

## LEGUME NITROGEN UTILISATION UNDER DROUGHT STRESS

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### Summary

Legumes, account for around 27% of the world's primary crop production and can be classified based on their use and traits into grain and forage legumes. Legumes can establish symbiosis with N-fixing soil bacteria. As a result, a new organ is formed, the nodule, where the reduction of atmospheric N<sub>2</sub> into ammonia is carried out catalyzed by the bacterial exclusive enzyme nitrogenase. The process, highly energy demanding, is known as symbiotic nitrogen fixation and provides all the N needs of the plant, thus avoiding the use of N fertilizers in the context of sustainable agriculture. However, legume crops are often grown under non-fixing conditions since legume nodulation is suppressed by high levels of soil nitrogen occurring in chemically fertilized agro-environment. In addition, legumes are very sensitive to environmental stresses, being drought one of the significant constraints affecting crop production. Due to their agricultural and economic importance, scientists have carried out basic and applied research on legumes to

better understand responses to abiotic stresses and to further comprehend plant-microbe interactions. An integrated view of nitrogen utilization under drought stress will be presented with particular focus on legume crops.

### **1. Drought stress**

Climate change is multi-faceted and includes changing concentrations of greenhouse gases in the atmosphere (like CO<sub>2</sub>), rising temperatures, changes in precipitation patterns, and increasing frequency of extreme weather events (Gray and Brady, 2016). Thus, Earth's climate is drastically changing leading to more intense and extended drought situations. Indeed, the area affected by drought has increased substantially since the middle 20<sup>th</sup> century (Dai, 2011), and the frequency of droughts is predicted to increase in regions that are already dry by the end of the 21<sup>st</sup> century. Drought observatories have estimated that around 40% of the land area is affected by drought and has an expectative in expansion due to the global climate change (Trenberth et al., 2013). It has been estimated that two-thirds of the potential yield of major crops are usually lost due to adverse growing

environments (Bajaj et al., 1999; Daryanto et al., 2016). Abiotic stresses, above all water deficit, are the most important factors limiting crop productivity, with a growing importance due to the increase in climate alterations such as reduced rainfall (Lesk et al., 2016). Indeed, most climate change studies predict an increase in arid areas worldwide (Shu et al., 2007), aggravated by the rapidly increasing world population, which puts pressure on food and water demands (Somerville and Briscoe, 2001). This problem not only arises from the limiting nature of water supplies, but also from the increased need for food production, which leads to an improper management of agricultural lands. For example, most crops are cultivated in lands and regions to which they are not optimally adapted, yielding up to 22% of their genetic potential due to improper climatic and soil conditions (Boyer, 1982). Therefore, the understanding of plant drought stress tolerance has become an urgent matter, since it can allow us a better management and to minimize its harmful effects on crops.

Drought is defined as "the decrease in water inputs into an agro/ecosystem over time that is sufficient to result in soil water

deficit (i.e., decrease in the available soil water)" (Gilbert and Medina, 2016). Therefore, drought is a condition of climatic dryness severe enough to reduce soil moisture and water below the minimums necessary for sustaining plant, animal and human life (Perez and Thompson, 1996). This stress interferes with the optimal plant growth, physiology, and reproduction, ultimately causing a significant reduction in plant productivity (Farooq et al., 2009). Water deficit can be defined as any water content of a tissue or cell below the highest water content exhibited in the most hydrated state. Although the terms "drought stress" and "water-deficit stress" are usually employed indistinctively, water does not only become limiting for plant communities as a result of inadequate rainfall but also due to other environmental conditions like excessive salinity in the soil solution or as a consequence of freezing temperatures. In this work, the term drought stress will be used referring to periods where water is withheld from the plant.

## **2. Drought is a major threat to legumes crops**

Grain and forage legumes are grown on around 15% of the arable surface of the Earth, being the second most important crop after cereals attending to world first crop production (FAOSTAT; Graham and Vance, 2003). The economic relevance of legume crops is related to both their importance as a protein source for animal feed and human nutrition and their use as raw material in the industry (Edgerton et al., 2008). Common bean, soybean, chickpea, pea and faba bean are some of the most widely cultivated grain legumes, while cowpea, pigeon pea, lentils and grass pea play an essential nutritional role in low-income regions of the world. Regarding forage legumes, plants in the *Medicago*, *Trifolium*, and *Lotus* genera are probably the most extended legumes for livestock production. Furthermore, the ability of legume plants to carry out nitrogen fixation in symbiosis with soil rhizobium bacteria provides an environmental-friendly source of reduced nitrogen in the biosphere, being an essential element in sustainable agriculture worldwide.

Despite the numerous advantages of the cultivation of legumes, one of the factors that limit their wider cultivation is the reduction of legume crop yields due to abiotic stress conditions, particularly drought.

Three are the main factors contributing to this limitation of productivity: i) in intensive crop-based agricultural systems worldwide, legumes are commonly grown under rain-fed conditions. This is the case in the Mediterranean area (Jacobsen et al. 2012), USA, Brazil and Argentina, the three countries responsible for 87% world's soybean production (FAOSTAT, 2013), or Asia (Kumar and Abbo, 2001); ii) legumes are often grown in rotation after cereal harvest towards the end of the growing season when environmental conditions are more limiting for plant growth; and iii) improvement in legume crop yields has not kept pace with those of cereals, for which higher yielding modern varieties have been developed (Jeuffroy and Ney, 1997). The limitations described above, along with the predictions of an increasing world food demand (Postel, 2000) and the rise in temperature at the global level, are driving forces for the investigation of legume responses to drought towards the ultimate development of new varieties with improved water use efficiency and drought tolerance.

### **3. Regulation of nitrogen fixation under drought**

Legumes can establish symbiosis with N-fixing soil bacteria. As a result, a new organ is formed, the nodule, where the reduction of atmospheric  $N_2$  into ammonia is carried out catalyzed by the bacterial exclusive enzyme nitrogenase. This process is known as symbiotic nitrogen fixation (SNF) and may provide all the N needs of the plant, avoiding the use of N fertilizers in the context of sustainable agriculture. The effects of drought on SNF occur at different steps of the symbiotic interaction: infection, nodule development, and nodule functioning. Under drought, both, the formation of new root hairs and the elongation of previously differentiated root hairs are limited and, as a consequence, the development of new plant-bacteria interactions and infection threads is greatly reduced (Worrall and Roughley, 1976). Moreover, SNF is one of the physiological processes to first show stress responses in nodulated legumes, a decline that cannot be explained by the relatively slow decline in photosynthetic rates (Durand et al., 1987).

Although several hypotheses have been proposed to explain the decline in SNF during drought, the origin of the inhibitory signals, the

molecular mechanisms involved and the interaction among the factors responsible for the inhibition of SNF are not yet fully understood. It has been postulated that drought stress provokes an increase in nodular oxygen diffusion resistance and thus, a decline in the oxygen level for bacteroid respiration (Durand et al., 1987). However, the increase of oxygen concentration in the rhizosphere of drought-stressed nodules does not fully restore NF rates, suggesting that other factors are also involved (Del Castillo et al., 1994; Del Castillo and Layzell, 1995).

The availability of carbon in nodules as supply for bacteroid respiration and nitrogenase activity is the second regulatory mechanism suggested (Figure 1). The main carbon source transported from the aerial part is sucrose, which is hydrolyzed in nodules by sucrose synthase (SuSy). The essential role of SuSy for NF has been shown for pea (Gordon et al., 1999) and the model legume *M. truncatula* (Baier et al., 2007). Indeed, Gordon et al showed a correlation between SuSy activity decline and NF inhibition in stressed soybean nodules (Gordon et al., 1997). Moreover, SuSy has been shown to be the first enzyme to decline under drought stress in soybean (Gonzalez et al., 1995), pea



(Gonzalez et al., 1998; Galvez et al., 2005) and common bean (Ramos et al., 1999), leading to the accumulation of sucrose and the depletion of organic acids, principally malate, in nodules. However, the SuSy mediated NF inhibition seems not to take place in forage legumes such as *M. sativa* (Naya et al., 2007) and *M. truncatula* (Larrainzar et al., 2009). In these studies, significant declines in the SuSy activity were found only after the inhibition of NF and concomitant to malate accumulation, suggesting that carbon availability is not the limiting factor for the inhibition of NF in these plants. Moreover, in a recent metabolomic approach, the limitation of respiratory carbon substrates was demonstrated not to be the cause of NF inhibition in drought-stressed *M. truncatula* nodules (Larrainzar et al., 2009).

The third suggested factor implies a N-feedback mechanism involving the N-status of the plant. This theory has received much attention in ureide-exporter tropical legumes, mostly due to studies conducted in soybean. Legumes can be classified into amide- or ureide-exporters according to the compounds used for the transport of fixed N compounds. In general, amide-exporter legumes, such as *M.*

*truncatula*, contain indeterminate type nodules and are originated from temperate regions. These plants transport fixed nitrogen in the form of amides, mainly asparagine and glutamine. On the other hand, ureide-exporter legumes, such as soybean, are mostly tropical legumes with determinate type nodules and transport mainly allantoin and allantoic acid. However, exceptions to this general pattern can be found. For instance, the temperate legume *Lotus* spp., with the determinate type of nodules, exports amides rather than ureides (Sprent, 2001). Several N compounds have been suggested as inhibitory signal molecules, such are the cases of ureides (Serraj et al., 1999; Vadez and Sinclair, 2000), glutamine (Neo and Layzell, 1997), asparagine (Bacanamwo and Harper, 1997; Vadez et al., 2000) and aspartate (King and Purcell, 2005). The restriction on the export of N compounds, with their subsequent accumulation in the nodules in water deficit conditions has also been postulated (Pate et al., 1969; Walsh, 1989a,b). Serraj et al. (2001a) refined the model by proposing two possible origins for the feedback inhibition: a direct feedback within the nodules and an indirect feedback due to N compound signals coming from the aerial part. A more

recent study showed that ureides were accumulated in soybean nodules and not in leaves, suggesting a local regulation of NF (Ladrera et al., 2007). Recent works using Split-Root-System-based approaches confirm the operation of local regulatory mechanisms controlling SNF in pea (Marino et al. 2007), *M. truncatula* (Gil-Quintana et al. 2013a), and soybean (Gil-Quintana et al. 2013b) under water deficit conditions. The concomitant reduction in nitrogenase activity, malate content and SuSy activity in the nodules of the unwatered split-root section supports the existence of a local carbon-based regulation of SNF in pea (Marino et al. 2007). In addition, the general variations in amino acid and ureide content in leaves, roots and nodules (Gil-Quintana et al. 2013a, b) challenged the widely accepted N-based systemic regulation hypothesis (King and Purcell 2005; Sulieman et al. 2010), reinforcing the direct feedback inhibition in the nodules hypothesis.

#### **4. Drought stress effect on the root system**

Although legume crops may lend to a sustainable use of nitrogen fertilizers, the nitrogen-fixing process is mostly suppressed in nitrogen

fertilized agro-environments (Murray et al., 2017). Under these conditions, legume response to drought would be similar to that of other cultivated crops, even though attention should be paid to specific features of legume plants (Figure 1). In herbaceous crops, most of the nitrate is reduced predominantly in the shoots via the reducing equivalents derived from photosynthesis (Scheurwater et al., 2002; Hachiya et al., 2016). Leaf nitrate reduction declines rapidly in response to drought in important crops such as maize (Foyer et al., 1998) or wheat (Fresneau et al., 2007) which correlate with the decline of the photosynthetic process. However, temperate legumes assimilate nitrate chiefly in the roots when growing under low N supply, while shoot nitrate assimilation becomes increasingly important as the nitrate concentration increases (Andrews, 1986). Conversely, tropical legumes exhibit constant ratios of the shoot to root nitrate assimilation where this ratio is specific for each species (Andrews, 1986). These features have not been tested for the current model plants for temperate and tropical legumes, *Medicago* and *Lotus*, respectively. Unlike nitrate, ammonium is chiefly assimilated in the roots by the coordinated

activities of GS and GOGAT (Funayama *et al.*, 2013; Guan *et al.*, 2015; Trepp *et al.*, 1999a, b). In the context of legume plants, ammonium nutrition would closely mimic the symbiotic N-fixing legumes since bacteroids assimilate very little of the fixed ammonia, which is mainly exported to the host plant (Brown and Dilworth, 1975; Vance *et al.*, 1994). In this context, legumes have been shown to be relatively tolerant to ammonium nutrition (Dominguez-Valdivia *et al.*, 2008; Ariz *et al.*, 2010).

Roots are the first organs that sense water deficit in soils and interact directly with edaphic water, and therefore drought responses of this organ are highly important. Several studies try to dissect the molecular response of roots of different legumes to drought stress (Micheletto *et al.*, 2007; Zhang *et al.*, 2014). In this context, the primary nitrogen assimilation pathway does not seem to be severely affected under drought stress conditions. This response seems coherent since drought affects cell growth and protein synthesis even at a very moderate level (Hsiao, 1973) and hence nitrogen demand is expected to be reduced. With regards to carbon economy, Muller *et al.*

(2011) highlighted a lack of correlation between carbon availability and sink organ growth under water-deficit stress.

Regarding nitrogen metabolism, drought provokes an overall accumulation of amino acids in roots of nodulated (Gil-Quintana et al., 2013) and non-nodulated plants (Frechilla et al., 2000) thereby dismissing a possible nitrogen starvation in drought-stressed plants. In addition, changes in protein synthesis and degradation may strongly affect the pool of free amino acids. Taking as reference the amino acid composition of the *Arabidopsis* proteome, Hildebrandt et al. (2015) estimated that the pool size of the protein-bound amino acids varied less than ten folds. Therefore, inhibition of protein synthesis (Lyon et al., 2016) or enhancement of proteolytic activities (Kohli et al., 2012) could influence the overall accumulation of the free amino acid pools in drought-stressed tissues. Lyon et al. (2016) highlight the importance of protein turnover dynamics in drought recovery processes. On the other hand, pool sizes of the free amino acids, which are around 100-1000-fold smaller than the corresponding pools of protein-bound amino acids, are highly diverse (Gil-quintana et al., 2013; Watanabe et al., 2013). This

reflect the various functional roles of these compounds and their interaction with the synthesis of other relevant compounds such as nucleotides or hormones. Amino acid synthetic pathways mainly consume intermediates from glycolysis, the pentose phosphate pathway, and the citric acid cycle and the involved enzymes are mostly located in the plastid with some of them addressed to the cytosol (reviewed in Pratelli and Pilot, 2014). In general, the primary products of nitrogen assimilation, Glu, Gln, Asp, and Asn, constitute the larger pools in plants (Coruzzi, 2003) although they are not much induced during stress, and accordingly, primary nitrogen assimilation enzyme activities rarely increase in response to drought stress (Larrainzar et al., 2009). Conversely, other less abundant amino acids under control conditions such as Pro (Jacoby et al., 2011), branched chain amino acids (Joshi et al., 2010), Lys and Thr (Obata and Fernie, 2012) and His and Trp (Larrainzar et al., 2009) respond individually to drought. Accordingly, the expression of different enzymes involved in the synthesis of some amino acids is eventually affected (Pratelli and Pilot, 2014). Unlike the amino acid synthesis, catabolism is mainly addressed to the

mitochondria or the cytosol (Hildebrandt et al., 2015). The involvement of mitochondria favors the nitrogen and carbon recycling during the senescence processes occurring under drought stress since mitochondria functionality remains longer than that of other organelles (Avila-Ospina et al., 2014). Araújo et al. (2011) pointed to protein degradation and amino acid catabolism as an alternative carbon source for respiratory processes in stressed plants. Research on amino acid metabolism needs to be expanded for a better understanding on intracellular compartmentalization (Mintz-Oron et al., 2012) dealing not only to photosynthetic tissue but also to those exhibiting a heterotrophic metabolism such as roots.

Furthermore, an active long-distance transport of the amino acids between root and shoot occurs involving both, the phloem and the xylem vascular tissues (Jeschke and Hartung, 2000). In legumes, amino acids are mainly transported via the xylem (Atckings et al., 1983) but concomitantly some amino acids may be transferred to the phloem to supply nitrogen directly to the sink (Zhang et al., 2010; Together, 2014). Recent studies have shown that transport of amino acids between shoot



and roots determine nitrogen uptake and metabolism (Miller et al., 2008; Santiago and Tegeder, 2016). However, the role of long-distant transport of amino acids needs to be studied further to better understand the changes in the source-sink interactions occurring under drought. The amino acid exchange requires continuous inward and outward transport across membranes, and numerous genes encoding amino acid transporters have been described (Jack et al., 2000). The induction of proline transporters has been reported in *Arabidopsis* and rice exposed to drought and salt stress (Rentsch et al., 1996; Zhao et al., 2012). In addition, the expression level of different amino acid transporters showed a differential response to drought among shoots and roots in wheat, suggesting that they may play a role in the amino acid exchange among aerial and underground tissues (Wang et al., 2017).

## **5. Future prospects**

Legume crops can fix atmospheric nitrogen through their symbiotic association with N-fixing bacteria or by using chemical fertilizers. In this latter case, the legume root exhibits particular features such as a

higher tolerance to ammonium and the ability to carry out nitrate reduction to a greater extent than other non-legume crops. Regarding nodulated plants, the nitrogen fixation process has been shown to be rapidly inhibited under moderate drought stress conditions. For those nitrogen fertilized legumes, few studies have been carried out at root level, although it is widely known that nitrate reductase activity is severely affected in leaves. However, although the different nitrogen assimilation processes seem to be impaired in legumes, the general accumulation of nitrogen compounds occurring in the different tissues dismiss any possible nitrogen scarcity playing a pivotal role in the legume response to drought. Indeed, cell growth is one of the processes firstly affected by water deficit stress at a moderate level and hence nitrogen demand is presumed to be lower under drought stress. Further knowledge on long distant transport of nitrogen compounds and amino acid metabolism compartmentalization may contribute to improving legume nitrogen utilization under moderate water stress conditions.



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## Legend to Figures

Figure 1. A) Split-root system set up as a tool to study the local or systemic nature of plant signaling processes. B) Schematic representation of the main physiological and metabolic processes affected by drought at shoot, root and nodule level. AAT, aspartate aminotransferase; AS, asparagine synthase; GS, glutamine synthetase; GOGAT, glutamate synthase; NR, nitrate reductase; NiR, nitrite reductase; SNF, symbiotic nitrogen fixation; SuSy, sucrose synthase; TCA, tricarboxylic acid cycle.

