ROLE OF LIPID BODIES IN N₂-FIXING ROOT
NODULES OF PEANUT (Arachis hypogaea L.)

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BY

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ABSTRACT

The peanut plant (*Arachis hypogaea* L.), unlike other legumes, can sustain nitrogen fixation when prolonged periods of darkness or detoping 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 ß-oxidation pathway and glyoxylate cycle is shown by the release of $^{14}CO_2$ 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⁺fix⁻ strains of *Bradyrhizobium* sp. were compared with the ones induced by nod⁺fix⁺ strain NC92. Both fix⁻ 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

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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_3) 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_4); and iii) biological nitrogen fixation -
atmospheric nitrogen is fixed into NH_3 catalysed by the enzyme
complex, nitrogenase in prokaryotic organisms either symbioti-
cally 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 (NO_2^-) are used by most plants but under neutral or alkaline conditions use ammonium ions (NH_4^+) better.

Figure 1. Schematic diagram of nitrogen cycle in nature

(Pelczar *et al.*, 1986).
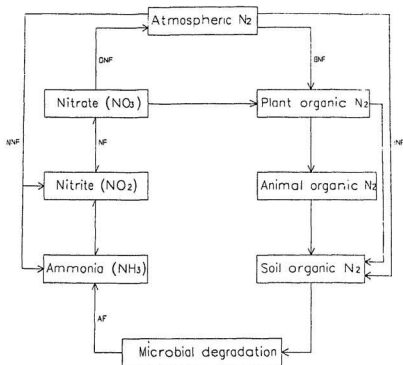
Symbol	Description
NNF	— Natural nitrogen fixation
BNF	— Biological nitrogen fixation
INF	— Industrial nitrogen fixation
AF	— Ammonification
NF	— Nitrification
DNF	— Denitrification

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

$$N_2 + 3H_2O \longrightarrow 2NH_3 + (3/2)O_2 \qquad \delta G^\circ = +340 \text{ KJ mol}^{-1}$$
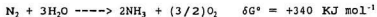
δG° is the standard free energy change of the reaction (Gallon and Chaplin, 1987). Energy required by the reaction is ultimately derived from the oxidation of the products of photosynthesis. The reaction is catalysed by nitrogenase, an enzyme unique to nitrogen-fixing organisms. Nitrogenase is extremely sensitive to oxygen. Biological nitrogen fixation is the major source of renewable combined nitrogen in the biosphere (Postgate, 1982). About 122×10^6 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).

I.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 μm X 1.2 - 3.0 μm), 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 rang of temperature under low oxygen tension. They do not produce

endospores. Glycogen and poly-β-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 penetra-
tion 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. Mag-
nesium-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:

$$N_2 + nATP + 6NADPH + 2H^+ \longrightarrow 2NH_4^+ + nADP + nPi + 6NADP + 6e^-$$

where, n = 6.5 (6.0 - 6.9) ATP/NH$_4^+$ (Rawsthrone *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 refer 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$^+$-specific iso-citrate 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 autoxidation, 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; Bergersen 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 synthe-
tase (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 substan-
tial 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, detoping, 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)
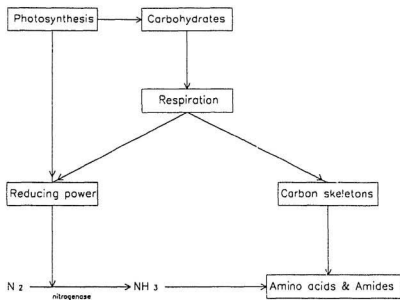
Figure 2

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 legnoceric acids as well as glyceride of oleic and leinoleic acids. The crop yield varies from 742 to 4400 kg/ha (Duke, 1981).

I.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 ß-oxidation sequence to produce acetyl-CoA, which is processed through the glyoxylate cycle. The enzymes of the ß-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 μm 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 contro-versial. 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 forma-tion of the membrane (Bergfield et al., 1978). The formation and function of lipid bodies have been well studied in different seeds (Warrner 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 maydelliana* Trautv. and *Astragulaus 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 β-oxidation pathway and glyoxylate cycle.

- cytochemical localization of lipase, catalase and malate synthase was conducted in nodule tissues as further evidence of β−oxidation pathway and glyoxylate cycles.

- morphometric comparison of lipid bodies was done in nitrogen-fixing (fix⁺) and non-fixing (fix⁻) 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), Patencheru, 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),
$MgSO_4.7H_2O$ (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 μmole m^{-2} 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 Detoping 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 (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 ultra-microtome. 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⁺) 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_2H_2) 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 μmoles of ethylene h^{-1} 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 μm) 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_{40} (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^2 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 μmole 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_2O_2 containing DAB solution (0.1 ml of 3% H_2O_2 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 Kornbery (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 μl 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 μmole 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 immedi-
ately 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 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 μCi 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 $^{14}CO_2$ 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% 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 μg 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 μg/ml) in 0.05 M potassium phosphate buffer
(pH 7.0) for 45 min. H_2O_2 was then added to a concentration of
5.0 mM and soaking was continued for 10 min. Higher levels of
H_2O_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 ± SE of the mean of
         three replicates.
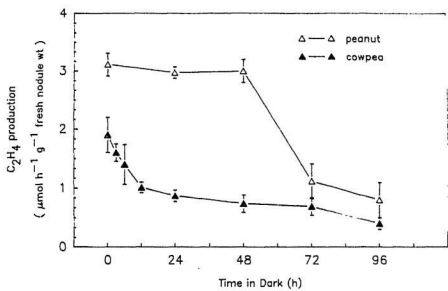
Figure 3

Figure 4. Acetylene reduction assay (ARA) of peanut and
cowpea nodules obtained from detopped plants.
Bar indicate ± SE of the mean of three repli-
cates.
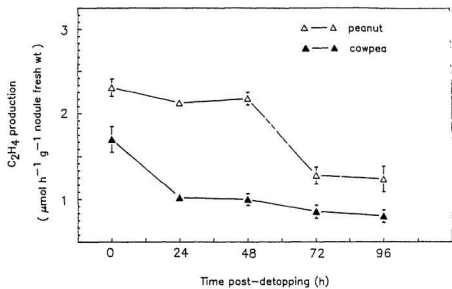
Figure 4
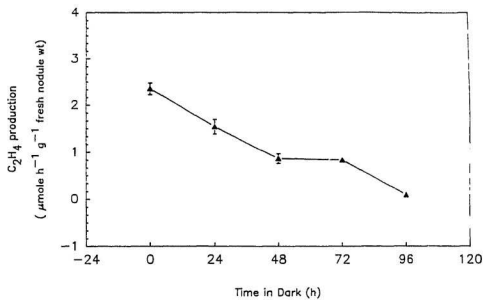
Figure 5

Figure 5. Acetylene reduction assay (ARA) of cowpea nodules obtained from dark-treated plants at constant temperature. Bars indicate ± 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 ± SE of the mean of
three replicates.
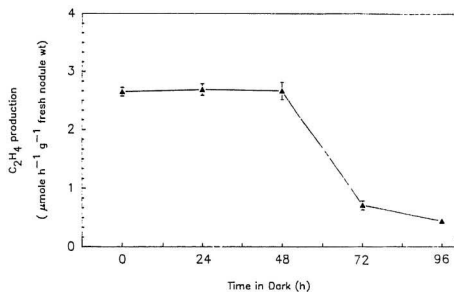
Figure 6

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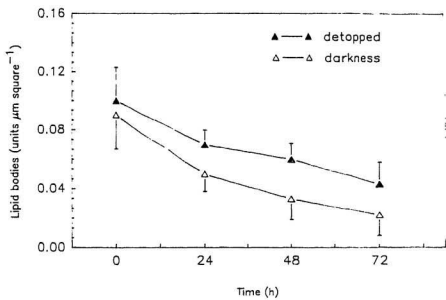
Figure 7

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 occuring 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	Catalase activity (units/mg protein)	
(hours)	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, ploy-β-hydroxy-butyrate.
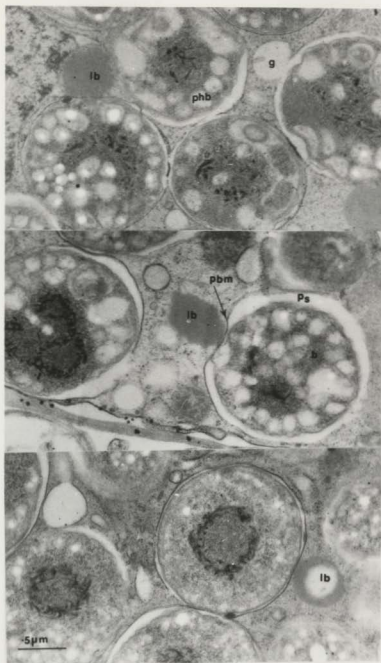
Figure 8

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-ß-
        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).
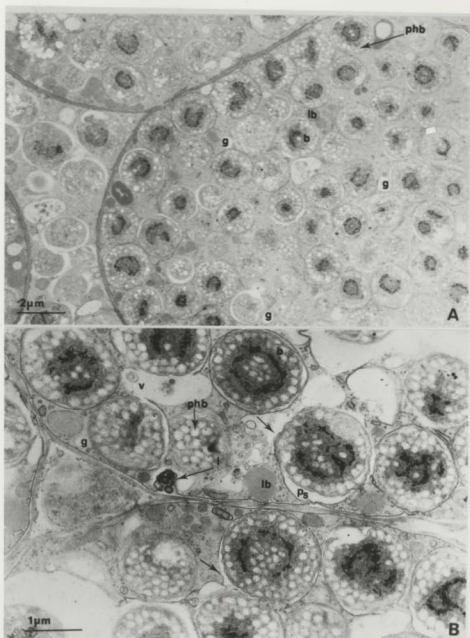
Figure 9

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 detoping experiment was conducted only with the peanut. The effects of detoping 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).
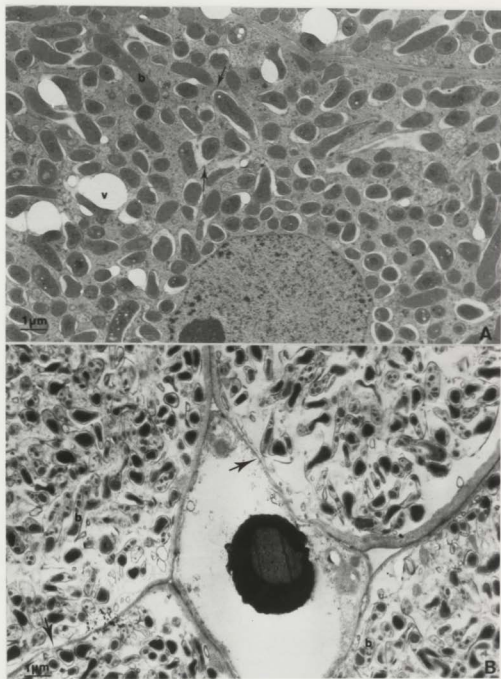
Figure 10

The results (Figs. 11 and 12) clearly indicate a gradual increase of $^{14}CO_2$ release with incubation time in the nodule homogenate, but a peak in the release of $^{14}CO_2$ by the bacteroid after 25 minutes of incubation time. The nodule cytosol showed very little $^{14}CO_2$ release, which remained constant throughout incubation. $^{14}CO_2$ 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 $^{14}CO_2$ 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 $^{14}CO_2$ release with incubation time but the amount of $^{14}CO^2$ release was higher in intact bacteria compared with the bacterial cell wall. On the other hand, very little $^{14}CO_2$ 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. $^{14}CO_2$ released from [^{14}C] linoleoyl coenzyme A
by the nodule homogenate of peanut and cowpea
nodules. Due to low sample volume, the experi-
ment was performed with a single replicate
each time but repeated twice for confirma-
tion. Similar trends in results were
obtained each time.
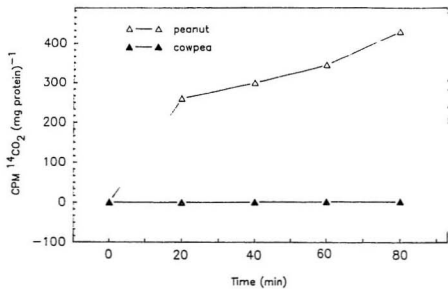
Figure 11

Figure 12. $^{14}CO_2$ released from [^{14}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.
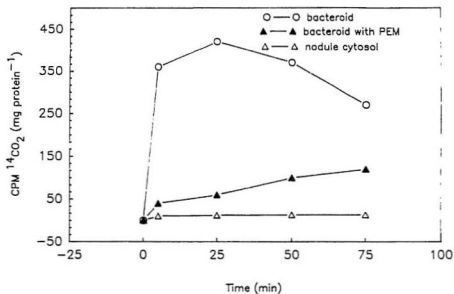
Figure 12

Figure 13. $^{14}CO_2$ released from [^{14}C] linoleoyl coenzyme A
by different fractions of cultured *Bradyrhi-*
zobium sp. 32H1. The experiment was repeated
three times for confirmation. Similar trends
in results were obtained each time.
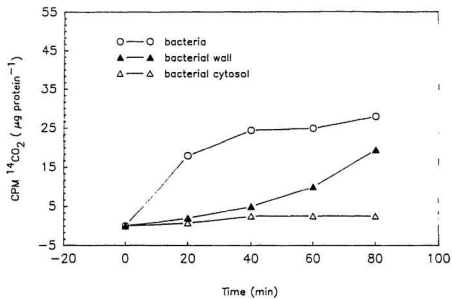
Figure 13

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 μmole/min/g protein) than the nodule cytosol fraction (7.74 μmole/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 triglyercides 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.
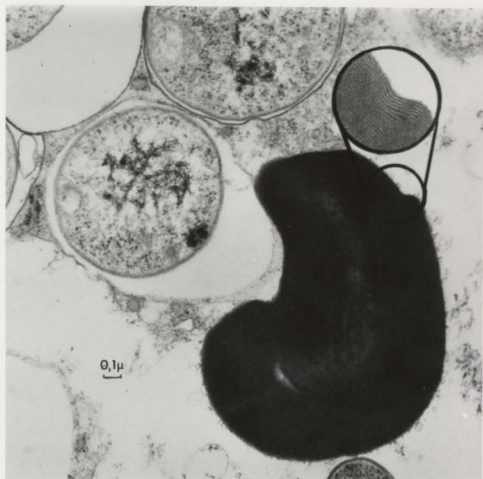
Figure 14

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

A) Electron micrograph of nodule tissues showing elec
        tron-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
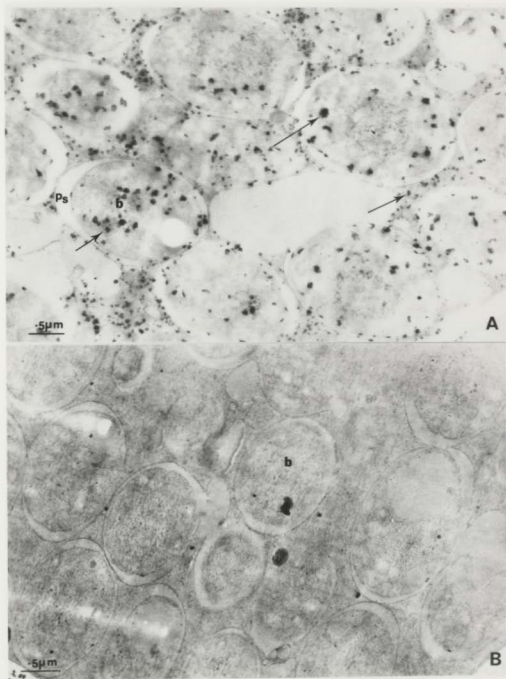
Figure 15

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)
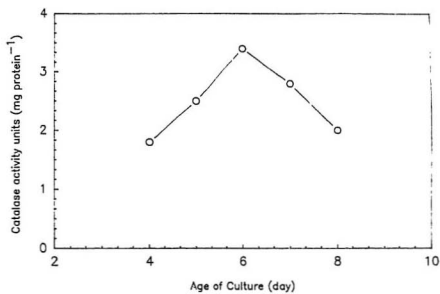
Figure 16

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)
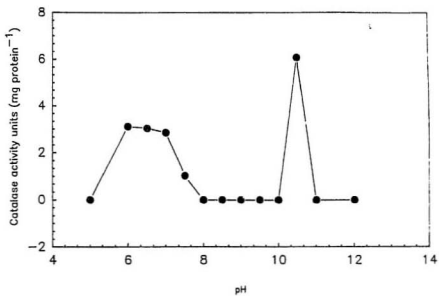
Figure 17

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)
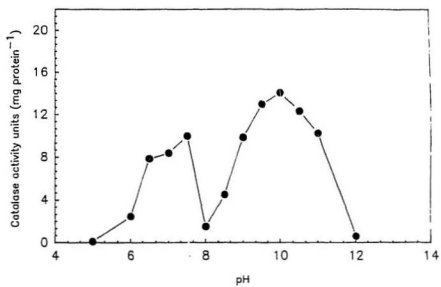
Figure 18

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)
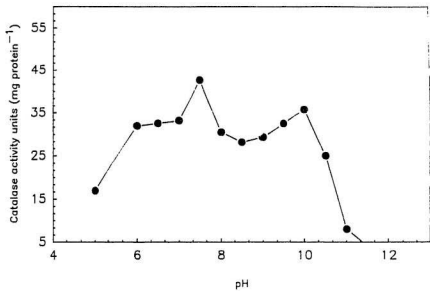
Figure 19

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)
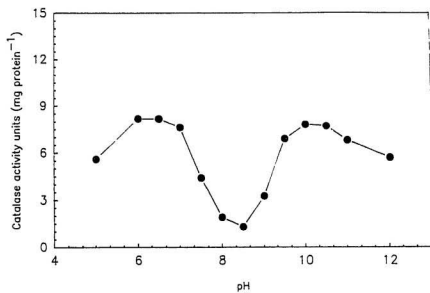
Figure 20

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

Sources	Enzyme activity (units/mg protein)		
	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⁻ and Fix⁺ Strains of *Bradyrhizobium* sp.

Nodules induced by the two fix⁻ 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⁺ 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).
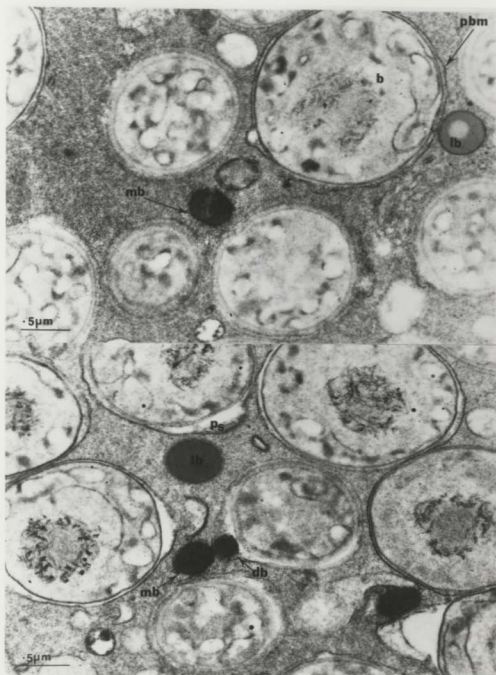
Figure 22

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 μm 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⁻) 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⁻) 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).
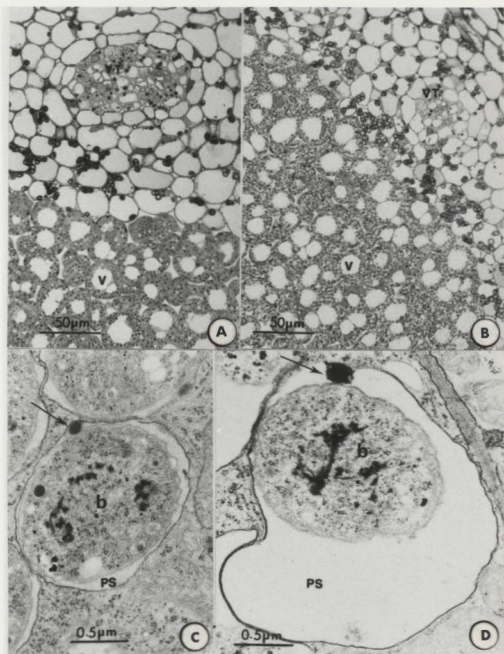
Figure 24

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 s nodule ce (PS).

C) Electron micrograph of nodule tissue (5 weeks post-inoculation) showing sensescing infected cells. The central vacuole (v) is enlarged. Note the abundance of lipid bodies (L).
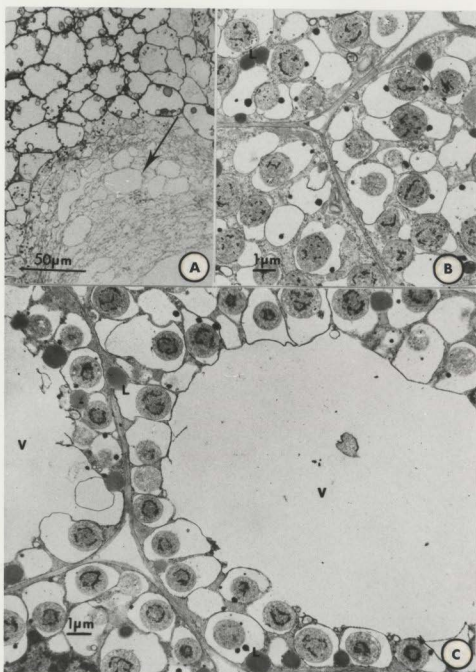
Figure 25

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).
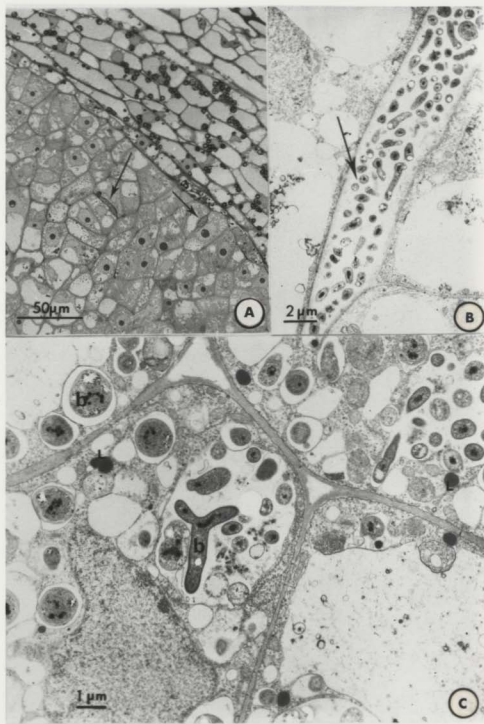
Figure 26

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 ± 32	pink/red	normal
7091	199 ± 23	white	soft
639	195 ± 11	white	soft, spongy
32H1	59 ± 8	pink/red	normal

*Mean ± 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 ± SE of the mean
(n = 20)
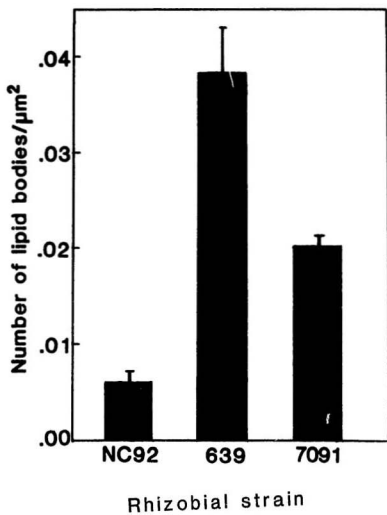
Figure 27

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 detoping 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 detoping 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). Detoping 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 (McNeil 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 detoping 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). There-fore, 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 ß-oxidation pathway and glyoxylate or TCA cycle to make organic acids available for bacteroids. Both the ß-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 ß-oxidation in lipid body catabolism, has been demonstrated in the bacteroid and nodule cytosol of peanut (Fig. 15). MS activity increases coincidently 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}C] LYL CoA to $^{14}CO_2$ (Fig. 11). The release of $^{14}CO_2$ is an indication that the β-oxidation pathway and glyoxylate cycle (Ward and Fairbairn, 1970) are operating in the peanut nodule (Fig. 11). Cowpea nodules show a complete lack of $^{14}CO_2$ release (Fig. 11). Catalase, which serves to detoxify H_2O_2 produced in β-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 (Kounchi 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 ß-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 detoping 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 ß-oxidation pathway. The by-product of this pathway is toxic H_2O_2 and accumulation of H_2O_2 suppresses the ß-oxidation pathway (Hashimoto and Hayashi, 1987). Therefore, to continue ß-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 ß-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; Kunce and Trelease, 1986; Drumm and Schopfer, 1974; Tsaftaris *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 ß-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⁻ and Fix⁺ Nodules

Nodules produced by the two fix⁻ 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 <u>early</u> 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⁻ 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⁻ 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[+] nodules of peanut supplement the high energy-demanding process of nitrogen fixation, while in fix 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 β-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 detoping treatments.

- Consistency in leghemoglobin and total protein of the nodule, even after 48 h of dark stress.

- Accumulation of lipid bodies in fix⁻ 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.
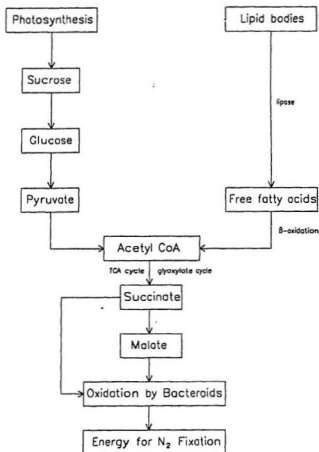
Figure 28

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