

Chapter 5

Oxygen and the Regulation of N₂ Fixation in Legume Nodules Under P Scarcity

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Abstract Oxygen (O₂) is vital for nodule metabolism owing to its role in mitochondrial respiration for ATP generation, a vital component in N₂ fixation. However, the concentration of O₂ must be carefully regulated, because O₂ can also reduce inhibit nitrogenase. Phosphorus (P) deficiency can increase the nodule's permeability to O₂ and thereby exert a deleterious effect on N₂ fixation. Although the mechanism by which the P deficiency increases the O₂ permeability is not known, it has been attributed to a reduction in the O₂ diffusion barrier within the nodule. In order to maintain N₂ fixation, the nodules have several adaptations at the structural and metabolic levels to prevent and ameliorate these negative impacts. These adaptations will be evaluated in terms of structural and metabolic responses to O₂ diffusion. The structural responses are based on the physical barrier of cortical cells and their intercellular spaces, while the metabolic responses include respiratory alteration and binding of O₂ by leghemoglobin. We provide a critical evaluation of the current knowledge regarding the P effect on O₂ permeability and propose new theories on potential mechanisms of P deficiency on the O₂ diffusion barrier.

Keywords Oxygen • P stress • N₂ fixation • Nodule • Structural adaptations • Metabolism

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5.1 Introduction

The reduction of atmospheric dinitrogen (N_2) by mature bacteroids is an extremely energy-intensive process fueled by photosynthetically derived carbon from the plant. Nodules require energy for the fixation of atmospheric N_2 (Kleinert et al. 2014), synthesis of exported organic solutes of nitrogen (N) (Magadlala et al. 2016), as well as for growth and maintenance (Kleinert et al. 2014). Large amounts of oxygen (O_2) are required to generate this energy, and nodules therefore have a considerably higher rate of O_2 consumption than other plant tissues (Mortimer et al. 2009). O_2 inhibits the functioning of nitrogenase and the regulation of bacteroid metabolism in the O_2 -limited environment of the nodule presents the bacteroid with problems. Therefore, energy, reductant, and carbon pools must be carefully balanced to ensure optimum rates of N_2 fixation (Lodwig and Poole 2003).

The O_2 concentration in the infection zone of nodules is maintained at approximately 18 nmol (Layzell and Hunt 1990). The mechanism of O_2 control in legumes has not yet been fully elucidated, but regulation appears to consist of three processes (Udvardi and Poole 2013):

1. An O_2 diffusion barrier exists in the outer cell layers of nodules which limits the diffusion of O_2 into the infected zone.
2. Bacteroids and plant mitochondria have high respiration rates for O_2 consumption.
3. Leghemoglobin has a high binding affinity for O_2 in the cytoplasm and delivers it to the infected cells for consumption by bacteroids and mitochondria.

The effect of O_2 on nodule physiology can be further exacerbated during phosphorus (P) deficiency. Many metabolites occur as orthophosphate (Pi) monoesters, while the phosphoanhydride bonds of compounds such as ATP function to transfer energy from energy-yielding process of photo-oxidative and substrate-level phosphorylation to the energy-dependent cellular processes of biosynthesis, ion pumping, and mechanical work (Plaxton and Tran 2011). The responses of nodules to O_2 and P stress may also depend on the morphological differences between nodules of different legumes.

5.2 Nodule Structure and Metabolism

The structures of nodules differ generally from legumes of tropical and temperate origin, although these morphological differences do not always follow this simple division. In tropical and subtropical legumes (e.g., soybean, common bean, and cowpea), the roots generally form determinate nodules (Fig. 5.1) with a closed meristem that at nodule maturity does not divide any further (Smith and Atkins 2002). Nodules from tropical legumes are spherically shaped and its infected cells lack vacuoles (Schubert 1986). Furthermore determinate nodules are known to harbor several bacteroids within a symbiosome, which results from fusion of separate

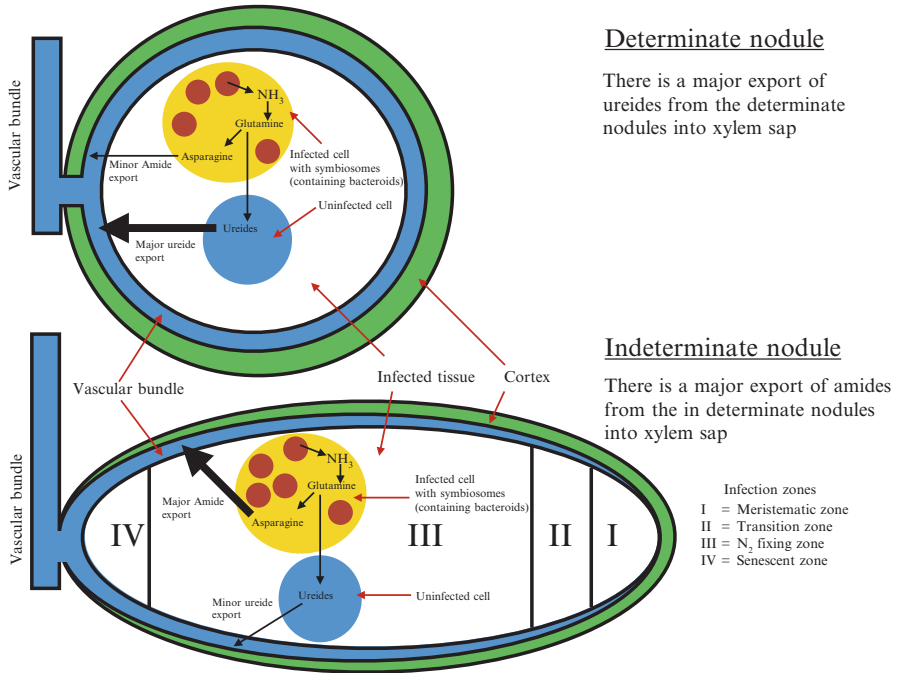


Fig. 5.1 The anatomical and metabolic differences between determinate and indeterminate legume nodules. Determinate nodules are generally from tropical origins and export fixed N₂ mostly as ureides. Indeterminate nodules generally originate from temperate regions and export fixed N₂ largely as amides (asparagine and glutamine)

symbiosomes and/or bacteroids dividing continually within the existing symbiosome (Prell and Poole 2006). In contrast, legumes from more temperate environments have nodules which are typically indeterminate (Fig. 5.1). These nodules are characterized by an open meristem, which allows for continual divisions right through the plant life cycle. This gives rise to a cylindrically shaped nodule in which the infected cells are vacuolated (Schubert 1986). Although various nodules differ in some structural details, the general organization of tissue regions is similar, with the infection zone normally confined to the central region of the nodule. The two types of nodules present an intriguing difference in metabolism.

In general, most temperate legumes (e.g., lupins, pea, clovers) usually transport their fixed N as amides, notably asparagine and glutamine (Fig. 5.1) (Streeter 1991). In comparison, the tropical legumes (e.g., soybeans, cowpea, common bean) export purine derivatives, most notably the ureides such as allantoin and allantoic acid (Fig. 5.1). The existence of these two metabolically distinct routes in legumes from different origins and nodule morphology is perhaps unsurpassed by other nutritional variations within plant systems (Streeter 1991; Le Roux et al. 2009). In terms of respiratory energy costs, the requirement of ATP and reductant per assimilated N does not differ significantly for ureide and amide

exporting legumes (Smith and Atkins 2002). Nonetheless, the organic N translocated as ureides has a lower C/N ratio than when N is exported as amino acids in amide exporting legumes (Smith and Atkins 2002). For these reasons, the ureide biosynthesis and export is commonly regarded as the more economical pathway in terms of C expenditure. Furthermore, photorespiration is generally promoted in tropical climates; so therefore the export of organic N as ureides may additionally serve to be more economical with C, compared to amino acid export. During P deficiency the export of more ureides, relative to amino acids, may be an adaptive advantage, especially when extremely low P supply can lead to a reduction in C supply. Although P stress will cause a decrease in the total N assimilation in the nodules and nodulated, the ratio of ureides being synthesized and exported, relative to amino acids, may change (Oliviera et al. 2004; Le Roux et al. 2009; Magadlela et al. 2016). The advantage of this adaptation during P stress is to export the form of organic N that is associated with a lower C economy. Compared to amino acids, the export of ureides represents a more N-dense form of organic N being exported to shoots (Atkins 1991; Todd et al. 2006). In spite of the anatomical and metabolic differences between these two groups of legumes, both amide and ureide exporting nodules are sensitive to O₂ supply. Moreover, P deficiency increases their respective O₂ permeabilities, so that biological N₂ fixation (BNF) is reduced (Ribet and Drevon 1995; Drevon and Hartwig 1997; Schulze and Drevon 2005; Le Roux et al. 2009).

5.3 Nodule O₂ Diffusion During P Deficiency

Irrespective of whether legumes have amide or ureide exporting nodules, the supply of P appears to be crucial to the control of O₂ permeability into the nodules. In this regard, these different types of legumes such as soybean (Ribet and Drevon 1995), common bean (Vadez et al. 1996), and alfalfa (Schulze and Drevon 2005) have all shown an increased O₂ diffusion into nodules during P stress. The effect of increases in O₂ flush inside the nodule resulting in the destruction the nitrogenase enzyme (Schulze and Drevon 2005; Avenhaus et al. 2016). This can occur in a relatively short time, from minutes to hours, with negative consequences to the capacity of nodules for fix N₂ (Schulze and Drevon 2005; Avenhaus et al. 2016). Besides P availability, nodule O₂ conductance is also affected by other abiotic stresses.

However, unlike drought or chilling stress, P deficiency has been repeatedly reported to increase nodule conductance to O₂ in many investigations, e.g., soybean (Ribet and Drevon 1995), common bean (Drevon et al. 2015; Bargaz et al. 2011), and alfalfa (Schulze and Drevon 2005). With one exception in common bean (Jebara et al. 2005), nodule O₂ conductance changes of five genotypes were compared under P deficiency conditions, and two genotypes showed decreased nodule conductance while the other three had no changes. Interestingly, in a similar study in common bean from the same research group (Bargaz et al. 2011), the nodule conductance of all five different genotypes were found to increase

significantly under P-deficient conditions. The reasons for this discrepancy are unknown; investigation of changes in nodule O_2 conductance under P limitation in genotypes of other legume species may help answer this question. The nodule conductance is calculated by dividing the permeability of the whole nodule population of one plant by the total nodule surface area (Schulze and Drevon 2005). Therefore, nodule O_2 conductance is affected by the nodule shape variance (few large nodules or many small nodules) when the nodule permeability is the same, which could partially explain the genotypic variance in nodule conductance in common bean under P limitation (Jebara et al. 2005). Given the negative impacts of an increase in O_2 permeability to nodule function, it stands to reason that legumes have several adaptations in place to reduce the effects of O_2 permeability. These adaptive mechanisms can be structural or functional and appear to operate at the levels of course and fine control.

The course control is thought to be a physical barrier to gas diffusion, which is located in the inner cortex (Fig. 5.2) of nodules (Hunt and Layzell 1993; Minchin 1997; Galvez et al. 2000), while the fine control of O_2 levels appears to reside in the oxygenated leghemoglobin gradients (Fig. 5.2) within the nodules (Thumfort et al. 1999). Broadly, these two levels of control can be separated into structural and metabolic adaptations, which will be evaluated in the following sections.

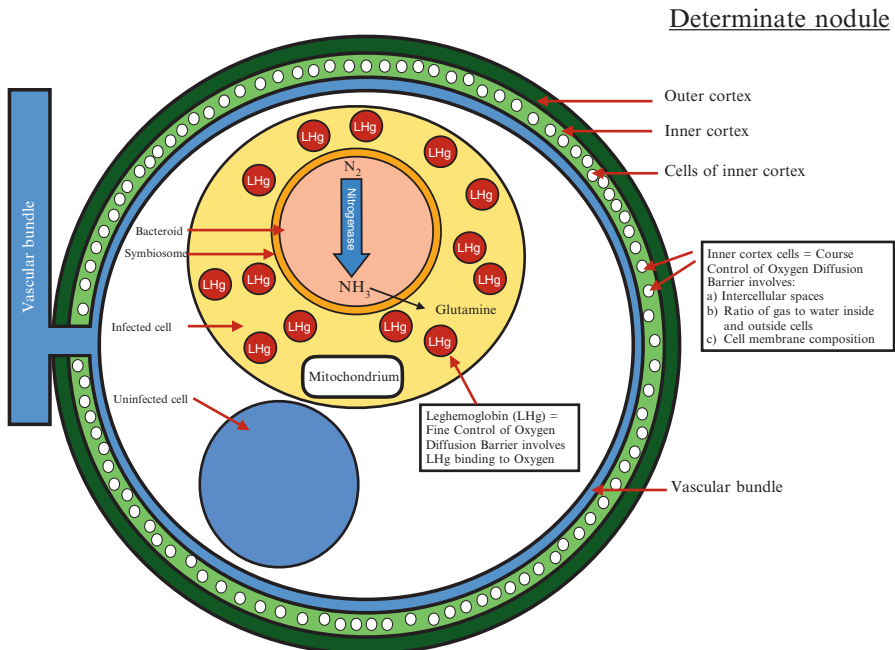


Fig. 5.2 The proposed O_2 diffusion barrier in legume nodules, using a determinate nodule as a model. The control of the O_2 diffusion barrier in the nodule is largely mediated by leghemoglobin- O_2 binding (fine control) and the physical barrier of inner cortical cells (course control)

5.4 Nodule Structural Adaptations to the Effect of P Stress on O₂ Permeability

N₂-fixing legumes have long been known to be sensitive to P deficiency, which probably is attributed to the key role of P in the cell energy metabolism and the high energy requirements for N₂ reduction by nitrogenase (Schulze and Drevon 2005). In nodulated plants, the nodule is usually the organ that has the highest P concentration (Bargaz et al. 2011; Schulze et al. 2006; Schulze and Drevon 2005). In multiple studies in various legume species, P limitation was generally shown to greatly reduce the number, size, biomass, as well as total surface area of nodules (Bargaz et al. 2011, 2013; Lazali et al. 2013; Sulieman et al. 2013; Schulze and Drevon 2005; Høgh-Jensen et al. 2002; Ribet and Drevon 1995). At the whole-plant level, however, the biomass ratio of nodule vs. shoot, root, or the entire plant could either increase, decrease, or remain relatively stable because the shoot and root growth, especially shoot growth, are similarly reduced by P limitation (Jebara et al. 2005; Kouas et al. 2005; Schulze and Drevon 2005). Differences in the nodule growth response to P deficiency appear to be related to plant species, genotypes, and the duration and the severity of the stress. Further structural control of the O₂ diffusion barrier is more apparent at the anatomical scale, involving the cortical cells of nodules (Fig. 5.2).

Drevon et al. (1998) suggested that osmoregulatory changes in nodule cortical cells may be responsible for the increased O₂ diffusion. The physiological purpose of this increased O₂ diffusion during P stress is unclear, but may be connected to ensuring sufficient adenylate levels for high N₂ fixation rates, in spite of the O₂ inhibition of nitrogenase (Schulze and Drevon 2005). Other legume species such as *Lupinus albus* control the O₂ diffusion in nodules during P stress by blocking the free spaces between cortical cells (Fig. 5.2) (De Lorenza et al. 1993; Iannetta et al. 1993; Schulze et al. 2006). In earlier studies, nodule O₂ conductance changes have been suggested to be regulated by variations of the intercellular spaces in the nodule inner cortex under conditions such as drought, chilling, salinity, or changes in rhizosphere O₂ concentrations (reviewed in Valentine et al. 2011; van Heerden et al. 2008; Serraj et al. 1995). The air space changes could result from occlusion of intercellular spaces and/or swelling or shrinking of the cells (Fig. 5.2). Speculations are that similar changes probably happen in the nodule under P-deficient stress. However, direct microscopic evidence is still lacking. The alterations in nodule water conductance under P deficiency have not been directly studied.

In one rare report, aquaporin transcripts were shown to increase by *in situ* hybridization in common bean (Drevon et al. 2015) under P stress. To our knowledge, independent studies on the changes in aquaporin function at transcript and protein levels under P limitation have not been performed. According to RNA-seq transcriptome profiling results of all the nodule aquaporins under P-deficient stress in *Medicago truncatula* (Cabeza et al. 2014), the majority of the nodule aquaporins are indeed up regulated, especially the very highly expressed aquaporins. Direct measurements on nodule water conductance changes under P limitation are required considering the possible indirect correlation between gene expression and protein

abundance/activity. The involvement of aquaporins poses an interesting possibility for the role of water relations as a possible mechanism of the O₂ barrier. This role of water relations is also supported by ion movements from the cortex during times of increased O₂ diffusion (Fig. 5.2).

The movement of K⁺ ions from the cortex to the central zone of nodules, such as *Glycine max*, was shown to increase the nodule permeability to O₂ (Wei and Layzell 2006). This is consistent with a mechanism of K⁺ acting in a water relations capacity within the cortex (Vessey et al. 1988; King and Layzell 1991; de Lima et al. 1994). In this regard, the removal of K⁺ from the nodule cortex can facilitate the loss of water from these cortical cells to the xylem stream. The consequence is a change in the proportion of gas (high permeability) to water (low permeability) in the cortex and thereby an increase in O₂ permeability (Fig. 5.2). In this regard, the effect of P deficiency may be linked to a decline of K⁺ influx into the cells via a ATP/ADP-dependent K-pump/channel or K⁺ efflux via an outward K-pump/channel.

A further potential physical barrier to O₂ diffusion may reside in the structure of the cell membranes of infected cells or the symbiosomes (Fig. 5.2). Recent work has shown that P-deficient nodules can reduce the phospholipid component of their cell membranes (Vardien et al. 2016) and possibly replace them with sulfolipids and galactolipids. It may therefore also be possible that the removal of membrane phospholipids during P deficiency can contribute to the increase in O₂ permeability. This is an intriguing possibility, which should be further explored.

5.5 Metabolic Adaptations to the Effect of P Stress on O₂ Permeability

The responses of nodule N₂ fixation to O₂ may also be greatly accentuated under P stress, where the consequent metabolic changes within the nodule will attenuate these responses. Le Roux et al. (2006) reported constant Pi levels as well as ADP/ATP ratios in nodules after 14 days of P stress, while in the host roots there was a decline in Pi concentrations and adenylate levels. Several other authors also reported stable nodular Pi levels indicating that nodules may function optimally at low Pi concentrations (Al Niemi et al. 1997, 1998; Kleinert et al. 2014) and that the bacteroid fraction of nodules are able to realize their own P requirements by scavenging from host cells and not readily releasing P reserves back to host roots (Al Niemi et al. 1997, 1998; Colebatch et al. 2004). BNF measured in *L. luteus* declined during P deficiency, but the decline was mostly likely due to an indirect N-feedback effect of accumulated amino acids caused by a decline in plant growth (Kleinert et al. 2014). The decline in BNF also corresponded with a reduction in root-nodule CO₂ release rates and nodule O₂ uptake rates. A declining BNF, usually an energy-intensive process, would cause a decrease in the sink strength of nodules, and the lower respiration rates under P stress is confirmation of this. Similar reductions in the respiration rates of *Phaseolus vulgaris* during decreased nodular BNF were reported by Mortimer et al. (2008, 2009).

5.5.1 *Alternative Glycolytic and Mitochondrial Electron Transport Chain Bypasses*

The increase in O_2 uptake during P stress has been linked to the increase in the permeability of the O_2 diffusion barrier (Schulze and Drevon 2005; Bargaz et al. 2011; Drevon et al. 2015). Under P stress, the limitation of P_i will reduce the ability of adenylate synthesis in nodules (Le Roux et al. 2006). However, since most of the research in area indicated that there is an increase in the O_2 uptake during P stress, this would imply that an alternative route to mitochondrial ATP phosphorylation might be in operation. This is supported by the engagement of the alternative oxidase during P stress, where a non-phosphorylating route is involved in O_2 consumption (Rychter et al. 1992).

The reduction in intracellular levels of ATP, ADP, and related nucleoside Ps which follows a drastic decline in cytoplasmic P_i levels, as experienced during prolonged P deficiency, would inhibit carbon flux through the ATP-dependent glycolytic steps (Plaxton and Podesta 2006). However, P_i -deficient plants need to generate energy as well as carbon skeletons to maintain their core metabolic processes. To this end, a cluster of at least six adenylate-independent glycolytic “bypass” enzymes have been identified in P-deficient plants (Plaxton and Podesta 2006) in addition to the inorganic pyrophosphate (PPi)-dependent H^+ -pump (H^+ -PPiase) of the tonoplast membrane (Plaxton and Tran 2011). These PSI bypasses facilitate glycolytic flux and vacuolar pH maintenance during periods of intense P_i stress when there is a decline in intracellular levels of adenylate and P_i levels (Plaxton and Tran 2011). Phosphoenolpyruvate carboxylase (PEPc) functions as the bypass enzyme together with malate dehydrogenase (MDH) and malic enzyme (ME) for the reaction catalyzed by ADP-limited cytosolic pyruvate kinase (PKc). The activity of this metabolic bypass during P_i stress when the ADP supply may be limiting for optimal PKc functioning would ensure continued pyruvate supply to the tricarboxylic acid cycle while at the same time releasing P_i back into the metabolic pool (Duff et al. 1989; Plaxton 2004). Several authors have reported an increase in PEPc activity for P_i -stressed samples compared to P_i -sufficient controls in *Brassica nigra* (Duff et al. 1989), *Brassica napus* (Nagano et al. 1994), and *Catharanthus roseus* suspension cells (Moraes and Plaxton 2000; Plaxton and Podesta 2006). Juszczuk and Rychter (2002) proposed that the increase in pyruvate synthesized via the alternative PEPc-MDH route could serve as a mechanism for oxidizing of reducing equivalents which accumulate during P stress. Schulze et al. (2006) found that P stress induced nodular enzyme activities of PEPc and MDH in *L. albus* plants. The two enzymes are central to carbon cycling and the energy substrates for N_2 fixation. Le Roux et al. (2006) reported no changes in nodular pyruvate levels synthesized from PEPc-derived malate during P deficiency, which implied that malate may have been used as a source for bacterial respiration inside the nodules due to the low nodular O_2 concentrations that would favor malate rather than pyruvate as the end product of glycolysis (Vance and Heichel 1991).

The decline in intracellular Pi and ADP levels during P deficiency will also impact on respiratory electron flow through the cytochrome pathway at sites of coupled ATP synthesis (Plaxton and Tran 2011). As with the glycolytic bypasses, the presence of nonenergy conserving pathways of mitochondrial electron transport provides a mechanism for respiratory flux to be maintained under limiting ADP and/or Pi conditions. Plants utilize the upregulation and/or increased engagement of nonenergy conserving (rotenone and/or cyanide-insensitive) pathways of the mitochondrial electron transport chain during P stress (Rychter and Mikulska 1990; Plaxton and Podesta 2006). This would allow the continued functioning of the mitochondrial citric acid cycle and electron transport chain with limited ATP production which would contribute to the survival of P-deficient plants (Plaxton and Tran 2011). The arrested growth and metabolism of P-deficient transgenic tobacco unable to synthesize a functional alternative oxidase (AOX) add weight to this idea (Sieger et al. 2005; Plaxton and Tran 2011). The lack of alternative oxidase during P stress appears to correlate with an increase in levels of proteins usually associated with oxidative stress. The continuation of respiration via the alternative oxidase which plays a role in maintaining the cellular redox and carbon balance also provides an essential adaptation whereby plant cells can control their response to Pi deficiency (Sieger et al. 2005).

5.5.2 Oxidative Stress Responses

The role of O₂ in oxidative stress is that the increase in O₂ permeability during P stress can lead O₂ being converted to reactive O₂ species (ROS). The study by Bargaz et al. (2013) is among the first to report on oxidative stress in nodules of N₂-fixing legumes during P deficiency. The limitation of P can result in imbalances in the antioxidant defense systems of plants, which lead to changes in mitochondrial membrane components and inhibition of electron transport through the cytochrome respiratory pathway (Juszczuk et al. 2001). This in turn can bring about an accumulation of ROS which can lead to oxidative stress (Bargaz et al. 2013). Plants employ antioxidative enzymes and nonenzymatic substances to act as free radical scavengers to detoxify ROS and protect cells from oxidative damage. Bargaz et al. (2013) found increased electrolyte leakage, malondialdehyde, and H₂O₂ in nodules of P-stressed plants, which all point to disturbances in cell membrane stability under P stress. Peroxidase activity was also found to be higher in P-stressed nodules than the P-sufficient treatment. This concurs with the findings of a 30% increase in peroxidase activity in *P. vulgaris* roots by Juszczuk et al. (2001). P-deficient nodules of *P. vulgaris* plants also exhibited an increase in phenol content under P deficiency (Bargaz et al. 2013). There have been reports that plants accumulating phenolic compounds are able to neutralize lipid radicals and therefore have important antioxidant properties for the protection of membranes (Bargaz et al. 2013).

5.5.3 Leghemoglobin and P Nutrition

The alteration of nodule metabolism during P-induced variations in O₂ supply represents a level of fine control which can be flexible over short or long periods of stress. In the area of control, the activity of leghemoglobin is vital because it binds to O₂. Leghemoglobin is localized within the cytosol of infected cells of the nodules and facilitates O₂ supply of the mitochondria of the bacteroids (Sherer et al. 2008). Moreover, it maintains a low-free O₂ concentration within these nodules, in order to protect the O₂-sensitive nitrogenase from irreversible inhibition by O₂ (Sherer et al. 2008, Schulze and Drevon 2005, Avenhaus et al. 2016). The short-term attenuation of the O₂ diffusion barrier by leghemoglobin is evident in a recent study of increased O₂ permeability into nodules (Fig. 5.2). It was found that the decline in N₂ was quickly followed by an upregulation in genes for nitrogenase formation. At the same time, a tightening of the O₂ diffusion barrier, presumably by leghemoglobin, reduced internal O₂ concentration and thereby protected the nitrogenase against further inhibition by O₂ (Avenhaus et al. 2016). During P deficiency, the role of leghemoglobin may become more important as the O₂ diffusion barrier is reduced during low P supply.

The interaction between P supply and leghemoglobin levels needs further investigation, since very little research has been focused on this. In one study, Miao et al. (2007) found that although P deficiency reduced N₂ fixation, it had no effect on leghemoglobin concentration in nodules of *G. max*. Interestingly, leghemoglobin may affect the levels of P compounds such as ATP. In a study on S deficiency, Sherer et al. (2008) found that reduced levels of leghemoglobin were also associated with a decline in ATP concentrations. This was attributed to the diminished leghemoglobin supply of O₂ to mitochondria, where the production of ATP was directly coupled to O₂.

5.6 Conclusions

The negative impact of excessive O₂ supply on nodule metabolism can be intensified during P deficiency. Although the precise mechanism of the O₂ inhibition has to date remained unclear, the exacerbated effects during P stress are even more elusive. Nonetheless, the adaptations to overcome this O₂ inhibition are known to be regulated at the structural and metabolic levels within nodules. Currently some of these mechanisms have been elucidated separately, but it is very likely that functional adaptation operates in a more integrated system. These integrated mechanisms may be the key to understanding the adaptations to P-induced O₂ stress. Clearly they need to be further explored with the modern tools of functional genomics, but in combination with classical approaches such as anatomy, physiology, and biochemistry.

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