GENETIC AND BIOCHEMICAL STUDIES ON BIOSYNTHESIS OF SOME AMINO ACIDS IN *RHIZOBIUM*

A THESIS

Submitted in fulfilment of the
requirements for the award of the degree
of
DOCTOR OF PHILOSOPHY
in
BIOSCIENCES AND BIOTECHNOLOGY

By

C. KRISHNA PRASAD

DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY
UNIVERSITY OF ROORKEE
ROORKEE-247 667 (INDIA)

SEPTEMBER, 1998

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"GENETIC AND BIOCHEMICAL STUDIES ON BIOSYNTHESIS OF SOME AMINO ACIDS IN *RHIZOBIUM*"**, in fulfilment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biosciences and Biotechnology** of the University is an authentic record of my own work carried out during a period from June 1994 to September 1998 under the supervision of **Dr. (Prof.) G.S. Randhawa.**

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

Ch. K. Prasad

Dated : **Sept. 16, 1998** **(C. KRISHNA PRASAD)**

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

G. S. Randhawa

Dated: **Sept. 16 , 1998**

(Dr. G.S. Randhawa)
Professor
Department of Biosciences &
Biotechnology
University of Roorkee
Roorkee - 247667
India

The Ph.D. Viva - Voce examination of ***C. Krishna Prasad***, Research Scholar, has been held on 22.9.99 .

G. S. Randhawa — Signature of Supervisor

Bm. M. J. Pant 22.09.99 — Signature of H.O.D.

Head
Deptt. of Biosciences and
Biotechnology
University of Roorkee
ROORKEE

Signature of External Examiner

ABSTRACT

The present work was taken up with an aim to focus on the influence of biosynthetic pathways of amino acids of *Rhizobium meliloti* on symbiotic nitrogen fixation. The objectives taken were generation of amino acid auxotrophs with defective symbiotic properties, their characterization and finding out the role of the intermediates of the affected biosynthetic pathways in normal symbiosis. To generate auxotrophs, random transposon mutagenesis of *R. meliloti* strain Rmd201 (Str^r derivative of AK631) was carried out with the help of transposon Tn5 delivery vector pGS9. Conjugations between *E. coli* strain WA803 (Met^-, Thi^-) harbouring the suicide plasmid pGS9 and *R. meliloti* Rmd201 yielded Tn5 induced kanamycin resistant transconjugants. Six thousand transconjugants were collected from 45 crosses, purified and screened on minimal medium for auxotrophs. The nutritional requirements of auxotrophs which failed to grow on minimal medium were determined on minimal medium supplemented with nutritional pools. Out of thirty auxotrophs obtained, ten aromatic amino acid auxotrophs - six tryptophan (VK1, H4, H6, VK15, VK28 and VK30), one phenylalanine (H38), one tyrosine (R1) and two *aro* auxotrophs requiring all three amino acids, viz., tryptophan, tyrosine and phenylalanine (VK18 and H5)- were chosen for further studies.

Biochemical characterization of tryptophan auxotrophs included intermediate feeding and intermediate accumulation studies, and cross-feeding assays. Based on the results of these experiments tryptophan auxotrophs were placed into three categories as follows :

(i) *trpE* mutants (H4, VK1 & H6) which grew on anthranilic acid and did

not accumulate any of the intermediates of the tryptophan biosynthetic pathway.

(ii) VK15, that grew on indole and accumulated anthranilic acid.

(iii) Tryptophan synthase mutants (VK28 & VK30) which grew only on minimal medium supplemented with tryptophan and accumulated anthranilic acid and indole glycerol phosphate.

The three *trp* mutants, viz., VK15, VK 28 and VK30 cross-fed the *trpE* mutants and VK28 & VK30 cross-fed VK15.

Symbiotic properties of aromatic amino acid auxotrophs were determined by inoculating them on alfalfa seedlings (*Medicago sativa* cv. T9) grown aseptically on nitrogen free agar slants. All auxotrophs induced nodulation but the mean shoot dry weight of the plants inoculated by these auxotrophs (except tyrosine auxotroph and tryptophan synthase mutants) were significantly less than the shoot dry weight of the parental strain inoculated plants indicating reduced nitrogen fixation abilities of these mutant strains. *trpE*, *aro* and *phe* mutants showed almost no nitrogen fixation (Fix$^-$), whereas VK15 was partially effective.

Early events in the nodulation process like root hair curling and infection thread formation were followed by observing the methylene blue stained root portions of alfalfa plants inoculated with the mutant strains. All mutants induced root hair curling and infection thread formation.

Nodules from six week old plants were fixed, post fixed and embedded in araldite epoxy resin. Semithin and ultrathin sections of the nodules were observed under light and transmission electron microscopes, respectively. Nodules elicited by Rmd201 contained four well formed zones: meristematic

region, infection zone, nitrogen fixation zone and senescence zone. TEM studies showed all stages of bacteroid development in these nodules. Tyrosine and tryptophan synthase mutants which were Fix^+ formed nodules whose histology resembled that of parental strain induced nodules in all aspects.

trpE and *aro* mutants formed nodules with unusual defects. The meristematic zone in these nodules was normal but the infection zone was very extensive and occupied most part of the nodule. The nitrogen fixing region was restricted to few layers at the base of the nodule. TEM studies revealed normal bacteroid development only in the nitrogen fixing zone. The bacteroids in the extended infection zone and in the zone between infection and nitrogen fixing zone showed no tendency towards maturation. The cytoplasm of these bacteroids was homogeneous unlike the bacteroids from the same zones of nodules induced by Rmd201 where heterogeneous cytoplasm was exhibited by the bacteroids.

VK15, which is a slightly leaky mutant, exhibited novel symbiotic defects. The plants inoculated with this strain were stunted but did not appear chlorotic presumably because traces of nitrogen fixation occurred. The nodules were elongate, cylindrical and slightly pinkish. Electron microscopic studies showed all stages of bacteroid development.

phe mutant induced nodules with normal meristematic and infection zones but the zone after infection region was very extensive. The cells in this region were studded with many amyloplasts. The nitrogen fixation zone was not observed. Bacterial release did occur in these nodules but soon after their release they degenerated.

The linkage of auxotrophy to Tn5 insertion was tested by transferring

the Tn5 inserted portion of the genome of the auxotrophs into another *R. meliloti* strain ZB555 with the help of plasmid pJB3JI. All Km^r transconjugants obtained showed the respective donor's auxotrophy. This confirmed that auxotrophy was due to Tn5 insertion. This also proved that no other independent Tn5 insertion occurred elsewhere in the genome of these auxotrophs.

The position of Tn5 insertion in VK15 was located using plasmid pJB3JI mediated mapping method. The Tn5 insertion in this mutant mapped at the *trp15* locus which has been reported earlier.

In order to find out the pleiotropic effects of Tn5 insertions, all auxotrophs were tested for cellulose fibrils, β-(1→2) glucans, acidic exopolysaccharides and lipopolysaccharides production and in every case their production seemed normal.

Dicarboxylic acids (malate, succinate, etc.) were effectively utilized by these auxotrophs confirming that the symbiotic lesions were not due to *dct* phenotype.

From the results it is obvious that a normal flow of metabolites through aromatic amino acid biosynthetic pathways is required for successful *R. meliloti*-alfalfa symbiosis. Tryptophan and tyrosine but not phenylalanine seem to be available to the bacteria *in planta*. Anthranilic acid appear to be required for proper nodule development. The fact that *aro* mutants resembled the *trpE* mutants rather than the *phe* mutant in their symbiotic defectiveness confirms this. Though anthranilic acid is required for symbiosis, a subsequent intermediate(s) in the tryptophan pathway also appear to influence nitrogen fixation process.

ACKNOWLEDGEMENTS

It is my privilege to have had the meaning of research exemplified to me by my mentor and supervisor, **Dr. G.S. Randhawa**, Professor, Department of Biosciences and Biotechnology, University of Roorkee, Roorkee, who through his supervision, constructive criticism, expert guidance and constant encouragement throughout has seen the completion of this work.

I express my sincere sense of gratitude and indebtness to Dr. B.M.J. Pereira, Head of the Department of Biosciences and Biotechnology, University of Roorkee, Roorkee and Prof. R. Barthwal, former H.O.D., for their useful discussions and providing me the required research facilities. I am also grateful to Prof. R.P. Mathur, Dr. R.P. Singh, Dr. R. Prasad, faculty members of this department for their valuable suggestions.

I wish to express my heartfelt veneration for Dr. Peter Putnoky, Biological Research Centre, Szeged, Hungary and Dr. A. Kondorosi, Director, Institut des Végtalés, CNRS, France for providing me the *Rhizobium* mapping strains and *Rhizobium* genomic library.

I am grateful to Dr. Rakesh Tuli, Deputy Director, NBRI, Lucknow; Dr. Aqbal Singh, Principal Scientist, Biotechnology Centre, IARI, New Delhi; Prof. (Retd) H.K. Das, Centre for Biotechnology, JNU, New Delhi for providing the necessary bacterial strains and plasmids.

I am deeply indebted to Prof. D.K. Maheswari, Department of Microbiology and Botany, Gurukul Kangri University, Hardwar and Dr. Purohit, Scientist, NEERI, Nagpur, for their valuable guidance.

I am short of words to express my tender feelings of gratitude for Mrs. Surinder Randhawa, and my colleague Ms. Vineetha and her husband Mr. Muralidharan who have given generously their time to help me in many ways. Here, I put on record my admiration for their assistance, support, without which the present work would not have seen the light of the day.

I am grateful to Dr. Taposh K. Das, Electron microscope facility, Deptt. of Anatomy, AIIMS, New Delhi for providing the EM facility. I am thankful to Mr. C.P. Sharma, Mr. Sandeep, Mr. Madan and Mr. Meharban Singh for their help in processing the samples for LM and TEM studies and in taking photographs.

I am deeply indebted to my seniors Dr. Umesh Kumar, Mr. Balyan, Dr. Manju Rani Agarwal, Dr. Abha Dubey for their valuable help and cooperation.

I wish to record my heartful thanks to my colleagues Ihsan, Basil, Hassan, Abbas, Raad, Dheeraj, Pradeep, Surabhi, Omvir, Raj Kumar and Sunita for providing me new ideas.

I owe a lot to my best friends duo Abdul Rafi Khwaja (B.D) and Neeraj Kumar Mittal who were always with me in difficult times.

I can not forget the helping hands of L. Ravi Kumar, M.N.V. Ravi Kumar, Tanveer, Prem, Bishwatosh, Bhowmik (Dada), Neeraj Vij, Pankaj Kumar, Suneel Chhatre, Mandal, Bhabani, D.P.S. Negi, R.M. Negi, Yoginder Kumar, Sanjay Jain, Piyush Kuchhal, Manoj Bansal, Naveen Srivastava, Sanjeet Dwivedi, Ashutosh and Aditya Gautam who helped me in many ways and with their good sense of humour made my life in Roorkee, a pleasant one.

My special word of thanks goes to Mr. Ved Pal Saini, Laboratory

Technician, for providing technical assistance and for the smooth progress of my work.

Financial assistance rendered by UGC, Govt. of India, is greatly acknowledged.

I know, no adequate way to express my indebtness to my beloved parents and sisters. Their love and affection - nothing short of a miracle, served as a dynamic impetus in spurring me on to greater self assurance. They have contributed much to the completion of this thesis. To all of them, goes my deepest sense of gratitude, love and thanks.

I gratefully acknowledge Mr. Kapil (Times Computers) for carefully typing this thesis.

Ch. K. Prasad

(C. KRISHNA PRASAD)

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS USED

a	=	amyloplast
bc	=	bacteroid
cw	=	cell wall
ec	=	empty cell
edm	=	electron dense material
er	=	endoplasmic reticulum
I	=	infection zone
is	=	intercellular space
it	=	infection thread
m	=	mitochondria
mg	=	milligram
ml	=	millilitre
n	=	nucleus
nm	=	nanometer
NZ	=	nitrogen fixation zone
pbm	=	peribacteroid membrane
phb	=	poly-β-hydroxybutyrate
r	=	rhizobia
rh	=	root hair
S	=	senescence zone
s	=	starch
sb	=	senescent bacteroid
sc	=	shepherd's crook
v	=	vacuole
VB	=	vascular bundle
vc	=	vesicle
µg	=	microgram
µm	=	micrometer

CONTENTS

Chapter 1

INTRODUCTION

Nitrogen, an important constituent of all living organisms, comprises 78% of the atmosphere. The atmospheric nitrogen occurs in gaseous form and being inert, is not available to most living organisms for use. Primary producers of the food chain, plants, can utilize only combined forms of nitrogen and it is their availability in the soil that limits the crop productivity. Industrially, nitrogen and hydrogen may be combined by the Haber-Bosch process to form ammonia which can be used as a fertilizer, either directly or after processing to produce other compounds such as urea. It has been estimated that to produce and deliver 1 kg of nitrogen fertilizer to farm 1.5 kg of fuel oil is required. Further, 50% of the applied fertilizer gets leached. This not only wastes energy and money but also leads to serious pollution problems, particularly in water supplies. In contrast, most nitrogen fixing organisms use light energy, directly or indirectly, to produce ammonia. The manufacture occurs *in situ* so there are no distribution costs. Further, biological nitrogen fixation may be tailored to the needs of the organism. Biological nitrogen fixation involves conversion of atmospheric nitrogen to ammonia by a group of prokaryotes called diazotrophs which include a small group of bacteria and blue green algae. The bacteria, on the basis of the extent of their association with plants, can be classified into three main groups, i.e., (i) Free living (*Klebsiella pneumoniae, Azotobacter vinelandii*), (ii) Associative (*Azospirillum spp.*) and (iii) Symbiotic (*Rhizobium meliloti, Bradyrhizobium japonicum, Frankia,* etc.). Rhizobia are of special significance to agriculture, since they contribute as much as 25% of the terrestrial fixed nitrogen in association with legumes.

Rhizobia enter into a symbiotic relationship with legume plants. This is a complex relationship in which free-living bacteria induce root nodules,

invade them, transform into bacteroids and fix nitrogen. Recent developments in recombinant DNA technology made it easy to characterize most of the rhizobial genes involved in this complex process. These include genes governing nodule formation (*nod*) (Kondorosi *et al.*, 1984; Fisher and Long, 1992), nodule invasion (*exo, ndv*) (Dylan *et al.*, 1986; Geremia *et al.*, 1987; Charles *et al.*, 1991; Leigh and Coplin, 1992; Leigh and Walker, 1994) and nitrogen fixation (*nif, fix*) (Forrai *et al.*, 1983; David *et al.*, 1988; Putnoky *et al.*, 1988; Batut *et al.*, 1989).

Nutritional conditions in the nodule or in the developing bacteroid affect development and nitrogen fixation. Bacteroid utilization of C_4-dicarboxylic acids, derived from plant photosynthate is essential for symbiotic nitrogen fixation (Watson *et al.*, 1988) but it is unknown what other metabolites bacteria need or are provided by the host *in planta*. The role of amino acids is less clear as auxotrophs for certain amino acids (glutamate, serine, glycine, glutamine, etc.) are not affected in nitrogen fixation (Kerppola and Kahn, 1985; de Bruijn *et al.*, 1989) whereas auxotrophs for asparagine, leucine, tyrosine and anthranilic acid yielded ineffective nodules (Kerppola and Kahn, 1988; Barsomian *et al.*, 1992). *Rhizobium meliloti ilvC* mutants (Aguilar and Grasso, 1991) and histidine auxotrophs of *Bradyrhizobium japonicum* (Sadowsky *et al.*, 1986) did not form nodules on their respective host plants. In most of the cases it is unknown whether the symbiotic defectiveness was merely due to unavailability of that particular amino acid to the bacteria *in planta* or due to role of intermediates in symbiosis. In most of the cases, ineffective nodules formed by the auxotrophs were not subjected to microscopy, and it is unclear if the symbiotic defect was due to lack of bacterial colonization of the nodule or due to a defect in

later steps of bacteroid development. Considering the above facts the study on the biosynthetic pathways of amino acids in *Rhizobium meliloti* was undertaken with the following objectives:

a) Generation and biochemical characterization of symbiotically defective auxotrophs.

b) Detailed study of symbiotic properties of the isolated auxotrophs including observation of early nodulation events, and light and electron microscopic studies of nodules.

c) Investigation of the role(s) of amino acids or their intermediates in symbiosis.

d) Mapping of the genes involved in the biosynthesis of amino acids of interest.

Chapter 2

Literature Review

Rhizobia are symbiotic nitrogen fixing bacteria which are able to form and invade nodules on the roots of leguminous plants. To date four genera of rhizobia have been well characterized: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Sinorhizobium*. Taxonomically *Rhizobium* appears more closely related to *Agrobacterium* than to *Bradyrhizobium*, while *Azorhizobium* lies between *Bradyrhizobium* and *Xanthobacter*.

2.1 Development of nodules and nitrogen fixation

2.1.1 Signals from plant to bacterium

Rhizobia respond positively to exudates from plant roots (especially components of exudates like sugars, amino acids, etc.). Studies revealed that rhizobia show strong chemotaxis towards specific flavonoid compounds (Caetano-Anolles *et al.*, 1988; Kape *et al.*, 1991) which are released by the roots of legumes (Maxwell and Philips, 1990). The *nodD* gene product (NodD) associates with the cytoplasmic membrane of rhizobia and interacts with flavonoids in root exudates (Schlaman *et al.*, 1989). Legume species exude a characteristic spectrum of flavonoid compounds and NodD proteins from different species of rhizobia recognize particular flavonoids preferentially. This molecular recognition is important in determination of host - *Rhizobium* specificity (Kondorosi *et al.*, 1989; McIver *et al.*, 1989). In presence of an appropriate flavonoid, NodD activates transcription of all other *nod* genes by binding to a region known as *nod* box (Fisher and Long, 1989).

2.1.2 Signals from bacterium to plant

R. meliloti bacteria respond to the plant flavonoids and secrete a product of *nod* genes, NodRm-1 which elicits root hair deformations (Lerouge *et al.*,

1990) and cortical cell divisions (Roche *et al.*, 1991). The *nodABC* genes appear to be functionally interchangeable among all species of *Rhizobium* and thus are called common *nod* genes. Other genes such as *nodFE*, *nodG*, *nodH*, and *nodL* are involved in specifying the type of host plant nodulated. Bacteria carrying mutations in these genes often show abnormalities in the root hair curling reactions elicited on their normal hosts and sometimes also infect hosts that would normally be unresponsive to them. For example, in *R. meliloti* mutations in *nodH* and *nodQ* lead to the production of signal substance (NodRm-2) that elicits root hair deformation on *Vicia* instead of alfalfa. NodRm-2 is structurally related to NodRm-1 but lacks the sulphate group at the reducing end of tetrasaccharide (Lerouge *et al.*, 1990) which indicates that host-specificity for alfalfa is determined by sulphation of NodRm-2 (Schwedock and Long, 1990). Although the signal molecules synthesized by the *nod* genes of other species of *Rhizobium* (Spaink *et al.*, 1989), *Bradyrhizobium* (Gottfert *et al.*, 1989) and *Azorhizobium* (Goethals *et al.*, 1990) have not yet been characterized, they are likely to be similar to those already described for *R. meliloti*.

2.1.3 Nodule development

In legumes, the region of the growing root that is most susceptible to *Rhizobium* infection is just behind the apical meristem at the site of emergence of root hairs (Bhuvaneswari *et al.*, 1981). In this region, there is induction of mitotic activity in the root cortex within 12-24 hrs. of inoculation with *Rhizobium* (Calvert *et al.*, 1984). Initially, the plane of cell divisions is oriented in such a way that the axis of new wall deposition is perpendicular to the longitudinal axis of the root. Subsequently, this nodule primordium

gives rise to an organized meristem consisting of a mass of small cells dividing in all planes.

The location of a nodule primordium in root cortex depends on the type of nodule formed by a particular plant (Newcomb, 1981). In general, 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 indeterminate cylindrical nodules and have a persistent apical meristem (Newcomb, 1976). This persistent activity of the meristem ensures nodule elongation since new cells are constantly added to the distal end of the nodule (Vasse *et al.*, 1990). While meristem is active, rhizobia are released from the infection threads into the nodule cell cytoplasm. In indeterminate nodules, the nodule growth and functioning occur simultaneously therefore and all developmental zones during differentiation can be observed in a single longitudinal section of nodule (Fig.1). On the other hand, in *Phaseolus* and *Lotus*, cortical cell divisions occur just beneath the epidermis (Mathews *et al.*, 1989). These cells are invaded by rhizobia before they become meristematic (Rolfe and Gresshoff, 1988), and therefore rhizobia can spread by dividing within the cytoplasm of host cells, which are themselves dividing. Thus the nodule meristem involves a mixture of infected and uninfected cells derived from the root cortex, together with uninfected cells derived from root pericycle. This form of nodule development results in the formation of spherical (determinate) nodules in which meristematic activity is only a transient phase.

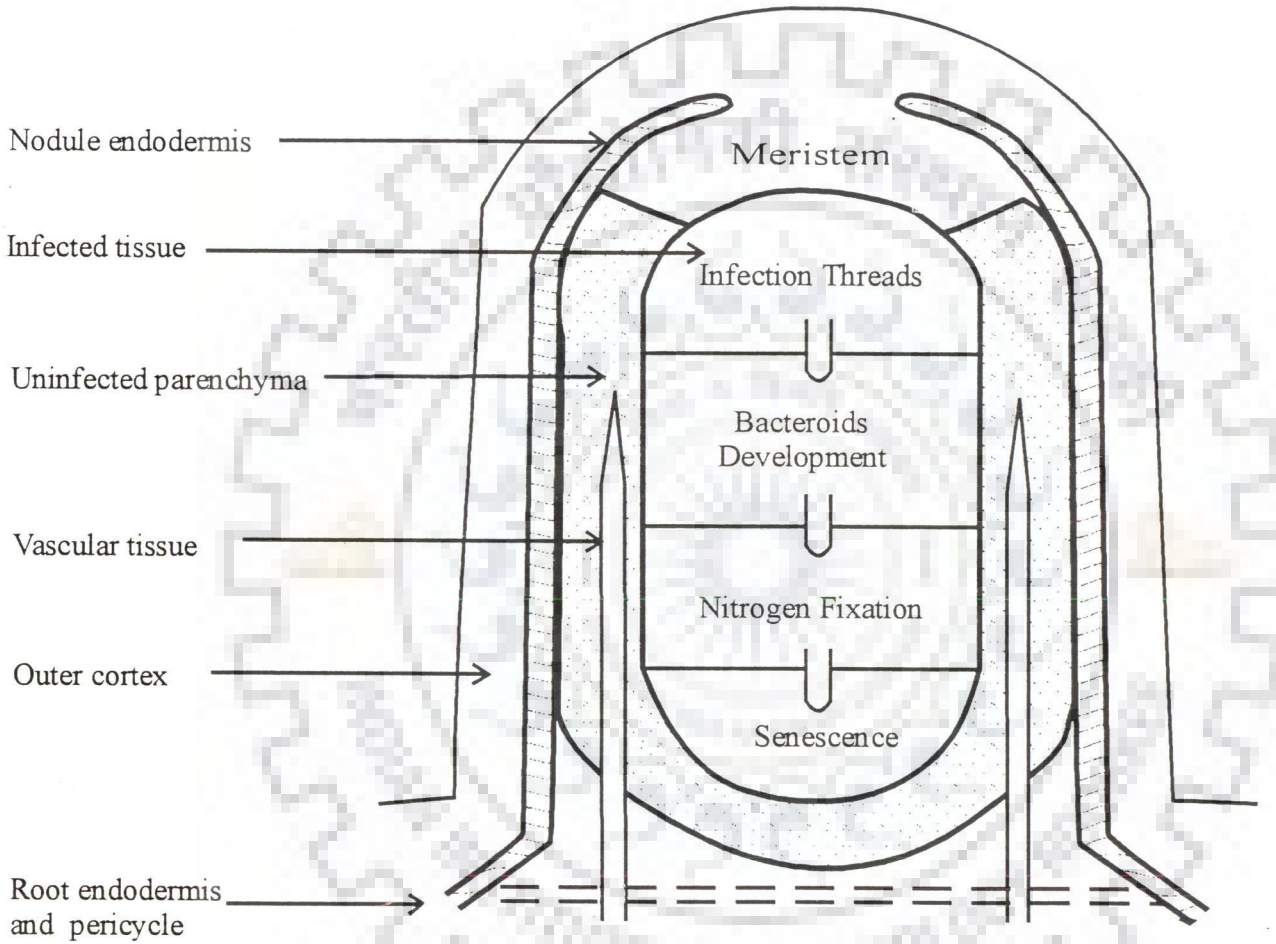

Fig. 1. Differentiation of cell types from the apical (uninfected) meristem of pea nodule. In the longitudinal axis of the nodule, the central mass of tissue shows successive stages of host cell invasion and differentiation by *Rhizobium* (after Brewin, 1991).

2.1.4 Nodule invasion

2.1.4.1 Role of the bacterial surface in early infection events

Cellulose fibrils produced by rhizobia may help to entangle the bacteria in the surface mucigel of the plant root and this binding may be enhanced by calcium-dependent proteins produced by rhizobia (Smit *et al.*, 1989). Thus *Rhizobium*-derived polysaccharides and proteins may be important in the extracellular environment.

The synthesis of cyclic β-(1→2) glucans by *Rhizobium* is also important for infection thread development, although in *Bradyrhizobium spp.* it appears that a β-(1→3), (1→6) glucan replaces the β-(1→2) glucan (Miller *et al.*, 1990). These periplasmic oligosaccharides may function in bacterial attachment or in osmotic adaptation (Dylan *et al.*, 1990a), but their exact role remains obscure (Dylan *et al.*, 1990b).

Mutants that lack extracellular polysaccharide (EPS) induce little or no invasion in indeterminate nodules. In *R. meliloti eps* mutants that fail to synthesize the normal acidic heteropolysaccharide (EPS I) can be complemented by an entirely different acidic heteropolysaccharide, termed EPS II (Glazebrook and Walker, 1989). Alternatively, a particular form of lipopolysaccharide specified by the *lpsZ* gene can substitute for the absence of EPS I and restore a symbiotically wild type phenotye (Williams *et al.*, 1990).

It is interesting that the symbiotic phenotype of extracellular polysaccharide-defective *Rhizobium* mutants differs, depending on whether the host plant gives rise to nodules with determinate or indeterminate morphology. On *Pisum, Medicago* and *Leucaena*, which all produce indeterminate nodules, *Rhizobium eps* mutants induce no nodule (Nod$^-$) or

empty nodules (Inf$^-$). The same or similar mutations do not, however, interfere with the development of determinate nodules on *Phaseolus*, *Glycine* and *Lotus* (Hotter and Scott, 1991). On the other hand lipopolysaccharide-defective mutants lacking O-antigen have a severe Inf$^-$ phenotype on *Phaseolus* and *Glycine*, which produce determinate nodules (Puvanesarajah *et al.*, 1987), but show impaired bacteroid release in peas (Brewin *et al.*, 1990) and no observed phenotype in alfalfa (Clover *et al.*, 1989), both of which produce indeterminate nodules. These differences probably reflect the fact that extracellular polysaccharide is more important for infection thread development and that the precise structure of lipopolysaccharide is more important for endocytosis and bacteroid differentiation.

2.1.4.2 Strategies of infections

In legume-*Rhizobium* symbiosis, there are three essential types of infection processes, i.e.,

(i) Infection thread entry via root hairs
(ii) Crack entry with no infection thread formation
(iii) Crack entry with infection thread formation

In the first type, bacteria gain access to the plant cortex via the infection thread which is made by the plant in response to rhizobia. 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, so that an outwardly growing cell wall cylinder is converted into an inwardly growing tunnel that follows the nucleus

towards the base of the root hair cell. Cytological examination of the apical growing tip of the uninfected root hair has revealed cytoskeletal connections between the nucleus and the growing root hair tip (Lloyd *et al.*, 1987). This infection thread grows between root cells and bacteria grow and multiply within its lumen. An extension of the plasmamembrane surrounds the infection thread. Therefore, the bacteria within the infection thread can still be considered to be topologically outside the plant cell though they are embedded in an extracellular matrix that contains plant made glycoproteins. This type of infection is observed in alfalfa, peas, soybean, etc.

In crack entry cases *Rhizobium* cells are thought to enter the plant tissue between cells at the base of multicellular root hairs which occur on emerging lateral roots. Although the details of actual infection process are not known, masses of bacteria enter the cortical cells of lateral roots and both the host cells as well as the membrane enveloped bacteria divide repeatedly to form nodules (Chandler *et al.*, 1982).

Third type of infection occurs in some rhizobia which invade certain plants of the non-legume group of *Parasponia*. Infection threads in this case ramify between and within plant cells setting up an extensive network of invaded tissue. Furthermore, these nodules are formed at the point of emergence of lateral roots.

2.1.4.3 Bacterial release and their differentiation to bacteroids

As infection thread approaches the region of the newly dividing nodule meristematic cells it branches and bifurcates, carrying the bacteria into many cells in developing nodules. Vesicles are then blebbed off into the plant

cell cytoplasm; each vesicle contains one bacterium surrounded by plant membrane. The bacteria are taken up into the cytoplasm by a process called phagocytosis. The bacteria, for sometime after release continue to divide and sometimes the growth is vigorous enough that multiple bacteria are seen bounded by a single peribacteroid membrane. The bacteria undergo important transformations and are known as bacteroids (Hennelke, 1990). 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, amyloplasts are pushed to the periphery.

2.1.5 Nitrogen fixation

The genes involved in nitrogen fixation are designated as *nif* and *fix*. *nif* genes are structural genes (*nifHDKE*) that encode nitrogenase enzyme and *fix* genes are those which do not have any homologous parts in *Klebsiella pneumoniae* but are important in symbiotic nitrogen fixation. In *R. meliloti*, two extremely large megaplasmids of about 1,400 kb (*pSym* a or megaplasmid 1) and 17,000 kb (*pSym* b or megaplasmid 2) are present (Banfalvi *et al.*, 1981; Hynes *et al.*, 1981; Rosenberg *et al.*, 1981; Burkhardt *et al.*, 1987; Sorbal *et al.*, 1991; Honeycutt *et al.*, 1993). The *nif* and *fix* genes of *R. meliloti* are organised in two distinct clusters whose structures and genomic locations are species specific; both cluster I (*nifHDKE, nif N, fixABCX, nifA, nifB*) and cluster II (*fixLJ, fixF, fixNOQP, fixGHIS*) are located on megaplasmid 1 (David *et al.*, 1987).

The fixed nitrogen produced in the bacteroids passes across the peribacteroid membrane, through the host cytoplasm and across the

plasmodesmata to the cytoplasm of adjacent uninfected cells. The fixed nitrogen enters the peroxisomes of these cells. Ureides, the form of nitrogen circulated in soybean (Newcomb and Kowal, 1985; Blevins, 1989) are synthesized and released into the cytoplasm. The ureides or asparagine (Blevins, 1989) can pass through successive uninfected cells and reach(es) vascular bundles at the periphery of the nodules, from where they can be circulated throughout the plant.

2.2 Metabolism of *Rhizobium* versus symbiosis

2.2.1 C_4-dicarboxylates transport mutants

Evidence uniquely implicating C_4-dicarboxylates (succinate, malate, etc.) as principle carbon and energy substrates provided by the plant to the mature bacteroids has come from the studies carried out on dicarboxylic acid transport (*dct*) mutants (Ronson *et al.*, 1981; Watson *et al.*, 1988; Engelke *et al.*, 1989; Jiang *et al.*, 1989; Wang *et al.*, 1989; Watson, 1990). The fact that the *dct* genes are present on one of the megaplasmids of *R. meliloti* (Engelke *et al.*, 1987; Finan *et al.*, 1988; Watson *et al.*, 1988) and are controlled by *ntrA* sigma factor (Ronson *et al.*, 1987) supports that these genes are essential for nitrogen fixation. *R. meliloti* mutants that are unable to grow on aspartate are symbiotically ineffective (Rastogi and Watson, 1991). It has been proposed that aspartate (also a dicarboxylate and requiring *dctA* functions for its uptake) rather than other dicarboxylates is the carbon and energy source to the bacteria *in planta*.

2.2.2 Auxotrophic mutants

The physiology of the symbiotic interactions between *Rhizobium* and the host legume was probed using auxotrophic mutants. Particularly, studies

on possible roles of specific bacteroid metabolites at various stages of symbiotic interaction were helped by the auxotrophic mutants and their prototrophic revertants.

Perhaps purine auxotrophs are the most affected in symbiosis. Purine auxotrophs of most species of *Rhizobium* are defective in symbiosis with their normal hosts (Scherrer and Dénarié, 1971; Pankhurst and Schwinghamer, 1974; Federov and Zaretskaya, 1979; Kerppola and Kahn, 1988; Kim *et al.*, 1988; Swamynathan and Singh, 1992). Externally supplemented adenine restored nodulation in some *ade* mutants of *R. leguminosarum* (Pankhurst and Schwinghamer, 1974) but addition of only an intermediate of adenine, i.e., 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) restored nodulation in *R. etli* (Newman *et al.*, 1994). In absence of AICAR usually pseudonodules resulted but addition of AICAR lead to proper nodulation, particularly infection process.

Auxotrophs of *R. meliloti* and *R. fredii* requiring both isoleucine and valine are nodulation defective (Denarie *et al.*, 1975; Kim *et al.*, 1988; Aguilar and Grasso, 1991). It has been found that the promoter of the common nodulation genes *nodABC* was not activated by luteolin in *R. meliloti ilvC* mutant (Aguilar and Grasso, 1991). Nodulation was not observed on supplementation of either isoleucine and valine or the intermediates. It was reasoned that the cellular concentrations of acetolactate in this *ilvC* mutant reaches levels that inhibit the expression of nodulation genes or antagonize the inducing effect of luteolin. An alternative hypothesis proposed by the same workers was that the product of *ilvC* gene also recognizes substrates other than those in the isoleucine-valine pathway and that the product of its conversion is required for the expression of nodulation genes.

Involvement of tryptophan biosynthetic pathway in nodulation and nitrogen fixation processes was demonstrated by Kummer and Kuykendall, 1989 and Barsomian *et al.*, 1992. Except tryptophan synthase mutants all other tryptophan auxotrophs of *B. japonicum* were found to be Nod$^-$. It seems that the bacterial synthesis of indole glycerol phosphate is necessary for nodulation in *Bradyrhizobium* (Kummer and Kuykendall, 1989). In *R. meliloti*-alfalfa symbiosis the picture is altogether different. All tryptophan auxotrophs (except *trpE*) of *R. meliloti* have been reported to be Nod$^+$, Fix$^+$; though *trpE* mutants were Nod$^+$, the nodules induced by them showed unusual defects in their histology and fixed no or less nitrogen (Barsomian *et al.*, 1992). During bacteroid transformation there is an increasing demand of iron. Anthranilic acid which can act as a siderophore may be coming to bacterial rescue in procuring iron for their transformation to bacteroids. Expression of *trpE* in wild type nodules confirmed this hypothesis (Barsomian *et al.*, 1992). Another interesting observation made on tryptophan pathway is that prototrophic revertants of a Nod$^-$ tryptophan auxotroph of *B. japonicum* showed enhanced nodulation and nitrogen fixation (Hunter and Kuykendall, 1990). The physiological basis for this increase in nodulation is not known, but enhanced tryptophan catabolism does not appear to be involved.

It appears that normal flux of metabolites through the aromatic biosynthesis pathway is essential for bacteroid development (Jelesko *et al.*, 1993). *R. meliloti* mutants isolated by these workers showed decreased deoxy arabino-heptonic acid-7-phosphate synthase (DAHP synthase) activity and formed ineffective nodules. Histology of nodules induced by these mutants showed extended invasion zones like the nodules induced by *trpE* mutants.

Interesting features of arginine biosynthetic pathway involvement in symbiosis was investigated by Kerppola and Kahn, 1988. Auxotrophs of *R. meliloti* blocked either in ornithine transcarbamylase or argino-succinate synthase are effective but mutants blocked in early part of arginine biosynthetic pathway that leads to ornithine are ineffective. It was proposed that ornithine is a precursor for polyamino synthesis and lack of polyamines directly lead to the symbiotic defect.

Leucine auxotrophs of *Rhizobium* are symbiotically ineffective (Truchet *et al.*, 1980; Kerppola and Kahn, 1988). The release of bacteria into nodule cells was not observed in these mutants. However, supply of leucine to the root environment restored symbiotic defectiveness (Truchet *et al.*, 1980).

Schwinghamer, 1970 and Pankhurst *et al.*, 1971 demonstrated the requirement of riboflavin for bacteroid development. The riboflavin requiring auxotroph failed to transform to bacteroids and thereby nodules were ineffective. When the plant growth medium was supplied with riboflavin bacteroid transformation occurred and even the removal of riboflavin later from the plant medium did not affect symbiosis.

Histidine auxotrophs of *B. japonicum* failed to induce nodules on their respective host plants (Sadowsky *et al.*, 1986; So *et al.*, 1987). Possibility of involvement of histidine biosynthetic pathway in nodulation has been proposed by Sadowsky *et al.*, 1986 as symbiotically competent as well as Nod$^-$ histidine auxotrophs were obtained. However, in these Nod$^-$ histidine auxotrophs external supplementation of histidine could restore nodulation.

2.3 Genetics of *Rhizobium*

2.3.1 Mutagenesis

To understand the complex set of interactions involved in the symbiosis, symbiotic mutants were generated through one or more of the following methods and studied.

2.3.1.1 Chemical mutagenesis

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

2.3.1.2 Random transposon mutagenesis

Suicidal plasmids carrying transposon Tn5 have been constructed by Beringer *et al.*, 1978; Selvaraj and Iyer, 1983 (Fig. 2); Simon *et al.*,1983, which were subsequently used for random transposon mutagenesis of *Rhizobium* (Meade *et al.*, 1981 and 1982; Forrai *et al.*, 1983; Sadowsky *et al.*, 1986; Barsomian *et al.*, 1992). In this, the mutagenesis was carried out by transferring a suicidal plasmid carrying transposon from *E. coli* to *Rhizobium* (Fig. 3). Transposon insertion into a gene leads to loss of its function (e.g. symbiotic function) and confers new phenotype to the mutant (antibiotic resistance) which makes its selection easy. Moreover, the transposon can be physically identified, either by restriction analysis or by DNA-DNA heteroduplexing. Many genes of symbiotic importance have been identified and studied (Noel *et al.*, 1982; Meade *et al.*, 1982; Forrai *et al.*, 1983; Hirsch *et al.*, 1984; Hom *et al.*, 1984; Leigh *et al.*, 1987).

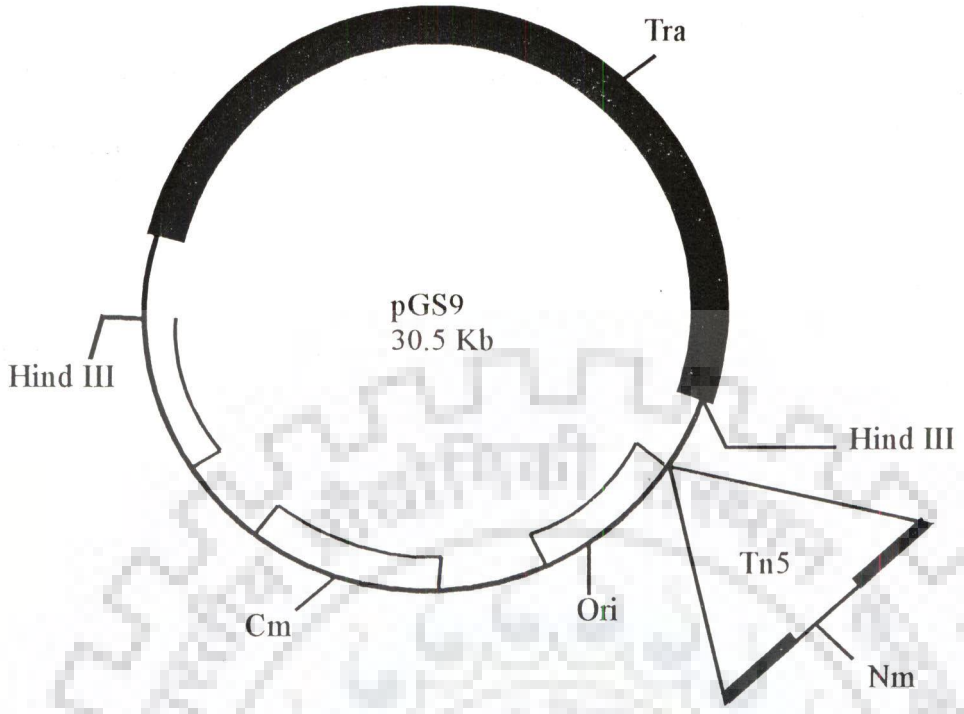

Fig. 2. Transposon Tn5-delivery vector pGS9. Abbreviations : Cm, Chloramphenicol; Nm, Neomycin; Ori, Origin of replication

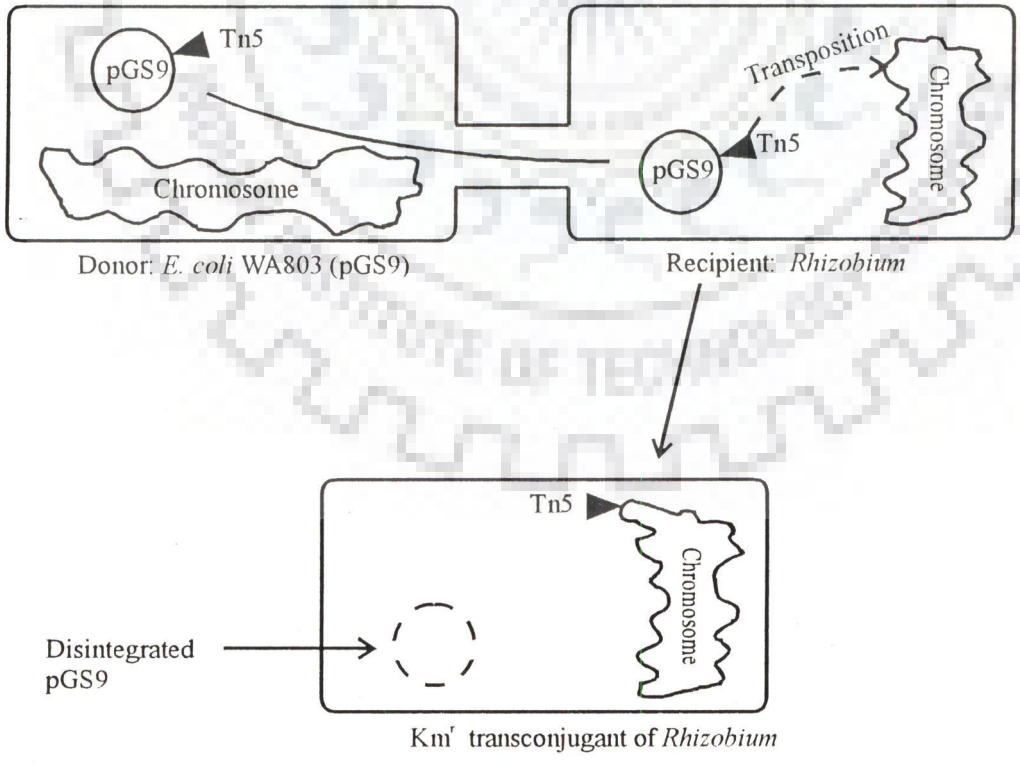

Fig. 3. Diagramatic representation of transposon Tn5 mutagenesis

2.3.1.3 Site directed mutagenesis

Ruvkun and Ausubel, 1981 extended the Tn5 mutagenesis methodology for *Rhizobium* by developing a general method for site-directed Tn5 mutagenesis. This involves the cloning of *Rhizobium* genes (*nif, nod* or *fix*) into multicopy plasmids, followed by Tn5 mutagenesis in *E. coli*, reintroduction of the Tn5-mutated sequences into *Rhizobium* and forced gene-replacement of the corresponding *Rhizobium* wild type sequences with the Tn5-mutated counterparts. Such analysis has made possible the combined physical and genetic characterization of additional *nod* and *nif* genes in a variety of *Rhizobium* species (Ausubel, 1982; Corbin *et al.*, 1983).

2.3.2 Mapping studies

Genetic studies of *Rhizobium* have been severely hampered by the lack of an indigenous conjugation system. Chromosomal transfer by conjugation allows rapid genetic mapping. The P1 incompatibility group of R plasmids have been shown to promote chromosome mobilisation in a number of gram-negative bacteria (Beringer and Hopwood, 1976; Kondorosi *et al.*, 1977; Meade and Signer, 1977). These plasmids generally do not promote transfer from single, distinct origins (Hass and Holloway, 1976; Meade and Signer, 1977) necessiating a measure of coinheritance frequencies (Kondorosi *et al.*, 1977; Beringer *et al.*, 1978a). Such a method has allowed the establishment of circular linkage maps of four nitrogen fixing *Rhizobium* strains. In all cases plasmids of the P1 incompatibility group were used for the mobilisation of the chromosome; plasmid RP4 for *R. meliloti* strain 2011 (Meade and Signer, 1977) and R68.45 for *R. meliloti* 41 (Kondorosi *et al.*, 1977), *R. meliloti* strain GR4 (Casadesus and Olivares, 1979) and

for *R. leguminosarum* 300 (Beringer and Hopwood, 1976; Beringer *et al.*, 1978b). Linkage maps of *R. meliloti* 2011, *R. meliloti* 41 (Fig. 4) and *R. leguminosarum* were compared using mapping function and by suppression tests of mutations (Kondorosi *et al.*, 1980).

A great deal of attention has been focussed on the two megaplasmids since they carry many of the genes required for symbiosis. For example, pRmeSU47a carries the *nod* genes required for nodule induction and the *nif* and *fix* genes which are required for nitrogen fixation (Batut *et al.*, 1985) while pRmeSU47b carries genes required for exopolysaccharide biosynthesis, dicarboxylic acid transport and bacteroid development (Charles and Finan, 1990). Both genetic and physical maps of the regions of pRmeSU47a carrying the *nod*, *nif* and *fix* loci have been published (Julliot *et al.*, 1984; Batut *et al.*, 1985; Swason *et al.*, 1987). A circular linkage map of pRmeSU47b (Charles and Finan, 1990), genetic and physical maps of the regions of the megaplasmid required for exopolysaccharide biosynthesis (Long *et al.*, 1988; Glazebrook and Walker, 1989) have been prepared.

With the advent of pulse field gel electrophoresis (PFGE), which is ideal for the separation of very large DNA restriction fragments on agarose gels, the physical mapping of bacterial genomes became much easier. Because of the strict correlation of physical and genetic maps of bacteria, physical maps aid in mapping new markers. A correlated physical and genetic map of the single, circular *B. japonicum* 110 chromosome was constructed (Kundig *et al.*, 1993). A total of 63 genes were placed precisely on this map by using genetically applicable mutagenesis techniques. The physical map of a 500 kb symbiotic plasmid from *Rhizobium spp.* strain

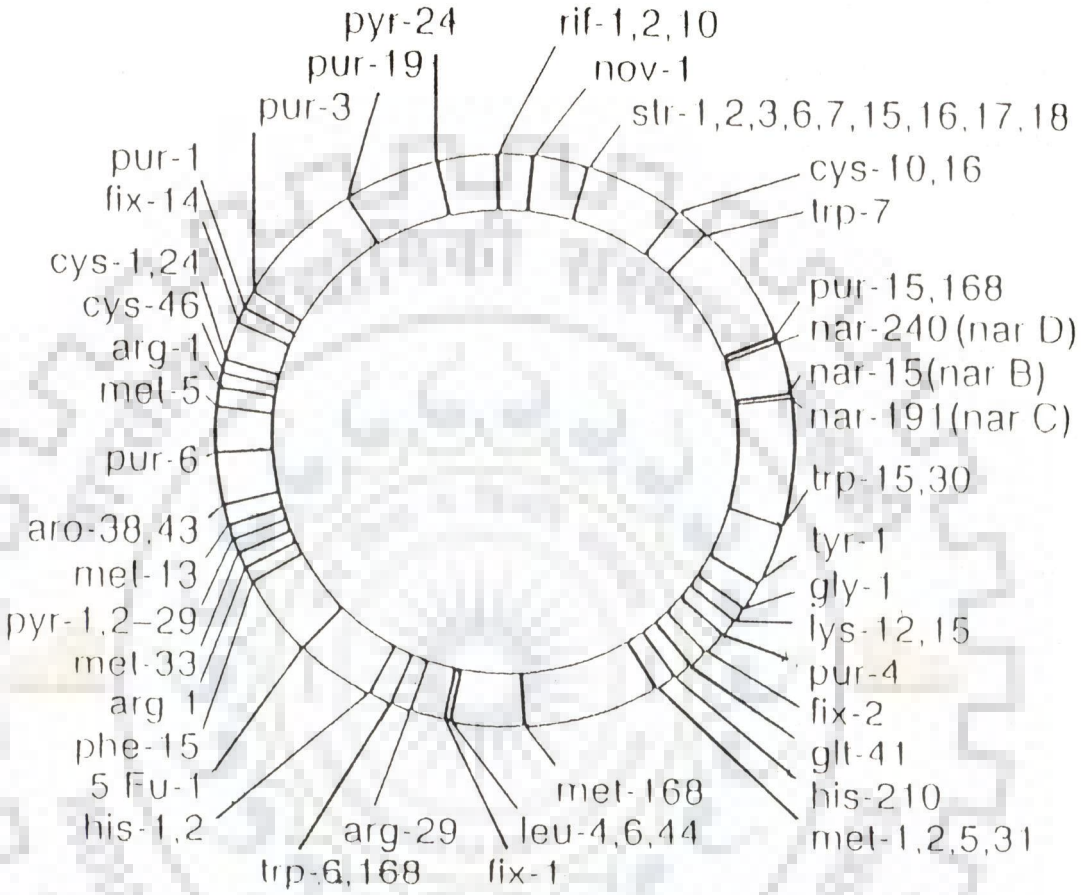

Fig.4. Chromosomal map of *Rhizobium meliloti* strain Rm41

NGR234 has been established (Perret *et al.*, 1991). Physical map of the genome of *R. meliloti*1021 including chromosome and two megaplasmids pRme1021a and pRme1021b were given by Honeycutt *et al.*, 1993. Recently complete sequencing of *Rhizobium* megaplasmid was done by Freiberg *et al.*, 1997.

Chapter 3

Materials And Methods

3.1 Materials

3.1.1 Bacterial strains

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

3.1.2 Plant cultivar

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

3.1.3 Growth media

3.1.3.1 Media for *R. meliloti*

3.1.3.1.1 Tryptone yeast extract medium (TY)

(Sikka and Kumar, 1984)

Constituent	Amount/litre
Tryptone	5 g
Yeast extract	3 g
$CaCl_2 . 2H_2O$	0.02 g
Water	to make 1 litre volume

The pH of the medium was adjusted to 7.0 with 0.1 N NaOH and to make solid medium 15 g of agar was added before autoclaving.

Table 1. Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant characteristics	Source / Reference
Rhizobium meliloti		
AK631	Nod$^+$, Fix$^+$, compact colony variant of wild type strain Rm41	Adam Kondorosi
Rmd201	Spontaneous Str^r derivative of AK631 (Nod$^+$, Fix$^+$)	Khanuja and Kumar, 1988
PP631	Rm41 (pJB3JI)	Peter Putnoky
ZB555	Rm41 *cys-46 phe-15 rif-1 str-1*	-do-
ZB556	Rm41 *gly-1 met-2 ade-4 str-1 rif-1*	-do-
ZB557	Rm41 *phe-15 leu-4 rif-1 str-1*	-do-
ZB205	Rm41 *ade-15 narB rif-1 5 fu^r*	-do-
VK1	Rmd201 *trpE*::Tn5	This study
VK15	Rmd201 *trp*::Tn5	-do-
VK18	Rmd201 *aro*::Tn5	-do-
VK28	Rmd201 *trp*::Tn5	-do-
VK30	Rmd201 *trp*::Tn5	-do-
H4	Rmd201 *trpE*::Tn5	Raad Hassani (This lab)
H5	Rmd201 *aro*::Tn5	-do-
H6	Rmd201 *trpE*::Tn5	-do-
H38	Rmd201 *phe*::Tn5	-do-
R1	Rmd201 *tyr*::Tn5	Ruma Gupta (This lab)
VK11	VK1 (pJB3JI)	This study
VK151	VK15 (pJB3JI)	-do-
VK181	VK18 (pJB3JI)	-do-
VK281	VK28 (pJB3JI)	-do-
VK301	VK30 (pJB3JI)	-do-
H41	H4 (pJB3JI)	-do-
H51	H5 (pJB3JI)	-do-
H61	H6 (pJB3JI)	-do-
H381	H38 (pJB3JI)	-do-
R11	R1 (pJB3JI)	-do-
Escherichia coli		
WA803 (pGS9)	Met$^-$ Thi$^-$ Cm^r Km^r	Selvaraj and Iyer, 1983
Plasmids		
pGS9	Inc N, rep P15A, Cm^r, Km^r	Selvaraj and Iyer, 1983
pJB3JI	Km^s derivative of pR68.45 capable of mobilising genomic segments of its host, Tc^r Cb^r Nal^r	Brewin *et al.*, 1980

3.1.3.1.2 Mannitol salts yeast extract medium (MSY)

(Khanuja and Kumar, 1989)

Constituent	Amount/litre
Mannitol	10 g
Yeast extract	1 g.
K_2HPO_4	0.2 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.8 mM
$CaCl_2.2H_2O$	0.4 mM
Water	to make 1 litre volume

The pH of the medium was adjusted to 6.8 with 0.1 N NaOH. To make solid medium 15 g of agar was added and autoclaved.

3.1.3.1.3 Rhizobium minimal medium (RMM)

(Khanuja and Kumar, 1989)

Constituent	Amount/litre
$Na_2HPO_4.12H_2O$	0.45 g
$(NH_4)_2SO_4$	2.0 g
$FeCl_3$	2.0 mg
$MgSO_4.7H_2O$	0.1 g
$CaCl_2.2H_2O$	0.04 g
Water	990 ml

20% glucose solution was prepared, filter sterilized and 10 ml from it was added to the autoclaved medium. The pH of the medium was adjusted to 7.0 with 0.01 N NaOH.

3.1.3.2 Medium for *E. coli*

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

Constituent	Amount/litre
Bactotryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g
Water	to make 1 litre volume

pH of the medium was adjusted to 7.0 with 0.01 N NaOH.

All growth media were autoclaved at 15 psi for 20 min. Wherever solid medium was used agar @ 15 g/litre was added before autoclaving.

3.1.3.3 Nitrogen free plant growth medium

The nitrogen free plant growth medium developed by Engelke *et al.*, 1987 was used for carrying out plant assays. Twelve stocks of following composition were prepared :

Solution	Salt	Amount of salt used (g)	Volume of water used (ml)
A	K_2HPO_4	2.0902	10
B	KH_2PO_4	0.544	10
C	$CaCl_2$	7.351	10
D	$C_6H_5O_7$ $Fe.3H_2O$	0.335	10
E	$MgSO_4$	6.162	10
F	K_2SO_4	4.3562	10
G	$MnSO_4$	0.034	20

Contd...

H	H_3BO_3	0.026	20
I	$ZnSO_4$	0.03	20
J	$CuSO_4$	0.002	20
K	$CaSO_4$	0.006	20
L	$NaMoO_4$	0.006	20

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

3.1.4 Supplements to media

3.1.4.1 Antibiotics

Antibiotics were dissolved as follows: Streptomycin sulphate (Himedia) and kanamycin acid sulphate (Sigma) in water; rifampicin (Sigma) in dimethyl sulphoxide, and tetracycline hydrochloride (Himedia) in 50% ethanol. The final concentrations of different antibiotics used in the media were as follows: Tetracycline (15 µg/ml), streptomycin (100 µg/ml), kanamycin (50 µg/ml for *E. coli* and 400 µg/ml for *R. meliloti*) and rifampicin (30 µg/ml).

3.1.4.2 Amino acids, nitrogenous bases and vitamins

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

To identify the nature of auxotrophy, Holliday pools (Holliday, 1956) with few modifications were used. The compositions of different pools were as follows:

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

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

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

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

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

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

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

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

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

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

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

3.1.4.3 Dicarboxylic acids

Maleic acid (Himedia) and sodium succinate (Himedia) were directly added to the medium @ 2 g/litre before autoclaving.

3.1.4.4 Intermediates

Anthranilic acid (Himedia), indole (Sisco) and shikimic acid (Sigma) were dissolved in water or dimethyl sulphoxide and added to the medium before plating.

3.1.4.5 Dyes

Calcofluor white (Sigma) and congo red (Himedia) were added to the medium at the rate of 0.02% before autoclaving. 0.01% methylene blue was used to stain infection threads in root hairs and 1% toluidine blue prepared in 1% borax was used for staining semithin sections for optical microscopy.

3.1.5 Reagents for intermediate accumulation studies

3.1.5.1 *p*-dimethyl amino benzaldehyde reagent (Snell and Snell, 1967)

6 g of *p*-aminobenzaldehyde was dissolved in 10 ml of water. Concentrated H_2SO_4 was added slowly to this mixture to make 100 ml volume.

3.1.5.2 Ferric chloride reagent (Yanofsky and Smith, 1962)

This reagent with the following composition was prepared and stored at room temperature in dark bottles.

1 ml of 0.5 M $FeCl_3$

50 ml of water

30 ml of Conc. H_2SO_4

3.1.6 Composition of solutions for light and electron microscopy of nodules of alfalfa

3.1.6.1 Preparation of blocks

(a) Karnovsky fixative (Karnovsky, 1965)

12.5 ml 8% paraformaldehyde (Sigma)

5.0 ml 12.5% glutaraldehyde (Sigma)

25.0 ml phosphate buffer

7.5 ml distilled water

(b) 0.2 M phosphate buffer

NaH_2PO_4 - 6.41 g

$Na_2HPO_4.7\ H_2O$ - 41.3 g

or

$Na_2HPO_4.12H_2O$ - 55.2 g

Double distilled water to make 1 litre.

(c) Post fixative (2% OsO_4)

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

(d) Araldite resin

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

Araldite Cy 212	-	10 ml
Hardener (Dodecenyl succinic anhydrite, DDSA; HY 964)	-	10 ml
Accelerator (Tridimethyl-amino-methyl phenol, DMP30; DYO64)	-	0.4 ml
Plasticizer (Dibutylphthalate)	-	1.0 ml

The ingredients were stirred vigorously to mix.

3.1.6.2 Solutions for staining ultrathin sections

(a) Uranyl acetate

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

(b) Lead citrate

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

3.2 Methods

3.2.1 General bacteriological procedures

3.2.1.1 Culturing of bacteria

A loopful of the bacterial culture was inoculated in 10 ml of TY (for *R. meliloti*) or LB (for *E. coli*) broth and the inoculated broth was kept in an orbital incubator shaker operating at 120 rpm. The temperatures set for *R. meliloti* and *E. coli* were 30°C and 37°C, respectively. 0.1 ml from the overnight grown culture was transferred to 10 ml of fresh broth and incubated for 8-10 hrs. for reculturing.

3.2.1.2 Serial dilutions

Physiological saline (0.9% NaCl) was used for carrying out serial dilutions. 0.1 ml from neat culture was added to 0.9 ml of saline for 10^{-1} dilution.

0.1 ml from 10^{-1} dilution was added to another tube containing 0.9 ml of saline to obtain 10^{-2} dilution. Similarly, further dilutions were made according to the need of the experiment.

3.2.2 Transposon mutagenesis

Log phase cultures of *R. meliloti* Rmd201 and *E. coli* WA803 were mixed in 2:1 ratio and 0.1 ml of this mixture was spread over half of the TY plate. The remaining half plate was divided into two parts, and 0.05 ml of Rmd201 culture into first part and 0.05 ml of WA803 culture into second part were spread as controls. Several such plates were prepared and incubated for 16-24 hrs. at 30°C. The growth obtained after incubation was scrapped and suspended in 1 ml of saline containing Tween 20 (0.02%). The suspension was thoroughly mixed with the help of a cyclomixer and 0.1 ml of suspension from each cross was spread on TY plates containing streptomycin (100 µg/ml) and kanamycin (400 µg/ml). In all matings, the donor and recipient suspensions were also spread on selective plates as controls. These plates were incubated for 3 to 5 days at 30°C. Transconjugants obtained after 3 to 5 days were streaked on TY medium containing streptomycin (100 µg/ml) and kanamycin (400 µg/ml). These streak purified Tn5 derivatives were screened for the auxotrophs.

3.2.3 Screening for auxotrophs

Transconjugants obtained by transposon Tn5 mutagenesis were patched on RMM and TY plates and incubated at 30°C for 2 to 3 days. Growth patterns were observed after every 6 hrs. to ensure the identification of all auxotrophs including the leaky ones. Mutants not growing on RMM plates were picked up as auxotrophs and saved for further characterization.

3.2.4 Determination of the nature of auxotrophy

To determine the nature of auxotrophic lesions, auxotrophs were streaked on nutritional pools and their growth patterns were observed over a period of 5 days at 30°C. Once an idea about the nature of auxotrophy was obtained, auxotrophs were streaked on minimal medium plates supplemented with suspected nutrients for confirmation.

3.2.5 Biochemical characterization of aromatic amino acid auxotrophs

3.2.5.1 Intermediate feeding studies

The tryptophan auxotrophs were streaked on RMM plates supplemented with anthranilic acid, indole or tryptophan (50 µg/ml each) and incubated at 30°C for 4-5 days.

RMM + shikimic acid (50 µg/ml) plates were used for streaking *aro* mutants which required all three aromatic amino acids, viz., tryptophan, tyrosine and phenylalanine. The growth on these plates was observed after an incubation period of 4-5 days at 30°C.

3.2.5.2 Intermediate accumulation studies

For intermediate accumulation studies a tryptophan auxotroph was grown in TY broth and the bacterial culture was centrifuged at 8,000 rpm for 10 min. The bacterial cells were washed twice with RMM, suspended in 10 ml of RMM and incubated. After an incubation period of 48 hrs. at 30°C the culture was centrifuged for 10 min. at 10,000 rpm. The supernatant was

a) Anthranilic acid and indole estimations

To 5 ml of supernatant 1 ml of *p*-dimethyl-aminobenzaldehyde reagent was added. The mixture was left undisturbed for 20 min. and then read at 420 nm for anthranilic acid and 550 nm for indole.

b) Indole glycerol phosphate

1.5 ml of ferric chloride reagent was added to 1 ml of sample and left for 5-10 min. The mixture was observed for appearance of pink to red colour.

3.2.5.3 Cross-feeding assays

Different combinations of tryptophan auxotrophs, two at a time were streaked on RMM medium containing limiting amounts of tryptophan (2 µg/ml) and incubated for 4-5 days at 30°C. It was observed that the strain that was being cross-fed showed growth nearer to the streak of the strain which was cross-feeding it.

3.2.6 Genetic experiments

3.2.6.1 Construction of donors

For checking linkage of Tn5 insertions to auxotrophy as well as for mapping experiments a donor strain was constructed for each auxotroph by introducing genome mobilising plasmid pJB3JI into it as follows: late log phase cultures of auxotroph and AK631 harbouring pJB3JI were taken, mixed in 1:1 ratio and spread on TY plate, and incubated at 30°C. After 18-24 hrs., growth was scrapped and suspended in 1 ml of saline. Serial dilutions were made upto 10^{-2} dilution. 0.1 ml from neat as well as from 10^{-1} and 10^{-2} dilutions were spread on TY plates containing kanamycin (400 µg/ml) and tetracycline (15 µg/ml) and incubated for 4 days at 30°C.

Out of several transconjugants obtained chromosome mobilising ability of five transconjugants was tested as described in the next section. The transconjugant showing maximum chromosome mobilising ability was used as a donor in the subsequent experiments. The donor strains constructed in this study are given in the Table 1.

3.2.6.2 Linkage of Tn5 insertions to auxotrophies

The donor strain of each auxotroph was conjugated on TY plates with the recipient *R. meliloti* strain ZB555. The mating mixture collected after a conjugation period of 24 hrs. at 30°C was plated on TY plates containing kanamycin (400 µg/ml) and rifampicin (40 µg/ml). The kanamycin resistant transconjugants obtained after 4-5 days of incubation period at 30°C were checked for donor's auxotrophy as described earlier.

3.2.6.3 Mapping experiments

The donor strain constructed for each auxotroph was conjugated with four *R. meliloti* recipient strains (ZB555, ZB556, ZB557 and ZB205) on TY medium for 24 hrs. at 30°C. The mating mixture was spread on TY plates containing kanamycin (400 µg/ml) and rifampicin (40 µg/ml). The coinheritance frequency or linkage of selected marker (Km^r phenotype) with different unselected markers was calculated and the distance between them was determined using the following formula:

$$c = (1-d)^3$$

where

c = linkage frequency of two markers

d = distance between them

3.2.7 Pleiotropic effects of Tn5 insertions

3.2.7.1 Dicarboxylic acid utilization

Auxotrophs were streaked on minimal medium supplemented with their nutritional requirements as well as maleic acid or sodium succinate as a sole carbon source. The plates were then incubated at 30°C. The growth was observed after 4-5 days of incubation period.

3.2.7.2 Production of cell surface molecules

(a) Test for the production of lipopolysaccharides (LPS)

The bacterial strains to be tested were streaked on TY plates containing sodium deoxycholate (1 mg/ml) and then incubated at 30°C for 2 to 3 days. Strains which showed growth on these plates were considered to be LPS producing.

(b) Test for production of cellulose fibrils and acidic exopolysaccharides

The production of cellulose fibrils and acidic exopolysaccharides in *Rhizobium* can be tested with dyes like congo red (Kneen and LaRue, 1983) and calcofluor white (Leigh *et al.*, 1987), respectively. Strains which produce cellulose fibrils form red colonies on congo red containing medium and fluoresce under long ultra violet light on calcofluor containing medium. MSY plates with congo red (0.02%) and with calcofluor white (0.02%) were prepared and the strains were streaked on these plates and incubated for 4 days at 30°C. Calcofluor plates were observed under long wave length ultra violet light whereas congo red plates were observed in normal light.

(c) Test for β-(1→2) glucans production

Motility of bacteria in swarm plates can be attributed to β-(1→2) glucans production (Geremia *et al.*, 1987). To test the production of β-(1→2) glucans in the aromatic amino acid auxotrophs, a drop of bacterial culture was spotted on a TY swarm plate (containing 0.3% agar) and incubated at 30°C for 2 days. Swarming of the bacterial strains was determined by the spread of the bacterial growth.

3.2.8 Methodology for plant experiments

3.2.8.1 Plant inoculation tests

For nodulation assays plant growth medium slants were prepared in 20 cm × 2.5 cm glass tubes. Alfalfa seeds were surface sterilized with 0.1% $HgCl_2$ for one minute followed by treatment with absolute alcohol for 30 seconds. The seeds were then rinsed four times with sterile distilled water and spread on 1% agar plates for germination. These plates were covered with black paper and incubated in dark at 25°C. Two days old seedlings were transferred to slants so that each tube received two seedlings.

Parental strain Rmd201 and aromatic amino acid auxotrophic strains of *R. meliloti* were cultured to log phase in 10 ml of TY broth. The culture was centrifuged at 5,000 rpm for 10 min. to obtain a pellet which, after washing with sterile distilled water, was suspended in 5 ml of sterile water. 0.5 ml of the suspension was dispensed into each tube containing two seedlings. Then tubes containing seedlings were kept under 2000 lux light with the roots of seedlings protected from direct exposure to light. A light period of 16 hrs., a dark period of 8 hrs. and 25°C temperature were maintained. The plants were collected after 6 weeks and data on mean shoot dry weight

and nodule characteristics were recorded. For determining mean shoot dry weight, plant top was harvested and dried in an oven at 65°C for 72 hrs. and then weighed.

3.2.8.2 Nodule occupancy tests

Nodule occupancy by the various strains was tested by reisolating rhizobia from the nodules and testing their auxotrophic and antibiotic markers. All the nodules from a plant were collected, surface sterilized, crushed in a drop of saline. The bacterial suspension thus obtained was diluted to obtain 10^{-1} and 10^{-2} dilutions. 0.1 ml from neat as well as each of the two dilutions was spread on RMM medium supplemented with respective auxotrophic requirement(s) and incubation was done at 30°C for 2-3 days. The colonies obtained were patched on RMM and RMM medium supplemented with kanamycin (400 µg/ml) and incubation was done as above at 30°C. The colonies which grew on RMM and did not grow on kanamycin supplemented medium were considered to be prototrophic revertants.

3.2.8.3 Light microscopy (LM) for observing infection thread formation

Six days after inoculation the root portions of plants to be examined were cut, stained in 0.01% methylene blue for 15 min., thoroughly washed in sterile water and placed on a clean slide. These slides were examined at 40× magnification under optical microscope (Leica *DM* LB).

3.2.8.4 Histology of nodules

3.2.8.4.1 Fixation

Nodules from plants inoculated with the parental strain and auxotrophs were excised and washed quickly in 2 to 3 changes of sterile distilled water

and dipped in Karnovsky fixative in 5 ml glass vials. Bigger nodules were cut longitudinally to 1 to 2 mm thickness and then placed in the fixative. To facilitate penetration additives like acrolein (at 0.1% final concentration) was added to the fixative. The vials were placed in vacuum chamber to prevent the material from floating on the surface. The fixation was carried at 4°C for 2 to 24 hrs.

3.2.8.4.2 Specimen block preparation for light and transmission electron microscopic studies

(a) Washing

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

(b) Post fixation

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

(c) Dehydration

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

30% acetone - 2 changes of 15 min. each

50% acetone - - do -

70% acetone - - do -

80% acetone - - do -

90% acetone - - do -

95% acetone - - do -

Dry acetone - 15 min.

The following step was carried at room temperature

Dry acetone - 2 changes of 30 min. each

(d) Clearing the tissues off acetone

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

(e) Infiltration and embedding

The following infiltration mixtures were prepared from the embedding medium.

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

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

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

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

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

3.2.8.4.3 Preparation of semithin sections for optical microscopy

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

3.2.8.4.4 Preparation of ultrathin sections

After scanning the sections under the optical microscope, the area to be examined under TEM was selected and the blocks were further hand trimmed. Ultrathin sections (60-90 nm thick) were cut using Ultracut E microtome (C.Reichert, austria). The silver coloured sections were lifted onto 200 mesh size copper grids. The grids were coated with 3% formvar (Polyvenyl formaldehyde) in ethylene dichloride and carbon by a process of evaporation under vacuum. After double coating, the formvar was dissolved either with ethylene dichloride or chloroform leaving behind the carbon film. To obtain a good contrast a double staining method using uranyl acetate and lead citrate was adopted. The grids carrying the sections were placed in uranyl acetate solution in a clean watch glass. A wooden or card-board cover was placed over the watch glass as the staining is effective when carried out in dark. The staining process was continued for 10 to 15 min. Then the grids were

washed in 2 lots of 50% ethanol and 2 lots of double distilled water with continuous agitation. The grids were then dried carefully on a filter paper and then stained with lead citrate for 5 to 10 min. After this, these were washed briefly in 0.1 N NaOH and then in 2 lots of double distilled water and dried. The dried grids were stored in grid holder at room temperature and viewed under TEM (Philips EM 300) and the areas of interest were photographed.

3.2.9 Statistical analysis

All the values were expressed as mean ± SEM (Standard error of the mean). Significance between controls vs experimental values was ascertained using student 't' test.

Chapter 4

RESULTS

Random transposon Tn5 mutagenesis of *Rhizobium meliloti* strain Rmd201 (Str^r derivative of AK631 which inturn is a compact colony variant of wild type strain Rm41) was carried out to generate auxotrophs. Out of thirty different auxotrophs generated, aromatic amino acid auxotrophs were selected for further studies. Biochemical and genetic characterization of these auxotrophs was done and their symbiotic properties were studied in detail, the results of which are presented here.

4.1 Transposon mutagenesis and isolation of auxotrophs

The suicide plasmid vector pGS9 used in this study successfully delivered the transposon Tn5 into *R. meliloti* Rmd201 genome and generated kanamycin resistant transconjugants at a frequency of 2×10^{-5} per recipient whereas the spontaneous kanamycin resistance in Rmd201 was $< 10^{-8}$. From 45 crosses six thousand transconjugants were collected and streak purified on TY medium containing streptomycin (100 µg/ml) and kanamycin (400 µg/ml). A loopful of cells from each streak was suspended in a drop of sterile saline and the cell suspension thus obtained was patched on minimal medium RMM and TY complete medium with sterile toothpicks. The bacterial growth was observed after 2 days of incubation at 30°C.

Thirty transconjugants which did not grow on RMM but grew on TY were selected as auxotrophs. Out of these thirty auxotrophs two were leaky as they grew slowly on RMM in comparison to the parental strain.

4.2 Nutritional requirements of auxotrophs

Holliday pools (Holliday, 1956) helped in determining the nutritional

requirements of auxotrophs. Out of thirty auxotrophs, three (VK4, VK5 and VK44) were auxotrophic for isoleucine and valine, eight (VK6, VK10, VK13, VK17, VK20, VK32, VK33 and VK42) for cysteine/methionine, five (VK21, VK29, VK31, VK36 and VK39) for methionine, four (VK1, VK15, VK28 and VK30) for tryptophan, one (VK18) for tryptophan, tyrosine and phenylalanine, four (VK27, VK37, VK38 and VK40) for adenine, one (VK41) for adenine and thiamine and three (VK12, VK19 and VK43) for uracil; the nutritional requirement(s) of one leaky mutant VK23 could not be determined. Tryptophan auxotroph VK15 was slightly leaky.

Preliminary plant inoculation tests with all the isolated auxotrophs showed interesting symbiotic properties of aromatic amino acid auxotrophs requiring either tryptophan, phenylalanine or tyrosine or all these three amino acids and hence these mutants were selected for further investigations. Five aromatic amino acid auxotrophs isolated by other workers in the lab were also included. Table 2 gives a list of aromatic amino acid auxotrophs chosen for the present studies.

4.3 Location of block in the biosynthetic pathway in each aromatic amino acid auxotroph

4.3.1 Intermediate feeding

The growth responses of tryptophan auxotrophs to supplementation of minimum medium with different intermediates of tryptophan are given in Table 3. Three tryptophan auxotrophs VK1, H4 and H6 grew on RMM supplemented with anthranilic acid, indole or tryptophan. VK15, which is a slightly leaky mutant, grew on indole or tryptophan but not anthranilic acid

Table 2. List of aromatic amino acid auxotrophs used in this study

S.No.	Strain	Auxotrophy
1.	VK1	Tryptophan
2.	VK15*	Tryptophan
3.	VK28	Tryptophan
4.	VK30	Tryptophan
5.	H4	Tryptophan
6.	H6	Tryptophan
7.	H38	Phenylalanine
8.	R1	Tyrosine
9.	VK18	Tryptophan+Tyrosine+Phenylalanine
10.	H5	Tryptophan+Tyrosine+Phenylalanine

* = Leaky mutant

supplementation whereas VK28 and VK30 grew only on RMM supplemented with tryptophan.

Two mutants, viz., VK18 and H5 which required the supplementation of RMM with all three aromatic amino acids for growth were streaked on RMM medium containing 50 µg/ml of shikimic acid and incubated for 4 days at 30°C. None of these two mutants could grow on this shikimic acid supplemented RMM medium.

4.3.2 Intermediate accumulation studies

Tryptophan auxotrophs, viz., H4, H6 and VK1 did not accumulate any of the tryptophan intermediates tested whereas VK15, VK28 and VK30 accumulated more than 15 µg/ml of anthranilic acid in minimal medium. VK28 and VK30 accumulated indole glycerol phosphate in addition to anthranilic acid (Table 4).

4.3.3 Cross-feeding assays

Three tryptophan auxotrophs VK1, H4 and H6 which grew on anthranilic acid supplemented RMM medium were cross-fed by VK15, VK28 or VK30. The mutants VK28 and VK30, which did not grow on indole supplemented RMM, cross-fed VK15. Cross-feeding among VK1, H4 and H6 and also among VK28 and VK30 was not observed.

Based on intermediate feeding, intermediate accumulation and cross-feeding studies the tryptophan auxotrophs were divided into three groups as follows:

(i) *trpE* mutants (H4, H6 and VK1) which grew on anthranilic acid supplemented RMM and did not accumulate anthranilic acid in RMM

Table 3. Growth responses of tryptophan auxotrophs to supplementation of anthranilic acid, indole and tryptophan in the minimal medium RMM

Strain	RMM	RMM supplemented with (50 µg/ml) of		
		Anthranilic acid	Indole	Tryptophan
VK1	–	+	+	+
H4	–	+	+	+
H6	–	+	+	+
VK15	–	–	+	+
VK28	–	–	–	+
VK30	–	–	–	+
Rmd201	+	+	+	+

+ = Growth

– = No growth

Table 4. Intermediate(s) accumulated by tryptophan auxotrophs in minimal medium RMM

Strain	Tryptophan intermediate		
	Anthranilic acid	Indole glycerol phosphate	Indole
VK1	–	–	–
H4	–	–	–
H6	–	–	–
VK15	+	–	–
VK28	+	+	–
VK30	+	+	–
Rmd201	–	–	–

+ = Accumulated

– = Not accumulated

(ii) VK15 which grew on indole supplemented RMM and accumulated anthranilic acid in RMM

(iii) Tryptophan synthase mutants VK28 and VK30 which grew only on tryptophan supplemented RMM and accumulated anthranilic acid and indole glycerol phosphate in RMM

Figure 5 shows the position of block in each of the aromatic amino acid auxotrophs in the biosynthetic pathways of aromatic amino acids.

4.4 Symbiotic properties of aromatic amino acid auxotrophs

4.4.1 Plant inoculation tests

The plants inoculated with the parental strain Rmd201, tryptophan synthase mutants VK28 and VK30 or tyrosine auxotroph R1 were healthy with no signs of nitrogen starvation. On the other hand plants inoculated with *aro* mutants H5 and VK18, *phe* auxotroph H38 or *trpE* mutants H4, H6 and VK1 appeared weak as compared to be parental strain inoculated plants and became chlorotic six weeks after inoculation. The plants inoculated with VK15 showed no shoot elongation four weeks after inoculation as a result of which they appeared stunted with shorter internodal regions (Plate 1).

The mean shoot dry weight of the plants inoculated with the parental strain or auxotrophic strains (except H38) was significantly more than the mean shoot dry weight of the uninoculated plants. Mean shoot dry weight of plants inoculated with VK28, VK30 or R1 did not differ significantly from the mean shoot dry weight of plants inoculated with Rmd201 whereas mean shoot dry weight of plants inoculated with *trpE* mutants VK1, H4 and H6, VK15, *aro* mutants H5 and VK18, and H38 were significantly less than the mean

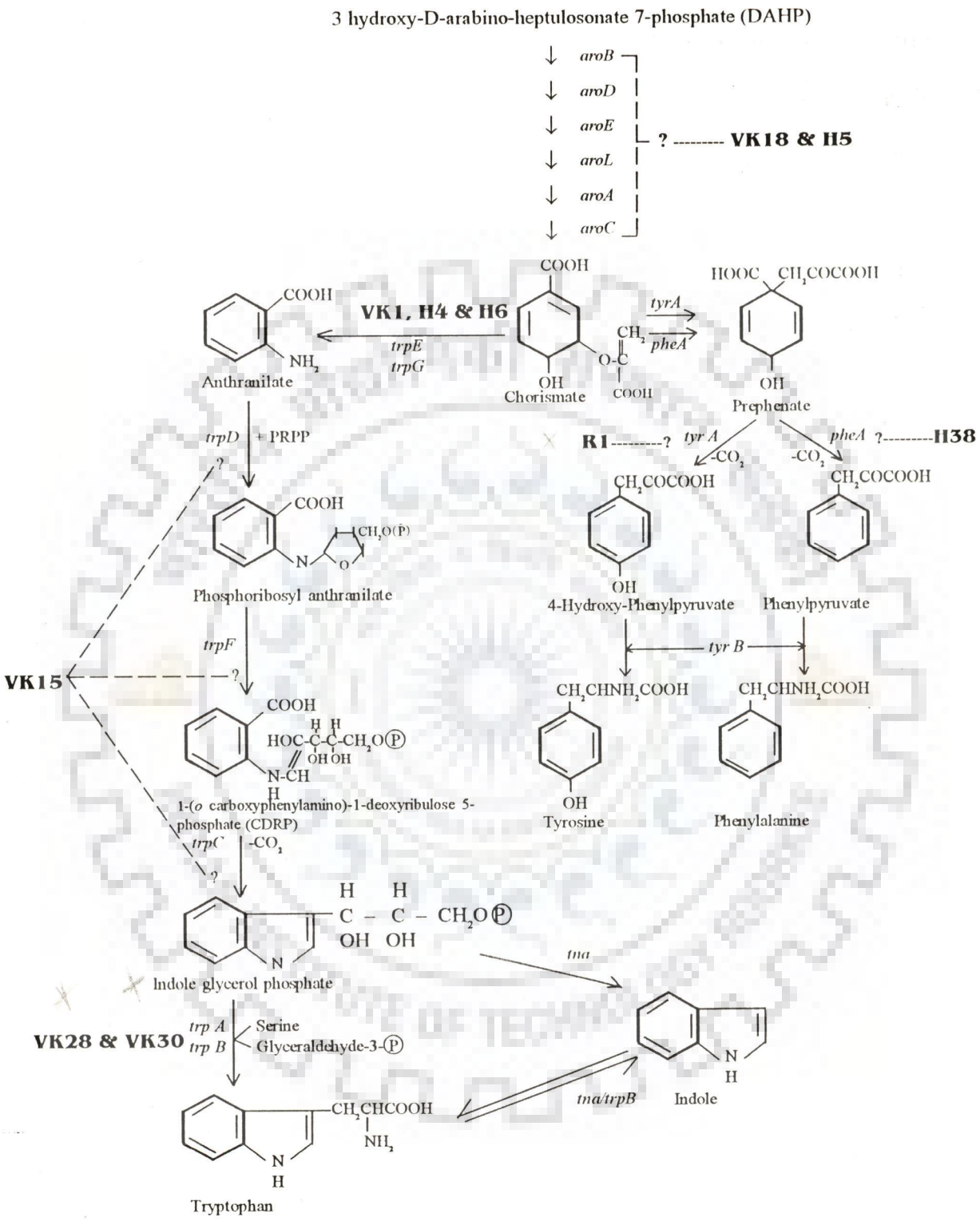

Fig. 5. The pathways of the synthesis of aromatic amino acids showing the positions of mutations in the auxotrophs obtained

Plate 1. Morphological features of representative plants of alfalfa inoculated with *Rhizobium meliloti* Rmd201 and its aromatic amino acid auxotrophic derivatives.

A. Rmd201 inoculated plant

B. *tyr* mutant R1 inoculated plant

C. *phe* mutant H38 inoculated plant

D. *aro* mutant inoculated plant

E. *trpE* mutant inoculated plant

F. VK15 inoculated plant

G. Tryptophan synthase mutant inoculated plant

shoot dry weight of plants inoculated with Rmd201. Mean shoot dry weight of VK15 inoculated plants was significantly less than the mean shoot dry weight of the parental strain inoculated plants but was significantly more than the mean shoot dry weight of the plants inoculated with *trpE* (VK1, H4 and H6), *aro* (VK18 and H5) and H38 mutants (Table 5).

Pink and cylindrical nodules were observed on roots of plants inoculated with Rmd201, tryptophan synthase mutants VK28 and VK30, or tyrosine mutant R1 whereas white and irregular nodules were observed on plants inoculated with phenylalanine auxotroph H38. Nodules on plants inoculated with *aro* mutants and *trpE* mutants were cylindrical and white whereas VK15 inoculated plants showed slightly pinkish and cylindrical nodules.

The nodules occupied by prototrophic revertants were always pink and cylindrical and the plants on which they were found showed mean shoot dry weight which did not differ significantly from the mean shoot dry weight of the parental strain inoculated plants.

4.4.2 Nodule occupancy tests

After recording the data on mean shoot dry weight and nodule characteristics of a particular plant bacteria were reisolated from nodules of this plant to test their authenticity. If the nodules were found to be occupied by the inoculated strain, the data on this plant was retained; in case prototrophic revertants were obtained the data was rejected.

4.4.3 Infection thread formation

Shepherd's crooks and infection threads were observed in root hairs of alfalfa plants inoculated with parental strain and aromatic amino acid

Table 5. Mean shoot dry weights and nodule characteristics of plants inoculated with aromatic amino acid auxotrophs

Strain	Mean shoot dry weight[a] (mg)	Nodule characteristics	
		Shape	Colour
Uninoculated control	6.2 ± 0.9	–	–
Rmd201	33.6 ± 2.5	Cylindrical	Pink
VK28	29.5 ± 3.2	Cylindrical	Pink
VK30	31.0 ± 2.7	Cylindrical	Pink
R1	28.7 ± 4.0	Cylindrical	Pink
VK1	11.8 ± 2.0 *	Cylindrical	white
H4	12.9 ± 1.6 *	Cylindrical	White
H6	12.0 ± 1.4*	Cylindrical	White
VK15	20.8 ± 1.2*	Cylindrical	Slightly pinkish
H5	11.2 ± 2.0*	Cylindrical	White
VK18	12.9 ± 1.9*	Cylindrical	White
H38	8.1 ± 1.5* †	Irregular	White

* = Significantly less than parental strain ($P < 0.05$)

† = No significant difference from uninoculated control

a = Each mean shoot dry weight value is a mean of eight plants

auxotrophs strains whereas the root hairs were straight and devoid of infection threads in case of uninoculated plants (Plate 2).

4.4.4 Histology of nodules

4.4.4.1 Light and electron microscopic studies of nodules induced by the parental strain Rmd201

A longitudinal cross section of nodule induced by Rmd201 (Plate 3: A) revealed four main zones: meristematic zone, infection zone, nitrogen fixation zone and senescence zone. Apical meristematic zone contained uninfected and constantly dividing plant cells. The infection zone lied just beneath the meristematic zone and the plant cells in this region contained large nucleus, many vacuoles and immature bacteroids (Plate 3: B). Few layers next to this zone constituted the interzone. Here the plant cells started getting filled with amyloplasts (Plate 3: C). The nitrogen fixation zone which formed the bulk of the nodule contained plant cells filled with mature bacteroids. The bacteroids in these plant cells were arranged perpendicular to the central vacuole (Plate 4: A). The senescence zone which occupied the basal portion of the nodule contained oldest nodule cells (Plate 4: B).

Electron microscopic studies showed that the released bacteria in the infection zone were electron dense (Plate 5: A). As a result of condensation of nucleic material the cytoplasm of the bacteroids started getting heterogenic in the infection region near to nitrogen fixation zone (Plate 5: B). The mature bacteroids in the nitrogen fixation zone exhibited more heterogeneous cytoplasm with electron dense and electron transparent regions (Plate 5: C). The plant cell organelles in the empty cells as well as

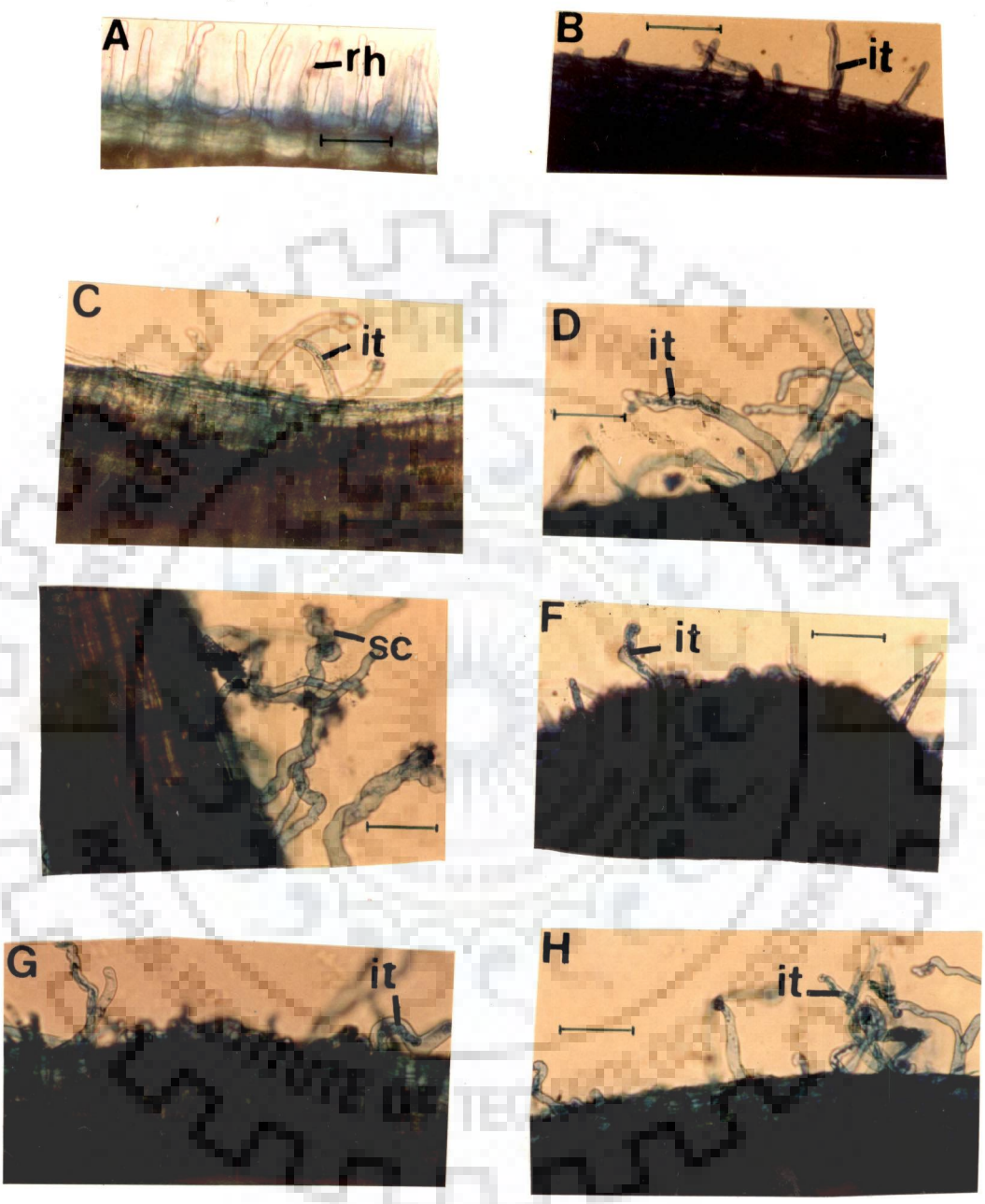

Plate 2. Root hair curling and infection thread formation in root hairs of alfalfa seedlings inoculated with *Rhizobium meliloti* Rmd201 and its aromatic amino acid auxotrophs. **A.** Uninoculated **B.** Rmd201 inoculated **C.** *tyr* mutant inoculated **D.** *phe* mutant inoculated **E.** *aro* mutant inoculated **F.** *trpE* mutant inoculated **G.** VK15 inoculated **H.** Tryptophan synthase mutant inoculated

Abbreviations: sc, shepherd's crook; it, infection thread; rh, root hair. Bar, 100 μm (×100).

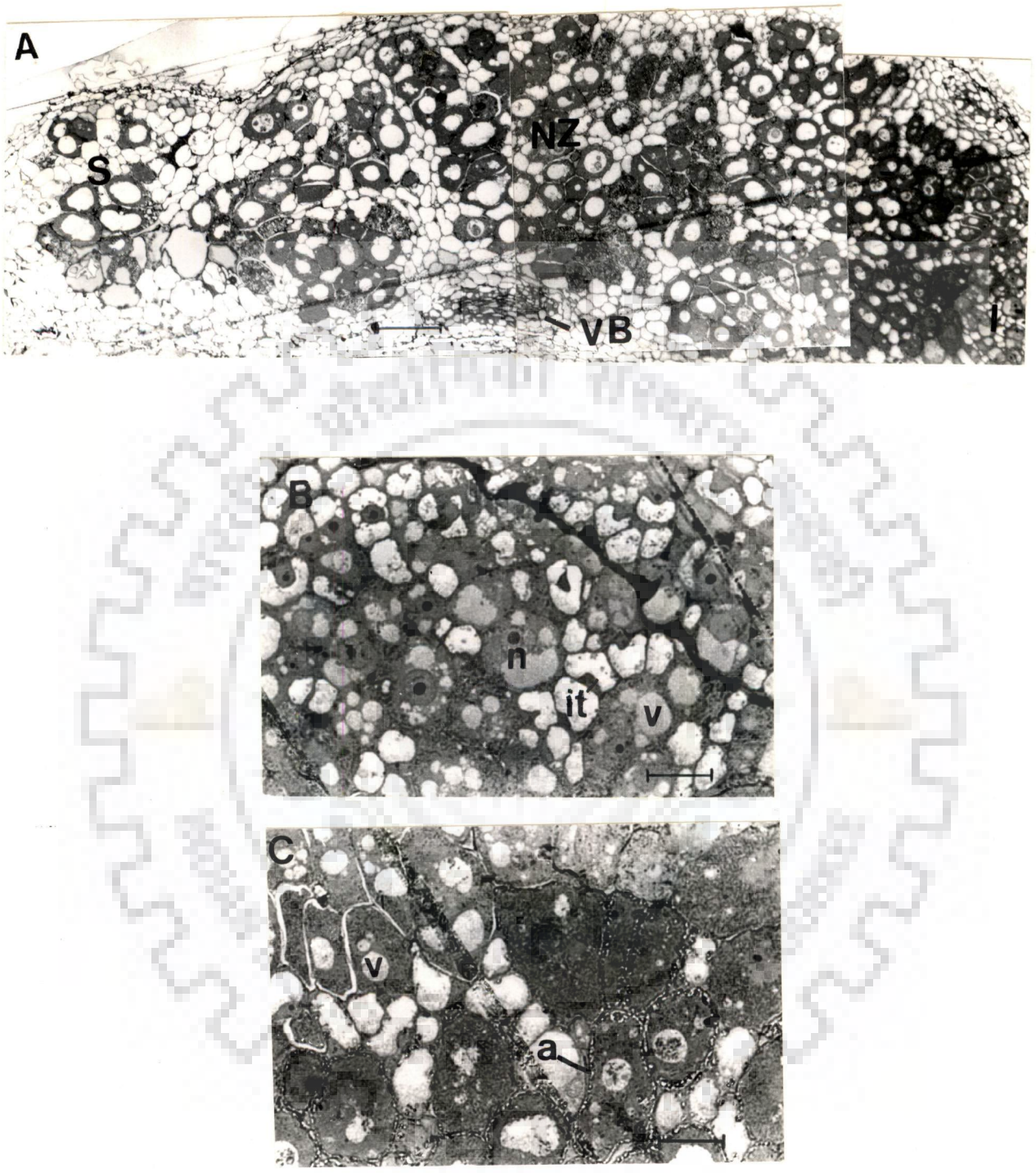

Plate 3. Light microscopic studies of semithin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** Longitudinal section of the whole nodule showing infection zone (I), nitrogen fixation zone (NZ), senescence zone (S) and peripheral vascular bundles (VB). Bar, 100 μm (×100). **B.** Infection zone showing cells containing infection threads (it), vacuoles (v) and prominent nucleus (n). Bar, 25 μm (×400). **C.** Zone between infection and nitrogen fixation zones. The cells in this zone are packed with amyloplasts (a). Bar, 25 μm (×400).

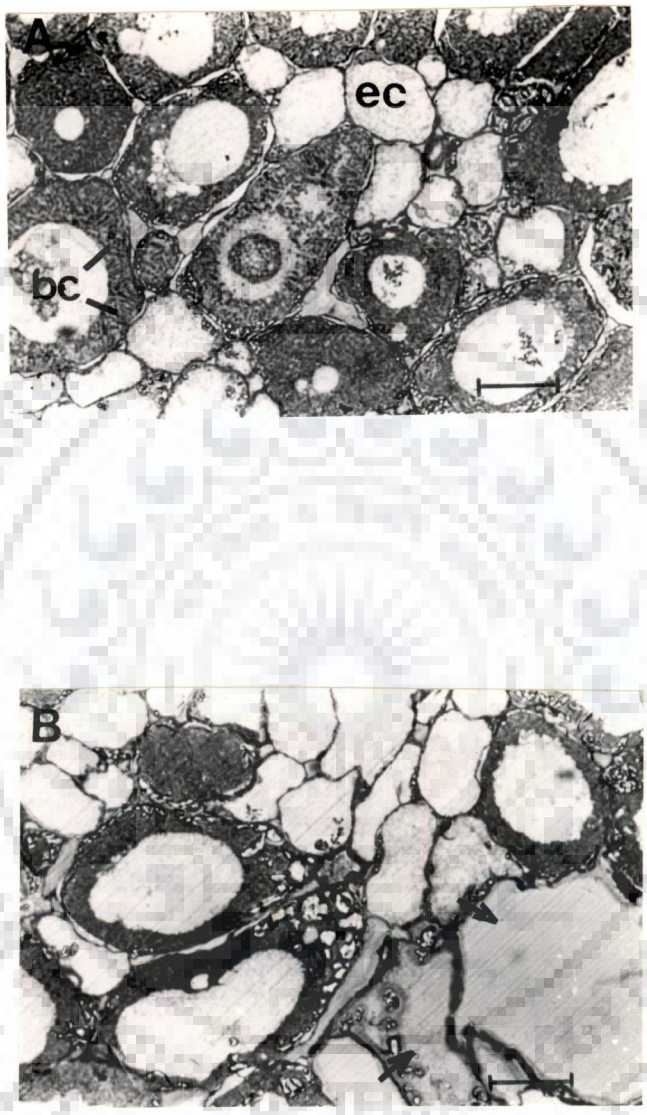

Plate 4. Light microscopic studies of semithin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** Nitrogen fixation zone showing cells filled with mature bacteroids (bc) and empty cells (ec). Bar, 25 μm (×400). **B.** Senescence zone showing in degenerated condition (indicated by arrows). Bar, 25 μm (×400).

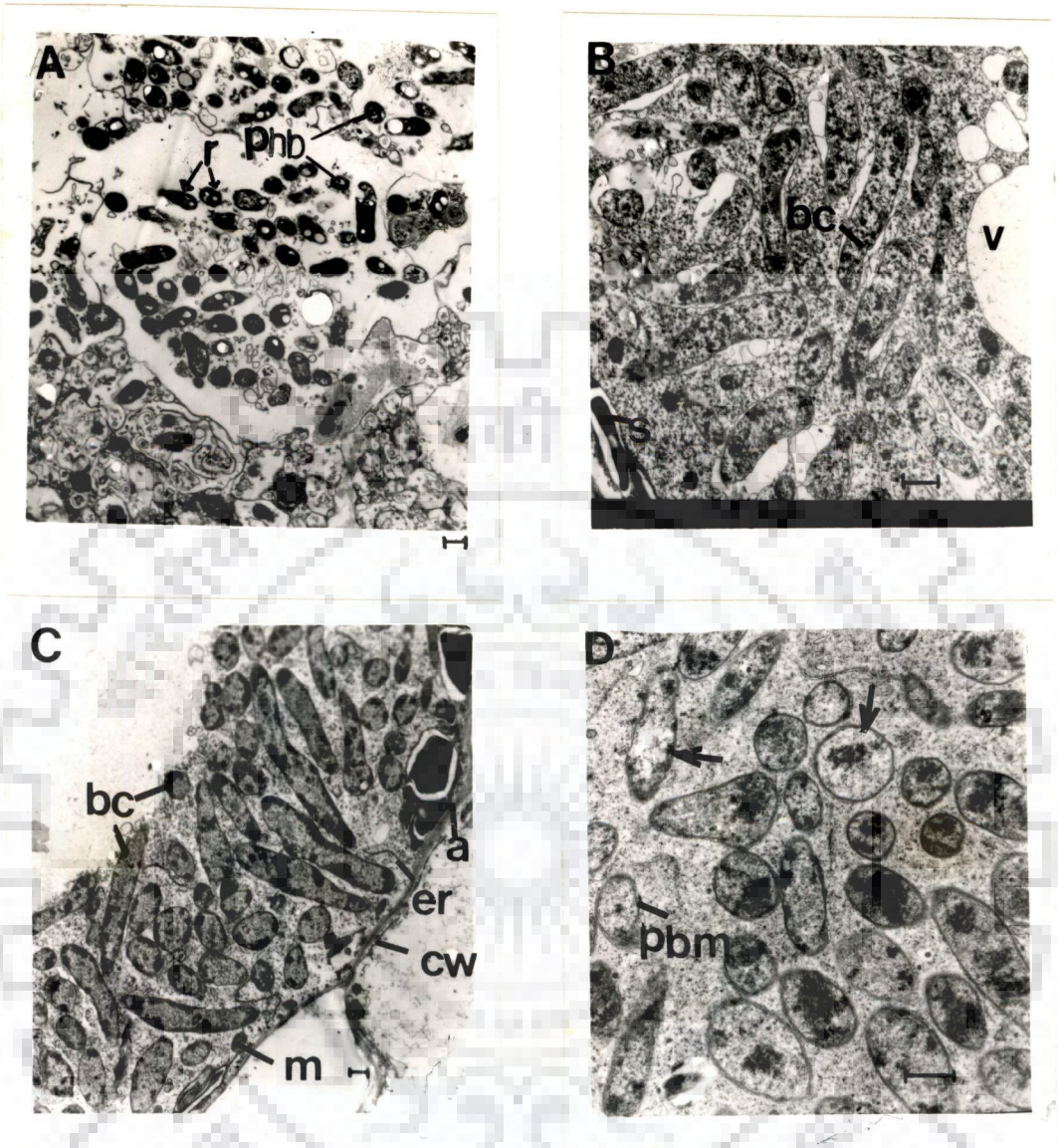

Plate 5. TEM studies of ultrathin sections of a nodule induced by *Rhizobium meliloti* Rmd201. **A.** Depicts freshly released rhizobia (r) which are electron dense and contain poly-β-hydroxybutyrate (phb) granules. Bar, 1 μm (×2,900). **B.** A nodule cell from zone between infection and nitrogen fixation zones showing bacteroids (bc) with less heterogeneous cytoplasm. Bar, 1 μm (×4,100). **C.** A nodule cell from nitrogen fixation zone containing mature bacteroids. The cytoplasm in these elongated bacteroids is heterogeneous. Cell organelles like mitochondria (m), amyloplasts (a) containing starch granules (s), endoplasmic reticulae (er) are displaced to the periphery, near to the cell wall (cw). Bar, 1 μm (×2,300). **D.** Degenerating bacteroids from senescence zone (indicated by arrows). The cytoplasm of these bacteroids is electron transparent and the peribacteroid membrane (pbm) is broken. Bar, 1 μm (×5,800).

in the cells filled with bacteroids took the peripheral position near to the cell wall. Degenerated bacteroids with transparent cytoplasm and broken peribacteroid membranes were seen in the senescence zone (Plate 5: D).

4.4.4.2 Light and electron microscopic studies of nodules induced by aromatic amino acid auxotrophs

(a) *trpE* mutants

The longitudinal section of nodule induced by a *trpE* mutant (Plate 6: A) deviated significantly from that of the nodule induced by parental strain. The plant cells in the infection zone, however, were morphologically similar in these both types of nodules as is evident from the comparison under high magnification (Plate 6: B); each of these cells contained a darkly staining nucleus, many large vacuoles and a few immature bacteroids. But unlike the parental strain induced nodules in which the infection zone was only a few layers thick, the infection zone in case of nodules induced by *trpE* mutants was very extensive encompassing almost half of the nodule. A few plant cells in the extended infection zone were filled with mature bacteroids. The nitrogen fixation zone was poorly developed; it was represented by very few plant cells filled with mature bacteroids which were surrounded by many infected cells containing immature bacteroids (Plate 6: C).

Electron microscopic picture (Plate 7: A) of a plant cell from the early infection zone showed an infection droplet containing bacteria with poly-β-hydroxybutyrate granules (phb). In the extended infection zone the bacteroids containing plant cells were devoid of electron dense material and the cytoplasm of bacteroids in this zone was not heterogeneous (Plate 7: B&C). In each uninfected plant cell the nucleus and mitochondria took the peripheral

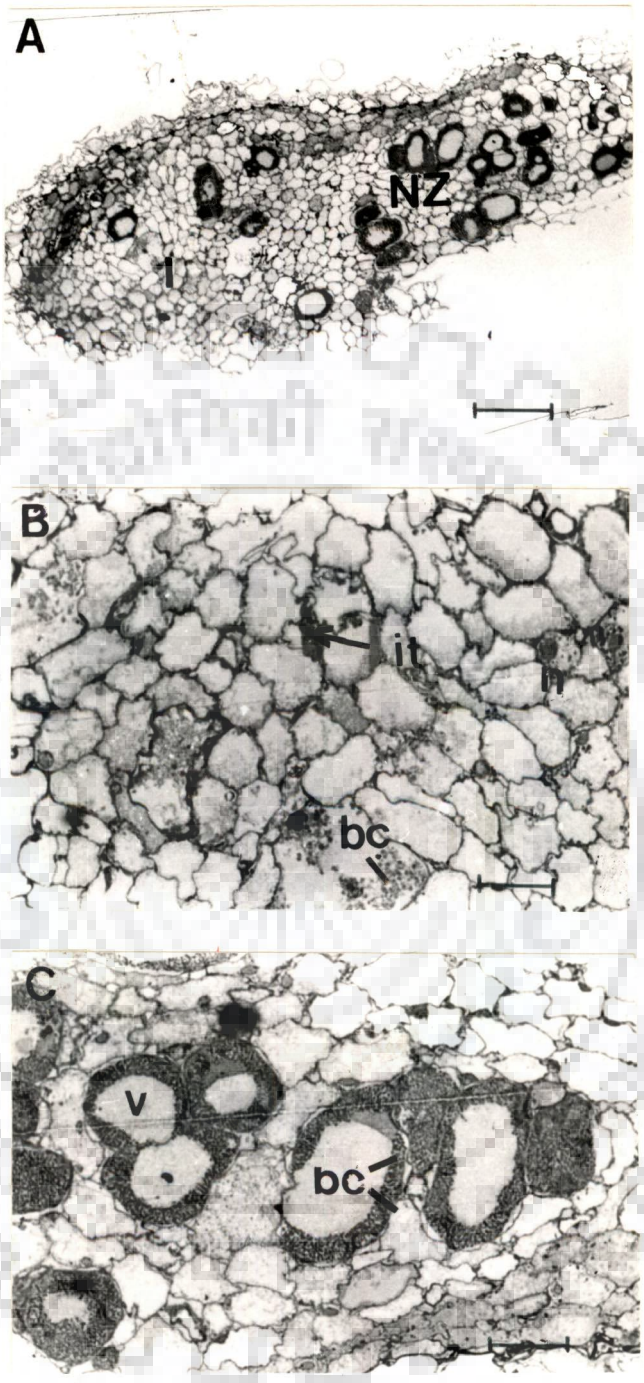

Plate 6. Light microscopic studies of semithin sections of a nodule induced by a *trpE* mutant of *Rhizobium meliloti* Rmd201. **A.** Longitudinal section of the whole nodule revealing an extensive infection zone (I) and a poorly developed nitrogen fixation zone (NZ), which is confined to the base of the nodule. Bar, 100 μm (×100). **B.** Infection zone showing cells containing infection threads (it), prominent nucleus (n) and immature bacteroids. Bar, 25 μm (×400). **C.** Nodule cells from nitrogen fixation zone with a central vacuole (v) and properly arranged bacteroids (bc). Bar, 25 μm (×400).

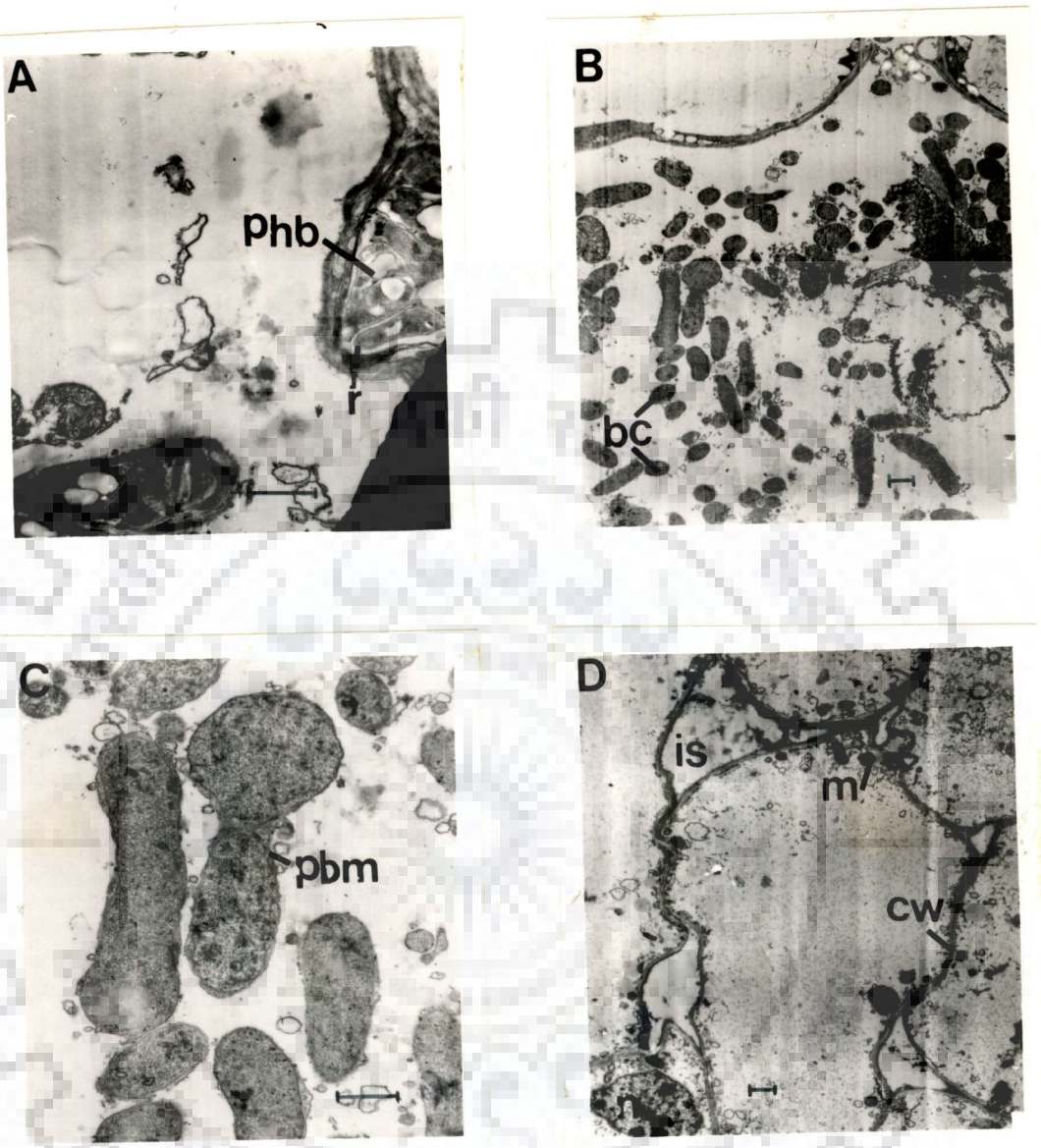

Plate 7. TEM studies of ultrathin sections of a nodule induced by a *trpE* mutant of *Rhizobium meliloti* Rmd201. **A.** A nodule cell of early infection zone showing the infection droplet containing rhizobia (r). These bacteria contain poly-β-hydroxybutyrate (phb) granules. Bar, 1 mm (×8,400). **B.** Bacteroids (bc) in a nodule cell from infection zone. Bar, 1 μm (×2,300). **C.** Higher magnification of same bacteroids showing that these are elongated and covered by peribacteroid membrane (pbm). The bacteroid cytoplasm is homogeneous. Bar, 1 μm (×8,400). **D.** An empty cell from the nitrogen fixation zone showing peripherally placed organelles like mitochondria (m) and nucleus (n). Intercellular spaces (is) and cell wall (cw) are clearly seen. Bar, 1 μm (×2,900).

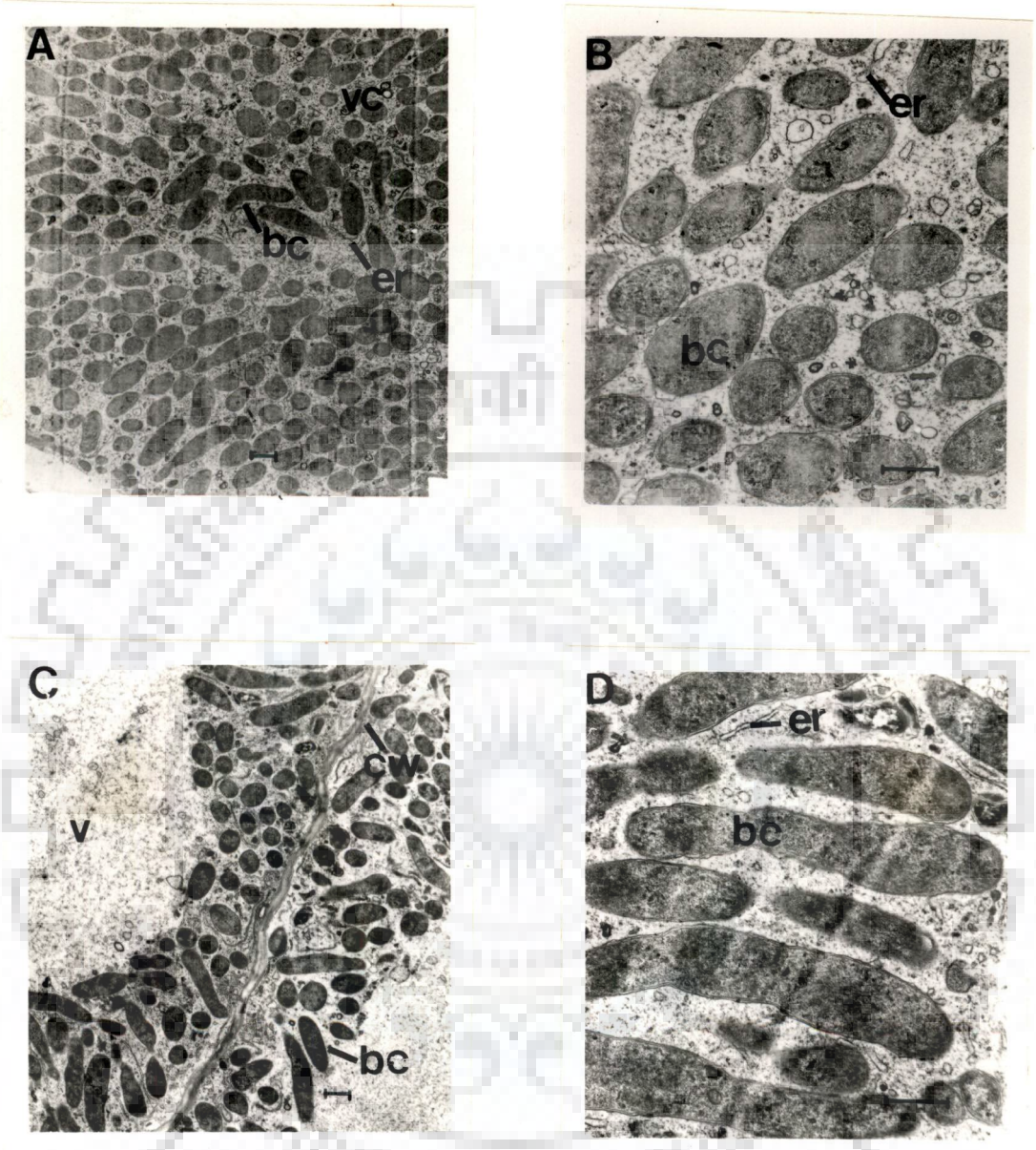

Plate 8. TEM studies of ultrathin sections of a nodule induced by a *trpE* mutant of *Rhizobium meliloti* Rmd201. **A.** A nodule cell from the zone near to the nitrogen fixation zone showing bacteroids (bc), numerous vesicles (vc) and endoplasmic reticulae (er). Bar, 1 μm (×2,900). **B.** Higher magnification of bacteroids from the same cell showing homogeneous nature of cytoplasm of the bacteroids. Bar, 1 μm (×8,400). **C.** Two nodule cells from nitrogen fixation zone containing polymorphic and mature bacteroids (bc) that are arranged around the central vacuole (v). Bar, 1 μm (×2,900). **D.** Higher magnification of mature bacteroids from nitrogen fixation zone showing heterogeneous cytoplasm. Bar, 1 μm (×8,400).

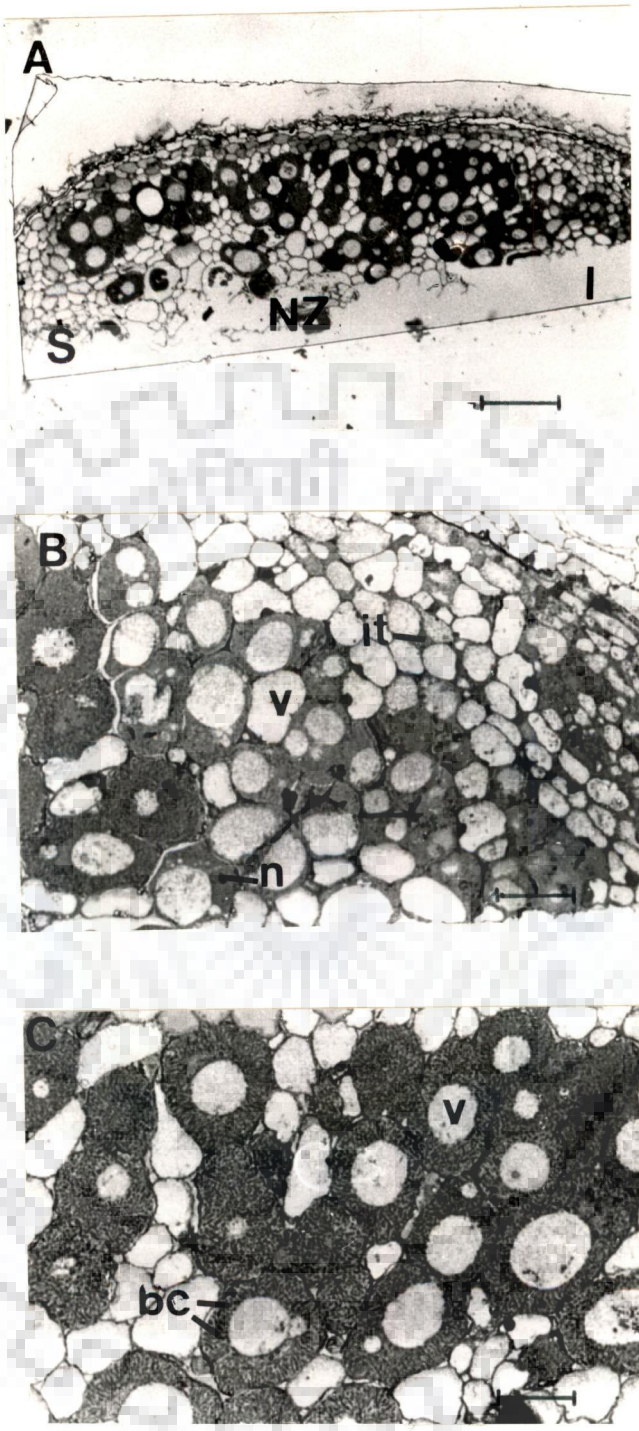

Plate 9. Light microscopic studies of semithin sections of a nodule induced by strain VK15, a tryptophan auxotroph of *Rhizobium meliloti* Rmd201. **A.** Longitudinal section of the whole nodule showing apical meristem (M), infection zone (I), nitrogen fixation zone (NZ) and senescence zone (S). Bar, 100 μm (×100). **B.** Infection zone at ×400 magnification. Numerous vacuoles (v) and prominent nucleus (n) are seen in the infected cells. Bar, 25 μm. **C.** Nodule cells from nitrogen fixation zone containing mature bacteroids (bc). Bar, 25 μm (×400).

position, near to the cell wall (Plate 7: D). The bacteroids in the zone between infection and nitrogen fixation zones did not show heterogeneous cytoplasm unlike the bacteroids from the same zone in case of nodule induced by the parental strain Rmd201. Even initiation of nuclear condensation was not observed in these bacteroids. (Plate 8: A & B). Very few plant cells filled with bacteroids contained fully mature bacteroids showing electron dense and electron transparent regions (Plate 8: C & D).

(b) VK15

Well formed meristematic, infection and nitrogen fixation zones were seen in longitudinal section of nodules induced by VK15 (Plate 9: A). The arrangement of these zones was exactly like that of nodule induced by the parental strain. The plant cells in the infection zone contained many vacuoles and enlarged nucleus (Plate 9: B). The nitrogen fixing region resembled the wild type in all aspects (Plate 9: C).

The released bacteria were rod shaped and each bacterium (bacteroid) was surrounded by a wavy peribacteroid membrane. An electron dense material was seen in bacteroids containing plant cells as well as in the intercellular spaces (Plate 10: A & B). The newly infected cells contained many mitochondria and endoplasmic reticulae (Plate 10: C). The bacteroids from the zone between infection zone and nitrogen fixation zone exhibited electron dense and electron transparent regions (Plate 10: D). In the nitrogen fixation region the bacteroids were fully mature exhibiting electron dense and electron transparent regions. The cytoplasm of these bacteroids was more heterogeneous and the peribacteroid membrane was straight (Plate 11: A&B).

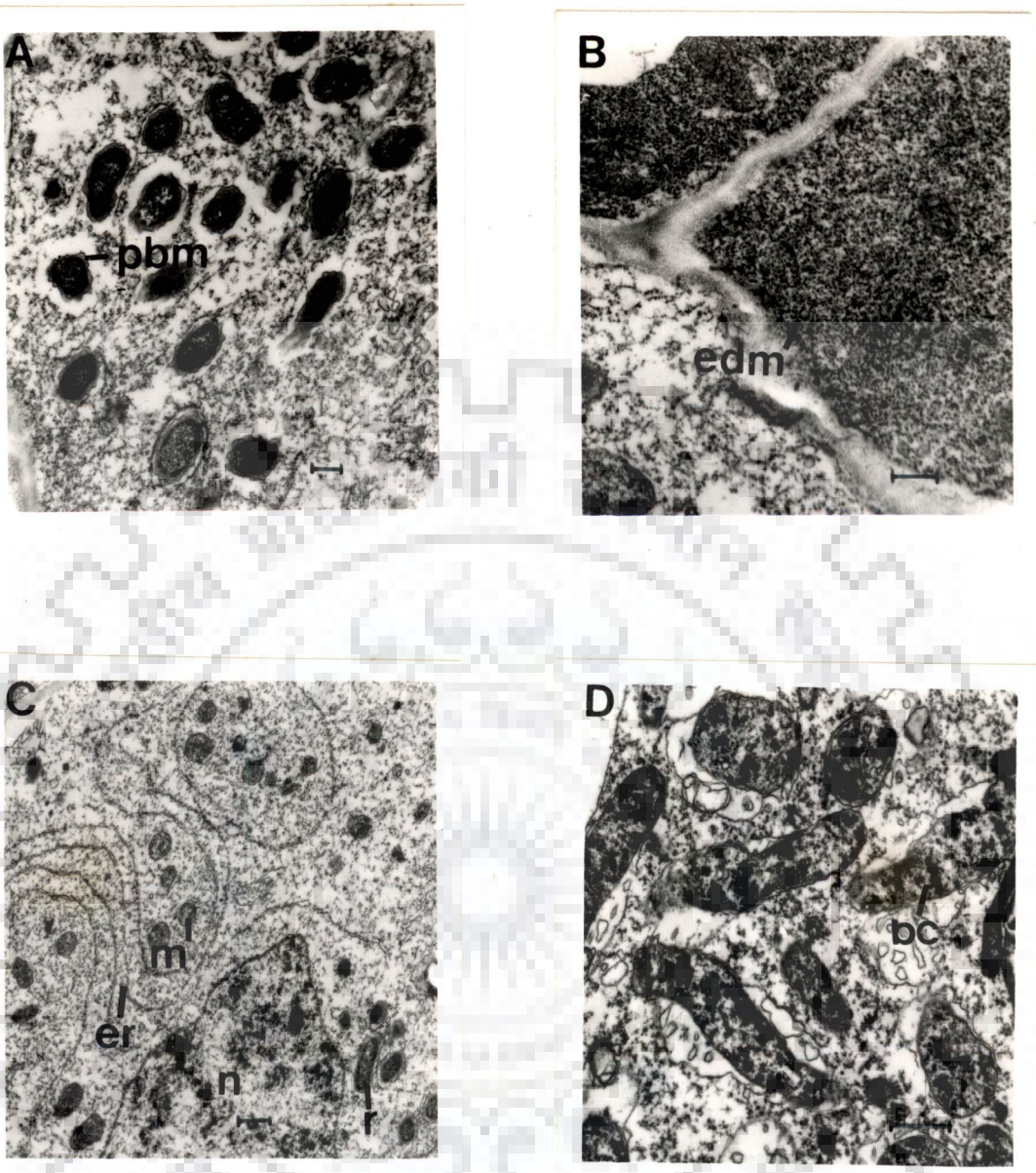

Plate 10. TEM studies of ultrathin sections of a nodule induced by strain VK15, a tryptophan auxotroph of *Rhizobium meliloti* Rmd201. **A.** A nodule cell from early infection zone showing released bacteria enclosed by a wavy peribacteroid membrane (pbm). Bar, 1 μm (×3,600). **B.** Intercellular space filled with electron dense material (edm). Bar, 1 μm (×5,470). **C.** A nodule cell from early infection zone displaying many mitochondria (m), endoplasmic reticulae (er), enlarged nucleus (n) and released rhizobia (r) covered with peribacteroid membrane (pbm). Bar, 1 μm (×3,600). **D.** A nodule cell from zone near to the nitrogen fixation zone showing bacteroids in transforming state. The cytoplasm is heterogeneous in these bacteroids as a result of nuclear condensation. Bar, 1 μm (×8,400).

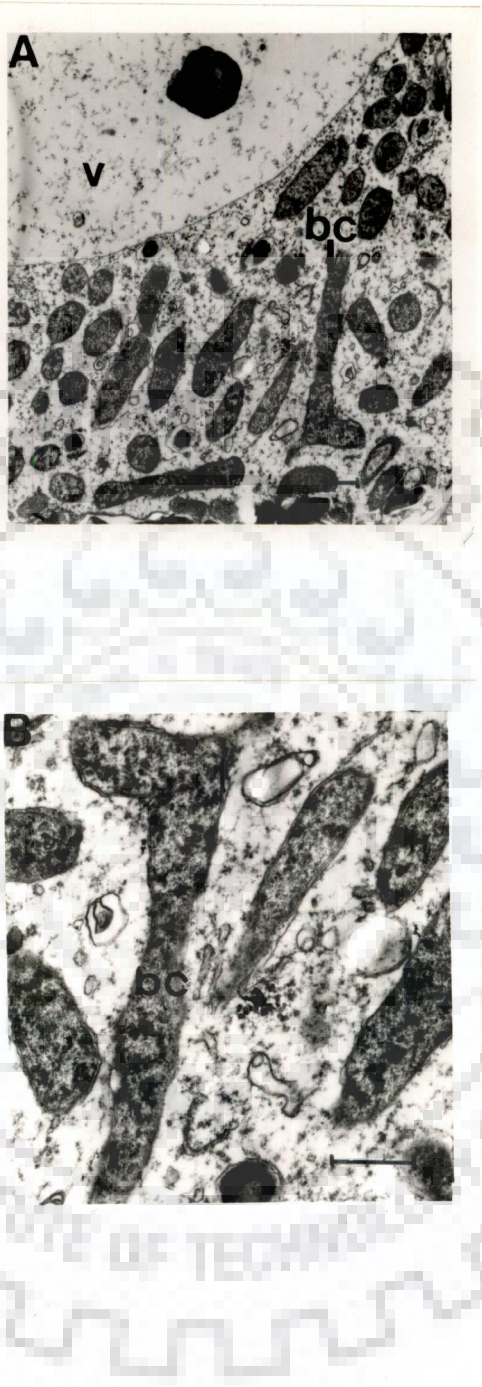

Plate 11. TEM studies of ultrathin sections of a nodule induced by strain VK15, a tryptophan auxotroph of *Rhizobium meliloti* Rmd201. **A.** A nodule cell from the nitrogen fixation zone displaying bacteroids in different shapes arranged around the central vacuole. Bar, 1 μm (×4,100). **B.** Mature bacteroids displaying heterogeneous cytoplasm and regular peribacteroid membrane (pbm). Bar, 1 μm (×10,800).

(c) Tryptophan synthase mutants

The histology of nodules induced by tryptophan synthase mutants (Plate 12: A) resembled the parental strain induced nodule's histology in having apical meristem, short infection zone and an extensive nitrogen fixing zone. Higher magnification of infection zone under light microscope showed prominent nucleus and many vacuoles in infected plant cells (Plate 12: B). Next to the infection zone and just before nitrogen fixation zone the plant cells started getting packed with amyloplasts (Plate 12: B). The bacteroids filled plant cells in the nitrogen fixing region were arranged radially with empty cells in between (Plate 12: C). Each of these bacteroids filled cells had a large central vacuole and perpendicularly arranged bacteroids.

The bacteroids containing plant cells in the infection zone contained many endoplasmic reticulae, vacuoles, prominent nucleus and electron dense material (Plate 13: A&B). In the empty plant cells the cytoplasm was restricted to the periphery of the cells and the cell organelles were seen very near to the cell wall. The bacteroids of the nitrogen fixation region resembled the nitrogen fixing bacteroids of the parental strain in all aspects (Plate 13: C&D).

(d) *aro* mutants

Striking similarities were seen in the histology of nodules induced by *aro* mutants and *trpE* mutants (Plate 14: A). The infection zone was many layers thick and the plant cells in this zone contained enlarged nucleus and immature bacteroids (Plate 14: B). The interzone between the poorly developed nitrogen fixation zone and infection zone contained plant cells

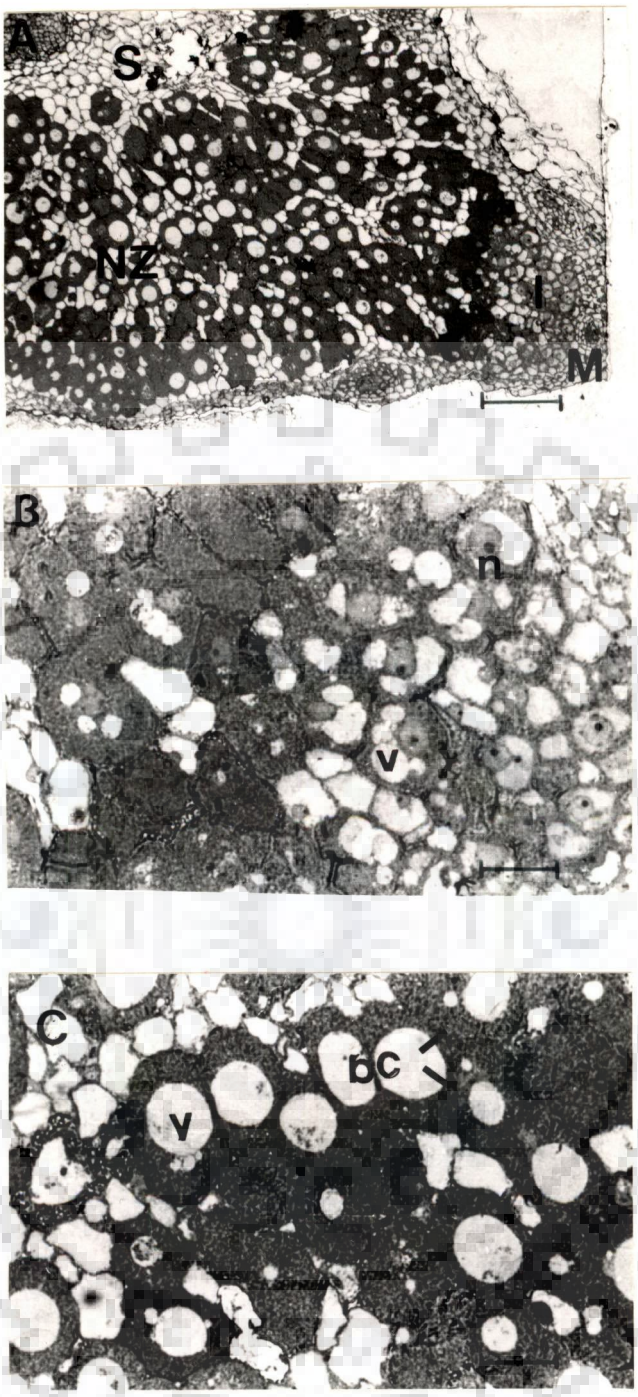

Plate 12. Light microscopic studies of semithin sections of a nodule induced by a tryptophan synthase mutant of *Rhizobium meliloti* Rmd201. **A.** The whole nodule section showing meristematic zone (M), infection zone (I), well developed nitrogen fixation zone (NZ) and senescence zone (S). Bar, 100 μm (×100). **B.** Infection zone cells containing enlarged nucleus (n) and many vacuoles (v). Bar, 25 μm (×400). **C.** Nitrogen fixation zone cells displaying large central vacuole (v) and properly arranged bacteroids (bc). Bar, 25 μm (×400).

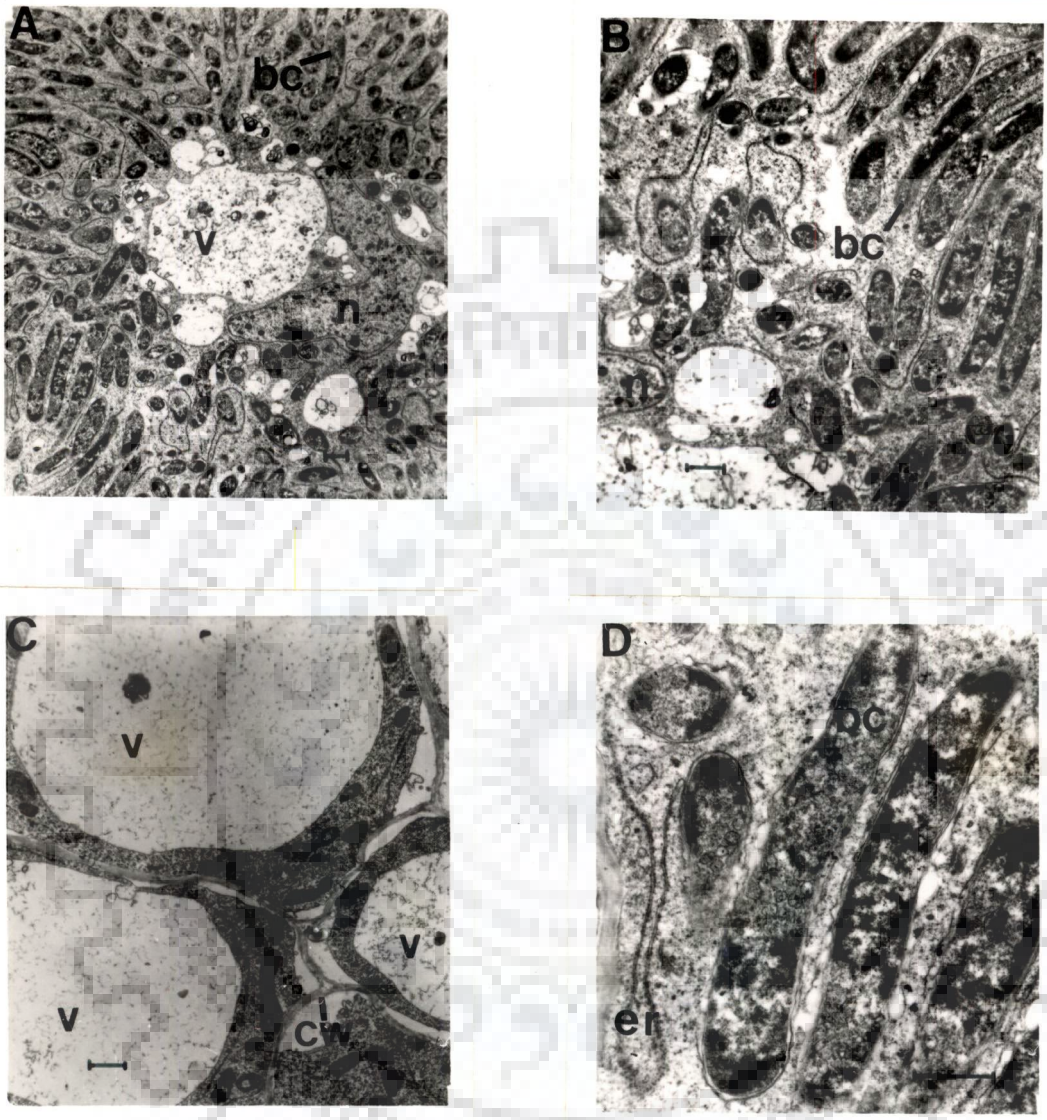

Plate 13. TEM studies of ultrathin sections of a nodule induced by a tryptophan synthase mutant of *Rhizobium meliloti* Rmd201. **A.** A nodule cell from infection zone displaying enlarged, prominent nucleus (n), vacuoles (v) and bacteroids (bc). Bar, 1 μm (×2,300). **B.** The same cell under higher magnification. Initiation of heterogeneity of cytoplasm has started in the bacteroids as a result of nuclear condensation. Bar, 1 μm (×4,100). **C.** The empty nodule cells showing the cytoplasm restricted to the periphery. All cell organelles are seen near to the cell wall. Bar, 1 μm (×4,100). **D.** The magnified image (×10,800) of mature bacteroids from nitrogen fixation zone showing distinct electron dense and electron transparent regions. Bar, 1 μm.

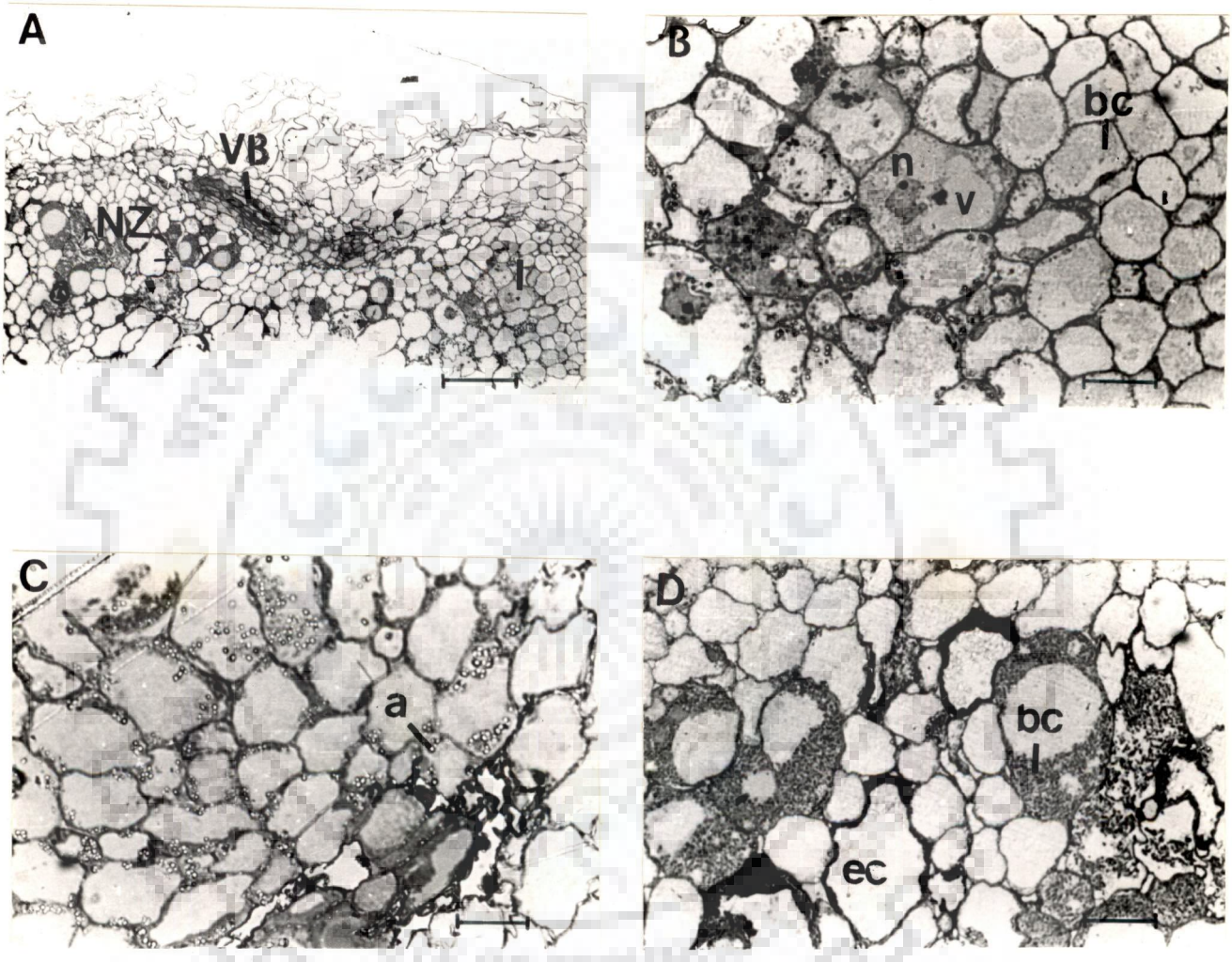

Plate 14. Light microscopic studies of semithin sections of a nodule induced by an *aro* mutant of *Rhizobium meliloti* Rmd201. **A.** The whole nodule section. The infection zone (I) is extensive and the nitrogen fixation zone (NZ) is poorly developed. Bar, 100 μm (×100). **B.** The infection zone cells containing prominent nucleus (n), numerous vacuoles (v) and immature bacteroids (bc). Bar, 25 μm (×400). **C.** Interzone cells filled with amyloplasts (a). Bar, 25 μm (×400). **D.** The poorly developed nitrogen fixation zone containing very few cells filled with bacteroids. Most of the cells in this region are empty. Bar, 25 μm (×400).

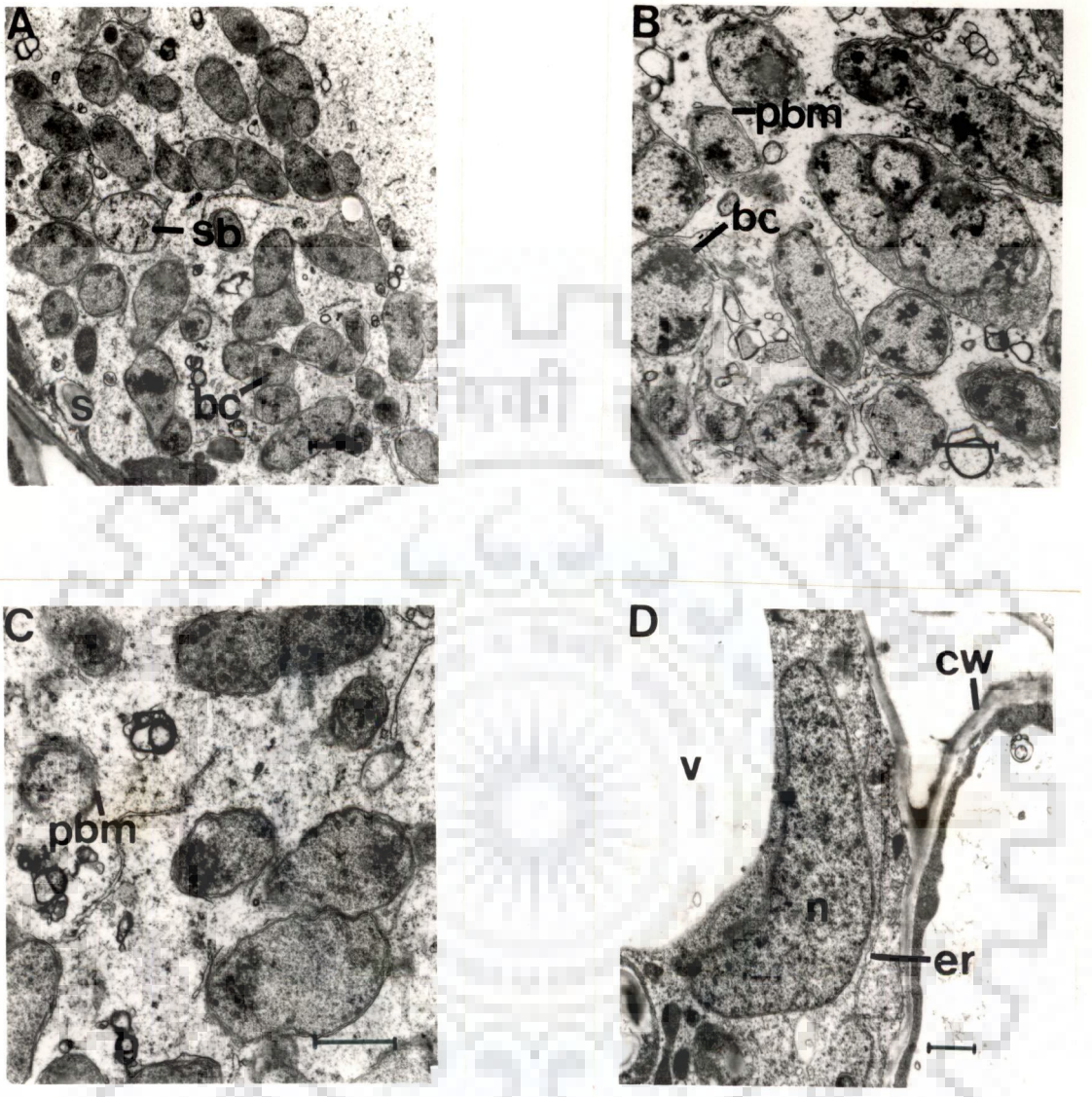

Plate 15. TEM studies of ultrathin sections of a nodule induced by an *aro* mutant of *Rhizobium meliloti* Rmd201. **A.** A nodule cell from the zone between infection and nitrogen fixation zones. Initiation of nuclear condensation is not seen in these bacteroids and as a result they exhibited homogeneous cytoplasm. Senescent bacteroids are also seen along with these immature bacteroids (sb). Bar, 1 μm (×5,800). **B.** Bacteroids from nitrogen fixation zone. These bacteroids are set for lysis as their cytoplasm become more homogeneous and electron transparent. Bar, 1 μm (×8,400). **C.** Lysing bacteroids from the so called nitrogen fixation zone. The peribacteroid membrane (pbm) is dissolved in some bacteroids. Bar, 1 μm (×10,800). **D.** Mitochondria (m), endoplasmic reticulae (er) and nucleus (n) are seen near to the cell wall (cw) in empty cells. Bar, 1 μm (×5,800).

packed with many amyloplasts (Plate 14: C). The nitrogen fixation region was confined to the base of the nodule and was poorly developed (Plate 14: D).

Nuclear condensation did not start in the bacteroids of the infection zone as a result of which they exhibited homogeneous cytoplasm (Plate 15: A). Most of the bacteroids from the nitrogen fixing zone were seen in degenerating condition. These bacteroids were not elongated and their peribacteroid membranes were broken. The cytoplasm of these bacteroids was more electron transparent like in the degenerating bacteroids (Plate 15: B&C). In uninfected and infected nodule cells the cell organelles were restricted to the periphery (Plate 15: D).

(e) Tyrosine mutant

tyr mutant R1 which was Fix^+ formed nodules whose histology (Plate 16: A) matched with that of the nodule induced by the parental strain. The infection zone was short and the plant cells in this zone contained vacuoles and prominent nucleus (Plate 16: B). The nitrogen fixing zone was well formed with radially arranged bacteroids filled cells (Plate 16: C).

Electron microscopic studies on bacteroids of different zones showed all stages of bacteroidal development (Plate 17: A-D).

(f) *phe* mutant

The ineffective nodules induced by *phe* mutant H38 showed two zones; an extensive infection zone with a profuse network of infection threads and a zone of plant cells flooded with amyloplasts (Plate 18: A). In the infection zone the plant cells contained large nucleus and few immature bacteroids. Ramifications of infection threads were seen all through the nodule. Most

Plate 16. Light microscopic studies of semithin sections of a nodule induced by *tyr* mutant of *Rhizobium meliloti* Rmd201. **A.** Longitudinal section of the whole nodule displaying infection (I), nitrogen fixation (NZ) and senescence zones (S). Bar, 100 μm (×100). **B.** Cells from infection zone with enlarged nucleus (n), immature bacteroids (bc) and infection threads (it). Bar, 25 μm (×400). **C.** Nitrogen fixation zone exhibiting empty cells (ec) and mature bacteroids filled cells. The amyloplasts (a) in the filled cells are pushed to the periphery. Bar, 25 μm (×400).

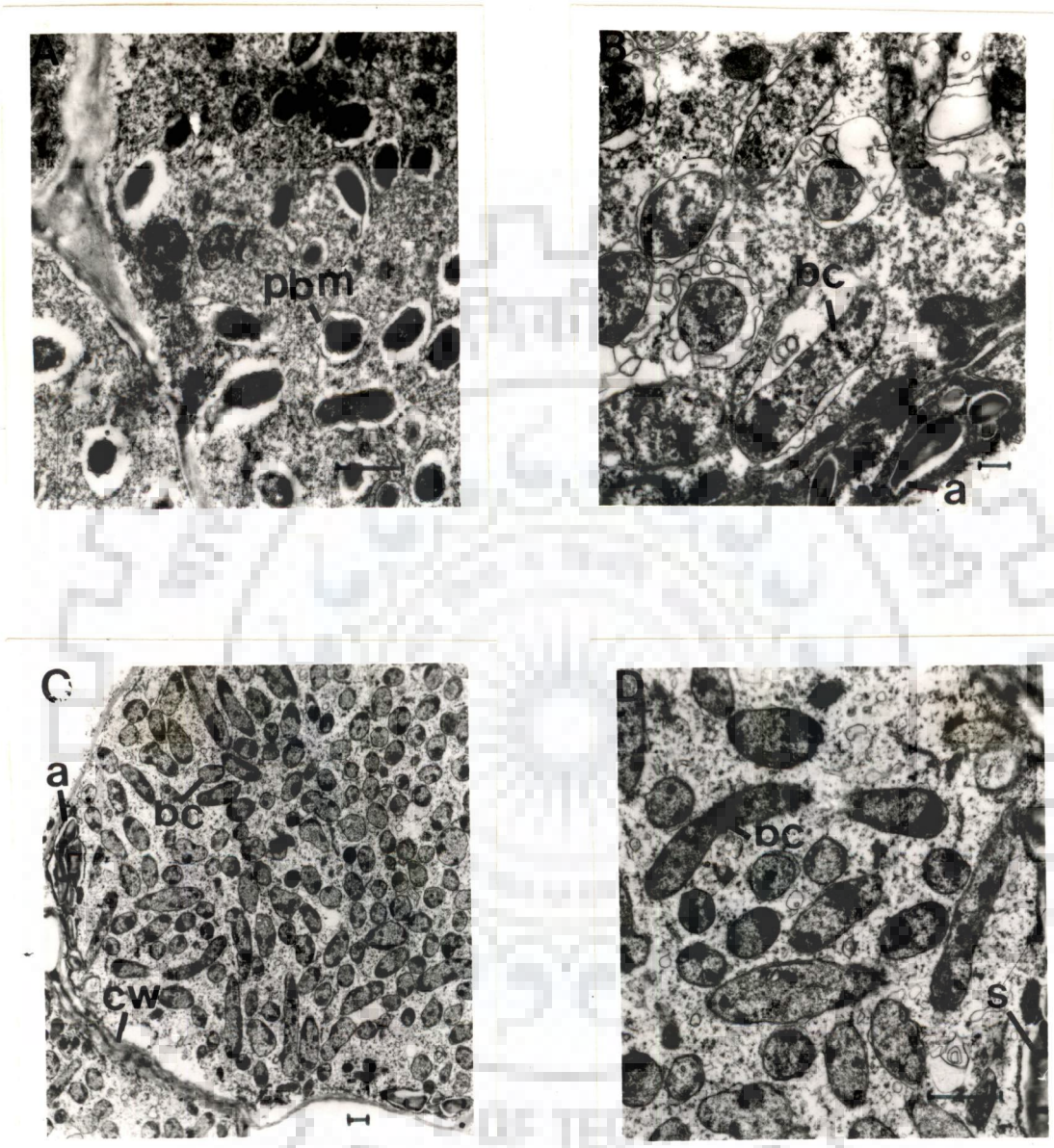

Plate 17. TEM studies of ultrathin sections of a nodule induced by *tyr* mutant of *Rhizobium meliloti* Rmd201. **A.** The released bacteria with peribacteroid membrane (pbm). Bar, 1 μm (×8,400). **B.** A nodule cell from the infection zone showing bacteroids. These bacteroids started showing heterogeneous cytoplasm. The amyloplasts are seen at the periphery. Bar, 1 μm (×4,100). **C.** A nodule cell from nitrogen fixation zone containing mature bacteroids. Bar, 1 μm (×2,300). **D.** Pleiomorphic bacteroids from nitrogen fixation zone. Please note the heterogeneity of cytoplasm of bacteroids. Bar, 1 μm (×9,800).

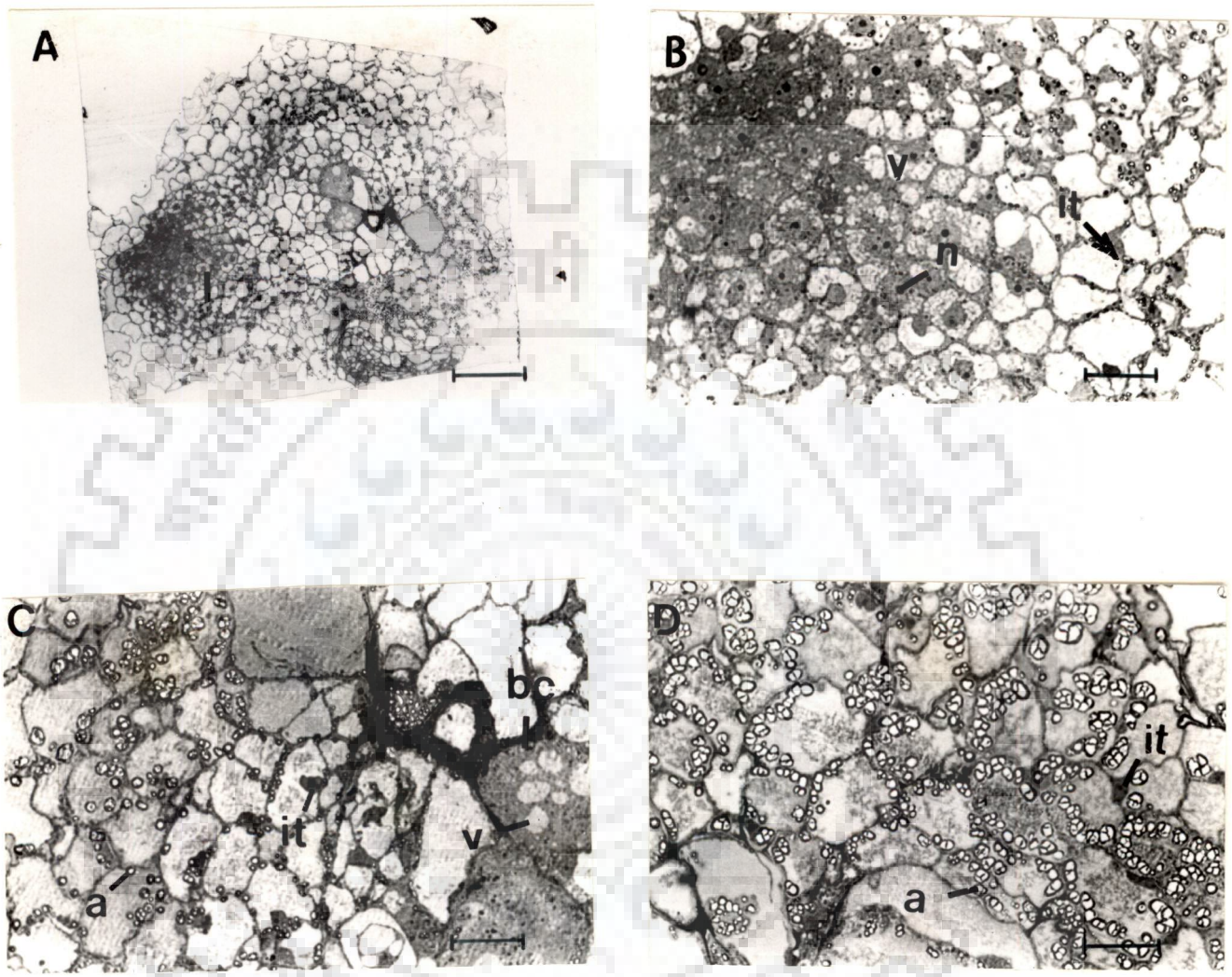

Plate 18. Light microscopic studies of a nodule induced by *phe* mutant of *Rhizobium meliloti* Rmd201. **A.** The whole nodule section revealing infection zone (I) and cells filled with amyloplasts (a). Nitrogen fixation zone is absent. Bar, 100 μm (×100). **B.** Infection zone showing cells filled with immature bacteroids. The cells also contain prominent nucleus (n) and many vacuoles (v). Bar, 25 μm (×400). **C.** The nodule cells from the late infection zone exhibiting many amyloplasts (a) and infection threads (it). Bar, 25 μm (×400). **D.** The nodules cells studded with many amyloplasts (a) are found next to the infection zone. Bar, 25 μm (×400).

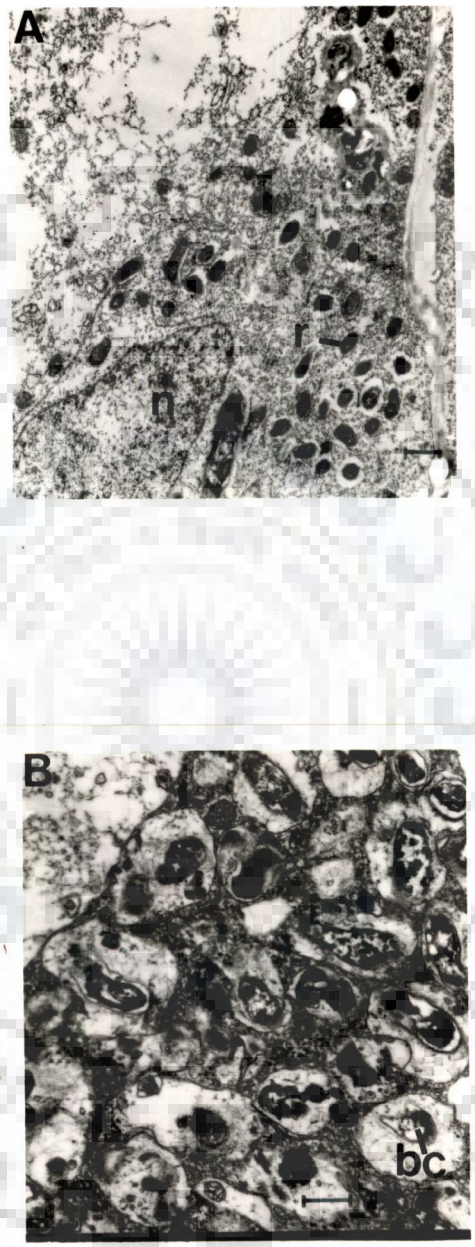

Plate 19. TEM studies of a nodule induced by *phe* mutant of *Rhizobium meliloti* Rmd201. **A.** The released bacteria (b) with peribacteroid membrane (pbm) in the infected cells of the early infection zone. Bar, 1 μm (×4,100). **B.** The degenerating bacteroids in the nodule cells from infection zone. Please note the rupture of peribacteroid membrane. Bar, 1 μm (×5,800).

of the plant cells were filled with many amyloplasts, a situation seen in case of ineffective nodules. Nitrogen fixation zone was not observed (Plate 18: B-D).

Proper release of bacteria occurred (Plate 19: A) but the bacteroids were seen in degenerated condition soon after their release (Plate 19: B).

4.5 Genetic studies on aromatic amino acid auxotrophs

4.5.1 Linkage of Tn5 insertion to auxotrophy

IncP group plasmid pJB3JI, which codes for tetracycline resistance, has the ability to mobilise the segments of rhizobial genome from donor cells to suitable recipient cells; integration of the mobilized donor DNA occurs in the recipient genome. This plasmid was introduced into each auxotroph by conjugation using its tetracycline resistance marker. The resulting strain was mated as a donor with the recipient strain ZB555 and 40 kanamycin resistant transconjugants were selected and tested for the presence of the donor's auxotrophic marker(s). In all crosses, all kanamycin resistant transconjugants showed respective donor's auxotrophy confirming 100% linkage of Tn5 insertion to auxotrophy.

4.5.2 Location of transposon Tn5 by plasmid-mediated mapping method

Donor strains of the auxotrophs constructed by the introduction of plasmid pJB3JI were also used to locate the position of transposon Tn5. Each donor was conjugated with four *R. meliloti* recipient strains ZB555, ZB556, ZB557 and ZB205, the markers of which cover most of the chromosome. In all crosses selection was made for the transfer of kanamycin resistance marker,

Table 6. Mapping of transposon Tn5 in *Rhizobium meliloti* auxotrophic strain VK15

Donor strain	Recipient strain	Selected marker	Unselected marker	No. of Km^r transconjugants analysed	No. of Km^r transconjugants showing unselected marker transfer	Linkage (c)	Distance (d)
VK151	ZB555	Km^r (*trp*$^-$)	cys^+	48	0	-	-
			phe^+	48	0	-	-
VK151	ZB556	Km^r (*trp*$^-$)	gly^+	132	41	0.31	0.32
			ade^+	132	31	0.24	0.38
			met^+	132	18	0.14	0.44
VK151	ZB557	Km^r (*trp*$^-$)	phe^+	45	0	-	-
			leu^+	45	0	-	-
VK151	ZB205	Km^r (*trp*$^-$)	ade^+	20*	0	-	-

Abbreviations: Km^r, kanamycin resistance; gly^+, glycine prototrophy; trp^-, tryptophan auxotrophy; cys^+ cysteine prototrophy; phe^+, phenylalanine prototrophy; ade^+, adenine prototrophy; met^+, methionine prototrophy; leu^+, leucine prototrophy.

* Due to the poor chromosome mobilising ability of plasmid pJB3JI available with us higher number of Km^r transconjugants could not be obtained.

which is encoded by Tn5. The cotransfer of other unselected markers was determined by patching the transconjugants on appropriate plates. In VK15 strain kanamycin resistance showed 31% cotransfer with *gly-1*, 24% with *ade-4* and 18% with *met-2*. The distances calculated based on linkage frequencies are given in the Table 6 and the possible position of *trp* marker of VK15 on the chromosome is given in Fig.6.

Because of poor chromosomal mobilisation ability of pJB3JI available in our lab mapping the position of Tn5 in other auxotrophs was not possible, however, the presence of Tn5 in some regions of the chromosome could be ruled out.

4.6 Pleiotropic effects of Tn5 insertions

4.6.1 Production of cell surface molecules

All aromatic amino acid auxotrophs and the parental strain Rmd201 took up the dye in congo red medium, grew in presence of sodium deoxycholate, showed motility in swarm medium and fluoresced in presence of calcofluor white under ultra violet light, indicating the normal production of cellulose fibrils, lipopolysaccharides, β-(1→2) glucans and exopolysaccharides. These results showed that the cell surface molecules in auxotrophs were like those of the parental strain.

4.6.2 Dicarboxylates uptake

Like the parental strain Rmd201 all auxotrophs showed growth on the minimal medium containing malate or succinate indicating that they could use either of these dicarboxylic acids as a sole carbon source.

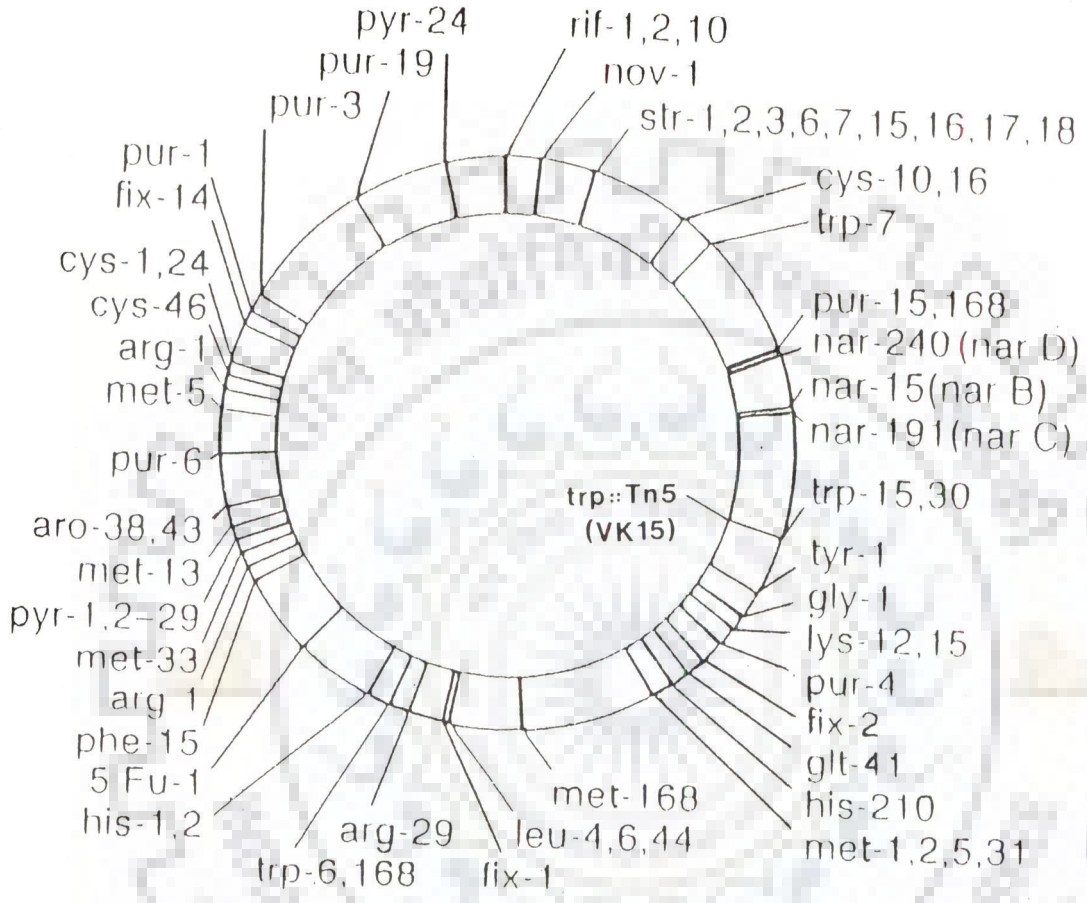

Fig.6. Chromosomal map of *Rhizobium meliloti* strain Rm41 showing the position of Tn5 insertion in auxotrophic strain VK15

Chapter 5

Discussion

Six thousand kanamycin resistant transconjugants were generated by random transposon Tn5 mutagenesis of *R. meliloti* strain Rmd201 (Nod^+, Fix^+, Str^r and compact colony morphology derivative of the wild type strain Rm41). The transposition frequency obtained was 2×10^{-5} which was far above the frequency of spontaneous resistance to 400 μg/ml of kanamycin in *R. meliloti* ($<10^{-8}$). Similar Tn5 transposition frequencies have been reported in *Rhizobium* by other workers (Meade *et al.*, 1982; Kim *et al.*, 1988). The screening of the Tn5-derivatives yielded 30 auxotrophs, the frequency of the isolation of auxotrophs being 0.5% which is the same as reported by Meade *et al.*, 1982. Isolation of auxotrophs requiring different amino acids/nitrogenous bases/vitamins indicated near randomness of Tn5 insertions. The high frequency of cysteine/methionine auxotrophy among the isolated auxotrophs may be due to the presence of a large number of genes involved in sulfur metabolism or preferential insertion of Tn5 into these genes. Similar observations were made by Meade *et al.*, 1982.

When these auxotrophs were tested for their symbiotic properties on alfalfa plants, quite interesting results were obtained from the auxotrophs of tryptophan, phenylalanine, tyrosine and those requiring all these three amino acids. It was found that out of six tryptophan auxotrophs two were as effective as the parental strain, three were ineffective and one was partially effective. The lone phenylalanine mutant and two *aro* mutants were ineffective whereas the tyrosine mutant was effective. These aromatic amino acid auxotrophs were chosen for biochemical characterization, genetic studies and thorough investigation of their symbiotic properties.

The location of block in the tryptophan biosynthetic pathway in each tryptophan auxotroph was determined by intermediate feeding, intermediate accumulation and cross-feeding assays. Three *trp* auxotrophs, viz., H4, H6 and VK1 are anthranilate synthase mutants *(trpE)* as they grew on minimal medium supplemented with anthranilic acid and did not accumulate anthranilic acid in minimal medium. It has been reported by Bae *et al.*, 1991 that in *R. meliloti trpE* and *trpG* are fused to form *trpE(G)*. So these mutants may represent *trpE(G)* class mutants. The mutant VK15, which is slightly leaky, has a block somewhere between anthranilic acid and indole as it grew on minimal medium supplemented with indole and accumulated anthranilic acid. Since the intermediates, viz., phosphoribosylanthranilic acid (PRA) and 1-(*o*-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP) are not commercially available it was not possible to determine precisely the gene(s) affected in this mutant by the techniques mentioned above; the affected gene(s) could be *trpF, trpC, trpD* or *trpCD* cluster. Tryptophan synthase mutants (VK28 and VK30) did not grow on indole supplemented minimal medium and accumulated anthranilic acid and indole glycerol phosphate but not indole. These mutants are expected to accumulate indole as they did not grow on indole but indole was not detected in the minimal medium in which they were grown. This could be due to too low accumulation of indole which was not detected by the method used or might be due to Tn5 insertion into *trpFBA* gene cluster in such a way that both *trpA* and *trpB* functions were lost.

Cross-feeding assays further confirmed the position of blocks in the tryptophan auxotrophs. VK15, VK28 and VK30 cross-fed the *trpE(G)* mutants. As VK15 secretes anthranilic acid and tryptophan synthase mutants

(VK28 and VK30) secrete anthranilic acid as well as indole glycerol phosphate it is obvious that the *trpE(G)* mutants use them for growth. VK15 was cross-fed by tryptophan synthase mutants possibly with indole glycerol phosphate. The absence of cross-feeding among *trpE(G)* mutants and also among tryptophan synthase mutants reiterated that they belong to the same group.

The two *aro* mutants which are expected to have block before chorismic acid did not grow on shikimic acid. This may be due to occurrence of block in between shikimic acid and chorismic acid or may simply be due to inability of *Rhizobium* to transport and utilize shikimic acid.

If the biosynthetic pathways leading to phenylalanine and tyrosine are assumed to be similar, then the affected gene in the phenylalanine auxotroph H38 is *pheA* and that in tyrosine auxotroph R1 is *tyrA*; these are the only genes whose disruption result in Phe^- and Tyr^- phenotypes, respectively. A single mutation in other gene (*tyrB*) result in double auxotrophic requirement for both phenylalanine and tyrosine. However, this assumption could be wrong if these biosynthetic pathways in *Rhizobium* are different from those of *E. coli*.

Tyrosine auxotroph showed normal symbiotic properties. This auxotroph induced root hair curling and infection thread formation in root hairs of alfalfa. The light and electron microscopic studies of nodules induced by this auxotroph showed similarities with nodules induced by Rmd201 in all aspects. The mean shoot dry weight of plants inoculated with this auxotroph did not differ significantly from the mean shoot dry weight of plants inoculated by the parental strain Rmd201 indicating the Fix^+ phenotype of this auxotroph. It seems that this auxotroph derives its amino acid

requirement from the host plant and hence is not affected in symbiosis.

Studies on the symbiotic properties of tryptophan auxotrophic mutants indicated the involvement of some trptophan biosynthetic intermediates in symbiosis. The plants inoculated with *trpE(G)* mutants were short and became chlorotic six weeks after inoculation. The mean shoot dry weight of a *trpE* mutant inoculated plants was significantly less than that of the plants inoculated with the parental strain but more than that of uninoculated plants indicating that only trace amounts of nitrogen fixation occurred in the plants inoculated with *trpE* mutant. The early nodulation events in these auxotrophs proceeded quite well with induction of root hair curling and infection thread formation but the nodules induced by these mutants showed unusual defects. In these nodules the infection zone was very long, extending upto half of the nodule and the poorly developed nitrogen fixation zone occupied the basal part of the nodule; these observations are consistent with an earlier report (Barsomian *et al.*, 1992). Electron microscopic studies showed that the bacteroids in the extended infection zone were not mature and did not contain heterogeneous cytoplasm. The bacteroids containing plant cells in this zone were devoid of electron dense material, likely to be leghaemoglobin. Even in the interzone between infection and nitrogen fixation zone nuclear condensation in bacteroids did not occur and as a result they exhibited homogeneous cytoplasm. There were very few nodule cells with mature bacteroids in the so called nitrogen fixation zone which might be responsible for traces of nitrogen fixation. Before explaining the reasons for these unusual defects in these nodules we have to consider two facts. First, the availability of tryptophan to the rhizobia from plant was

established from the fact that tryptophan synthase mutants were symbiotically effective; and second, VK15 and tryptophan synthase mutants have blocks in their tryptophan biosynthetic pathway after anthranilic acid and the nodules induced by them showed the histological aspects like those of the parental strain induced nodules. Considering these two facts and an earlier report about the ineffectiveness of *trpE(G)* mutants of *R. meliloti* strain 1021 (Barsomian *et al.*, 1992), it is possible to conclude that anthranilic acid has a role in nodule development and function at least in *R. meliloti*. To explain the role of anthranilic acid in proper nodule development and function the factors needed for bacteroid development and nitrogen fixation are to be considered. Iron requirement during bacteroid transformation and nitrogen fixation is more as it is needed for heme (thereby leghemoglobin synthesis) synthesis and nitrogenase enzyme (that is required for nitrogen fixation) synthesis. Based on the earlier reports (Rioux *et al.*, 1986a & 1986b) on secretion of anthranilic acid and its promoted iron uptake in *R. leguminosarum* Barsomian *et al.*, 1992 proposed that anthranilic acid may be acting as *in planta* siderophore, promoting iron uptake needed for bacteroid development and the absence of this factor may be leading to undifferentiation of bacteroids. They also explained the occurrence of nitrogen fixation zone at the base of the nodule by proposing that bacteria are able to get plant supplied anthranilic acid at the base of the nodule.

We agree to this hypothesis as we have found out the bacteroids from the infection zone and zone between infection and nitrogen fixation zones did not show tendency towards maturation as initiation of nuclear condensation in these bacteroids did not occur and as a result they exhibited

homogeneous cytoplasm. Absence of electron dense material which is presumably leghaemoglobin in the bacteroids filled cells of the extended infection zone can be explained by this hypothesis.

Plants inoculated with VK15 strain showed surprising defects. Though these plants were not chlorotic but proper shoot elongation did not occur and as a result they looked stunted. The mean shoot dry weight of these plants were significantly less than the parental strain inoculated plants but more than those of the plants inoculated with *trpE(G)* and *aro* mutants thereby indicating reduced nitrogen fixation in these plants. Though the histology of VK15 induced nodules resembles that of the parental strain, it seems that the nodules induced by VK15 mutant could not fix nitrogen properly because of the non-availability of some tryptophan intermediate(s) which may be PRA, CDRP or indole glycerol phosphate or a combination of these. Indole acetic acid (IAA) production by *Rhizobium* has been proposed to play a role in proper nodule function and the source(s) of IAA production in *R. meliloti* are not fully understood. Though the indole-pyruvate pathway has been proposed as a source of IAA production, the possibility of IAA production in *R. meliloti* through another pathway can not be ruled out. In this context it seems that the major contribution of tryptophan biosynthetic pathway of *Rhizobium* is to provide compound(s) containing indole ring which is/are needed for IAA production. Since VK15 is a leaky mutant, the amount of indole compounds contributed by it may not be sufficient enough to meet the requirements of plant for indole acetic acid and it may be the reason for reduced nitrogen fixation and hence stunted growth. Absence of anthranilic acid as well as other tryptophan intermediates synthesis in *trpE* and *aro* mutants would have severely

hampered their symbiotic properties whereas partial symbiotic effectiveness of VK15 may be merely due to its leakiness which made available small amounts of the required tryptophan biosynthetic intermediates.

The plants inoculated with *aro* mutants showed significantly less mean shoot dry weight and the nodules induced by these mutants showed similarities in their histology with the nodules induced by *trpE(G)* mutants. These nodules had extended invasion zone and poorly developed nitrogen fixing zone that too at the base of the nodule. The mean shoot dry weight of plants were significantly lower than that of mean shoot dry weight of plants inoculated with Rmd201. Absence of anthranilic acid production in these mutants might have lead to extended infection zone and poorly developed nitrogen fixation zones. *R. meliloti* mutants with decreased deoxy arabino-heptonic acid-7-phosphate synthase (DAHP) activity because of which the metabolite flow through aromatic pathway is low induced nodules with extended infection zones (Jelesko *et al.*, 1993). This defect can be attributed to the decreased synthesis of anthranilic acid because of the fact the host plant provides tyrosine (because *tyr* mutants were symbiotically effective) and the nodules elicited by *aro* mutants showed similarities with the nodules induced by *trpE(G)* mutants. It is surprising to know how these mutants are able to manage phenylalanine as the *phe* mutants have severely affected symbiotic properties and nodules formed by them showed different histological defects from that of *aro* mutants induced nodules. It may be that plant supplies intermediates of phenylalanine and from them *aro* mutants synthesize phenylalanine to meet their phenylalanine requirement.

Phenylalanine requiring mutants showed severely affected symbiotic

properties. The nitrogen fixation zone was not observed in the nodules elicited by them and the electron microscopic studies showed degeneration of bacteria soon after release. In comparison to the defects in *aro* mutants where bacteroid transformation did occur, the defect in *phe* mutant could be merely due to unavailability of phenylalanine to the bacteria.

Cell surface molecules (cellulose fibrils, β-(1→2) glucans, lipopolysaccharides, acidic exopolysaccharides, etc.) production and transport of dicarboxylic acids by *Rhizobium* are important for successful symbiosis. Production of cell surface molecules was normal in all aromatic amino acid auxotrophs and they could transport and utilize dicarboxylates as carbon source as well. From this it is clear that symbiotic defectiveness in these auxotrophs is not due to absence of cell surface molecules production or the inability of auxotrophs to transport dicarboxylates.

The position of Tn5 insertion in VK15 was located to be at the *trp15* locus mapped by Kondorosi *et al.*, 1977. 100% linkage of auxotrophy to Tn5 insertion was observed in all auxotrophs and all prototrophic revertants showed kanamycin sensitiveness indicating the precise excision of Tn5. These revertants showed normal symbiotic properties. The above results indicated that in each mutant the same Tn5 insertion was responsible for auxotrophy as well as the symbiotic defects.

Overall the present results represent an other example in which *R. meliloti* utilizes the general metabolic pathways that are common to a large number of bacteria for the active expression of specific symbiotic functions.

References

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.

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

Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I. and Kondorosi, A. 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. Mol. Gen. Genet. **184:**314-325.

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., Terzaghi, B., Gherardi, 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.

Batut, J., Daveran-Mingot, M.-L., David, M., Jacobs, J., Garnerone, A. M. and Kahn, D. 1989. *FixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. EMBO J. **8:**1279-1286.

Beringer, J. E. and Hopwood, D. A. 1976. Chromosomal recombination and mapping in *Rhizobium leguminosarum*. Nature **264:**291-293.

Beringer, J. E., Beyon, J. L., Buchanan-Wollaston, V. and Johnston, A. W. B. 1978a. Transfer of drug resistance transposon Tn5 to *Rhizobium*. Nature **276:**633-634.

Beringer, J. E., Hoggan, S. A. and Johnston, A. W. B. 1978b. Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid mediated recom bination. J. Gen. Microbiol. **104:**201-207.

Bhuvaneswari, T. V., Bhagwat, A. A. and Bauer, W. D. 1981. Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. Plant physiol. **68:**1144-1149.

Blevins, D. G. 1989. An overview of nitrogen metabolism in higher plants. *In*: Plant Nitrogen Metabolism. (eds) Poulton, J. E., Romeo, J. T. and Conn, E. E., Plenum Publishing Corp., New York, pp. 1-41.

Brewin, N. J., Beringer, J. E. and Johnston, A. W. B. 1980. Plasmid mediated transfer of host range specificity between seven strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. **120:**413-420.

Brewin, N. J., Rae, A L., Perotto, S., Knox, J. P., Roberts, K., *et al.* 1990. Immunological dissection of the plant-microbial interface in pea nodules. *In:* Nitrogen Fixation: Achievements and Objectives. (eds) Gresshoff, P. M., Roth, J., Stacey, G. and Newton, W. E. New York, London: Chapman & Hall. pp. 227-234.

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

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

Caetano-Anolles, G., Crist-Estes, D. K. and Bauer, W. D. 1988. Chemotaxis of *Rhizobium meliloti* towards the plant flavone luteolin and dependence on functional nodulation genes. *In*: Nitrogen Fixation: Hundred Years After, (eds) Bothe, H., de Bruijn, F. J. and Newton, W. E., Stuttgart, New York: Fischer. p. 460.

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S. A. and Bauer, W. D. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can. J. Bot. **62:**2375-2384.

Casadesus, J. and Olivares, J. 1979. Rough and fine linkage mapping of the *Rhizobium meliloti* chromosome. Mol. Gen. Genet. **174:**203-209.

Cava, J. R., Elias, P. M., Turowski, D. A. and Noel, K. D. 1989. *Rhizobium leguminosarum* CFN42 genetic regions encoding lipopolysaccharide structures essential for complete nodule development on bean plants. J. Bacteriol. **171:**8-15.

Chandler, M. R., Date, R. A. and Roughlay, R. J. 1982. Infection and root nodule development in *Stylosanthes* species of *Rhizobium*. J. Exp. Bot. **33:**47-57.

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

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.

Clover, R. H., Kieber, J. and Signer, E. R. 1989. Lipopolysaccharide mutants of *Rhizobium meliloti* are not defective in symbiosis. J. Bacteriol. **171:**3961-3967.

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.

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

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.

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.

Dénarié, J., Truchet, G. and Bergeron, B. 1975. Effects of some mutations on symbiotic properties of *Rhizobium*. *In:* Symbiotic nitrogen fixation in plants. Vol. 7. (ed) Nutman, P. S., Cambridge University Press, Cambridge. p. 47.

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.

Dylan, T., Lelpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E., Helinski, D. R. and Ditta, G. S. 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.

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

Dylan, T., Nagpal, P., Helinski, D. R. and Ditta, G. S. 1990b. Symbiotic pseudorevertants of *Rhizobium meliloti ndv* mutants. J. Bacteriol. **172:**1409-1417.

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 Puhler, A. 1989. Identification and sequence analysis of the *Rhizobium meliloti dctA* gene encoding the C_4-dicarboxylate carrier. J. Bacteriol. **171:**5551-5560.

Federov, S. N. and Zaretskaya, A. N. 1979. Ethylmethanesulfonate induction of auxotrophic mutants of *Rhizobium meliloti* and their

characteristics. Mikrobiologiya **47:**728-732.

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

Fisher, R. F. and Long, S. R. 1989. DNA footprint analysis of transcriptional activator proteins NodD1 and NodD3 on inducible *nod* gene promoters. J. Bacteriol. **171:**5492-5502.

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

Forrai, T., Vincze, E., Banfalvi, Z., Kiss, G. B., Randhawa, G. S. and Kondorosi, A. 1983. Localization of symbiotic mutations in *Rhizobium meliloti*. J. Bacteriol. **153:**635-643.

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.

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 beta (1-2) glucan. J. Bacteriol. **169:**880-884.

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.

Goethals, K., van den Eede, G., van Montagu, M. and Holsters, M. 1990. Identification and characterization of a functional *nodD* gene in *Azorhizobium caulinodans* ORS571. J. Bacteriol. **172:**2658-2666.

Gottfert, M., Lamb, J. W., Gasser, R., Semeza, J. and Hennecke, H. 1989. Mutation analysis of the *Bradyrhizobium japonicum* common *nod*

genes and further *nod* box-linked genomic DNA regions. Mol. Gen. Genet. **215:**407-415.

Hass, D. and Holloway, B. W. 1976. R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **144:**243-251.

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

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.

Holliday, R. 1956. A new method for the identification of biochemical mutants of micro-organisms. 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.

Honeycutt, R. J., Mc Clelland, M. and Sobral, B. W. S. 1993. Physical map of the genome of *Rhizobium meliloti* 1021. J. Bacteriol. **157:**6945-6952.

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 effective nodulation of alfalfa. Mol. Gen. Genet. **202:**356-362.

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.

Jiang, J., Gu, B., Albright, L. M. and Nixon, B. T. 1989. Conservation between coding and regulatory elements of *Rhizobium meliloti* and *Rhizobium leguminosarum dct* genes. J. Bacteriol. **171:**5244-5253.

Julliot, J. S., Dusha, I., Renalier, M. H., Terzaghi, B., Garnerone, A. M. and Boistard, P. 1984. An RP4-prime containing a 285 kb fragment of *Rhizobium meliloti pSym* megaplasmid: structural characterization and utilization for genetic studies of symbiotic functions controlled by *pSym*. Mol. Gen. Genet. **193:**17-26.

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.

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

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

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

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

Khanuja, S. P. S. and Kumar, S. 1989. Symbiotic and galactose utilization properties of phages 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* HH 303 by Transposon Mutagenesis. Appl. Environ. Microbiol. **54:**423-427.

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

Kondorosi, A., Kiss, G. B., Forrai, T., Vincze, E. and Banfalvi, Z. 1977. Circular linkage map of *Rhizobium meliloti* chromosome. Nature **268:**525-527.

Kondorosi, A., Vincze, E., Johnston, A. W. B. and Beringer, J. E. 1980.A comparison of three *Rhizobium* linkage maps. Mol. Gen. Genet. **178:**403-408.

Kondorosi, A., Kondorosi, E., Gyorgypal, Z., Banfalvi, Z., Gyuris, J., *et al.* 1989. Molecular genetic basis of *Rhizobium*-legume interactions. Genome **31:**350-353.

Kondorosi, E., Banfalvi, Z. and Kondorosi, A. 1984. Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: identification of nodulation genes. Mol. Gen. Genet. **193:**445-452.

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

Kundig, C., Hennecke, H. and Gottfert, M. 1993. Correlated physical and genetic map of the *Bradyrhizobium japonicum* 110 genome. J. Bacteriol. **175:**613-622.

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

Leigh, J. A., and Walker, G. C. 1987. Exopolysaccharides of *Rhizobium*: Synthesis, regulation and symbiotic functions. Trends Genet. **10:**63-67.

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

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.

Lloyd, 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 the root hairs, but f-actin is involved in basipetal migration. Cell Motil. Cytoskeleton. **8:**27-36.

Long, S., Reed, J. W., Himawan, J. W. and Walker, G. C. 1988. Genetic analysis of a cluster of genes required for synthesis of the calcofluor binding exopolysaccharide of *Rhizobium meliloti*. J. Bacteriol. **170:**4329-4248.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: A laboratory mannual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, A.1.

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

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.

Mclever, J., Dyordjevic, 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. and Signer, E. R. 1977. Genetic mapping of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **74:**2076-2078.

Meade, H. M., Long, S. R., Ruvkun, 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.

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.

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

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

Newcomb, E. H. and Kowal, R. R. 1985. Ultrastructural specialization for ureide production in uninfected cells of soybean nodules. Protoplasma **125:**1-12.

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.

Noel, K. D., Stacey, G., Tandon, S. R., Silver, L. E. and Brill, W. J. 1982. *Rhizobium japonicum* mutants defective in symbiotic nitrogen fixation. J. Bacteriol. **152:**485-494.

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

Pankhurst, C. E., and Schwinghamer, E. A. 1974. Adenine requirement for nodulation of pea by an auxotrophic mutant of *Rhizobium leguminosarum*. Arch. Microbiol. **100:**219-238.

Perret, X., Broughton, W. J. and Brenner, S. 1991. Canonica ordered cosmid library of the symbiotic plasmid of *Rhizobium* species NGR234. Proc. Natl. Acad. Sci. USA. **88:**1923-1927.

Putnoky, P., Grosshopf, E., Cam Ha, D. T., Kiss, G. B. and Kondorosi, A. 1988. *Rhizobium fix* genes mediate at least two communication steps in symbiotic nodule development. J. Cell Biol. **106:**597-607.

Puvanesarajah, V., Schell, F. M., Gerhold, D. and Stacey, G. 1987. Cell surface polysaccharides from *Bradyrhizobium japonicum* and a non-

nodulating mutant. J. Bacteriol. **169:**137-141.

Rastogi, V. K. and Watson, R. J. 1991. Aspartate aminotransferase activity is required for aspartate catabolism and symbiotic nitrogen fixation in *Rhizobium meliloti*. J. Bacteriol. **173:**2879-2887.

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.

Roche, P., Lerouge, P., Prome, J. C., Faucher, C., Vasse, J., *et al.* 1991. NodRm-1, A sulphated lipo-oligosaccharide signal of *Rhizobium meliloti*, elicits hair deformation, cortical cell divisions and nodule organogenesis on alfalfa roots. *In:* Advances in Molecular Genetics of Plant-Microbe Interactions. (eds). Hennecke, H. and Verma, D. P. S. Dordrecht & Kluwer. pp. 119-126.

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

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. **78:**4284-4288.

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. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. Mol. Gen. Genet. **184:**326-333.

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

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.

Scherrer, A. and Dénarié, J. 1971. Symbiotic properties of some auxotrophic mutants of *Rhizobium meliloti* and their prototrophic revertants. Plant Soil **(Special vol.):**39-45.

Schlaman, H. R. M., Spaink, H. P., Okker, R. J. H. and Lugtenberg, B. J. J. 1989. Subcellular localization of the *nodD* gene product in *Rhizobium leguminosarum*. J. Bacteriol. **171:**4686-4693.

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

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

Sikka, V. K. and Kumar, S. 1984. Underexpression of Ap from R-plasmids in fast growing *Rhizobium* species. Appl. Environ. Microbiol. **48:**1248-1250.

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

Singh, A., Ram, J., Sikka, V. K. and Kumar, S. 1984. Derivation of marked strains in *Rhizobium leguminosarum* R1d1 by nitrosoguanidine and transposon mutagenesis. Indian J. Exp. Biol. **22:**239-247.

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 adhesin, 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., New Jersey.

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

Spaink, H. P., Weinman, J., Djordjevic, M. A., Wijffelman, C. A., Okker, R. J. H. and Lugtenberg, B. J. J. 1989. Genetic analysis and cellular localization of the *Rhizobium* host specificity-determining NodE protein. EMBO J. **8:**2811-2818.

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

Swanson, J. A., Tu, J. K., Ogawa, J., Sanga, R., Fisher, R. F. and Long, S. R. 1987. Extended region of nodulation genes in *R. meliloti* 1021. Genetics **117:**181-189.

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

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.

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., 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. 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.

Williams, M. N. W., Hollingsworth, R. I., Klein, S. and Signer, E. R. 1990. The symbiotic defect of *Rhizobium meliloti* exopolysaccharide mutants is supressed by *lpsZ*$^+$, a gene involved in lipopolysaccharide biosynthesis. J. Bacteriol. **172:**2622-2632.

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.