OLEOSOMES IN SOME NITROGEN-FIXING ROOT NODULES

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Oleosomes in some nitrogen-fixing root nodules.

By

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ABSTRACT

The high energy-demanding process of nitrogen fixation in symbiotic root nodules is generally supported by a supply of carbon compounds derived from current photosynthate of the host plant. However, in *Arachis hypogaea* L (peanut) nodules, which have oleosomes (lipid bodies) in the infected cells, the lipid catabolism may supplement the energy supply in case of photosynthate stress. The present investigation was undertaken to further study oleosomic metabolism in *Arachis hypogaea* and four other legumes: *A. pintoi* L., *A. duranensis* L., *A. batizocoi* L. and *Lathyrus maritimus* L. (Bigel) (beach pea) nodules where oleosomes are present.

The oleosomes of *A. hypogaea* root nodules contained diacylglycerol (DAG), triacylglycerol (TAG), phospholipids (PL) and oleosins. The oleosomes varied in size, electron density and in the width of a less electron-dense peripheral layer. Four oleosin bands having molecular weights 66.0 KD, 61.1 KD, 56.3 KD and 10.0 KD could be resolved by polyacrylamide gel electrophoresis.

The development of symbiosis and oleosome distribution was studied in three wild species of *Arachis* i.e. *A. pintoi*, *A. duranensis* and *A. batizocoi*. Oleosomes were present in the infected cells of *A. pintoi* during the infection process and before establishment of symbiosis. In *A. duranensis* and *A. batizocoi* oleosomes persisted during symbiosis in mature nodules. *A.pintoi* mature nodules were devoid of oleosomes in infected cells, but reappeared during senescence. Another interesting feature in this species was the reversion of spherical bacteroids into rod-forms within the confines of the senescent nodule tissue.

Studies on the distributional pattern of oleosomes in the root nodules of naturally growing *L. maritimus* (beach pea) revealed that the pre-winter nodules were filled with large numbers of oleosomes and amyloplasts in uninfected interstitial and parenchyma cells. These storage organelles could not be seen in the cells of nodule sampled during post-winter periods before aerial shoots emerged. The results indicate that either the oleosomes are catabolized slowly during the winter months, to allow the nodules to survive the extreme cold temperatures or they are rapidly mobilized just before the growing season. The olesomes in beach pea nodules seem to serve as storage organelles in the uninfected and parenchyma cells and not directly related to nitrogen fixation

per se. The overwintered nodules are capable of resuming nitrogen fixation due to the presence of persistent infection threads with rhiozobia and many rod-shaped *Rhizobium* among the senescent infected cells.

Dedicated to the memory of

my beloved mother

(*Aai : Anusaya*)

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ABBREVIATIONS

BSA	Bovine serum albumin
DAG	Diacylglycerides
DAI	Days after inoculation
DTT	Dithiothreiotol
ER	Endoplasmic reticulum
KD	Kilodalton
PAGE	Polyacrylamide gel electrophoresis
PL	Phospholipids
pPD	p-phenylenediamine
TAG	Triacylglycerides
TLC	Thin layer chromatography
SDS-Page	Sodium dodecyl sulphate - polyacrylamide gel elctrophoresis

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I INTRODUCTION

I.1 General introduction

Nitrogen (N_2), in its elemental gaseous form, constitutes almost four-fifths of the world's atmosphere (Allen and Allen, 1981). This is virtually an inexaustible supply, yet plants and animals can not assimilate nitrogen in its free form. Because nitrogen is an essential component of the proteins and nucleic acids necessary for cell protoplasm, all organisms are dependent on having it available in a form which they can utilize. The relative stability and inertness make it unavailable to eukaryotic organisms. Nitrogen is combined with other elements like hydrogen and oxygen by an endergonic reaction called nitrogen fixation (Gallon and Chaplin, 1987).

Fixation, including industrial nitrogen fixation may be either biological or non-biological. Biological nitrogen fixation involves the reduction of nitrogen to ammonia catalysed by the enzyme complex, nitrogenase in prokaryotic organisms. Diazotrophic bacteria can fix nitrogen at atmospheric temperatures and pressures, whereas the Haber-Bosch process for industrial production of ammonia requires pressure of 200 atm and 800°C - data that provide eloquent testimony to the extraordinary nature of nitrogenase (Glenn and Dilworth, 1991). The net result of these processes is that

nitrogen compounds are added to soil or water; these are then assimilated by plants and microorganisms. This assimilated nitrogen is returned to the soil on the death and decay of these organisms. Within the soil, ammonia and nitrate are reversibly interchangeable by the action of various microorganisms. Animals are able to utilize only nitrogen compounds which have been previously assimilated by plants. Figure 1 depicts the simplest form of nitrogen cycle in which combined form of nitrogen can be lost to the atmosphere by a process known as denitrification (Gallon and Chaplin, 1987).

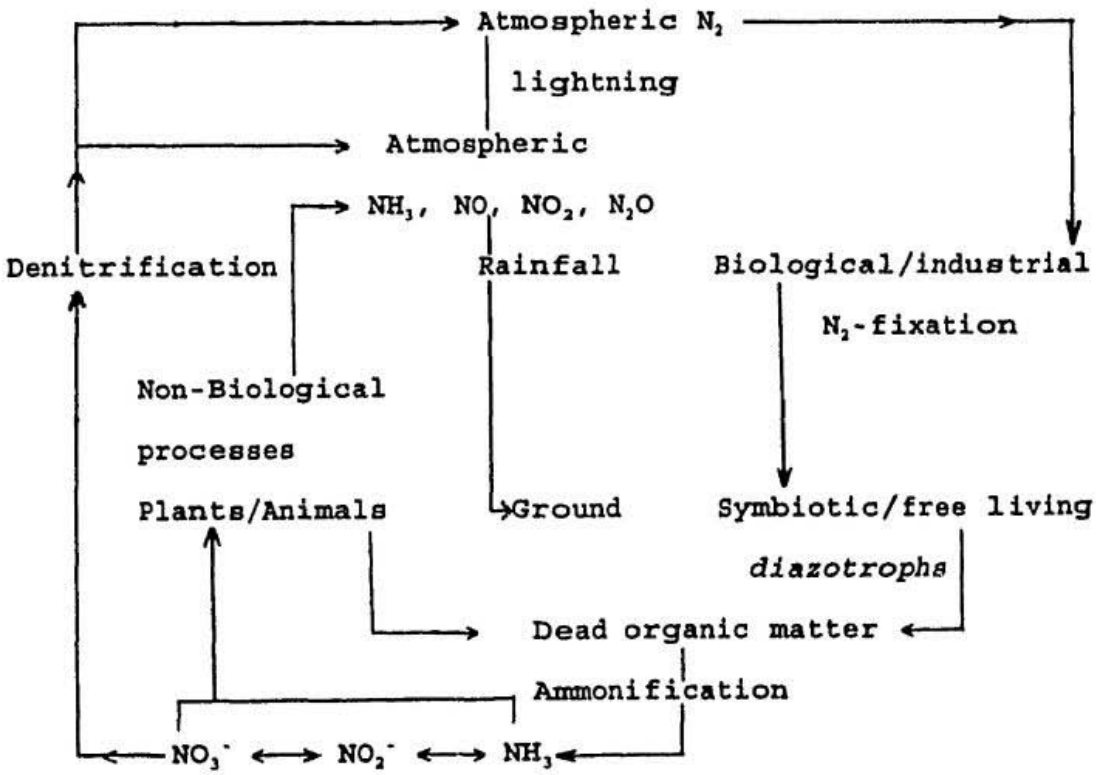


Fig. 1. The nitrogen cycle (modified from Gallon and Chaplin, 1987).

I.2 Biological nitrogen fixation

Biological nitrogen fixation involves the reduction of nitrogen to ammonia by the enzyme complex, nitrogenase. The overall reaction can be represented as:

$$N_2 + 3H_2O \text{ ------> } 2NH_3 + 3/2\ O_2 \quad \Delta G^\circ = +340 \text{ kJ mol}^{-1} \text{ NH}_3$$

The standard free energy change of the reaction is positive, indicating that the reaction requires the input of energy. This must be ultimately derived from the oxidation of carbohydrates which have been produced either directly or indirectly by photosynthesis. Diazotrophic organisms are able to couple the oxidation of carbohydrate to the reduction of the nitrogen (Gallon and Chaplin, 1987). There are no nitrogen fixing higher plants nor indeed any eukaryotic microorganisms that can fix nitrogen. Where plants are involved in the nitrogen-fixing process, it is as partners in a symbiotic association with diazotrophic prokaryotes, the latter being responsible for nitrogen fixation. In fact, the ability to fix nitrogen seems to be exclusively the property of a limited number of prokaryotic species, some of which are free living (asymbiotic) while others fix nitrogen in symbiotic association with plants.

I.2.1 Asymbiotic nitrogen fixation

Asymbiotic nitrogen fixation includes the diverse group of prokaryotes, such as strict anaerobes, *Clostridium*; facultative anaerobes/microaerobes, *Klebsiella*, *Azospirillum*; obligate aerobes, *Azotobacter*, *Beijerinckia*; and some of the chemoautotrophs and photosynthetic bacteria. The nitrogen fixation rates of free living diazotrophs are usually very low, ranging from 0.1 to 100 kg N ha^{-1} yr^{-1} (Boring *et al.*, 1988; Knowles, 1977; Waughman *et al.*, 1981)as compared to the symbiotic association with legumes, 30-300 kg N ha^{-1} yr^{-1} as the requirement of plant growth (La Rue and Patterson, 1981). The major factor limiting asymbiotic nitrogenase is carbon energy supply.

Of these free living diazotrophs *Azotobacters* were the first nitrogen-fixing organisms found to contain more than one type of nitrogenase enzyme (Bishop *et al.*, 1980). The well known molybdenum nitrogenase is synthesized if the metal is present in the environment. If molybdenum concentrations are less than about 10 nM, a vanadium-based enzyme is available for nitrogen fixation in both *A. chroococcum* and *A. vinelandii* (Hales *et al.*, 1986 and Robson *et al.*, 1986). The latter

species may synthesize a third nitrogenase under these conditions (Jacobson *et al.*, 1986). The other important and unique feature of *Azotobacter* is their extreme tolerance to oxygen while fixing nitrogen; mechanisms for protecting nitrogenase against O_2 damage exist alongside a dependence on aerobic metabolism for energy and growth. Additionally, the H_2 evolved during nitrogen fixation can be catabolised by the enzyme hydrogenase (Kennedy and Toukdrian, 1987).

I.2.2 Symbiotic nitrogen fixation

Nitrogen fixing symbioses fall into two main types. One involves interactions between legumes and bacteria of the genus *Rhizobium*. Only one non-legume plant, *Parasponia*, a member of family Ulmaceae has been found to form symbiotic root nodules with *Rhizobium* (Trinick, 1973). The second type involves associations between non-leguminous angiosperms and the actinomycete *Frankia* sp. (Baker and Mullin, 1992 and Simonet *et al.*, 1990). Common to both legume and non-legume symbioses is the fact that the bacteria are housed in specialised structures called nodules. Although the 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 in association with rhizobia. These

nodules have been shown to be capable of high rates of nitrogen fixation (Subba Rao and Yatazawa, 1984).

I.2.3 *Rhizobium* - legume symbiosis

Rhizobium bacteria stimulate leguminous plants to develop root nodules, which the bacteria infect and inhabit. Ultimately, the two organisms establish metabolic cooperation. The bacteria reduce (fix) molecular nitrogen into ammonia, which they export to the plant for assimilation; the plant reduces carbon dioxide into sugars during photosynthesis and translocates these to the root where the bacteria use them as fuel (Long, 1989) and the breakdown products, i.e. organic acids, as metabolites that combine with ammonia.

The plant family Leguminoseae (Fabaceae) is the third largest family in the Angiosperms, 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). The legumes are economically important and unique in having symbiotic associations with nitrogen-fixing rhizobia. There is also considerable specificity of individual strains or species of *Rhizobium* for particular groups of plants, as shown in Table 1.

During a complex series of developmental steps, the bacteria and the plants each influence such fundamental activities as cell division, gene expression, metabolic function and cell morphogenesis. Analyses of the bacterial influence on these processes have led to the identification of otherwise elusive components that are parts of the indigenous plant systems for signal transduction, gene regulation, cell division and cell wall formation. The driving forces of recent study of *Rhizobium* - legume symbioses include bacterial genetics, plant molecular biology and detailed microscopy of the bacteria - plant interaction (Long, 1989).

Table 1 *Rhizobium* - plant association

Rhizobium	Plant
Rhizobium meliloti	Alfalfa (*Medicago*)
Rhizobium leguminosarum	
biovar *viciae*	Pea (*Pisum*)
biovar *trifolii*	Clover (*Trifolium*)
biovar *phaseoli*	Bean (*Phaseolus*)
Rhizobium fredii (*R. japonicum*)	soybean (*Glycine*)
Bradyrhizobium japonicum	soybean (*Glycine*)
Rhizobium loti	Lotus (*Lotus*)

Azorhizobium caulinodans	Sesbania (*Sesbania*)
Rhizobium NGR234	Siratro (*Macroptilium*)
Bradyrhizobium sp.	Peanut (*Arachis*), Cowpea (*Vigna*), Parasponia (*Parasponia*, a non-legume)

(Roth and Stacey, 1991)

I.2.4 *Rhizobium* - the nitrogen fixing bacteria

Rhizobia are gram negative, rod shaped bacteria (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 over a wide range of temperatures under low oxygen tension. They do not produce endospores. Glycogen and poly-β-hydroxybutyric acid are formed as storage granules. Most species of *Rhizobium* are specific in their association with legumes. Rhizobia have been taxonomically grouped and designated particular species based on a cross-inoculation concept proposed by Fred, Baldwin and McCoy (1932) similar to those in Table 1.

Metabolically, the genus *Rhizobium* can be divided into two

broad groups. There are fast growers and slow growers (Allen and Allen, 1950; Elkan, 1981). *Rhizobium* isolated from legumes of temperate origin such as *R.trifolii*, *R. leguminosarum*, *R. phaseoli* and *R. meliloti* are designated fast growers, having generation times of less than six hours, whereas slow growers, such as *R. japonicum* and *R. lupini* isolated from legumes of tropical origin, have generation times exceeding six hours. Differences in carbohydrate nutrition have been reported by several groups (Chakrabarti *et al.*, 1981; Martinez-Drets and Avias, 1974; Parke and Ornston, 1984; Skotnicki and Rolfe, 1977; Stowers and Eglesham, 1983). Enzymatic distinctions have also been made (Hernandez and Focht, 1984). Enzymatic criteria have recently been used to establish the taxonomic relationship of new *Rhizobium* germ plasm such as the stem-nodulating *Rhizobium* (Stowers and Eaglesham, 1983) and fast growing *R. japonicum*. Distinct biochemical properties of fast- and slow-growing rhizobia has led Jordan (1982) to propose that slow growing rhizobia represent a separate genus, *Bradyrhizobium*.

Rhizobium genetics has been greatly advanced by transposon mutagenesis, recombinant cloning and plasmid transfer experiments (Denarie *et al.*, 1981; Kondorosi and Johnston, 1981; and Long 1984). The fast growing *Rhizobium* species typically have large plasmids, one or more of which carry symbiotic genes and are designated p*Sym*. These vary from

R. leguminosarum plasmids of about 200-300 kilobases (kb) to the large "megaplasmids" (1200-1500 kb) of *R. meliloti*. In some other symbionts, such as *Bradyrhizobium*, symbiotic genes are apparently not located on plasmids. Several groups of symbiotic genes -*nod,exo,nif* and *fix*- have been defined (Long, 1989).

I.2.5 Nodule initiation and development

Establishment of a nitrogen fixing symbiosis between rhizobia and legumes is a complex developmental process that involves constant communication between the partners. A series of steps involved in these process have been studied by using bacterial genetics, microscopy and molecular biology to assay the success of interaction.

Nodule development can be divided into stages of pre-infection, nodule initiation and differentiation. The pre-infection stages commence even before the host plant and its compatible *Rhizobium* strain recognize each other as potential partners on a cellular basis. Flavonoids released by the plant serve as chemoattractants and also induce *Rhizobium nod* genes product which associate with the cytoplasmic membranes of rhizobia and appears to interact with the specific flavonoids in root exudates (Maxwell and Phillips, 1990). After

chemotaxis, rhizobia attach to the responsive root hairs (Bhuvaneswari *et al.*,1980). The rhizobia attach to susceptible root hairs via a two step attachment process (Dazzo *et al.*, 1984; Smit *et al.*,1987). First, they loosely attach to a plant receptor via a protein on the bacterial surface known as rhicadesin. Rhicadesin is a calcium binding protein that appears to be common among Rhizobiaceae. Then, tighter adherence occurs either by means of cellulose fimbrils (Smit *et al.*, 1987) or fimbriae (Vesper and Bauer, 1986). Often, the rhizobia are seen to attach to the root hair in a polar or end on fashion (Hersch, 1992). Lectins of the legume root hair have also been implicated at these stages of infection. However, lectins are more likely to be involved in invasion rather than attachment of rhizobia (Kijne,1992; Roth and Stacey,1991). Entry of bacteria appears to occur at the root hair tip, probably because the cell wall is thinner and less cross-linked there than elsewhere. Susceptible root hairs deform into a number of unusual shapes after inoculation with rhizobia, including corkscrews, branches, twists, and spirals. A few of the deformed root hairs coil 360^0 and form diagnostic curls known as 'shepherd's crooks'. Root hair deformation is dependent on the presence of functional *Rhizobium nod* genes (Hersch, 1992).

A sulphated and acylated tetraglucosamine glycolipid called NodRm-1 was identified as the secreted product of the

nod gene activity in *R. meliloti* (Lerouge *et al.*, 1990). NodRm-1 elicits root hair deformations when added in nanomolar concentrations to asceptically grown seedlings of alfalfa. This compound also stimulates cortical cell divisions (Roche *et al.*, 1991) which establish the nodule primordium (*Noi*; nodule initiation). In the fast growing *Rhizobium* sp., the *nod* genes are located on a plasmid whereas in the slow growing *Bradyrhizobium*, they are chromosomally borne (Gyorgypal *et al.*, 1991).

After inducing 'shepherd's crook' formation, the rhizobia penetrate the root hair cell by means of an infection thread. An electron microscopic study (Callaham and Torrey, 1981) has shown that rhizobia cause the dissolution of the plant cell wall at a specific point, while others (Nutman, 1956) proposed earlier that the infection thread forms via a process of cell wall invagination (Pueppke, 1986). Recent studies by Bakhuizen (1988) support Callaham and Torrey's observations. Following dissolution of cell wall, the plasma membrane of root hair invaginates, and cell wall material is deposited around it and the rhizobia within. The host cell nucleus is attached by microtubules to the infection thread as it passes through the root hair cell (Bakhuizen, 1988; Lloyd *et al.*, 1987). The bacteria travel from host cell to host cell via the infection thread and its branches. However, some tropical legumes such as *Arachis* (peanut) and *Stylosanthes*, rather promiscuous host

plants, do not form infection threads in the root hairs, instead they are nodulated from sites of lateral root emergence where epidermal cracks are the points of entry into the intercellular space; cell divisions are induced in the cortex of emerging lateral root (Chandler, 1978 and Chandler *et al.*, 1982). After the preinfection stages, cortical cell divisions take place several cells distant from the infection thread. Cell divisions occur either in the outer or inner cortex of the root. The type of nodule that develops depends on the host plant, not on the rhizobial strain (Dart, 1977; Newcomb 1981). Two major types of nodules are found on the roots of legumes. The indeterminate type is characterized by a persistent apical nodule meristem, while the determinate type has diffuse meristematic activity which may cease after a certain period. The persistent apical meristem causes indeterminate nodules to be elongated and club-shaped because new cells are constantly being added to the distal end of nodule. All stages of nodule development are represented in one nodule because an age gradient occurs from the distal meristem to the proximal point of the attachment to the parent root. Plants having indeterminate nodules include clover, alfalfa and pea. In contrast, determinate nodules are spherical. Cell divisions cease early during nodule development and the final form of the nodule results from cell enlargement rather than cell division. Nodules of soybean,

mungbean and peanut are examples of determinate nodules (Hersch 1992).

During symbiosis, the host plant expresses a certain number of proteins specific to nodule development and nitrogen fixation called nodulins (Legocki and Verma, 1980). Many early *nodulin* genes are expressed sequentially during nodule differentiation, and some of these genes can be induced in nodules devoid of bacteria (Nap and Bisseling, 1990). These studies showed that some of the early nodulins are involved in the early infection process, whereas others participate in root nodule morphogenesis. The late *nodulin* genes are expressed concomitant with or following the release of bacteria from the infection thread but prior to the induction of nitrogenase and the commencement of nitrogen fixation (Verma and Delauney, 1988). One late nodulin, leghemoglobin, is largely reponsible for transporting oxygen throughout the infected region of the nodule (Appleby, 1984).

I.2.6 Internal compartmentalization

The formation of a subcellular compartment housing the bacteria inside the infected cell is the final stage of successful infection. The failure to form this membrane compartment or its disintegration renders the association

pathogenic (Werner *et al.*, 1985). Infection by rhizobia triggers the proliferation of the membrane system that generates components of the peribacteroid membrane (PBM). Continued proliferation of this membrane is essential to enclose rhizobia so that direct contact of the bacteria with the host cytoplasm is avoided. In soybean root nodules, almost 30 times more membrane is generated in the form of PBM than in the form of plasma membrane (Verma *et al.*, 1978). Because the rhizobia are enclosed whithin the PBM, the space between the bacteria and the PBM, which is known as the peribacteroid space, must be equilibrated with certain metabolites, including dicarboxylic acids (used as carbon sources by bacteroids), to eliminate the concentration gradient between the host and the rhizobia. In a sense, this internalizes the organism and brings it into the closest association possible with the host. Equilibration of the peribacteroid space is apparently accomplished by opening some specific channels in the PBM (Verma, 1992). The PBM is relatively impermeable to various sugars and amino acids that have been tested (Udvardi *et al.*, 1988a; Udvardi *et al.*, 1990) but a dicarboxylate carrier in the PBM facilitates rapid transport of dicarboxylic acids to the bacteroids (Ou Yang *et al.*, 1990; Udvardi *et al.*, 1988b). Nodulin-26 is probably the PBM protein responsible for dicarboxylate transport (Ou Yang *et al.*, 1991). Protein phosphorylation stimulates the rate of malate uptake across

the PBM of soybean nodules and this may be important in controlling the bacteroid carbon supply during symbiotic nitrogen fixation (Ou Yang *et al*, 1991). There is also an electrogenic H^+-ATPase in the PBM that could be involved in metabolite transport across this membrane (Udvardi and Day 1989; Udvardi *et al*., 1991). It is becoming clear that the PBM defines a cellular compartment where *Rhizobium* can function as an "organelle" (Verma and Long, 1983). A term 'symbiosome' has been introduced to describe the bacteroid in the PBM including the peribacteroid space (Mellor, 1989).

I.2.7 The bacteroid

The term bacteroid has been used to describe a variety of structures, including swollen forms of *Rhizobium* in cultures and some or all the cells present in legume nodules. According to Sutton *et al*.(1981) "bacteroids" refer to all *Rhizobium* cells found within the central tissue cells of legume root nodules, without regard to morphology or physiology. *Rhizobium* cells located in infection threads or nodule intercellular spaces are regarded as extracellular and are therefore referred to as bacteria. The size and shape of bacteroids and the number enclosed in each peribacteroid membrane are largely determined by the plant, since bacteroids of different

Rhizobium strains in effective nodules of a given host nearly always take on the same morphology. In some cases it has been shown that a single *Rhizobium* strain forms bacteroids of differing morphology in different hosts. The most dramatic example being rod-shaped bacteroids in peribacteroid membranes in cowpea root nodule as contrasted with large, spherical bacteroids enclosed singly in peribacteroid membranes in peanut (Dart, 1977; Sen *et al.*, 1986). The surface structure of *Rhizobium* is similar to other gram-negative bacteria in having a complex outer membrane around the cell membrane (Vincent, 1977). Inside the nodule cells of the host, the rhizobia undergo transformation into nitrogen-fixing bacteroids with a modifications in their outer membrane (Bergerson, 1974; Brussel *et al.*, 1977). Such modifications of the outer membrane of *Rhizobium* bacteroids are reflected in their response to osmotic shock and chemical composition (Brussel *et al.*, 1977). Bal and co-workers (1980, 1982 and 1985) have shown that the rhizobia shed their outer membranes which are then replaced by a new outer membrane soon after their release into host cells. Bacteroids from *Rhizobium* as well from *Bradyrhizobium* strains can redifferentiate to free living viable cells (Sutton *et al.*, 1977 and 1981).

I.2.8 Nitrogenase and nitrogen fixation

Nitrogenase is the unique enzyme involved in reduction of nitrogen. In rhizobia, this enzyme is expressed during symbiotic nitrogen fixation. The nitrogenase is also expressed in certain free living bacteria such as *Azotobacter, Klebsiella, Clostridium* and Cyanobacteria. Nitrogenase is comprised of two easily separable proteins designated the iron protein (Fe protein) and the molybdenum-iron protein (MoFe protein). The iron protein (encoded by the *nif H* gene) is a homodimer with a native molecular weight of 60-80 KD and subunit molecular weight of 30-32 KD (Burgess, 1984). The larger (200 KD) subunit is a MoFe protein which binds the reducible substrate (Hagerman and Burris, 1978) and the smaller (60 KD) the Fe-protein, interacts with ATP and Mg^{2+} in a hydrolytic reaction. Both proteins are irreversibly inactivated by oxygen, the Fe protein most rapidly in vitro. Protection of these proteins from damage by oxygen is one of the overriding challenges in the physiology of nitrogen fixation (Postgate, 1974).

Thorneley and Lowe (1985) proposed a model which attempts to describe the mechanism of nitrogenase action in the fixation of nitrogen and production of hydrogen. This model

consists of two cycles. In the Fe protein cycle, electrons are passed first to the Fe protein, and then from the Fe protein to the MoFe protein. Experimental evidence suggests that two MgATP must be bound to the reduced Fe protein before this molecule can form a reversible complex with the MoFe protein. Once complexed, the reduced Fe protein can transfer the electron to the MoFe protein. This oxidation-reduction step is coupled to the MgATP hydrolysis, and is effectively irreversible. The Fe protein:MgADP:reduced MoFe protein complex can undergo a reversible dissolution. The oxidized Fe protein:MgADP complex can be reduced and the ADP exchanged for ATP, while the reduced MoFe protein can return to acquire additional electrons from the Fe protein through the Fe protein cycle. In total,8 electron transfers to the MoFe protein are required to reduce N_2 and produce H_2, and between each electron transfer the Fe protein:MoFe protein complex dissociates completely. This cycle of 8 electron transfers has been called the MoFe protein cycle (Layzell, 1990).

I.3 Nitrogen assimilation and transportation

Bergersen (1965) and Kennedy (1966) demonstrated that NH_4^+ is the stable product of nitrogen fixation in legume nodules. The NH_4^+ produced is excreted by bacteroids (Bergerson and

Turner, 1967) into the host cell cytoplasm where it is assimilated and used in the synthesis of organic nitrogen for transport.

Meeks *et al.*(1978) showed by using $[^{13}N]N_2$, a short-lived radioactive isotope of N, that NH_4^+ is first incorporated into the amide position of glutamine in the reaction catalysed by glutamine synthetase. The amide group is subsequently transferred to the 2-carbon of oxoglutarate in the reductive amination reaction carried out by glutamate synthase. Both enzymes have been isolated and purified from legume nodules (Boland and Benny 1977; Cullimore *et al;* 1983; McParland *et al.*, 1976). Glutamine synthetase is localised totally in the cytoplasm while glutamate synthase exists in the cytoplasm and in the plastid (Awonaike *et al.*, 1981; Boland et al; 1982; Shelp & Atkins 1984; Shelp et al; 1983). Both enzymes are induced during nodule development (Atkins et al; 1984; Boland *et al.*, 1978; Groat and Vance, 1981; Reynolds *et al.*, 1982; Robertson *et al.*, 1975; Schubert *et al.*, 1981).

Nitrogen-fixing plants can be classified as amide exporters or ureide exporters based on the composition of the xylem fluid collected from excised nodules or nodulated root systems. The amide exporters transport asparagine, glutamine or 4-methyleneglutamine while ureide exporters transport either allantoin and allantoic acid or citrulline. Legumes of tribes Vicieae, Genisteae, and Trifolieae are generally amide

exporters. These tribes of more temperate origin include pɔa, lupin, broad bean, alfalfa and clover. Based on results of $^{15}N_2$-labelling studies, asparagine is synthesized from the product of recent nitrogen fixation in nodules of amide-exporting symbioses (Aprison *et al.*, 1954, Kennedy, 1966; Leaf *et al.*, 1959).

Fowden (1954) reported an unusual nonprotein amino acid amide 4-methyleneglutamine in peanuts. Tropical legumes of the tribe Phaseoleae synthesize and transport the ureids allantoin and allantoic acid from recently fixed nitrogen. These two compounds account for 60 to 90 % of the total nitrogen in the xylem sap of soybeans (McClure and Israel, 1979), cowpeas (Pate *et al.*, 1980), garden beans (Pate, 1973) and other legumes (Pate *et al.*, 1980). One tropical legume, peanut (*Arachis hypogaea*), is reportedly an amide exporter.

I.4 Symbiotic nitrogen fixation by Peanut and Beach Pea

I.4.1 Peanut

The genus *Arachis* belongs to the family Fabaceae (Leguminosae) and sub-family Papilionoideae. It is found in tropical and the 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. Peanut 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 structures 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 the 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 nector for bees. The seeds are a rich source of vitamin B complex, especially thiamine, 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 glycerides of oleic and linoleic acids. The crop yield varies from 742 to 4400 kg/ha (Duke and

Henson, 1985).

The genus *Arachis* has long been known to nodulate with rhizobia from diverse species of plant hosts but relatively few strains of rhizobia are capable of high levels of fixation (Allen and Allen 1940; 1981). In spite of these early observations *Arachis* has been considered by some as a promiscuous and an effective nitrogen fixer (Date, 1977; Peoples *et al.*, 1989). According to Graham and Hubbell (1975) and Date (1977), the species will nodulate effectively with a range of rhizobia from many different legumes. However, as pointed out by Gillar and Wilson (1991) and Singleton *et al.* (1992), the classification of tropical legumes and especially forage legumes, as promiscuous effective, promiscuous ineffective or specific as defined by (1977), tends to lose its usefulness as greater ranges of rhizobial strains are tested and increasing numbers of exceptions to the classification scheme are reported (Thomas, 1993).

The mode of infection of *Arachis* is, like that of *Stylosanthes*, rather unusual as entry of rhizobia into the plant is via wound or crack infections at the junction of lateral roots rather than via the classical mode of entry through root hairs (Chandler, 1978; Sprent and Sprent 1990). In stoloniferous species such as A.*pintoi*, a perennial forage, nodules can be observed frequently in the axils of roots emerging from stolons (Thomas, 1993).

The nodules of *Arachis* are further distinguished from those of other legumes in that they have an unusually low number of viable rhizobial cells per unit biomass of nodule tissue. In these nodules and those of *A.erecta*, *A.nambyquarae*, and *A.villosulicarpa*, the bacteroids appear to take on the unusual appearance of spheroplast-like structures (Staphorst and Strijdom, 1972). These structures were later found to have a distinct cell wall or outer membrane (Bal *et al.*, 1985). Peanut also shows distinctly different patterns of lectin accumulation during symbiotic interactions with homologous strains of (brady)rhizobia than do other legumes (VandenBosch *et al*, 1994). Presence of lipid bodies, more appropriatly called oleosomes, have also been reported in peanut nodules (Jayaram and Bal, 1991) and their role in providing supplementary source of energy for nitrogen fixation during photosynthate stress has been suggested (Siddique and Bal, 1992).

Rates of nitrogen fixation for peanut nodules (*Arachis hypogaea)* range from 68-206 kg/ha nitrogen with the proportion of the plant's nitrogen obtained from fixation ranging from 47-92% (Gillar and Wilson, 1991). The literature on the wild species of *Arachis*, which were used in this study, is very limited. Recent emphasis has been on the use of wild *Arachis* species for genetic improvement of cultivated or forage peanut because some of them posses superior characters. The present

investigation is an attempt to understand the *Rhizobium*-peanut symbiosis with special reference to oleosomes.

I.4.2 Beach Pea

Lathyrus maritimus (L.) Bigel, commonly known as beach pea, grows along the shorelines of arctic and subarctic regions from Greenland to Siberia and Japan (Fernald, 1950). In Canada, it is found in Newfoundland, Nova Scotia and Quebec (Hitchcock 1952; Lamourex and Grandtner 1977, Scoggan 1950). There are about 130 species in the genus *Lathyrus* consisting of climbing and herbaceous perennials. This genus belongs to the tribe Vicieae of the subfamily Papilionoideae and the family Leguminoseae (Fabaceae). *Lathyrus* belongs to the so-called pea cross-inoculation group. The work of Carrol (1934) and Wilson (1939) confirmed the mutual relatedness of *Lathyrus*, *Pisum*, *Vicia* and *Lens* and their rhizobia.

According to Allen and Allen (1981), comparatively few members of the genus have been studied. The literature on the species *L.maritimus*, which is used in these studies, is very limited. Most of the work done on this genus has been concentrated on those species that cause lathyrism. Symptoms of lathyrism in man usually appear after eating seeds of the plant, commonly occuring as a paralysis of the muscles below

the knee, pains in the back followed by weakness and stiffness of the legs and progressive locomotive incoordination (Kuo et al., 1994). Priliminary assay has shown the neurotoxin to be extremely low in the seeds of *L. maritimus* seems (Shahidi 1995, personal communication).

Recently the symbiotic association of *Rhizobium* strains and *L. maritimus* inhabitant of subarctic region has been reported by Barimah-Asare and Bal (1994). The isolate of *L.maritimus* is reported to have a fast-growing *Rhizobium leguminosarum* biovar *vicia*, which has a wide range of pH and salt tolerance, and could infect only *Vicia cracca* but not *Vicia faba* (faba bean) or *Pisum sativum* (pea). Nitrogenase activity in *L. maritimus* was found to be highest at 20^{0}C but could be maintained with lower levels of activity at 5 ^{0}C (Barimah-Asare, 1991).

Barimah-Asare and Bal (1994) recently reported that the nodule anatomy and the fine structure of beach pea is similar to other perennial indeterminate forms and further suggested that the oleosomes present in the uninfected parenchyma cells of these nodules may serve as a food reserve, to be mobilized during activation of the nodule meristem at the beginning of growing season.

I.4.3 Oleosomes

Plant seeds store triacylglycerols (TAG) as food reserves for germination and postgerminative growth of the seedlings (Ching, 1970). The TAGs are present in small discrete intracellular organelles called oil bodies (Huang, 1985; Huang *et al.*, 1991; Yatsu and Jacks, 1972), lipid bodies (Ching, 1972; Lin and Huang, 1983), or oleosomes (Yatsu *et al*, 1971). In this text the term oleosome has been used. Isolated oleosomes have a spherical shape and possess diameters ranging from 0.5 to 2.5 μm. They contain mostly TAG and small amounts of phospholipids (PL) and proteins. Electron microscopy of the oleosomes shows that the organelle has an electron-opaque matrix of TAG surrounded by one electron-dense layer, a half-unit membrane of one phospholipid layer (Yatsu and Jack, 1972) with unique proteins termed oleosins. These oleosomes are remarkably stable either inside the cell or in isolated preparations. The physiological significance of maintaining the population of small descrete oleosomes is to provide ample surface areas for the attachment of lipase to the organelles during postgerminative growth so that the reserve TAG can be mobilized rapidly. How the oleosomes maintain their small sizes without coalescing is unknown (Tzen and Huang, 1992). Oleosomes are abundant in plant seeds, and are among the

simplest organelles in eukaryotes. Similar organelles can be found in the pollen (Stanley and Linskens, 1974), and the peanut root nodule (Jayaram and Bal, 1991) of angiosperms as well as in tissues of more primitive plants, such as the megagametophytes of gymnosperms (Ching, 1970) and the spores of ferns (Gemmrich, 1981). Intracellular storage lipid organelles of similar structure are also present in tissues of nonplant species, including the brown adipose(Gurr, 1980) and other tissues of mammals (Fawcett, 1966), eggs of some nematodes and other nonmammals (Rubin and Trelease, 1976), and unicellular organisms such as yeast (Clausen *et al.*,1974), *Euglena* (Osafune *et al.*, 1980) and algae (Roessler 1988).

Ultrastructural investigations of maturing embryos of different oil producing plants and of anise cell-suspension cultures indicated that lipid bodies originate from the endoplasmic reticulum (ER) by insertion of TAGs into the hydrophobic space of the phospholipid bilayer (Werner *et al.*, 1985). In contrast, other studies have shown that oleosomes develop in the cytoplasm without contact with the ER (Bergfeld *et al.*, 1978). The biosynthesis of TAG has been analysed in different plant cell (Dutta and Appelqvist, 1989; Kleinig *et al.*, 1978) and embryo cultures (Dutta *et al.*, 1991). Several breeding programmes focus on the development of oil plants producing TAGs with an altered composition of acyl moities (Dutta and Appelqvist, 1989; Ellenbracht *et al.*, 1980; Pence

et al., 1981; Weber and Taylor, 1990).

There is evidence that TAGs and oleosins are synthesized concomitantly in the ER, from which a nascent, mature oleosome is formed by budding (Loer and Herman, 1993; Tzen *et al.*, 1993). Other investigations, however, show that the accumulation of oleosins lags temporally behind that of TAG (Cummins and Murphy, 1990; Hills *et al.*, 1991).

I.4.4 Oleosomes in nitrogen fixation

Carbon compounds derived from the host cells are essential for symbiotic nitrogen fixation. Carbohydrate metabolism provides ATP for the high-energy requiring process of nitrogen fixation, electrons for the reduction process, and carbon skeleton for the incorporation and transport of fixed nitrogen (Dilworth and Glenn, 1984).

Bal and coworkers (see below), recently reported the presence of oleosomes in temperate and tropical root nodules and their possible involvement in nodule function and nitrogen fixation is being investigated.

In tropical legumes such as peanut, the root nodule oleosomes were found to be different than seed oleosomes with respect to the presence of an electron-dense rim, showing lipolytic activity and higher amounts of saturated fatty acids

(Jayaram and Bal, 1991). In nodules induced by fix⁻ strain of *Bradyrhizobium*, oleosomes accumulate in the order of two to three times higher than in the nodules induced by effective wild-type strains (Bal and Siddique, 1991). Results from experiments done in dark treated and detopped peanut plants, where nitrogenase activity was maintained for prolonged periods, the number of oleosomes was found to decrease. The correlation between nitrogen fixation (acetylene reduction) and oleosome degradation in the absence of photosynthate has been clearly demonstrated (Siddique and Bal, 1992). These and other results (Bal *et al.*, 1989; Hameed and Bal, 1985) support the hypothesis that oleosomes serve as a supplementary source of energy in peanut root nodules during photosynthate stress.

In temperate legumes such as beach pea and *Oxytropis arctobia* the oleosomes disappear in the symbiotic stage from infected cells, but remain in the nodule parenchyma. It has been suggested that in arctic legumes, lipids may be involved in protecting the host tissues from low temperature stress (Newcomb and Wood, 1986) and also for membrane proliferation, growth and development of symbiosomes (Barimah-Asare and Bal, 1994; Prevost and Bal, 1994).

I.5 Objectives

To study the oleosomic metabolism in symbiotic nitrogen-fixing root nodules.

Oleosomes have been involved as a supplementary source of energy and carbon in the root nodules of *Arachis hypogaea*. Their metabolism during symbiosis provides an additional advantage for the energy demanding process of nitrogen fixation. It is therefore necessary to evaluate other legume nodules for this trait and to understand in detail the functions of oleosome at different stages of development and in different tissues. The following are the specific objectives for this investigation.

- to isolate the oleosomes and characterize neutral lipids, phospholipids and oleosins from nitrogen-fixing root nodules of *A. hypogaea*.

- to study the oleosome distribution in wild species of *Arachis* i.e., *A. pintoi*, *A. duranensis* and *A. batizocoi* root nodule in relation to nodule development.

- to study the oleosome distribution in the perennial root nodules of *L. maritimus*.

II. MATERIALS AND METHODS

II. 1 Seed source and planting

Seeds of the diploid perennial peanut (*Arachis pintoi* L cv. No. 17434), recently developed at Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia were donated by Dr. R.J. Thomas. Seeds of other diploid annual peanuts (*A. batizocoi* and *A. duranensis* L.)were obtained through the courtesy of Dr. H. Thomas Stalker at North Carolina State University, USA, and seeds of the tetraploid cultivated *A. hypogaea*, cv. Jumbo Virginia were purchased from W. Atlee Burpee Co., Warminster, PA., USA (Table 2). Peanut seeds were either germinated first or directly planted in sterile vermiculite and inoculated with *Bradyrhizobium* sp. from a broth culture as described by Sen and Weaver (1980). The planted pots (6" STD) 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 nitrogen free nutrient solution (Elfolk, 1960).

II. 2. *Bradyrhizobium* cultures

Bradyrhizobium strain 32H1 was obtained from Nitragin, Milwaukee and 7091 (Nod^+ Fix^-) was obtained from Dr. P.T.C. Nambiar, International Crop Research Institute for Semi-arid Tropics (ICRISAT), Patancheru, India. The other effective

strain of *Bradyrhizobium* sp. CIAT 3101 recommended for *A.pintoi* (Thomas, 1993) was donated by Dr. R.J. Thomas, CIAT, Cali, Columbia (Table 2). All the rhizobial strains were maintained on yeast extract mannitol (YEM) broth medium containing, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4$, $7H_2O$; 0.1 g of NaCl, 0.4 g of yeast extract, 10 g mannitol and 1 L of distilled water at pH 6.8- 7.0. (Vincent, 1970) with constant shaking (140- 150 rpm) at 30°C in an Orbit Environshaker, Lab-line Instrument Inc.

Table 2 Source and relevant characteristics of the legume species and *Bradyrhizobium* strains.

Species/ Strain	Genotypic/ Phenotypic characters	Source
a) Legume		
A. *hypogaea*	4n, seasonal, cultivated	W.Atlee Burpee Co.,PA.,USA
A. *duranensis*	2n, annual, wild	H. Stalker
A. *batizocoi*	2n, annual, wild	H. Stalker
A. *pintoi*	2n, perennial, wild	R. Thomas
L. maritimus	2n, perennial, wild	This study

b) *Bradyrhizobium*

32 H1	nod$^+$ fix$^+$ for *A. hypogaea*	Nitragin
CIAT 3101	nod$^+$ fix$^+$ for *A. pintoi*	R. Thomas
7091	nod$^+$ fix$^-$ for *Arachis*	P.Nambiar

II.3 Isolation of oleosomes from *A. hypogaea* root nodule and seed.

Arachis hypogaea (Jumbo Virginia) plants, inoculated with *Bradyrhizobium* effective strain 32H1 were grown as mentioned in Section II.1. All the plants were uprooted at 35 DAI and nodules were collected and frozen at -70°C until use.

The method employed for isolation of the oleosomes was essentially of Tzen and Huang (1992) with minor modification. The nodule and seed material was homogenized at 4°C in grinding medium (10g of nodules/20 mL, and 2g of peanut seed/20 mL) with pestal and mortar. The grinding medium contained 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 2mM DTT, and 0.15 M TRICINE adjusted to pH 7.5 with KOH. The homogenate was filtered through cheesecloth. After filtration, each 20 mL portion of the homogenate was placed at the bottom of a 40 mL centrifuge tube, and 10 mL of floatation medium (grinding medium containing 0.4 instead of 0.6 M sucrose) was

layered on top. The tubes were centrifuged at 10,000g for 30 min in a Sorvall SA-600 rotor. The oleosomes floating as a thin white layer on top were collected, and resuspended in 10 mL of grinding medium containing an additional 2M of NaCl. The resuspension was placed at the bottom of a 40 mL centrifuge tube, and 10 mL of floating medium (grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top. The tubes were centrifuged again as above. The fat pad on top was collected, and resuspended in 10 mL grinding medium. The resuspension was placed at the bottom of a 40 mL centrifuge tube, and 10 mL of floating medium (grinding medium containing 0.4 instead of 0.6 M sucrose) was layered on top. The contents was centrifuged as above. The fat pad on top was collected, and resuspended with grinding medium.

II.3.1 Separation of neutral lipids, phospholipids and proteins from isolated oleosomes of *A. hypogaea* root nodule and seed.

The method employed for this experiment was also essentially that of Tzen and Huang (1992) with modifications as below. All the steps were carried at 4°C. A 500 μl preparation of isolated oleosome was extracted with 750 μl diethyl ether in a 1.5 mL Eppendorf tube. After centrifugation at 13,600g for 4 min, the upper ether layer was

collected. The lower aqueous layer and the interfacial materials were extracted with 750 μl diethyl ether two additional times. The ether fractions (which contained neutral lipids) were pooled (2.25 mL), and the ether was evaporated under nitrogen gas. The aqueous layer, together with the interfacial materials, was placed under nitrogen in order to evaporate the remaining ether. A volume of 500 μl chloroform/methanol (2:1 v/v) was added. After gentle shaking, the tube was centrifuged at 13,000g for 4 min the lower chloroform layer (which contained PL), the upper methanol/water layer, and the interfacial materials (which contained oleosin proteins) were collected individually.

The chloroform fraction (which contained PL) was washed two times each with 1 mL methanol/water (1:1 v/v) followed by centrifugation.

The interfacial materials were washed three times by the following procedure. The interfacial fraction from root nodule (150 μL) and mixed with 300 μL chloroform/methanol (2:1 v/v). The interfacial fraction of seed (250 μL) was mixed with 500 μL chloroform/methanol (2:1 v/v). These mixtures were then centrifuged at 4°C. The interfacial materials were collected and resuspended in 150 μL of water in case of nodule and 250 μL of water in case of seed. Final interfacial material remained in aggregates; it was vigourously vortexed for dissolution.

II.3.2 Analyses of oleosome constituents isolated from *A. hypogaea* root nodule and seed.

The lipid and protein contents in the above ether fraction, the chloroform fraction, the methanol/water fraction, and the interfacial fraction were analysed by thin layer chromatography (TLC) and SDS-PAGE, respectively. The molecular weight of purified protein was determined by using Bio-Rad low molecular weight standards. Lipid standards obtained from Sigma for neutral lipids and phosphatidylethanolamine or phosphatidylecholine for PL were run along with the sample for TLC. The TLC plate (Silica Gel 60A from Whatman) was developed in hexane/diethyl ether/acetic acid (80:20:2;v/v/v) for the separation of neutral lipids. After drying, the plate was further developed briefly in chloroform/acetic acid/methanol/water (70:25:5:2; v/v/v/v) in order to allow the separation of PL from the origin. The plate was allowed to react with iodine (Tzen *et al.*, 1992).

For SDS-PAGE, the separating gel and the stacking gel consisted of 12.5 and 4.75% polyacrylamide respectively. After electrophoresis, the gel was stained with Coomassie blue R-250 and destained according to Laemmali (1970).

The neutral lipids in the ether fraction were weighed gravimetrically. The quantity PL was not determined. The proteins in the oleosome fractions after ether extraction were quantitated by the Lowry method (Lowry *et al*, 1951) using BSA as a standard.

II. 4 Peanut root nodule samples for microscopy

Based on general morphological observations two similar plants were selected for nodule sampling at weekly intervals up to the 10th week post-inoculation. From these plants, two nodules at three different locations on the tap root were selected for microscopical analysis. These three locations were at the collar region (about 2 cm. below the vermiculite), middle of the tap root (about 5 cm below the vermiculite) and at the growing end of the tap root. This selection of nodule samples was done because at any given time nodules at different stages of development may be present in the root system.

The root nodules were classified in three different developmental growth stages based on the color and size of the nodule as : (1) immature/small/white: 1.0 - 1.5 mm in dia., (2) mature/medium/pink: 1.5 - 2.0 mm in dia. and (3) mature/large/red : > 2.0 mm in dia.

II.4.1 Light, scanning and transmission electron microscopy (LM, SEM and TEM)

Thin slices of nodules of different stages of development were fixed in a mixture of glutaraldehyde (5%) and paraformaldehyde (4%) in 0.1 M phosphate buffer, pH 7.2 for 2h (Karnovsky, 1965), washed in the buffer and post-fixed in 1% osmium tetroxide (OsO_4) in the same buffer for 1 h at 4^0C. The samples were then washed three times with buffer and dehydrated through an ethanol series up to 100% and embedded in Spurr's embedding medium (Spurr, 1969). For lipid preservation, nodule samples were *en bloc* stained with 1% P-phenylenediamine (pPD) in 70% ethanol for 1 h during dehydration (Bal, 1990). Control samples were treated with hexane for 45 minutes after fixation in the aldehyde mixture and subsequently dehydrated in ethanol series prior to OsO_4 treatment. The samples were then rehydrated, treated with OsO_4 and 1 % pPD and processed as described above.

Some of the nodule samples were also sliced and fixed as above for scanning electron microscopy. After dehydration the samples were dried at critical point and gold coated in a sputter coating unit. The observations were made using an Hitachi S570 scanning electron microscope.

The semi-thin sections (1.5 μm) of pPD-stained samples were viewed with a light microscope without further staining.

Ultrathin sections were post-stained with uranyl acetate and lead citrate and viewed with a Zeiss EM 109 transmission electron microscope. The area occupied by oleosomes in the cells in magnified photomicrographs was measured using a Zeiss MOP-3. For oleosome counts, two slides of each block of each tretments with several sections were used.

The olecsome fraction (fat pad) (section II.3) isolated from peanut root nodules was also processed for electron microscopic studies as described earlier.

II.4.2 Induced senescence in A. *pintoi*

The naturally occurring senescence was observed in 70 DAI plant nodules with LM, SEM and TEM. Isolation of the viable cells of *Bradyrhizobium* from the senescent and effective nodules were made and viability counts were enumerated by dilution plate technique. The squash preparations were also performed from the same nodules.

To induce premature senescence, 5 plants at 42 DAI (day after inoculation) were subjected to detopping by cutting the shoot 5 cm above soil level and after two days nodules were fixed for microscopical studies (section II.3). Control plants were left intact. In another treatment plants were irrigated with 20 mM KNO_3 added to Ellfolk nutrient solution (500

mL/pot) (De Lorenzo *et al*, 1994) at 12 DAI and then the plants were irrigated with nitrogen free Ellfolk nutrient solution upto 42 DAI; nodules were sampled for microscopy (section II.3). Control plants received no added combined nitrogen.

II.5 Seasonal effect on storage organelles in naturally grown *L. maritimus* root nodule.

We studied the seasonal effect on oleosomes and also on amyloplasts in *L. maritimus* root nodules from Newfoundland under natural environmental conditions. Bellevue beach in Newfoundland, on the shoreline of the Atlantic Ocean (Trinity Bay), provided an ideal site for naturally growing beach pea plants; the substratum consists mainly of gravel. The site is easily accessible by the Trans Canada Highway.

Nodules were collected by gently removing the gravel and exposing the underground rhizomatous stem which had adventitious roots with nitrogen-fixing nodules. Trips were made to the site in the beginning of the spring session before the aerial shoots of plants were visible. Plants were selected from three to four differerent spots and were marked by wooden sticks which made it possible to sample nodules from the same plants each time.

In samples taken in May 30, 1993, April 30, 1994 and May 22, 1995 the nodules were collected from the underground frost

in the gravel, the air temperature being -1°C to 4°C. Samples were also taken on October 16, 1993, October 2, 1994 and October 6, 1996, when the plants were preparing for overwintering, after the fruiting season. Nodules were sampled in June, July and August as well.

Nodules were sliced longitudinally with a sharp razar blade and immediately fixed in a mixture of paraformaldehyde and glutaraldehyde in phosphate buffer, PH 7.0 (Karnovsky, 1965) and were brought back to the laboratory for subsequent processing for light and electron microscopy as decribed in Section II.3. *En bloc* staining for oleosomes was achived by using p-Phenylendiamine (Bal, 1990).

III RESULTS

III.1 Neutral lipids, phospholipids and oleosins in root nodule and seed oleosome fractions of *Arachis hypogaea*.

Isolated oleosomes from *A.hypogaea* root nodules and seed were fractionated into neutral lipids (TAG & DAG), PL and *oleosins as mentioned in section* II.1.1. The total lipids and proteins obtained are presented in Table 3. The different fractions i.e., ether fraction (neutral lipids), chloroform fraction (PL), methanol/water fraction and interfacial fraction (oleosins), were analysed by SDS-PAGE and TLC (Fig. 2 and 3). In SDS-PAGE analysis the oleosomes showed four protein bands (oleosins) in the interfacial fraction and also similar three bands in methanol/water fraction from both root nodule and seed oleosome fractionations. The sub-molecular weight of nodule oleosins were 10.0 KD,56.3 KD, 61.1 KD and 66.0 KD in weight (Fig.2), whereas seed oleosins were as 25.5 KD, 35.2 KD, 40.0 KD and 59.5 KD in weight (Fig. 3). The TLC analysis showed three PL bands from nodule oleosomes and two from seed oleosomes in ether fractions. Some of the PL bands could also be seen in chloroform fraction. PL bands were not

observed in interfacial fraction. Further TLC showed two bands of neutral lipids (TAG & DAG) in nodule and seed oleosomes only in the ether fraction and none in any other fractions (Fig. 4).

Table 3 Yield of total protein (oleosins) and neutral lipids from interfacial fraction and diethyl ether of isolated oleosomes of peanut root nodules and seeds.

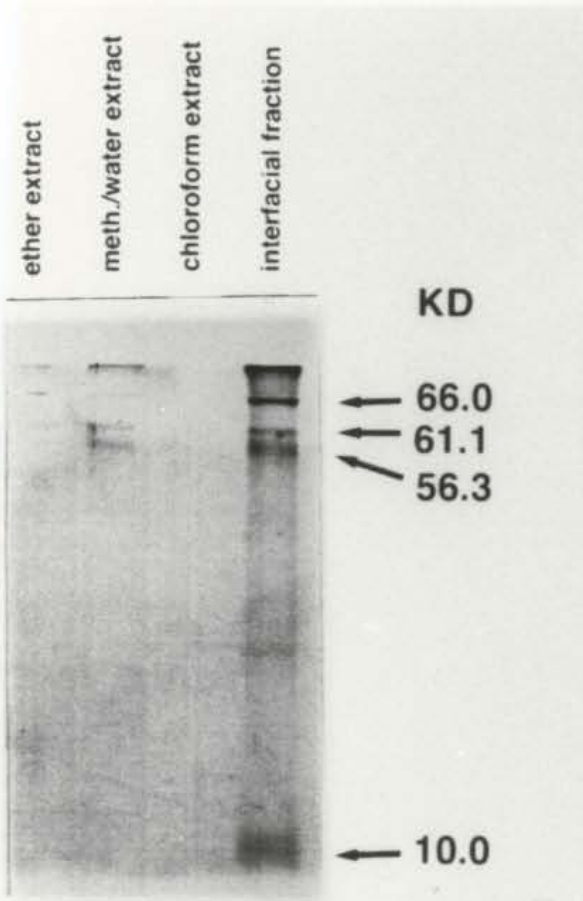
oleosome fraction	nodule oleosomes*	seed oleosomes**
Interfacial fraction (oleosins)	60 μg/mL	240 μg/mL
Ether fraction (neutral lipids)	0.05 g/mL	0.7 g/mL

* from 10 g nodule (w/w)

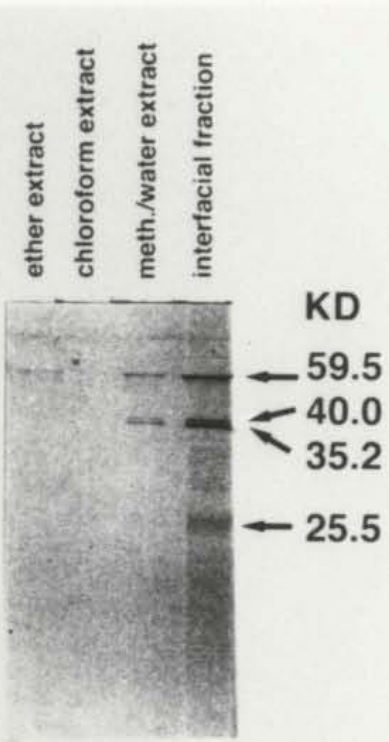
** from 2 g soaked peanut seed for 2h.

Fig.2 SDS-PAGE of fractions obtained in a fractionation of *A. hypogaea* root nodule oleosome preparation. Note the four protein bands (oleosins: 66.0 KD, 61.1 KD, 56.3 KD and 10.0 KD)in interfacial fraction. Three similar bands can also be seen in methanol/H_2O fraction. Gel was loaded with 3.0 μg of protein. Gel electrophoresis (polyacrylamide 12.5 %) was performed as described in Material and Methods.

Fig.3 SDS-PAGE of fractions obtained in a fractionation of *A. hypogaea* seed oleosome preparation. Note the four protein bands (oleosins: 59.5 KD, 40.0 KD, 35.2 KD and 25.5 KD) in interfacial fraction. Similar protein bands could also be seen in methanol/H_2O fraction. Gel was loaded with 24 μg of protein. Gel electrophoresis (12.5 % polyacrylamide) was performed as described in Material and Methods.

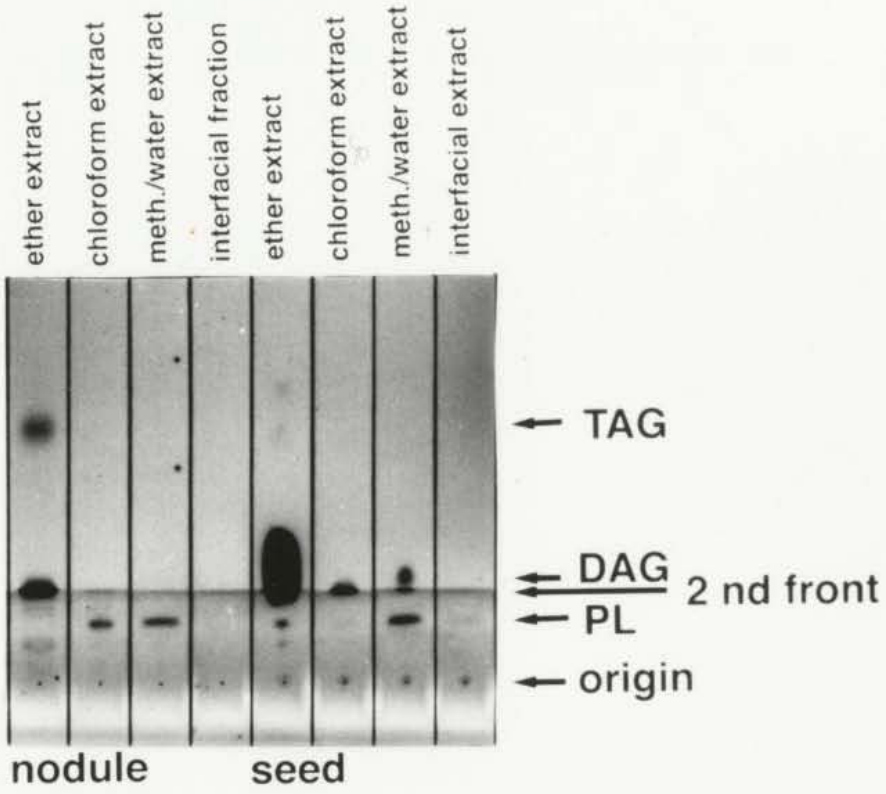


nodule 2



seed 3

Fig.4 Thin layer chromatography of the fractions of a *A. hypogaea* root nodule and seed oleosome preparations. Note the presence of TAG and DAG in only ether fraction (neutral lipids) of root nodule and in seed oleosomes. PL bands could be seen in ether and chloroform fraction.


ether extract
chloroform extract
meth./water extract
interfacial fraction
ether extract
chloroform extract
meth./water extract
interfacial extract
TAG
DAG
2 nd front
PL
origin
nodule
seed

III.2 Morphology of root nodule oleosomes from *Arachis hypogaea*.

The oleosomes in the isolated fractions of the nodules showed considerable size differences and variation in their electron density (Fig. 5 and Fig. 6). The electron micrographs revealed three distinct forms with respect to size (Table 3) and in many cases a peripheral layer of less electron density was apparent. An irregular periphery around a large oleosome shown in Fig. 5 also showed the less electron-dense layer. Such a layer (arrow) was present in many of the oleosomes (Fig. 5). In some cases there seemed to be an out-growth of this layer (arrowhead) as shown in Fig. 5. There were electron-dense deposits (d) and localised scouring of the oleosome surface which made the oleosomes pointed in some places (Fig.5). Electron transparent furrowing (arrows) could be seen in some oleosomes (Fig. 6). The larger oleosomes (asterix) seemed to have less electron-density.

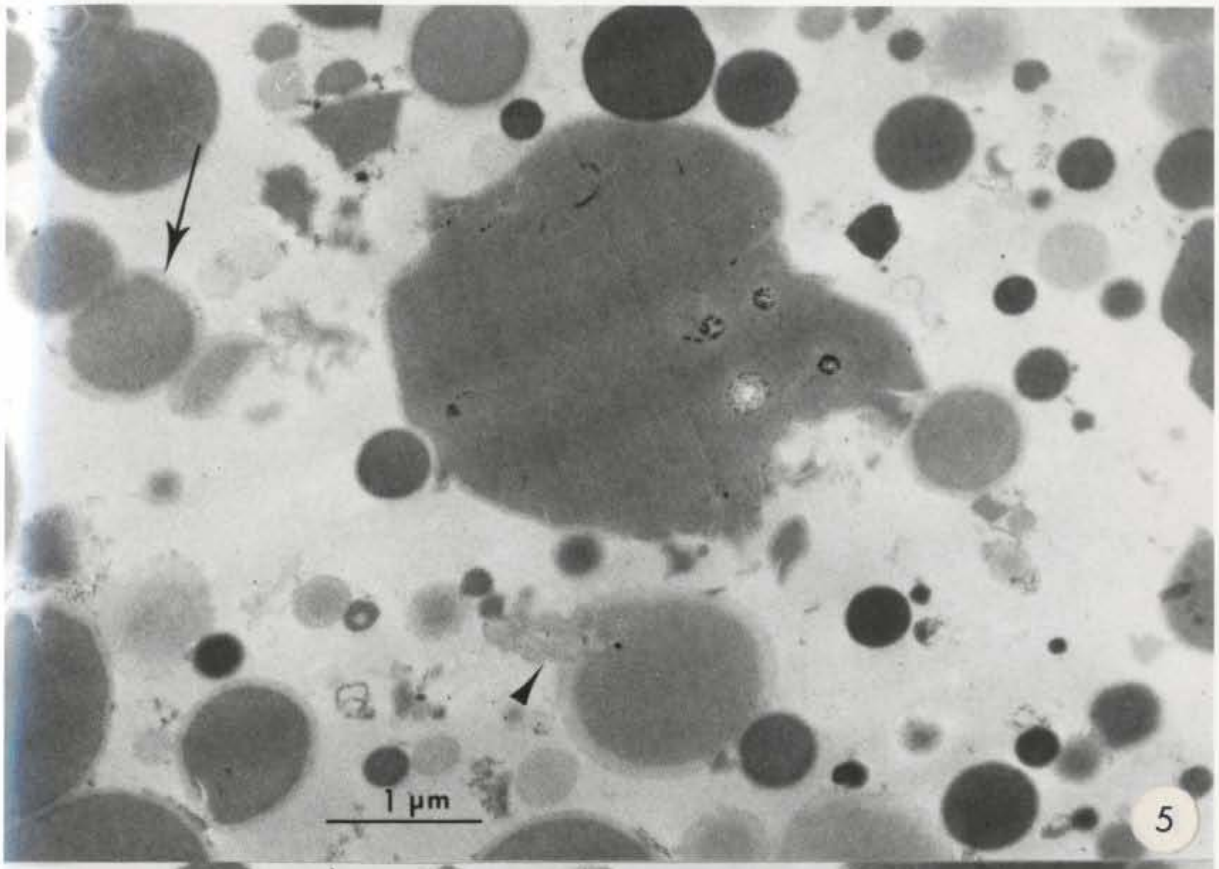
Table 4 Variation in size and peripheral layer of isolated oleosomes from peanut root nodule.

oleosome class	oleosome size (μm)*	width of peripheral layer (nm)*
1) Large	1.8 ± 1.0	70 ± 18
2) Medium	0.8 ± 0.3	42 ± 16
3) Small	0.4 ± 0.2	18 ± 6

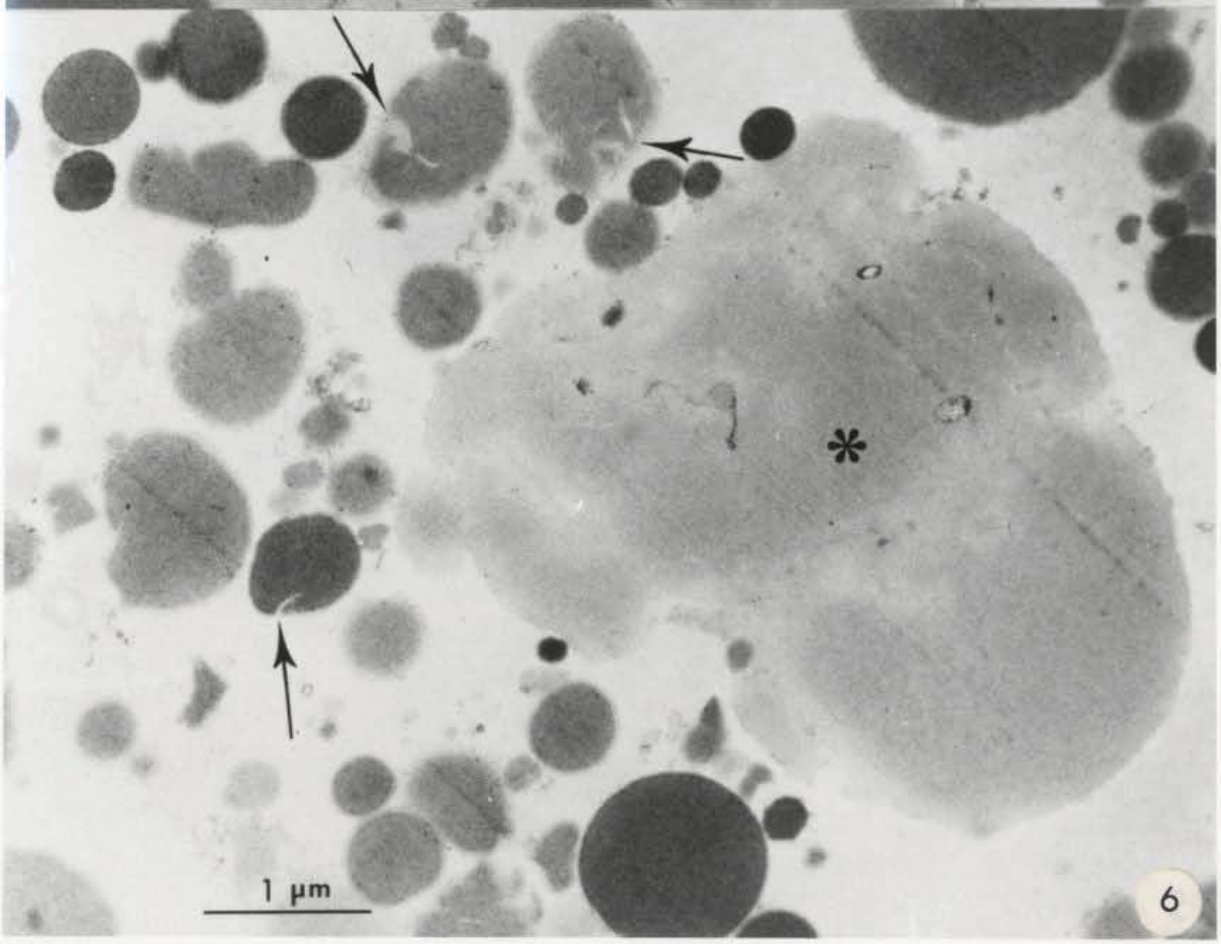
* SE ± n=80

Fig.5 Ultrathin section of the isolated oleosome fraction of *A. hypogaea* root nodule showing the size differences in the oleosome population. Variation in electron density is also notable alongwith the less electron-dense layer (arrow) around some of the oleosomes. Note the irregular out-growth of the layer (arrowhead)

Fig.6 Ultrathin section of the isolated fat pad of *A. hypogaea* root nodule showing a large oleosome (*) with a less electron-dense matrix indicating possible lipolytic activity. Note the oleosomes that show furrowing (arrows).



5



6

III.3 Distribution of oleosomes in *A. pintoi*, *A. duranensis* and *A. batizocoi*

During the early stages of development in immature/small /white nodules, oleosomes were observed in the infected cells, where rhizobia were released and proliferation of both host and rhizobia were underway (Fig.7). Oleosomes also occurred in some nodule parenchyma cells. Hexane solubilization in control preparations confirmed the presence of oleosomes. As the rhizobia differentiated into spherical forms of bacteroids in mature/ medium/pink nodules, oleosomes disappeared from the infected cells (Fig.8), but could be seen in the nodule parenchyma. The lack of oleosomes in infected cells continued throughout the symbiotic phase of the nodules up to mature/large/red ones sampled 60 DAI (days after inoculation) (Fig.9). In 70-day old nodules undergoing senescence, oleosomes reappeared in the infected cells. The spherical bacteroids at this stage seemed to have disappeared and large vacuoles were found in the infected cells, which contained rod-shaped rhizobia (Fig.10,11,12 and 13). The senescing cells showed disruption of the tonoplasts. Both transmission and scanning electron microscopic observations confirm the presence of rod-shaped rhizobia during senescence (Fig.11,12 and 13). The light microscopic observations in squash preparation of senescing nodules also revealed both rod and

spherical forms with some forms intermediate between spheres and rods. Senescing nodules of *A.hypogaea* did not reveal such change in bacteroid forms.

The infected cells of *A. duranensis* and *A. batizocoi* showed the presence of oleosomes, when the nodules were induced with the same strain of *Bradyrhizobium* (Fig.14). When the nodules of *A.pintoi* were induced by the ineffective strain, $nod^{+}fix^{-}$, nodules of this species showed accumulation of oleosomes (Fig. 15).

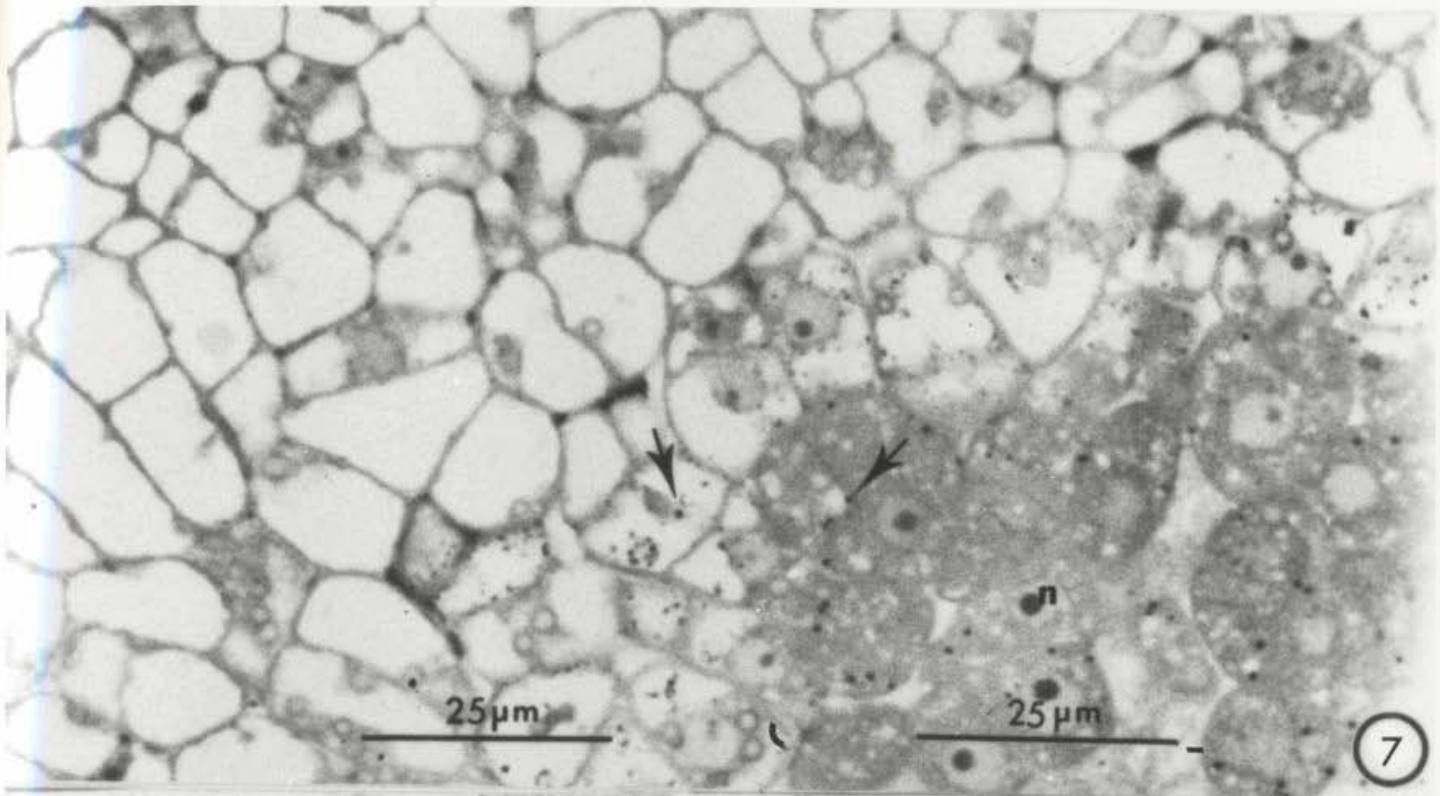
The percent area of infected cells occupied by oleosomes at different stages of development is summarized in Fig.16, which also shows accumulation and increase of the oleosome population in nod^{+} fix^{-} ineffective nodules of *A.pintoi*. Nodules from the two other diploid species, *A. duranensis* and *A. batizocoi* showed presence of oleosomes in the infected cells throughout their developmental stages (Fig.17). Percent areas of oleosomes in nodule parenchyma cells of *A. pintoi* are plotted in figure 18. The mature/medium/pink nodules showed more oleosomes than any other stages.

Although in *A. pintoi* oleosomes were absent from mature nodules, their presence was noted in the nodule parenchyma cells mainly 2-3 layers around the infected zone. These cells show decreased oleosome populations when compared to *A. duranensis* and *A. batizocoi* nodules at 42 DAI (Fig.19).

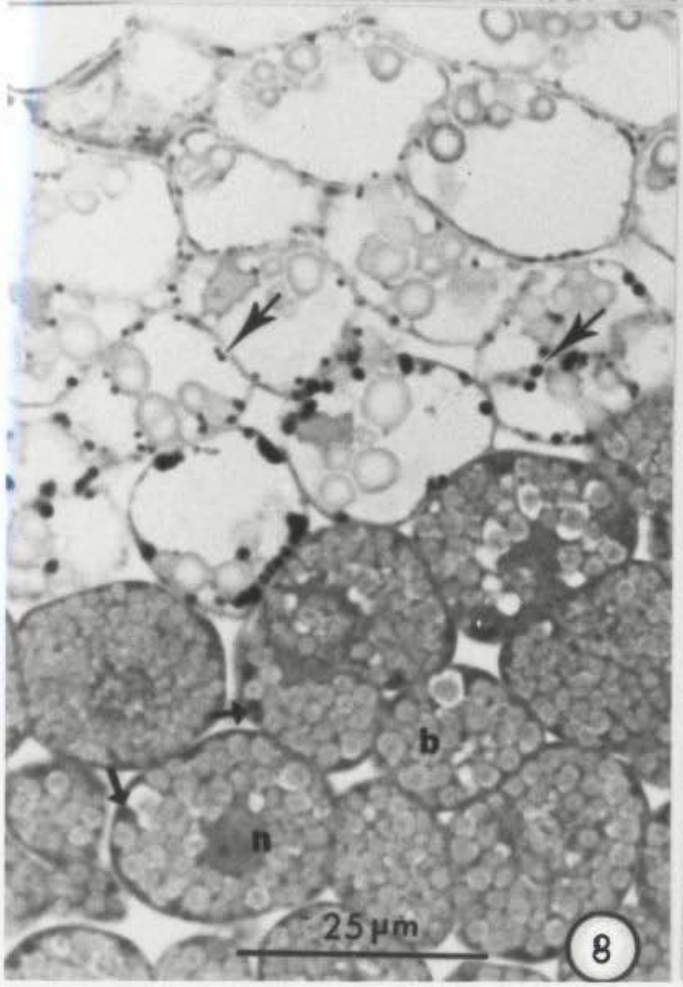
Fig.7 Photomicrograph of p-phenylenediamine (pPD)-stained semi-thin section of immature/small/white (1.0-1.5 mm) nodule of *A. pintoi* (21 DAI) showing oleosomes (arrows) in the undifferentiated infected cells (lower right) and also in the adjacent layers of nodule parenchyma cells. Nucleoli (n) within nucleus are densely stained.

Fig.8 Photomicrograph of pPD-stained semi-thin section of mature/medium/pink (1.5-2.0 mm) nodule of *A. pintoi* (35 DAI) showing nucleus (n), bacteroids (b) in infected cells and oleosomes (large arrows) in parenchyma cells only. Infected cells are devoid of oleosomes. Note the three layers of parenchyma cells adjacent to the infected zone containing many and large oleosomes. The small arrows indicate amyloplasts in infected cells (confirmed by transmission electron microscopy).

Fig.9 Photomicrograph of pPD-stained semi-thin section of mature/large/red (>2.0 mm) nodule of *A. pintoi* (49 DAI) showing nucleus (n), bacteroids (b) in infected cells. Note the infected zone is devoid of oleosomes and the parenchyma cells adjacent to the infected zone show fewer and smaller oleosomes.



7



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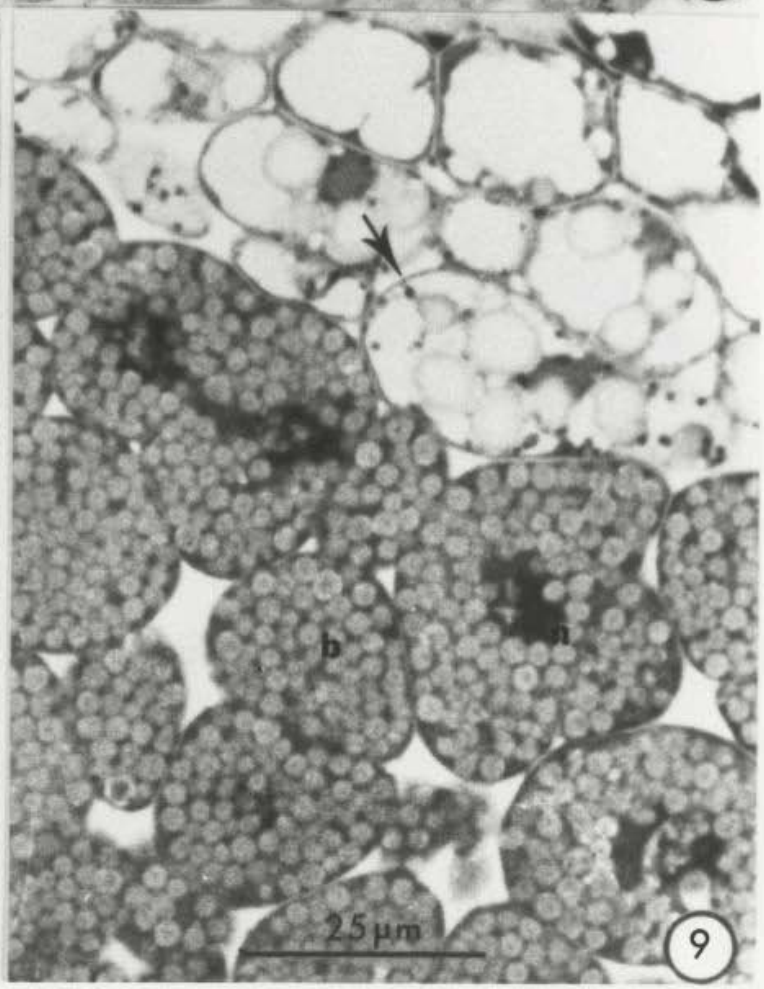


9

Fig.10 Photomicrograph of semi-thin section of senescing nodule (70 DAI) of *A. pintoi* showing rod-forms (R) in the vacuolar space of the infected cells. The characteristic spherical forms (S) of bacteroids are in the periphery of the cells. Note the intact uninfected nodule parenchyma and the dark-stained oleosomes (o) in both the infected and nodule parenchyma cells.

Fig.11 Transmission electron micrograph of ultrathin section of the senescing nodule of *A. pintoi*. Note both longitudinal and cross sectional profiles of rod-forms of rhizobia (R) in the vacuolar space, disruption of the tonoplast, oleosomes (o) and the spherical forms (S) of bacteroids.

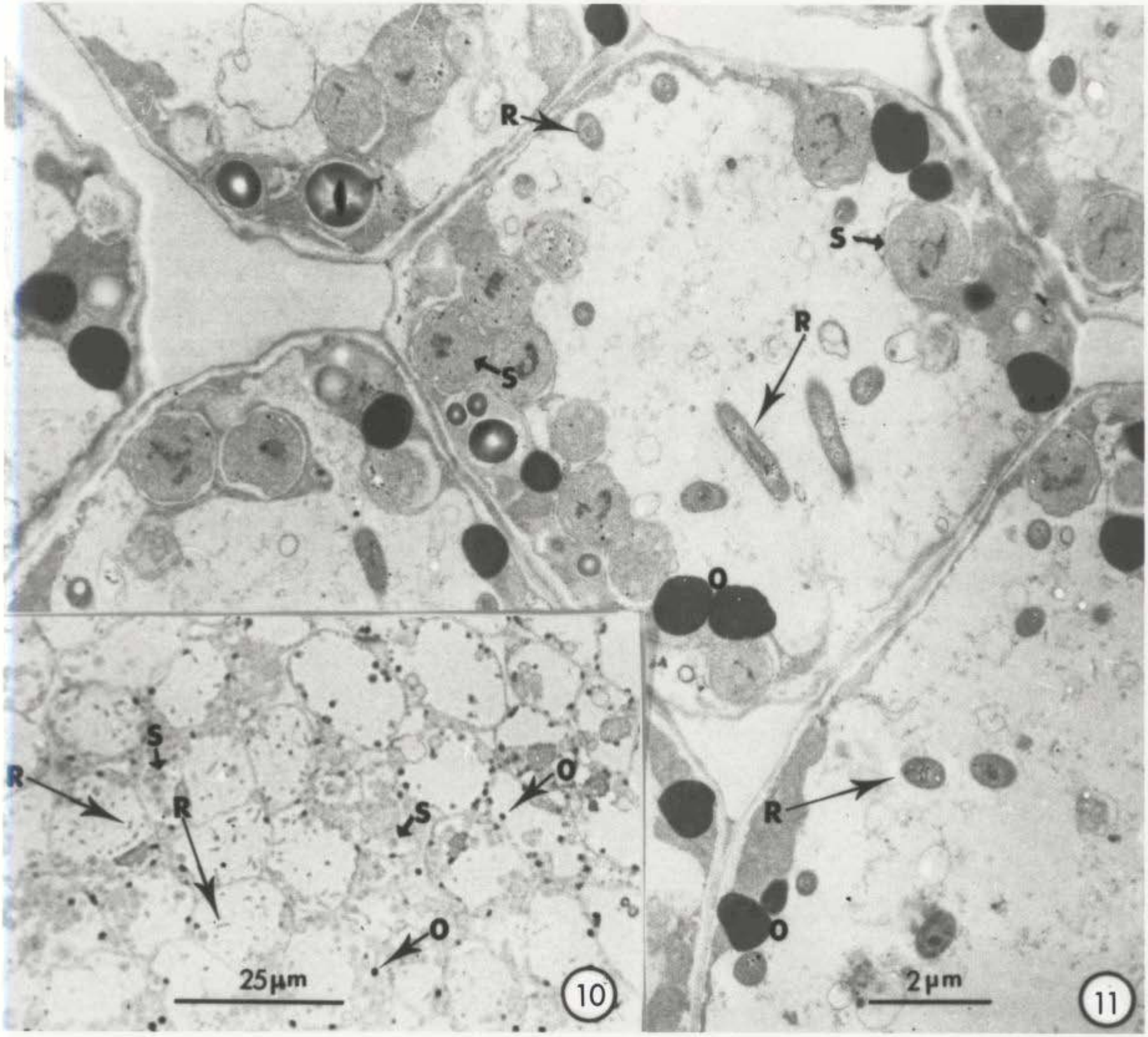
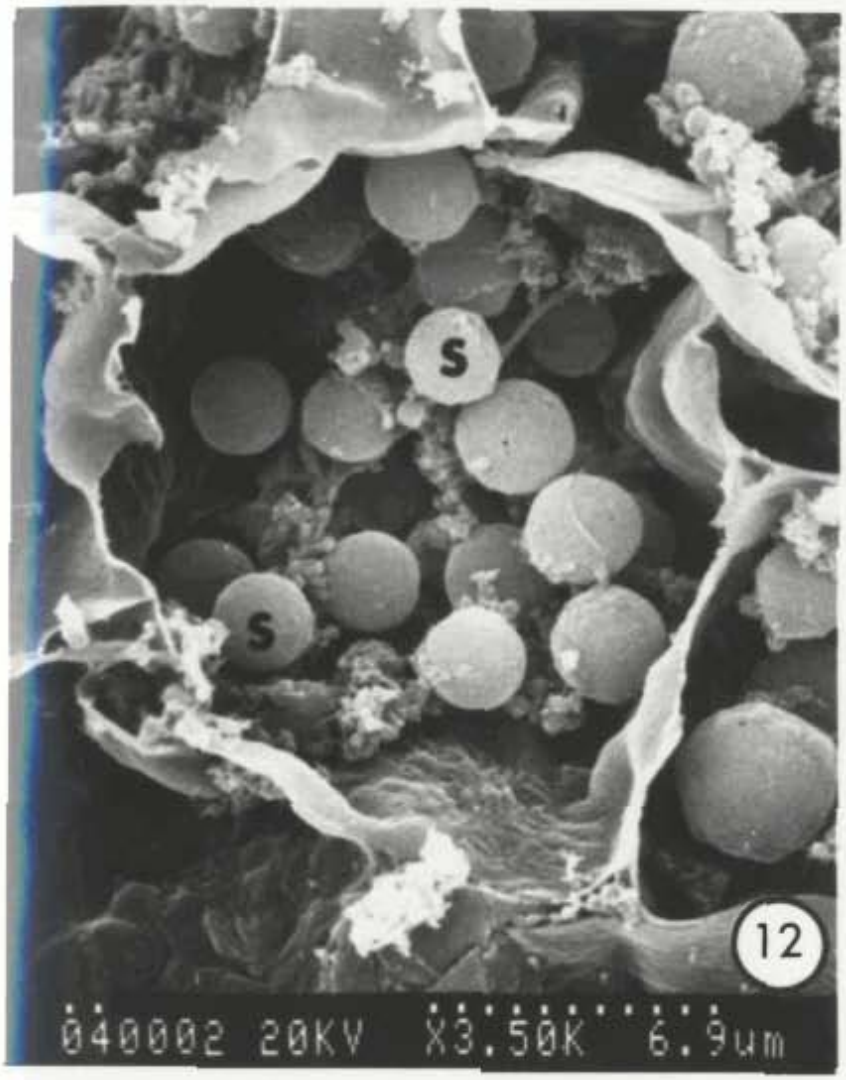

R
S
R
S
O
O
S
R
R
S
O
O
25µm
10
R
O
2µm
11

Fig.12 Scanning electron micrograph of mature infected cells of mature/large/red nodule (42 DAI) of *A. pintoi* showing spherical bacteroids (S) only. x 3500.

Fig.13 Scanning electron micrograph showing senescent infected cells of mature/large/red (old) nodule (70 DAI) of *A. pintoi* with reverted rod-forms of rhizobia (R) along with spherical bacteroids (S). x 7000.


S
S
12
040002 20KV X3.50K 6.9um

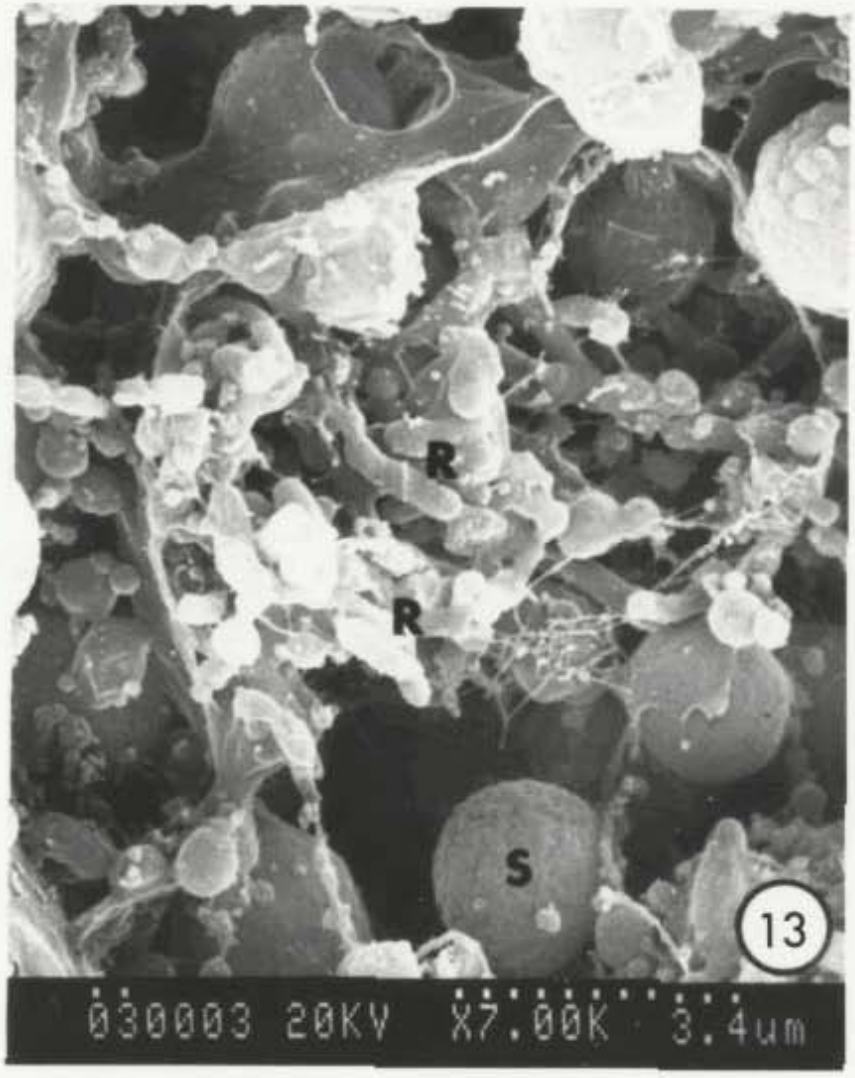
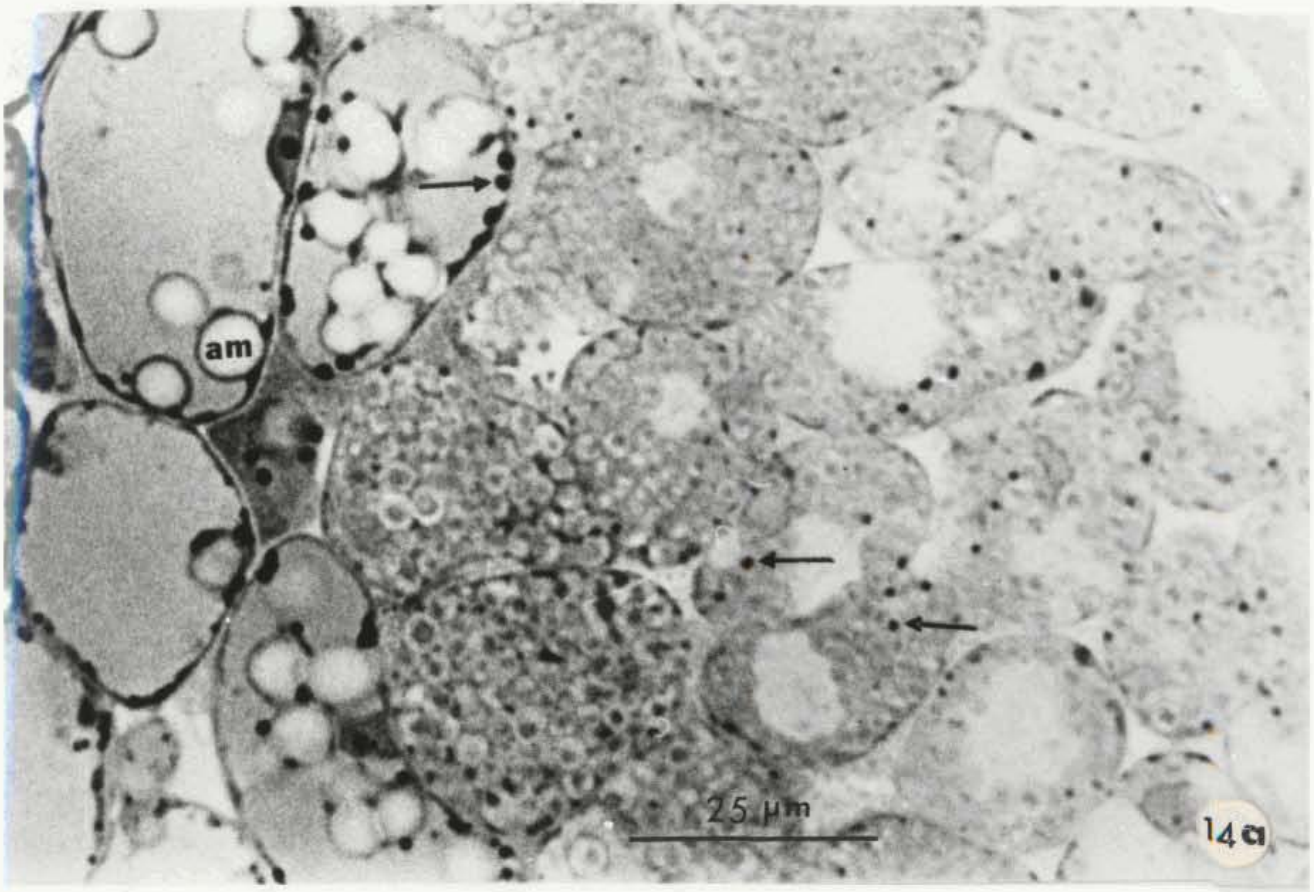

R
R
S
13
030003 20KV X7.00K 3.4um

Fig.14 Photomicrograph of pPD-stained semi-thin section of mature/big/red nodule of *A. duranensis* (a) and *A.batizocoi* (b) at 42 DAI. Note the presence of oleosomes (arrows) in the infected cells of both the species. There are fewer amyloplasts (am) in parenchyma cells of *A. batizocoi*.


am
25 µm
14a

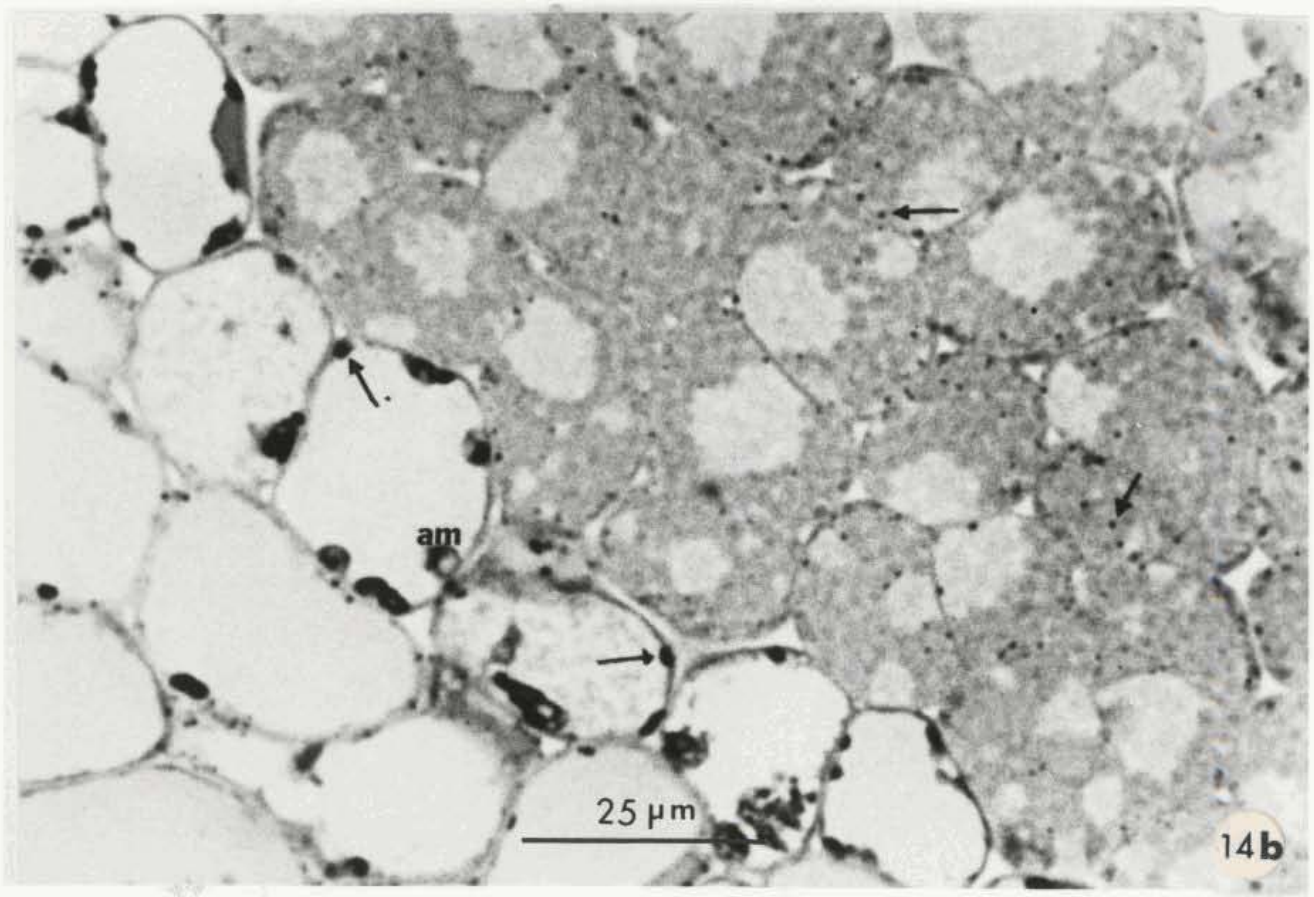

am
25 µm
14b

Fig. 15 Photomicrograph of pPD-stained semi-thin section of ineffective nodule of *A.pintoi* induced by Nod^{+}fix^{-} strain of *Bradyrhizobium* (7091). Note the presence of oleosomes (arrows) and undifferentiated infected cells.

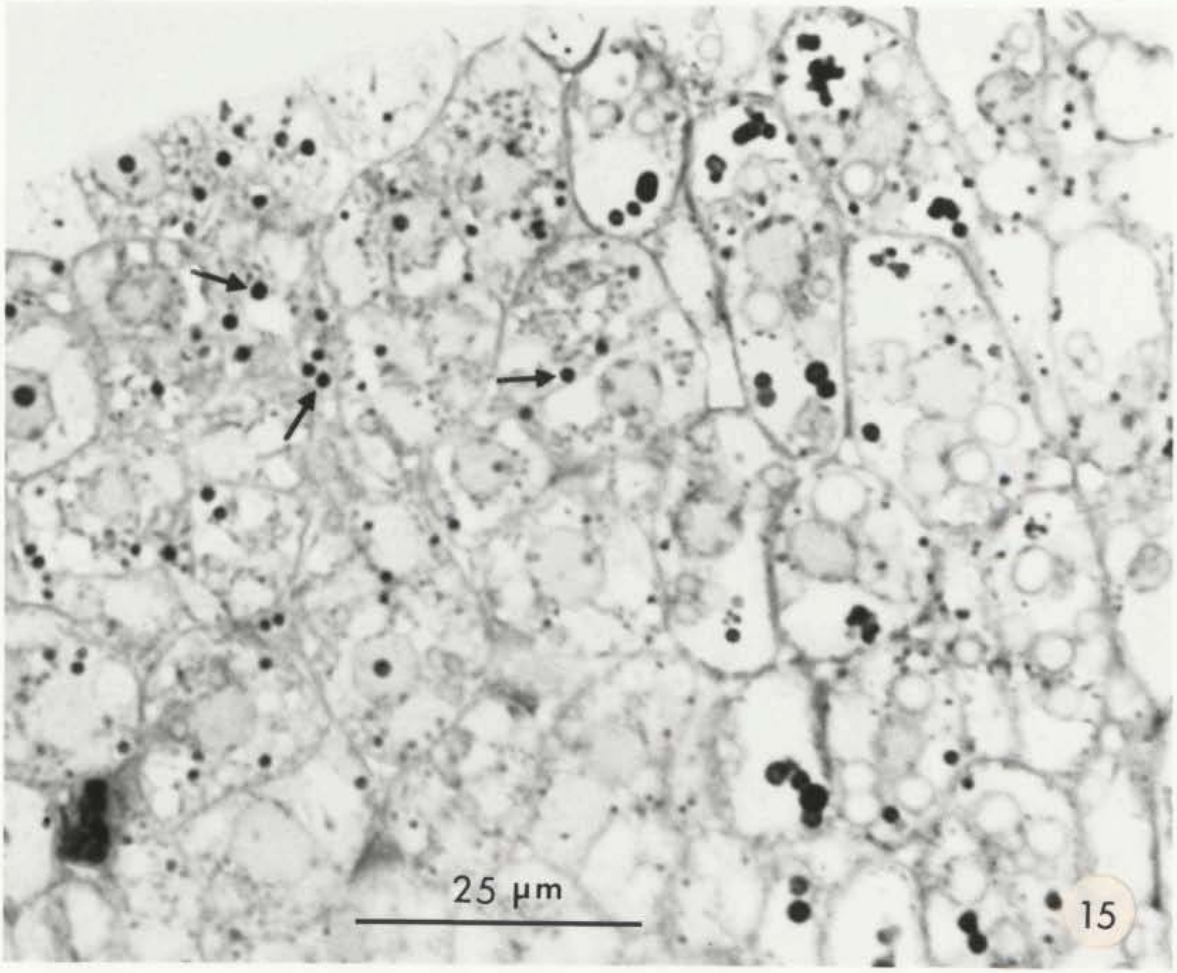
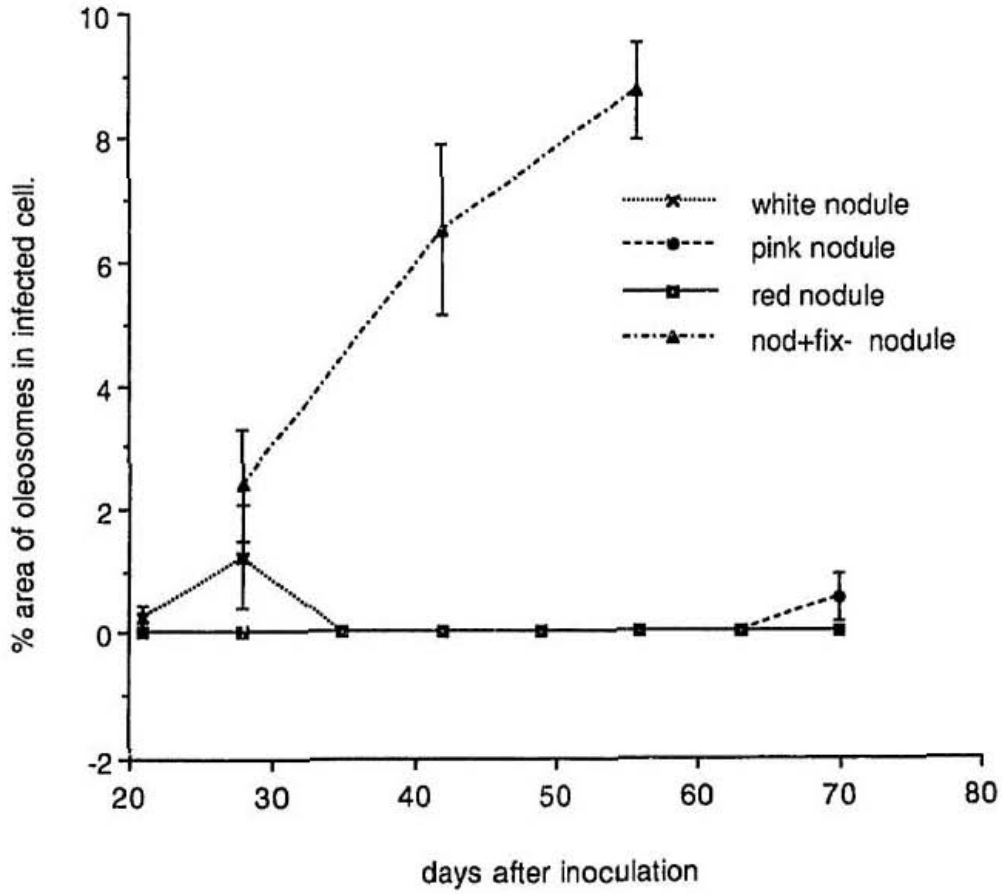
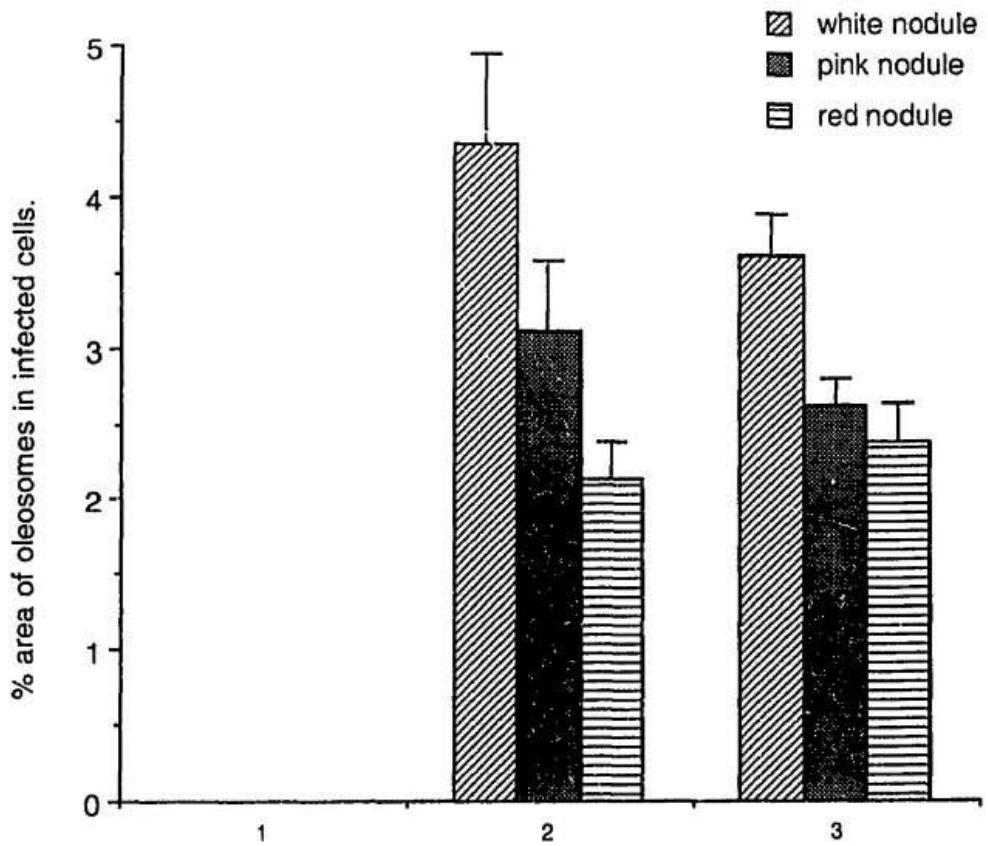

25 µm
15

Fig.16 Percent area of oleosomes in the infected cells of *A. pintoi* nodules at different stages of development. Note the complete lack of oleosomes in the symbiotic stages (mature/pink and red nodules). Oleosomes appear only in the asymbiotic stages of the nodule i.e. in immature/white, senescing and ineffective (nod$^+$ fix$^-$) nodules. Note the increasing accumulation of oleosomes in the ineffective nodules in absence of nitrogen fixation. Bars represent the ± SE. n=80



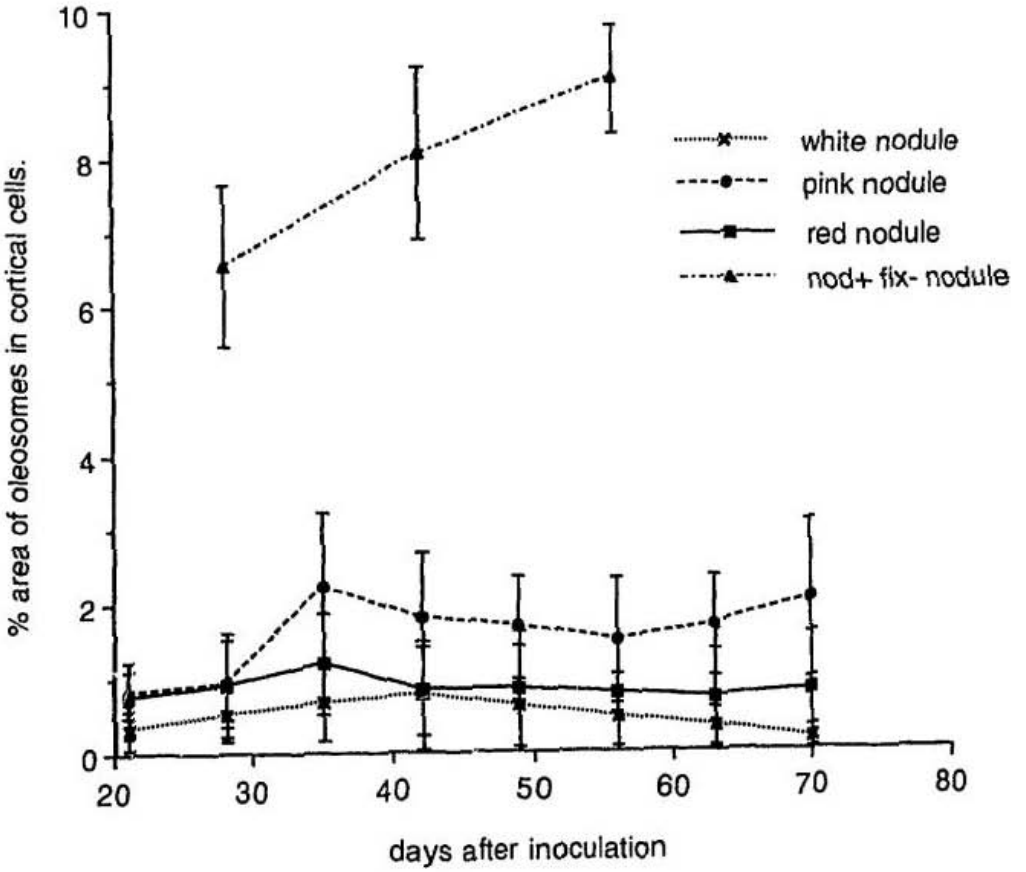
16 Percent area of oleosomes in infected cells of *A.pintoi* nodules at different stages of development.

Fig.17 Percent area of oleosomes in infected cells of nodules from *A.pintoi*, *A.batizocoi* and *A.duranensis*. Note the complete lack of oleosomes in infected cells of *A. pintoi* nodules as compared with two other diploid *Arachis* nodules at the symbiotic stage (42 DAI). Note the decreasing trend of oleosomes in pink and red nodules. Bars represent the ± SE. n=80



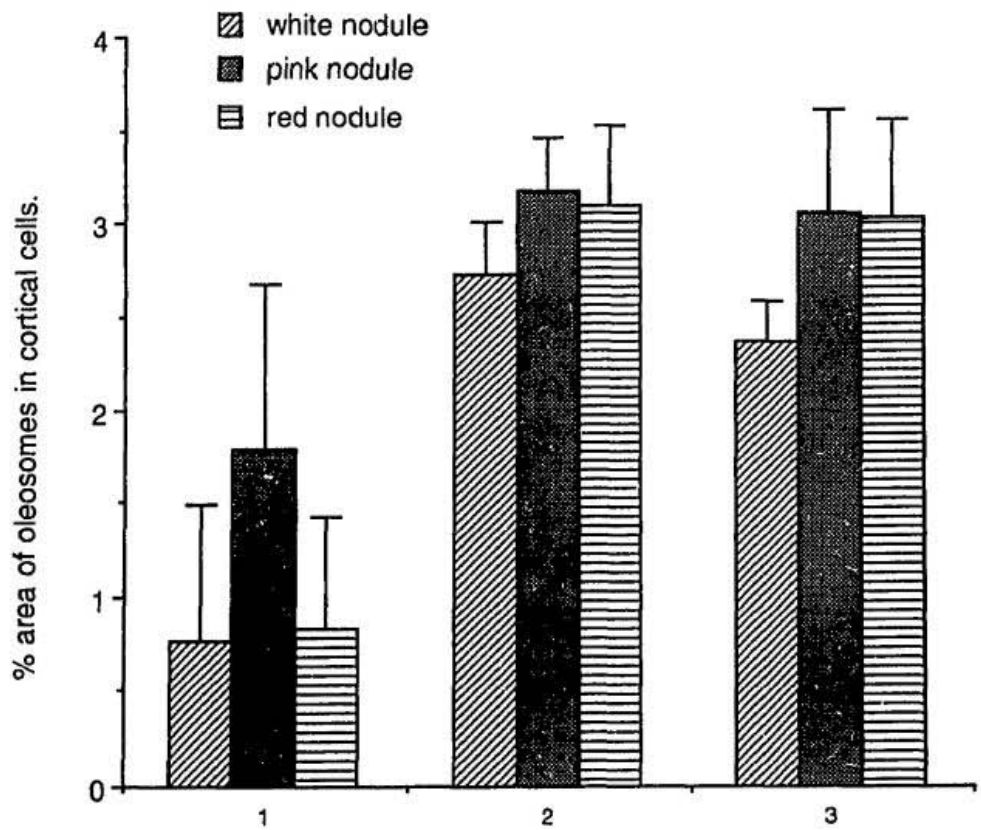
17 Percent area of oleosomes in infected cells of nodules from *A.pintoi*, *A.batizocoi* and *A.duranensis*.

Fig.18 Pattern of oleosome distribution in the nodule parenchyma cells of *A. pintoi* at different stages of development.Oleosome content is higher in mature/pink nodules compared to immature/white and mature/red nodules. Note increasing oleosome population in the parenchyma cells of ineffective (nod^+ fix^-) nodules. Bars represent the ± SE. n=80



18 Percent area of oleosomes in parenchyma cells of A.pintoi nodules at different stages of development.

Fig.19 Distribution pattern of oleosomes in the nodule parenchyma cells of three diploid species during the symbiotic stage (42 DAI). Oleosome content is significantly lower in the nodule parenchyma of *A. pintoi*. Bars represent the ± SE. n=80



1=A. pintoi, 2=A. batizocoi & A. duranensis (42 DAI).

19 **Percent area of oleosomes in nodule parenchyma cells of *A.pintoi*, *A.batizocoi* and *A.duranensis*.**

III.4 Induced senescence in *A.pintoi*.

It was observed that *A. pintoi* showed the presence of oleosomes along with redifferentiation of spherical bacteroids to viable rod-forms in the vacuole during natural senescence which occured in plants at 70 DAI. A short experiment was therefore conducted to see the effect of artificially induced senescence on the reappearance of rod-forms and the oleosomes.

The results depicted in Table 5, indeed showed the cells undergoing senescence in KNO_3 (20 mM) and detopping (2^{nd} day) treatments with the presence of oleosomes and rod-forms of bacteria (Fig.20a and 20b). The percent area occupied by oleosomes in control and other treatments is given in Table 5. However, such redifferentiation of bacteroids was not found in the senescing cells of *A. hypogaea*. The viable count from such nodules did not show any significant difference in viability of bacteroids from non-senescent and senescent nodules (Table 6).

Table 5 Percent area of oleosomes in senescent/infected cells of *A.pintoi* root nodules induced by KNO3 (20 mM) and detopping.

Treatments (nodule describtion)	% area of oleosomes*	shape of bacteria/bacteroid
1 KNO3 (immature/medium/pink)	2.8±0.81	spherical and rod-forms.
2 Detopping (2nd day) (mature/big/red)	2.3±0.63	spherical and rod-forms.
3 Control (mature/big/red)	0.0±0.0	only spherical

n=80 cells

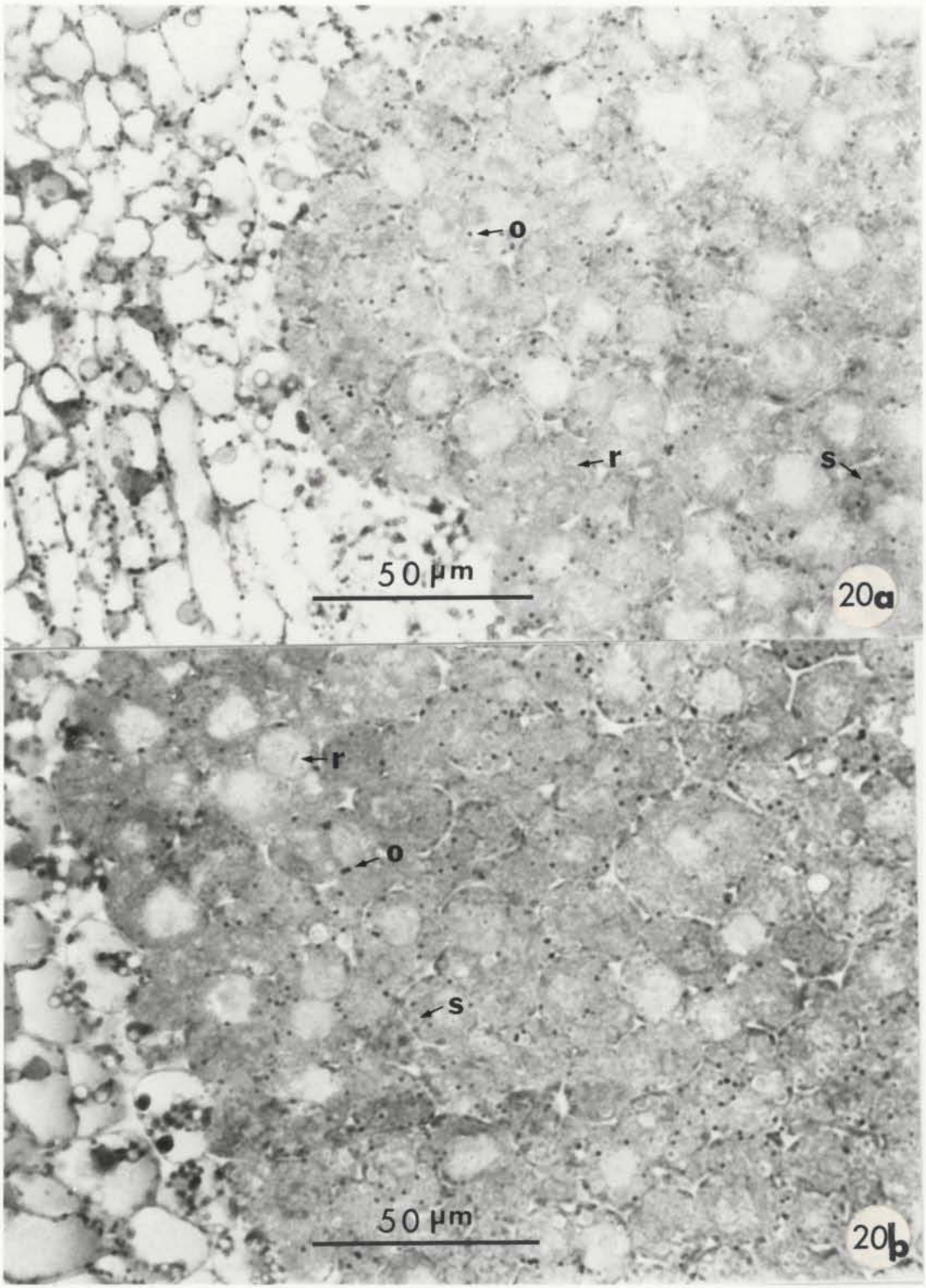
Table 6 Enumeration of rhizobia (cells/mL) by the plate count method, cultured from mature and senescing nodules of *A.pintoi*.

mature nodule 35 DAI*	senescing nodule 120 DAI**
9.0×10^3 ± 1.7	8.0×10^3 ± 2.3

* containing only spherical bacteroids

** containing a mixed population of spherical, intermediate-forms and rod-forms. Verified in smear preparation in both cases. (SE ± 5 plates).

Fig.20 Photomicrograph of pPD-stained semi-thin section of prematurely induced senescence in *A. pintoi* root nodules by KNO_3 (a) and detopping (b). Note the presence of oleosomes (o), spherical (s) bacteroids and rod-forms (r) bacteria in both the treatment.


o
r
s
50 µm
20a
r
o
s
50 µm
20b

III.5 Seasonal effect on storage organelles in *L. maritimus* root nodule.

Nodules of *L. maritimus* are of the indeterminate type. They were mostly elongate, sometimes branched but very young nodules were spherical and different types of nodules could be seen at any given time (Fig. 21). In senescent nodules the pink/red colour of the symbiotic zone had a blackish green tint. As complete degeneration of internal tissues took place the nodules became black in color. Some of the nodules showed notches. In our observations we have noticed only one notch. The indeterminate nodules of beach pea in summer months were differentiated into distinct zones as shown in Fig. 22.

Pre-winter nodules

The samples taken in early October, when the pods had matured and opened up for seed dispersal, were from the plants preparing for overwintering. The nodules at this stage were undergoing senescence. Histological analysis showed arrest of cell division in the meristem (Fig. 23), the lack of infection threads in the invasive zone (Fig. 24 and 25), the disappearance of the presymbiotic zone and senescence of the symbiotic cells (Fig. 26). The senescence was characterized

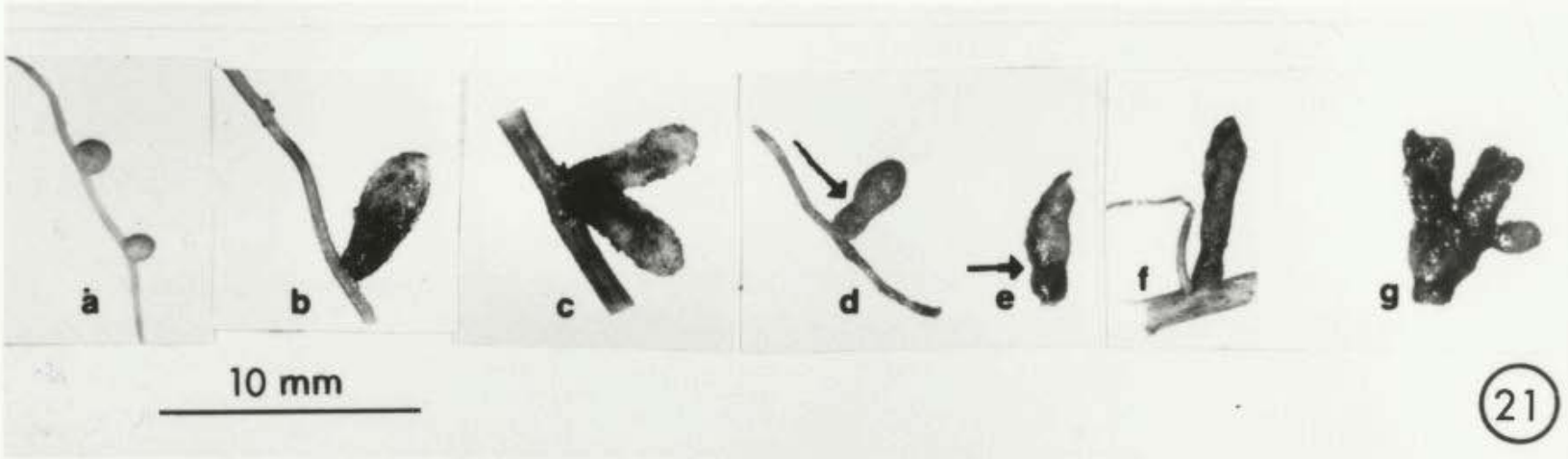
macroscopically by a change in leghemoglobin color to green and microscopically by filling of the bacteroids with poly β-hydroxybutyric acid granules making them opaque (Fig. 26). This is followed by degeneration of bacteroids and cell contents. At the same time an increase in the accumulation of amyloplasts with starch grains in the interstitial uninfected cells of the symbiotic zone and nodule parenchyma became apparent (Fig. 23,24,25 and 26). The nodule parenchyma (outer and inner cortex) also became heavily filled with amyloplast starch grains. Oleosomes became abundant in all the parenchyma cells (Fig.27 and 28), including those in the vascular parenchyma. The vascular tissue also showed the presence of protein bodies which resisted solubilization in hexane treatment. The observations of samples from year to year did not show any significant variation.

Post-winter nodules

In samples taken during March and April, before the shoots appeared above ground, striking changes in the histology of the nodules were evident. The starch grains and oleosomes had disappeared (Fig.29,30,31 and 32) and the provascular tissue had become active (Fig. 32). Oleosomes could be seen only in the vascular parenchyma (Fig. 29). The provascular tissue at the distal part of the nodule showed

meristimatic activity in cells with considerable cytoplasmic content and very small vacuoles (Fig. 32). Some of the oleosomes could be seen in the endodermal cells around the vascular tissue (Fig. 33) and protein bodies persisted in the vascular parenchyma cells (Fig. 34). The cells of the senescent zone had clumped and degenerated bacteroids, but some of the cells contained rod-shaped bacteria, which did not show any morphological sign of degeneration (Fig.35 and 36). Some persistent infection threads could be detected containing rhizobia in the invasive zone (Fig. 37). No significant variation could be observed in the samples taken in different years.

Fig.21 *Lathyrus maritimus* L (beach pea) nodules at different stages of development found during all times from late March to middle of October. (a) Spherical young nodules, (b) and (c) mature elongate and branched nodules, (d) notched (arrow) nodule, (e) notched (arrow) but decaying nodule, (f) decayed nodule and (g) decayed branched nodule.



21

Fig.22 A diagram showing longitudinal section of *L. maritimus* nodule. Note the meristem (m) at the distal end, invasion zone (iv), early symbiotic zone (es), late symbiotic zone (ls)and senescent zone (sn). There are five vascular tissue strands (vt) of which one is shown in longitudinal plane and the other in cross section. Provascular cells are close to the meristem. A distinct exodermis (ex)is present. The uninfected cells of the symbiotic zone are called interstitial cells (not shown in diagram).Vascular tissue is surrounded by an endodermis (not shown in diagram).

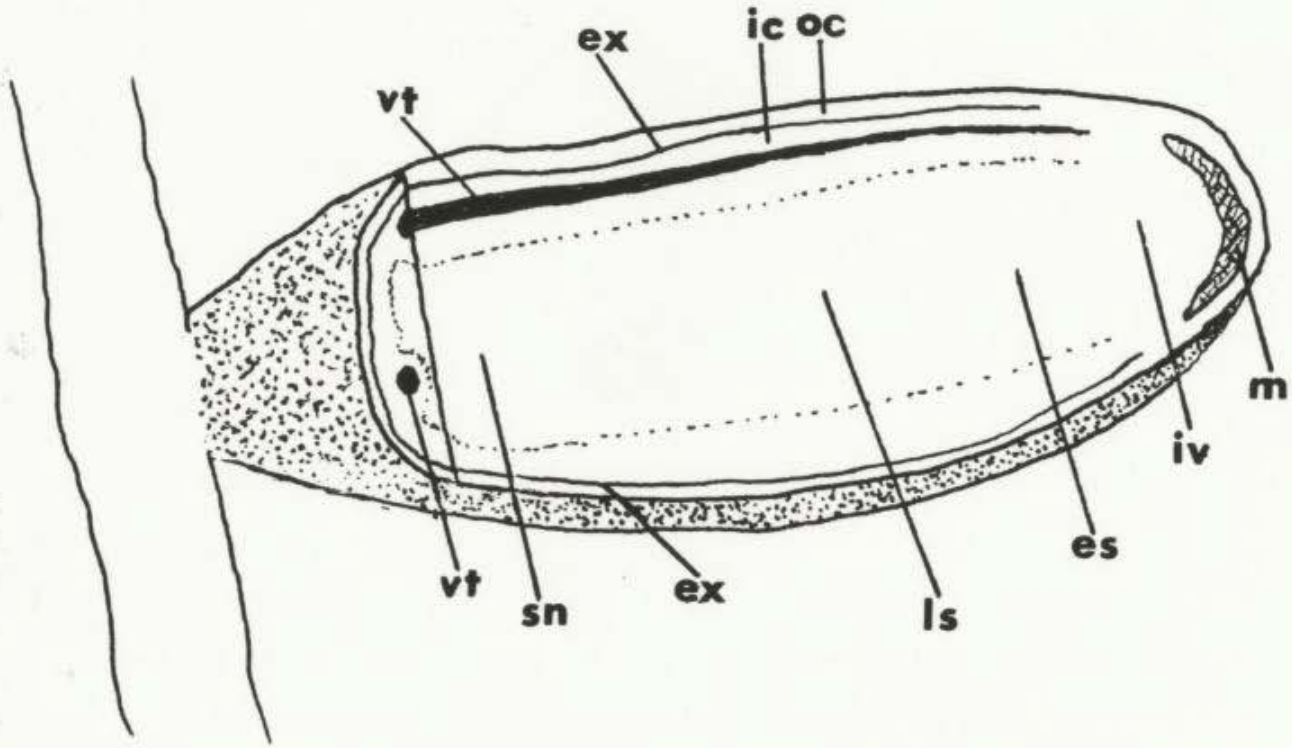

ex
ic oc
vt
m
iv
es
vt
sn
ex
ls

Fig. 23, 24, 25, 26, 27 and 28

Photomicrographs of pre-winter nodule samples of *L. maritimus*.

Fig.23 Meristem (m) of the nodule showing non-dividing cells surrounded by cells containing starch (small arrows) and oleosomes (large arrows).

Fig.24 Nodule parenchyma (inner and outer cortex) and interstitial uninfected cells showing heavy deposits of starch grains (small arrows) and oleosomes (large arrows). Note the exodermis (Ex), and the infected cells showing opaque bacteroids due to poly β-hydroxybutyric acid granules.

Fig.25 Innner cortex of nodule showing the endodermal layer (En) around the tangentially cut vascular tissue (Vt) and the invasive zone (iv) lacking any infection threads.

Fig.26 Mature symbiotic infected cells (*) and senescent infected cells (Sn). Note the change in appearance of bacteroids.

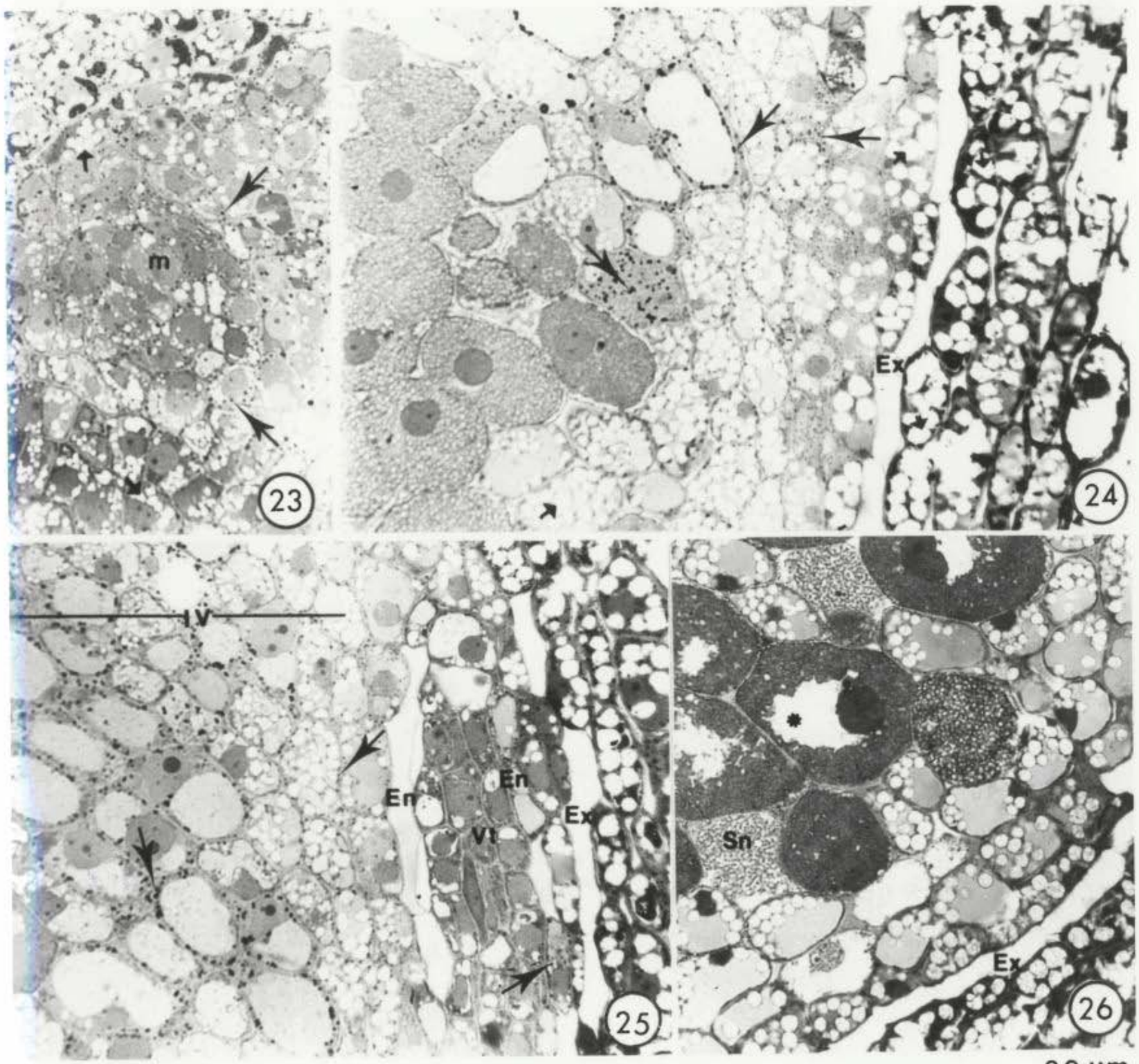

m
23
Ex
24
IV
En
En
Ex
Vt
25
Sn
Ex
26
30 µm

Fig.27 Electron micrograph of a parenchyma cell showing starch grain (Sg) and oleosomes (large arrow) in pre-winter nodule of *L. maritimus*.

Fig.28 Electron micrograph showing associations of starch grains (Sg), mitochondria (Mt), oleosomes (arrow) and rough endoplasmic reticulum (er) in pre-winter nodule of *L. maritimus*.


Sg
2µm
27

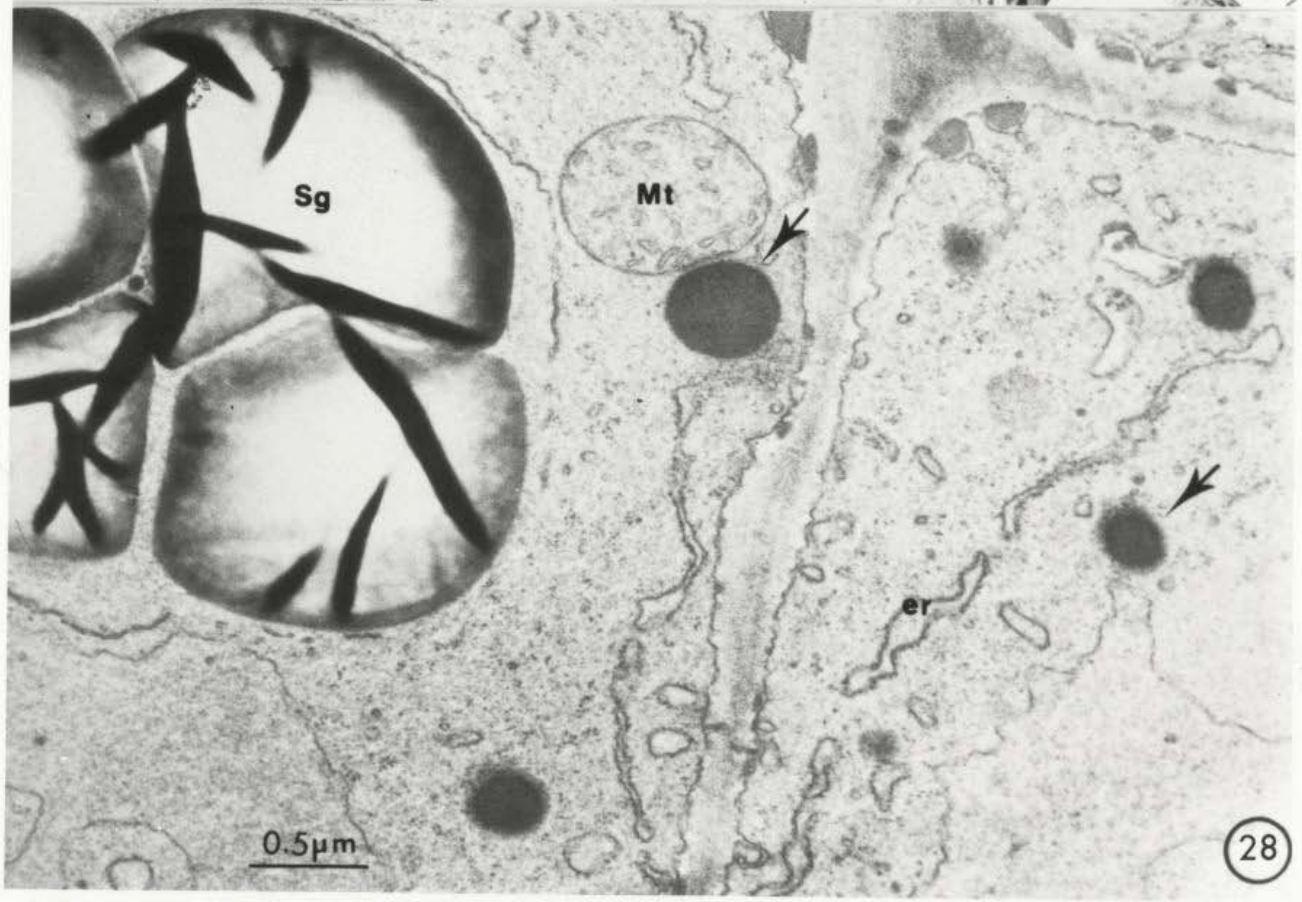

Sg
Mt
er
0.5µm
28

Fig.29, 30, 31 and 32

Photomicrographs of post-winter nodule samples of *L. maritimus*

Fig.29 Photomicrograph showing the vascular tissue.Note the vascular parenchyma showing oleosomes (arrow) en=endodermis.

Fig.30 A view of the senescent zone (Snz).

Fig.31 The invasive zone (inv), showing no starch grains or oleosomes. Note very clear presence of infection threads (arrow).

Fig.32 Provascular parenchyma showing a dividing cell (arrow), and the other cells of the meristem (m). Note the lack of storage granules in the parenchyma surrounding the meristem.

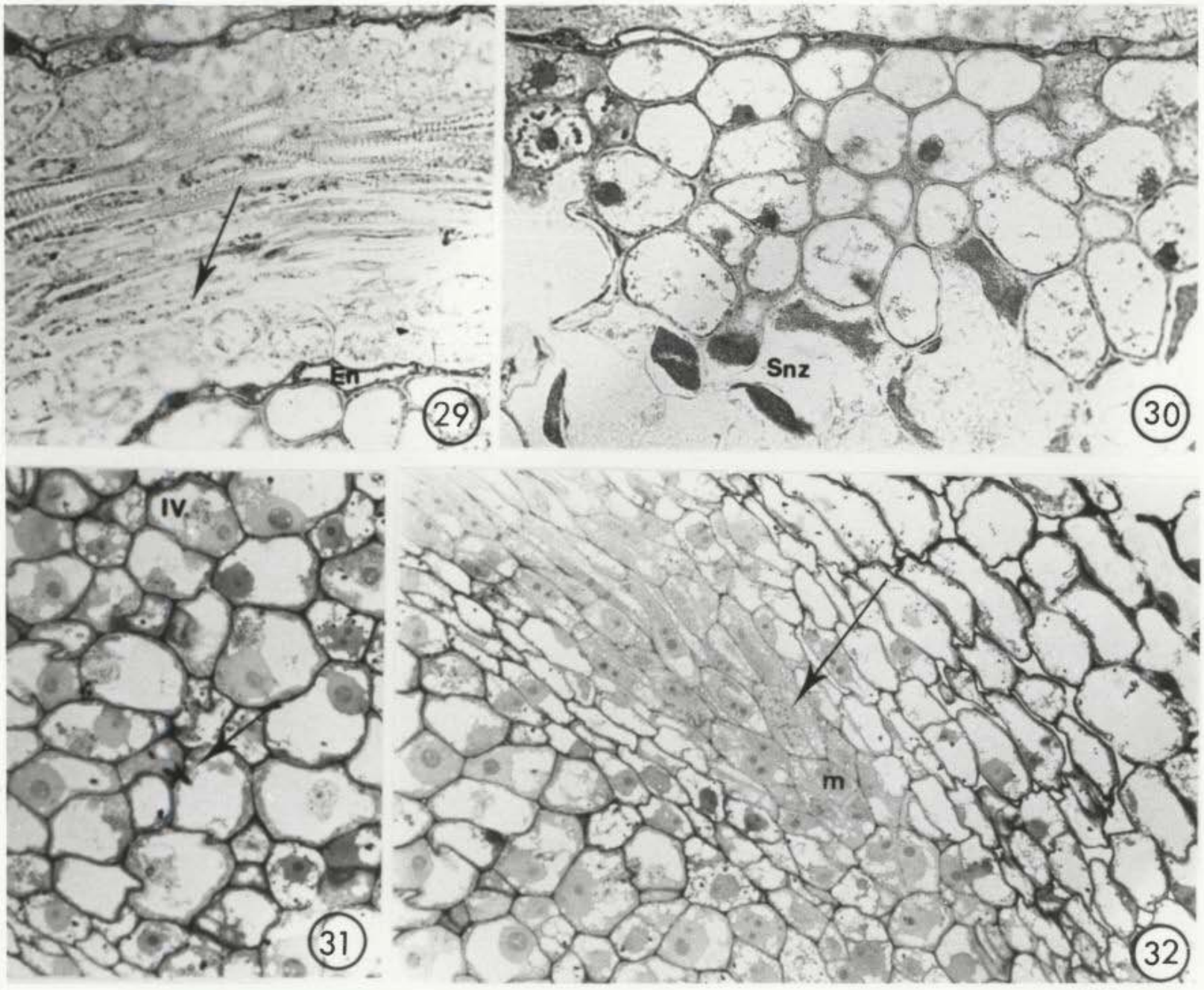
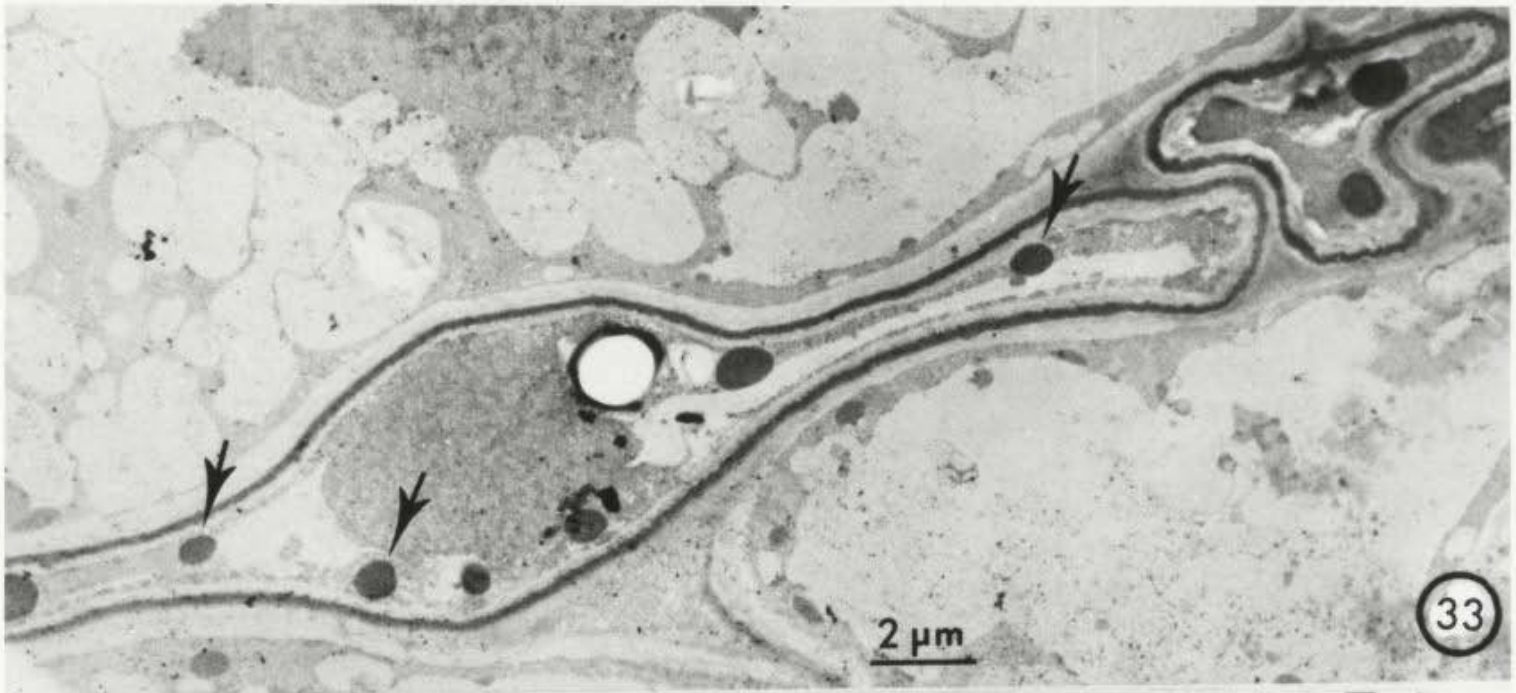

En
29
Snz
30
IV
31
m
32
30 µm

Fig.33 Electron micrograph of the endodermal layer showing the presence of oleosomes (arrow) in post-winter nodule of *L. maritimus*. Note the characteristic thickening of the cell wall.

Fig.34 Electron micrograph of a xylem parechyma cell showing protein bodies (Pb) in post-winter nodule of *L. maritimus*.

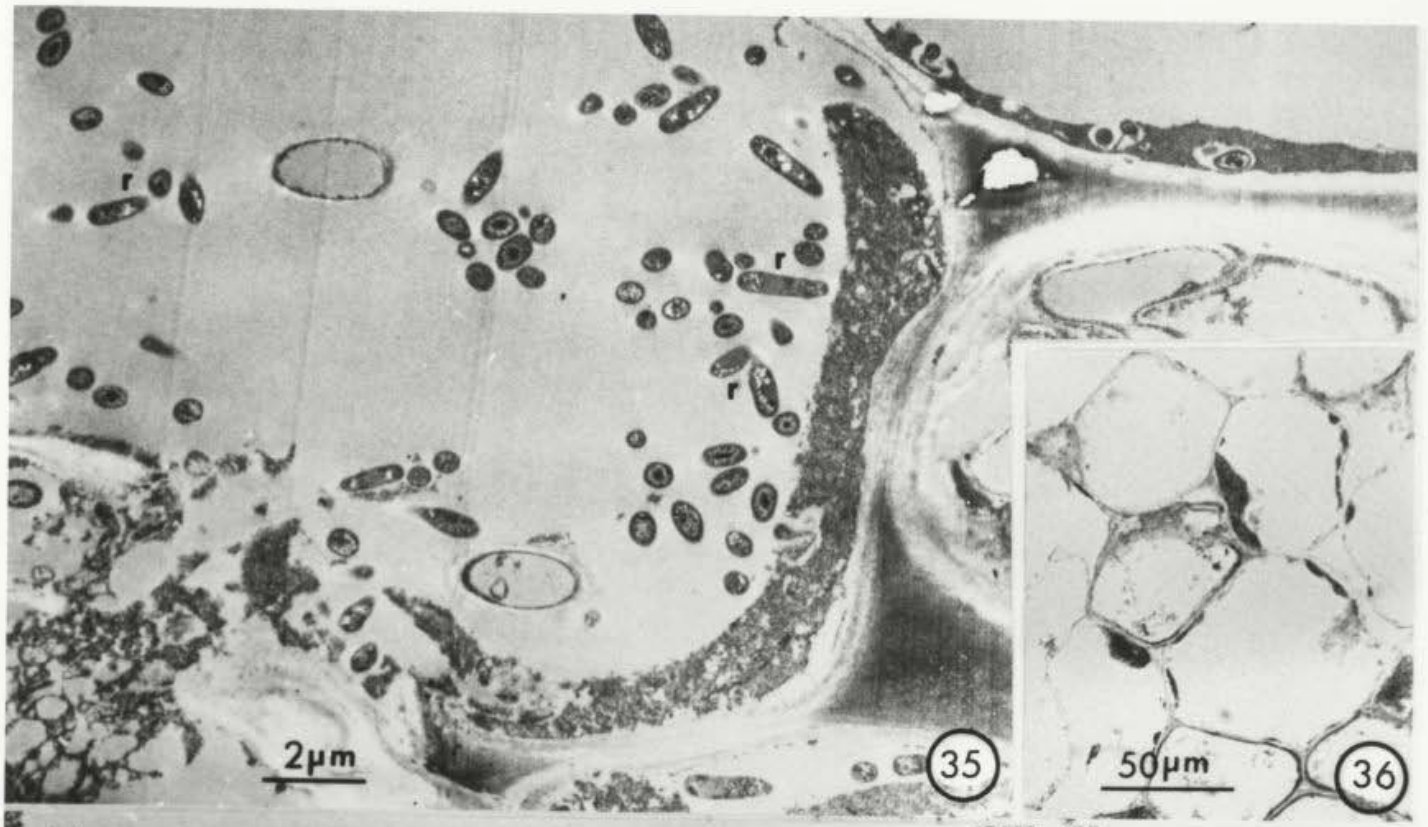

2 µm
33

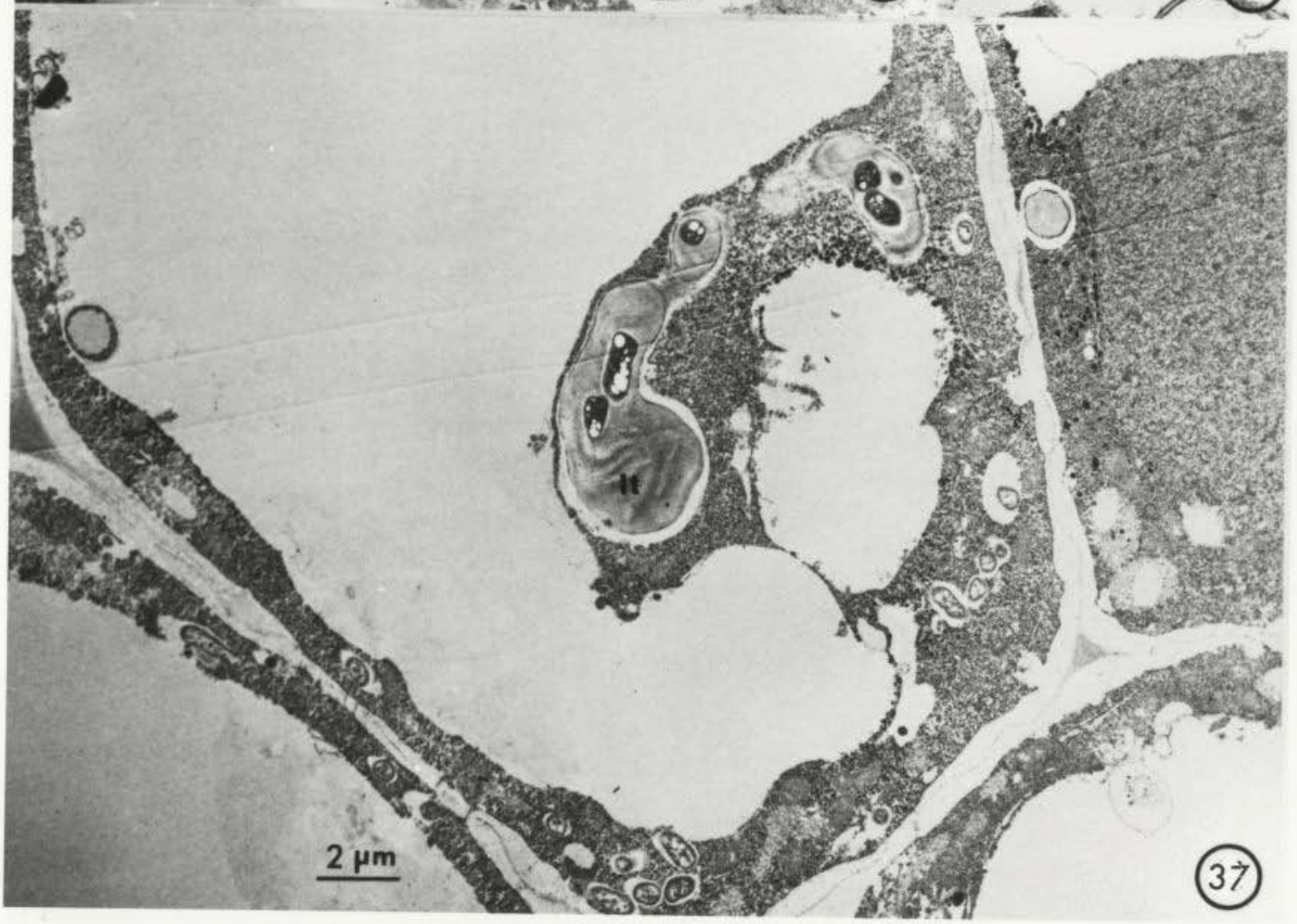

Pb
Pb
2µm
34

Fig.35 Electron micrograph of the senescent zone cell showing some rod-shaped bacteria (r) in post-winter nodule of *L. maritimus*.

Fig.36 Photomicrograph of the senescent tissue from where the Fig. 35 is taken.

Fig.37 Electron micrograph showing persistent infection thread (It) in the invasive zone of post-winter nodule of *L. maritimus*.


r
r
r
2µm
35
50µm
36


It
2 µm
37

IV DISCUSSION

IV. 1 Oleosomes and oleosins in *A. hypogaea*.

The recent studies on the composition, structure, synthesis and degradation of oleosomes have been made on seeds or germinating embryos (Huang, 1992). Most seed oils are TAG; the sole well known exception is in jojoba, which stores wax esters instead. In general, the oleosomes contain about 92.98% (w/w) neutral lipids, and 1-4% proteins. Most of the neutral lipids are TAG; the minor neutral lipid components include diacylglycerols and free fatty acids.

The composition of the TAG, including their acyl constituents and the acyl positional specificity, are highly species-specific and have been analysed extensively owing to the importance of seed oils in nutrition and industry. The major PL in the oil bodies is phosphatidylcholine, and the minor PL include phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol (Tzen and Huang, 1992).

The oleosome proteins, termed oleosins, have special characteristics and are unique to the organelles. Oleosomes in seeds are degraded during germination and post-germinative growth. TAG in oleosomes are hydrolysed to glycerol and fatty

acids, which are then converted to carbohydrates for the growth of the seedling. The subcellular location of the lipase varies according to species (Huang *et al.*, 1987). In most species, the glyoxysomes, which β-oxidize the TAG derived fatty acids for gluconeogenesis, contain a lipase in their membrane.

The oleosome associated proteins, 'oleosins' are usually a class of low-molecular weight proteins and it has been suggested that structural function of the oleosins is to prevent contact with cytosolic enzymes and the coalescence of lipid bodies. A putative lipase-attachment site on the oleosins implicated their involvement in the process of lipid degradation (Vance and Huang, 1987; Murphy *et al.*, 1991). The most extensively studied oleosins from maize showed four major polypeptides are being associated with oleosomes, one of higher molecular weight (45 KD) called H protein and three of lower molecular weight (15.5, 18.0, and 19.5 KD) called L3,L2,L1 proteins respectively (Vance and Huang, 1987). The amino acid sequences of 16 and 18 KD from the maize embryo oleosins from their gene or cDNA nucleotide sequences reveal unique secondary structures in the protein (Qu and Huang, 1990; Vance and Huang, 1987). Each oleosin molecule contains a relatively hydrophilic N-terminal domain, a central totally hydrophobic domain and amphipathic α-helical domain at or near the c-terminus. Although both maize oleosins contain these

three structural domains, their amino acid sequences as well as gene nucleotide sequences are similar only in the central hydrophobic domain. The maize oleosin 16 KD and 18 KD have been cosidered as isoforms encoded by two different genes derived from a common ancestor gene (Qu and Huang, 1990). Tzen *et al.* (1990) suggested that there are at least two immunologically distinct isoforms of oleosins present in diverse seed species, one of lower molecular weight and another one of higher molecular weight.

The results of TLC from peanut root nodules show the presence of one TAG band, one DAG band, three PL bands and SDS-PAGE shows four oleosins. However, this pattern was found to be different when compared with the seed oleosomes of the same species. The root nodule oleosins(10.0 KD, 56.3 KD, 61.1 KD & 66.0 KD) are of high molecular weight than seed oleosins (25.5 KD, 35.2 KD, 40.0 KD and 59.5 KD).

It is also interesting that these high molecular weight oleosins are present in the highly metabolically active tissue of the root nodule. The function of such high molecular weight oleosins in the root nodule remains to be elucidated. One of the possible functions could be to regulate the constant supply of carbon/energy in the process of nitrogen fixation, through the oleosome degradation. Lipase has been cytochemically localized in nodule oleosomes, where the reaction product could be seen on the periphery, while seed

oleosomes showed activity only in glyoxysomes (Jayaram and Bal, 1991). These oleosins may have lipolytic activity, which remains to be determined.

IV.2 Morphology of root nodule oleosomes from *A. hypogaea*.

The major constraint in this study was acquiring gram quantities of nodules. The fat pad was more like a film on the flotation medium as compared to the ones obtained from seed, the oleosome amounts being low i.e. only about 4-5 % of the total cell area in the nodule (Jayaram and Bal, 1991). As reported by Huang (1992), if a seed contains 40 % oil and 30 % proteins, it will have 0.4 to 1.6 % oleosins; 2-8 % of the seed protein is composed of oleosins. So, if we consider 4 % oleosomes of the nodule cell, then the oleosins would be approximately 0.1 % only.

Oleosomes from diverse species are 0.2 - 2.5 μm in diameter; the average size is species dependent and is likely affected by nutritional and environmental factors (Huang, 1992). It has been reported that, within the same seed, oleosomes in different tissues may be of different sizes. For example, in maize embryo, the oleosomes in scutellum are larger than in the embryonic axis (Trelease, 1969). Similarly

the oleosome size has also been observed to be different in different tissues of the peanut root nodules, as shown in the parenchyma cells and infected cells by Jayaram and Bal (1991). The oleosomes of the nodule fat pad are heterogenous; some are derived from the infected nitrogen-fixing cells while others, mostly the larger ones are from the uninfected parenchyma cells. The less electron-dense outer layer in the oleosome is most likely proteinaceous. This peripheral layer has shown lipolytic activity (Jayaram and Bal, 1991). This is possibly the site for lipase receptor proteins. Signs showing furrowing of the oleosomes (Fig. 6) is indicative of their catabolic process and it is assumed that with the progress of lipolysis the oleosomes become less elctron-dense and possibly appear to be swollen into larger entities as seen in the electron micrographs.

IV.3 Oleosome distribution in *A pintoi*

Recently the significance of oleosomes in root nodule of *A. hypogaea* (peanut) (Bal *et al.*, 1989; Jayaram and Bal, 1991) and their possible involvement as a supplimentary source of carbon have been documented (Siddique and Bal, 1991 and 1992). The results presented here (Fig. 8, 9 and 16) reveal the complete absence of oleosomes in the infected cells of effective, mature/red and pink nodules at all the stages of

growth in *A.pintoi*. The lack of oleosomes in the infected cells of effective nodule suggests the inability of the host to provide any additional energy and carbon source (oleosomes), which may affect nitrogen fixation and symbiotic interaction under photosynthetic stress conditions. Interestingly oleosomes (0.24 and 1.24 percent of the cell area) are present in the infected cells of immature/small/white nodules at 21 and 28 DAI and again reappear at senescing stage, when no effective symbiosis occurs. It can be therefore assumed that oleosomes are essential for the early stages of nodule differentiation, when some 30 fold increase in membranes takes place in the nodules (Verma *et al.*, 1978). A recent study on *Sesbania rostrata* nodules also confirms transient appearence of oleosomes in the early stages of nodule differentiation (Bal and Dendaluri, 1996). There seems to be no mechanism to maintain a dynamic equilibrium level of oleosomes at all stages of development in the nodules of *A.pintoi*, as in effective nodules of *A.hypogaea* (peanut) (Jayaram and Bal, 1991). However, in *A.pintoi*, nod^{+} fix^{-} nodules indicate that the infected cells are capable of synthesizing and accumulating oleosomes. This increase in oleosomes population suggested that they are not utilizaed in the absence of effective nodulation, as has been also shown in peanut (Bal and Siddique, 1991).

Lack of oleosomes at the effective stages of the nodule in *A. pintoi* suggests that these nodules may be at a disadvantage in terms of having a ready supply of additional supplimentary energy. The significance of oleosomes in the nodule parenchyma is not clear. In the uninfected parenchyma cells there is a decreasing trend in the amount of oleosomes in the mature/red nodules, compared to the mature/pink ones. The other two diploids and cultivated sp. of *Arachis* showed the presence of oleosomes in infected cells at all the developmental stages when inoculated with the same strain of *Bradyrhizobium*, suggesting that it is a host-specific characteristic of the symbiotic interactions.

The other observations that could be related to support the above hyphothesis is that large amounts of amyloplasts in parenchyma cells were observed only in this perennial peanut (Fig.8) in comparison to others. The presence of increased number of amyloplasts suggest the relative ineffectiveness of the nodule for nitrogen fixation (Bergersen, 1957; Chandler *et al.*, 1974; Newcomb *et al.*, 1977). These storage granules have to be mobilized to maintain the equilibrium of the carbon skeleton for protein and lipid synthesis.

IV.4 Nodule senescence in A. *pintoi*

Studies of the A. *pintoi* nodule have shown characteristic internal organizatio: - the large spherical bacteroids, in contrast with the elongated and branched ones of cowpea, which differentiate from the same strain of rhizobia (Sen and Weaver, 1984). In this perennial wild diploid species, A. *pintoi*, all the bacteroids in effective pink/red nodules were found to be typically spherical like the tetraploid A.*hypogaea*. However, the immature/small/white nodules show rod shaped bacteroids during early developmental stages of growth as in A. *hypogaea*. The senescing stage of the nodule in A. *pintoi* revealed a reversion of spherical bacteroids into the rod-shaped form. It is generally believed that the bacteroids of symbiotic nitrogen-fixing root nodules revert back to their asymbiotic forms when released in soil or in culture, although some nodule bacteroids lack this capability (Zhou *et al*, 1985). Carefully controlled studies have shown that bacteroids became progressively less viable with age (Sutton *et al*., 1977). The loss of viabilty has been attributed to the degree of dedifferentiation as indicated by detergent-sensitivity (Sutton and Paterson, 1980). This investigation shows that the large spherical bacteroids of A. *pintoi* nodules revert to rod-forms during senescence within the intact nodule. The

transformation of rhizobia into spherical forms in these nodules is complete as the nodule matures. Absence of any infection thread and its persistence in the mature nodule does not allow any contamination from undifferentiated rod-forms in a mature nodule. The spherical forms are so distinct in morphology that the rod-forms must arise by dedifferentiation of the mature spherical bacteroids during senescence. The rod-forms were generally found in the large vacuole of the senescing cells. This particular species, *A. pintoi*, allows fully differentiated bacteroids to revert back within the confines of its nodule tissue. This may insure survial of the bacteria when they are released in the soil.

The *A. pintoi* root nodules were subjected to premature senescence to confirm the redefferentiation of bacteroids into rod-forms, either by treatment of plants with nitrate or by detopping the plants. Indeed, the presence of rod-forms and also spherical bacteroids alongwith the presence of oleosomes could be confirmed in these nodules. The senescence induced artficially was similar to that occurred naturally or with the nod$^+$ fix$^-$ strain with the notable presence of rod-forms and oleosomes. This senescing phenomenon is interesting in *A. pintoi* and needs to be investigated in more detail because this species is recently becoming introduced as a forage crop (Thomas, 1993). Cutting of the tops will take place during grazing the animals. The nitrate induced senescence has

recently been reported by De Lorenzo *et al.* (1994) in root nodules of *Lupinus albus*; they have suggested that nitrate treatment induces a decrease in the activity of the main enzymes catalase and ascorbate peroxidase that scavenge H_2O_2 in the nodule cytosol.

Appearance of oleosomes during senescence is possibly due to conservation of degraded products of membrane, which are recycled into storage triacylglycerides. It is interesting that both reversion of rhizobia and possible reconversion of degraded products occur during senescence in these nodules. Both these phenotypic traits may be related to the perennial habit of *A.pintoi*. Senescing nodules of *A. hypogaea* (peanut) do not exhibit such characteristics.

IV.5 Seasonal effect on storage organelles in *L. maritimus* root nodule.

The histological status of the nodules of the perennial *Lathyrus maritimus* L., before and after winter throws considerable light on their survival strategy during the winter months. Throughout the growing season nodules develop continuously and therefore enter the overwintering process at various stages of development. As the nodules became active in spring, new growth takes place resulting in a constriction or

a notch in these nodules. As more than one notch was never been seen in this study, we suggest that the nodules persist only for two successive years.

During post-pod filling at the beginning of the cold season the plant undergoes a storage program in preparation for the winter. Large quantities of starch are deposited in the amyloplasts along with oleosomes, and the nodule essentially becomes a storage organ. The infected cells of the symbiotic zone show senescing bacteroids, which are at first recognizable by the β-hydroxybutyric acid granules, and finally represent degenerated clumps. However, some bacteria remain undifferentiated and protected within infection threads, and some bacteroids revert into rod-shaped bacteria. Such reversion has been reported also in the senescing nodules of perennial species of *Arachis pintoi* L (Khetmalas and Bal, 1994 and the present study).

Overwintered vascular parenchyma and endodermal cells seem to retain reasonably good ultrastructural morphology including the storage organelles. The fact that water can remain in the supercooled state within the xylem ray parenchyma (Fujikawa *et al.*, 1994), possibly allows these cells to remain metabolically active over the winter. The regeneration of the meristem therefore is likely to be initiated in the provascular parenchyma close to the distal part of the nodule. The supply of cell division - inducing

substances is likely to be transported through this tissue also.

It can be concluded that in the perennial beach pea plant a substantial amount of its energy is allocated into oleosomes and amyloplast storage organelles of the nodules after pod filling. Like the seed, the nodule becomes a storage organ, in preparation for the winter. Materials in the storage organelles are mobilized for regenerating the nodule activity even before the aerial parts of the plant have grown. Pre-winter appearance and post-winter disappearance of oleosomes and starch granules is very striking, but whether they are slowly metabolised during the winter or rapidly metabolised and exhausted within days in spring remains to be determined.

V SUMMARY

Nitrogen fixation in legume root nodules is believed to be supported by the supply of carbon compounds derived from the current photosynthate of the host cells. The presence of storage organelles such as oleosomes in the infected cells may serve as a supplementary source of carbon and energy during photosynthate stress as has been suggested for *Arachis hypogaea* root nodules (Bal, 1990; Bal et al., 1989; Bal and Siddique, 1991; Siddique and Bal, 1991, 1992).

The present investigation further showed that the peanut root nodule oleosomes has constituents DAG, TAG, PL and oleosins similar to those reported from other sources (Huang, 1992). Peanut root nodule oleosomes revealed four oleosin bands. The molecular weights were 66 KD, 61.1 KD, 56.3 KD and 10 KD. The isolated oleosomes showed considerable variation in size, electron density and in the presence of a less electron-dense peripheral layer.

The development of symbiosis in wild species of legumes such as *Arachis* and beach pea in relation to oleosomes and other ultrastructural features was studied. Microscopical observations at different developmental stages of nodules of *Arachis pintoi* have revealed that oleosomes were present only during early stages of the infection process and development

before the establishment of symbiosis, and during senescence. Oleosomes were absent in the infected cells of effective nodules of *A. pintoi* whereas similar cells in the nodules of other wild diploid speceis of *Arachis* (*A. batizocoi* and *A. duranensis*) and tetraploid *A. hypogaea* showed presence of oleosomes. Another interesting feature in this species was the reversion of spherical bacteroids into rod-forms within the confines of the senescent nodule tissue.

The histological and ultrastructural studies of the root nodules of naturally growing beach pea revealed considerable seasonal variation in the presence of storage organelles, such as amyloplasts and oleosomes. After fruiting (pre-winter) large numbers of amyloplasts with starch grains and oleosomes filled the uninfected interstitial cells and parenchyma cells of the nodule tissues. These storage organelles could not be seen in the cells of nodules sampled during post-winter periods before aerial shoots emerged, indicating their importance in overwintering. Persistent infection threads with rhizobia could be seen and rod-shaped rhizobia in senescent cells were indicative of reversion of bacteroids to rod-forms within the nodule tissue.

vi CONCLUSIONS

The peanut root nodule oleosomes are composed of DAG, TAG, PL and oleosins as reported in oleosomes from other sources (Huang, 1992). This is the first report to demonstrate the presence of oleosins in the root nodule oleosomes, which are of 66 KD, 61.1 KD, 56.3 KD and 10 KD molecular weight. The isolated oleosomes showed considerable variation in size, electron density and in the presence of a less electron-dense peripheral layer.

During the symbiotic stages, one of the species of *Arachis*, *A. pintoi* (2n, perennial) was found to be devoid of oleosomes in the mature infected cells of their nitrogen-fixing nodules. The appearance of oleosomes in white/immature nodules at the early plant growth stage, naturally and prematurally senescing nodules of *A. pintoi* indicates that the infected cells are capable of synthesizing and accumulating oleosomes.

The reversion of bacteroids into viable rod-forms in the senescing nodules was observed and reported for the first time. In the perennial beach pea plant a substantial amount of its energy is allocated into oleosomes and other storage organelles of the nodules after pod filling; starch and lipids comprise the major storage materials. Like the seed the nodule becomes a storage tissue which allows it to overwinter.

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