GENETIC AND SYMBIOTIC STUDIES ON PURINE AUXOTROPHS OF *Sinorhizobium meliloti*

A THESIS

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

DOCTOR OF PHILOSOPHY

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BIOSCIENCES AND BIOTECHNOLOGY

By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**GENETIC AND SYMBIOTIC STUDIES ON PURINE AUXOTROHS OF *Sinorhizobium meliloti***" in fulfilment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biosciences and Biotechnology** of the University is an authentic record of my own work carried out during a period from February 1998 to April 2001 under the supervision of **Dr. R. Prasad (Asst. Prof.)**.

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.

Dated: April 2001 **(ABASS TALI AL JUDI)**

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

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ABSTRACT

Sinorhizobium meliloti Rmd201 (Nod^+,Fix^+,Str^r) strain, a derivative of the wild type strain Rm41, was subjected to random Tn5 mutagenesis with the help of suicide plasmid pGS9, carrying transposon Tn5. Two thousand and two hundred kanamycin resistant transconjugants were obtained from crosses between *E. coli* WA803 (pGS9) and *S. meliloti* strain Rmd201. These transconjugants were streaked on minimal medium and twelve auxotrophs were identified on the basis of their inability to grow in this medium. Streaking of these auxotrophs on nutritional pools yielded two adenine auxotrophic mutants. Two adenine+thiamine and eight adenine auxotrophs isolated by other research workers were also included in further studies.

The positions of blocks in the purine biosynthetic pathway for these auxotrophs were determined by intermediate feeding and cross feeding studies and based on these results the purine auxotrophs were divided into four groups as follows: Group (I) Purine auxotrophs, NV2 and NV24, which require both adenine and thiamine for their growth and each of these, has a block in the adenine-thiamine pathway before amino imidazole riboside (AIR). Group (II) Purine auxotrophs, NV10, NV28, and AL7 that require amino imidazole carboxamide riboside (AICAR) for their growth and each of these have a block in the adenine pathway before AICAR. Group (III) Purine auxotrophs, AL3, VK37, VK38, VK40 and RH13 which require inosine or adenine for their growth and each of these has a block in the adenine pathway before inosine monophosphate (IMP) and group (IV) Purine auxotrophs, VK27 and RH19, which require adenine for their growth and each of these, has a block in the pathway before adenosine monophosphate (AMP).

In order to find out the pleiotropic effects of Tn5 insertions, all purine auxotrophs were tested for production of exopolysaccharides, lipopolysaccharides, cellulose fibrils, β (1-2) glucans and utilization of dicarboxylic acids. All these auxotrophs were found to utilize dicarboxylic acids as a sole carbon source and produce exopolysaccharides, lipopolysaccharides and cellulose fibrils as the parental strain Rmd201, whereas, these mutants showed less motility in swarm medium than the parental strain which related to production of β (1-2) glucans.

The symbiotic properties of different purine auxotrophs along with the parental strain *S. meliloti* Rmd201 were studied by inoculating them on alfalfa (*Medicago sativa*) seedlings grown on nitrogen free agar slants. The nodules induced by the parental strain Rmd201 were of cylindrical shape and pink in color. The mutants induced-nodules were round /irregular in shape and white in color. The mean shoot dry weights and mean shoot high of the plants inoculated with purine auxotrophs did not differ significantly from those of uninoculated alfalfa plants. These indicate the ineffectiveness of these mutants in symbiotic nitrogen fixation. The ineffectiveness of these mutants in nitrogen fixation were also reflected by comparing total nitrogen content of plant inoculated with parental strain and that inculated with purine auxotrophs. All purine auxotrophs were found to induce root hair deformation and infection thread formation. The addition of adenine and other intermediates to plant growth medium did not restore effectiveness in these mutants.

For histological studies, the nodules from six weeks old plants were cut, fixed and embedded in araldite epoxy resin. Thin and ultrathin sections of nodules were cut and examined under light and transmission electron microscopes, respectively. The nodules induced by the parental strain Rmd201 had four zones: meristematic zone,

infection zone, nitrogen fixation zone and senescence zone. Cells of meristematic zone were found to be typically small and isodiametric in shape. This region was devoid of rhizobia. A network of infection threads was seen in the infection zone. Poly-β-hydroxybuterate (phb) inclusions were found in the bacterial cytoplasm. In the nitrogen fixation zone of nodule, the mature bacteroids were elongated. The younger bacteroids exhibited a relatively homogeneous cytoplasm. In the distal part of the nitrogen fixation zone, the bacteroids had proliferated to the extent that the most of the host cell cytoplasm was occupied by elongated bacteroids. The organelles of host cell and bacteroids were found at the peripheral position around the centrally located vacuole. In mature bacteroids, which were present in the proximal part of nitrogen fixation zone, the cytoplasm was heterogeneous due to condensation of nucleic acid material. The transition from the nitrogen fixation zone to senescence zone occurred abruptly.

Light microscopic studies of the nodules induced by purine auxotrophs did not show significant differences among themselves. The longitudinal section of whole nodule in each case showed the lack of distinct zones like the parental strain induced nodules; most of the cortical cells were devoid of bacteroids, while a few contained amyloplasts. Infection threads and many peripheral vascular bundles were commonly seen in the nodule sections.

At ultrastructural level, the nodules induced by group I auxotrophic mutant NV2 showed infection threads containing bacteria in lytic condition. The released bacteroids started degrading at this stage. The nodules induced by group II (AL7) and group III (VK38) auxotrophs showed a large number of uninvaded cells with starch granules. Some of the cortical cells contained bacteroids with heterogeneous cytoplasm and normal shape, while some others contained bacteroids with heterogeneous

cytoplasm and were abnormal in shape. It seems that the lysis of bacteroids occurred immediately after their release. In the nodules induced by group IV auxotroph VK 27 bacteroidal development appeared to be relatively more advance as compared to that in the nodules induced by the purine auxotrophs of groups I, II and III.

These results showed that a normal flow of metabolites through purine biosynthetic pathway is required for successful *S. meloliti*-alfalfa symbiosis. Some intermediates and enzymes of purine biosynthetic pathway may have a role in bacteroidal development and maturation.

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LIST OF PUBLICATIONS / PAPERS PRESENTED IN CONFERENCES / SYMPOSIA

1. Al-Judi, A.T. and Prasad, R. (2000) Genetic and Biochemical studies of purine auxotrophic mutants of *Rhizobium meliloti*: Role in Symbiosis. In : National Symposium on Recent Trends in Plant Science Research, Department of Botany, University of Kerala, Thiruvananthapuram, April, 17-19-2000, Kerala, India.

2. Al-Judi, A.T. and Prasad, R. (2000) Isolation and characterization of purine auxotrophic mutants of *Sinorhizobium meliloti*. In: Conference of 69th Annual Meeting of Biological Chemists, December,7-9-2000, Calcutta, India.

3. Al-Judi, A.T. and Prasad, R. (2001) Mutation in the purine pathway of *Sinorhizobium meliloti* lead to defect in symbiosis with alfalfa. In: Indian Science Congress (88th Session) Jan. 3-7-2001, Indian Agricultural Research Institute, New Delhi.

4. Al-Judi, A.T. and Prasad, R. (2001) Ultrastructural studies of alfalfa root nodules formed by symbiotically defective purine auxotrophic mutants of *Sinorhizobium meliloti*. In: National Symposium on Relevance of Plant Biochemistry and Biotechnology – Modern Trends, March 2001, Department of Biotechnology, The American College, Madurai, India.

5. Al-Judi, A.T. and Prasad, R. (2001) Studies on *Sinorhizobium meliloti* purine auxotrophic mutants that are symbiotically defective. In: National Conference on Environment Conservation, Sustainable Development and Industrial Expansion in 21 Centuary, March 3-4, 2001, Allahabad (U.P.) (Communicated).

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS USED

a	-	amyloplast
bc	-	bacteroid
CW	-	cell wall
GB	-	golgi bodies
MZ	-	meristimetic zone
IZ	-	infection zone
NZ	-	nitrogen fixation zone
SZ	-	senescence zone
it	-	infection thread
n	-	nucleus
m	-	mitochondria
RER	-	rough endoplasmic reticulum
S	-	starch granule
IC	-	intracellular space
r	-	rhizobia
pbm	-	peribacteroidal membrane
rh	-	root hair
sc	-	shepherd's crook
V	-	vacuoles
VB	-	vascular bundles
ml	-	millilitre
mg	-	milligram
µg	-	microgram
µm	-	micrometer

CONTENTS

Chapter 1

INTRODUCTION

Nitrogen (N), being a constituent of major biological macro molecules (protein, nucleic acids and other essential molecules), plays important role in nutrition of almost all organisms. In nature nitrogen is present in various forms. In atmosphere it exists in gaseous form and constitutes about 78% (by volume). The plants, which are the primary producers of food chain, are unable to utilize this atmospheric nitrogen. Instead they use combined form of nitrogen (ammonia, nitrate and nitrite) present in soil. The soil becomes deficient in nitrogen due to continuous cereals cropping and depletion. This nitrogen deficiency in soil is replenished by adding chemical fertilizers to the soil. Besides the industrial production of nitrogen fertilizers is costly. Approximately 50% of these applied fertilizers get leached which not only leads to pollution but also wastes energy and money. Therefore, there is a need to look for other sources of nitrogen supply to plant. In this context one alternative source of nitrogen is biological nitrogen fixation. It involves the conversion of atmospheric gaseous nitrogen to ammonia by group of prokaryotes, collectively termed diazotrophs, which include bacteria and blue green algae. A number of bacterial species are known to have the ability to fix atmospheric nitrogen. Based on their association with plants, these bacteria can be classified into three main groups.

(1) Free living (*Klebsiella pneumoniae, Azotobacter vinelandii, Rhodospirillum rubrum, etc.)*

(2) Associative *(Azospirillum* spp.*)*, and

(3) Symbiotic *(Rhizobium, Bradyrhizobium, Azohrizobium, Frankia, etc.*).

The symbiotically fixed nitrogen is the largest source of nitrogen in the global nitrogen cycle. It has been estimated that bacteria belonging to Rhizobiaceae fixed up to 120 million tones of atmospheric nitrogen annually (Freiberg *et al.*,1997).

Among these nitrogen fixed by legume-*Rhizobium* symbiosis account for more than 25% and is an important primary candidate for sustainable agriculture. Historically, the ability of leguminous plant to restore soil fertility has been known for nearly 2000 years ago and formed the basis of crop rotation. This knowledge was purely empirical until Bossingault (1830) produce evidence showing the value of leguminous plants ability to fix nitrogen. Subsequently Hellriegel and Wifarth (1988) related the ability of leguminous plants to fix nitrogen with the presence on their roots of localized swellings called nodules. Rhizobia reduced the atmospheric dinitrogen to ammonia, which is assimilated by plants and in turn it gets plant photosynthates to meet their energy requirement. Rhizobia are classified into two groups: fast growing and slow growing (Masterson *et al.,* 1982). The fast growing rhizobia like *Sinorhizobium meliloti, R. leguminosarum, etc.* are found to be more typical system due to relatively lesser time of doubling (2 to 4h). The growth of certain strain of *S. meliloti* is known to be fastest among the fast growing rhizobia. *S. meliloti* forms symbiotic relation with three genera of medicago plant group, *Medicago, Melilotus* and *Trigonella.* The fast growth of *S. meliloti* and the small size of the alfalfa plants facilitate the study of bacterium-plant interactions under laboratory conditions. Due to above advantage, *S. meliloti*-alfalfa system was chosen for this study.

The symbiotic nitrogen fixation by rhizobia is carried out inside a special structure called nodules on the roots of leguminous plants. The nodule provides the suitable environment for reduction of dinitrogen to ammonia. The formation of nodules are the result of a multi-step process requiring the specific gene products of both partners (Vincent, 1980). The most critical steps of the nodule development are colonization, recognition and infection of the host by the bacterium (Newcomb, 1981).

The rhizobial-legume symbiosis starts with the interaction between the bacteria and the root epidermis. First reaction of root system to the presence of rhizobia is the curling and deformation of root hairs. The bacteria get entrapped in the curl; the host cell wall is hydrolyzed and a tubular structure called infection thread is formed. The bacteria invade root hair and root cortex with the help of infection thread. The nucleus at the tip of the curled root hair guides the path of the infection thread. Meanwhile, cells of root cortex are mitotically activated and form nodule primordium (Truchet *et al.*, 1985; Werner, 1992; Devlin and Witham, 1999). As the primordium develops to a nodule, bacteria are released from the tip of the infection thread by endocytosis and differentiate into bacteroids which are surrounded by plant derived peribacteriod membrane (Schultize and Kondorosi, 1998). Based on their differentiation features bacteroids are called bacteroid type 1-5 (Vasse *et al.*, 1990). Specific signal from the plant coupled with the appropriate physiological environment is thought to stimulate the expression of nitrogenase and other bacterial genes involved in nitrogen fixation. The heme produced by rhizobia combines with globin produced by the plant, to form leghemoglobin that is required for nitrogen fixation. This protein plays an important role in regulation of the free oxygen concentration inside nodule and protect the nitrogenase enzyme which is very sensitive to oxygen (de Bruijn *et al.*, 1994).

Recent advances in recombinant DNA technology have made it easy to understanding the complexity of symbiotic association of rhizobia with the host plant. Several rhizobial and plant genes involved in this process have been identified from different species. Rhizobial genes responsible for nodule development (*nod, nol*) (Kondorosi *et al.*, 1984; Fisher and Long, 1992; Orenik *et al.*, 1994; Bellato *et al.*, 1996; Freiberg *et al.*, 1997; Kiss *et al.*, 1998; Cabanes *et al.*, 2000), nitrogen fixation

(*nif, fix*) (Batut *et al.*, 1985; Fischer, 1996; Bauer *et al.*, 1998) and nodule invasion (*exo, lps, kps*., etc) (Borthakur and Johnston,1987; Reed *et al.*,1991; Reuhs *et al.*, 1995; Cheng and Walker,1998[a]; Kereszt *et al.*,1998; York and Walker,1998[a,b]), have been cloned, sequenced and characterized.

In addition, it has also been reported that bacteroid development and nitrogen fixation depend on nutritional conditions of nodule. It is well established that bacteroids depend on their host plant for carbon and energy and can utilize C_4-dicarboxylic acids (Watson *et al.*, 1990; Finan *et al.*, 1991; Østeras *et al.*, 1997; Boesten *et al.*, 1998). Beside *nod, nol* and *fix* genes which play important role, several other functions of rhizobia were also found to influence symbiosis directly or indirectly. These include metabolic pathways for synthesis of amino acids (Sadosky *et al.*, 1986; Yadav *et al.*, 1998; Prasad *et al.*, 2000), nucleotide base (Kerppola and Kahn, 1988[a]; Newman *et al.*, 1992; Djordjovic *et al.*, 1996; Vineetha, 1998) and vitamins (Pankhurst *et al.*, 1972 ; Kim *et al.*, 1988). Anthranilic acid, an intermediate of the tryptophan biosynthetic pathway and tryptophan synthase enzyme have been found to be essential for effective symbiosis in *S. meliloti* and *R. etli* (Barsomian *et al.*, 1992; Taté *et al.*, 1999[a]; Prasad *et al.*, 2000). *S. meliloti ilvC* and histidine auxotrophs of *B. japonicum* did not induce nodule formation (Nod$^-$) on their respective host plants (Sadowsky *et al.*, 1986; Aguilar and Grasso, 1991). However, histidine auxotrophs of *R. etli* were found to be effective in nodule formation with pea plant (Newman *et al.*,1995). Studies on nodules induced by pyrimidine auxotrophs indicated that some of the pyrimidine intermediates/enzymes might have some role in symbiosis (Vineetha, 1998). *S. meliloti* and *R. leguminosarum* pyrimidine auxotrophs have been reported to form ineffective nodules (Kerppola and Khan,1988[b]; Noel *et al.*, 1988).

Role of purine biosynthetic pathway in symbiosis has been established in several *Rhizobium*-legume systems (Pain, 1979; Pankhurst and Schwinghamer, 1974; Noel *et al.*, 1988; Newman *et al.*, 1992; 1995; Stevens *et al.*, 2000). In general purine auxotrophs of several *Rhizobium* spp. appeared to be defective in early infection process (Noel *et al.*, 1988; Djordjevic *et al.*, 1996; Newman *et al.*, 1992; 1994). Symbiotic defects in these purine auxotrophs were restored on supplementation of plant nutrition medium with purine intermediate AICAR (5 aminoimidazole-4-carboxamide riboside) (Newman *et al.*, 1994,1995). *S. meliloti* purine auxotrophs are found to be $Nod^{+}Fix^{-}$ (Swamynathan and Singh,1992; Gupta,1996). However, in all these cases no detailed histological studies of nodules induced by purine auxotrophs were performed and it is unclear if symbiotic ineffectiveness is due to unavailability of purine or purine intermediate(s) which is/are responsible for defects in bacteroids development and function. There is also a possibility that some enzyme(s) of the purine biosynthetic pathway has (have) a dual role i.e. apart from its role in the biosynthesis of purine. These enzymes may also convert some unknown precursor into a product necessary for symbiosis. Keeping these observations in mind, the study of purine auxotrophs of *S. meliloti* was performed with following objectives.

1. To generate purine auxotrophs mutants of *S. meliloti* by random transposon Tn5 mutagenesis.
2. To study the biochemical and symbiotic characteristics of purine auxotrophs.
3. To study the peliotropic effect of Tn5 mutagenesis in purine auxotrophs.
4. To study the histology of nodules induced by purine auxotrophs.

Chapter 2

LITERATURE REVIEW

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

I. Taxonomy of rhizobia

II. Genetics of rhizobia

III. Development of the legume root nodule

IV. Genes involved in nitrogen fixation

V. Other genes involved in symbiosis

2.1 TAXONOMY OF RHIZOBIA

In plant kingdom a number of plants are benefited from plant-prokaryotes association. One of such association is symbiosis between plants and a group of bacteria, which form nodule. The bacteria that can establish symbiosis by forming nodules, belong to two phylogenetic groups, namely *Rhizobium* (Van Berkun and Eardly, 1998) and *Frankia* (Benson and Silvester, 1993). *Rhizobium* are Gram-negative soil bacteria that have the ability to interact with plant species belonging to the leguminaceae family. In contrast, *Frankia* Gram positive actinomycetes nodulate with some families of angiosperms collectively called actinorhizal plants (Benson and Silvester, 1993;Van Berkun and Eardly, 1998). The ability of legumes to fix atmospheric nitrogen and cultivation of bacteria isolated from legume nodules, were reported long back in 18th century (Burris, 1977). These bacteria were called *Rhizobium* (rhizo = root and bio = living) by Frank, (1989). On the basis of growth rate rhizobia are classified into two goups: slow growers (with generating time more 6 hours) and fast growers (with generating time less than 6 hours). Later on, with further developments in rhizobial research, they were regrouped into three genera; *Rhizobium,*

Bradyrhizobium and *Azorhizobium*. *Bradyrhizobium* (a slow growing) in general have broder host-range than *Rhizobium,* which is fast growing and nodulate only limited temperate legumes. *Azorhizobium* which are more related to *Bradyrhizobium* than to *Rhizobium,* can utilize gaseous nitrogen as a sole nitrogen source during growth in free living conditions (Rinaudo *et al.*, 1991; Dowine and Brewin, 1992; Willems and Collins, 1993). Further development in *Rhizobium* taxonomy were based on, numercal taxonomy, 16s rRNA analysis and nucleic acid hybridization studies, which have been helpful to infer the phylogenetic diversity in the Rhizobiaceae family (Selander *et al.*, 1986; Jarivs *et al.*, 1992; Jarvis and Tighe, 1994; Martinez-Romero, 1994; Gualtieri and Bisseling, 2000). Recently fast growing rhizobia has been reclassified and two genera called *Sinorhizobium* and *Mezorhizobium* have been recognized, (Chen *et al.*, 1988; de Lajudie *et al.*, 1994; Lindstrom *et al.,* 1995;Young *et al.*, 1996). The current taxonomical classification of rhizobia with their host specificity and phylogenetic diversity has been presented in Fig-1 and Table-1)

2.2. GENETICS OF RHIOZOBIA

2.2.1 MUTAGENESIS

The primary focus of present day rhizobial research is the identification of symbiotically significant genes and elucidation of their role in symbiosis. One approach to understand the function and organization of this was by generating symbiotically defective mutants. Several mutants with altered symbiotic features were generated by using various methods of mutagenesis such as:

(i) chemical mutagenesis (ii) transposon mutagenesis, and (iii) site-directed Tn5 mutagenesis.

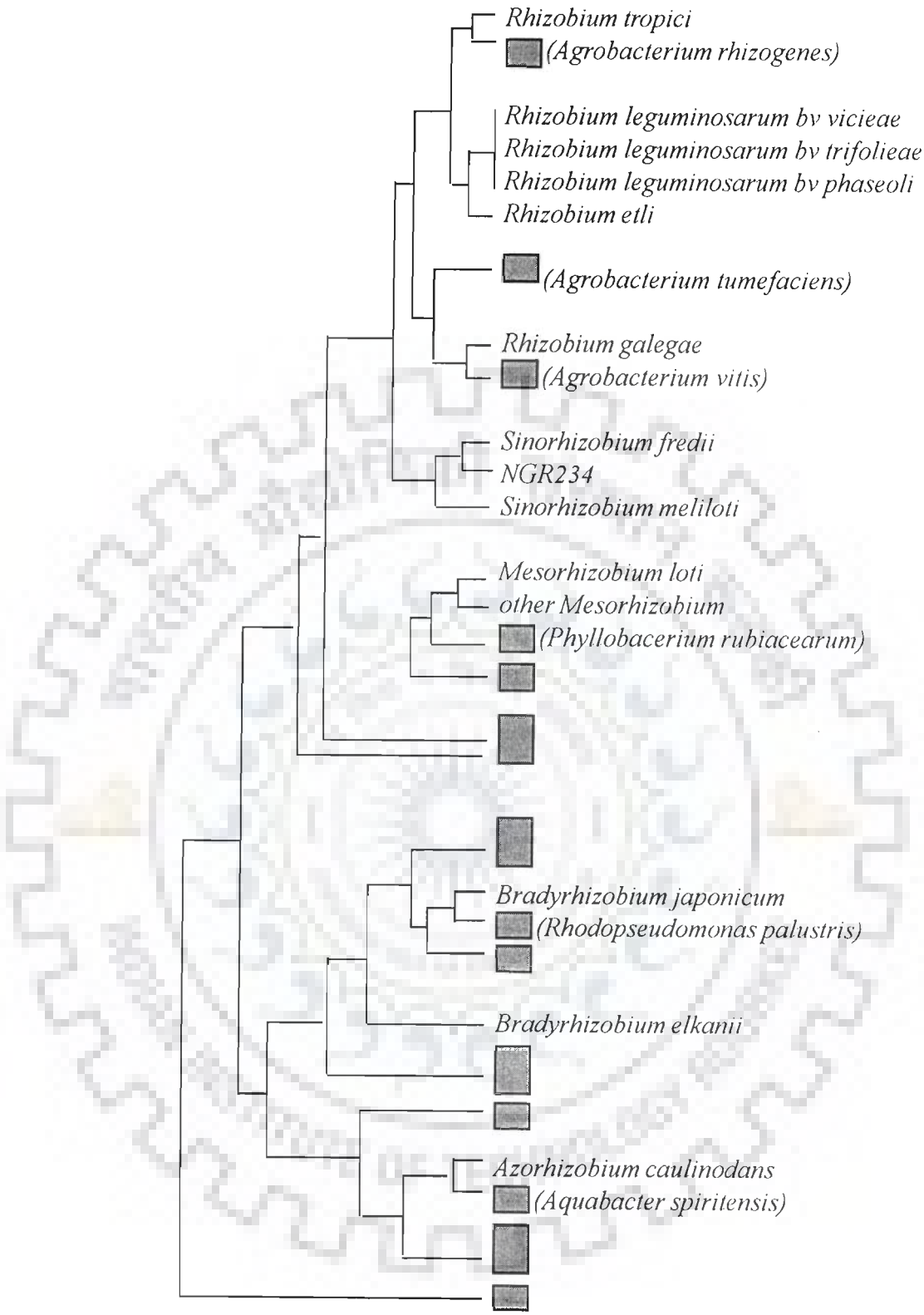


Figure 1: Phylogeny of rhizobia as deduced from 16S ribosomal RNA gene sequences. Lineages of non-symbiotic bacteria are shown by boxes, with a representative given in parenthesis for lineages closely related to symbiotic groups, (after Gualtieri and Bisseling, 2000).

Table 1: Taxonomic classification of rhizobia (based on Scholla and Elkan, 1984; Eardley *et al.*, 1990; Martinez-Romero,1994; Nour *et al.*, 1995; Xu *et al.*, 1995; Chen *et al.*, 1995; 1997; Amarger *et al.*, 1997; de Lajudie *et al.*, 1994; 1998; Terefework *et al.*, 1998; Nick *et al.*, 1999 ; Vij, N., 2000)

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

2.2.1.1 Chemical Mutagenesis

A number of mutagens like ethyl methane sulphonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (NTG), nitric acid, etc. have been used to generate auxotrophs and symbiotically defective mutants of different *Rhizobium* strains (Noel *et al.*, 1982; Forrai *et al.*, 1983; Singh *et al.*, 1984; Cava *et al.*, 1989; Kumar and Kuykendall, 1989 Swamynathan and Singh, 1992).

2.2.1.2 Random Transposon Mutagenesis

Due to carcinogenic nature of chemical mutagens and other limitations, transposon mutagenesis is preferable for generation of auxotrophic mutants. Transposon induced mutants have several advantage overs mutants developed by chemical mutagenesis: (i) mutation are relatively random due to little insertion specifeity (ii) reversion frequency is low in the transposon mutants (iii) antibiotic resistance maker of the transposon work as a positively selection marker (iv) molecular cloning of transposon marker gene is relatively easier (v) the mutated symbiotic gene tagged byTn5 can easily be identified by hybridization with Tn5 probe.

Due to above advantages random transposon mutagenesis has been described as a general method for inducing transposon in genome of wide variety of Gram-negative bacteria (Johnston *et al.*, 1978; Beringer *et al.*, 1978; Meade *at al.*, 1982; Selvaraj and Iyer, 1983; Ditta, 1986). Transposon Tn5 conferring Km^r/Nm^r has been used to study the localization and preliminary analysis of important genes (Selvaraj and Iyer, 1983). It involves insertion of Tn5 from a suicide plasmid vehicle (pGS9) into rhizobial genome. The suicide plasmid is introduced into rhizobia by conjugation from an *E. coli* donor (Fig-2a and 2b). Since the suicide plasmid is not stable in rhizobia, selection of

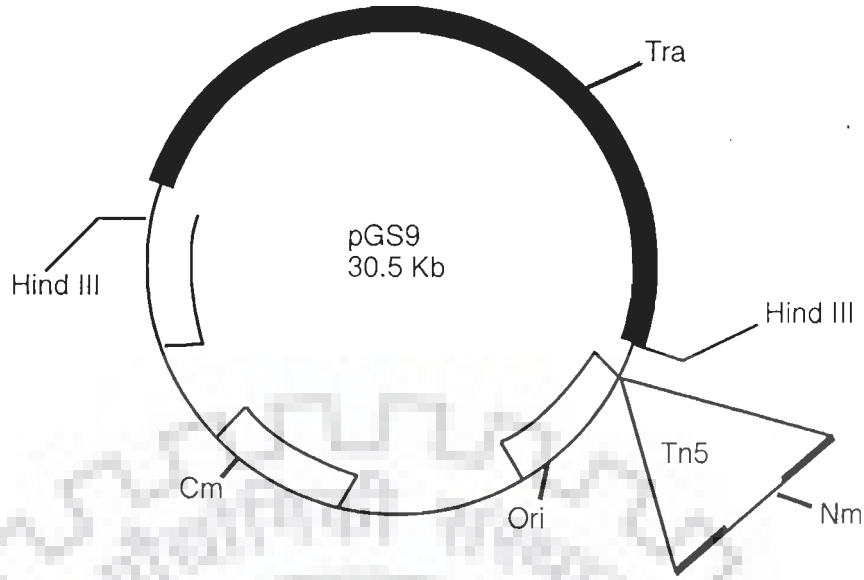


Fig. 2a: Transposon Tn-5 delivery vector pGS9. Abbreviations : Cm, Chloramphenicol; Nm, Neomycin; Ori, Origin of replication

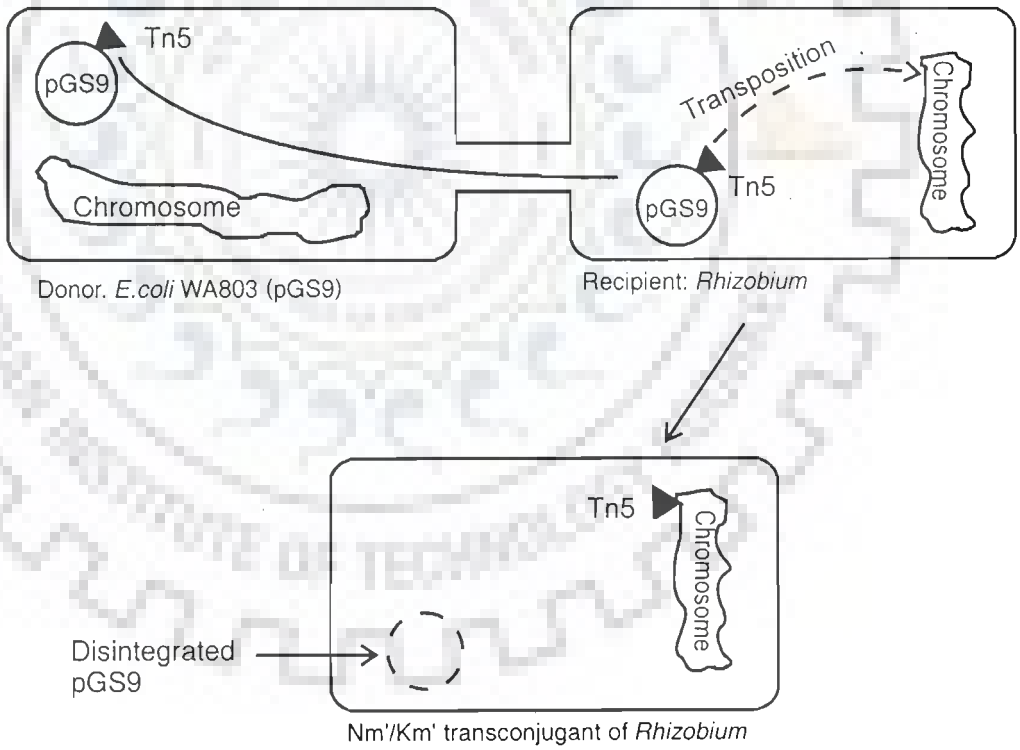


Fig. 2b: Diagramatic representation of transposon Tn5 mutagenesis

neomycin/ kanamycin resistant transconjugants leads to isolation of Tn5 insertion mutants (Beringer *et. al.* 1978; Selvaraj and Iyer, 1983; Simon *et. al.*, 1983). The technique has been used successfully to generate symbiotic defective mutants of different rhizobial species (Duncan, 1981; Forrai *et al.*, 1983; Kerppola and Khan, 1988[b]; Khanuja and Kumer, 1989; Barsomian *et al.*, 1992). Many symbiotically important genes have been identified and characterized (Hirsch *et al.*, 1984; Ali *et al.*, 1984; Hom *et al.*, 1984; Leigh *et al.*, 1987).

2.2.1.3 Site-Directed Tn5 Mutagenesis

Site-directed mutagenesis is one of the recent technique and very useful for fine structural analysis of a specific gene. This involves the cloning of *Rhizobium* gene (such as *nif* and *fix* genes) into a suitable multi copy plasmid. The cloned genes were then subjected to Tn5 mutagenesis in *E. coli* and reintroduced into *Rhizobium* spp. by recombination (Ditta, 1986). Site-directed mutagenesis has been used for the genetic and physical characterization studies of *nod* and *nif* genes clusters in variety of *Rhizobium* species (Corbin *et al.*, 1983; Zimmerman *et al.*, 1983; Szeto *et al.*, 1984; Quinto *et al.*, 1985; Castillo *et al.*, 1999). A variant of Tn5, Tn5 PhoA has been used to mutate a specific class of genes incoding secretory or membrane spanning proteins (Long *et al.*, 1988[b]). Recently in vitro Tn5 transposon system for site-directed mutagenesis has been developed (Goryshin and Reznikoff, 1998; Goryshin *et al.*, 2000).

2.3 DEVELOPMENT OF LEGUME ROOT NODULES

Establishment of an effective legume *Rhizobium*-symbiosis is complex process and involves several steps from initial recognition and infection to nodule formation

and development. The literature on the legume root nodule development has been reviewed (Brewin, 1991). A lot of diversity in the morphology of legume root nodules has been reported (Gualtieri and Bisseling, 2000). In spite of the diversity four anatomical features are generally common in all legume nodules (i) Induction of new plant meristem, (ii) 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. 3).

2.3.1 RECOGNITION AND INITIATION

Rhizobia, a Gram negative bacteria, live saprophytically in soil. To colonize the plant roots, these bacteria are supposed to reach near it by the process called chemotaxis (Maxwell and Phillips, 1990; Kape *et al.,* 1991). Colonization and recognition depend on signals both from bacteria and its host.

2.3.1.1 Diffusible Signals from Plant to Bacteria

Early events of legumes nodules formations require expression of nodulation genes in different *Rhizobium* species through the cooperative action of the protein product of *nodD* gene and components of root and seeds exudates (Spaink *et al.*, 1989; Long 1989; Zuanazzi *et al.*, 1998). Chemotaxis and motility have been found to play important contributions to the symbiotic interaction of rhizobia with their hosts (Ames and Bergman 1981; Soyby and Bergman 1983; Caetano-Anolles *et al.*, 1988[a]). Legumes plant root exudate has strong chemotaxic effect in *Rhizobium* due to presence of specific flavonoids, besides sugars, amino acids and other components (Caetano-Anolles *at al* 1988[ab]; Kape *et al.*, 1991). In alfalfa root exudate, several flavonoids

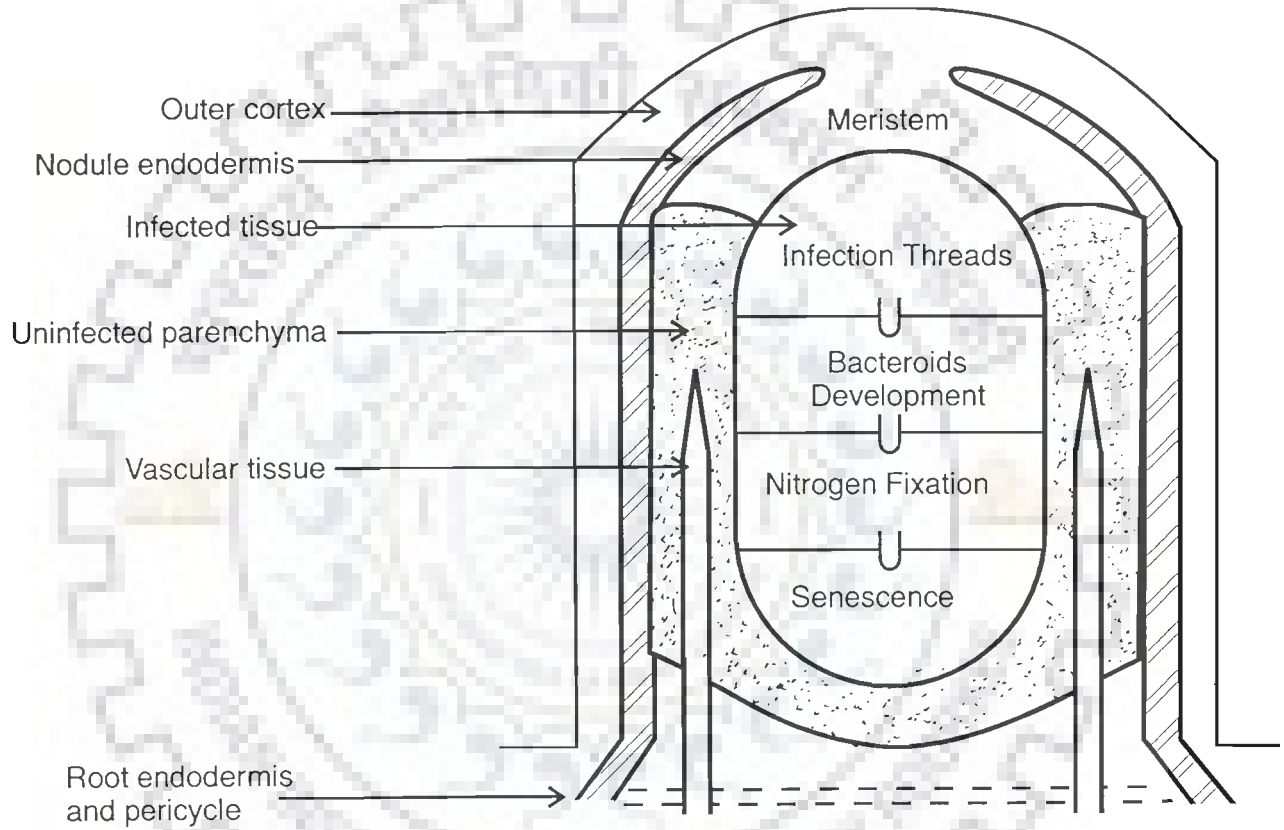


Fig.3: Differentiation of cell types from the apical (uninfected) meristem of pea nodule. In the longitudinal axis of the nodule. The central mass of tissue shows successive stages of host cell invasion and differentation by *Rhizobium* (after Brewin 1991;Werner,1992).

(leteolin and other flavonoids) have been identified (Kapulnik *et al.*, 1987; Hartwig *et al.*, 1990; 1991; Hubac *et al.*, 1993). Besides working as a potent chemoatractants, flavonoids in host root exudate stimulate the transcription of *nodD* genes of *Rhizobium* (Parke *et al.*, 1985 Caetano-Anolles *et al.*, 1988[b]; Aguilar *et al.*, 1988). These flavonoids are species specific and *nodD* genes from different species of rhizobia interact preferentially with specific flavonoid. This specific interaction between flavonoids of the host plant and *nodD gene* is an important determinant of host-*Rhizobium* specificity (Peters *et al.*, 1986; Zaat *et al.*, 1988; Györypel *et al.*, 1991, Graham, 1991; Long, 2001). These plant exudates are syntheszied as glycones in the cytoplasm of root cells and exuded into rhizosphere through specific process or stored in vacuoles as conjugates and can be released later through specific hydrolases (Maxwell and Phillips, 1990).

In addition to flavonoids, other chemical classes of *nod* gene inducers have been identified in alfalfa root exudate (Phillips *et al.*, 1992; McKhann *et al.*, 1997). It was found that alfalfa seeds (*Medicago sativa*) release two betaine that induce the *nod* genes in *S. meliloti* (Phillips *et al.*, 1994).

2.3.1.2 Diffusible Signals from Bacteria to Plant

Rhizobia approaching the roots of compatible host plants respond to plant derived signal compound (usually flavonoids) by expression of its nodulation (*nod*) genes which leads to production and secretion of return signal (Nod factors) (Spaink *et al.* 1991; Schultze *et al.*, 1995). Nodulation genes play key role for plant roots recognition and invasion. They are designated as *nod A.....Z*, *nol* and *noe* genes. In general rhizobia *nod* genes are organized in several operons and localized on large

(Sym) plasmids. The complete sequence of the Sym plasmid has been determined (Freiberg *et al.*, 1997). The nodulation genes can be divided into common *nod* genes, regulatory *nod* genes and host specific nodulation genes. *nod* genes *ABC* belong to common *nod* genes and are present in all rhizobia. These genes are functionally interchangeable among different species (Kondorosi *et al.*, 1984) and usually exist as a single operon with some exception (Scott *et al.*, 1996). The *nod ABC* genes code for enzymes required for the synthesis of core Nod factors (Fisher and Long, 1992). Mutation in these genes resulted in the strains that are unable to curl root hairs (Hac^-) of their respective hosts (Long *et al.,* 1982; Kondorosi *et al.,* 1984; Long and Atkinson 1990). *nodA* and *nodB* genes in *S. meliloti* are involved in formation of cytokinin like compound that stimulate mitosis of plant cell (Schmidt *et al.*, 1988). The *nodC* protein suggested to be receptor, transducing signal molecules from bacteria to plant cell (Schmidt *et al.*, 1986; Mergaet *et al.,* 1993; Geremia *et al.*, 1994). Besides *nodABC, nodMN* and *nodFE* have also been identified as common *nod* genes. *NodM* code for signal production and bacteroids maturation (Baev *et al.,* 1991; 1992). The *nodI* and *nodJ* genes found to exist down stream of the *nodABC* genes and probably part of *nodABC* operon (Evans and Dowine, 1986).

Host specific-*nod* genes (*hsn*) are required to determine specific Nod signals for *Rhizobium*-host plant recognition. Mutation in (*hsn*) genes lead to altered infection and nodulation, and some times results in infection of host plant which are normally unresponsive (Lerouge *et al.,* 1990; Schwedock and Long.,1990). This includes *nodH, nodFEG* and *nodQP* genes which are clustered in 6 kb away from common *nod* genes in *S. meliloti* toward the *nifHDK* region (Long *et al.,* 1982; Kondorosi, *et al.,* 1984).

The expressions of *nod* genes are regulated by *nodD* gene. The *nodD* gene product is a transcriptional activator which binds to the promoter of other *nod* genes on the so-called *nod* box (Honma *et al.*, 1990; Baev *et al.*, 1992; Fisher and Long, 1992). The *nodD* proteins activate transcription of *nod* genes in the presence of inducer molecule that is specific flavonoid released from host plant. The *nodD* gene is highly conserved within *Rhizobium* species and there found to be 75% homology in *nodD* genes of *R. leguminosarum* bv. *trifolii and S. meliloti* (Van Brussel *et al.*, 1986; Schofield and Watson, 1986). *S. meliloti* has three *nodD* genes which are activated by different flavonoids (Spaink *et al.*,1990; Honma *et al.*, 1990; Machdo *et al.*, 1998). While the expression of the most common and host specific *nod* genes are regulated by *nodD* gene. The host specific *nodHPQ* genes are organized as an operon that is transcribed in a *nodD*-independent manner and do not get affected by flavonoids (Folch-Mallol *et al.*, 1996; 1998). These are required for production of sulfated intermediate of Nod factor (Ehrhardt *et al.*, 1995). In *S. meliloti* the main host determinate is a sulfate group at the reducing sugar residue (Lerouge *et al.*, 1990). The functions of *nol* and *nod* genes are summarized in Table-2.

In *Rhizobium* common *nod* genes as well as host-specific (*hsn*) genes are involved in the production of extracellular Nod signals (factors) which are chitooligosaccharide with variable structure (Lerouge *et al.*, 1990; Spaink *et al.*, 1991; Carlson *et al.*, 1993; Geiger *et al.*, 1994; Röhrig *et al.*, 1994; Ovtsyna *et al.*, 2000). These factors act as external growth signals triggering an endogenous nodulation program in the host-plant and eliciting several key developmental responses in the root of legume hosts (Schultze and Kondorosi, 1998;Catoira *et al.*, 2000). In *S. meliloti* this is called NodRm-1 (Fig.4) which carry sulfate group to C-6 position of reducing acetyl

Table 2: Functions and properties of *nod* genes (based on Fisher and Long, 1992; Werner, 1992; Sharma *et al.*, 1993; Gillette and Elken 1996; Bellato *et al.* 1996; Gu *et al.* 1997; Kiss 1998;)

Genes	Function and properties
nod	
nodAB	Required for Nod factor production
nodC	Homology to chitin and cellulose synthase; proposed to form β-1,4-glycosyl bond
nodD	Transcriptional activator of inducible *nod* genes
nodE	Host -specific;homology to β-ketoacyl synthase(condensing enzyme) of fatty acyl synthase; proposed to synthesize Nod factor acyl chain
nodF	Host-specific; homology to acyl carrier protein; proposed to synthesize Nod factor fatty acyl chain
nodG	Host-specific; homology to reductases; proposed to modify Nod factor fatty acyl side chain
nodH	Host-specific; required for formation of sulphated Nod factor ;proposed to transfer activated sulphate to Nod factor
nodI	Homology to ATP-binding active transport proteins; proposed to form membrane transport complex with *nodJ*
nodJ	Homology to transmembrane proteins; proposed to form membrane transport complex with *nodI*
nodK	Unknown
nodL	Host-specific; homology to acetyl transferase; required for formation of 6-O-acetyl Nod factor; proposed to add O-acetyl group to Nod factor
nodM	Host-specific glucosamine synthase; proposed to synthesize Nod factor sugar subunits
nodN	Host-specific; involved in *Vicia hirsuta* nodulation
nodO	Exported Ca^{2+} -binding; homology to haemolysin; proposed to mediate in early stage in rhizobia-legume interaction
nodP	Host-specific ATP sulphurylase; proposed to provide activated sulphate for transfer to Nod factor
nodQ	Host-specific ATP sulphurylase and APS kinase; together with NodP makes activated sulphate; proposed to provide activated sulphate for transfer to Nod factor
nodR	Reported only in *R. leguminosarum* bv. *trifolii*. Host range
nodS	Function unknown
nodT	Host-specific; involved in *Trifolium subterranean* nodulation; proposed to be membrane protein
nodU	Function unknown.
nodV	Homology to two-component regulatory system sensor proteins; proposed to regulate unknown target genes
nodW	Reported only in *B. japonicum*, Important for host range, sequence similarity to family of transcriptional regulatory proteins.
nodX	Reported only in *R. leguminosarum* strains capable of nodulating Afghanistan pea.
nod1A	Host range. Essential for nodulation of selected soybean cultivers. Reported only from *B. japonicum*.
nodY Z	Host-specific nodulation

Genes	Function and properties
nol	
nolA	Extension of genotype-specific nodulation, required for efficient nodulation of host plant.
nolB	Unknown
nolC	Extension of genotype-specific nodulation
nolE	Unknown
nolFGHI	Production of nod metabolites
nolP	Unknown
nolR	Repressor of *nodD*, required for optimal nodulation
nolW	Cultiver specificity gene.
nolX	Cultiver specificity gene.
NolD ,nolE	Host rang in *S.meliloti*
nolF, nolG	

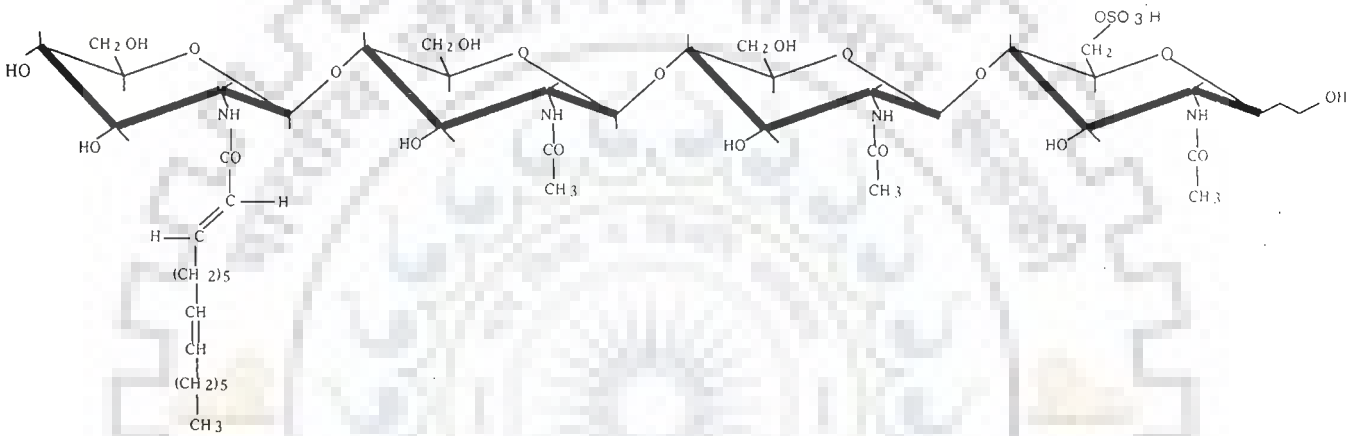


Fig. 4: The proposed NodRm-1 structure (Nod factors) a host specific signal made by *S. meliloti* after (Lerouge *et al.*, 1990; Spaink *et al.*, 1991).

glucosamine residue (Schultze *et al.*, 1992; 1995). This substitution is necessary for biological activity of the host plant alfalfa (Spaink *et al.*, 1991).

2.3.2 ROLE OF BACTERIAL SURFACE IN EARLY INFECTION EVENTS

Rhizobial cell surface components (Exopolysaccharide, lipopolysaccharide, β(1,2) glucans, cellulose fibrils and capsular polysaccharide) are essential for infection. Besides exopolysaccharides are required for infection thread formation, while lipopolysaccharides have a role in endocytosis and bacteroids differentiation. β(1-2) glucans are found to affect early infection process.

2.3.2.1 β(1-2) Glucans

β(1-2) glucans are localized predominantly with the periplasmic compartment of rhizobial cell (Miller *et al.*, 1986; 1990) and in Rhizobiaceae .β(1-2) glucans are believed to provide roles during hypoosmotic adaptation as well as during plant infection process (Dylan *et al.*, 1990[a,b]; Cangelosi *et al.*, 1990; Zorreguieta *et al.*, 1990; Breedveld *et al.*, 1995).

β(1-2) glucans were found to play important role in the first step of symbiotic process by attachment of bacteria with the host plant which related with the motility of bacteria. β(1-2) glucans residues are linked solely by β(1-2) glucosidic bond in *Rhizobium* and *Agrobacterium* spp. While in *Bradyrhizobium* spp. glucose residues are linked by both β(1-3) and (1-6) glucosidic bonds (platt *et al.*, 1990; Breedveld and Miller, 1994; Castro *et al* ., 1996). The activity of the motile bacteria were found to be more efficient that non motile in nodule formation (Ames and Bergman, 1981; Soby

and Bergaman, 1983). In *S. meliloti* strain MVII-1 it is accomplished by a complex flagella (Götz and Schmitt, 1987) and the absence of β(1-2) glucans correlated with the absence of motility.There are two genetic loci in *S. meliloti* (*ndvA, ndvB*) which encoding function required for β(1-2) glucans, these were found to be functionality and structurally homologous to *chvA* and *chvB* loci of *Agrobacterium tumefaciens* (Breedveld and Miller, 1994).

The *ndvA* (*chvA*) gene encodes a protein involved in the transport of the cyclic β-glucans to the periplasmic and extracellular medium (Stanfield *et al.*, 1988; Cangelosi *et al.*, 1989). Mutant in *ndvA* and *ndvB* loci result in impairment of host plant infection. The *ndvB* locus in *S. melioti* encodes a 319-Kilodalton protein involved in the prediction of β(1-2) glucans (Gastro *et al.*, 1996). Mutation in this loci produces mutants that have reduced amount of periplasmic β(1-2) glucans and form bacteroids that are morphologically abnormal and exhibit several vegetative defects (Ielpi *et al.*, 1990). Additional loci (*ndvB* like locus *ndvC* and *cgmB*) linked to cyclic β-glucan biosynthesis have been identified in *B. japonicum* and *S. meliloti* (Bhagwat *et al.* 1993; 1996; Wang *et. al.*, 1999). The cyclic β-glucans may function during several stages of plant infection process (Miller *et al.*, 1994; Breedveld and Miller, 1994).

2.3.2.2 Cellulose Fibrils

Cellulose fibrils have been reported to play a role in the attachment to the plant root in several bacteria including *Rhizobium* (Smit *et al.*, 1986; 1992). It has been reported that some species of *Rhizobium* synthesis cellulose fibrils that were involved in subsequent tighter binding of bacteria to root surface (Napoli *et al.* 1975; Dazzo, 1984). This binding appear to be reinforced by calcium dependent protein (Ca^{+2} depend

adhesin). The calcium binding protein is important for rhicadhesin that is involved in the first step of rhizobial attachment to root hair (Kijne *et al.* 1988, Smit *et al.* 1987, 1989). The gene involved in cellulose fibrils synthesis has been cloned, characterized and found to be located at two operon (*celABC, celDE*) in *Agrobacterium tumeficaiens* (Mathysse *et al.* 1995[b]). Recently six genes involved in cellulose synthesis (*cel genes*) from *R.leguminosarum* bv. *trifolii* has been cloned and characterized (Ausmees *et al.*, 1999). The primary sequence and organization of these genes are similar to *Agrobacterium tumeficaiens* (Matthysse *et al.*, 1995[ab]). These genes were reported to be functionally interchangeable between *Agrobacterium* and *Rhizobium.* Besides, two additional genes found to be putative regulator (*CelR1* and *CelR2*) have been identified. They exist as a single operon and controlled cellulose synthesis (Ausmees *et al.*, 1999).

2.3.2.3 Exopolysaccharide

Exopolysaccharide (EPS) of bacterial surface have shown to be important in early infection events for rhizobia (Chen *et al.*, 1985; Borthakur *et al.*, 1986; Doherty *et al.*, 1988). Genes responsible for EPS production were found to be located on the second megaplasmed in *S. meliloti* (Finan *et al.*, 1986; Long *et al.*, 1988[a]; Zhan and Leigh, 1990). Mutant defective in EPS production induced little or no invasion in their respective hosts (Leigh *et al.*, 1985). The exo mutants which are deficient in (EPS) production in *S. meliloti* formed ineffective nodule on alfalfa plant without bacteroids, and infection thread (Finan *et al.*, 1986; Leigh *et al.*, 1987; Leigh and Lee, 1988). The major polysaccharides in *S. meliloti* are succinoglycan (EPSI) (Fig.5). This acidic polysaccharide is needed for invasion and nodule development. Its function is

primarily in the penetration of infection thread (Glucksmann *et al.*, 1993[a] ; Reinhold *et al.*, 1994). *S. meliloti* mutants deficient in (EPSI) production were defective in invasion of alfalfa nodule and formed Fix$^-$ nodules that are devoid of bacteroids (Leigh *et al.*, 1985; Glusksmann *et al.*, 1993[b]) these nodule formed without infection threads (Niehaus *et al.*, 1993). Reed and his coworkers (1991) suggested that at least 13 loci are involved in (EPSI) production, these were previously mapped to cluster in second magaplasmid in *S. meliloti* Rmd1021 (Leigh and Lee 1988).

In addition to (EPSI) *S. meliloti* produced a galactoglucan (EPSII) which acts as a specific signal as does the signal factor NodRm1. (Lerouge *et al.* 1990; Truchet *et al.*, 1991). The biosynthesis of (EPSII) requires at least seven *exo* loci on second megaplasmid (Glazebrook and Walker, 1989; Gonzalez *et al.*, 1996). This molecule is proposed to be contributed by *ExoK* and *ExsH* glyconases, (Battisti *et al.*, 1992; York and Walker 1998[ab]). Two other genes *exoS* and *exoR* found to regulate succinoglycan production are located on chromosome and are not related to *exo* genes that located in megaplasmid (Doherty *et al.*, 1988). These regulations were carried through *Exo-S-chv* l two component regulatory system in *S. meliloti* (Cheng and Walker, 1998[a]). Recently some other genes involved in the production of (EPS) have been reported and studied (Zhan and Leigh, 1990; Battisti *et al.*, 1992; Kereszt *et al.*, 1998).

2.3.2.4 Capsular Polysaccharide

It has been demonstrated that some rhizobia possess surface polysaccharide that analogues to group II K antigen (KPS) of *E. coli* (Reuhs *et al.* 1993). It has also been reported that capsular polysaccharide (KPS) can replace (EPS) during symbiotic development (Petrovics *et al.*, 1993).

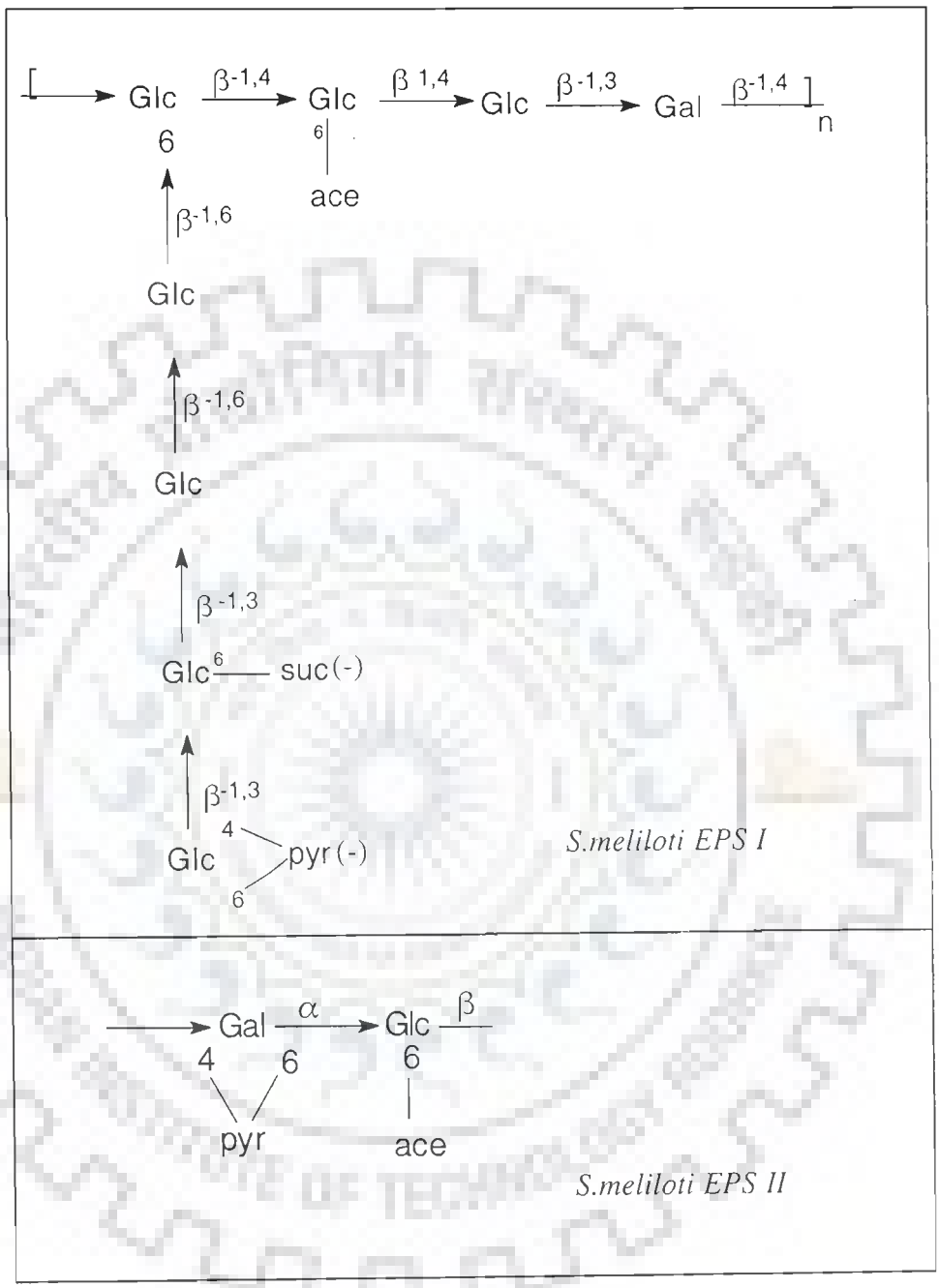


Fig. 5 : Structure of succinoglycan (EPS I) and (EPS II) of *S. meliloti*. Abbreviations : Glc, glucose; Gal, galactose; pyr, pyruvate; suc, succinyl; ace, acetyl (after Glucksmann and his coworkers, 1993[b]).

KPS of *Rhizobium* have been shown to be composed of disaccharide repeating units of Kdo (3-deoxy-D-manno-2-octulosonic acid) (Reuhs *et al.,* 1995; 1997). Two novel gene clusters designated as *rkp-2* and *rkp-3* involved in the production of KPS have been identified in *S. meliloti* (Kereszt *et al.,* 1998). It has been suggested that the *rkp-3* region carries the majority of the genes necessary for biosynthesis of disaccharide sub-unit of (KPS) and mutation in (KPS) production were found to defective in nodule invasion.

Recent reports showed that capsular polysaccharide (K-antigens) and lipopolysaccharide (LPS) are important surface antigens of *S. meliloti* spp. (Reuhs *et al.,* 1999[a]), and it has been shown that K- antigens may be involved in early recognition step in *S. meliloti* -alfalfa interaction (Beequart - de Kozak *et al.,* 1997; Reuhs *et al.,* 1999[b]).

2.3.2.5 Lipopolysaccharide

Lipopolysaccharide (LPS) plays an essential role in rhizobial-legume symbiosis. The mutants deficient in LPS biosynthesis were defective in the infection process during the initial infection thread formation and consequently there is no detectable deposition of bacteria into nodule cells (Carlson *et al.*, 1987[ab]; Carrion *et al.,* 1990; Dazzo *et al.,* 1991; Reuhs *et al.,* 1993).

The mutants deficient in (LPS) biosynthesis have been reported to be impaired in *R. leguminosarum* and *R. japonicum* (de-Maaged *et al.*, 1989). Lipopolysaccharide mutants of *S. meliloti* and *R. leguminosarum* bv. *phaseoli* mostly form abortive infection threads which do not release bacteria or defective in nodule entry, there was root hair deformation and cell division but no invasion of nodule (Kieber *et al.,* 1987;

Williams *et al.,* 1990[b]; Lagares *et al.,* 1992; Forsberg and Carlson 1998). Recently it has been reported that *S. meliloti* Rm6963 LPS mutant, form effective nodules in *Medicago sativa,* but ineffective nodules in *M. truncalula* (Niehus *et al.*, 1998).

2.3.3 ROOT HAIR DEFORMATION AND INFECTION THREAD DEVELOPMENT

The rhizobia can infect in different ways; (a) infection through infection thread and (b) infection via so-called crack entry, which takes place by the entry of bacteria into epidermal cells through gaps in epidermis (Chandler *et al.*, 1982; de Faria *et al.,* 1988). The infection through infection thread involves root hair deformation and formation of root hair curl called shepherd's crook. This process of root hair deformation involves host genes like *Mtrip1* encoding peroxidase (Cook *et al.*, 1995) and nodulin genes *ENOD5* and *ENOD12* that encode cell wall components (Vijn *et al.*, 1993). Microscopical studies have shown that root hair deformation is due to induction of root hair tip growth by Nod factors (Heidstra *et al.*, 1994). Purified Nod factors can induce expression of above mentioned plant genes and hence root hair deformation (Maxwell and Phillips, 1990; Journet *et al.*, 1994;Dazzo *et al.,* 1996). Besides, Nod factors are also involved in infection by crack entry (Goethals *et al.*, 1994).

Rhizobia entrapped in root hair curl (shepherd's crook) induce infection thread formation by hydrolysis of plant cell wall (Callaham and Torrey, 1981). It has been shown that invagination of plasma membrane at the site of hydrolysis results in formation of tubular structure called infection thread (Devlin and Witham, 1999). The infection thread grows towards the root hair base and subsequently the nodule primordium (Turgeon and Bauer, 1985; Long and Atkinson, 1990; Mathesius *et al.,*

2000). The polygalactouronase enzyme (PG) from the plant root is found to play role in infection process and hair invasion by rhizobia. The production of these enzymes is found to be increased during rhizobial infection (Munoz *et al.*, 1998).

2.3.4 NODULE DEVELOPMENT AND MATURATION

The region of 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). Concomitment with infection of root hair, root cortical cells are activated and start dividing to produce nodule primordium (Calvert, 1984). The threads penetrate deeply in the root cortex inducing a proliferation of the cells (Dart, 1975; Fahn, 1989). The number of the cells in the nodule primordium increases first by division throughout a spherical mass of host cells and later by activity of an apically localized meristematic zone region which is not penetrated by bacteria (Rolf and Gresshoff, 1988). The location of the nodule primordia in the inner cortex depend on the type of nodule formed by a particular plant and were found to have a large degree of variations in the final nodule depending host species (Newcomb, 1981; Dudley *et al.*, 1987). In temperate plants like pea, alfalfa inner cortex cells divide and form primordium. The nodule meristem differentiate during the whole nodule life into different cell types that results in the formation of indeterminate cylindrical nodules (Newcomb, 1976; Vasse *et al,* 1990). In tropical legumes such as glycine, phaseolus etc, the outer cortical cells of root divide to form nodule's primordium (Rolfe and Gresshoff; 1988, Mathews *et al.,* 1989). The resulting nodules are spherical with determinate growth and commonly known as determinate nodules (Pawlowski *et al.,* 1996).

Bacterial release from the infection thread is not a prerequisite for the development of nitrogen fixing bacteroids. In the *Parasponia* and *Andira* spp. Symbiosis, the microsymbiont is retained in modified infection threads called fixation threads (de Faria *et al.*, 1986; 1987). The root epidermis is broken as the nodule enlarges but the nodules do not emerge from the cortex. The cells of most cortex divide and stretch considerably so that they remain as the outer most layer of nodule.

The infection thread after approaching the region of newly dividing nodule meristematic cells, branches and vesicles are blabbed off into the cell cytoplasm of plant cells and result in the release of rhizobial bacteria into the cells (Calvert *et al.*, 1984; Rolfe and Gerasshoff, 1988). The released bacteria are surrounded by plant derived peribacteridal membrane (pbm) and the membrane enclosed bacteria are known as symbiosomes. The membrane enclosing the bacteria are found to be antigenically similar to plant cell membrane (Perotto *et al.*, 1991). The bacteria after release, divide and undergo transformation to become mature bacteroids. Nodule's cells increase in volume to enclose many bacteroids and supply them with nutrients. When the nodule cells mature, the host cells organelles like mitochondria, amyloplasts displaced to the peripheral position as the centrally located vacuole enlarge. The mature bacteroids are enlarged and found in the peripheral cytoplasm of host cell. Nod factors and several host genes (nodulin genes) like *ENOD12, ENOD14* etc. are found to play significant role in nodule development and bacteroids maturation (Vijn *et al.*, 1993; Charon *et al.*, 1997). The important nodulin genes and their functions are listed in Table 3.

Table 3: Expression of early nodulins, after (Werner, 1992; Vijn *et al.* 1993; de Bruijn *et al.* 1994; Karlowski *et al.* 1997).

Nodulin	Protein characteristic	Appearance (days after inoculation)	Tissue organ specificity
ENOD 12	hydroxyproline-rich cell wall protein	8-10	Root hairs, cortical cells with infection threads, related to infection process and able to express *ENOD 14* in the inner cortex of the root
ENOD 5	arabinogalactan protein	10-13	Early symbiotic zone, related to infection process and able to express *ENOD 14* in the inner cortex of the root
ENOD 2	hydroxyproline-rich cell wall protein	10-13	Symbiotic zone, after disappearance of *ENOD 12*
ENOD 14	Cysteine clustered protein	13-17 just before start of nitrogenase activity	Symbiotic zone peribact-eroid space
ENOD 3	Cysteine clustered	Just before start of nitrogenase activity	Symbiotic zone peribact-eroid space
Leghaemoglobin (for comparison)		13-17	Host cell cytoplasm

2.4 GENES INVOLVED IN NITROGEN FIXATION

Genes involved in the final stages of symbiosis are identified and are known as nitrogen fixation genes (*nif* and *fix* genes). These genes are mainly concerned with enzymology of nitrogen fixation (Beringer, 1980). In *Klebsiella pneumoniae* which fix nitrogen under nitrogen limited anaerobic conditions, 21 adjacent *nif* genes are organized in eight operons within a 24 kb region of DNA (Arnold *et al.*, 1988). The nitrogen fixation genes in *Rhizobium, which* has been shown to be equivalent to *K. pneumoniae nif* genes, are assigned the same names. The *nif* genes are found to be one of the highly conserved prokaryotic translated genes (Mazur *et al.*, 1980). The location of *nif* and *fix* genes are found to differ among rhizobial species. In *Rhizobium loti*, *Bradyrhizobium* spp. and *Azorhizhobium* sp. *nod, nif* and *fix* genes are located on chromosome, while in S. *meliloti, R. leguminosarum* and *Rhizobium* spp. NGR234 these genes are located on the symbiotic plasmids (Beringer *et al.*, 1990). *S. meliloti* carries two megaplasmids pSyma (1400kb) and pSymb(1700kb), respectively (Banfalvi *et al.*, 1981; Batut *et al.*, 1985; Burkhardt *et al.*, 1987). Plasmid Syma carries two clusters of symbiotic genes. The cluster I includes *nifHDKE, nifN, fixABCX, nifA* and *nifB* genes while the cluster II has *fixLJ*, *fixK*, *fixNOQP* and *fixGHIS* genes (David *et al.*, 1987). The cluster II genes are located at about 220kb downstream of the *nifHDKE* operon and are transcribed in opposite orientations (Renalier *et al.*, 1987; David *et al.*, 1987). The *nif HDK* (structural genes) are located on a single operon on Syma in *S. meliloti* and other rhizobial species and code for nitrogenase polypeptides (Renalier *et al.*, 1987). The *nifA* gene located upstream of *nifHDK*, is a regulatory gene and act as transcriptional activator for all other *nif* and *fix* genes (Ditta *et al.*, 1987).

In *Rhizobium*, *Bradyrhizobium*, *Azorhizobium and Sinorhizobium*, in addition to *nif* genes, a numbers of *fix* genes which are important for nitrogen fixation in symbiosis and have unknown functions at present, have been identified. *fixABCX* genes were identified in *S. meliloti*, *R. leguminosarum* b.v. *viciae*, *trifolii* and *phaseolii* and *B. japonicum* (Gulber *et al.*, 1989; Stock *et al.*, 1990) and found to be organized in a single operon in all species except in *B. japonicum* (Earl *et al.*, 1987; Fischer, 1994). *fixABCX* genes are required for nitrogenase activity, and are located between *nifHDK* and *nifA* operons (Ruvkun *et al.*,1982). *fixGHIS* genes are predicted to code transmembrane proteins as deduced from amino acid sequences (Fisher and Long, 1992; Fischer, 1994). *fixR* and *fixX* genes code for a ferrodoxin like proteins and involved in oxidation-reduction process (Thony *et al.*, 1987). The predicted amino acid sequences of *fixNOQP* genes suggested that they encode membrane bound cytochrome c-containing heme/copper cytochrome oxidase (Mandon *et al.*, 1993). The *fixNOQP* genes were described in *S. meliloti* as a duplicated *fix* region that is linked to the regulatory genes *fixLJ* and *fixK*, whose expression is induced under symbiotic conditions. Homologous genes were identified in other *Rhizobium* species and *B. japonicum* (Renalier *et al.*, 1987; Preisig *et al.*, 1993). The products and proposed functions of *nif* and *fix* genes are given in table 4. Some other additional genes required for effective symbiosis, are located on Symb and on chromosome (Watson *et al.*, 1988; Reuber *et al.*, 1991; Honeycutt *et al.*, 1993; Bauer, *et. al.*, 1998).

2.5 ROLE OF BACTEROIDAL METABOLISM IN SYMBIOSIS

2.5.1 C_4-Dicarboxylate Transport Mutants

Metabolic exchanges between bacteroids and host plant are essential for understanding the symbiosis and its regulation. C_4-dicarboxylic acids (succinate,

Table 4: Functions of *nif* and *fix* genes in *Rhizobium leguminasorum*, *Sinorhizobium meliloti*, *Bradyrhizobium japonicum* or *Azorhizobium caulinodans* (Werner, 1992; Sharma *et al.*, 1993; Fischer, 1994; Schlüter *et al.*, 1997).

Gene	Proposed function and/or product
***nif* genes**	
nifA	Positive regulator of *nif, fix* and additional genes
nifH,D,K	Structural genes for dinitrogenease reductase (*nifH*) and dinitrogenase (*nifD, nifK*)
nifB,E,N	Synthesis of the FeMo cofactor of dinetrogenase
nifS	Maturation of dinitrogenase
nifW	Required for full activity of FeMo protein
nifX	Unknown function
***fix* genes**	
*fix*ABCX	may function in electron transport to nitrogenase : *fix X* shows similarity to ferridoxins
fixNOQP	Microaerobically-induced, membrane-bound cytrochrome oxidase
fixGHIS	Proposed to function as a membrane bound complex coupling a cation pump to redox process
fixLJ	Oxygen-responsive two component regulatory system involved in positive control of *fixK* (*S. meliloti, B. japonicum* and *A. caulinodans*) and *nifA* (*S. meliloti*)
fixK/*fixK*$_2$	Positive regulator of *fixNOQP* (*S. meliloti, B. japonicum, A. caulinodans*), *nifA* (*A. caulinodans*), nitrate respiration (*B. japonicum*) and negative regulator of *nifA* and *fixK* (*S. meliloti*)
fixR	Similar to NAD-dependent hydrogenases
fixU	Unknown
fixW	May participate in a nitrogenase specific e$^-$ transport

aspartate, malate etc.) supplied by the plants, were found to be the primary carbon and energy source to mature bacteroids in leguminous root nodules (Stowers *et al.*, 1985; Finan *et al.*, 1991). Studies of C_4-dicarboxylic acids transport (dct) mutants of different *Rhizobium* species, have provided evidence that dicarboxylates found to be the major carbon and energy source for bacteroids in the nodules (Engelke *et al.*, 1987; Watson *et al.*, 1988). The mutants defective in dicarboxylic acids transport (*dctA*) unable to grow in dicarboxylates are found to induce nodules which are symbiotically ineffective with poorly developed symbiotic zones and carried numerous starch granules (Watson *et al.*, 1988; Engelke *et al.*, 1989; Østeras *et al.*, 1995; Mortimer, 1999). In *S. meloliti* the dct system is regulated by C_4-dicarboxylic acid regulatory genes (*dctB* and *dctD*) and *ntrA* gene products (Watson *et al.*, 1990, 1993). The *ntrA* is also required for *nifH* expression and nitrate assimilation (Ronson *et al.*, 1984; 1987, Boesten *et al.*, 1998).

2.5.2 Symbiotic Role of Biosynthetic Pathways of Amino Acids, Vitamins and Nucleotide Bases

Rhizobial metabolic mutants have been found to be symbiotically defective. A number of genes involved in the biosynthesis of amino acids, vitamins and nucleotide bases in rhizobia appear to play a role in symbiosis (Denarie *et al.*, 1976; Forrai *et al.*, 1983; Kim *et al.*, 1988; Aguilar and Gasso, 1991; Barsomian *et al.*, 1992; Newman, 1994; 1995; Taté *et al.*, 1999[a,b]; Stevens *et al.*, 2000). Symbiotic phenotypic features varies with the type of mutation and are characterized by loss of nodulation capability (Nod^-) or by formation of ineffective nodules (Fix^-) (Vincent, 1980).

Kumar and Kuykendall (1989) demonstrated the involvement of tryptophan biosynthetic pathway in symbiosis and observed that except tryptophan synthase

mutants all other tryptophan auxotrophs of *B. japonicum* were Nod$^-$. It has also been reported that the normal flow through of metabolites of aromatic acid biosynthetic pathway is required for development of bacteroids in *S. meliloti* (Jelesko *et al.*, 1993). In *S. meliloti trpE* mutants were found to be Nod$^+$ Fix$^+$ but the nodule showed unusual defects in internal morphology and were able to fix only trace amount of nitrogen (Barsomian *et al.,* 1992; Prasad, 1998). It has also been reported that the *trpB* gene of *R. etli* is essential for effective of symbiosis (Taté, *et al.*, 1999[a]). The *trpE* mutants are characterized by its inability to synthesize anthranilic acid. During bacteroids transformation there is an increasing demand of iron. It has been observed that with the secretion of anthranilic acid there was increase in iron uptake in *R. leguminosarum* (Rioux *et al.*, 1986). It has been further proposed that anthranilic acid may be acting as *planta* siderophore (Barsomian *et al.* 1992). The siderophores were found to mediate the iron metabolism during growth and nitrogen fixation in bacteroids (Barton *et al.*, 1996). Recently it was found that in *R. leguminosarm* b.v. *viciae,* the adenine auxotrophs which are defected in symbiosis with pea plant, also showed reduced production of siderophores (Stevens *et al.,* 2000).

Histidine auxotrophs of *B. japonicum* was also found to be Nod$^-$ (Sadowsky *et al.*, 1986; Yadav, *et al.*, 1998). However, external supplementation of histidine could restore nodulation in these mutants indicating unavailability of histidine in *planta*. It has been observed that the histidine auxotroph CE230 of *R. etli*, was found to nodulate normally in pea plant which indicated that there is sufficient histidine in the root environment to support the growth of this mutant (Newman *et al.*, 1995).

Isoleucine, valine (ilv) mutants of *S. ferdii* and *S. meliloti* were found to be Nod$^-$ (Kim *et al.*, 1988; Aguilar and Grasso, 1991). These mutants were found to be

unable to nodulate either on supplementation of isoleucine and valine or their intermediates. It has been hypothesed that the cellular concentration of acetolactate in these *ilvC* mutants might have reached level, which inhibited the expression of nodulation genes.

S. meliloti arginine auxotrophs which blocked either in ornithine transcarbamylase or arginosuccinate synthase were effective in symbiosis but mutants which were blocked in early steps of arginine biosynthetic pathway were ineffective. It has been suggested that since ornithine is a precursor for synthesis of polyamines, the lackness of this polyamine may be directly leading to the symbiotic defect(Kerppola and Kahn, 1988[a]).

Methionine auxotrophs have been found to be Nod^+ Fix^+ in *R. leguminosarum* and Nod^+ Fix^- in *S. meliloti* (Pain 1979; Kerppola and Kahn, 1980[b]). The differences in symbiotic behavior of the methionine auxotrophs between these two micro-symbionts may be due to mutation in different genes of methionine biosynthetic pathway. Recently, *metZ* gene in *R. etli* has been found to be essential in nodulation (Tate' *et al.*, 1999[b]).

Vitamins have also been shown to play a role in symbiosis (Schwinghamer *et al.*, 1969; 1970; Kim *et al.*, 1988). Pankhurst *et al.* (1972) demonstrated the requirement of riboflavin for bacteroid development in *R. leguminosarum* bv. *trifolii*. The defect was restored by external supplementation of riboflavin to plant growth medium. It has been indicated that nicotinic acid auxotrophs of *S. ferdii* have been reported to induce ineffective nodules in soybean plant (Kim *et al.*, 1988). Biotin and other vitamins have also been reported to act as a growth factor for colonization of roots by *S. meliloti* 1021 (Streit *et al.*, 1996).

Pyrimidine auxotrophs of *S. meliloti* and *R. leguminosarum* were found to be defective in symbiosis. They produce ineffective nodules in their hosts (Scherrer and Denarie, 1971; Pain 1979). *R. leguminosarum* bv. *phaseoli* strain CFN-42 pryimidine auxotroph was found to be Ndv$^-$ (nodule development deficient). The addition of uridine to plant growth medium could not restore the phenotype defect (Noel *et al.*, 1988).

Kerppola and Kahn (1988[a]) reported that mutation in carbomyl phsophate synthetase and other steps of pyrimidine biosynthesis before orotic acid resulted in ineffective symbiosis in *S. meliloti* strain 104A14. It has also been reported recently that pyrimidine auxotrophs of *S. meliloti* Rmd201 are defective in symbiosis with alfalfa plant (Vineetha, 1998).

2.5.3 Purine Biosynthesis and Symbiosis

Purine auxotrophs of various *Rhizobium* species have been reported to be symbiotically defective and are usually unable to initiate or complete the infection process (Scherrer and Denarie, 1971; Fedorv and Zaretskaya, 1977; Malek and Kowalski, 1983; Kerppola and Kahn, 1988[b]; Kim *et al.*, 1988; Dickstein *et al.*, 1991). The purine auxotrophs (pur$^-$) of *R. leguminosarum* bv. *viciae* strains have been described as noninfective (Pain, 1979) or non nodulating with pea plant (Pankhurst and Schwinghamer, 1974). These mutants induced bumps like structures and the biochemical block in purine biosynthesis for these mutants were found before 5-aminoimidazole 4-carboxamide ribonucleotide (AICAR). Purine auxotrophs of *R. etli* were found to elicit pseudonodules on bean plants without infection thread, and as a result no bacteria could be isolated from these pseudonodules (Noel *et al.*, 1988). In

soybeans, purine auxotrophs of *S. fredii* was found to induce pseudonodules that do not contain bacteria (Kim *et al*, 1988). Purine auxotroph of broad host range *Rhizobium* strain NGR234 elicits root hair curling and nodule meristem initiation on siratro plant, but no infection thread were formed (Djordjevic *et al.*, 1988). These mutants also elicited poorly developed nodules on *Leucaena leucocephala* and *Lablab purpureus* (Chen *et al.*, 1985). It has been speculated that symbiotic defect in this case was due to overproduction of EPS. Although anatomical developments were not blocked at identical stage in these different mutants mentioned above, the underlying molecular basis for limited development supposed to be quite similar and purine auxotroph appears specifically to affect the infection process with exception of *S. meliloti*.

Supplementation of purines or purines nucleosides to the plant growth medium did not restore or enhance the symbiotic proficiency or nodulation phenotype of purine auxotrophs in all cases except for *R. leguminosarum* (Pankhurst and Schwinghamer, 1974). In *R. leguminosarum* it has been reported that addition of adenine to plant growth medium enhanced nodulation of peas by purine auxotrophs. However, while purines are ineffective, the riboside of the purine biosynthetic intermediate AICAR has been reported to restore infection by purine auxotrophs on bean, pea, and soybean (Noel *et al.*, 1988, Newman *et al.*, 1992; 1994; 1995). It has been reported that the addition of 5-aminoimidazole 4-carbaximide (AICA) riboside, the unphosphorylated derivative of the purine precursor AICAR, to the plant growth medium significantly enhanced nodule development in both *R. etli* and *R. leguminosarum* (Newman *et al.*, 1992; 1994). Although infection threads were seen but nodule lack detectable nitrogenous activity and no bacteroids could be seen in the cells of the nodules, which are normally infected. It has been suggested that AICA riboside restored almost

complete infection thread development but cannot overcome a second limitation that prevents the proliferation of bacteroids (Newman *et al.,* 1994). On the basis of the ability of AICA riboside to promote infection by purine auxotrophic strains, despite being serving as a very poor purine source, it has been hypothesized that AICAR plays a role in infection other than merely promoting bacterial growth (Newman *et al.,* 1994; 1995). Recently it has been reported that in *S. meliloti,* (AICA) riboside the precursor of AICAR prevent *fixNOQP* expression by inhibition of *fixK* a transcriptional activator (Soberon et al., 2001).

On the other hand, purine auxotrophs of *S. meliloti* also reported to be symbiotically ineffective (Nod^+, Fix^-) but defects are usually at later stage of infection (Kerppola and Kahn ,1988[b]; Dickstein *et al.,* 1991; Swamynathan and Singh,1992; Gupta, 1996). Supplementation of plant growth medium with adenine or other derivatives were found to have no discernible effect on either nodule development or symbiotic efficiency of these auxotrophs. It has been reported that some cases of deficiency of purine auxotrophs were associated with change on the cell surface molecules (Swamynathan and Singh, 1995; Djordjevic *et al.,* 1996; Gupta, 1996). More recently, Stevens *et al.*, (2000) have isolated purine auxotrophs of *R. leguminosarum* bv. *viciae* which were having mutation in *purM* and *purN* genes, which specify for early steps in purine biosynthesis prior to AICAR, and are needed for nodulation of pea plants (Stevens *et al.*, 2000). These auxotrophs also showed pleiotropic effect and found to be defective in siderophore (vicibactin) synthesis.

Chapter 3

MATERIALS & METHODS

3.1 MATERIALS

3.1.1 BACTERIAL STRAINS

The bacterial strains and plasmid constructs used in this study are listed in Table 5.

Table 5 :Bacterial strains and plasmids used and constructed in this study.

Strains/plasmids	Relevant Characteristics	Source/Reference
Sinorhizobium meliloti AK631	Nod^+ Fix^+ compact colony varirant of wild type strain Rm41	Adam Kondorosi
Rmd 201	Spontaneous Sm^r derivative of AK631, Nod^+Fix^+	Khanuja and Kumar (1988)
VK27	Rmd201 Pur^- (*pur*::Tn5)	This lab
VK37	-do-	-do-
VK38	-do-	-do-
VK40	-do-	-do-
RH13	-do-	This lab
RH19	-do-	-do-
AL3	-do-	This work
AL7	-do-	-do-
NV10	-do-	This lab
NV28	-do-	-do-
NV24	-do-	-do-
NV2	-do-	-do-
Escherichia coli WA803 (pGS9)	Met^-, Thi^-, Cm^r, Km^r	Selvaraj and Iyer (1983)
Plasmids		
pGS9	Inc N rep P15A, Cm^r, Km^r	Selvaraj and Iyer (1983)

3.1 PLANT SEEDS CULTIVAR

Seeds of alfalfa plants (*Medicago sativa* cv. T9) used in the study were obtained from National Seed Corporation, New Delhi, India.

3.1.3 CULTURE MEDIA

3.1.3.1 Media for *Sinorhizobium meliloti*

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

Constituent	Amount (g)/liter
Bactotryptone	5
Yeast extract	3
$CaCl_2.2H_2O$	0.12
The above component were dissolved in distilled water	

pH of the medium was adjusted to 7.0 with 0.1 N NaOH.Volume was make to make 1 liter, and 20 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 (g)/liter
Mannitol	10
Yeast extract	0.2
$K_2HPO_4.3H_2O$	0.2
KH_2PO_4	0.2
$MgSO_4.7H_2O$	0.1
$CaCl_2.2H_2O$	0.05
Distilled water were added to make 1 liter volume	

The pH of the medium was adjusted to 6.8 with 0.1 N NaOH solution. To make solid medium 20 g agar was added and autoclaved.

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

Constituent	Stock solution	Amount of stock solution added/liter	Amount/liter
$Na_2HPO_4.12H2O$	0.45g/10 ml	10 ml	0.45 g
$(NH_4)_2SO_4$	2.0g/10 ml	10 ml	2.0 g
$FeCl_3$	2.0mg/10 ml	1 ml	2.0 mg
$MgSO_4.7H_2O$	0.1g/ml	2.0 mg	0.1 g
$CaCl_2.2H_2O$	0.04g/ml	1 ml	0.04 g
Distilled water were added to make upto1 liter volume			

pH of the medium was adjusted to 7.0 with 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***

L.B. Mediu·m (Luria Bertani) Medium (Sambrook *et al.*, 1989)

Constituent	Amount (g)/liter
Bactotryptone	10
Yeast extract	5
Sodium chloride	10
Distilled water were added to make 1 liter volume	

Components were dissolved and final volume was adjusted to 1 liter with distilled water. 20g/liter agar was added for preparing solid medium.

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. Stock solutions of the individual components were prepared as per list.

Solution	Salt	Amount of salt Used/g	Volume of water Used/ml
A	K_2HPO_4	2.0902	10
B	KH_2HPO_4	0.544	10
C	$CaCl_2$	7.351	10
D	$C_6H_5O_7Fe.3H_2O$	0.335	10
E	$MgSO_4$	6.162	10
F	K_2SO_4	4.3562	10
G	$MnSO_4$	0.034	20
H	H_3BO_4	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 liter of plant growth medium, 10g 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 0.1 N NaOH solution or 0.1N HCl. The resulting medium was then used to prepare slants.

3.1.3.4 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 SUPPLEMENTS TO MEDIA

3.1.4.1 Antibiotics

All antibiotics used were purchased from sigma USA. Stock solutions of streptomycin sulphate (10mg/ml) and kanamycin acid sulphate (40mg/ml) were prepared in distilled water and filter sterilized . The final concentration of different antibiotics used in the media were as; streptomycin sulphate (100 μg/ml), kanamycin acid sulphate (40 μg/ml) for *E. coli* and (400 μg/ml) for *S. meliloti* respectively.

3.1.4.2 Amino Acids, Nitrogenous Bases and Vitamins

Stock solutions of amino acids, nitrogenous bases and vitamins were made in distilled water and filter sterilized. The finial concentrations of amino acids, nitrogenous bases and vitamins were 50 μg/ml, 20 μg/ml and 5 μg/ml, respectively. 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: Clutamine, asparagine, uracil, aspartic acid and arginine.

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

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

3.1.4.3 Dicarboxylic Acid

Dicarboxylic acids such as (malate, sodium succinate and asparate) were purchased from HiMedia and added to the RMM (without glucose) @ 2g per litre as a sole carbon before autoclaving.

3.1.4.4 Purine pathway Intermediate

Purine intermediates used in this study were purchased from sigma USA. Distilled sterile water was used as solvent for preparing stock solution of intermediates (amino imidazole riboside- AIR, Amino imidazole carboxamide riboside- AICAR, inosine, thiamine, adenine or guanine). Intermediates were added to the medium at 50 µg/ml and 20 µg/ml for thiamine concentration with RMM or the plant growth media.

3.1.4.5 Dyes

Calcofluor white (Sigma), congo red and aniline blue (HiMedia) were added to the MSY medium up to 0.02% before autoclaving. 0.01% methylene and 1% toluidine blue in 1% borax was prepared and used for staining of infection threads in root hairs

and semithin sections for optical microscopy, respectively. Sodium deoxycholate (DOC) (HiMedia) was added at the rate of 1 mg/ml before autoclaving the TY medium.

3.1.5 BACTERIAL GROWTH-CONDITIONS

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

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

3.1.6.1 Preparation of Blocks

(a) Karnovaky Fixative (Karnovsky, 1965)

12.5 ml 8% paraformaldehyde (Sigma)

5.0 ml 12.5% glutaraldehyde (Sigma)

25.0 ml phosphate buffer (PH 7.4)

7.5 ml distilled water

(b) 0.2 M Phosphate Buffer

NaH_2PO_4 - 6.41 g

$Na_2HPO_4.7H2O$ - 41.3 g

Or

$Na_2HPO_4.12H_2O$ - 55.2 g

Double distilled water to make 1 liter.

(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°C in a tightly stoppered brawn coloured bottle.

(d) **Araladite 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 (Dibutylphthlate)	-	1.0 ml

The ingredients were stirred vigorously to mix.

3.1.6.2 Solution for Staning 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°C.

(b) Lead Citrate

To 12 ml of mild alkaline water in centrifuge tube. 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°C.

3.2 METHODS

3.2.1 MAINTENANCE OF BACTERIAL CULTURE

The pure bacterial cultures were maintained in the TY medium containing (15%) of glycerol. For routine use strains were also maintained in slants of TY agar medium for *S. meliloti* and LB agar medium for *E. coli* strains and then stored at 4°C in refrigerator.

Single colonies were obtained by plating or streaking cells of strain on complete or minimal medium. Plates were incubated at 2-3 days for complete medium and 7 days for minimal medium at 30°C for growth. A fully-grown. *S. meliloti* colony usually contained about 10^7 cells.

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

3.2.2 BACTERIAL CONJUGATIONS

Patch mating were done according to Kondorosi *et al.*, (1977). TY broth cultures of *S. meliloti* and *E. coli* were grown for 48 and 16 hrs. respectively. From cross between *E. coli* and *S. meliloti*, they were mixed in the ratio 3:7(0.03 ml of *E. coli* culture and 0.07 ml of *S. meliloti* culture) and several agar TY plates with the help of sterile glass rod. Then the plate was incubated at 30°C 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 selective medium. The plates were examined for mutants after five days of inoculation at 30°C.

3.2.3 SERIAL DILUTION

Serial dilution were made in saline solution (0.9% NaCl) to obtain single colonies for many experiments, 10^{-2},10^{-3},10^{-4} and other dilution were made according to the requirement of experiment and 0.1ml of these were used for streaking the plates.

3.2.4 ISOLATION OF MUTANTS AND SCREENING FOR AUXOTROPHS

3.2.4.1 Transposon Tn5 Mutagenesis

Cultures of *E. coli* strain WA803 carrying plasmid (pGS9) and *S. meliloti* strain Rmd201 were grown to log phase in TY broth, 0.03 ml of *E. coli* and 0.07 ml of *S. meliloti* were delivered on the surface of TY medium in a Petriplate. The two cultures were mixed and spread with the help of a sterile glass rod. Several patches were made, and incubated at 30° C 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 μg/ml) and Sm (100μg/ml). In all mating, the donor and recipient cultures were also plated on TY medium supplemented with Km(400mg/ml) and Sm (100 mg/ml). The plates were incubated for 5 days.

There were around 50-200 colonies on each plate when the neat suspension from a patch was plated on 2-3 plates. Usually from a patch 40-50 colonies were purified twice on TY plates having Km(400 μg/ml) and Sm (100 μg/ml) the collection of two thousand two hundred mutants of *S. meliloti* were done from different patch mating, these mutants were maintained on TY plates having Km(400 μg/ml) and Sm (100μg/ml).

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 mutants on TY plate.

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 minimal (RMM) and complete (TY) media with sterile toothpicks. 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 population obtained from a single colony was again checked for auxotrophy. The natures of auxotrophy of mutants were determined subsequently.

3.2.5 DETERMINATION OF AUXOTROPHY

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

3.2.6 REVERISION ANALYSIS

The cells of an auxotrophic mutant were grown to late log phase in TY medium at 30° C, washed twice with RMM medium and resuspended in the same medium up to 10^9 cells per ml. 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°C. These prototrophs were replica plated on RMM agar plates supplemented with kanamycin to test 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 supplement. 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.

The plates were then incubated for 2-3 days at 30°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 purine biosynthetic pathway in each purine auxotroph was determined by feeding of the intermediates of the pathway. RMM medium was supplemented with (amino imadazole ribodside -AIR, amino imidazole carboxamide riboside -AICAR, thiamine, inosine, guanine), RMM medium supplemented with adenine or adenine + thiamine was used as a positive control. A cell suspension of each auxotroph was streaked on each supplemented medium. The parental cell suspension was also streaked on various plates as control, growth pattern was observed after incubation at 30°C for 3-4 days.

3.2.9 UPTAKE OF DICARBOXYLIC ACIDS

RMM (without glucose) supplemented with adenine/adenine + thiamine and malate, aspartate or sodium succinate as a sole carbon source was used for this study. Auxotrophs and parental strain (as a positive control) were streaked on the above media and growth pattern of auxotrophs was compared with parental strain after incubation at 30°C for 3-4 days.

3.2.10 CELL SURFACE CHARACTERISTICS

3.2.10.1 Test for production of exopolysaccharide

The production of exopolysaccharide (EPS) was tested using calcoflour stain (Leigh *et al.*, 1987). Auxotrophs and parental strains Rmd201 were streaked on MSY medium containing calcoflour and inoculated at 30°C for four days. The strains, which

produce EPS, are fluorescent under long wavelength UV light on calcofluor containing medium.

3.2.10.2 Test for Production of β (1→2) glucans

The motility of bacteria in swarm plats can be attributed to β (1→2) glucans productions (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°C for 2 days. Parental strain Rmd201cell suspension also was spotted as a positive control. Swarming of the bacterial strains was determined by the spread of the bacterial growth.

3.2.10.3 Test for production of cellulose fibrils

Cellulose fibrils in deferent purine auxotrophs and parental strains Rmd201 were tested using congo red stain (Kneen and La Rue, 1983). Strains produce cellulose fibrils can form red colonies on congo red MSY containing medium. Plates were incubated at 30°C for four days and result can observe in normal light.

3.2.10.4 Test for Production of lipopolysaccharide (LPS)

Cell suspension of each purine auxotroph were streaked on TY plates containing sodium dexyochcolate (1 mg/ml) and then incubated at 30°C for 2 to 3 days. Parental strain also was streaked on these plates as a positive control. Growth pattern was compared after the incubation period, strains that grown on this plates were considered to be LPS producing.

3.2.10.5 Test for production of β(1→3) glucans

The production of β(1→3) glucans was tested using aniline blue (Nikanishi *et al.*, 1976). The plates were inoculated at 30^0C for four days and observed for β(1→3) glucans production in normal light. The strain produced β(1→3) glucans form blue colonies on aniline blue MSY containing medium.

3.2.11 METHODOLOGY FOR PLANT EXPERIMENTS

Plant growth mediums were prepared as described before in 20cmx2.5 cm glass tubes whenever needed depending upon experimental condition. Preparations of media were carried out in sterile condition. Plant growth mediums supplemented with purine intermediate were also used for test of recover symbiotic efficiency of these auxotrophs.

Alfalfa *(Medicago sativa* cv *T9)* seeds used in this study 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 for tow days at 25°C in a dark place for germination. Two germinated seeds were transferred to each tube under sterile condition and keep in plant growth chamber.

For inculcation, fresh liquid cultures of *S. meliloti* Rmd201 and purine auxotrophic strains in TY medium were prepared. Each culture was centrifuged (5000 rpm for 10 min), washed twicely with sterile water and suspended in 4 ml of sterile

water. 0.5 ml of this suspension was dispensed into each tube containing two-day 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°C 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 shoot high and mean shoot dry weight. Dry shoot weight was recorded after drying the plant in an oven at 85° C for 72 hrs.

3.2.11.1 Estimation of Total Nitrogen Amount

Total nitrogen contain for six weak alfalfa plant inoculated by different purine auxotrophs were estimated. For each sample, eight plants were collected completely dried and crushed by porcelain to make homogeneous powder. Total nitrogen content for samples have been performed by elemental analyzer Carlo Erba 1108 Heraeus at RSIC,Central Drug Research Institute, Lucknow.

3.2.11.2 Nodule Occupancy Tests

Nodule occupancy for 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 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°C. The colonies obtained were patched on RMM medium, RMM supplemented with the nutrient requirement(s) of the auxotrophs and TY medium supplemented with kanaymacin (400 μg/ml) and

streptomycin (100 μg/ml). Plates were incubated at 30°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.

3.2.11.3 Light Microscopy of Observing Infection Threads

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 plant roots were then washed twice in sterile water and cut into 1 cm long pieces. Each roots piece was placed on a clean and sterile slide, and covered with a coverslip. The slide was then examined at 40 x and 100 x magnification under light microscope (Leica DM LB).

3.2.11.4 Histology of Nodules

Histological studies were carried out using light and electron microscopy. Nodules were collected after 6 weeks of plant growth and methodology for these experiments consisted of fixation, block preparation, and preparation of semithin and ultrathin sections of the specimen.

3.2.11.4.1 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 additive 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 out at 4C° overnight.

3.2.11.4.2 Specimen Block Preparation for Light and Transmission Electron Microscopic Studies

(a) Washing, Post fixation and Dehydration

After fixation the nodules were washed twice in 0.1 M phosphates buffer and then left in the same buffer overnight at 4°C. Final trimming of the nodules to appropriate size was done when these were in the buffer. The nodules after fixation were taken out and placed in 1% OsO_4 for 2 hrs, at 4°C.

Dehydrating of the sample was carried out using dry acetone. The samples were passed through the various dehydration series. (30%. 50%, 70%, 80%, 90%, 95% acetone and dry acetone) two change for 15 min. at 4°C. The samples were further dehydrated by dry acetone two changes of 30 min. each at room temperature.

(b) 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.

(c) Infiltration and Embedding

The following infiltration mixtures were prepared from the embedding medium.

a. 1 part of embedding medium 3 parts of toluene

b. 2 parts of embedding medium and 2 part 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 over 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°C in a special oven for 12 to 24 hrs. The temperature of the oven was then raised to 60° C and the embedded tissues were kept for 24 to 48 hr. For complete polymerization of the embedding medium.

3.2.11.4.3 Preparation of Semithin Sections for light Microscopy

The resin blocks containing specimens were fitted in the specimen block holder. The block holder was attached to Ultra cut 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 clean glass slide. The slide was placed on a hot plate (at about 80°C) for drying. The sections were then stained in 1% toluidine blue for 30 seconds. Later the sections washed with distilled water. These slides were then observed under light microscope (Leica *DM* LB) and areas of interest were photographed.

3.2.11.4.4 Preparation of Ultrathin Sections

After scanning the sections under the light microscope, the area to be examined under TEM was selected and blocks were further hand trimmed. Ultrathin sections (60 - 90 nm thick) were cut using Ultracut E macrotome (C. Reichert, Austria). The silver coloured sections were lifted on to 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 cardboard 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 (Phillips EM 300) and the areas of interest were photographed.

3.2.12 STATISTICAL ANALYSIS

The data related to plant characteristics of parental and its purine auxotrophs inoculation were statistically analysis using student's 't' test (Bailey, 1995). The means of eight plants for different characters were used.

Calculation of critical difference (C.D).

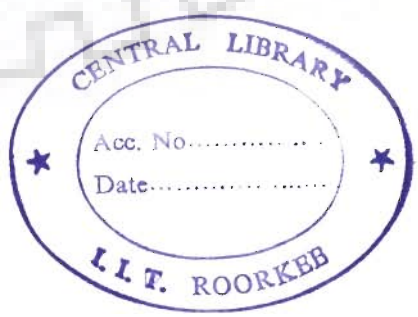
$$C.D = t_{0.05} \times \overline{Sd}$$

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 = number of replications.



Chapter 4

RESULTS

To focus on the nature and the extent of purine biosynthetic pathway involvement in symbiosis of *Sinorhizobium meliloti.* Purine auxotrophic mutants of *Sinorhizobium meliloti* Rmd201 strain were generated through transposon mutagenesis. The biochemical and symbiotic characterization of purine mutants isolated during the course of this study and by other researcher in the lab were done and nodules induced by these auxotrophs were subjected to microscopic studies. The results of this study are presented here.

4.1 Tn5 MUTAGENISIS AND ISOLATION OF PURINE AUXOTROPHS

S. meliloti Rmd201 which is $Nod^{+}Fix^{+}$ was mutagenised with transposon Tn5 mutagenesis which codes for resistance to kanamycin .The conjugation between *S. meliloti* and *E. coli* WA803 strain was carried out with suicide Tn5 delivery vector pGS9 .Tn5 derivative selected by plating the mating mixture on TY medium containing kanamycin (400μg/ml) and streptomycin (100μg/ml). The Km^{r} trasconjugants were obtained at frequency of $4x10^{-5}$ per recipient. In order to avoid sibling not more than 100 Tn5 derivative were selected from each cross. 2200 Km^{r} colonies from 39 cross were collected and purified on TY medium containing kanamycin and streptomycin. All Tn5 transconjugant were kanamycin resistant are chloramphenicol sensitive, this confirming disintegration of Tn5 delivering vector pGS9 in these mutants. .All Tn5 mutant obtained above were streaked on minimal medium RMM and TY complete medium. Twelve mutants did not grow on minimal medium, these were assume to be auxotrophs and further purified for single colony on TY medium containing

kanamycin and streptomycin antibiotic. Nutrition requirement of these auxotrophs were determine by patching these mutants on nutrition pool (Holliday, 1965). Out of 12 auxotrophs, two auxotrophs (NV2 and NV24) found to had double nutritional requirement for (adenine + thiamine), while other purine auxotrophs had single nutritional requirement for the adenine.

4.2 CHARACTERIZATION OF PURINE AUXORTOPHS

4.2.1 REVERSION ANALYSIS

In order to check the stability of auxotrophic mutant reversion analysis were carried out. Spontaneous excision of Tn5 in all purine auxotrophs resulting in the formation of Km^s prototrophic revertants. Minimum reversion frequency was observed in auxotrophic mutant NV24 ($1.01x10^{-11}$), were the maximum frequency was observed in auxotrophic mutant VK40 ($2.06x10^{-8}$). The result of reversion analysis frequency of various mutants is presented in Table- 6.

4.2.2 BIOCHEMEICAL CHARACTERIZATION

Biochemical studies were carried out to identify the block in purine auxotrophs within the purine biosynthetic pathway by intermediate feeding and cross feeding tests.

4.2.2.1 Intermediate feeding and cross-feeding tests

It was observed by intermediate feeding experiment that purine auxotrophs (NV2 and NV24) showed growth on RMM minimal medium supplemented with (adenine + thiamine), but no growth occurred when adenine alone were added to RMM minimal medium. This indicates that the block occur before amino imidazole riboside-

Table 6: **Reversion frequencies of purine auxotrophic mutants of *Sinorhizobium meliloti* strain Rmd201.**

S.No	Auxotrophic mutant	Spontaneous reversion frequency
1.	VK27	2.00×10^{-8}
2.	VK37	1.04×10^{-11}
3.	VK38	1.95×10^{-11}
4.	VK40	4.60×10^{-8}
5.	RH13	1.10×10^{-10}
6.	RH19	1.40×10^{-11}
7.	AL3	1.02×10^{-11}
8.	AL7	1.90×10^{-9}
9.	NV10	1.06×10^{-11}
10.	NV28	1.30×10^{-9}
11.	NV24	1.01×10^{-11}
12.	NV2	2.05×10^{-9}

AIR. Auxotrophs (NV28, NV10 and AL7) showed growth on RMM minimal medium supplemented with either amino imidazole carboxamide riboside - AICAR or inosine or adenine, indicating that the block in these auxotrophs are before amino imidazole carboxamide riboside- AICAR (Table -7).

Auxotrophs viz, VK37, VK38, VK40, RH13 and AL3 showed growth on RMM minimal medium supplemented with inosine or adenine whereas, auxotrophs (RH19, VK27) showed growth on RMM medium supplemented with only adenine, indicating that block in these groups are before inosine and adenine respectively. The respective block of each mutant is shown in Fig- 6,Table- 7. It was feather observe that all these purine auxotrophs were unable to grow in RMM minimal medium supplemented with guanine or guanosine which supported the idea that the conversion pathway of GMP to IMP is absent while from AMP to IMP is present in *S. meliloti* Fig- 6, Table- 7.

To detect cross feeding among purine auxotrophs, all these strains were streaked near each other in all possible combination on RMM medium supplemented with minimal quantity of their nutritional requirement. Result indicated that none of the auxotrophs could feed any other auxotrophs.

4.3 PLEIOTROPIC EFFECT OF PURINE BIOSYNTHETIC GENES

4.3.1 CELL SURFACE CHARACTERIZATION

The parental strain Rmd201 and all purine auxotrophs used in this study found to be normal in cellulose fibriles, lipopolysaccharide and exopolysaccharide production as indicated from took up the dye in congo red containing medium, growth in presence

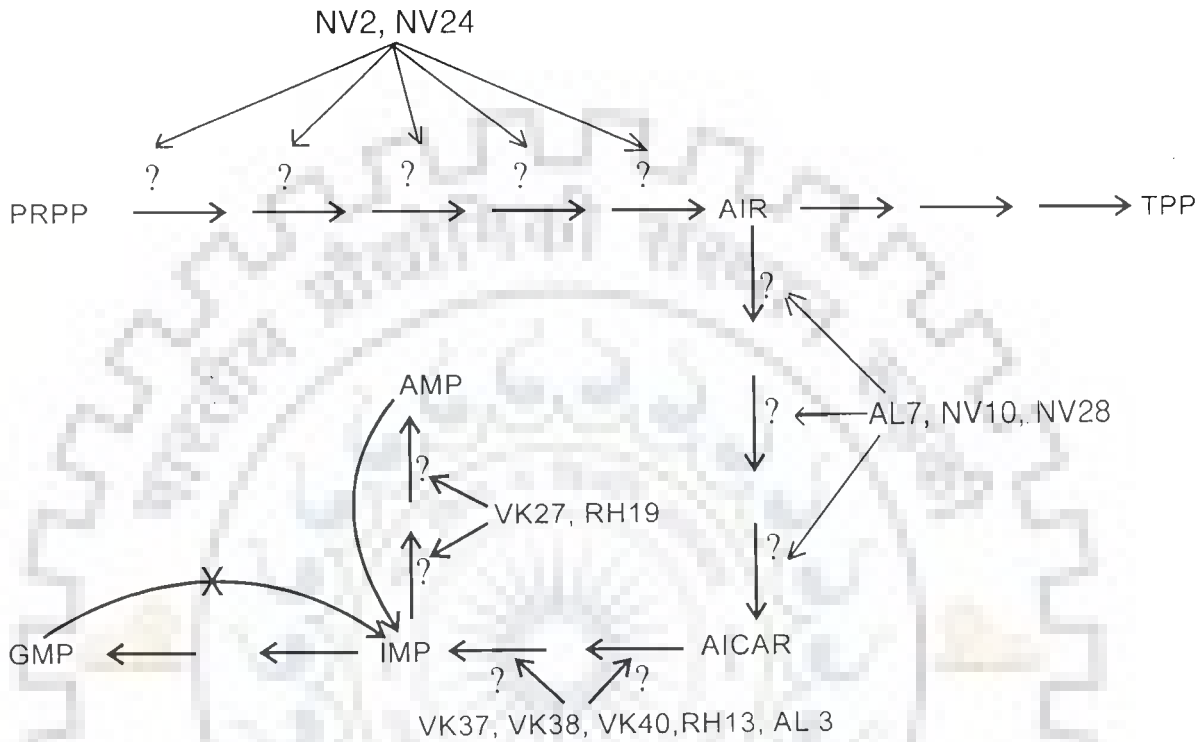


Fig. 6: Pathways of the synthesis of purine showing the positions of mutation in the purine auxotrophs of *Sinorhizobium meliloti* Rmd201.

Abbreviations :

PRPP - Phosphoribosyl pyrophosphate
AIR - Amino imidazole riboside
TPP - Thiamine pyrophosphate
AICAR- Amino imidazole carboxamide riboside
IMP - Inosine monophosphate
AMP - Adenosine monophosphate
GMP - Guanosine monophosphate

Note : That GMP is not converted to IMP in *S. meliloti*.

Table 7: Growth response of purine auxotrophs of *Sinorhizobium meliloti* Rmd201 to different intermediates of purine biosynthetic pathways.

Strains	RMM supplied with 50 µg/ml of								RMM
	AIR	Th*	AICAR	In	Gu	Gun	Ad	Ad+Th*	
Rmd201	+	+	+	+	+	+	+	+	+
VK27	–	–	–	–	–	–	+	+	–
RH19	–	–	–	–	–	–	+	+	–
VK37	–	–	–	+	–	–	+	+	–
VK38	–	–	–	+	–	–	+	+	–
VK40	–	–	–	+	–	–	+	+	–
RH13	–	–	–	+	–	–	+	+	–
AL3	–	–	–	+	–	–	+	+	–
AL7	–	–	+	+	–	–	+	+	–
NV10	–	–	+	+	–	–	+	+	–
NV28	–	–	+	+	–	–	+	+	–
NV24	+	–	–	–	–	–	–	+	–
NV2	+	–	–	–	–	–	–	+	–

RMM - Rhizobium minimal medium, AIR - Amino imidazole riboside, Th - Thiamine, AICAR - Amino imidazole carboxamide riboside, In - Inosine, Gu - Guanine, Gun - Guanosine, Ad+Th - Adenine + Thamine, * - All strains were treated with 20 µg/ml.

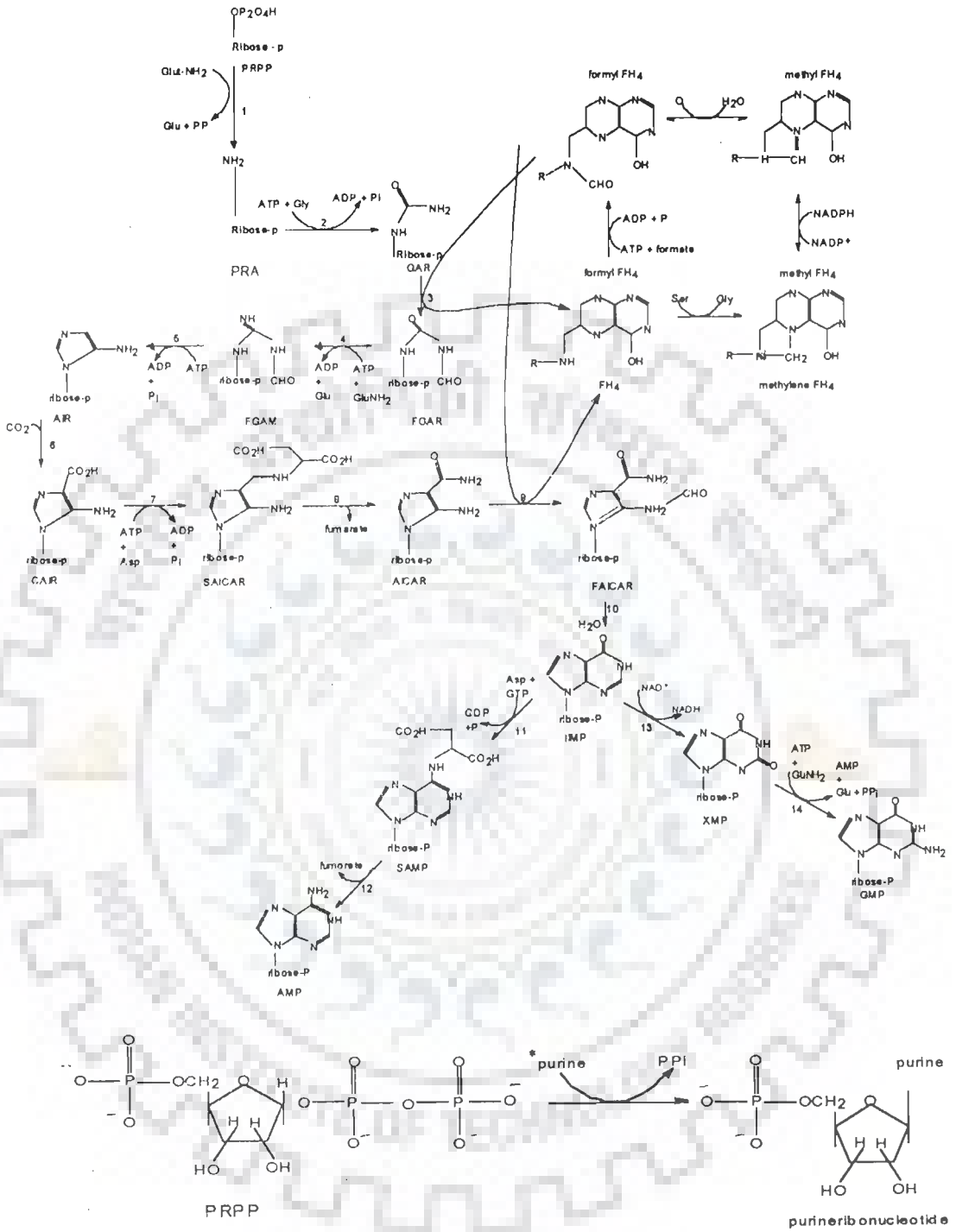


Fig. 6a. The purine *de novo* biosynthetic and salvage pathways. Chemical structures of intermediates where new bonds formed at each step are in boldface. Intermediate and products are abbreviated as follows: PRPP. 5-phosphoribosy1-1-pyrophosphate; PRA, 5-phosphoribesy1-1-amine: GAR, glycinamide ribotide: FGAR, N-formylglycinamide ribotide: FGAM, N-formylglycinamide ribotide: AIR, 5-aminoimidazole ribotide: CAIR, 5-amino-4-imidazole-carboxylic acid ribotide; SAICAR, 5-aminoimidazole-4-N-succinocarboxamide ribotide: AICAR, 5-aminoimidazole-4-carbocamide ribotide: FAICAR, 5-formaminoimidazole-4-carboxamide ribotide: IMP, inosinic acid: SAMP, adenylosuccinic acid: AMP, adenylic acid: XMP, xanthylic acid: GMP, guanylic acid: FH4, tetrahdrofolate. Enzymatic activities for purine synhesis are 1, amidophosphoribosyl transferase (phosphoribosylpyrophosphate amidotransferase EC 2.4.2.14) 2,GAR synthetase, or GARS (phosporibosylglycinamide synthetase, EC 6.3.4.13); 3. GAR transformylase or GART (phosphoribosylgylcinamide formyltransferase. EC 2.1.2.2); 4. FGAR amidotransferase (phosphoribosylgylcinamide amidotransferase, EC 6.3.5.3); 5,AIR synthetase or AIRS phosphoribosylaminomidazole synthetase, EC 6.3.3.1); 6,AIR carboxylase (phosphoribosylaminomidazole carboxylase, EC 4.1.1.21);7, SAICAR synthetase(phosphoribosylaminomidazole succinocarboximide synthetase, EC 6.3.2.6); 8, 12, adenylosuccinate lyase (EC 4.3.2.2); 9, AICAR transformylase (phosphoribosylaminomidazole carboxamide formyltransferase, EC 2.1.2.3); 10, inosinicase (inosinate cyclohydrolase, EC 3.5.4.10); 11, adenylosuccinate synthetase (EC 6.3.4.4); 13, IMP dehydrogenase (EC 1.2.1.14); 14, GMP synthetase (EC 6.3.4.1); *Free purine basses are formed by hydrolytic dehydration of nuclic acid and nucleotides (Stryer, 1975; Henikoff, 1987).

of sodium deoxycholate (DOC) in medium and fluoresced under ultraviolet light in calcoflour containing medium. On other hand all purine auxotrophs showed less motility in swarm medium than the parental strain Rmd201 which related to production of β(1-2) glucans. Cyclic β(1-2) glucans play important role in flagellum motility and less motility of these mutants reduce their infection ability. It was further observed that similar to parental strain, all purine auxotrophs showed as serve of normal production of β(1-3) glucans as they did not took up the dye in aniline blue containing medium

4.3.2 UPTAKE OF DICARBOXYLIC ACID

Similar to parental strains Rmd201 all purine auxotrophs were able to grow in minimal medium containing dicarboxylic acid (malate, sodium succinate or aspartate). Indicating that these auxotrophs were able to use these dicarboxylic acid as a sole of carbon source.

4.4 SYMBIOTIC PROPERTIES OF PURINE AUXOTROPHS

4.4.1 SYMBIOTIC PROPERTIES OF PLANTS INOCULATED WITH PURINE AUXOTROPHS

Symbiotic studies were carried out by inoculating alfalfa seedlings with *S. meliloti* parental strain Rmd201 and purine auxotrophs Table - 8.

Six weaks after inoculation the symbiotic features were minimized on nodule characterization, shoot height, shoot dry weight and total nitrogen contain Table- 8. In order to find the authenticity of nodulation by a particular strain the bacteria were re-isolated from nodules and checked for their markers. Plant data were taken only if the nodules were found to be occupied by the same inoculated strain. Data presented in

Table 8: The characteristic of alfalfa plants inoculated with *Sinorhizobium meliloti* strain Rmd201 and its purine auxotrophic mutants.

S.No	*S.meliloti* strain	No. of days to fist nodule appearance	Nodule shape	Nodule colour	Nodule location	Mean shoot height (cm)*	Mean shoot dry weight (mg)*	Total nitrogen contain per plant (mg)
1.	Rmd201	7-9	Cylindrical	Pink	PR + LR	21.0 ± 1.9	25.5 ± 2.5	0.4107
2.	Uninoculated control		-	-	-	6.8 ± 1.5	7.7 ± 0.7	0.1743
3.	VK27	9-11	Irregular	White	PR + LR	8.6 ± 0.8 N.S	9.9 ± 0.8 N.S	0.2337
4.	VK37	-do-	Irregular Round	-do-	-do-	7.5 ± 1.0 -do-	9.0 ± 0.5 -do-	0.1965
5.	VK38	10-12	-do-	-do-	-do-	8.4 ± 1.8 -do-	9.5 ± 0.9 -do-	0.1996
6.	VK40	-do-	-do-	-do-	-do-	7.3 ± 1.2 -do-	9.0 ± 0.6 -do-	0.1988
7.	RH13	9-11	Irregular	-do-	-do-	7.8 ± 1.3 -do-	9.8 ±1.1 -do-	0.1968
8.	RH19	-do-	-do-	-do-	LR	9.1 ± 1.1 -do-	8.8 ± 0.1 -do-	0.2009
9.	AL 3	10-12	Round	-do-	PR + LR	8.3 ± 0.4 -do-	9.1 ± 2.6 -do-	0.1995
10.	AL 7	9-11	Irregular Round	-do-	-do-	8.5 ± 1.5 -do-	9.1 ± 2.3 -do-	0.1704
11.	NV10	-do-	-do-	-do-	-do-	8.6 ± 0.8 -do-	9.8 ± 1.8 -do-	0.1702
12.	NV28	-do-	Irregular	-do-	-do-	8.8 ± 1.0 -do-	10.0 ± 1.2 -do-	0.1716
13.	NV24	-do-	Round	-do-	-do-	8.0 ± 0.0 -do-	8.3 ± 1.6 -do-	0.1860
14.	NV2	-do-	-do-	-do-	-do-	8.2 ± 0.9 -do-	9.3 ± 1.4 -do-	0.1802

Abbreviations :
N.S - Non significant difference from uninoculated control value ($p<0.05$), * Each value is man of eight plants.
PR - Primary root, LR - Lateral root

Table-8 showed that there is a difference in initiation of nodule formation of alfalfa plants (number of days to first nodule appearance) between the parental strain and the purine auxotrophs. Also clear differences emerged in the morphology of the nodules elicited by these mutants from those that induced by the parental strains. Results showed that the nodules induced by parental strain were cylindrical in shape and pink in color Table - 8, (Plate 1). Whereas these induced by purine auxotrophs were small round or irregular in shape and white in color Table - 8, (Plate 1:a,b,c,d). The symbiotic properties is present in Table- 8 showed that there is no significant difference in the mean shoot height and mean shoot dry weight between the uninoculated plants and alfalfa plants inoculated by purine auxotrophs. These indicate the ineffectiveness of these mutants in symbiotic nitrogen fixation. The ineffectiveness of purine auxotrophs in nitrogen fixation were also reflected by comparing total nitrogen contain of plants inoculated with parental strain Rmd201 and that inoculated with different purine auxotrophs Table -8. The result showed that there were no differences in nitrogen content between the uninoculated plants with various plants inoculated with various auxotrophs. Indicate that these purine auxotrophs are ineffective in nitrogen fixation. The little difference of purine auxotrophs with uninoculated plant in the plant height, shoot dry weight and total nitrogen contain were because in general rhizobia are known to produce the plant hormone indole acetic acid (IAA) in the plant medium which induce the plant growth to some extent.

Plate 1: Alfalfa plants (8 week old) inoculated with wild type *Sinorhizobium meliloti* Rmd 201 and purine auxotrophs.

(a) Alfalfa plants

A. Inoculated with NS24
B. Inoculated with NS2
C. Inoculated with Rmd201
D. Uninoculated (control)

(b) Alfalfa plants

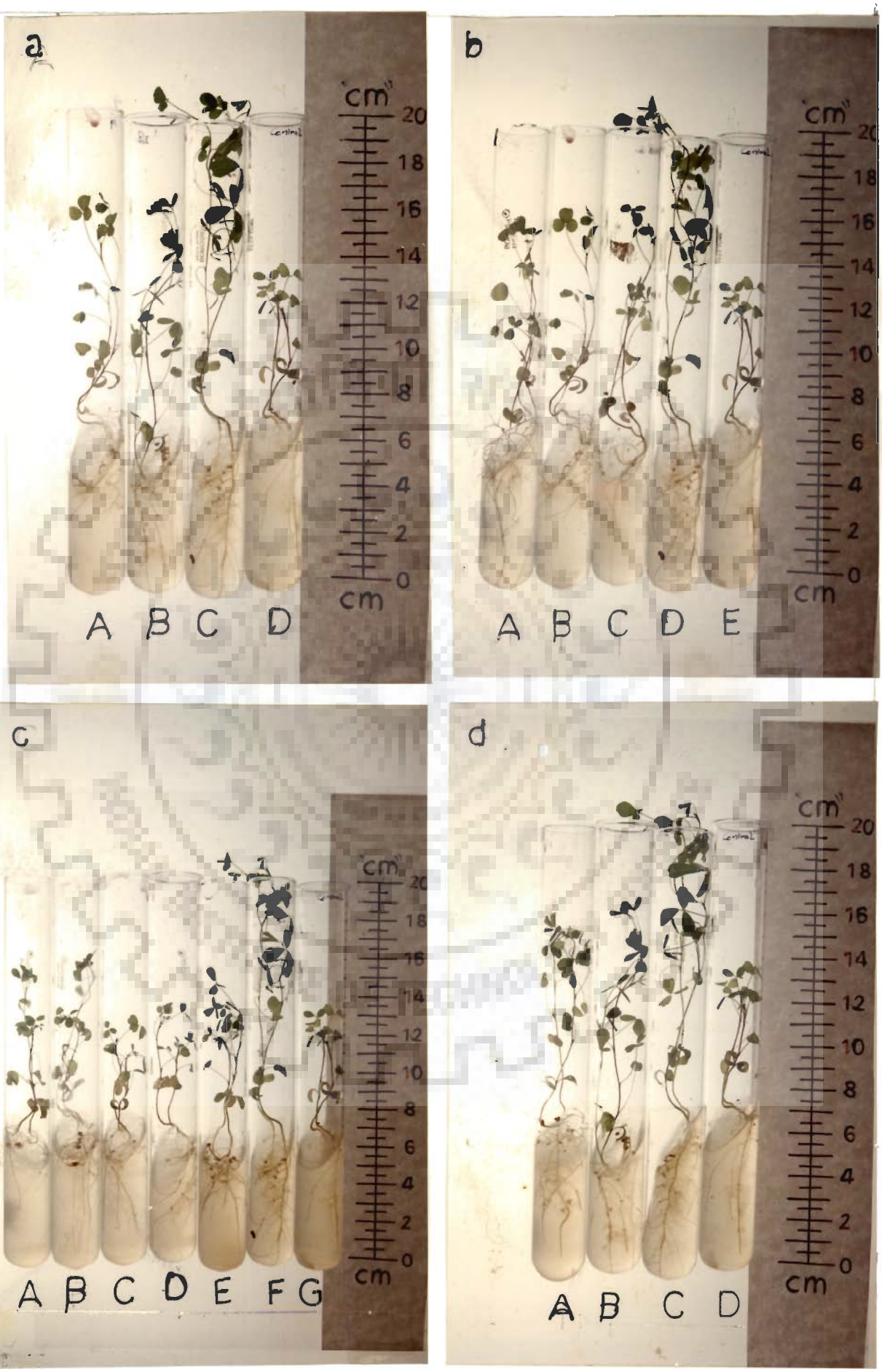
A. Inoculated with AL7
B. Inoculated with NS10
C. Inoculated with NS28
D. Inoculated with Rmd201
E. Uninoculated (control)

(c) Alfalfa plants

A. Inoculated with RH13
B. Inoculated with VK37
C. Inoculated with VK38
D. Inoculated with VK40
E. Inoculated with AL3
F . Inoculated with Rmd201
G. Uninoculated (control)

(d) Alfalfa plants

A. Inoculated with VK27
B. Inoculated with RH19
C. Inoculated with Rmd201
D. Uninoculated (control)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4.4.2 SYMBIOTIC PROPERTIES OF PLANTS INOCULATED WITH REVERTANT PURINE AUXOTROPHS

The symbiotic characteristics of alfalfa plants inoculated with the revertants of purine auxotrophs and parental strain Rmd201 were similar. They induced nodules are cylindrical in shape, pink in color effective in nitrogen fixation Table- 9. Revertants inoculated plants did not different significantly from the plant inoculated with parental strain in mean shoot height and mean dry weight characteristic Table- 9. Results indicate that the revertants of these auxotrophs formed normal nitrogen fixing nodules like the *S. meliloti* parental strain in alfalfa plant.

4.4.3 EXOGENOUS SUPPLEMENTATION OF ADENINE OR PURINE INTERMEDIATE TO PLANTS INOCULATED WITH PURINE AUXOTROPHS

The plant culture medium supplemented with adenine or other purine intermediates were used to study the symbiotic interaction of purine auxotrophs with alfalfa plants in the presence of these intermediates. The supplementation of plant culture medium with adenine did not restore the symbiotic defects in purine auxotrophs (adenine defective mutants), but enhanced the growth of plants root and shoot system to some extant. Similarly the supplementation of inosine, amino imidazole carboxamide riboside - AICAR or (adenine + thiamine) to plant growth medium could not restore the symbiotic defective of (VK37, VK38, VK40, RH13 and AL3) mutants or (NV28, NV10 and AL7) mutants or (NV24 and NV2) mutants respectively. It was determine from the result that in all these different supplementation studies, nodules induced by

Table 9: The characteristic of alfalfa plants inoculated with revertants of purine auxotrophs of *Sinorhizobium meliloti* strain Rmd201.

S.No	*S.meliloti* strain	No. of days to fist nodule appearance	Nodule shape	Nodule colour	Nodule location	Mean shoot height (cm)*	Mean shoot dry weight (mg)*
1.	Rmd201	7-9	Cylindrical	Pink	PR + LR	21.0 ± 1.9	25.5 ± 2.5
2.	Uninoculated control		-	-	-	6.8 ± 1.5	7.7 ± 0.7
3.	VK27	7-9	Cylindrical	Pink	PR + LR	19.6 ± 1.2 N.S	23.2 ± 2.0 N.S
4.	VK37	-do-	-do-	-do-	-do-	17.9 ± 3.5 -do-	21.3 ± 4.3 -do-
5.	VK38	-do-	-do-	-do-	-do-	18.3 ± 3.6 -do-	19.6 ± 3.0 -do-
6.	VK40	-do-	-do-	-do-	-do-	17.2 ± 2.4 -do-	19.0 ± 2.4 -do-
7.	RH13	-do-	-do-	-do-	-do-	19.5 ± 1.7 -do-	20.2 ± 2.7 -do-
8.	RH19	-do-	-do-	-do-	-do-	20.1 ± 0.9 -do-	23.1 ± 1.2 -do-
9.	AL3	-do-	-do-	-do-	-do-	18.3 ± 2.1 -do-	22.3 ± 2.3 -do-
10.	AL7	-do-	-do-	-do-	-do-	16.7 ± 4.1 -do-	18.4 ± 3.2 -do-
11.	NV10	-do-	-do-	-do-	-do-	17.3 ± 2.1 -do-	18.6 ± 3.2 -do-
12.	NV28	-do-	-do-	-do-	-do-	18.1 ± 1.4 -do-	19.5 ± 1.2 -do-
13.	NV24	-do-	-do-	-do-	-do-	18.3 ± 0.9 -do-	20.3 ± 1.8 -do-
14.	NV2	-do-	-do-	-do-	-do-	19.1 ± 2.1 -do-	20.8 ± 2.5 -do-

Abbreviations :
N.S - Non significant difference from parental Rmd201 strain inoculated plant value ($p<0.05$),
* Each value is man of eight plants.
PR - Primary root, LR - Lateral root

purine auxotrophs were still small and white in color and ineffective in nitrogen fixation with alfalfa plants.

4.5 NODULE OCCUPANCY TESTS

Nodule occupancy was estimated by isolating rhizobial bacteria from the nodules of each plant inoculated with particular purine auxotroph and checking for the auxotrophic marker(s). Bacteria with nutritional requirements similar to those used for inoculation of the plants were obtained from nodules. This confirmed that the nodules were occupied by the same strain with which the plant was inoculated; on the other hand, plant tests indicated that more than 90% of plants inoculated with auxotrophs VK40, RH13, RH19, AL3 and NV24 showed 100% occupancy of nodules by respective auxotrophs. Other plants showing less occupancy with their auxotrophs are shown in Table-10.

4.6 MICROSCOPIC STUDIES

4.6.1 Infection Thread Formation

Light microscopic studies were carried out to observed early steps of infection process (root hair curling, infection thread formation) for all *S. meliloti* purine auxotrophs and the parental strain. Root hair curling, shepherd's crooks and infection thread formation were observed in root hairs of alfalfa plants inoculated with *S. meliloti* parental strain (Plate 2: A, B, C and D) and all purine auxotrophs (Plate 3: A, B, C and D). Also bright refractive spots can be seen in the root hairs which representing localized alterations in the refractive index of the root hair wall that occur prior to

Table 10: Noudule occupancy characteristics of plants inoculated with purine auxotorphic mutants of *Sinorhizobium meliloti* Rmd201.

S.No	Purine 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	VK27	24	21	3	87.5
2.	VK37	38	34	4	89.5
3.	VK38	33	27	6	81.8
4.	VK40	28	26	2	92.9
5.	RH13	30	28	2	93.3
6.	RH19	37	35	2	94.6
7.	AL3	28	27	1	96.4
8.	AL7	32	24	8	75.0
9.	NV10	30	25	5	83.3
10.	NV28	27	21	6	77.8
11.	NV24	26	25	1	96.2
12.	NV2	32	27	5	84.4

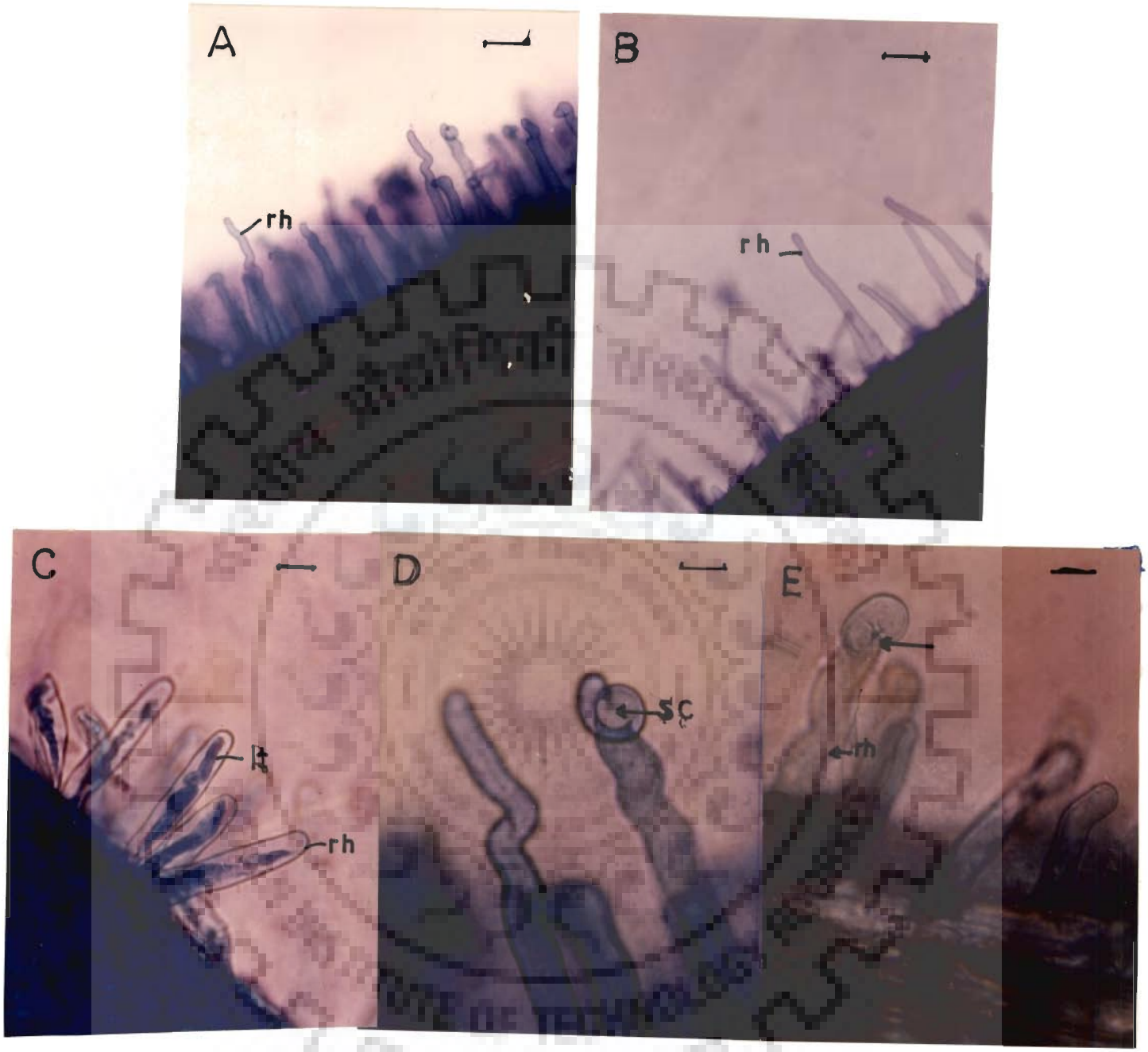

Plate 2: Root hair curling and infection thread formation in root hair of alfalfa plant (seedlings) inoculated with *S. meliloti* Rmd201.

- **A.** Root hairs deformation in plant inoculated with parental strain Rmd201. Bar, 50 μm(x100)
- **B.** Control plant uninoculated with straight root hair. Bar, 50 μm(x100)
- **C.** Multiple infection thread (it) in root hairs (rh) of alfalfa seedling inoculated with parental Rmd201. Bar, 25 μm(x400)
- **D.** Root hair with typical shepherd's crook (sc) showing 360° curvature of the root hairs tip and hyaline spot in the middle of the curvature. Bar, 25 μm(x400)
- **E.** Deformation of root hair with bright refractile spot (arrow) of plant inoculated with parental strain Rmd201. Bar, 25 μm(x400)

Abbreviation : rh-root hair, it-infection thread, sc-shepherd's crook

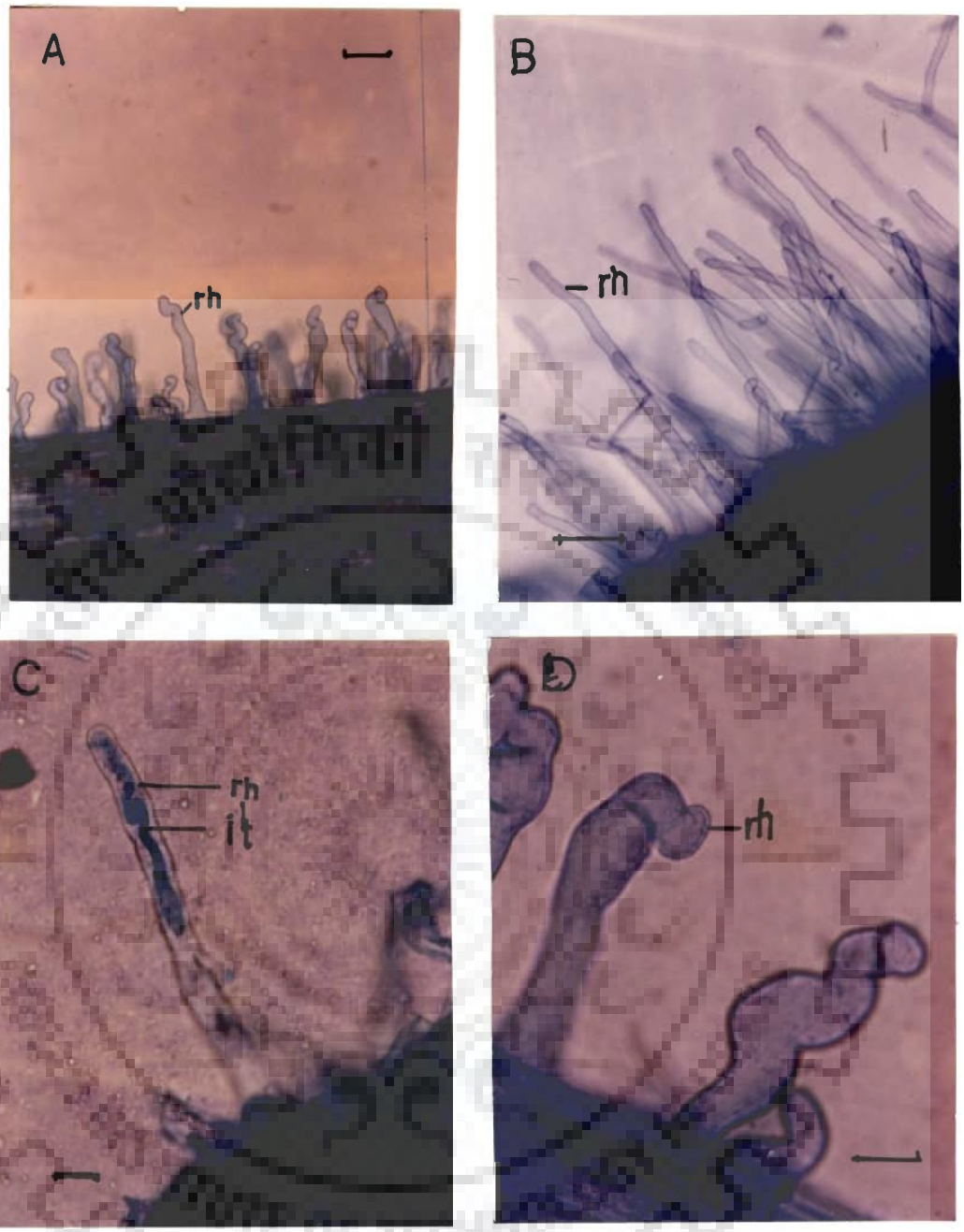

Plate 3: Root hair curling and infection thread formation in root hair of alfalfa plant (seedlings) inoculated with *S. meliloti* purine auxotrophic mutants.

A. Root hairs deformation in plant inoculated with auxotrophic mutants VK37. Bar, 50 μm(x100).

B. Control uninoculated plants showing straight root hairs. Bar, 50 μm(x100)

C. Root hair with infection thread of plant inoculated with auxotrophic mutant VK38. Bar, 25 μm(x400).

D. Different type of root hair deformation in plant root inoculated with VK27. Bar, 25 μm(x400).

Abbreviation : rh-root hair, it-infection thread

penetration by rhizobia early in the infection process. The uninoculated plant root hairs were seen to be straight (Plate 3: B). These results indicated that all these purine auxotrophs were effective in early stages of root hairs infection of alfalfa plants.

4.7 HISTOLOGICAL STUDIES OF NODULES

4.7.1 Light and Electron Microscopic Studies of Nodules Induced by the Parental Strain *S. meliloti* Rmd201

To determine the effect of purine auxotrophic mutation in the morphology and developmental feature of plant nodules induce by these auxotrophs, light and electron microscopic TEM studied were carried out.

Light microscopic studies of longitudinal cross section of nodule induced by the parental strain Rmd201 revealed that these nodules are elongated cylindrical shapes. From the distal (apical) to the proximal (basal) region of nodule four distinguished zones were observed, apical meristematic zone, infection zone, nitrogen fixation zone and senescent zone (Plate 4: A). Except for apical meristematic zone, lateral nodular tissues such as parenchyma (i.e., the inner cortex) surrounded the other three zones. Also from the section we can observed the elongated nitrogen fixation zone and reduce senescence zone in the basal part of nodule. Some variation may occur depending on the strain of *S. meliloti* and variety of alfalfa plants.

The meristematic zone contained constantly dividing uninfected cells isodiametric in shape with large single nucleus and numerous small vacuoles, mitochondria and other granules. This region is devoid of rhizobia (Plate 4: A, B). Next to this is, infection zone that can be seen with cells increased in size (Plate 4: A & Plate 5: A). The infection threads are common in this region. The nitrogen fixation zone

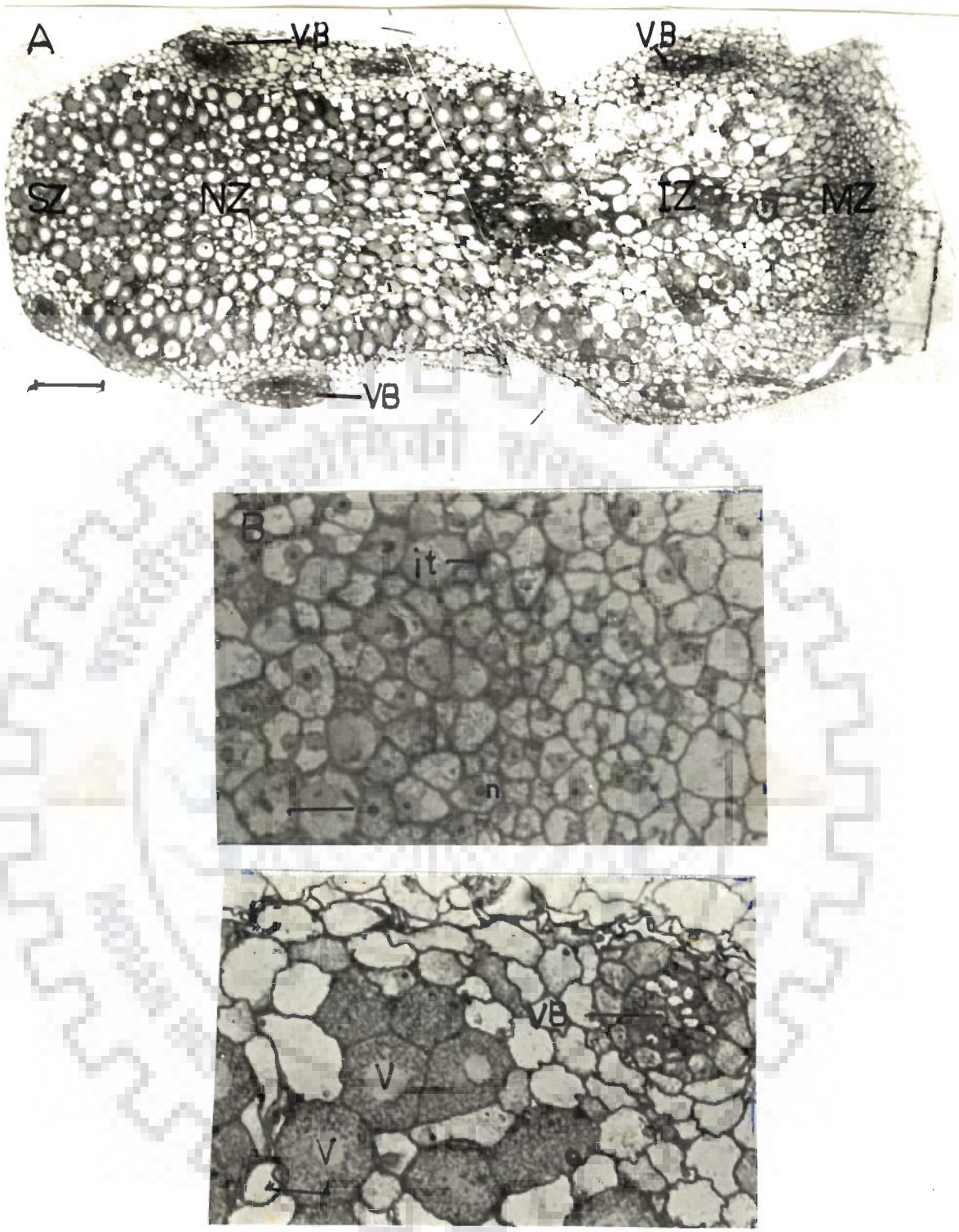

Plate 4: Light microscopic studies of longitudinal and semithin section of nodule induced by *S. meliloti* Rmd201.

A. L.S of nodule showing meristematic zone (M), infection zone (IZ), nitrogen fixation zone (NZ), senescence zone (SZ), and peripheral vascular bundle (VB). Bar, 100 μm(x100).

B. Nodule section in meristematic zone (M) and pre-infection zone showing nuclei (n) with nucleolus and infection thread (it). Bar, 25 μm(x400).

C. A part of nodule showing nitrogen fixation zone: Infected cells filled with bacteriods (bc) and central vacule (V) alongwith empty cells and vascular bundle (VB). Bar, 25 μm(x400).

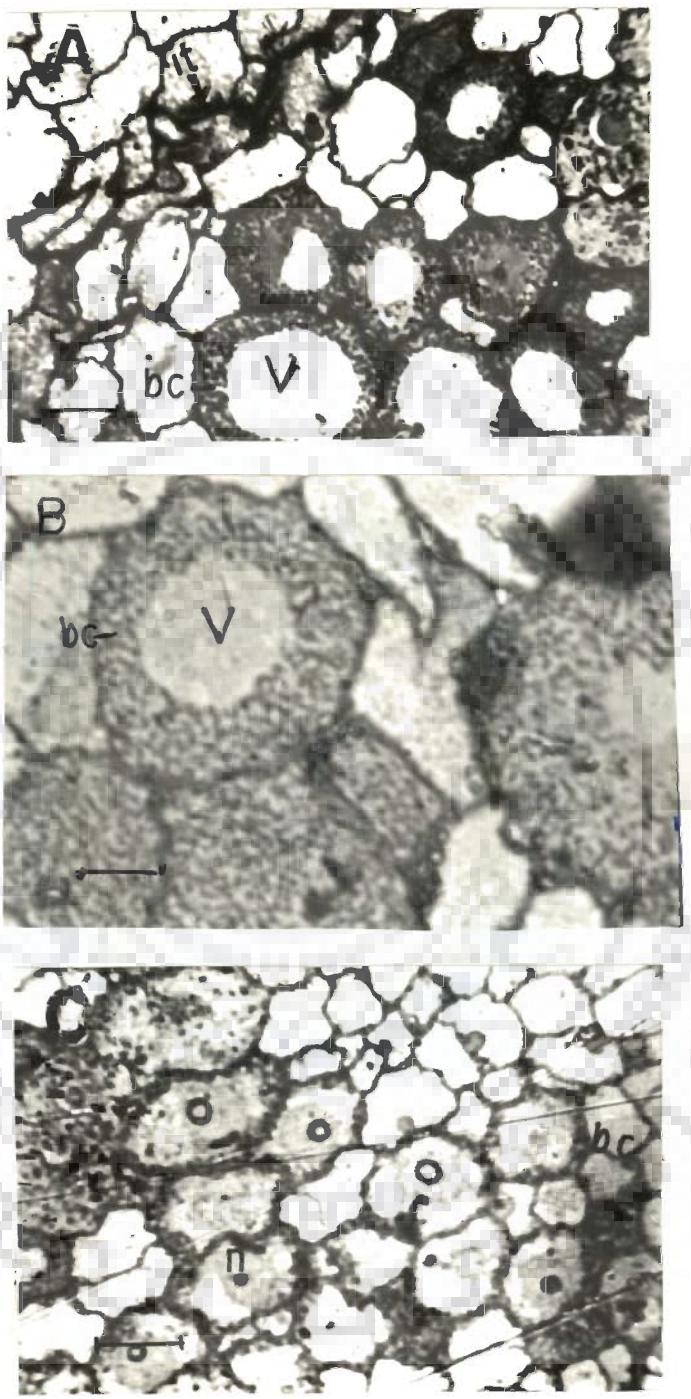

Plate 5: Light microscopic studies of longitudinal and semithin section of nodule induced by *S. meliloti* Rmd201.

A. Nodule section showing infection zone and nitrogen fixation zone with infection thread (it), cells containing mature bacteriods (bc) and empty cells (UC). Bar, 25μm(x400).

B. Nitorgen fixzation zone with cell containing mature bacteriods (bc) and central vacuole (V). Bar, 25 μm(x400).

C. Senescence zone showing cells with degenerated bacteroids (bc) and nuclei (n) Bar, 25 μm(x400).

that occupied the major part of the nodule follows the infection zone. The cells of this region were filled with mature bacteroids, which were organized around the central vacuole in each cell (Plate 4: C & Plate 5: A, B). The basal part of nodule (the senescence zone) which occupied the basal portion of the nodule was very short and contained older nodule cells (Plate 5: C).

Transmission electron microscopic studies (TEM) of nodules showed the presence of infection thread and cells within the infection zone, which were field with rod shaped electron dense bacteroids (Plate 6: A, B and C). The bacteroids within the infection thread and released ones were found to contain clear poly-β-hydroxybutyrate (phb) granules in their cytoplasm. Each freshly released bacteroides were surrounded by host derived peribacteroidal membrane (pbm) which isolate it from the host cytoplasm (Plate 6: B and C). Few small amyloplasts and starch granules were observed along the host cell wall. These were found more frequently near intercellular spaces which related to the healthy of nodules (Plate 6: A & Plate 7: A).

In late infection and nitrogen fixation zone, the nodule cells were seen with mitochondria, RER and Golgi bodies. The bacteroids were elongated and exhibited partial heterogeneous cytoplasm (Plate 7: B, C and D). The plant cells organelles in the uninfected cell as well as in the one filled with bacteroids took the peripheral position near to the cell wall as the centrally located vacuole enlarged (Plate 7: A and B). The mature bacteroids which present in the more proximal part of the nitrogen fixation zone can seen with heterogeneous cytoplasm because of condensation in nucleic acid material (Plate 8: A). The old cells have less RER and Golgi bodies present in their cytoplasm. Due to bacteroidial differentiation a large portion the cell cytoplasm was occupied by the endosymbiont. The last type of bacteroids differentiation took place in

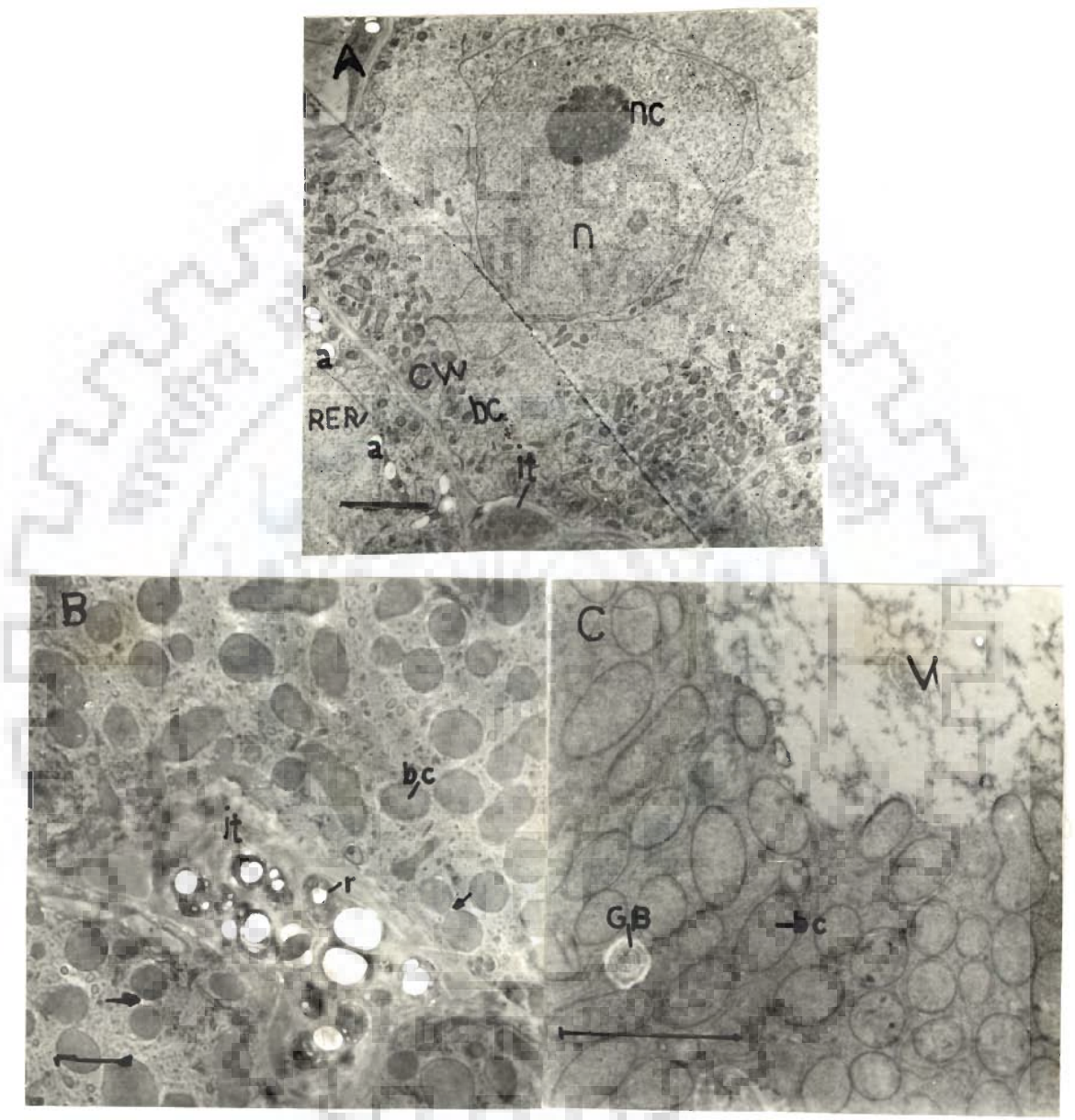

Plate 6: **TEM studies of longitudinal and ultrathin section of a nodule induced by** *S. meliloti* **Rmd201.**

A. Part of nodule cells showing cell wall (CW) and a large member of freshly released bacteriod (bc). Cell cytoplasm also contains numerious rough endoplesmac reticulum (RER), and amyloplasts (a). Bar, 1 μm(x3,480).

B. Part of cells in the infection zone showing cells with bacteriods (bc) and infection thread containing rhizobia (r) with (phb) granules. Cell were seen with newly devided bacteria. Bar, 1 μm(x10,600).

C. Part of cell in infection zone showing freshly released bacteriods with electron dense cytoplasm, around a central vacule (V), Golgibodies (GB) and rough endoplasmic reticulum (RER) could also be seens. Bar 1 μm (x10600).

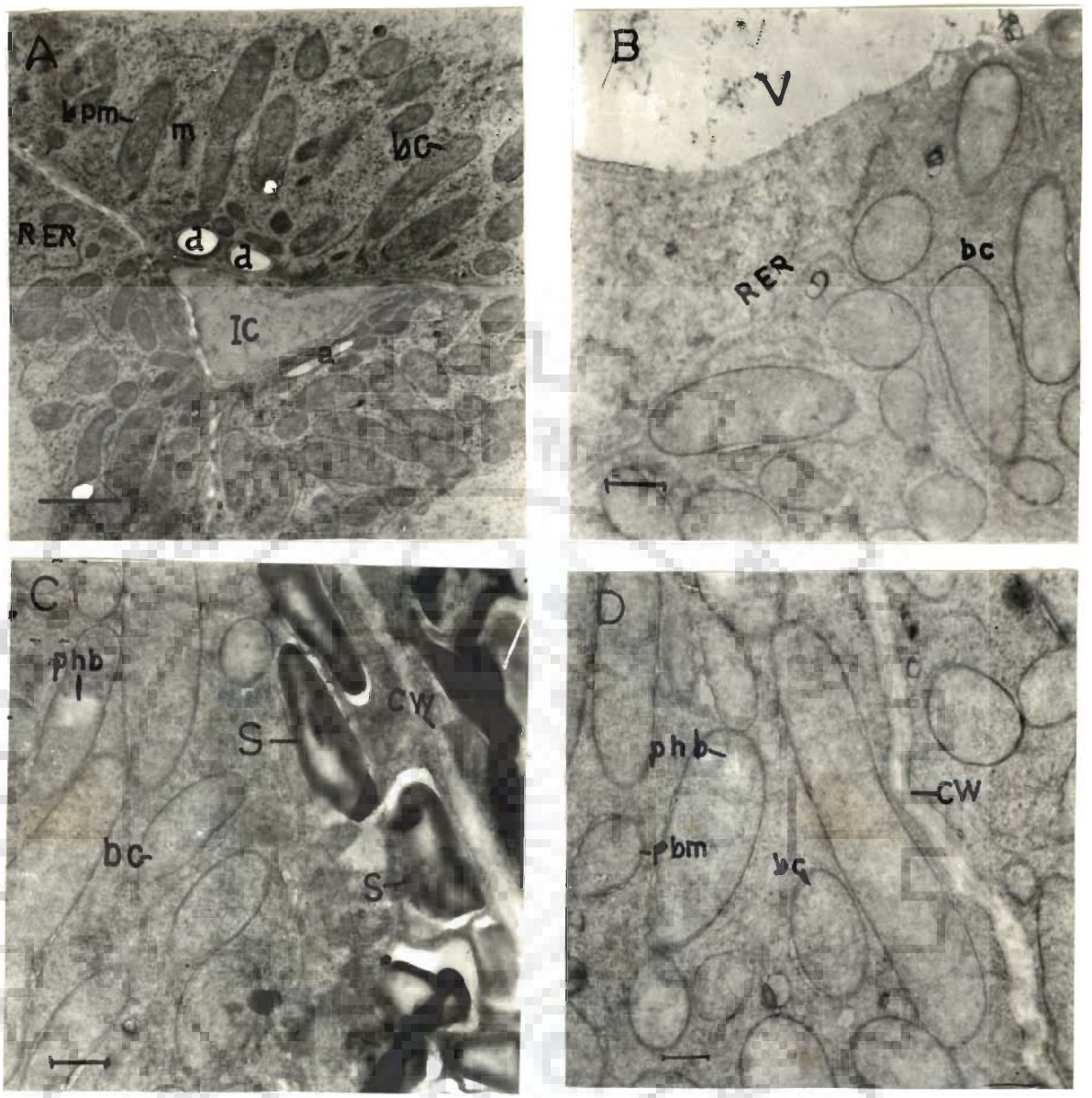

Plate 7: **TEM studies of longitudinal and ultrathin section of a nodule induced by *S. meliloti* Rmd201.**

A. Part of nodule cells showing bacteroids (bc) surrounded by weavy peribacteroidal membrane (pbm), amyloplst (a), rough endoplasmic reticulum (RER) and intracellular space (IC). Bar, 1 μm(x10,600).

B. Part of nodule cells with freshly released bacteroids (bc) around a central vacuole(V) and homogeneous cytoplasm containing rough endoplasmic reticulum (RER). Bar, 1 μm(x13,600).

C. Part of nodule cell showing bacteroids (bc) with (phb) granules and cell cytoplasm with peripheral large starch granules (S) near cell wall (CW). Bar, 1 μm(x13,840).

D. Part of nodule cells from nitrogen fixation zone showing mature bacteroids with homogeneous cytoplasms containing (phb) granules. Rough endoplasmic reticulum (RER), bacteroids with peribacteroidal membrane (pbm) and plant cell wall (CW) are shown. Bar, 1 μm(x18,600).

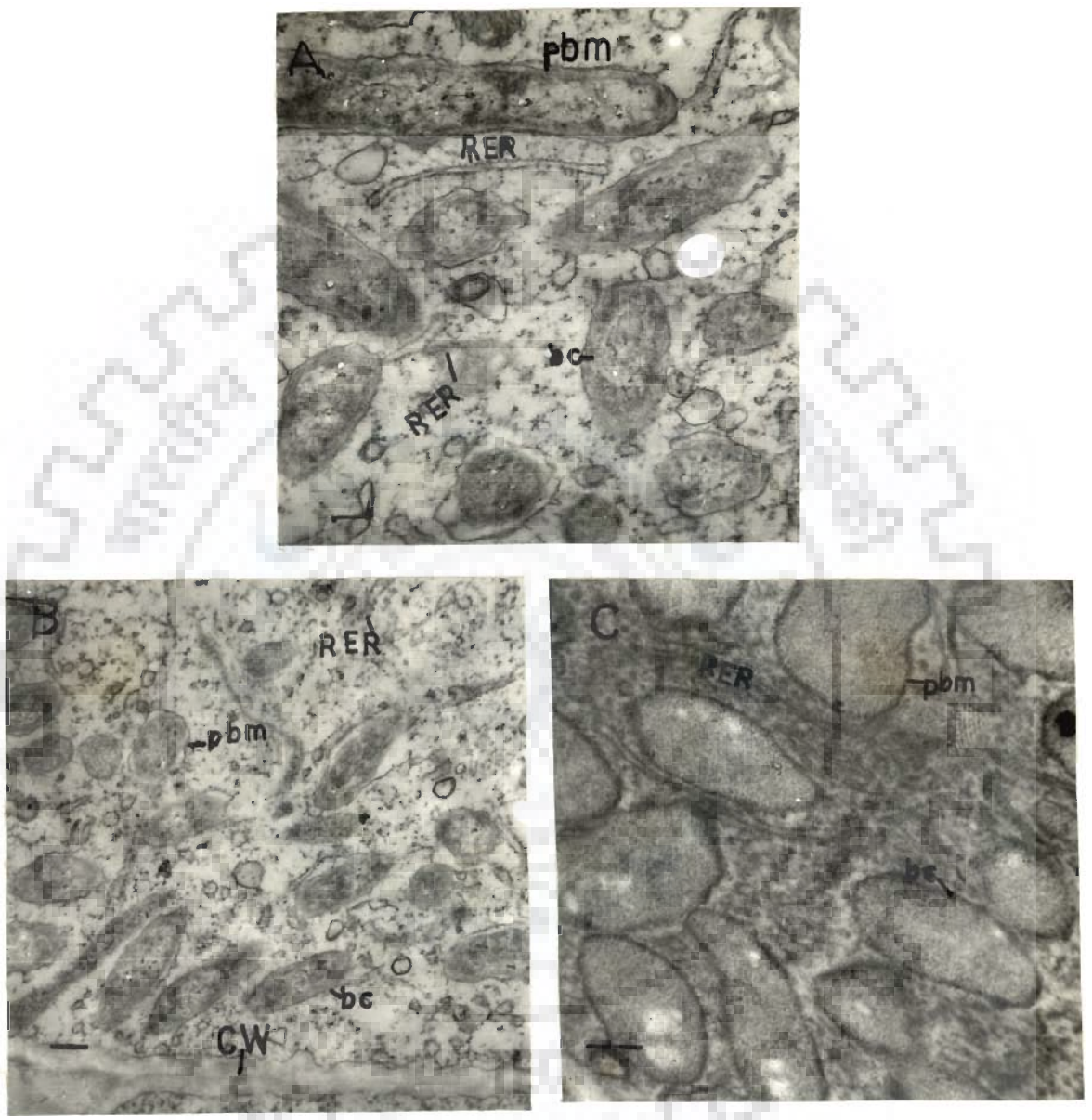

Plate 8: **TEM studies of longitudinal and ultrathin sectionof a nodule induced by *S. meliloti* Rmd201.**

A. Part of nodule cell in late nitrogen fixation and senescence zone showing bacteroids (bc) with heterogenous cytoplasm and some with ruptured peribacteroidal membrane (pbm). Cell cytoplasm is shown with rough endoplasmic reticulum (RER). Bar, 1 μm(x33,600).

B. Part of nodule cell in senescence zone showing bacteroids with heterogenous cytoplasm and ruptured peribacteroidal membrane (pbm). Bar, 1 μm(x18,400).

C. Part of nodule cell in senescence zone showing degenerating bacteroids, the cytoplasm of each bacteroid is electron transparent and broken peribacteroidal membrane (pbm) are also seen in some bacteroids. Bar, 1 μm(x33,600).

the proximal nitrogen fixation zone, which occur near the senescence zone, the bacteroids varied in morphology and showed a progressive loss of cytoplasmic heterogeneity and stained weakly (Plate 8: A, B and C). The bacteroids decrease gradually in number and ghost membranes of the bacteroids were the ultimate result of the senescing process (Plate 8: C). The senesce zone contained degenerated bacteroids which had transparent cytoplasm, The degenerating bacteroids appear to break apart with the confines of peribacteroidal membrane and different degree of deterioration were observed in this zone (Plate 8: A and B).

4.7.2 Light and Electron Microscopic Studies of Nodules Induced by NV2, AL7 and VK38 Mutants

Light microscopic studies of longitudinal section of nodules induced by NV2 mutant that are (adenine + thiamine) auxotroph revealed poorly morphological developed nodules in comparison to the parental strains induced nodules. In light microscopic studies the nodules were seen round in shape instead of elongated, and lacked the four distinct zones that were present in parental strain nodule (Plate 9: A). The nodules were found to have many layers of parenchyma cells around the peripheral region and few layers of meristematic cells with nucleus and number of small vacuoles (Plate 9: B). The poorly developed infection zone with a lot of amyloplasts and infection thread can be seen in the section. A few cells with released bacteroids can be seen in section while the majority of the cells were found to be devoid of endosymbionts (Plate 9: C).

Electron microscopic studies showed that most of cells were without bacteroids and having a large centrally located vacuole and peripheral cytoplasm with number of

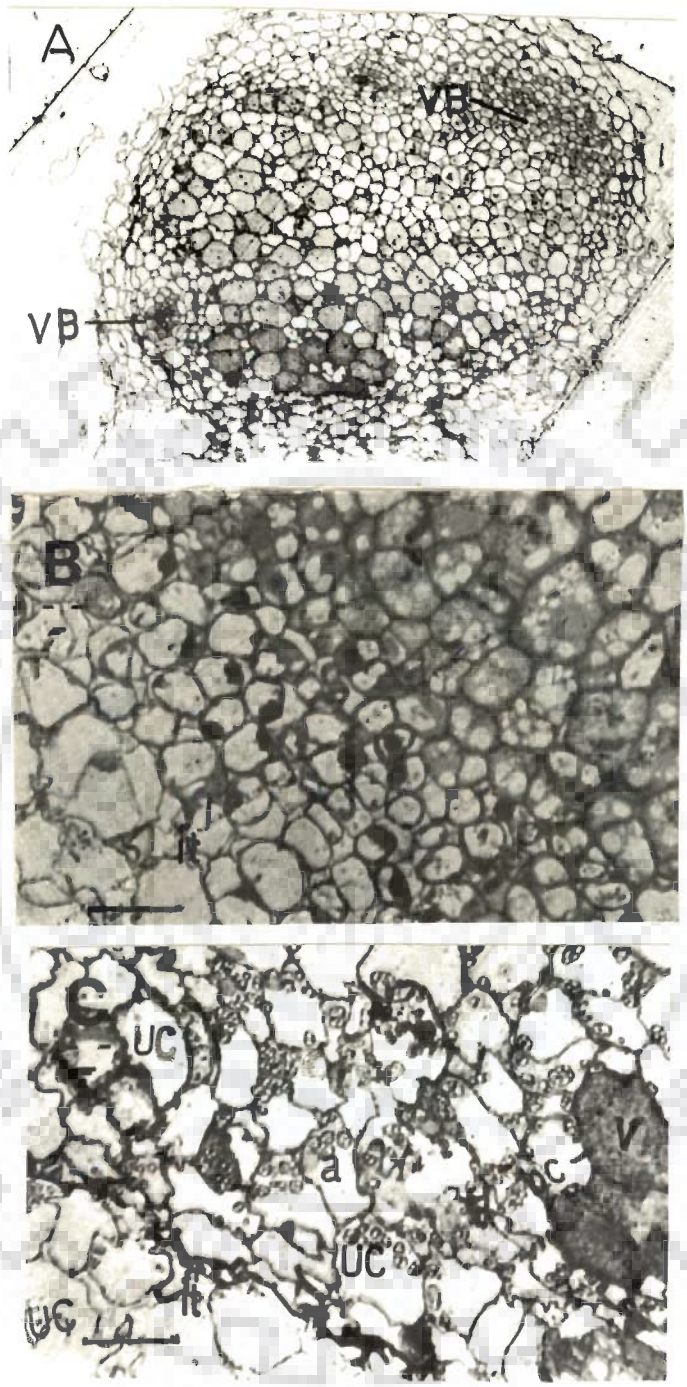

Plate 9: **Light microscopic studies of longituidinal and semithin sections of a nodule induced by the purine auxotroph NV2 of *S. meliloti* Rmd201.**

A. L.S. of whole nodule section showing indistinct zones along with peripheral vascular bundle (VB) and infection thread (it). Bar, 100 μm(x100).

B. Nodule section showing meristimatic zone cells with clear nuclei (n), cytoplasm with number of small vacuoles (V) and infection thread (it). Bar, 25 μm(x400).

C. Nodule section showing infection zone with infection thread (it), uninfected cell (UC) containing number of amyloplasts (a) and some cells filled with bacteroids (bc) around vacuole (V). Bar, 25 μm(x400).

mitochondria (Plate 10: A and B). Occasionally few cells with released bacteroids could also be seen (Plate 10: C). The infection thread with lying rhizobia having poly-β-hydorxybutyrate (phb) granules was also observed in some cells (Plate 10: C). Unlike the parental strain most of the freshly released bacteroids found to be abnormal in shape with electron dense and heterogeneous cytoplasm. It seems that the bacteroids started degradation at this stage.

Light and electron microscopic studies of nodules induced by purine auxotrophs AL7 (having blocked before AICAR-amino imidazole carboxamide riboside) and VK38 (having block before IMP-inosine monophosphate), respectively; also revealed somewhat similar anatomical observation. In general these nodules were irregular or round in shape with peripheral vascular bundles and lack the distinct zones that were present in parental strain induced nodules. The histological features of nodule induced by AL7 and VK38 are presented in (Plate 11: A, B and C & Plate 14: A, B and C), respectively. No significant differences were observed among histological features of nodule induced by AL7 and VK38 as reveled from light and electron microscopic studies. In the sections of nodule induced by the both auxotrophs the majority of cells found to be devoid of bacteroids while a few cells with occasionally released bacteroids were also seen (Plate 11: B, C; Plate 14: B & Plate 15: A). A large amount of amyloplasts were found to be accumulated in these empty cells (Plate 11: C & Plate 14: C). The release of bacteria found to be normal and the bacteroids were seen with peribacteroidal membrane and heterogeneous cytoplasm (Plate 13: A and B & Plate 16: A and B). The infection thread filled with bacteroids containing poly-β-hydroxybutyrate (phb) granules were also seen in some cells (Plate-13: C & Plate 15: B). The majority of bacteroids were abnormal in shape which indicate that further

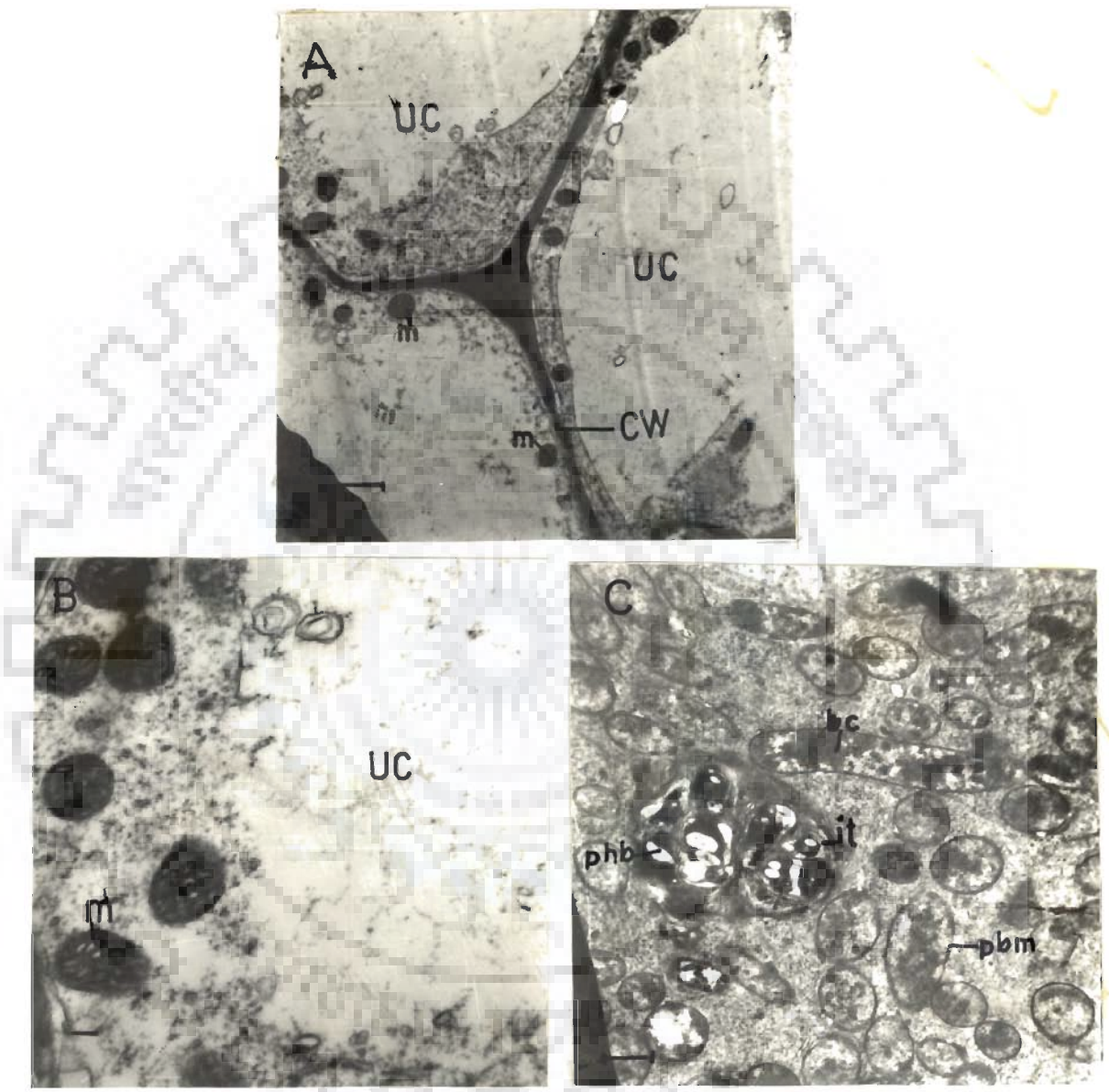

Plate 10: TEM studies of longitudinal and ultrathin section of a nodule induced by the strain NV2, a purine auxotroph of *S. meliloti* Rmd201.

A. Part of uninfected nodule cells (UC) showing central vacuole (V), cell wall (CW) and mitochondria (m). Bar, 1 μm(x10,600).

B. Part of uninfected cell (UC) nodule under higher magnification showing central vacuole (V) and peripheral mitochondria (m). Bar, 1 μm(x33,600).

C. Part of cells filled with bacteroids (bc) and infection thread (it) with bacteroids containing poly-β-hydroxybutyrate granules (phb). The cytoplasm of released bacteroids found to be electron transparent and peribacteroidal membrane (pbm) of many bacteroids were ruptured. Bar, 1 μm(x13,600).

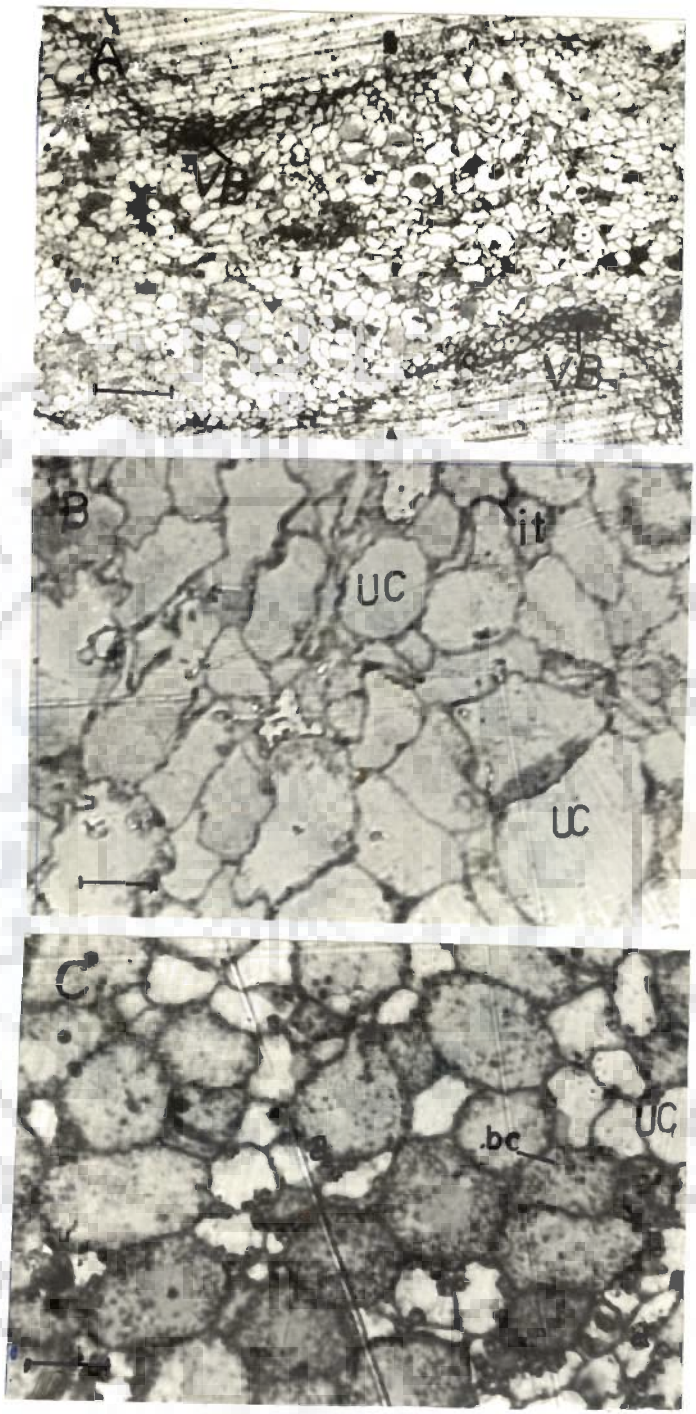

Plate 11: Light microscopic studies of longituidinal and semithin section of nodule induced by the strain AL7, a purine auxotrophs of *S. meliloti* Rmd201.

A. L.S of whole nodule showing indistinct zones with peripheral vascular bundle (VB). Bar, 100 μm(x100).

B. Nodule section showing pre-infection zone with infection thread (it) and uninfected cells (UC). Bar, 25 μm(x400).

C. Nodule section showing poorly developed infection zone with uninfected (UC) containing amyloplasts (a) and infected cells with released bacteroids (bc). Bar, 25 μm(x400).

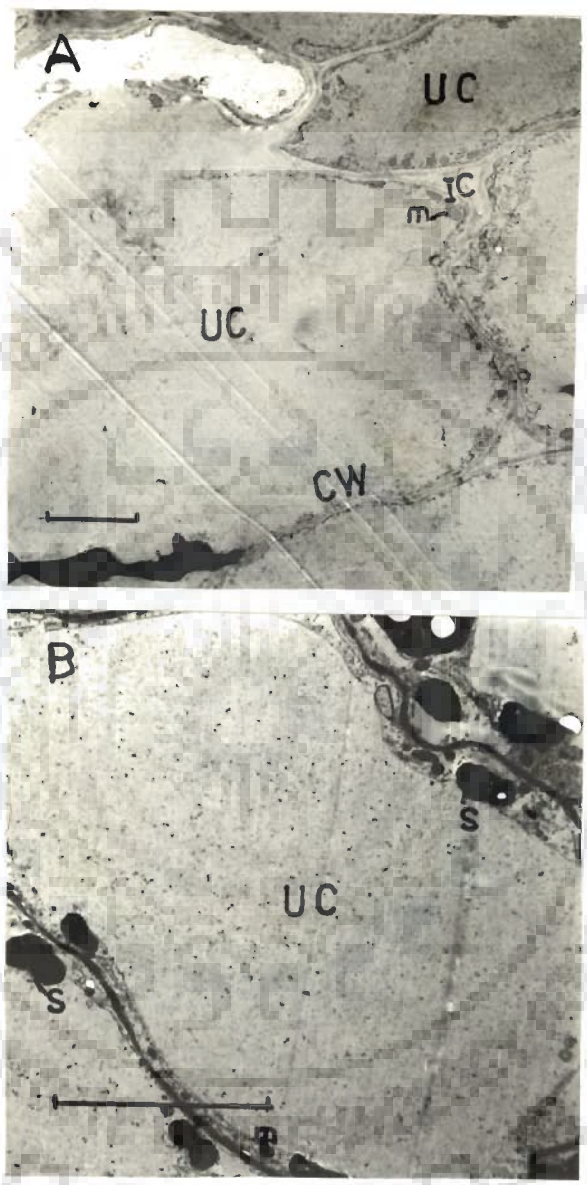

Plate 12: TEM studies of longitudinal and ultrathin section of a nodule induced by the strain AL7, a purine auxotroph of *S. meliloti* Rmd201.

A. Part of uninfected nodule cells (UC) showing central vacuole, peripheral mitochondria (m), intercellular spaces and cell wall (CW). Bar, 1 μm(x3,480).

B. Part of uninfected nodule cells (UC) under higher magnification showing large starch granules (S) and mitochondria (m) within peripheral cytoplasm. Bar, 1 μm(x3,480).

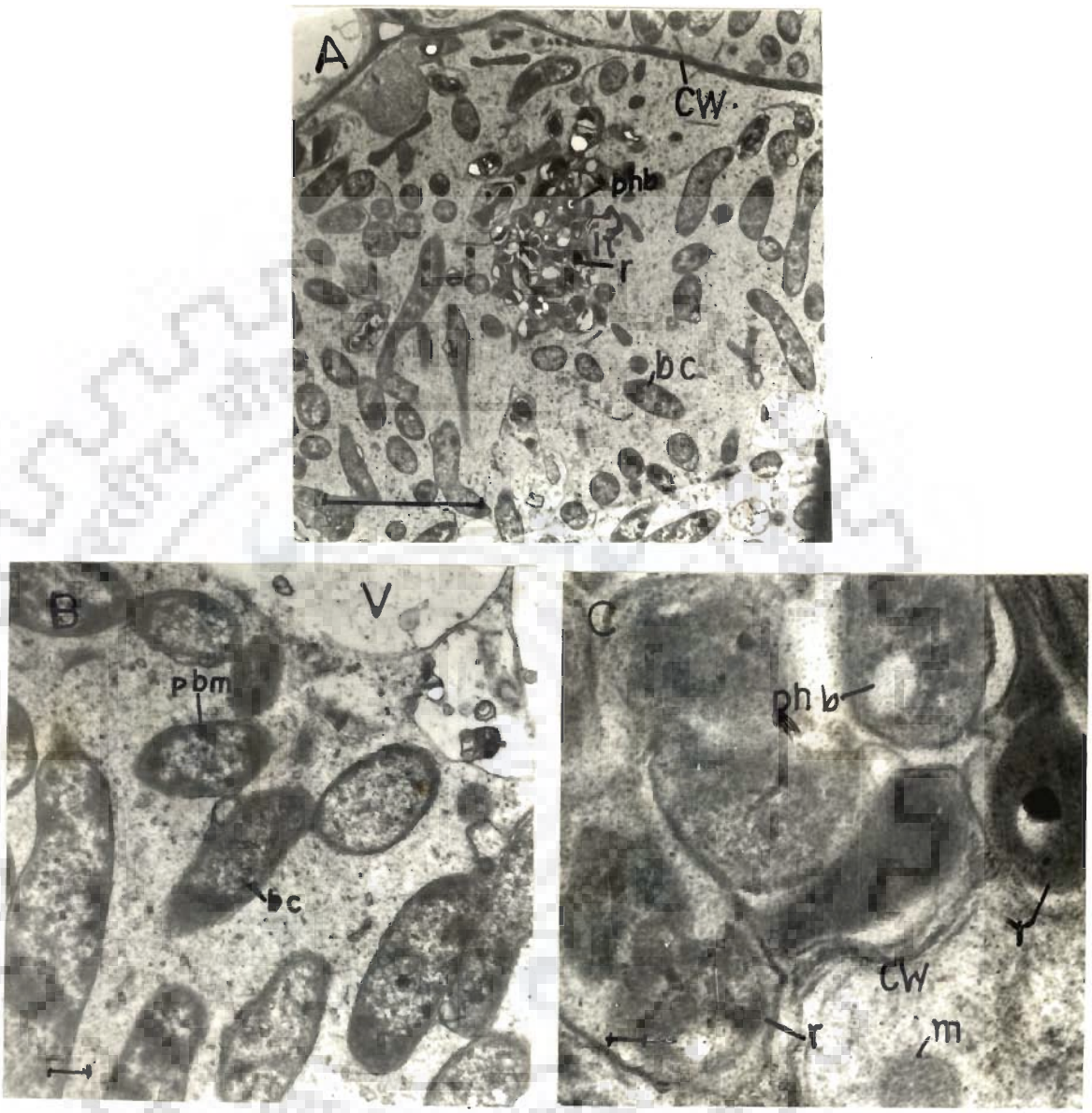

Plate 13: **TEM studies of longitudinal and ultrathin section of a nodule induced by the strain AL7, a purine auxotroph of *S. meliloti* Rmd201.**

A. Part of uninfected nodule cell showing cell cytoplasm filled with poorly developed bacteriods with heterogenous cytoplasm. Infection thread (it) with rhizobia (r) containing phb granules and plant cell wall (CW) are seen. Bar, 1 μm(x4,600).

B. Part of nodule cell showing degenerating bacteroids (bc) around central vacuole (V). Bar, 1 μm(x18,400).

C. Part of nodule cell in infection zone showing infection thread containing rhizobia (r) with phb granules under higher magnification Mitochondria in the adjacent cells could be easily seen. Bar, 1 μm(x25,000).

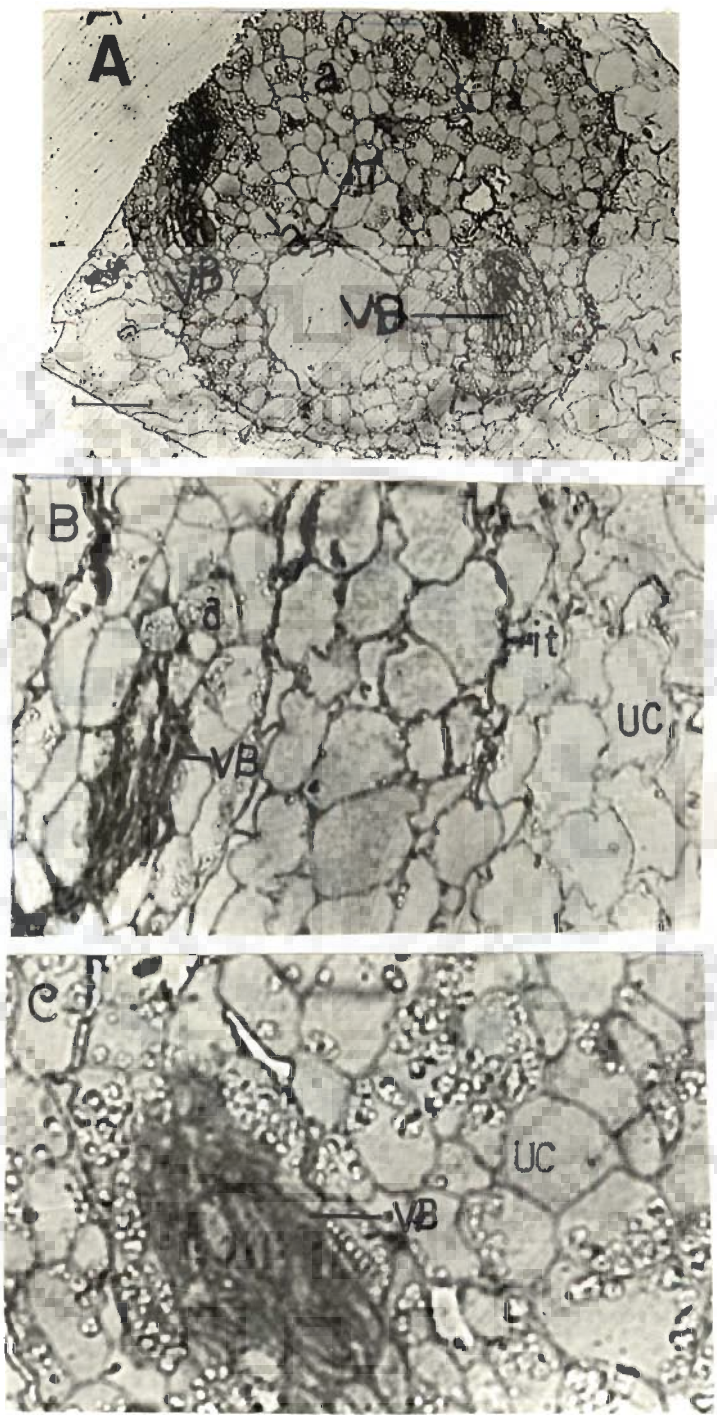

Plate 14: **Light microscopic studies of longituidinal and semithin sections of nodule induced by the strain VK38, a purine auxotrophs of *S. meliloti* Rmd201.**

A. L.S of whole nodule showing poorly developed indistinct zones with peripheral vascular bundles (VB) and uninfected cells (UC) containing number of amyloplasts (a). Bar, 100 μm(x100).

B. Nodule section showing infection zone with infection thread (it). Uninfected cells containing number of amyloplasts (a) and vascular bundle (VB) are also shown. Bar, 25 μm(x400).

C. Nodule section showing uninfected cells (UC) with aggregated amyloplasts (a) and peripheral vascular bundles (VB). Bar, 25 μm(x400).

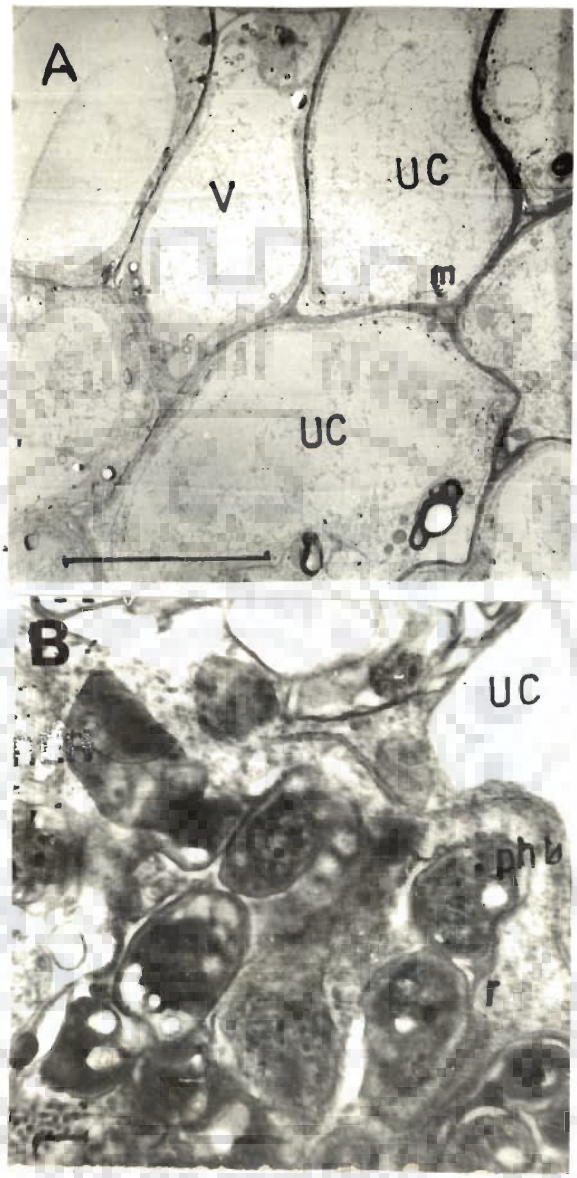

Plate 15: TEM studies of longitudinal and ultrathin section of a nodule induced by the strain VK38, a purine auxotroph of *S. meliloti* Rmd201.

A. Part of uninfected nodule cells with peripheral cytoplasm, central vacule (V) and mitochondria (m). Bar, 1 μm(x3,480).

B. Part of nodule in the infection zone under higher magnification showing infection thread with rhizobia containing clear (phb) granules and uninfected cell (UC). Bar, 1 μm(x18,400).

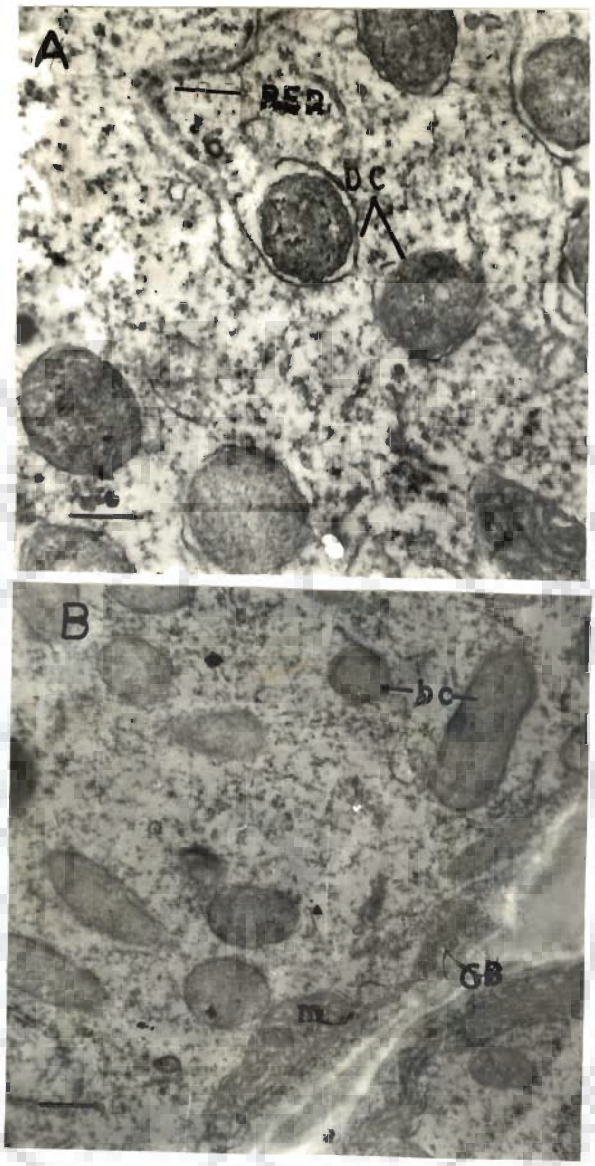

Plate 16: TEM studies of longitudinal and ultrathin section of a nodule induced by the strain VK38, a purine auxotroph of *S. meliloti* Rmd201.

A. Part of nodule cell containing released bacteroids (bc) with clear peribacteroidal membrane (pbm) and heterogeneous cytoplasm, alongwith rough endoplasmic reticulum (RER) and mitochondria (m). Bar, 1 μm(x33,600).

B. Part of nodule cell showing degrading bacteroids. Some bacteroids with electron transparent cytoplasm and ruptured peribacteroidal membrane could be seen. Cell organelles i.e. mitochondria (m) and golgi bodies (GB) were found to be present in cell cytoplasm. Bar, 1 μm(x18,400).

development of bacteroids were impaired. Large periplasmic space was observed between slightly convoluted inner and outer membranes (Plate 16: A). Cell organelles i.e. mitochondria, rough endoplasmic reticulum RER etc. were observed in the cell cytoplasm. The released bacteroids started degrading and could be seen with transparent cytoplasm and ruptured peribacteroidal membrane (pbm) (Plate 16: B).

4.7.3 Light and Electron Microscopic Studies of Nodule Induced by the Mutant VK27

The histological studies of nodule induced by VK27 showed some what advanced features as compared to other auxotrophs (NV2, AL7 and VK38), induced nodules described before. The nodules induced by VK27 were also irregular in shape and the four clear distinct zones present in parental strain were not observed. However, in these nodules poorly developed infection, nitrogen fixation and senescent zone along with peripheral vascular bundles could be seen in the nodule section (Plate 17: A). Infection threads of some cells with prominent nuclei and amyloplasts were observed in the section (Plate 17: B). Infection and nitrogen fixation zones were observed with few-aggregated cell along the cross section unlike the parental strain induced nodules. Only a few cells were found filled with bacteroids, which were organized around a large central vacuole (Plate 17: A and C). The senescent zone could be seen in the basal part of the nodule (Plate 17: A). Electron microscopic study of sections of poorly developed infection zone showed the presence of uninfected cells with large central vacuole, peripheral cytoplasm, several mitochondria and starch granules (Plate 18: A and B). Infection thread could also be seen with layed rhizobia.

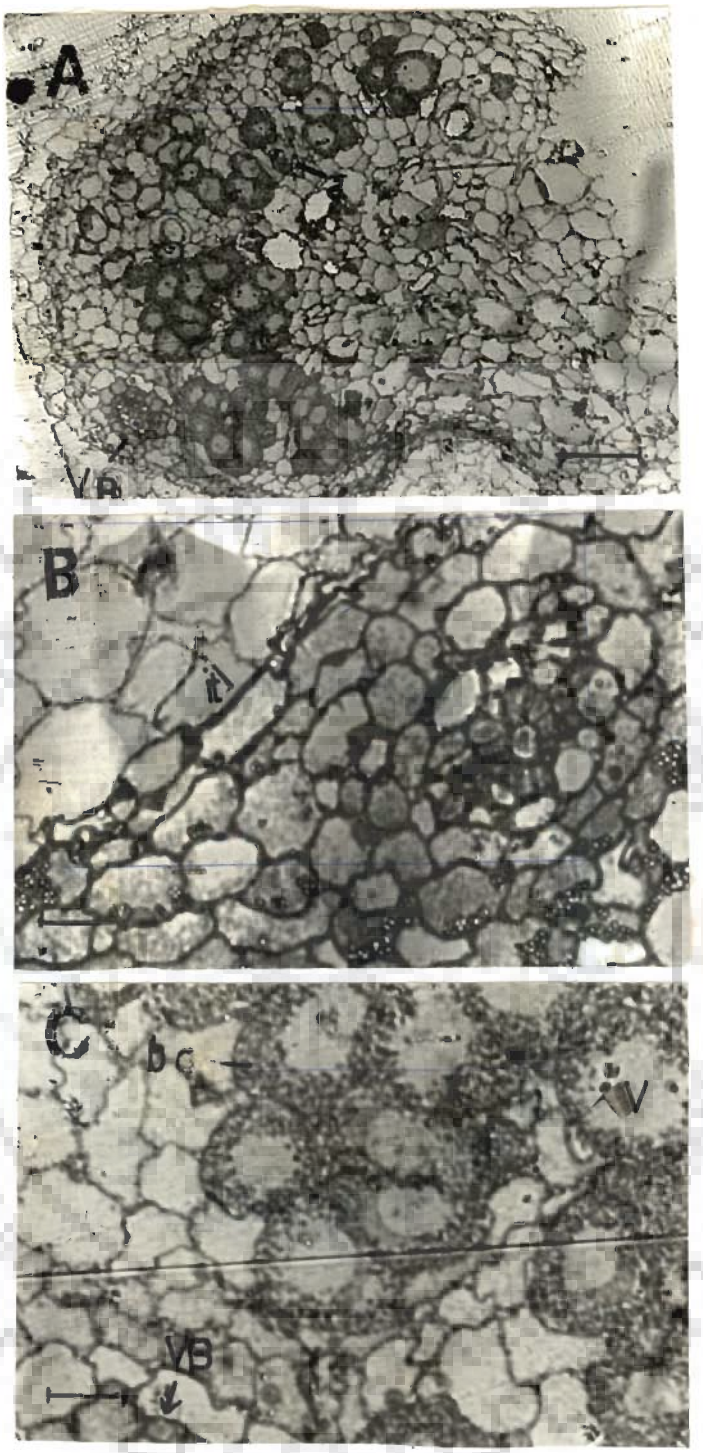

Plate 17: Light microscopic studies of longituidinal and semithin section of nodule induced by the strain VK27, a purine auxotrophs of *S. memiloti* Rmd201.

A. L.S of whole nodule showing poorly developed zones, infected cells filled with bacteroids (bc) around central vacuole (V), vascular bundle (VB) and uninfected cells containing numerous amyloplasts (a). Bar, 100 μm(x100).

B. Nodule section showing infection zone wih infection thread (it) passing across the section, some cells containing amyloplasts (a) and vascular bundle (VB). Bar, 25μm(x400).

C. Nodule section showing poorly developed nitrogen fixation zone with empty cells (UC) and some cells filled with bacteroids (bc) around central located vacuoles (V). Bar, 25μm(x400).

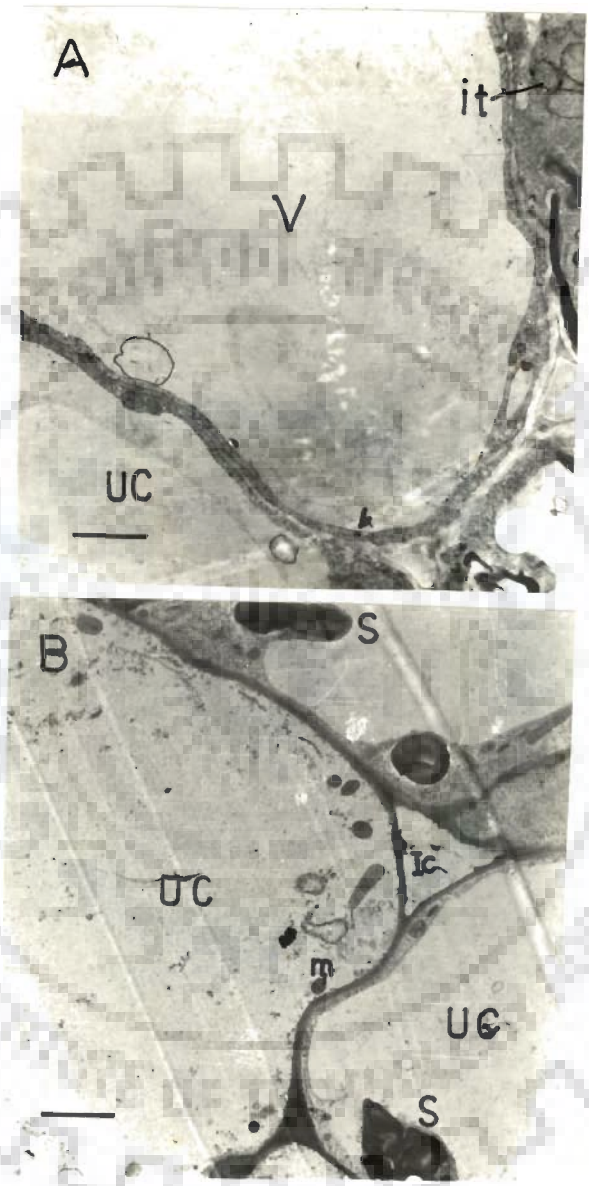

Plate 18: TEM studies of longitudinal and ultrathin section of a nodule induced by the strain VK27, a purine auxotroph of *S. meliloti* Rmd201.

A. Part of nodule uninfected cells (UC) in early infection zone with central vacuole (V) and infection thread containing rhizobia (r). Bar, 1 μm (6,200).

B. Part of uninfected nodule cells (UC) showing large starch granules (S) and mitochondria (m) toward periphery near cell wall (CW). Bar, 1 μm(x4,600).

In some cells of the nodule freshly released bacteroids were seen with a wavy peribacteroidal membrane (pbm), poly-β-hydroxybutyrate granules (phb) and heterogeneous cytoplasm as seen in the nodule induced by parental strain. Whereas in some other cells the bacteroids were seen with electron dense and heterogeneous cytoplasm containing phb granules (Plate 19: B). Large periplasmic spaces with irregular peribacteridal membrane were observed between the inner and outer membranes reflecting abnormal feature of bacteroids (Plate 19: B). The further development of bacteroids seem not normal as lesser number and mixed population were present compared to densely packed elongated bacteroids in parental strain induced nodules. The majority of bacteroids were of abnormal shapes and poorly organized around central vacuole (Plate 19: C) and the degrading bacteroids with clear transparent cytoplasm and ruptured peribacteroidal membrane (pbm) could be observed (Plate 19: D).

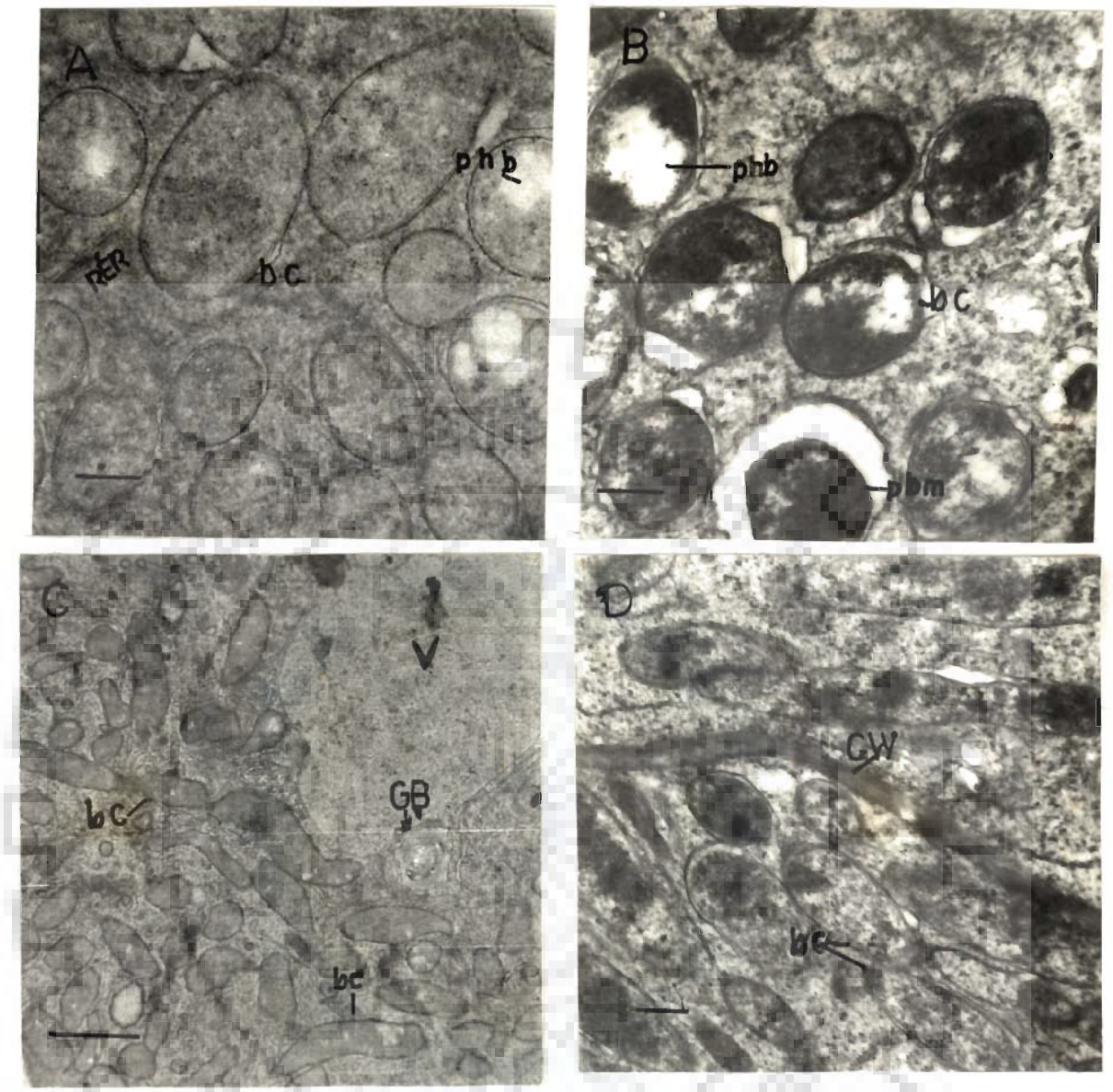

Plate 19: TEM studies of longitudinal and ultrathin section of a nodule induced by the strain VK27, a purine auxotroph of *S. meliloti* Rmd201.

A. Part of infected nodule cell filled with released bacteroids containing phb granules and surrounded by peribacteroidal membrane phb. Besides bacteroids some RER were also seen. Bar, 1μm(x25,200).

B. Part of nodule cell filled with released bacteroids (bc) with phb granules and heterogeneous cytoplasm. These bacteroids are of round shape and enclosed by peribacteroidal membrane (pbm) with unusual large periplasmic space. Bar1 μm(x33,600).

C. Part of nodule cell showing cell filled with mix population of poorly developed abnormal shaped bacteroids (bc) with electron transparent cytoplasm around a central vacuole (V). A number of cell organelles such as RER and golgi bodies are present in cell cytoplasm. Bar, 1 μm(x18,400).

D. Part of nodule cell in senscence zone showing abnormal shaped degenerating bacteroids (bc) with electron transparent cytoplasm and ruptured peribacteroidal membrane (pbm). Bar, 1 μm(x18,400).

Table 11 : Comparison of Phenotypic Characteristics of Purine auxotrophs of *Rhizobium Leguminosarum* bivoar *Phaseoli* (Noel *et al* 1984, 1988) and *S. meliloti* mutants used in this study.

Strains	Phenotypic characteristics	References
R. Leguminosarum bv. *phaseoli*		
CE3	Sm^r NdV^+ Fix^+	Noel *et al* 1984
CE 106	Pur-106::Tn5 Sm^r Ndv^-	”
CE 107	Pur-107::Tn5 Sm^r Ndv^-	”
CE 110	Pur-110:: Tn5 Sm^r Ndv^-	”
CE 115	Pur-115::Tn5 Sm^r Ndv^-	”
CE 122	Pur-122:: ery-1 Ndv^-	Noel *et al.* 1988
CE 124	Pur-124::Tn5 Ndv^-	”
CE 170	Pur-170::Tn5 Ndv^-	”
CE 165	Pur-165::Tn5 Ndv^-	”
S. meliloti		
Rmd201	Sm^r Nod^+, Fix^+	Use in this study
VK 27	Pur-27 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
VK 37	Pur-37 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
VK 38	Pur-38 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
VK 40	Pur-40 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
H 13	Pur-13 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
H 19	Pur-19 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
AL 3	Pur-3 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
AL 7	Pur-7 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
NV 10	Pur-10 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
NV 28	Pur-28 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
NV 24	Pur-24 ::Tn5 Sm^r Km^r Nod^+ Fix^-	”
NV 2	Pur-2 ::Tn5 Sm^r Km^r Nod^+ Fix^-	

Abbreviations

Km = kanamycin, Sm = streptomycin ery: erthomycin, Ndv: nodule development

Chapter 5

DISCUSSION

Establishment of normal symbiosis between rhizobial strains and its legume hosts involves complex physiological and biochemical interactions. A number of plant and bacterial genes are involved in this process. These include the genes responsible for nodule developmental (*nod*), nitrogen fixation (*nif and fix*), and host range determination (*hsn*) genes (Fischer, 1994). Besides, *nif, fix and nol* genes, the metabolic pathway and their intermediates are found to indirectly influence symbiosis. The useful information regarding the role of a particular metabolic pathway (intermediate /enzyme and its genes) were obtained from the studies of auxotrophic mutants (nutritional deficiency). Mutation (auxotrophy) sometimes results in loss of symbiotic effectiveness. The degree of association between auxotrophy and symbiotic effectiveness varies with the type of mutation and with *Rhizobium* species. It has been reported that nitrogen based (purine and pyrimidine) auxotrophs of several *Rhizobium* species including *S. meliloti* are defective in symbiosis (Scherrer and Denarie, 1971; Kim *et al.*, 1988; Kerppola and Kahn, 1988[b]; Swamynathan and Singh, 1992; Djordjevic *et al.*, 1996; Stevens *et al.*, 2000). Less is known regarding interactions, which occur in the subsequent stages of nodule development particularly when rhizobia are released from infection thread into host cell and change from vegetative into nitrogen fixing bacteroids. In present study an attempt has been made to understand the role of purine metabolic pathway in nodule development and nitrogen fixation in alfalfa plant by obtaining purine auxotrophic mutants of *S. meliloti*.

Due to several advantages over chemical and physical method and easy selection, random transposon mutagenesis using Tn5 delivery vector was used to isolate purine auxotrophs. Random mutagenesis using Tn5 delivery vector pGS9 gave kanamycin resistant derivatives of *S. meliloti* strain Rmd201. The percentage frequency of auxotrophs obtained was upto 0.54%. Auxotrophs were presumably Tn5 induced since the spontaneous

kanamycin resistance frequency was very low of the order 10^{-8} and similar frequency of mutagenesis has also been reported in other studies (Pain, 1979; Meade *et al.*, 1982; Ali *et al.*, 1984; Kim *et al.*, 1988; Vineetha, 1998). Nutritional requirements of the auxotrophic mutants showed the randomness of Tn5 insertion. Most of purine auxotrophs isolated were defective in single nutritional requirement (VK27, VK37, VK38, VK40, RH19, RH13, AL3, NV10, NV28 and AL7) that for adenine, except (NV2 and NV24) mutants which had double nutritional requirement both for adenine and thiamine (Fig.6). An attempt was made to determine the position of biochemical block in each purine auxotroph by intermediate and cross feeding assay. Intermediate feeding test showed that purine auxotrophs (NV2, NV24) could grow only on adenine + thiamine supplemented minimal media. The location of biochemical block in these mutants is likely to be at any one of the five steps before AIR (amino imidazole riboside). Purine auxotrophs (NV10, NV28 and AL7) could grow in AICAR supplemented minimal medium. The defects in these mutants may be it any one of the steps between AIR and AICAR. Purine auxotrophs (VK37, VK38, VK40, RH13 and AL3) and (VK27 and RH19) could grow in minimal media supplemented with inosine and adenine, respectively. This shows that the location of biochemical blocks in these mutants was at any one of the two steps between AICAR and IMP and between IMP and AMP, respectively (Fig. 6). It seems that the defects in these mutants may due to mutation in the genes of enzymes that are related to synthesis of intermediates in purine pathway.

An interesting feature of the rhizobial purine biosynthetic pathway which is supported by the present study and also from earlier studies, is that the pathway for conversion of GMP to IMP is absent in *S. meliloti* while the pathway from AMP to IMP is present (Neuhard and Nygaard, 1987). This was evident from the fact that the addition of guanine or guanosine to RMM minimal media could not support the growth of these mutants

while adenine could do so. These results were in agreement with those of other earlier studies (Noel *et al.*, 1988; Swamynathan and Singh, 1992).

Spontaneous excision of Tn5 took place in some cells of each auxotroph leading to formation of kanamycin sensitive prototrophic revertant cells. Prototrophic revertants of all purine auxotrophs formed normal nitrogen fixing nodule as the parental strain. However, the frequency of reversion differs from one type of auxotrophs to another. These results show that symbiotic defects of each purine auxotroph can be attributed to the insertion of transposon Tn5 in one of the purine biosynthetic pathway genes. This further indicates that single mutation was responsible for purine auxotrophy and their symbiotic defects because the prototrophic revertants of these mutants are found to have symbiotic characteristics similar to that of parental strain induced nodules.

Production of cell surface molecules like exopolysaccharide, lipopolysaccharide, cellulose fibrils and β(1-2) glucans are essential for successful symbiosis and were found to play significant role in the initial steps of infection process. The mutants isolated in present study seem normal and similar to the parental strain Rmd201 in the production of exopolysaccharides, lipopolysaccharides and cellulose fibrils except for β(1-2) glucans production. Less motility in swarm plates shows that there is deficiency in β(1-2) glucans production in these mutants. Auxotrophic mutants defective in β(1-2) glucans production have also been reported earlier (Geremia *et al.*, 1987; Dylan *et al.*, 1990[a]). Normal root hair curling, root hair deformation and infection thread formation in alfalfa plants inoculated by these mutants were similar to the parental strain inoculated plants, which shows that early

infection process seems to be normal in these mutants. However, the delay in nodule initiation could be due to less motility of these mutants, which reduced their infection ability.

Similar to parental strain, all purine auxotrophs were able to utilize dicarboxylic acid as a carbon source. The utilization of dicarboxylic acid by bacteroids is found to play an important role in the establishment of successful symbiosis process (Engelke *et al.*, 1987; Boesten *et al.*, 1998). It seems that the symbiotic defectiveness of the present purine auxotrophs does not appear to be due to the absence of any cell surface molecules or inability to transport dicarboxylic acid.

These mutants were found to be symbiotically defective since they produce white and round or irregular shaped nodules unlike parental strain induced nodules, which are pink in colour and cylindrical in shape. Significant differences were obtained in shoot height, shoot dry weight and nitrogen content between the purine auxotrophs and parental strain inoculated plants. The differences were also seen in time of nodule appearance. Time of nodule appearance was relatively delayed in purine auxotrophs compared to parental strain Rmd201 inoculated plants (Table-8). These observations reflect the ineffectiveness of these mutants to fix nitrogen (Fix^-) in alfalfa plant. Purine auxotrophs with defective symbiotic features have also been reported earlier from different *Rhizobium* species (Scherrer and Denarie, 1971; Kerppola and Kahn 1988[b]; Swamynathan and Singh 1992; Gupta, 1996).

Exogenous supplementation of adenine and other intermediates to plant growth medium showed no discernable effect on either nodule development or restoration of symbiotic efficiency of the purine auxotrophs used in the present study. The failure in restoration of the symbiotic features by exogenous supplementation in *S. meliloti* has also been reported earlier (Swamynathan and Singh, 1992). Purine auxotrophs with symbiotic defects have been observed in numerous studies using rhizobia with different host ranges. In

contrast to *S. meliloti*-Alfalfa symbiosis (Nod^+, Fix^-) (Dickeston et al, 1991, Kerppola and Kahn 1988[b]; Swamynathan and Singh 1992), in general purine auxotrophs appear specifically to be defective in infection process (Nod^-, Fix^-) (Table-11) in other *Rhizobium* species (Pankhurst and Schwinghamer, 1974; Djordjevic *et al.*, 1988; 1996; Noel *et al.*, 1988; Newman *et al.*, 1994). It has been suggested that failure of purine auxotrophs in infection does not seem due to requirement for purine, because they grow in the presence of root exudate from pea and bean. Exogenous addition of purine does not restore the symbiotic defect (Pankhurst and Schwinghamer, 1974; Noel *et al.*, 1988; Newman *et al.*, 1994). However, exogenous feeding of purine intermediates such as (AICA) riboside and AICAR were found to restore infection (Nod^+) by these auxotrophs on bean, pea and soybean plants (Noel *et al.*, 1988; Newman *et al.*, 1992; Newman, 1995; Djordjevic *et al.*, 1996). It has been suggested that the ability of rhizobia to produce AICA and AICAR is important for the initiation, sustained infection thread growth and subsequent nodule development (Noel *et al.*, 1988; Newman *et al.*, 1992; 1994; 1995). Newman and his coworker (1994) further proposed that AICAR is diverted from purine pathway and metabolized in a manner that is central to the effective developmental of nodule (Newman *et al.*, 1992). In contrast, exogenous supplementation of purine or its specific intermediates in *S. meliloti*-alfalfa symbiosis could not confirm the precise role and seems relatively difficult due to Nod^+ Fix^- nature compare to other species which are Nod^- Fix^-.

Various possibilities by which purine auxotrophs can influence symbiosis have been explained earlier (Pankhurst and Schwinghamer 1974; Noel *et al.*, 1988; Swamynathan and Singh, 1992). The inability to synthesize cytokinin has been suggested in one of the explanation. It has been suggested that adenine acts more as a growth promoter, since cytokinin which stimulates polyploid mitosis (an essential feature of nodule development) is

synthesized from AMP (Morris and Powell, 1987). Since the purine auxotrophs of *S. meliloti* form nodule in alfalfa plant, preclude this possibility. Other alternative explanation suggests that mutant bacteria are unhealthy because of insufficient adenine availability to them. However, exogenous supply of adenine and other intermediates to plant growth substrate could not restore effectiveness. It seems that either exogenously supplied intermediates are not accessible to the auxotrophs in the nodule or not taken up by these mutants in symbiotic state (Noel *et al.*, 1988; Swamynathan and Singh, 1992).

In an attempt to identify the exact defect in the nodule development due to block at a particular step of purine biosynthesis and assigning role of a particular intermediate in *S. meliloti*, ultra structural analysis of nodules induced by purine auxotrophs were carried out. Light and transmission electron microscopic (TEM) studies of nodule induced by auxotrophs (NV2, AL7 and VK38) which have block before AIR, AICAR and IMP, respectively, showed poorly developed zone as compared to parental strain induced nodules (Plate 4: A; Plate 9: A; Plate 11: A & Plate 14: A). A few infection threads along some nodule's cell with rhizobia were observed. Most of the cells in poorly developed infection and nitrogen fixation zone were uninfected and devoid of rhizobia (Plate 9: C; Plate 10: A; Plate 11: B; Plate 12: A and B; Plate 13: C; Plate 14: B & Plate 15: B). Some cells which filled with mix shapes released bacteria and in lesser density compared to parental strain cells, were also seen. This shows that further development (differentiation) of released bacteroids did not take place. Instead, they start degrading and resulted in Fix$^-$ nodules (Plate 10: C;Plate 13: A and B & Plate 16: B). In uninfected cells of these nodules, cells were seen with peripheral cytoplasm, central vacuole and cell organelles, mitochondria, RER and amyloplasts, etc. In all these nodules, vascular bundles can be seen in the peripheral region of the nodule which is a sign of true nodule structure and not root outgrowth or

pseudonodules as seen in other *Rhizobium* purine auxotrophs (Pankhurst and Schwinghamer, 1974, Noel *et al.,* 1998; Djordjevic *et al.,* 1996). It seems that defects in these nodules were related to lesser number of infection thread formations, their growth and to a great extent in bacteroid maturation (differentiation). Compared to the above-discussed purine auxotrophs (NV2, AL7 and VK38) relative improvement in development was observed in VK27 purine auxotrophs induced nodules. VK27 induced nodules were also poorly developed and lacked distinct zones, but relatively more nodular cells were seen with released bacteroids. The cytoplasm of the released bacteroids were heterogeneous in nature (Plate 19: A and B) and these bacteroids did not follow the normal developmental stages as occurred in bacteroids of parental strain induced nodule as reported (Vasse *et al.,* 1990). In addition, Light and Transmission Electron Microscopic (TEM) studies of nodules cells infected by *S. meliloti* purine auxotrophs showed presence of a lot of cell organelles i.e. increased number of RER, amyloplasts, mitochondria and starch granules in younger tissues of ineffective nodules. Similar observation has also been made in earlier studies (Mackenzie and Jordan, 1974; Hirsch *et al.*, 1983). It was suggested that these cellular changes appear to reflect a response to nitrogen starvation in the ineffective nodules.

From the present study, it is concluded that the nodules induced by *S. meliloti* purine auxotrophic mutants in alfalfa plant, are symbiotically defective and defects are at slightly later stages of bacteroids maturation. That is during one of the stages of development from vegetative rhizobia to functional bacteroids that are active in nitrogen fixation. Although present study confirms the role of purine biosynthetic pathway intermediate(s)/enzyme(s) in *S. meliloti* alfalfa symbiosis and also indicates that the undiminished metabolic flow through of the *de novo* purine biosynthesis or a particular intermediate in the pathway is essential for symbiotic interaction. But the

precise role or the importance of a particular intermediate / enzyme could not be established conclusively which needs further detail investigation. However, very recently is has been reported that ACIA riboside, the precursor of ACIAR prevents *S. meliloti fixNOQP* induction by the inhibition of *fixK* transcription, a transcritional activator (Soberon *et al.*, 2001). This further confirms the importance of purine biosynthetic pathway in *S. meliloti*-alfalfa Symbiosis.

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