

ROLE OF LIPID BODIES IN  $N_2$ -FIXING ROOT  
NODULES OF PEANUT (*Arachis hypogaea* L.)

CENTRE FOR NEWFOUNDLAND STUDIES

**TOTAL OF 10 PAGES ONLY  
MAY BE XEROXED**

(Without Author's Permission)

ABU-BAKER M. SIDDIQUE, B.Sc.(Hons.), M.Sc.









ROLE OF LIPID BODIES IN N<sub>2</sub>-FIXING ROOT  
NODULES OF PEANUT (*Arachis hypogaea* L.)

BY

©ABU-BAKER M. SIDDIQUE, B. Sc. (Hons.), M. Sc.

A thesis submitted to the School of Graduate  
Studies in partial fulfilment of the  
requirements for the degree of  
Doctor of Philosophy

Department of Biology  
Memorial University of Newfoundland  
July 1991

St. John's

Newfoundland

Canada

## ABSTRACT

The peanut plant (*Arachis hypogaea* L.), unlike other legumes, can sustain nitrogen fixation when prolonged periods of darkness or detopping curtail the supply of photosynthate to the nodule. This ability to withstand photosynthate stress is attributed to the presence of lipid bodies in infected nodule cells. In both dark-treated and detopped plants, the lipid bodies show a gradual decrease in numbers, which suggests their utilization as a source of energy and carbon for nitrogen fixation. Lipolytic activity can be localized in the lipid bodies and the existence of the  $\beta$ -oxidation pathway and glyoxylate cycle is shown by the release of  $^{14}\text{CO}_2$  from linoleoyl Coenzyme A by the nodule homogenate. In addition, the biochemical assay and cytochemical localization of catalase and malate synthase also confirm lipid catabolism in the nodule.

Catalase from cultured *Bradyrhizobium* sp. 32H1, root nodules and seeds of peanut is active in a wide range of pH, having two pH optima. The enzyme activity is associated with both the bacteroids and the host cytosol. In isolated nodule bacteroids the presence of catalase is restricted to the bacteroid surface only, whereas in the rhizobia grown in culture, the activity remains inside the cells. Triazole-

sensitive DAB reaction revealed microbodies in the host cytoplasm, often lying close to the peribacteroid membrane. DAB-positive dense bodies are also found on the bacteroid surface at the host-symbiont interface.

Ineffective nodules of peanut induced by two nod<sup>+</sup>fix<sup>-</sup> strains of *Bradyrhizobium* sp. were compared with the ones induced by nod<sup>+</sup>fix<sup>+</sup> strain NC92. Both fix<sup>-</sup> strains (639 and 7091) induce small nodules lacking leghemoglobin and nitrogen-fixing activity. Ultrastructural observations revealed that the nodules of 639 have an enlarged peribacteroid space and lack persistence of nodule function. The 7091-induced nodules showed an impediment to bacteroid release and differentiation. In the ineffective nodules, larger numbers of lipid bodies were found to accumulate, compared to the effective NC92 nodules. The large lipid accumulation in the absence of nitrogen fixation serves as further evidence to confirm that the nodule lipid bodies are utilized as a supplementary source of carbon and energy for nitrogen fixation in peanut root nodules.

*To My Beloved Parents*

## ACKNOWLEDGEMENTS

I would like to express my deep sense of gratitude to Dr. A. K. Bal, Professor of Biology, for his constant guidance, wise council and partial financial support throughout the program. Thanks are due to my supervisory committee members, Dr. P. J. Scott and Dr. T. R. Patel, for their valuable suggestions and friendly discussions during the research. I am grateful to the School of Graduate Studies and Department of Biology for providing the graduate student support and teaching assistantship throughout the program.

I am also thankful to the technical staff of Biology Department, particularly, C. Emerson, R. Ficken, E. Fitzgerald and W. Brown for their excellent help. Thanks are due to my friends Jayaram, John, Ganpati, Ravi and my Burton's pond room-mate Fidzi, Sharr and Shafiq for their lovely company.

A special thanks to my brother, Dr. Azim U. Mallik, for his wise advice and constant inspiration in my academic career. I owe an intellectual debt to him.

Finally I would like to express my profound thanks to my special friend for her precious love and support.

# TABLE OF CONTENTS

	page
ABSTRACT .....	ii
ACKNOWLEDGEMENTS .....	v
TABLE OF CONTENTS .....	vi
LIST OF TABLES .....	x
LIST OF FIGURES .....	xi
<b>I. INTRODUCTION</b>	
I.1. General Introduction .....	1
I.2. Nitrogen-fixing Organisms .....	5
I.3. Biological Nitrogen Fixation .....	5
I.3.1. Asymbiotic Nitrogen-fixation .....	6
I.3.2. Symbiotic Nitrogen-fixation .....	7
I.3.3. <i>Rhizobium</i> -legume Symbiosis .....	8
I.3.4. <i>Rhizobium</i> - the Nitrogen-fixing Bacterium .....	8
I.3.5. The Infection and Nodulation Process ..	11
I.3.6. Mechanism of Nitrogen Fixation .....	12
I.3.7. Hemoglobin in Nitrogen Fixation .....	14
I.3.8. Ammonium Assimilation and Transportation	16
I.4. Photosynthesis in Nitrogen-fixation .....	17
I.5. Peanut ( <i>Arachis hypogaea</i> ), Taxonomy, Distribution and Importance .....	21
I.5.1. Specificity of Peanut- <i>Rhizobium</i> Symbiosis	22

I.6. Catalase in Nitrogen Fixation .....	23
I.7. Lipase and Lipid Catabolism .....	24
I.7.1. Lipid in Nitrogen Fixation .....	25
I.8. Objectives .....	27
I.9. Outline of Methods Employed .....	28
<b>II. MATERIALS AND METHODS</b>	
II.1. <i>Rhizobium</i> Strains and Medium .....	30
II.2. Plant Materials and Growing Conditions .....	31
II.3. Dark Treatment and Detoping of Plants .....	31
II.4. Electron Microscopy (EM) of Samples .....	32
II.5. Nodule Numbers .....	33
II.6. Nitrogenase Assay by the Acetylene ( $C_2H_2$ ) Reduction Technique .....	33
II.7. Lipid Body Preservation, Staining and Counting	34
II.8. Sample Preparation .....	35
II.8.1. Nodule and Bacteroid Fractions .....	35
II.8.2. Cell-free Extract of Cultured <i>Rhizobium</i>	36
II.8.3. Seed Cytosol .....	36
II.9. Determination of Leghemoglobin (LHb) .....	37
II.10. Catalase .....	37
II.10.1. Assay ... ..	37
II.10.2. Assay at Different Temperatures ...	39
II.10.3. Cytochemical Localization .....	39
II.11. Malate Synthase ... ..	40

II.11.1. Assay	40
II.11.2. Cytochemical Localization	41
II.12. Linoleoyl Coenzyme A (LYL-CoA) Oxidation	42
II.12.1. Sample Preparation	42
II.12.2. Determination of LYL-CoA Oxidation	43
II.13. Localization of Lipolysis	43
II.14. Gel Electrophoresis	44
II.14.1. Preparation and Running of Gel	44
II.14.2. Gel Staining	45
<b>III. RESULTS</b>	
III.1. Acetylene Reduction Assay (ARA) and Lipid Bodies (LB)	46
III.2. Leghemoglobin (LHb), Total Protein (TP) and Catalase in Dark-stressed and Detopped Nonnodules	57
III.3. Dark- and Detopp- Stressed on Nodule Ultrastructure	57
III.4. Linoleoyl Coenzyme A (LYL-CoA) Oxidation	66
III.4.1. <i>In vivo</i> Sample	66
III.4.2. <i>In vitro</i> Grown <i>Bradyrhizobium</i> sp. 32H1.	69
III.5. Lipase Localization	76
III.6. Malate Synthase Assay and Localization	76
III.7. Catalase Assay and Localization	79
III.8. Gel Electrophoresis	93



III.9. Ultrastructure of Peanut Nodules Induced by Fix <sup>-</sup> and Fix <sup>+</sup> Strains of <i>Bradyrhizobium</i> sp. ...	93
<b>IV. DISCUSSION</b>	
IV.1. Acetylene Reduction Assay (ARA) and Lipid Bodies (LB) .....	111
IV.2. Lipase Localization .....	115
IV.3. Malate Synthase Assay and Localization .....	116
IV.4. Linoleoyl Coenzyme A (LYL-CoA) Oxidation .....	117
IV.5. Leghemoglobin (LHb) and Total Protein (TP) .....	118
IV.6. Catalase in Stressed Nodules .....	119
IV.7. Ultrastructure of Stressed Nodules .....	121
IV.8. Catalase Assay and Localization .....	123
IV.9. Ultrastructure and Lipid Bodies of Fix <sup>-</sup> and Fix <sup>+</sup> Nodules .....	125
<b>V. SUMMARY</b> .....	129
<b>VI. CONCLUSIONS</b> .....	130
<b>VII. REFERENCES</b> .....	133

# LIST OF TABLES

	page
Table 1. Leghemoglobin and total protein content in the root nodules of dark-treated peanut plant .....	58
Table 2. Leghemoglobin and total protein content in the root nodules of detopped peanut plants .....	59
Table 3. Leghemoglobin and total protein content in the root nodules of dark-treated cow pea plants .....	60
Table 4. Catalase activity in peanut and cowpea root nodule homogenate obtained from plants subjected to different periods of darkness .....	61
Table 5. Catalase activity in different fractions of bacteroids and bacteria .....	92
Table 6. Number, size and texture of peanut nodules produced by the different strains of rhizobia .....	108

## LIST OF FIGURES

	page
Figure 1. Schematic diagram of nitrogen cycle .....	4
Figure 2. Schematic diagram showing relationship between photosynthesis and nitrogen fixation	20
Figure 3. Acetylene reduction assay (ARA) of peanut and cowpea nodules obtained from dark-treated plants .....	48
Figure 4. Acetylene reduction assay (ARA) of peanut and cowpea nodules obtained from detopped plants .....	50
Figure 5. Acetylene reduction assay (ARA) of cowpea nodules obtained from dark-treated plants at constant temperature .....	52
Figure 6. Acetylene reduction assay (ARA) of the nodules of dark-treated peanut plants (cv. Early Spanish) .....	54
Figure 7. Lipid body counts in 1.5- $\mu$ m thick sections of nodules from dark-treated and detopped peanut plants .....	56
Figure 8. An electron micrograph of peanut nodule tissues obtained from 24 h dark-stressed plant .....	63
Figure 9. An electron micrograph of peanut nodule	

tissues obtained from 48 h and 72 h dark-stressed plants .....	65
Figure 10. An electron micrograph of cowpea nodule tissues obtained from 24 h and 48 h dark-stressed plants .....	68
Figure 11. $^{14}\text{CO}_2$ released from [ $^{14}\text{C}$ ] linoleoyl coenzyme A by the nodule homogenate of peanut and cowpea nodules .....	71
Figure 12. $^{14}\text{CO}_2$ released from [ $^{14}\text{C}$ ] linoleoyl coenzyme A by peanut nodule cytosol and bacteroids .....	73
Figure 13. $^{14}\text{CO}_2$ released from [ $^{14}\text{C}$ ] linoleoyl coenzyme A by different fractions of cultured <i>Bradyrhizobium</i> sp. 32H1 .....	75
Figure 14. Cytochemical localization of lipase in peanut root nodule tissue .....	78
Figure 15. Cytochemical localization of malate synthase in peanut nodule tissues .....	81
Figure 16. Graph showing catalase activity at different times in <i>in vitro</i> culture of <i>Bradyrhizobium</i> sp. 32H1 growing in YEM medium .....	83
Figure 17. Graph showing catalase activity of the bacterial cell content at different pH values .....	85

Figure 18. Graph showing catalase activity of the peanut nodule cytosol at different pH values .....	87
Figure 19. Graph showing catalase activity of the isolated bacteroid fraction from peanut nodule at different pH values .....	89
Figure 20. Graph showing catalase activity of the 3-day germinated peanut seed cytosol at different pH values .....	91
Figure 21. Cytochemical localization (DAB positive) of catalase in <i>Bradyrhizobium</i> sp. 32H1 ....	95
Figure 22. Cytochemical localization (DAB positive) of catalase in root nodule tissues of peanut .	97
Figure 23. DAB stained native polyacrylamide (10%) gel showing three distinct catalase bands in 3-day germinated seed and one band in nodule cytosol of peanut .....	99
Figure 24. Photo-and electron micrographs of nodules (3 week post-inoculation) induced by <i>Bradyrhizobium</i> strains NC92 and 639 .....	103
Figure 25. Photo-and electron micrographs of nodules (4,5 and 6 weeks post-inoculation) induced by <i>Bradyrhizobium</i> strain 639 .....	105
Figure 26. Photo-and electron micrographs of nodules (5 weeks post-inoculation) induced by	

<i>Bradyrhizobium</i> strain 7091 .....	107
Figure 27. A graph showing lipid body counts in the infected cells of the three nodules formed by <i>Bradyrhizobium</i> strains NC92, 639 and 7091 .....	110
Figure 28. Schematic diagram showing the role of lipid bodies in $N_2$ -fixing root nodules of peanut .....	132

# Chapter I

## INTRODUCTION

### I.1. General Introduction

Amino acids are key building blocks of proteins which are basic to cellular functions. In proteins, nitrogen is present in its combined form. Although molecular nitrogen is abundant in the earth's atmosphere, accounting for 78% by weight, its relative stability and inertness make it unavailable to eukaryotic organisms. Nitrogen is combined with other elements like hydrogen and oxygen in an endergonic reaction called nitrogen fixation.

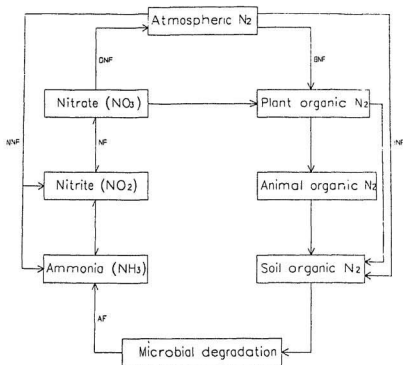
There are basically three different ways by which atmospheric nitrogen is fixed into a combined form: i) atmospheric fixation - nitrogen reacts with oxygen ( $O_2$ ) due to lightning discharge or ultraviolet radiation to form oxides, e.g. nitric oxide ( $NO_2$ ) which is carried to the soil with rain; ii) industrial fixation (Haber-Bosch process) - nitrogen and  $H_2$  react at high temperature and pressure to yield ammonia ( $NH_3$ ); and iii) biological nitrogen fixation - atmospheric nitrogen is fixed into  $NH_3$  catalysed by the enzyme complex, nitrogenase in prokaryotic organisms either symbiotically or in association with higher organisms.

In nature, nitrogen balance is maintained by its cycling through plants, animals and microbes. This nitrogen cycle (Fig. 1) involves the recovery of combined nitrogen from dead plants and animals by microbial action, its passage through the soil in various forms and its final reabsorption by plants. In this cycle, combined nitrogen can be lost to the atmosphere by denitrification and gained from it by natural and artificial (industrial) nitrogen fixation.

Most plants take up nitrogen in a combined form, usually as nitrate or ammonia, via their root system, sometimes assisted by mycorrhizal fungi. A small amount of ammonia from animal excrement or other sources may volatilize from soil and be absorbed by the plant canopy (Sprent, 1987). The nitrite ions ( $\text{NO}_2^-$ ) are used by most plants but under neutral or alkaline conditions use ammonium ions ( $\text{NH}_4^+$ ) better.



Figure 1. Schematic diagram of nitrogen cycle in nature  
(Pelczar et al., 1986).



NNF - Natural nitrogen fixation  
 BNF - Biological nitrogen fixation  
 INF - Industrial nitrogen fixation  
 AF - Ammonification  
 NF - Nitrification  
 DNF - Denitrification

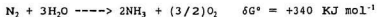
Figure 1

### **I.2. Nitrogen-fixing Organisms**

Nitrogen fixation is confined to certain group of prokaryote, which are distributed among Eubacteria and Cyanobacteria. The taxonomy of these organisms is continuously under revision. They are difficult to classify as they may have characters which span many different taxa.

### **I.3. Biological Nitrogen Fixation**

Biological nitrogen fixation involves the reduction of atmospheric nitrogen to ammonia, which is incorporated into the organic nitrogen of the nitrogen-fixing organism or its host. The overall reaction can be represented as



$\Delta G^\circ$  is the standard free energy change of the reaction (Gallon and Chaplin, 1987). Energy required by the reaction is ultimately derived from the oxidation of the products of photosynthesis. The reaction is catalysed by nitrogenase, an enzyme unique to nitrogen-fixing organisms. Nitrogenase is extremely sensitive to oxygen. Biological nitrogen fixation is the major source of renewable combined nitrogen in the biosphere (Postgate, 1982). About  $122 \times 10^6$  metric tons of nitrogen is fixed per annum (Burris, 1980) which is almost 71% of the world's fixed nitrogen in the soil and water. Industrial nitrogen-fixation contributes approximately 15% and

environmental factors contribute the other 10% of fixed nitrogen (Bray, 1983).

Although biological nitrogen-fixation is limited to a few prokaryote, some higher plants can also profit from this process in loose association with such prokaryote (associative symbiosis) or by harbouring the prokaryote inside root nodules (symbiosis) (Stewart, 1977). Biological nitrogen-fixation can occur in prokaryote when they are also free-living (asymbiotic).

### 1.3.1. Asymbiotic Nitrogen-fixation

Free-living nitrogen-fixing organisms can be aerobic (*Azotobacter*), anaerobic (*Clostridium*) or photosynthetic (*Nostoc*). They are found in both soil and aquatic environments. Nitrogen fixed asymbiotically is not directly available to the plants until the nitrogen-fixing organisms die and decompose in the soil. These organisms are approximately 1000 times less effective in their contribution of fixed nitrogen to the soil than symbiotic bacteria (Bray, 1983). There are some free-living nitrogen-fixing bacteria which are abundant in the rhizosphere of different plants (Vose, 1983; Haahtela and Kari, 1986). Such an association suggests a form of mutualism (You and Zhou, 1988). Newman and Bowen (1974) established that associated bacteria were randomly distributed on roots and that 4-10% of a root's surface was covered by micro-organisms.

Colonization of the root interior has also been reported (Patriquin and Dobereiner, 1978; Patriquin, 1981).

### **I.3.2. Symbiotic Nitrogen-fixation**

Symbiotic nitrogen-fixation is another form of biological nitrogen-fixation, where the bacteria inhabit in nodule cells formed by the host plant. A very common symbiotic association occurs between leguminous plants and *Rhizobium* spp. In nature, nodulation of *Parasponia* sp. (a member of Ulmaceae) by *Rhizobium* indicates the possibility of extending *Rhizobium* symbiosis beyond the Leguminosae. Nodules have been induced on the non-legume, oilseed rape, following enzyme treatment of the seedling roots and inoculation with *Rhizobium* sp. (Al-Mallah et al., 1990). In the same way, nodule-like structures have also been induced on rice and wheat plants roots in recent years (Al-Mallah et al., 1989a,b), but little is known about the efficiency of their function in terms of nitrogen fixation. Although vast majority of nodules occur on the roots of the host plant, certain aquatic and water-tolerant species of legumes develop nodules on their stems (Alazard, 1985; Dreyfus and Dommergues, 1981; Arora, 1954) or trunk (Prin et al., 1991). These nodules have been shown to be capable of a high rates of nitrogen-fixation (Subba Rao and Yatazawa, 1984).

### **I.3.3. Rhizobium-legume Symbiosis**

Legumes are placed in the family Leguminosae, recently renamed as Fabaceae, with sub-families Caesalpinoideae, Mimosoideae and Papilionoideae. The family Fabaceae consists of about 750 genera with 16,000-19,000 species (Allen and Allen, 1981) and is widely distributed from the tropics to the arctic. Cultivated species such as peas, beans, alfalfa, clover, lupin, soybean, cowpea, peanut, etc. have the characteristic legume fruit. Most members form a symbiotic association with a *Rhizobium* sp. and play a crucial ecological role in maintaining adequate nitrogen resources in the earth. About 10% of the legumes have been examined for nodulation. Among those, 85% of the species belong to the Papilionoideae and 25% to the Mimosoideae. Nodulation is rare in the Caesalpinoideae (Postgate, 1982).

### **I.3.4. Rhizobium - the Nitrogen-fixing Bacteria**

The rhizobia are a group of bacteria belonging to the genus *Rhizobium* that fix atmospheric nitrogen in symbiotic associations with specific host plants, almost exclusively legumes. They are gram negative, rods ( $0.5 - 0.9 \mu\text{m} \times 1.2 - 3.0 \mu\text{m}$ ), occur singly or in pairs and are generally motile. The flagella are either peritrichous, polar or sub-polar (Jordan and Allen, 1974). Rhizobia usually grow in a wide range of temperature under low oxygen tension. They do not produce

endospores. Glycogen and poly- $\beta$ -hydroxybutyric acid are formed as storage granules. The free-living rhizobia can infect a specific host and produce nodules in which they are transformed into bacteroids after undergoing both morphological and biochemical changes. The bacteroids, that fix atmospheric nitrogen and supply it to the host, are characterized by a highly irregular shape caused by outer cell wall changes (Van Brussel, 1973; Sutton and Peterson, 1979; Bal et al., 1980). The electrophoretic profile of protein in bacteroids is different from free-living rhizobia (Sen and Weaver, 1988).

There are two major groups of rhizobia, commonly known as fast- and slow-growing species. The generation time for fast-growers is 2-4 h while it is 6-8 h for slow-growers (Vincent, 1977). These two groups differ biochemically and physiologically and have been placed in two separate genera, *Rhizobium* and *Bradyrhizobium* (Jordan, 1982). Unlike *Rhizobium*, *Bradyrhizobium* does not produce a lot of mucoids (extracellular polysaccharide) in agar. The two groups also differ in several symbiotic properties; for example, *Bradyrhizobium* strains can be induced to fix nitrogen in free-living cultures (Kurz and LaRue, 1975; McComb et al., 1975; Pagan et al., 1975). *Rhizobium* species fix nitrogen only symbiotically. These fast-growing species generally infect only a few closely related legumes whereas *Bradyrhizobium* species of the cowpea cross-inoculation group can infect a broad range of diverse legume

hosts (Elkan, 1981). Fast-growing species almost invariably infect their host via the root hairs whereas slow-growing species infect by root hairs (in pigeonpea) or direct penetration of epidermal cells (in groundnut), the so-called 'crack entry' (Chandler, 1978; Dart, 1977; Kapil and Kapil, 1971). Many sugars, polyols and organic acids can be utilized by the fast-growing species but the slow-growers are more specialized in their requirements and generally prefer pentoses (Vincent, 1977). Although one or more vitamins are required by several strains for growth (Graham, 1963), *Bradyrhizobium* is less vitamin demanding than fast-growing ones. Mineral requirement for rhizobial growth is important and optimal concentrations for different minerals vary among the strains. The viability of a culture is reduced in the absence of Mg and Ca. Magnesium-limited cells become elongated and sometimes branched. Calcium-limited cells become irregular, swollen, or roughly spherical (Vincent, 1977). Salts of both Na and K elements are toxic at higher concentrations but omission of K from the basal medium restricts the growth of *R. trifolii* and *R. meliloti* (Vincent, 1977).

Indole acetic acid (IAA) and gibberellin-like substances are produced by rhizobia (Katznelson and Cole, 1965). Both fungicides and insecticides can be toxic to rhizobia (Vincent, 1958), but hormone herbicides generally do not have any effect on rhizobia (Fletcher and Alcron, 1958). Rhizobia are suscep-



tible to broad spectrum antibiotics and the effect of such antibiotics cuts across specific boundaries so that strains and sub-strains within one species show a considerable range of sensitivity and resistance. Micro-organisms found in soil and in the rhizosphere can be synergistic or antagonistic to rhizobial growth.

#### **I.3.5. The Infection and Nodulation Process**

The legume-*Rhizobium* interaction involves a series of plant-bacterium signals which activate the successive steps in nodulation (Faucher et al., 1988; Halverson and Stacey, 1986; Lerouge et al., 1990; Peters et al., 1986; Truchet et al., 1980). After introduction of the bacteria to the root surface of young seedlings, multiplying bacteria colonize the root and especially adhere to emerging root hairs. During subsequent growth of these root hairs, different types of deformations occur, of which marked curling or shepherd's crooks types are most notable. Following root deformation, the bacteria penetrate the root cells where they are immediately surrounded by a membrane. This leads to the formation of the infection thread which penetrates the root cortical cells. Coincident with the development of the infection thread and the passage of rhizobia through, stimulates cortical cell division resulting in the formation of the nodule. The bacteria are released into the nodule cells by a process of endocytosis but

they remain surrounded by a host cell cytoplasmic membrane, called the peribacteroid membrane. The peribacteroid membrane protects the bacteroid from plant defence mechanisms (Vance, 1983). Inside the peribacteroid membrane the bacteria undergo morphological and biochemical changes and become transformed into bacteroids. At this stage nitrogen fixation begins. Meanwhile, the host cells have synthesized a series of nodule-specific proteins, the nodulins (Downie et al., 1988; Kouchi et al., 1989). Among these nodulins, the apoproteins of the leghemoglobins are most obvious. This sequence of steps is representative for many plants and their corresponding bacteria. However in some plants, like the peanuts, infection through root hairs or the formation of infection threads is not observed. Instead, direct entry occurs through weak places of the epidermis such as at the junction of lateral roots. The bacteria multiply in the intercellular space (Chandler, 1978) and from there enter the root cells (crack infection).

### **I.3.6. Mechanism of Nitrogen Fixation**

The basic mechanism of nitrogen fixation is similar in all cases studied: atmospheric nitrogen is reduced to ammonia inside the bacteroid of the nodule by the following reaction:

$$\text{N}_2 + \text{nATP} + 6\text{NADPH} + 2\text{H}^+ \rightarrow 2\text{NH}_4^+ + \text{nADP} + \text{nPi} + 6\text{NADP} + 6\text{e}^-$$

where,  $n = 6.5$  ( $6.0 - 6.9$ )  $\text{ATP}/\text{NH}_4^+$  (Rawsthorne et al., 1980).

This reaction requires i) nitrogenase, ii) a strong reducing agent, iii) an energy source such as ATP and iv) low oxygen tension.

Nitrogenase is located inside the bacteroid and its production is coded by the *nif* gene complex. The structure of nitrogenase varies little among nitrogen-fixing organisms. In all cases there are two distinct components to the active enzyme complex; the Fe protein and the Mo-Fe protein. The Fe protein consists of two subunits (mol. wt. 60,000 Da) and the Mo-Fe protein consists of four subunits (mo. wt. 200,000 Da). The Mo-Fe protein is regarded as the true dinitrogenase because the reduction of nitrogen takes place on this protein and the Fe protein is designated as the dinitrogenase reductase (Hageman and Burris, 1978, 1979). The functioning system is referred to as 'nitrogenase'.

Depending upon the nitrogen-fixing organism, the source and nature of electron donors are variable. NADPH is utilized as a reductant (Wong et al., 1971; Yates, 1977) in *Rhizobium*. The use of NADPH as an electron donor in nodule metabolism becomes very clear by the presence of NADP<sup>+</sup>-specific isocitrate dehydrogenase in the bacteroid of the nodules (Bray, 1983).

Nitrogenase requires a lot of energy (ATP is the major source) to function in the symbiotic process. Each mole of nitrogen reduced to ammonia consumes 12-15 moles of ATP making biological nitrogen fixation a bioenergetically expensive process (Gutschick, 1980). Photosynthesis and respiration of the host plant provide the ATP.

Nitrogenase is very sensitive to oxygen so it is very important that low level of oxygen be maintained in the nodule for the activation of the nitrogenase. Leghemoglobin, an oxygen-binding protein, controls the oxygen flow to the bacteroid in the nodule (Appleby, 1984). The nodule anatomy also plays an important role in the regulation of oxygen concentration (Hunt et al., 1987; Witty et al., 1987; Dakora and Atkins, 1989).

#### **I.3.7. Hemoglobin in Nitrogen Fixation**

The red pigment, hemoglobin, found in all nitrogen-fixing nodules of both legumes and non-legumes plants (Wittenberg et al., 1974; Bergersen and Turner, 1975; Appleby, 1983) is called leghemoglobin (LHb) in the legume nodule. Recently, hemoglobin gene has also been reported in the root tissues of plants which are not involved in any symbiotic associations with bacteroids for nitrogen-fixation (Appleby et al., 1988). The oxygen-binding nature of LHb plays an important role in nitrogen fixation by controlling the oxygen flux to the

bacteroid where oxygen-sensitive nitrogenase is present. The amino acid sequence of LHB shows extensive analogies to those of myoglobin and human hemoglobin (Ellfolk, 1972) and the tertiary structure of LHB bears a close resemblance to that of animal myoglobin (Vainshtein et al., 1977). LHBs generally show structural and functional heterogeneity for their oxygen-binding functions (Uheda and Syono, 1982a,b; Holl et al., 1983; Kortt et al., 1987; Kuhse and Puhler, 1987) except in a few *Phaseolus* spp. (Lulsdorf and Holl, 1991). As with other oxygen-carrying hemoproteins, LHB is sensitive to autooxidation, which results in the formation of FerriLHB, the oxidation form of LHB. This form of LHB is unable to bind oxygen and is inactive in the nitrogen fixation process (Wittenberg et al., 1974). During the aging of the nodules the LHB is irreversibly converted to the green non-functional form and nitrogen fixation ceases. The total amount of protein and LHB was found to be high in the red nodules, but both dwindle with the onset of nodule senescence (Swaraj and Garg, 1977).

During LHB synthesis, the heme part of LHB is derived either from the bacteroid of the infected cell (Appelby, 1984) or the mitochondria of an uninfected cell (Dimitrijevic et al., 1989) while the globin part is synthesized in the host cytoplasm. Both heme and globin are assembled in cytoplasm of the host cell (Verma et al., 1979). The synthesis of functional nitrogenase by legume nodule bacteroids is dependent on

the prior appearance of LHB (Appleby, 1984).

Previously it was thought that LHB was only restricted to the cytoplasm of infected cells (Verma and Bal, 1976; Berger-sen and Appleby, 1981; Robertson et al., 1984) but, recently, LHB has been localized in the ground cytoplasm of both infected and uninfected cells of soybean nodules (VandenBosch and Newcomb, 1988).

Numerous procedures have been used to estimate the LHB content of nodules. These are generally modifications of clinical techniques developed for hemoglobin and are based on the optical absorption of deoxygenated LHB (Keilin and Wang, 1945; Johnson and Hume, 1973), pyridine hemochromogen (Virtanen et al., 1947) or cyanomet LHB (Wilson and Reisenauer, 1963). LHB content has also been measured fluorometrically (LaRue and Child, 1979).

### **I.3.8. Ammonium Assimilation and Transportation**

The first stable product of nitrogenase activity is the ammonium ion. It is generally accepted that ammonium must be assimilated quickly. In nitrogen-fixing systems there are two reasons for this - ammonium is toxic and it suppresses synthesis of nitrogenase. Although there is some evidence that rhizobia can metabolize a high concentration of ammonium (Dilworth and Glenn, 1982), their host cells probably cannot. In the nodule, ammonium is exported from the bacteroid and

assimilated by the nodule-specific enzyme, glutamine synthetase (GS) in the nodule cytosol (Duke and Henson, 1985; Pate and Atkins, 1983). The presence of GS in the bacteroid is also reported but there is no evidence that this plays a substantial role in the assimilation of fixed nitrogen. In summary, the bacteroids reduce nitrogen gas into ammonium which passed to the host cell for assimilation - a good division of labour between the symbiont and the host (Sprent, 1987).

Following the assimilation of ammonium into glutamine, it is transported to other parts of the plant through the xylem (Walsh et al., 1989). The legume nodule transports its nitrogenous compounds mainly as amide (asparagine and glutamine) or ureides (allantoin or allantoic acid), depending on the legume species. Usually, temperate legumes export amide (Shelp and Atkins, 1984) and tropical and subtropical ones export ureide (Schubert, 1986; Atkins, 1987) from their nodules. Apart from amides and ureides, many other nitrogen-containing compounds are exported, but in smaller quantities (Pate and Atkins, 1983).

#### **I.4. Photosynthesis and Nitrogen-fixation**

Photosynthates provide the energy and carbon skeletons for nitrogen fixation (Fig. 2). In addition to this, the nodule uses energy for its growth and maintenance. The total cost is 15-30% of the total assimilation capacity of the plant; for

example, 12 g of carbohydrate is required for every gram of nitrogen fixed (Gutschick, 1980). A continuous supply of photosynthate to the nodule is essential for continued nitrogen fixation in intact plants (Bach et al., 1958). The close link between nitrogen fixation and photosynthesis has been investigated in different legumes (Lawn and Burn, 1974; Streeter, 1974; Hardy and Havelka, 1976; Rainbird et al., 1983a; Ching et al., 1975; Swaraj et al., 1986, 1988; Gordon et al., 1986; Ryle et al., 1985a; Pfeiffer et al., 1983; Wheeler, 1971). All these studies indicate that nitrogen fixation gradually decreases under photosynthetic stress conditions such as darkness, defoliation, detopping, shading and cloudiness. Ching et al. (1975) observed a reduction of ATP by 70%, sucrose by 60%, ADP by 60%, nitrogenase activity by 50% and energy charged by 15% in soybean root nodules kept in the dark for one day. The leghemoglobin and total protein of the nodule also decrease under photosynthetic stress (Pfeiffer et al., 1983; Swaraj et al., 1986). Reserve carbohydrates of the nodule may also be used in the absence of photosynthesis to support nitrogen fixation (Gerson et al., 1978; Hostak et al., 1987). Nodular disintegration due to photosynthetic stress has also been demonstrated in soybean (Cohen et al., 1986) and in white clover (Gordon et al., 1986).



Figure 2. Schematic diagram showing relationship between  
photosynthesis and nitrogen fixation (Bidwell,  
1974)

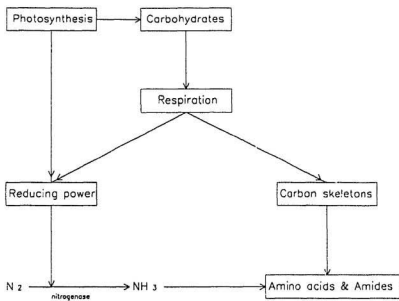


Figure 2

### **I.5. Peanut (*Arachis hypogaea* L.): Taxonomy, Distribution and Importance**

The genus *Arachis* belongs to the family Fabaceae (Leguminosae) and sub-family Papilionoideae and occurs in tropical and subtropical regions. Based on morphology and cross-compatibility the genus *Arachis* has been divided into several sections (Wynne and Halward, 1989). They are native to South America but have been introduced into many other areas. *A. hypogaea*, called by different popular names such as peanut, groundnut, goober etc, is the only species in cultivation. According to archaeological data, peanuts have been cultivated for over 3,500 years, during which time numerous morphological forms have evolved (Hammons, 1982). Plants are annual or perennial herbs and have a well-developed taproot system with many lateral roots emerging from the hypocotyl and aerial branches. The roots are soft, cylindrical and lack root hairs, but root hair-like structure were found by Nambiar et al. (1983). The depth of primary roots can be 90-120 cm with extensive lateral roots. Peanuts are warm-season plants, preferring 50-100 cm of rainfall/year, and are best suited to well-drained, friable, loamy soil containing adequate amounts of phosphates, potash and calcium. Propagation by cuttings is possible, but the plant is usually grown from seeds.

Peanuts are important to humans as a source of nutrition. Its fresh foliage is fed to hogs and cattle, produces high

quality hay and has value as a green manure for soil improvement. The flowers furnish rich nectar for bees. The seeds are a rich source of vitamin B complex, especially thiamin, riboflavin and nicotinic acids, and are a source of protein and oil (Ahmed and Young, 1982). Peanuts rank second to soybean in commercial importance as a source of high quality oil characterized by the presence of arachidic and legnocerlic acids as well as glyceride of oleic and leinoleic acids. The crop yield varies from 742 to 4400 kg/ha (Duke, 1981).

#### **1.5.1. Specificity of Peanut-*Rhizobium* Symbiosis**

Unlike many legumes that are nodulated only by specific groups of rhizobia, peanuts are nodulated by rhizobia that also nodulate several other species of tropical leguminous plants (Allen and Allen, 1981). The following special features of the peanut-*Rhizobium* symbiosis make peanut root nodules distinct:

- i) Lectins are generally important in the specific *Rhizobium* recognition for the host (Dazzo, 1982). In peanut, lectin is not important (Pueppke et al., 1980).
- ii) Normal root hairs are absent in peanuts. Instead, tufted clusters or rosettes of hairs are frequently found in the junction of root axiles (Gorbet and Burton, 1979).
- iii) No infection threads are observed in peanuts (Chandler, 1978). Peanut nodules are believed to develop from a single infected cell by subsequent cell divisions. In

isozymes (CAT-1, CAT-2 and CAT-3) are encoded on the three unlinked structural genes, Cat-1, Cat-2 and Cat-3, respectively (Chandlee et al., 1983) and the expression of these genes is highly regulated both temporally and spatially (Redinbaugh et al., 1988).

Virtanen (1956) hypothesized that catalase activity is responsible for preventing the accumulation of  $H_2O_2$ , which otherwise may result in the oxidation and inhibition of the synthesis of some of the essential substances for nitrogen fixation. Recent studies have shown that leghemoglobin appears to be susceptible to breakdown by  $H_2O_2$  (Puppo and Halliwell, 1988). A high level of catalase is found in the heterocysts of Cyanobacteria (Henry et al., 1978) and in the *Frankia* vesicles (Puppo et al., 1989) where it protects the overall nitrogen-fixation process against  $H_2O_2$ . Francis and Alexander (1972) tried to clarify the role of catalase in nitrogen-fixing root nodules of white clover and soybean and found a positive correlation between nitrogen fixation and catalase activity.

### **I.7. Lipase and Lipid Catabolism**

Lipase, the first enzyme in lipid catabolism, catalyses the hydrolysis of reserve triacylglycerol to fatty acids and glycerol (Beevers, 1969; Hutton and Stumpf, 1969). Depending on the plant species, the lipase may be localized, either in

contrast with other legumes, no uninfected interstitial cells are present in the infected zone (Chandler, 1978).

iv) The bacteroids are large and spherical (Staphorst and Strijdom, 1972) with lipid and dense bodies (Bal et al., 1989).

#### **I.6. Catalase in Nitrogen Fixation**

The enzyme, catalase (EC. 1.11.1.6;  $H_2O_2$  oxidoreductase), is widely distributed in nature and is found in all aerobic micro-organisms as well as plant and animal cells containing a cytochrome systems (Deisseroth and Dounce, 1970). All forms of catalase isolated to date have been shown to consist of four subunits of about 60,000 Da each, which gives a protein of approximately 240,000 Da (Aebi, 1983). The enzyme functions as a scavenger of toxic  $H_2O_2$  produced by the cells in various metabolic reactions. The targets of this  $H_2O_2$  are enzymes, lipids, membranes and nucleic acids (Morgan et al., 1976; Chance et al., 1979). Multiple forms of catalase have been identified in several plants, such as tobacco (Havir and McHale, 1987), cotton (Kunce and Trelease, 1986), barley (Kendall et al., 1983) and also in some bacteria (Meir and Yagil, 1984; Seah and Kaplan, 1973). The physiological significance of these multiple isoenzymes is not yet clear. In maize (*Zea mays* L.), three biochemically distinct catalase

the membrane of lipid bodies, or in other subcellular compartments. With few exceptions, lipase activity is absent in ungerminated seeds, but increases rapidly during the germination period. The mechanisms of lipolysis vary greatly among different oil seeds. The oilseed lipases that have been studied differ widely in characteristics such as the optimal pH for maximal activity, molecular weight, substrate specificity and subcellular location (Huang, 1983). Lipases are widely distributed in animals, plants, fungi and bacterial cells (Huang, 1983).

The fatty acids released by lipase activity are metabolized by the  $\beta$ -oxidation sequence to produce acetyl-CoA, which is processed through the glyoxylate cycle. The enzymes of the  $\beta$ -oxidation sequence and glyoxylate cycles are localized exclusively in the glyoxysomes, which are special types of peroxisomes (Beevers, 1969; Hutton and Stumpf, 1969). The lipid bodies and glyoxysomes are in physical contact with one another (Frederick et al., 1975), which facilitates transport of acylglycerols or fatty acids from the lipid bodies to the glyoxysomes. Succinate, generated in the glyoxylate cycle, is released from the glyoxysomes and is converted eventually to sucrose and other metabolites (Huang, 1983).

#### **I.7.1. Lipid in Nitrogen Fixation**

Lipids are generally important components of cellular

organelles and are also used for energy storage in most of the seeds, especially oil seeds. Lipids are synthesized in plastids and stored in special structures 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 surrounded by a half-unit membrane of one phospholipid layer (Huang, 1985; Yatsu and Jacks, 1972). They are spherical bodies about 1  $\mu$ 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, the formation of lipid bodies is still controversial. It has been proposed that lipid bodies originate from the ER by the accumulation of triacylglycerol between the two layers of the ER unit membrane and finally pinch off to form the lipid bodies (Wanner and Theimer, 1978; Wanner *et al.*, 1981). Alternatively, lipid bodies may arise in the cytoplasm by an accumulation of triacylglycerol followed by the formation of the membrane (Bergfield *et al.*, 1978). The formation and function of lipid bodies have been well studied in different seeds (Warnner *et al.*, 1981; Huang *et al.*, 1986). 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.*, 1986). But the



function of lipid bodies in nitrogen-fixing root nodules has not been elucidated since they were reported in the peanut (Hameed and Bal, 1985), in arctic legumes *Oxytropis maydeliana* Trautv. and *Astragulus alpinus* L. (Newcomb and Wood, 1986; Prevost and Bal, 1989) and in the sub-arctic legume, *Lathyrus maritimus* (Bal and Barimah-Asare, 1991). In the peanut root nodule, lipid bodies are present in both infected and uninfected cells of all developmental stages of nodules, including those which are senescent. Compared with mature nodules (active in  $N_2$  fixation), immature ones (those which lack leghemoglobin) contained the maximum number of lipid bodies (Jayaram and Bal, 1991). This is probably due to a lack of  $N_2$  fixation in the absence of leghemoglobin (Bergersen, 1982). It has been speculated that lipids may support nitrogen fixation and/or help in increasing the temperature within the cell to facilitate growth and development at cold temperatures in arctic legumes (Newcomb and Wood, 1986). In peanut nodules, they may provide a supplementary energy source for nitrogen fixation (Bal et al., 1989).

### **I.8. Objectives**

This study was undertaken to determine the role of lipid bodies in nitrogen fixation in the root nodules of the peanut.

### **I.9. Outline of Methods Employed**

Nodulated plants were subjected to stress conditions so as to create an extra demands for energy and carbon required in nitrogen fixation. Ineffective nodules were used to investigate the status of lipid bodies without nitrogen fixation. Stress conditions were imposed by keeping plants in dark and by detoping.

- at intervals during the period of stress, the rate of nitrogen fixation (as measured by  $C_2H_2$  reduction), the number of lipid bodies, leghemoglobin and total protein contents were measured to determine any correlation with nodule function.
- the ultrastructural changes of nodules due to stress were observed to determine if structural integrity is linked to nodule function.
- catalase activity at different intervals of darkness, oxidation of exogenous linoleoyl coenzyme A (LYL-CoA) and malate synthase were assayed in nodule homogenate to determine operation of  $\beta$ -oxidation pathway and glyoxylate cycle.

- cytochemical localization of lipase, catalase and malate synthase was conducted in nodule tissues as further evidence of  $\beta$ -oxidation pathway and glyoxylate cycles.
- morphometric comparison of lipid bodies was done in nitrogen-fixing (fix<sup>+</sup>) and non-fixing (fix<sup>-</sup>) nodules to clarify the involvement of lipid bodies in nitrogen fixation.

## Chapter II

### MATERIALS AND METHODS

#### II.1. Rhizobium Strains and Medium

*Bradyrhizobium* sp. strain 32H1 was obtained from Nitragin, Milwaukee and strains NC92, 639 and 7091 were obtained from Dr. P. T. C. Nambiar, International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India. The strain 639 is a kanamycine resistant Tn5 mutant of the wild type NC92 (Wilson et al., 1987). The strain 7091 is an ineffective bradyrhizobial isolate from ICRISAT. Both NC92 and 7091 were found to be kanamycin sensitive. All strains were cultured in broth yeast extract mannitol (YEM) medium at pH 6.8 - 7.0 (Vincent, 1970) with constant shaking (140 - 150 rpm) at room temperature in an orbit Environ-shaker (Lab-line Institutions Inc). Six-day old cultures were used in all experiments. The YEM medium consists of mannitol (10 g),  $K_2HPO_4$  (0.5 g),  $MgSO_4 \cdot 7H_2O$  (0.2 g), yeast extract (0.4 g) and distilled water (1 litre). Rhizobial cultures were maintained on YEM agar slants in culture tubes and stored at 4°C (or lyophilized the culture) for future use.

## II.2. Plant Materials and Growing Conditions

Seeds of peanut (*Arachis hypogaea* L. cv. 'Jumbo Virginia' and 'Early Spanish') and cowpea (*Vigna unguiculata* L. cv. 'California Blackeye') were purchased from W. Atlee Burpee Co., Warminster, PA, USA. The peanut seeds were placed on moist paper towel in a tray for 5 - 6 days for germination and then the seeds were inoculated with *Bradyrhizobium* sp. from a broth culture as described by Sen and Weaver (1980). In the case of cowpea, the seeds were washed (3 - 4 times) and soaked in distilled water for approximately twelve hours before inoculation. The inoculated seeds of both peanut and cowpea were planted individually in pots (6" STD) with autoclaved vermiculite. The potted plants were kept in an environment chamber with approximately  $700 \mu\text{mole m}^{-2} \text{s}^{-1}$  PPFD (photosynthetic photon flux density) under day/night conditions of 16h/8h, 27°C/22°C and 70%/50% relative humidity and irrigated with a nitrogen-free nutrient solution (Ellfolk, 1960).

## II.3. Dark treatment and Detopping of Plants

After 45 days for peanut and 28 days for cowpea plants, the lights in the environment chambers were turned off. Half of the plants were detopped (the stem was cut off at ground level). Nodulated plants were sampled from dark and detopped treatments after 0 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96

h for the measurement of acetylene reduction, catalase assay, total protein and leghemoglobin. Samples were also processed for cytological and ultrastructural studies. Three plants were used at each time interval for each sample.

#### **II.4. Electron Microscopy (EM) of Samples**

Nodules from three plants were sliced 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 ( $\text{OsO}_4$ ) 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% and absolute respectively. After dehydration, the sections 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 sections 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 ultramicrotome. The sections were post-stained with lead citrate and uranyl acetate before observation under a Zeiss 109 transmission electron microscope.

#### II.5. Nodule Number

Nodules induced by different strains (fix and fix<sup>+</sup>) of *Bradyrhizobium* sp. were counted on 42-day old plants. Fifteen healthy plants were selected for each sampling and the nodules were counted. Mean values and standard errors of mean were calculated.

#### II.6. Nitrogenase Assay by the Acetylene (C<sub>2</sub>H<sub>2</sub>) Reduction Technique

Nitrogenase activity of nodules was assayed by measuring reduction of acetylene to ethylene (Hardy et al., 1968). Each root system with nodules (about 100 nodules) was enclosed singly in 13 ml vacutainer tubes fitted with airtight rubber stoppers. Using a pressure lock gas syringe (Precision Sampling Corporation), 0.1 atm of air was replaced by 0.1 atm of acetylene (freshly generated in the laboratory from calcium carbide). The nodulated root was incubated for 3 h at 23 ± 2°C. Control tubes: (a) 0.1 atm acetylene without the nodulated root and (b) nodulated root without acetylene. Gas samples

were analyzed in a Basic™ Gas Chromatography GC 9700. Nitrogenase activity was calculated by using standard curves for acetylene and ethylene and was expressed as  $\mu$ moles of ethylene  $\text{h}^{-1} \text{g}^{-1}$  fresh weight of nodule. Each assay was done in triplicate.

#### II.7. Lipid Body Preservation, Staining and Counting

For lipid body preservation, nodule slices were fixed and processed as described for EM (in II.4) except samples were treated with 1% p-Phenylenediamine (pPD) in 70% ethanol for 1 h during dehydration (*en bloc* staining, Boshier et al., 1984; Bal et al., 1989 and Bal, 1990). For controls, the samples were treated with hexane for 45 min after fixation and dehydration (Bal, 1990). After dehydration, the samples were embedded in Spurr's medium as mentioned above.

The semi-thin sections (1-2  $\mu\text{m}$ ) were cut from both treated and control blocks, viewed under light microscope at 40x and the number of lipid bodies per unit area was determined. For each treatment, several sections were used from five different tissue blocks.

A new technique involving staining of the sections (from blocks not *en bloc* stained) with pPD was also developed. Thick sections were cut from the tissue block, placed on a slide and dried by gentle heating. After cooling, the sections were



dipped in 1% PPD in 70% ethanol for about 3-5 min in the dark and then washed, first with 70% alcohol and then with distilled water. The slide was passed over a flame to dry the section and then mounted with one drop of histoclad and a cover slip to make a permanent preparation.

## **II.8. Sample Preparation**

### **II.8.1. Nodule and Bacteroid Fractions**

Fresh and healthy root nodules were removed from 45-day old plants, washed with distilled water and homogenized gently with 0.05 M buffer containing 5 mM EDTA at desired pH in a mortar. The homogenate was then centrifuged at 265 xg for 10 min and the supernatant was further centrifuged at 14,000 xg for 10 min. The resultant pellet and supernatant were considered bacteroid and nodule cytosol fractions, respectively after microscopical observation. Before using the bacteroid fraction, it was washed with buffer until the supernatant from washing showed no catalase activity. To solubilize the peribacteroid membrane (PBM), the pellet was treated with 0.1% Nonidet P<sub>40</sub> (Bal et al., 1980), washed and resuspended in buffer.

The bacteroid fraction was then passed through a modified Hugh's press (Model 9AB Biotec, Sweden) at 2.6 metric tons/cm<sup>2</sup> to break the cells. The cell walls were pelleted at 200,000 xg

for 1 h in a Sorvall OTD-5 ultracentrifuge and washed with buffer to produce a cell-free extract. Throughout the experiments, phosphate and glycine-sodium hydroxide buffers were used and every step was carried out at 4°C.

#### **II.8.2. Cell-free Extract of Cultured *Rhizobium***

The 6-day old cultured cells were collected by centrifugation, washed with buffer and disrupted by sonication for 3-5 min at short intervals or with the modified Hugh's press (as above). The sample was centrifuged at 200,000 xg to separate cell walls (pellet) and cell contents (supernatant).

#### **II.8.3. Seed Cytosol**

Peanut seeds were prepared for germination (see II.2) and after three days the testa and embryo were removed. One cotyledon was ground in 5 ml of appropriate buffer at the various experimental pH. The homogenate was centrifuged at 6500 rpm (regular centrifuge) for 10 min. The centrifugation produced three distinct layers: a thick lipid layer (top), seed cytosol (middle) and cell debris (bottom) from which the seed cytosol was removed with a glass pipette and was used for the catalase assay and gel electrophoresis. Phosphate and sodium hydroxide buffers (0.05 M) were used throughout the experiments.

## **II.9. Determination of Leghemoglobin (LHb)**

A colorimetric assay of LHb in fresh nodules was carried out using the procedure of Wilson and Reisenauer (1963). The nodules (0.05 g) were ground in 3 ml Drabkin's solution (52 mg potassium cyanide, 198 mg potassium ferricyanide and 1 g sodium bicarbonate to 1000 ml of distilled water). The nodule sample was centrifuged (15 min at 500 xg) and the supernatant was saved. The LHb was extracted twice more from the nodule tissue (pellet), the supernatants were combined and the volume adjusted up to 10 ml with Drabkin's solution. The resulting supernatant was centrifuged at 20,000 xg for 30 min, producing a clear solution of host cell cytoplasm that was used for LHb determination.

A colorimetric assay of HLb was performed in a 1.0 cm cell at 540 nm against Drabkin's solution with a Spectronic 1001 (Bausch & Lomb). A standard curve was prepared with known amounts of hemoglobin (Hb) in Drabkin's solution (0.5, 1.0, 2.0, 4.0 and 6.0 mg per 10 ml) so that the absorbance of the sample readings could be extrapolated from the standard curve to find the amount of LHb in the sample. The final result was expressed in mg of LHb/gm fresh weight of nodules.

## **II.10. Catalase**

### **II.10.1. Catalase Assay**

The enzyme, catalase, was assayed spectrophotometrically

according to the method of 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 3 ml cuvette and made up to 2 ml with the same buffer. In case of activity inhibition, 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 starting the reaction (zero time). As a control, only buffer was used. The reactants were mixed thoroughly and the change in absorbance was recorded every 10 seconds intervals 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 (Worthington Enzyme Manual, 1972). Protein was measured in the sample by Lowry's method (1951) calibrated with bovine serum albumin (BSA).

### II.10.2. Assay at Different Temperatures

The sonicated bacterial cells (in 0.05 M phosphate buffer, pH 7.0) were treated at different temperatures (70°C, 65°C, 60°C, 55°C, 50°C and 40°C) for 5 min in a water bath. After cooling to room temperature, the catalase activity was measured along with a control (without treatment).

### II.10.3. Cytochemical Localization

Cytochemical localization of catalase in peanut root nodules and in *Bradyrhizobium* sp. 32H1 was done by using the 3,3'-diaminobenzidine (DAB) reaction following the procedure described by Frederick and Newcomb (1969). The samples (small segments of nodule tissues and bacterial pellet) were fixed in a Karnovsky in 50 mM potassium phosphate buffer at pH 8.0 (Karnovsky, 1965) 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 DAB solution (2 mg/ml of 50 mM 2-amino-methyl-1,3-propanediol buffer at pH 9.5). Preincubation was followed by the incubation of samples in a H<sub>2</sub>O<sub>2</sub> containing DAB solution (0.1 ml of 3% H<sub>2</sub>O<sub>2</sub> in 5 ml) 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% osmium tetroxide in 50 mM potassium phosphate buffer (pH 6.8) for 2 h, washed with buffer and processed for electron

microscopy (section II.4).

Three controls were run along with the treatments: a) 0.02 M 3-amino-1,2,4-triazole (a competitive catalase inhibitor) was added to the DAB solution during pre-incubation time, b) pre-incubation and incubation were both done without DAB, c) incubation without the substrate,  $H_2O_2$ .

## **II.11. Malate Synthase**

### **II.11.1. Assay**

The presence of malate synthase was determined in the nodule homogenate using the method of Dixon and Kornberg (1959). This method measures the decrease in the rate of optical density (OD) at 232 nm due to breakage of the thioester bond of acetyl Coenzyme A in the presence of glyoxylate. The reaction mixture consists of 3 ml Tris HCl buffer (0.1 M, pH 8.0), 0.15 ml  $Mg_2Cl$  (0.1 M), 0.10  $\mu$ l acetyl CoA (10 mM), 0.10 ml nodule homogenate (contained 0.4-0.5 mg protein) and 0.02 ml Na-glyoxylate (0.02 M). The reaction mixture, except for Na-glyoxylate, was placed in a 3 ml cuvette and the spectrophotometer was set at zero and allowed to stabilize for several minutes. Then glyoxylate was added in the cuvette and O.D. changes were recorded for 2 minutes at 30 sec intervals. Specific activity is expressed as units of enzyme/mg protein. One unit of enzyme is defined as the amount that catalyses the

cleavage of 1  $\mu$ mole of acetyl coenzyme A per minute.

### II.11.2. Cytochemical Localization

Cytochemical localization of malate synthase in nodule tissues was done according to Trelease et al. (1974). Mature nodule slices were fixed in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 50 mM phosphate buffer (pH 6.8) for 20-30 min at 4°C. After washing thoroughly with 3-4 changes of the above buffer (20 mM) for 15-20 min, the samples were pre-incubated with 3 mM potassium ferricyanide in washing buffer for 30 min at room temperature. The samples were washed again for 15-20 min with the same buffer and then incubated in an incubation medium for 40 min at 37°C in a water bath with constant shaking. The incubation medium was prepared immediately prior to use by adding the following compounds in the order indicated below with constant stirring between each addition : 0.30 ml K-phosphate buffer (65 mM, pH 6.9), 0.20 ml copper-tartrate solution (pH 6.9), 0.25 ml distilled water, 0.03 ml potassium ferricyanide (150 mM) and 0.10 ml acetyl CoA (1.0 mM). As a control, samples were incubated in media from which either acetyl CoA or glyoxylate or both had been omitted. After the incubation, samples were rinsed for 20-30 min with several changes of the buffer and post-fixed in  $\text{OsO}_4$  (2% solution made up in 50 mM K-phosphate buffer, pH 7.0) for

1 h at room temperature. They were washed in buffer and dehydrated through an alcohol series and embedded in Spurr's medium (section II.4). Ultra-thin sections were cut and observed under TEM with or without being post-stained in uranyl acetate and lead citrate.

All solutions were prepared one day before use and stored (4°C), except for ferrocyanide which was made up in a dark bottle immediately before use. The copper-tartrate solution was prepared by slowly adding copper sulphate crystals (0.624 g) to 500 mM Na,K-tartrate solution and the pH was adjusted to 6.9 with 1N NaOH. The volume was brought up to 50 ml with distilled water. The final concentration of the copper-tartrate solution was 50 mM copper sulphate and 500 mM Na,K-tartrate.

## II.12. Linoleoyl Coenzyme A (LYL-CoA) Oxidation

### II.12.1. Sample Preparation

Oxidation of LYL-CoA was determined in different fractions of the nodule (eg. nodule homogenate, nodule cytosol, bacteroid, bacteroid with PBM, bacteroidal cell content and bacteroidal cell wall) and *in vitro* grown *Bradyrhizobium* sp. 32H1 (whole cell, cell wall and cell content), individually. All the samples were prepared as described in sections II.8.1 and II.8.2, except buffer, which was used at pH 7.0. Each sample was standardized so that constant amounts of protein



(0.5 mg/ml) in the samples were used throughout the experiments.

#### **II.12.2. Determination of LYL-CoA Oxidation**

One ml of sample was placed in a 60-ml reaction flask with 0.4 ml of 1 N KOH in a center cup suspended from the rubber stopper. The reaction was started by injecting 0.1  $\mu$ Ci of [ $^{14}$ C] LYL CoA (Dupont NEN products). Incubation was carried out for 0, 20, 40, 60 and 80 min at 30°C in a Dubnoff metabolic shaker. Three controls were used. The reaction mixtures were as described above except that they (a) were kept at 0 - 4°C, (b) were kept at 30°C but did not contain [ $^{14}$ C] LYL CoA or (c) contained a blank with only the KOH. Incubation was terminated with 0.4 ml of 4 N HCl. The  $^{14}\text{CO}_2$  was allowed to be absorbed by the KOH overnight and radioactivity was measured in a Beckman LS-3150T Scintillation Counter. Protein from the samples was measured by the method of Lowry et al. (1951).

#### **II.13. Localization of Lipolysis**

Lipolytic activity in the lipid bodies of nodule cells was demonstrated by a method modified from that of Blanchette-Mackie and Scow (1981). Nodule slices were fixed with a aldehyde fixative (Karnovsky, 1965) in cacodylate buffer (pH 7.2) at 4°C for 1 h, washed thoroughly in buffer at 4°C and

incubated at 37°C for 18 h in 1% tannic acid dissolved in the buffer. The control was nodule slices incubated in the tannic acid/buffer solution at 4°C. The samples were then treated with 1%  $\text{OsO}_4$  in K-phosphate buffer (pH 7.0) for 1 h at 4°C followed by routine dehydration and embedding (section II.4).

## **II.14. Gel Electrophoresis**

### **II.14.1. Preparation and Running of Gel**

This was performed according to the method of Laemmli (1970) with a few modifications. Slab gel (15 X 12 cm) was used with 4% acrylamide for stacking and 10% acrylamide for the separating gel. The final concentrations in the separating gel were as follows: 0.375 M Tris-HCl (pH 7.0), 0.025% (volume) of tetramethyl-ethylenediamine (TEMED) and ammonium persulphate (APS). The stacking gel contained 0.125 M Tris-HCl (pH 7.0) and 0.025% TEMED and APS. The running buffer, Tris-glycine (pH 8.3), contained 0.0625 M Tris-HCl (pH 7.0), 10% glycerol and 0.001% bromophenol blue as the tracking dye. 50  $\mu\text{g}$  of protein was loaded in each well. Electrophoresis was carried out with a current of 15 mA for stacking gel and 30 mA for running gel until the bromophenol blue marker reached the end of the gel. The total time required for running was about 7 h.

#### II.14.2. Gel Staining

Native gel was stained according to the method described by Clare *et al.* (1984). The gel was soaked in horseradish peroxidase (50  $\mu\text{g}/\text{ml}$ ) in 0.05 M potassium phosphate buffer (pH 7.0) for 45 min.  $\text{H}_2\text{O}_2$  was then added to a concentration of 5.0 mM and soaking was continued for 10 min. Higher levels of  $\text{H}_2\text{O}_2$  reduced the sensitivity of the stain. The gels were then quickly rinsed twice with distilled water and placed in DAB (0.5 mg/ml) in potassium phosphate buffer until staining was completed. DAB solution was freshly prepared and kept in the dark.

## Chapter III

### RESULTS

#### III.1. Acetylene Reduction Assay (ARA) and Lipid Bodies (LB)

Peanut plants maintained a normal rate of ARA values in the nodules for up to 48 h in the dark. After 48 h there was a drop in ARA values (Fig. 3). Similar trends in ARA values were observed in detopped peanut nodules (Fig. 4). In nodulated cowpea plants, the ARA activity started to decline within 3 h of the beginning of darkness and there was a 40% reduction of ARA values after 12 h of darkness (Fig. 3). Detopped cowpea nodules behaved in a similar fashion (Fig. 4). Cowpea plants maintained at constant temperature (day/night) also showed a gradual decline of ARA activity (Fig. 5).

Another set of experiments was conducted with the peanut variety 'Early Spanish' (Fig. 6) to confirm the results obtained with the variety 'Jumbo virginia' (Fig. 3). The results presented in Figure 6 indicate that there was no reduction in ARA values until after 48 h of darkness. The number of lipid bodies in the nodule tissues of dark treated and detopped peanut plants gradually declined under the experimental conditions (Fig. 7).

Figure 3. Acetylene reduction assay (ARA) of peanut and cowpea nodules obtained from dark-treated plants. Bars indicate  $\pm$  SE of the mean of three replicates.

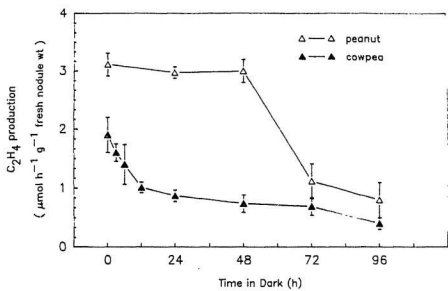


Figure 3

Figure 4. Acetylene reduction assay (ARA) of peanut and cowpea nodules obtained from detopped plants. Bar indicate  $\pm$  SE of the mean of three replicates.

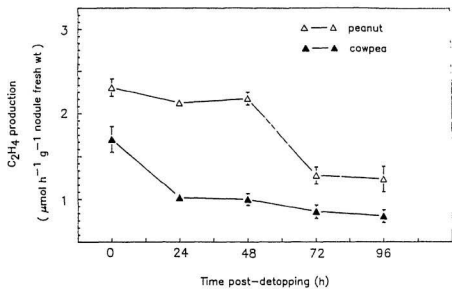


Figure 4



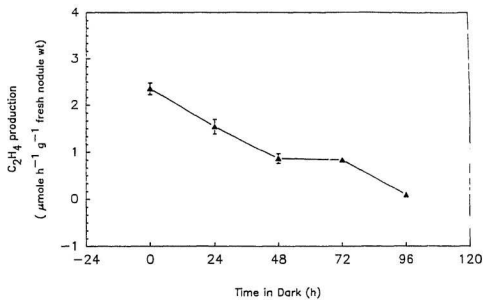


Figure 5

Figure 5. Acetylene reduction assay (ARA) of cowpea nodules obtained from dark-treated plants at constant temperature. Bars indicate  $\pm$  SE of the mean of three replicates.

Figure 6. Acetylene reduction assay (ARA) of the nodules of dark-treated peanut plants (cv. Early Spanish). Bars indicate  $\pm$  SE of the mean of three replicates.

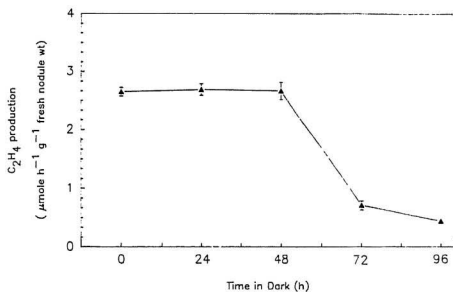


Figure 6

Figure 7. Lipid body counts in 1.5- $\mu$ m thick sections (five blocks in each sample) of nodules from dark-treated and detopped peanut plants. Bars indicate  $\pm$  SE of the mean (n=20).

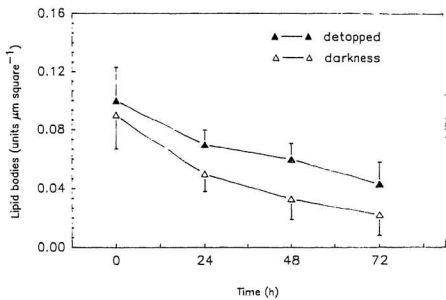


Figure 7

## **II.2. Leghemoglobin (LHb), Total Protein (TP) and Catalase in Nodule of Dark-stressed and Detopped Plants.**

No considerable changes of total LHb content were observed in the nodule cytosol of dark-stressed and detopped peanut plants for 48 h of treatment (Tables 1 and 2). However, after 48 h, (both dark-stressed and detopped) the LHb content decreased by a small amount. In the case of cowpea plants, a gradual decline of LHb and also the total protein content of the nodule cytosol was observed with the increase of the dark period (Table 3). The total protein content of the peanut nodule cytosol remained more or less the same for up to 96 h in dark-treated and detopped plants (Tables 1 and 2).

The nodule homogenate of dark-treated peanut plants showed a gradual increase of catalase activity with an increase of the dark period (Table 4) without real changes in cowpea.

## **III.3. Dark-stressed and Detopped on Nodule Ultrastructure**

Ultrastructural changes in peanut and cowpea root nodules were observed in relation to the length of dark treatment of the plants. In peanut nodules, the only structural changes occurring up to 48 h of darkness were the lipid bodies became more closer to the peribacteroid membrane, and empty lipid bodies (ghosts) could be observed in the infected cells (Figs. 8 and 9). At 72 h, degeneration of both cytoplasm and bacte-

Table 1. Leghemoglobin and total protein content in the root nodules of dark-treated peanut plants.

Darkness (hours)	Leghemoglobin (mg/g.F.wt.nodule)	Total protein (mg/g.F.wt.nodule)
0	21.40	50.40
24	20.90	48.50
48	20.00	49.00
72	19.00	49.75

Note: Due to low sample volume, the experiment was performed with two replicates each time, but repeated three times for confirmation



Table 2. Leghemoglobin and total protein content in the root nodules of detopped peanut plants.

Time after detoping (hours)	Leghemoglobin (mg/g./F.wt.nodule)	Total protein (mg/g.F.wt.nodule)
0	21.40	50.40
24	21.10	50.60
48	21.58	51.00
72	19.29	50.00
96	19.40	50.00

Note: Due to low sample volume, the experiment was performed with two replicates each time, but repeated three times for confirmation

Table 3. Leghemoglobin and total protein content of the root nodule of dark-treated cowpea plants

Darkness (hours)	Leghemoglobin (mg/g.F.wt. nodule)	Total protein (mg/g.F.wt.nodule)
0	8.70	27.82
24	7.70	18.0
48	7.00	15.5
72	5.81	10.22
96	4.20	9.01

Note: Due to low sample volume, the experiment was performed with two replicates each time, but repeated three times for confirmation

Table 4. Catalase activity in peanut and cowpea root nodule homogenate obtained from plants subjected to different periods of darkness.

Darkness (hours)	<u>Catalase activity (units/mg protein)</u>	
	peanut	cowpea
0	2.72	1.72
24	3.30	1.78
48	3.56	1.71
72	4.00	1.90
96	4.86	1.61

Note: Due to low sample volume, the experiment was performed with two replicates each time, but repeated three times for confirmation

Figure 8. Three electron micrographs of peanut nodule tissues obtained from 24 h dark-stressed plants. Note the lipid body (lb) and ghost lipid body (g) are in close association with the peribacteroid membrane (pbm); lipid body in the bottom micrograph is partly empty (lb'). ps, peribacteroid space; phb, poly- $\beta$ -hydroxybutyrate.

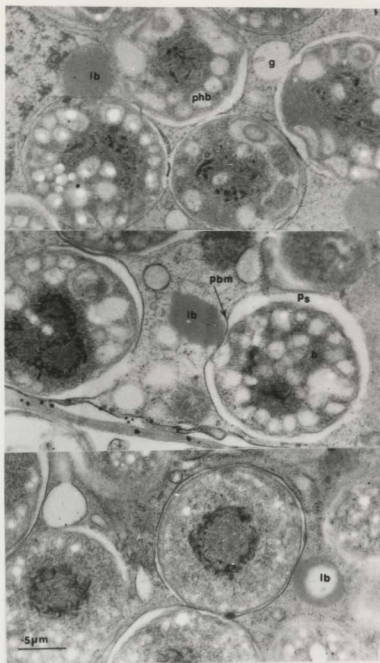


Figure 3

Figure 9. An electron micrograph of peanut nodule tissues obtained from 48 h and 72 h dark-stressed plants.

- A) Nodule tissue from 24 h stressed plant showing healthy bacteroid (b) with full of poly- $\beta$ -hydroxybutyrate granules (phb) and more ghost lipid bodies (g) than usual lipid bodies.
- B) Nodule tissue from 72 h stressed plants, showing break down of peribacteriod membrane (arrow), vacuolation (v) in host cytoplasm, lomasome-like structure (l) and ghost lipid body (g).

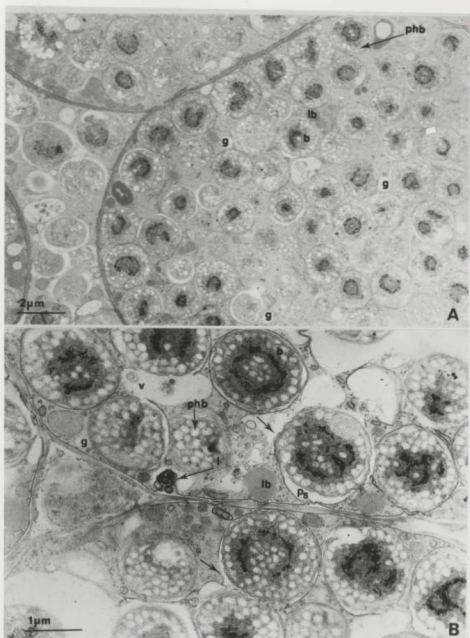


Figure 9

roids with peribacteroid membrane had started in the infected cell of the peanut nodule. The production of vacuoles and lomasome-like structures were also noticed at this stage of the stress conditions (Fig. 9B). Some lipid bodies still remained intact at 72 h dark-stressed nodules. In the case of the cowpea nodules, the effects of darkness at the ultrastructural level manifested themselves earlier and were very clear. Within 24 h, the bacteroids had started to disorganize with the initiation of lysis of the host cytoplasm and peribacteroid membrane (Fig. 10A). The breakdown of bacteroids, peribacteroid membrane, host cell wall and extensive cytoplasmic degeneration of the infected cell became quite obvious at 48 h (Fig. 10B). The detopping experiment was conducted only with the peanut. The effects of detopping on the nodule at the ultrastructural level were more or less similar to the dark effect on nodules except that the disorganization of the bacteroids and the vacuolation in the cytoplasm of infected cells seemed to be more rapid at 72 h in detopped plants.

### **III.4. Linoleoyl Coenzyme A (LYL-COA) Oxidation**

#### **III.4.1. *In vivo* Sample**

The oxidation of LYL-COA was determined in different fractions of the peanut nodule sample (eg. nodule homogenate, nodule cytosol, bacteroid with and without peribacteroid membrane).



Figure 10. An electron micrograph of cowpea nodule tissues obtained at 24 h and 48 h from dark-stressed plants.

- A) Nodule tissue at 24 h, showing cytoplasmic vacuolation (v), peribacteroid membrane breakdown (arrow).
- B) Nodule tissue at 48 h, showing degeneration of cytoplasm, bacteroid (b), and host cell wall (arrows).

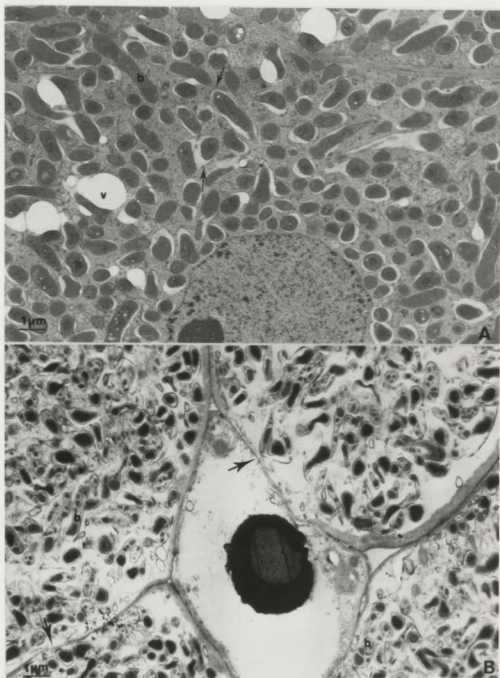


Figure 10

The results (Figs. 11 and 12) clearly indicate a gradual increase of  $^{14}\text{CO}_2$  release with incubation time in the nodule homogenate, but a peak in the release of  $^{14}\text{CO}_2$  by the bacteroid after 25 minutes of incubation time. The nodule cytosol showed very little  $^{14}\text{CO}_2$  release, which remained constant throughout incubation.  $^{14}\text{CO}_2$  release by the bacteroid with a peribacteroid membrane gradually increased throughout incubation (Fig. 12). This may be due to the presence of fatty acid oxidizing enzymes inside the peribacteroid space or the peribacteroid membrane. It is important to note that the cowpea nodule homogenate showed no detectable  $^{14}\text{CO}_2$  release (Fig. 11).

#### III.4.2. In *in vitro* Grown *Bradyrhizobium* sp. 32H1.

As with the nodule fractions, the oxidation of LYL-COA was also determined in different fractions of *in vitro* grown *Bradyrhizobium* sp. 32H1 (Fig. 13). Both the intact bacteria and bacterial cell wall showed an increase of  $^{14}\text{CO}_2$  release with incubation time but the amount of  $^{14}\text{CO}_2$  release was higher in intact bacteria compared with the bacterial cell wall. On the other hand, very little  $^{14}\text{CO}_2$  release was observed by the bacterial cytosol even after 80 minutes of incubation. It seems that the oxidizing enzymes are located in the cell wall of the bacteria.

Figure 11.  $^{14}\text{CO}_2$  released from [ $^{14}\text{C}$ ] linoleoyl coenzyme A by the nodule homogenate of peanut and cowpea nodules. Due to low sample volume, the experiment was performed with a single replicate each time but repeated twice for confirmation. Similar trends in results were obtained each time.

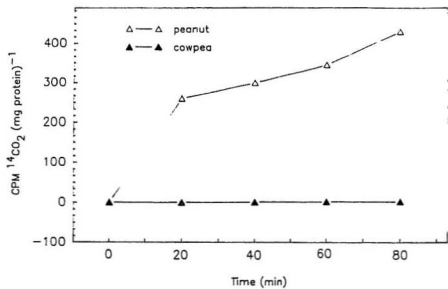


Figure 11

Figure 12.  $^{14}\text{CO}_2$  released from [ $^{14}\text{C}$ ] linoleoyl coenzyme A by peanut nodule cytosol and bacteroids. The experiment was repeated three times for confirmation. Similar trends in results were obtained each time.

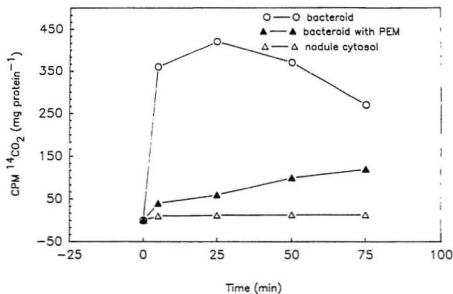


Figure 12

Figure 13.  $^{14}\text{CO}_2$  released from [ $^{14}\text{C}$ ] linoleoyl coenzyme A by different fractions of cultured *Bradyrhizobium* sp. 32H1. The experiment was repeated three times for confirmation. Similar trends in results were obtained each time.



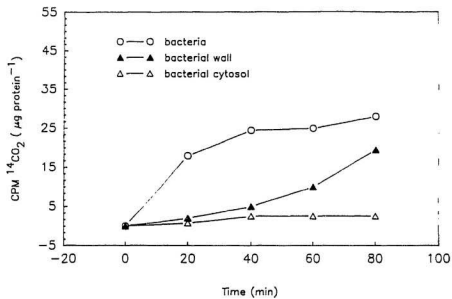


Figure 13

### III.5. Lipase Localization

Lipase, the enzyme responsible for the release of fatty acids from triacylglycerol, was demonstrated in the lipid bodies of peanut root nodules (Fig. 14). The endogenous substrate of the lipid bodies and lipase were allowed to react in the presence of tannic acid after proper fixation. The tannic acid served as a capturing agent of released fatty acids which formed 4 nm diameter micelles, clearly revealed by osmium tetroxide post-fixation. In the present preparation, the lipid bodies were all converted into highly osmiophilic, massive micellular structures (Fig. 14). Control preparations kept at 0 - 4°C did not show such transformations of the lipid bodies.

### III.6. Malate Synthase (MS) Assay and Localization

Malate synthase, a key enzyme of the glyoxylate cycle, catalyses the condensation of acetyl CoA with glyoxylate to form malate. The present studies demonstrate the existence of MS in both the nodule cytosol and the bacteroid fractions of peanut root nodule. The enzymatic assay clearly indicates that the bacteroid fraction possesses more activity (20.18  $\mu\text{mole/min/g protein}$ ) than the nodule cytosol fraction (7.74  $\mu\text{mole/min/g protein}$ ).

Cytochemical localization of MS enzyme activity, based on the reduction of ferricyanide to ferrocyanide clearly showed

Figure 14. Cytochemical localization of lipase in peanut root nodule tissue. The endogenous lipases are activated during incubation in this technique and fatty acids are released from the triglycerides of lipid bodies. In the presence of tannic acid and after osmium treatment, the fatty acids are revealed as 4 nm diameter lamellar micelles as shown in the inset. The inset is a magnified view of the circled portion of the lipid body printed to show the lamellar micelles. The lipid body in the entire micrograph is too dark due to the affinity of osmium to the fatty acids and therefore the lamellar micelles can be only visualized in lighter print.

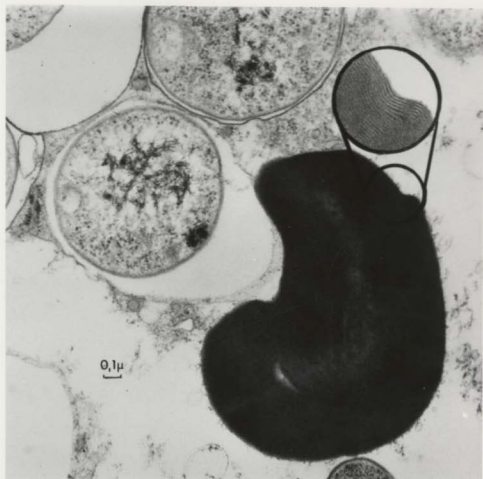


Figure 14

Figure 15. Cytochemical localization of malate synthase  
in peanut nodule tissues.

A) Electron micrograph of nodule tissues showing electron-dense reaction product (arrow) inside bacteroids (b) and nodule cytoplasm.

B) Control preparation. no reaction product.

electron-opaque reaction product (ferrocyanide) in the bacteroids as well as in cytosol (Fig. 15A). Control preparation (without acetyl CoA and glyoxylate) did not show any reaction products (Fig. 15B).

### III.7. Catalase Assay and Localization

The catalase activity in *Bradyrhizobium* sp. 32H1 could only be detected after the cells were sonicated. The intact bacteria did not show any catalase activity. Maximum activity was reached in 6 day old cultures (Fig. 16). Assays made at different pH values produced two peaks (Fig. 17), one at pH 6.0 and the other at 10.5. In the peanut nodule tissue, the nodule cytosol and the bacteroid fraction also showed two optima one at pH 7.5 and the other at 10.0 (Fig. 18 and 19). Similarly, peanut seed cytosol also exhibited two pH optima, one at pH 6.0 and another at pH 10.0 (Fig. 20). The intact bacteroids, isolated from peanut nodules, had considerable catalase activity but none could be detected in the sonicated bacteroid cell contents. The intact bacteroids, isolated from cowpea nodules, showed no catalase activity although, after breaking the cell wall by sonication, activity was obtained in the cell contents (Table 5). In the cultured bacteria, the activity was present only in the cell contents and not in the cell walls (Table 5). The catalase activity was sensitive to triazole, showing 97% inhibition. Studies of enzyme activity

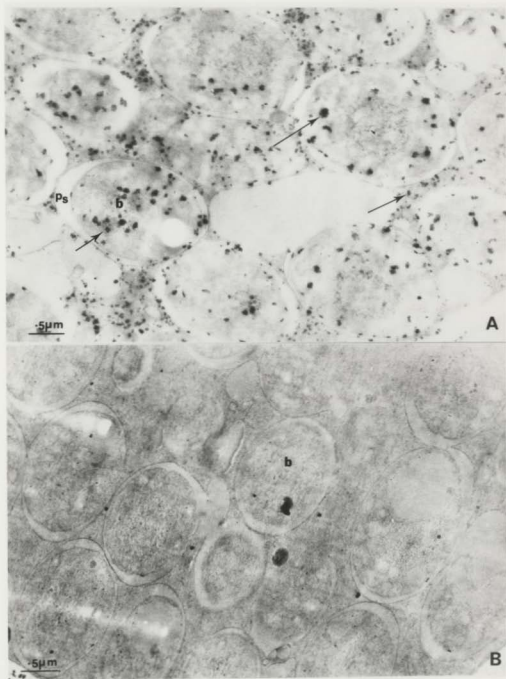


Figure 15

Figure 16. Graph showing catalase activity at different times in *in vitro* culture of *Bradyrhizobium* sp. 32H1 growing in YEM medium. Note that maximum activity is obtained on the 6th day. (Experiment was repeated three times for confirmation)



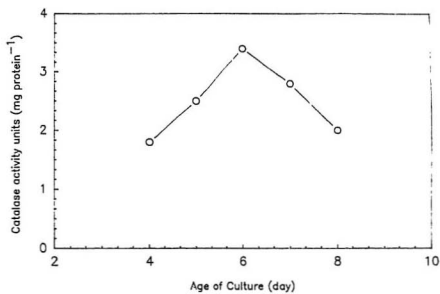


Figure 16

Figure 17. Graph showing catalase activity of bacterial cell contents at different pH values. Note the presence of two distinct peaks at pH 6 and 10.5. (Experiment was repeated three times for confirmation)

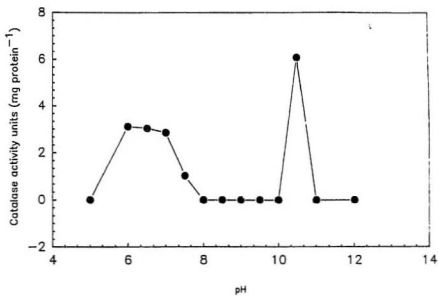


Figure 17

Figure 18. Graph showing catalase activity of the peanut nodule cytosol at different pH values. Note the two distinct peaks at 7.5 and 10. (Experiment was repeated three times for confirmation)

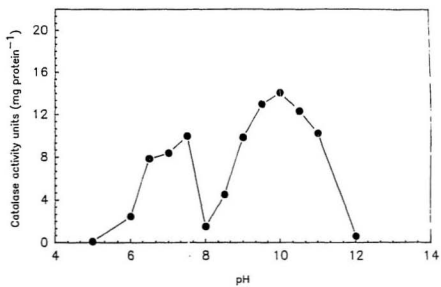


Figure 18

Figure 19. Graph showing catalase activity of the isolated bacteroid fraction from peanut nodule at different pH values. Note the two peaks at 7.5 and 10. (Experiment was repeated three times for confirmation)

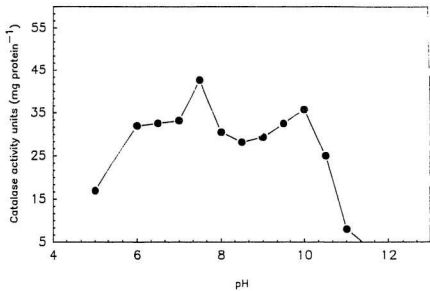


Figure 19

Figure 20. Graph showing catalase activity of the 3-day germinated peanut seed cytosol at different pH values. Note the two distinct peaks at pH 6 and 10. (Experiment was repeated three times for confirmation)



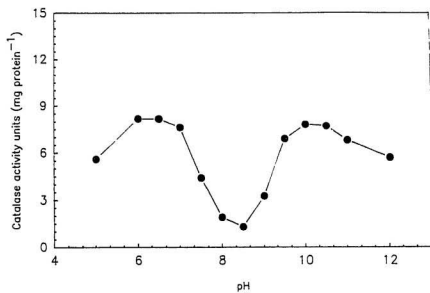


Figure 20

Table 5. Catalase activity in different fractions of bacteroids and bacteria.

Sources	<u>Enzyme activity (units/mg protein)</u>		
	Intact cell	Cell wall	Cell content
Peanut bacteroids	28.44	20.97	0.00
Cowpea bacteroids	0.00	ND*	4.70
Cultured bacteria	0.00	00	4.50

\*Not determined

Note: Due to low sample volume, the experiment was performed with two replicates each time, but repeated three times for confirmation

inhibition at different temperatures revealed complete inhibition above 40°C. Cytochemical localization of catalase by DAB reaction as positive for the cytosol of cultured *Bradyrhizobium* (Fig. 21) but, in the nodule bacteroid, only dense bodies on the cell walls were stained by the DAB reaction (Fig. 22). Microbodies close to peribacteroid membranes were also positive for the DAB reaction (Fig. 22). Sensitivity to triazole was evident for the DAB reaction in control samples.

### III.8. Gel Electrophoresis

Peanut seeds and nodule homogenates were run in tris-glycine buffered native polyacrylamide gel electrophoresis and stained for catalase activity (Fig. 23). Three distinct catalase bands were evident in the seed homogenate (Fig. 23, lane A) whereas nodule homogenate showed only one band (Fig. 23, lane B) which had different mobility from that of the seed homogenate.

### III.9. Ultrastructure of Peanut Nodules Induced by *Fix*<sup>-</sup> and *Fix*<sup>+</sup> Strains of *Bradyrhizobium* sp.

Nodules induced by the two *fix*<sup>-</sup> strains were smaller in size and white inside showing no indication of the presence of leghemoglobin during the course of this study, whereas the ones induced by *fix*<sup>+</sup> strain NC92 became pink within 3 weeks

Figure 21. Cytochemical localization (DAB positive) of catalase in *Bradyrhizobium* sp. 32H1.

- A) An electron micrograph showing electron dense reaction products (arrow) inside the bacteria (b). Not post stained.
- B) Control preparation (without DAB), lack of reaction product. Note that the section was post stained.



Figure 21

Figure 22. Cytochemical localization (DAB positive) of catalase in the root nodule tissues of peanut. Note the catalase site (electron-dense) in dense body (db) and microbody (mb), but not in lipid body (lb) attached to the peribacteroid membrane (pbm).

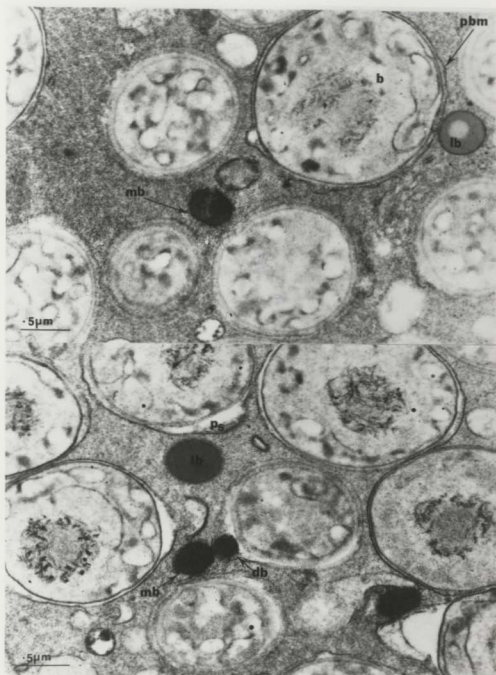


Figure 22

Figure 23. DAB stained native polyacrylamide (10%) gel showing three distinct catalase bands in 3-day germinated seed (lane A) and one in nodule cytosol (lane B) of peanut.





Figure 23

post-inoculation and the colour persisted (Table 6). The effective NC92 nodules had fully differentiated spherical bacteroids by that time. The bacteroids had characteristic dense bodies in the peribacteroid space and a close-fitting peribacteroid membrane not more than  $0.2\ \mu\text{m}$  from the bacteroid outer membrane (Fig. 24C). Lipid bodies (oleosomes) were observed in close association with the peribacteroid membrane, as shown earlier (Bal et al., 1989). In the infected cells, such morphological characteristics were shown up to the 6th week of the post-inoculation period and beyond. As is typical of peanut nodules, interstitial uninfected cells were absent. Prominent vascular strands traversed the cortical cells which contained amyloplasts (Fig. 24A).

Nodules induced by *Bradyrhizobium* strain 639 (fix<sup>-</sup>) differentiated into large spherical bacteroids within 3 weeks post-inoculation. Nodule tissue with extensive mitotic activity, in cells already invaded by the bradyrhizobia, was observed in 2 week post-inoculation samples. The infection process and release of bacteria of strain 639 resembled that of the wild type NC92 (fix<sup>-</sup>) nodules. The dense bodies on the bacteroid outer membranes were larger and, in some cases, revealed electron-transparent areas. The peribacteroid space became highly hypertrophied (Figs. 24D, 25B and 25C), which is apparent also in photomicrographs (Fig. 24B and cf Fig. 24A).

The vascular tissue was smaller in size (Fig. 24B) than in NC92 nodules. The general histology in both appeared similar at this early stage, except for the highly enlarged peribacteroid space and the large numbers of lipid bodies in the host cytoplasm (Figs. 25B and 27). The central vacuole of infected cells was very prominent in both NC92 and 639 nodules. At the 4th week, the vacuole enlarged further as most of the bacteroids disappeared and by the 5th and 6th week the cells were left with only their walls remaining (Fig. 25A). The cortex however remained unchanged, resulting in nodules with a hollow interior.

The 7091 nodules never reached a state of proper differentiation. Even at 5/6 weeks post-inoculation the nodule tissue showed bacteria trapped in the intercellular spaces (Figs. 26A and 26B). Release of bacteria was not uniform and very few cells possessed bacteroids that were spherical. In many cells, branched/elongated bacteroids were also seen (Fig. 26C).

Nodules induced by different strains of *Bradyrhizobium* sp. (NC92, 32H1, 7091 and 639) were counted and found that the highest number of nodules per plant was produced by NC92, followed by 7091, 639 and 32H1 (Table 6).

An estimation of the number of lipid bodies in the infected cells of 4 weeks post-inoculation nodules was made. Large numbers of lipid bodies were found in both 639- and 7091-induced nodules (Fig. 27)

Figure 24. Photo-and electron micrographs of nodules (3 week post-inoculation) induced by bradyrhizobial strains NC92 and 639.

- A) Photomicrograph showing the cortical and infected zone of the NC92 nodule. The infected cells on the lower half of the micrograph show large central vacuoles (v) and spherical bacteroids. The upper half shows cortical cells with densely-stained amyloplasts; the vascular tissue (VT) is prominent.
- B) Photomicrograph of 639 nodule showing the organization of cortical and infected zones. Note the less prominent vascular tissue (VT) and gaps between the dark bacteroids and the host cytoplasm with vacuoles (V)
- C) Electron micrograph taken from the same tissue as A showing the NC92 bacteroids (b), dense bodies (arrow) and the peribacteroid space (PS) enclosed by the peribacteroid membrane.
- D) Electron micrograph taken from the same tissue as B showing a 639 bacteroid (b) with its dense body (arrow) and highly enlarged peribacteroid space (PS).

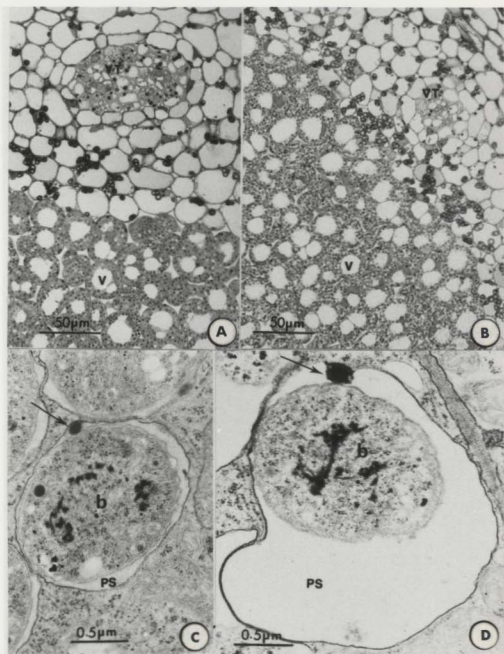


Figure 24

Figure 25. Photo-and electron micrographs of nodules (4,5 and 6 weeks post-inoculation) induced by strain 639.

- A) Photomicrograph of a nodule (6 weeks post-inoculation) showing degeneration of both bacteroids and host cytoplasm in the infected zone (arrow) which is left with only the cell wall material. Note the unchanged cortical zone on upper half of the micrograph.
- B) Electron micrograph of nodule tissue (4 weeks post-inoculation) showing the general ultrastructural pattern. Note the abundance of lipid bodies (L) and the enlarged peribacteroid space (PS).
- C) Electron micrograph of nodule tissue (5 weeks post-inoculation) showing senescing infected cells. The central vacuole (v) is enlarged. Note the abundance of lipid bodies (L).

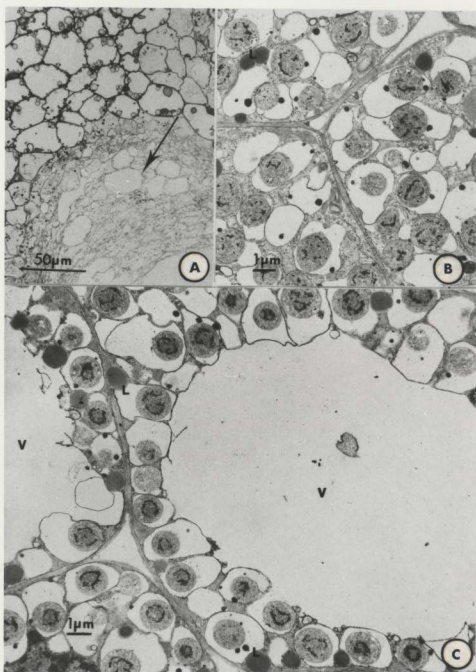


Figure 25

Figure 26. Photo-and electron micrographs of nodules (5 weeks post-inoculation) induced by strain 7091.

- A) Photomicrograph of the cortical and the infected zone showing intercellular spaces in the infected zone with trapped bradyrhizobia (arrows).
- B) Electron micrograph of the same tissue as A showing the intercellular bradyrhizobia (arrow).
- C) Electron micrograph of infected cells showing branched and elongated bacteroids (b) within a lytic vacuole. Some bacteroids (b) are spherical. Note the abundance of lipid bodies (L).





Figure 26

Table 6. Number, size and texture of peanut nodules produced by the different strains of rhizobia.

Strain	Nodule number* per plant	Internal colour of nodule	Texture
NC 92	386 $\pm$ 32	pink/red	normal
7091	199 $\pm$ 23	white	soft
639	195 $\pm$ 11	white	soft, spongy
32H1	59 $\pm$ 8	pink/red	normal

\*Mean  $\pm$  SEM (N = 15)

Figure 27. Graph showing lipid body counts in the infected cells of nodules formed by NC92, 639 and 7091. Note the striking accumulation in 639 and 7091. Bars indicate  $\pm$  SE of the mean (n = 20)

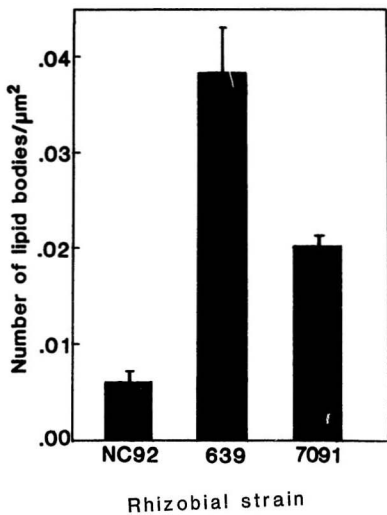


Figure 27

## Chapter IV

### DISCUSSION

#### IV.1. Acetylene Reduction Assay (ARA) and Lipid Bodies (LB)

Nitrogen fixation by nodulated peanut and cowpea plants under photosynthetic stress is clearly different early in the stress period. The maintenance of a normal rate of ARA in peanuts for up to 48 h of darkness is a very uncommon phenomenon amongst legumes. Cowpea plants infected with the same strain of *Bradyrhizobium* showed greater than a 40% reduction in ARA activity within 12 h (Fig. 3). Soybean (Ching et al., 1975), chickpea (Swaraj et al., 1986) and cowpea (Swaraj et al., 1988) show similar declines in ARA values when the plants are kept in the dark. Cutting off the photosynthate supply by detopping the plants also results in ARA values similar to the dark treatment in both peanut and cowpea (Fig. 4). Dabas et al. (1988) reported a sharp decline of ARA within 48 h of detopping in pigeon peas as was found in the cowpea, but not in the peanut, in this study. Defoliation (Mederski and Streeter, 1977; Ryle et al., 1985, 1988; Culvenor et al., 1989) and stem-girdling (Walsh et al., 1987) also caused severe reduction of ARA in nodules. Any photosynthetic stress involves an interruption in the flow of photosynthate to the nodules. Hence, the decline of nitrogen fixation under stress is, in

general, attributed to the lack of photosynthate (Minchin and Pate, 1974; Murphy, 1986). Storage materials like PHB granules (Wong and Evans, 1971), starch granules (Hostak et al., 1987) and soluble sugars present in the nodules are not sufficiently mobilized to support high ARA values, although some early reports indicate the utilization of PHB granules in certain species of lupin (Gerson et al., 1978).

The decline of nitrogen fixation in the dark in cowpea and soybean has been attributed to lower temperatures during the night (Rainbird et al., 1983; Schweitzer and Harper, 1980). They observed that ARA values remain unchanged under a constant temperature during the dark period. Contrary to these observations, the present investigation has found a decline of ARA values during the dark period in cowpea nodules kept under a constant temperature regime (Fig. 5). Detopping experiments with peanut and cowpea served as an excellent control since they disrupted photosynthate translocation as well as preventing any interaction between nodular function and shoot temperatures. The ARA values in both detopped peanut and cowpea were similar to those of the dark treatment.

It has also been claimed that the decline of ARA in cowpea, soybean and white clover is mainly due to increased  $O_2$  resistance in nodules under stress conditions (Hartwig et al., 1987; Vessey et al., 1988; Dabas et al., 1988; Hartwig et al.,

1990; Witty et al., 1984; Minchin et al., 1985; Carroll et al., 1987). The present study is intended to demonstrate lipid body catabolism in nodules stressed to create an extra demand for carbon and energy for nodular function. It was not intended to investigate the effect of other factors involved in nitrogen fixation under photosynthetic stress. However, in the light of peanut nodule characteristics, it would be interesting to justify the high resistance of  $O_2$  diffusion in nodules under stress conditions. As suggested earlier, the diffusion of  $O_2$  is restricted by the nodular structure (Sinclair and Goudriaan, 1981; Tjepkema and Yocum, 1974). Therefore, in contrast to peanut nodules, the thicker cortex (Sen et al., 1986) and lignified endodermis in both cowpea and soybean (Tjepkema and Yocum, 1974) are believed to be barriers to  $O_2$  diffusion in those nodules. Another reason for  $O_2$  deficiency in stressed nodules of cowpea and others is probably their lower LHB content (Table 4) compared with peanut nodules (Table 2). LHB, the  $O_2$ -binding protein, stores excess  $O_2$  and regulates  $O_2$  flow to the bacteroids of functional nodules (Appleby, 1984). The amount of  $O_2$  used in functional nodules also varies from host to host depending upon the nature of organic carbon that participates in bacteroidal metabolism. Recently, Trinchant and Rigaud (1990) proposed that malate and lactate oxidation require a lower  $O_2$  supply

than other organic acids such as succinate. Isolated soybean bacteroids consume varying amounts of  $O_2$  in  $C_2H_2$  reduction, depending upon the carbon source supplied during the assay (McNeill et al., 1984). Additionally, it can also be noted that questions have been raised about the validity of ARA of detopped plants on the grounds of disturbed oxygen flux to the bacteroids (Minchin et al., 1986). The repeated assays confirm the patterns of ARA values in this study.

Prolonged maintenance of  $N_2$  fixation in the absence of photosynthate can be explained by the utilization of metabolic reserves present in the peanut nodule (Bal et al. 1989). Unlike those of most tropical and temperate legumes, peanut nodules have lipid bodies which occupy about 5% of the total volume in the infected cell (Jayaram and Bal, 1991) and are in close contact with the peribacteroid membrane (Fig. 8). There is a gradual decrease in the number of lipid bodies (observed per unit area) in the infected cells in both dark-treated and detopped plants (Fig. 7). These results indicate that lipid bodies are consumed during photosynthetic stress in peanut nodules to support  $N_2$  fixation for at least 48 h. The proximity of lipid bodies to the bacteroids and the presence of lipolytic activity make the lipid bodies readily available for utilization. Cowpea nodules, on the other hand, are totally devoid of lipid bodies (Bal, unpublished) and are unable to



maintain high ARA values in the dark as well as after detopping for a prolonged period. Both cowpea and peanut plants were kept under identical environmental conditions, including temperature. Starch depletion has been recently reported in dark-treated alfalfa nodules (Hostak et al., 1987) but no correlation has been established with  $N_2$  fixation. Starch granules are not found in the infected cells of the peanut nodule (Bal et al., 1989). The uninfected cortical cells contain amyloplasts with starch which show no apparent change during the dark periods. The electron micrographs of nodules from 72 h dark-treated plants do not show any apparent loss of PHB granules.

#### **IV.2. Lipase Localization**

The first step in lipid body utilization is the mobilization of fatty acids from the triacylglycerides by the action of lipases, which are located inside the lipid bodies (Fig. 14). 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 (Wang and Huang, 1987) and transported to the lipid body where it is bound to a membrane protein (Vance 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 (section IV.4) as well as the TCA cycle (Gallon and Chaplin, 1987).

#### IV.3. Malate Synthase (MS) Assay and Localization

The enzyme, malate synthase, which is involved in the glyoxylate cycle following the  $\beta$ -oxidation in lipid body catabolism, has been demonstrated in the bacteroid and nodule cytosol of peanut (Fig. 15). MS activity increases coincidentally in germinating seeds with the decrease of lipid which is located in the glyoxysome (Cooper and Beevers, 1969; Beevers, 1979), the site of the glyoxylate cycle. So far, there has been no documentation of glyoxysomes in the nodules. No glyoxysomes were found in the nodule cells in the present investigation. Therefore, the localization of MS inside the bacteroids lead to the conclusion that the glyoxylate cycle operates inside the bacteroids (in the absence of glyoxysomes) of peanut nodule. This statement is supported further by the work of Stovall and Cole (1978) and Johnson et al. (1966) in soybean. They detected MS in the bacteroid fraction of the soybean nodules. Unfortunately the data on its activity in the nodule

cytosol is lacking in their work. The MS activity in the nodule cytosol of peanut may not be involved with the glyoxylate cycle because this cycle usually takes place within membrane-bound organelles, not in the ground cytosol. The low activity of MS in the cytosol could be due to possible leakage from bacteroids through autolysis or disruption during sample preparation.

#### IV.4. Linoleoyl Coenzyme A (LYL-CoA) Oxidation

Evidence for fatty acid oxidation was obtained from the oxidation of exogenous [ $^{14}\text{C}$ ] LYL CoA to  $^{14}\text{CO}_2$  (Fig. 11). The release of  $^{14}\text{CO}_2$  is an indication that the  $\beta$ -oxidation pathway and glyoxylate cycle (Ward and Fairbairn, 1970) are operating in the peanut nodule (Fig. 11). Cowpea nodules show a complete lack of  $^{14}\text{CO}_2$  release (Fig. 11). Catalase, which serves to detoxify  $\text{H}_2\text{O}_2$  produced in  $\beta$ -oxidation (Tolbert, 1981), has also been found to be active in the peanut nodule (Fig. 22).

Complete oxidation of fatty acids released from lipid bodies results in dicarboxylic acid, which is believed to be the most probable substrate for bacteroids in the nodule (Kouchi and Yoneyama, 1984a,b; Reibach and Streeter, 1984). Appleby (1984) and Mellor (1989) stated that isolated bacteroids favour malate and succinate to support nitrogen fixation. This is supported by the existence of dicarboxylic acid

transporter in symbiotic *Bradyrhizobium* (Udvardi et al., 1988; Reibach and Streeter, 1984) which catalyses the uptake of malate and succinate, and whose expression is essential for an effective symbiosis (Ronson, 1988). Lipid body catabolism providing succinate and malate via the  $\beta$ -oxidation pathway and glyoxylate cycle, may be the main source of energy for nitrogen fixation under photosynthetic stress.

#### IV.5. Leghemoglobin (LHb) and Total Protein (TP)

The efficiency of nodule function largely depends upon the LHb and TP content of nodules. The presence of LHb in the nitrogen-fixing organ is considered a prerequisite for nitrogen fixation (Appleby, 1984). Considerable evidence confirms that the main function of LHb is to facilitate  $O_2$  diffusion to the respiring bacteroid as free  $O_2$  at an extremely low, non-toxic concentration (Appleby, 1984). A direct correlation has been drawn between the LHb content and nitrogen fixation of nodules in different legumes (Graham and Parker, 1961; Johnson and Hume, 1973; Appleby, 1984). The present work demonstrates the status of LHb and TP content of cowpea and peanut root nodules following dark and detopping treatments of plants. The results show that LHb and TP remain constant in peanut root nodules with up to 48 h of treatment, whereas, in cowpea nodules both LHb and TP start to decline

along with nitrogen fixation soon after the treatment begins. Compared to LHB, TP declines more rapidly in cowpea nodules confirming earlier work by Swaraj et al. (1986). A slow decrease of LHB compared to TP in cowpea has been described to the hydrolysis of a protein other than LHB (Swaraj et al., 1986). Protein hydrolysis following treatment causing an increase in amino acids have been reported in pea (Roponen, 1970) and chickpea (Swaraj et al., 1986) nodules. Proteolytic activity, responsible for protein hydrolysis, is common in different legume nodules such as soybean (Malik et al., 1981; Pfeiffer et al., 1983), French bean (Pladys and Rigaud, 1982), alfalfa (Vance et al., 1979) and white clover (Gordon and Kessler, 1990). In contrast to other legumes, consistency of LHB and PT concentrations in peanut nodules may be explained by the presence of lipid bodies in the nodule tissues. Lipid body catabolism protects the nodule from starvation without any drastic changes under stress conditions.

#### **IV.6. Catalase in Stressed Nodules**

Catalase activity in peanut nodule homogenate was found to be slightly higher following dark treatment, whereas, in cowpea it remained the same. An increase of catalase activity in peanut may be linked to lipid body catabolism in nodule tissues. As mentioned earlier, under photosynthetic stress, the number of lipid bodies decreases which indicates a higher

rate of lipid body catabolism through the  $\beta$ -oxidation pathway. The by-product of this pathway is toxic  $H_2O_2$  and accumulation of  $H_2O_2$  suppresses the  $\beta$ -oxidation pathway (Hashimoto and Hayashi, 1987). Therefore, to continue  $\beta$ -oxidation and normal nodule functions, an increase of catalase in the peanut nodule microenvironment under darkness is probably a defense mechanism of the nitrogen-fixing organ to protect it from the toxicity of  $H_2O_2$ . In addition to these effects,  $H_2O_2$  can also inactivate LHB (Puppo and Halliwell, 1988) and reduce the efficiency of the nodule. According to these authors, catalase protects the LHB from  $H_2O_2$ . Effective nodules of pea (Virtanen, 1956), clover and soybean (Francis and Alexander, 1972) contain more catalase than ineffective nodules. Puppo *et al.* (1989) demonstrated a significant increase of catalase in *Frankia* vesicles under  $N_2$ -fixing conditions. Similarly a high level of catalase was found in heterocysts of Cyanobacteria (Henry *et al.*, 1978). All these findings indicate a relationship between catalase content and  $N_2$  fixation of nodules. Increased catalase in peanut, compared to cowpea, may be a sign of  $\beta$ -oxidation operation under stressed condition, when lipid bodies undergo increased catabolism in the peanut nodules.

#### IV.7. Ultrastructure of Stressed Nodules

The structural integrity of the nodule cell is important for the  $N_2$ -fixing process. Any damage to the ultrastructure will negatively affect  $N_2$  fixation. Many environmental factors are responsible for this kind of damage, such as drought, salinity, temperature and photosynthetic stress. Photosynthetic stress occurs quite often in nature due to defoliation, cloudiness, shading and darkness. Nodulated peanut and cowpea plants, that were subjected to darkness or detopped, has shown that peanut plants can retain ultrastructural integrity of the nodules along with undiminished  $N_2$  fixation for up to 48 h. In cowpea, nodular disintegration starts within 24 h of darkness. In nodular disintegration, the infected cell generally develops a large central vacuole and the peribacteroid membrane starts to disintegrate. Disintegration of the peribacteroid membrane disturbs the interaction between host and symbiont (Werner *et al.*, 1984) which leads to the premature senescence of the nodule (Baird and Webster, 1982). The failure of proper peribacteroid membrane formation causes ineffective nodules in soybean as reported earlier (Bassett *et al.*, 1977; Mollar *et al.*, 1985 and Werner *et al.*, 1985). Soybean (Cohen *et al.*, 1986) and white clover (Gordon *et al.*, 1986) nodules respond in the same way as cowpea to photosynthetic stress. Recently, Vikman *et al.*, (1990)

demonstrated structural damage of the infected host cell and *Frankia* cell in response to dark treatment of *Alnus* root nodules. Structural degradation was observed in infected root nodule cells of *Alnus* after exposing the plant to ozone (Greitner and Winner, 1989). Remarkably vacuolations appeared in infected cells of the cowpea nodule following dark treatment of plants. The vacuoles contain lytic enzymes such as hydrolases and protease (Boller and Wiemken, 1986) which cause cellular damage. Cohen et al. (1986) observed repair of ultrastructural damage to dark-induced soybean plants after returning them to a normal photoperiod. Similar results were obtained with defoliated white clover (Gordon et al., 1986) where recovery started as new leaves appeared on the plants. This ultrastructural damage of nodules is most probably due to deficiency of carbohydrate metabolism under photosynthetic stress. Unlike other legumes, the integrity of the nodular structure in peanut under prolonged photosynthetic stress is most likely related to lipid metabolism in the nodule tissue, which provides sufficient energy to maintain nodular structure and function. The appearance of lipid bodies ghost (empty or partially empty lipid bodies) under photosynthate stress (Figs. 8 and 9) is another clear morphological indication of their utilization in nodule metabolism.



#### IV.8. Catalase Assay and Localization

Biochemical assay of catalase from peanut seed homogenate, nodule cytosol, bacteroid and rhizobia showed that the enzyme from all four fractions is active in a wide range of pH with two pH optima. Although the nodule cytosol and bacteroid catalase have the same pH optima, specific activity is greater in the nodule cytosol. One pH optimum was in common in seed homogenate, nodule cytosol and the bacteroid fraction. On the other hand, the two pH optima in *Rhizobium* grown in culture were different. The similarity between the pH optima of the nodule cytosol and the bacteroid fraction is remarkable. The bacteria which have different pH optima must have modified the enzyme while in symbiotic association with the host to provide greater harmony in a common environment. Multiple pH optima also occur in *E. coli* (Meir and Yagil, 1984) and baker's yeast (Seah and Kaplan, 1973) where two peaks of catalase activity have been reported over a wide range of pH. The presence of two distinct pH optima is an indication of multiple forms of catalase which have been reported in other forms of bacteria (Meir and Yagil, 1985; Wayne and Diaz, 1982; Loewen and Switala, 1987; Gregory and Fridovich, 1974; Seah and Kaplan, 1973) as well as in several vascular plants (Havir and McHale, 1987; Kuncze and Trelease, 1986; Drumm and Schopfer, 1974; Tsafaris et al., 1983). Like other plants and bacteria, germinated peanut seeds and its nodule fraction also showed

multiple catalase bands on negative polyacrylamide gels (Fig. 23). This suggests that peanut has three different forms of catalase and all of these are different from the nodule catalase. Havir and McHale (1987) observed changes in catalase profiles during seedling growth and leaf maturation of tobacco plants. Plant age and environmental conditions also influence the catalase pattern (Drumm and Schopfer, 1974). Little information is available about the properties of these multiple forms of catalase, an understanding of which would help explain their function. It is assumed that these multiple forms of catalase evolved to serve different physiological functions required in cellular development. While the functions of multiple forms of catalases in peanut root nodules remain to be explored, the sites of catalase activity demonstrated in this study provide some insights into the physiological interaction between the two partners, the host and the symbiont.

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. All such 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

peanut root nodule and rhizobia. The present investigation, which used both biochemical and cytochemical methods, indicates that 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 (Hameed, 1986; Bal et al., 1989) at the host-symbiont interface (Fig. 22). Furthermore, catalase activity is absent in the cell content of bacteroids whereas in cultured bacteria the enzyme is localized in the cell content only. In nodule tissue, the aerobic functions are restricted to the host-symbiont interface so that the  $O_2$ -sensitive  $N_2$  fixation process may continue within the bacteroid (Bergersen, 1982). It is likely that  $H_2O_2$  will be formed at this site on the surface of the bacteroid cell wall due to  $\beta$ -oxidation of lipids (Hameed and Bal, 1985; Hameed, 1986). Catalase is needed to detoxify the activity of  $H_2O_2$ . This could explain its presence on the cell wall and not within the bacteroid. On the other hand, in *in vitro* *Bradyrhizobium*, which does not fix nitrogen, aerobic conditions within the cell necessitate the presence of catalase inside and not on the surface of the cell wall.

#### IV.9. Ultrastructure and Lipid Bodies of *Fix<sup>-</sup>* and *Fix<sup>+</sup>*

##### Nodules

Nodules produced by the two *fix<sup>-</sup>* strains show marked differ-

ences in their phenotype and bacterial differentiation. Strain 639 is a mutant of NC92 isolated and characterized by Wilson et al. (1987) as showing no detectable acetylene reduction activity; i.e., 0% relative nitrogen fixation. The strain induces nodulation in the early stages with the same efficiency as the wild type NC92, but is distinctive by the presence of large numbers of lipid bodies in the host cytoplasm and by the enlarged peribacteroid space around the bacteroids. The accumulation of lipid bodies may be a result of lower rates of catabolism in the absence of nitrogen fixation. At the same time, an enlarged peribacteroid space widens the interface between the bacteroids and the host cytoplasm, a characteristic reflecting failure of symbiosis (Bal et al., 1989). Breakdown and lysis of bacteroids start within the peribacteroid space by the 4th week of the post-inoculation period and the large central vacuoles continue to enlarge until the infected cells are left with only the cell wall material. However, the uninfected cortical cells remain unchanged. The poor vasculature observed, compared to the wild type nodule, is possibly due to the lower demand for translocation of material in the absence of nitrogen fixation and symbiosis. This kind of phenotype compares with the failure of 'nodule function' as proposed by Vincent (1980) and further elaborated by Sprent (1989). It seems that the lack of nitrogen fixation (*nif*) and complementary functions (*cof*) lead

to an impediment of nodule persistence (nop), resulting in an early senescence of both the host cell and the rhizobia.

Strain 7091 is also totally ineffective as it has 0% relative nitrogen fixation and would therefore be useful in evaluating the lipid body status of infected cells. As well, in the absence of nitrogen fixation, more lipid bodies accumulate. The phenotype of nodules formed by this strain is clearly expressed as a partial failure to release rhizobia (bar) and their characteristic differentiation into bacteroids (bad) (Vincent, 1980). Bacterial release is partial and bacterial differentiation is not fully realized. The nodules formed are maintained in this state for a long period (at least 6 weeks in the present study) with no clear characteristic differentiation. Bacteria which invade the cells do not fully differentiate into spherical forms; some become elongated and branched. While it is thought that the peanut plant (macrosymbiont) influences the characteristic spherical bacteroid shape (Sen and Weaver, 1984), in the case of strain 7091, the influence is only partial.

In the nodules induced by both fix<sup>-</sup> strains, large accumulations of lipid bodies become apparent from estimates of their numbers as well as the area occupied in the infected cells. The larger numbers of lipid bodies in the nodules of fix<sup>-</sup> strains indicate an accumulation in the absence of

nitrogen fixation. The low numbers in the wild type (NC92), on the other hand, represent possibly a steady state situation where lipid bodies may be catabolized at a slightly lower rate than they are synthesized. The low lipid body counts may therefore be a reflection of their involvement in nitrogen fixation as a supplementary energy source. These observations support the view that lipid bodies in the effective fix<sup>+</sup> nodules of peanut supplement the high energy-demanding process of nitrogen fixation, while in fix<sup>-</sup> strains they are not utilized and therefore accumulate.

## V. SUMMARY

The study of the role of lipid bodies in nitrogen-fixing root nodules of peanut can be summarized by the following points:

- Maintenance of nitrogen fixation with a gradual decline of lipid bodies in nodule tissues under prolong photosynthetic stress.
- Existence of lipase, catalase and malate synthase activity in the nodule to operate the  $\beta$ -oxidation pathway and the glyoxylate cycle.
- Increase of catalase activity in nodule homogenate following the dark treatment of plants.
- Oxidation of exogenous linoleoyl coenzyme-A by nodule bacteroids.
- Maintenance of ultrastructural integrity of the nodule for up to 48 h of dark and detopping treatments.
- Consistency in leghemoglobin and total protein of the nodule, even after 48 h of dark stress.
- Accumulation of lipid bodies in fix<sup>-</sup> nodule.

## VI. CONCLUSIONS

It is concluded that lipid bodies are used as a supplementary source of energy in the metabolism of peanut root nodules and having lipid bodies in the symbiotic organ is an added advantage in nitrogen fixation. The role of lipid bodies in  $N_2$ -fixing root nodules of peanut is schematically represented in figure 28.



Figure 28. Schematic diagram showing the role of lipid bodies in  $N_2$ -fixing root nodules of peanut.

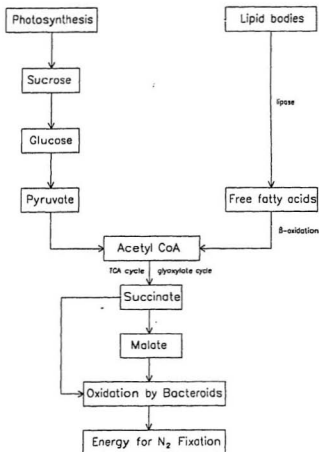


Figure 28

## VI. REFERENCES

- Aebi H, (1983) In: Bergmeyer HU (ed) Methods of Enzymatic Analysis, Vol 3, Verlag Chemie, Weinheim, pp 273-286
- Ahmed EM, Young CE (1982) Composition, quality and flavor of peanut. In: Pattee HE, Young CT (eds) Peanut Science and Technology. American Peanut Research and Education Society, Inc. Yoakum, Texas, USA, pp 655-688.
- Al-Mallah MK, Davey MK, Cocking EC (1989a) Formation of nodular structures on rice seedlings by rhizobia. J Expt Bot 40: 473-478
- Al-Mallah MK, Davey MK, Cocking EC (1989b) A new approach to the nodulation of non-legumes by rhizobia and the transformation of cereals by agrobacteria using enzymatic treatment of root hairs. Int J Genetic Manipul Plants 5: 1-7
- Al-Mallah MK, Davey MR, Cocking EC (1990) Nodulation of Oilseed Rape (*Brassica napus*) by Rhizobia. J Expt Bot 41: 1567-1572

- Alazard D (1985) Stem and root nodulation in *Aeschynomene* spp. *Appl Environ Microbiol* 50: 732-734
- Allen ON, Allen EK (1981) *The Leguminosae*. University of Wisconsin Press, Madison. Macmillan Publishing Co., London
- Appleby CA (1983) Haemoglobin in a non leguminous plant. *Parasponia*: possible genetic origin and function in nitrogen fixation. *Science (Washington)* 220: 951-954
- Appleby CA, (1984) Leghemoglobin and *Rhizobium* respiration. *Annu Rev Plant Physiol* 35: 443-478
- Appleby CA, Bogusz D, Dennis ES, Pacock WJ (1988) A role for haemoglobin in all plant roots? *Plant Cell Environ* 11: 359-367
- Arora N (1954) Morphological development of the root and stem nodules of *Aeschynomene indica* L. *Phytomorphology* 4: 211-216
- Atkins C (1987) Metabolism and translocation of fixed nitrogen in the nodulated legume. *Plant and Soil* 100: 157-169

- Bach MK, Magee WE, Burris RH (1958) Translocation of photosynthetic products to soybean nodules and their role in nitrogen fixation. *Plant Physiol* 33: 118-124
- Baird LM, Webster BD (1982) Morphogenesis of effective and ineffective root nodules in *Phaseolus vulgaris* L. *Bot Gazt* 143: 41-51
- Bal AK (1990) Localization of plant lipids for light microscopy using p-phenylenediamine in tissues of *Arachis hypogaea* L. *Stain Technol* 65: 91-94
- Bal AK, Barimah-Asare J (1991) Lipid bodies in rhizobia-infected root nodules of beach pea (*Lathyrus maritimus*). *Proc Can Soci Microbiol.* p. 78 (Abst)
- Bal AK, Hameed S, Jayaram S (1989) Ultrastructural characteristics of the host-symbiont interface in nitrogen-fixing peanut nodules. *Protoplasma* 150: 19-26
- Bal AK, Shantharam S, Verma DPS (1980) Changes in the outer cell wall of *Rhizobium* during development of root nodule symbiosis in soybean. *Can J Microbiol* 26: 1096-1103

- Barry AL, Thornsberry C (1980) Susceptibility testing: Diffusion test procedures. In: Lennette EH (ed) Manual of Clinical Microbiology. American Society for Microbiology, Washington, DC, pp 463-474
- Bassett B, Goodman RN, Novacky A (1977) Ultrastructure of soybean nodules II: deterioration of symbiosis in ineffective nodules. Can J Microbiol 23: 873-883
- Beers RFJ, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195: 133-140
- Beevers H (1969) Glyoxysomes of castor bean endosperm and their relation to glycogenesis. Annu. N.Y. Acad. Sci. 168: 313-324
- Beevers H (1979) Microbodies in higher plants. Annu Rev Plant Physiol 30: 150-193
- Bergersen FJ (1982) Root nodules of legumes: Structure and Functions. Research Studies Press, Letchworth, England. 97-121
- Bergersen FJ, Turner GL (1975) Leghemoglobin and the supply of O<sub>2</sub> to nitrogen-fixing root nodule bacteroids: presence

of two oxidase systems and ATP production at low free  $O_2$  concentration. J Gen Microbiol 91: 345-354

Bergersen FJ, Appleby CA (1981) Leghaemoglobin within bacteroid-enclosing membrane envelopes from soybean root nodules. Planta 152: 534-543

Bergfield R, Hong YN, Kuhn T, Schopfer P (1978) Formation of oleosomes (storage lipid bodies) during embryogenesis and their breakdown during seedling development in cotyledons of *Sinapis alba* L. Planta 143: 297-303

Bidwell RGS (1974) Nitrogen metabolism. In: Bidwell RGS (ed) Plant Physiology. Collier Macmillan Publishers, London, pp. 173-206.

Blanchette-Mackie EJ, Scow RO (1981) Lipolysis and lamellar structures in white adipose tissue of young rats: Lipid movement in membranes. J Ultrastruct Res 77: 295-318

Boller T, Wiemken A (1986) Dynamics of vacuolar compartmentation. Annu Rev Plant Physiol 37: 137-164

Boshier DP, Holloway H, Kitchin LF (1984) A comparison of standard lipid staining technique used in electron microscopic studies of mammalian tissues. Stain Technol

59: 83-89

Bray CM (1983). Interconversion of nitrogen. Nitrogen Metabolism in Plants, pp. 1-28. Longman Inc., New York.

Burris RH (1980) The Global Nitrogen Budget - Science or Seance? In: Newton WE, Orme-Johnson WH (Eds) Nitrogen Fixation, Vol II, University Park Press, Baltimore, pp 7-16

Carroll BJ, Hanssen AP, McNeil DL, Gresshoff PM (1987) Effect of oxygen supply on nitrogenase activity of nitrate- and dark-stressed soybean (*Glycine max* (L.) Merr.) plants. Plant Physiol 14: 679-687

Chance B, Sies H, Boveris A (1979) Hydrogenperoxide metabolism in mammalian organs. Physiological Rev 59: 527-605

Chandlee JM, Tsiftaris AS, Scandalios JG (1983) Purification and partial characterization of three genetically defined catalases of maize. Plant Sci Lett 29: 117-131

Chandler MR (1978) Some observations on infection of *Arachis hypogaea* L. by *Rhizobium*. J Expt Bot 29: 749-755



- Ching TM, Hedtke S, Russell SA, Evans J (1975) Energy state and dinitrogen fixation in soybean nodules of dark grown plants. *J Plant Physiol* 55: 796-798
- Ching TM (1972) Metabolism of germinating seeds. In: Koslowski TT (ed) *Seed Biology*. Academic Press, New York, pp 103-228
- Clare DA, Duong MN, Darr D, Archibald F, Fridovich I (1984) Effect of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal Biochem* 140: 532-537
- Cohen HP, Sarath G, Lee K, Wagner FW (1986) Soybean root nodule ultrastructure during dark-induced stress and recovery. *Protoplasma* 132:69-75
- Cooper TG, Beevers H (1969b) Mitochondria and glyoxysomes from castor bean endosperm. *J Biol Chem* 244: 3514-3520
- Culvenor RA, Davidson IA, Simpson RJ (1989) Regrowth by swards of subterranean clover after defoliation. 2. Carbon exchange in shoot, root and nodule. *Ann Bot* 64: 557-567

Dabas S, Swaraj K, Sheoran IS (1988) Effect of source removal on functioning of the nitrogen fixation in pigeonpea (*Cajanus cajanus* L.) nodules. J Plant Physiol 132: 690-694

Dakora FD, Atkins CA (1989) Diffusion of oxygen in relation to structure and function in legume root nodules. Aust J Plant Physiol 16: 131-140

Dart P (1977) Infection and development of legumes nodules. In: Hardy RWF, Silver WS (eds) A Treatise of Dinitrogen Fixation. John Willey & Sons, New York, pp 367-472

Dazzo FB (1982) The mechanism of recognition between legume root and rhizobia: Some implications for biological nitrogen fixation in tropics. In: Graham PH, Harris SC (eds) Biological Nitrogen Fixation Technology for Tropical Agriculture. Cali, Colombia

Deisseroth A, Dounce A (1970) Catalase: Physical and chemical properties, mechanism of catalysis and physiological role. Physiol Rev 50: 319-375

Dilworth MJ, Glenn AR (1982) Movement of ammonia in *Rhizobium leguminosarum*. J Gen Microbiol 128: 29-37

- Dimitrijevic L, Puppo A, Trinchant JC, Rigaud J (1989) Ferrochelatase activities and heme contents in purified mitochondria from soybean roots and root nodules. *J Plant Physiol* 134: 642-644
- Dixon GH, Kornberg HL (1959) Assay methods for key enzymes of the glyoxylate cycle. *Biochem J* 72: 3p
- Downie JA, Johnston AWB (1988) Nodulation of legumes by rhizobium. *Plant Cell Envi* 11: 403-412
- Dreyfus BL, Dommergues YR (1981) Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata*. *FEMS Microbiol Lett* 10: 313-317
- Drumm H, Schopfer P (1974) Effect of phytochrome on development of catalase activity and isoenzyme pattern in mustard (*Sinapis alba* L.) seedlings. A reinvestigation. *Planta* 120: 13-30
- Duke SH, Henson CA (1985) Legume nodule carbon utilization in the synthesis of organic acids for the production of transport amides and amino acids. In: Ludden PW, Burris JE (eds) Nitrogen fixation and CO<sub>2</sub> metabolism. Elsevier, New York, pp 293-302

- Duke JA (1981) *Arachis hypogaea* L. In: Duke JA (ed) Handbook of Legumes of World Economic Importance. Plenum Press, New York and London, pp 18-22
- Ellfolk N (1960) Crystalline haemoglobin 1. Purification procedure. Acta Chem Scand 14: 609-616
- Ellfolk N (1972) Leghaemoglobin, a plant haemoglobin. Endeavour 31: 139-142
- Elkan GH (1981) The taxonomy of the Rhizobiaceae. In: Giles K, Atherly A (eds) Biology of the Rhizobiaceae. Academic Press, New York, pp 191-224
- Fahimi HD, Yokota S (1981) Ultrastructural and cytochemical aspects of animal peroxisomes - Some recent observations. In: Schweiger HG (ed) International Cell Biology. Springer-Verlag, Berlin Heidelberg, New York, pp 640-650
- Faucher C, Maillet F, Vasse J, Rosenberg C, Brussel AAN, Truchet G, Denarie J (1988) *Rhizobium meliloti* host range nodH gene determines production of an alfalfa-specific extracellular signal. J Bacteriol 170: 5489-5499
- Fletcher WW, Alcorn JWS (1958) In " Nutrition of the legume" E.G. Hallsworth (ed.), pp. 284-288, Butterworths, London

- Francis AJ, Alexander M (1972) Catalase activity and nitrogen fixation in legume root nodules. *Can J Microbiol* 18: 861-864
- Frederick SE, Newcomb EH (1969) Cytochemical localization of catalase in leaf microbodies (Peroxisomes). *J Cell Biol* 43: 343-353
- Frederick AC, Gruber PJ, Newcomb EH (1975) Plant microbodies. *Protoplasma* 84: 1-29
- Frey-wyssling A, Grieshaber E, Muhlethaler, K (1963) Origin of sphaerosomes in plant cells. *J Ultra Res* 8: 506-516
- Gallon JR, Chaplin AE (1987) *An Introduction to Nitrogen Fixation*. Cassel, London
- Gerson T, Patel JJ, Wong MN (1978) The effects of age, darkness and nitrate on poly- $\beta$ -hydroxybutyrate levels and nitrogen-fixing ability of *Rhizobium* in *Lupinus angustifolius*. *Physiol Plant* 42: 420-424
- Gorbet DW, Burton JC (1979) A non-nodulating peanut. *Crop Sci.* 19: 727-728

- Gordon AJ, Ryle GJA, Mitchell DF, Lowry KH, Powell CE (1986)  
The effect of defoliation on carbohydrate, protein and  
leghaemoglobin content of white clover nodules. *Ann Bot*  
58: 141-154
- Gordon AJ, Kessler W (1990) Defoliation-induced stress in  
nodules of white clover. II. Immunological and enzymatic  
measurements of key proteins. *J Expt Bot* 41: 1255-1262
- Graham PH, Parker CA (1961) Leghemoglobin and symbiotic  
nitrogen fixation. *Aust J Cell Sci* 23: 231-232
- Graham PH (1963) Vitamin requirements of root nodule bacteria.  
*J Gen Microbiol* 30: 245-268
- Gregory EM, Fridovich I (1974) Visualization of catalase on  
acrylamide gels. *Anal Biochem* 58: 57-62
- Greitner CS, Winner WE (1989) Effects of O<sub>2</sub> on alder  
photosynthesis and symbiosis with *Frankia*. *New Phytol*  
111: 647-656
- Gutschick VP (1980) Energy flows in the nitrogen cycle,  
especially in fixation. In: Newton WE, Orme-Johnson WH  
(eds) *Nitrogen Fixation* (vol. I). University Park Press,  
Baltimore, pp. 17-27.

- Haahtela K, Kari K (1986) The role of root-associated *Klebsiella pneumoniae* in the nitrogen nutrition of *Poa pratensis* and *Triticum aestivum* as estimated by the method of  $^{15}\text{N}$  isotope dilution. *Plant Soil* 90: 245-254
- Hageman RV, Burris RH (1979) Changes in the EPR signal of dinitrogenase from *Azotobacter vinelandii* during the lag period before hydrogen evolution begins. *J Bot Chem* 254: 11189-11192
- Hageman RV, Burris RH (1978) Nitrogenase and nitrogenase reduction associate dissociate with each catalytic cycle. *Proc Natl Acad Sci USA* 75: 2699-2702
- Halverson LJ, Stacey G (1986) Signal exchange in plant-microbe interactions. *Microbiol Rev* 50: 193-225
- Hameed S (1986) Involvement of microbodies in symbiotic nitrogen-fixing *Arachis hypogaea* L. (peanut) root nodules. M. Sc. thesis, Memorial University of Newfoundland, Canada
- Hameed S, Bal AK (1985) Involvement of microbodies in peanut-*Rhizobium* symbiosis. In: Evans HJ, Bottomley PJ,

Newton WE (Eds.) Nitrogen fixation research progress.  
Martinus Nijhoff Publishers, Dordrecht, pp 310

Hammons RO (1982) Origin and early history of the peanut.  
In: Pattee HE, Young CT (eds) Peanut Science and Technology. American Peanut Research Education Society, Yoakum, TX, pp 1-20

Hardy RWF, Holsten RD, Jackson EK, Burns RE (1968). The acetylene-ethylene assay for  $N_2$  fixation: Laboratory and field valuation. Plant Physiol 43: 1185-1207

Hardy R, Havelka UD (1976) Photosynthate as a major factor limiting nitrogen fixation by field-grown legumes with emphasis on soybeans. In: Nutman PS (ed) Symbiotic Nitrogen Fixation in Plants. International Biology Program Series, Vol 7, Cambridge University Press, pp. 421-439

Hartwig U, Boller BC, Baur-Hösch B, Nösberger J (1990) The infection of carbohydrate reserves on the response of nodulated white clover defoliation. Ann Bot 65: 97-105

Hartwig U, Boller B, Nosberger J (1987) Oxygen supply limits nitrogenase activity of clover nodules after defoliation. Ann Bot 59: 285-291



- Hashimoto F, Hayashi H (1987) Significance of catalase in peroxisomal fatty acyl-CoA  $\beta$ -oxidation. *Biochem Biophys Acta* 921: 142-150
- Havir EA, McHale NA (1987) Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiol* 84: 450-455
- Henry LEA, Gogotov IN, Hall DO (1978) Superoxide dismutase and catalase in the protection of the proton-donating systems of nitrogen fixation in the blue-green algae *Anabaena cylindrica*. *Biochem J* 174: 373-377
- Herman EM (1987) Immunogold-localization and synthesis of an oil-body membrane in developing soybean seeds. *Planta* 172: 336-345
- Holl FB, Milliron ML, Delafield SJ (1983) Qualitative variation in root nodule leghemoglobins: intra-specific variation in component profiles. *Plant Sci Lett* 32: 321-326
- Hostak MS, Henson CA, Duke SH, VandenBosch KA (1987) Starch granule distribution between cell types of alfalfa nodules as affected by symbiotic development. *Can J Bot*

65: 1108-1115

Hrsel I (1966) The morphology and function of vesicles and vesicle related formations in cell ultrastructure. *Biologia Plantarum* (Praha) 8: 263-267

Huang AHC, Qu R, Wang SM, Vance VB, Cao YZ, Lin YH (1986) Synthesis and degradation of lipid bodies in the scutella of maize. In: Stumpf PK, Mudd JH, Nes WD, (eds) *T. Ple metabolism, structure and function of plant lipid*. Plenum Press, New York, pp 239-246

Huang AHC (1985) In: Linskens HF, Jackson JF, (eds) *Modern Methods of Plant Analysis vol I*. Springer Verlag, Berlin, pp 145-151

Huang AHC, Trelease RN, Moore TS (1983) *Plant Peroxisomes*. Academic Press, New York, pp 16-19

Huang AHC (1983) In: Brockman HL, Borgstrom B (eds) *Lipases*. Elsevier Publishing, Amsterdam, pp 419-442

Hunt S, King BJ, Canvin DT, Layzell DB (1987) Steady and nonsteady state gas exchange characteristics of soybean nodules in relation to the oxygen diffusion barrier. *Plant Physiology* 84: 164-172

- Hutton D, Stumpf PK (1969) Characterization  $\beta$ -oxidation system from maturing and germinating castor bean seeds. *Plant Physiol* 44: 508-516
- Jayaram S, Bal AK (1991) Oleosomes (lipid bodies) in nitrogen-fixing peanut nodules. *Plant Cell and Environ* 14: 195-203
- Johnson HS, Hume DJ (1973) Comparisons of nitrogen fixation estimates in soybeans by nodule weight, leghemoglobin content, and acetylene reduction. *Can J Microbiol* 19: 1165-1168
- Johnson GV, Evans HJ, Ching T (1966) Enzymes of the glyoxylate in rhizobia and nodules of legumes. *Plant Physiol* 41: 1330-1336
- Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov. a genus slow-growing, root ndule bacteria from leguminous plants. *Int J Syst Bacteriol* 32: 136-139
- Jordan DC, Allen ON (1974) Gram-negative aerobic rods and cocci - Family III. Rhizobiaceae. In: Buchanan RE, Gibbons NE (eds) *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore, Williams and Wilkins,

261-264

Kapil RN, Kapil N (1971) Root nodules of *Cajanus cajan* : origin, structure and ontogeny. *Phytomorphology* 21: 192-202

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J Cell Biol* 27: 137a-8a

Katznelson H, Cole SE (1965) Production of gibberellin-like substances by bacteria and actinomycetes. *Can J Microbiol* 11: 733-741

Keilin D, Wang YL (1945) Hemoglobin in the root nodules of leguminous plants. *Nature* 155: 227-229

Kendall AC, Keys AJ, Turner JC, Lea PJ, Mifflin BJ (1983) The isolation and characterization of a catalase-deficient mutant of barley (*Hordeum vulgare* L.). *Planta* 159: 505-511

Kortt A, Strike PM, Bogusz D, Appleby CA (1987) The amino acid sequence of leghemoglobin II from *Sesbania rostrata*-stem nodules. *Biochem Int* 15: 509-516

- Kouchi H, Yoneyama T (1984a) Dynamics of carbon photosynthetically assimilated in nodulated soybean under steady state conditions. 1. Development and application of  $^{13}\text{CO}_2$  assimilation system at a constant  $^{13}\text{C}$  abundance. Ann Bot 53: 875-882
- Kouchi H, Yoneyama T (1984b) Dynamics of carbon photosynthetically assimilated in nodulated soybean under steady state conditions. 2. The incorporation of  $^{13}\text{C}$  into carbohydrates, organic acids and some storage compounds. Ann Bot 53: 883-896
- Kouchi H, Tsukamoto M, Tajima S (1989) Differential expression of nodule-specific (nodulin) genes in the infected, uninfected and cortical cells of soybean (*Glycine max*) root nodules. J Plant Physiol 135: 608-617
- Kuhse J, Puhler A (1987) Conserved sequence motifs in the untranslated 3'-end of leghemoglobin transcripts isolated from broad bean nodules. Plant Sci 49: 137-143
- Kunce CM, Trelease RN (1986) Heterogeneity of catalase in maturing and germinated cotton seeds. Plant Physiol 81: 1134-1139

Kurz GW, LaRue TA (1975) Nitrogenase activity in rhizobia in the absence of plant host. *Nature (Lond)* 256: 407-409

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* 227: 680-685

LaRue TA, Child JJ (1979) Sensitive fluorometric assay for leghemoglobin. *Anal Biochem* 92: 11-15

Lawn RJ, Burn WA (1974) Symbiotic nitrogen fixation in soybeans I. Effect of photosynthetic source-sink manipulations. *Crop Sci* 14: 11-16

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC, Denarie J (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature (Lond)* 344: 781-784

Lin Y, Huang AHC (1983) Lipase in lipid bodies of cotyledons of rape and mustard seedlings. *Arch Biochem Biophys* 225: 360-369

Loewen PC, Switala J (1987) Multiple catalases in *Bacillus subtilis*. *J Bacteriol* 169: 3601-3607

- Lowry OH, Rosebrough NJ, Farr A, Randall RJ (1951) Protein measurement with the Folin reagent. J Biol Chem 193: 265-275
- Lulsdorf MM, Holl FB (1991) Leghemoglobin variability in the genus *Phaseolus*. Can J Bot 69: 353-358
- Malik NSA, Pfeiffer NE, Williams RD, Wagner FW (1981) Peptidohydrolases of soybean root nodules. Plant Physiol 68: 386-392
- McComb JA, Elliott J, Dilworth MJ (1975) Acetylene reduction by *Rhizobium* in pure culture. Nature (London) 256: 409-410
- McNeil D, Carroll BJ, Gresshoff PM (1984) The nitrogen fixation capacity of bacteroids extracted from soybean nodules inhibited by nitrate, ammonia or dark treatments. In: Ghai BS (ed) Symbiotic Nitrogen Fixation, Vol 1. USG Publishers and Distributors, Ludhiana, India, pp 79-88
- Mederski HJ, Streeter JG (1977) Continuous, automated acetylene reduction assays using intact plants. Plant Physiol 59: 1076-1081

- Meir E, Yagil E (1985) Further characterization of two catalases in *Escherichia coli*. *Curr Microbiol* 12: 315-320
- Meir E, Yagil E (1984) Catalase-negative mutants of *Escherichia coli*. *Curr Microbiol* 11: 13-18
- Mellor RB (1989) Bacteroids in *Rhizobium*-legume symbiosis inhabit a plant internal lytic compartment: Implication for other microbial endosymbioses. *J Expt Bot* 40: 831-839
- Minchin FR, Sheehy JE, Witty JF (1986) Further errors in the acetylene reduction assay: Effects of plant disturbance. *J Expt Bot* 37: 1581-1591
- Minchin F, Pate JS (1974) Diurnal functioning of legume root nodule. *J Expt Bot* 25: 295-308
- Minchin FR, Sheehy JE, Mingeuz MI, Witty JF (1985) Characterization of the resistance to oxygen diffusion in legume nodules. *Ann Bot* 55: 53-60
- Moller RB, Christensen TMIE, Bassarab S, Werner D (1985) Phospholipid transfer from ER to the peribacteroid membrane in soybean nodules. *Zeitschrift für Naturforschung* 40: 73-79



- Morgan AR, Cove RL, Elgert TM (1976) The mechanism of DNA strand breakage by vitamin C and superoxide and the protective role of catalase and superoxide dismutase. *Nucleic Acid Res* 3: 1139-1149
- Muhlethaler K (1955) Untersuchungen uber die struktur and entwicklung der proplastiden. *Protoplasma* 45: 264-279
- Murphy PM (1986) Effect of light and atmospheric carbon dioxide concentration on nitrogen fixation by herbage legumes. *Plant and Soil* 95: 399-409
- Nambiar PTC, Nigam SN, Dart PJ, Gibbons RW (1983) Absence of root hairs in non-nodulating groundnut *Arachis hypogaea* L. *J Expt Bot* 34: 484-488
- Newcomb W, Wood SM (1986) Fine structure of nitrogen-fixing leguminous root nodules from the Canadian arctic. *Nordic J Bot* 6: 609-926
- Newcomb EH, Tandon SR (1981) Uninfected cells of soybean root nodules: Ultrastructure suggests key role in ureide production. *Science* 212: 1394-1396
- Newman EI, Bowen HJ (1974) Patterns of distribution of bacteria on root surfaces. *Soil Biol Biochem* 6: 205-210

Pagan JD, Child JJ, Scowcroft WR, Gibson AH (1975). Nitrogen fixation by *Rhizobium* cultured on defined medium. *Nature* (London) 256: 406-407

Pate JS, Atkins CA (1983) Nitrogen uptake, transport and utilization. In: Broughton WJ (ed) *Nitrogen Fixation*. Vol 3, Clarendon Press, Oxford, pp 245-298

Patriquin DG (1981) New developments in grass-bacteria associations. In: Subba Rao NS (ed) *Advances in agricultural microbiology*. Oxford and IBH Publishing Co., New Delhi, pp 139-190

Patriquin DG, Dobereiner J (1978) Light microscopy observation of tetrazolium reducing bacteria in endorhizosphere of maize and other grasses in Brazil. *Can J Microbiol* 24: 734-742

Pelczar MJ, Chan ECS, Krieg NR (1986) *Micobiology of Soil*. In: *Microbiology*. McGraw Hill Book company, pp. 543-568

Peters NK, Frost JW, Long SR (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* (Washington) 233: 977-980

Pfeiffer NE, Torres CM, Wagner FW (1983) Proteolytic activity in soybean root nodules. *Plant Physiol* 71: 797-802

Pladys D, Rigaud J (1982) Acid protease and leghemoglobin digestion in French bean root nodule. *Z Pflanzenphysiol* 108: 163-169

Posgate JR (1982) *The fundamentals of Nitrogen Fixation*, Cambridge University Press, Cambridge, pp 138-168

Prevost D, Bal AK (1989) Morphological development and lipid composition of nodules from the arctic legume *Oxytropis arctobia*. *Proc North Ame Symbio Nitro Fixa Confer.* p 112

Prin Y, Duhoux E, Diem HG, Roederer Y, Dommergues YR (1991) Aerial nodules in *Casuarina cunninghamiana*. *Appl Environ Microbiol* 57: 871-874

Pueppke SG, Freund TG, Schulz BC, Friedman HP (1980) Interaction of lectins from soybean and peanut with rhizobia that nodulated soybean, peanut, or both plants. *Can J Microbiol* 26: 1489-1497

Puppo A, Dimitrijevic L, Rigaud J (1989) Superoxide dismutase and catalase activities in purified *Frankia* vesicles. *Physiol Plantarum* 77: 308-311

- Puppo A, Halliwell B (1988) Generation of Hydroxyl radicals by soybean nodule leghaemoglobins. *Planta* 173: 405-410
- Rainbird RM, Atkins CA, Pate JS (1983a) Effect of temperature on nitrogenase functioning in cowpea nodules. *Plant Physiol* 73: 392-394
- Rainbird RM, Atkins CA, Pate JS (1983b) Diurnal variation in functioning of cowpea nodules. *Plant Physiol* 72: 308-312
- Rawsthorne S, Minchin FR, Summerfield RJ, Cookson, C, Coombs J (1980) Carbon and nitrogen metabolism in legume root nodules. *Phytochem* 19: 341-355
- Redinbaugh MG, Wadsworth GJ, Scandalios JG (1988) Characterization of catalase transcripts and differential expression in maize. *Biochem Biophys Acta* 951: 104-116
- Reibach PH, Streeter JG (1984) Evaluation of active versus passive uptake of metabolites by *Rhizobium japonicum* bacteroids. *J Bacteriol* 159: 47-52
- Robertson JG, Wells B, Bisseling T, Farnden KJF, Johnston WAB (1984) Immunogold localization of leghaemoglobin in cytoplasm in nitrogen-fixing root nodules of pea. *Nature* 311: 254-256

Ronson, CW (1988) Genetic regulation of  $C_4$ -dicarboxylate transport mutants of *Rhizobium trifoli* from ineffective nodules of *Trifolium repens*. Proc Natl Acad Sci USA 78: 4284-4288

Roponen, I (1970) The effect of darkness on the leghemoglobin content and amino acid levels on the root nodules of pea plants. Physiol Plantarum 23: 452-460

Roughan PG, Slack CR (1982) Cellular organization glycerolipid metabolism. Annu Rev Plant Physiol 33: 97-132

Ryle GJA, Powell CE, Gordon AJ (1988) Responses of  $N_2$  fixation-linked respiration to host-plant energy status in white clover acclimated to a controlled environment. J Expt Bot 39: 879-887

Ryle GJA, Powell CE, Gordon AJ (1985a) Short-term changes in  $CO_2$  evolution associated with nitrogenase activity in white clover in response to defoliation and photosynthesis. J Expt Bot 36: 634-643

Schubert KR (1986) Products of biological nitrogen fixation in higher plants: Synthesis, Transport and Metabolism.

Ann Rev plant Physiol 37: 539-574

Schweitzer LE, Harper JE (1980) Effect of light, dark and temperature on root nodule activity (acetylene reduction) of soybean. Plant Physiol 65: 51-56

Seah TCM, Kaplan JG (1973) Purification and properties of catalase of baker's yeast. J Biol Chem 248: 2889-2893

Sen D, Weaver RW (1980) Nitrogen fixing activity of rhizobial strain 32H1 in peanut and cowpea nodules. Plant Sci Lett 18: 315-318

Sen D, Weaver RW (1988) Protein profiles of bacteroids of *Bradyrhizobium* from peanut and cowpea. Microbios Lett 38: 61-64

Sen D, Weaver RW (1984) A basis for different rates of N<sub>2</sub> fixation by the same strains of *Rhizobium* in peanut and cowpea root nodules. Plant Sci Lett 34: 239-246

Sen D, Weaver RW, Bal AK (1986) Structure and organization of effective peanut and cowpea root nodules induced by rhizobial strain 32H1. J Expt Bot 37: 356-363

- Shelp BJ, Atkins CA (1984) Subcellular locations of enzymes of ammonia assimilation and asparagine synthesis in root nodules of *Lupinus albus* L. *Plant Sci Lett* 36: 225-230
- Sinclair TR, Goudriaan J (1981) Physical and morphological constraints on transport in nodules. *Plant Physiol* 67: 143-145
- Somerville C, Browse J (1991) Plant lipids : Metabolism, mutants and membranes. *Science* 252: 80-87
- Sorokin HP (1967) The spherosomes and the reserve fat in the plant cells. *Amer J Bot* 54: 1008-1016
- Sprent, JI (1987) Nitrogen fixation. In: Wilkins MB (ed) *Advanced plant physiology*. John Wiley & Sons, New York, pp 249-276
- Sprent, JI (1989) Which steps are essential for the formation of functional legume nodules? *New Phytology* III: 129-153
- Spurr AR (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *J Ultra Res* 26: 31-43
- Staphorst JL, Strijdom, B.W. (1972). Some observations on the bacteroids in nodules of *Arachis* sp. and isolation

of rhizobia from these nodules. *Photophylactica* 4: 87-92

Stewart WDP (1977) Present-day nitrogen-fixing plants. *AMBIO*  
6: 166-173

Stovall I, Cole M (1978) Organic acid metabolism by isolated  
*Rhizobium japonicum* bacteroids. *Plant Physiol* 61: 787-790

Streeter JG (1974) Growth of two soybean shoots on a single  
root. *J Expt Bot* 25: 189-98

Subba Rao NS, Yatazawa M (1984) Stem nodules. In: Subba Rao  
NS (ed) *Current Developments in Nitrogen Fixation*. Edward  
Arnold, London, 101-110

Sutton WD, Peterson AD (1979) The detergent sensitivity of  
*Rhizobium* bacteroids and bacteria. *Plant Sci Lett* 16:  
377-385

Swaraj K, Sheoran IS, Garg OP (1988) Dark-induced changes in  
the functioning of root nodules of *Vigna unguiculata*.  
*Plant Physiol Biochem* 26: 79-84

Swaraj K, Kuhad MS, Garg OP (1986) Dark treatment effects on  
symbiotic nitrogen fixation and related processes in  
*Cicer arietinum* (chickpea). *Environ Expt Bot* 26: 31-38



- Swaraj K, Garg OP (1977) The effect of ageing on the leghemoglobin of cowpea nodules. *Physiol Plantarum* 39: 185-189
- Tjepkema JD, Yocum CS (1974) Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. *Planta* 119: 351-360
- Tolbert NE (1981) Metabolic pathways in peroxisomes and glyoxysomes. *Annu Rev Biochem* 50: 133-157
- Trelease RN, Becker WM, Burke JJ (1974) Cytochemical localization of malate synthase in glyoxysomes. *J Cell Biol* 60: 483-495
- Trinchant JC, Rigaud J (1990) Occurrence of two pathways for malate oxidation in bacteroids isolated from *Sesbania rostrata* stem nodules during  $C_2H_2$  reduction. *Plant Physiol* 94: 1002-1008
- Truchet G, Michel M, Denarie J (1980) Sequential analysis of the organogenesis of lucerne (*Medicago sativa*) root nodules using symbiotically-defective mutants of *Rhizobium meliloti*. *Differentiation* 16: 163-172
- Tsaftaris AS, Bosabalidis AM, Scandalios JG (1983) Cell-type-specific gene expression and a catalasemic peroxisomes in

a null Cat2 catalase mutant of maize. Proc Natl Acad Sci  
USA 80: 4455-4459

Udvardi MK, Price GD, Gresshoff PM, Day DA (1988) A dicarbo  
xylate transporter on the peribacteroid membrane of  
soybean nodules. FEBS Lett 231: 36-40

Uheda E, Syono K (1982a) Physiological role of leghemoglobin  
heterogeneity in pea root nodule development. Plant cell  
Physiol 23: 75-84

Uheda E, Syono K (1982b) Effects of leghemoglobin components  
on nitrogen fixation and oxygen consumption. Plant Cell  
Physiol 23: 85-90

Vainshtein BK, Harutyunyan EH, Kuranova IP, Borisov VV,  
Sosfenov NI, Pavlovsky AG, Grevenko AI, Konareva NV,  
Nekrasov YV (1977) Steric structure of lupine leghemo-  
globin with 2.8 Å resolution. Dokl Acad Nauk SSSR 233:  
238-241

Van Brussel AAN (1973) The cell wall of bacteroids of *Rhizo-  
bium leguminosarum* Frank. PhD thesis, University of  
Leiden, The Netherlands

Vance CP (1983) *Rhizobium* infection and nodulation: A beneficial plant disease ? Ann Rev Microbiol 37: 399-424

Vance VB, Huang AHC (1987) The major protein from lipid bodies of maize, characterization and structure based on cDNA cloning. J Biol Chem 262: 11275-11279

Vance CP, Johnson LEB (1983) Plant determined ineffective nodules in alfalfa (*Medicago sativa*): Structural and biochemical comparisons. Can J Bot 61: 93-106

Vance CP, Heichel GH, Barnes DK, Bryan JW, Johnson LE (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. Plant Physiol 64: 1-8

VandenBosch KA, Newcomb EH (1988) The occurrence of leghemoglobin protein in the uninfected interstitial cells of soybean root nodules. Planta 175: 442-451

Vaughn KC (1985) Structural and cytochemical characterization of three specialized peroxisome types in soybean. Physiol Plant 64: 1-12

Verma DPS, Bal AK (1976) Intercellular site of synthesis and localization of leghemoglobin in soybean root nodules.

Proc Natl Acad Sci USA 73: 3843-3847

Verma DPS, Ball S, Guerin C, Wanamaker L (1979) Leghemoglobin biosynthesis in soybean root nodules. Characterization of the nascent and released peptides and the relative rate of synthesis of the major leghemoglobins. *Biochem* 18: 476-483

Vessey JK, Walsh KB, Layzell DB (1988) Oxygen limitation of  $N_2$  fixation in stem-girdled and nitrate-treated soybean. *Physiologia plantarum* 73: 113-121

Vikman P-Å, Lundquist PO, Huss-Danell K (1990) Respiratory capacity, nitrogenase activity and structural changes of *Frankia*, in symbiosis with *Alnus incana*, in response to prolonged darkness. *Planta* 182: 617-625

Vincent JM (1970) A Manual for the Practical Study of the Root-Nodule Bacteria, Blackwell Scientific Publication, Oxford

Vincent JM (1980) Factors controlling the legume *Rhizobium* Symbiosis. In: Newton WN, Ore-Johnson WH (eds) Nitrogen Fixation vol 2, University Park Press, Baltimore, 103-127

Vincent JM (1977) *Rhizobium*: General microbiology. In: Hardy RWF, Silver WS(eds) A Treatise on Dinitrogen Fixation, Section III, Willey & Sons, New York, pp 277-366

Vincent JM (1958) In: Hallsworth EG (ed) Nutrition of the legumes. Butterworths, London, 108-123

Virtanen AI (1956) Proc 3rd Int Congr Biochem Brussels, Belgium 425-433

Virtanen AI, Jorma J, Linkola H, Linnasalmi A (1947) On the relation between nitrogen fixation and leghemoglobin content of leguminose root nodules. Acta Chem Scand 90-111

Vose PB (1983) Developments in nonlegume  $N_2$ -fixing systems. Can J Microbiol 29: 837-850

Walsh KB, Mccully ME, Canny MJ (1989) Vascular transport and soybean nodule function: nodule xylem is a blind alley, not a throughway. Plant cell Envi 12: 395-405

Walsh KB, Vessey JK, Layzell DB (1987) Carbohydrate supply and  $N_2$  fixation in soybean. The effect of varied day-length and stem girdling. Plant Physiol 85: 137-144

- Wang S, Huang AHC (1987) Biosynthesis of lipase in the scutellum of maize kernel. *J Biol Chem* 262: 2270-2274
- Wang S, Lin Y, Huang AHC (1984) Lipase activities in scutella of maize lines having diverse kernel lipid content. *Plant Physiol* 76: 837-839
- Ward CW, Fairbairn D (1970) Enzymes of  $\beta$ -oxidation and their functions during development of *Ascaris lumbricoides* eggs. *Dev Biol* 22: 366-387
- Warnner G, Theimer RR (1978) Membranous appendices of spherosomes (oleosomes): Possible role in fat utilization in germinating oil seeds. *Planta* 140: 163-169
- Warnner G, Formanek H, Theimer RR (1981) The ontogeny of lipid bodies (spherosomes) in plant cells. Ultrastructural Evidence *Planta* 151: 109-123
- Wayne LG, Diaz GA (1982) Serological, taxonomic and kinetic studies of the T and M classes mycobacterial catalase. *Int J Syst Bacteriol* 32: 296-304
- Werner D, Morschel E, Kort R, Mellor RB, Bassarab S (1984) Lysis of bacteroids in the vicinity of the host cell nucleus in an ineffective (fix<sup>-</sup>) root nodule of soybean

(*Glycine max*). *Planta* 162: 8-16

Werner D, Mellor RB, Hahn MG, Grisebach H (1985) Soybean root response to symbiotic infection glyceollin accumulation in an ineffective type of soybean nodules with an early loss of the peribacteroid membrane. *Zeitschrift für Naturforschung* 40c: 179-181

Wheeler CT (1971) The causation of the diurnal changes in nitrogen fixation in the nodules of *Alnus glutinosa*. *New Phytol* 70: 487-495

Wilson KJ, Anjaiah V, Nambiar PTC, Ausubel FM (1987) Isolation and characterization of symbiotic mutants of *Bradyrhizobium* sp. (*Arachis*) strain NC92: Mutants with host specific defects in nodulations and nitrogen fixation. *J Bacteriol* 169: 2177-2186

Wilson DO, Reisenauer, HM (1963) Cobalt requirement of symbiotically grown alfalfa. *Plant Soil* 19: 364-373

Wittenberg JB, Bergersen J, Appleby CA, Turner GH (1974) Facilitated oxygen diffusion. *J Biol Chem* 249: 4057-4066

Witty JF, Skot L, Revsbech NP (1987) Direct evidence for changes in the resistance of legume root nodules to

oxygen diffusion. J Expt Bot 38: 1129-1140

Witty JF, Minchin FR, Sheehy JE, Minguez MK (1984) Acetylene-induced changes in the oxygen diffusion resistance and nitrogenase activity of legume root nodules. Ann Bot 53: 13-20

Wong PP, Evans HJ (1971) Poly- $\beta$ -hydroxybutyrate utilization by soybean (*Glycine max* Merr.) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol 47: 750-755

Wong P, Evan HT, Klucas R, Russel S (1971) Biological nitrogen fixation. Plant and Soil (special edition) pp 525-543.  
Lie TA, Mulder EG (eds)

Wynne JC, Halward T (1989) Cytogenetics and genetics of *Arachis*. Crit Rev Plant Sci 8: 189-220

Yates MG (1977) Physiological aspects of nitrogen fixation.  
In: Newton W, Postgate JR, Rodriguez-Barrueco C (eds)  
Recent Developments in Nitrogen Fixation. Academic Press,  
New York, pp 219-270



Yatsu LY, Jacks TJ (1972) Spherosome membranes: Half unit-membranes. Plant Physiology 49: 937-943

Yatsu LY, Jacks TJ, Hensarling TP (1971) Isolation of s spherosomes (olesomes) from onion, cabbage and cotton seed tissues. Plant Physiol 48: 675-682

You C, Zhou F (1988) Non-nodular endorhizospheric nitrogen fixation in wetland rice. Can J Microbiol 35: 675-682





