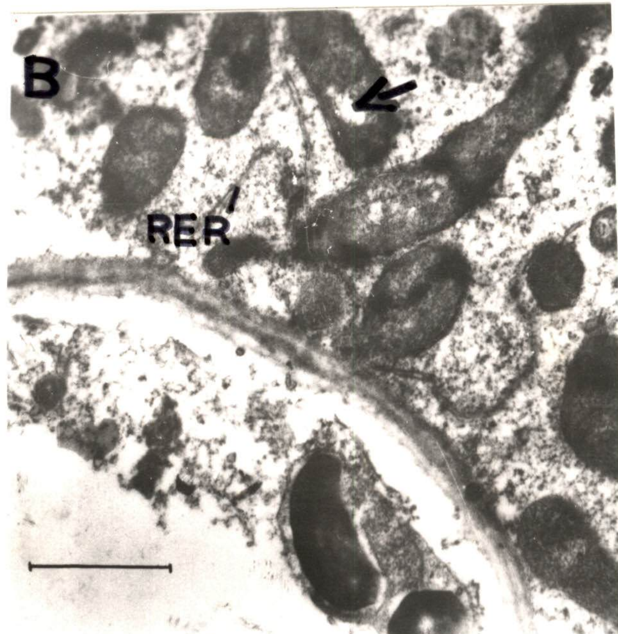
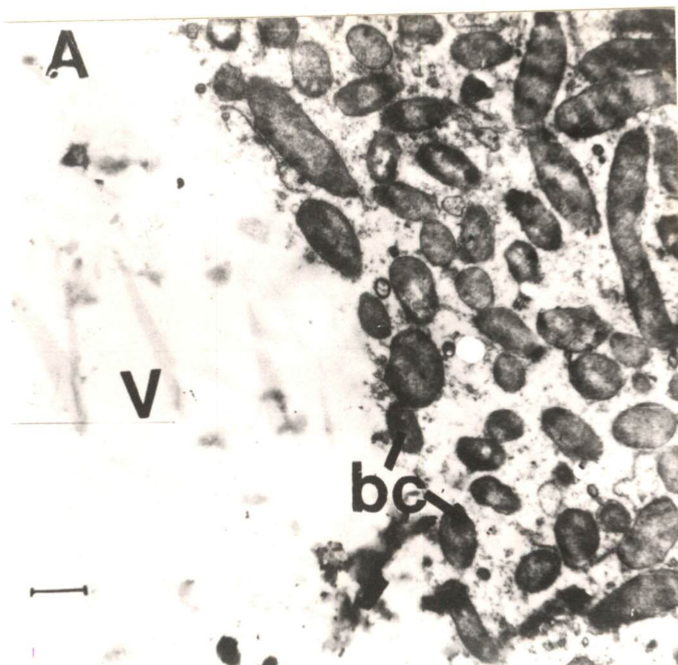


Plate 25. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of nodule cell from poorly developed nitrogen fixation zone showing partially mature bacteroids (bc). Nuclear condensation in these bacteroids is not proper and some regions of bacteroidal cytoplasm are electron transparent (indicated by arrows). Bar, 1μm (x10,600). **B.** Same field higher magnification showing RER and electron transparent cytoplasm (indicated by arrow) in some bacteroids (bc). Bar, 1μm (x 19,800).



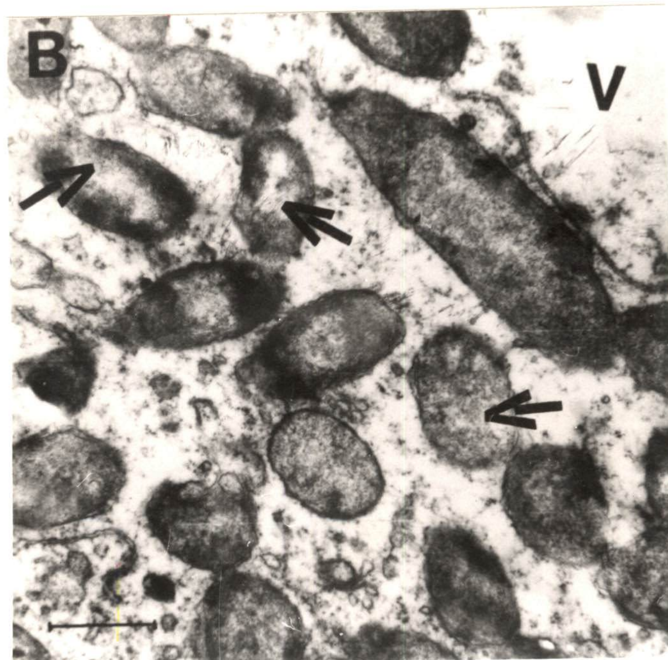
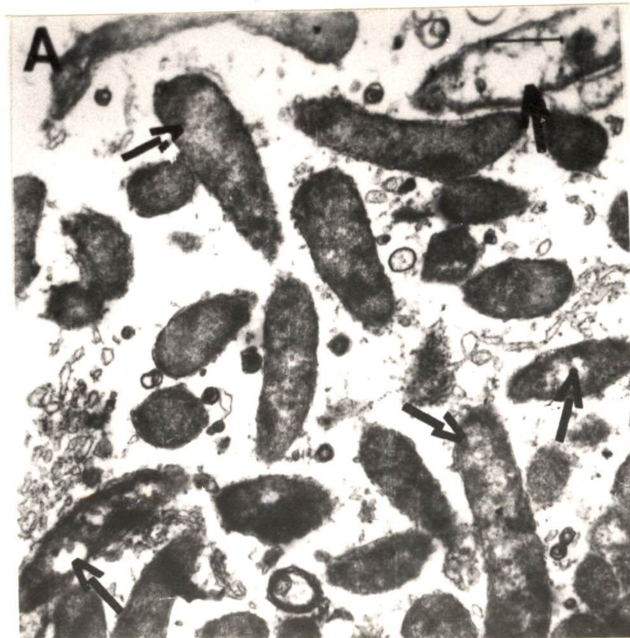


Plate 26. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell from poorly developed nitrogen fixation zone showing properly organised bacteroids (bc) around the vacuole (V). Bar, 1μm (x6,200). **B.** Same field higher magnification showing electron transparent cytoplasm (indicated by arrows) in some bacteroids. Bar, 1μm (x 14,000).



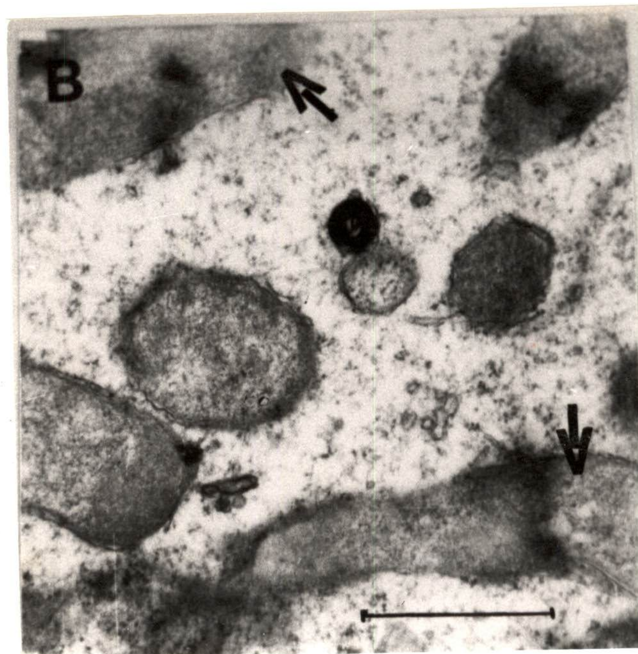


Plate 27. TEM studies of longitudinal and ultrathin sections of a nodule induced by the strain H36, a pyrimidine auxotroph of *Rhizobium meliloti* Rmd201. **A.** A part of the nodule cell from poorly developed nitrogen fixation zone showing electron transparent cytoplasm and ruptured peribacteroidal membrane (indicated by arrows) in some bacteroids. Bar, 1 μm (x 10,000). **B.** A part of the nodule cell from senescence zone showing the above mentioned characteristics (indicated by arrows) of degenerated bacteroids. Bar, 1μm (x 26,900).

many vacuoles and golgi bodies just like the Rmd201 induced nodules (Plate 24:A). The bacteroids in the late infection zone were elongated but the cytoplasm of these bacteroids appeared to be electron dense in nature (Plate 24:B). The bacteroids in the poorly developed nitrogen fixation zone were elongated and organised properly around the vacuole; their cytoplasm was partially heterogeneous (with little nuclear condensation) (Plates 25 & 26). Most of the bacteroids exhibited clear electron transparent cytoplasm and ruptured pbm (Plate 27:A). The bacteroids in the senescence zone also showed similar morphological characteristics (Plate 27:B).

Chapter 5

DISCUSSION

Random transposon mutagenesis using Tn5 delivery vector pGS9 yielded 6000 kanamycin resistant derivatives of *R.meliloti* strain Rmd201 (Nod^+ Fix^+). The frequency of occurrence of these kanamycin resistant derivatives, which were presumably Tn5 induced since the spontaneous kanamycin resistance frequency was very low of the order 10^{-8}, was $3x10^{-5}$ per recipient. The frequency of occurrence of auxotrophs among the Tn5 derivatives was 0.48%. Similar frequencies of Tn5 transposition and occurrence of auxotrophs among the Tn5 derivatives in rhizobia have been reported by Meade *et al.* (1982); Hom *et al.* (1984) and Kim *et al.* (1988). Diverse nutritional requirements of the auxotrophic mutants indicated near randomness of Tn5 insertions. The isolated auxotrophs included 9 cysteine/methionine and 7 methionine auxotrophs. Similar results have also been reported by the earlier workers (Meade *et al.*, 1982; Hom *et al.*, 1984). The high frequency of these auxotrophs may be due to the presence of a large number of genes involved in sulphur metabolism or preferential insertion of Tn5 into the genes involved in the biosynthesis of these amino acids.

Three auxotrophs requiring both isoleucine and valine were obtained in this study. All these mutants did not nodulate alfalfa plants. Aguilar and Grasso (1991) have also reported non-nodulation characteristics of isoleucine and valine requiring auxotrophic mutants. Probably there is non-availability of isoleucine and valine in root exudate or a gene involved in the biosynthesis of these amino acids has a role in the nodule formation process. Cysteine/methionine and methionine auxotrophs were Nod^+ and Fix^+. The cysteine/methionine auxotrophs obtained by Hom *et al.* (1984) were also unaffected in symbiosis. It seems that sulphur-containing amino acids are available to rhizobia from the host plant. Consistent with the earlier reports (Kerppola and Kahn, 1988a; 1988b) purine and pyrimidine auxotrophs were Nod^+ but

Fix⁻. It seems that either these metabolites are not available to rhizobia *in planta* or the enzyme/intermediates of the biosynthetic pathways of these metabolites have a role in the symbiotic process. Since very little work has been done in past on the role of the pyrimidine biosynthetic pathways in symbiosis and there was the availability of nine pyrimidine auxotrophic mutants in this work, it was decided to carry out detailed genetic and symbiotic investigations on these mutants. An attempt was made to determine the position of biochemical block in each pyrimidine auxotroph by intermediate feeding, intermediate accumulation and cross feeding studies.

Carbamoyl phosphate, synthesized by carbamoyl phosphate synthetase enzyme,is a common intermediate for arginine and pyrimidine biosynthetic pathways. A mutation in the genes for carbamoyl phosphate synthetase enzyme (*car A* & *car B*) leads to auxotrophies for arginine and uracil. In the present work these mutants, viz., H33, H37 & H47, each of which required the supplementation of minimal medium with both arginine and uracil for its growth,were obtained. The growth of H33, H37 and H47 mutants on minimal medium supplemented with carbamoyl phosphate indicated the lack of carbamoyl phosphate synthetase activity in these mutants. Hence each of these mutants had a Tn5 insertion in *car A* & *B* genes.

The mutants with defect in carbamoyl phosphate synthetase enzyme were expected to grow on minimal medium supplemented with arginine and any of the intermediates of pyrimidine biosynthetic pathways. Positive results were obtained with orotic acid but not with carbamoyl aspartate or dihydroorotic acid indicating some problem to rhizobial bacteria in the uptake/ utilization of these compounds.

Five pyrimidine auxotrophs, viz., VE12, VE43, VE19, H9 and H7 could grow on orotic acid supplemented minimal medium. The location of

biochemical block in these mutants is likely to be at any of the three steps between carbamoyl phosphate and orotic acid. The exact location of block could not be determined by intermediate feeding studies since it was found as described above that uptake of carbamoyl aspartate and dihydroorotic acid from the growth medium was not successful.

The pyrimidine auxotrophic mutant H36 did not grow on minimal medium supplemented with orotic acid and it accumulated orotic acid in minimal medium. These results show that this mutant has a biochemical block at either of the two steps between orotic acid and uridine monophosphate. Since the mutant H36 accumulates orotic acid it was expected to cross-feed the pyrimidine auxotrophs which were able to grow on minimal medium supplemented with orotic acid. But such cross feeding was not observed. The lack of cross feeding may be due to the low amounts of orotic acid secreted by H36 mutant.

Production of cell surface molecules like cellulose fibrils, β(1→2) glucans, lipopolysaccharides and acidic exopolysaccharides; and utilization of dicarboxylic acids are important for successful symbiosis. Cell surface molecules of all pyrimidine auxotrophic mutants were similar to those of the parental strain Rmd201 and all these mutants could utilize dicarboxylic acids as a carbon source like the parental strain. Hence symbiotic defectiveness of these auxotrophs does not appear to be due to the absence of any of the cell surface molecules or inability to transport dicarboxylic acids.

Kondorosi *et al.* (1977a) constructed a circular linkage map of *R.meliloti* strain Rm41 and found that the pyrimidine mutations mapped at two regions, the first consisting of mutations pyr 1, 2-29 and the second consisting of mutation pyr 24. The present work has been carried out with a derivative of the above mentioned strain. The mutations of the pyrimidine auxotrophs of

this work have been found not mapped in the 2/3 region, which includes the above mentioned first region of already mapped pyrimidine mutations of the chromosome. Hence the location of the pyrimidine mutations of the present work are likely to be at or near the already mapped pyr-24 locus.

100% cotransfer of the Tn5 encoded kanamycin resistance and auxotrophy in all pyrimidine auxotrophs showed that each of these auxotrophs carried only one Tn5 insertion. Spontaneous excision of this Tn5 took place in some cells of each auxotroph leading to the formation of kanamycin sensitive prototrophic revertant cells. Prototrophic revertants of all pyrimidine auxotrophs formed normal nitrogen fixing nodules like the parental strain Rmd201. On the basis of these results symbiotic defects of each pyrimidine auxotroph can be attributed to the insertion of transposon Tn5 in one of the pyrimidine biosynthetic genes.

When alfalfa plants were inoculated with the pyrimidine auxotrophs H33, H37, H47, H7 or H36, all the nodules on about one third of the inoculated plants were found to be occupied by the prototrophic revertant of the respective auxotroph. The spontaneous reversion frequencies of these auxotrophs indicated that the inocula of these strains used in plant experiments contained extremely small numbers of prototrophic revertant cells. The high frequencies of nodule occupancy by the prototrophic revertants in contrast to their low numbers in the inocula used were apparently due to the selective advantage of the prototrophs over the auxotrophs in nodule formation.

In case of inoculation with the pyrimidine auxotrophs VE12, VE43, VE19 and H9, the nodules on all the inoculated plants were occupied by the prototrophic revertants. One may conclude from these results that these auxotrophs are *nod*$^-$. But this conclusion does not seem to be true because of two reasons, (i) the pyrimidine auxotrophs having a biochemical block at an

earlier step formed nodules and (ii) the mutant H7 which has like these mutants a block between carbamoyl phosphate and orotic acid also induced nodules. Relatively high frequencies of spontaneous reversion of these auxotrophs in comparison to those of the other pyrimidine auxotrophs may be the reason for the above result. A large number of plants should be inoculated to obtain nodules occupied by these auxotrophs. Alternatively mutants with deletions in pyrimidine biosynthetic genes can be used.

The formation of nodules, which were found to contain rhizobial bacteria and bacteroids, by the pyrimidine auxotrophic mutants of *R.meliloti* demonstrated that small amounts of the pyrimidines were available to these bacteria from the plant source since there was no possibility of the multiplication of these auxotrophs without the supply of pyrimidines from outside. The symbiotic defects of these auxotrophs were not restored on plant nutrient medium supplemented with uracil (or uracil + arginine in case of carbamoyl phosphate synthetase mutants). This non restoration of the symbiotic defects may be due to the non availability of uracil from the nutrient medium to the rhizobial mutants in the nodules. Another reason for these results may be the absence in these auxotrophs of a pyrimidine intermediate/ enzyme having a role in symbiosis. None of these intermediates of pyrimidine biosynthetic pathway restored the symbiotic defects of any of the pyrimidine auxotroph. These results do not rule out the involvement of the pyrimidine intermediates in symbiosis since it has already been shown that this rhizobial strain has a problem in the uptake of atleast two of the pyrimidine intermediates. Further sufficient amounts of the intermediates, which are taken up by this strain, may not be available to the auxotrophic cells in the nodules. Even one of the enzymes of the pyrimidine biosynthetic pathway may be responsible for the conversion of some unknown-precursor into a

product having a role in symbiosis. Thus the lack of activity of such an enzyme due to a mutation will result in pyrimidine auxotrophy as well as defective symbiosis.

Histological studies of the pyrimidine auxotrophs H33, H7 and H36 showed the involvement of the pyrimidine biosynthetic enzymes and/or intermediates in determining the nodule structure; though all these mutants induced ineffective nodules, yet the extent of nodule development varied according to the position of biochemical block in the pyrimidine biosynthetic pathway. The auxotroph H33 has the block in the first step, H7 in the step number 2/3/4 and H36 in one of the last two steps. The structure of the nodules induced by the mutant H7 showed advanced features over that of the H33 induced nodules. Similarly the nodules induced by the mutant H36 had advanced structural features as compared to the H7 induced nodules. The nodules induced by the auxotrophs H33 lacked peripheral vasculature and proper zones whereas the mutant H7 induced nodules had peripheral vasculature and well developed infection zone. As compared to the mutant H7 further development occurred in the nodules induced by the auxotrophic mutant H36 to the extent that four distinct zones were seen. The rough endoplasmic reticulum (RER) and golgi complex (gc) contents were high in cells of nodules induced by the three pyrimidine auxotrophs, viz., H33, H7 and H36. High RER and gc contents were observed in younger tissues of effective nodules in *R.meliloti* and ineffective nodules of *R. meliloti* (Mackenzie and Jordan, 1974; Hirsch *et al.*, 1983). These cellular responses appear to reflect the plant's response to nitrogen starvation.

Carbamoyl phosphate is a common intermediate in the biosynthesis of arginine and pyrimidines. Therefore a defect in the biosynthesis of carbamoyl phosphate leads to simultaneous auxotrophies for arginine and pyrimidines.

Carbamoyl phosphate enters the arginine biosynthetic pathway at the penultimate step. Since *R. meliloti* auxotrophs blocked in the last two steps were found to be effective in symbiosis (Kerppola and Kahn 1988a) it appears that the plant supplies arginine to rhizobia inside the nodules. Therefore the defects in symbiosis of mutants which lack carbamoyl phosphate synthetase activity may be attributed to defective pyrimidine biosynthetic pathway only rather than both arginine and pyrimidne biosynthetic pathways. Carbamoyl phosphate synthetase activity and/or carbamoyl phosphate may be playing some role in nodule development.

The above findings indicate the role of the pyrimidine biosynthetic enzymes/intermediates in symbiosis. These studies could not be narrowed down to a particular enzyme or intermediate for such a role. Further work needs to be done in this direction. Mutants for all the steps of this pathway are needed. The problem of the spontaneous reversion of auxotrophs can be solved with the use of deletion mutants.

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