Chapter 3
Strategies for Adaptation to Waterlogging and Hypoxia in Nitrogen Fixing Nodules of Legumes

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Abstract Symbiotic nitrogen fixation between legumes and rhizobia bacteria occurs in a microaerobic environment within a specialized organ, the root nodule. The fixation of dinitrogen requires a considerable energy input and a high respiratory rate, but the fundamental nitrogen fixation enzyme, nitrogenase, is inactivated by free oxygen. Because of this apparent conundrum, the diffusion of oxygen into the nodule infection zone is exquisitely regulated in response to multiple environmental cues, and becomes sensitive to alterations in the external rhizosphere oxygen tension. As a result, most legumes are sensitive to waterlogging, showing reductions in nodulation and productivity in flooded soils. Nevertheless, certain legumes have evolved developmental strategies to modulate the pathway of oxygen diffusion to the nodule, the patterns of nodule formation on roots and stems, and altered pathways of bacterial invasion to adapt to flooding conditions. In the present chapter, the regulation of oxygen diffusion and adaptations to waterlogged conditions by nitrogen fixing nodules of flooding-sensitive and flooding-tolerant legumes are discussed.

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylate
GA	Gibberellic acid
IT	Infection thread

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K_m	Michaelis constant
kPa	kilopascal
LRB	Lateral root boundary
O_i	Free oxygen concentration within the infected cells
PIP	Plasma membrane intrinsic protein
P_f	Osmotic water permeability
RHC	Root hair curling
ROS	Reactive oxygen species
TIP	Tonoplast intrinsic protein

3.1 Introduction: The Oxygen Diffusion Barrier in Nodules

Most terrestrial plants are sensitive to flooding stress, which is principally the result of hypoxia and reduced respiration due to low O_2 diffusion coefficients in water (Ferrell and Himmelblau 1967) and poor radial diffusion of O_2 within the mature root (Ober and Sharp 1996; van Dongen et al. 2003). In many legumes, flooding drastically reduces root growth, suppresses nodulation and nitrogen fixation, and reduces photosynthesis and overall yield (Minchin and Pate 1975; Minchin and Summerfield 1976; Scott et al. 1989; Sung 1993; Linkemer et al. 1998; Bacanamwo and Purcell 1999a). Due to the high energy requirement associated with symbiotic nitrogen fixation, legume root nodules are particularly sensitive to changes in oxygen concentration in the soil, and in general legumes relying on nitrogen fixation are more sensitive to flooding stress than plants grown on other alternative sources of nitrogen, such as nitrate (Minchin and Pate 1975; Buttery 1987; Bacanamwo and Purcell 1999a, b). The problem is exacerbated by the microaerobic environment in which symbiotic nitrogen fixation takes place (Minchin et al. 2008). Nevertheless, all legumes show adaptive strategies that allow tolerance to fluctuations of oxygen concentrations in the rhizosphere by controlling the rate of oxygen diffusion into the core of the nodule, and in extreme cases such as wetland legumes, these strategies have allowed flooding tolerance. Thus, a consideration of the basis of nodule sensitivity to flooding and waterlogging stress must first begin with an analysis of the importance of gas diffusion as a global regulatory mechanism that buffers nitrogen fixation rates in legume nodules in response to metabolic and environmental cues.

3.1.1 *Nodule Morphology and the Gas Diffusion Barrier*

The basic need for a gas diffusion barrier is apparent from the balancing act that nodules play in: (1) providing adequate oxygen for the substantial respiratory and energy needs of the nitrogen fixation process, while (2) mitigating the damaging effects of free oxygen on the fundamental fixation enzyme, the bacterial nitrogenase. The role of the nodule gas diffusion barrier in the modulation of oxygen entry and the efflux of other gases (e.g., H_2 and CO_2) has been discussed extensively in several reviews (Witty et al. 1986; Hunt and Layzell 1993; Bergersen 1997; Minchin 1997; Layzell 1998; Minchin et al. 2008). For the purpose of this chapter, only the fundamental characteristics of the gas diffusion barrier relevant to adaptation to external oxygen concentrations will be summarized since flooding stress will largely impact these.

The oxygen/gas diffusion barrier is an outcome of the overall cellular and tissue morphology of the legume nodule. In a mature nitrogen-fixing nodule, the infected zone at the core of the nodule consists predominantly of two cell types: (1) the enlarged infected cells, containing the rhizobia bacteroids enclosed within symbiosome organelles, and (2) smaller companion uninfected interstitial cells (Bergersen 1982). Early observations with oxygen electrodes (Tjepkema and Yocum 1974; Witty et al. 1987), followed by accurate estimations of pO_2 by nodule oximetry (based on spectroscopic analysis of fractional leg hemoglobin oxygenation in intact nodules) (Denison and Layzell 1991), revealed that the internal infected zone of legume nodules is microaerobic. For example, the free oxygen concentration within the infected cells (O_i) of soybean nodules is maintained at approximately 20 nM under atmospheric concentrations of oxygen (Layzell et al. 1990). To accommodate the high respiratory need associated with the nitrogen fixation process, several adaptations have evolved to allow high energy metabolism under microaerobic conditions, including the synthesis of leghemoglobin that serves as a free oxygen buffer as well as a facilitated carrier of oxygen for bacteroid respiration (Bergerson, 1982). In addition, the architecture of the infected cell is arranged such that mitochondria are clustered at the periphery of the infected cell near the intercellular spaces which contain higher pO_2 to facilitate host cell respiration (Bergerson, 1982). Also, the terminal oxidase of the bacteroid electron chain has a low K_m (20 nM) for oxygen, allowing respiration under microaerobic conditions (Bergerson and Turner 1993).

The centralized infected zone is surrounded by a cortical tissue layer consisting of several organized cell layers that differ in number and complexity depending upon the nature of the particular legume–rhizobia association (Minchin 1997). The region of the inner cortex surrounding the infected zone consists of small cells with restricted intercellular spaces (Witty et al. 1987; Parsons and Day 1990). Measurements with oxygen microelectrodes reveal that the pO_2 drops precipitously across this inner cortex (Tjepkema and Yocum 1974; Witty et al. 1987). This region has generally been considered the major boundary of the gas diffusion barrier in nitrogen-fixing nodules. However, while the inner cortex is a critical barrier for gas diffusion, O_2 diffusion from the rhizosphere to the bacteroid is likely a more complex process that depends upon multiple additional factors including intercellular gas spaces in the infected zone, the rate of O_2 consumption in mitochondria in the infected cell, and the rates of leghemoglobin-O_2 diffusion in the infected cell cytosol (reviewed in Minchin 1997; Bergersen 1997; Minchin et al. 2008).

3.1.2 Modulation of the Gas Diffusion Barrier

Early support that oxygen diffusion into the nodule is a rate-limiting factor in regulation of nitrogen fixation came from the observation by Pankhurst and Sprent (1975a) that drought stress-induced inhibition of nitrogenase activity can be partially restored by the experimental elevation of external pO_2. The implication was that the reduction of oxygen diffusion into the infected zone due to osmotic stress resulted in an inhibition of the rate of nitrogen fixation based on the reduced availability of oxygen for respiration and energy metabolism. Ultimately, the idea of a variable gaseous diffusion barrier that regulates O_i and serves the dual role of controlling respiratory metabolism/nitrogen fixation while preventing nitrogenase inactivation was proposed (Sheehy et al. 1983).

Subsequent work demonstrated that a wide variety of environmental and metabolic cues modulate the gas diffusion barrier. For example, under conditions of reduced carbon availability, induced by a variety of conditions such as darkness (Drevon et al. 1991), nodule excision (Ralston and Imsande 1982; Sung et al. 1991), stem girdling (Vessey et al. 1988), or detopping (Denison et al. 1992), the lack of carbon induces a rapid reduction in respiration and the rate of nitrogen fixation resulting from a reduction in O_2 diffusion into the infected zone and reduced O_i. Such modulation of the oxygen diffusion barrier is proposed to prevent nitrogenase inactivation by O_2 accumulation in response to reduced oxygen consumption by the bacteroids (Witty et al. 1986). Other environmental factors that regulate the diffusion barrier by restricting oxygen diffusion include nitrate fertilizers, water deficit induced by drought or hyperosmotic stress, and temperature (reviewed in Witty et al. 1986; Hunt and Layzell 1993; Minchin 1997; Layzell 1998). In most cases, experimental elevation of the external pO_2 at least partially restores the inhibitory effects of these treatments, suggesting that a reduction in O_2 flux through the diffusion barrier is in part responsible for the modulation of respiration and nitrogen fixation by these environmental paramenters.

3.1.3 Control of the Gas Diffusion Barrier in Response to Sub-Ambient O_2 and Flooding

Tissues that are naturally hypoxic, even under plant growth in normoxic conditions, are particularly sensitive to oxygen deprivation resulting from flooding stress (Bailey-Serres and Voesenek 2008). Thus, even under mild hypoxic conditions, these tissues can experience severe hypoxia or even anoxia because of the slow diffusion rate of O_2 and the low gas porosity of these naturally hypoxic tissues. As discussed above, the gas diffusion barrier of nitrogen-fixing nodules maintains a microaerobic state (~20 nM O_2) in the infected zone, and analyses of the rate of respiration and nitrogen fixation under normal atmospheric oxygen conditions show that the nitrogenase activity and respiratory metabolism are maintained at a suboptimal level by this concentration of O_2 (Hunt et al. 1989). Exposure of nodules to 10% O_2 results in a rapid reduction of the adenylate energy charge with the bacteroid fraction being particularly sensitive to this hypoxic stress (Kuzma et al. 1999). Nodules show two modes of adaption to changes in external oxygen concentrations: (1) a short term modulation of the gas permeability barrier; and (2) longer term developmental/genetic alterations to enhance gas permeability involving changes in tissue, cellular and subcellular morphology (Minchin 1997).

With respect to the short-term adaptive strategies, reduction of the rhizophere pO_2 results in a rapid reduction in nitrogenase activity that over time eventually recovers to the initial level (Criswell et al. 1976). Comparison of the respiratory rate (CO_2 evolution) and nitrogenase activity (H_2 evolution or acetylene reduction) in intact nodules in response to changes in external oxygen concentrations showed a transient decline in both, but an eventual recovery to rates approximating or even exceeding pretreatment levels (Hunt et al. 1987; 1989; Weisz and Sinclair 1987a). Measurements of internal O_i by nodule oximetry reveal that the infected zone is able to rapidly buffer O_i in response to changes in external oxygen tension. For example, when administered an abrupt change of external oxygen from 20 to 25 kPa, soybean nodules show a transient increase in O_i followed by a rapid ($<$10 min) return to the original steady state level (King et al. 1988). This change is likely due to reversible modulation of the rate of O_2 flux through the diffusion barrier in response to changes in the external rhizosphere oxygen concentration. In the case of supra-ambient oxygen conditions the nodule resistance to gas diffusion is enhanced compared to normoxic controls (Weisz and Sinclair 1987b; King and Layzell 1991). In contrast, sub-ambient oxygen conditions decrease the resistance of the gas diffusion barrier (Weisz and Sinclair 1987b). Thus, in response to flooding conditions and a drop in oxygen content of the soil, nodules would initially enhance the rate of oxygen uptake by modulation of the gas diffusion barrier, stimulating oxygen uptake as part of a short-term response.

3.1.4 Mechanism of Regulation of the Gas Diffusion Barrier in Response to pO_2

Mechanistic and molecular features of how gas diffusion is regulated in response to fluctuations in external oxygen concentration, and as well as how the oxygen signal is perceived and transduced, remain a subject of debate (Minchin et al. 2008). Nodules exposed to supra-ambient oxygen concentrations show a rapid decrease in intercellular gas spaces within the inner cortex of the nodule that accompanies increased resistance to gas permeability (Witty et al. 1987). This lead to the hypothesis that the osmotically-driven flux of water from inner cortical cells to the apoplastic intercellular space is responsible for restricting the rate of gas diffusion in response to elevated external pO_2 (Hunt and Layzell 1993; Purcell and Sinclair 1994). On the basis of an anatomical investigation, stresses that restrict oxygen flux into the nodule (e.g., supra-ambient pO_2 or salt stress) cause an apparent collapse of the intercellular spaces of the inner cortex that likely further contributes to a restricted pathway for gas diffusion (Serraj et al. 1995). Noting a similarity to other osmocontractile cells, such as pulvini motor cells that undergo turgor-driven changes in cell shape, it was proposed that cells of the nodule inner cortex could respond in an analogous manner to external stimuli that restrict gas entry into the nodule. Electrophysiological observations show that a brief exposure of nodules to elevated pO_2 results in a transient membrane depolarization with normal negative membrane potential re-established upon return to ambient pO_2 conditions (Denison and Kinraide 1995). Based on this observation, an "osmoelectrical" model was proposed through which movement of K^+ into the apoplastic space accompanies the efflux of water from nodule cells which increases the water-filled spaces. This in turn causes a change in cell turgor and shape which increase the resistance to gas flow in response to elevated rhizosphere O_2 (Denison and Kinraide 1995). Hypoxic conditions would be expected to have the reverse effect with low pO_2 causing an increase in gas filled extracellular spaces in the inner cortex. This observation has been confirmed in nodules cultured under sub-ambient concentrations of oxygen (Parsons and Day 1990; Dakora and Atkins 1991).

Additional support for osmotic regulation of gas diffusion through the inner cortex comes from the analysis of the subcellular localization of tonoplast and plasma membrane aquaporins (TIPs and PIPs) in nodules. High densities of these proteins increase the osmotic water permeability (P_f) of biological membranes and are characteristic of membranes associated with the rapid movement of bulk water in response to osmotic and pressure gradients (Maurel et al. 2008). Subcellular localization of γTIP and PIP1 and PIP2 in soybean nodules shows that these aquaporins are expressed at much higher levels on cells of the inner cortex compared to cells in the infected zone and vascular transfer cells (Serraj et al. 1998; Fleurat-Lessard et al. 2005). By analogy to motor cells of the pulvini, Fleurat-Lessard et al. (2005) postulated that this density of aquaporins is necessary to mediate rapid and reversible water movements accompanying ion fluxes to and from the apoplastic space to mediate changes in cell shape, intercellular space water content, and oxygen permeability in response to environmental cues.

Recent measurements of the cortical and infected zone fractions of soybean nodules treated with supra-ambient O_2 (30%) show a change in the localized K^+ concentrations that is consistent with the movement of K^+ from the infected zone to the cortex (Wei and Layzell 2006). It is postulated that water movement into the cortical region accompanying the movement of K^+ ions may flood the intercellular spaces of the inner cortex decreasing the gas permeability of this critical region in response to elevated O_2. This is consistent with observations that artificial elevation of KCl concentrations in the nodule cortex transiently decrease the O_2 permeability of nodules (Purcell and Sinclair 1994). Conversely, Wei and Layzell (2006) showed that hypoxic concentrations of O_2 (10%) result in elevated concentrations of K^+ in the infected zone compared to the nodule cortex. This in turn could result in the movement of water from intercellular spaces of the nodule cortex to cells within the infected zone, increasing the gas permeability of the inner cortex.

3.2 Developmental and Morphological Adaptations of Nitrogen-Fixing Nodules to Low Oxygen Stress

3.2.1 Secondary Aerenchyma Formation

While rapid modulation of the gas diffusion barrier may allow adaptations to reduced soil oxygen accompanying short term flooding, legumes respond to longer term waterlogging and hypoxic oxygen concentrations by triggering a number of developmental adaptations that lead to altered morphology designed to increase the flow of oxygen to the submerged roots and nodules. For example, soybean nodules in waterlogged soils (Pankhurst and Sprent 1975b; Shimamura et al. 2003; Thomas et al. 2005) or grown under conditions of limiting rhizosphere pO_2 (Parsons and Day 1990; Dakora and Atkins 1991) show drastic changes in morphology, with large quantities of lenticels and secondary aerenchyma tissue covering the nodule surface. Secondary aerenchyma differs from the cortical aerenchyma that is commonly formed in root and stem tissues, and is derived from differentiation of secondary meristematic cells (Jackson and Armstrong 1999). Secondary aerenchyma of nodules consists of loosely packed, white parenchymous tissue with large unoccluded intercellular spaces (Parsons and Day 1990) which enhance the gas porosity of the nodule under conditions of low rhizosphere oxygen (Thomas et al. 2005). The formation of secondary aerenchyma in response to waterlogging or reduced concentrations of rhizosphere oxygen is observed in a wide variety of legume nodules (Minchin and Summerfield 1976; Arrese-Igor et al. 1993; Dakora and Atkins 1990a; Pugh et al. 1995; James and Sprent 1999), suggesting that this is a common adaptive response to low pO_2 stress. In contrast, nodules grown under supra-ambient pO_2 conditions show a drastic reduction in the number of aerenchymous lenticels (Parsons and Day 1990; Dakora and Atkins 1991), suggesting that this developmental mechanism is part of the adaptation of the nodule to elevated external pO_2 conditions to restrict oxygen porosity.

In soybean, waterlogging triggers the development of adventitious roots as well as aerenchyma in the hypocotyl, roots and nodule tissues (Pankhurst and Sprent 1975b; Bacanamwo and Purcell 1999b; Shimamura et al. 2003; Thomas et al. 2005). These morphological changes are observed within 1 day after initiation of the flooding, and persist throughout prolonged waterlogging stress (Shimamura et al. 2003; Thomas et al. 2005). Analysis of nitrogen fixation and assimilation based on the xylem sap content of glutamine and ureides (assimilation products) and alanine (a sign of root hypoxia) shows a drastic reduction in the glutamine and ureides, and a concomitant accumulation of alanine within 1 day after waterlogging (Thomas et al. 2005). This trend is reversed by 7 days post flooding, correlating with the development of nodule and root aerenchyma and an increase in the gas porosity of these organs, suggesting a critical role for aerenchyma in adaptation (Thomas et al. 2005).

3.2.2 The Inner Cortex and Infected Zone

In addition to increases in secondary aerenchyma/lenticel formation on the surface of the nodule, additional alterations in the morphology of the inner cortex and the infected zone are also triggered to increase the oxygen porosity of the nodule in response to hypoxic conditions. For example, the analysis of nodules from various legumes cultured under rhizosphere oxygen concentrations below 5% show substantial developmental changes including: (1) reduced size and dry matter of the nodule as well as reduction in the size of the centralized infected zone (Dakora and Atkins 1990a, b; Dakora and Atkins 1991); (2) thickening of the inner cortical region and a more spherical cortical cell morphology resulting in direct connections between the extracellular spaces of adjacent cells (Dakora and Atkins 1990a; Dakora and Atkins 1991; Arrese-Igor et al. 1993); (3) increased extracellular gas spaces in the inner cortex (Parsons and Day 1990; Dakora and Atkins 1990a; Dakora and Atkins 1991; Arrese-Igor et al. 1993); and (4) a reduction in the volume and size of the infected cells, and an increased ratio of uninfected interstitial cells to infected cells (Dakora and Atkins 1990a; Dakora and Atkins 1991; Arrese-Igor et al. 1993). Together with the surface changes and aerechymous lenticel formation discussed above, these alterations are proposed to reduce the resistance of the gas diffusion pathway and account for an enhanced permeability of oxygen into the infected zone observed under conditions of severe hypoxia (Dakora and Atkins 1990a; 1990c). Further, the reduction in the cell size and number within the infected zone would be expected to reduce the respiratory needs of the nodule under conditions of low oxygen.

In addition, changes in the organization of the infected and uninfected cells within the central zone are observed in some legumes under severely hypoxic conditions. For example, when grown under rhizosphere O_2 concentrations of 1%, the morphology of the infected zone of alfalfa nodules is altered with the infected cells clustered in a ring at the periphery of the infected zone, presumably closer to the inner cortex and the oxygen barrier (Arrese-Igor et al. 1993). A comparable strategy is used in white clover (*Trifolium repens* L.) which displays an unusual tolerance to growth under continuously flooded conditions, and actually shows an increase in yield under these conditions compared to aerated control plants (Pugh et al. 1995). Besides the typical formation of lenticels and aerenchyma on the nodule surface, the infected cells of white clover cultured under waterlogged conditions become enlarged and show a more prominent central vacuole compared to aerated controls. The larger central vacuole presses the cytosolic layer to the outer periphery of the infected cell, increasing the cell surface area/volume ratio. This is postulated to increase the accessibility of the infected cell cytosol to oxygen in the intercellular gas spaces (Pugh et al. 1995).

With respect to the inner cortex, additional changes associated with hypoxic rhizosphere oxygen concentrations include alterations in intercellular “occlusions”. Ultrastructural analyses showed that nodules accumulate electron dense material that apparently occludes the intercellular gas spaces of the inner cortical region when plants are grown at elevated oxygen concentrations (Parsons and Day 1990; Dakora and Atkins 1990b; 1991). These occlusions become less apparent in nodules from plants cultured at sub-ambient oxygen concentrations. The intercellular spaces of soybean and cowpea nodules of soybean and cowpea contain a glycoprotein that is recognized by a monoclonal antibody (MAC236) that reacts with the carbohydrate moiety (Bradley et al. 1988; VandenBosch et al. 1989), and it has been proposed that this glycoprotein is responsible for the intercellular occlusions. Immunolocalization of the MAC236 glycoprotein antigen in soybean nodules exposed to 40%, ambient, and 10% oxygen shows a correlation between the accumulation of the MAC236-glycoprotein antigen in nodule cortical cells and oxygen concentration (James et al. 1991). At high oxygen concentrations (40%) intercellular spaces become occluded with this glycoprotein and it was proposed that its accumulation and deposition in these spaces, together with the decrease in the actual volume of these spaces, may contribute to the reduction in gas diffusion within the nodule cortex (James et al. 1991). In support of this, a variety of stresses that restrict gas permeability in nodules (e.g., low temperature, darkening, detopping and nitrate fertilizers) also lead to the accumulation of the MAC 236 glycoprotein antigen, as well as an additional lower molecular weight glycoprotein (MAC265 antigen), in the intercellular spaces of soybean (James et al. 2000) and lupin (Ianetta et al. 1993; de Lorenzo et al. 1993) nodules. Extracellular glycoprotein also accumulates at other locations within the nodule, including the intercellular spaces of cells in the infection zone (James et al. 2000), suggesting a possible role in regulation of gas permeability in the central zone of the nodule as well.

3.2.3 Influence of Adaptive Changes on Nitrogen Fixation Under Altered Rhizosphere pO_2 Conditions

Various “flooding sensitive” legumes, show a remarkable ability to adapt to growth at low oxygen within a tolerable range. For example, soybean nodules cultured at subambient pO_2 as low as 2.5% O_2 show similar levels of nitrogen fixation (acetylene reduction), respiration, and the ureide content of phloem sap compared to control nodules grown under ambient oxygen conditions (Dakora and Atkins 1991; Parsons and Day 1990). At extreme hypoxic levels of O_2 (1%) a decrease in these parameters was observed, suggesting that the ability to buffer changes in oxygen content by increasing the gas permeability of the nodule may be exceeded. Studies with cowpea nodules show a similar effect of reduced oxygen, with plants able to adapt to oxygen as low as 10%. At 5% O_2 or lower, a reduction in the rate of nitrogen fixation, as well as a decrease in the nodule and whole plant dry weight was observed (Dakora and Atkins 1990a, b). This may be due to decreased numbers of nodules, and a reduced size of the infected tissue as discussed above. However, the plants partially compensate for this by increasing the efficiency of nitrogen fixation (nitrogenase activity/bacteroid) at extremely low O_2 by an unknown mechanism (Dakora and Atkins 1990a), an observation that was also made in alfalfa plants grown at 1% O_2 (Arrese-Igor et al. 1993). In alfalfa, nodules grown under 8% O_2 show little differences in nitrogenase activity and respiration compared to control nodules (Wycoff et al. 1998). However, plants grown at more severe hypoxic concentrations (1% O_2) show reduced number of infected cells, reduced yield of roots and nodules, and lower nodule respiration based on the rate of CO_2 evolution (Arrese-Igor et al. 1993). Overall these observations suggest that flooding sensitive legumes possess the ability to adapt to mild sub-ambient oxygen conditions but that this capacity is exceeded in conditions of extreme hypoxia.

While nodulated plants show the ability to adapt to long term growth under reduced oxygen conditions, and retain the ability to exert short term adjustments in the gas diffusion barrier to mitigate the effects of modest changes in oxygen concentration (Atkins et al. 1993), plants grown under severe hypoxia (<2.5% O_2) were much more sensitive to acute increases in rhizosphere O_2 associated with the abrupt return of plants to ambient oxygen (Dakora and Atkins 1990c; Dakora and Atkins 1991). For example, nodulated cowpea plants cultured under 1 or 2.5% O_2 show severe loss of nitrogenase activity upon transfer to ambient O_2 which did not recover for 15 days (Dakora and Atkins 1990c). Thus in addition to severe restriction of oxygen resulting from waterlogging, an additional stress associated with re-oxygenation of the nodule during recovery from hypoxia stress and its effects on nitrogenase also needs to be considered. This re-oxygenation sensitivity phenomena has also been observed for flood-adapted nodules of aquatic legumes such as *Neptunia patens* (James et al. 1992b), suggesting that: (1) the nodules of flood-adapted legumes have a reduced resistance to oxygen diffusion into the nodule; and (2) this higher oxygen permeability may lead to sensitivity to oxidative stress upon rapid transition from a flooded to a well-drained habitat.

3.3 Strategies of Adaptation: Flood-Tolerant Legumes and Oxygen Diffusion

3.3.1 Tropical Wetland Legumes

Although legume nodules in general have short and long term adaptive programs that allow growth over a broad range of rhizosphere oxygen concentrations, the oxygen content of waterlogged soils can lead to severe hypoxia which would exceed this range of adaptation (Pugh et al. 1995), and it is acknowledged that most legumes are sensitive to prolonged flooding (Loureiro et al. 1998). Nevertheless "flooding tolerant" aquatic and semiaquatic legumes have developed additional strategies to allow adaption to extreme conditions associated with growth in wetland environments (Justin and Armstrong 1987; Loureiro et al. 1998; Sprent 1999; James et al. 2001; Koponen et al. 2003; Den Herder et al. 2006). For example, nodulated legume trees and shrubs are widespread in a number of tropical wetlands where they play an important role in providing fixed nitrogen to permanently or seasonally flooded tropical forests (Loureiro et al. 1998; Saur et al. 2000; James et al. 2001; Koponen et al. 2003). Indeed, soils of both seasonally and permanently flooded wetlands are nitrogen deficient because of leaching of nitrogenous compounds, a lack of mineralization of organic matter and increased denitrification (Loureiro et al. 1998). This results in positive selection of nodulated, nitrogen-fixing legumes in these environments.

Tropical wetland legumes adapt to waterlogged environments by using a variety of strategies including: (1) the formation of extensive interconnected aerenchyma or other pathways limiting oxygen diffusion resistance in stem, root and nodule systems; and (2) alterations in the nodulation patterns with the formation of nodules just below the waterline, or on adventitious roots and stems above the waterline, as well as the nodulation of floating root systems. These strategies are discussed extensively for a wide variety of tropical legumes from several wetland ecosystems (Loureiro et al. 1998; Saur et al. 2000; James et al. 2001; Koponen et al. 2003), and for the purpose of this review, examples with representative wetland or aquatic legumes are discussed below.

3.3.1.1 Nodulation of Submerged Stems and Roots: Increased Porosity Mechanisms

Discolobium is a hydrophytic leguminous shrub that is common to the Brazilian Pantanal wetlands where it is found in permanently flooded environments (James et al. 2001). *D. pulchellum* is nodulated on stems and roots under flooding conditions (Loureiro et al. 1994), and unlike avoidance adaptive measures typical of many wetland legumes (see Sect. 3.3.1.2), *D. pulchellum* nodules can be found at depths exceeding 2 m suggesting that they survive and function under severely low oxygen concentrations (Loureiro et al. 1998). To increase air porosity into submerged tissues, *D. pulchellum* stems are hollow, and stem, root and nodule tissues are interconnected by a highly developed network of aerenchyma that penetrates to the mid cortex of the nodule (Loureiro et al. 1994). *D. pulchellum* forms determinant aeschynomenoid type of nodules (Sprent and James 2007) with an infected zone consisting solely of infected cells with large unoccluded intercellular air spaces, and with no interstitial uninfected cells (Loureiro et al. 1994). The internal structures of stem and root nodules of flooded *D. pulchellum* are unique, showing a direct vascular connection between the nodule to stem or root vasculature, and the presence of an internal vascular bundle surrounded by infected cells (Loureiro et al. 1994). An interesting observation is that *D. pulchellum* stem nodules senesce rapidly and aerenchyma tissue collapses upon exposure to air (Loureiro et al. 1994). This suggests that the nodules are terminally adapted to a permanently flooded environment, possibly by maximizing a constitutive low resistance oxygen diffusion pathway from the stem to the nodule. If this is the case, exposure of *D. pulchellum* to atmospheric oxygen concentrations could lead to nitrogenase inactivation or oxidative damage due to the accumulation of reactive oxygen species (ROS).

Development of networks of stem and root aerenchyma, and increases in intercellular air spaces in flooded nodules seem to be a common adaptation mechanism in variety of flood-tolerant, semi-aquatic legumes including *Mimosa pellita* (James et al. 2001), *Neptunia* (Schaede 1940; James et al. 1992a), *Alnus rubra* (Batzli and Dawson 1999), *Pentraclethra macroloba* (Walter and Bien 1989), and *Viminaria juncea* (Walker et al. 1983). These morphological changes are important to maintain nitrogen fixing activity (Walter and Bien 1989; Walker et al. 1983) or for recovery of nitrogen-fixation rates to levels of non-flooded nodules during prolonged waterlogging stress (Batzli and Dawson 1999).

3.3.1.2 Aerial Nodulation of Stems and Adventitious Roots: Avoidance Mechanisms

An additional flooding-tolerance strategy exhibited by semi-aquatic tropical legumes is the alteration in the pattern of root and stem nodulation to avoid the low oxygen environment associated with submergence. For example, under flooding conditions, the nodulation of semi-aquatic species of *Neptunia* occurs at the top of the tap root, as well as on spongy lateral roots that float at the water surface (Schaede 1940; Allen and Allen 1981). In the case of flood-tolerant species of *Aeschynomene* (Alazard 1985) and *Sesbania* (Dreyfus and Dommergues 1981), waterlogging results in the induction of stem nodulation. This commonly occurs on the aerial portion of the stem above the water line (e.g., *S. rostrata* and several species of *Aeschynomene*), and is considered a nodulation strategy to avoid the restricted oxygen environment associated with submersion. The cortical cells of stem nodules on these plants contain chloroplasts and are photosynthetic (James et al. 1998), which may contribute to the high nitrogen fixation activity of stem-nodulated legumes under conditions of flooding (Boivin et al. 1997). In the case of *Aeschynomene fluminensis*, stem nodules form only on submerged roots and show morphological adaptations including large intercellular air spaces in stems that are connected via aerenchymous tissue to the base of the nodules (Loureiro et al. 1995). *A. fluminensis* nodules are also photosynthetic, and it was proposed that photosynthetic evolution of of O_2 could represent an additional mechanism to augment low pO_2 in a submerged environment (Louriero et al. 1995, 1998). In addition, the rhizobia bacteroids in *A. fluminensis* nodules are also photosynthetic and light enhances the nitrogenase activity of the endosymbiont, suggesting an alternative source of biosynthetic energy to drive nitrogen fixation under oxygen limiting conditions (Evans et al. 1990).

3.3.2 Lotus uliginosus: *A Temperate Wetland Legume*

While flooding tolerance is more typically observed in tropical wetland legumes, examples of adaptation to flooding among selected temperate species have also been observed. *Lotus uliginosus* (marsh birdsfoot trefoil) is a wetland forage legume that shows a high tolerance to flooding compared to *L. corniculatus* (birdsfoot trefoil), the more commonly used non-wetland species (Justin and Armstrong 1987). Since *L. uliginosus* is considered to be a parent of the more common *L. corniculatus*, the comparison of the two species has been instructive for examination of adaptive strategies of nodulation and nitrogen fixation in flooding-tolerant legumes. *L. uliginosus* successfully forms active nitrogen fixing nodules upon infection with *Mesorhizobium loti* under waterlogged conditions (James and Crawford 1998; James and Sprent 1999). Comparison of the plant yield and nodulation under conditions of root submergence provided the surprising observation that flooded *L. uliginosus* plants show increased yield (shoot and nodule dry weight, nodule numbers, carbon and nitrogen) when cultured under conditions with reduced dissolved oxygen (roots flooded with N_2-bubbled water for 60 days compared to roots flooded with air-saturated water for 60 days). In comparison, *L. corniculatus* showed a lower yield, higher levels of ethylene gas release, and signs of nodule senescence in response to submergence compared to *L. ulginosus* (James and Crawford 1998).

The morphology of the nodules of the two *Lotus* species grown under low oxygen conditions show typical developmental adaptations including the presence of profuse secondary aerenchyma/lenticel development on the surface of flooded-nodules and development of nodules primarily on the tap root close to the hypocotyl, modifications designed to lower the resistance and distance of gas diffusion (James and Crawford 1998). However, despite the similarity in nodule morphology, the root porosity of *L. ulginosus* nodules cultured under conditions of low oxygen was fourfold higher than *L. corniculatus* nodules. This suggests that a less restricted oxygen diffusion pathway is responsible for increased survival and adaptation to this stress. Consistent with the proposal of a less restricted oxygen diffusion pathway, the cortical cells of *L. ulginosus* nodules showed lower amounts of the MAC265 glycoprotein antigen compared to *L. corniculatus* nodules cultured under low oxygen conditions (James and Crawford 1998).

In addition, *L. ulginosus* plants show adaptations to flooding with respect to the rhizobia infection pathway. Similar to other terrestrial legume species (reviewed in Oldroyd and Downie 2008), *L. ulginosus* is principally infected with *Mesorhizobium loti* through the classical root hair curling (RHC) pathway. However, in flooded plants, the bacteria infect *L. ulginosus* by a “crack-based” infection mechanism (see next section) by accumulating within intercellular spaces in the tap root aerenchyma (James and Sprent 1999). In addition, functional nodules are formed on the adventitious roots which emerge from the stems of flooded plants, as well as on the stem itself (James and Sprent 1999), which likely help obviate the problems of oxygen diffusion to nodules in a flooded environment.

3.4 Strategies of Adaptation: Alternate Nodulation Pathways for Flooding Tolerant Legumes

3.4.1 Intercellular-Based Mechanism of Nodulation: The Lateral Root Boundary Pathway

A hallmark of flood-stressed legumes is the reduction in the numbers of nodules formed on waterlogged roots. Problems that compromise nodulation under conditions of flooding include increased levels of ethylene gas, which inhibit nodulation in several terrestrial legume/rhizobia associations (Penmetsa and Cook 1997; Guinel and Sloetjes 2000), as well as the inhibition of root hair growth. In order to cope with these problems, flooding-tolerant legumes such as *L. ulginosus* (James and Sprent 1999), *Neptunia* sp. (James et al. 1992a; Subba-Rao et al. 1995; Goormachtig et al. 2004a), and *Sesbania rostrata* (Ndoye et al. 1994; Goormachtig et al. 2004a) alter the pathway of rhizobial infection.

Nodulation in most legumes normally takes place by a "root hair curling" (RHC) mechanism (reviewed in Patriarca et al. 2004; Oldroyd and Downie 2008) that involves intracellular entry of the invading rhizobia bacteria through root hairs normally found in a region ("zone 1") immediately above the root tip. Soil rhizobia bind to the tips of the root hairs of the host legume and induce morphological changes including swelling, deformation, and curling, with the later causing entrapment of the bacteria in a microcolony at the root hair tip. Localized cell wall hydrolysis leads to the invasion of the bacterial microcolony and the formation of infection thread (IT) tubes which penetrate the root epidermis and move transcellularly through the root cortex. Interaction of the rhizobia with the root hair simultaneously triggers cortical cell division and the formation of nodule primordia. Upon reaching the nodule primordia, ITs release the rhizobia bacteria which are taken up by endocytosis. Bacterial initiation of these events is stimulated by the release of nodulation factors ("nod factors") which are lipochitooligosaccharide signaling molecules that consist of a tetrameric backbone of β1-4 *N*-acetyl glucosamine residues, an N-linked fatty acyl group, and a variety of chemical modifications that provide host–bacteria specificity (Geurts and Bisseling 2002; D'Haeze and Holsters 2002; Oldroyd and Downie 2008).

In contrast to the RHC-mechanism, a more rudimentary "lateral root boundary" (LRB) mechanism of infection is commonly used by aquatic legumes. LRB infection is often referred to as "crack invasion" or "intercellular infection", and involves the entry of rhizobia bacteria in breaks in the epidermis, generally at secondary root/primary root boundaries, and accumulation within the extracellular spaces of the root cortex in zones referred to as "infection pockets" (Goormachtig et al. 2004b; Den Herder et al. 2006; Oldroyd and Downie 2008). Similar to the RHC-mechanism, nod factor-induction of cortical cell division and nodule primordia formation take place. Infection of the nodule primorida by bacteria in the infection pocket takes place by the formation of an extracellular IT which, unlike RHC infection, penetrates the root through the intercellular spaces prior to bacterial release into the host cells of the nodule primordium (Goormachtig et al. 2004b). However, depending on the specific legume/rhizobial association, in some cases direct uptake of rhizobia by nodule cells from the cortical infection pocket can occur without IT formation (Goormachtig et al. 2004b).

The primary role of root curling (RHC mechanism) or infection pocket formation (LRB mechanism) is to provide a colony of bacteria entrapped within the plant, presumably to provide a critical "signaling center" to facilitate signal exchange between the plant and symbiont (Goormachtig et al. 2004a). In the case of RHC infection, two legumes have emerged as model systems for the investigation of molecular genetics and functional genomics associated with bacteria/host signal transduction and nodule development: *Lotus japonicus,* a model for legumes that form determinant nodules, and *Medicago truncatula*, a model for legumes that form indeterminant nodules (Udvardi et al. 2005; Stacey et al. 2006; Young and Udvardi 2009). With respect to flood-tolerant species that utilize LRB infection, the tropical legume *Sesbania rostrata* has become a model system for investigation of "crack nodulation" (reviewed in Goormachtig et al. 2004b; Den Herder et al. 2006). Also, since *S. rostrata* exhibits both RHC and LRB nodulation strategies, it also has been an instructive model for how tropical legumes cope with flooding stress by using different infection signaling pathways.

3.4.2 **Sesbania rostrata:** ***A Model Legume for Aquatic Nodulation***

Sesbania rostrata is considered a semi-aquatic tropical legume, and attracted initial interest because of its agronomic potential as a flood tolerant "green manure" legume, especially for nitrogen enrichment of rice fields (Becker et al. 1995). *S. rostrata* can be infected by *Azorhizobium caulinodans* by either the RHC or LRB pathway (Ndoye et al. 1994; Goormachtig et al. 2004a). As part of the adaptation strategy to a semi-aquatic lifestyle, the *S. rostrata* stem contains rows of lateral root primordia located along the stem length. When grown on aerated soils these stem root primordia remain dormant and *S. rostrata* nodulation occurs by the classical RHC pathway, resulting in the formation of indeterminant nodules with a persistent meristerm. However, root submergence triggers the emergence and growth of lateral adventitious roots from these stem root primorida, and the LRB infection pathway is promoted and spherical determinant nodules are formed. Simultaneously, flooding causes a suppression of root hair growth in zone 1 of the root, and the arrest of *A. caulinodans* nodulation by the RHC pathway (Goormachtig et al. 2004a).

LRB nodulation of epidermal breaks at the boundary of the stem and the emerging adventitious roots occurs by a series of steps, including: (1) initiation of a localized cortical cell death response generating the "infection pocket" cavity within the root cortex; (2) entry and colonization of the infection pocket by azorhizobia bacteria; (3) induction of cortical cell division in the root and formation of nodule primordia; (4) formation of intercellular ITs that penetrate the root and release the symbiont into infected cells of the nodule primordia (Den Herder et al. 2006). Infection also takes place at the base of submerged lateral roots by a similar pathway, with *S. rostrata* forming nodules on roots as well as on stems on emerging lateral adventitious roots under waterlogged conditions (Ndoye et al. 1994).

Similar to the RHC-based pathway, LRB infection in *S. rostrata* requires the *A. caulinodans* nod factor (D'Haeze et al. 1998, 2003). However, analysis of *A. caulinodans* mutants that produce nod factors lacking specific modifications of the lipochitinoligosaccharide backbone show that while these mutants are incapable of RHC infection, they can still generate functional nodules through the LRB pathway, although with less efficiency and lower nodule numbers (D'Haeze et al. 2000). This argues that the nod factor receptor and signaling networks for the two infection pathways likely share some properties but that LRB infection has a less stringent nod factor structural requirement than the RHC pathway, and also that the two pathways involve some additional non shared components as well. This is supported by transcriptional profiling analyses. For example, by using cDNA-amplified fragment length polymorphism analysis, Capoen et al. (2007) compared the transcript profiles of roots from the site of *A. caulinodans* infection under hydroponic (LRB) and aeroponic (RHC) conditions. A large number of genes were found that are common to both nodulation pathways. In general these genes are proposed to be associated with common events in both pathways such as nodule primordia formation and later stages of nodule development. However, clusters of genes that were unique to either the RHC or LRB pathways were also identified, several of which encode proteins proposed to be specific to the invasion process of each pathway (Capoen et al. 2007).

The elevation of the levels of ethylene is a common response during flooding stress (Perata and Voesenek 2007; Bailey-Serres and Voesenek 2008), and it is clear that this is a critical signal that reciprocally regulates the path of nodulation in *S. rostrata* (Goormachtig et al. 2004a). Upon flooding of *S. rostrata* roots, the enzymes for ethylene biosynthesis such as *S*-adenosyl methionine synthetase, ACC synthase and ACC oxidase, as well as an ethylene response element transcripion factor, are upregulated suggesting that the production and perception of ethylene is induced (Schroeyers et al. 2004). By using a pharmacological strategy, it was shown that application of ethylene biosynthesis inhibitors results in an increase in zone 1 root hair nodulation whereas the addition of the ethylene precursor ACC shows the opposite effect with zone 1 nodulation inhibited (Goormachtig et al. 2004a). Conversely, inhibitors of ethylene action block nod factor-mediated infection pocket formation and nodule primordia formation, and in general inhibit the nodulation of *S. rostrata* under aquatic growth conditions (D'Haeze et al. 2003). In addition, ethylene apparently controls the type of nodule formed under aquatic and nonaquatic conditions (Fernandez-López et al. 1998). Ethylene biosynthetic inhibitors applied during aquatic *S. rostrata* nodule formation result in the persistence of an indeterminant nodule, typical of RHC infections, suggesting a role of ethylene in regulating the meristematic activity of the nodule and whether an indeterminant or determinant nodule type is formed.

A critical element of LRB nodulation that is lacking in RHC nodulation is the formation of the cortical infection pocket for the accumulation of *A. caulinodans* bacteria. The induction of this pocket involves nod factor and ethylene-induced programmed cell death of cortical cells localized adjacent to the epidermal crack at the lateral root base. In addition, basal root infection was associated with the release of high levels of H_2O_2 which is proposed to mediate the programmed cell death response as part of the infection pocket formation (D'Haeze et al. 2003). Nod factor-induced responses in hydroponic *S. rostrata* are inhibited by the pre-application of H_2O_2 scavengers or inhibitors of ROS production, suggesting that H_2O_2 plays a role as a second messenger of the nod factor signal, selectively in the LRB pathway (D'Haeze et al. 2003).

Besides ethylene gas, additional crosstalk with other hormone-signaling pathways also appears to be important in determining the infection pathway for *S. rostrata* nodulation. For example, a synergistic role in aquatic nodulation has also been proposed for gibberellin in mediating the formation of infection pockets during aquatic nodulation since GA biosynthetic inhibitors block infection pocket and IT formation (Lievens et al. 2005). Also, jasmonic acid has been shown to have the opposite effect, with this hormone triggering the RHC infection pathway while suppressing the LRB pathway (Capoen et al. 2009).

From these observations, a model (Holsters et al. 2005; Den Herder et al. 2006) has been proposed for the regulation of the path of rhizobial infection of semi-aquatic legumes. The RHC pathway is proposed to be the default pathway in flood-tolerant legumes under normal growth in well-aerated media. However, upon flooding, ethylene accumulates due to: (1) the low diffusion co-efficient of ethylene in aqueous media; and (2) increased induction of genes coding for ethylene biosynthetic enzymes and signaling proteins as a general response to flooding stress (Schroeyers et al 2004). This triggers a shift in nod factor signaling from a RHC-based intracellular infection mechanism through epidermal root hairs, to a LRB-invasion mechanism through epidermal cracks in adventitious and lateral root boundaries. Since ethylene is commonly involved in the adaptation of a number of growth responses in flood tolerant nonlegumes, it is proposed that flood-tolerant legumes coupled elements of the ethylene response network to traditional nod factor signaling pathways to enable an alternative mode of nodulation. In addition, the ethylene-induced switch to a crack infection pathway avoids the negative effects of ethylene-sensitive arrest of root hair development and RHC-based infection observed in flooding intolerant legumes (Holsters et al. 2005; Den Herder et al. 2006).

3.5 Summary and Concluding Remarks

The nodule represents a unique and specialized organ that has evolved to maintain a microaerobic symbiotic environment designed to simultaneously support a high respiratory rate while preventing the inactivation of oxygen-sensitive nitrogenase.

The regulation of oxygen entry through a high resistance gas diffusion barrier is critical for the maintenance and regulation of this environment. The diffusion rate is modulated both by short term and long term adaptations in response to environmental and metabolic cues, and this represents a critical control of the energetically expensive nitrogen fixation process in response to stress and metabolic need. From the perspective of waterlogging stress, the rhizosphere oxygen concentration is a key signal that rapidly and reversibly stimulates oxygen diffusion in response to hypoxia or inhibits diffusion in response to elevated pO_2.

With respect to short term regulation of gas diffusion, osmotically-driven water flow between the cells of the inner cortex and the apoplastic spaces has emerged as a promising model to explain the reversible modulation of gas diffusion through the inner nodule cortex. However, mechanistic details of the molecular components of this apparatus and the signaling process through which oxygen signal is sensed and transduced are lacking. Questions remain regarding the relative contribution of the inner cortex and the infected zone to the control of oxygen diffusion to the bacteroid under conditions of limiting rhizosphere oxygen and flooding. In addition, the induction of the synthesis and deposition of extracellular glycoproteins appear to be correlated with the degree of resistance of the gas diffusion pathway, however how these proteins aid in short and long term regulation of gas diffusion through the intercellular cortical and infected cell spaces remains to be addressed.

With respect to long-term adaptations, both flooding-tolerant and flooding-sensitive legumes have general developmental programs to adapt to waterlogged conditions. In response to flooding and/or reduced external oxygen concentrations, an increase in the gas porosity of the nodule by the formation of secondary aerenchyma and surface lenticels is triggered. In the case of aquatic wetland legumes, this network of aerenchyma is extensive and is continguous with stems, roots and nodules. In extreme cases, such as the tropical aquatic legume *Discolobium*, this may have led to an “obligate flooding” nodule, which is terminally adapted to a submerged state and cannot survive return to ambient oxygen conditions. Flood-tolerant legumes have also developed additional developmental strategies including the formation of nodules on stems and adventitious roots that avoid hypoxia by growing on aerial tissues or on floating root or stem systems. In these cases, an alternative nodulation strategy involving “crack infection” of epidermal breaks at the point of lateral root or adventitious root emergence has been employed. Interestingly nod factor signaling is also involved in this process but the pathway is distinct from the classical nod factor/RHC signal transduction pathway, and involves crosstalk with other signaling pathways, particularly with the stress hormone ethylene. *Sesbania rostrata* has emerged as the model legume for this flooding-tolerant nodulation pathway, and remains a promising system for future elucidation of the mechanistic features of crack nodulation, and the role of ethylene signaling in switching the mechanism of nodulation that is critical to adaptation to waterlogging stress.

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