GENETIC AND SYMBIOTIC STUDIES ON AROMATIC AMINO ACID AUXOTROPHS OF *Sinorhizobium meliloti*

A THESIS

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

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OCTOBER, 2001

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "GENETIC AND SYMBIOTIC STUDIES ON AROMATIC AMINO ACID AUXOTROPHS 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 Institute is an authentic record of my own work carried out during a period from February, 1998 to October, 2001 under the supervision of **Dr. R. Prasad**.

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

Dated : **Oct.,22, 2001** **(HASSAN FADHIL NAJI)**

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

Random Tn5 insertion mutants of *Sinorhizobium meliloti* strain Rmd201 were isolated by transposon Tn5 mutagenesis. Conjugation between donor *Escherichia coli* strain WA803, harbouring the Tn5 on its suicide plasmid pGS9, and recipient *S. meliloti* Rmd201 resulted in the generation of five thousand kanamycin resistant transconjugants from 85 crosses. Screening these transconjugants yielded in the isolation of twenty two auxotrophs, unable to grow on *Rhizobium* minimal medium (RMM). Streaking these auxotrophs on nutritional pools resulted in the determination of four auxotrophs of aromatic amino acid biosynthetic pathways (two of tryptophan, one of tyrosine and one of phenylalanine). Two auxotrophs requiring all three aromatic amino acids, viz., tryptophan, tyrosine and phenylalanine, and three tryptophan auxotrophs of *S. meliloti* Rmd201, isolated by other researchers in the lab were also included in this study.

Based on the location of metabolic block in the aromatic amino acid biosynthetic pathways, these auxotrophs were classified into four groups:

i. *aro* mutants (NV3 and BA2): Grew on RMM supplemented with all three aromatic amino acids.

ii. *trp* mutants which were further classified into three subgroups :

a. *trpE(G)* mutants (FN2 and FN3): Grew on RMM supplemented with anthranilic acid, indole or tryptophan and did not accumulate anthranilic acid and indole-3-glycerol phosphate in RMM.

b. *trpD, trpF, or trpC* mutants (NV7 and NV31): Grew on indole or tryptophan supplemented RMM and accumulated anthranilic acid in RMM.

c. *trpB* mutant (BA6): Grew only on tryptophan supplemented RMM and accumulated anthranilic acid and indole-3-glycerol phosphate in RMM.

iii. *tyrA* mutant (FN4): Grew only on tyrosine supplemented RMM.

iv. *pheA* mutant (FN9): Grew only on phenylalanine supplemented RMM.

Similar to the parental strain Rmd201, all aromatic amino acid auxotrophs were able to infect the root hairs and form nodules on the roots of alfalfa (*Medicago sativa* cv. T9) plants (Nod^+). However, the nodules induced by *aro, trpE(G)* and *pheA* mutants were symbiotically ineffective (Fix^-). The plants nodulated by these mutants were weak, stunted and became chlorotic six weeks after inoculation which showed the inability of these auxotrophs to fix nitrogen. The remaining Tn5 insertion mutants, represented by *trpD, trpF or trpC, trpB* and *tyrA* mutants elicited fully effective nodules on the roots of alfalfa plants. These plants were healthy, green (indicating that nitrogen was being fixed) and resembled the parental strain Rmd201 inoculated plants in all respects.

The symbiotic ability of *trpE(G)* mutants was restored on supplementation of plant growth medium with anthranilic acid (upto 10 µg/ml). However, supplementation of plant growth medium with indole (upto 5 µg/ml) or tryptophan (upto 20 µg/ml) could not restore the symbiotic ability of *trpE(G)* mutants. At 2.5 µg/ml supplementation of anthranilic acid, only partial restoration was observed, whereas complete restoration took place at 5, 7.5 and 10 µg/ml supplementation of anthranilic acid. The *trpE(G)* mutants, supplemented with 2.5 µg/ml of anthranilic acid, formed slightly pink nodules on alfalfa roots. The mean height and dry shoot weight of these plants differed significantly from those of the parental strain Rmd201 inoculated plants and with the uninoculated controls. However, the *trpE(G)* mutants inoculated plants, supplemented with 5-10 µg/ml of anthranilic acid, resembled those of the parental strain Rmd201 inoculated plants in all aspects. Atomic absorption spectrometer analysis of iron uptake by auxotrophs revealed that the *trpE(G)* mutants,

grown on minimal medium having iron (supplemented with minimal nutritional requirements of the auxotrophs), took up less amount of iron than anthranilic acid-producing mutants. These findings suggest that anthranilic acid has a role in symbiotic process. The symbiotic functions of *pheA* and *aro* mutants were not restored by addition of phenylalanine (upto 30 μg/ml) and aromatic amino acids (upto 5 μg/ml), respectively, to the plant growth medium.

Light microscopic observations of nodules induced by the parental strain Rmd201 showed normal developmental stages of the distinct zones, viz., distal meristematic, infection, amyloplast-rich inter, nitrogen-fixing and proximal senescence zones. Transmission electron microscopy of these zones revealed all stages of bacteroid development.

The microscopic observations of nodules elicited by *aro* and *trpE(G)* mutants exhibited striking similarities. Each of these nodules showed extensive infection zone which occupied most part of the middle nodule tissues, while the nitrogen-fixing region was poorly developed and restricted to a few layers just beneath the infection zone. The bacteroids in these zones did not show functional maturation. The senescence zone occupied almost one third of the nodules and contained deteriorating bacteroids.

Histology of nodules formed by *trpE(G)* mutants, supplemented with 2.5 μg/ml of anthranilic acid, showed that the rhizobial release into the nodule cells was normal and in most of the rhizobial cells differentiation into bacteroidal state was almost complete. Nitrogen-fixing zones of these nodules were not fully developed and contained bacteroids in a degenerating condition. However, in the nodules induced by the same mutants, supplemented with 5, 7.5 or 10 μg/ml of anthranilic acid, the nitrogen-fixing zones were fully developed.

Light and electron microscopy of nodules elicited by *pheA* mutant exhibited no distinctive cellular zones. The release of rhizobial bacteria into nodule cells was normal, but the differentiation of released bacteria into bacteroids was not complete and they were observed to be in a degenerating condition after their release.

Microscopy of *trpD, trpF or trpC, trpB* and *tyrA* mutant-nodules showed normal developmental stages. The internal histological features of these nodules at structural and ultrastructural levels resembled those of the nodules induced by Rmd201 strain in all respects.

The aromatic amino acid auxotrophs were similar to the parental strain Rmd201 with respect to the production of cell surface carbohydrate molecules (β-glucans, cellulose fibrils, lipopolysaccharides and succinylated exopolysaccharides), utilization of carbon sources (C_4-dicarboxylic acids and sugars) and production of cytochrome c oxidase indicating that the symbiotic defects of *aro, trpE(G)* and *pheA* mutants were not caused by a change in any of the above mentioned characteristics. The defective symbiosis of these auxotrophs could be merely due to the loss of biosynthetic gene functions through Tn5 insertion into *aro, trpE(G)* and *pheA* genes. Hence, the functions of *aro*, *trpE(G)* and *pheA* genes involved in aromatic amino acid biosynthetic pathways of *S. meliloti* are required for an effective nodule development and optimal symbiotic nitrogen fixation. However, the informations regarding the role they play in symbiosis are not well established. The restoration of the symbiotic ability of *trpE(G)* mutants with exogenous supplementation of anthranilic acid confirmed its role in symbiosis, most probably by facilitating iron uptake which plays significant role in nitrogen fixation.

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LIST OF IMPORTANT ABBREVIATIONS

A	=	Amyloplast-rich interzone
a	=	Amyloplast
aro	=	Aromatic amino acids
b	=	Bacteroid
bp	=	Base pair
bv	=	Biovar
c	=	Cortex
CF	=	Cellulose fibrils
CFU	=	Colony forming units
CG	=	Cyclic β-glucans
cm	=	Chloramphenicol
cv	=	Cultivar
cw	=	Cell wall
ec	=	Empty cell
EPS	=	Exopolysaccharides
er	=	Endoplasmic reticulum
Fix	=	Nitrogen fixation
gm	=	Gram
hs	=	Hyaline spot
I	=	Infection zone
IR	=	Inverted repeat
IS	=	Insertion sequence
is	=	Intercellular space
it	=	Infection thread
kb	=	Kilobase
Km	=	Kanamycin
KPS	=	Capsular polysaccharides
LB	=	Luria-Bertani
LCO	=	Lipochitin oligosaccharides
LPS	=	Lipopolysaccharides
M	=	Meristematic zone

m	=	Mitochondria
mg	=	Milligram
ml	=	Millilitre
MSY	=	Mannitol salt yeast extract
N	=	Nitrogen-fixing zone
n	=	Nucleus
Nm	=	Neomycin
nm	=	Nanometer
Nod	=	Nodulation
pbm	=	Peribacteroid membrane
phb	=	Poly-β-hydroxybutarate
phe	=	Phenylalanine
r	=	Root hair
rh	=	Rhizobia
RMM	=	*Rhizobium* minimal medium
rpm	=	Round per minute
S	=	Senescence zone
s	=	Symbiosome
sb	=	Senescent bacteroid
sc	=	Shepherd's crook
Sm	=	Streptomycin
Tn	=	Transposon
trp	=	Tryptophan
TY	=	Tryptone yeast extract
tyr	=	Tyrosine
v	=	Vacuole
vb	=	Vascular bundle
vc	=	Vesicle
µg	=	Microgram
µl	=	Microlitre
µm	=	Micrometer
YEM	=	Yeast extract mannitol

Chapter 1

INTRODUCTION

Light and electron microscopy of nodules elicited by *pheA* mutant exhibited no distinctive cellular zones. The release of rhizobial bacteria into nodule cells was normal, but the differentiation of released bacteria into bacteroids was not complete and they were observed to be in a degenerating condition after their release.

Microscopy of *trpD, trpF or trpC, trpB* and *tyrA* mutant-nodules showed normal developmental stages. The internal histological features of these nodules at structural and ultrastructural levels resembled those of the nodules induced by Rmd201 strain in all respects.

The aromatic amino acid auxotrophs were similar to the parental strain Rmd201 with respect to the production of cell surface carbohydrate molecules (β-glucans, cellulose fibrils, lipopolysaccharides and succinylated exopolysaccharides), utilization of carbon sources (C_4-dicarboxylic acids and sugars) and production of cytochrome c oxidase indicating that the symbiotic defects of *aro, trpE(G)* and *pheA* mutants were not caused by a change in any of the above mentioned characteristics. The defective symbiosis of these auxotrophs could be merely due to the loss of biosynthetic gene functions through Tn5 insertion into *aro, trpE(G)* and *pheA* genes. Hence, the functions of *aro*, *trpE(G)* and *pheA* genes involved in aromatic amino acid biosynthetic pathways of *S. meliloti* are required for an effective nodule development and optimal symbiotic nitrogen fixation. However, the informations regarding the role they play in symbiosis are not well established. The restoration of the symbiotic ability of *trpE(G)* mutants with exogenous supplementation of anthranilic acid confirmed its role in symbiosis, most probably by facilitating iron uptake which plays significant role in nitrogen fixation.

Nitrogen gas (N_2) or azote, meaning without life, as described by Antonie Lavoisier about 200 years ago, has been proved to be anything but lifeless (Zahran, 1999). It is an important biological macroelement and an essential macronutrient for life in all organisms. Though atmosphere contains about 10^{15} tonnes of N_2, only a fraction is made available for utilization by plants through transformation of $3x10^9$ tonnes of N_2 per year on a global basis (Postgate, 1982). Primary producers of food chain, plants, can utilize only combined forms of nitrogen (ammonia, nitrate, nitrite, etc.), therefore, N_2 must first be converted into fixed or combined forms. This takes place by spontaneous, chemical and biological processes. Of these, lightening contributes about 10% (Sprent and Sprent, 1990), chemical fertilizers account for about 25% and biological processes of nitrogen fixation account for about 60% of the world's supply of nitrogen (Zahran, 1999). The chemical fertilizers commonly used to replenish the nitrogen deficient soil, are costly and their continuous use pollutes the ecosystem. Therefore, it has become essential to look for other alternative sources of nitrogen supply. The biological nitrogen fixation is one of the plausible solution due to its great potential. However, nearly 87 species in 2 genera of archaea, 38 genera of bacteria and 20 genera of cyanobacteria have been identified as diazotrophs or prokaryotes that have the ability to fix nitrogen (Dixon and Wheeter, 1986; Sprent and Sprent, 1990; Zahran *et al.*, 1995). These are being used as biofertilizers to increase the crop productivity, decrease the environmental pollution and improve the soil fertility to benefit the subsequent crop. Furthermore, the cost is so low that every farmer can afford using them, and these are the best alternatives to the inorganic (synthetic) fertilizers. Based on the extent of their association with plants, the

diazotrophs are classified into asymbiotic or free-living (*Azotobacter, etc.*), associative (*Azospirillum, etc.*) and symbiotic (*Rhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium* and *Mesorhizobium*, "collectively called rhizobia", and *Frankia*, "actinomycetes"). All these organisms possess the ability of enzymatic conversion of N_2 to ammonia (NH_3), by a process known as biological nitrogen fixation, which is of paramount economical and ecological significance. Among these, the symbiotic associations are responsible for reducing 120 million tonnes of N_2 to NH_3 each year (Freiberg *et al.*, 1997).

Based on growth rate, rhizobia are classified as fast-growers (*Rhizobium, Sinorhizobium, Azorhizobium* and *Mesorhizobium*) and slow-growers (*Bradyrhizobium*). Rhizobia are able to establish an endosymbiotic interaction with leguminous plants that results in the formation of specialized organs called nodules on the roots of these plants. These mutualistic associations are rather species-specific and complex. They are dependent on the rhizobial strain, the host plant genotype and the interaction of these symbionts with the pedoclimatic factors and the climatic conditions (Bordeleau and Prévost, 1994). Among the fast-growing rhizobia, *S. meliloti* forms symbiotic association with species of *Medicago, Melilotus* and *Trigonella*, members of the family Leguminosae. Establishment of symbiosis (nodule formation) entails a complex series of events that requires a coordinated temporal and spatial expression of both macro-and microsymbiont genes (Brewin, 1991; Fisher and Long, 1992; Brewin, 1993; Fischer, 1994; Mylona *et al.*, 1995; Dénarié *et al.*, 1996; Long, 2001). Symbiotic process can be divided into three major stages: preinfection, infection with concomitant nodule formation and nodule function. This multistage process involves

bacterial multiplication in the rhizosphere, recognition, which includes exchange of specific signal molecules (flavonoids and lipochitin oligosaccharides or Nod factors) between compatible symbiotic partners, and infection of the root hairs. Furthermore, several cell surface carbohydrate molecules, such as cyclic β-glucans, cellulose fibrils, lipopolysaccharides, exopolysaccharides and capsular polysaccharides (K antigen), have a role in early infection events to increase the efficiency of infection (Bauer,1981; Carlson, 1982; Long, 1989b; Franssen *et al.*, 1992; Hirsch, 1992; Reuhs *et al.*, 1993; Breedveld and Miller, 1994; Pellock *et al.*, 2000).

Among the first indications of the interaction between the rhizobia and the legume is the deformation of root hairs (Bauer, 1981). The rhizobia get entrapped in a curled root hair, or shepherd's crook. The host cell wall is hydrolyzed and an invasion tube called infection thread is induced which carries the invading bacteria towards the base of root hair. The cells of root cortex are mitotically activated and form nodule primordium. The bacteria are released endocytotically from infection thread into the cytoplasm of invaded host cells. These bacteria are surrounded by host-derived peribacteroid membrane which controls the nutrient transfer between the symbionts, divide and then differentiate into nondividing bacteroids (symbiosomes). They are able to reduce N_2, through the activation of the nitrogenase complex, to NH_3 which is then assimilated by the plant (Verma, 1992; Panagiota *et al.*, 1995; van Rhijn and Vanderleyden, 1995; Taté *et al.*, 1999a). In turn, the plant provides the bacteroids with carbon and energy sources. But it is largely unclear which sources (such as carbon, sulfur or nitrogen) are required during bacteroid development.

Symbiosis requires a respiratory chain that has a high affinity for oxygen and is efficiently coupled to ATP production (Batut and Boistard, 1994). Since, nitrogen fixation is an energy-consuming process, requiring upto 20 ATP molecules to reduce just one molecule of N_2, therefore, bacteroid utilizes organic compounds such as sucrose, derived from plant photosynthate, to provide the energy (Streeter, 1991). The C_4-dicarboxylic acids, intermediates of the tricarboxylic acid cycle, are likely to be the major carbon sources used by bacteroids for the reduction of N_2 (Streeter *et al.*, 1987; Rosendahl *et al.*, 1990; Fougere *et al.*, 1991; Reid and Poole, 1998).

The recent advances in molecular biology and recombinant DNA technology have helped in understanding the symbiotic process. However, attempts are being made to improve the efficiency of nitrogen fixation by genetic engineering of symbiotic gene expression. Based on their function(s), the symbiotic genes have been broadly classified. In rhizobia, some of these genes belong to the group of cell surface carbohydrate molecule (*cgm*, *exo*, *pss*, *ndv*, *rkp lps*, etc.) (Borthakur and Johnston, 1987; Priefer, 1989; Charles *et al.*, 1991; Brzoska and Singer, 1991; Kereszt *et al.*, 1998; Król *et al.*, 1998; Wang *et al.*, 1999; So *et al.*, 2000; Lagares *et al.*, 2001), nodulation (*nod*, *nol* and *noe*) (Fisher and Long, 1992; Long, 2001), nitrogen fixation (*nif and fix*) (Sharma *et al.*, 1993; Fischer, 1994) and bacteroid metabolism (*dct, ntr,* etc.) genes (Bolton *et al.*, 1986; Ronson *et al.* 1987; Finan *et al.*, 1988; Watson *et al.*, 1988; Engelke *et al.*, 1989; Wang *et al.*, 1989; Watson *et al.*, 1990; Patriarca *et al.*, 1993; Boesten *et al.*, 1998; Reid and Poole, 1998; Oláh *et al.*, 2001). In the plant nodules, symbiotic genes belong to the group of early and late nodulin (*ENOD5*,

ENOD12, *GmN93*, *MtN6* etc.) genes (van Kammen, 1984; Scheres *et al.*, 1990; Kouchi and Hata, 1993; Mathis *et al.*, 1999).

In addition to the symbiotic genes which play a key role in nitrogen fixation, some biosynthetic pathways of rhizobia also appear to have a role in the symbiotic process. Auxotrophs of the metabolic pathways of vitamins, nucleotide bases and some amino acids (aspartate, asparagine, arginine, histidine, leucine, isoleucine and valine, methionine, proline, phenylalanine, tryptophan and tyrosine) have been found to be defective in symbiosis (Schwinghamer, 1967; 1970; Pankhurst *et al.*, 1972; Sadowsky *et al.*, 1986; Noel *et al.*, 1988; Kerppola and Kahn, 1988a; Kummer and Kuykendall, 1989; Aguilar and Grasso, 1991; Barsomian *et al.*, 1992; Jelesko *et al.*, 1993; Newman *et al.*, 1994; Yadav *et al.*, 1998; Taté *et al.*, 1999 b & c; King *et al.*, 2000; Prasad *et al.*, 2000; Ferraioli *et al.*, 2001; López *et al.*, 2001; Vineetha *et al.*, 2001). The defective symbiosis is not due to the unavailability of the end products only; the metabolic intermediate(s) of a particular pathway appear to contribute to symbiosis. It has been shown that the tryptophan auxotrophs of *B. japonicum* which have defects earlier in the tryptophan biosynthetic pathway did not nodulate soybean plants (Wells and Kuykendall, 1983). However, Kummer and Kuykendall (1989) found that the tryptophan auxotrophs of *B. japonicum* were symbiotically defective. Anthranilic acid, an intermediate of the tryptophan biosynthetic pathway, has been shown to be involved in symbiosis of *S. meliloti* (Barsomian *et al.*, 1992; Prasad *et al.*, 2000). Normal metabolic flow through the aromatic amino acid biosynthetic pathways of *S. meliloti* have been found to be required for the development of bacteroids (Jelesko *et al.*, 1993). Recently, it has been reported that the *trpB* gene of *R. etli* is essential for

functional nitrogen-fixing symbiosis (Taté *et al.*, 1999b). However, in all these cases, no detailed studies of exogenous feeding and histology of nodules of the symbiotically defective auxotrophs of aromatic amino acid biosynthetic pathways were performed. Considering the above facts, the present study was undertaken to determine the role of aromatic amino acid biosynthetic pathways in *S. meliloti* Rmd201-*M. sativa* cv. T9 symbiosis with the following objectives:

a. Isolation and characterization of Tn5 insertion mutants of the aromatic amino acid biosynthetic pathways of *S. meliloti* Rmd201.
b. Determination of metabolic block in the aromatic amino acid biosynthetic pathways of each auxotroph.
c. Investigation of the symbiotic properties, including infection phenotypes on alfalfa root hairs, formation of nodules and plant characteristics.
d. Study of the effect of exogenous feeding on the restoration of the symbiotic ability of the symbiotically defective auxotrophs.
e. Exploration of the histology of nodules induced by the auxotrophs.

Chapter 2

LITERATURE REVIEW

2.1 Historical perspective of rhizobia

The story of the bacteria that are able to form nitrogen-fixing nodules on the roots of leguminous plants has recently been reviewed by Perret *et al.* (2000). It began in 1542, when Leonhard Fuchsius published drawings of nodulated legumes. Malpighi (1675) observed nodules on the root of beans (*Phaseolus vulgaris* and *Vicia faba*, members of the family Leguminosae). Woronin (1866) noted that the nodules of *Alnus glutinosa* (Betulaceae) and *Lupinus mutabilis* (Leguminosae) were filled with minute bodies resembling bacteria. In 1879, Frank found nodules on the roots of all healthy legumes and demonstrated that incinerating soil prevented the nodulation of *Pisum sativum*. Hellriegel (1886), and Hellriegel and Wilfarth (1888) showed that the nodule formation results from external infection of *Lupinus* spp., *P. vulgaris*, *P. sativum*, *Ornithopus sativa, Trifolium* spp. and *Vicia sativa*. However, it was Beyerinck (1888) who furnished the first proof that bacteria provoke nodules; he did this by preparing pure cultures of nodule organisms from *V. faba* and using them to infect Faba beans growing in sterile soil (Beyerinck., 1890). He proposed the name *Bacillus radicicola* for these organisms, but they were renamed as *Rhizobium* (rhizo = root and bios = living) by Frank (1889).

2.2 Taxonomy of rhizobia

Rhizobia are taxonomically diverse members of the alpha subdivisions of the class Proteobacteria. This group of bacteria offered a taxonomic challenge since for many years their characterization was based on a selective interaction with a plant host. Early researchers considered all rhizobia to be a single species capable of nodulating all legumes. Lohnis and Hansen (1921) divided the root nodule bacteria, based on growth

rate into two groups: fast-growers with generation time less than 6 hours (which nodulate alfalfa, clover, bean and pea plants), and slow-growers with generation time more than 6 hours (which provoke nodules on the roots of soyabean and cowpea plants). In 1982, Jordan proposed the genus *Bradyrhizobium* for the slow-growing root nodule bacteria. Later, the genus *Azorhizobium* (a stem-nodulating nitrogen-fixing bacterium) was described by Dreyfus *et al.* (1988). The use of modern methods of bacterial systematic such as numerical taxonomy, nucleic acid hybridization, 16S rRNA analysis, etc., has demonstrated the existence of marked genetic diversity among rhizobial bacteria. Therefore, two genera *Sinorhizobium* (Chen *et al.*, 1988) and *Mesorhizobium* (Jarvis *et al.*, 1997) were proposed as new members of rhizobia. The species *Rhizobium meliloti* has been reclassified as *Sinorhizobium meliloti* (de Lajudie *et al.*, 1994). Recently, Gualtieri and Bisseling (1999) published the phylogenetic tree of rhizobia as deduced from 16S rRNA gene sequences.

The genus *Rhizobium* is phylogenetically heterogeneous with two different groups: *Rhizobium leguminosarum* (Jordan, 1984), *Rhizobium tropici* (Martínez-Romero *et al.*, 1991), *Rhizobium etli* (Segovia *et al.*, 1993), *Rhizobium gallicum* (Amarger *et al.*, 1997), *Rhizobium hainanense* (Chen *et al.*, 1997) and *Rhizobium mongolense* (van Berkum *et al.*, 1998) form one rRNA branch and *Rhizobium galegae* (Lindström, 1989), *Rhizobium giardinii* (Amarger *et al.*, 1997) and *Rhizobium Huautlense* (Wang *et al.*, 1998) are in a separate branch together with *Agrobacterium biovar* 1 (Kersters and De Ley, 1984) and *Agrobacterium vitis* (Ophel and Kerr, 1990). The genus *Bradyrhizobium*, with the species *Bradyrhizobium japonicum* (Jordan, 1984), *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992) and *Bradyrhizobium liaoningense* (Xu

et al., 1995) are root-nodulating but slow-growing bacteria. *Azorhizobium caulinodans*, a stem-nodulating species, is the only species of the genus *Azorhizobium* (Dreyfus *et al.*, 1988). The genus *Mesorhizobium* consists of the species *Mesorhizobium loti* (Jarvis *et al.*, 1982), *Mesorhizobium huakuii* (Chen *et al.*, 1991), *Mesorhizobium ciceri* (Nour *et al.*, 1994), *Mesorhizobium mediterraneum* (Nour *et al.*, 1995), *Mesorhizobium tianshanense* (Chen *et al.*, 1995) and *Mesorhizobium plurifarium* (de Lajudie *et al.*, 1998). From the fast-growing rhizobia, the genus *Sinorhizobium*, consists of the species *Sinorhizobium fredii* (Scholla and Elkan, 1984), *Sinorhizobium meliloti* (Jordan, 1984), *Sinorhizobium saheli* and *Sinorhizobium terangae* (de Lajudie *et al.*, 1994) and *Sinorhizobium medicae* (Rome *et al.*, 1996), recently, Nick *et al.* (1999) described the new members of this genus, *Sinorhizobium arboris* and *Sinorhizobium kostiense*, and demonstrated the phylogenetic tree of rhizobia and some related bacteria in the α-subclass of the Proteobacteria (Fig. 1.).

2.3 Genetics of rhizobia

Rhizobial genome can be considered as a complex structure, formed by the chromosome and high molecular weight plasmids, the megaplasmids. These harbour the genes involved in symbiosis. In *S. meliloti* typically, two megaplasmids of approximately 1,400 and 1,600 kb have been reported (Banfalvi *et al.*, 1981; Burkardt and Burkardt, 1984; Banfalvi *et al.*, 1985; Finan *et al.*, 1986; Hynes *et al.*, 1986; Burkardt *et al.*, 1987; Sobral *et al.*, 1991). These plasmids have been termed as pRmeSU47a and pRmeSU47b or pSymA and pSymB, respectively. The larger of these megaplasmids, pRmeSU47b has been shown to carry determinants for exopolysaccharide synthesis, thiamine biosynthesis,

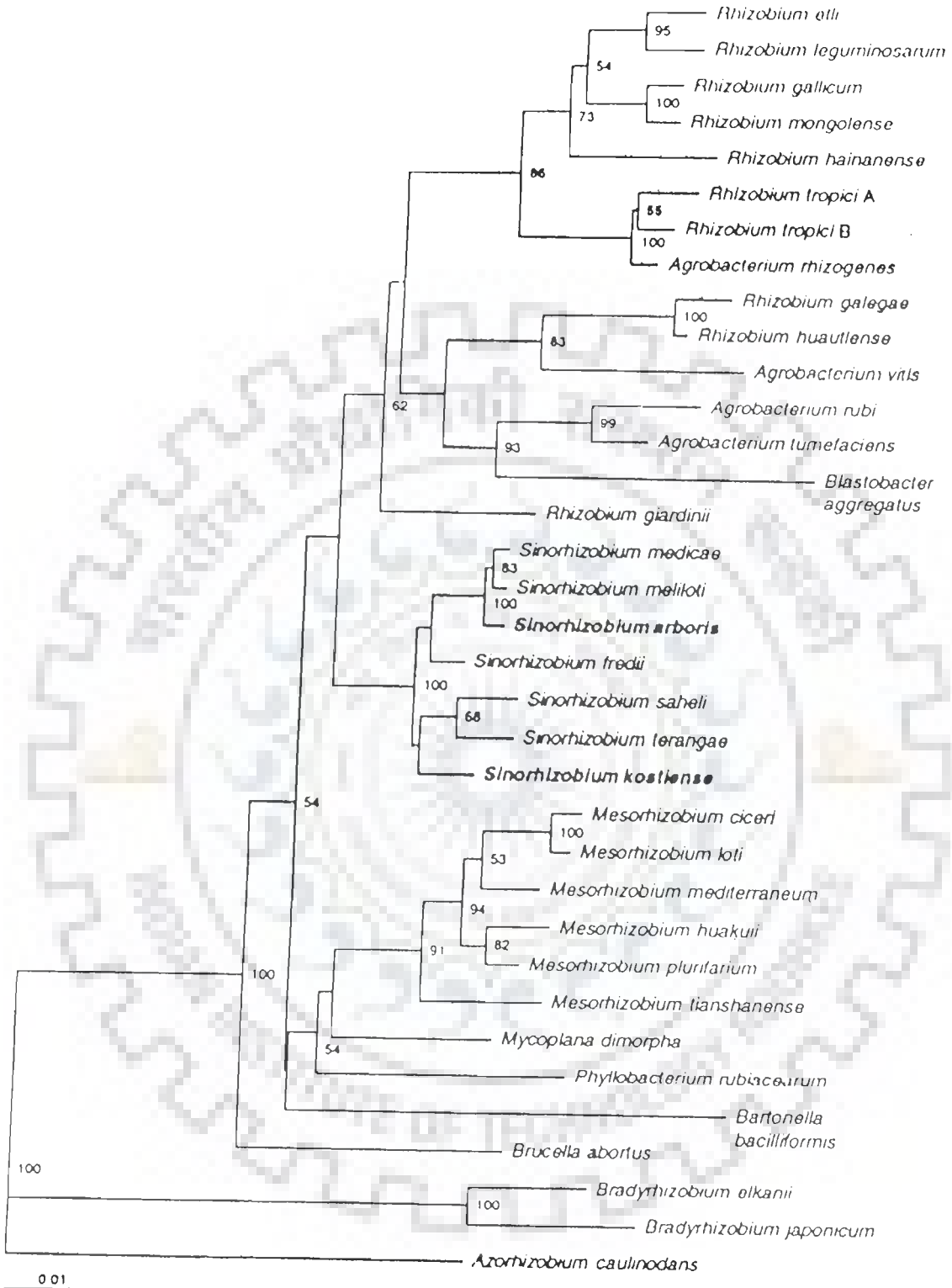

Fig. 1. Phylogenetic tree of rhizobia and some related bacteria in the α-subclass of the Proteobacteria. The tree was constructed by the neighbour-joining method from 16S rRNA sequences. Bootstrap probability values greater than 50% are indicated at the branch-points. Bar, 0.01 substitution per site (Nick *et al.*, 1999).

high-affinity phosphate transport and dicarboxylic acid transport (Finan *et al.*, 1986; Hynes *et al.*, 1986; Watson *et al.*, 1988; Glazebrook and Walker, 1989; Yarosh *et al.*, 1989; Charles and Finan, 1990; 1991; Bardin *et al.*, 1996). The other megaplasmid, pRmeSU47a has been shown to carry two determinants for nodulation and nitrogen fixation (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Batut *et al.*, 1985; Renalier *et al.*, 1987; David *et al.*, 1988; Boivin *et al.*, 1990; Ogawa and Long, 1995; Barnett and Long, 1997; Oresnik *et al.*, 2000). However, Flores *et al.* (1998) reported that, in different *Rhizobium* and *Sinorhizobium* spp., most nodulation and nitrogen fixation genes are plasmid borne, while in *Bradyrhizobium*, *Azorhizobium* and *Mesorhizobium* spp. these genes are located on the chromosome.

2.3.1 Mutation

Any heritable permanent change in the nucleotide sequence of a gene (modification of DNA), through spontaneous errors in DNA replication, or as a consequence of the damaging affects of physical, chemical and biological agents on the genome, lead to mutation. Restoration of the original phenotype of mutant is called reversion (back) mutation. The rate of mutation can be increased by employing the following mutagenesis.

2.3.2 Mutagenesis

2.3.2.1 Physical mutagenesis

Absorption of high-energy ionizing radiations such as Roentgen (X) rays, Gamma (γ) rays and cathode rays, causes the target molecules to lose electrons. These electrons can cause extensive chemical alterations in DNA, including strand breaks, base and sugar

destruction. Non-ionizing radiations such as ultraviolet (UV) light, overexposure to sunlight which contains UV light, produce pyrimidine dimers from adjacent pyrimidine bases of DNA. Karanja and Wood (1988) found that a high percentage of the *R. leguminosarum* bv. *phaseoli* strains that persisted at 45°C lost their infectivity. They attributed these losses in infectiveness to plasmid curing. Heat treatment of *R. leguminosarum* bv. *phaseoli* at 35 and 37°C resulted in mutant strains lacking a plasmid DNA implicated in the synthesis of melanin and is related to the loss of symbiotic properties of these bacteria (Beltra *et al.*, 1988).

2.3.2.2 Chemical mutagenesis

A wide range of chemical mutagens having different mode of action can react with DNA and alter its properties, such as, base analogs (5-bromouracil, 2-aminopurine, etc.), intercalating agents (acridine orange, proflavine, acriflavine, ethidium bromide, etc.) and several natural and synthetic organic and inorganic chemicals (nitrous acid, hydroxylamine, ethylmethane sulfonate, ethylnitrosourea, N-methyl-N′-nitro-N-nitrosoguanidine, etc.). Some of these mutagens have been employed to generate auxotrophic and symbiotic mutants of rhizobia (Beringer, 1974; Fedorov and Zaretskaya, 1977; Pain, 1979; Noel *et al.*, 1982; Well and Kuykendall, 1983; Singh *et al.*, 1984; Noel *et al.*, 1988; Cava *et al.*, 1989; Kummer and Kuykendall, 1989; McIver *et al.*, 1989).

2.3.2.3 Biological mutagenesis

Several mobile segments called transposable elements are widely used to induce mutations. Some of these mutagens can be inserted artificially at desired sequence and used to construct a mutated gene directly or to change specific base pairs within a gene.

This gene can also be linked into a vector and cloned. The transposable elements have played a major role in understanding the genetic analysis of symbiotic process (Kleckner *et al.*, 1977; Beringer *et al.*, 1978; Rolfe *et al.*, 1980; Meade *et al.*, 1982; Forrai *et al.*, 1983). These elements can be divided into three types: insertion sequences, transposons and some bacteriophages like Mu.

2.3.2.3.1 Transposon Tn5

Transposons are discrete DNA segments which insert into many sites of bacterial genome, often giving rise to strong polar mutations (Kleckner, 1977). Tn5 is a composite transposon of about 5,700 base pairs (bp) element, conferring kanamycin or neomycin resistance (Berg *et al.*, 1975; 1980). It has a central unique region of about 2700 bp with two insertion sequences IS50L and IS50R of about 1535 bp each, at its left and right ends (Fig. 2). These contain the genes necessary for transposition of Tn5 and its regulation (Selvaraj and Iyer, 1983).

The bacterial transposon Tn5 encodes two proteins, the transposase and a related protein, the transposition inhibitor, whose relative abundance determines, in part, the frequency of Tn5 transposition (Reznikoff, 1993). However, the transposase apparently recognizes, cuts and eventually ligates the target DNA during transposition, while the IS50L is not capable of independent transposition because there is a nonsense mutation in its transposase gene. In *Rhizobium* spp., Tn5 also confers resistance to streptomycin (Putnoky *et al.*, 1983; Selvaraj and Iyer, 1983). The streptomycin determinant lies in the central region of Tn5, between the identical insertion sequences distal to Km^r/Nm^r gene and encodes a streptomycin phosphotransferase which is not expressed in *E. coli*. The selectable resistance phenotype, high frequency of transposition and degree of

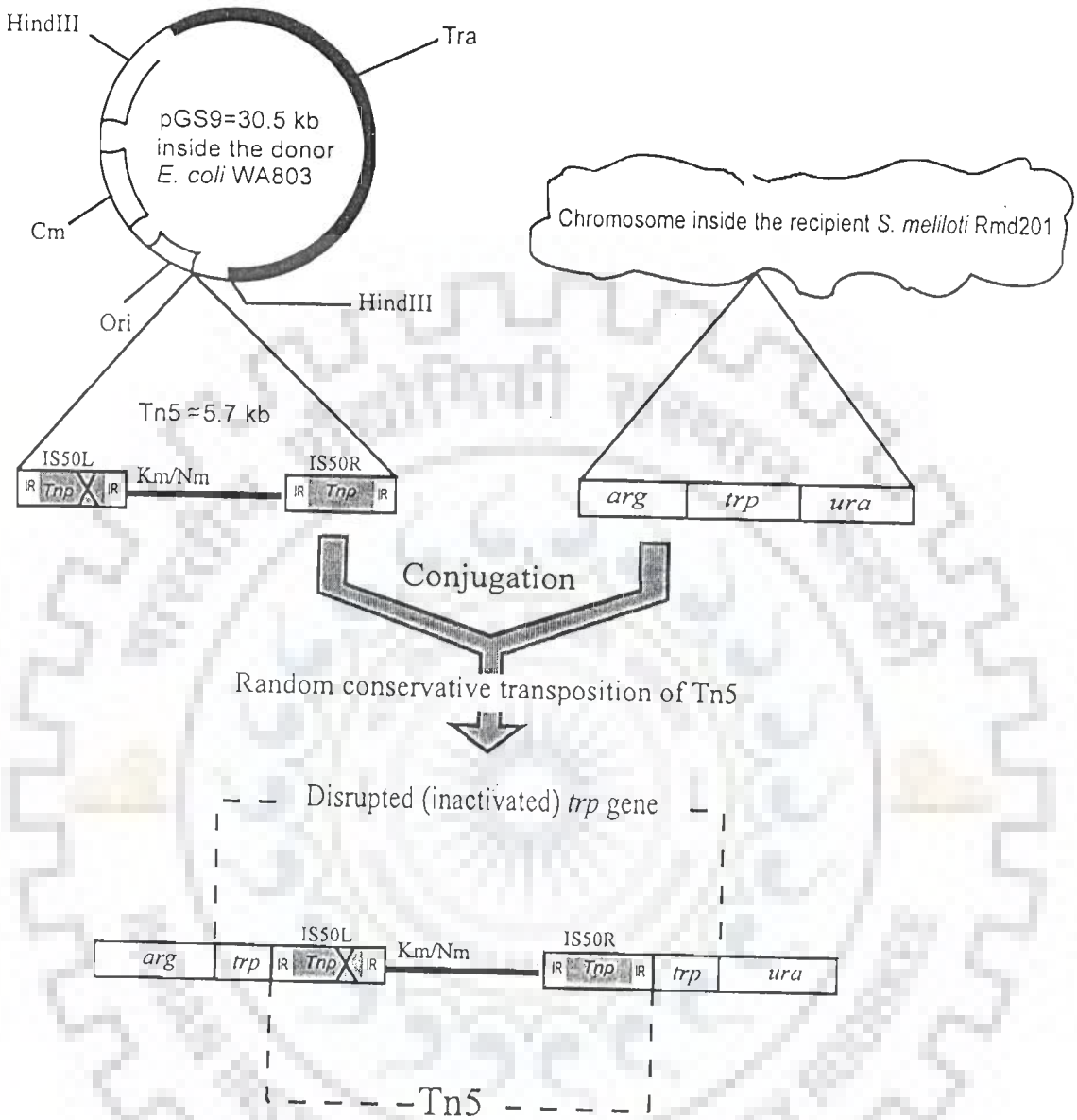

Fig. 2. Schematic representation of the transposon Tn5 mutagenesis.
Abbreviations: Cm, chloramphenicol; Nm, neomycin; Km, kanamycin; Ori, origin of replication; Tra, conjugal transfer; IS, insertion sequence; IR, inverted repeat; *Tnp*, transposase; X, nonsense mutation; *arg*, arginine; *trp*, tryptophan; *ura*, uracil.

randomness in insertion specificity have made Tn5 the most widely used transposon in genetic manipulation in different bacteria (Kleckner *et al.*, 1977; Beringer *et al.*, 1978; Shaw and Berg, 1979; Meade *et al.*, 1982).

2.3.2.3.1.1 Random transposon mutagenesis

Many suicidal plasmids carrying transposon Tn5 were constructed and used for random transposon mutagenesis (Beringer *et al.*, 1978; Selvarag and Iyer, 1983; Simon *et al.*, 1983). These plasmids have helped to generate several types of auxotrophic and symbiotic mutants (Meade *et al.*, 1982; Forrai *et al.*, 1983; Ali *et al.*, 1984; Noel *et al.*, 1984; Hirsch *et al.*, 1984; Hom *et al.*, 1984; Sadowsky *et al.*, 1986; Kummer and Kuykendall, 1989; Aguilar and Grasso, 1991; Barsomian *et al.*, 1992; Jelesko *et al.*, 1993, Taté *et al.*, 1997; Yadav *et al.*, 1998; Taté *et al.*, 1999 b & c; Prasad *et al.*, 2000; Ferraioli *et al.*, 2001). The mutagenesis was carried out by transferring the suicidal plasmid from *E. coli* to rhizobial genome by conjugation process (Fig. 2). The vector after entering can not survive itself but Tn5 can survive by jumping into the rhizobial genome. The insertion into a gene lead to loss of its function and confers new phenotype (kanamycin/neomycin resistance) to the mutant which makes its selection easy and to mark their location in the bacterial DNA.

2.3.2.3.1.2 Site-directed mutagenesis

Three different methods of site-directed mutagenesis for mutagenizing and cloning genes have been identified. These include cassete mutagenesis (Wells *et al.*, 1985), primer extension mutagenesis (Gillam *et al.*, 1980; Zoller and Smith, 1983) and polymerase chain reaction (Scharf *et al.*, 1986). Site-directed mutagenesis is extremely useful for fine structural genetic analysis as it allows mutation of a specific

gene. It involves the Tn5 mutagenesis of cloned DNA in *E. coli*, followed by transfer of the selected insertion sequence into rhizobia involving recombination. This procedure of recombination has been called by several names *viz.*, homogenotization, gene replacement and marker exchange (Ditta, 1986). Site-directed mutagenesis has been used for physical and genetic characterization of several rhizobial genes (Ruvkun and Ausubel, 1981; Ausubel, 1982; Ruvkun *et al.*, 1982, Corbin *et al.*, 1983; Watson and Rastogi, 1993; Kereszt *et al.*, 1998). Recently, *in vitro* Tn5-transposition system for directed mutagenesis has been developed (Goryshin and Reznikoff, 1998).

2.4 Rhizobia-legume symbiosis

Two distinct phylogenetic groups are rhizobia and *Frankia*, which fix atmospheric nitrogen in symbiotic association with higher plants, leading to rhizobia-legume symbiosis and *Frankia*-actinorhizal symbiosis, respectively (Young, 1992). The interaction between rhizobia and legume has been divided into three major stages: preinfection (recognition), infection with concomitant nodule formation, and nodule function.

2.4.1 Preinfection (recognition)

2.4.1.1 Legume root exudates

Rhizobia can live saprophytically in soil and in order to infect the legume roots, these bacteria must first recognize the symbiotic partner. Therefore, rhizobia respond chemotactically towards two classes of chemicals exudated by legume root cells. Firstly, some nutrients such as carbohydrates, organic acids, vitamins and amino acids serve as chemotactic agents to support the growth of rhizobia (Rovira and Davey, 1971; Bushby, 1982; Gaworzewska and Carlile, 1982; Götz *et al.*, 1982; Aguilar *et al.*, 1988; Helal and

Sauerbeck, 1989; Barbour *et al.*, 1991). Secondly, specific flavonoids such as flavones, isoflavones, flavonone/flavonols, coumestans and some other phenolic compounds that do not serve as nutrients, but can interact with the *nodD* gene product, NodD protein, to activate expression of other nodulation (*nod*, *nol* and *noe*) genes of rhizobia to synthesize signal molecules, the lipochitin oligosaccharides (LCO) or Nod factors (NF) (Peters *et al.*, 1986; Kosslak *et al.*, 1987; Caetano-Anollés *et al.*, 1988; Aguilar, 1988; Maxwell and Phillips, 1990; Kape *et al.*, 1991; Fisher and Long, 1992; Dénarié *et al.*, 1996; Heidstra and Bisseling, 1996; Long, 1996; Schultze and Kondorosi, 1996; Spaink, 1996; 2001). Other molecules, such as the betaines, erythronic acids may also act as inducers for nodulation genes of rhizobia (Phillips *et al.*, 1992; Gagnon and Ibrahim, 1998). The spectrum of attractants may vary, not just at the species level but also from strain to strain and can also vary with the host genotypes, which allows, rhizobia to be strongly attracted to specific sites on the legume root surface, presumably because these are the sites of metabolite secretion (Gulash *et al.*, 1984). These interactions are an important determination of host-rhizobia specificity (Horvath *et al.*, 1987; McIver *et al.*, 1989; Spaink *et al.*, 1989; Fisher and Long, 1992; Dénarié *et al.*, 1996; Long, 1996; Long, 2001). Furthermore, before reaching the root legume, rhizobia struggle against different conditions within the soil, such as presence of inhibitory substances or competitive microorganisms.

2.4.1.2 Rhizobial Nod factor synthesis

Legume root exudates induce nodulation genes of rhizobia to produce and release extracellular signal molecules, the LCO or NF. NF chemical structure consists of a chitin oligosaccharide backbone substituted by a N-acyl chain at the non-reducing end and by

various other decorations at the two glucosamine-terminal residues. The substitutions attached to the core structure are dependent on each species or strain and make the Nod factors plant specific (Spaink, 1994; Dénarié *et al.*, 1996; Long, 2001). The enzymes necessary for the production of the NF backbone are encoded by the *nodABC* genes found in all nodulating rhizobial strains. These genes are referred to as common *nod* genes (Kondorosi, 1991). The common *nod* genes (*nodM* and *nodN*) have been identified, *nodM* encodes functions for efficiency of Nod signal production and bacteroid maturation (Baev *et al.*, 1992). Host specific (*hsn*) genes such as *nodFE*, *nodG*, *nodH*, *nodL*, etc. (Fisher and Long, 1992) are involved in specifying the type of host plant to be nodulated. Mutation in these genes lead to abnormalities in the root hair curling reactions elicited on their normal hosts and some times also result in the infection of host plants which are normally unresponsive (Lerouge *et al.*, 1990; Schwedock and Long, 1990). *nodHPQ* genes are organized as an operon that is transcribed in a *nodD*-independent manner and is not regulated by flavonoids (Folch-Mallol *et al.*, 1998). Recently, it has been reported that *nodX* gene from different *R. leguminosarum* strains is temperature regulated (Olsthoorn *et al.*, 2000).

Ritsema *et al.* (1996) found that the replacement of *R. leguminosarum* bv. *viciae* *nodA* by its *B. japonicum* homologue resulted in the loss of *vicia* spp. nodulation. Additionally, Débellé *et al.* (1996) proved that the NodA protein of *S. meliloti* contribute to host range by determining the oligomerization of the NF back bone. The host-specific nodulation genes are responsible for the side groups encountered on the GlcNAc residues of the NF core molecule (Dénarié *et al.*, 1996; Mergaret *et al.*, 1997). Olsthoorn *et al.* (1998) reported that two novel NF structures were discovered in *M. loti*. One NF

structure consisted of only two GlcNAc sugar units while the other was an NF carrying a fucosyl group at a nonterminal GlcNAc . Recently, Taté *et al.* (1999 b & c) and Ferraioli *et al.* (2001) demonstrated that *R. etli* mutant strains CTNUX4, CTNUX23 and CTNUX5 that were mutated in the *trpB*, *metZ* and *argC* genes, respectively, were unable to produce NF, unless tryptophan, methionine and arginine respectively, were added to the growth medium. However, little is known about how these signals are specifically preceived by host plant cells. It has been demonstrated that a plasma membrane depolarization (Ehrhardt *et al.*, 1992; Kurkdjian, 1995; Felle *et al.*, 1995) and intracellular alkalinization (Felle *et al.*, 1996) of alfalfa root hairs take place in response to NF produced by *S. meliloti*.

2.4.2 Infection and nodule formation

2.4.2.1 Cell surface carbohydrate molecules and their role in early infection events

Several biochemical interactions that take place in early infection events are probably involved in the synthesis of several cell surface carbohydrate molecules such as cyclic β-glucans, cellulose fibrils, lipopolysaccharides, exopolysaccharides and capsular polysaccharides. These components have an important role in performing various functions during the plant infection process and increase the efficiency of infection.

2.4.2.1.1 Cyclic β-glucans (CG)

A major type of curdlans, β-(1,3)-glucans can be specifically stained with aniline blue dye (Nikanishi *et al.*, 1976). The genes *ndvA* and *ndvB* encoding functions required for cyclic β-(1,2)-glucans synthesis have been identified in *S. meliloti* (Carlson, 1982; Dylan *et al.*, 1986). However, these genes have homology with agrobacterial

chromosomal virulence loci, the *chvA* and *chvB*, respectively (Dylan *et al.*, 1986). Geremia *et al.* (1987) observed that the absence of β-(1,2)-glucans is correlated with absence of motility. The synthesis of β-(1,2)-glucans was found to be required for infection thread development. However, a β-(1,3) and β-(1,6)-glucan appears to replace β-(1,2)-glucan in *Bradyrhizobium* species (Dudman and Jones, 1980; Miller *et al.*, 1990). In *B. japonicum* and *S. meliloti*, additional loci linked to CG biosynthesis have been identified. These include the identification of *ndvB*-like locus and the *ndvC* locus in *B. japonicum* (Bhagwat *et al.*, 1993; Bhagwat and Keister, 1995; Bhagwat *et al.*, 1996). The *ndvC* locus appears to be involved in the biosynthesis of β-(1,6) linkages within the *B. japonicum* CG backbone (Bhagwat *et al.*, 1993). It has been suggested that the periplasmic oligosaccharides have a role in performing important functions during plant infection and hypoosmotic adaptation (Dylan *et al.*, 1990; Breedveld and Miller, 1994). Recently, the *cgmB* gene of *S. meliloti* involved in CG biosynthesis has been cloned, sequenced and characterized (Wang *et al.*, 1999).

2.4.2.1.2 Cellulose fibrils (CF)

CF are thought to be helping rhizobia to entangle in the surface mucigel of the plant root. This binding may be reinforced by calcium-dependent adhesion proteins called rhicadhesions produced by rhizobia (Smit *et al.*, 1989), thus leading to an increase in the efficiency of infection. These molecules can be identified by their capacity to bind congo red dye which results in the formation of red colonies on the culture media (Kneen and La Rue, 1983).

2.4.2.1.3 Lipopolysaccharides (LPS)

LPS are an important component of the outer membranes in rhizobial cell walls, which are similar to LPS of Gram-negative bacteria (Mayer *et al.*, 1985). It consists of lipid A and polysaccharides. The lipid A portion of the molecule consists of a backbone of alternating pyrophosphate units and glucosamine, to which long fatty acid side chains are attached. Lipid A is a toxic substance that contributes to the danger of infection by Gram-negative bacteria. Polysaccharide side chains extending outward from glucosamine units make up the remainder of the molecules. It consists of O-specific antigen, also called somatic antigen, which is a long chain polysaccharide, and the oligosaccharide core (Hitchcock *et al.*, 1986). It has previously been reported that *R. leguminosarum* bv. *phaseoli* mutants which have defects in production of LPS resulted in deformation of root hairs and stimulation of cell division but did not invade cortical cells and formed nodules which were not able to fix nitrogen (Noel *et al.*, 1986). The LPS of *R. leguminosarum* and *S. meliloti* were found to be actually involved in the interaction of these rhizobia with their host plants (Carlson *et al.*, 1987; Brink *et al.*, 1990; Lagares *et al.*, 1992). It has been shown that the *S. meliloti* mutant Rm6963 (a LPS mutant) form effective nodules with *M. sativa* but failed to establish a symbiosis with *M. truncatula* (Niehaus *et al.*, 1998). In *S. meliloti*, *lpsZ*, a LPS gene involved in symbiosis has been cloned and sequenced (Brzoska and Signer, 1991). Recently, the genetic characterization of a gene region involved in lipopolysaccharide biosynthesis in *B. japonicum* (So *et al.*, 2000) and *S. meliloti* (Lagares *et al.*, 2001) has been reported.

2.4.2.1.4 Exopolysaccharides (EPS)

EPS are complex sugar polymers secreted from rhizobia that loosely encapsulate the cell surface. These molecules have a role in infection thread development. Genes controlling their biosynthesis in *S. meliloti* have been mapped on a megaplasmid pRmeSU47b (Banfalvi *et al.*, 1985; Finan *et al.*, 1986; Glazebrook and Walker, 1989; Charles and Finan, 1990; 1991). *S. meliloti* produces two major EPS molecules, EPSI and EPSII (Leight and Coplin, 1992). EPSI (a succinoglycan) is constitutively expressed under normal laboratory growth conditions, and its presence can be conveniently detected by the binding of the fluorescent compound calcofluor. When grown on agar media containing calcofluor, EPSI-producing strains fluoresce brightly (Doherty *et al.*, 1988). EPSII (a galactoglucan) is not produced at detectable levels in growing culture, but the genes coding for EPSII synthesis are upregulated in response to phosphate starvation (Zhan *et al.*, 1991; Summers *et al.*, 1998; Ruberg *et al.*, 1999). Recently, the *pssA* and *pssCDE* genes of *R. leguminosarum* bv. *trifolii* which are involved in EPS biosynthesis have been characterized (van Workum *et al.*, 1997; Król *et al.*, 1998; Pollock *et al.*, 1998).

It has been reported that *S. meliloti* mutants in production of the CG (*ndv* mutants) or the EPS (*exo* mutants), form small white nodules devoid of bacteria and bacteroids indicating that these genes are required for nodule invasion (Leigh *et al.*, 1985; Long, 1989a; Cheng and Walker, 1998). In other studies, it has been shown that *exo* mutants of *M. loti* were able to induce fully effective nodules on the roots of *Lotus pedunculatus*, but they provoke small and ineffective nodules on the roots of *L. leucocephala* (Hotter and Scott, 1991). Similarly, *exo* mutants of *Rhizobium* sp. strain NGR234 (Chen *et al.*, 1985),

R. leguminosarum bv. *viciae* (Borthakur *et al.*, 1986) and *R. leguminosarum* bv. *trifolii* (van Workum *et al.*, 1997) formed ineffective nodules on the roots of their respective hosts, *L. leucocephala*, *P. sativum* and *T. repens*, respectively. In contrast, *exo* mutants of *R. leguminosarum* bv. *phaseoli* (Diebold and Noel, 1989) and *S. fredii* (Kim *et al.*, 1989) induced fully effective nodules on the roots of determinate legumes, *P. vulgaris* and *G. max*, respectively. One possible explanation of these data is that EPS is required for fully effective symbiosis on plants that produce indeterminate nodules but not on legumes which form determinate nodules. However, Parniske *et al.* (1994) showed that *exo* mutant of *B. japonicum* which form nitrogen-fixing nodules on the roots of *G. max* was ineffective on *G. soja*. Furthermore, a mutant of *Rhizobium* strain TAL1145 that is deficient in EPS synthesis still nodulates various hosts, independent of their nodule type (Parveen *et al.*, 1997).

2.4.2.1.5 Capsular polysaccharides (KPS)

Rhizobia possess surface polysaccharides analogous to group II K antigens (KPS) of *E. coli* (Reuhs *et al.*, 1993); these *E. coli* polysaccharides can replace EPS of *S. meliloti* in infection process (Petrovics *et al.*, 1993; Reuhs *et al.*, 1995). KPS isolated from *S. meliloti* have shown banding patterns on PAGE that correspond to different degrees of polymerization controlled by *lpsZ* gene (Reuhs *et al.*, 1995). KPS of rhizobia have been shown to be composed of a disaccharide repeating unit containing glucuronic acid and a modified pseudaminic acid residue (Reuhs *et al.*, 1998). Two novel gene clusters, designated as *rkp-2* and *rkp-3* regions, involved in production of KPS have been identified in *S. meliloti*, genetic and biochemical analysis of *rkp-2* region was also done (Kereszt *et al.*, 1998). Recently, Pellock *et al.* (2000) reported that the polysaccharides:

succinoglycan, EPSII and K antigen (KPS) of *S. meliloti*, do not function equally well in mediating root nodule invasion. However, these researchers have also observed that succinoglycan is more efficient than K antigen and much more efficient than EPSII in mediating the growth of infection threads on the root hairs of alfalfa plants.

2.4.2.2 Kinetics of infection and nodule formation

The kinetics of infection and nodule formation are presented in Fig. 3. Root hair deformation (curling, branching, waviness, bulging, swelling, etc.) occurs within a few hours of rhizobial exposure to root hairs (Bauer, 1981). It has been proposed that attached rhizobia induce a local stimulation in the rate of plant cell wall expansion (van Batenburg *et al.*, 1986). This process involves host genes like *MtripI* (Cook *et al.*, 1995), *ENOD5* and *ENOD12* (Scheres *et al.*, 1990) and *MtN6* (Mathis *et al.*,1999). During the initiation of an infection thread, the normal process of apical cell wall growth in the root hair seems to be turned inside out in such a way that the outwardly growing cell wall cylinder gets converted into an inwardly growing tunnel. This tunnel has been found to follow the nucleus towards the base of the root hair cell and cytoskeletal connections have been observed between the nucleus and the growing root hair tip (LIoyd *et al.*, 1987). While meristem is active, rhizobia are released from the infection threads in to the nodule cell cytoplasm. The location of the nodule primordium in the root cortex depends on the type of nodule formed by a particular plant (Newcomb, 1981). In temperate legumes such as pea, vetch and alfalfa, the primordium is formed from the cells in the inner cortex (Libbenga and Harkes, 1973; Dudley *et al.*, 1987). These legumes form cylindrical (indeterminate) nodules and have a persistent apical meristem (Newcomb, 1976). The persistent activity of the meristem ensures nodule elongation since new cells are

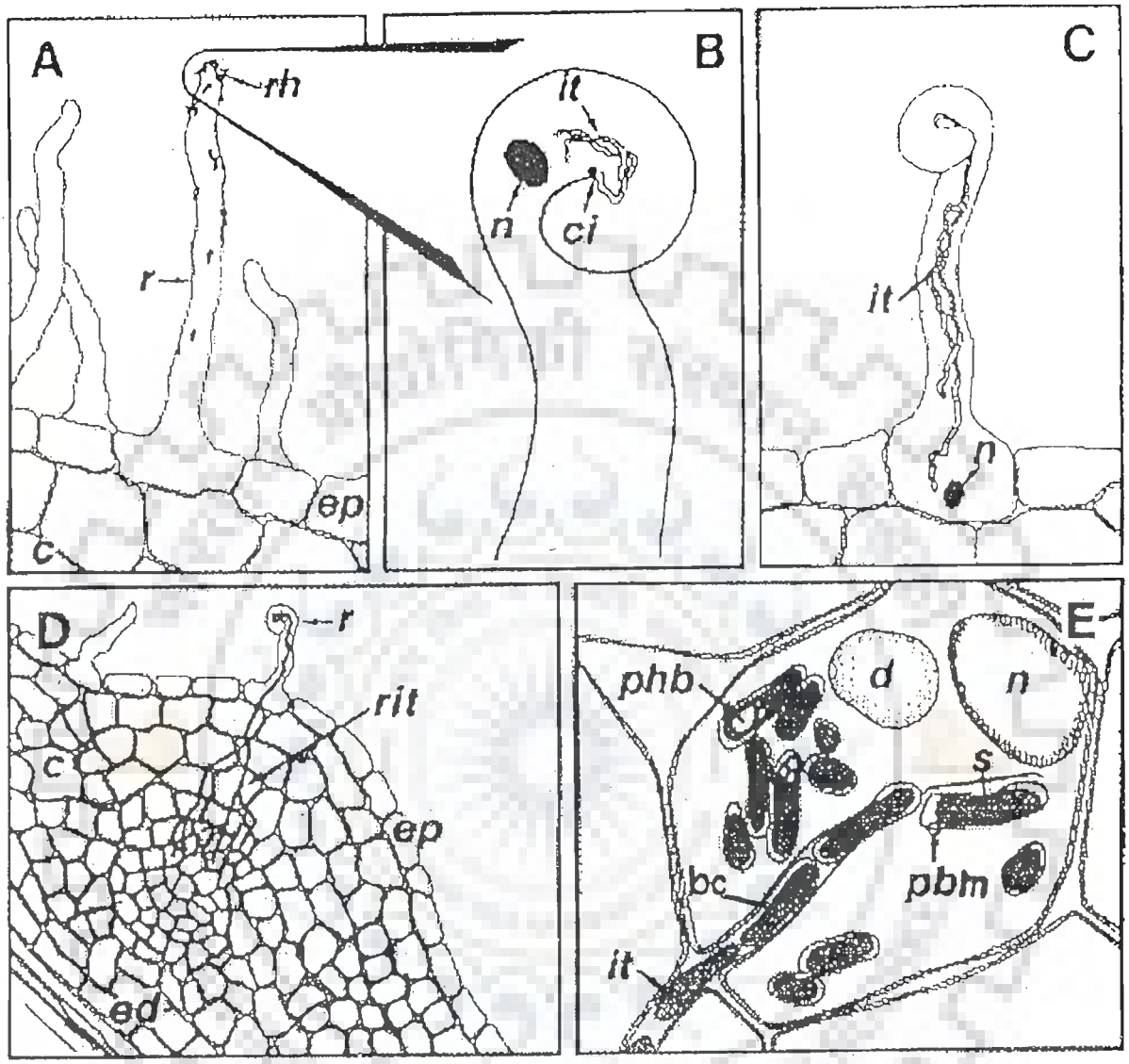

Fig. 3. Schematic representation of the kinetics of infection and nodule formation by rhizobia. A. Rhizobia (*rh*) multiply in the rhizosphere and attach to the root hairs (*r*). B. Nod factors induce root hair curling and permit bacterial penetration at the center of infection (*ci*), the plant nucleus (*n*) precedes the growing infection thread(s) (*it*). C. Infection thread reaches the base of the root hair cell. D. A developing infection thread ramifies (*rit*) near the nodule primordia formed by dividing cortical cells. E. Bacteria (*bc*) are released from the infection thread and form bacteroids or symbiosomes (*s*) in nodule cells, granules of poly-β-hydroxybutarate (*phb*) accumulate in bacteroids surrounded by the peribacteroid membrane (*pbm*). Other abbreviations: *c*, cortex; *d*, digestive vacuole; *ep*, epidermis; *ed*, endodermis (Perret *et al.*, 2000).

contently add to the distal end of the nodules. However, for the differentiation of several of the central tissues found in mature indeterminate nodule, which consist of the distal meristematic, infection, amyloplast-rich inter, nitrogen-fixing and proximal senescence zones. With the exception of the apical meristem, the tissues of these zones are surrounded by peripheral tissues, which include vascularized parenchyma, endodermis and cortex (Vasse *et al.*, 1990). In indeterminate nodules, the nodule growth and functioning occur simultaneously and all developmental zones during differentiation can be observed in a single longitudinal section of nodule. In the plants like *Phaseolus* and *Lotus*, cortical cell divisions occur just beneath the epidermis (Mathews *et al.*, 1989) and these cells are invaded by rhizobia before they become meristematic (Rolfe and Gresshoff, 1988), thus the rhizobial cell divisions occur simultaneously with the plant cell divisions. The nodule meristem involves a mixture of infected and uninfected cells derived from root cortex together with uninfected cells derived from root pericycle, and results in the formation of spherical (determinate) nodules in which meristematic activity is only a transient phase (Pawlowski *et al.*, 1996). Furthermore, there are several reports that provided evidence that the legume ethylene regulates nodule number (Fearn and La Rue, 1991; Penmetsa and Cook, 1997), nodule position (Heidstra *et al.*, 1997), and the morphology of nodules (Fernandez-lópez *et al.*, 1998).

2.4.2.3 Bacterial differentiation and nodule organogenesis

The bacteria are taken up endocytotically from infection thread into the cytoplasm of invaded host cells. It has been reported that the rhizobial bacteria inside the infection thread and after release (before differentiation) contained poly-β-

hydroxybutarate granules (Paan *et al.*, 1980; Hirsch *et al.*, 1982; 1983). For sometime after release, the bacteria divide and some times the growth is vigorous enough so that multiple bacteria are seen bounded by a single peribacteroid membrane. This has been found to be antigenically similar to the plant cell membrane (Perotto *et al.*, 1991). The bacteria undergo important changes and eventually differentiate into nondividing, nitrogen-fixing endosymbionts known as bacteroids (symbiosomes) (Hennecke, 1990). The bacteroids increase in size, become pleomorphic, undergo several changes in nucleic acid content and ultrastructural levels. Meanwhile, the host cells greatly increase their volumes to accommodate many bacteroids and the host plant also supplies nutrients to rapidly increasing number of bacteroids. The plant cell organelles like mitochondria and amyloplasts are pushed to the periphery of these cells. However, several nodulin genes like *ENOD12*, *ENOD40* and *GmN93* are expressed during nodule organogenesis (Kouchi and Hata, 1993).

2.4.3 Nodule function

2.4.3.1 Regulation of nitrogenase complex activity in bacteroids

The bacteroids synthesize the nitrogenase complex, which is responsible for fixation of nitrogen into ammonia. This enzyme system is very similar in composition and function in all prokaryotes which produce it. It consists of two component proteins: a reductase is a 64 KDa dimer of identical subunits that contains one iron-sulfer cluster and two ATP binding sites which provides electrons with high reducing power, and a nitrogenase which uses these electrons to reduce nitrogen to ammonia. This enzyme is 22 KDa, consisting of two α and β ($\alpha_2 \beta_2$) components and an iron-molybdenum complex. The electron transfer carriers such as flavodoxin or ferredoxin, from the

reductase to the nitrogenase is coupled to the hydrolysis of ATP by reductase. Together, these two enzymes catalyze the following reaction:

$$N_2 + 8H^+ + 8e^- \xrightarrow[\text{Nitrogenase complex}]{16\ Mg\ ATP \longrightarrow 16\ Mg\ ADP + 16\ Pi} 2NH_3 + H_2\uparrow$$

The reduction of acetylene (HC≡CH) to ethylene ($H_2C=CH_2$) has become a standard technique for determining nitrogen fixation activities (Dilworth, 1966; Schollhorn and Burris, 1967). These two gases are easily separated and determined quantitatively by gas-chromatography where less than 10 pmol of C_2H_2 in the assays can be detected accurately. The acetylene-reducing capacity of the nodule was correlated with the differentiation of bacteroids (Vasse *et al.*, 1990). Nitrogenase complex is irreversibly inhibited by oxygen, so the enzyme must be protected from this reactive substance. In nodules, bacteroids must first be maintained in a low oxygen environment, but supplied with a high oxygen flux. The protection is afforded by the symbiotic synthesis of leghaemoglobin. The globin part of this monomeric oxygen-binding protein is synthesized by the host plant (Dilworth, 1969; Cutting and Schulman, 1971; Verma *et al.*, 1974; Sidloi-Lumbroso *et al.*, 1978), while the haem group is synthesized by the rhizobial bacteroids (Cutting and Schulman, 1969; Godfrey and Dilworth, 1971; Nadler and Avissar, 1977). The haem is transported out of the bacteroids into the plant cytoplasm, where it combines with the plant produced globin to form leghaemoglobin. The leghaemoglobin facilitates diffusion of oxygen to the bacteroids for respiration without endangering the activation of oxygen labile nitrogenase complex.

2.4.3.1.1 Nitrogen fixation (*nif* and *fix*) genes

Several types of nitrogen fixation (*nif* and *fix*) genes which have different mode of functions, are directly concerned with the nitrogenase complex. Twenty adjacent *nif* genes are organized in eight operons in a 24 kb region of DNA in *Klebsiella pneumoniae*. *Rhizobium fix* genes, shown to be equivalent to *K. pneumoniae nif* genes are assigned the same names (Beringer *et al.*, 1980; Arnold *et al.*, 1988). The *nif* and *fix* genes location is species-specific. In *S. meliloti*, two clusters of these genes have been located on a megaplasmid pRmeSU47a. The cluster I includes *nifHDKE*, *nifN*, *fixABCX*, *nifA* and *nifB* genes whereas the cluster II has *fixLJ*, *fixK*, *fixNOQP* and *fixGHIS* genes. The position of cluster II is 220 kb downstream of *nifHDKE* operon and it is transcribed in direction opposite to *nifHDKE* (Batut *et al.*, 1985; David *et al.*, 1987; 1988).

nifHDK genes are located in a single operon in *Rhizobium* spp. These genes code for nitrogenase polypeptides (Ruvkun and Ausubel, 1980; Rosenberg *et al.*, 1981). *nifA* is located upstream of *nifHDK* (Dixon, 1984) and is a regulatory gene of *nif* and *fix* genes (Ditta et al., 1987).

fixABCX genes required for nitrogenase activity are located between *nifHDK* and *nifA* operons (Ruvkun *et al.*, 1982). *fixGHIS* gene products are predicted to be transmembrane proteins on the basis of amino acid sequences (Fischer, 1994). *fixR* is located upstream of regulatory *nifA* gene and is involved in oxidation-reduction process (Thöny *et al.*, 1987). The predicted amino acid sequences of *fixNOQP* proteins imply that they encode membrane-bound cytochrome c-containing haem/copper cytochrome oxidase (Mandon *et al.*, 1993). The proposed function(s) of various *nif* and *fix* genes in rhizobia (Sharma *et al.*, 1993; Fischer, 1994) are given in Table 1.

Table 1. Functions of *nif* and *fix* genes in rhizobia

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

2.4.3.2 Cytochromes and their role in nitrogen fixation

Cytochromes are electron transfer proteins that carry haem as a prosthetic group, their redox function is intimately related to the change in valency of haem iron (IUB, 1992; Yamanaka, 1992). Cytochrome c oxidase can be used as a simple, rapid screening test in the diagnosis of rhizobia (Sadowsky *et al.*, 1983). Since respiration takes place in the cytoplasmic membrane, cytochromes are often localized in this compartment. In addition, they are found in the periplasmic space, where their electron transfer function is connected with that of membrane-bound cytochromes. In *S. meliloti*, *B. japonicum* and *R. leguminosarum*, the *cycHJKL* genes are found to be in a single operon (Kereszt *et al.*, 1995; Ritz *et al.*, 1995; Delgado *et al.*, 1995). This operon is essential for the biogenesis of c-type cytochromes. Mutations in *cycHJKL* of rhizobia, completely abolish symbiotic nitrogen fixation. Recently, Yeoman *et al.* (1997) found that *R. leguminosarum* strains that were mutated in the *cycHJKL* operon were defective in Fe uptake and in the production of vicibactin.

2.4.3.3 Molecular mechanism of ammonium transport

This topic has recently been reviewed by Taté *et al.* (1999a; Fig. 4). The identification of the molecular mechanism by which bacteroids are able to fix nitrogen but, unlike free-living diazotrophs, are unable to assimilate the NH_3 produced, is one of the most important aspects of endosymbiotic association. Although, most of the fixed-nitrogen produced by the symbiosomes is exported to the host plant to satisfy its demands of nitrogen (Glenn and Dilworth, 1984), the form (e.g., NH_3/NH_4^+ or organic compounds, such as amino acids) and the mechanism (passive diffusion or active transport) by which nitrogen is exported are still largely unclear. However, Udvardi and Day (1997) proposed

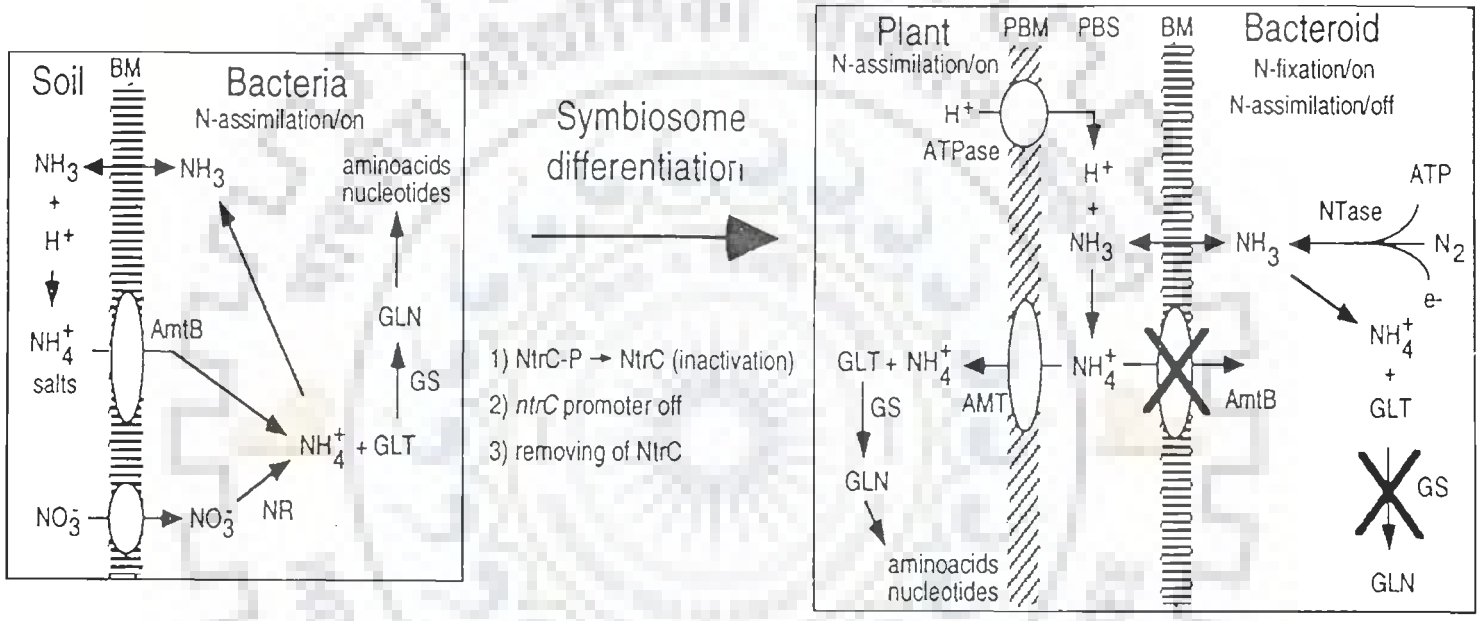

Fig. 4. Schematic representation of the ammonium transport from the N_2-fixing symbiosome to the cytoplasm of the invaded plant cell (Taté *et al.*, 1999a).

Abbreviations : AMT, ammonium transport; BM, bacteroid membrane; GLN, glutamine; GLT, glutamate; GS, glutamine synthetase; NTase, nitrogenase; PBM, peribacteroid membrane; PBS, peribacteroid space.

that NH_3 might passively diffuse across the bacteroid membrane into the relatively acidic peribacteroid space where it is converted to NH_4^+, which enters the plant cytosol through a channel located on the peribacteroid membrane. This model was based on previous observation indicating that the NH_4^+ uptake and glutamine synthase activities, are expressed at very low levels in symbiosomes and on the identification of an NH_4^+ carrier (identified by the patch clamp technique) located on the peribacteroid membrane (Tyerman *et al.*, 1995; Kaiser *et al.*, 1998).

Merrick and Edwards (1995) demonstrated that the transcription of genes involved in NH_4^+ assimilation in *R. etli*, is regulated by the two-component Ntr system. These include the histidine protein kinase NtrB and the transcriptional regulator NtrC (Patriarca *et al.*, 1992; 1993; 1994). Furthermore, Patriarca *et al.* (1996) and Taté *et al.* (1998) observed that in *R. etli* bacteria growing inside the nodule, this system is switched off by a three-step mechanism of gene regulation acting; firstly by inactivation of the NtrC protein (most probably by de-phosphorylation), secondly by down-regulation of *ntrC* transcription occurring in bacteroids of young symbiosomes, and thirdly by the complete removal of NtrC protein by an unknown mechanism of protein degradation. Therefore, Taté *et al.* (1998) suggested that the inactivation of NtrC may be the first step of a regulatory mechanism of gene regulation acting during bacteroid differentiation to uncouple N_2 fixation and NH_4^+ assimilation activities.

2.5 Bacteroidal metabolism and their role in symbiosis

2.5.1 C_4-dicarboxylic acid transport system

Succinate, malate, fumarate and aspartate are considered substrates for the rhizobial dicarboxylic acid transport (Dct) system (Watson *et al.*, 1988; 1993). In

S. meliloti, the Dct is encoded by three genes located on a megaplasmid pRmeSU47b, *dctA*, *dctB* and *dctD* (Watson *et al.*, 1988). The *dctA* gene codes for a high-affinity permease. It has been shown that mutation in *dctA* gene, do not generally disturb nodule development, but block nitrogen fixation (Ronson *et al.*, 1981; Finan *et al.*, 1983; Arwas *et al.*, 1985; Bolton *et al.*, 1986; Engelke *et al.*, 1987; Watson *et al.*, 1988). The *dctB* and *dctD* genes encode a two-component sensor regulator system, which activates the transcription of *dctA* in response to the presence of dicarboxylates in the periplasm, where the sensor domain of DctB is located (Watson, 1990; Reid and Poole, 1998). *dctBD* genes are controlled by *ntrA* gene that codes for sigma factor (Ronson *et al.*, 1987). DctBD-dependent and independent *dctA* expression in *S. meliloti* has been identified (Boesten *et al.*, 1998). Recently, Yurgel *et al.* (2000) reported that the permease component of the Dct system, DctA, of *S. meliloti* can transport orotate, a monocarboxylic acid.

2.5.2 Biosynthetic pathways of vitamins, nucleotide bases and amino acids

The symbiotic role of the biosynthetic pathways of vitamins, nucleotide bases and amino acids of rhizobia have been identified by using auxotrophic mutants as markers for probing the nature of symbiosis. In some cases, some metabolic intermediates or end products affect the symbiotic interaction.

2.5.2.1 Biosynthetic pathways of vitamins

Early researchers (Levin *et al.*, 1954) attributed the synthesis of large amounts of vitamin B_{12} by the rhizobia to leghaemoglobin production in the nodule. Graham (1963) found that the root nodule bacteria have different requirements of vitamins. Schwinghamer (1970) and Pankhurst *et al.* (1972) demonstrated the requirement of

riboflavin biosynthesis for bacteroid development of *R. leguminosarum* bv. *trifolii*. The riboflavin requiring auxotrophs isolated by these researchers were unable to differentiate into bacteroids and resulted in the formation of ineffective nodules. Recently, it has been found that biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *S. meliloti* 1021 (Streit *et al.*, 1996).

2.5.2.2 Biosynthetic pathways of nucleotide bases

Nucleotide bases (purines and pyrimidines) also have a role in rhizobia-legume symbiosis. Purine auxotrophs of *S. meliloti* (Scherrer and Dénarié, 1971; Fedorov and Zaretskaya, 1977; Malek and Kowalski, 1983; Kerppola and Kahn, 1988a; Swamynathan and Singh, 1992; 1995), *R. leguminosarum* (Schwinghamer, 1967; Pankhurst and Schwinghamer, 1974; Pain, 1979), *S. fredii* HH303 (Kim *et al.*, 1988), *Rhizobium* sp. strain NGR234 (Chen *et al.*, 1985) and *R. etli* (Noel *et al.*, 1988) have been reported to be defective in symbiosis. Supplementation of adenine or adenine containing-compounds to the roots of pea seedlings would allow a non-nodulating mutant of *R. leguminosarum* to elicit ineffective nodules (Schwinghamer, 1967). Chen *et al.* (1985) speculated that two adenine auxotrophs of *Rhizobium* sp. strain NGR234 were symbiotically defective because of EPS overproduction. Purine auxotrophs of *R. etli* were found to elicit pseudonodules on bean plants. These auxotrophs lead to root hair curling and cortical cell division but did not elicit infection thread formation and as a result no bacteria could be isolated from the resulting pseudonodules (Noel *et al.*, 1988). Supplementation of the plant growth medium with adenosine had no effect on nodule phenotype while addition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) significantly enhanced root nodule development (Noel *et al.*, 1988; Newman *et al.*, 1992). Purine auxotrophs of

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

Pyrimidine auxotrophs of *S. meliloti* (Scherrer and Dénarié, 1971; Vineetha *et al.*, 2001) and *S. fredii* (Kim *et al.*, 1988) have been isolated and found to be ineffective in nitrogen fixation. Nodule development defective phenotype of pyrimidine auxotrophs of *R. leguminosarm* bv. *phaseoli* CFN42 could not be suppressed by pyrimidine addition to the plant growth medium (Noel *et al.*, 1988). Mutations in carbamoylphosphate synthetase and steps of pyrimidine biosynthesis before orotic acid have been found to result in ineffective symbiosis in *S. meliloti* strain 104A14 (Kerppola and Kahn, 1985; 1988a & b).

2.5.2.3 Biosynthetic pathways of amino acids

Histidine auxotrophs of *B. japonicum* (Sadowsky *et al.*, 1986; So *et al.*, 1987) and *R. leguminosarum* bv. *trifolii* (Yadav *et al.*, 1998) failed to induce nodules on the roots of their respective plant hosts. However, histidine supplementation restored nodulation ability of these mutants indicating unavailability of histidine *in planta*.

Leucine auxotrophs of *R. leguminosarum* reported by Pain (1979) were symbiotically effective, while of *S. meliloti* were symbiotically ineffective (Truchet *et al.*, 1980; Kerppola and Kahn, 1988a). However, supplementation of leucine to the

plants inoculated with the leucine mutants isolated by Truchet *et al.* (1980) could restore symbiotic effectiveness of these auxotrophs. In case of leucine auxotrophs, isolated by Kerppola and Kahn (1988a), addition of leucine did not restore symbiotic ability of these auxotrophs.

Isoleucine and valine auxotrophs of *S. fredii* HH303 (Kim *et al.*, 1988) and *S. meliloti* (Aguilar and Grasso, 1991; López *et al.*, 2001) have been shown to have symbiotically defective phenotype. It has been found that the promotor of the common nodulation genes (*nod ABC*) in *S. meliloti ilvC* mutants was not activated by luteolin (Aguilar and Grasso, 1991). These mutants were unable to nodulate either on supplementation of isoleucine and valine or their intermediates. It has been hypothesised that the cellular concentrations of acetolactate in *ilvC* mutants might have reached levels which inhibited the expression of nodulation genes. However, López *et al.* (2001) proposed that the pleiotropic action of the *ilvC* gene in *S. meliloti* may reveal novel complexities involved in the symbiotic interaction.

Arginine auxotrophs of *S. meliloti* were investigated by Dénarié *et al.* (1976), Fedorov and Zaretskaya (1977) and Kerppola and Kahn (1988a). It has been demonstrated that the mutants blocked in the later step of the arginine biosynthetic pathway, in either ornithine transcarbamylase or arginosuccinate synthetase, were effective. In contrast, mutants blocked in the early step that leads to ornithine, were ineffective. It was proposed that since ornithine is a precursor in polyamines synthesis (Tabor and Tabor, 1985) and the lack of polyamines may directly lead to the symbiotic defect. Recently, Ferraioli *et al.* (2001) reported that the *argC* gene of *R. etli* strain CTNUX5 is essential for functional symbiosis.

Aspartate auxotrophs of *S. fredii* HH303 reported by Kim *et al.* (1988) and asparagine auxotrophs of *S. meliloti* 104A14 isolated by Kerppola and Kahn (1988a) formed ineffective nodules on the roots of soybean and alfalfa plants, respectively.

Scherrer and Dénarié (1971), Pain (1979) and Kim *et al.* (1988) reported that the methionine auxotrophs of *S. meliloti*, *R. leguminosarum* and *S. fredii*, respectively, formed effective nitrogen-fixing nodules on the roots of their respective hosts. However, Kerppola and Kahn (1988a) observed that methionine auxotrophs of *S. meliloti* 104A14, elicited ineffective nodules on the roots of alfalfa plants. Recently, Taté *et al.* (1999c) found that the *R. etli metZ* gene is essential for nodulation. It has been shown that cysteine or glutamate, but not methionine, is supplied by the root cells to *R. etli* bacteroids located inside the nodules (Taté *et al.*, 1997; 1999b). However, these researchers suggested that the different symbiotic behavior of the methionine-requiring strains of rhizobia may be due to the step affected by the mutations or it may be due to strain-specific requirements.

Proline auxotrophs of *R. leguminosarm* bv. *viciae* strain C1204b reported by Te-Chein *et al.* (1991) were ineffective in nitrogen fixation, while the *B. japonicum proC* gene has been found to be essential for symbiosis (King *et al.*, 2000). However, the role of some amino acids like serine, glycine, glutamine and glutamate in symbiosis is still not clear (de Bruijn *et al.*, 1989).

The aromatic amino acid biosynthesis (Moat and Foster, 1995; Pittard *et al.*, 1996; Xiu *et al.*, 1997; Fig. 6) begins with the condensation of erythrose-4-phosphate and phosphoenolpyruvate to form 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) which is then converted into shikimate and to chorismate, a common precursor for

aromatic amino acids. Auxotrophs of these acids were demonstrated by Jelesko *et al.* (1993) and Prasad *et al.* (2000). It appeared that normal metabolic flow through the aromatic amino acid biosynthetic pathways of *S. meliloti* was essential for the bacteroid development. However, the mutants isolated by Jelesko *et al.* (1993) showed decreased DAHP synthase activity and formed ineffective nodules.

The synthesis of tryptophan, is catalyzed by five enzymes: anthranilate synthase, anthranilate phosphoribosyl transferase, phosphoribosyl anthranilate isomerase, indole-3-glycerol phosphate synthase and tryptophan synthase. The levels of these enzymes depend on six structural genes present in tryptophan operon. In *S. meliloti*, these genes are present in three unlinked chromosomal groups, containing *trpE*, *trpC*, and *trpD*, and *trpA*, *trpB*, and *trpF*, respectively (Johnston *et al.*, 1978). The *trpE* locus, identified by heterologous complementation, has been shown by Bae *et al.* (1989) to be a fusion between the *trpE* and *trpG* coding sequences and has been named *trpE(G)*.

Pain (1979) reported that the tryptophan auxotrophs of *R. leguminosarum* were symbiotically effective. However, Wells and Kuykendall (1983) demonstrated that out of eleven tryptophan auxotrophs of *B. japonicum* strain I-110ARS, eight mutants which had defects earlier in the tryptophan biosynthetic pathway did not nodulate soybean plants. The remaining mutants which had defects in tryptophan synthase, the last step of tryptophan biosynthetic pathway, nodulated soybean plants. Kummer and Kuykendall (1989) reported that the tryptophan auxotrophs of *B. japonicum* were symbiotically defective, while the lone double auxotroph of tryptophan and histidine did not form any nodule-like structure on the roots of soybean plants. The interesting observation of tryptophan biosynthesis is that the prototrophic revertants of Nod^- tryptophan auxotroph

of *B. japonicum* showed enhanced nodulation and nitrogen fixation (Hunter and Kuykendall, 1990). Barsomian *et al.* (1992) showed that except *trpE(G)* mutants of *S. meliloti*, all other tryptophan auxotrophs were found to be Nod^+, Fix^+, though *trpE(G)* mutants form two types of nodules showing unusual defects in their histological features. One type contains bacteroids in its base and was capable to fix only trace amount of nitrogen, while the other lacks bacteroids and could not fix nitrogen. Taté *et al.* (1999b) demonstrated that *R. etli* strain CTNUX4 that was mutated in the *trpB* gene induced ineffective nodules on the root of *P. vulgaris*.

Phenylalanine and tyrosine biosynthesis begins with the conversion of chorismate to prephenate by chorismate mutase. Prephenate is converted to phenylpyruvate by prephenate dehydratase and then to phenylalanine, or to 4-hydroxyphenylpyruvate by prephenate dehydrogenase and then to tyrosine. Alternatively, prephenate may be converted to arogenate by transamination with arogenate dehydratase forming phenylalanine or arogenate dehydrogenase forming tyrosine. It has been reported that the phenylalanine auxotrophs of *R. leguminosarum* isolated by Pain (1979) provoked nodules that were symbiotically effective. Tyrosine auxotrophs of *S. meliloti* 104A14, isolated by Kerppola and Kahn (1988a) elicited ineffective nodules on the roots of alfalfa plants. However, Prasad *et al.* (2000) reported that the *phe* and *tyr* mutants of *S. meliloti* Rmd201 were Nod^+, Fix^- and Nod^+, Fix^+, respectively.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial strains and plasmid

The relevant characteristics and sources or references of bacterial strains and plasmid used in this study are demonstrated in Table 2.

Table 2. Bacterial strains and plasmid used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
Sinorhizobium meliloti Rmd201	Spontaneous Sm^r derivative of AK631 (Nod^+, Fix^+)	Khanuja and Kumar (1988)
NV3	Rmd201, *aro*::Tn5, Sm^r, Km^r	This lab
BA2	Rmd201, *aro*::Tn5, Sm^r, Km^r	This lab
FN2	Rmd201, *trpE(G)*::Tn5, Sm^r, Km^r	This study
FN3	Rmd201, *trpE(G)*::Tn5, Sm^r, Km^r	This study
NV7	Rmd201, *trpD, trpF* or *trpC*::Tn5, Sm^r, Km^r	This lab
NV31	Rmd201, *trpD, trpF* or *trpC*::Tn5, Sm^r, Km^r	This lab
BA6	Rmd201, *trpB*::Tn5, Sm^r, Km^r	This lab
FN4	Rmd201, *tyrA*::Tn5, Sm^r, Km^r	This study
FN9	Rmd201, *pheA* :: Tn5, Sm^r, Km^r	This study
Escherichia coli		
WA803 (pGS9)	Met^-, Thi^-, Cm^r, Km^r	Selvaraj and Iyer (1983)
Plasmid		
pGS9	Inc. N, rep, P15A, Cm^r, Km^r	Selvaraj and Iyer (1983)

Abbreviations: Nod^+, ability to nodulate; Fix^+, ability to fix nitrogen, Sm^r, streptomycin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Met^- inability to produce methionine; Thi^-, inability to produce thiamine; Inc. N, incompatibility group N; rep, replication and maintenance.

3.1.2 Plant cultivar

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

3.1.3 Chemicals

The chemicals used in this investigation were of analytical grade obtained from Hi-Media Laboratories, Mumbai, India; G.S. Chemical Laboratories, New Delhi, India; Sisco Research Laboratories, Mumbai, India; Merck Laboratories, Mumbai, India; Polypharm Laboratories, Mumbai, India and Sigma Chemical Company, USA.

3.1.4 Composition of growth media

3.1.4.1 Media for *S. meliloti* strains

3.1.4.1.1 Complete media

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

Constituent	Amount(gm)
Tryptone	5.0
Yeast extract	3.0
$CaCl_2.2H_2O$	0.12

Distilled water to make 1.0 litre volume.

3.1.4.1.1.1.1 TY swarm plates (Dylan *et al.*, 1990)

The composition of this medium was the same as TY medium except for reduction of agar concentration to 0.3% (w/v). This medium was used to test the ability of *S. meliloti* strains to produce β-(1,2)-glucans.

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

Constituent	Amount (gm)
Mannitol	10
Yeast extract	1.0
$K_2HPO_4.3H_2O$	0.2
KH_2PO_4	0.2
$MgSO_4.7H_2O$	0.1
$CaCl_2.2H_2O$	0.05

Distilled water to make 1.0 litre volume.

3.1.4.1.1.3 Yeast extract mannitol (YEM) medium (Vincent, 1970)

Constituent	Amount (gm)
Mannitol	10.0
Yeast extract	10.0
$K_2HPO_4.3H_2O$	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$CaCO_3$*	3.0

Distilled water to make 1.0 litre volume.

* Added when excess neutralizing agent was required.

3.1.4.1.2 Minimal medium

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

Constituent	Amount (gm)
$Na_2HPO_4.12H_2O$	0.45
$(NH_4)_2SO_4$	2.0

$FeCl_3$	0.002
$MgSO_4.7H_2O$	0.1
$CaCl_2.2H_2O$	0.04

Distilled water to make 1.0 litre volume

Glucose solution (20% w/v) was prepared and filter sterilized using 0.2 μm membrane filter (Schleicher & Schüll). 10 ml from this solution was added to make 1 litre volume of the autoclaved medium.

3.1.4.2 Medium for *E. coli* strain

3.1.4.2.1 Luria-Bertani (LB) medium (complete medium)(Sambrook *et al.*, 1989)

Constituent	**Amount (gm)**
Tryptone	10
Yeast extract	5.0
NaCl	10

Distilled water to make 1 litre volume.

For making solid medium 16 gm of agar (Hi-Media) was added to the above media before autoclaving.

3.1.4.3 Media for alfalfa (*M. sativa*) plants

3.1.4.3.1 1% (w/v) water agar medium

1.0 gm of agar was added to 100 ml of distilled water and autoclaved. This medium was used for germination of alfalfa seeds.

3.1.4.3.2 Nitrogen free plant growth medium

The nitrogen free plant growth medium developed by Engelke *et al.* (1987) was used for growing alfalfa plants. Twelve stock solutions of following composition were prepared.

Stock solution	Constituent	Amount (gm)	Distilled water (ml)
A	$K_2HPO_4.3H_2O$	2.090	10
B	KH_2PO_4	0.544	10
C	$CaCl_2.2H_2O$	7.351	10
D	$C_6H_5O_7.Fe.3H_2O$	0.335	10
E	$MgSO_4.7H_2O$	6.162	10
F	K_2SO_4	4.356	40
G	$MnSO_4.H_2O$	0.034	20
H	H_3BO_3	0.026	20
I	$ZnSO_4.7H_2O$	0.030	20
J	$CuSO_4.5H_2O$	0.002	20
K	$CaSO_4.2H_2O$	0.006	20
L	$Na_2MoO_4.2H_2O$	0.006	20

These stock solutions were autoclaved. To make one litre of plant growth medium, 10 gm of agar was added to 985 ml of distilled water and the resulting water agar medium was autoclaved. Four ml of stock solution F and one ml each of the remaining stock solutions were added to autoclaved agar solution. Whenever nutritional supplementations of the auxotrophs were required, the required amount of sterile nutrients were added to the medium. This medium was then used to prepare slants for growing alfalfa plants.

The pH of the above media was adjusted to 6.8 with 0.1 N NaOH or 0.1 N HCl solutions before adding agar and then autoclaved. All growth media were autoclaved at 15 psi (121°C) for 20 minutes.

3.1.5 Supplements to media

3.1.5.1 Antibiotics

The antibiotics used in this study were streptomycin sulphate (Hi-Media), kanamycin acid sulphate (Sigma) and chloramphenicol (Hi-Media). Stock solutions of streptomycin sulphate and kanamycin acid sulphate were prepared in sterile distilled water, while that of chloramphenicol was prepared in 50% (w/v) ethanol. Filter sterilization was done by passing them through 0.2 μm membrane filters (Schleicher & Schüll). Required volume from the sterile stock solution was added to the autoclaved medium after cooling it to about 50°C just before plating. The final concentrations of antibiotics added to the media were as follows: streptomycin sulphate, 100 μg/ml, for *S. meliloti* Rmd201; kanamycin acid sulphate and chloramphenicol, 100 and 40 μg/ml, respectively, for *E. coli* WA803 and both streptomycin sulphate and kanamycin acid sulphate, 100 and 400 μg/ml, respectively, for auxotrophic mutants.

3.1.5.2 Vitamins, nucleotide bases and amino acids

Stock solutions of vitamins, nucleotide bases and amino acids (these chemicals were purchased from Hi-Media Laboratories) were prepared in distilled water. The solutions were filter sterilized using 0.2 μm membrane filters (Schleicher & Schüll). The final concentrations used in this study for vitamins, nucleotide bases and amino acids were 10, 20 and 50 μg/ml, respectively. Required volumes from the sterile stock solutions were added to the autoclaved media. To identify the nature of auxotrophy of each strain, nutritional pools as described by Holliday (1956) with some modifications were used. The compositions of modified Holliday pools are listed in Table 3.

Table 3. Nutritional pool compositions of RMM plates

Pools	1	2	3	4	5
6	Adenine	Guanine	Cysteine	Methionine	Thiamine
7	Histidine	Leucine	Isoleucine	Lysine	Valine
8	Phenylalanine	Tyrosine	Tryptophan	Threonine	Proline
9	Glutamine	Asparagine	Uracil	Aspartic acid	Arginine
10	Thymine	Serine	Glutamic acid	Alanine	Glycine
11	Pantothenic acid	Riboflavin	Biotin	Cobalamine	Nil

3.1.5.3 Intermediates

For preparing stock solutions of intermediates, shikimic acid (Sigma), anthranilic acid (Hi-Media) and indole (Sisco) were dissolved in distilled water. The solutions were filter sterilized by passing them through 0.2 μm membrane filters (Schleicher & Schüll). These solutions were added to minimal medium to make final concentration of 50 μg/ml after autoclaving.

3.1.5.4 C_4-dicarboxylic acids

2 gm of C_4-dicarboxylic acids, viz., malic acid, succinic acid, fumaric acid and aspartic acid (Hi-Media) were added to 1 litre of the RMM (without glucose and enriched with 50 μg/ml of respective nutritional requirements of the auxotrophs), as sole carbon source, before autoclaving.

3.1.5.5 Sugars

20% (w/v) solutions of different sugars, such as, arabinose, glucose, galactose, mannose, xylose, lactose, maltose, sucrose, fructose, mannitol and sorbitol (Hi-Media)

were prepared. These solutions were filter sterilized by passing them through 0.2 μm membrane filters (Schleicher & Schüll). 10 ml from each sugar solution was added to 1 litre of autoclaved RMM, containing 50 μg/ml of auxotrophic requirements, as sole carbon source.

3.1.5.6 Sodium deoxycholate (DOC)

DOC (Hi-Media) was added to TY medium at the rate of 1 mg/ml before autoclaving and used to test the ability of *S. meliloti* strains to produce lipopolysaccharides.

3.1.5.7 Dyes

Calcofluor white (Sigma), congo red (Hi-Media) and aniline blue (Hi-Media) were added to the MSY medium at the rate of 0.02% (w/v) before autoclaving. These dyes were used to test the ability of *S. meliloti* strains to produce succinylated exopolysaccharides, cellulose fibrils and β-(1,3)-glucans, respectively. Methylene blue (0.01% w/v), prepared in distilled water, was used to stain root hairs of alfalfa plants and toluidine blue (1% w/v), from Hi-Media Laboratories, prepared in 1% (w/v) borax (Sigma) was used to stain semithin sections of nodules for light microscopy.

3.1.6 Diluents

0.85% (w/v) NaCl (physiological saline solution) and RMM (without sugar) were used for carrying out the serial dilutions and washing bacterial cultures, respectively.

3.1.7 Reagents for measurement of tryptophan intermediate accumulation

3.1.7.1 6% (w/v) *p*-dimethyl amino benzaldehyde reagent (Snell and Snell, 1967)

6 gm of *p*-dimethyl amino benzaldehyde (G.S.C) was dissolved in 100 ml of

90% (v/v) sulfuric acid. This reagent was used for detection of anthranilic acid.

3.1.7.2 Ferric chloride reagent (Yanofsky and Smith, 1962)

This reagent with the following composition was prepared and stored at room temperature in a glass-stoppered brown coloured bottle:

1 ml of 0.5 M $FeCl_3$

50 ml of distilled water

30 ml of conc. H_2SO_4

This reagent was used for detection of indole-3-glycerol phosphate.

3.1.8 Reagent for detection of cytochrome c oxidase (Kovaks, 1956; Bradshaw, 1992)

1 gm of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma) was dissolved in 100 ml of distilled water in a dark, glass-stoppered dropping bottle and kept at 4°C in refrigerator. The reagent was prepared fresh after every two weeks.

3.1.9 Composition of solutions for preparation of histological sections of nodules

3.1.9.1 Requirements for preparation of blocks

i. 0.2 M phosphate buffer (pH 7.2) (Bollag *et al.*, 1996).

This buffer was prepared as follows:

Two stock solutions A and B of following compositions were prepared:

Stock solution A (0.2 M NaH_2PO_4):

Dissolve 27.6 gm of NaH_2PO_4 in 1 litre of distilled water.

Stock solution B (0.2 M Na_2HPO_4):

Dissolve 28.4 gm of Na_2HPO_4 in 1 litre of distilled water.

28 ml of solution A was mixed with 72 ml of solution B. The pH of this buffer was adjusted to 7.2 and autoclaved at 15 psi (121°C) for 20 min.

ii. **Fixatives**

a. **Primary fixative** (Karnovsky, 1965)

This fixative with the following composition was prepared and used for the primary fixation of nodules.

12.5 ml of 8% (w/v) paraformaldehytde (Sigma) (added 4 gm of paraformaldehyde in 50 ml of distilled water and heated the solution to 60-70°C followed by the addition of few drops of 1N NaOH solution).

5.0 ml of 12.5% (v/v) glutaraldehyde (Sigma)

25.0 ml of 0.2 M phosphate buffer (pH 7.2)

7.5 ml of double distilled water

b. **Secondary (post) fixative**

Stock solution of 2% (w/v) osmium tetroxide (OsO_4) was prepared by dissolving 1 gm of OsO_4 in 50 ml of double distilled water. This solution was stored at 4°C in a tightly stoppered brown coloured bottle.

iii. **Acetone series**

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

iv. **Araldite CY212 (resin) embedding medium**

This medium with the following composition was used for embedding of nodules.

Araldite CY 212	10 ml
Hardener (Dodecenyl succinic anhydrite, DDSA; HY 964)	10 ml

Accelerator (Tridimethyl aminomethyl phenol, DMP30;DY064) 0.4 ml

Plasticizer (Dibutylphthalate) 1.0 ml

The above ingredients were mixed and vigorously stirred.

3.1.9.2 Solutions for staining ultrathin sections of nodules

i. Uranyl acetate solution

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

ii. Lead citrate solution

To 12 ml of filtered 50% (v/v) ethanol in a 15 ml centrifuge tube, one and half pellets of NaOH were added. After dissolution of NaOH, 50 mg of lead citrate was added, shaken well to assist in dissolution and centrifuged at 5,000 rpm for 2 minutes. The supernatant was stored at 4°C in refrigerator.

3.2 METHODS

3.2.1 General bacteriological procedures

3.2.1.1 Bacterial growth conditions

S. meliloti and *E. coli* strains were grown in their respective media as described previously. The incubation temperatures for *S. meliloti* and *E. coli* strains were 28 and 37°C, respectively. For subculturing, 0.1 ml of overnight incubated broth culture was transferred to 100 ml Erlenmeyer flask containing 10 ml of respective broth medium and incubated for 12-14 hours. The incubation times to obtain log phase cultures of *S. meliloti* and *E. coli* were 24-32 hours and 14-16 hours, respectively. For single

colony isolation of *S. meliloti* strains incubation time was 3 days on complete medium and 5 days on minimal medium.

3.2.1.2 Preparation of serial dilutions

Physiological saline solution was used for carrying out serial dilutions for single colony isolation. 1 ml from a neat culture was added to the first tube containing 9 ml of diluent to obtain 10^{-1} dilution. After proper mixing, 1 ml of bacterial suspension from this tube was added to next tube containing 9 ml of diluent to obtain 10^{-2} dilution. Further dilutions were made in this pattern according to the requirements of the experiment.

3.2.1.3 Enumeration of colony forming units (CFU)

CFU per milliliter were calculated by serial dilution technique. 0.2 ml of each dilution was spread, with the help of a sterile glass rod, on the surface of TY agar medium in Petri dishes during the growth period of bacteria. Three plates were used for each dilution. The plates were incubated at 28°C for 3 days and then the plates, of each dilution, where the colonies were between 50-300 were selected. The average number of the colonies in these plates was multiplied by 5 and dilution factor to enumerate the viable count of bacteria per ml.

3.2.2 Isolation of aromatic amino acid auxotrophs

3.2.2.1 Transposon Tn5 mutagenesis

Transposon Tn5 induced random mutagenesis of *S. meliloti* strain Rmd201, a derivative of strain AK631, was performed as described by Selvaraj and Iyer (1983) with slight modifications. Log phase cultures of donor *E. coli* strain WA803, harbouring the Tn5 on its suicide plasmid pGS9, and recipient *S. meliloti* Rmd201

were mixed in a 3:7 ratio, respectively. 0.1 ml of the biparental mating mixture was delivered on the surface of TY agar medium in a Petri dish and spreaded with the help of a sterile glass rod. Several patches were made as well as the parental strains Rmd201 and WA803 were also spreaded individually on TY agar medium as controls. These plates were incubated at 28°C for 24 hours. The growth obtained after incubation time was scrapped using a sterile inoculation loop and suspended in 2 ml of physiological saline solution in a 5 ml sterile test tube. The suspension of biparental mated cells was thoroughly mixed with the help of a cyclomixer and 0.1 ml of this suspension from each cross was spread on TY plates supplemented with streptomycin (100 μg/ml) and kanamycin (400 μg/ml). In all matings, the donor and recipient cultures were also plated on TY medium supplemented with streptomycin (100 μg/ml) and kanamycin (400 μg/ml)) as controls. The plates were incubated at 28°C for five days. The transconjugants obtained after five days were purified twice on TY agar plates containing streptomycin (100 μg/ml) and kanamycin (400 μg/ml). All kanamycin resistant (Km^r) transconjugants were checked for the loss of the suicide plasmid by looking for the absence of vector encoding chloramphenicol resistance in these transconjugants. The above cross was repeated 85 times and the transconjugants obtained from these crosses were screened for auxotrophs.

3.2.2.2 Screening for auxotrophs

All the Tn5 induced Km^r transconjugants obtained by transposon Tn5 mutagenesis were screened for auxotrophs by replica plating method. These were streaked, by using sterile applicator sticks, on RMM and TY agar plates. The plates were incubated at 28°C for 3 days. The Tn5 derivatives which grew on TY medium

but not on RMM were considered to be auxotrophs. These auxotrophs were streaked on TY medium containing streptomycin (100 μg/ml) and kanamycin (400 μg/ml) for single colony isolation. The nature of auxotrophy of each Tn5 insertion mutant was determined subsequently.

3.2.2.3 Determination of the nature of auxotrophy

A loopful of fresh single colony of each Tn5 insertion mutant was suspended in 50 μl of physiological saline solution on a sterile glass slide. The bacterial suspension was patched on nutritional pools (agar plates of RMM supplemented with eleven nutritional pools, Table 3) with the help of an inoculation loop. The plates were incubated at 28°C for 5 days. The nature of auxotrophy was determined on the basis of the growth pattern on these plates.

Generally, a colony will respond on a plate containing one of the pools from 1 to 5 and on another plate containing one of the pools from 6 to 11, as described in Table 3, thus allowing for direct identification of a single growth factor requirement. For example, a colony growing on pool 1 and 8 requires phenylalanine, a colony growing on pool 2 and 8 requires tyrosine and a colony growing on pool 3 and 8 requires tryptophan. If a colony grows on only pool 8, it requires more than one of the nutrients in that pool. The auxotrophic requirement(s) of each strain was further confirmed by streaking on agar plates containing RMM supplemented with suspected nutrient(s).

3.2.3 Maintenance of bacterial cultures

Purified isolated strains were streaked on slants of YEM (for *S. meliloti* strains) or LB (for *E. coli* WA803) agar medium. After growth periods of 3 and 2 days at 28

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and 37°C for *S. meliloti* and *E. coli* strains, respectively. These slants were kept at 4°C in refrigerator. Each strain was subcultured at an interval of two months.

3.2.4 Reversion analysis of auxotrophs

Each aromatic amino acid auxotrophic mutant was grown in 100 ml Erlenmeyer flask containing 10 ml of TY broth medium. The flasks were incubated at 28°C in an orbital incubator (GALLENKAMP, USA) operating at 120 rpm, to a late log phase of growth. The broth cultures were washed twice (to remove residual TY medium) with 10 ml of RMM (without sugar) by centrifuging at 5,000 rpm for 5 minutes in a refrigerated centrifuge (BECKMAN, J_2-21, USA) at 4°C and then resuspended in the same volume of RMM (without sugar) to a concentration of about 10^9 CFU/ml. The bacterial suspension was spread on RMM agar plates, the prototrophic colonies were enumerated after five days of incubation at 28°C and the frequency of spontaneous reversion was calculated. The prototrophs were streaked on RMM agar plates supplemented with kanamycin (400 μg/ml) to confirm the excision of Tn5 in these prototrophs.

3.2.5 Determination of metabolic block in the aromatic amino acid biosynthetic pathways of each auxotroph

3.2.5.1 Intermediate feeding

The aromatic amino acid auxotrophs were streaked on RMM plates supplemented with 50 μg/ml of shikimic acid or all three aromatic amino acids, viz., tryptophan, tyrosine and phenylalanine. These auxotrophic mutants were also streaked on RMM plates supplemented with 50 μg/ml of anthranilic acid, indole or tryptophan. RMM plates supplemented with 50 μg/ml of tyrosine or phenylalanine were also

streaked with these mutants. The parental strain Rmd201 was streaked on each plate. The growth pattern was observed after an incubation period of 3-5 days at 28°C.

3.2.5.2 Intermediate accumulation

The aromatic amino acid auxotrophs and parental strain Rmd201 were grown in 100 ml Erlenmeyer flasks containing 10 ml of TY broth medium. The flasks were incubated in an orbital incubator (GALLENKAMP, USA) operating at 120 rpm for 24 hours at 28°C. The broth cultures were washed twice (to remove residual TY medium) with 10 ml of RMM (without sugar), by centrifuging at 5,000 rpm for 10 minutes in a refrigerated centrifuge (BECKMAN, J_2-21, USA) at 4°C and then resuspended in the same volume of RMM (without sugar) in 100 ml Erlenmeyer flasks. These flasks were incubated in an orbital incubator at 28°C for 48 hours. The cultures were centrifuged at 5,000 rpm for 10 minutes and the supernatants were tested for presence of the following intermediates:

3.2.5.2.1 Anthranilic acid and indole

Anthranilic acid and indole were determined by the method of Snell and Snell (1967). 1 ml of 6% (w/v) *p*-dimethyl amino benzaldehyde reagent was added to 5 ml of supernatant. The mixture was left undisturbed for 20 minutes and then the optical density was read at 420 nm for anthranilic acid and at 550 nm for indole using DU-6 spectrophotometer (BECKMAN, USA). The presence of anthranilic acid in cultures was also determined by visual observation of a blue flourescence emission spectrum under ultraviolet light at wave length 239-390 nm (Aoki *et al.*, 1984).

3.2.5.2.2 Indole-3-glycerol phosphate

The production of indole-3-glycerol phosphate was determined by the method

of Yanofsky and Smith (1962). 1.5 ml of ferric chloride reagent was added to 1 ml of supernatant, left for 5-10 minutes and then the mixture was observed for appearance of pink to red colour.

3.2.5.3 Cross feeding

Phenotypic complementation of auxotrophic mutants was done with cross feeding experiments in which a loopful of fresh single colony of each mutant was suspended in 50 µl of physiological saline solution, on a sterile glass slide. Three strains at a time were streaked on RMM plates enriched with minimal nutritional requirements of the auxotrophs (3 µg/ml). The streaked lines maintained a narrow gap of about 2 mm in a manner that each streak formed a side of an equilateral triangle. The parental strain Rmd201 was also streaked on these plates. The plates were incubated at 28°C for 4 days. Growth pattern was observed that the strain which was being cross fed showed growth nearer to the streak of the strain which was cross-feeding it.

3.2.6 Pleiotropic effects of transposon Tn5 insertion into the aromatic amino acid biosynthetic genes of auxotrophs

3.2.6.1 Detection of cell surface carbohydrate molecules

3.2.6.1.1 Test for detection of β-glucans

3.2.6.1.1.1 Test for detection of cyclic β-(1,2)-glucans

Motility of rhizobial strains in swarm plates (TY medium containing 0.3% (w/v) agar) can be attributed to cyclic β-(1,2)-glucans production (Geremia *et al.*,1987). 50 µl of cell suspension of each auxotroph along with the parental strain

were spotted on TY swarm plates. These plates were incubated at 28°C for 2 days. Swarming of the strains were determined by the spread of the bacterial growth.

3.2.6.1.1.2 Test for detection of β-(1-3)-glucans

β-(1,3)-glucans-producing strains form blue colonies on medium containing aniline blue dye (Nikanishi *et al.*, 1976). The auxotrophs and the parental strain were streaked on MSY agar plates containing 0.02% (w/v) aniline blue. These plates were incubated at 28°C for 3 days and then examined under normal visible light.

3.2.6.1.2 Tests for detection of cellulose fibrils and succinylated exopolysaccharides (EPS I)

Rhizobial strains which produce cellulose fibrils and EPS I can be tested on media supplemented with congo red dye (Kneen and La Rue, 1983) and calcofluor white dye (Leight *et al.*, 1987), respectively. Strains producing cellulose fibrils form red colonies on medium containing congo red, while EPS I-producing strains show fluorescence under ultraviolet light. MSY plates having 0.02% (w/v) of congo red or calcofluor white were streaked with the aromatic amino acid auxotrophic strains and parental strain Rmd201. Incubation was done at 28°C for 3 days. Calcofluor white plates were observed under long wave length of UV light whereas congo red plates were observed under normal visible light.

3.2.6.1.3 Test for detection of lipopolysaccharides (LPS)

All auxotrophs along with the parental strain Rmd201 were streaked on MSY agar plates containing sodium deoxycholate (1 mg/ml). The growth of the strains was observed after incubation time at 28°C for 3 days. The strains which showed growth on these plates were considered to be LPS producing (Swamynathan and Singh, 1995).

3.2.6.2 Utilization of carbon sources

3.2.6.2.1 C_4-dicarboxylic acid

All aromatic amino acid auxotrophs and parental strain Rmd201 were streaked on minimal medium (without glucose), supplemented with respective nutritional requirements of the auxotrophs (50 µg/ml) and malic acid, succinic acid, fumaric acid or aspartic acid. Growth pattern of the auxotrophs was compared with the parental strain Rmd201 after 3-5 days of incubation period at 28°C.

3.2.6.2.2 Sugars

The bacterial strains were streaked on RMM supplemented with the nutritional requirements of the auxotrophs and various sugars (arabinose, glucose, galactose, mannose, xylose, lactose, maltose, sucrose, fructose, mannitol and sorbitol). The plates were examined after 3-5 days of incubation time at 28 °C.

3.2.6.3 Test for detection of cytochrome c oxidase

Cytochrome c oxidase test was performed according to the method described by Kovaks (1956) and Bradshaw (1992). A Whatman's No.1 filter paper was laid in a sterile Petri dish. 50 µl of cytochrome c oxidase reagent (1% w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride was dropped on the centre of the filter paper. A little portion from fresh single colony of each aromatic amino acid auxotroph was removed by a sterile applicator stick and smeared thoroughly on the reagent-impregnated filter paper in a line 3-6 mm long. The parental strain Rmd201 was also smeared on this filter paper. The change in colour of the smeared colonies

from creamish-white to purple or violet within 10 seconds indicated that these strains have the ability to produce cytochrome c oxidase.

3.2.7 Symbiotic properties of aromatic amino acid auxotrophs

3.2.7.1 Preparation of seedlings

Normal and undamaged seeds of alfalfa (*M. sativa* cv. T9) plants were selected, washed and soaked in sterile distilled water for 10 minutes. These seeds were then surface sterilized by treatment with absolute alcohol for one minute. Followed by treatment with 0.1 % (w/v) mercuric chloride acidified with concentrated HCl ($HgCl_2$, 1.0 gm; conc. HCl, 5 ml; distilled water, 1 litre) for one minute (Vincent, 1970). The seeds were then rinsed thoroughly with at least five changes of sterile distilled water. Surface sterilized seeds were spread on 1.0 % (w/v) water agar medium. The plates were incubated inverted at 25°C for two days in dark so as to induce straight radicals and uniform seedlings.

3.2.7.2 Transfer of seedlings to slants of nitrogen free plant growth medium

For growing the seedlings, 20 x 2.5 cm glass tubes containing 25 ml of autoclaved nitrogen free plant growth medium were used (Engelke *et al.*, 1987). These tubes were closed with cotton plugs before autoclaving. Two healthy grown seedlings, each two days old, were transferred to the slants with the help of a sterile forcep and an inoculation loop. The lower portion of each tube was wrapped by black paper to protect the root system of the growing seedlings from direct exposure to light.

3.2.7.3 Inoculation of seedlings with rhizobial strains

A loopful of a fresh single colony of each rhizobial strain was inoculated into

100 ml Erlenmeyer flask containing 10 ml of MSY broth and incubated at 28°C for 24 hours in an orbital incubator (GALLENKAMP, USA) operating at 120 rpm. The broth cultures were washed twice (to remove residual MSY medium) with 10 ml of sterile physiological saline solution by centrifuging at 3,000 rpm for 5 minutes in a refrigerated centrifuge (BECKMAN, J_2-21, USA) at 4°C and then resuspended in 5 ml of sterile physiological saline solution. 0.5 ml of the rhizobial cell suspension was dispensed into each tube having two days old seedlings. Control tubes containing two seedlings each received 0.5 ml of physiological saline solution. Five tubes containing two seedlings each were used for each treatment.

3.2.7.4 Plant growth conditions

After inoculation of seedlings, the slants were incubated in plant growth chamber (COLTON, NSW-INDIA). Fluorescent tube lights were used for providing the growing plants approximately 2000 lux of visible light with roots protected from direct exposure to light. 16 hours light and 8 hours dark cycle was maintained. The air temperature and relative humidity of the growth chamber were maintained at 25°C and 70-80%, respectively. Six weeks after inoculation with rhizobial strains, many parameters characteristic of each plant were recorded, such as time of appearance of first nodule, nodule numbers, nodule shapes, nodule colours, shoot length and shoot dry weight. To measure the latter, plant shoots were collected, dried in an oven at 60°C for 3 days and then weighed. Nitrogen fixation was indirectly evaluated by scoring the plants Fix^+ or Fix^- on the basis of nodule colour and/or plant matter production.

3.2.7.5 Light microscopic observations of alfalfa root hairs

The root hairs of 4-10 days old plants after inoculation with rhizobial strains,

were prepared and examined as described by Vasse and Truchet (1984), Truchet *et al.* (1985) and Taté *et al.* (1999c). The root portions of the plants were removed, washed with sterile water (to remove residual nitrogen free plant growth medium) and cut into 1 cm long pieces. The root pieces were stained with methylene blue (0.01 % w/v) for 15 minutes, washed with two changes of sterile water, placed on a clean glass slide and covered with a cover slip. The slide was then examined at 10X and 40X magnifications under light microscope (LEICA, DM LB, GERMANY) for observing root hair deformations (curling, branching, waviness, bulging, swelling, etc.), shepherd's crook with hyaline spot, infection thread and ability of the rhizobial strains to nodulate alfalfa root hairs.

3.2.7.6 Nodule occupancy tests

Rhizobial strains were reisolated from six weeks old nodules and tested for their auxotrophic and antibiotic markers (Koch's postulate). All nodules from a plant were harvested and surface sterilized by immersion in 95% (v/v) ethanol for one minute. These nodules were then washed in sterile distilled water and immersed in 0.1% acidified $HgCl_2$ for one minute. The nodules were thoroughly washed 5 times with sterile distilled water (to remove residual alcohol and mercuric chloride). The surface sterilized nodules were then crushed aseptically in 100 µl of sterile physiological saline solution with the help of a sterilized glass rod. The resulting bacterial suspension was diluted with physiological saline solution to obtain 10^{-1} and 10^{-2} dilutions. 0.1 ml of a neat suspension and 0.1 ml of each dilution were spread on TY medium supplemented with streptomycin (100 µg/ml). Three replicates were used for neat suspension and each of the two dilutions. These plates were incubated at 28°C

for 3 days. The colonies obtained were streaked on agar plates containing RMM and RMM supplemented with kanamycin (400 µg/ml) and streptomycin (100 µg/ml) plus respective nutritional requirements of the auxotrophs. These plates were incubated at 28°C for 3 days. The colonies which showed growth on RMM but not on RMM containing Km and Sm, plus auxotrophic requirements, were considered to be prototrophic revertants. The colonies which did not grow on RMM but grew on RMM supplemented with Km and Sm plus respective nutritional requirements of the auxotrophs were recorded as auxotrophs.

3.2.8 Preparation of histological sections of nodules

Six weeks old nodules induced by the parental strain Rmd201 and its aromatic amino acid auxotrophs were harvested from alfalfa roots (ten nodules were taken for each strain). The nodule were washed thrice with sterile distilled water (to remove residual plant growth medium). These were then prepared for light and transmission electron microscopy as described by Weibel (1973), Hirsch *et al.* (1983), Vasse *et al.* (1990), Baev *et al.* (1992) and Taté *et al.* (1999a).

3.2.8.1 Primary fixation of nodules

The washed nodules were transferred to 5 ml glass vials containing 2 ml of the primary fixative. To facilitate penetration of the fixative, acrolein (0.1% w/v) was added to the fixative. The vials were placed in a vacuum chamber to prevent the floating of the nodules on the surface. These were then kept at 4°C for 24 hours.

3.2.8.2 Preparation of specimen block

3.2.8.2.1 Washing of nodules

Following primary fixation, the nodules were washed twice in 0.2 M phosphate

buffer (pH 7.2). These nodules were left in the same buffer overnight at 4°C.

3.2.8.2.2 Secondary fixation of nodules

The nodules after washing were post-fixed in 2% (w/v) osmium tetroxide at 4°C for 2 hours and subsequently washed with 0.2 M phosphate buffer (pH 7.2).

3.2.8.2.3 Dehydration of nodules

To remove water, the nodules were passed through a graded acetone series (30, 50, 70, 80, 90, 95% (v/v) and dry acetone). A sample was kept in each of the acetone concentrations at 4°C for 15 minutes. Finally, two changes of 30 minutes each in dry acetone were given at room temperature.

3.2.8.2.4 Removal of acetone from nodules

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

3.2.8.2.5 Infiltration

The following infiltration mixtures were prepared:

i. One part of embedding medium and three parts of toluene

ii. Two parts of embedding medium and two parts of toluene

iii. Three parts of embedding medium and one part of toluene

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

3.2.8.2.6 Embedding of nodules

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

3.2.8.3 Preparation of semithin sections for light microscopy

The block holder was attached to Ultracut E microtome (C. REICHERT, OMU3, AUSTRIA) and semithin sections (0.5 to 2.0 μm thick) were cut using a glass knife. The sections floating in distilled water in attached metallic trough were removed by using a thin brush and placed on a clean glass slide. The slide was dried by placing on a hot plate at 80°C for 30 seconds. The sections were stained with 1% (w/v) toludine blue for 1 minute, washed with distilled water and observed under light microscope (LEICA, DM LB, GERMANY). The areas of interest were photographed.

3.2.8.4 Preparation of ultrathin sections for transmission electron microscopy

After scanning the sections under the light microscope, the area to be examined under transmission electron microscope was selected and the blocks were further hand trimmed. Ultrathin sections (60-90 nm thick) were cut using Ultracut E microtome (C. REICHERT, OMU3, AUSTRIA). These sections were lifted onto 200 mesh size copper grids. The grids were coated with 3% (w/v) formvar (polyvenyl formaldehyde) in ethylene dichloride and carbon by a process of evaporation under vacuum. After double coating, the formvar was removed by dissolving in ethylene dichloride or

chloroform to leave behind the carbon film. Double staining of sections with uranyl acetate and lead citrate was done to obtain a good contrast. The grids carrying the sections were placed in 0.5 ml of uranyl acetate solution on a watch glass for 15 minutes. A cardboard cover was placed over the watch glass to provide dark condition for staining. The grids were washed twice with 50% (v/v) ethanol and twice with double distilled water. After washing, the grids were dried on a filter paper and then placed in 0.5 ml of lead citrate solution on clean watch glass for 10 minutes. The grids, after removing from the above solution, were washed with 0.1 N NaOH solution and then twice with double distilled water. After washing, the grids were dried, stored in a grid holder at room temperature and viewed under transmission electron microscope (PHILIPS, CM10, HOLLAND). The areas of interest were photographed.

3.2.9 Measurement of iron

The rhizobial strains were grown in 100 ml Erlenmeyer flasks containing 10 ml of TY broth medium to late log phase of growth. The broth cultures were washed twice (to remove residual TY medium) with 10 ml of RMM (without $FeCl_3$ and sugar). The viable count of each strain was enumerated by serial dilution technique. Approximately 10^6 CFU/ml was added in each case into 100 ml Erlenmeyer flask containing 10 ml of minimal medium, which was modified to have 10 µg/ml of $FeCl_3$ and supplemented with minimal nutritional requirements (3 µg/ml) of the auxotrophs. The flasks were incubated at 28°C in an orbital incubator (GALLENKAMP, USA) operating at 120 rpm. After a growth period of two days, the broth cultures were centrifuged at 5,000 rpm for 5 minutes. The supernatants were then analysed for

measurement of iron using atomic absorption spectrometer (AVANTA-M, GBC, AUSTRALIA).

The iron uptake by the rhizobial cells of the aromatic amino acid auxotrophs along with the parental strain Rmd201 was measured using the procedure of the American Public Health Association (1998) with some modifications. The pellets were resuspended in 10 ml of deionized water and the final viable count of each rhizobial strain was calculated by serial dilution technique. Each suspension having approximately 1.2 x 10^9 CFU was dispensed into a 50 ml beaker and 1 ml of conc. HNO_3 was added. These were then placed on a hot plate and evaporated (without boiling) to nearly dryness until digestion was completed. This is indicated by the light coloured and clear solution. The samples were cooled, diluted with deionized water to 10 ml and analysed for measurement of iron using atomic absorption spectrometer.

3.2.10 Statistical analysis

According to the nature of the data obtained from the present study, student's 't' test, as described by Bailey (1995), was used for statistically analysing these data. All values were expressed as mean ± standard error of mean.

Chapter 4

RESULTS

The objectives of the present study were investigated in detail and the results are presented below.

4.1 Isolation of aromatic amino acid auxotrophs

4.1.1 Transposon Tn5 mutagenesis and screening for auxotrophs

Random transposon Tn5 mutagenesis was employed to generate aromatic amino acid auxotrophs of *S. meliloti* Rmd201. The suicide plasmid vector pGS9 delivered the transposon Tn5 from *E. coli* WA803 into *S. meliloti* Rmd201 genome, as a random conservative transposition, by conjugation process (Fig. 2). This process resulted in the generation of 5000 kanamycin resistant transconjugants from 85 crosses at a frequency of 3.2×10^{-5} per recipient. The frequency of spontaneous resistance to kanamycin (400 μg/ml) of *S. meliloti* Rmd201 was much lower ($<10^{-8}$). The transconjugants were collected and streak-purified on TY medium having streptomycin (100 μg/ml) and kanamycin (400 μg/ml). All Tn5 induced kanamycin resistant transconjugants were chloramphenicol sensitive indicating disintegration of Tn5 delivery vector pGS9 in these transconjugants. The single colony of each Tn5 derivative obtained was screened for auxotrophy by patching on RMM and TY media. Twenty two Tn5 derivatives which did not grow on RMM but showed growth on TY medium supplemented with streptomycin (100 μg/ml) and kanamycin (400 μg/ml), were selected as auxotrophs.

4.1.2 Determination of the nature of auxotrophy

Nutritional pools of RMM plates as described by Holliday (1956) with some modifications were used. These pools helped in determining the nature of auxotrophy. Out of the twenty two Tn5 insertion mutants, four were found to be auxotrophic mutants of the aromatic amino acid biosynthetic pathways, two of tryptophan (FN2 and

FN3), one of tyrosine (FN4) and one of phenylalanine (FN9). Two auxotrophs (NV3 and BA2) requiring all three aromatic amino acids, viz., tryptophan, tyrosine and phenylalanine, and three others (NV7, NV31 and BA6) of *S. meliloti* Rmd201 requiring only tryptophan, isolated by other researchers in the lab were also included for detailed investigations. A total of nine aromatic amino acid auxotrophs are listed in Table 4.

4.2 Characterization of aromatic amino acid auxotrophs

4.2.1 Reversion analysis of auxotrophs

Prototrophic colonies of each aromatic amino acid auxotroph were counted on RMM agar plates having kanamycin (400 μg/ml) and the frequency of spontaneous reversion was calculated. Spontaneous excision of transposon Tn5 in these auxotrophs occurred and resulted in the formation of kanamycin sensitive prototrophic revertants. Differences in the spontaneous reversion frequencies of these auxotrophs were observed. The maximum reversion frequency was found in NV3 mutant (9.5×10^{-8}), whereas the minimum was observed in FN2 mutant (1.3×10^{-9}). Table 5 gives the rates of spontaneous reversion frequencies of auxotrophic mutants. These results suggest that the Tn5 insertion into the aromatic amino acid biosynthetic genes of auxotrophs was stable and the kanamycin sensitive revertants of auxotrophs were caused by excision of Tn5 insertion from these genes.

4.2.2 Location of metabolic block in the aromatic amino acid biosynthetic pathways of each auxotroph.

Intermediate feeding and accumulation, and cross feeding experiments were conducted for determining the metabolic block in the aromatic amino acid biosynthetic pathways of each auxotroph.

Table 4. Nutritional requirements of the aromatic amino acid auxotrophic mutants of *S. meliloti* Rmd201

S. No.	Strain	Auxotrophy
1	NV3*	Tryptophan, tyrosine and phenylalanine
2	BA2*	Tryptophan, tyrosine and phenylalanine
3	FN2	Tryptophan
4	FN3	Tryptophan
5	NV7*	Tryptophan
6	NV31*	Tryptophan
7	BA6*	Tryptophan
8	FN4	Tyrosine
9	FN9	Phenylalanine

* Isolated by other researchers in the lab.

Table 5. Spontaneous reversion frequencies of aromatic amino acid auxotrophic mutants of *S. meliloti* Rmd201.

S. No.	Strain	Spontaneous reversion frequency
1	NV3	9.5×10^{-8}
2	BA2	2.1×10^{-9}
3	FN2	1.3×10^{-9}
4	FN3	9.1×10^{-9}
5	NV7	9.5×10^{-9}
6	NV31	1.8×10^{-8}
7	BA6	2.7×10^{-8}
8	FN4	8.2×10^{-9}
9	FN9	9.3×10^{-9}
10	Rmd201	None (parental strain)

4.2.2.1 Intermediate feeding

The results of intermediate feeding are presented in Table 6. Two mutants (NV3 and BA2) did not grow on minimal medium supplemented with 50 μg/ml of tryptophan, tyrosine or phenylalanine, or their intermediates (used in the study). However, these mutants showed growth on RMM agar plates supplemented with all three aromatic amino acids (tryptophan, tyrosine and phenylalanine). Two mutants (FN2 and FN3) grew on RMM agar plates supplemented with 50 μg/ml of anthranilic acid, indole or tryptophan. NV7 and NV31 mutants showed growth on RMM agar plates supplemented with 50 μg/ml of indole or tryptophan. The mutant BA6 grew only on RMM having 50 μg/ml of tryptophan. FN4 and FN9 mutants showed growth only on RMM agar plates having 50 μg/ml of tyrosine and phenylalanine, respectively.

4.2.2.2 Intermediate accumulation

Among the five *trp* mutants tested for tryptophan intermediate accumulation in RMM supplemented with growth-limiting concentrations of trytophan (3 μg/ml), the auxotrophs NV7, NV31 and BA6 showed accumulation of anthranilic acid (more than 15 μg/ml). The mutant BA6 also accumulated anthranilic acid (more than 15 μg/ml) in addition to indole-3-glycerol phosphate. On the other hand, the auxotrophs FN2 and FN3 did not accumulate any intermediate of tryptophan biosynthetic pathway. The intermediate accumulation of *aro* (NV3 and BA2), *tyrA* (FN4) and *pheA* (FN9) mutants could not be determined because of unavailability of the intermediates or the reagents required for their detection.

Table 6. Growth responses of aromatic auxotrophs of *S. meliloti* Rmd201 to aromatic amino acids or their intermediates.

S. No.	Strain	RMM	Growth on RMM supplemented with 50 μg/ml of :						
			Shikimic acid	Anthranilic acid	Indole	Tryptophan	Tyrosine	Phenylalanine	**All aromatic amino acids**
1	NV3	–	–	–	–	–	–	–	+
2	BA2	–	–	–	–	–	–	–	+
3	FN2	–	–	+	+	+	–	–	+
4	FN3	–	–	+	+	+	–	–	+
5	NV7	–	–	–	+	+	–	–	+
6	NV31	–	–	–	+	+	–	–	+
7	BA6	–	–	–	–	+	–	–	+
8	FN4	–	–	–	–	–	+	–	+
9	FN9	–	–	–	–	–	–	+	+
10	Rmd201	+	+	+	+	+	+	+	+

Abbreviations: +, growth ; – , no growth ; RMM, *Rhizobium* minimal medium

4.2.2.3 Cross feeding

When the aromatic amino acid strains grew on RMM agar plates supplemented with minimal nutritional requirements of the auxotrophs, it was observed that the tryptophan mutants showed cross feeding. *trpE(G)* mutants (FN2 and FN3) were cross-fed by *trpD*, *trpF* or *trpC* mutants (NV7 and NV31), and *trpB* mutant (BA6). *trpD*, *trpF* or *trpC* mutants were cross-fed by *trpB* mutant. On the other hand, *aro* (NV3 and BA2), *tyrA* (FN4) or *pheA* (FN9) mutants could not cross feed any mutant. Cross feeding in the same group and also among groups (except *trp* mutants) did not occur.

Based on the above results, the location of metabolic block in the aromatic amino acid biosynthetic pathways of each auxotroph was determined (Fig. 5). The aromatic amino acid auxotrophs were classified into four groups:

i. *aro* mutants (NV3 and BA2): Grew on RMM supplemented with all three amino acids, viz. tryptophan, tyrosine and phenylalanine.

ii. *trp* mutants which were further classified into three subgroups:

a. *trpE(G)* mutants (FN2 and FN3): Grew on RMM supplemented with anthranilic acid, indole or tryptophan and did not accumulate anthranilic acid and indole-3-glycerol phosphate in RMM.

b. *trpD*, *trpF* or *trpC* mutants (NV7 and NV31): Grew on indole or tryptophan supplemented RMM and accumulated anthranilic acid in RMM.

c. *trpB* mutant (BA6): Grew only on tryptophan supplemented RMM and accumulated anthranilic acid and indole-3-glycerol phosphate in RMM.

iii. *tyrA* mutant (FN4): Grew only on tyrosine supplemented RMM.

iv. *pheA* mutant (FN9): Grew only on phenylalanine supplemented RMM.

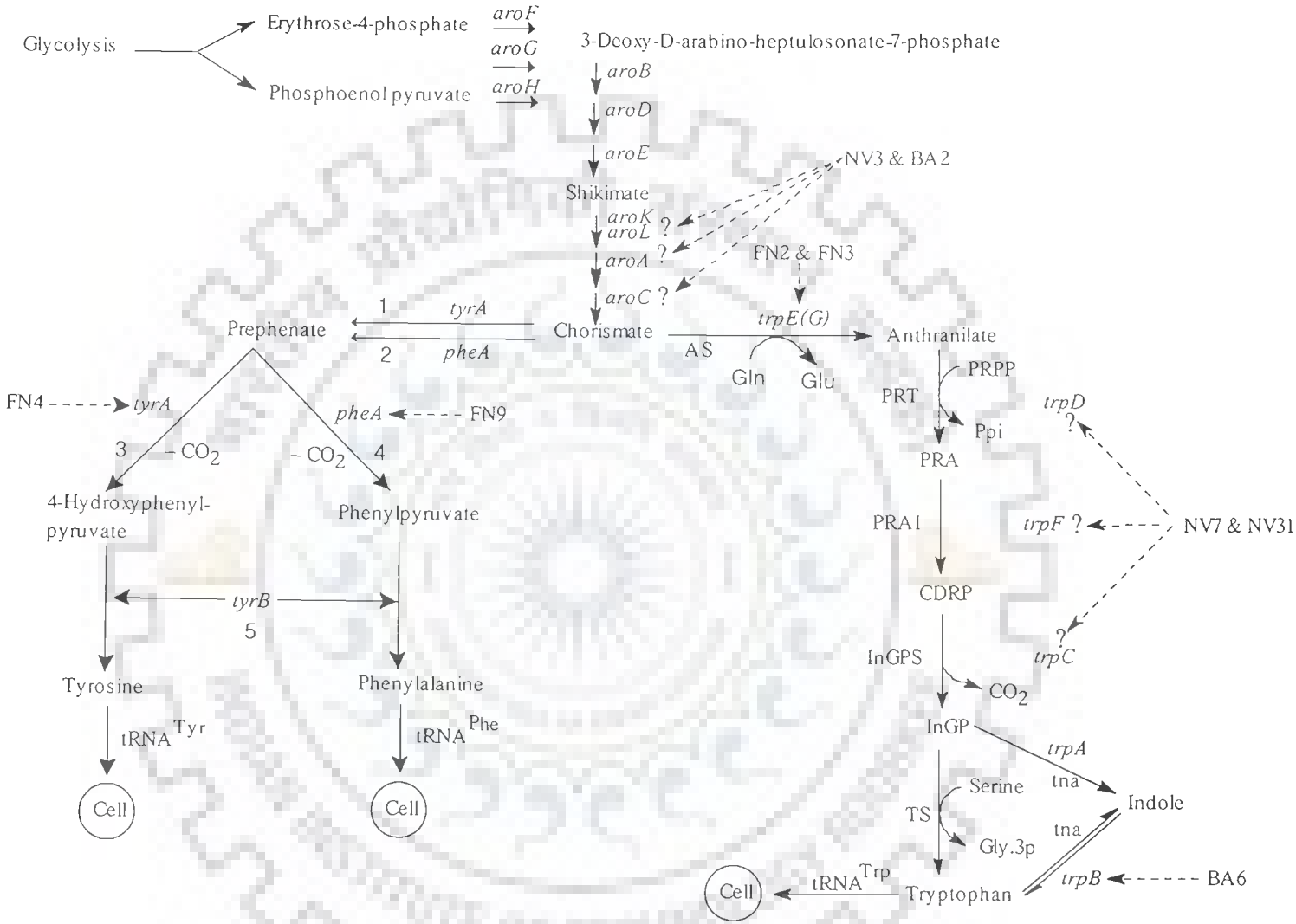

Fig. 5. The biosynthetic pathways of the aromatic amino acids showing the location of metabolic block in the auxotrophic mutants [based on Moat and Foster (1995), Pittard (1996) and Xiu *et al.* (1997)].

Abbreviations : Gln, glutamine; Glu, glutamate; PRPP, 5-phosphoribosyl-1-pyrophosphate; Ppi, pyrophosphate; tna, tryptophanase; PRA, 5-phosphoribosyl anthranilate; CDRP, 1-(o-carboxy phenyl amino)-1-deoxyribulose-5-phosphate; InGP, indole-3-glycerol phosphate; Gly.3p, glyceraldehyde-3-phosphate; AS, anthranilate synthase; PRT, anthranilate phosphoribosyl transferase; PRAI, phosphoribosyl anthranilate isomerase; InGPS, indole-3-glycerol phosphate synthase; TS, tryptophan synthase; 1, chorismate mutase T; 2, chorismate mutase P; 3, prephenate dehydrogenase; 4, prephenate dehydratase; 5, tyrosine aminotransferase

4.3 Pleiotropic effects of transposon Tn5 insertion into the aromatic amino acid biosynthetic genes of auxotrophs.

4.3.1 Production of cell surface carbohydrate molecules

All rhizobial strains (auxotrophs and parental strain Rmd201) showed growth in presence of sodium deoxycholate, took up the congo red dye, fluoresced in presence of calcoflour white under UV light and showed swarming on swarm plates indicating the normal production of lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β-(1,2)-glucans, respectively. All these auxotrophs and the parental strain Rmd201 did not show binding with aniline blue dye in medium which indicated absence of β-(1,3)-glucans production. These results showed normal production of cell surface carbohydrate molecules in auxotrophs like the parental strain Rmd201.

4.3.2 Utilization of carbon sources

4.3.2.1 C_4-dicarboxylic acids

All aromatic auxotrophs and the parental strain Rmd201 exhibited normal growth on minimal medium containing the nutritional requirements of the auxotrophs and malic acid, succinic acid, fumaric acid or aspartic acid instead of glucose. These results indicate that the auxotrophs and parental strain Rmd201 were able to utilize C_4-dicarboxylic acids as carbon sources.

4.3.2.2 Sugars

When the aromatic amino acid mutants and the parental strain Rmd201 were tested for growth on minimal medium containing the nutritional requirements of the auxotrophs and different sugars (arabinose, glucose, galactose, mannose, xylose, lactose, maltose, sucrose, fructose, mannitol and sorbitol). It was observed that similar

to the parental strain Rmd201, all aromatic amino acid auxotrophic mutants were able to utilize these sugars as carbon sources to satisfy their demands of energy.

4.3.3 Production of cytochrome c oxidase

The smeared colonies of the parental strain Rmd201 and its aromatic amino acid auxotrophs on the cytochrome c oxidase reagent-impregnated filter paper (see section 3.2.6.3) showed change in colour from creamish-white to purple or violet. These results indicated that in these strains there were no defects in production of the cytochrome c oxidase which is one of the main enzyme of the respiratory electron transport chain.

4.4 Symbiotic properties of aromatic amino acid auxotrophs

4.4.1 Infection phenotypes on alfalfa root hairs and formation of nodules

When the methylene blue stained root portions of alfalfa plants inoculated with the auxotrophs and parental strain Rmd201 were observed under light microscope, it was found that similar to the parental strain Rmd201, all the auxotrophs were normal in elicitation of root hair deformations (i.e., curling, branching, waviness, bulging, swelling, etc.), shepherd's crook with hyaline spot and infection thread, and formation of nodules. However, the root hairs of uninoculated controls were straight and devoid of the infection phenotypes (Plate 1; 2).

4.4.2 Characteristics of alfalfa plants nodulated by *S. meliloti* Rmd201 and its auxotrophs or prototrophic revertants

The nodule and shoot characteristics of *M. sativa* cv. T9 nodulated by the rhizobial strains are given in Table 7. The data presented in this Table are being listed

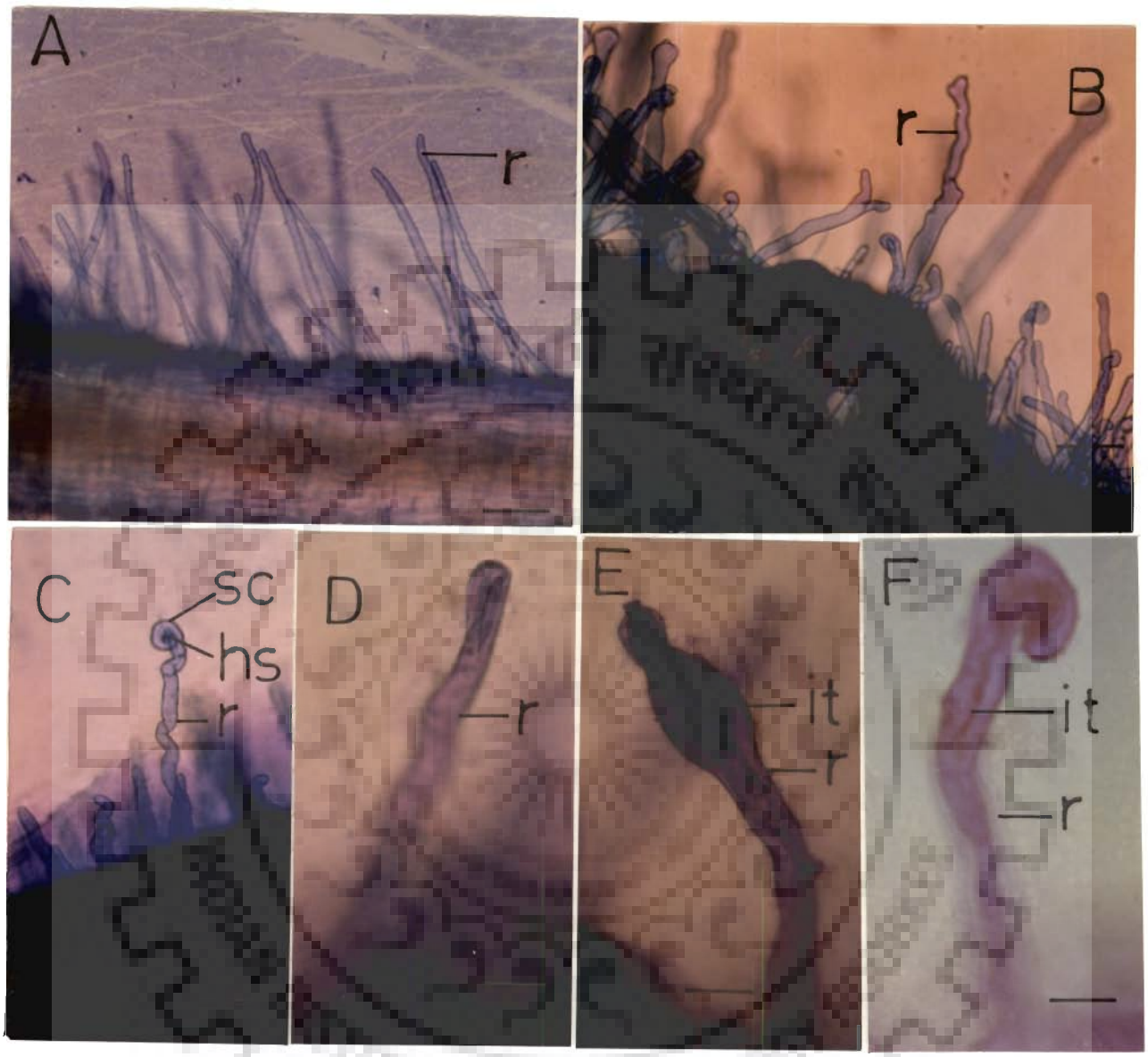

Plate 1. Light microscopic observations of root hairs of *M. sativa* cv. T9 inoculated with *S. meliloti* Rmd201. **A.** Uninoculated control showing intact, straight root hairs. **B.** Root hair deformations: i.e., curling, branching, waviness, bulging, swelling, etc. **C.** Formation of shepherd's crook showing the 360° curvature of the root hair tip with hyaline spot. **D.** Infection of the root hair. **E.** Root hair penetration at the center of infection and formation of infection thread. **F.** Elongating infection thread at the base of the root hair cell. Bars: **A, B & C**, 100 μm (x 100); **D, E & F**, 25 μm (x 400). Abbreviations: r, root hair; it, infection thread; sc, shepherd's crook; hs, hyaline spot.

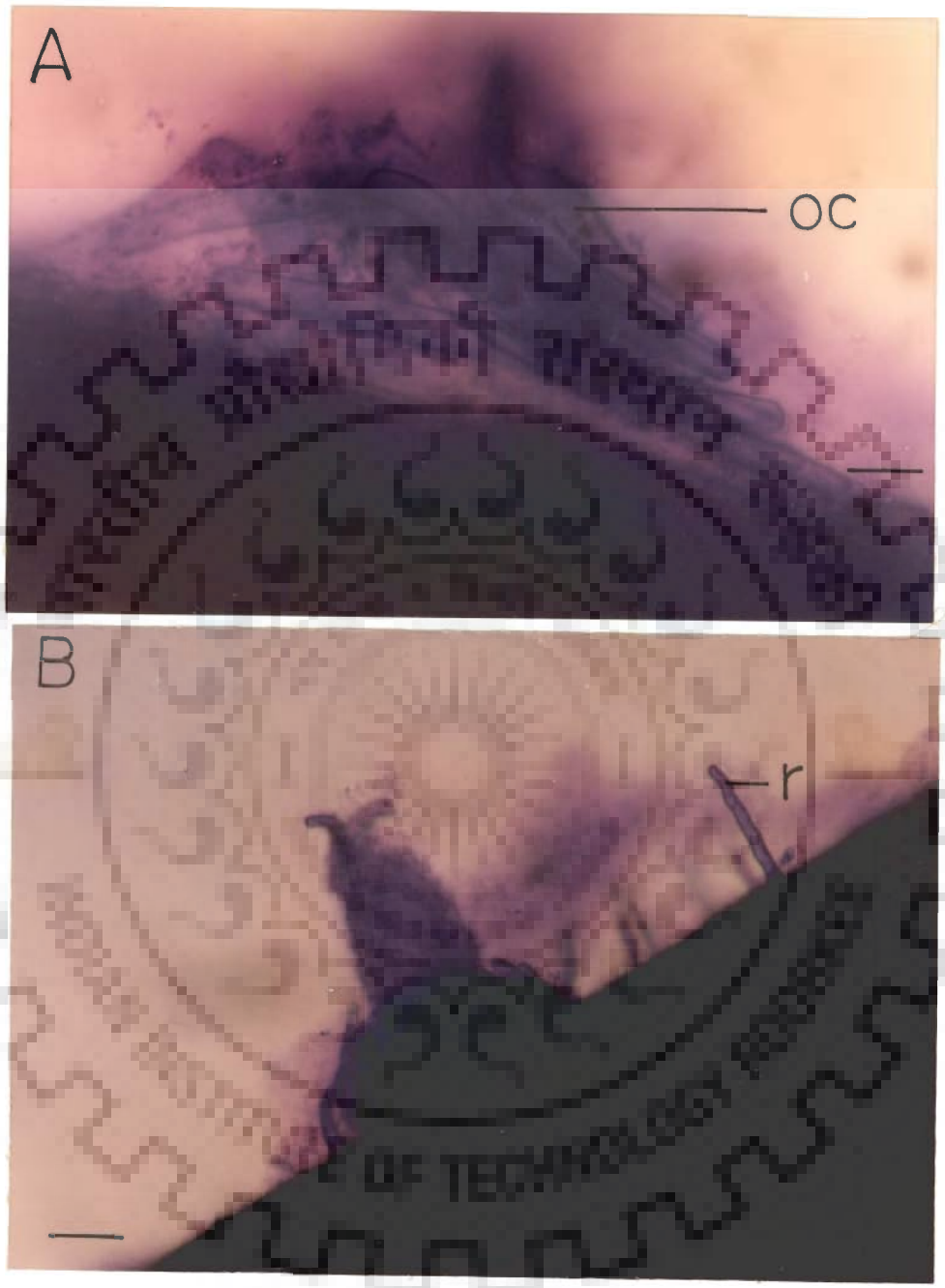

Plate 2. Light microscopic observations of root hairs of *M. sativa* cv. T9 inoculated with *S. meliloti* Rmd201. **A.** Initial nodule structure. **B.** Formation of nodule. Bars: **A**, 25 μm (x 400); **B**, 100 μm (x 100). Abbreviations: oc, outer root cortex cells; r, root hair.

Table 7. Characteristics of *M. sativa* cv. T9 nodulated by *S. meliloti* Rmd201 and its aromatic amino acid auxotrophs.

S. No.	Strain	Nodule characteristics				Shoot characteristics	
		Time of first nodule appearance in days (mean ± SE)	Nodule/plant (mean ± SE)	Shape	Colour	Shoot length cm/plant (mean ± SE)	Dry shoot weight mg/plant (mean ± SE)
Control	No inoculum	Nil	0.0	Nil	Nil	6.7 ± 0.5*	7.1 ± 0.8*
1	NV3	9.0 ± 1.2	7.5 ± 2.2	Cylindrical	White	9.2 ± 1.4*	10.9 ± 1.4*
2	BA2	9.5 ± 0.4	7.2 ± 1.0	Cylindrical	White	9.8 ± 1.2*	10.5 ± 2.2*
3	FN2	9.3 ± 0.5	7.5 ± 1.1	Cylindrical	White	10.1 ± 2.1*	11.3 ± 1.6*
4	FN3	9.6 ± 1.0	7.8 ± 1.4	Cylindrical	White	10.6 ± 1.8*	10.8 ± 1.9*
5	NV7	9.3 ± 0.6	8.0 ± 2.5	Cylindrical	Pink	20.5 ± 2.5	23.3 ± 4.5
6	NV31	9.3 ± 0.7	7.8 ± 1.3	Cylindrical	Pink	21.3 ± 3.1	22.9 ± 2.8
7	BA6	8.5 ± 1.0*	8.0 ± 1.0	Cylindrical	Pink	19.4 ± 1.7	23.0 ± 0.9
8	FN4	9.2 ± 0.2	8.2 ± 0.2	Cylindrical	Pink	20.8 ± 1.5	24.4 ± 1.3
9	FN9	10.5 ± 1.7*	4.5 ± 0.5*	Irregular	White	7.1 ± 1.2*†	7.4 ± 1.2*†
10	Rmd201	9.1 ± 1.2	8.5 ± 2.5	Cylindrical	Pink	21.2 ± 2.3	24.8 ± 3.1

ϕ Each value is a mean of ten plants ± standard error, * significant differences with parental strain Rmd201 inoculated plant ($P<0.05$); † No significant difference with uninoculated controls

only after reisolation of the particular strain from nodules and checking for their markers (Koch's postulate).

aro, *trpE(G)* and *pheA* mutants formed small and white (suggesting a lack of leghaemoglobin) nodules on the roots of alfalfa plants that were symbiotically ineffective. The mean height and dry shoot weight of plants nodulated by these mutants differed significantly from those of the parental strain Rmd201 inoculated plants and with the uninoculated controls ($P<0.05$), except for the plants nodulated by *pheA* mutant which resembled uninoculated controls in all aspects. These plants appeared weak, stunted and became chlorotic six weeks after inoculation, indicating the inability of these auxotrophs to fix nitrogen. The remaining Tn5 insertion mutants, represented by *trpD*, *trpF* or *trpC*, *trpB* and *tyrA* mutants elicited normal, cylindrical and pink (indicating the presence of leghaemoglobin) nodules on the roots of alfalfa plants that were symbiotically fully effective. The mean height and dry shoot weight of these plants did not differ significantly from those of the parental strain Rmd201 inoculated plants. These plants appeared healthy, green (indicating that nitrogen was being fixed) and resembled the parental strain Rmd201 inoculated plants in all respects (Plate 3).

The mean time taken for the appearance of first nodule after inoculation with the rhizobial strains ranged from 8.5 (BA6) to 10.5 days (FN9); mean number of nodules per plant varied from 4.5 (FN9) to 8.5 (Rmd201); mean shoot length per plant differed from 7.1 (FN9) to 21.3 cm (NV31) whereas 6.7 cm for the uninoculated controls; mean dry shoot weight per plant varied from 7.4 (FN9) to 24.8 mg (Rmd201) whereas 7.1 mg for the uninoculated controls.

Plate 3. Morphological features of *M. sativa* cv. T9 nodulated by *S. meliloti* Rmd201 and its aromatic amino acid auxotrophs, grown in 20 x 2.5 cm test tubes. **A.** Rmd201 inoculated plant. **B.** *aro* mutant (NV3) inoculated plant. **C.** *trpE(G)* mutant (FN2) inoculated plant. **D.** *trpD*, *trpF* or *trpC* mutant (NV7) inoculated plant. **E.** *trpB* mutant (BA6) inoculated plant. **F.** *pheA* mutant (FN9) inoculated plant. **G.** *tyrA* mutant (FN4) inoculated plant. **H.** Uninoculated control.

When the kanamycin sensitive prototrophic revertants of the aromatic amino acid auxotrophs were tested on the roots of *M. sativa* cv. T9, it was found that similar to the parental strain Rmd201, all the revertants elicited normal, cylindrical and pink nodules on the roots of alfalfa plants that were symbiotically fully effective. The plants nodulated by the revertants were healthy, green with no signs of nitrogen starvation and resembled those of the parental strain Rmd201 inoculated plants in all respects.

4.4.3 Occupancy of nodules by aromatic amino acid auxotrophs

To test the authenticity of nodulation by the auxotrophic strains, nodule occupancy tests were carried out. The rhizobial bacteria were reisolated from the nodules of each inoculated plant with a particular auxotroph or parental strain Rmd201 and checked for their markers (Koch's postulate). Differences in the number of plants showing 100% occupancy of nodules by the auxotrophs were found. The mutant BA2 showed maximum percentage (90%) of plants showing 100% occupancy of nodules, whereas the minimum (63.3%) was found in the FN9 mutant. The parental strain Rmd201 exhibited all plants showing 100% occupancy of nodules. The results of nodule occupancy tests are summarized in Table 8.

4.5 Exogenous feeding of the symbiotically defective auxotrophs

4.5.1 The effect of addition of nutritional requirements of the auxotrophs on the growth of alfalfa plants

In order to test the effect of addition of aromatic amino acids or their intermediates on alfalfa plant growth, plant growth medium was supplemented with various concentrations (0-50 μg/ml) of the nutritional requirements of the auxotrophs. The optimal concentrations (concentration with no adverse effect on root and shoot

Table 8. Occupancy of nodules by aromatic amino acid auxotrophs of *S. meliloti* Rmd201

S. No.	Strain	No. of plants showing 100% occupancy of nodules by the auxotroph*	Percentage of plants showing 100% occupancy of nodules by the auxotroph
1	NV3	23	76.6
2	BA2	27	90.0
3	FN2	21	70.0
4	FN3	25	83.3
5	NV7	20	66.6
6	NV31	22	73.3
7	BA6	24	80.0
8	FN4	23	76.6
9	FN9	19	63.3
10	Rmd201 (parental strain)	30	100

*Thirty plants were inoculated with each strain

developments) of each nutritional supplement were determined, which were used in further studies of supplementation experiments. The effect of aromatic amino acids or their intermediates on growth of alfalfa plants are given in Table 9. It was found that the optimal concentrations of anthranilic acid and indole were upto 10 and 5 μg/ml, respectively, while that of tryptophan, phenylalanine and tyrosine were upto 20, 30 and 50 μg/ml, respectively. Upto 5 μg/ml of all three aromatic amino acids (tryptophan, phenylalanine and tyrosine) did not affect root and shoot developments of alfalfa plants. However, other higher concentrations of the nutritional requirements of the auxotrophs were found adverse to the root and shoot developments.

4.5.2 Restoration of the symbiotic ability of the symbiotically defective auxotrophs

After determining the concentrations of aromatic amino acids or their intermediates with no adverse effect on root and shoot developments, the effect of exogenous feeding on the restoration of the symbiotic ability of the symbiotically defective auxotrophs were studied. It was found that the symbiotic ability of *trpE(G)* mutants was restored on supplementation of plant growth medium with anthranilic acid (upto 10 μg/ml). At 2.5 μg/ml supplementation of anthranilic acid, only partial restoration was observed, whereas complete restoration took place at 5, 7.5 and 10 μg/ml supplementation of anthranilic acid. However, supplementations of plant growth medium with indole (upto 5 μg/ml) or tryptophan (upto 20 μg/ml) could not restore the symbiotic ability of *trpE(G)* mutants. The symbiotic functions of *pheA* and *aro* mutants were also not restored by supplementation of plant growth medium with phenylalanine

Table 9. Growth responses of *M. sativa* cv. T9 to aromatic amino acids or their intermediates

Aromatic amino acids or their intermediates	**Growth on plant growth medium supplemented with : (μg/ml)**						
	0	**5**	**10**	**20**	**30**	**40**	**50**
Anthranilic acid	+	+	+	–	–	–	–
Indole	+	+	–	–	–	–	–
Tryptophan	+	+	+	+	–	–	–
Phenylalanine	+	+	+	+	+	–	–
Tyrosine	+	+	+	+	+	+	+
All aromatic amino acids	+	+	–	–	–	–	–

Abbreviations : +, no adverse effect on root and shoot developments; –, adverse effect on root and shoot development

(upto 30 μg/ml) and all three aromatic amino acids (tryptophan, tyrosine and phenylalanine) upto 5 μg/ml, respectively. The nodule and shoot characteristics of alfalfa plants nodulated by the parental strain Rmd201 and its symbiotically defective auxotrophs grown with different concentrations of nutritional supplementations, are given in Table 10. The data were retained only when the defective mutants were reisolated from nodules and checked for their markers (Koch's postulate). It was found that when the plant growth medium was supplemented with 2.5 μg/ml of anthranilic acid, *trpE(G)* mutants were able to induce slightly pink nodules on the roots of alfalfa plants,. The mean height and dry shoot weight of these plants differed significantly from those of the parental strain Rmd201 inoculated plants ($P<0.05$). However, with further increase in concentration of anthranilic acid to 5, 7.5 and 10 μg/ml, *trpE(G)* mutants elicited pink nodules on the root of alfalfa plants that were symbiotically effective. The plants nodulated by these mutants resembled those of the parental strain inoculated plants in all respects (Plate 4). The characteristics of *trpE(G)* mutants inoculated plants supplemented with 5 μg/ml of indole or 20 μg/ml of tryptophan resembled those of *trpE(G)* mutants inoculated plants, without supplementation, in all respects. However, the *pheA* and *aro* mutants inoculated plants supplemented with 30 and 5 μg/ml of phenylalanine and all three aromatic amino acids, (tryptophan, tyrosine and phenylalanine) respectively, also resembled those of the *pheA* and *aro* mutants inoculated plants, without supplementation, in all respects.

Plate 4. Morphological features of *M. sativa* cv. T9 nodulated by *S. meliloti* Rmd201 and its *trpE(G)* mutant (FN2), grown in 20 x 2.5 cm test tubes, with different concentrations of anthranilic acid. **A.** Rmd201 inoculated plant. **B.** FN2 mutant inoculated plant without anthranilic acid. **C.** FN2 mutant inoculated plant with 2.5 μg/ml of anthranilic acid. **D.** FN2 mutant inoculated plant with 5 μg/ml of anthranilic acid. **E.** FN2 mutant inoculated plant with 7.5 μg/ml of anthranilic acid. **F.** FN2 mutant inoculated plant with 10 μg/ml of anthranilic acid. **G.** Uninoculated control.

Table 10. Characteristics of *M. sativa* cv. T9 nodulated by *S. meliloti* Rmd201 and its symbiotically defective auxotrophs grown with their nutritional requirements ϕ.

Strain	Nutritional Requirement	Concentration (μg/ml)	Nodule characteristics				Shoot characteristics	
			Time of first nodule appearance in days (mean ± SE)	Nodule/plant (mean ± SE)	Shape	Colour	Shoot length cm/plant (mean ± SE)	Dry shoot weight mg/plant (mean ± SE)
No inoculum#	Nil	0	Nil	0.0	Nil	Nil	6.7 ± 0.5*	7.1 ± 0.8*
FN2	Ant., Ind. or Trp.	0	9.3 ± 0.5	7.5 ± 1.1	Cylindrical	White	10.1 ± 2.1*	11.3 ± 1.6*
FN3	Ant. Ind. or Trp.	0	9.6 ± 1.0	7.8 ± 1.4	Cylindrical	White	10.6 ± 1.8*	10.8 ± 1.9*
FN2	Ant.	2.5	9.2 ± 1.2	6.9 ± 0.8	Cylindrical	Slighty pink	14.6 ± 1.2*	18.7 ± 0.8*
FN3	Ant.	2.5	9.7 ± 0.9	7.3 ± 2.1	Cylindircal	Slighty pink	15.2 ± 1.6*	17.6 ± 2.2*
FN2	Ant.	5	9.5 ± 0.6	7.5 ± 1.8	Cylindrical	Pink	21.1 ± 0.9	22.5 ±2.5
FN3	Ant.	5	9.4 ± 1.1	7.2 ± 0.9	Cylindrical	Pink	20.0 ± 1.3	23.8 ± 1.9
FN2	Ant.	7.5	9.3 ± 0.3	8.0 ± 0.6	Cylindrical	Pink	20.7 ± 1.8	24.7 ± 2.1
FN3	Ant.	7.5	9.1 ± 0.5	7.5 ± 0.1	Cylindrical	Pink	21.2 ± 1.6	25.2 ± 2.6
FN2	Ant.	10	9.2 ± 0.7	7.2 ± 0.7	Cylindrical	Pink	21.4 ± 2.1	24.9 ± 2.8
FN3	Ant.	10	8.9 ± 1.5	7.7 ± 1.5	Cylindrical	Pink	21.3 ± 2.4	25.3 ± 2.6
FN2	Ind.	5	8.5 ± 0.3	7.0 ± 0.5	Cylindrical	White	10.6 ± 1.3*	11.1 ± 1.2*
FN3	Ind.	5	9.0 ± 0.8	7.5 ± 1.2	Cylindrical	White	11.0 ± 0.9*	11.6 ± 0.8*
FN2	Trp.	20	9.0 ± 1.0	6.3 ±1.7	Cylindrical	White	11.0 ± 2.7*	10.9 ± 2.0*
FN3	Trp.	20	9.5 ± 0.3	6.5 ± 1.0	Cylindrical	White	12.8 ± 1.5*	11.7 ± 1.6*
FN9	Phe.	0	10.5 ± 1.7*	4.5 ± 0.5*	Irregular	White	7.1 ± 1.2*†	7.4 ± 1.2*†
FN9	Phe.	30	10.0 ± 1.3*	5.0 ± 1.2*	Irregular	White	6.9 ± 0.8*†	7.7 ± 1.0*†
NV3	Aro.	0	9.0 ± 1.2	7.5 ± 2.2	Cylindrical	White	9.2 ± 1.4*	10.9 ± 1.4*
BA2	Aro.	0	9.5 ± 0.4	7.2 ± 1.0	Cylindrical	White	9.8 ± 1.2*	10.5 ± 2.2*
NV3	Aro.	5	9.4 ± 0.5	7.0 ± 1.5	Cylindrical	White	9.6 ± 0.9*	11.2 ± 0.8*
BA2	Aro.	5	9.2 ± 1.2	7.5 ± 0.5	Cylindrical	White	9.3 ±1.3*	11.4 ± 1.2*
Rmd201#	Nil	0	9.1 ± 1.2	8.5 ± 2.5	Cylindrical	Pink	21.2 ± 2.3	24.8 ± 3.1

ϕ Each value is a mean of ten plants ± standard error, * significant differences with parental strain Rmd201 inoculated plant ($p<0.05$). † No significant difference with uninoculated controls. # The characteristics of uninoculated controls and parental strain Rmd201 inoculated plants grown without nutritional supplementations were the same to those supplemented with optimal concentrations of the nutritional requirements.
Abbreviations: Ant, anthranilic acid; Ind, indole; Trp, tryptophan; Phe, phenylalanine, Aro, aromatic amino acids (tryptophan, tyrosine and phenylalanine).

4.6 Histology of nodules induced by the parental strain Rmd201 and its aromatic amino acid auxotrophs

In order to understand the cause of symbiotic defects of *aro*, *trpE(G)* and *pheA* mutants, light and transmission electron microscopic observations of nodules induced by the parental strain Rmd201 and its aromatic amino acid auxotrophs were carried out. These studies have helped to follow the differentiation and/or deterioration of released rhizobia inside the cells of nodules elicited by these strains.

4.6.1 Parental strain Rmd201 nodules

4.6.1.1 Light microscopy

The light microscopic observations of the longitudinal-semithin sections of nodules induced by the parental strain Rmd201 exhibited normal developmental stages of the distinct zones, viz., distal meristematic, infection, nitrogen-fixing and proximal senescence zones. In addition to these zones, there is a region located between infection and nitrogen-fixing zones extending over one or three layers of cells, is called amyloplast-rich interzone. With the exception of the apical meristem, the tissues of these zones were surrounded by peripheral tissues, which included vascularized parenchyma, endodermis and cortex (Plate 5A). The meristematic zone located at the distal forward portion of the nodules, contained dividing, uninfected cells. The infection zone that lied next to meristematic zone comprised of cells having large number of rhizobial bacteria, which were released from infection threads. The centres of the nodule cells in this zone were frequently occupied by large vacuoles (Plate 5B). The peripheral vascular bundle, surrounded by an endodermal layer was seen in this zone (Plate 6A). Few layers next to short infection zone constituted the amyloplast-rich interzone. Here the nodule cells

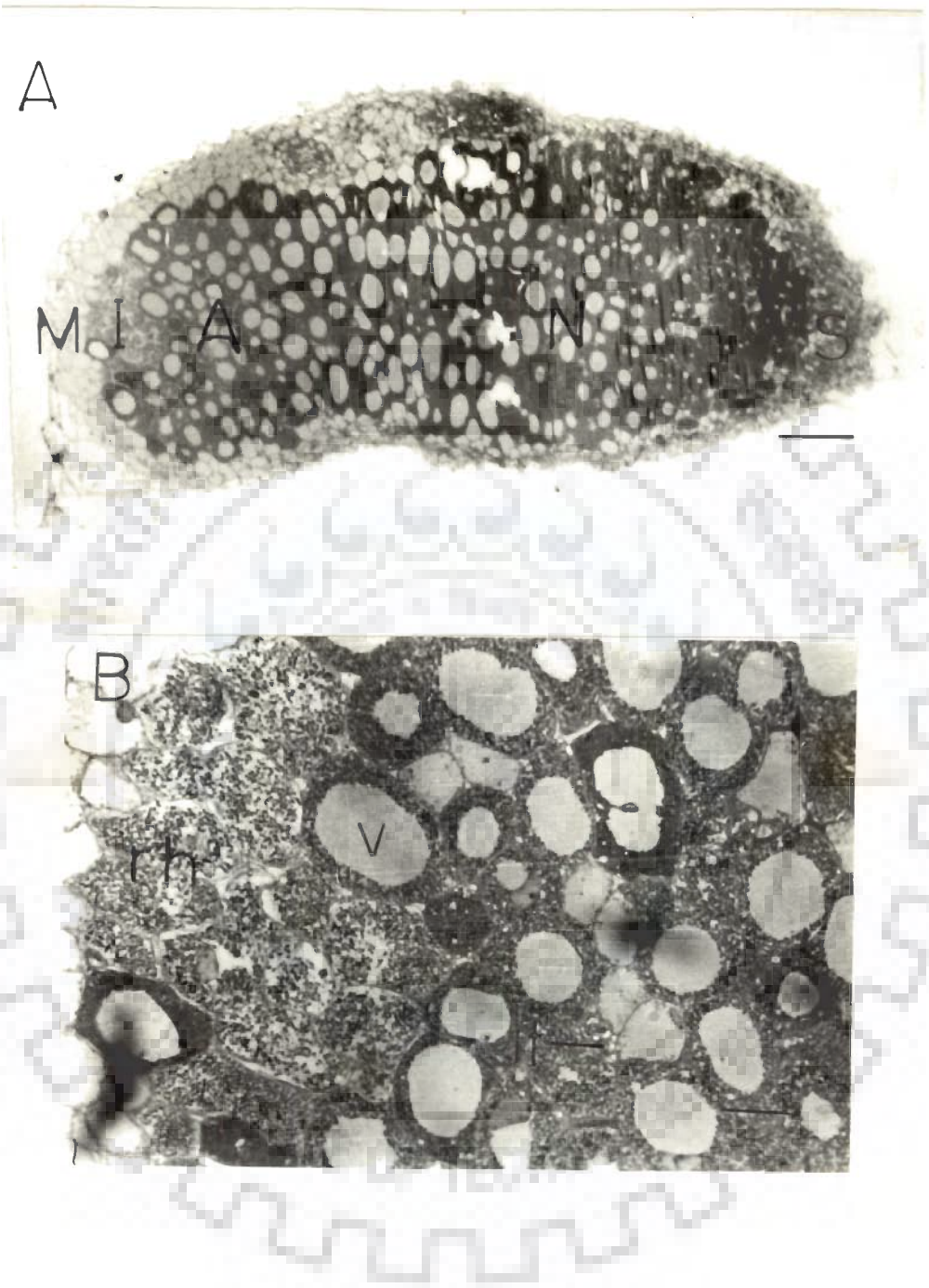

Plate 5. Light microscopic observations of longitudinal-semithin sections of a nodule induced by *S. meliloti* Rmd201. **A.** The whole nodule section showing distal meristematic zone (M), infection zone (I), amyloplast-rich interzone (A), nitrogen-fixing zone (N) and senescence zone (S). Bar, 100 μm (x 100). **B.** Infection zone cells showing infection threads (it), rhizobia (rh) and vacuoles (v). Bar: 25 μm (x 400).

were studded with large numbers of amyloplasts near an intercellular space (Plate 6B). The nitrogen-fixing zone formed the major percentage of the nodule, their cells were filled with large number of bacteroids which were organised around the central vacuole in each cell (Plate 6C). The senescense zone which occupied the basal portion of the nodule contained oldest nodule cells, filled with deteriorated bacteroids (Plate 6D).

4.6.1.2 Transmission electron microscopy

Transmission electron microscopic observations of longitudinal-ultrathin sections of nodules induced by *S. meliloti* Rmd201 revealed all stages of bacteroid development. The rhizobia in the infection zone were rod-shaped, electron dense and most of the bacterial cytoplasm was found to contain clear poly-β-hydroxybutarate granules indicating that these bacteria were freshly released. These bacteroids were surrounded by host-derived peribacteroid membrane (Plate 7A). In the same zone the nodule cells contained endoplasmic reticulum, Golgi complex and bacteroids (Plate 7B). The mature bacteroids in the nitrogen-fixing zone were polymorphic but most of these bacteroids were elongated and had more heterogeneous cytoplasm which had electron dense and electron transparent regions. Golgi complex and endoplasmic reticulum were rarely seen in this region in comparison to those of the infection zone because of bacteroid differentiation (Plate 7C). In the late nitrogen-fixing zone, the differentiated bacteroids exhibited greater heterogenity in the cytoplasm. This appeared to be the result of condensation of nuclear material. Deteriorated bacteroids having electron transparent cytoplasm, surrounded by broken peribacteroid membrane were seen in senescence zone (Plate 7D).

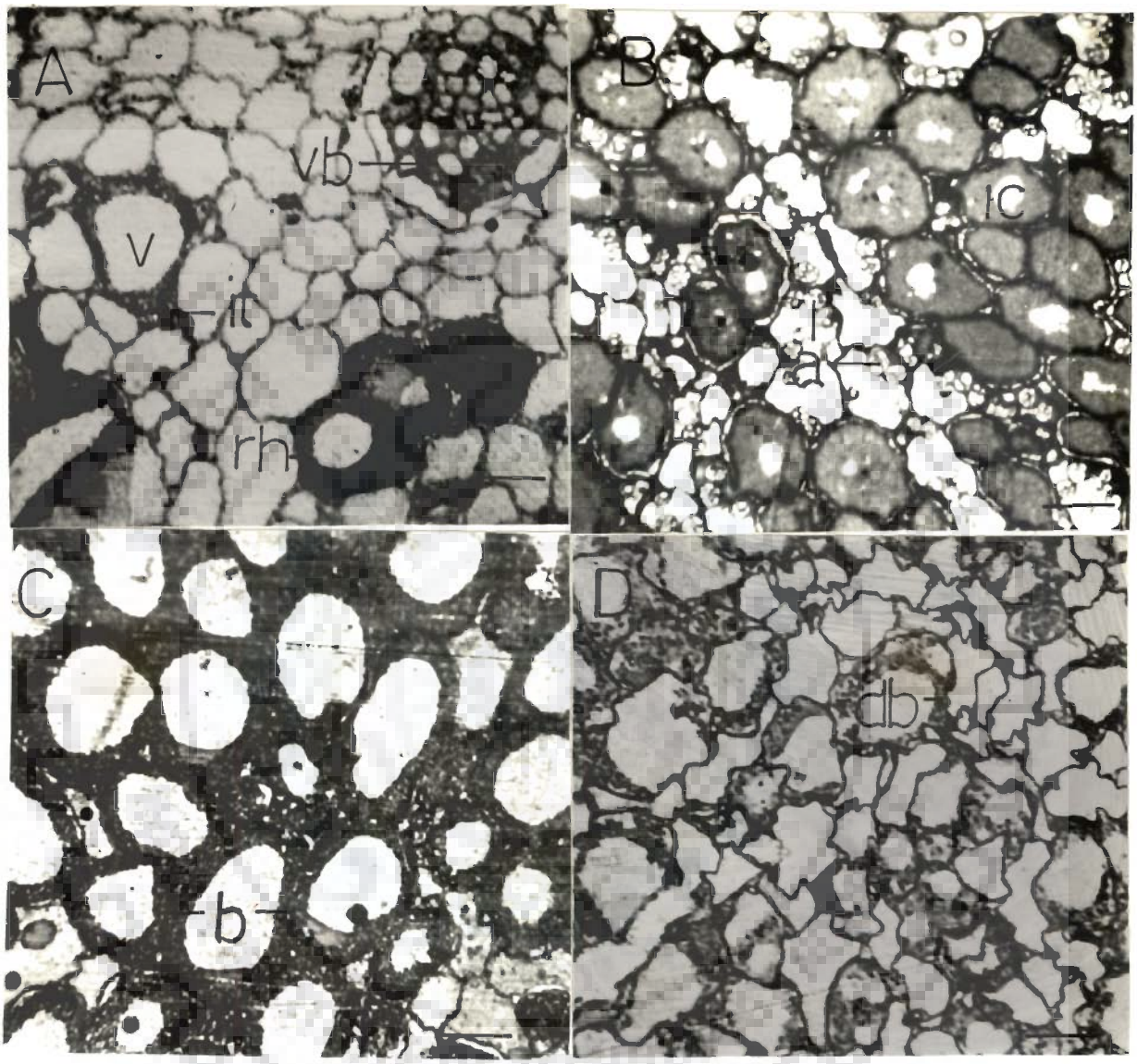

Plate 6. Light microscopic observations of longitudinal-semithin sections of a nodule induced by *S. meliloti* Rmd201. **A.** Nodule cells of infection zone showing infection thread (it), rhizobia (rh), vacuoles (v) and peripheral vascular bundle (vb). **B.** Interzone cells showing infected cells (ic) and the cell walls were studded with amyloplasts (a). **C.** Nitrogen-fixing zone cells filled with bacteroids (b). **D.** Senescence zone cells occupied by deteriorated bacteroids (db). Bars: **A**, **B**, **C** & **D**, 25 μm (x 400).

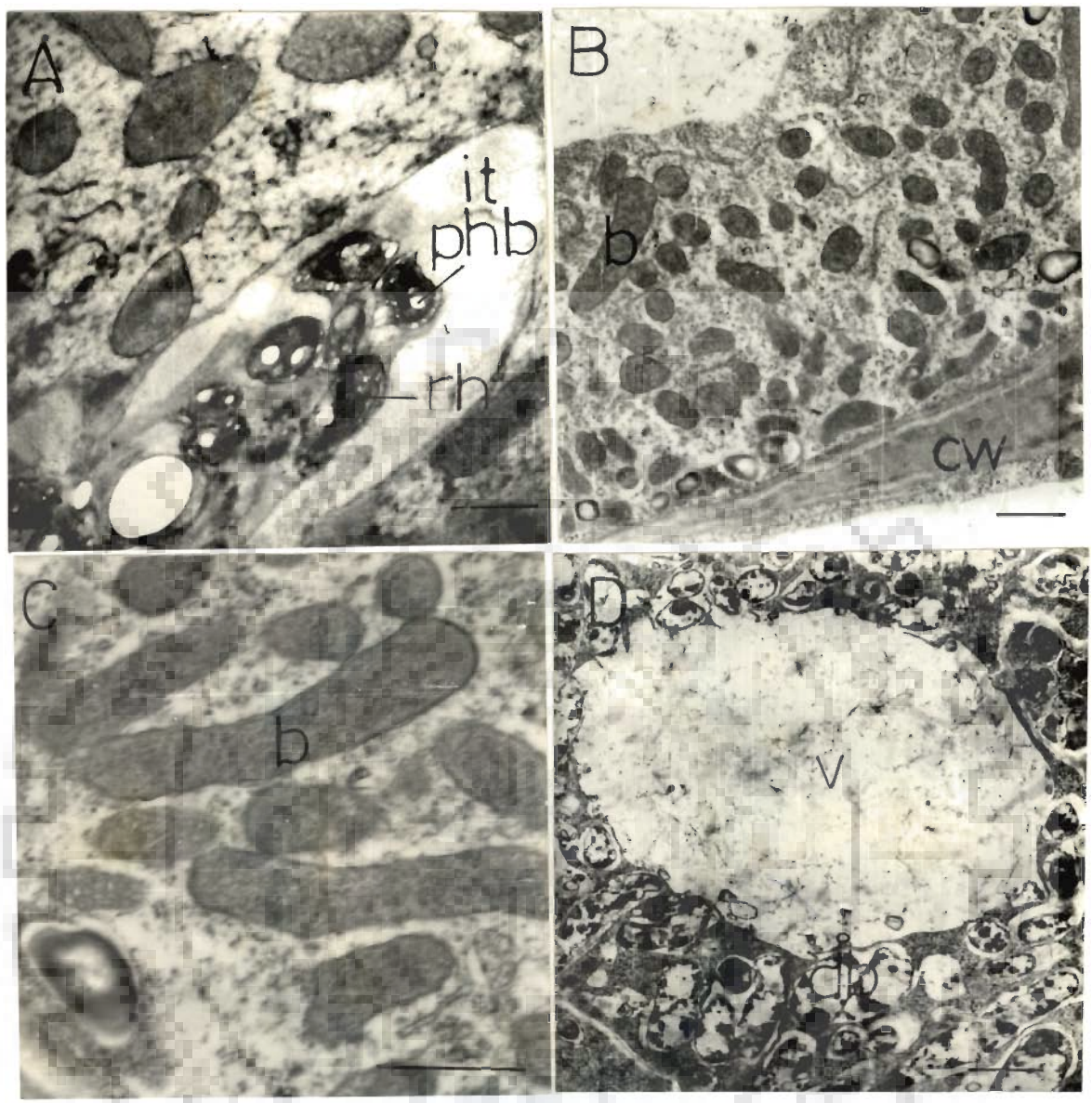

Plate 7. Transmission electron microscopic observations of longitudinal-ultrathin sections of a nodule induced by *S. meliloti* Rmd201. **A.** A part of nodule cell of infection zone showing infection thread (it) containing rhizobia (rh) having poly-β-hydroxybutarate (phb) granules. Bar: 1 μm (x 11500). **B.** Nodule cell cytoplasm of the same zone showing partially heterogeneous bacteroids (b) and the cell organelles were displaced near to the cell wall (cw), arranged around central vacuoles (v). Bar. 1 μm (x 9000). **C.** A part of nodule cell of nitrogen-fixing zone showing elongated mature bacteroids (b). Bar: 1 μm (x 21000). **D.** Nodule cell of senescence zone showing deteriorated bacteroids (db) arranged around central vacuole (v) Bar: 1 μm, (x 10500)

4.6.2 *aro* and *trpE(G)* mutant nodules

The general internal histological features of the nodules induced by *aro* mutants (NV3 and BA2) exhibited striking similarities to those of the nodules formed by *trpE(G)* mutants (FN2 and FN3). As a representative, the microscopic observations of FN2 mutant-induced nodules are presented here.

4.6.2.1 Light microscopy

The nodules induced by FN2 mutant were poorly developed in comparison to the parental strain Rmd201 nodules (Plate 8A). The infection zone was many layers thick and very extensive, encompassing most part of the middle nodule tissues. The bacteria were mostly confined to the infection threads but occasional release of bacteria was found in some cells, while the majority of nodule cells were empty (Plate 8B). The cell walls were studded with amyloplasts (Plate 9A). In the interzone, the nodule cells contained large number of amyloplasts and few bacteroids (plate 9B). Unlike the parental strain Rmd201-induced nodules, only a few nodule cells in the nitrogen-fixing zone were seen with bacteroids. This zone was confined to the basal portion of the nodule (Plate 9C). Next to this region, the senescence zone having deteriorated bacteroids was seen (Plate 9D).

4.6.2.2 Transmission electron microscopy

The freshly released rhizobia containing poly-β-hydroxybutarate granules were seen in nodule cells of extended infection zone. Beside these bacteria, the cytoplasm of nodule cells contained cell organelles like mitochondria, endoplasmic reticulum and Golgi complex (Plate 10A). Some bacteroids in poorly developed nitrogen-fixing zone were observed to be in lysing conditions as their peribacteroid membranes were also

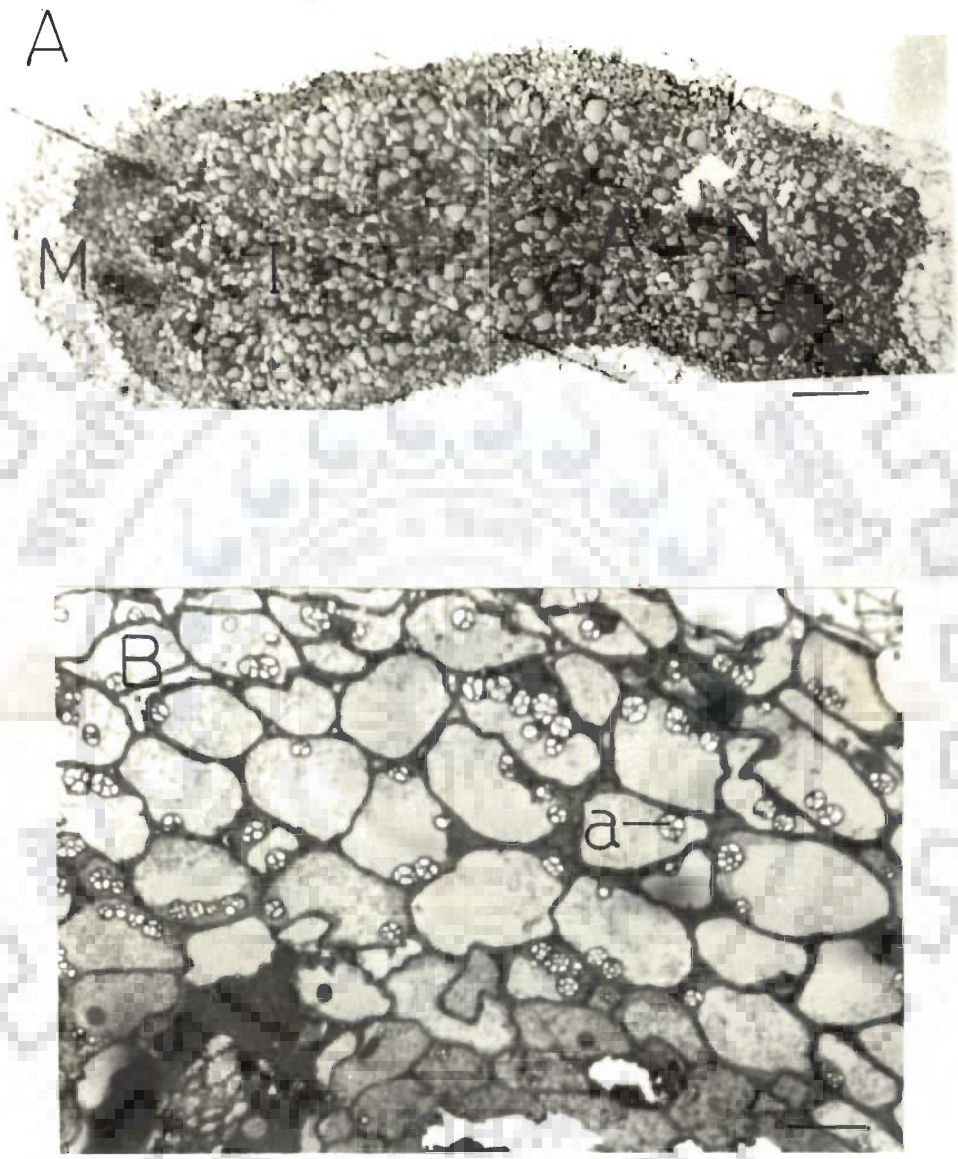

Plate 8. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant of *S. meliloti* Rmd201. **A.** The whole nodule section showing poorly developed nodule regions, viz., distal meristematic zone (M), infection zone (I), amyloplast-rich interzone (A), nitrogen-fixing zone (N) and senescence zone (S). Bar, 100 μm (x 100). **B.** Early infection zone cells showing the cell walls studded with amyloplasts (a). Bar: 25 μm (x 400).

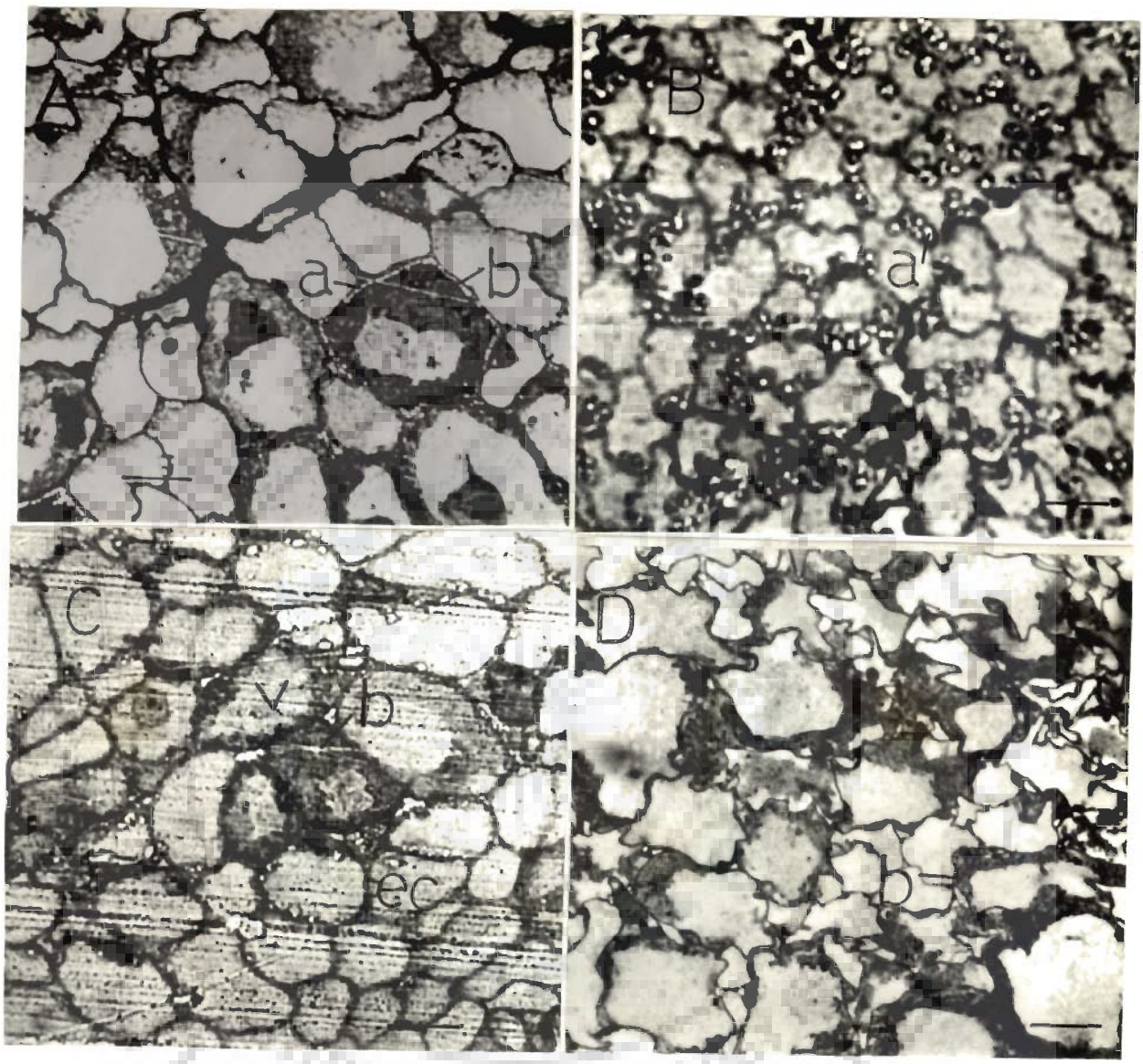

Plate 9. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant of *S. meliloti* Rmd201. **A.** Nodule cells of late infection zone having bacteroids (b) and the cell walls were studded with amyloplasts (a). **B.** Interzone cells packed with large number of amyloplasts (a). **C.** Some nodule cells of poorly developed nitrogen-fixing zone containing bacteroids (b) arranged around central vacuoles (v), while the others were empty cells (ec). **D.** Packed senescence zone having senescent bacteroids (b). Bars: **A, B, C & D**,25 μm (x 400).

broken and the cytoplasm of these bacteroids became electron transparent (Plate 10B). Unlike the parental strain Rmd201-induced nodules, the infected cells in this region contained senescence bacteroids, which have clear electron transparent cytoplasm (Plate 10C). In the senescence zone the bacteroids were observed to be in a degenerating condition which showed morphological characteristics similar to those of the bacteroids in poorly developing nitrogen-fixing zone (Plate 10D).

4.6.3 *trpD*, *trpF* or *trpC* and *trpB* mutant nodules

The nodules induced by *trpD*, *trpF* or *trpC* (NV7 and NV31) and *trpB* (BA6) mutants had similar internal histological structure. As a representative, the microscopic observations of NV7 mutant-induced nodules are presented below.

4.6.3.1 Light microscopy

The histological features of nodules induced by the strain NV7 were matched to those of the nodules formed by the parental strain Rmd201, having normal distinct zones, viz., distal meristematic, infection, amyloplast-rich inter, nitrogen-fixing and senescence zones (Plate 11A). The meristematic zone was devoid of infection threads and rhizobial bacteria (Plate 11B). In transverse section of the whole nodules of infection zone, some nodule cells were filled with bacteria (Plate 12A). In the interzone, the nodule cells started getting filled with rhizobia and amyloplasts (Plate 12B). The nitrogen-fixing zone which formed the bulk of the nodule, contained most of the cells filled with large number of nitrogen-fixing bacteroids. The symbiosomes in these cells were arranged around the central vacuole (Plate 12C). The senescence region quite resembled to that of the parental strain Rmd201 nodules in all aspects (Plate 12D).

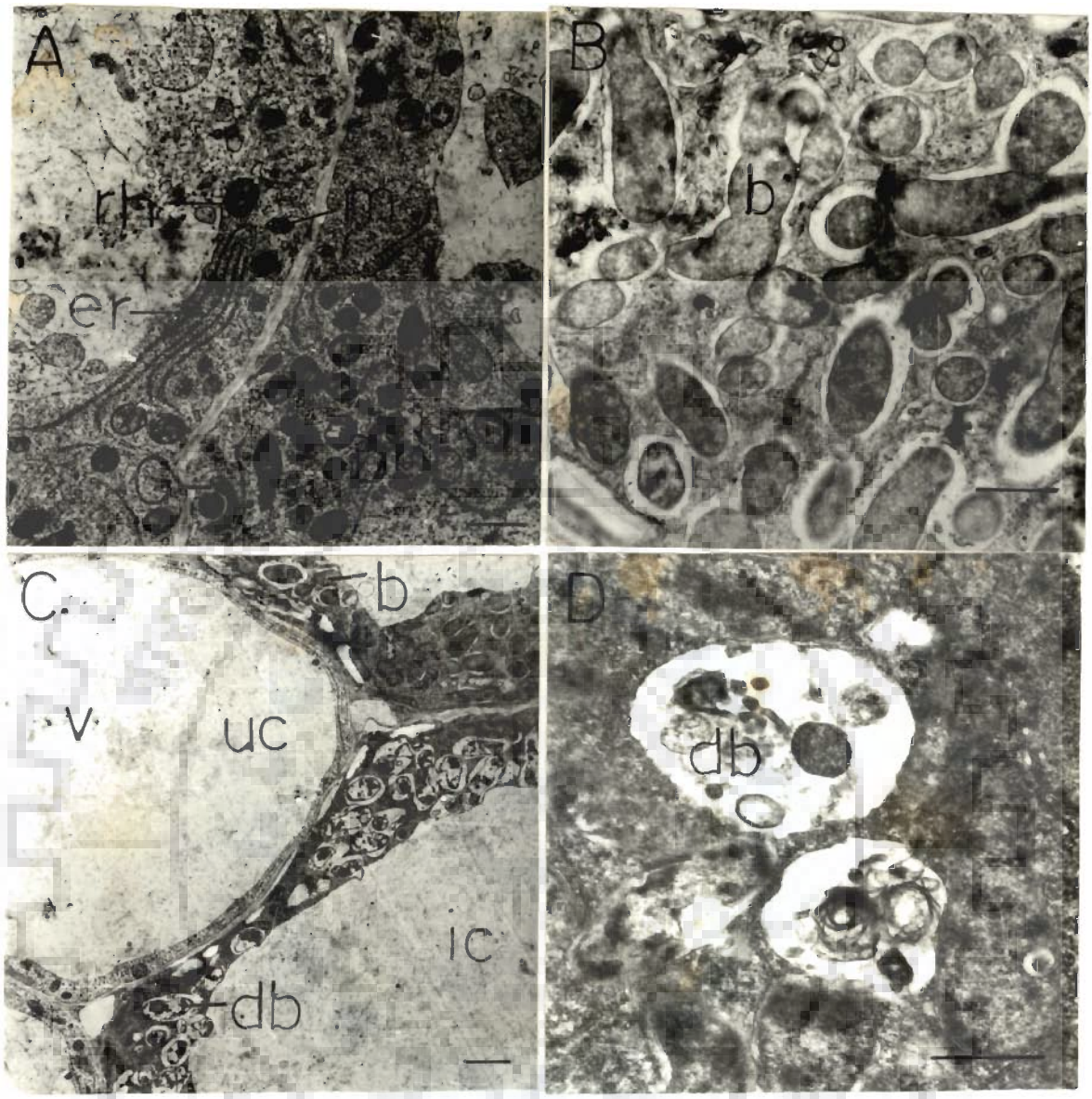

Plate 10. Transmission electron microscopic observations of longitudinal-ultrathin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant of *S. meliloti* Rmd201. **A.** A part of nodule cells of infection zone showing freshly released rhizobia (rh) having poly-β-hydroxybutarate (phb)granules, mitochondria (m), endoplasmic reticulum (er) and Golgi body (G). Bar: 1 μm (x 7750). **B.** A part of nodule cell of poorly developed nitrogen-fixing zone showing polymorphic bacteroids (b). Bar: 1 μm (x 11500). **C.** Cells of the same zone showing two parts of uninfected cells (uc) and other two parts of infected cells (ic) having mature bacteroids (b) and deteriorated bacteroids (db) arranged around central vacuoles (v). Bar: 1 μm (x 6625). **D.** Complete degenerated bacteroids (db) of senescence zone. Bar: 1 μm (x 15750).

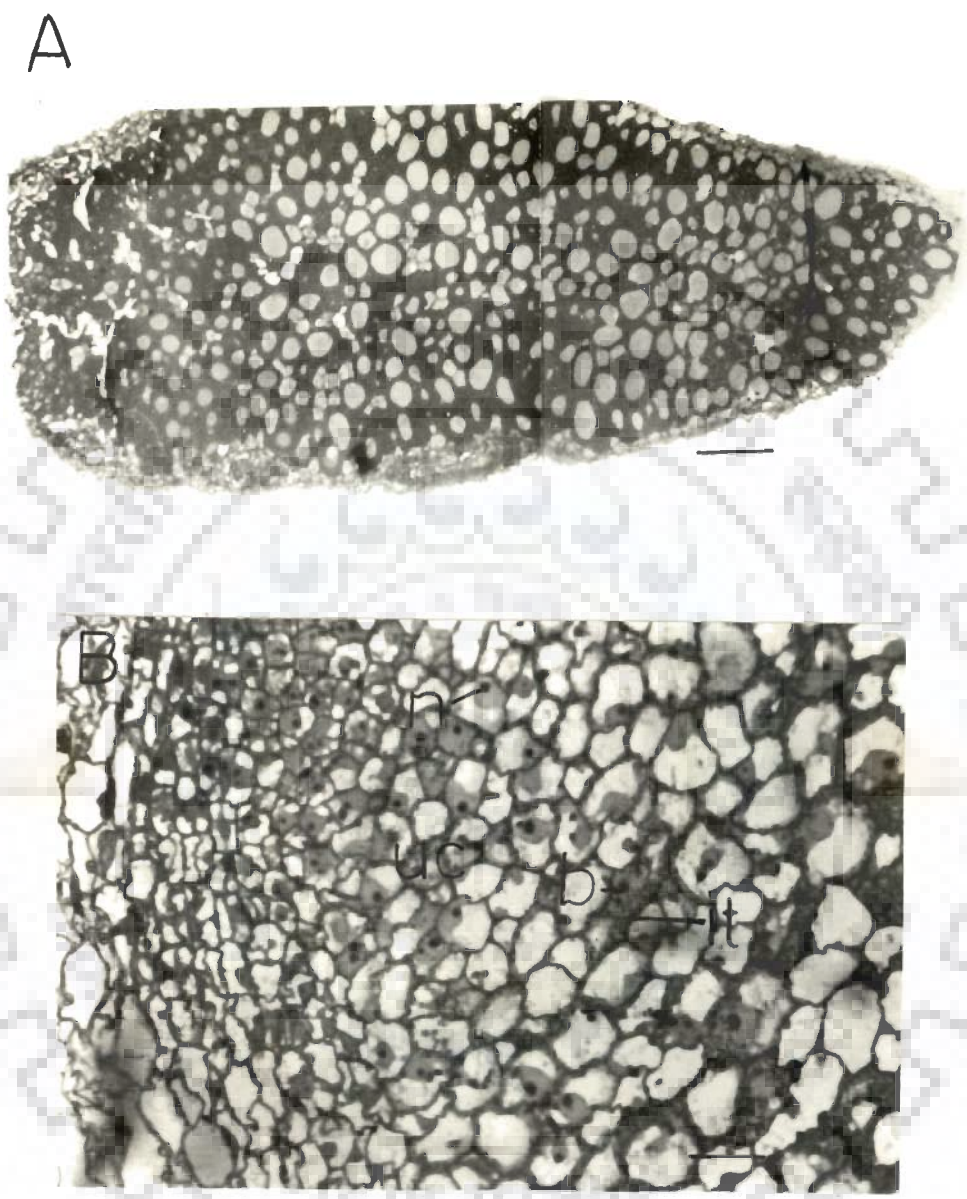

Plate 11. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain NV7, a *trpD trpF* or *trpC* mutant of *S. meliloti* Rmd201. **A.** The whole nodule section showing normal distinct zones. Bar: 100 μm (x100). **B.** Meristematic zone contained uninfected cells (uc) having prominent nuclei (n), while the early infection zone showing infection thread (it) and bacteroids (b). Bar: 25 μm (x 400).

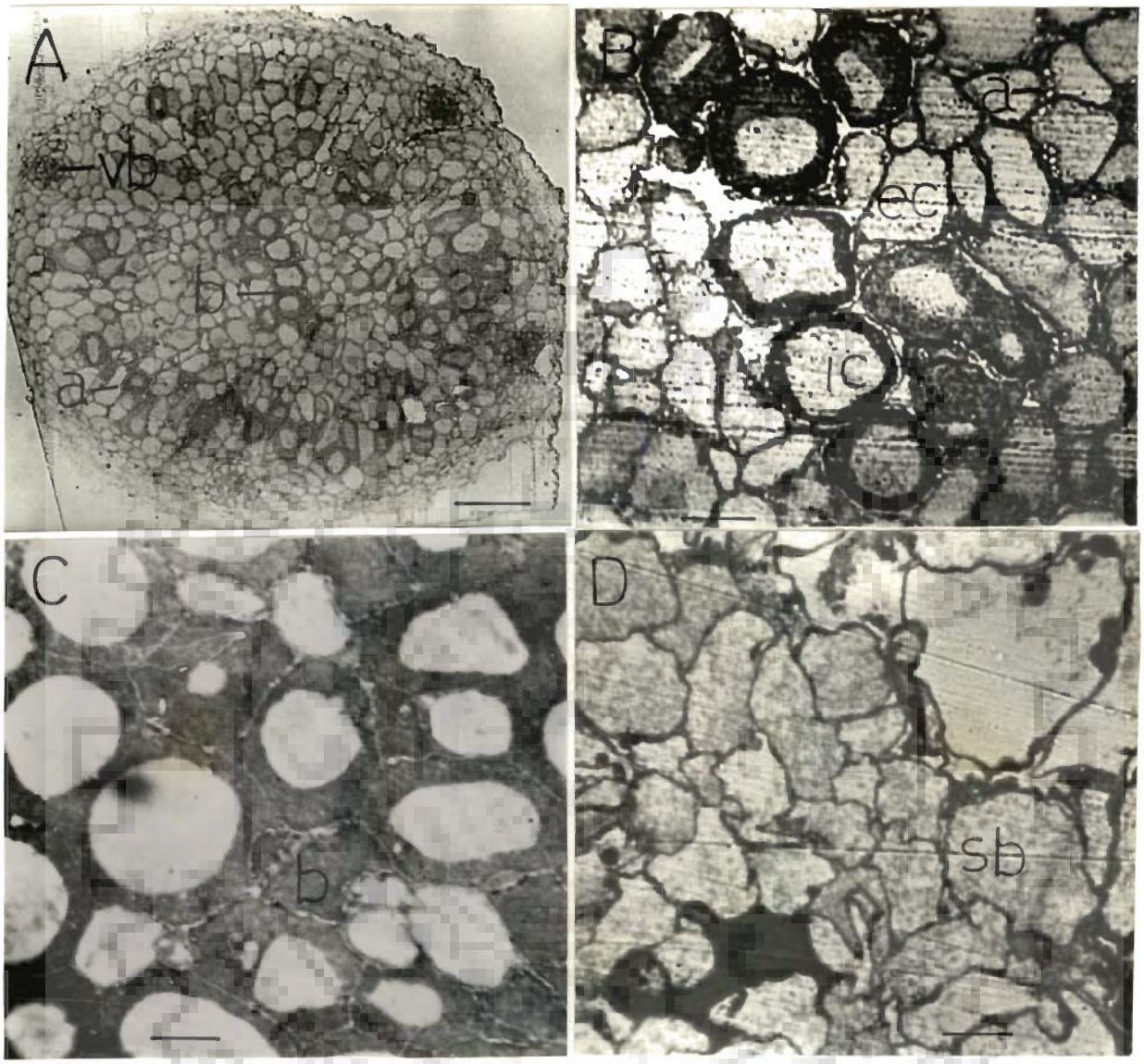

Plate 12. Light microscopic observations of semithin sections of a nodule induced by the strain NV7, a *trpD*, *trpF* or *trpC* mutant of *S. meliloti* Rmd201. **A.** Transverse section of the whole nodule of infection zone showing the bacteroids (b), four peripheral vascular bundles (vb) and the cells in this region were studded with amyloplasts (a). Bar: 100 µm (x100). **B.** Nodule cells of the interzone showing infected cells (ic), empty cells (ec) and the cell walls were studded with large number of amyloplasts (a). **C.** Nitrogen-fixing zone cells filled with bacteroids (b). **D.** Oldest nodule cells having senescent bacteroids (sb). Bar: **B**, **C** & **D**, 25 µm (x 400).

4.6.3.2 Transmission electron microsocpy

The transmission electron microscopic observations revealed that the bacteroids in the infection region were similar to those described previously in parental strain Rmd201, being electron dense as a result of condensation of nucleic acid material. The endoplasmic reticulum and other cell organelles were apparent in the cells of this zone (Plate 13A). Elongated bacteroids with heterogeneous cytoplasm were seen in the late infection region near nitrogen-fixing zone (Plate 13B). The elongated nitrogen-fixing bacteroids surrounded by peribacteroid membrane in the nitrogen-fixing zone exhibited more heterogeneous cytoplasm with electron dense and electron transparent regions (Plate 13C). Majority of bacteroids in the cells of senescence zone were observed to be in a degenerating state (Plate 13D).

4.6.4 *pheA* mutant nodules

4.6.4.1 Light microscopy

FN9 mutant (a *pheA* mutant) induced nodules on the root of alfalfa plants were found to be symbiotically ineffective. Unlike the parental strain Rmd201 the nodules induced by this mutant lacked distinctive cellular zones (Plate 14A). The frontal portion of these nodules was composed of uninfected, dividing cells containing large number of amyloplasts (Plate 14B). Only a few nodule cells contained bacteroids, while the others were devoid of bacteroids (Plate 14C).

4.6.4.2 Transmission electron microscopy

The transmission electron microscopic observation revealed that the release of rhizobial bacteria from infection threads to the cytoplasm of nodule cells was normal. But the differentiation of released bacteria into functional bacteroids was not complete

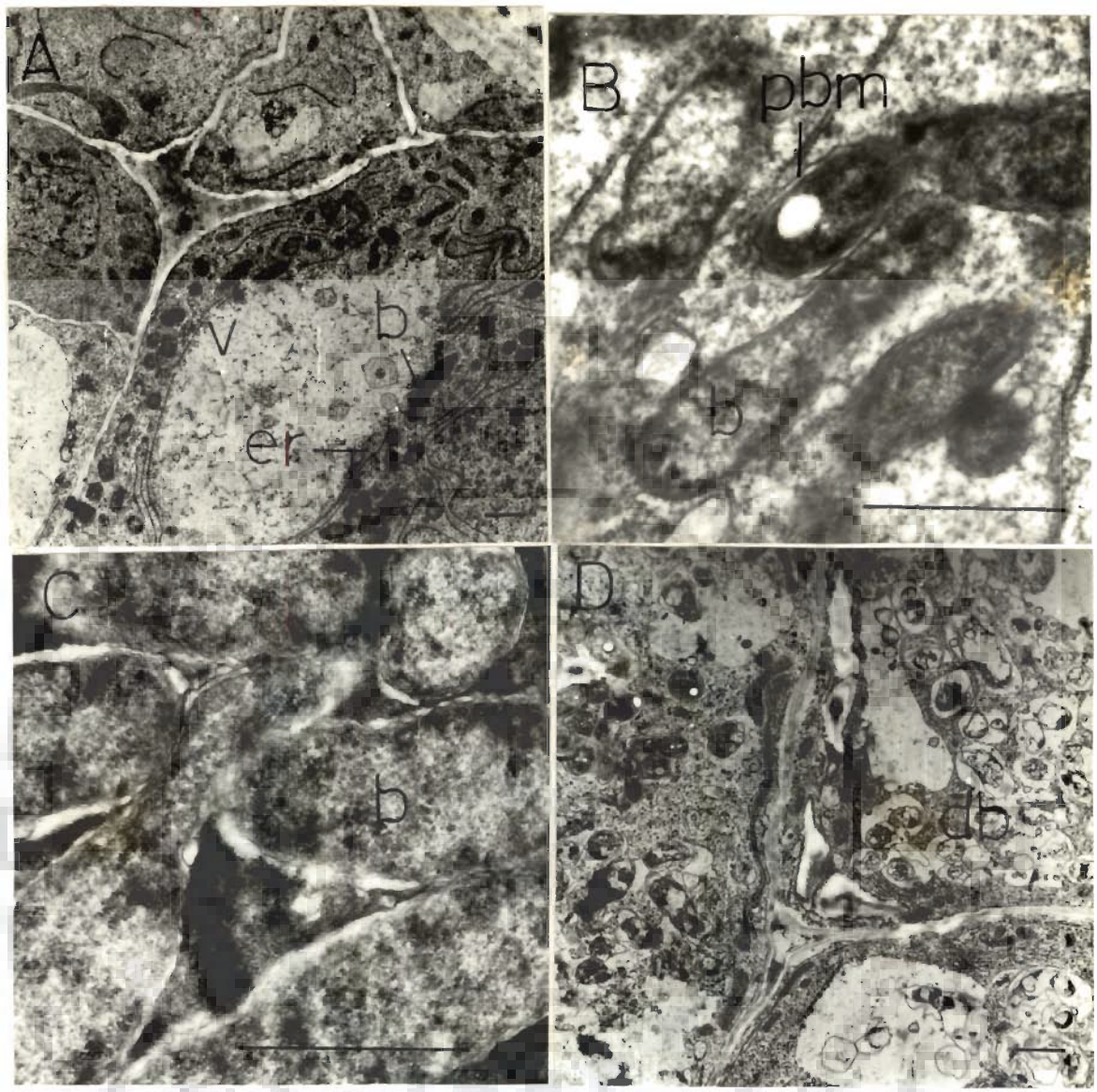

Plate 13. Transmission electron microscopic observation of longitudinal-ultrathin sections of a nodules induced by the strain NV7, a *trpD*, *trpF* or *trpC* mutant of *S. meliloti* Rmd201. **A.** Fine structure characteristics of the nodule cells of infection zone showing released bacteria (b), large number of endoplasmic reticulum (er) and the cell organelles organized around cellular vacuoles (v). Bar: 1 μm (x 4875). **B.** A part of nodule cells of late infection zone showing elongated bacteroids (b) surrounded by peribacteroid membrane (pbm). Bar: 1 μm (x 28750). **C.** Bacteroids (b) of nitrogen-fixing zone exhibited heterogenous cytoplasm with electron dense and electron transparent regions. Bar: 1μm (x 33750). **D.** A part of nodule cells of senescence zone showing deteriorated bacteroids (db). Bar: 1 μm (x 6625).

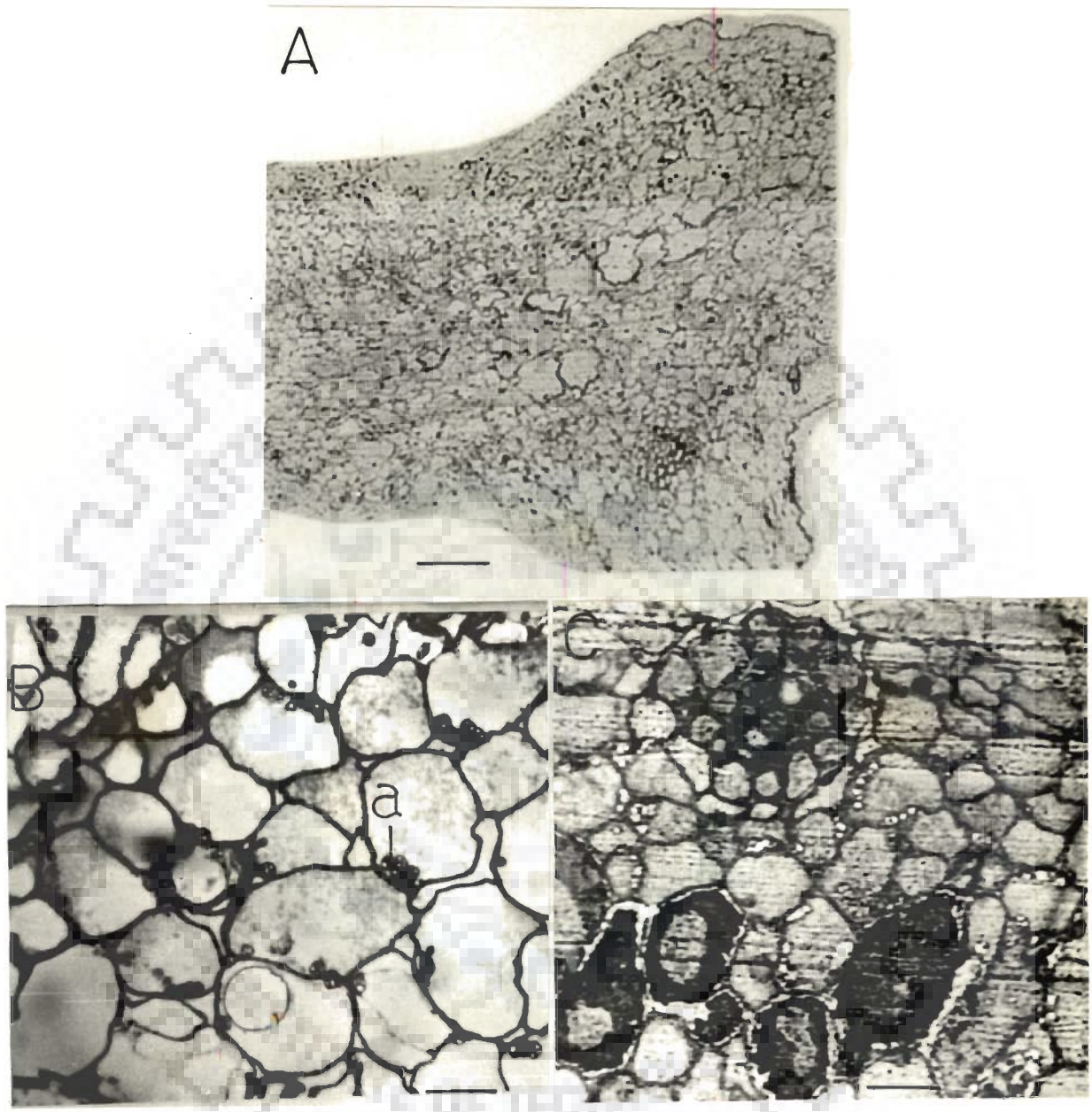

Plate 14. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN9, a *pheA* mutant of *S. meliloti* Rmd201. **A.** The whole nodule section showing no distinctive cellular zones. Bar: 100 μm (x 100). **B.** Cells of the frontal portion of nodule contained large number of amyloplasts (a). Bar: 25 μm (x 400). **C.** A few nodule cells were filled with bacteroids, while the others were devoid of bacteroids. Bar: 25 μm (x 400).

and they were observed to be in a degenerating condition after their release (Plate 15A; B). The cell organelles like mitochondira and amyloplasts were clearly observed displacing the nodule cells to periphery (Plate 15C; D).

4.6.5 *tyrA* mutant nodules

4.6.5.1 Light microscopy

Similar to the parental strain Rmd201 induced-nodules distinct zones were present in the nodules induced by the strain FN4 (a *tyrA* mutant) (Plate 16A). The meristematic zone contained uninfected, dividing cells having prominent nuclei (Plate 16B), while some cells of infection zone were seen having released bacteria and infection threads (Plate 17A). Most of the cells in the late infection and nitrogen-fixing zones were filled with bacteroids, arranged around central vacuoles (Plate 17B; C). The senescence zone which occupied the basal portion of the nodule contained deteriorated bacteroids (Plate 17D).

4.6.5.2 Transmission electron microscopy

Transmission electron microscopic observations of longitudinal-ultrathin sections of nodules induced by *tyrA* mutant showed that similar to the parental strain Rmd201 induced nodules, the nodule cells of infection and nitrogen-fixing zones were filled with bacteroids. The bacteroids in these cells were elongated having partial heterogeneous cytoplasm (Plate 18A). In the nitrogen-fixing zone cells, the bacteroids exhibited clear electron transparent cytoplasm (Plate 18B). The senescence zone cells were seen having lysed bacteroids (Plate 18C).

4.6.6 Histology of nodules induced by *trpE(G)* mutants supplemented with 2.5 μg/ml of anthranilic acid

The light microscopic observations of the longitudinal-semithin sections of nodules

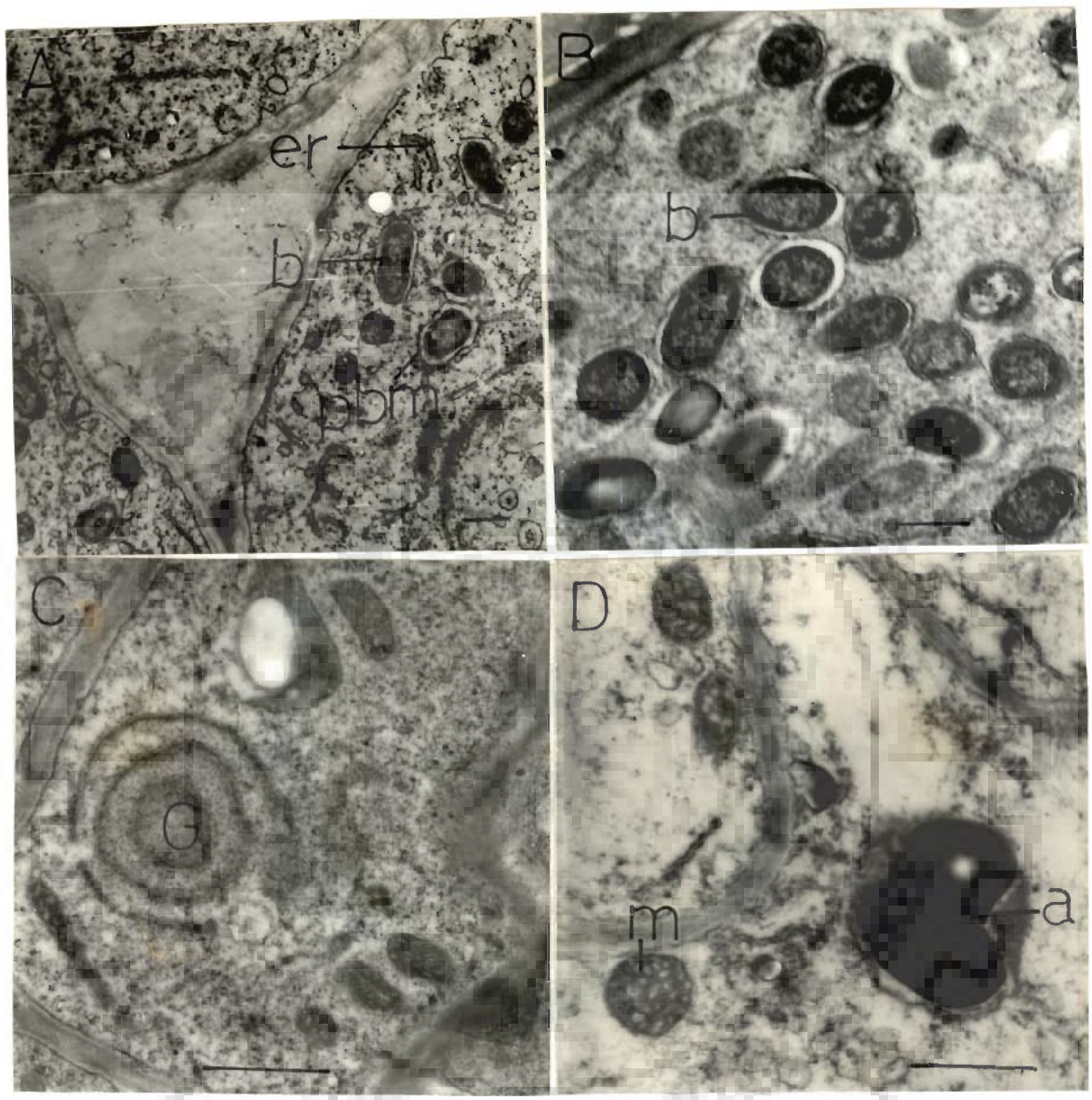

Plate 15. Transmission electron microscopic observations of longitudinal-ultrathin sections of a nodule induced by the strain FN9, a *pheA* mutant of *S. meliloti* Rmd201. **A.** A part of nodule cell of the frontal part of nodule displaying many bacteroids (b) surrounded by reptured peribacteroid membrane (pbm), the nodule cells also having mitochondria and endoplasmic reticulum (er). Bar: 1 μm (x 6625). **B.** Nodule cell containing bacteroids (b) in degenerating state. Bar: 1 μm (x 9500). **C.** A part of nodule cell showing Golgi complex (G) and mitochondria (m). Bar: 1 μm (x 17125). **D.** Uninfected nodule cells having mitochondria (m) and amyloplasts (a) Bar: 1 μm(x 18050).

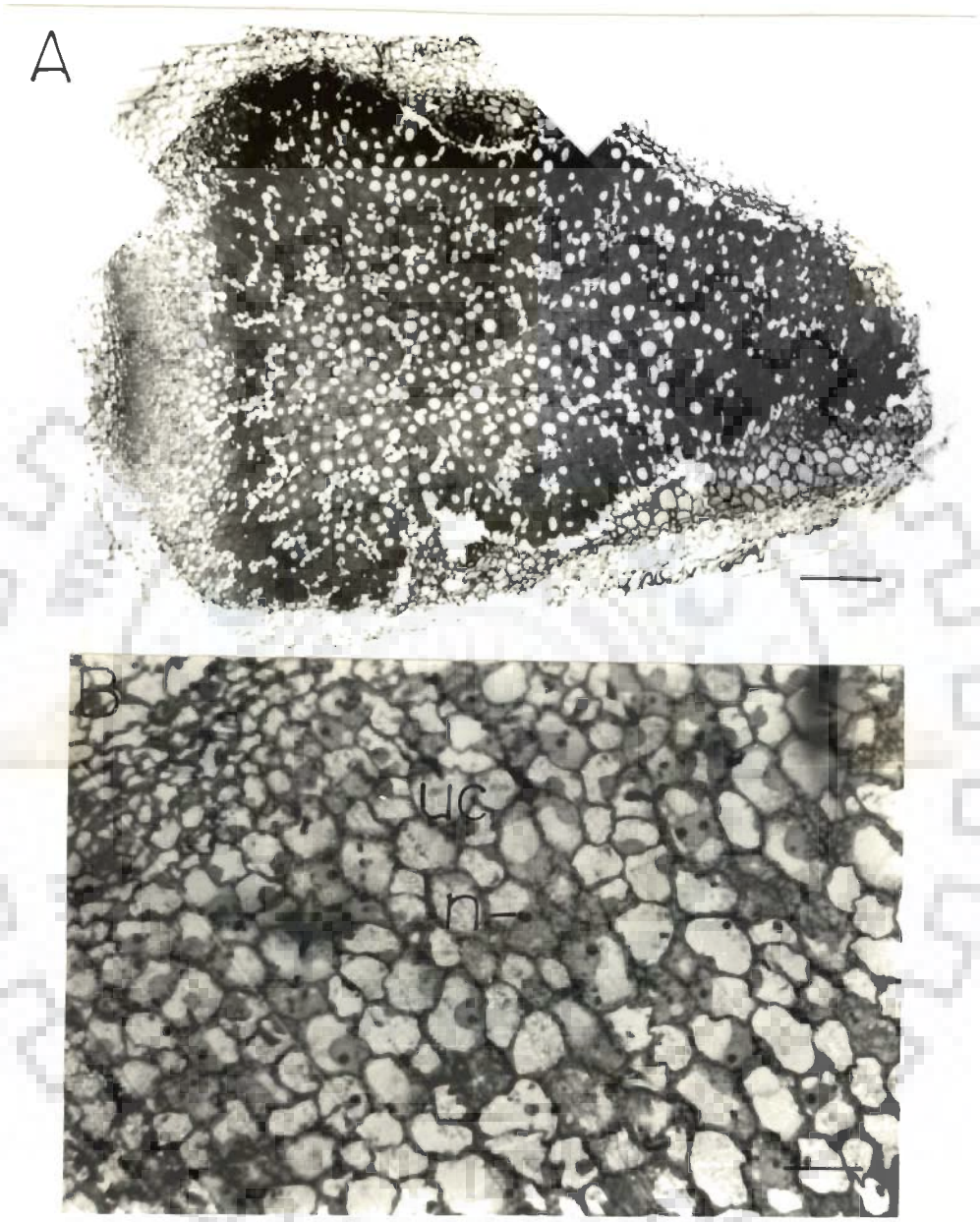

Plate 16. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN4, a *tyrA* mutant of *S. meliloti* Rmd201. **A.** The whole nodule section exhibited all nodule zones. Bar: 100 μm (x 100). **B.** Nodule cells of distal meristematic zone showing uninfected cells (uc) having prominent nuclei (n). Bar: 25 μm (x 400).

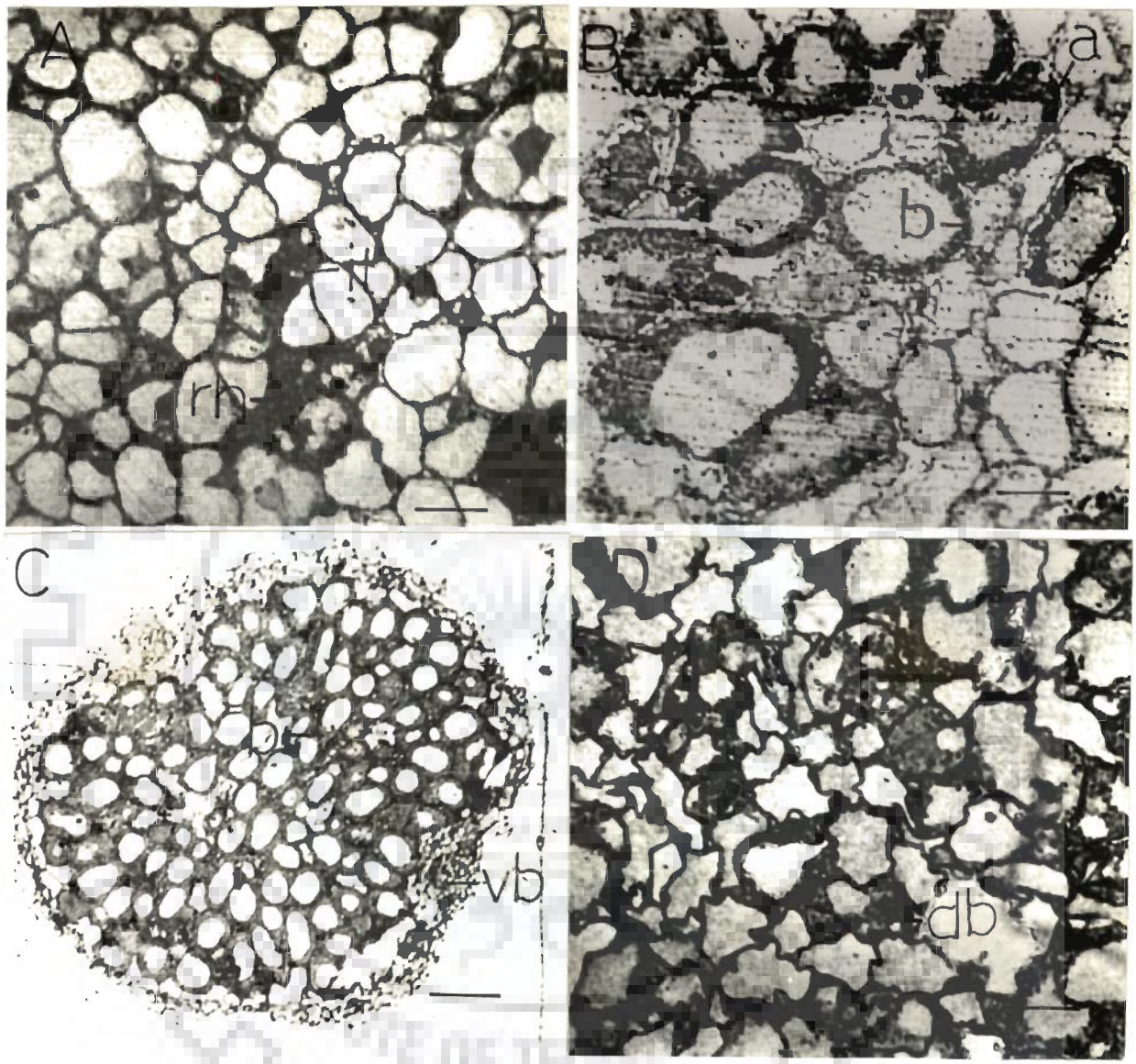

Plate 17. Light microscopic observations of semithin sections of a nodule induced by the strain FN4, a *tyrA* mutant of *S. meliloti* Rmd201. **A.** Infection zone cells showing infection thread (it) and rhizobia (rh). **B.** Interzone cells displaying large number of amyloplasts (a) and bacteroids (b). **C.** Transverse section of the whole nodule of nitrogen-fixing zone showing peripheral vascular bundles (vb) and the nodule cells were filled with bacteroids (b). Bar: 100 μm (x 100). **D.** Basal portion of the nodule displaying senescence cells having deteriorated bacteroids (db). Bar: **A**, **B**, & **D**, 25 μm (x 400).

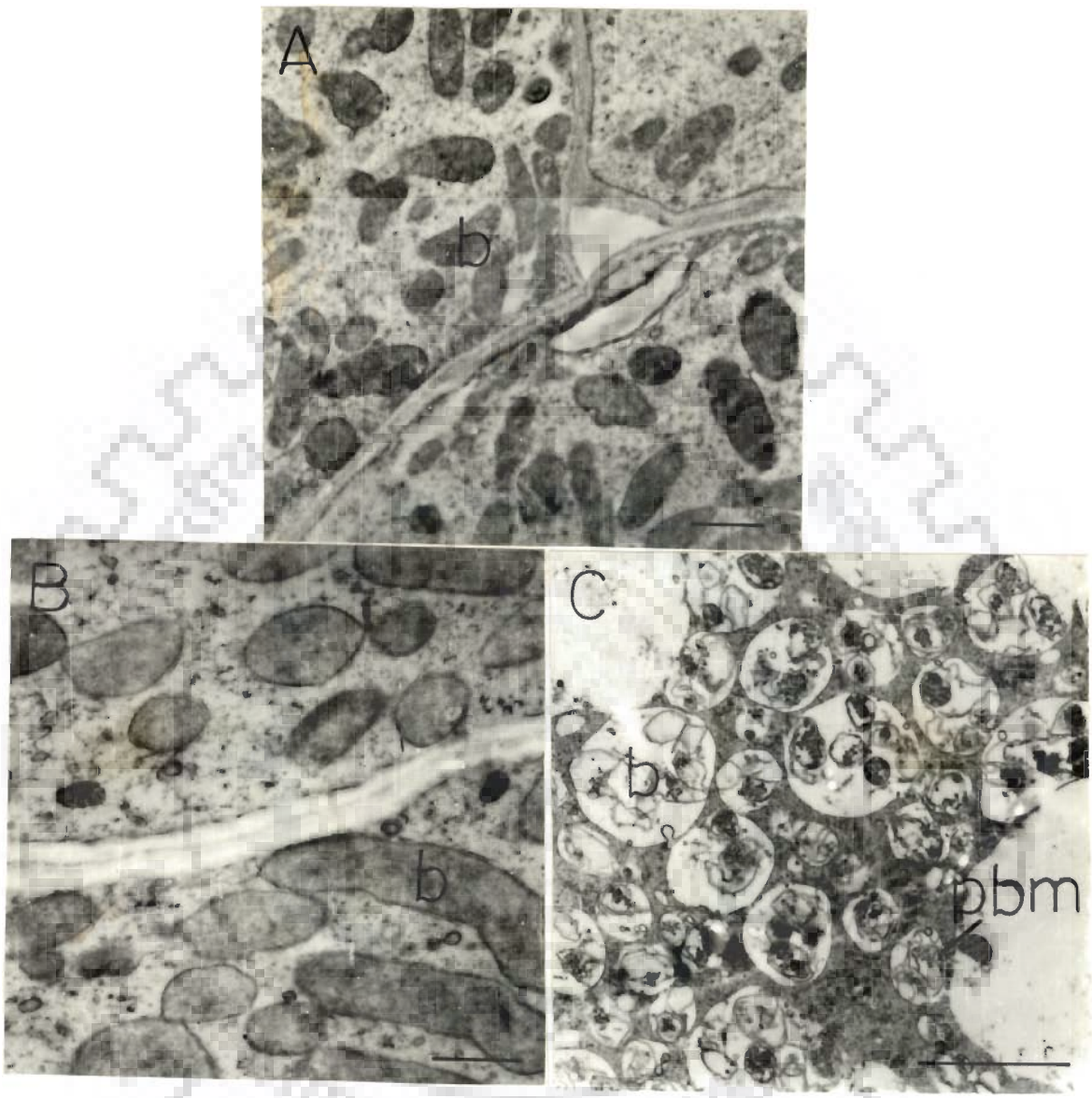

Plate 18. Transmission electron microscopic observations of longitudinal-ultrathin sections of a nodule induced by the strain FN4, a *tyrA* mutant of *S. meliloti* Rmd201. **A.** A parts of three nodule cells of nitrogen-fixing zone showing bacteroids (b) having partially heterogeneous cytoplsm. Bar: 1 μm (x 10250). **B.** A parts of nodule cells of the same zone displaying elongated bacteroids (b) having clear electron transparent cytoplasm. Bar: 1 μm (x 11790). **C.** Higher magnification of senescence zone showing lysed bacteroids (b) surrounded by reptured peribacteroid membrane (pbm). Bar: 1 μm (x 21000).

induced by *trpE(G)* mutants (FN2 and FN3), grown with 2.5 μg/ml of anthranilic acid showed normal developmental stages of the nodule zones (Plate 19A). As a representative, the microscopic observations of FN2 mutant-induced nodules are demonstrated below.

Most of the nodule cells were filled with bacteroids compared to those of FN2 mutant-induced nodules without supplementation. Large number of amyloplasts were also observed in these cells (Plate 19B). Although, most of the nodule cells of nitrogen-fixing zone were filled with bacteroids, but there was only partial restoration of the nitrogen fixation ability as indicated from the morphological features of alfalfa plants grown with 2.5 μg/ml of anthranilic acid (Plate 4). It seemed that there was still some defect in bacteroids development (differentiation and maturation). Lysed nodule cells having deteriorated bacteroids were seen in the senescence zone (Plate 19C).

Transmission electron microscopic observations of longitudinal-ultrathin sections of these nodules showed that the rhizobial release into nodule cells was normal. The released rhizobia of infection zone were rod-shape. The cell organelles were also seen in the nodule cells of this zone (Plate 20A). In most cases, the differentiation of rhizobial cells to bacteroid state was complete. Under higher magnification of nitrogen-fixing zone, some of the bacteroids were also observed in abnormal form or in degenerating state (Plate 20B; C). Perhaps the partial restoration of nitrogen-fixation ability could be merely due to the presence of abnormal bacteroids in nitrogen-fixing zone that were unable to fix nitrogen. In the senescence zone, rhizobial cells having broken peribacteroid membrane and electron transparent cytoplasm were seen (Plate 20D).

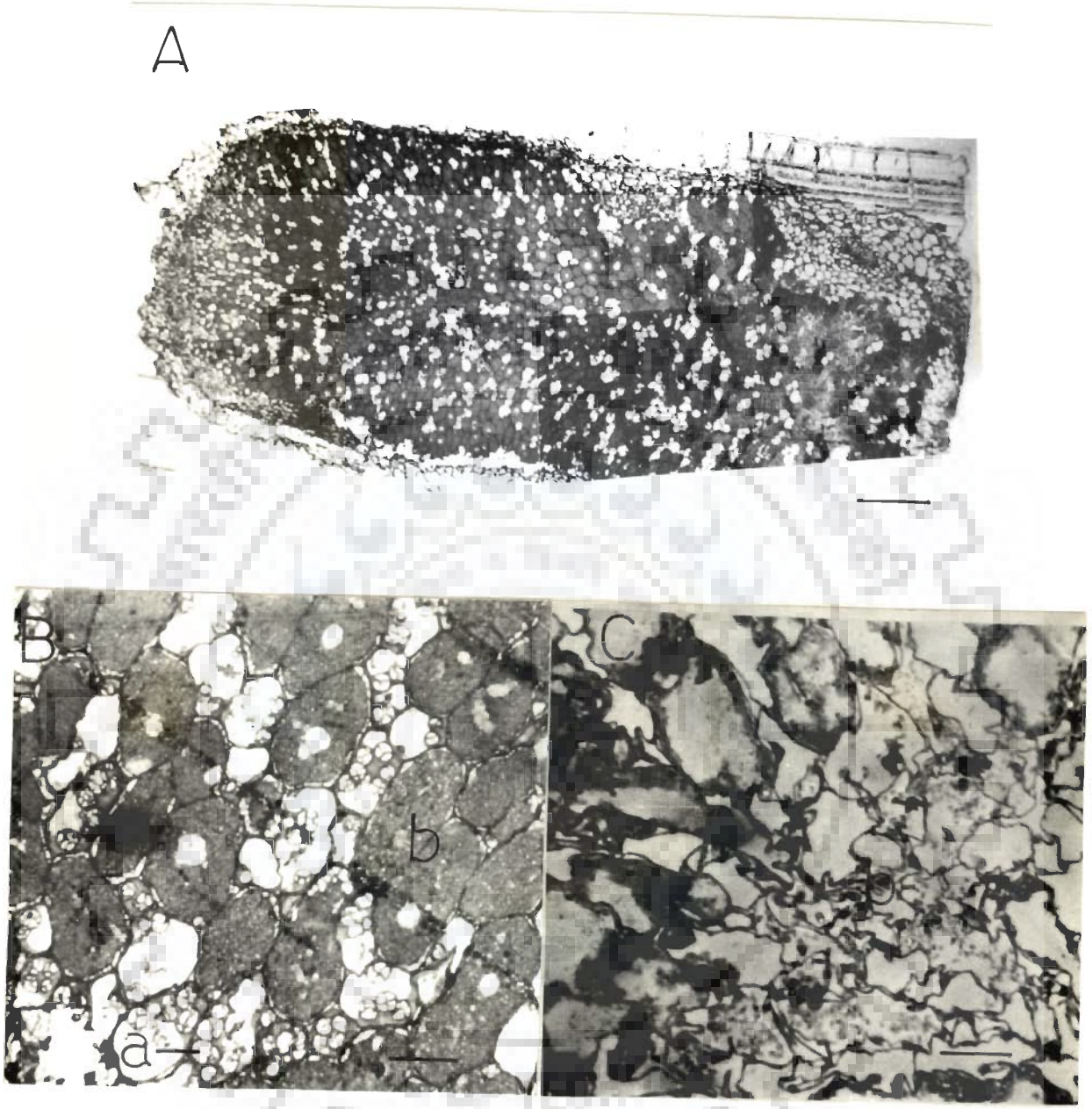

Plate 19. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant supplemented with 2.5 µg/ml of anthranilic acid. **A.** The whole nodule section showing the nodule zones. Bar: 100 µm (x 100). **B.** Nitrogen-fixing zone showing nodule cells filled with bacteroids (b) and the others studded with amyloplasts (a). **C.** Senescence zone exhibited bacteroids (b) in degenerating condition. Bars: B & C, 25 µm (x 400).

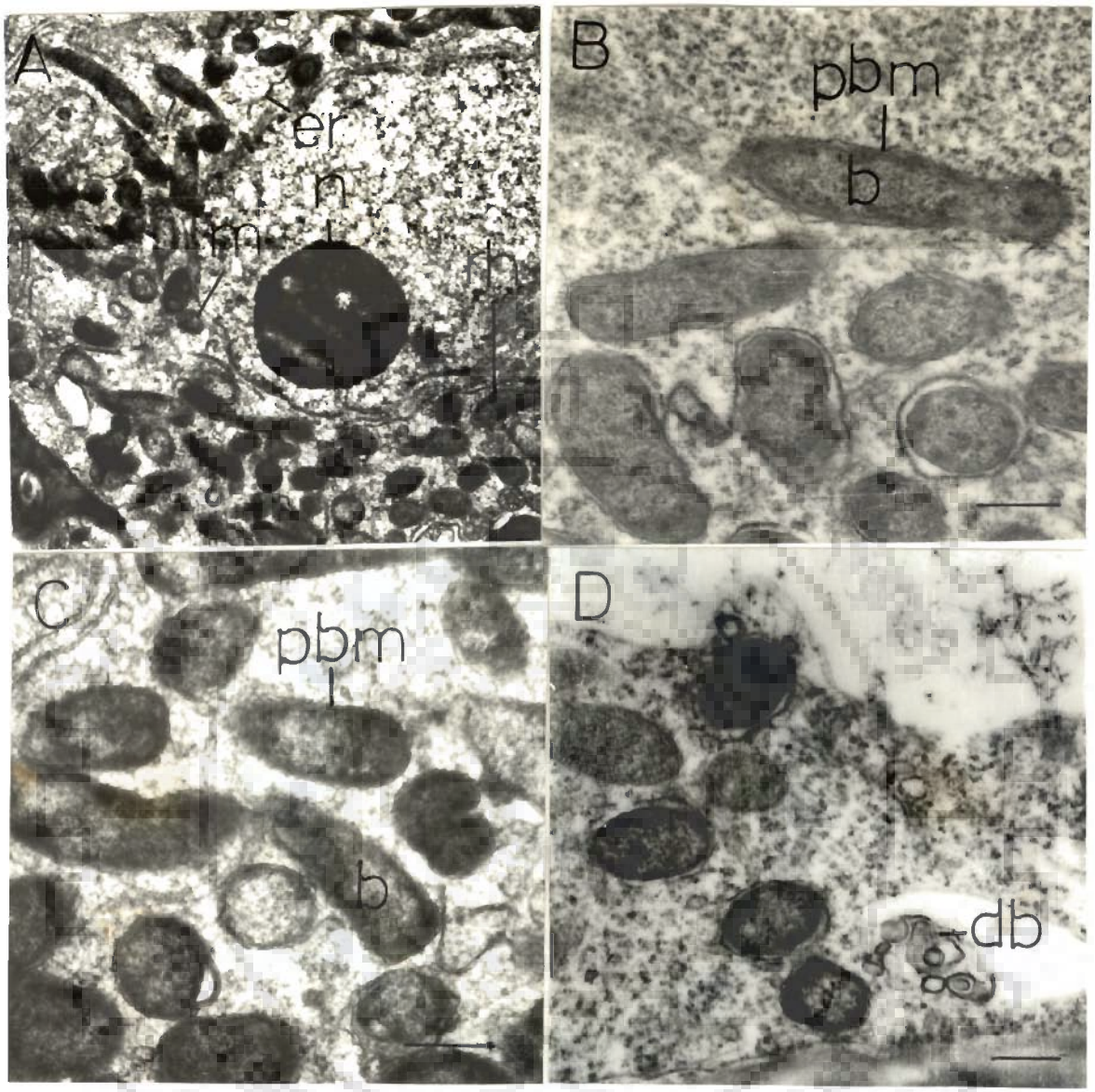

Plate 20. Transmission electron microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant supplemented with 2.5 µg/ml of anthranilic acid. **A.** A part of nodule cell of infection zone showing released rhizobia (rh), large number of endoplasmic reticulum (er), mitochondria (m) and big nucleus (n). Bar: 1µm (x 6625). **B.** A part of nodule cell of nitrogen-fixing zone showing bacteroids (b) surrounded by peribacteroid membrane (pbm). Bar: 1µm (x 12375). **C.** Bacteroids (b) of same zone having electron dense and electron transparent regions surrounded by reptured peribacteroid membrane (pbm). Bar: 1 µm (x 12000). **D.** Degenerated bacteroids (db) were observed in the nodule cells of senescence zone. Bar: 1 µm (x 9125).

4.6.7 Histology of nodules induced by *trpE(G)* mutants supplmented with 5, 7.5 or 10 μg/ml of anthranilic acid

With further increase in concentration of anthranilic acid to 5, 7.5 and 10 μg/ml, *trpE(G)* mutants (FN2 and FN3) induced fully effective nodules on the roots of alfalfa plants. The nodules induced by these mutants had similar internal histological structure. As a representative, the results of the strain FN2 grown with 5 μg/ml of anthranilic acid are presented here.

Like the parental strain Rmd201-induced nodule, the nodules induced by FN2 mutant grown with 5 μg/ml of anthranilic acid had normal distinct zones (Plate 21A). The meristematic zone was composed of uninfected cells having prominent nuclei (Plate 21B). Infection threads were visible in intercellular spaces of nodule cells along with freshly released bacteroids in infection zone (Plate 22A). Large number of bacteroids and amyloplasts were observed in the interzone cells (Plate 22B). The cells of nitrogen-fixing zone were filled with bacteroids (Plate 22C). Lysed bacteroids were observed in the senescence zone (Plate 22D).

It was clear from the transmission electron microscopic observations that the nodule cells of infection zone were filled with large number of freshly released bacteroids beside other cell organelles (Plate 23A). Differentiated bacteroids having distinct peribacteroid membrane and electron transparent patches in the electron dense cytoplasm were seen in the nodule cells of nitrogen-fixing zone (Plate 23B; C). Deteriorated rhizobial bacteria were seen in the nodule cells of senescence zone (Plate 23D).

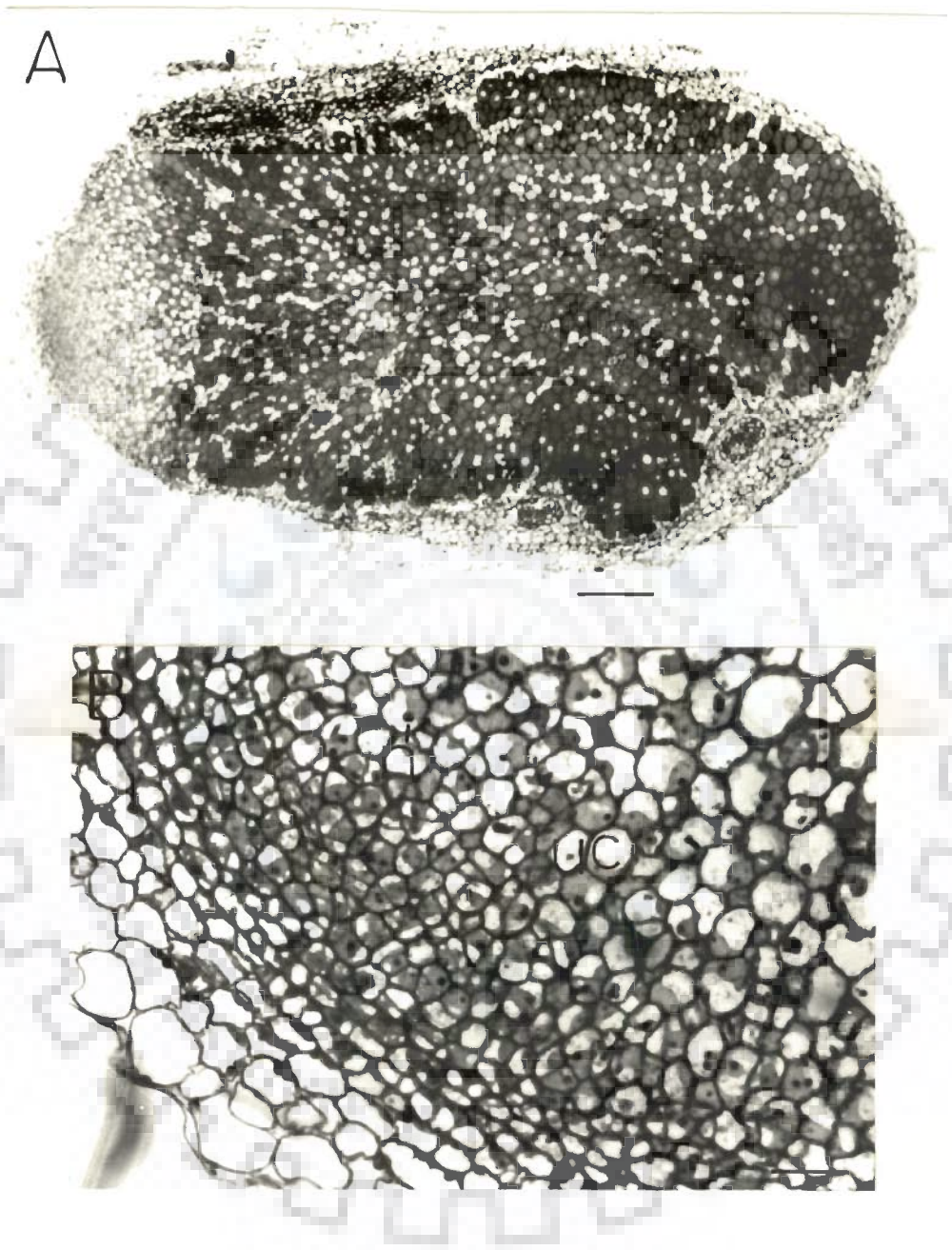

Plate 21. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant supplemented with 5 µg/ml of anthranilic acid. **A.** The whole nodule section showing normal development of the nodule zones. Bar: 100 µm (x 100). **B.** Meristematic zone cells showing uninfected cells (uc) having prominent nuclei (n). Bar: 25 µm (x 400).

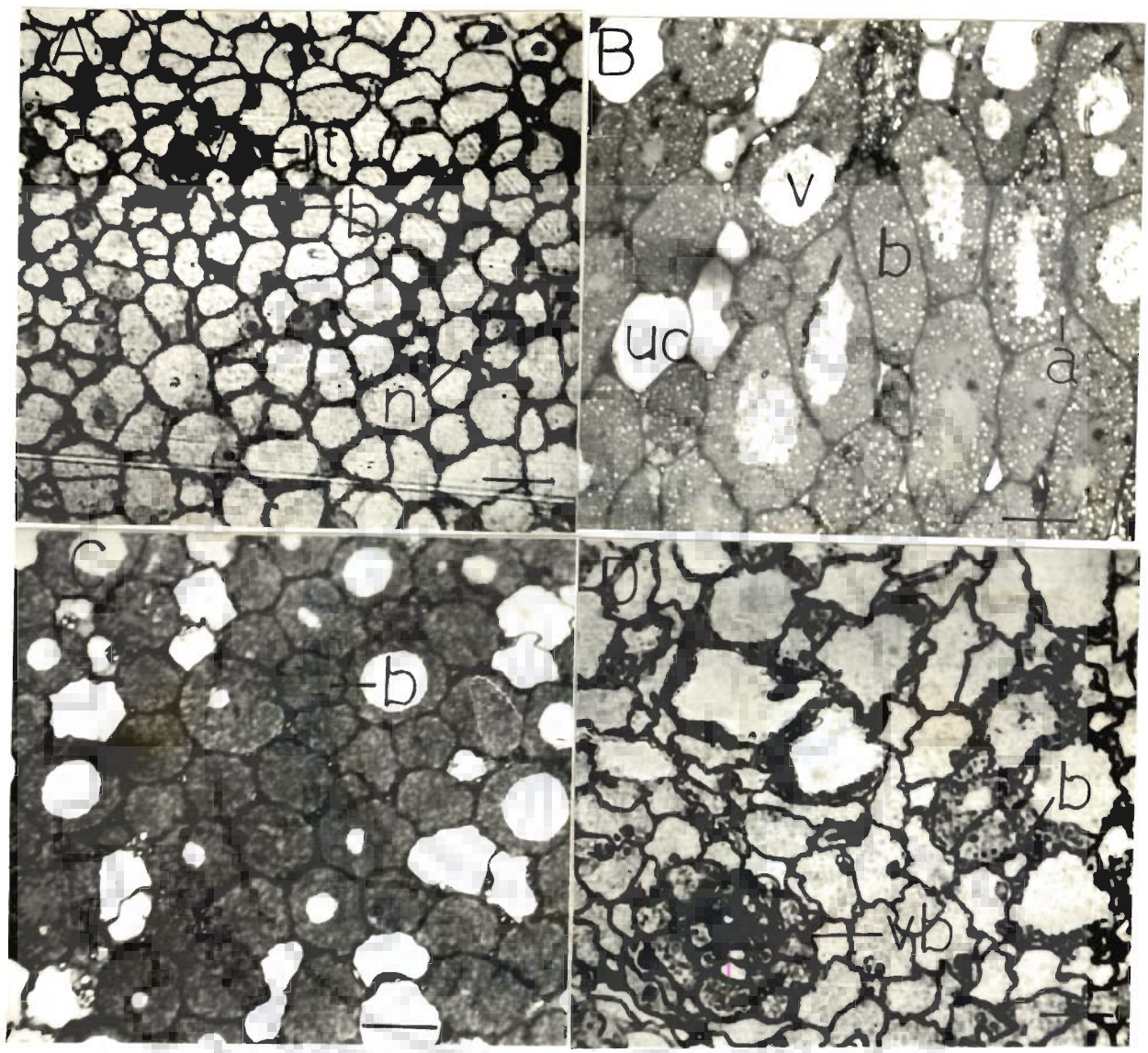

Plate 22. Light microscopic observations of longitudinal-semithin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant supplemented with 5 µg/ml of anthranilic acid. **A.** Nodule cells of infection zone showing infection threads (it), bacteroids (b), prominent nuclei (n) and vacuoles (v). **B.** Interzone cells showing uninfected cells (uc), the amyloplasts (a) and bacteroids (b) arranged around central vacuoles (v). C. Nitrogen-fixing zone cells filled with bacteroids (b). **D.** Senescence zone displaying bacteroids (b) in degenerating state and peripheral vascular bundle (vb). Bars: **A**, **B**, **C** & **D**, 25 µm (x 400).

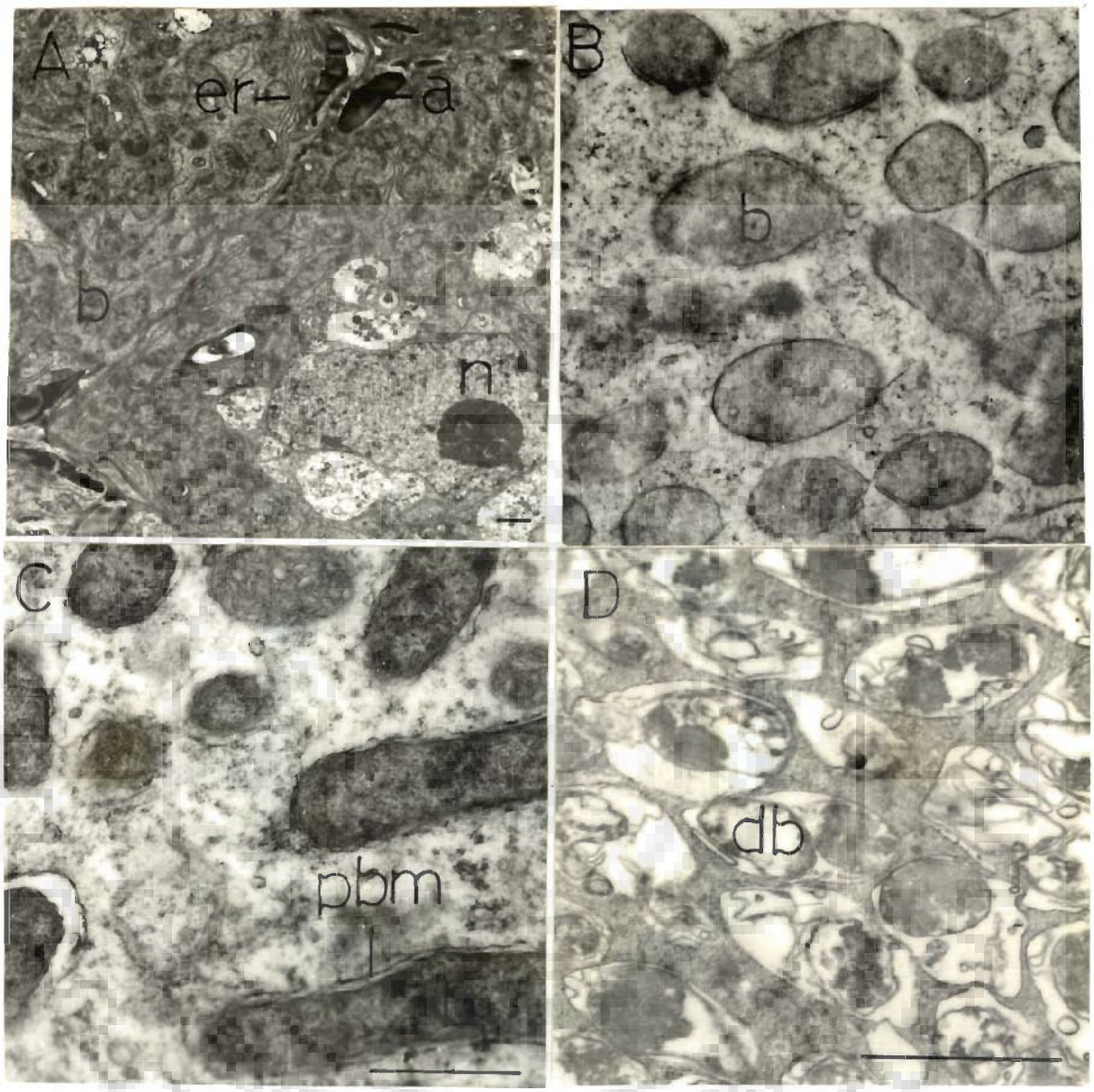

Plate 23. Transmission electron microscopic observations of longitudinal-ultrathin sections of a nodule induced by the strain FN2, a *trpE(G)* mutant supplemented with 5 µg/ml of anthranilic acid. **A.** Infection zone cells showing freshly released bacteroids (b), big nucleus (n), endoplasmic reticulum (er) and amyloplasts (a). Bar: 1 µm (x 3875). **B.** Differentiated bacteroids (b) of nitrogen-fixing zone. Bar: 1µm (x 15750). **C.** Higher magnification of a part of nodule cell of the same zone showing mature bacteroids (b) surrounded by distinct peribacteroid membrane (pbm). Bar: 1µm (x 20600). **D.** A part of nodule cell of senescence zone showing deteriorated bacteroids (db). Bar: 1µm (x 28250).

4.7 Iron uptake by aromatic amino acid auxotrophs of *S. meliloti* Rmd201

Since the anthranilic acid supplementation restored the symbiotic ability of *trpE(G)* mutants, therefore, an attempt was made to determine the role of anthranilic acid in facilitating iron uptake. Mimimal medium having iron, supplemented with minimal nutritional requirements of the auxotrophs was inoculated with various rhizobial strains. The iron uptake by these strains was measured by atomic absorption spectrometer and the results are demonstrated in Fig. 6. It was shown that the amount of iron taken up by *aro* and *trpE(G)* mutants were close to each other and less than the amount taken up by the anthranilic acid-producing mutants. These amounts ranged between 0.391 μg/ml (13.75%) for the strain FN3, a *trpE(G)* mutant and 0.523 μg/ml (18.39%) for the strain BA2, an *aro* mutant. While the amount of iron uptake by other aromatic amino acid auxotrophs ranged between 1.082 μg/ml (38.05%) of strain FN9, a *pheA* mutant and 1.261 μg/ml (44.35%) for the strain BA6, a *trpB* mutant. The parental strain Rmd201 took up 1.384 μg/ml (48.68%) of total iron (2.843 μg/ml) from minimal medium.

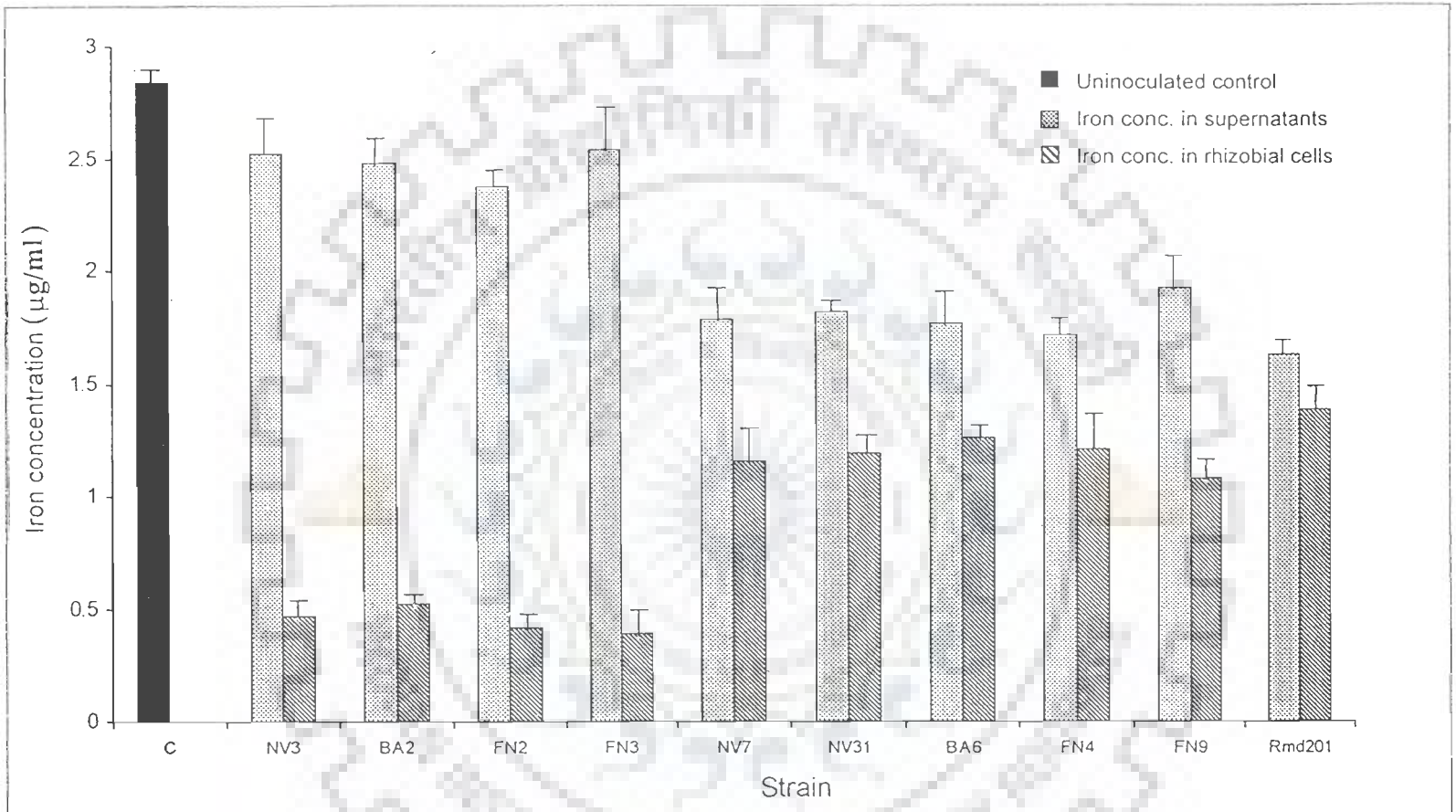

Fig. 6. Iron uptake by aromatic amino acid auxotrophs of *S. meliloti* Rmd201

Chapter 5

DISCUSSION

Since transposon Tn5 mutagenensis has certain advantages over the chemical and physical mutagenesis due to the selectable resistance phenotype, high frequency of transposition and degree of randomness in insertion specificity, therefore, it has been employed for generation of auxotrophic mutants of the aromatic amino acid biosynthetic pathways of *S. meliloti* Rmd201. From 85 crosses, total 5000 kanamycin resistant transconjugants were generated. The transconjugants were detected at a frequency of 3.2×10^{-5} per recipient that was many times greater than the rate of spontaneous mutation frequency ($<10^{-8}$). The frequency of occurrence of auxotrophs among the Tn5 derivatives was 0.44%. Somewhat similar frequencies of Tn5 transposition and occurrence of auxotrophs among the Tn5 derivatives in rhizobia have been reported in earlier studies (Meade *et al.*, 1982; Ali *et al.*, 1984; Hom *et al.*, 1984; Kim *et al.*, 1988; Kummer and Kuykendall, 1989; Saha and Singh, 1999). The diverse nutritional requirements of the isolated auxotrophs indicated the relative randomness of Tn5 insertions with the fact that these mutations are a result of a single Tn5 insertion in one gene. The sensitivity of isolated Tn5 insertion mutants to chloramphenicol indirectly indicated the loss of the suicide plasmid (pGS9) inside the cells of *S. meliloti* Rmd201.

The Tn5 insertion into the genome of the aromatic amino acid auxotrophs was found to be stable as revealed from the reversion analysis. The kanamycin sensitive prototrophic revertants of auxotrophs were detected at frequencies of 10^{-8} to 10^{-9}. Similar frequencies were reported for *R. leguminosarum* (Pain, 1979), *S. meliloti* (Meade *et al.*, 1982; Ali *et al.*, 1984), *B. japonicum* (Home *et al.*, 1984) and *S. fredii* HH303 (Kim *et al.*, 1988). It is suggested that the sensitivity of prototrophic revertants

to kanamycin was due to the excision of the Tn5 from the aromatic amino acid biosynthetic genes of the auxotrophs.

The inability of the NV3 and BA2 mutants to grow on RMM supplemented with shikimic acid and the growth on RMM having all three aromatic amino acids, viz., tryptophan, tyrosine and phenylalanine were identified as *aro* biosynthesis defective mutants and the mutations in these were between chorismic acid and shikimic acid most probably in *aroK*, *aroL*, *aroA* or *aroC* gene. The lack of cross feeding between NV3 and BA2 reiterated that they belong to the same group, either in same or different locations but not in subsequent locations.

The growth of the FN2 and FN3 mutants on RMM supplemented with anthranilic acid, indole or tryptophan and the lack of accumulation of the intermediates of tryptophan biosynthetic pathway, when grown with growth-limiting concentrations of tryptophan in RMM, indicated that these mutants had Tn5 insertion in *trpE(G)* gene. Thus, they had block at the first step of tryptophan biosynthetic pathway and are deficient in the *trpE(G)* gene product, anthranilate synthase. The mutants NV7 and NV31 showed growth on RMM supplemented with indole or tryptophan and accumulated anthranilic acid when grown with growth-limiting concentrations of tryptophan. Thus, the genes affected in these mutants could be *trpD*, *trpF* or *trpC*. However, their precise locations could not be determined by the methods used in this study due to commercial unavailability of the intermediates, viz., 5-phosphoribosyl anthranilate and 1-(o-carboxy phenyl amino)-1-deoxyribulose-5-phosphate. The mutant BA6 showed growth on anthranilic acid or indole supplemented RMM, and accumulated anthranilic acid and indole-3-glycerol phosphate under tryptophan-

limiting growth condition. This mutant was expected to accumulate indole as it did not grow on indole supplemented RMM, but indole could not be detected in RMM in which they were grown. This could be due to the too low amount of indole which could not be detected by the method used in this study. In enteric bacteria, the *trpB* gene product (the β subunit) together with the *trpA* gene product (the α subunit) forms the tryptophan synthase complex (Moat and Foster, 1995; Pittard, 1996; Xiu *et al.*, 1997). Therefore, it is likely that the mutant BA6 may be deficient in *trpB* gene product, the last step of tryptophan biosynthetic pathway. The cross-feeding experiments confirmed the location of metabolic block in the tryptophan biosynthetic pathway of each *trp* mutant. Since the mutants NV7 and NV31 produce anthranilic acid, they cross-fed FN2 and FN3 mutants. As BA6 mutant secrete anthranilic acid and indole-3-glycerol phosphate, it cross-fed FN2, FN3, NV7 and NV31 mutants. The lack of cross-feeding between FN2 and FN3 mutants and also between NV7 and NV31 mutants indicated that FN2 and FN3 mutants, and also NV7 and NV31 mutants belong to the same group, respectively.

The growth of the FN4 and FN9 mutants on RMM supplemented with tyrosine and phenylalanine, respectively, were identified as *tyrA* and *pheA* biosynthesis defective mutants, respectively. A single mutation in *tyrB* gene results in double auxotrophic requirement of both tyrosine and phenylalanine. Neither *tyrA* nor *pheA* mutants could cross-feed any other auxotrophs, indicating that these auxotrophs belong to different groups.

The symbiotic properties of the rhizobial strains revealed that the parental strain

Rmd201 and the aromatic amino acid auxotrophs were normal in elicitation of infection phenotypes on alfalfa root hairs and formation of nodules. These observations indicated that the strains were able to produce and release flavonoid-inducible lipochitin oligosaccharides (Nod factors), that are essential for recognition of the symbiotic partner, infection and formation of nodules. It has been reported in earlier studies that the LCO from *S. meliloti*, *R. leguminosarum* bv. *viciae* and *B. elkanii* induced nodule structures on the roots of their respective plant hosts (Truchet *et al.*, 1991; Spaink *et al.*, 1991; Stokkermans and Peters, 1994).

The morphological characteristics showed that the alfalfa plants inoculated with *aro*, *trpE(G)* and *pheA* mutants had symbiotic deficiencies compared to the parental strain Rmd201 inoculated plants. These mutants induced ineffective nodules on the roots of alfalfa plants that were unable to fix nitrogen. Thus, it is clear that some intermediates or end products of aromatic amino acid biosynthetic pathways play a role in symbiotic process. With the exception of the *trpE(G)* mutants, the other tryptophan mutants and tyrosine auxotroph elicited fully effective nodules on the roots of alfalfa plants. The symbiotic properties of these mutants resembled those of the parental strain Rmd201 in all respects. However, Prasad *et al.* (2000) reported that a leaky mutant of *S. meliloti* Rmd201 having mutation in *trpD*, *trpF* or *trpC* gene, was partially effective in nitrogen fixation. The histology of the nodules induced by this strain was normal but the inoculated plants were stunted. It is quite likely that tryptophan, but not anthranilic acid, and tyrosine, but not phenylalanine, are provided by the root cells to rhizobial bacteria located inside the nodules of alfalfa plants.

The characteristics such as production of cell surface carbohydrate molecules (β-glucans, cellulose fibrils, lipopolysaccharides and succinylated exopolysaccharides), utilization of carbon sources (C_4-dicarboxylic acids and sugars) and production of cytochrome c oxidase of auxotrophs were similar to those of the parental strain Rmd201. These results indicated that the symbiotic defects of *aro*, *trpE(G)* and *pheA* mutants were not due to the change in any of the above mentioned characteristics. Thus, it is likely that the defective symbiosis of these auxotrophs could be merely due to the loss of biosynthetic gene functions through Tn5 insertion into *aro*, *trpE(G)* and *pheA* genes.

The supplementation studies showed that the addition of aromatic amino acids and their intermediates beyond certain concentrations had adverse effects on the growth of roots of alfalfa plants (increased number of secondary roots and abnormal elongation of the root hairs). However, the concentrations below these levels were found to have no adverse effects on root and shoot developments and were used for further studies. It has also been reported by Taté *et al.* (1999b) that the addition of tryptophan (from 5 to 20 μg/ml) affects the root growth of bean and alters the distribution of root hair lengths, but such phenotypic changes were not observed with other amino acids such as cysteine, methionine or arginine (Taté *et al.*, 1997 & 1999C). The adverse effects of the addition of aromatic amino acids or their intermediates over optimal concentrations, may be due to alteration in the phytohormone homeostasis (biosynthesis/degradation) of growing alfalfa roots. Previously, it has been observed that the tryptophan is the biosynthetic precursor of phytohormone auxin (indole-3-acetic acid) in the axes of

germinating seedlings of *P. vulgaris* (Bialek *et al.*, 1992). Furthermore, it is also interesting to note that the anthranilate represents the first step towards the synthesis of an indole ring and is a regulator of root development, stimulating both the formation of lateral root primordia and the root hair elongation (Schiefelbein *et al.* 1997).

Restoration of the symbiotic ability of *trpE(G)* mutants on supplementation of plant growth medium with anthranilic acid suggests that it plays an important role in symbiosis, most probably by facilitating iron uptake. This is supported by the fact that less iron uptake occurs by the *aro* and *trpE(G)* mutants compared to anthranilic acid producing mutants and the observation that *aro* mutants resembled the *trpE(G)* mutants rather than the *pheA* mutant in their symbiotic deficiencies. Hence, an undiminished metabolic flow of anthranilic acid through tryptophan biosynthetic pathway, appears to be essential for an effective nodule development and functional nitrogen-fixing symbiosis. On the other hand, there was no restoration of the symbiotic ability of *trpE(G)*, *pheA* and *aro* mutants on supplementation of plant growth medium with indole or tryptophan, phenylalanine and all aromatic amino acids, respectively. This could be either due to the failure of the rhizobial mutants located inside the cells of nodules, to utilize these compounds from plant growth medium or in case of *aro* mutants, also due to failure to synthesize anthranilic acid, since their metabolic blocks are prior to chorismic acid. This supports that anthranilic acid has a role in symbiosis.

Light microscopic observations of unusual defective nodules induced by the *trpE(G)* mutants (FN2 and FN3) showed that the infection zone was long, extending upto half of the middle nodule tissues, while the poorly developed nitrogen-fixing zone occupied only the basal part of the nodules. Ultrastructural observations of transmission

electron microscope revealed that the bacteria in the extended infection zone as well as in the amyloplast-rich interzone were not able to differentiate into functional bacteroids. The various stages of early development are present but the bacteroids were unable to achieve the maturity as seen in the parental strain Rmd201. Furthermore, some bacteroids located in young invaded nodule cells and appeared irregular in shape, are thinner than the parental strain type bacteroids and apparently undergo premature senescence. This suggested that the bacteroid differentiation process is delayed or not completed. These bacteroids exhibited very less nucleic acid condensation as compared to that in the parental strain Rmd201-induced nodules. It seems that the absence of some factor halted bacterial growth, division as well as maturation process in young invaded nodule cells. However, some nodule cells were seen having few bacteroids in nitrogen-fixing zones which might be responsible for the trace amount of fixed nitrogen. It is concluded that anthranilic acid or some factor derived from it has a role in nodule development in *S. meliloti*. Based on earlier reports, anthranilic acid has been shown to function as siderophore for *R. leguminosarum* (Rioux *et al.*, 1986a & b). Siderophore production has been reported for strains belonging to *B. japonicum* (Modi *et al.*, 1985; Guerinot *et al.*, 1990), *R. leguminosarum* biovars *phaseoli* (Carillo and Peralta, 1988), *trifolii* (Ames-Gottfred *et al.*, 1989; Skorupska *et al.*, 1989) and *viciae* (Yeoman *et al.*, 1999), *S. meliloti* (Reigh and O'Connell, 1993) and *R. ciceri* (Suneja *et al.*, 2000). The iron-containing enzymes and proteins involved in nitrogen fixation include nitrogenase complex, ferredoxin, cytochromes and leghaemoglobin. Nitrogenase complex and leghaemoglobin constituted upto 12 and 30% of total soluble protein in the bacterial and infected-plant cells, respectively (Verma and Long, 1983;

Guerinot, 1991). Therefore, there is high demand for iron during the bacteroid development because of the need to synthesize the above enzymes and proteins necessary for nitrogen fixation. Since, the iron uptake studies showed that the *trpE(G)* mutants took up less amount of iron from minimal medium as compared to the anthranilic acid-producing mutants, hence, it is quite likely that the symbiotic defect of these mutants could be merely due to non-production of anthranilic acid which leads to unavailability of the iron necessary for differentiation of bacteroids. The restoration of the symbiotic ability of *trpE(G)* mutants on supplementation of plant growth medium with anthranilic acid confirmed this fact. It has previously been reported that iron deficiency specifically limits nodule development in the soybean-*Bradyrhizobium* sp. symbiosis (O'Hara *et al.*, 1988). There are evidences which confirm the presence of an iron transport system in *R. leguminosarum* bacteroid (Nadler *et al.*, 1990) and *S. meliloti* 1021 which significantly increase the ability of the differentiated bacterium to fix nitrogen and induce an increase in plant growth (Gill *et al.*, 1991). In addition, iron is also found to play regulatory role, since it is involved in the oxygen-sensitive expression of the nitrogen fixation regulatory protein NifA and required for respiratory electron transport chain (Fischer, 1994). Our results further support the hypothesis suggested by Barsomian *et al.* (1992) that the anthranilic acid may be acting as an *in planta* siderophore, facilitating the iron uptake required for the development of bacteroids and in the absence of this factor the bacteroids fail to differentiate.

In case of *aro* mutants (NV3 and BA2) which also induced nodules with similar histological defects of *trpE(G)* mutants, it is likely that the anthranilic acid unavailability resulted in extended infection and poorly develped nitrogen-fixing zones.

Similar explanation for extended invasion zones in the nodules induced by 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase mutants has been proposed (Jelesko *et al.*, 1993). In these mutants the metabolic flow through the aromatic amino acid biosynthetic pathways was less. It is not known why the nodules induced by the *aro* mutants did not show histological aspects similar to that of *pheA* mutant (FN9)-induced nodules where the defect was severe and the bacteria lysed after their release from infection threads without proceeding to any of the committed differentiation stages. Similar observations have been made in an earlier study (Prasad *et al.*, 2000). It has been suggested that the plant host may be provided one or more intermediates (most probably prephenate) of phenylalanine from which the *aro* mutants could synthesize phenylalanine, whereas, the *pheA* mutant could not do so due to the position of mutation in the biosynthetic pathway. Hence, the defects of *pheA* mutant could be merely due to the unavailability of phenylalanine to this mutant. These observations indicated that the bacterial nutrient(s) are specific for each symbiotic relationship, as previously postulated (Schubert, 1986).

Thus, it is concluded that the functions of *aro, trpE(G)* and *pheA* genes involved in aromatic amino acid biosynthetic pathways of *S. meliloti* are required for functional nitrogen-fixing symbiosis. Anthranilic acid or its derivatives in particular were found to play significant roles in development (differentiation and maturation) of bacteroids by facilitating iron uptake, most probably acting as siderophore. However, still there are many gaps in our knowledge about its actual role which needs further investigations.

References

Aguilar, J.M.M.; Ashby, A.M.; Richards, A.J.M.; Loake, G.J.; Watson, M.D. and Shaw, C.H. 1988. Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes. J. Gen. Microbiol. *134*:2741-2746.

Aguilar, O.M. and Grasso, D.H. 1991. The product of the *Rhizobium meliloti ilvC* gene is required for isoleucine and valine synthesis and nodulation of alfalfa. J. Bacteriol. *173*:7756-7764.

Ali, H.; Béchet, M.; Niel, C. and Guillaume, J.-B. 1984. *Rhizobium meliloti* Tn5 induced auxotrophic mutants responding to different biosynthetic intermediates. Can. J. Microbiol. *30*:507-511.

Amarger, N.; Macheret, V. and Laguerre, G. 1997. *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int. J. Syst. Bacteriol. *47*:996-1006.

American Public Health Association. 1998. Standard methods for the examination of water and wastewater. 20th ed. American Public Health Association, pp:3-8.

Ames-Gottfred, N.P.; Christie, B.R. and Jordan, D.C. 1989. Use of the chrome azurol S agar plate technique to differentiate strains and field isolates of *Rhizobium leguminosarum* biovar *trifolii*. Appl. Environ. Microbiol. *55*:707-710.

Aoki, K.; Shinke, R. and Nishira, H. 1984. Anthranilic acid production from aniline by *Rhodococcus erythropolis* AN-13. Agric. Biol. Chem. *48*:2309-2313.

Arnold, W.; Rump, A.; Klipp, W.; Periefer, V.B. and Pühler, A. 1988. Nucleotide sequence of a 24,206 base pair DNA fragment carrying entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. J. Mol. Biol. *203*:715-738.

Arwas, R.; Mckay, I.A.; Rowney, F.R.P.; Dilworth, M.J. and Glenn, A.R. 1985. Properties of organic acid utilization mutants of *Rhizobium leguminosarum* strain 300. J. Gen. Microbiol. *131*:2059-2066.

Ausubel, F.M. 1982. Molecular genetics of symbiotic nitrogen fixation. Cell *29*:1-2.

Bae, Y.M.; Holmgren, E. and Crawford, I.P. 1989. *Rhizobium meliloti* anthranilate synthase gene: Cloning, sequence, and expression in *Escherichia coli*. J. Bacteriol. *171*:3471-3478.

Baev, N.; Schultze, M.; Barlier, I.; Ha, D.C.; Virelizier, H.; Kondorosi, É. and Kondorosi, Á. 1992. *Rhizobium nodM* and *nodN* genes are common *nod* genes: *nodM* encodes functions for efficiency of Nod signal production and bacteroid maturation. J. Bacteriol. *174*:7555-7565.

Bailey, N.T.J. 1995. Statistical methods in biology. 3rd ed. Cambridge University Press, Cambridge, pp:50-60.

Banfalvi, Z.; Kondorosi, É., Kondorosi, Á. 1985. *Rhizobium meliloti* carries two megaplasmids. Plasmid *13*:129-138.

Banfalvi, Z.; Sakanyan, V.; Koncz, C.; Kiss, A.; Dusha, I. and Kondorosi, Á. 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol. Gen. Genet. *184*:318-325.

Barbour, W.M.; Hattermann, D.R. and Stacey, G. 1991. Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates. Appl. Environ. Microbiol. *57*:2635-2639.

Bardin, S.; Dan, S.; Osteras, M. and Finan, T.M. 1996. A phosphate transport system is required for symbiotic nitrogen fixation by *Rhizobium meliloti*. J. Bacteriol. *178*:4540-4547.

Barnett, M.J. and Long, S.R. 1997. Identification and characterization of a gene on *Rhizobium meliloti* pSyma, *syrB*, that negatively affects *syrM* expression. Mol. Plant-Microbe Interact. *10*:550-559.

Barsomian, G.D.; Urzainqui, A.; Lohman, K. and Walker, G.C. 1992. *Rhizobium meliloti* mutants unable to synthesize anthranilate display a novel symbiotic phenotype. J. Bacteriol. *174*:4416-4426.

Batut, J. and Boistard, P. 1994. Oxygen control in *Rhizobium*. Antonie van Leeuwenhoek *66*:129-150.

Batut, J.; Terzaghi, B.; Ghérardi, M.; Huguet, M.; Terzaghi, E.; Garnerone, A.-M.; Boistard, P. and Huguet, T. 1985. Localization of a symbiotic *fix* region on *Rhizobium meliloti* pSym megaplasmid more than 200 kilobases from the *nod-nif* region. Mol. Gen. Genet. *199*:232-239.

Bauer, W.D. 1981. Infection of legumes by rhizobia. Ann. Rev. Plant Physiol. *32*:407-449.

Beltra, R.; Del-Solar, G.; Sanchez-Serrano, J.J. and Alonso, E. 1988. Mutants of *Rhizobium phaseoli* HM Mel(-) obtained by means of elevated temperatures. Zentbl. Mikrobiol. *143*:529-532.

Berg, D.E.; Davies, J.; Allet, B. and Rochaix, J.D. 1975. Transposition of R factor genes to bacteriophage λ. Proc. Natl. Acad. Sci. USA *72*:3628-3632.

Berg, D.E.; Weiss, A. and Crossland, L. 1980. Polarity of Tn5 insertion mutations in *Escherichia coli*. J. Bacteriol. *142*:439-446.

Beringer, J.E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. *84*:188-198.

Beringer, J.E.; Beynon, J.L.; Buchanan-Wollaston, A.V. and Johnston, A.W.B. 1978. Transfer of the drug-resistance transposon Tn5 to *Rhizobium*. Nature (London) *276*:633-634.

Beringer, J.E.; Brewin, N.J. and Johnston, A.W.B. 1980. The genetic analysis of *Rhizobium* in relation to symbiotic nitrogen fixation. Heredity *45*:161-186.

Beyerinck, M.W. 1888. Die bacterien der Papilionaceen-Knöllchen. Bot. Zeitung *46*:725-740 [cited by Perret *et al.* (2000)].

Beyerinck, M.W. 1890. Künstilicke infection von *Vicia faba* mit *Bacillus radicicola*. Ernährungsbeding ungen dieser bacterie. Bot. Zeitung *52*:837-843[cited by Perret *et al.* (2000)].

Bhagwat, A.A. and Keister, D.L. 1995. Site-directed mutagenesis of the β-(1,3), β-(1,6)-D-glucan synthesis locus of *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. *8*:366-370.

Bhagwat, A.A; Gross, K.C.; Tully, R.E. and Keister, D.L. 1996. β-glucan synthesis in *Bradyrhizobium japonicum*: Characterization of a new locus (*ndvC*) influencing β-(1→6) linkages. J. Bacteriol. *178*:4635-4642.

Bhagwat, A.A; Tully, R.E. and Keister, D.L. 1993. Identification and cloning of cyclic β-(1,3), β-(1,6)-D-glucan synthesis locus from *Bradyrhizobium japonicum*. FEMS Microbiol. Lett. *114*:139-144.

Bialek, K.; Michalczuk, L. and Cohen, J.D. 1992. Auxin biosynthesis during seed germination in *Phaseolus vulgaris*. Plant Physiol. *100*:509-517.

Boesten, B.; Batut, J. and Boistard, P. 1998. DctBD-dependent and-independent expression of *Sinorhizobium (Rhizobium) meliloti* C_4-dicarboxylate transport gene (*dctA*) during symbiosis. Mol. Plant-Microbe Interact. *11*:878-886.

Boivin, C.; Camut, S.; Malpica, C.A.; Truchet, G. and Rosenberg, C. 1990. *Rhizobium meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. Plant Cell *2*:1157-1170.

Bollag, D.M.; Rozycki, M.D. and Edelstein, S.J. 1996. Protein methods. 2nd ed. John Wiley & Sons. Inc., New York, pp:6.

Bolton, E.; Higgisson, B.; Harrington, A. and O'Gara, F. 1986. Dicarboxylic acid transport in *Rhizobium meliloti*: Isolation of mutants and cloning of dicarboxylic acid transport genes. Arch. Microbiol. *144*:142-146.

Bordeleau, L.M. and Prévost, D. 1994. Nodulation and nitrogen fixation in extreme environments. Plant and Soil *161*:115-125.

Borthakur, D. and Johnston, A.W.B. 1987. Sequence of *psi*, a gene on symbiotic plasmid of *Rhizobium phaseoli* which inhibits exopolysaccharide synthesis and nodulation and demonstration that its transcription is inhibited by *psr*, another gene on the symbiotic plasmid. Mol. Gen. Genet. *207*:149-154.

Borthakur, D.; Barbur, C.E.; Lamb, J.W.; Daniels, M.J.; Downie, J.A. and Johnston, A.W.B. 1986. A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *R. phaseoli* and is corrected by cloned DNA from *Rhizobium* or the phytopathogen *Xanthomonas*. Mol. Gen. Genet. *203*:320-323.

Bradshaw, L.J. 1992. Laboratory microbiology. 4th ed. Saunders College Publishing, California, USA, pp:211-212.

Breedveld, M.W. and Miller, K.J. 1994. Cyclic β-glucans of members of the family Rhizobiaceae. Microbiol. Rev. *58*:145-161.

Brewin, N.J. 1991. Development of the legume root nodule. Ann. Rev. Cell. Biol. *7*:191-226.

Brewin, N.J. 1993. The *Rhizobium*-legume symbiosis : Plant morphogenesis in a nodule. Semin. Cell Biol. *4*:149-156.

Brink, B.A.; Miller, J.; Carlson, R.W. and Noel, K.D. 1990. Expression of *Rhizobium leguminosarum* CFN42 genes for lipopolysaccharide in strains derived from different *R. leguminosarum* soil isolates. J. Bacteriol. *172*:548-555.

Brzoska, P.M. and Singer, E.R. 1991. *lpsZ*, a lipopolysaccharide gene involved in symbiosis of *Rhizobium meliloti*. J. Bacteriol. *173*:3235-3237.

Burkardt, B. and Burkardt, H.J. 1984. Visualization and exact molecular weight determination of *Rhizobium meliloti* megaplasmid. J. Mol. Biol. *175*:213-218.

Burkardt, B.; Schillik, D. and Pühler, A. 1987. Physical characterization of *Rhizobium meliloti* megaplasmids. Plasmid *17*:13-25.

Bushby, H.V.A. 1982. Ecology. In: Nitrogen fixation: *Rhizobium*, vol. 2. Broughton, W.J. (ed.). Clarendon Press. Oxford, pp:35-75.

Caetano-Anollés, G.; Crist-Estes, D.K. and Bauer, W.D. 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J. Bacteriol. *170*:3164-3169.

Carillo, G.C. and Peralta, J.R.V. 1988. Siderophore-like activities in *Rhizobium phaseoli*. J. Plant Nutr. *11*:935-944.

Carlson, R.W. 1982. Surface chemistry. In: Nitrogen fixation: *Rhizobium*, vol. 2. Broughton, W.J. (ed.). Clarendon Press. Oxford, pp:199-234.

Carlson, R.W.; Kalembasa, S.; Turowski, D.; Pachori, P. and Noel, K.D. 1987. Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. J. Bacteriol. *169*:4923-4928.

Cava, J.R.; Elias, P.M.; Turowski, D.A. and Noel, K.D. 1989. Genetic regions encoding lipopolysaccharide structures essential for complete nodule development on bean plants. J. Bacteriol. *171*:8-15.

Charles, T.C. and Finan, T.M. 1990. Genetic map of *Rhizobium meliloti* megaplasmid pRmeSU47b. J. Bacteriol. *172*:2469-2476.

Charles, T.C. and Finan, T.M. 1991. Analysis of a 1600-kilobase *Rhizobium meliloti* megaplasmid using defined deletions generated *in vivo*. Genetics *127*:5-20.

Charles, T.C.; Newcomb, W. and Finan, T.M. 1991. *ndvF*, a novel locus located on megaplasmid pRmeSU47b, (pEXO) of *Rhizobium meliloti*, is required for normal nodule development. J. Bacteriol. *173*:3981-3992.

Chen, H.; Batley, M.; Redmond, J. and Rolfe, B.G. 1985. Alteration of the effective nodulation properties of a fast-growing broad host range *Rhizobium* due to changes in exopolysaccharide synthesis. J. Plant Physiol. *120*:331-349.

Chen, W.X.; Li, G.S.; Qi, Y.L.; Wang, E.-T.; Yuan, H.L. and Li, J.L. 1991. *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. Int. J. Syst. Bacteriol. *41*:275-280.

Chen, W.X.; Tan, Z.Y.; Gao, J.L.; Li, Y. and Wang, E.-T. 1997. *Rhizobium hainanense* sp. nov., isolated from tropical legumes. Int. J. Syst. Bacteriol. *47*:870-873.

Chen, W.X.; Wang, E.-T.; Wang, S.; Li, Y.; Chen, X. and Li, Y. 1995. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. Int. J. Syst. Bacteriol. *45*:153-159.

Chen, W.X.; Yan, G.H. and Li, J.L. 1988. Numerical taxonomic study of fast growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. *38*:392-397.

Cheng, H.-P. and Walker, G. 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. *180*:5183-5191.

Cook, D.; Dreyer, D.; Bonnet, D.; Howell, M.; Nony, E. and VandenBosch, K. 1995. Transient induction of a peroxidase gene in *Medicago truncatula* precedes infection by *Rhizobium meliloti*. Plant Cell *7*:43-55.

Corbin, D.; Barron, L. and Ditta, G.S. 1983. Organization and expression of *Rhizobium meliloti* nitrogen fixation genes. Proc. Natl. Acad. Sci. USA *80*:3005-3009.

Cutting, J.A. and Schulman, H.M. 1969. The site of heme synthesis in soybean root nodules. Biochim. Biophys. Acta. *192*:486-493.

Cutting, J.A. and Schulman, H.M. 1971. The biogenesis of leghemoglobin: The determinant in the *Rhizobium* legume symbiosis for legume specificity. Biochim. Biophys. Acta. *229*:58-62.

David, M.; Daveran, M.L.; Batut, J.; Dedieu, A.; Domergue, O.; Ghai, J.; Hertig, C.; Boistard, P. and Kahn, D. 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. Cell *54*:671-683.

David, M.; Domergue, O.; Pognonec, P. and Kahn, D. 1987. Transcription patterns of *Rhizobium meliloti* symbiotic plasmid pSym: Identification of *nifA*-independent *fix* genes. J. Bacteriol. *169*:2239-2244.

Débellé, F.; Plazanet, C.; Roche, P.; Pujol, C.; Savagnac, A.; Rosenberg, C.; Promé, J.-C and Dénarié, J. 1996. The NodA proteins of *Rhizobium meliloti* and *Rhizobium tropici* specify the N-acylation of Nod factors by different fatty acids. Mol. Microbiol. *22*:303-314.

de Bruijn, F.J.; Rossbach, S.; Schneider, M.; Ratet, P.; Messmer, S.; Szeto, W.W.; Ausubel, F.M. and Schell, J. 1989. *Rhizobium meliloti* 1021 has three differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. J. Bacteriol. *171*:1673-1682.

de Lajudie, P.; Willems, A.; Nick, G. and 9 other authors. 1998. Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. Int. J. Syst. Bacteriol. *48*:369-382.

de Lajudie, P.; Willems, A.; Pot, B.; Dewettinck, D.; Maestrojuan, G.; Neyra, M.; Collins, M.D.; Dreyfus, B.; Kersters, K. and Gillis, M. 1994. Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Bacteriol. *44*:715-733.

Delgado, M.-J.; Yeoman, K.H.; Wu, G.; Vargas, C.; Davies,A.E.; Polle, R.K.; Johnston, A.W.B. and Downie, J.A. 1995. Characterization of the *cycHJKL* genes invoved in cytochrome c biogenesis and symbiotic nitrogen fixation in *Rhizobium leguminosarum*. J. Bacteriol. *177*:4927-4934.

Dénarié, J.; Débellé, F. and Promé, J.-C. 1996. *Rhizobium* lipo-chitooligosaccharide nodulation factors: Signalling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. *65*:503-535.

Dénarié, J.; Truchet, G. and Bergeron, B. 1976. Effects of some mutations on symbiotic properties of *Rhizobium*. In: Symbiotic nitrogen fixation. Nutman, P.S. (ed.). Cambridge University Press. Cambridge, pp:47-61.

Diebold, R. and Noel, K.D. 1989. *Rhizobium leguminosarum* exopolysaccharide mutants: Biochemical and genetic analyses and symbiotic behaviour on three hosts. J. Bacteriol. *171*:4821-4830.

Dilworth, M.J. 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. Biochim. Biophys. Acta. *127*:285-294.

Dilworth, M.J. 1969. The plant as the genetic determinant of leghemoglobin production in the legume root nodule. Biochim. Biophys. Acta. *184*:432-441.

Ditta, G. 1986. Tn5 mapping of *Rhizobium* nitrogen fixation genes. Methods Enzymol. *118*:519-528.

Ditta, G.; Stanfield, S.; Corbin, D. and Helinski, D.R. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA *77*:7347-7351.

Ditta, G.; Virts, E.; Palomares, A. and Kim, C. 1987. The *nifA* gene of *Rhizobium meliloti* is oxygen regulated. J. Bacteriol. *169*:3217-3223.

Dixon, R.A. 1984. The genetic complexity of nitrogen fixation. J. Gen. Microbiol. *130*:2745-2755.

Dixon, R.O.D. and Wheeter, C. T. 1986. Nitrogen fixation in plants. Blackie, Glasgow, United Kingdom.

Doherty, D.; Leigh, J.A.; Glazebrook, J. and Walker, G.C. 1988. *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic calcofluor-binding exopolysaccharide. J. Bacteriol. *170*:4249-4256.

Dreyfus, B.; Garcia, J.L. and Gillis, M. 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem nodulating nitrogen fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bacteriol. *38*:89-98.

Dudley, M.E.; Jacobs, T.W. and Long, S.R. 1987. Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*. Planta *171*:289-301.

Dudman, W.F. and Jones, A.J. 1980. The extracellular glucans of *Bradyrhizobium japonicum* strain 311b71a. Carbohydr. Res. *84*:358-364.

Dylan, T.; Helinski, D.R. and Ditta, G.S. 1990. Hypoosmotic adaptation in *Rhizobium meliloti* requires β-(1→2)-glucan. J. Bacteriol. *172*:1400-1408.

Dylan, T.; Ielpi, L.; Stanfield, S.; Kashyap, L.; Douglas, C. Yanofsky, M.; Nester, E.; Helinski, D.R. and Ditta, G. 1986. *Rhizobium meliloti* genes required for nodule

development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA *83*:4403-4407.

Ehrhardt, D.W.; Atkinson, E.M. and Long, S.R. 1992. Depolarizaton of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science *256*:998-1000.

Ehrhardt, D.W.; Atkinson, E.M.; Faull, K.F.; Freedberg, D.I.; Sutherlin, D.P.; Armstrong, R. and Long, S.R. 1995. *In vitro* sulfotransferase activity of NodH, a nodulation protein of *Rhizobium meliloti* required for host-specific nodulation. J. Bacteriol. *177*:6237-6245.

Engelke, T.; Jagadish, M.N. and Pühler, A. 1987. Biochemical and genetical analysis of *Rhizobium meliloti* mutants defective in C_4-dicarboxylate transport. J. Gen. Microbiol. *133*:3019-3029.

Engelke, T.; Jording. D.; Kapp, D. and Pühler, A. 1989. Identification and sequence analysis of the *Rhizobium meliloti dctA* gene encoding the C_4-dicarboxylate carrier. J. Bacteriol. *171*:5551-5560.

Fearn, J.C. and La Rue, T.A. 1991. Ethylene inhibitors restore nodulation to *Sym5* mutants of *Pisum sativum* L. cv. Sparkle. Plant Physiol. *96*:239-244.

Fedorov, S.N. and Zaretskaya, A.N. 1977. Ethylmethane sulfonate induction of auxotrophic mutants of *Rhizobium meliloti* and their characteristics. Mikrobilogiya *47*:728-732.

Felle, H.H.; Kondorosi, É.; Kondorosi, Á. and Schultze, M. 1995. Nod signal-induced plasma membrane potential changes in alfalfa root hairs are differentially sensitive to structural modifications of the lipochitooligosaccharide. Plant J. *7*:939-947.

Felle, H. H.; Kondorosi, É.; Kondorosi, Á. and Schultze, M. 1996. Rapid alkalinization in alfalfa root hair in response to rhizobial lipochitooligo-saccharide signals. Plant J. *10*:295-301

Fernandez-lópez, M.; Goormachtig, S.; Gao, M.; D'Haeze, W.; van Montagu, M. and Holsters, M. 1998. Ethyline-mediated phenotypic plasticity in root nodule development on *Sesbania rostrata*. Proc. Natl. Acad. Sci. USA *95*:12724-12728.

Ferraioli, S.; Taté, R.; Caputo, E.; Lamberti, A.; Riccio, A. and Patriarca, E. J. 2001. The *Rhizobium etli argC* gene is essential for arginine biosynthesis and nodulation of *Phaseolus vulgaris*. Mol. Plant-Microbe Interact. *14*:250-254.

Finan, T.M.; Kunkel, B.; De Vos, G.F. and Signer, E.R. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. *167*:66-72.

Finan, T.M.; Oresnik, I. and Bottacin, A. 1988. Mutants of *Rhizobium meliloti* defective in succinate metabolism. J. Bacteriol. *170*:3396-3403.

Finan, T.M.; Wood, J.M. and Jordan, D.C. 1983. Symbiotic properties of C_4-dicarboxylic acids transport mutants of *Rhizobium leguminosarum*. J. Bacteriol. *154*:1403-1413

Fischer, H.M. 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. *58*:352-386.

Fisher, R.F. and Long, S.R. 1992. *Rhizobium*-plant signal exchange. Nature *357*:655-660.

Flores, M.; Mavingul, P.; Girard, L.; Perret, X.; Broughton, W.J.; Martinéz-Romero, E.; Davila, G. and Palacios, R. 1998. Three replicons of *Rhizobium* sp. strain NGR234 harbor symbiotic gene sequences. J. Bacteriol. *180*:6052-6053.

Folch-Mallol, J.L.; Manyani, H.; Marroqui, S.; Sousa, C.; Vargas, C.; Nava, N.; Colmenero-Flores, J.M.; Quinto, C. and Megias, M. 1998. Sulfation of Nod factors via *nodHPQ* and *nodD* independent in *Rhizobium tropici* CIAT899. Mol. Plant-Microbe Interact. *11*:979-987.

Forrai, T.; Vincze, E.; Banfalvi, Z.; Kiss, G.B.; Randhawa, G.S. and kondorosi, Á. 1983. Lacalization of symbiotic mutation in *Rhizobium meliloti*. J. Bacteriol. *153*:635-643.

Fougere, F.; Le Rudulier, D. and Streeter, J.G. 1991. Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa* L.) Plant Physiol. *96*:1228-1236.

Frank, B. 1879. Über die parasiten in den Wurzelans-chwellungen der Papilionaceen. Bot. Zeitung *24*:377-388 [cited by Perret *et al.* (2000)].

Frank, B. 1889. Uber die pilzsymbiose der leguminosen. Ber. Dtsch. Bot. Ges. *7*:332-346 [cited by Perret *et al.* (2000)].

Franssen, H.J.; Vijn, I.; Yang, W.C. and Bisseling, T.1992. Developmental aspects of the *Rhizobium*-legume symbiosis. Plant Mol. Biol. *19*:89-107.

Freiberg, C.; Fellay, R.; Bairoch, A.; Broughton, W.J.; Rosenthal, A. and Perret, X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature *387*:394-401.

Fuchsius, L. 1542. De historia stirpium commentarii isignes. Michael Isingrin, Basel, Switzerland [cited by Perret *et al.* (2000)].

Gagnon, H. and Ibrahim, R.K. 1998. Aldonic acids: A novel family of *nod* gene inducers of *Mesorhizobium loti*, *Rhizobium lupini*, and *Sinorhizobium meliloti*. Mol. Plant-Microbe Interact. *11*:988-998.

Gaworzewska, E.T. and Carlile, M.J. 1982. Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. J. Gen. Microbiol. *128*:1179-1188.

Geremia, R.A.; Cavaignac, S.; Zorreguieta, A.; Toro, N.; Olivares, J. and Ugalde, R.A. 1987. A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form β-(1→2) glucan. J. Bacteriol. *169*:880-884.

Gill, P.R.; Barton, L.L.; Scoble, M.D. and Neilands, J.B. 1991. A highe affinity iron transport system of *Rhizobium meliloti* may be required for efficient nitrogen fixation *in planta*. In: Iron nutrition and interactions in plants. Chen, Y. and Hadar, Y. (ed.). Kluwer Academic Publishers. The Netherlands, pp:251-257.

Gillam, S.; Astell, C.R. and Smith, M. 1980. Site-specific mutagensis using oligo-deoxyribonucleotides: Isolation of a phenotypically silent ØX174 mutant, with a specific nucleotide deletion, at very high efficiency. Gene *12*:129-137.

Glazebrook, J. and Walker, G.C. 1989. A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell *56*:661-672.

Glenn, A.R. and Dilworth, M.J. 1984. Methylamine and ammonium transport systems in *Rhizobium leguminosarum* MNF 3841. J. Gen. Microbiol. *130*:1961-1968.

Godfrey, C.A. and Dilworth, M.J. 1971. Haem biosynthesis from ^{14}C-δ-amino-levulinic acid in laboratory-grown and root nodule *Rhizobium lupini*. J. Gen. Microbiol. *69*:385-390.

Goryshin, I.Y. and Reznikoff, W.S. 1998. Tn5 *in vitro* transposition. J. Biol. Chem. *273*:7367-7374.

Götz, R.; Limmer, N.; Ober, K. and Schmitt, R. 1982. Motility and chemotaxis in two strains of *Rhizobium* with complex flagella. J. Gen. Microbiol. *128*:789-798.

Graham, P.H. 1963. Vitamin requirements of root nodule bacteria. J. Gen. Microbiol. *30*:245-248.

Gualtieri, G. and Bisseling, T. 1999. The evolution of nodulation. Plant Mol. Biol. *42*:181-194.

Guerinot, M.L. 1991. Iron uptake and metabolism in the rhizobia/legume symbiosis. Plant Soil *130*:199-209.

Guerinot, M.L.; Meidl, E.J. and Plessner, O. 1990. Citrate as a siderophore in *Bradyrhizobium japonicum*. J. Bacteriol. *172*:3298-3303.

Gulash, M.; Ames, P., Larosiliere, R.C. and Bergman, K. 1984. Rhizobia are attracted to localized sites on legume roots. Appl. Environ. Microbiol. *48*:149-152.

Heidstra, R. and Bisseling, T. 1996. Nod factor-induced host responses and mechanisms of Nod factor perception. New Phytol. *133*:25-43.

Heidstra, R.; Yang, W.C.; Yalcin, Y.; Peck, S.; Emons, A.M.; van Kammen, A. and Bisseling, T. 1997. Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*-legume interaction. Develop. *124*:1781-1787.

Helal, H.M. and Sauerbeck, D. 1989. Carbon turnover in the rhizosphere. Z. Pflanzenernaehr. Bodenkd. *152*:211-216.

Hellriegel, H. 1886. Welche Stickstoffquellen stehen der Pflanze zu Gebote? Landwirtsch. Versuchstat Dresden. *33*:464-465 [cited by Perret *et al.* (2000)].

Hellriegel, H. and Wilfarth, H. 1888. Untersuchungen über die stickstoff-nahrung der Gramineen und Leguminosen. Beilageheft zu der Zeitschrift des Vereins für die Rübenzucker-Industrie des Deutschen Reiches, Buchdruckerei der "Post". Kayssler & Co., Berlin, Germany [cited by Perret *et al.* (2000)].

Hennecke, H. 1990. Nitrogen fixation genes involved in the *Bradyrhizobium japonicum*-soybean symbiosis. FEBS Lett. *268*:422-426.

Hirsch, A.M. 1992. Tansley review No. 40. Developmental biology of legume nodulation. New Phytol. *122*:211-237.

Hirsch, A.M.; Bang, M. and Ausubel, F.M. 1983. Ultrastructural analysis of ineffective alfalfa nodules formed by *nif*::Tn5 mutants of *Rhizobium meliloti*. J. Bacteriol. *155*:367-380.

Hirsch, A.M.; Long, S.R.; Bang, M.; Haskins, N. And Ausubel, F.M. 1982. Structural studies of alfalfa roots infected with nodulation mutants of *Rhizobium meliloti*. J. Bacteriol. *151*:411-419.

Hirsch, A.M., Wilson, K.J.; Jones, D.G.; Bang, W.; Walker, G.C. and Ausubel, F.M. 1984. *Rhizobium meliloti* genes allow *Agrobacterium tumefaciens* and *Escherichia coli* to form pseudonodules on alfalfa. J. Bacteriol. *158*:1133-1143.

Hitchcock, P.J.; Leive, L.; Mäkelä, P.H.; Reitschel, E.T.; Strittmatter, W. and Morrison, D.C. 1986. Lipopolysaccharide nomenclature-past, present and future. J. Bacteriol. *166*:699-705.

Holliday, R. 1956. A new method for the identification of biochemical mutants of microorganisms. Nature *178*:987.

Hom, S.S.M.; Uratsu, S.L. and Hoang, F. 1984. Transposon Tn5-induced mutagenesis of *Rhizobium japonicum* yielding a wide variety of mutants. J. Bacteriol. *159*:335-340.

Horvath, B.; Bachem, C.W.B.; Schell, J. and Kondorosi, Á. 1987. Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal, interacting with the *nodD* gene product. EMBO J. *6*:841-848.

Hotter, G.S. and Scott, D.B. 1991. Exopolysaccharide mutants of *Rhizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. J. Bacteriol. *173*:851-859.

Hunter, W.J. and Kuykendall, L.D. 1990. Enhanced nodulation and nitrogen fixation by a revertant of a nodulation-defective *Bradyrhizobium japonicum* tryptophan auxotroph. Appl. Environ. Microbiol. *56*:2399-2403.

Hynes, M.F.; Simon, R.; Müller, P.; Niehaus, K.; Labes, M. and Pühler, A. 1986. The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. *202*:356-362.

International Union of Biochemistry. 1992. Nomenclature of electron transfer proteins. Recommendations (1989) of the nomenclature committee of the International Union of Biochemistry. J. Biol. Chem. *267*:665-677.

Jarvis, B.D.W.; Pankhurst, C.E. and Patel, J.J. 1982. *Rhizobium loti*, a new species of legume-root nodule bacteria. Int. J. Syst. Bacteriol. *32*:378-380.

Jarvis, B.D.W.; van Berkum, P.; Chen, W.-X.; Nour, S.M.; Fernandez, M.P.; Cleyet-Marel, J.C. and Gillis, M. 1997. Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum* and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. Int. J. Syst. Bacteriol. *47*:895-898.

Jelesko, J.G.; Lara, J.C. and Leigh, J.A. 1993. *Rhizobium meliloti* mutants with decreased DAHP synthase activity are sensitive to exogenous tryptophan and phenylalanine and form ineffective nodules. Mol. Plant-Microbe Interact. *6*:135-143.

Johnston, A.W.B.; Bibb, M.J. and Beringer, J.E. 1978. Tryptophan genes in *Rhizobium*-their organization and their transfer to other bacterial genera. Mol. Gen. Genet. *165*:323-330.

Jordan, D.C. 1982. Transfer of *Rhizobium japonicum* to *Bradyrhizobium* gen. nov., a genus of slow growing, root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. *32*:136-139.

Jordan, D.C. 1984. Family III. Rhizobiaceae Conn 1938. 321^{AL}. In: Bergey's manual of systematic bacteriology, vol. 1. Krieg, N.R. and Holt, J.G. (ed.) The Williams & Wilkins Co., Baltimore, pp:234-256.

Kaiser, B.N.; Finnegan, P.M.; Tyerman, S.D.; Whitehead, L.F.; Bergersen, F.J.; Day, D.A. and Udvardi, M.K. 1998. Characterization of an ammonium transport protein from the peribacteroid membrane of soybean nodules. Science *281*:1202-1206.

Kape, R.; Parniske, M. and Werner, D. 1991. Chemotaxis and *nod* gene activity of *Bradyrhizobium japonicum* in response to hydroxycinnamic acids and isoflavonoids. Appl. Environ. Microbiol. *57*:316-319.

Karanja, N.K. and Wood, M. 1988. Selecting *Rhizobium phaseoli* strain for use with beans (*Phaseolus vulgaris L.*) in Kenya. Tolerance of high temperature and antibiotic resistance. Plant Soil *112*:15-22.

Karnovsky, M.J. 1965. A Formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. *27*:137A.

Kereszt, A.; Kiss, E.; Reuhs, B.L.; Carlson, R.W. Kondorosi, Á. and Putnoky, P. 1998. Novel *rkp* gene clusters of *Sinorhizobium meliloti* involved in capsular polysaccharide production and invasion of the symbiotic nodule: The *rkpK* gene encodes a UDP-glucose dehydrogenase. J. Bacteriol. *180*:5426-5431.

Kereszt, A.; Slaska-Kiss, K.; Putnoky, P.; Banfalvi, Z. and Kondorosi, Á. 1995. The *cycHJKL* genes of *Rhizobium meliloti* involved in cytochrome c biogenesis are required for 'respiratory' nitrate reduction *ex planta* and for nitrogen fixation during symbiosis. Mol. Gen. Genet. *247*:39-47.

Kerppola, T.K. and Kahn, M.L. 1985. Characterization of auxotrophs of *Rhizobium meliloti* 104A14 and cloning of the genes for carbamoylphosphate synthase, In: Nitrogen fixation research progress. Evans, H.J., Bottomly, P.J. and Newton, W.E. (ed.) Martinus Nijhoff Publishers, Dordrecht. The Netherlands, pp:223-234.

Kerppola, T.K. and Kahn, M.L. 1988a. Symbiotic phenotypes of auxotrophic mutants of *Rhizobium meliloti* 104A14. J. Gen. Microbiol. *134*:913-919.

Kerppola, T.K. and Kahn, M.L. 1988b. Genetic analysis of carbamoylphosphate synthesis in *Rhizobium meliloti* 104A14. J. Gen. Microbiol. *134*:921-929.

Kersters, K. and De Ley, 1984. Genus III. *Agrobacterium* conn 1942. In: Bergey's manual of systematic bacteriology, vol. I. Krieg, N.R. and Holt, J.G. (ed.) The Williams & Wilkins Co., Baltimore, pp:244-254.

Khanuja, S.P.S. and Kumar, S. 1988. Isolation of phages for *Rhizobium meliloti* AK631. Ind. J. Exp. Biol. *26*:665-667.

Khanuja, S.P.S. and Kumar, S. 1989. Symbiotic and galactose utilization properties of phage RMP64 resistant mutants affecting three complementation groups in *Rhizobium meliloti*. J. Genet. *68*:93-108.

Kim, C.H.; Kuykendall, L.D.; Shah, K.S. and Keister, D.L. 1988. Induction of symbiotically defective auxotrophic mutants of *Rhizobium fredii* HH303 by transposon mutagenesis. Appl. Environ. Microbiol. *54*:423-427.

Kim, C.H.; Tully, R.E. and Keister, D.L. 1989. Exopolysaccharide-deficient mutants of *Rhizobium fredii* HH303 which are symbiotically effective. Appl. Environ. Microbiol. *55*:1852-1854.

King, N.D.; Hojnacki, D. and O'Brian, M.R. 2000. The *Bradyrhizobium japonicum* proline biosynthesis gene *proC* is essential for symbiosis. Appl. Environ. Microbiol. *66*:5469-5471.

Kleckner,N.; Roth, J. and Botstein, D. 1977. Genetic engineering *in vivo* using translocatable drug-resistance elements: New methods in bacterial genetics. J. Mol. Biol. *116*:125-159.

Kneen, B.E. and La Rue, T.A. 1983. Congo red absorption by *Rhizobium leguminosarum*. Appl. Environ. Microbiol. *45*:340-342.

Kondorosi, Á. 1989. *Rhizobium*-legume interactions: Nodulation genes. In: Plant-microbe interactions, vol. 3. Kosuge, T. and Nester, E.W. (ed.). McGraw-Hill Book Co., New York, pp:383- 420.

Kondorosi, Á. 1991. Regulation of nodulation genes in rhizobia. In: Molecular signals in plant-microbe communication. Verma, D.P.S. (ed.) CRC press, Boca Raton, F.L., pp:325-340.

Kosslak, R.M.; Bookland, R.; Barkei, J.; Paaren, H.E. and Applebaum, E.R. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA *84*:7428-7432.

Kouchi, H. and Hata, S. 1993. Isolation and characterization of novel cDNAs representing genes expressed at early stages of soybean nodule development. Mol. Gen. Genet. *238*:106-119.

Kovaks, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature(London) *178*:703.

Król, J.; Wielbo, J.; Mazur, A.; Kopcinska, J. Lotocka, B.; Golinowski, W. and Skorupska, A. 1998. Molecular characterization of *pssCDE* genes of *Rhizobium leguminosarum* bv. *trifolii* strain TA1: *pssD* mutant is affected in exopolysaccharide synthesis and endocytosis of bacteria. Mol. Plant-Microbe interact. *11*:1142-1148.

Kummer, R.M. and Kuykendall, L.D. 1989. Symbiotic properties of amino acid auxotrophs of *Bradyrhizobium japonicum*. Soil. Biol. Biochem. *21*:779-782.

Kurkdjian, A.C. 1995. Role of the differentiation of root epidermal cells in Nod factor from *Rhizobium meliloti*-induced root depolarization of *Medicago sativa*. Plant Physiol. *107*:783-790.

Kuykendall, L.D.; Saxena, B.; Devine, T.E. and Udell, S.E. 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. *38*:501-505.

Lagares, A.; Caetano-Anollés, G.; Niehaus, K.; Lorenzen, J.; Ljunggren, H.D.; Pühler, A. and Favelukes, G. 1992. A *Rhizobium meliloti* lipopolysaccharide mutant altered in competitiveness for nodulation of alfalfa. J. Bacteriol. *174*: 5941-5952.

Lagares, A.; Hozbor, D.F.; Niehaus, K.; Otero, A.J.; Lorenzen, J.; Arnold, W. and Pühler, A. 2001. Genetic characterization of a *Sinorhizobium meliloti* chromosomal region in lipopolysaccharide biosynthesis. J. Bacteriol. *183*:1248-1258.

Leigh, J.A. and Coplin, D.L. 1992. Exopolysaccharides in plant-bacterial interactions. Annu.Rev. Microbiol. *46*:307-346.

Leigh, J.A.; Reed, J.W.; Hanks, J.F.; Hirsch, A.M. and Walker, G.C. 1987. *Rhizobium meliloti* mutants that fail to succinylate their calcofluor-binding exopolysaccharide are defective in nodule invasion. Cell *51*:579-587.

Leigh, J.A.; Signer, E.R. and Walker, G.C. 1985. Exopolysaccharide deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA *82*:6231-6235.

Lerouge, P.; Roche, P.; Faucher, C.; Maillet, F.; Truchet, G.; Promé, J.-C. and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and a cylated glucosamine oligosaccharide signal. Nature *344*:781-784.

Levin, A.P.; Funk, H.B. and Tendler, M.D. 1954. Vitamin B_{12}, rhizobia, and leguminous plants. Science *120*:784.

Libbenga, K.R. and Harkes, P.A.A. 1973. Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum*. L. Planta *114*:17-28.

Lindström, K. 1989. *Rhizobium galegae*, a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. *39*:365-367.

LIoyd, C.W.; Pearce, K.J.; Rawlins, D.J.; Ridge, R.W. and Shaw, P.J. 1987. Endoplasmic microtubules connect the advancing nucleus to the tip of legume root hairs, but F-actin is involved in basipetal migration. Cell. Motil. Cytoskeleton *8*:27-36.

Lohnis, F. and Hansen, R. 1921. Nodule bacteria of leguminous plants. J. Agric. Res. *20*:543-556.

Long, S.R. 1989a. *Rhizobium* genetics. Annu. Rev. Genet. *23*:483-506.

Long, S.R. 1989b. *Rhizobium*-legume nodulation: Life together in the undrground. Cell *56*:203-214.

Long, S.R. 1996. *Rhizobium* symbiosis: Nod factors in perspective. Plant Cell *8*: 1885-1898.

Long, S.R. 2001. Genes and signals in the *Rhizobium*-legume symbiosis. Plant Physiol. *125*:69-72.

López, J. C.; Grasso, D.H.; Frugier, F.; Crespi, M.D. and Aguilar, O.M. 2001. Early symbiotic responses induced by *Sinorhizobium meliloti ilvC* mutants in alfalfa. Mol. Plant-Microbe Interact. *14*:55-62.

Malek, W. and Kowalski, M. 1983. Symbiotic properties of adenine requiring mutants of *Rhizobium meliloti* strain L5-30. Acta. Microbiol. Pol. *32*:19-24.

Malpighi, M. 1675. Anatome plantarum. Regiae Societati (J. Martyn), London, United Kingdom [cited by Perret *et al.* (2000)].

Mandon, K.; Hillebrand, H.; Mougel, C.; Desnoues, N.; Dreyfus, B.; Kaminiski, P.A. and Elmerich, C. 1993. Function analysis of the *fixNOQP* region of *Azorhizobium caulinodans*. J. Bacteriol. *176*:2560-2568.

Martínez-Romero, E.; Segovia, L.; Mercante, F.M.; Franco, A.A.; Graham, P. and Pardo, M.A. 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. *41*:417-426.

Mathews, A.; Carroll, B.J.; Gresshoff, P.M. 1989. Development of *Bradyrhizobium* infections in supernodulating and non-nodulating mutants of soybean (Glycine max [L.] Merrill). Protoplasma *150*:40-47.

Mathis, R.; Grosjean, C; de Billy, F.; Huguet, T. and Gamas, P. 1999. The early nodulin gene *MtN6* is a novel marker for events preceding infection of *Medicago truncatula* roots by *Sinorhizobium meliloti*. Mol. Plant-Microbe Interact. *12*:544-555.

Maxwell, C.A. and Phillips, D.A. 1990. Concurrent synthesis and release of *nod* gene inducing flavonoids from alfalfa roots. Plant Physiol. *93*:1552-1558.

Mayer, H.; Tharanathan, R.N. and Weckesser, J. 1985. Analysis of lipopoly-saccharides of Gram-negative bacteria. Methods Microbiol. *18*:157-207.

McIver, J.; Djordjevic, M.A.; Weinman, J.J.; Bender, G.L. and Rolfe, B.G. 1989. Extension of host range of *Rhizobium leguminosarum* bv. *trifolii* caused by point

mutations in *nodD* that result in alterations in regulatory function and recognition of inducer molecules. Mol. Plant-Microbe Interact. *2*:97-106.

Meade, H.M.; Long, S.R.; Ruvkin, G.B.; Brown, S.E. and Ausubel, F.M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. J. Bacteriol. *149*:114-122.

Mergaert, P.; van Montagu, M. and Holsters, M. 1997. Molecular mechanisms of Nod factor diversity. Mol. Microbiol. *25*:811-817.

Merrick, M. and Edwards, R. 1995. Nitrogen control in bacteria. Microbiol. Rev. *59*:604-622.

Miller, K. J.; Gore, R.S.; Johnson, R.; Benesi, A.J. and Reinhold, V.N. 1990. Cell-associated oligosaccharides of *Bradyrhizobium* spp. J. Bacteriol. *172*:136-142.

Moat, A.G. and Foster, J.W. 1995. The aromatic amino acid pathway. In: Microbiol physiology, 3rd ed. John Wiley & Sons. Inc., New York, pp:484-491.

Modi, M.; Shah, K.S. and Modi, V.V. 1985. Isolation and characterization of a catechol-like siderophore from cowpea *Rhizobium* RA-1. Arch. Microbiol. *141*:156-158.

Mylona, P.; Pawlowski, K. and Bisseling, T. 1995. Symbiotic nitrogen fixation. The Plant Cell *7*:869-885.

Nadler, K.D. and Avissar, Y. 1977. Heme biosynthesis in soybean root nodules. 1. On the role of bacteroid δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydrase in the synthesis of the heme of leghemoglobin. Plant Physiol. *60*:433-436.

Nadler, K.D.; Johnston, A.W.B.; Chen, J.-W. and John, T.R. 1990. A *Rhizobium leguminosarum* mutant defective in symbiotic iron acquisition. J. Bacteriol. *172*:670-677.

Newcomb, W. 1976. A correlated light and electron microscopy study of symbiotic growth and differentiation in *Pisum sativum* root hair nodules. Can. J. Bot. *54*:2163-2186.

Newcomb, W. 1981. Nodule morphogenesis and differentiation, In: Biology of Rhizobiaceae. Giles, K.L. and Atherly, A.G. (ed.). Academic Press. New York, pp:247-298.

Newman, J.D.; Diebold, R.J.; Schultz, B.W. and Noel, K.D. 1994. Infection of soybean and pea nodules by *Rhizobium* spp. purine auxotrophs in the presence of 5-aminoimidazole-4-carboxamide riboside. J. Bacteriol. *176*:3286-3294.

Newman, J.D.; Schultz, B.W. and Noel, K.D. 1992. Dissection of nodule development by supplementation of *Rhizobium leguminosarum* biovar *phaseoli* purine auxotrophs with 5-aminoimidazole-4-carboxamide riboside. Plant Physiol. *99*:401-408.

Nick, G.; de Lajudie, P.; Eardly, B.D.; Suomalainen, S.; Paulin, L.; Zhang, X.; Gillis, M. and Lindström, K. 1999. *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. Int. J. Syst. Bacteriol. *49*:1359-1368.

Niehaus, K.; Lagares, A. and Pühler, A. 1998. A *Sinorhizobium meliloti* lipopolysaccharide mutant induces effective nodules on the host plant *Medicago sativa* (alfalfa) but fails to establish a symbiosis with *Medicago truncatula*. Mol. Plant-Microbe Interact. *11*:906-914.

Nikanishi, I.; Kimura, K.; Suzuki, T.; Ishikawa, M., Banno, I.; Sakane, T. and Harada, T. 1976. Demonstration of curdlan type polysaccharides and some other beta (1→3) glucan in microorganisms with aniline blue. J. Gen. Appl. Microbiol. *22*:1-11.

Noel, K.D.; Carneol, M. and Brill, W.J. 1982. Nodule protein synthesis and nitrogenase activity of soybeans exposed to fixed nitrogen. Plant Physiol. *70*:1236-1241.

Noel, K.D.; Diebold, R.J.; Cava, J.R. and Brink, B.A. 1988. Rhizobial purine and pyrimidine auxotrophs: Nutrient supplementation, genetic analysis, and the symbiotic requirement for de novo purine biosynthesis. Arch. Microbiol. *149*:499-506.

Noel, K.D.; Sanchez, A.; Fernandez, L.; Leemans, J. and Cevallos, M. A. 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn5 insertions. J. Bacteriol. *158*:148-155.

Noel, K.D.; Vandenbosch, K.A. and Kulpaca, B. 1986. Mutations in *Rhizobium phaseoli* that lead to arrested development of infection threads. J. Bacteriol. *168*:1392-1401.

Nour, S.M.; Cleyet-Marel, J.-C.; Normand, P. and Fernandez, M.P. 1995. Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. Int. J. Syst. Bacteriol. *45*:640-648.

Nour, S.M.; Fernandez, M.P.; Normand, P. and Cleyet-Marel, J.-C. 1994. *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). Int. J. Syst. Bacteriol. *44*:511-522.

Ogawa, J. and Long, S.R. 1995. The *Rhizobium meliloti groE1c* locus is required for regulation of early *nod* genes by the transcription activator NodD. Genes Dev. *9*:714-729.

O'Hara, G.W.; Dilworth, M.J.; Boonkerd, N. and Parkpian, P. 1988. Iron-deficiency specifically limits nodule development in peanut inoculated with *Bradyrhizobium* sp. New Phytol. *108*:51-57.

Ola'h, B.; Kiss, E.; Györgypál, Z.; Borzi, J.; Cinege, G.; Csanádi, G.; Batut, J.; Kondorosi, Á and Dusha, I. 2001. Mutation in the *ntrR* gene, a member of the *vap* gene family, increases the symbiotic efficiency of *Sinorhizobium meliloti*. Mol. Plant-Microbe Interact. *14*:887-894.

Olsthoorn, M.M.A.; López-Lara, I.M.; Peterson, B.O.; Bock, K.; Haverkamp, J.; Spaink, H.P. and Thomas-Oates, J.E. 1998. Novel branched Nod factor structure results from α-(1→3) fucosyl transferase activity: The major lipo-chitin oligosaccharides from *Mesorhizobium loti* strain NZP2213 bear an α-(1→3) fucosyl substituent on a nonterminal backbone residue. Biochem. *37*:9024-9032.

Olsthoorn, M.M.A.; Stokvis, E.; Haverkamp, J.; Spaink, H.P. and Thomas-Oates, J.E. 2000. Growth temperature regulation of host-specific modifications of rhizobial lipo-chitin oligosaccharides: The function of *nodX* is temperature regulated. Mol. Plant-Microbe. Interact. *13*:808-820.

Ophel, K. and Kerr, A. 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. Int. J. Syst. Bacteriol. *40*:236-241.

Oresnik, I. J.; Liu, S-L.; Yost, C.K. and Hynes, M. F. 2000. Megaplasmid pRme2011a of *Sinorhizobium meliloti* is not required for viability. J. Bacteriol. *182*:3582-3586.

Paau, A.S.; Bloch, C.B. and Brill, W.J. 1980. Developmental fate of *Rhizobium meliloti* bacteroids in alfalfa nodules. J. Bacteriol. *143*:1480-1490.

Pain, A.N. 1979. Symbiotic properties of antibiotic-resistant and auxotrophic mutants of *Rhizobium leguminosarum*. J. Appl. Bacteriol. *47*:53-64.

Panagiota, M.; Pawlowski, K. and Bisseling, T. 1995. Symbiotic nitrogen fixation. Plant Cell *7*:869-885.

Pankhurst, C.E.; Schwinghamer, E.A. and Bergersen, F.J. 1972. The structure and acetylene reducing activity of root nodules formed by a riboflavin-requiring mutant of *Rhizobium trifolii*. J. Gen. Microbiol. *70*:161-177.

Parniske, M.; Schmidt, P.E.; Kosch, K. and Müller, P. 1994. Plant defense responses of host plants with determinate nodules induced by EPS-defective *exoB* mutants of *Bradyrhizobium japonicum*. Mol. Plant -Microbe Interact. *7*:631-638.

Parveen, N.; Webb, D.T. and Borthakur, D. 1997. The symbiotic phenotypes of exopolysaccharide-defective mutants of *Rhizobium* sp. strain TAL1145 do not differ on determinate-and indeterminate-nodulating tree legumes. Microbiol. *143*:1959-1967.

Patriarca, E.J.; Chiurazzi, M.; Manco, G.; Riccio, A.; Lamberti, A.; De Paolis, A.; Rossi, M.; Defez, R. and Iaccarino, M. 1992. Activation of the *Rhizobium leguminosarum glnII* gene by NtrC is dependent on upstream DNA sequences. Mol. Gen. Genet. *234*:337-345.

Patriarca, E.J.; Riccio, A.; Colonna-Romano, S.; Defez, R. and Iaccarino, M. 1994. DNA binding activity of NtrC from *Rhizobium* grown on different nitrogen sources. FEBS Lett. *354*:89-92.

Patriarca, E.J.; Riccio, A.; Taté, R.; Colonna-Romano, S.; Iaccarino, M. and Defez, R. 1993. The *ntrBC* genes of *Rhizobium leguminosarum* are part of a complex operon subject to negative autoregulation. Mol. Microbiol. *9*:569-577.

Patriarca, E.J.; Taté, R.; Fedorova, E.; Riccio, A.; Defez, R. and Iaccarino, M. 1996. Down-regulation of the *Rhizobium* ntr system in the determinate nodule of *Phaseolus vulgaris* identifies a specific developmental zone. Mol. Plant-Microbe Interact. *9*:243-251.

Pawlowski, K.; Ribeiro, A. and Bisseling, T. 1996. Nitrogen fixing root nodule symbioses: Legume nodules and actinorhizal nodules. In: Biotechology annual review, vol. 2. El-Gewely, M.R. (ed.). Elsevier Science, B.V., pp:151-184.

Pellock, B.J.; Cheng, H-P, and Walker, G.C. 2000. Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. J. Bacteriol. *182*:4310-4318.

Penmetsa, R.V. and Cook, D.R. 1997. A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. Science *275*:527-530.

Perotto, S.; VandenBosch, K.A.; Butcher, G.W. and Brewin, N.J. 1991. Molecular composition and development of the plant glycocalyx associated with the peribacteroid membrane of pea root nodules. Develop. *112*:763-776.

Perret, X.; Staehelin, C. and Broughton, W.J. 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. *64*:180-201.

Peters, N.K.; Frost, J. W. and Long, S.R. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science *233*:977-980.

Petrovics, G.; Putonky, P.; Reuhs, B.; Kim, J.; Thorp, T.A.; Noel, K.; Carlson, R.W. and Kondorosi, Á. 1993. The presence of a novel type of surface polysaccharide in *Rhizobium meliloti* requires a new fatty acid synthase like gene cluster involved in symbiotic development. Mol. Microbiol. *8*:1083-1094.

Phillips, D.A.; Joseph, C.M. and Maxwell, C.A. 1992. Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. Plant Physiol. *99*:1526-1531.

Pittard, A.J. 1996. Biosynthesis of the aromatic amino acids. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, vol. I. Neidhardt, F.C. and nine other authors. ASM Press, Washington, D.C., pp:458-484.

Pollock, T.J.; van Workum, W.A.T.; Thorne, L.; Mikolajczak, M.J.; Yamazaki, M.; Kijne, J.W. and Armentrout, R.W. 1998. Assignment of biochemical functions to glycosyl transferase genes which are essential for biosynthesis of exopolysaccharides in *Sphingomonas* strain S88 and *Rhizobium leguminosarum*. J. Bacteriol. *180*:586-593.

Postgate, J.R. 1982. The fundamentals of nitrogen fixation, Cambridge University Press, Cambridge, United Kingdom.

Prasad, C.K.; Vineetha, K.E.; Hassani, R.; Gupta, R. and Randhawa, G.S. 2000. Isolation and symbiotic characterization of aromatic amino acid auxotrophs of *Sinorhizobium meliloti*. Ind. J. Exp. Biol. *38*:1041-1049.

Priefer, U.B. 1989. Genes involved in lipopolysaccharide production and symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* biovar *viciae* VF39. J. Bacteriol. *171*:6161-6168.

Putnoky, P.; Kiss, G.B.; Ott, I. and Kondorosi, Á. 1983. Tn5 carries a streptomycin resistance determinant downstream from the kanamycin resistance gene. Mol. Gen. Genet. *191*:288-294.

Reid, C.J. and Poole, P.S. 1998. Roles of DctA and DctB in signal detection by the dicarboxylic acid transport system of *Rhizobium leguminosarum*. J. Bacteriol. *180*:2660-2669.

Reigh, G. and O'Connell, M. 1993. Siderophore-mediated iron transport correlates with the presence of specific iron-regulated proteins in the outer membrane of *Rhizobium meliloti*. J. Bacteriol. *175*:94-102.

Renalier, M.H.; Batut, J.; Ghai, J.; Terzaghi, B.; Gherardi, M.; David, M.; Garnerone, A.-M.; Vasse, J.; Truchet, G.; Huguet, T. and Boistard, P. 1987. A new symbiotic cluster on the pSym megaplasmid of *Rhizobium meliloti* 2011 carries a functional *fix* gene repeat and a *nod* locus. J. Bacteriol. *169*:2231-2238.

Reuhs, B.L.; Carlson, R.W. and Kim, J.S. 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-*D*-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. J. Bacteriol. *175*:3570-3580.

Reuhs, B.L.; Geller, D.P.; Kim, J.S.; Fox, J.E.; Kolli, V.S. and Pueppke, S.G. 1998. *Sinorhizobium fredii* and *Sinorhizobium meliloti* produce structurally conserver lipopolysaccharides and strain-specific K antigens. Appl. Environ. Microbiol. *64*:4930-4938.

Reuhs, B.L.; Williams, M.N.V.; Kim, J.S.; Carlson, R.W. and Cóté, F. 1995. Suppression of the Fix$^-$ phenotype of *Rhizobium meliloti exoB* mutants by *lpsZ* is correlated to a modified expression of the K-polysaccharide. J. Bacteriol. *177*:4289-4296.

Reznikoff, W.S. 1993. The Tn5 transposon. Annu. Rev. Microbiol. *47*:945-963.

Rioux, C.R; Jordan, D.C. and Rattray, J.B.M. 1986a. Iron requirement of *Rhizobium leguminosarum* and secretion of anthranilic acid during growth on an iron-deficient medium. Arch. Biochem. Biophys. *248*:175-182.

Rioux, C.R.; Jordan, D.C and Rattray, J.B.M. 1986b. Anthranilate promoted iron uptake in *Rhizobium leguminosarum*. Arch. Biochem. Biophys. *248*:183-189.

Ritsema, T.; Wijfjes, A.H.M.; Lugtenberg, B.J.J. and Spaink, H.P. 1996. *Rhizobium* nodulation protein NodA is a host-specific determinant of the transfer of fatty acids in Nod factor biosynthesis. Mol. Gen. Genet. *251*:44-51.

Ritz, D.; Thöny-Meyer, L. and hennecke, H. 1995. The *cycHJKL* gene cluster plays an essential role in the biogenesis of c-type cytochromes in *Bradyrhizobium japonicum*. Mol. Gen. Genet. *247*:27-38.

Rolfe, B.G. and Gresshoff, P.M. 1988. Genetic analysis of legume nodule initiation. Ann. Rev. Plant. Physiol. Plant Mol. Biol. *39*:297-319.

Rolfe, B.G.; Gresshoff, P.M. and Shine, J. 1980. Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci. Lett. *19*:277-284.

Rome, S.; Fernandez, M.P.; Brunel, B.; Normand, P. and Cleyet-Marel, J.-C. 1996. *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. Int. J. Syst. Bacteriol. *46*:972-980.

Ronson, C.W.; Lyttleton, P. and Robertson, J.G. 1981. C_4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. Proc. Natl. Acad. Sci. USA *82*:6231-6245.

Ronson, C.W.; Nixon, B.T.; Albright, L.M. and Ausubel, F.M. 1987. *Rhizobium meliloti ntrA* (*rpoN*) gene is required for diverse metabolic functions. J. Bacteriol. *169*:2424-2431.

Rosenberg, C.; Boistard, P.; Dénarié, J. and Casse-Delbart, F. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *R. meliloti*. Mol. Gen. Genet. *184*:326-333.

Rosendahl, L.; Vance, C.P. and Pedersen, W.B. 1990. Products of dark CO_2 fixation in pea root nodules support bacteroid metabolism. Plant Physiol. *93*:12-19.

Rovira, A.D. and Davey, C.B. 1971. Biology of the rhizosphere. In: The plant root and its environment. Carson, E.W. (ed.). University Press of Virginia. Charlottesville, pp:153-204.

Ruberg, S.; Pühler, A. and Becker, A. 1999. Biosynthesis of the exopolysaccharide galactoglucan in *Sinorhizobium meliloti* is subject to a complex control by the phosphate-dependent regulator PhoB and the proteins ExpG and MucR. Microbiol. *145*:603-611.

Ruvkum, G.B. and Ausubel, F.M. 1980. Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. USA *77*:191-195.

Ruvkun, G.B. and Ausubel, F.M. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature *289*:85-89.

Ruvkun, G.B.; Sundaresan, V. and Ausubel, F.M. 1982. Directed transposon Tn5 mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell *29*:551-559.

Sadowsky, M.J.; Keyser, H.H. and Bohlool, B.B. 1983. Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. Int. J. Syst. Bacteriol. *33*:716-722.

Sadowsky, M.J.; Rostas, K.; Sista, P.R.; Bussey, H. and Verma, D.P.S. 1986. Symbiotically defective histidine auxotrophs of *Bradyrhizobium japonicum*. Arch. Microbiol. *144*:334-339.

Saha, D. and Singh, A. 1999. Tn5 induced mutants of iron assimilation pathway in *Cicer Rhizobium* strain, COBE13. Ind. J. Exp. Biol. *37*:691-695.

Sambrook, J.; Fritsch, E.F.; and Maniatis, T. 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harber Laboratory Press, Cold Spring Harbor, New York.

Scharf, S.J.; Horn, G.T. and Erlich, H.A. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science *233*:1076-1078.

Scheres, B.; van Engelen, F.; van der Knaap, E.; van de Wiel, C.; van Kammen, A. and Bisseling, T. 1990. Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell *2*:687-700.

Scherrer, A. and Dénarié, J. 1971. Symbiotic properties of some auxotrophic mutants of *Rhizobium meliloti*. Plant Soil. Special volume:39-45.

Schiefelbein, J.W.; Masucci, J.D. and Wang, H. 1997. Building a root: The control of patterning and morphogenesis during root development. Plant Cell *9*:1089-1098.

Scholla, M.H. and Elkan, G.H. 1984. *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. Int. J. Syst. Bacteriol. *34*:484-486.

Schollhorn, R. and Burris, R.H. 1967. Study of intermediates in nitrogen fixation. Ferderation Proc. *25*:710.

Schubert, K.R. 1986. Products of biological nitrogen fixation in higher plants: Synthesis transport and metabolism. Annu. Rev. Plant Physiol. *37*:539- 574.

Schultze, M. and Kondorosi, Á. 1996. The role of lipochitooligosaccharides in root nodule organogenesis and plant cell growth. Curr. Opin. Genet. Dev. *6*:631- 638.

Schwedock, J. and Long, S.R. 1990. ATP sulphurylase activity of the *nodp* and *nodQ* gene products of *Rhizobium meliloti*. Nature *348*:644-647.

Schwinghamer, E.A. 1967. Effectiveness of *Rhizobium* as modified by mutation for resistance to antibiotics. Antonie van Leeuwenhoek *33*:121-36.

Schwinghamer, E.A. 1970. Requirement of riboflavin for effective symbiosis on clover by an auxotrophic mutant strain of *Rhizobium trifolii*. Aust. J. Biol. Sci. *23*:1187-1196.

Segovia, L.; Young, J.P.W. and Martínez-Romero, E. 1993. Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. *43*:374-377.

Selvaraj, G. and Iyer, V.N. 1983. Suicide plasmid vehicles for insetion mutagenesis in *Rhizobium meliloti* and related bacteria. J. Bacteriol. *156*:1292-1300

Sharma, S.B.; Khanuja, S.P.S.; Singh, A. and Kumar, S. 1993. Genetics and molecular biology of *Rhizobium*. Proc. Ind. Natn. Sci. Acad. *B59*:419-440.

Shaw, K.J. and Berg, C.M. 1979. *Escherichia* K12 auxotrophs induced by the insertion of the transposon element Tn5. Genetics *92*:741-747.

Sidloi-Lumbroso, R.; Kleiman, L. and Schulman, H. M. 1978. Biochemical evidence that leghaemoglobin genes are present in the soybean but not in the *Rhizobium* genome. Nature (London) *273*:558-560.

Simon, R.; Prriefer, U. and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in gram-negative bacteria. Biotech. *1*:784-791.

Singh, A.; Ram, J.; Sikka, V.K. and Kumar, S. 1984. Derivation of marked strains in *Rhizobium leguminosarum* RIdI by nitrosoguanidine and transposon mutagenesis. Ind. J. Exp. Biol. *22*:239-247.

Skorupska, A.; Derylo, M. and Loriewicz, Z. 1989. Siderophore production and utilization by *Rhizobium trifolii*. Biol. Metals *2*:45-59.

Smit, G.; Logman, T.J.J.; Berrigter, M.E.T.I.; Kijne, J.W. and Lugtenberg, B.J.J. 1989. Purification and partial characterization of the *Rhizobium leguminosarum* biovar *viciae* Ca^{2+}-dependent adhesion, which mediates the first step in attachment of cells of the family Rhizobiaceae to plant root hair tips. J. Bacteriol. *171*:4054-4062.

Snell, F.D. and Snell, C.T. 1967. Colorometric methods of analysis including photometric methods. vol. IVA. D.van Nostrand Company Inc., London. pp:384.

So, J.-S.; Hodgson, A.L.M.; Haugland, L.; Leavitt, M.; Banfalvi, Z.; Nieuwkoop, A.J. and Stacy G.1987. Transposon induced symbiotic mutants of *Bradyrhizobium japonicum*: Isolation of two gene regions essential for nodulation. Mol. Gen. Genet. *207*:15-23.

So, J.-S.; Kim, W.-S. and Stacey, G. 2000. Molecular characterization of a gene region involved in lipopolysaccharide biosynthesis in *Bradyrhizobium japonicum*: Cloning, sequencing and expression of *rfaf* gene. FEMS Microbiol. Lett. *190*:109-114.

Sobral, B.W.S.; Honeycutt, R.J.; Atherly, A.G. and McClelland, M. 1991. Electrophoretic separation of the three *Rhizobium meliloti* replicons. J. Bacteriol. *173*:5173-5180.

Spaink, H.P. 1994. The molecular basis of the host specificity of the *Rhizobium* bacteria. Antonie van Leeuwenhoek *65*:81-98.

Spaink, H.P. 1996. Regulation of plant morphogenesis by lipo-chitin oligosaccharides. Crit. Rev. Plant Sci. *15*:559-582.

Spaink, H.P.; Okker, R.J.H.; Wijffelman, C.A.; Tak, T.; Goosen-de Roo, L.: Pees, E.; van Brussel; A.A.N. and Lugtenberg, B.J.J. 1989. Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. J. Bacteriol. *171*:4045-4053.

Spaink, H.P.; Sheeley, D.M.; van Brussel, A.A.N.; Glushka, J.; York, W.S.; Tak,T.; Geiger, O.; Kennedy, E.P.; Reinhold, V.N. and Lugtenberg, B.J.J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host-specificity of *Rhizobium*. Nature *354*:125-130.

Sprent, J.I. and Sprent, P. 1990. Nitrogen fixing organisms. Pure and applied aspects. Chapman & Hall, London.

Stokkermans, T.J.W. and Peters, N.K. 1994. *Bradyrhizobium elkanii* lipo-oligosaccharides signals induce complete nodule structure on *Glycine soja*. Siebold et Zucc. Planta *194*:413-420.

Streeter, J.G.1987. Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. Plant Physiol. *85*:768-773.

Streeter, J.G. 1991. Transport and metabolism of carbon and nitrogen in legume nodule. Advances in Bot. Res. *18*:130-187.

Streit, W.R.; Joseph, C.M. and Phillips, D.A. 1996. Biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *Rhizobium meliloti* 1021. Mol. Plant-Microbe Interact. *9*:330-338.

Summers, M.L.; Elkins, J.G.; Elliot, B. and McDermott, T.R. 1998. Expression and regulation of phosphate starvation inducible genes in *Rhizobium meliloti*. Mol. Plant-Microbe Interact. *11*:1094-1101.

Suneja, S.; Dhul, M. and Anand, R.C. 2000. Screening of *Rhizobium ciceri* for siderophore production and iron availability. Ind. J. Plant. Physiol. *5*:198-202.

Swamynathan, S.K.and Singh, A. 1992. *Rhizobium meliloti* purine auxotrophs are Nod^+ but defective in nitrogen fixation. J. Genet. *71*:11-21.

Swamynathan, S.K. and Singh, A. 1995. Pleiotropic effects of purine auxotrophy in *Rhizobium meliloti* on cell surface molecules. J. Biosci. *20*:17-28.

Tabor, C.W. and Tabor,H. 1985. Polyamines in microorganisms. Microbiol. Rev. *49*: 81-99.

Taté, R.; Cermola, M.; Riccio, A.; laccarino, M.; Merrick, M.; Favre, R. and Patriarca, E.J. 1999a. Ectopic expression of the *Rhizobium etli amtB* gene affects the symbiosome differentiation process and nodule development. Mol. Plant-Microbe Interact. *12*:515-525.

Taté, R.; Riccio, A.; Caputo, E.; Cermola, M.; Favre, R. and Patriarca, E.J. 1999b. The *Rhizobium etli trpB* gene is essential for an effective symbiotic interaction with *Phaseolus valgaris*. Mol. Plant-Microbe Interact. *12*:926-933.

Taté,R.; Riccio, A.; Caputo, E.; Iaccarino, M. and Patriarca, E.J. 1999c. The *Rhizobium etli metZ* gene is essential for methionine biosynthesis and nodulation of *Phaseolus vulgaris*. Mol. Plant-Microbe Interact. *12*:24-34.

Taté, R., Riccio, A.; Iaccarino, M. and Patriarca, E.J. 1997. A *cysG* mutant strain of *R. etli* pleiotropically defective in sulfate and nitrate assimilation. J. Bacteriol. *179*:7343-7350.

Taté, R.; Riccio, A.; Mirrick, M. and Patriarca, E.J. 1998. The *Rhizobium etli amtB* gene coding for an NH_4^+ transporter is down-regulated early during bacteroid differentiation. Mol. Plant-Microbe Interact. *11*:188-198.

Te-Chein, C.; Rupp, R.; Beck, S. and Orser, C.S. 1991. Proline auxotrophic and catabolic mutants of *Rhizobium leguminosarum* biovar *viciae* strain C1204b are unaffected in nitrogen fixation. FEMS Microbiol. Lett. *77*:299-302.

Thöny, B.; Fischer, H.M.; Anthamatten, D.; Bruderer, T. and Hennecke, H. 1987. The symbiotic nitrogen fixation regulatory operon (*fixRnifA*) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nifA*-independent type of activation. Nucleic Acids Res. *15*:8479-8499.

Truchet, G.; Débéllé, F.; Vasse, J.; Terzaghi, B.; Garnerone, A.-M.; Rosenberg, C.; Batut, J.; Maillet, F. and Dénarié, J. 1985. Identification of a *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. J. Bacteriol. *164*:1200-1210.

Truchet, G., Michel, M. and Dénarié, J. 1980. Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically-defective mutants of *Rhizobium meliloti*. Differnt. *16*:163-172.

Truchet, G.; Roche, P.; Lerouge, P.; Vasse, J.; Camut, S.; de Billy, F.; Promé, J.-C. and Dénarié, J. 1991. Sulfated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature *351*:670-673.

Tyerman, S.D.; Whitehead, L.F. and Day, D.A. 1995. A channel-like transporter for NH_4^+ on the symbiotic interface of N_2-fixing plants. Nature *378*:629-632.

Udvardi, M.K. and Day, D.A. 1997. Metabolite transport across symbiotic membranes of legume nodules. Annu. Rev. Plant Physiol. Plant Mol. Biol. *48*:493-523.

van Batenburg, F.H.D.; Jonker, R. and Kijne, J.W. 1986. *Rhizobium* induces marked root hair curling by redirection of tip growth: A computer stimulation. Physiol. Plant *66*:476-480.

van Berkum, P.; Beyene, D.; Bao, G.; Campbell, T.A. and Eardly, B.D. 1998. *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. Int. J. Syst. Bacteriol. *48*:13-22.

van Kammen, A. 1984. Suggested nomenclature for plant genes involved in nodulation and symbiosis. Plant Mol. Biol. Rep. *2*:43-45.

van Rhijn, P. and Vanderleyden, J. 1995. The *Rhizobium*-plant symbiosis. Microbiol. Rev. *59*:124-142.

van Workum, W.A.T.; Canter-Cremers, H.C.J.; Wijfjes, A.H.M.; van der Kolk, C.; Wijffelman, C.A. and Kijne, J.W. 1997. Cloning and characterization of four genes of *Rhizobium leguminosarum* bv. *trifolii* involved in exopolysaccharide production and nodulation. Mol. Plant-Microbe Interact. *10*:290-301.

Vasse, J.M. and Truchet, G.L. 1984. The *Rhizobium*-legume symbiosis: Observation of root infection by bright-field microscopy after staining with methylene blue. Planta *161*:487-489.

Vasse, J.; de Billy, F.; Camut, S. and Truchet, G. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. *172*:4295-4306.

Verma, D.P.S. 1992. Signals in root nodule organogenesis and endocytosis of *Rhizobium*. Plant Cell *4*:373-382.

Verma, D.P.S. and Long, S. 1983. The molecular biology of *Rhizobium*-legume symbiosis. Int. Rev. Cytol. 14 (Suppl.):211-245.

Verma, D.P.S.; Nash, D.T. and Schulman, H.M. 1974. Isolation and *in vitro* translation of soybean leghemoglobin mRNA. Nature (London) *251*:74-77.

Vincent, J.M. 1970. A manual for the practical study of root-nodule bacteria. International Biological Programme Handbook No.15. Blackwell Scientific Publications Ltd., Oxford.

Vineetha, K.E.; Vij. N.; Prasad, C.K.; Hassani, R. and Randhawa, G.S. 2001. Ultrastructural studies on nodules induced by pyrimidine auxotrophs of *Sinorhizobium meliloti*. Ind. J. Exp. Biol. *39*:371-377.

Wang, E.-T.; van Berkum, P.; Beyene, D.; Sui, X.H.; Dorado, O.; Chen, W.X. and Martínez-Romero, E. 1998. *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. Int. J. Syst. Bacteriol. *48*:687-699.

Wang, P.; Ingram-Smith, C.; Hadley, J.A. and Miller, K.J. 1999. Cloning, sequencing, and characterization of the *cgmB* gene of *Sinorhizobium meliloti* involved in cyclic β-glucan biosynthesis. J. Bacteriol. *181*:4576-4583.

Wang, Y.P.; Birkenhead, K.; Boesten, B.; Sundaram, M. and O'Gara, F. 1989. Genetic analysis and regulation of the *Rhizobium meliloti* genes controlling C_4-dicarboxylic acid transport. Gene *85*:135-144.

Watson, R.J. 1990. Analysis of the C_4-dicarboxylate transport genes of *Rhizobium meliloti*: Nucleotide sequence and deduced products of *dctA*, *dctB* and *dctD*. Mol. Plant-Microbe Interact. *3*:174-181.

Watson, R.J. and Rastogi, V.K. 1993. Cloning and nucleotide sequencing of *Rhizobium meliloti* aminotransferase genes: An aspartate amino transferase requried for symbiotic nitrogen fixation is a typical. J. Bacteriol. *175*:1919-1928.

Watson, R.J.; Chan, Y.-K.; Wheatcroft, R.; Yang, A.-F. and Han, S. 1988. *Rhizobium meliloti* genes required for C_4-dicarboxylate transport and symbiotic nitrogen fixation are located on a megaplasmid. J. Bacteriol. *170*:927-934.

Watson, R.J.; Rastogi, V.K. and Chan, Y.-K. 1993. Aspartate transport in *Rhizobium meliloti*. J. Gen. Microbiol. *139*:1315-1323.

Weibel, E.R. 1973. Stereologic techniques for electron microscopy. In: Principles and techniques of electron microscopy. Hayat, M. (ed.). von Nostrand Reinhold. New York, pp:237-296.

Wells, J.A.; Vasser, M. and Powers, D.B. 1985. Cassette mutagenesis : An efficient method for generation of multiple mutations at defined sites. Gene *34*:315-325.

Wells, S.E. and Kuykendall, L.D. 1983. Tryptophan auxotrophs of *Rhizobium japonicum*. J. Bacteriol. *156*:1356-1358.

Woronin, M.S. 1866. Über die bei der Schwarzerle (*Alnus glutinosa*) und bei der gewöhnlichen Gartenlupine (*Lupinus mutabilis*) auftretenden Wurzelanschwellungen. Mémoires de l' Academie Impériale des Sciences de St. Pétersbourg, VII Series, vol. X [cited by Perret *et al.* (2000)].

Xiu, Z.-L.; Zeng, A.-P.; and Deckwer, W.-D. 1997. Model analysis concerning the effects of growth rate and intracellular tyrptophan level on the stability and dynamics of tryptophan biosynthesis in bacteria. J. Biotech. *58*:125-140.

Xu, L.M.; Ge, C.; Cui, Z.; Li, J. and Fan, H. 1995. *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Int. J. Syst. Bacteriol. *45*:706-711.

Yadav, A.S.; Vashishat, R.K.; Kuykendall, L.D. and Hashem, F.M. 1998. Biochemical and symbiotic properties of histidine-requiring mutants of *Rhizobium leguminosarum* biovar *trifolii*. Lett. Appl. Microbiol. *1*:22-27.

Yamanaka, T. 1992. The biochemistry of bacterial cytochromes. Japan Scientific Societies Press, Tokyo.

Yanofsky, C. and Smith, O.H. 1962. Enzymes involved in the biosynthesis of tryptophan. Methods Enzymol. *5*:794-806.

Yarosh, O.K.; Charles, T.C. and Finan, T.M. 1989. Analysis of C_4-dicarboxylate transport genes in *Rhizobium meliloti*. Mol. Microbiol. *3*:813-823.

Yeoman, K.H.; Delgado, M.-J.; Wexler, M.; Downie, J.A. and Johnston, A.W.B. 1997. High affinity iron acquisition in *Rhizobium leguminosarum* requires the *cycHJKL* operon and the *feuPQ* gene products, which belong to the family of two-component transcriptional regulators. Microbiol. 143:127-134.

Yeoman, K.H.; May, A.G.; deLuca, N.G.; Stuckey, D.B. and Johnston, A.W.B. 1999. A putative ECF σ factor gene, *rpoI*, regulates siderophore production in *Rhizobium leguminosarum*. Mol. Plant-Microbe Interact. *12*:994-999.

Young, J.P.W. 1992. Phylogenetic classification of nitrogen-fixing organisms. In: Biological nitrogen fixation. Stacey, G.; Burris, R.H. and Evans, H.J. (ed.). Chapman & Hall. New York, pp:43-86.

Yurgel, S.; Mortimer, M.W.; Rogers, K.N. and Kahn, M.L. 2000. New substrates for the dicarboxylate transport system of *Sinorhizobium meliloti*. J. Bacteriol. *182*:4216-4221.

Zahran, H. H. 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol. Mol. Biol. Rev. *63*:968-989.

Zahran, H.H.; Ahmed, M.S. and Afkar, E.A. 1995. Isolation and characterization of nitrogen-fixing moderate halophilic bacteria from saline soils of Egypt. J. Basic Microbiol. *35*:269-275.

Zhan, H.; Lee, C.C. and Leigh, J.A. 1991. Induction of the second exopolysaccharide (EPSb) in *Rhizobium meliloti* SU47 by low phosphate concentrations. J. Bacteriol. *173*:7391-7394.

Zoller, M.J. and Smith, M. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. *100*:468-500.