

Physiological Responses of Legume Nodules to Drought

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ABSTRACT

Legumes include important agricultural crops, as their high protein content is of primary importance for human food and animal feed. In addition, the ability of most of them to establish symbiotic relationships with soil bacteria allows them to obtain their N requirements from nitrogen fixation in nodules and, therefore, avoids the use of nitrogen fertilizers. Thus, legumes are also essential to improve the soil fertility and quality of agricultural lands and to reclaim eroded or barren areas, making them crucial for agricultural and environmental sustainability. However, legume nitrogen fixation in crop species is very sensitive to environmental constraints and drought, in particular. The present contribution reviews our current knowledge on the processes involved in this inhibition, with particular emphasis on oxygen, nitrogen and carbon physiology. Emerging aspects such as oxidative damage, C/N interactions and sulphur metabolism together with future prospects are also discussed.

Keywords: carbon flux, C/N interactions, environmental physiology, nitrogen fixation, sucrose synthase, water stress

Abbreviations: BNF, biological nitrogen fixation; Lb, leghemoglobin; NO, nitric oxide; ODB, oxygen diffusion barrier; PEP, phosphoenolpyruvate; ROS, reactive oxygen species; SS, sucrose synthase

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AN INTRODUCTION TO DROUGHT AND NITROGEN FIXATION

Crop yields are more dependent on an adequate supply of water than on any other single environmental factor (Kramer and Boyer 1997). Even plants with an optimum water supply experience transient periods of water shortage, where water absorption cannot compensate for water loss by transpiration, a situation that largely depends on environmental factors such as temperature, relative humidity and wind speed. In addition, many other environmental stresses, such as cold, salt and high temperature, have a water-stress component. However, as Passioura (2002) pointed out, drought may have very different connotations and time-scales for different practitioners, the latter ranging from years to decades for meteorologists, insurers and farmers, to a few hours for molecular biologists (with agronomists and crop and plant physiologists somewhere in between in a timeframe of days to weeks, and most rarely months). The real significance of drought can also be quite different depending on the level of study. While most molecular studies generally focus on survival from severe shocks

(for example, drying on filter paper or application of a osmoticum), in a viable agriculture, other than subsistence conditions where survival may represent an added value, reduction of yield is the main focus and this generally develops in a more gradual, slow-acting way (Passioura 2002; Purcell 2009). The framework of this contribution is confined to the above-mentioned “physiological” time-scale of days.

The sequence of events that occurs when water stress develops starts with cellular growth as the most sensitive response, followed by a wide range of biochemical and physiological events, the more negative the water potential becomes (Hsiao 1973). Events occurring later on during the water stress period or at very negative water potential are often indirect responses to earlier events rather than direct responses to water stress itself. This picture is further complicated because the sensitivity of some responses is highly dependent on the plant species. Intense research on the molecular basis of plant responses to drought has been carried out over the last years, but this is outside the scope of this review and can be found elsewhere (Chinnusamy *et al.* 2004; Zhang *et al.* 2004; Bartels and Sunkar 2005;

Vinocur and Altman 2005; Bohnert *et al.* 2006; Maggio *et al.* 2006; Valliyodan and Nguyen 2006; Yamaguchi-Shinozaki and Shinozaki 2006; McDowell *et al.* 2008; Morison *et al.* 2008; Neumann 2008; Lawlor and Tezara 2009).

Biological nitrogen fixation (BNF) in legumes occurs in symbiosis with prokaryotes originally classified in the genus *Rhizobium*. Although these organisms are still designated by the common name rhizobia, according to recent molecular studies, the list of rhizobia actually comprises 76 species found in 13 genera belonging to the α - and β - subgroups of the proteobacteria (Vinueza 2007; Weir 2008), some of which may have arisen through lateral transfer of symbiotic genes. This situation suggests that the ability to fix N_2 in symbiosis with legumes could be much more widespread than previously believed.

Although there are several reviews on different aspects of the effect of drought on nodule performance (Serraj *et al.* 1999a; Zahran 1999; Schulze 2004; Purcell 2009), BNF is commonly overlooked in most general overviews on water stress effects on plant functioning, because of its restriction to leguminous plants or non-leguminous symbioses with *Frankia* bacteria. Although some authors (Frechilla *et al.* 1999, 2000; Kirova *et al.* 2008) have pointed out that the different products of BNF and nitrate assimilation may have a differential effect on photorespiratory nitrogen metabolism which might be enhanced under drought, these suggestions have yet to be further explored. Therefore, most responses of legumes to drought other than those associated with BNF are not significantly different from other plant families. Thus, the present contribution is focused on the effects on legume nodules.

The classical view of the inhibition of BNF by drought has been ascribed to a lack of carbohydrate supply to the nodules, resulting from a reduction in shoot photosynthesis (Huang *et al.* 1975). In the long-term, it is evident that a lack of photoassimilates will contribute to a decline in BNF, and it is quite unlikely that the latter will recover upon rehydration without a restoration of photosynthetic rates. However, it has been shown that BNF is more sensitive than photosynthesis to moderate water deprivation. BNF decreases steadily throughout the water deficiency period whilst photosynthesis decreases only slightly during the first days under drought (Durand *et al.* 1987; Djekoun and Planchon 1991). This higher sensitivity of BNF to water stress compared to photosynthetic carbon assimilation has led to a widespread assumption that BNF is "very sensitive to water stress" and this statement is further sustained when BNF is compared to nitrate assimilation. However, direct comparisons between both processes are scarce, with information obtained using methods not considered reliable nowadays in view of the *in vivo* regulation of both nitrogen assimilation pathways (Obaton *et al.* 1982). Under field conditions, the addition of N fertilizers to soybean (*Glycine max* (L.) Merr.) plants partly ameliorates the effect of drought compared to plants depending primarily on BNF (Purcell and King 1996). However, recent reports have suggested that N_2 -fixing plants of alfalfa (*Medicago sativa* L.; Antolín *et al.* 1995), common bean (*Phaseolus vulgaris* L.; Lodeiro *et al.* 2000), pea (*Pisum sativum* L.; Frechilla *et al.* 2000), and soybean (Kirova *et al.* 2008) can be more tolerant to mild drought than nitrate-reducing plants, at least under controlled conditions. Obviously, these comparisons largely depend on the genetic capability of plants in terms of nitrate reduction and the extent of the adverse effect of drought not only on BNF, but also on nitrate transport and nitrate reduction in the particular species (Salvagiotti *et al.* 2008).

Furthermore, it should be noted that the negative effect of drought on BNF is the sum of three different responses: effects on the infection of legumes by rhizobia, effects on nodule growth and development and, finally, direct effects on nodule functioning. Assessment of the latter is further complicated due to the fact that a large number of studies have been performed with the traditional closed acetylene reduction assay, which has been shown to be unreliable for

assessing absolute rates of BNF (Minchin *et al.* 1983, 1986). It can be argued that the technique may still be valid for comparative purposes (control versus water-stressed plants). However, this hypothesis has not been experimentally proven and, at least theoretically, would be wrong if nodules subjected to different environmental conditions displayed significantly different resistances to oxygen diffusion (Minchin *et al.* 1986). For example, it has been shown that specific BNF in pea under high CO_2 appeared to be 2.5-times higher, according to the standard acetylene reduction assay, whereas a more accurate method of BNF measurements, based on H_2 evolution in a symbiosis lacking hydrogenase activity, showed that specific BNF rates were the same in both ambient and high- CO_2 concentrations (Cabrerizo *et al.* 2001).

THE METABOLIC REGULATION OF NODULE NITROGEN FIXATION UNDER DROUGHT

The role of the bacterial strain in the response of nodulated legumes to drought

In legume nodules, BNF is carried out by differentiated forms of rhizobia, termed bacteroids, within specialised structures, called symbiosomes, inside the plant host cells (Roth *et al.* 1988). As such, the main metabolic factors that regulate BNF are not expected to differ much from those widely studied controlling the nitrogenase of free-living diazotrophs: (a) the occurrence of an energy-yielding substrate, (b) an adequate oxygen balance, allowing optimal use of energy-yielding substrates whilst preventing damage to the oxygen sensitive nitrogenase complex, and (c) the maintenance of an adequate nitrogen status, as free-living prokaryotes switch off the energy-demanding BNF if nitrogen is plentiful (Merrick 2005). However, it should be noted that, conversely to the situation of free-living diazotrophs, the perception of environmental constraints in the legume-rhizobial symbiosis is carried out by the host plant and not by the N_2 -fixing bacteroids.

There are several reports of genetically modified rhizobial strains being more efficient in BNF under optimal conditions compared to their parental strains (Yurgel *et al.* 1998; Soberón *et al.* 1999; Marroquí *et al.* 2001). Unfortunately, most of these reports are incomplete and there are no integrated projects that have evaluated the overall plants' performance in terms of photosynthetic capacity and carbon allocation to nodules and/or assessment of their BNF capacity. Once the efficiency of a particular strain has been assessed, a further problem that needs to be overcome is competition with native rhizobia, which are usually better competitors for nodule occupancy compared to the rhizobial strain applied in the inoculum (Dowling and Broughton 1986).

The bacterial partner is, in general, much more tolerant to abiotic stresses than its respective plant host, although diversity in the response of different strains to abiotic stresses is widely acknowledged (Zahran 1999). There are many reports of strains displaying salt or osmotic stress tolerance under free-living conditions. However, the rationale for using the tolerance to environmental constraints of a free-living organism as a test for fitness in a symbiosis is not evident, and it remains to be demonstrated that a rhizobial strain tolerant to drought actually confers this property to the symbiosis. Furthermore, for field experiences, unless the efficiency and the benefits of introducing allochthonous strains can guarantee an improvement in plant performance, such inoculations might be ecologically unsustainable and even detrimental (Castro-Sowinski *et al.* 2007), although their ecological impact is mostly of times limited because of their lack of competitiveness compared to native populations.

Another approach is the overexpression of a bacterial feature that may enhance the plants responses to drought. Thus, Suárez *et al.* (2008) reported that improvement of drought tolerance and grain yield in common bean by over-

expression of trehalose-6-phosphate synthase in rhizobia. Trehalose, and its closely related metabolites, is known to be involved in osmoprotection (as opposed to osmoregulation) which confers drought tolerance (Garg *et al.* 2002). Unfortunately, this tolerance seems to be related, in most cases, with improving survival to terminal drought, rather than reducing the adverse physiological processes involved in early or temporary drought. Some influence of the bacterial partner has been recently reported in the *Medicago truncatula*-*Sinorhizobium meliloti* symbiosis (Mhadhbi *et al.* 2009), although it remains to be determined whether this behaviour can be extended to other symbioses in view of the characteristic features of this symbiosis (see section on *Medicago* species). Thus, although the bacterial partner may have a role in the response of the symbiosis to drought, this seems to be rather limited compared to the nodule plant responses. Not surprisingly most of the physiological control involving the plant also depends, as in prokaryotes, on an adequate coordination of oxygen, nitrogen and carbon fluxes.

The role of oxygen

Oxygen is critical for BNF since most nitrogenases are very sensitive to its presence, being irreversibly inhibited within a very short time (seconds to minutes, Hill 1988; Burris 1991). Therefore, legume nodules need to maintain a micro-aerobic environment within their central, bacteroid-containing zone. However, BNF requires a large energy supply and, although the plant shoot may provide carbon in excess via the phloem, an anaerobic metabolism would not provide enough energy to support BNF. This situation is resolved by the presence of leghemoglobin (Lb), which maintains a very low oxygen concentration within the infected cells (~50–100 nM), while providing a high flux of oxygen to the high affinity cytochromes of the bacteroids. In addition, this oxygen flux must be balanced against carbohydrate availability to the bacteroids and is maintained by a variable oxygen diffusion barrier (ODB). The ODB was discovered in nodules following pioneering microelectrodes work (Tjepkema and Yocum 1974; Witty *et al.* 1987) coupled with physiological studies, involving simultaneous measurements of BNF (using acetylene reduction or hydrogen production in a flow-through system), oxygen interchange (as CO₂ production) and calculations of oxygen diffusion resistance using mathematical models based on Fick's first law of diffusion (Minchin *et al.* 2008). The responses of nodule functioning to most environmental constraints, including defoliation, darkness, heat stress, phosphate deficiency, nitrate supply (which is not a stress for the whole plant, but severely impairs BNF), salt stress and drought can be related to the operation of the ODB: a closure of the ODB represents a decrease in oxygen availability for bacteroids and, therefore, a lack of energy to support the highly demanding BNF. Indeed, it has been suggested that decreased oxygen permeability is a universal stress response in legume root nodules and the key factor in the inhibition of BNF (Denison 1998). If this was indeed the only causative factor then the inhibition of BNF caused by drought should be completely reversible by increasing the external oxygen availability. However, Hunt and Layzell (1993) and Diaz del Castillo *et al.* (1994) found that most of the reduction in BNF under drought could not be restored by raising the external oxygen concentration. This implies that nodules of water-stressed plants no longer had the metabolic capacity to support optimal levels of BNF, even in the presence of sufficient oxygen. It is evident that a decreased oxygen flux in nodules is critical in the nodule responses to environmental constraints and, in particular, to drought, but further metabolic constraints must be involved. Nitrogen and carbon are the obvious candidates.

Nitrogen status and feedback inhibition of BNF

As in free-living diazotrophs, it has been suggested that the nitrogen status of the plant may be regulating nodule BNF. This is largely based on indirect evidence (Oti-Boateng and Silsbury 1993; Parsons *et al.* 1993; Hartwig 1998) including known facts such as the inhibition of BNF by inorganic nitrogen (Streeter 1988). Several molecules have been suggested to act as the "signal molecule" to link "nitrogen status" and the inhibition of BNF (presumably by N feedback) following experiments with external supplies of NH₄⁺ to the leaves, applying different molecules to the root system or amino acids analyses following detopping. These include ureides (Atkins *et al.* 1992; Serraj *et al.* 1999b; Vadez *et al.* 2000), glutamine (Neo and Layzell 1997), asparagine (Bacanamwo and Harper 1997; Vadez *et al.* 2000), and the ratio of glutamic acid/glutamine (Curioni *et al.* 1999) or aspartic acid/asparagine (Lima and Sodek 2003).

The N feedback hypothesis has also been proposed to be involved in the regulation of BNF under drought conditions with several candidate N molecules suggested to act as a signal molecule (King and Purcell 2005). Moreover, it has been reported that BNF is a more drought-sensitive process in ureide-exporting nodules, such as those on soybean and common bean, where allantoin and allantoate are the main nitrogenous compounds exported, than in the amide-exporters, such as those on pea, lupin (*Lupinus albus* L.), faba-bean (*Vicia faba* L.) and chickpea (*Cicer arietinum* L.) (Sinclair and Serraj 1995). Most of the research carried out so far on the possible mechanisms involved in the inhibition of BNF by drought has been made in soybean plants, which is a ureide exporter. The involvement of shoot ureides in BNF is supported by studies showing that a supply of external ureides to soybean plants increased the concentration in leaves and inhibited nitrogenase activity (Serraj *et al.* 1999b; Vadez *et al.* 2000). Also, Vadez and Sinclair (2001) reported an inverse relationship between shoot ureide concentrations in well-watered plants and drought sensitivity of BNF among soybean cultivars. Furthermore, drought tolerance in soybean has been associated with concentrations of ureides in shoots, with sensitive lines showing a higher accumulation compared to tolerant lines (Serraj and Sinclair 1996; Purcell *et al.* 1998). This differential accumulation of ureides was associated with distinct ureide catabolism pathways in leaves (Vadez and Sinclair 2002), supporting the idea of a systemic regulation of BNF. However, Todd and Polacco (2004) have suggested that soybean cultivars with contrasting tolerance to drought have the same enzymatic pathway for allantoate degradation in shoots. Recently, Charlson *et al.* (2009) showed that allantoate amidohydrolase expression is not associated with the increased ureide catabolism observed in drought-tolerant genotypes.

A possible role of increased nodular N content (ureides or N compounds in general) in the regulation of soybean BNF has received much less attention, despite the fact that Minchin and Pate (1974) recorded an increase in the soluble amino acid content of pea nodules under low transpiration conditions more than 30 years ago and that amino acid accumulation has been shown in nodules of alfalfa plants subjected to salt stress (Fougère *et al.* 1991). A recent comparative study on nodule ureide content profiles between drought-tolerant and -sensitive cultivars showed that nodular ureide accumulation mirrored the decline in BNF (King and Purcell 2005). Moreover, Ladrera *et al.* (2007a) showed that, in the short-term, an accumulation of ureides in leaves of tolerant and sensitive soybean lines could not be detected despite the fact that BNF was already inhibited. This strongly suggests that leaf ureides are not involved in the early stages of BNF inhibition under drought, although a role in the later stages of a more severe drought (Serraj *et al.* 1999b) can not be discounted. Furthermore, Ladrera *et al.* (2007a) showed genotypic variation in nodule accumulation of ureides under drought, which was closely correlated with levels of BNF inhibition. Therefore, it was concluded that ureides, along with other N-compounds, are potential candi-

dates for inducing a nodule-based feedback inhibition of BNF. In this localised, as opposed to shoot-based, BNF inhibition it would appear that export from nodules is impaired, resulting in the accumulation of N compounds.

This also opens the question as to whether nodular transport is driven by transpiration or by translocation from shoots. Under conditions of non-limiting water availability, it is generally assumed that most of the water transport to nodules takes place via the phloem, and therefore, the export of N products from the nodules through the xylem is dependent on this water influx from the phloem (Streeter 1993; Walsh 1995; Serraj *et al.* 1999a). However, using split-root systems, where each half-root was either water-deprived or irrigated at field capacity, nodule water potential was remarkably different between the two half-roots, even though there was no difference between the water potential of control and treated shoots (Marino *et al.* 2007a). Indeed, in the water-deprived split-root system, a decline in BNF was monitored before any decrease in shoot transpiration rate (Gil-Quintana *et al.* 2009). However, BNF can be modulated by changes in vapour pressure deficit under conditions in which plants have ample water availability (Arrese-Igor and Sinclair, unpublished results).

Based on all these considerations, it is tempting to suggest that BNF can be directly disrupted by a drought-induced accumulation of N-compounds, provoked by an inhibition of nodular export. Furthermore, the possibility of a localised control is also in agreement with studies using split-root systems, where inhibition of BNF under drought only occurred in the half-roots that were water-deprived, whilst BNF of the half-roots irrigated at field capacity was maintained at control values (Marino *et al.* 2007a).

However, there is no evidence for nodule ureides being the actual compounds that induce the decrease in BNF. In fact, as pointed out by Sprent (2001), it is unlikely that ureides are a general signal among leguminous plants, since amides are the main nitrogenous export product of nodules, with ureide export being the exception. This leads to the crucial question as to which is the "signal molecule" which accumulates in legume nodules to invoke the N-feedback mechanism. As already pointed out, free-living prokaryotes have a very stringent control of the cell nitrogen status (*ntr* genes), so as to maximise the physiological benefits from diazotrophy. This nitrogen control involves members of the PII protein family, such as GlnB, GlnK, and NifI, which play a pivotal role in nearly all organisms (Merrick 2005). Indeed, PII is now recognised as the critical signal-transduction protein in numerous aspects of bacterial nitrogen metabolism (Arcondéguy *et al.* 2001). The situation in nodules is far from being clearly ascertained and, in the classical model of a simple, cross membrane, interchange of carbon (malate) from the host-plant cytosol for NH_4^+ coming from bacteroids, it is difficult to foresee how the plant nitrogen status might have a direct effect on nitrogenase activity. Nevertheless, Lodwig *et al.* (2003) have suggested that a complex amino-acid cycling occurs in nodules, in which the plant provides amino acids to the bacteroids, enabling them to shut down their ammonium assimilation, so bacteroids can thus be considered as plant organelles to cycle amino acids for asparagine synthesis. It is tempting, although not yet experimentally proven, to suggest that this exchange could be directly disrupted by a drought-induced accumulation of N-compounds, provoked by an inhibition of nodular export.

The involvement of carbon metabolism in nodules

Carbon metabolism in nodules, recently reviewed by Vance (2008), has been largely neglected in relation to the importance of BNF to N supply to the plant and the ecosystem. This is rather surprising as nodule BNF depends on the supply of sucrose delivered from the host plant phloem, a similar situation to other sink tissues in plants (Avigad 1982; Hawker 1985). However, the fact that BNF declines before any significant change in photosynthesis under drought

seemed to dismiss any relevant role of carbohydrate metabolism in drought stressed nodules. It is known that sucrose can be hydrolysed by either sucrose synthase (SS) or alkaline invertase, and that nodules use the glycolytic pathway to provide energy and carbon skeletons for bacteroid respiration and ammonia assimilation. Based on enzyme kinetic data, Morell and Copeland (1984, 1985) concluded that alkaline invertase in nodules had a predominant role in providing hexoses from sucrose, relating SS activity to the production of UDP-glucose for the formation of other nucleotide sugars and polysaccharides, such as starch and cellulose.

However, González *et al.* (1995) reported a strong decline in SS activity following drought in soybean nodules, with a subsequent potential decrease in the glycolytic flux. The same observation was also reported in pea (González *et al.* 1998) and common bean nodules (Ramos *et al.* 1999). This was coincident with work with pea mutants, which showed that SS is essential for normal nodule development and function: plants with this low level of SS (10% of wild-type SS activity) were unable to fix N_2 (Craig *et al.* 1999; Gordon *et al.* 1999). Using an antisense approach to knock down the nodule-enhanced SS, a similar conclusion was recently reached for the model legume *M. truncatula* (Baier *et al.* 2007), whilst in *Lotus japonicus*, since the predominant SS form (*LjSUS3*) only accounts for 60% of the total nodule SS transcripts, only the double mutant in the two most abundant SS isoforms showed severely impaired BNF (Horst *et al.* 2007). It is now clearly established that there is a strong correlation between drought, nodule SS activity and BNF for a number of grain legumes (Arrese-Igor *et al.* 1999). Furthermore, it has been shown that a decline in SS content and activity leads to a dramatic decrease in nodule malate content, which induces an impairment of bacteroid functioning, including BNF (González *et al.* 2001b; Gálvez *et al.* 2005). Malate deprivation under drought could also be due to the impairment of HCO_3^- binding to phosphoenolpyruvate (PEP) to form oxaloacetate and, subsequently, malate. Although both carbonic anhydrase (Coba de la Peña *et al.* 1997; Gálvez *et al.* 2000) and PEP carboxylase (Schulze 2004; Nomura *et al.* 2006) have been shown to be essential for nodule performance, it is still to be shown that these enzyme activities are affected or play any significant role in BNF regulation under drought.

Additional evidence for the involvement of carbon metabolism within nodules in the response to drought comes from the fact that lines showing contrasting tolerance also display different responses in their nodule SS behaviour, in both soybean (Ladrera *et al.* 2007a) and common bean (Sassi *et al.* 2008). Finally, new proteomic approaches have further confirmed the involvement of SS in response to drought (Larrainzar *et al.* 2007).

In this way, when an environmental stress, particularly drought, severely impairs the overall plant performance, this strict dependence on SS activity in nodules would ensure a decrease of nodular sink strength and prevents nodules from becoming an excessive burden for the plant, even prior to any detectable changes in photosynthesis. This scenario has been suggested as an evolutionary mechanism developed to aid plant survival under stress (Arrese-Igor *et al.* 1999; Marino *et al.* 2008). In addition, the impairment of sucrose cleavage, coincident with a sustained sucrose flux from the shoots, leads to the accumulation of sucrose in nodules. Although these concentrations are not high enough to guarantee any osmotic adjustment, they may be sufficient to provide osmoprotection against damage caused by reactive oxygen species (ROS) to proteins and membranes (Cuin and Shabala 2005, 2007).

THE RESPONSE TO DROUGHT IN MEDICAGO SPECIES

Recent experiments with alfalfa showed that exposure of plants to moderate drought had no effect on SS activity, but caused inhibition of nitrogenase activity, and up-regulation

of genes encoding most of the enzymatic antioxidant defences. Moderate drought also decreased the content of MoFe- and Fe-nitrogenase proteins in bacteroids (Naya *et al.* 2007). This decrease in nitrogenase proteins, the accumulation of respiratory substrates and the oxidation of lipids and proteins, and the up-regulation of antioxidant genes showed that bacteroids have their respiratory activity impaired and that oxidative stress occurs in nodules under drought conditions prior to any detectable effect on SS or Lb. Intensification of stress led to further decreases in nitrogenase activity, a decline in SS activity and an increase in malate, succinate, and sucrose content. Thus, the metabolic capacity of bacteroids and oxidative damage of cellular components are contributory factors to the drought-induced inhibition of BNF in alfalfa nodules (Naya *et al.* 2007). More recently, it has been shown that the response to salt stress of BNF in *M. ciliaris* lines with contrasting tolerance does not show such a clear trend in relation to nodule carbohydrate metabolism (Ben-Sala *et al.* 2009). Furthermore, lines of *M. truncatula*, selected on the basis of a contrasting tolerance to salt, seem to display a response to drought that does not directly involve the decline of SS (Ladrera *et al.* 2007b). Although a proteomic analysis of the response of *M. truncatula* to drought revealed that the decline of SS is one of the most remarkable changes in the plant fraction of nodules (Larrainzar *et al.* 2007), a plant-system approach involving the proteome and metabolome responses of *M. truncatula* nodules to drought showed that the decline in SS content was not correlated with a decrease in malate concentration (Larrainzar *et al.* 2009), in contrast to reports for nodules of grain legumes (González *et al.* 2001b; Gálvez *et al.* 2005). This relatively contrasting behaviour of *Medicago* species to other legumes is intriguing. One possible explanation is that, in pasture legumes such as *Medicago*, grazing has produced a strong evolutionary selective pressure on continued nodular SS activity during drought stress. Although this is a theoretical proposal, this hypothesis would make sense in terms of nodule regrowth/reactivation following grazing, since SS activity is required to maintain a supply of reductants for the regulation of oxidative damage during stress and it could also explain why nodules of grain legumes show rapid signs of stress-induced senescence under nitrate, whilst alfalfa nodules do not.

The possibility of the medics displaying a substantially different response to drought compared to other relevant, cultivated plants raises a big question as to how conclusions derived from studies on the response of *M. truncatula* to drought can be widely generalised to other plant species. Indeed, although some authors are very enthusiastic in terms of applying the advances obtained by means of -omics technologies from *M. truncatula* to crop legumes (Young and Udvardi 2009), others have recently raised some doubts in this regard (Moreau *et al.* 2008; Sprent and James 2008; Terpolilli *et al.* 2008).

OTHER FACTORS INFLUENCING BNF UNDER DROUGHT

Oxidative damage in nodules can be induced by environmental constraints (Gogorcena *et al.* 1995, 1997; Matoros *et al.* 1999) and this causes a detrimental effect on nodule performance and BNF. In alfalfa nodules, this seems to be of major importance (Naya *et al.* 2007), but, in other species, it is yet to be shown whether oxidative stress is a primary response or it occurs later on in the drought stress response, corresponding to a late, irreversible stage of nodule senescence. Although the external supply of antioxidant ascorbate fails to revert the drought-induced oxidative damage (Zabalza *et al.* 2008), recent reports suggest that nodules having an increased enzymatic antioxidant defence can display a higher tolerance to osmotic/salt stress in common bean (Sassi *et al.* 2008) and chickpea (Kaur *et al.* 2009). In addition, Verdoy *et al.* (2006) have reported increased tolerance to osmotic stress in *M. truncatula* by overexpression of Δ -pyrroline-5-carboxylate synthetase,

leading to the accumulation of high proline levels. As with trehalose in bacteroids, it is unlikely that proline contributes to osmotic adjustment, but may be involved into some yet unknown osmoprotection. Furthermore, Redondo *et al.* (2009) showed that overexpression of a flavodoxin in bacteroids induces changes in antioxidant metabolism leading to delayed senescence in alfalfa root nodules. This may be relevant since, in the *Medicago* symbiosis, oxidative stress on nitrogenase activity and protein content seems to be one of the earliest drought effects (Naya *et al.* 2007). However, it remains to be determined whether this is so in other symbioses, where C and N regulation seems to precede oxidative stress. Despite these promising results, it remains to be tested whether these symbioses actually outperform their wild parents under drought, with BNF being measured using unbiased, reliable methods.

Studies on C/N interactions are common place in most physiological approaches to plant performance, particularly under non-optimal conditions. However an integrated approach to C/N interactions in nodules is still lacking. There is recent evidence of cytosolic isocitrate dehydrogenase, a major component in the C/N balance, playing an important role in nodules in response to carbon shortage (Gálvez *et al.* 2005) or oxidative stress (Marino *et al.* 2007b). Also, a statistical analysis of the various components involved in the response of nodules to drought showed that the malate/ureide ratio had a better correlation with BNF than malate or ureide content alone (Ladrera *et al.* 2007a). How such a C/N balance might control BNF has yet to be addressed.

Recent proteome analysis of the plant fraction from nodules of *M. truncatula* provided a novel evidence of drought effects on the enzyme Met synthase (Larrainzar *et al.* 2007). Decreased Met availability would have a major effect on both protein synthesis and sulphur metabolism in nodules. The latter is a largely unexplored field, except for the evidence of glutathione involvement in the antioxidant defence system and of S-adenosyl-Met in ethylene biosynthesis. Certainly, more information is needed regarding the involvement of nodule sulphur metabolism in the response to drought.

SIGNALS INVOLVED IN THE INHIBITION OF BNF UNDER DROUGHT

The pathway linking stress perception by plants to the inhibition of BNF is a rather unexplored field. Whether signals are derived from systemic communication, are self-contained in nodules or respond to bacteroid perception of a changing environment remains unknown. As discussed above, the situation in nodules largely differs from the one free-living diazotrophs may face in an open environment. Recent experiments showed that the SS-mediated decline of BNF under drought does not involve systemic signals, such as abscisic acid (González *et al.* 2001a), and using split-root systems, it has been shown that this response has a local origin (Marino *et al.* 2007a). An earlier attempt was made to integrate the various proposed components of the ODB, termed the "rolling wave" hypothesis (Minchin 1997), suggested the involvement of H₂O₂ as an early signal molecule following environmental changes. In view of the above mentioned regulation of carbon flux in nodules by the cell redox state it is likely that common signals might affect carbon flux and the ODB simultaneously. Thus, ROS seem to play a crucial role in oxidative signalling during drought stress, as the physiological nodular responses can be mimicked using a local application of a ROS-generator, such as paraquat (Marino *et al.* 2006), and ROS also affect SS expression at both the transcriptional and post-translational levels (Marino *et al.* 2008). Redox signals are now recognized as having a crucial role in establishing and maintaining symbiosis between rhizobia and legumes (Chang *et al.* 2009; Marino *et al.* 2009). It is important to note that this oxidative signalling would occur in nodules prior to any oxidative damage to proteins or lipids.

Nitric oxide (NO) has recently gained interest as a

major signaling molecule during plant development and response to environmental changes (Delledonne *et al.* 1998). Its role is crucial for plant-pathogen interactions, participating in the control of plant defense response and resistance. The presence of NO in legume nodules was first reported by Maskall *et al.* (1977) based on the identification of Lb-NO complexes. Subsequent studies have reported the presence of NO using fluorescent dyes and confocal microscopy in *M. truncatula* nodules (Baudouin *et al.* 2006) and a wide modulation of NO-related genes was detected during nodule formation (Ferrari *et al.* 2008). NO production has been also detected in functional nodules, being localized in the bacteroid-containing cells of the nodule fixation zone. The location of such a production in fully metabolically active cells raises the hypothesis of a new function for NO in this symbiosis (Baudouin *et al.* 2006).

Although abscisic acid does not appear to be involved in the decline of SS, it was shown that it might detrimentally affect the Lb/O₂ diffusion regulation of BNF (González *et al.* 2001a), leading to a decrease in BNF. Lb can also be a target for oxidative signalling at higher ROS concentrations than those required for SS inhibition (Marino *et al.* 2006). Obviously, this is an emerging area that should provide further insights into nodular stress responses.

FUTURE PROSPECTS

Recent technologies such as genomics, proteomics and metabolomics may facilitate understanding of the regulation of BNF under drought. However, they should be applied to gain new insights, and not just in a descriptive manner, so as to create a system approach for a full understanding of nodule functioning. In this sense, the recent description of a gene expression atlas of the model legume *M. truncatula* represents an unparalleled resource for legume functional genomics, which, hopefully, may accelerate discoveries in this field (Benedito *et al.* 2008). Another important issue is the recent possibility of absolute quantification of nodule proteins (Wienkoop *et al.* 2008), which can be of interest in relation to changes triggered by drought or any other stress. However, it should be noted that, because of the abundant post-translational modification of proteins, the information obtained from these approaches needs to be complemented by other techniques in order to determine precisely its actual physiological relevance.

Another issue is that most of our current knowledge of metabolic pathways and their regulation derives from information obtained “*in vitro*” using optimal conditions for enzyme activity that are far from those in living cells. A first approach to circumvent this limitation can be made in coordinating data from enzyme activities and metabolite availability. The development of metabolic flux analysis coupled to metabolomics in order to obtain metabolite flux networks would be helpful to our understanding and to potentially engineer the nodular systems in a rational manner.

Finally, progress in our understanding of the effects of drought on BNF, which is essential for improving legume yield and quality under limiting conditions, may require the scientific will to integrate data at very different levels, using interdisciplinary approaches. However, this can only be achieved with the extended commitment from funding agencies at the national and international levels to provide the appropriate conditions for this research to progress and produce useful results in this critical field for human food, animal feed and sustainable agriculture.

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