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NITROGEN FIXATION BY LEGUMES IN FLOODED REGIONS

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Resumo:

“Fixação de Nitrogênio por Legumes em Regiões Inundadas.”

As áreas alagadas estão, com frequência, sujeitas a perdas líquidas anuais de N do sistema via lixiviação do solo, não recompensadas pelas entradas de N via mineralização da matéria orgânica. Estes ecossistemas oligotróficos são amplamente dependentes dos acréscimos de N provenientes da fixação biológica de nitrogênio (FBN), onde as simbioses rizóbio-leguminosas são os principais contribuintes. Entretanto, leguminosas são geralmente sensíveis às inundações, e o alagamento do solo pode limitar seu crescimento pois os nódulos precisam de O_2 para manter a respiração aeróbica necessária para suprir a grande quantidade de ATP indispensável à atividade da nitrogenase. Apesar de terem adaptações como a proteína condutora de O_2 , a leghemoglobina (Lb), para otimizar o suprimento de O_2 , a maioria das leguminosas terrestres não são suficientemente capazes de aumentar o fornecimento de O_2 para dentro de seus nódulos enquanto inundadas. Portanto a FBN dessas plantas é reduzida ou completamente bloqueada. Em contraste, a maioria das leguminosas que fixam N_2 em regiões alagadas desenvolveram mecanismos adicionais que aumentam o suprimento de O_2 aos seus nódulos, e por isso são capazes de manter a FBN. Nesta revisão, examinamos as estratégias de crescimento, como a nodulação caulinar, assim como as adaptações morfológicas, estruturais e bioquímicas que permitem a essas leguminosas fixarem o N_2 enquanto inundadas.

Abstract:

Wetlands are often subject to annual net losses of N from the system via leaching of the soil, which are not balanced by inputs of N via the mineralisation of organic matter. These oligotrophic ecosystems are largely dependent on N inputs from biological nitrogen fixation (BNF), and legume-rhizobial symbioses are some of the main contributors. However, legumes are often sensitive to flooding, and soil inundation can have a serious effect on their growth. This is because legume nodules have a requirement for O_2 to maintain the aerobic respiration necessary to supply the high amounts of ATP needed for nitrogenase activity. Despite having adaptations such as the O_2 -carrying protein leghaemoglobin (Lb) to optimise O_2 supply, most terrestrial legumes are not capable of increasing the O_2 supply to and within their nodules sufficiently whilst flooded, and hence BNF is reduced or completely curtailed. By contrast, most legumes that fix N_2 in flooded regions have evolved additional mechanisms to optimise the O_2 supply to their flooded nodules, and are thus able to maintain BNF. In this review, we examine the growth strategies, such as stem-nodulation, as well as the morphological, structural and biochemical adaptations that allow these legumes to fix N_2 whilst flooded.

Introduction

Many plants in the family Leguminosae (and also the non-legume *Parasponia*; Trinick, 1979) are capable of forming N₂-fixing symbioses with soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, and *Sinorhizobium* (collectively termed rhizobia) (for recent reviews see Sprent & Sprent, 1990; Brewin, 1991; Hirsch, 1992). The ability to fix N₂ has allowed these legumes to colonise soils with low N-status and hence, by making a positive contribution to the N-cycle of N-deficient regions, are of great importance to their ecology (Sprent & Sprent, 1990). A number of N₂-fixing legumes are also important as crops *e.g.*, soybean (*Glycine max*), beans (*Phaseolus vulgaris*), clover (*Trifolium* spp.) and peanut (*Arachis hypogaea*). These plants can fix substantial quantities of N₂ and therefore they do not need costly and potentially harmful (to the environment) applications of mineral N-fertilisers (mostly nitrates or urea). For example, in Brazil, the soybean crop is worth more than 1.8 billion US dollars per year, and all its N requirements are met by N₂ fixation (Döbereiner *et al.*, 1995). Moreover, in the tropics, N₂-fixing legumes *e.g.*, cowpea (*Vigna unguiculata*) are frequently used as "green manures" which replace the need for N-fertiliser by actually adding N to the soil for the use of subsequent, non-N₂-fixing crops *e.g.*, maize (*Zea mays*) (Giller & Wilson, 1991; Döbereiner *et al.*, 1995).

The importance of N₂-fixing legumes in tropical wetlands

Seasonally flooded ecosystems such as the Pantanal Mato-Grossense and the Amazon in Brazil, and the flooded forests of the Orinoco basin, are of regional and global importance due to their contributions to the hydrological and biogeochemical cycles, as well as being areas of high biodiversity, with many plant and animal species threatened with extinction (Salati *et al.*, 1982; Allem & Valls, 1987; Junk *et al.*, 1989; Groombridge, 1992; Barrios & Herrera, 1993a, b). Indeed, Crawford (1992) has recently stressed the importance of seasonal flooding in maintaining the biodiversity of wetland plants. Therefore, an understanding of the processes underlying the plant diversity *e.g.*, nutrient and O₂ availability (Crawford, 1992), as well as the net contribution by nodulated legumes to the N-cycle (Sprent & Sprent, 1990), is essential for the maintenance of their ecosystems.

In tropical wetlands, many of the plant species are nodulated legumes. Recent work has shown that not only will these fix substantial amounts of N₂ whilst flooded, but there may even be positive selection pressure for them (Allem & Valls, 1987; Walter & Bien, 1989; de Souza-Moreira *et al.*, 1992; Barrios & Herrera, 1993a, b; Loureiro *et al.*, 1994, 1995; James *et al.*, 1995a, b). This selection pressure may be due to the inherently low N-status of the heavily leached soils in tropical wetlands, and also because, under flooded conditions, there is a decrease in the mineralisation of organic matter, which consequently results in a shortage of available N (Bennett & Albrecht, 1984; Walter & Bien, 1989; Barrios & Herrera, 1993b). As N₂-fixing legumes contribute

significantly to the N-balance of tropical wetlands (Salati *et al.*, 1982; Barrios & Herrera, 1993b), they are of obvious importance in studies on their preservation and sustainability (Sprent & Sprent, 1990; Sprent, 1995). Moreover, many of the N₂-fixing legumes are themselves under pressure from logging, and from grazing by indigenous and agricultural fauna (Allem & Valls, 1987; Barrios & Herrera, 1993a, b). For example, Barrios & Herrera (1993a, b) have highlighted the selective logging of the N₂-fixing trees, *Acosmium nitens* and *Campsiandra laurifolia*, from the flooded forests of the Orinoco. In addition, smaller legumes such as *Discolobium pulchellum* and *Aeschynomene* spp. are preferentially grazed by animals (*e.g.*, cattle, capybara, various fish species) in the Pantanal due to their high protein content (Allem & Valls, 1987). Therefore, in addition to the stresses imposed by their seasonally flooded environments (Crawford, 1992), N₂-fixing legumes are also under threat from animal and man-made pressures, and the consequences of a subsequent decline in their number/diversity may have serious and unforeseen effects on the N-cycle (Sprent & Sprent, 1990).

Not only are wetland legumes ecologically important, but they are also of agricultural importance. For example, there has been much recent interest in using nodulated wetland legumes, particularly stem-nodulated ones, as green manures in the cultivation of lowland rice due to their high rates of N₂ fixation under flooded conditions (Dreyfus *et al.*, 1985; Morris *et al.*, 1989; Ladha *et al.*, 1992a, b; Murooka *et al.*, 1993; Gutteridge, 1994; Becker & George, 1995; Boivin *et al.*, 1997). Therefore, in this paper we review the various adaptations (mostly related to O₂ supply) which allow wetland legumes to nodulate, grow and fix N₂ whilst inundated for all or part of the year. In the final part of the review, we examine in detail stem-nodulation as an adaptation to flooding tolerance, and how it may allow for high N₂-fixation rates, particularly in "green manure" legumes.

The role of O₂ in nitrogen fixation by legume nodules

In order for the process of N₂-fixation to occur, and for there to be efficient exchange of metabolites between host and microsymbiont (*i.e.*, photosynthate in exchange for fixed N-compounds), legumes have evolved a complex organ, the nodule. Nodules develop on the host legume roots, and also the stems of some species (see later), after they are infected by compatible rhizobia. The infection process involves a complex series of exchanges of chemical signals between the plant and the rhizobia (Brewin, 1991; Hirsch, 1992), and usually (but not always; see review by Sprent, 1989) involves the rhizobia entering a root hair and subsequently infecting a nodule meristem developing within the infected root. After the nodule is initiated it grows out from the root, the bacteria multiply rapidly within the host cells, and the rhizobia (now termed *bacteroids*) begin to fix N₂ within 1-3 weeks of nodule initiation, depending on species. The central, N₂-fixing, infected zone is surrounded by uninfected cell layers, collectively termed the cortex. The cortex contains the vascular system by which the nodule is connected to the parent root, and hence is able to exchange metabolites with the host.

N_2 -fixation is an energy hungry process requiring 16 molecules of ATP for every molecule of ammonia produced (Gallon, 1992). For the bacteroids to supply sufficient ammonia to the plant they must use aerobic respiration to produce this ATP from the C-substrates provided by the host plant (Witty *et al.*, 1986; Hunt & Layzell, 1993). This requirement for aerobic bacteroid respiration has created an apparent paradox in the functioning of legume nodules as nitrogenase, the enzyme responsible for the N_2 fixation, is readily denatured by pO_2 s greater than 5 mmol m^{-3} (Sheehy & Thornley, 1988; Gallon, 1992). To resolve this paradox the nodule has a gaseous diffusion barrier in the cortex which prevents the inward diffusion of atmospheric O_2 (Tjepkema & Yocum, 1974; Witty *et al.*, 1987), and hence maintains the pO_2 within the infected tissue to a concentration at which nitrogenase expression and activity can occur ($20\text{-}30 \text{ mmol m}^{-3}$; King *et al.*, 1988). Moreover, nodules also contain a high concentration of the O_2 -carrying protein, leghaemoglobin (Lb), which surrounds the bacteroids within the host cells (Appleby, 1984). As Lb allows low concentrations of dissolved O_2 to be conveyed to the N_2 -fixing bacteroids at a rapid rate, their respiratory requirements are satisfied without incurring damage to nitrogenase.

Nodules are able to regulate further the O_2 supply to the bacteroids through controlling the permeability of the cortical O_2 diffusion barrier (Witty *et al.*, 1986; Witty & Minchin, 1994; Hunt & Layzell, 1993; Minchin, 1997). This regulation is probably brought about via the variable occlusion of intercellular air spaces in the mid- and inner cortices (Parsons & Day, 1990; James *et al.*, 1991, 1996, 1997; Webb & Sheehy, 1991; Witty & Minchin, 1994; Iannetta *et al.*, 1995) and/or pulvinar movements in the inner cortex (Serraj *et al.*, 1995). The ability to regulate O_2 supply is critical to the functioning of the symbiosis as it allows it to adapt rapidly to alterations in energy supply caused by environmental perturbations such as temperature changes, darkness and defoliation (Witty *et al.*, 1986; Hunt & Layzell, 1993). For example, a reduction in energy supply to the bacteroids brought about via shoot darkening would result in a decrease in bacteroid respiration, and hence (according to Ficks law of gaseous diffusion) a potentially damaging increase in the pO_2 of the infected tissue would occur if the nodule was not capable of increasing rapidly the resistance of the cortex to O_2 diffusion (Witty *et al.*, 1986; Iannetta *et al.*, 1995). Conversely, at the end of a period of darkness, when photosynthate supply is restored, the O_2 diffusion barrier opens to allow the bacteroids to take advantage of the increase in carbon substrates, and consequently there is an observed increase in bacteroid respiration and N_2 fixation (Witty *et al.*, 1986; Hunt & Layzell, 1993).

The variable O_2 diffusion barrier also allows some legume nodules to adapt to artificially imposed increases and decreases in external pO_2 (compared to ambient), over both the short- and long-term in greenhouse and controlled environment experiments (Minchin *et al.*, 1983, 1992; Weisz & Sinclair, 1987a, b; Hunt *et al.*, 1987; Witty *et al.*, 1987; Drevon *et al.*, 1988; Rosendahl & Jakobsen, 1988; Parsons & Day, 1990; Dakora & Atkins, 1990, 1991; James *et al.*, 1991; Dalton *et al.*, 1991; Atkins *et al.*, 1993;

Iannetta *et al.*, 1995). However, in nature, variations in rhizosphere pO_2 will usually only mean *decreases* in pO_2 , and these decreases will often be brought about via flooding of the soil surrounding the nodulated roots (Walker *et al.*, 1983; Morris *et al.*, 1989; James *et al.*, 1992a, b; Arrese-Igor *et al.*, 1993; Becker & George, 1995; Pugh *et al.*, 1995). Indeed, soil inundation can impose major constraints on the growth of N_2 -fixing crops as most species used in agriculture are not adapted to flooding. Even with the remarkable adaptability shown by nodules to variations in O_2 supply (see references above), the majority of terrestrial legumes (and even some wetland legumes) so far examined showed decreases in N_2 fixation after prolonged flooding or exposure of their nodules to lower than ambient pO_2 *e.g.*, soybean, *Vigna* spp., *Vicia faba*, lupin (*Lupinus* spp.), lucerne (*Medicago sativa*), *Lotus* spp., pea (*Pisum sativum*), *Leucaena leucocephala*, *Aeschynomene* spp., *Sesbania* spp. and *Viminaria juncea* (Bond, 1951; Mague & Burris, 1972; Minchin & Pate, 1975; Minchin & Summerfield, 1976; Trinick *et al.*, 1976; Hong *et al.*, 1977; Gallacher & Sprent, 1978; Zablutowicht & Focht, 1979; Bisseling *et al.*, 1980; Hunter *et al.*, 1980; Albrecht *et al.*, 1981; Walker *et al.*, 1983; Bennett & Albrecht, 1984; Morris *et al.*, 1989; Trinchant & Rigaud, 1989; Dakora & Atkins, 1990, 1991; Dalton *et al.*, 1991; Shiferaw *et al.*, 1992; Atkins *et al.*, 1993; Zhang *et al.*, 1995). However, there are exceptions, such as white clover (Pugh *et al.*, 1995), and even some studies on soybean (Nathanson *et al.*, 1984; Parsons & Day, 1990; James *et al.*, 1991) and lucerne (Arrese-Igor *et al.*, 1993).

Growth strategies and morphological adaptations used by flooded legumes

In swamplands such as the Pantanal Mato-Grossense, much of the vegetation exists as mats, floating on the surface of the water. This type of vegetation simply moves up and down with the water level as it fluctuates through the year. Many of the plants on these mats in the Pantanal are nodulated legumes. For example, Loureiro *et al.* (1996) have recently reported nodules on adventitious roots arising from floating, aerenchymatous stems of *Vigna longifolia* and *Sesbania exasperata*; both the (submerged) roots and the stems of these species were nodulated. Moreover, *Neptunia* species are very common in tropical wetlands, including the Pantanal, and most of the wetland species have nodulated adventitious roots arising from hollow stems, and a similar floating growth habit to that described above for *V. longifolia* and *S. exasperata* (Schaeede, 1940; James *et al.*, 1992a, b; Subba-Rao *et al.*, 1995). Indeed, after a series of experiments, James *et al.* (1992a, b) concluded that it was partly these adaptations that enabled submerged *Neptunia plena* nodules to fix the same amount of N_2 as terrestrially grown nodules on the same species. James *et al.* (1992b) also showed that a reduction in root mass and number of nodules on submerged *N. plena* compared to vermiculite-grown plants, helped to increase the proportion of nodulated root respiration devoted to nitrogenase activity in the former. Therefore, it appears that a combination of a floating growth habit, a reduction in root/nodule mass, and diffusion of air down aerenchymatous stems and lenticels to submerged nodulated roots may be sufficient to allow nodules on some legume species to obtain O_2 for N_2 fixation.

The main advantage of having nodules on roots arising from floating stems is that air pathways are kept to a minimum (James *et al.*, 1992b) *i.e.*, the species employing this strategy do not have nodules which are profoundly submerged, and hence simple diffusion of O₂ through the interconnected pathway of stem/root/nodule aerenchyma/lenticels is sufficient to supply the respiratory needs of the nodules. Indeed, James *et al.* (1992a, b) reported that *N. plena* nodules tended to be concentrated at the top of the tap root near the base of the stem. However, there are a number of wetland legumes which have nodules more profoundly submerged than *Neptunia*, and other floating species. For example, *Discolobium*, a legume from the Pantanal newly-reported as being both root and stem nodulated (Loureiro *et al.*, 1994), can have its stem submerged by over 2 m (Loureiro, James & Pott, unpubl.). As most of the nodules on *Discolobium* are on the roots and the lower stem it is likely that for much of the year these nodules will experience severe constraints on O₂ supply (Armstrong, 1979). Nevertheless, submerged *Discolobium* nodules are obviously active, with visible Lb and significant nitrogenase activity (as determined by the acetylene reduction assay and ¹⁵N₂ incorporation; Loureiro *et al.*, 1994). Loureiro *et al.* (1994) concluded that the nodules of *Discolobium* probably received an air supply via the profuse lenticellular tissue down the submerged stem, which connected with lenticels on the nodules themselves. In addition, *Discolobium* stems are very light and made up of cells with many large intercellular spaces. This latter adaptation is very common in wetland plants (Armstrong, 1979; Justin & Armstrong, 1987; Crawford, 1992; Armstrong & Armstrong, 1994), and has also been observed in nodulated *Neptunia* (James *et al.*, 1992a, b), *Sesbania* (Morris *et al.*, 1989; Becker & George, 1995), *V. juncea* (Walker *et al.*, 1983) and *Aeschynomene* species *e.g.*, *A. fluminensis* (Loureiro *et al.*, 1995). Indeed, in the case of *A. fluminensis*, another stem and root-nodulated species from the Pantanal, Loureiro *et al.* (1995) observed that intercellular air spaces in roots and stems were particularly large at the base of the nodules that they subtended. This suggested that the "standard" air pathways to the submerged roots of non-nodulated wetland plants (Armstrong, 1979; Justin & Armstrong, 1987) had been adapted in this species to supply the high respiratory demands of the submerged N₂-fixing stem and root nodules (Loureiro *et al.*, 1995). It would be interesting to quantify, perhaps using ¹⁵N₂ and/or ¹⁸O₂ (Walker *et al.*, 1983; James *et al.*, 1992b), and assess whether the passive diffusion of air down the stem to the submerged nodules on *e.g.*, *Discolobium* and *Aeschynomene* spp., is adequate to support biological nitrogen fixation (BNF). In addition, high rates of *facilitated*, as opposed to purely *passive*, gaseous diffusion down stems to submerged roots/rhizomes have been demonstrated to occur in water lilies (Dacey, 1980), reeds (*Phragmites australis*) (Armstrong *et al.*, 1992), and also trees such as *Alnus* (Grosse & Schroder, 1985). These processes include thermal diffusion, humidity-induced pressure flow, and Knudsen diffusion, and are reviewed by Crawford (1992). It is reasonable to suggest that some, or all, of these processes may also occur in nodulated wetland legumes, particularly those which are rhizomatous (Armstrong *et al.*, 1992) *e.g.*, *Discolobium* (Pott, Loureiro & James, unpubl.).

A very common strategy of wetland plants is to produce adventitious roots on the submerged stem in order to overcome the O₂ constraints suffered by the submerged main root system (Jackson, 1985; Morris *et al.*, 1989; Crawford, 1992). We have mentioned previously that legume species with a floating growth habit also produce adventitious roots, and that these will nodulate *e.g.*, *Neptunia* (Schaeede, 1940; James *et al.*, 1992a, b; Subba-Rao *et al.*, 1995). In the case of *Neptunia*, as the nodules are so close to the stem, they have often been mistaken for stem nodules *e.g.*, Ladha *et al.* (1992a). However, Schaeede (1940) in his seminal paper on *Neptunia* states that the nodules he observed were on the adventitious roots, and this has since been confirmed by James *et al.* (1992a) and Subba-Rao *et al.* (1995). A more detailed description of "true" stem nodules is given in a later section.

Nodulated adventitious roots (but not stem nodules) have also been reported on *Campsiandra laurifolia* (Barrios & Herrera, 1993a) and *Mimosa pellita* (James *et al.* 1995a, b). Both these trees are abundant along the margins of tributaries of the Orinoco (Barrios & Herrera, 1993a, b) and, in the case of *C. laurifolia*, may be seasonally submerged by up to 12 m during the wet season (Barrios & Herrera, 1993a). Indeed, *Campsiandra* nodules were examined by Barrios & Herrera (1993a) throughout the year and were always found to have significant nitrogenase activity (determined by acetylene reduction and ¹⁵N studies). This was not just the case with nodules on the adventitious roots, but also included those nodules which were obtained by diving 12 m down to the submerged soil, which was likely to be virtually anaerobic at the height of the flooded season (Crawford, 1992). Initial experiments with *M. pellita* suggest that it is not as tolerant of flooding as *Campsiandra* (James *et al.*, 1995a, b), which may be the most extreme case of a flooding tolerant, N₂-fixing legume so far reported. However, *M. pellita* is extremely common in South American wetlands (Barrios & Herrera, 1993b) *e.g.* it is ubiquitous in the Pantanal (Pott & Pott, 1994) and was recently observed growing in great numbers alongside partially-submerged *Discolobium* (Loureiro *et al.*, 1996). James *et al.* (1995b) have recently reported that nodules on *M. pellita* adventitious roots are highly lenticellular and are connected via root lenticels to lenticels on the submerged stem, thus suggesting a continuous air pathway from the aerial stem to the submerged adventitious root nodules. Similar air pathways to submerged nodules have also been demonstrated with those on the roots/adventitious roots of *V. juncea* (Walker *et al.*, 1983), *Neptunia* (James *et al.*, 1992a, b), and the temperate wetland legume, *Lotus uliginosus* (James & Crawford, 1998).

Another symbiosis which is worthy of mention in respect to nodulation of adventitious roots is that between *Pentaclethra* and rhizobia (Walter & Bien, 1989). This unusual species will form adventitious roots on flooded stems which remain nodulated as the water level recedes resulting in apparently fully functional "aerial" root nodules. The consequences of such an aerial position as regards nodule O₂ relations is discussed later in this review.

Nodules on flooding-tolerant legumes: structural, physiological and biochemical adaptations

There have been a number of recent reports on the structure of root and stem nodules on flooding tolerant legumes (James *et al.*, 1992a, b, 1995b, 1996; Loureiro *et al.*, 1994, 1995; Subba-Rao *et al.*, 1995). Although submerged nodules on *Neptunia*, *Mimosa pellita*, *Campsiandra*, *Sesbania* and *Aeschynomene* tend to have highly lenticellular cortices, in general they have structures similar to those of terrestrial nodules (James *et al.*, 1992a, 1995b, 1996; Barrios & Herrera, 1993a; Loureiro *et al.*, 1995), with the obvious exception of the chloroplast-containing, photosynthetic stem nodules on *Sesbania rostrata* (Dreyfus & Dommergues, 1981; Duhoux, 1984) and some *Aeschynomene* species (Yatazawa & Yoshida, 1979; Vaughn & Elmore, 1985; Alazard & Duhoux, 1987; Loureiro *et al.*, 1995). Indeed, if nodules from terrestrial species *e.g.*, soybean, cowpea and lucerne are grown in below-ambient pO_2 s they resemble aquatic nodules (*i.e.*, with increases in lenticellular and outer cortical tissue), and hence it has been concluded that even terrestrial nodules can adapt their structures to low rhizosphere pO_2 s, if given sufficient time to develop (Parsons & Day, 1990; Dakora & Atkins, 1990, 1991; James *et al.*, 1991; Arrese-Igor *et al.*, 1993; Pugh *et al.*, 1995). For example, James *et al.* (1992a) and Loureiro *et al.* (1995) recently concluded that the structure of submerged *Neptunia* root and *A. fluminensis* root/stem nodules, respectively, differed little from nodules of these species grown under well-drained, terrestrial conditions, and concluded that rates of O_2 supply to the submerged nodules (via the adaptations discussed in the previous section) were probably similar to those of terrestrial ones.

At this point it is worth considering how legume nodules, both tolerant and non-tolerant to flooding, avoid an influx of water into the N_2 -fixing tissue when they are inundated. Webb & Sheehy (1991) have reported a hydrophobic surface on a number of terrestrial nodule types, and Parsons *et al.* (1993) and Loureiro *et al.* (1994) have suggested that an impermeable, "waxy" or "corky" epidermis covers the stem nodules of *S. rostrata* and *Discolobium*, respectively. It remains to be established whether this is true of other nodules as well.

Of all the flooding tolerant, N_2 -fixing legumes so far examined, one of the most interesting is *Discolobium* (Loureiro *et al.*, 1994) as, unlike those on *Neptunia* and *A. fluminensis* described above, the nodules on this plant differ greatly from terrestrial nodules. Indeed, it is impossible to properly compare aquatically and terrestrially-grown *Discolobium* nodules as the nodules senesce rapidly if the water surrounding them is removed (Loureiro *et al.*, 1994). *Discolobium* stem and root nodules, uniquely among legume nodules, have a vascular system which penetrates the infected tissue of the nodules within cortical ingrowths. Loureiro *et al.* (1994) suggested that these ingrowths may be an adaptation to low pO_2 as they could provide a routeway for O_2 diffusion (via air supplied by stem and nodule lenticellular pathways) into the centre of the infected tissue. Another very interesting legume in this respect is *Campsiandra laurifolia* (Barrios &

Herrera, 1993a). The O_2 pathways to, and within, the very profoundly submerged nodules on this species have yet to be investigated, although *C. laurifolia* nodules are perennial and appear to survive the flooded period by maintaining their meristems (most of the N_2 -fixing infected cells senesce), which subsequently produce new infected cells when the floodwater recedes in the dry season (Barrios & Herrera, 1993a).

Interestingly, all the submerged nodules from flooding tolerant species so far examined in respect to O_2 diffusion (e.g., *A. fluminensis*, *Discolobium*, *Lotus uliginosus*, *Mimosa pellita*, *Neptunia*) have shown structural evidence of a "typical" oxygen diffusion barrier (James *et al.*, 1992a; 1995b; Loureiro *et al.*, 1994, 1995; James & Crawford, 1998) i.e., layers of interlocking cells with few, or no, radially-orientated intercellular spaces in the inner cortex immediately adjacent to the infected zone (Parsons & Day, 1990; Brown & Walsh, 1994), and glycoprotein occlusions of intercellular spaces of the inner and mid-cortex (James *et al.*, 1991, 1994). At first such a barrier seems to be an anachronism as most of these species appear to have a need for an *increased* O_2 supply to the infected zone and a *fixed* O_2 diffusion barrier will obviously restrict this. However, it must be remembered that all the above-mentioned species are subjected to *fluctuating* water levels and hence the O_2 supply to their submerged nodules will vary greatly during the growth of the plant. Therefore, a *variable* O_2 diffusion barrier (Witty *et al.*, 1986; Hunt & Layzell, 1993) will be of obvious use in regulating the supply of O_2 to the infected zone. For example, whilst flooded the nodules are able to maintain maximum aeration of the infected tissue by keeping the barrier "open" but, upon exposure to the air after a flooding period, the barrier can "close" and so prevent immediate denaturation of nitrogenase via O_2 and toxic oxygen radicals (Gallon, 1992). Indeed the O_2 diffusion barrier in flooding-tolerant nodules (e.g., those on *Neptunia*; James *et al.*, 1992a, b) may act as a defence against post-anoxic stress, which is a major problem experienced by other organs on flooding-tolerant plants upon re-exposure to air (Crawford, 1992; and see later). James *et al.*, (1991, 1997), de Lorenzo *et al.* (1993) and Iannetta *et al.* (1993a, b; 1995) have presented evidence suggesting that the O_2 diffusion barrier in terrestrial nodules may be altered by varying the extent to which intercellular air spaces in the cortex are occluded by material, including glycoproteins. The presence of intercellular space-occluding material in many flooding- and hypoxia-tolerant nodules (James *et al.*, 1992a; 1994, 1995b, 1996; Arrese-Igor *et al.*, 1993; Loureiro *et al.*, 1994, 1995; James & Crawford, 1998) suggests that it may also be involved in O_2 regulation in these as well.

A recent example of flooding tolerant nodules experiencing post-anoxia comes from the study of James *et al.* (1992b) in which nodulated roots of water-cultured *Neptunia* were immediately drained and hence subjected rapidly to full atmospheric pO_2 . This resulted in an immediate cessation of nitrogenase activity which did not recover. By contrast, when submerged nodulated roots were drained, but exposed only to 1% O_2 followed by gradual, incremental (30 min steps) increases in rhizosphere pO_2 of 5% to 21% O_2 , the nitrogenase activity of the nodules increased gradually to the same level as

nodules grown in 21% O₂. These experiments demonstrated that, although the structural and physiological characteristics of the O₂ diffusion barrier of flooded *Neptunia* nodules did not differ from those of the non-flooded nodules (James *et al.*, 1992a, b) the flooded nodules did differ to the extent in which the variable component of the O₂ diffusion barrier was "open" (Minchin *et al.*, 1985, 1992). This meant that, as O₂ supply to the flooded nodules was largely dependent on air diffusing down stem aerenchyma and lenticels, the diffusion barrier of the nodules was set to a "minimum" level such that O₂ influx across the barrier could be maximised under the O₂-limiting growth conditions. This strategy resulted in the destruction of nitrogenase upon the rapid exposure of flooded nodules to air because the variable diffusion barrier of the previously flooded nodules could not accommodate such a rapid increase in pO₂ (James *et al.*, 1992b). By contrast, the variable component of the diffusion barrier in flooded *N. plena* nodules which were subjected to *gradual* increases in pO₂, was able to adapt to these, in common with nodules on terrestrial legume species (Minchin *et al.*, 1983, 1992; Hunt *et al.*, 1987, 1989; Weisz & Sinclair, 1987; Drevon *et al.*, 1988; Rosendahl & Jakobsen, 1988; Dakora & Atkins, 1991; Diaz del Castillo *et al.*, 1992; Atkins *et al.*, 1993). Further evidence about the finely-balanced nature of the O₂ relations of submerged *Neptunia* nodules came from studies of direct aeration or agitation of the water surrounding the nodulated roots. Direct aeration, in a manner commonly used in hydroponics, had an adverse effect on growth and N₂ fixation whereas agitation (stirring) of the water was beneficial (James *et al.*, 1992b).

Other than the studies of James *et al.* (1992a, b) there has been relatively little work on the physiology of flooding tolerant legumes, particularly on the O₂ relations of their nodules. Walker *et al.* (1983) examined *Viminaria juncea*, an herbaceous legume from Australia that is seasonally flooded by shallow water, and showed that, although it produced aerenchyma and pneumatophores (vertical tubes connecting diageotropic roots to the atmosphere above the floodwater) to assist in aeration of the nodulated roots, its N₂ fixation was still reduced by over 50% during the wet season. By contrast, Loureiro *et al.* (1995) have shown that with *A. fluminensis*, which is submerged for much of the year by over 2 m, there was an actual increase in plant dry weight with flooding. Similar results have been obtained with *Sesbania rostrata* (Ndoye & Dreyfus, 1988; Becker & George, 1995), and with the "terrestrial" species, *Trifolium repens* (Pugh *et al.*, 1995) and soybean (Nathanson *et al.*, 1984). James & Crawford (1998) have recently obtained some interesting data by comparing the growth and N₂ fixation of two temperate species of *Lotus* after exposing their (flooded) nodulated roots to two levels of hypoxia. Surprisingly, even *Lotus corniculatus*, which is of "intermediate" flooding tolerance (Justin & Armstrong, 1987; Shiferaw *et al.*, 1992) grew well and incorporated equal amounts of N when the growth medium was bubbled with either air or N₂ (giving dissolved O₂ concentrations of 0.241 and 0.094 $\mu\text{mol ml}^{-1}$, respectively). However, even more strikingly, when the highly flooding-tolerant species *Lotus uliginosus* (Justin & Armstrong, 1987) was treated in this manner it showed a significant increase in growth and N₂ fixation compared to *L. corniculatus* under both aeration regimes and, moreover,

fixed significantly more N_2 under the more hypoxic (N_2 -bubbled) compared to the less hypoxic (air-bubbled) regime. Both *Lotus* species produced ethylene when flooded (Jackson, 1985), and developed lenticels/aerenchyma around the submerged portion of the stem (Justin & Armstrong, 1987; Armstrong & Armstrong, 1994), although the latter were considerably more profuse on *L. uliginosus*. The enhancement of N_2 fixation under hypoxia shown by *L. uliginosus* was attributed partly to its increased nodulation, and partly to its apparent increased ability to transport O_2 to the N_2 -fixing cells within the nodules (James & Crawford, 1998).

There have also been few studies on the occurrence of the O_2 -carrying protein, leghaemoglobin (Lb), within flooding-tolerant nodules. Lb is critical to the functioning of legume-rhizobium symbioses and its occurrence is closely correlated with nitrogenase activity (Appleby, 1984; King *et al.*, 1988; Dakora *et al.*, 1991; Hunt & Layzell, 1993; Dakora, 1995). It is located within the cytoplasm of the infected cells (Robertson *et al.*, 1984; VandenBosch & Newcomb, 1988), and it acts by rapidly conveying dissolved O_2 to the bacteroids so that they have adequate concentrations to support the high respiration associated with nitrogenase activity (Appleby, 1984). Considering the O_2 -carrying role of Lb in nodules grown under ambient pO_2 , it might be expected that concentrations of Lb would be increased in nodules grown in below-ambient pO_2 s. However, there is discrepancy in the literature regarding this. For example, although James *et al.* (1991) reported an increase in Lb concentration in soybean nodules grown in 10% O_2 (compared to those grown in 21% O_2), Parsons & Day (1990) showed no difference in haem content of nodules grown at 4.7 kPa, and Dalton *et al.* (1991), Dakora *et al.* (1991), and Arrese-Igor *et al.* (1993) all reported *decreases* in Lb concentrations in nodules at various below-ambient pO_2 s. However, both Dakora *et al.* (1991) and Arrese-Igor *et al.* (1993) emphasise that on a *per bacteroid* basis, the amount of Lb available was actually increased in the low O_2 nodules in their studies.

James *et al.* (1992b) showed no differences between flooded and non-flooded *Neptunia* nodules in their Lb concentrations, suggesting that the flooded *Neptunia* nodules had an adequate O_2 supply (via the various adaptations discussed previously), and thus precluded the need for extra Lb synthesis. James & Crawford (1998) found that *L. uliginosus* nodules had decreased concentrations of Lb compared to *L. corniculatus* nodules, and this was decreased in both species by a prolonged period of hypoxia (bubbling of the growth medium by N_2). However, on a nodule protein basis, Lb concentration was actually increased in *L. corniculatus* nodules by the hypoxic treatment, and James & Crawford (1998) concluded that this indicated that O_2 supply to *L. corniculatus* nodules was reduced when they were grown under hypoxia compared to the flooding-tolerant *L. uliginosus* nodules. It would be interesting to examine the Lbs in nodules which are more profoundly flooded, and thus which may have more acute O_2 constraints than *Neptunia* and *L. uliginosus* nodules, such as those on *Discolobium* and *Campsiandra* (Barrios & Herrera, 1993a; Loureiro *et al.*, 1994). The Lb in stem nodules on *S. rostrata* and *Aeschynomene* spp. is discussed in a later section.

We have mentioned previously that nodules growing in flooded environments will be subjected to fluctuating water levels and that these will result in periods of anoxia/hypoxia, followed by post-anoxic periods. Crawford (1992) suggested that the post-anoxic periods are more hazardous to plants than the actual flooded periods, as the rapid influx of O₂ into tissues that are under a highly reduced state results in the rapid production of potentially devastating O₂ radicals, such as superoxide. Some plants which are adapted to prolonged periods of flooding *e.g.*, *Iris pseudacorus*, have rhizomes which can withstand being buried in anaerobic mud for many weeks, followed by re-exposure to air. *I. pseudacorus* rhizomes tolerate the post-anoxic shock by accumulating large concentrations of O₂ defence enzymes such as superoxide dismutase (SOD) (Crawford, 1992) which converts superoxide to the less harmful hydrogen peroxide. The latter is then generally disposed of via catalase, peroxidases or the ascorbate-glutathione pathway (Gallon, 1992). It is well known that nitrogenase is extremely sensitive to O₂ and O₂-derived radicals, and the rhizobial nitrogenase within legume nodules is no different in this respect (see reviews by Becana & Rodriguez-Barrueco, 1989; Gallon, 1992). Indeed, the presence of Fe-containing compounds, such as nitrogenase and Lb, makes the highly-reducing conditions in the centre of legume nodules particularly conducive to the production of toxic oxygen species (Puppo *et al.*, 1982; Becana & Rodriguez-Barrueco, 1989; Gallon, 1992). However, nodules contain high concentrations of antioxidants *i.e.*, SOD, catalases, peroxidases, ascorbate, glutathione and *α*-tocopherol, to combat these radicals (Puppo *et al.*, 1982; Becana & Rodriguez-Barrueco, 1989; Dalton *et al.*, 1991, 1993; Gallon, 1992; Escuredo *et al.*, 1996). Recently, Dalton *et al.* (1991, 1993) have shown that concentrations of glutathione and ascorbate are positively correlated with effectiveness of nodules on soybean and other terrestrial legumes, and are also altered in response to changes and fluctuations in ambient pO₂. Therefore, nodules on flooding-tolerant legumes that are subjected to fluctuating water levels may also have an enhanced requirement for such defences, in common with other organs on flooding-tolerant plants (Crawford, 1992). However, the only recent work published on this subject has been that of Puppo *et al.* (1986) with aerial stem nodules on *S. rostrata* (see later).

Plants subjected to prolonged periods of anoxia/hypoxia often convert their respiratory metabolism from aerobic pathways to fermentative ones (Crawford, 1992). It is generally considered that, although the N₂-fixing region of legume nodules is inherently hypoxic and O₂-limited, aerobic respiration by the bacteroids is supported by Lb-facilitated transport of dissolved O₂ through the host cytoplasm to the bacteroids (Witty *et al.*, 1986; Hunt & Layzell, 1993), and by ATP supplied by microaerobic respiration by mitochondria within the infected cells (Rawsthorne & LaRue, 1986; Millar *et al.*, 1995). However, there is evidence that fermentative respiration also occurs within nodules, particularly within the host cell cytosol (Witty *et al.*, 1986; Vance & Gantt, 1992). For example, nodules have been shown to accumulate ethanol and acetaldehyde (Sprent & Gallacher, 1976; DeVries *et al.*, 1980; Tajima & LaRue, 1982; Witty *et al.*, 1983; Smith, 1985), indicating that glycolysis is occurring. Indeed, phosphoenolpyruvate carboxylase (PEPC) activity is high in legume nodules (Sprent & Gallacher, 1976; King *et al.*, 1986; Vance & Gantt, 1992),

fixing CO₂ (non-photosynthetically) to produce organic acids, particularly malate, for use in nitrogenase-linked, bacteroid respiration (Day & Copeland, 1991; Vance & Gantt, 1992). Few studies have been made of the effects of flooding/O₂ stress on fermentation in legume nodules, except for those by Sprent & Gallacher (1976), DeVries *et al.* (1980) and Witty *et al.* (1983) who showed an increase in ethanol accumulation. Trinchant & Rigaud (1989) also showed that root and stem nodules on the flooding-tolerant legume, *S. rostrata*, had increased activity of malate and lactate dehydrogenases when they were submerged, and suggested that fermentative malate and lactate metabolism helped maintain their nitrogenase activity under flooded conditions.

Stem nodulation, and the infection of flooded stems and roots by rhizobia

Nodulation of the stem, as opposed to the hypocotyl (Fyson & Sprent, 1980; Nambiar *et al.* 1982), has been confirmed only in some hydrophytic species in the genera *Aeschynomene*, *Discolobium* and *Sesbania* (see recent review by Boivin *et al.*, 1997). *Aeschynomene* stem nodules were first reported by Hagerup (1928) and there have been many subsequent reports in this widely-distributed genus (Suessenguth & Beyerle, 1936; Arora, 1954; Jenik & Kubikova, 1969; Barrios & Gonzalez, 1971; Yatazawa & Yoshida, 1979; Allen & Allen, 1981; Eaglesham & Szalay, 1983; Alazard, 1985; Alazard & Duhoux, 1987; Loureiro *et al.*, 1995), including stem "collar" nodules on *A. sensitiva* (Boivin *et al.*, 1997). Stem nodules on *Sesbania rostrata*, a native of West Africa, were first described by Dreyfus & Dommergues (1981), and there have been recent reports of four more stem-nodulated species in this genus (Ladha *et al.*, 1992a; Boivin *et al.*, 1997). *Discolobium*, a relative of *Aeschynomene* and a native to the Pantanal, is the latest addition to the list. Two species of *Discolobium* are reported to have stem nodules (*D. pulchellum* and *D. psoraleaeifolium*; Loureiro *et al.*, 1994), although probably at least one more of the eight species is stem-nodulated (*D. leptophyllum*; Loureiro, Pott & James, unpubl.). As we mentioned previously, reports that aquatic *Neptunia* species are stem-nodulated (*e.g.*, Ladha *et al.*, 1992a) are likely to be erroneous. For example, after serial-sectioning *N. plena* "stem nodules" James *et al.* (1992a) found that the nodules were always connected vascularly to the adventitious roots and not to the subtending stem, even though the latter was connected to the nodule via parenchyma and aerenchyma. This was also true of *N. natans* (synonym *N. oleracea*) (Schaede, 1940; Subba-Rao *et al.*, 1995). James *et al.* (1992a) hence suggested that the anatomy of the vascular system could be used as the basis of an unambiguous definition of "true" stem nodules *i.e.*, they are nodules that are connected *directly* to the vascular system of the stem itself and not via that of an adventitious root. Therefore, the recent observations of "stem nodules" on the submerged, adventitious-rooted, stems of *Vigna longifolia*, *Sesbania exasperata* (Loureiro *et al.*, 1996), and *Lotus uliginosus* (1998) need to be verified by microscopy.

The formation of aerial or submerged stem nodules seems to depend on the type of adventitious root primordia which form on the stem (Alazard, 1985; Alazard &

Duhoux, 1987, 1988; Ladha *et al.*, 1992a; Lorquin *et al.*, 1993; Boivin *et al.*, 1997), as these are the sites of rhizobial infection and subsequent nodule formation (Tsien *et al.*, 1983; Duhoux, 1984; Alazard & Duhoux, 1990). *S. rostrata*, *A. afraspera* and *A. nilotica* stems have very prominent root primordia which do not require inundation for infection (*i.e.*, Group I stem-nodulators; Boivin *et al.*, 1997), but can be infected by airborne rhizobia, and hence these plants will nodulate profusely all over the aerial stem. For example, the root primordia on *S. rostrata* stems will either develop into an adventitious root upon inundation or exposure to high humidity (Duhoux, 1984; de Lajudie & Huguet, 1988; Ladha *et al.*, 1992a; Parsons *et al.*, 1993), or into a nodule upon infection by *Azorhizobium caulinodans* (Tsien *et al.*, 1983; Duhoux, 1984; Olsson & Rolfe, 1985). By contrast, *A. crassicaulis*, *A. elaphroxylon*, *A. pfundii* (Alazard & Duhoux, 1988; Ladha *et al.*, 1992a; Boivin *et al.*, 1997) and *A. fluminensis* (Loureiro *et al.*, 1995) have less prominent adventitious root primordia which require complete inundation before their dormancy can be broken and they can be subsequently infected by (Brady)rhizobia (Alazard & Duhoux, 1987, 1988; Ladha *et al.*, 1992a). Hence, the latter species (*i.e.*, Group III stem-nodulators; Boivin *et al.*, 1997) will only form stem nodules underwater (Vaughn & Elmore, 1985; Alazard & Duhoux, 1988; Ladha *et al.*, 1992a; Loureiro *et al.*, 1995). Intermediate between Group I and Group III are the Group II stem-nodulators, such as *A. aspera*, *A. indica*, *A. scabra* and *A. sensitiva*, which form mainly submerged stem nodules, but can also form some nodules on the aerial stem (Alazard, 1985; Boivin *et al.*, 1997).

The bacteria which infect the stems and roots of flooding tolerant legumes are also of considerable interest as they often differ markedly, both genetically and physiologically, from those infecting the roots of terrestrial legumes (de Bruijn, 1989; Ladha *et al.*, 1992a; Loureiro, 1994). Moreover, flooding-tolerant legumes also tend to have rhizobial infection processes which differ from the "usual" root hair infection pathway seen in most terrestrial legumes so far examined (Sprent, 1989; Brewin, 1991). For example, the bacterium originally isolated from *S. rostrata* stem and root nodules by Dreyfus *et al.* (1983) was found to be in a new, hitherto undiscovered, genus of rhizobia *i.e.*, *Azorhizobium*. Only a single species, *Az. caulinodans*, has so far been characterised (Dreyfus *et al.*, 1988). Two of the distinguishing features of *Az. caulinodans* in free-living culture is its ability to live on N₂ as the sole N source, and also its ability to fix N₂ in a relatively high pO₂ compared to other free-living diazotrophs (Dreyfus *et al.*, 1983, 1988). Recently, another bacterium from *S. rostrata*, *Sinorhizobium teranga*, has been isolated and characterised (de Lajudie *et al.*, 1994). However, this bacterium does not fix N₂ *ex planta*, and stem nodulation of *S. rostrata* by *Sinorhizobium* is suppressed by prior root nodulation by *Az. caulinodans* (Tomekpe *et al.*, 1996). Free-living *Az. caulinodans* is present as an epiphyte on *S. rostrata* (Adebayo *et al.*, 1989; Robertson *et al.*, 1995), and it has been suggested that these epiphytic bacteria are a major source of inocula for the infection of successive generations of this plant (Ladha *et al.*, 1992a). Other sources of inoculum may be *Azorhizobium* within the floodwater, the level of which fluctuates up and down the stem, and also wind-borne bacteria (de Bruijn, 1989; Ladha *et al.*, 1992a).

Azorhizobia infect the stems of *S. rostrata* at the adventitious root primordia by entering the fissures created by the root primordium as it breaks through the stem epidermis, or by entering dead root cells at this point (Tsien *et al.* 1983; Duhoux, 1984). The bacteria multiply within the fissures/dead cells and infect the developing meristematic tissue in the incipient root cortex via infection threads. The nodule primordia then develop in a manner similar to that of indeterminate root nodules (Brewin, 1991) *i.e.*, new cells produced by an apical meristem are continually infected via infection threads as the nodule grows (Duhoux, 1984), although Ndoye *et al.* (1994) have since shown that meristem activity ceases two weeks after inoculation and that the later stages of *S. rostrata* nodule development are more similar to those of determinate nodules. The infection of *S. rostrata* roots by *Az. caulinodans* and the subsequent development of root nodules has also been described (Olsson & Rolfe, 1985; Ndoye *et al.*, 1994), and these processes generally resemble those involved in stem nodulation, except that the bacteria (*Az. caulinodans*, or possibly *Si. teranga*) infect *S. rostrata* at the base of lateral roots by entering between epidermal cells and/or short, thick root hairs (Ndoye *et al.*, 1994). A similar process is also likely to occur in the infection of the roots of other *Sesbania* species *e.g.*, *S. aculeata* (Rana & Krishnan, 1995). Neither of the published studies on root nodulation of *S. rostrata* (Olsson & Rolfe, 1985; Ndoye *et al.*, 1994) examined the formation of root nodules under flooded conditions. However, considering that intercellular root infection processes are common in other flooding tolerant legumes *e.g.*, *Neptunia* (Schaede, 1940; James *et al.*, 1992a; Subba-Rao *et al.*, 1995) and *Aeschynomene* (Alazard & Duhoux, 1990), it is possible that the infection process described for non-flooded *S. rostrata* by Ndoye *et al.* (1994) also occurs when its roots are submerged. More work is needed to confirm this.

Most of the bacteria which nodulate the roots and stems of *Aeschynomene* spp. are in the genus *Bradyrhizobium*. This includes strains which have been shown to be photosynthetic (Evans *et al.*, 1990; Young *et al.*, 1991; Lorquin *et al.*, 1993; Loureiro *et al.*, 1993; Loureiro, 1994; So *et al.*, 1994; van Berkum *et al.*, 1995), and to also fix N_2 *ex planta*. (Alazard, 1990). NB: none of the rhizobial strains from the closely related genus, *Discolobium*, have so far been shown to be either photosynthetic or to fix N_2 *ex planta* (Loureiro *et al.*, 1994; Loureiro, 1994). As with *Az. caulinodans*, the ability to fix N_2 *ex planta* may give rhizobia from *Aeschynomene* stem nodules a competitive advantage in the rhizosphere or phyllosphere (Ladha *et al.*, 1992a), and this has also been suggested as an explanation for the occurrence in some of these strains of bacteriochlorophyll (Bchl) (van Berkum *et al.*, 1995). As mentioned earlier, *Aeschynomene* spp. form nodules on the aerial and/or the submerged stem, depending on the plant species. Most studies on the infection and development of *Aeschynomene* stem nodules has been with those species that form aerial stem nodules *e.g.*, *A. indica*, *A. afraspera*, *A. scabra* (Arora, 1954; Yatazawa & Yoshida, 1979; Eaglesham & Szalay, 1983; Vaughn & Elmore, 1985; Alazard & Duhoux, 1987, 1990), although Loureiro *et al.* (1995) have recently published a detailed investigation into the submerged stem and root nodules on *A. fluminensis*. Alazard & Duhoux (1990) have shown that the infection

of adventitious root primordia on the aerial stem of *A. afraspera* by rhizobia is very similar to that of *S. rostrata* stems by *Az. caulinodans* (Tsien *et al.*, 1983; Duhoux, 1984) *i.e.*, bacteria accumulated in cavities in the stem epidermis around the root primordium and subsequently entered into the incipient nodule meristem developing in the root. However, the process differed from *S. rostrata* in that infection threads were never seen in any part of the nodule infection/development and bacteria penetrated the stem/root tissues by invagination of the host cell walls (Alazard & Duhoux, 1990); a process also observed by Vaughn & Elmore (1985) in *A. indica* stem nodules. In the early stages of infection of *A. afraspera*, after the host cells were infected, they collapsed and, by the "progressive collapse" of a pre-determined file of invaded cells, the host tissue directed the bacteria to the developing meristem (Alazard & Duhoux, 1990). This process has some similarities with the non-root hair pathways observed in the infection of the roots of the terrestrial Aeschynomeneoid legumes, *Arachis hypogaea* (Chandler, 1978) and *Stylosanthes* (Chandler *et al.*, 1982). Indeed, it is generally considered that roots of legumes in the tribe Aeschynomeneae are infected via cracks at lateral/adventitious root junctions, and that their nodules do not have infection threads (Sprent *et al.*, 1989), although Loureiro *et al.* (1994, 1995) have recently shown small infection threads in nodules of *Discolobium* and *A. fluminensis*.

Non-root hair infection processes have also been reported for the rhizobia infecting the roots of the aquatic mimosoid legumes, *Neptunia natans* (Schaeede, 1940; Debelle *et al.*, 1986; Subba-Rao *et al.*, 1995) and *N. plena* (James *et al.*, 1992a). Indeed, root hairs do not form on aquatically grown *Neptunia* spp. and so cannot be involved in the infection of these plants when their roots are flooded (Schaeede, 1940; Debelle *et al.*, 1986; James *et al.*, 1992a; Subba-Rao *et al.*, 1995). As with legumes in the Aeschynomeneae, these plants are infected at cracks in lateral/adventitious root junctions. However, a potential problem with an aquatic environment is that the plant-bacterial recognition signals necessary for the induction of the nodulation process (Sprent, 1989; Brewin, 1991; Hirsch, 1992) may be too greatly diluted. Subba-Rao *et al.* (1995) have hence suggested that the cracks or wounds produced by aquatic legumes with non-root hair infection processes may allow for the accumulation of (plant-produced) nod-gene-inducing factors (*e.g.*, flavonoids) and (bacterial-produced) nodule-inducing factors *e.g.*, lipo-chitin oligosaccharides (LCOs; Spaink, 1995). It is also possible that the dense mats of vegetation (composed mainly of floating stems) in which many of these plants live (see earlier) will also allow for the accumulation of these chemicals. This is supported by the work of James *et al.* (1993) which showed that stem extracts from *N. plena* had substantial *nod* gene-inducing activity. Interestingly, James *et al.* (1993) have also demonstrated that *N. plena* can be infected by *Rhizobium* strain DUS239 (WPBS3942) via root hairs when grown *terrestrially*, as well as via cracks when flooded (James *et al.*, 1992a, 1993). Therefore, the unusual *N. plena*-DUS239 symbiosis could be used to compare the exchange of recognition signals between host and rhizobia in legumes with non-root hair infection pathways with those with "conventional" root hair infection processes.

The extraordinary nature of the rhizobia isolated from aquatic *Neptunia* spp. has recently been supported by unpublished data by Wang, James, Whitty & Sprent which shows that they are probably in a new, hitherto unrecognised, species of *Rhizobium*. Another "new" (semi) aquatic species of *Rhizobium* is *R. huakuii*, isolated from root nodules of the herbaceous legume, *Astragalus sinicus*, which is used as a green manure in lowland rice production in Japan and southern China (Murooka *et al.*, 1993). As with stem-nodulated legumes (see later), *A. sinicus* can fix high quantities of N₂ under flooded conditions making it ideal for use in paddy fields. However, very little has been published on the physiology, growth and development of *A. sinicus* nodules apart from the recent study by Murooka *et al.* (1993) which showed that when grown under non-flooded conditions seedlings were infected via root hairs. James & Sprent (unpubl.) have since shown that subsequent development of *A. sinicus* nodules under these conditions is similar to that described for other indeterminate nodules (Brewin, 1991). It remains to be seen whether the infection process/nodule development is different when *A. sinicus* is flooded.

James, Crawford & Sprent (unpubl.) have recently examined the infection and nodulation of *Lotus* species under flooded conditions. *L. uliginosus* seeds germinated in flooded soil and the roots of the seedlings readily nodulated under these conditions. Moreover, when *L. uliginosus* stems were flooded they formed nodulated adventitious roots, and also a few nodules on the highly-aerenchymatous, submerged stem. The vascular systems of many of the "stem nodules" were connected to those of nearby adventitious roots. However, the vascular systems of others appeared to arise directly from the stem vascular system via traces which bifurcated into both a nodule and a root, and further studies are needed to determine if these are "genuine" stem nodules (see earlier). James *et al.* (1998) have shown that the processes involved in the infection of *L. uliginosus* stems and adventitious roots appear to involve the infection of enlarged epidermal cells at the tips of nodule primordia which are emerging from within root/stem aerenchyma; it is possible that the large air spaces in the aerenchyma allows for the accumulation of plant flavonoids/rhizobial LCOs which would normally be diluted under flooded conditions (Subba-Rao *et al.*, 1995). In contrast to *L. uliginosus*, *L. corniculatus* seeds would not germinate in flooded soil. However, roots of *L. corniculatus* when grown in well-drained soil which was subsequently flooded, continued to nodulate, although to a lesser extent than flooded *L. uliginosus*.

Some of the more profoundly flooding-tolerant legumes from the seasonally-flooded forests of South America may also have unusual infection processes *e.g.*, *Discolobium* (Loureiro *et al.*, 1994) and *Campsiandra laurifolia* (Barrios & Herrera, 1993a), and these await further studies. However, Barrios & Herrera (1993a) have shown that *C. laurifolia* roots developed new nodules in the dry season, when the rhizobia in the soil were more concentrated, but could also form nodules on flooded adventitious roots. *Mimosa pellita*, which shares habitats with *Discolobium* (Loureiro

et al., 1994) and *Campsiandra* (Barrios & Herrera, 1993a, b) in South America, also forms nodules on flooded adventitious roots (James *et al.*, 1995a, b), and James *et al.* (1995b) have shown that the rhizobia infect enlarged epidermal (lenticellular) cells before penetrating the root via infection threads.

The oxygen relations of photosynthetic and non-photosynthetic stem nodules

The formation of nodules on the stems of some hydrophytic legumes is considered to be an adaptation to raise the N₂-fixing organs above the level of the flooded roots, thus avoiding, or reducing, the oxygen constraints afflicting submerged root nodules (Ladha *et al.*, 1992a). In the case of *S. rostrata*, *A. afraspera*, *A. indica*, *A. nilotica* and *A. scabra*, this involves the formation of nodules on the aerial stem above the floodwater (Yatazawa & Yoshida, 1979; Dreyfus & Dommergues, 1981; Eaglesham & Szalay, 1983; Alazard & Duhoux, 1987, 1990; Ladha *et al.*, 1992a; Boivin *et al.*, 1997) whereas other species will form nodules only on the submerged stem (see earlier). However, submerged *Aeschynomene* stem nodules *e.g.*, those on *A. fluminensis*, will also continue to function after the water level decreases and consequently exposes the previously flooded nodules on the (by now aerial) stem (Loureiro *et al.*, 1995). On the other hand, *Discolobium* stem nodules are a special case in this respect as they can only form *and function* whilst submerged, senescing rapidly upon exposure to air (Loureiro *et al.*, 1994).

Although flooded stem nodules are likely to suffer the same type of O₂ constraints as submerged root nodules (James *et al.*, 1992a, b; Shiferaw *et al.*, 1992; Arrese-Igor *et al.*, 1993; Becker & George, 1995; Pugh *et al.*, 1995), Loureiro *et al.* (1995) suggested that by growing just beneath the water surface as the flood level rises, the nodules on *A. fluminensis* stems were effectively reducing the pathlength for O₂ diffusion down the stem. Moreover, stem nodules on most species of *Aeschynomene* so far reported as being stem-nodulated, contain chloroplasts in the cortical cells adjacent to the infected, N₂-fixing tissue (Dreyfus & Dommergues, 1981; Duhoux, 1984; Vaughn & Elmore, 1985; de Bruijn, 1989; Evans *et al.*, 1990; Hungria *et al.*, 1992; Loureiro *et al.*, 1995; Boivin *et al.*, 1997). Therefore, if, as has recently been demonstrated by James *et al.* (1996) and James *et al.* (1998) with aerial *S. rostrata* stem nodules, submerged *Aeschynomene* stem nodules can also photosynthetically evolve O₂, then this may offset the low pO₂ of their submerged environment, but only so long as they receive sufficient irradiance for significant photosystem II (PS_{II}) activity (Loureiro *et al.*, 1995).

In addition to cortical chloroplasts, both aerial and submerged stem nodules on *Aeschynomene* species often contain (Brady)rhizobia with Bchl (see earlier) and, if given light of the correct wavelength, the bacteroids themselves show light-enhanced CO₂ incorporation and light-decreased uptake of O₂ (Evans *et al.*, 1990; Hungria *et al.*, 1992, 1993; Loureiro *et al.*, 1993; Lorquin *et al.*, 1993; Loureiro, 1994; Ladha & So, 1994; Boivin *et al.*, 1997). Furthermore, Evans *et al.* (1990) have shown that exposing *A. indica* stem nodules to near infra-red light (*i.e.*, of the wavelength needed

to stimulate Bchl) will produce an immediate increase in nitrogenase (acetylene reduction) activity. Evans *et al.* (1990) speculated that the increase in nitrogenase activity was likely to be due to an increase in O₂ available to the O₂-limited bacteroids (Hunt & Layzell, 1993). Indeed, James *et al.* (1998) have recently demonstrated that *S. rostrata* stem nodules (which do not have Bchl, but do have cortical chloroplasts) also show (reversible) light-enhanced nitrogenase activity, and also suggested that this was due to an increase in O₂ availability to the bacteroids. James *et al.* (1998) had three lines of evidence to support this contention: 1) *S. rostrata* stem nodules contain the components of PS_{II} necessary for O₂ evolution (James *et al.*, 1996) and these components are active; 2) O₂-specific microelectrodes (Witty *et al.*, 1987) showed an increase in cortical pO₂ (up to 23.4%) when *S. rostrata* stem nodules were exposed to supplemental light and 3) exposing *S. rostrata* stem nodules to stepwise increases in external pO₂ to 60% produced an increase in nitrogenase activity similar to that achieved with supplemental light.

The high N₂ fixation rates reported for stem-nodulated legumes such as *S. rostrata* (Dreyfus & Dommergues, 1981; Rinaudo *et al.*, 1983; Dreyfus *et al.*, 1985; Ndoye & Dreyfus, 1988; Ladha *et al.*, 1992b; Kwon & Beevers, 1992; Hungria *et al.*, 1992) and *Aeschynomene* spp. (Yatazawa & Yoshida, 1979; Eaglesham & Szalay, 1983; Alazard & Becker, 1987; Alazard & Duhoux, 1987; Hungria *et al.*, 1992; Loureiro *et al.*, 1995) have been linked with the apparent ability of the stem nodules to photosynthesise, either via cortical chloroplasts (de Bruijn, 1989; Evans *et al.*, 1990; Hungria *et al.*, 1992; James *et al.*, 1996) or via photosynthetic microsymbionts (Evans *et al.*, 1990; Hungria *et al.*, 1992, 1993). It has been hypothesised that *in vivo* photosynthesis would supplement directly the supply of carbohydrates for N₂ fixation (de Bruijn, 1989; Hungria *et al.*, 1992; Ladha *et al.*, 1992a; Boivin *et al.*, 1997). Moreover, it has also been suggested that, regardless of the ability of the stem nodules themselves to photosynthesise, their closer proximity (compared to root nodules) to the plants main site of photosynthesis (*i.e.*, the leaves) means that they are in an advantageous position to receive photosynthate compared to purely root-nodulated symbioses (Ladha *et al.*, 1992a; Parsons *et al.*, 1992). With regard to *S. rostrata* stem nodules, this latter point may be more important than *in vivo* photosynthesis, as Parsons *et al.* (1992) have recently demonstrated that the nitrogenase activity of controlled environment-grown stem nodules declines rapidly if they are disconnected from the external source of photosynthate *i.e.*, if the stem is girdled so that phloem inputs above and below the nodules are cut. In addition, de Lajudie & Huguet (1988) and Parsons *et al.* (1993) showed that if nodules are grown on stems which are not exposed to light they do not develop chloroplasts but they still have nitrogenase activities comparable to normal, light-developed ones. On the other hand, the study of James *et al.* (1998) shows that if *S. rostrata* stem nodules are subjected to very high irradiances, more closely resembling those that they may receive in their native West Africa, then they may photosynthesise significantly more than greenhouse and controlled environment-grown nodules (Parsons *et al.*, 1992, 1993). Hence, the latter type of studies may underestimate the relationship between

photosynthesis and N₂ fixation by stem-nodulated legumes in their native tropical environments (*e.g.*, see Becker *et al.*, 1990).

We mentioned earlier that, although N₂-fixing bacteroids have a need for O₂ to supply them with ATP, nitrogenase itself is denatured by it (Gallon, 1992). The nitrogenase of *Azorhizobium* in its symbiotic, bacteroid, form, as opposed to its free-living form (see earlier), is no more tolerant of O₂ than that of other (root nodule) bacteroids (Bergersen *et al.*, 1988), and hence stem nodule bacteroids have as much of a requirement for O₂ protection as those in root nodules. However, a necessary component of CO₂ fixation via PS_I is electron transport to PS_I from PS_{II}, the latter deriving electrons from the oxidation of water and the consequent release of O₂ (Ghanotakis & Yocum, 1990). Photosynthetic CO₂ incorporation by stem nodule chloroplasts (Hungria *et al.*, 1992) is no exception to this, and PS_{II} has been shown to be present and active within *S. rostrata* stem nodules (James *et al.*, 1996; 1998). How then do stem nodules, which have the potential to produce photosynthetic O₂ (to hyperoxic levels; James *et al.*, 1998) within the very cortical tissues that would normally act as a O₂ diffusion barrier in root nodules (see earlier), protect their nitrogenase from O₂-denaturation?

This question may be answered by the study of James *et al.* (1996) that showed that concentrations of the intercellular space/cell wall glycoprotein recognised by the monoclonal antibody MAC265, and involved in the regulation of O₂ diffusion in some root nodules (James *et al.* 1991, 1997; de Lorenzo *et al.*, 1993; Iannetta *et al.*, 1993a, b, 1995), was greatly enhanced in (photosynthetic) *S. rostrata* stem nodules compared to (non-photosynthetic) *S. rostrata* root nodules. The MAC265 glycoprotein was localised in cortical intercellular spaces/cell walls adjacent to chloroplasts, and few spaces were left unoccluded with glycoprotein/cell wall material. In comparison, the intercellular spaces in the cortices of *S. rostrata* root nodules were relatively unoccluded and resembled the cortices of other flooding-tolerant root nodules (James *et al.*, 1992a; Loureiro *et al.*, 1994, 1995). James *et al.* (1996) suggested that the occlusions prevented external, as well as photosynthetically-derived O₂, from entering the infected zone of the stem nodules in concentrations which would denature nitrogenase. Indeed, James *et al.* (1998) have since demonstrated using O₂ micro-electrodes that, although the pO₂ within the outer cortex of stem nodules rose significantly upon the application of supplemental light, it did not affect the pO₂ within the infected zone, which remained below the detection limit of the electrodes throughout the experiments. This suggested strongly that a variable O₂ diffusion barrier was present in the stem nodules, in common with root nodules (see earlier). James *et al.* (1998) hypothesised that the barrier in stem nodules may operate via the rapid oxidative cross-linking of the MAC265 glycoprotein, as Bradley *et al.* (1992) have shown that cross-linking of this material (leading to insolubilisation and stiffening of cell walls) is triggered by fungal elicitors, glutathione or hydrogen peroxide as a host-defence response in *Phaseolus vulgaris* protoplasts. Stem nodule chloroplasts contain SOD (Puppo *et al.*, 1986) and this will react with superoxide to produce hydrogen peroxide which may then react with nearby cell wall/intercellular space MAC265

glycoprotein (James *et al.*, 1996). The resulting cross-linking and stiffening of cortical cell walls/intercellular material may hence increase O₂ diffusion resistance (Brown & Walsh, 1996).

Further evidence about the possible role of cell wall glycoproteins in stem nodule O₂ regulation comes from Dehio & de Bruijn (1992) who showed that expression of the *S. rostrata* early nodulin 2 gene (*SrEnod2*) was increased in stem nodules compared to root nodules. *Enod2* was originally localised within the inner cortex of pea and soybean nodules, and it has been suggested that the hydroxyproline-rich glycoprotein that it encodes is involved in the construction of an O₂ diffusion barrier (Van de Wiel *et al.*, 1990).

It has also been hypothesised that Lb components specific to stem nodules (Bogusz *et al.*, 1987) may allow for O₂ protection via "buffering" excess (photosynthetically-derived) O₂ which reaches the infected zone (Szabados *et al.*, 1990). However, there is as yet no evidence for such a mechanism. Moreover, Bergersen *et al.* (1986) have shown that *S. rostrata* stem and root nodule Lb has the highest dissolved O₂ affinity of any symbiotic Lb, suggesting that symbiotic N₂ fixation in both nodule types is actually operating at a lower pO₂ than in other legume nodules (de Bruijn, 1989).

Regarding stem nodule O₂ relations and their effects on nitrogenase activity, *Discolobium* stem nodules may again be a special case. For example, although they have nitrogenase activities which are comparable to those on *S. rostrata* and *Aeschynomene* spp., *Discolobium* stem nodules are never aerial, are relatively deeply submerged (compared to those on *Aeschynomene* spp.; Loureiro *et al.*, 1995), and do not have chloroplasts to supplement their O₂ supply (Loureiro *et al.*, 1994). Indeed, as we have suggested earlier, the O₂ relations and N₂-fixing capabilities of *Discolobium* stem and root nodules are worthy of much further research.

Flooding tolerant, root-nodulated legumes have long been used as green manures in wetland rice production, particularly in Asia (Albrecht *et al.*, 1981; Murooka *et al.*, 1993; Gutteridge, 1994). However, in recent years there has been much interest in using stem-nodulated species because of their high rates of BNF under both flooded and non-flooded conditions (Dreyfus *et al.*, 1985; Morris *et al.*, 1989; Ladha *et al.*, 1992a; Boivin *et al.*, 1997) (Table 1). For example, Dreyfus & Dommergues (1981) and Dreyfus *et al.* (1985) reported exceptionally high nitrogenase activity by stem-nodulated *S. rostrata* using the inaccurate (Witty & Minchin, 1988) acetylene reduction assay (ARA), and high values have also been reported for *Aeschynomene* spp. (Eaglesham & Szalay, 1983; Alazard & Duhoux, 1987; Hungria *et al.*, 1992; Loureiro *et al.*, 1995). Since then, there have been many studies of the potential contribution of N to succeeding rice crops by, in particular, *S. rostrata*, *A. afraspera* and *A. nilotica*, using more accurate, and field-based techniques such as ¹⁵N isotope dilution and ¹⁵N natural abundance (Table 1). These have been recently thoroughly reviewed by Ladha *et al.* (1992a).

Table I. Nitrogen accumulation by stem-nodulated legumes grown for 45-65 days.

Species	N accumulation (kg N.ha ⁻¹)	Country	Reference
<i>Sesbania rostrata</i>	272	Senegal	Rinaudo <i>et al.</i> (1983)
	131	Thailand	Crozat & Sangchyo-Sawat (1985)
	60	Philippines	Morris <i>et al.</i> (1986)
	79	Philippines	Ventura <i>et al.</i> (1987)
	83-109	Senegal	Ndoye & Dreyfus (1988)
	68-219	Philippines	Morris <i>et al.</i> (1989)
	100-160	Philippines	Furoc & Morris (1989)
	160	Philippines	Ladha <i>et al.</i> (1989)
	324-458	Philippines	Pareek <i>et al.</i> (1990)
	70-175	Philippines	Becker <i>et al.</i> (1990)
	194	Philippines	Becker <i>et al.</i> (1991)
68-154	Philippines	George <i>et al.</i> (1994)	
<i>Aeschynomene afraspera</i>	423	Senegal	Alazard & Becker (1987)
<i>A. indica</i>	41	Thailand	Crozat & Sangchyo-Sawat (1985)
	105-145	Philippines	Becker <i>et al.</i> (1990)
<i>A. nilotica</i>	532	Senegal	Alazard & Becker (1987)

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