

DIFFERENTIATION OF NITROGEN-FIXING NODULES OF  
SESBANIA ROSTRATA WITH SPECIAL REFERENCE TO  
OLEOSOMES (LIPID BODIES)

CENTRE FOR NEWFOUNDLAND STUDIES

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**DIFFERENTIATION OF NITROGEN-FIXING  
NODULES OF *SESBANIA ROSTRATA* WITH  
SPECIAL REFERENCE TO OLEOSOMES (LIPID  
BODIES)**

BY

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## ABSTRACT

Development and differentiation in the early stages of nitrogen-fixing stem and root nodules was studied in *Sesbania rostrata*. Correlated light and electron microscopic observations have revealed that the bacterium *Azorhizobium caulinodans* IRG 46, which nodulates *Sesbania* possibly enters the host through fissures encircling the secondary roots or the root initials present on the stems. Root hair infection threads could not be seen, although root hair growth inhibition was induced by the bacteria. The bacteria passed through the intercellular space before producing infection thread-like invaginations in the target cells, where they were released.

One of the striking features of the early stages of differentiation in the infected cell is the occurrence of oleosomes (lipid bodies) in both root and stem nodules. Even more remarkable is the size of the oleosomes in the stem nodule cells, which are in some cases eight times larger than those in the root oleosome ( 0.5 - 1.0  $\mu\text{m}$  in diameter) population. This increased size is thought to be due to the oversupply of photosynthate from the chlorophyllous cortex of the stem nodule. The photosynthate is possibly stored in triacylglycerol form within oleosomes for short term use.

The oleosomes disappear in both root and stem nodules after maturation of the infected cells. In both nodules maturation leads to multiple occupancy of bacteroids within peribacteroid membranes, which proliferate during nodule maturation.

Lipolytic activity could be localised in the infected zone of the nodule by the Nile Blue dyeing method. Catalase activity was determined by spectrophotometry of the bacteroid and the host cytosol fractions, the latter showing higher activity. Catalase was localized in vesicles budding off the perisymbiotic membrane by the DAB reaction.

Total lipid estimation by the gravimetric method indicated that the seeds have a higher total lipid content than the stem and root nodules. Gas chromatographic analyses of the lipids from the isolated oleosomes indicate that the dominant unsaturated fatty acids are  $C_{14:1}$ ,  $C_{16:1}$  in the stem nodule, whereas  $C_{18:1}$ ,  $C_{18:2}$  are dominant in the root nodule and seeds. Among saturated fatty acids  $C_{16:0}$  and  $C_{18:0}$  in the stem nodule, and  $C_{16:0}$  in the root nodule and the seed are dominant respectively.

In *Sesbania* nodules the oleosomes seem to be used up for the growth and development of the infected cells; they are not involved in nitrogen fixation *per se*.

**Dedicated to the memory of my  
beloved father**

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# TABLE OF CONTENTS

	Page
ABSTRACT .....	ii
ACKNOWLEDGEMENTS .....	v
TABLE OF CONTENTS .....	vi
LIST OF TABLES .....	ix
LIST OF FIGURES .....	x
<b>I. INTRODUCTION</b>	
1.1. Nitrogen Cycle .....	4
1.1.1. Ammonification .....	7
1.1.2. Nitrification .....	7
1.1.3. Denitrification .....	7
1.1.4. Nitrogen Fixation and Soil Bacteria .....	8
1.2. Source of Nitrogen for Plants without Nodules .....	9
1.3. Relationship of Nitrate Assimilation and Photosynthesis .....	13
1.4. Nodulation: A Complex Developmental Sequence .....	16
1.4.1. Significance of Infection: Recent Genetic Advances .....	18
1.5. Nodulins .....	20
1.5.1. Early Plant Nodulins .....	23
1.5.2. Late Nodulins .....	23
1.6. Nodule Metabolism in Relation to $N_2$ -Fixation .....	24
1.6.1. Nitrogen-Fixation by Bacteroids .....	24

1.6.2. Bacteroid Nitrogenase .....	24
1.6.3. Bacteroid Carbohydrate Metabolism.....	27
1.6.4. Leghemoglobin .....	28
1.7 Diversity of Nodulating Legumes .....	28
1.7.1 Infection Process, Nodule Formation and ontogeny of Stem Nodules .....	34
1.7.2 Oleosomes: Its Structure and Role.....	42
1.7.3 Role of Oleosomes in Nitrogen-Fixing Nodules.....	44
1.7.4 Objectives of the Present Study.....	45
1.7.5 Hypothesis.....	46
<b>II. MATERIALS AND METHODS</b>	
2.0. Plant Materials .....	47
2.1. Azorhizobium Cultures .....	47
2.2. Infection of root hairs by <i>Azorhizobium</i> .....	48
2.3. Electron Microscopy of Samples .....	48
2.4. Oleosome Preservation, Localization, Staining and Counting .....	49
2.5. Cytochemical Localization of Catalase Activity .....	50
2.5.1. Separation of Nodule and Bacteroid Fractions....	51
2.5.2. Catalase Assay .....	51
2.6. Histochemical Demonstration of Lipase.....	52
2.7. Total Lipid Estimation .....	53
2.8. Oleosome Fraction .....	54
2.9. Fatty Acid Analysis .....	54

**III. RESULTS**

3.0. Infection Process .....	56
3.1. Early Stages of Differentiation .....	61
3.2. Histochemical Localization of Lipase .....	74
3.3. Catalase Activity .....	79
3.4. Lipid Estimation and Oleosome Fraction .....	79
3.5. Fatty Acid Analyses of Oleosomes .....	86

**IV. DISCUSSION**

4.0 Infection Process .....	90
4.1. Stem Infection Site .....	91
4.2. Presence of Oleosomes in the Early Stages of Nodulation.....	94
4.3. Fatty Acid Composition of Oleosomes of <i>Sesbania</i> Nodules and Seeds.....	97
4.4. Lipolytic Activity .....	99
4.5. Catalase Activity .....	100
4.6. Function of Oleosomes in Nitrogen-Fixing Nodules	
4.6.1. Arctic and Alpine Legumes.....	102
4.6.2. Peanut Nodules .....	103
4.6.3. <i>Sesbania</i> Nodules.....	103
<b>V. CONCLUDING REMARKS.....</b>	<b>105</b>
<b>VI. REFERENCES .....</b>	<b>107</b>

## LIST OF TABLES

Table 1. Catalase activity in bacteroid and host cytosol fractions of <i>S. rostrata</i> root nodules.....	82
Table 2. Amount of total lipid of root nodule and stem nodule and seed of <i>S. rostrata</i> .....	85
Table 3. Major fatty acids of oleosomes of root and stem nodules and seed of <i>S. rostrata</i> .....	89

# LIST OF FIGURES

	Page
Figure 1. Distribution of nitrogen in dead and living organic material in the terrestrial biosphere .....	3
Figure 2. The recycling of nitrogen in the biosphere.....	6
Figure 3. Probable mode of action of nitrogenase.....	11
Figure 4. The relationships between nitrate estimation and photochemical reactions in the chloroplast...	15
Figure 5. Organization and regulation of the nod and nif genes on the sym plasmid of <i>Rhizobium meliloti</i> ...	22
Figure 6. A schematic representation showing the interaction between the microorganism (bacteroid) and the host plant (legume) and the biological components involved in symbiotic nitrogen fixation.....	26
Figure 7. <i>Sesbania rostrata</i> showing root and stem nodules..	31
Figure 8. <i>Sesbania rostrata</i> stem nodule ontogeny.....	41
Figure 9. Roots of inoculated seedlings showing short root hairs.....	58
Figure 10. Root hairs of uninoculated plants with long root hairs.....	58
Figures 11. & 12. Light and scanning electron micrographs	

- showing a fissure (see arrow) encircling the emerging secondary root at the point of emergence through the epidermis of the root..... 58
- Figure 13a. Photomicrograph showing the arrangement of primordia in vertical rows on the stem .....60
- Figure 13b. Longitudinal section of primordium of *Sesbania* stem showing a fissure (see arrow) encircling the root primordia of the stem .....60
- Figure 14. Entry of bacteria (see arrow) through intercellular spaces and infection thread development by invaginations of intercellular spaces showing bacteroids (b) and nucleus (n)...62
- Figure 15a. Photomicrograph of a pPD stained semithin section of a stem nodule. The younger infected cells contain oleosomes (heavily stained) of various sizes.....65
- Figure 15b. Histogram showing the different sizes of oleosomes in root and stem nodules .....67
- Figure 16. Electron micrograph showing lack of oleosomes in the root primordia (stained with pPD) .....69
- Figure 17. Photomicrograph showing the oleosomes in infected cells .....71
- Figure 18.- Control preparations treated with hexane to dissolve lipids .....71
- Figure 19. Photomicrograph of a root nodule showing

	oleosomes at early stages of nodule growth . . . . .	73
Figure 20.	Showing the disappearance of oleosomes at the late stages of nodule growth . . . . .	73
Figure 21.	Ultrastructure of an infected cell showing one bacteroid (b) per perisymbiotic membrane with a large oleosome. Note the presence of endoplasmic reticulum (er) close to it . . . . .	75
Figure 22.	Ultrastructure of an infected cell showing oleosomes and appearance of numerous vesicles (v) . . . . .	75
Figures 23. & 24.	Ultrastructure of infected cells showing the single and multiple occupancy of bacteroids in the perisymbiotic membrane sacs of the host cell . . . . .	78
Figures 25.	Histochemical demonstration of lipase activity stained by Nile Blue . . . . .	81
Figure 26.	Control preparation of lipase activity. . . . .	81
Figures 27 & 28.	Ultrastructure showing positive DAB reaction in the vesicles seemed to be budding off the perisymbiotic membrane of the symbiosome . . . . .	84
Figures 29.	Ultrastructure of the control preparation with an inhibitor triazole showing lack of DAB reaction. . . . .	84
Figure 30.	Ultrastructure of a disrupted infected cell stained	

	with DAB reaction.....	84
Figure 31.	Scanning electron micrograph of an isolated stem nodule oleosome fraction showing "megaoleosomes".....	88
Figure 32.	Scanning electron micrograph of an isolated oleosome fraction from the seed.....	88

# I. INTRODUCTION

Although more than two thirds of the earth's atmosphere consists of nitrogen, this element is often in short supply to organisms, particularly to plants. But without nitrogen life on this planet, as we know it today, could not exist. This is because nitrogen is the major component of a number of compounds which are essential for the structure and functions of all organisms, compounds including for example DNA and proteins. Apart from water, nitrogen is the key substance that limits the primary productivity of plants in most parts of the world. The plant has to rely on nitrogen occurring in more reactive combinations, e.g. with hydrogen (as in ammonia) or oxygen (as in nitrate or nitrite) to build its organic nitrogen containing molecules. Such nitrogen is referred to as "combined nitrogen" and is not nearly as common as the dinitrogen state. Biologically available nitrogen forms only a minute fraction of the total present on earth. Furthermore, much of this nitrogen is temporarily inaccessible to plants as it is present in the form of dead organic material from which it is only slowly released by microbial action. Figure 1 shows the quantitative relationship between the distribution of nitrogen in dead (necromass) and living material (biomass) in the terrestrial biosphere. Thus, we find in the biosphere there is a real shortage of nitrogen that can be made use of

Figure 1. Distribution of nitrogen in dead and living organic material in the terrestrial biosphere. The left hand circle shows the quantitative relationship between the nitrogen of the biomass (4%) compared with the nitrogen of the necromass (96%). The right hand circle shows the distribution of nitrogen between the plants (94%), animals (2%) and microorganisms (4%) which make up the biomass (Lewis, 1986).

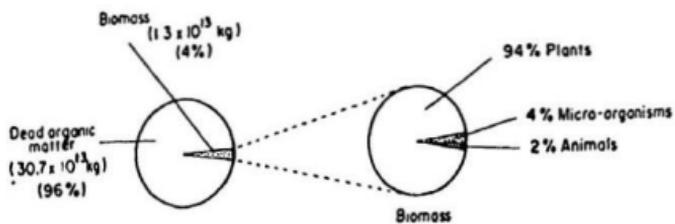


Figure 1

by living organisms and a necessity exists for the conservation of biomass complexed nitrogen. Nature responds to this necessity by recycling combined nitrogen in a complex process known as the "Nitrogen Cycle", a simplified version of which is shown in Figure 2 (Lewis, 1986).

### 1.1 NITROGEN CYCLE

The nitrogen cycle involves the scavenging of combined nitrogen from dead organisms and atmospheric dinitrogen-fixation by microbial activity, its passage through the soil in various forms and its final re-utilization by plants to form proteins, nucleic acids and other nitrogenous compounds of living cells of plants and animals.

Microorganisms perform essential functions within ecosystems, the integrated systems of biological populations and abiotic factors that comprise the global biosphere by biogeochemical cycling. Biogeochemical cycling is the movement of materials via biochemical reactions through the global biosphere and is a consequence of exchange of elements between the biotic and abiotic portions of the biosphere that occurs through the characteristic cyclic pathways as a result of metabolic reactions of living organisms. The major processes of the nitrogen cycle are ammonification, nitrification, denitrification and dinitrogen fixation.

Figure 2. The recycling of nitrogen in the biosphere (Lewis, 1986).

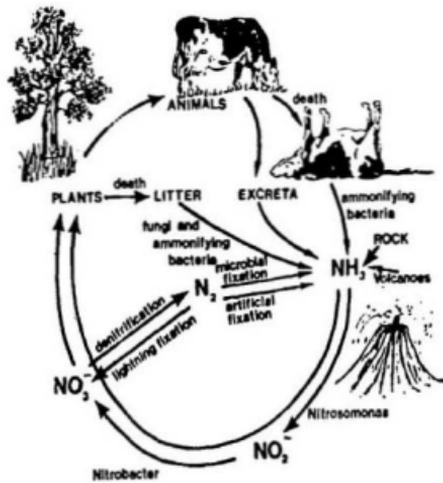


Figure 2

### 1.1.1 AMMONIFICATION

When organisms die, their nitrogenous remains become mineralized i.e. converted into inorganic forms of nitrogen, mainly ammonia, by a host of bacteria, fungi and actinomycetes. Certainly without microbial mineralization of nitrogen in humus, back to soluble ammonia, the world's nitrogen would soon be tied up in the biosphere (Gutshick, 1978). Examples of species capable of ammonification are found in the genera *Bacillus*, *Streptomyces*, and *Aspergillus*.

### 1.1.2 NITRIFICATION

Ammonia formed as a result of ammonification is a rich source of potential energy which does not go unexploited and bacteria like *Nitrosomonas* oxidize ammonia to nitrite and *Nitrobacter* further oxidize nitrite to nitrate within the upper aerobic regions of the soil. The ammonia and nitrate can be taken up by the plant roots from the upper layers of the soil and then incorporated into proteins and other nitrogen containing substances.

### 1.1.3 DENITRIFICATION

The major loss of combined nitrogen to the atmospheric dinitrogen pool is by the process of denitrification. Since nitrate is not absorbed on the soil particles it is readily leached to lower level and eventually finds itself below the area of exploitation by the roots of plants. Denitrifying

bacteria attack nitrate using it as a terminal electron acceptor in the place of oxygen and dinitrogen gas is produced as an end product of the reaction. The nitrogen gas readily diffuses back into the atmosphere and some of it is made available again as fixed nitrogen by free living or symbiotic bacteria using the nitrogenase reaction (Whatley, 1981).

#### 1.1.4 NITROGEN FIXATION AND SOIL BACTERIA

The process by which nitrogen is reduced to ammonia is called nitrogen fixation. Only certain prokaryotic organisms are able to fix molecular nitrogen and to grow in the absence of combined nitrogen. These have been included as Gram-negative aerobic/microaerophilic bacteria belonging to the group 4 and subgroup 4A in the Bergey's Manual of Determinate Bacteriology (Holt et al. 1994). Their generic names are i) *Azorhizobium* ii) *Bradyrhizobium* and iii) *Rhizobium*. Commonly present in most soils, they are able to live in symbiotic associations forming a specialized organ, the nodule, in the roots of legumes and to fix atmospheric nitrogen. This symbiotic association places the legumes in an advantageous situation as far as their nitrogen nutrition is concerned. Like other plants they can also utilize combined nitrogen present in the soil.

Of these genera mentioned above, *Azorhizobium* was only characterized in the mid-eighties (Dreyfus et al. 1988). It is fast growing and has the ability to form stem nodules in

*Sesbania* and other legumes. *Bradyrhizobium*, the slow growing soil bacterium which nodulates roots of various legumes, was recognized as a separate genus in early eighties (Jordan, 1984 in Bergey's Manual of Determinate Bacteriology, 1984). *Rhizobium* is now considered to be the fast growing genus inducing nodules in various legumes. Comparative gel electrophoresis of whole-cell proteins and deoxyribonucleic acid (DNA)-DNA and DNA-ribosomal ribonucleic acid (rRNA) hybridizations suggest that *Azorhizobium* is different from *Rhizobium* and *Bradyrhizobium* (Dreyfus et al. 1988).

Nitrogen fixation requires the presence of a special enzyme system, nitrogenase and particular conditions such as a reductant, a reducible substrate, an ATP generating system, and an anaerobic environment to function (Yates, 1980) (Fig. 3). The ability to fix nitrogen is distributed among many species of oxygenic and anoxygenic phototropic bacteria, chemoautotrophic and chemoheterotrophic bacteria, aerobic and anaerobic bacteria, in short among representatives of all physiological groups.

The actual reduction of atmospheric nitrogen to ammonia (Lewis, 1986) catalyzed by nitrogenase is as follows:



(where Pi represents inorganic phosphate)

## 1.2 SOURCE OF NITROGEN FOR PLANTS WITHOUT NODULES

Nitrate reduction by higher plants is one of the most

Figure 3. Probable mode of action of nitrogenase (Lewis,  
1986).

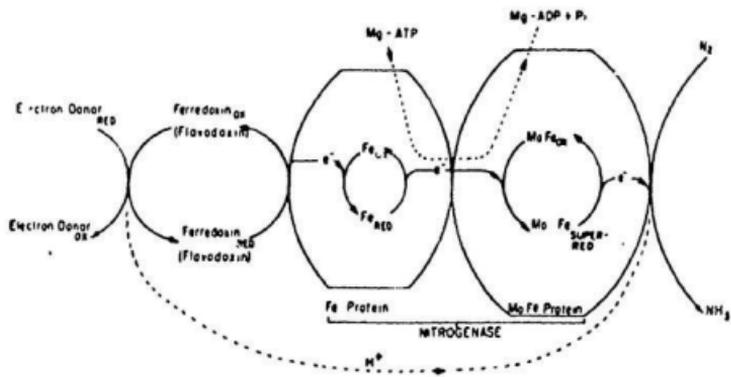


Figure 3

fundamental biological processes by which oxidized inorganic nitrogen is reduced to ammonia with water as the ultimate reductant at the expense of solar energy (Losada et al. 1981). With the exception of some free living blue-green algae and some plants that have a symbiotic association with nitrogen fixing prokaryotes (section 1.4), photosynthetic organisms derive most of the nitrogen from nitrate.

Utilization of nitrate by higher plants includes several processes, any one of which could limit nitrate assimilation (Huffaker and Rains, 1978). The first control point for nitrate assimilation is the uptake system. Nitrate absorbed by roots may be reduced by nitrate reductase (NR) in the roots or accumulated in the storage sites (vacuoles) or transported via the xylem to the shoots. After absorption a significant amount of nitrate is stored in the roots, some can be reduced and the remainder is translocated to the leaves (Huffaker and Rains, 1978).

The enzyme involved in the assimilation of nitrate is the NR (Lee, 1980). Some plants reduce considerable amounts of nitrate-nitrogen in the roots but the majority of the plants reduce it in the shoots (Lee, 1980). Ammonia formed during reduction of nitrate in roots or in shoots must be combined with a carbon skeleton before it can be transported to other parts of the plant cell (Lee, 1980). Each process in the uptake, distribution, reduction and incorporation of nitrogen

into organic forms is under genetic influence (Goodman, 1979) and thus may be the control point.

Nitrate assimilation includes both reductive and non-reductive processes in converting nitrate-nitrogen to amino-nitrogen. The four enzymes involved are nitrate reductase (NR) nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT). Three of these reactions are reductive and a total of 10 electrons must be provided for the assimilation of nitrate ion to glutamate (section 1.3) (Fig. 4).

### 1.3 RELATIONSHIP OF NITRATE ASSIMILATION AND PHOTOSYNTHESIS

Nitrate assimilation in green tissue is dependent on the photochemical reactions of the chloroplast to provide the reductant and ATP to convert nitrate to amino acids (Figure 4) (Schrader and Thomas, 1981). Nitrate reduction in the cytosol can be provided indirectly with reductant (NADH) from photosynthesis by oxidation of substrates such as dihydroxy acetone phosphate (DHAP) or malate are transported from the chloroplasts to the cytosol by the phosphate translocator (Heber, 1974). Inside the chloroplasts the reduction of nitrite to ammonia can be coupled directly to light reactions through reduced ferredoxin as an electron donor. ATP from photophosphorylation can be used for GS reaction and reduced ferredoxin can be used for GOGAT reaction as shown in the Figure 4. The quantum requirement for the reduction of nitrite

Figure 4. The relationships between nitrate estimation and photochemical reactions in the chloroplast. PT = phosphate translocator; NR = nitrate reductase; NiR = nitrite reductase; GS = glutamine synthetase; GOGAT = glutamate synthase; GLN = glutamine; GLU = glutamate;  $Fd_{red}$  = reduced ferredoxin;  $Fd_{ox}$  = oxidized ferredoxin; DHAP = dihydroxyacetone phosphate; PGald = 3-phosphoglyceraldehyde; 1,3 diPGA = 1,3 biphosphoglycerate; PGA = 3-phosphoglycerate; RuBp = ribulose, 1,5 biphosphate;  $\alpha$ kg =  $\alpha$ -ketoglutarate (Schrader and Thomas, 1981).



to ammonia is 12. The conversion of glutamate to glutamine requires 4 photons. If a triose phosphate (DHAP) is translocated from the chloroplast to the cytosol to provide the NADH for nitrate reduction, the reduction of phosphoglyceraldehyde to glyceraldehyde in the chloroplast will require 4 photons (Fig 4). Therefore in illuminated green tissues the total quantum requirement for nitrate reduction to ammonia is 16 photons per nitrate ion and for conversion of nitrate to glutamate is 20. For the non-chlorophyllous tissue or dark reduction of nitrate in green tissues, the reduction for nitrate assimilation must come from oxidation of carbohydrates or organic acids via the oxaloacetate-malate shuttle and most of the carbon is provided by sucrose transported to site of reduction.

#### 1.4 NODULATION: A COMPLEX DEVELOPMENTAL SEQUENCE

The complex series of events leading from bacterial colonization of the legume root to the export of symbiotically fixed nitrogen compounds from active nitrogen-fixing nodules require controlled coordinated genetic expression of both bacterial and plant partners (Long, 1989). Recognition of a specific host by rhizobia<sup>1</sup> is a prelude to infection. The recognition by rhizobial cells of legume roots is likely to be one of the first steps in the required sequence of

---

<sup>1</sup> rhizobia has been used in a broad sense to mean all nitrogen fixing symbiotic bacteria

interactions leading to infection and nodulation. Lectins, a group of plant proteins, may be involved in this recognition process. These carbohydrate binding proteins interact selectively with the microbial cell surface. Bacterial exopolysaccharides (EPS) also play a role in nodule formation. Nodule formation is stimulated at the root cortex by EPS which requires the nod D gene product for their synthesis (Leigh and Coplin, 1992) (section 1.4.1 and Fig 5.). The initial interactions involve attachment of the rhizobia to the surface of the root hairs and the formation of a thread-like structure called the infection thread. Rhizobia usually attach to the surface of the roots and root hairs in an end-on position apparently because the most active binding component on the cell wall or in the capsule occurs near the pole of the rhizobia (Bal et al. 1978).

Initiation of the infection thread is first observed as a swelling and the appearance of callose in the cell wall at the site of infection and an increase in the opacity and cytoplasmic streaming of the associated cytoplasm (Kumarasinghe and Nutman, 1977). During the period of growth of the infection thread through three to six layers of root outer cortical cells, meristematic activity is initiated in a small group of cells of the root cortex (Libenga and Harkes, 1973). Growth of the thread continues into this meristematic region where rhizobia are released from the tip into the

innermost meristematic cells which are pre-dominantly polyloid (Dart, 1977). The remainder of the meristematic zone consists of diploid cells which divide to form a radial system of noninfected cells in the mature nodule. These cells appear likely to enhance gaseous diffusion through the nodule (Vance, 1990). A distinctive feature of the effective nodule is the separation of bacteroid ( $N_2$ -fixing symbiotic bacterium) from the host cell cytoplasm by the peribacteroid membrane (Verma et al. 1978, Choong et al. 1993, Robertson and Farnden, 1980). Recently the term "symbiosome" has been introduced to refer to the bacteroid including the perisymbiotic membrane (Mellor, 1989).

Morphologically nodules are distinguished into two types namely, determinate and indeterminate. Determinate nodules are spherical and develop from a non-persistent hemispherical meristem, while indeterminate nodules are elongated cylindrical (finger-like) and develop by distal growth from a apical meristem (Allen and Allen, 1981).

#### **1.4.1 SIGNIFICANCE OF INFECTION: RECENT GENETIC ADVANCES**

Since most soils contain numerous types of different bacteria, the host plant and rhizobia must have mechanisms that allow homologous (compatible) rhizobial strains to penetrate and subsequently develop nodules, whereas heterologous (incompatible) strains and other soil bacteria are not allowed entry (Vance, 1990). The initial signalling

between the host plant and *Rhizobium* involves the exudation of low molecular weight flavonoids and isoflavonoids from roots that induce the expression of bacterial nodulating genes. This communication results in curling of root hairs and entrapment of bacteria within the curl, and the active penetration of bacteria through the cell wall (Djordjevic et al. 1987).

Advances in molecular genetics have provided a mechanistic explanation for host plant-rhizobia specificity. Symbiotic genes of rhizobia are categorized as those affecting nodulation genes (nod genes) and those controlling nitrogenase (nif genes). The symbiotic genes of *R. meliloti* have been analyzed in detail and serve as the foundation of understanding of the biochemical and molecular control of nodulation (Vance, 1990).

Mutation studies of the "nod A,B,C" genes indicate that these genes are involved in the earliest steps of symbiotic interaction i.e. root hair curling, infection and nodulation (Vance, 1990). The nod A,B,C genes in most rhizobia are functionally identical. Nodulation by rhizobial species is very host specific. For example *R. meliloti* nodulates alfalfa but not clover or soybean. This host specific nodulation characteristic is regulated by two operons containing Nod E, F, G and Nod H in *R. meliloti* (Vance, 1990).

Regulation of nod genes has been particularly revealing because experiments have demonstrated that the transcription

of Nod genes is activated by secondary plant products found in root exudates (Mulligan and Long, 1985). This control is affected by the interaction of the nod D gene product with plant root exudates. The induction of operons nod ABCIJ and nod EFGH requires the presence of Nod D gene product as shown in Fig. 5 (Vance, 1990) and the compounds in root exudates responsible for induction of nod gene transcriptions are flavones, flavonones and isoflavonones.

### 1.5. NODULINS

Specific expression of plant and bacterial genes accompanies the development of the symbiosis. Infection of legume roots by *Rhizobium* bacteria and the subsequent formation of the root nodule requires the expression of specific genes and the synthesis of unique proteins by both host and invading rhizobia. Plant proteins synthesized directly in response to infection and nodule formation (not in roots or in free-living rhizobia) are nodule specific proteins called nodulins (Legocki and Verma, 1979). Nodulins are distinguished from bacteroid and bacterial proteins; they are synthesized from poly (A)<sup>+</sup> RNA on 80 S ribosomes. Complementarity of nodulin mRNA to host genomic DNA is additional evidence for the plant origin of nodulins (Vance, 1990). The nodulin genes have been operationally defined as early and late nodulin genes according to the timing of their expression during nodule development (Govers et al. 1987).

Figure 5. Organization and regulation of the nod and nif genes on the sym plasmid of *Rhizobium meliloti* (Vance, 1990).

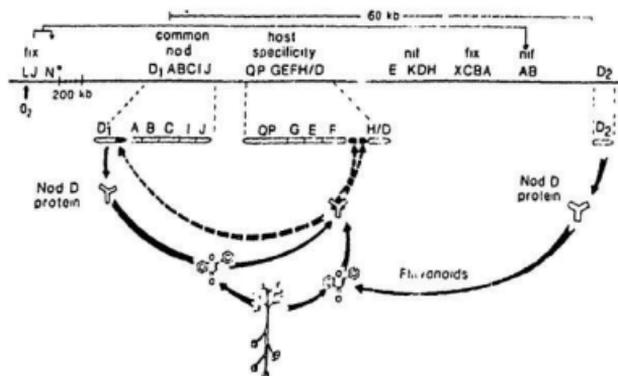


Figure 5

### 1.5.1 Early Plant Nodulins

Early plant nodulins which are expressed in roots within a few days of exposure to *Rhizobium* are most likely involved in the infection process and development of the nodule structure (Nap and Bisseling, 1990). Amongst the most characterized is soybean nodulin N-75, carried in part on a cDNA clone pENOD2 (Franssen et al. 1987). The sequence of this gene reveals a highly proline-rich repeat motif, [Pro-Pro-X-Glu-Lys-Pro-Pro], which suggests that it may be related to the (hydroxy)- proline-rich glycoproteins characterized as plant cell wall components. The trigger for early nodulin expression is produced by plant-bacteria interactions at the surface, probably involving the function of the known nod genes (Govers et al. 1987) and the function of N-75 and other early nodulins is most likely associated with nodule development, rather than with later differentiation (Franssen et al. 1987).

### 1.5.2 LATE NODULINS

The late nodulin genes comprise a large group of genes that is expressed around the onset of nitrogen fixation. Late nodulins aid in the function of a root nodule by creating the physiological conditions required within the nodule for nitrogen fixation, ammonium assimilation, and transport (Nap and Bisseling, 1990). Among some of the identified late nodulins are the leghemoglobins (oxygen carrier), sucrose synthetase (carbon metabolism), uricase (nitrogen

assimilation, ureides), glutamine synthetase (nitrogen assimilation) (Vance, 1990). In terms of development, late nodulins are truly late, because the full nodule structure with all its defining characteristics has developed before late nodulin gene expression becomes detectable. Late nodulins are also associated with the peribacteroid membrane (Nap and Bisseling, 1990; Choong et al. 1993) and all late nodulin genes appear to be coordinately expressed in time and development. These genes might thus all be activated as a result of a single signal, perhaps related to release of bacteria from the infected cells. The nature of this presumed signal, however, and the involvement of bacterial genes remain unclear.

## **1.6 NODULE METABOLISM IN RELATION TO $N_2$ FIXATION**

**1.6.1 Nitrogen fixation by bacteroids:** The bacteroids of legume nodules contain all the enzyme systems necessary for the reduction of  $N_2$  to ammonia (Robertson and Farnden, 1980). This fixation of nitrogen in bacteroids is catalyzed by the enzyme nitrogenase which requires energy, obtained by the metabolism of carbohydrates in the form of a reductant and ATP (Fig. 6) (Verma, 1982).

**1.6.2 Bacteroid nitrogenase:** The role of nitrogenase in reducing the atmospheric nitrogen is described in 1.1.4 section.

Figure 6. A schematic representation showing the interaction between the microorganism (bacteroid) and the host plant (legume) and the biological components involved in symbiotic nitrogen fixation (Verma, 1982).

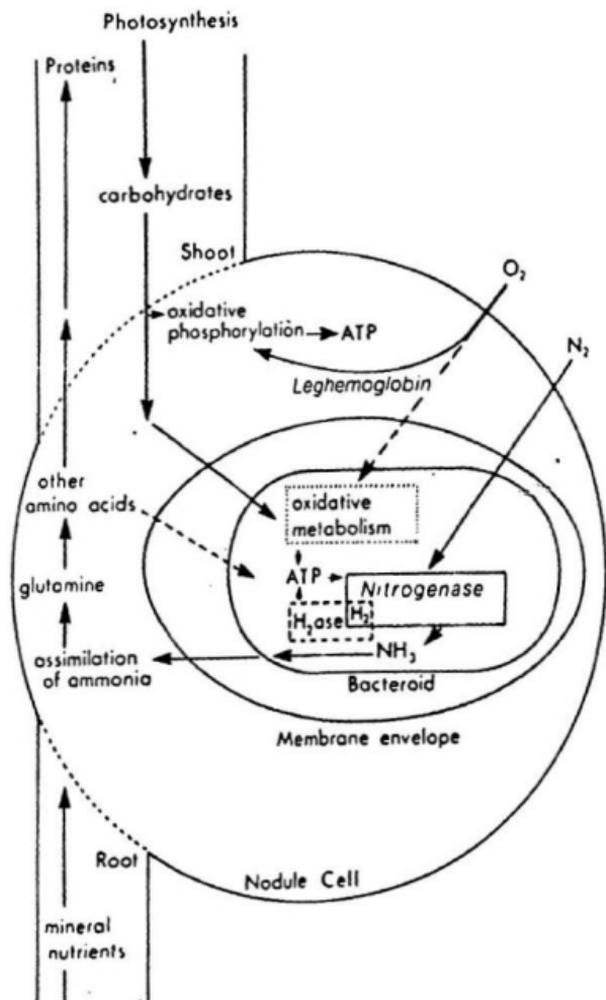


Figure 6

### 1.6.3 Bacteroid carbohydrate metabolism and ATP production and electron transport to nitrogenase:

ATP for the nitrogenase reaction is generally assumed to be produced by bacteroid oxidative phosphorylation (Robertson and Farnden, 1990). The main substrates supplied to bacteroids by the plant for the generation of ATP and reductant for nitrogenase fixation are probably sugars and/or organic acids. Sucrose appears to be the major metabolite translocated from the leaves to the roots and nodules (Robertson and Farnden, 1980). The tricarboxylic acid cycle (Kreb's cycle) has been reported to occur in free living *Rhizobium spp.*, *R. japonicum* and *Pisum sativum* bacteroids (Kurz and Larue, 1977).

The dependence of the bacteroid nitrogen fixation upon a steady supply of photosynthate from the host is well documented. Pate (1977) has stressed the importance of "current photosynthesis" in maintaining the optimum rates of nitrogen fixation. Bal and Siddique (1991) and Siddique and Bal (1991) reported that lipids can also be supplementary substrates that provide ATP, reducing power and the carbon skeleton through its beta oxidation pathway.

The product of nitrogen fixation (ammonia) is released into the host cell cytoplasm and converted to glutamine by glutamine synthetase. In the symbiotic situation of the nodule, the ammonia assimilation genes of rhizobia are repressed (Robertson and Farnden, 1980), as a result, release

of ammonia to the host cytoplasm is enhanced, and readily converted to amide by glutamine synthetase.

#### 1.6.4 LEGHEMOGLOBIN (Lb)

The nitrogenase enzyme complex is extremely sensitive to oxygen and yet the large ATP requirement for nitrogenase activity is believed to be derived from oxygen dependent oxidative phosphorylation within rhizobial cells (Appleby et al. 1988). An important solution to this problem in legume nodules is provided by leghemoglobin, a plant protein found outside the rhizobial cell. Leghemoglobin is thought to facilitate the diffusion of oxygen through the plant cell cytoplasm to the bacterial cells at concentrations which allow oxidative phosphorylation to occur without destroying nitrogenase activity (Appleby et al. 1988). This protein is truly symbiotic in that the globin portion is derived from the plant while the heme portion is derived from the rhizobial partner (Appleby et al. 1988).

#### 1.7 DIVERSITY OF NODULATING LEGUMES

Nodule formation is the essential feature of the legume-*Rhizobium* symbiosis, as nodules are the sites where the energy-requiring reduction of molecular nitrogen takes place. Most species in the family Leguminosae (now called Fabaceae) form nodules on roots. However, only a few species exhibit nodule formation on above-ground stems and are therefore referred to as stem-nodulating legumes.

*Sesbania*, an annual legume which grows in flooded soils in the Sahel region of West Africa during the rainy season is characterized by profuse nodulation along the stem and roots (Fig. 7) (Dreyfus and Dommergues, 1981a). *Rhizobium* and *Bradyrhizobium* strains are quite different from bacterial strains that nodulate *Sesbania*. Strains that nodulate *Sesbania* constitute a separate rRNA subbranch on the *Rhodopseudomonas palustris* rRNA branch in rRNA superfamily IV; and the closest relative of these organisms is *Xanthobacter*, but they are phenotypically and genotypically sufficiently different from the latter genus to deserve a separate generic rank (Dreyfus et al. 1988). A new genus, *Azorhizobium*, has been proposed, with one species, *Azorhizobium caulinodans*, which is a fast growing soil bacteria (Dreyfus et al. 1988). This bacterium is responsible for the induction of the two types of nodules (root and stem nodules). The phenomenon of nodulation on stems was first observed at the end of the last century on *Aeschynomene elaphroxylon*, but its significance was not recognized until recently (Allen and Allen, 1981). The term "stem-nodule" first appeared in histological studies on *Aeschynomene aspera* by Hagerup in 1928 (Ladha et al. 1992). He first associated the formation of bacteria-filled host cells on stems with nitrogen assimilation. Further reports on stem-nodule formation followed for *Ae. paniculata*, *Neptunia*

Figure 7. *Sesbania rostrata* showing root and stem nodules.



oleracea, *Ae. indica* (Arora, 1954), *Ae. elaphroxylon*, *Ae. evenia*, and *Ae. villosa* (Jenik and Kubikova, 1969). But it was only in the 1980s when Dreyfus and Dommergues (1981b) reported the profuse stem nodulation on fast growing *Sesbania rostrata* that the potential importance of this unique feature was recognized. The recognition of the agronomic potential of stem-nodulating legumes as green manure for lowland rice areas (Dreyfus et al. 1985) gave further impetus to the research activity in this field (Ladha et al. 1992).

A most distinctive characteristic of stem-nodulating legumes is the presence of predetermined nodulation sites on the stems. The formation of these sites is independent of infection with rhizobia (Dreyfus et al. 1984). They comprise primordia of adventitious roots that are able to grow out under waterlogged conditions (Tsien et al. 1983). These primordia can be distributed over the whole length of the stem or appear only on lower stem portions. They are arranged in straight vertical rows (*Sesbania*) or in spiral-shaped rows winding around the stem (*Aeschynomene*), giving the appearance of random distribution.

However, even though the presence of predetermined nodulation site is a distinct feature of stem-nodulating legumes, it may not be sufficiently discriminative for classification purposes of plants. Adventitious root primordia can also be found on other legumes. Some cultivars of *Arachis*

*hypogaea* bear nodules around the first node of the stem which is a site for adventitious root formation (Nambiar et al. 1982). A few *Vicia faba* cultivars and tree species *Cassia tota* and *Parkinsonia aculeata* L. (Yatazawa et al. 1987) also form nodules on the lower stem portion. Eaglesham and Ayanaba (1984) reported that after flooding, when parts of stem are under water *Glycine max* produced new roots on submerged stem portions. These can be infected with rhizobia to form nodules that are functional even after drainage of water. Furthermore, effective green nodules could be induced on aerial stem portions of young *S. speciosa* plants (Ladha et al. 1990). Generally the formation of nodules on root primordia will appear after 7 to 10 days after inoculation. The induced nodules increase in number and form an effective N<sub>2</sub>-fixing mass of green nodules on the stem. This particular situation may not be specific to *S. speciosa* and stem-nodule formation can probably be induced in other aquatic legumes. However, it was neither observed nor was it possible to induce stem nodules in *S. cannabina* (Synonym *S. aculeata*) (Ladha et al. 1990).

What appears important in the case of induced stem nodules is the existence of well-developed primordia that break through the epidermis at the point of forming an adventitious root. At this point specific *Azorhizobium* infection can take place. What happens in fact, is the

rhizobial infection of an outgrowing adventitious root and not of a primordium on the stem. Therefore, induced nodules on *S. speciosa* stems are, anatomically speaking, root nodules (Ladha et al. 1990).

#### 1.7.1 INFECTION PROCESS, NODULE FORMATION AND ONTOGENY OF STEM NODULES

Stem-nodulating rhizobia are true soil bacteria without the appropriate macrosymbiont they thrive saprophytically on soil organic matter. Due to their unipolar flagellum they are highly mobile in the aquatic phase (Ladha et al. 1992). Besides the soil, the phyllosphere of the host plant seems to be an alternate ecological niche for nonsymbiotic growth of stem-nodule rhizobia. Adebayo et al. (1989) reported the presence of large *Azorhizobium* populations on leaves of *S. rostrata*. Similarly, homologous (compatible) rhizobia were also found on the leaves of *Ae. afraspera*, *Ae. pratensis*, and *Ae. sensitiva*. Inoculum for stem infection can therefore come from two sources: the soil and the phyllosphere. In both cases, rain seems to play a major role for stem inoculation. Rainwater can wash the epiphytically-growing rhizobia down the stems and thereby infect nodulation sites. Furthermore, soil splash due to rain may bring rhizobia to the lower stem. This view is supported by the high correlation found between stem nodule numbers on *S. rostrata* and *Ae. afraspera* and the rainfall pattern (Becker et al. 1990). Dreyfus and Dommergues

(1981a) considered ants as a possible vector to inoculate primordia on the stem of *S. rostrata* under nonflooded conditions. Wind may be another important factor; often a high nodulation rate can be observed on stems of stem-nodulating legumes along dusty roads. Wind may transport contaminated soil particles on above-ground plant parts thus favouring stem inoculation.

After the rhizobia have reached the nodulation site, they penetrate into the cortical tissues of the stem. This depends on the anatomy of the root primordium. Three different situations can be found:

1. In *S. rostrata*, *Ae. afraspera*, and *Ae. nilotica* primordia are well developed and protrude distinctly out of the epidermis. Primordia are rod- or dome-shaped and are encircled by a 50 - 80  $\mu\text{m}$  wide circular cavity, which seems to be the entry point for rhizobia (Tsien et al. 1983; Alazard and Duhoux, 1987).
2. In the second situation, a less-developed root primordium underlies the epidermis of the stem. The epidermal cells generally cover the root primordium without formation of an epidermal dome and with no circular cavity at primordial base. The species belonging to this type are *Ae. indica*, *Ae. schimperi*, and *Ae. scabra* (Ladha et al. 1992).
3. The least-developed type of primordium has been observed in *Ae. crassicaulis*, *Ae. elaphroxylon*, and *Ae. pfundii*. The root

primordia, although clearly visible, remain embedded in the cortical tissues of the stem. External factors like waterlogging or mechanical scarification are needed to trigger primordial development and to allow the access of rhizobia (Alazard and Duhoux, 1987).

The anatomy of the adventitious root primordia on the stem may explain why *S. rostrata*, *Ae. afraspera*, and *Ae. nilotica* show generally profuse nodulation, whereas other species nodulate scarcely or only on submerged stem portions.

Upon penetration of rhizobia into the intercellular space, infection pockets are formed at the basal layers of the root primordium, which then resume their meristematic activity (Alazard, 1985). As a result, cell division starts in the infective center and the nodule begins to form. From the infected basal cells of the primordium, intracellular infection threads are formed, as in the case of *S. rostrata*. Infection threads are about two bacterial rod lengths wide and surrounded by electron-dense material (Duhoux and Dreyfus, 1982). In *Aeschynomene* spp. and *N. oleracea*, intercellular infection takes place without the formation of infection threads (Dreyfus et al. 1984). It is most likely that the mode of infection is controlled by the host plant's genome as reported for root-nodulating legumes. After the infective stage, rhizobia change in shape and size. They are called bacteroids and are surrounded by a peribacteroid membrane. As

the growing infective center forms the nodule, the majority of the cells in stem nodules are infected (Dart, 1977).

The formation of stem nodules becomes macroscopically visible within 5 to 7 days after inoculation and the nodules reach their full size in 15 to 20 days. In *S. rostrata*, stem nodules are spherical protrusions, 5 to 12 mm in diameter. They can easily be detached from the stem, as the basal portion of the nodule forms a narrow neck. In *Aeschynomene*, flattened hemispheric nodules are formed. Because no neck formation is found, the nodules are hard to remove. Stem nodules of *N. oleracea* are 3 to 5 mm ovoid structures, largely protruding from the stem. Their length is 1.5 to 3 times the nodule width. The fine structure of mature stem nodules is similar to that found in root nodules (Yatazawa et al. 1987; de Bruijn, 1989). Presence of a red pigment (leghemoglobin) is apparent, about 9 to 11 days after inoculation.

In general terms, stem nodulation on *S. rostrata* occurs by a mechanism closely related to the crack-entry mode of infection, described for roots of tropical legumes such as *Arachis* spp. (Chandler, 1978) and *Stylosanthes* spp. (Chandler et al. 1982). While the mode of entry of bacteria in the roots has not been given enough importance, Olsson and Rolfe (1985) has reported root hair curling and infection thread formation in root hairs of *S. rostrata* seedlings by *Rhizobium* strain ORS-571 or WE7. This is contrary to the crack entry mode

described for stem nodules. Therefore it remains to be seen if *Azorhizobium* strains show similar path of infection.

The stages of the infection and nodule formation process *S. rostrata* are summarized below and are diagrammatically represented in Figure 8 (de Bruijn, 1989).

#### Stage 1

*A. caulinidans* bacteria colonize the nodulation site at the base of the root primordium after gaining entry through the fissure surrounding it. The microenvironment present stimulates extensive proliferation of the invading bacteria. By the second day after infection, the nodulation site has already increased in diameter, suggesting the onset of meristematic activity (and/or cell expansion) in response to the bacterial presence (Figs. 8 a and b).

#### Stage 2

The azorhizobia penetrate the intercellular spaces of the meristematic tissue at the base of the root primordium and form infection pockets. The envelope of these pockets consists of fibrillar, parietal material, newly formed at the exterior of the host cells. At this time (4 to 5 days after infection) incipient infection centres (regions of actively dividing cells which appear as a half-moon shape in a longitudinal section (Figs. 8 b and c) can be observed in the meristematic zone of the inner cortex of the root primordium.

#### Stage 3

The intercellular infection pockets invade the basal cell layers of the root primordium and develop into intercellular infection thread-like structure one bacterium wide.

#### Stage 4

Bacteria are released into the cells of the infective center after penetration of the target cells by the infection thread-like structure and the bacteria become surrounded by a membrane envelope (Fig. 8c).

#### Stage 5

Further enlargement of the infective center occurs through successive cortical cell divisions and (massive) new intracellular infections. Eventually the infective centres merge in the mature stem nodule, whereby the vast majority of the cells are infected and densely packed with bacteroids (Fig. 8 d).

The resulting stem nodules (Fig. 8e) are usually spherical or ovoid, symmetric or with irregular lobes, and 3 to 8 mm in diameter (Tsien et al. 1983; Dreyfus et al. 1984). The stem nodules are structurally similar to synchronous (determinate) nodules formed on the roots of a variety of other tropical legumes (Sprent, 1980). A notable difference is the presence of a layer of cortical cells containing chloroplasts that surround the infected center of the nodule.

The stem nodules differ from root nodules due to their

Figure 8. *Sesbania rostrata* stem nodule ontogeny. (a) The stem infection site (dormant root primordium); (b) early stages of stem nodule ontogeny; (c) mode of infection; (d) developing stem nodule; (e) mature stem nodule (de Bruijn, 1989).

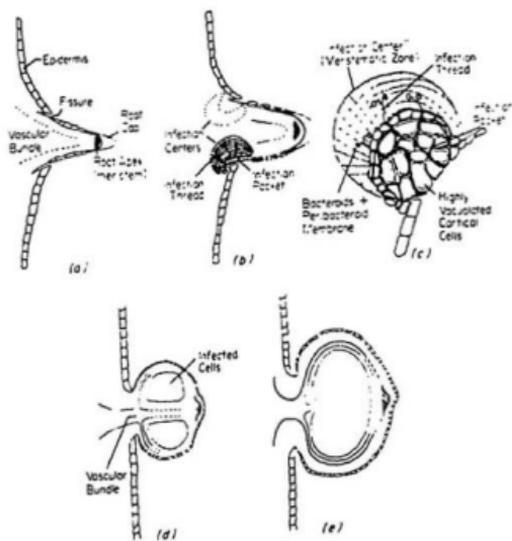


Figure 8

aerial location and the presence of a chloroplast-containing cortical cell layer around the infected tissue. Their specific location prevents the contact of stem nodules with soil solution or flood water. A photosynthetically active tissue around the nodule allows cyclic photophosphorylation as well as synthesis of carbohydrates and may therefore improve the energy supply for  $N_2$  fixation. Photosynthetically active tissues generate oxygen. Because electron transport in nitrogenase enzyme complex is sensitive to oxygen, additional mechanisms to protect bacteroids from oxygen may be needed in stem nodules. Trinchant and Rigaud (1987) showed that a high tolerance of nitrogenase in bacteroids of *S. rostrata* towards increased oxygen concentrations was due to the presence of a highly efficient superoxide dismutase. Furthermore, *Azorhizibium caulinodans* ORS 571 had a high  $O_2$  consumption in continuous culture. However, this  $O_2$ -tolerant,  $N_2$ -fixing system is not present if the same strain is grown at steady-state conditions (Bergersen et al. 1988).

#### 1.7.2 OLEOSOMES: ITS STRUCTURE AND ROLE

Lipids are generally important components of cellular organelles and are also used for energy storage in most seeds, especially oil seeds. In plants lipids are synthesized in plastids and stored in the cytoplasm in special structures. These lipid storing structures have been called lipid bodies (Ching, 1972; Lin and Huang, 1983), oil bodies (Roughan and

Slack, 1982; Herman, 1987), spherosomes (Muhlethaler, 1955; Frey-Wyssling et al. 1963; Hrsel, 1966; Sorokin, 1967) or oleosomes (Yatsu et al. 1971). A lipid body has a matrix of triacylglycerol (TAG) surrounded by a half-unit membrane of one phospholipid layer (Huang, 1985; Yatsu and Jacks, 1972). They are spherical bodies about 1  $\mu\text{m}$  in diameter and highly refractile under the light microscope (Yatsu et al. 1971). Although the synthesis of lipids (triacylglycerol) in plastids is well known, it has also been proposed that lipid bodies might originate from the endoplasmic reticulum by the accumulation of TAG between the two layers of the endoplasmic reticulum unit membrane and finally pinch off to form the lipid bodies (Warnner and Theimer, 1978; Warnner et al. 1981). Alternatively, lipid bodies may arise in the cytoplasm by an accumulation of TAG followed by the formation of the membrane (Bergfield et al. 1978). The formation and function of lipid bodies have been studied in different seeds (Warnner et al. 1981; Huang et al. 1983). It has been well documented that lipid bodies in seeds provide energy and carbon skeletons for the growth and development of new cells during germination (Huang et al. 1983.)

Oleosomes<sup>2</sup> contain a few major and related proteins unique to the organelles (Tzen et al. 1990). These proteins

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<sup>2</sup>In this investigation lipid bodies, on wards will be referred to as oleosomes

are called oleosins which are alkaline proteins of small molecular mass, ranging from 15 to 26 kD. Oleosomes remain stable inside the cells as well as in isolated preparations. In both situations, the organelles occur as individual entities, and when they are pressed against one another *in vivo* due to seed desiccation or *in vitro* after flotation centrifugation, they do not aggregate or coalesce, even after prolonged storage (Huang, 1985). The physiological significance of maintaining the oleosomes as small entities is to provide ample surface areas for the attachment of lipase to the organelles during post germinative growth so that the reserve TAG can be mobilized rapidly.

A model of the oleosome structure has been proposed by Tzen and Huang (1992). The basic structural unit of the oleosome surface consists of 13 phospholipid molecules to one oleosin molecule. About 2/5 of the oleosin is the hydrophobic stalk embedded in the hydrophobic acyl moieties of phospholipid and the TAG matrix; this stalk is depicted as an hairpin structure of 70 amino acid residues. The remaining 3/5 of the oleosin molecules cover or protrude outward from the oleosome.

### 1.7.3 ROLE OF OLEOSOMES IN NITROGEN-FIXING NODULES

Recently oleosomes have been reported in nitrogen-fixing nodules of arctic legumes (Newcomb and Wood, 1986) and in the tropical crop legume, peanut (Bal et al. 1989). Although their

function in the nodules of arctic legumes remains to be clarified, their presence in peanut is thought to have an added advantage when the beta oxidation pathway is operative along with the lipolytic activity, so that large amounts of energy stored in the oleosomes can be mobilized either for the energy demanding process of nitrogen fixation or for other metabolic functions of the nodule. Evidence for such utilization of supplementary energy from oleosomes in nitrogen fixation has been forthcoming through recent experiments by Siddique and Bal (1991, 1992). These oleosomes have been called short-term storage organelles (Jayaram and Bal, 1991) as distinct from long-term storage organelles of the seed. The enzyme, catalase which is a scavenger of hydrogen peroxide produced in the beta oxidation pathway was localized in microbodies as well in dense bodies in association with the bacteroids (Bal et al. 1989).

#### 1.7.4 OBJECTIVES OF THE PRESENT STUDY

Amongst the tropical legumes to date, only peanut has revealed the presence of oleosomes in the nodule tissue. The observation of oleosomes in the infected and uninfected cells of root and the stem nodules of *S. rostrata* (Denduluri and Bal, 1992) prompted this investigation to characterize the oleosomes and to determine their role in the root and stem nodules of *S. rostrata*. The oleosomes present in the root nodules appear to be different from those of the stem nodules.

The distribution, composition and the functional involvement of oleosomes in the root and stem nodules of *S. rostrata* during different stages of nodule development has been studied in this investigation.

#### 1.7.4 HYPOTHESIS

That nodule oleosomes serve as a source of energy for the differentiating nodule cells thus participating in its metabolic activity. To test the hypothesis the parameters studied were

- infection process in the formation of root nodules
- histochemical identification of oleosomes and their distribution in the nodule tissue
- ultrastructural characterization of the nodule
- localization of lipolytic activity
- localization and assay of catalase, an enzyme involved in beta oxidation pathway
- determination of the fatty acid composition of nodule oleosomes

## II. MATERIALS AND METHODS

### 2.0 PLANT MATERIALS

Seeds of *Sesbania rostrata* were obtained through the courtesy of Dr. J.K.Ladha, International Rice Research Institute (IRRI), Manila, Philippines. Seeds were germinated on wet paper towels and the seedlings were transferred to pots filled with vermiculite and were inoculated with the suspension of *Azorhizobium caulinodans* IRG 46 in distilled water containing  $10^8$  cells per ml. The potted seedlings were grown in environmental chambers under day/night conditions of 16 h/8 h, 27° C/22° C and 70%/50% relative humidity and were irrigated with nitrogen free nutrient solution (Ellfolk, 1960). The root primordia before inoculation were observed for its arrangement on the stem. With the advent of the fifth internode, the root initials became apparent on the stem. At this stage the internodes with root initials were painted with the bacterial culture (see below) to form the stem nodules. The stem nodules which developed (1.0 mm in size) within 15 - 20 days after inoculation were used in this study. Root nodules were sampled at 20 days and 40 days after inoculation. Size of the root nodules ranged between 1.0 - 1.5 mm at 20 days old and 2.0 - 2.5 mm at 40 days respectively.

### 2.1 AZORHIZOBIUM CULTURES

*Azorhizobium caulinodans* IRG 46 was also obtained from

Dr. J. K. Ladha. The cultures were maintained in yeast extract mannitol agar and cultured in a broth containing 10 g mannitol, 0.5 g  $K_2HPO_4$ , 0.2 g  $MgSO_4 \cdot 7H_2O$ , 0.1 g NaCl, 0.4 g yeast extract in 1 litre of distilled water at pH 6.8 - 7.0 with constant shaking in an Orbit Environs Shaker, Lab-Line Inc. at 28° C (Vincent, 1970).

## 2.2 INFECTION OF ROOT HAIRS BY AZORHIZOBIUM

The early root hair infection process in *S. rostrata* by *Azorhizobium* was studied in aseptically germinated seeds grown in petri dishes. Seedlings after 4 days were transferred to plastic bags containing the broth culture as described above. The seedlings were observed for changes in root hairs during 1-3 days after transfer to the bacterial broth. The root hair region was sliced longitudinally with a razor blade and stained in 1% methylene blue for observation under the light microscope.

## 2.3 ELECTRON MICROSCOPY OF SAMPLES

Thin slices of root and stem nodules were cut with a clean razor blade and fixed in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde (Karnovsky, 1965) in 0.1M Sorensen's buffer for 60 min at 4° C. After fixing, the slices were washed with the same buffer (pH 7.2) at least 3 times for 15 - 20 min at 4° C. The washing was followed by post fixation in 1% osmium tetroxide ( $OsO_4$ ) in the same buffer for 60 min at 4° C. The samples were washed again in the

buffer and dehydrated in an ethanol series (35%, 50%, 70%, 80%, 95% and absolute) with 5 min exposures in each concentration except 20 and 60 min in 95 percent and absolute respectively. After dehydration, the slices were passed through the series 1:1, 1:2 and 1:3 (v/v) of absolute ethanol and Spurr's embedding resin mixture (Spurr, 1969), left for 30 min in each mixture under vacuum. The slices were then placed in 100% resin for approximately twelve hours under vacuum, and embedded and polymerized at 70° C for at least 8 hours. Ultrathin sections were cut with a Sorvall MT-1 ultra-microtome. The sections were post-stained with lead citrate and uranyl acetate before observation under a Zeiss 109 transmission electron microscope.

For scanning electron microscopy of root infection process by bacteria, the fixed samples were critical point dried in 100 percent ethanol, mounted on aluminium stubs and coated with gold for observation by a Hitachi S570 scanning electron microscope.

#### **2.4 OLEOSOME PRESERVATION, LOCALIZATION, STAINING AND COUNTING**

For oleosome preservation, nodule slices of stem and root, and slices of root primordia of the stem (before and after inoculation) were fixed and processed as described above for electron microscopy except samples were treated with 1% p-phenylenediamine (pPD) in 70% ethanol for 1h during dehydration (en bloc staining, Bal et al. 1989; Bal, 1990).

For controls, the samples were treated with hexane for 45 min after fixation and dehydration and rehydrated before osmium tetroxide treatment (Bal, 1990). After rehydration, the samples were embedded in Spurr's medium as mentioned above.

Semi-thin sections (1-2  $\mu\text{m}$ ) were cut from both treated and control blocks, viewed with a light microscope at 400 X and the diameter of oleosomes were measured. Percentage of different sizes of oleosomes from 50 cells (n=50) were made into a histogram.

## 2.5 CYTOCHEMICAL LOCALIZATION OF CATALASE ACTIVITY

Cytochemical localization of catalase in *S. rostrata* stem nodule and root nodule was done using the 3,3-diaminobenzidine (DAB) reaction (Frederick and Newcomb, 1969). The nodule slices were fixed in aldehyde mixtures (Karnovsky, 1965) in 50 mM potassium phosphate buffer at pH 8.0 for 1 h at 4° C and then washed thoroughly with the same buffer for at least 20 min (3 - 4 times) at 4° C. After washing, the samples were pre-incubated in a medium containing DAB and 2mg/ml of 50 mM 2-amino-methyl-1,3-propanediol buffer at pH 9.5. Preincubation was followed by incubation of the samples in a  $\text{H}_2\text{O}_2$  (0.1 ml of 3%  $\text{H}_2\text{O}_2$  in 5 ml) containing DAB medium for 60 min at 37° C with constant shaking. After this, the samples were washed with buffer (3 - 4 times) and post-fixed in 2%  $\text{OsO}_4$  in 50 mM potassium phosphate buffer (pH 6.8) for 2 h, washed with buffer and processed for electron microscopy.

Controls were run along with the treatments with a addition of 0.02 M 3-amino-1,2,4-triazole ( a competitive catalase inhibitor) to the incubation medium during pre-incubation and incubation time.

#### 2.5.1 SEPARATION OF NODULE AND BACTEROID FRACTIONS

Root nodules (1.0 - 1.5 mm) were removed from the 20 day old plants, washed with distilled water and homogenized gently with 0.05M buffer containng 5mM EDTA in a mortar. The homogenate was then centrifuged at 265 xg for 10 min and the supernatant was further centrifuged at 1400 xg for 10 min. The resultant pellet and supernatant was considered as bacteroid and nodule cytosol fraction respectively. To solubilize the peribacteroid membrane (PBM), the pellet was treated with 0.1% Nonidet P<sub>40</sub> (Bal et al. 1980), washed thoroughly and resuspended in buffer.

The bacteroid fraction was then passed through a modified Hugh's Press (model 9 AB Biotech, Sweden) operated at a pressure 2.6 Metric tons/cm<sup>2</sup> to break the cells. The cell walls were pelleted at 20,000 xg for 1h in a Sorvall OTD-5 ultracentrifuge and washed with the buffer. The cell contents of bacteroids were in the supernatant. All fractions of nodule homogenate thus prepared were assayed for catalase activity.

#### 2.5.2 CATALASE ASSAY

The enzyme catalase was assayed spectrophotometrically (Beers and Sizer, 1952). During the assay procedure, a

constant amount of protein (0.5 mg) in the sample (in 50 mM phosphate buffer) was taken in a 3 ml cuvette and made up to 2 ml with the same buffer. In case of control preparation, 0.2 M aminotriazol in phosphate buffer was used along with the sample. To this sample, 1 ml of 59 mM  $H_2O_2$  in the same buffer was added just before the starting the reaction (zero time). As a control, only buffer was used. The reactants were mixed thoroughly and the change in absorbance was recorded at 10 seconds interval for 70 seconds at 240 nm on a Spectronic 1001 (Bauch and Lomb). The specific activity of catalase was calculated as units/mg of protein:

$$\text{Specific activity} = \frac{\text{changes in absorbance /minutes} \times 1000}{43.6 \times \text{mg protein/ml of reaction mixture}}$$

where 43.6 = Molar Absorbance Index for  $H_2O_2$  and one enzyme unit is equal to one  $\mu\text{mole}$  of  $H_2O_2$  decomposed per minute. Protein was measured in the sample by the method described by Lowry et al. (1951).

## 2.6. HISTOCHEMICAL DEMONSTRATION OF LIPASE

For a histochemical demonstration of lipase, thin slices (hand-made with a sharp razor blade) of 20 days old stem nodules (1.0 - 1.5 mm in size) were incubated at 37° C for 30-60 mins in a medium containing 1 ml of 5% (v/v) tween 80 (Polyoxyethylene 20 sorbitan mono-oleate, J.T. Baker Chemical Co., N.J., USA.), 2.5 ml of 0.2 M tris-maleate buffer pH

(7.2), 1 ml of 20 mM calcium chloride, 2.5 ml of 2.5% (w/v) sodium taurocholate and 18 ml of distilled water, after fixation in 2.5 to 5.0% glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.2) for 1 hr. at 4° C and washing in the same buffer (Nagata, 1974). The slices were then rinsed with distilled water and immersed at room temperature for 5 minutes in the colloidal Nile Blue solution. Again the slices were washed in distilled water and mounted in glycerine. The Nile Blue solution was prepared by dissolving 20mg of Nile Blue in 20ml of absolute ethanol and then filtered. The filtrate was added drop by drop to the colloidal solution of 5 gm of arabic gum dissolved in 200ml of boiling distilled water. Then the solution was evaporated to a total volume of 100ml. This viscous colloidal solution was used for the dyeing method (Takeuchi and Furuta, 1956).

For ultrastructural localization of lipase, the tannic acid method (Siddique and Bal, 1991) and strontium chloride method (Jayaram and Bal, 1991) was tried. However difficulties in ultrathin sectioning did not allow the evaluation of the results in a clear fashion. Therefore, the histochemical technique only is presented as an evidence for lipolytic activity in the nodule.

## 2.7 TOTAL LIPID ESTIMATION

The total lipid present in *S. rostrata* root and stem nodules as well as seed was estimated by the gravimetric

method described by Bligh and Dyer (1959). One gram of 20 day old nodules (1.0 - 1.5 mm), and seeds were homogenized separately in a mixture of chloroform and methanol in a ratio of 2:1 (v/v) to extract lipids. The chloroform layer was separated by centrifugation at 400 xg. A small amount of chloroform containing extracted lipid was taken in a pre-weighed vial and evaporated under nitrogen gas. The vial with the lipid residue was weighed again to estimate the amount of total lipid. Protein present in the nodules was estimated according to Lowry et al. (1951).

## 2.8 OLEOSOME FRACTIONATION

Nodules of stem and root, and seeds were homogenized separately in 50 mM tris.HCl buffer containing 0.5 M NaCl, pH 7.2 (Yatsu and Jacks, 1972), centrifuged at 400 xg for 5 min. or filtered through cheese cloth and the supernatant was centrifuged at 30,000 g for 20 min. A tiny fat pad floating on top contained the oleosomes and was stained with Sudan III and observed with the light microscope.

The fat pad fraction isolated from the stem nodule and the seed was also observed under a scanning electron microscope. The fat pad was smeared on a coverslip, fixed in aldehyde fixative and critical-point dried in 100 percent ethanol, mounted on aluminium stubs and coated with gold for observation using a Hitachi S570 scanning electron microscope.

## 2.9 FATTY ACID ANALYSES

The fat pad isolated from the above preparations was used to determine the fatty acid composition. Chloroform: methanol (2:1 v/v) was used as a solvent mixture. (Bligh and Dyer, 1959). A small quantity (few crystals) of hydroquinone was added as an antioxidant to the solvent mixture. The chloroform was evaporated under nitrogen gas. The samples were transmethylated by the addition of 2 ml of sulphuric acid and methanol mixture (1:15 v/v) and heated at 55-60° C for 4-5 h. Then the methylesters were extracted three times with 1.5 ml of hexane. The hexane layers were combined and rinsed twice with distilled water to remove residual sulphuric acid. This was followed by evaporation of hexane under nitrogen and the residual samples were reconstituted in carbon disulphide for gas chromatographic analyses. The samples were analyzed in a Perkin Elmer 8310 gas chromatograph (Department of Biochemistry, MUN) at 185° having a wall coated open tubular column. (Supelco sp-2330, 0.25 mm in diameter. x 30 m long). The fatty acid peaks were identified by comparing the "Relative Retention Time" of unknown fatty acids with the known fatty acids.

### III. RESULTS

#### 3.0 INFECTION PROCESS

Seedlings inoculated with *Azorhizobium* culture showed inhibited root hair growth and as a result the root hairs were shorter (Fig. 9) than the controls without the bacteria (Fig. 10). No invaginations of root hair wall or infection threads were found. Light and scanning electron microscopic studies showed that a fissure in the epidermis encircling the emerging secondary root exists at the point of emergence. (Figs. 11 & 12). Infection threads could not be located in the root hairs at any time.

In the stem, root primordia could be seen in young plants, when 5 internodes are formed. These primordia in uninoculated plants are arranged in vertical rows (Fig. 13a) and do not form nodules until they are inoculated. A fissure encircling root primordia in the stem has been clearly observed in histological preparations (Fig. 13b - arrow). The entry of bacteria probably takes place through the fissure and the intercellular spaces. The bacteria from the intercellular spaces gain entry into the target cell by infection thread-like invagination of the intercellular spaces (Fig. 14).

#### 3.1 EARLY STAGES OF DIFFERENTIATION

Within 5-7 days after inoculation, nodule primordia start to develop at the base of the root primordium of the stem.

Figure 9. Roots of inoculated seedlings showing short root hairs

Figure 10. Root hairs of uninoculated plants with long root hairs

Figures 11. & 12. Light and Scanning Electron Micrographs showing a fissure (see arrow) encircling the emerging secondary root at the point of emergence through the epidermis of the root

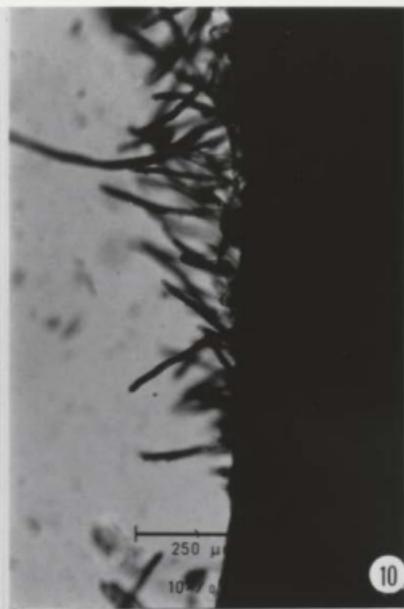
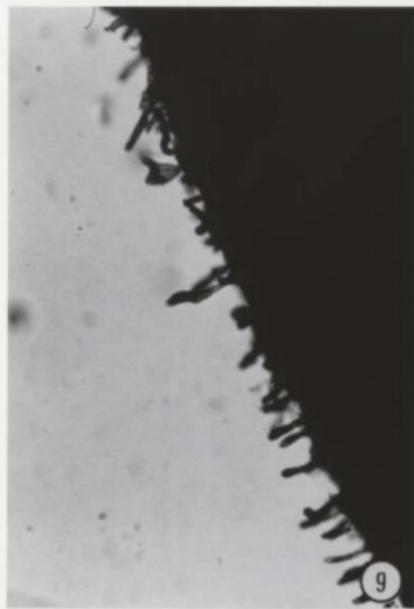


Figure 13a. Photomicrograph showing the arrangement of primordia in vertical rows on the stem.

Figure 13b. Longitudinal section of primordium of *Sesbania* stem showing a fissure (see arrow) encircling the root primordia of the stem

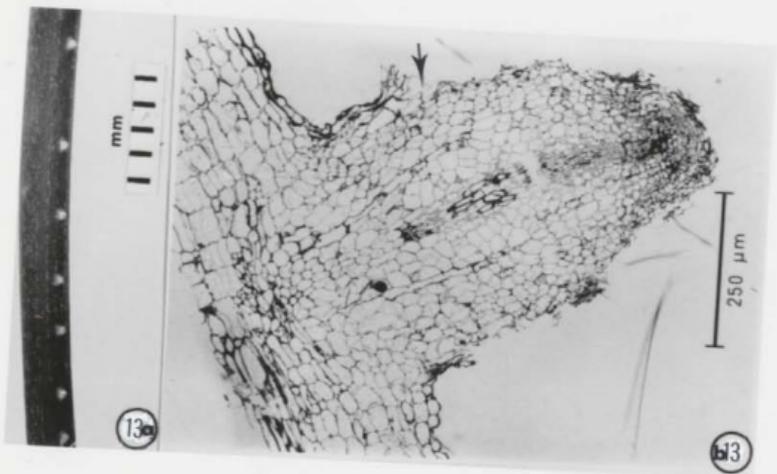


Figure 14. Entry of bacteria (see arrow) into the stem nodule cells through intercellular spaces and infection thread-like structure produced by invaginations of intercellular spaces showing bacteroids (b) and nucleus (n).



Meristematic activity is followed by infection thread-like structure mediated bacterial invasion and subsequent release of bacteroids in the infected cells (Fig. 14). One of the striking features of the stem nodules at this stage is the presence of large number of oleosomes readily visible in the infected cells stained with pPD at the light microscope level (Fig. 15a and 17). Only the cells which are small and undifferentiated show the presence of oleosomes. The cells that are differentiated fully do not have any oleosomes. Oleosomes have also been seen in root nodules during these stages. The oleosomes in the stem nodules are remarkably larger ( $1\mu\text{m}$ -  $8\mu\text{m}$  in diameter) ( $n=50$ ) than the ones in the root nodules (Fig. 15b). The absence of oleosomes in the root primordium of the stems were confirmed by electron microscopy of ultrathin sections (Fig. 16). Oleosome staining with pPD could be further confirmed by control preparations where hexane was used to extract the lipids (Fig. 18). In Figure 17 the oleosomes (arrows) are prominent in infected cells that are young and close to the nodule cortex of the stem nodule. In hexane extracted preparations (Fig. 18) no oleosomes can be stained. In the root nodule, a similar pattern of oleosome distribution is found but they are smaller in size ( $> 1\mu\text{m}$  in diameter) (Figs. 15b and 19). The mature infected cells (Fig. 20) show very few or no oleosomes.

An examination of the ultrastructure of the infected

Figure 15a. Photomicrograph of a pPD stained semithin section of a stem nodule. The younger infected cells contain oleosomes (heavily stained) of various sizes. Note the lack of oleosomes in the more elongated and mature cells.



Figure 15b. Histogram showing the different sizes of oleosomes  
in root and stem nodules.

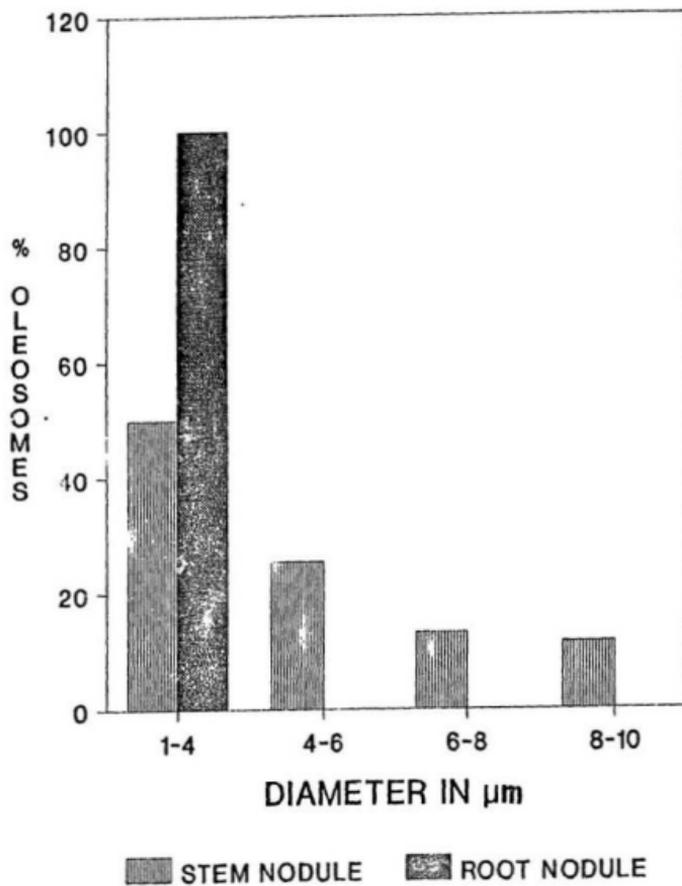


Figure 16. Electron micrograph showing lack of oleosomes in the root primordia of the stem before inoculation with *Azorhizobium*.

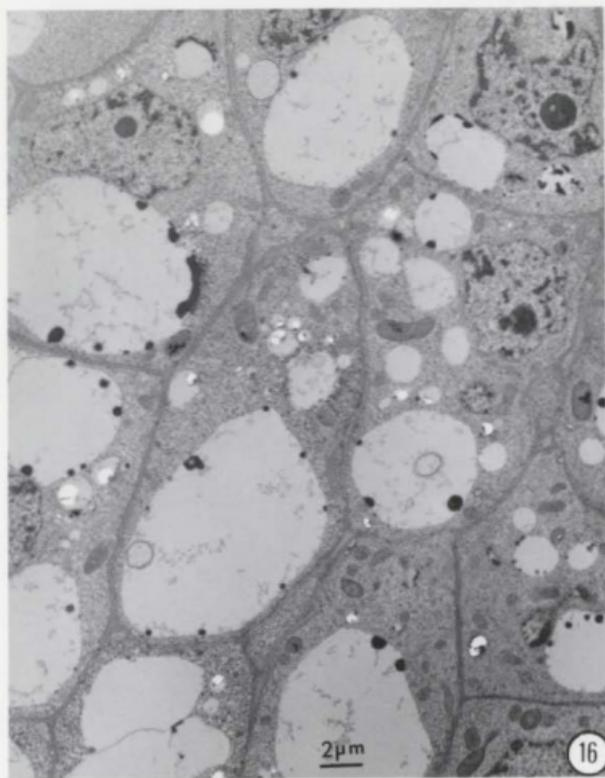


Figure 17. Photomicrograph of semithin sections of stem nodule showing the oleosomes in infected cells (see arrow) (p-phenylenediamine enbloc staining). Note the size differences in oleosomes.

Figure 18. Photomicrograph of semithin sections of stem nodule in control preparations treated with hexane showing the lack of oleosomes.

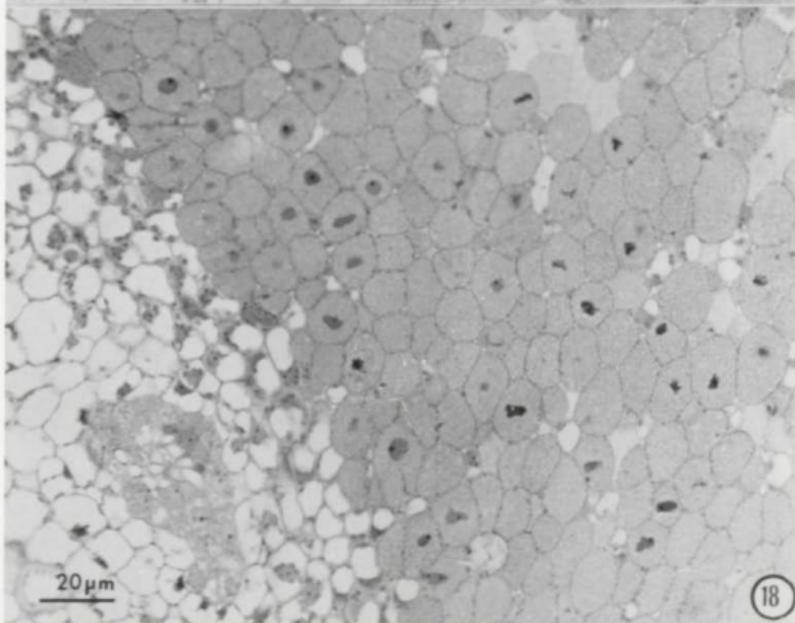
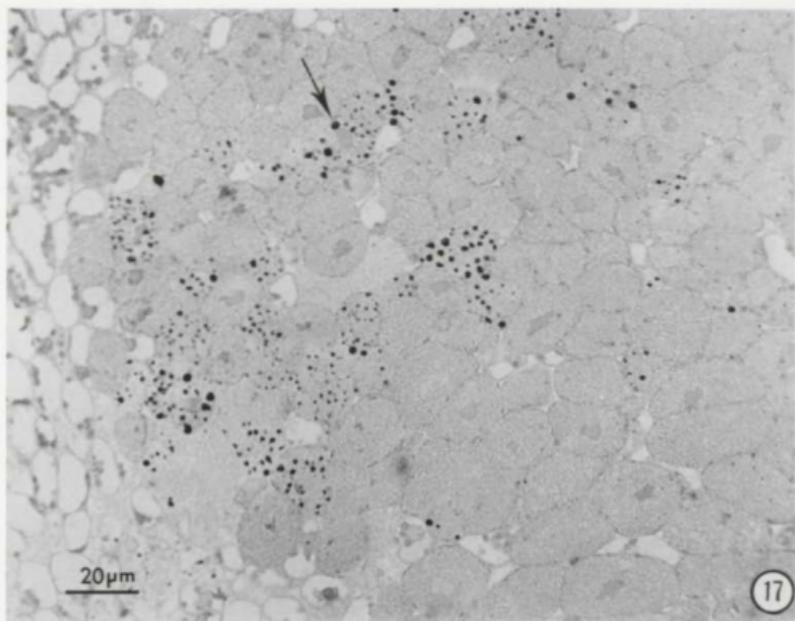
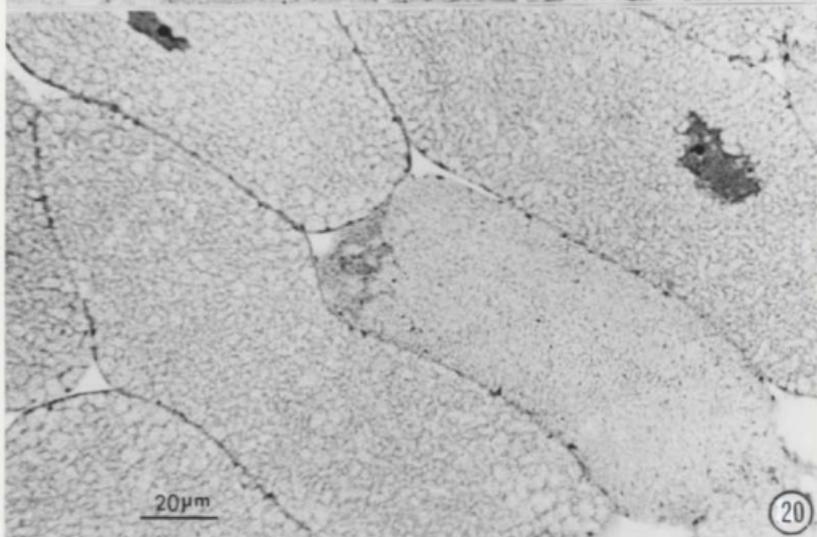
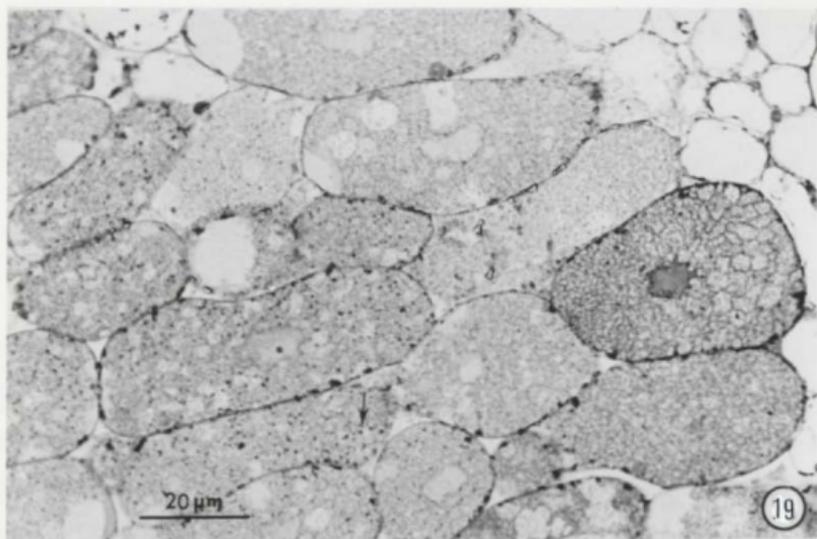


Figure 19. Photomicrograph of a root nodule (20 days old) showing oleosomes at early stages of nodule growth (see arrow).

Figure 20. Photomicrograph of a root nodule (40 days old) showing the disappearance of oleosomes at the late stages of nodule growth.



cells in the both root nodule and stem nodule show that the immature cells, which have only one bacteroid per perisymbiotic membrane, contain oleosomes in the cytoplasm (Fig. 21). The oleosomes are seen close to endoplasmic reticulum and numerous vesicles are found in the cytoplasm (Fig. 22).

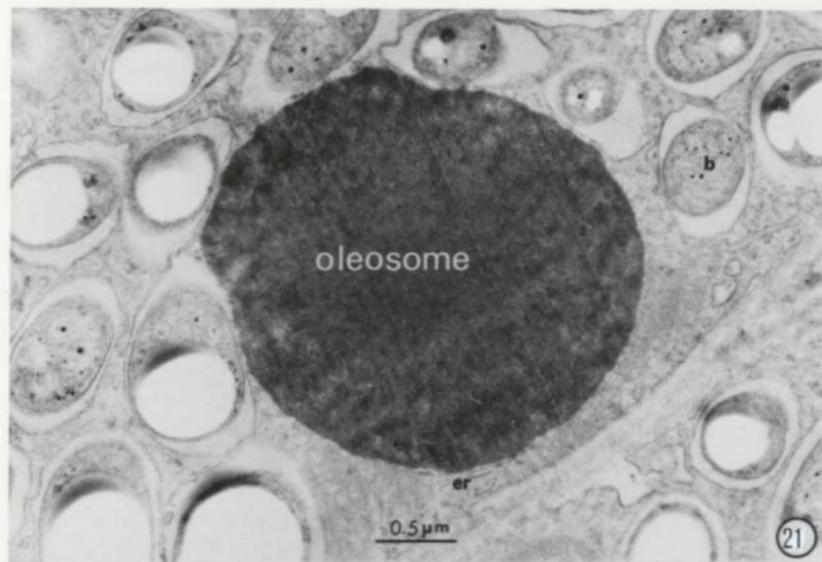
In both stem and root nodules, maturation of infected cells is characterized by multiple occupancy of bacteroids in the perisymbiotic membrane sacs (Figs. 23 and 24) which fills up the host cell. Numerous vesicles (Fig. 23 - arrows) appear close to the perisymbiotic membrane which seem to fuse resulting in proliferation of membrane. Oleosomes are generally absent in the mature infected cells with multiple occupancy of perisymbiotic membrane, but in rare cases a few small oleosomes may be seen.

### 3.2 HISTOCHEMICAL LOCALIZATION OF LIPASE

The ultrastructural localization was hampered by the constraints imposed by the tissue itself. Techniques used for localization of lipase did not yield results due to difficulties in embedding, which made ultrathin sectioning impossible. Therefore, lipase was localized at the light microscope level by the method of Takeuchi and Furuta (1956) which produced consistent results. The liberated fatty acids by the enzyme activity were stained by the colloidal Nile Blue solution at the light microscopic level. The sites of

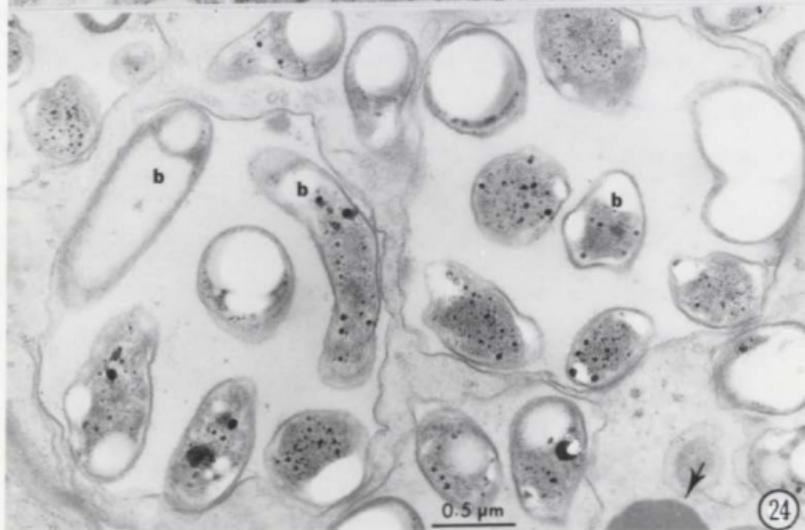
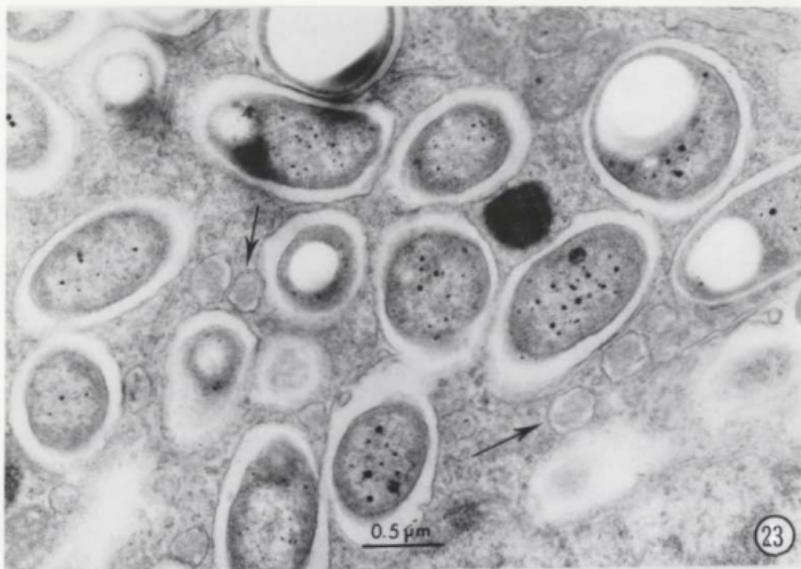
Figure 21. Ultrastructure of an infected cell of the stem nodule (20 days old) showing one bacteroid (b) per perisymbiotic membrane with a large oleosome. Note the presence of endoplasmic reticulum (er) close to it.

Figure 22. Ultrastructure of an infected cell of the stem nodule showing oleosomes and appearance of numerous vesicles (v).



Figures 23. Ultrastructure of an infected cell in early stages of stem nodules showing the single bacteroid (b) in the perisymbiotic membrane sacs of the host cell. Note the oleosome (o) and vesicles (arrow).

Figure 24. Ultrastructure of an infected cell at later stages showing multiple occupancy of the peribacteroid membrane sacs of the host cell by bacteroids (b). The arrow indicates part of an oleosome.



lipolytic activity appeared deep or bright violet (Fig. 25.). Control preparation did not show any reaction product and hence did not show the characteristic color when stained with Nile Blue (Fig. 26). The brown color in the infected cells is due to leghemoglobin.

### 3.3 CATALASE ACTIVITY

Catalase activity was detected in both bacteroidal and host cytosol fractions of two replicates (Table 1) of the root nodules. The host fraction showed higher activity than the bacteroidal fraction. A positive DAB reaction was observed in the vesicles which seemed to be budding off the perisymbiotic membrane of the symbiosome (Figs. 27 and 28). The DAB positive dense bodies were found only in early stages where mostly single bacteroids were present in the symbiosomes. Control preparations using triazole as inhibitor showed no DAB reaction (Fig. 29 arrow). Microbodies in the host cytoplasm showed the characteristic electron dense DAB reaction (Fig. 30). In this electron micrograph (Fig. 30) the microbody is shown in disrupted cell at the edge of the cut surface of the nodule tissue. Due to difficulties in sectioning the data from stem nodules were not obtained.

### 3.4 LIPID ESTIMATION AND OLEOSOME FRACTION

Estimation of total lipids by the gravimetric method for two replicates are shown in Table 2. The trend for both the replicates is the same. During the growth and differentiation

Figures 25. Histochemical demonstration of lipase activity in hand section of nodule tissue stained by Nile Blue. Note the blue-violet staining of the infected cells. The nodule cortex remains unstained. Some reaction is visualized in the epidermal cells and vascular bundles.

Figure 26. Control preparation of similar nodule section without substrate showing no blue-violet colour in the tissue..

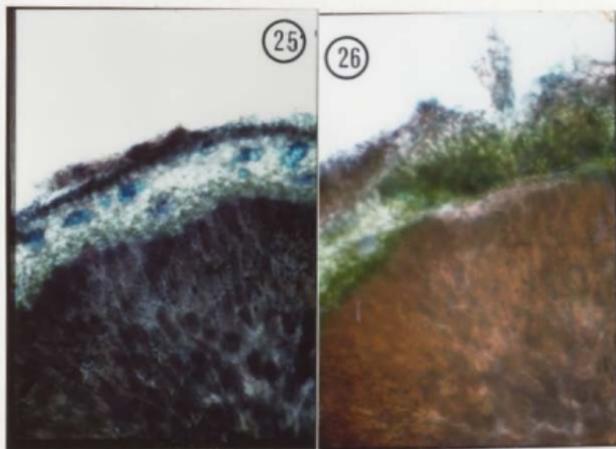


Table 1. Catalase activity<sup>a</sup> in bacteroid and host cytosol fractions of *Sesbania* root nodules

Replicate	Bacteroid fraction	Host cytosol fraction
1	0.698	2.59
2	0.925	3.88

<sup>a</sup> Catalase activity in enzyme units/mg protein

Figures 27 & 28. Ultrastructure of root nodules showing positive DAB reaction in the vesicles seemed to be budding off the perisymbiotic membrane of the symbiosome (see arrows) (no post staining).

Figures 29. Ultrastructure of the control preparation with an inhibitor triazole showing lack of DAB reaction (arrow) in root nodules (no post-staining).

Figure 30. Ultrastructure of a disrupted infected cell of a root nodule stained with DAB reaction. Note the microbody-like structure showing electron density due to better penetration of the reagent (no post-staining).

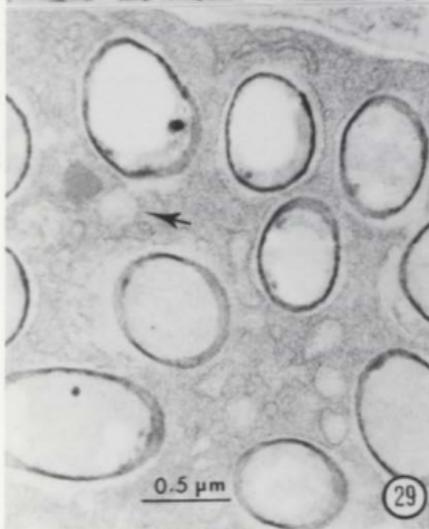
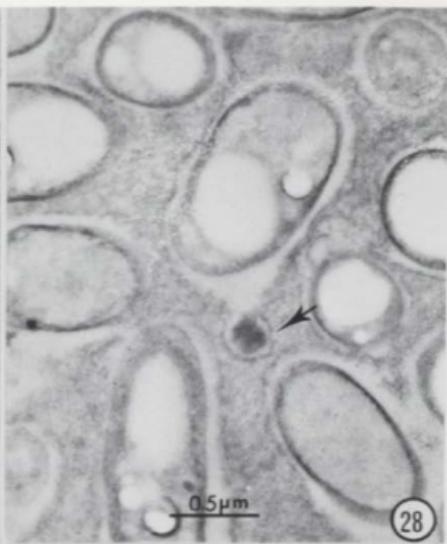
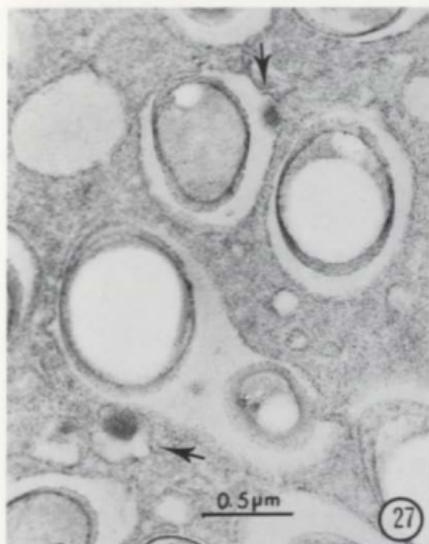


Table 2. Amount of total lipid<sup>a</sup> in root nodule and stem nodule and seed of *S. rostrata*

Replicate	Root nodule	Stem nodule	Seed
1	338.3	513.8	659.2
2	469.8	605.7	765.3

<sup>a</sup> mg/g Fresh Weight

of the infected cell the number and size of oleosomes appeared to be decreased as shown in Figures 19 and 20 for root nodules. A similar trend has been seen already in the stem nodule (Fig. 15b).

### 3.5 FATTY ACID ANALYSES OF OLEOSOMES

Fatty acid analyses of the oleosomes of *S. rostrata* root nodule and stem nodules, and seeds were carried out on isolated fractions, which stained positive for lipids with Sudan III and showed the presence of oleosomes under the scanning electron microscope (Figs. 31 & 32). They also retain their original configuration after isolation. Fatty acid composition of these oleosomes reveal that the percentage and the amount of fatty acids in seed, stem and root nodules show differences (Table 3). The major fatty acids traced by the chromatograph were C14:1 (stem nodule), C16:0 (root nodule, stem nodule and seed), C16:1 (stem nodule), C18:0 (stem nodule), C18:1 (root nodule and seed) and C18:2 (root nodule and seed). C14:1 was present in relatively higher amounts in stem nodules than in root nodules and seed. C20:1 which was present in relatively small amounts in the seed was totally undetectable in the nodule oleosome fraction. Overall, the total unsaturated fatty acid proportion was higher than the saturated fraction. The ratios of saturated versus unsaturated fatty acids in root nodule, stem nodule and seed oleosomes are 0.528, 0.495 and 0.406 respectively.

Figure 31. Scanning electron micrograph of an isolated stem nodule oleosome fraction showing "mega oleosomes".

Figure 32. Scanning electron micrograph of an isolated oleosome fraction from the seed. Note the size difference between stem oleosomes and seed oleosomes.



Table 3. Major fatty acids of oleosomes of root nodule and stem nodule and seed of *S. rostrata*

Fatty acid	Root nodule	Stem nodule	Seed
	oleosomes	oleosomes	oleosomes
C <sub>14:0</sub>	0.603 ± 0.05	1.212 ± 0.42	0.897 ± 0.14
C <sub>14:1</sub>	1.476 ± 0.21	34.022 ± 11.8	2.013 ± 0.34
C <sub>16:0</sub>	13.681 ± 0.98	10.895 ± 3.52	15.282 ± 0.69
C <sub>16:1</sub>	1.389 ± 0.57	12.935 ± 4.66	0.291 ± 0.04
C <sub>17:0</sub>	8.688 ± 2.18	5.455 ± 2.46	4.011 ± 0.88
C <sub>18:0</sub>	4.055 ± 0.24	11.378 ± 3.80	4.743 ± 0.55
C <sub>18:1</sub>	26.041 ± 2.65	3.875 ± 0.89	17.068 ± 0.81
C <sub>18:2</sub>	20.559 ± 1.86	5.939 ± 1.85	38.134 ± 1.46
C <sub>18:3</sub>	3.657 ± 0.19	4.370 ± 2.07	6.189 ± 0.09
C <sub>20:0</sub>	1.064 ± 0.04	0.384 ± 0.18	1.043 ± 0.21
C <sub>20:1</sub>	ND	ND	0.447 ± 0.01
C <sub>20:2</sub>	ND	ND	ND
C <sub>20:4</sub>	2.143 ± 0.92	ND	ND
C <sub>22:0</sub>	0.949 ± 0.10	1.654 ± 0.52	1.429 ± 0.81
C <sub>22:5</sub>	2.701 ± 0.57	1.371 ± 0.40	2.433 ± 0.34

ND = not detectable

Values represent percent fatty acids

## IV. DISCUSSION

### 4.0 INFECTION PROCESS

One of the first interactions between free-living rhizobia and the roots of the host legume, apart from stimulation of growth of the rhizobia in the rhizosphere, is marked deformation or curling of root hairs (Nutman, 1956; Dart, 1977). Deformation and infection of root hairs appear to be separate processes since deformation can be caused by some non-infective or heterologous strains of *Rhizobium* and by substances present in cell-free filtrates of cultures of *Rhizobium* (Yao and Vincent, 1969; Hubbell, 1970; Solheim and Raa, 1973; Dart, 1977). Deformation is not observed when species of non-legumes are grown in the presence of different strains of *Rhizobium* (Dart and Mercer, 1964). The significance of root hair deformation which results from uneven rates of growth of different areas of the root hair cell wall, is not understood.

Earlier studies (Olsson and Rolfe, 1985) of root infection in *S. rostrata* were done with *Rhizobium* strains ORS-571 or WE7 before the characterization and establishment of the genus *Azorhizobium*. Root hair curling was observed within 24 hours and infection threads, originating at the curled root hair, were also observed with fluorescent microscopy within 48

hrs. In the present investigation, the root hair curling or infection threads were not observed but the root hairs were inhibited in growth. It is possible that the strain of *Azorhizobium caulinodans* IRG 46 is different from the *Rhizobium* strain used in the earlier study. As shown in sections of photomicrograph and scanning electron micrographs (Figs. 11 and 12) the fissure encircling emerging secondary roots is most likely the path of entry for *Azorhizobia* under the conditions of the present investigation.

Infection in genera such as *Arachis* occurs through such fissure encircling the secondary roots i.e. through breaks in the epidermis and is characterised by the absence of infection threads. Intercellular infection is initiated at the basal junction between the emerging secondary root and the cracks in the adjacent epidermal cells. Rhizobia invade the host cell by localized cell wall invagination and degradation. Further development of the nodule occurs by repeated division of the infected host cells (Chandler, 1978).

#### 4.1 STEM INFECTION SITE

Stem nodulation is initiated at predetermined sites that are formed independently of *Azorhizobial* infection, which represents a distinct characteristic of stem-nodulating systems (Dreyfus et al. 1984). Although it was postulated initially that lenticels represented the actual infection site (Dreyfus and Dommergues, 1981a), it has been shown

subsequently that infection occurs at sites where adventitious root primordia emerge (Duhoux and Dreyfus, 1982). These preformed root primordia are dome shaped, slightly swollen areas, spaced 4 to 5 mm apart in vertical rows around the circumference of the stem. They seem to be quiescent, non-elongating, encircled by a fissure (Duhoux, 1984). Histological preparations in the present study strongly supports this site of infection in the stem (Fig. 13b) through breaks in epidermis and the cortex. This appears to be important in relation to the sensitivity to bacterial infection of the respective stem-nodulating system. Both *S. rostrata* and *Ae. asfraspera* exhibit clearly protruding root primordia on their stems and are readily infected and efficiently nodulated. In contrast, a group of *Aeschynomene* species (e.g. *Ae. indica*, *Ae. paniculata*, and *Ae. sensitiva*), with root primordia which hardly emerge, are less readily nodulated, while a third group, consisting of *Ae. crassicaulis* and *Ae. elephroxylas*, having the least emergent root primordia, are only poorly nodulated. In the latter case, nodules appeared only on those segments of the stem where waterlogging had induced the elongation of the root primordia (Dreyfus et al. 1984). Thus the nature of the infection site (degree of dormancy of the root primordia) appears to be an important factor in the stem nodulation process.

*S. rostrata* stem nodules can also form on shoot initials

at the apex of stem and leaves and at the cotyledonary nodes of young plantlets (Olsson and Rolfe, 1985). This may suggest that any undifferentiated meristematic (embryonic) tissue can be induced by the invading bacteria to become nodule tissue (Olsson and Rolfe, 1985).

The initial infection site, namely, the fissure surrounding the dormant root primordium, appears to be a convenient and physiologically beneficial environment for (airborne) azorhizobia to begin colonizing the plant tissue. The plant cells which are exposed to the invading bacteria at the inside of the fissure are differentiated, highly vacuolated, non dividing cells (Tsien et al. 1983; Duhoux, 1984). It is not known whether the bacteria gain entry through cells which are already broken as a result of the mechanical forces associated with the penetration of the epidermal dome by the root primordium (Duhoux, 1984). In any case, the bacteria rapidly proliferate on the bottom of the fissure which has been clearly shown in electron micrographs by Tsien et al. (1983). Nodulation of the stems of *S. rostrata* is affected by external conditions, such as humidity (rain) and wind, which facilitate airborne infection (Duhoux, 1984). Biological agents, such as insects, and windborne sand particles may also play a role. Mechanical wounding of various parts of the stem, prior to azorhizobial infection, does not result in nodule formation at those sites; nor does

*Agrobacterium*-induced tumour formation take place preferentially at root primordium emergence sites on the stem (Vlachova et al. 1987). Infection appears to be initiated "from the outside"; i.e., each adventitious root site must be infected separately. No spread of azorhizobia via the vascular system has been observed (Duhoux, 1984). This is in contrast with the case of secondary tumour formation induced by *Agrobacterium* on *S.rostrata*.

#### 4.2 PRESENCE OF OLEOSOMES IN THE EARLY STAGES OF NODULATION

Amongst the tropical legumes to date only peanut has revealed the presence of oleosomes. The presence of oleosomes in the infected cells of roots and stem nodules of *S. rostrata* is unique. Our investigations also suggest that the synthesis of oleosomes is under control of both the host and the symbiont. No oleosomes are found in the root primordia without inoculation and induction of nodulation (Figs. 13b and 16). This suggests that the synthesis of oleosomes requires the specific expression of both plant and bacterial genes. The trigger for the expression may be produced by plant-bacteria interactions at the surface involving the functioning of nod genes. The presence of oleosomes is normally restricted to growing and undifferentiated infected cells and none are present in the cells that are fully differentiated (Figs. 15a and 17). This observation suggests that oleosomes are used up in the proliferation of the enormous membrane system needed

for housing bacteroids and maintaining their growth, and perhaps to provide energy to the growing infected cells. In the root nodules also oleosomes disappear from in the infected cells of the nodules as they mature (Fig. 20). The oleosomes that are found during early differentiation are possibly consumed as the infected cells mature. The proximity of the oleosomes to the bacteroids and the presence of lipolytic activity make the TAG readily available for utilization.

The unusual presence of larger oleosomes in stem nodules is unique to *S. rostrata* (Figs. 15b, 17, 21 and 31). Size of oleosomes of the seeds in the literature is reported to be 0.5 - 2.5  $\mu\text{m}$  in diameter (Tzen and Huang, 1992). Such unusually large oleosomes deserve to be designated as "megaoleosomes" which are almost three times the size of normal oleosomes that we know about. These enormous oleosomes may be responsible for the difficulties in tissue embedding and sectioning for the electron microscope observations.

Stem nodules differ most strikingly from root nodules in that they have chloroplasts throughout the cortex (Dreyfus and Dommergues, 1981a; Legocki et al. 1983) including cells contiguous with the bacteroid zone. Avjioglu and Knox (1989) observed that the concentration of sucrose in the medium altered the pattern of lipid accumulation in zygotic and somatic embryos of *Brassica napus* L. They observed that the concentration of sucrose, at which the highest amount of dry

weight was accumulated after the 2-week culture period (that is 10% sucrose) was also the concentration at which the highest amount of storage lipid (41.2 mg/g) accumulated. As *S. rostrata* stem nodules have chloroplasts throughout the cortex, photosynthate in the form of sucrose must be in ready supply and thus helps in accumulation of storage TAG in oleosomes. The TAG is synthesized from sucrose through acetyl coenzyme A which forms fatty acids (Stumpf and Pollard, 1983). The photosynthetic machinery, being so close to the infected cells possibly results in an oversupply of sucrose, and consequently overproduction of TAG. This might be the reason for "megaoleosomes" formation in the stem nodules. In the root nodules on the other hand translocation of photosynthate from leaves takes a longer route and perhaps excessive accumulation is prevented (Rawsthorne et al. 1980).

*S. rostrata* stem nodules contain relatively a higher amount of total lipid than root nodules as estimated by gravimetric method (Table 2). The oleosomes may probably account for the observed higher total lipid content of stem nodules. However when compared to seeds both *S. rostrata* stem nodule and root nodule contain relatively less amount lipid. This has been one of the major constraints in obtaining oleosome fractions for analyses in this investigation. Similar difficulties were also reported by Jayaram and Bal (1991) in peanut root nodule where the oleosomes covered only some 5% of

the cell area.

Earlier studies in morphogenesis of *S.rostrata* nodules were done only upto 6-8 days after inoculation (Tsien *et al.* 1983, Duhoux, 1984). Oleosomes were not reported. However Duhoux (1984) has shown an electron micrograph (Fig. 8 in his article) where he labels a "spheroid" in close contact with cisternae of endoplasmic reticulum. It is likely that this spheroid is an oleosome. The presence of oleosomes in nodules is visualized only after the initial stages of morphogenesis (1-6 days old). The nodules studied in this investigation were 15-20 days old. Therefore this is a distinct stage where oleosomes are formed and TAG synthesized and rapidly used up. Due to oleosomic metabolism the nodule tissue at this stage was very difficult for electron microscopic observations, due to improper infiltration of embedding media.

#### 4.3 FATTY ACID COMPOSITION OF OLEOSOMES OF *SESBANIA* NODULES AND SEEDS

The present investigation shows that the nodule oleosomes differ from the seed oleosomes in their fatty acid composition (Table 3). Our observations on isolated oleosomes of *S. rostrata* nodules and seeds by scanning electron microscope indicate that large oleosomes were present in stem nodule fractions, whereas in the seed the smaller oleosomes dominate (Figs. 31 and 32). Alterations in the fatty acid composition of soybean seeds inoculated with *Bradyrhizobium* have been

reported (Pacovsky and Fuller, 1986). These authors have found that the nitrogen-fixing soybeans had higher amounts of  $C_{16:0}$  and  $C_{18:0}$  fatty acids than the non-nodulated nitrogen-fertilized plant in the seed as well as higher amounts of  $C_{15:1}$ ,  $C_{18:2}$  and  $C_{18:3}$  fatty acids. These authors have suggested that the nutrient requirement, amount of lipid storage material and membrane composition in symbiotically grown plants may have led to different modes of metabolism favoured by the host.

Jayraman and Bal (1991) reported peanut nodule and seed oleosomes contained relatively higher amount of saturated fatty acids than unsaturated fatty acids. Among the saturated ones fatty acid  $C_{18:0}$  was more in nodule oleosomes followed by fatty acid  $C_{16:0}$  respectively. In contrast in the present investigation on fatty acid composition of oleosomes in *S. rostrata* has revealed that unsaturated fatty acids are present in higher amounts than saturated ones (Table 3). Fatty acids  $C_{18:1}$  and  $C_{18:2}$  dominated in isolated root nodule oleosome fraction,  $C_{14:1}$  in stem oleosome fraction and  $C_{18:2}$  in seed oleosome fraction. Fatty acids, as such, are not taken up by the bacteroids. Lipid catabolism in the infected cells could generate organic acids such as succinate or malate and provide a source of energy.

It is well recognized that the photosynthate supplied in the form of sucrose is converted to organic acids, such as succinate or malate, and play an essential role in the

nitrogen fixation of bacteroids (Finan *et al.* 1983; Streeter and Salminen, 1985; Dilworth and Glenn, 1985; Kouchi and Yoneyama, 1986; Humbeck and Werner, 1989). Organic acids have also been shown to support higher rates of respiration as well as nitrogenase activity in the bacteroids (Ramaswamy and Bal, 1986; Streeter and Salminen, 1988). Recently, a dicarboxylate transporter has been identified and described in soybean nodules (Udvardi *et al.* 1988; Day *et al.* 1989) which is capable of transporting succinate or malate at rates sufficient to support the measured nitrogenase activity. This is further supported by the identification of malate synthase, a key enzyme for succinate or malate oxidation in bacteroids via tricarboxylic acid cycle (Day and Mannix, 1988; McKay *et al.* 1988; Kouchi *et al.* 1988; Kimura and Tajima, 1989).

#### 4.4 LIPOLYTIC ACTIVITY

The first step in oleosome utilization is the mobilization of fatty acids from the triacylglycerides by the action of lipases, which are located inside the oleosomes. Lipase, in association with lipid bodies, has also been reported in rape, mustard and maize seeds (Lin and Huang, 1983; Wang *et al.* 1984). Studies of seed lipases revealed that the enzyme is synthesized in the free polyribosomes and transported to the lipid body where it is bound to a membrane protein (Wang and Huang, 1987). Therefore, the release of fatty acids from lipid bodies can easily take place *in situ*.

The bacteroids are unable to utilize the fatty acid as such. It requires further breakdown through the beta oxidation pathway and glyoxylate or TCA cycle to make organic acids available for bacteroids. Both the beta oxidation pathway and glyoxylate cycle seem to operate in nodules in addition to the TCA cycle (Gallon and Chaplin, 1987).

In the present investigation the lipase activity was demonstrated histochemically (Fig. 25). The fatty acids liberated from the substrate by the enzyme activity were stained by Nile Blue. The lipase reactions demonstrated with Nile Blue dyeing were considered more suitable for the histochemical demonstration of lipase. Lipolytic activity demonstrated in the infective tissue of the nodule indicate that the oleosomes are possibly catabolized and free fatty acids are released. Free fatty acids are possibly oxidized through the beta oxidation pathway (Tolbert, 1981) and the presence of microbodies and catalase activity points to that direction. Dense bodies showing positive catalase activity may also be involved in lipid catabolism as shown by Bal et al. (1989).

#### 4.5 CATALASE ACTIVITY

The enzyme catalase plays an important role in the beta oxidation pathway of lipids (Tolbert, 1981). The enzyme functions within microbodies as a scavenger of the toxic hydrogen peroxide produced in the catabolic reactions.

Catalase activity detected in both bacteroidal and host cytosol fractions (Table 1) showed that the host cytosol had higher activity. This study shows a correlation between the presence of lipid bodies and the presence of catalase.

Numerous studies have been carried out on catalase localization in kidney and liver microbodies of animal tissues (Fahimi and Yokota, 1981) as well as in plants (Huang et al. 1983) using the DAB reaction. Cytochemical studies on root nodules have been done mainly on soybean, where microbodies occur in the uninfected interstitial cells of the nodule (Newcomb and Tandon, 1981; Vaughn, 1985). However, very little is known about the location of catalase activity in the *S. rostrata* nodules. The present investigation indicates that the catalase activity is not only restricted to the microbodies as in other tissues of plants and animals but also in dense bodies that are attached to the bacteroid wall (Bal et al. 1989) at the host-symbiont interface (Fig 28).

Catalase activity has been detected in the perisymbiont space of the symbiosomes. Careful study of many sectional profiles of the symbiosome/host interface strongly suggest that the perisymbiotic membrane with the catalase is endocytosed into the host cytosol. The enzyme catalase is present in both the prokaryotic rhizobia and eukaryotic plant cell (Siddique and Bal, 1989). However in symbiotic association within the nodule, catalase can be localized only

in the peribacteroid space, the enzyme is secreted from the bacteroid into the host cytosol, being delivered by the perisymbiotic membrane vesicle.

#### 4.6 FUNCTION OF OLEOSOMES IN NITROGEN-FIXING NODULES

4.6.1 ARCTIC AND ALPINE LEGUMES In arctic and alpine legume nodules large quantities of lipid bodies (oleosomes) have been reported (Newcomb and Wood, 1986; Barimah-Asare and Bal, 1994). In beach pea (*Lathyrus maritimus* L.) oleosomes are found to occur only in the early symbiotic stages of infected cells along with the amyloplasts. The oleosomes become scarce as amyloplasts disappear during the late symbiotic stage. These observations suggest that (1) the oleosomes may be metabolized in the presence of amyloplasts as plastids are known to be sites of fatty acids synthesis (Andrews and Ohlrogge, 1990) and (2) the disappearance of the oleosomes and the amyloplasts is an indication of their possible consumption during the membrane and rhizobial proliferation as the infected cell matures. Newcomb and Wood (1986) speculated that lipid bodies and amyloplasts may provide a ready source of energy for the process of nitrogen fixation by the bacteroids, or that they may represent some mechanism for increasing temperature within the cell to facilitate growth and development within the infected cells. Absence or scarcity of oleosomes from mature nitrogen-fixing symbiotic zones may indicate the lack of any role in the nitrogen fixation process per se.

#### 4.6.2 PEANUT NODULES (PERSISTENT OLEOSOMES)

Unlike most of the tropical and temperate legumes, peanut nodule oleosomes are persistent throughout all developmental stages and occupy about 5% of the total volume in the infected cells (Jayaram and Bal, 1991). They are in close contact with the peribacteroid membrane (Bal et al. 1989).

The peanut nodules with persistent oleosomes in root nodules of peanut are thought to have an added advantage when the beta oxidation pathway is operative along with the lipolytic activity, so that large amounts of energy stored in the oleosomes can be mobilized for nitrogen fixation and other metabolic functions. Evidence for such utilization as a supplementary source of energy has been forthcoming through recent experimental studies (Siddique and Bal, 1991, 1992; Bal and Siddique, 1991).

#### 4.6.3 SESBANIA NODULES

Like in beach pea, oleosomes in *S.rostrata* (both in stem and root nodules) are found to occur in the early symbiotic stages of infected cells. Early stages of *S. rostrata* stem nodules contain large oleosomes (1-8  $\mu\text{m}$  in diameter) and disappear when the cells differentiate. These observations suggest that, as in beach pea nodules, the disappearance of oleosomes in the infected cells indicate their possible consumption during the membrane and rhizobial proliferation as the infected cell matures. In the stem nodules oleosomes

become enormous in size possibly due to over supply and piling up of photosynthate which is converted to triacylglycerol for a very short-term storage in the early stages of nodule differentiation.

## V. CONCLUDING REMARKS

The study of "Differentiation of nitrogen-fixing nodules of *Sesbania rostrata* with special reference to oleosomes can be summarized by the following statements:

- the bacterium *Azorhizobium caulinodans* most likely enters the host through fissures encircling the root initials of the stem and the secondary roots of the root.
- oleosomes are present during early differentiating infected cells of both stem and root nodules.
- oleosomes present in the stem nodules are remarkably larger in size ( $< 8 \mu\text{m}$  in diameter) than the ones in the root nodules.
- oleosomes disappear in both root and stem nodules as the infected cell matures.
- oleosomes seem to be used up for the growth and development of the infected cells.
- lipolytic activity could be localised in the infection zone of the nodule by the Nile Blue dyeing method.

- unsaturated fatty acids such as  $C_{14:1}$ ,  $C_{16:1}$  are dominant in the stem nodules whereas  $C_{18:1}$ ,  $C_{18:2}$  are dominant in root nodules and in the seed.

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