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Nodule performance within a changing environmental context

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ABSTRACT

Global climate models predict that future environmental conditions will see alterations in temperature, water availability and [CO_2]. Climate Change will reinforce the need to develop highly productive crops. For this purpose it is essential to identify target traits conditioning plant performance in changing environments. N_2 fixing plants represent the second major crop of agricultural importance worldwide. The current review provides a compilation of results from existing literature on the effects of several abiotic stress conditions on nodule performance and N_2 fixation. The environmental factors analysed include water stress, salinity, temperature, and elevated [CO_2]. Despite the large number of studies analysing [CO_2] effects in plants, frequently they have been conducted under optimal growth conditions that are difficult to find in natural conditions where different stresses often occur simultaneously. This is why we have also included a section describing the current state of knowledge of interacting environmental conditions in nodule functioning. Regardless of the environmental factor considered, it is evident that some general patterns of nodule response are observed. Nodule carbohydrate and N compound availability, together with the presence of oxygen reactive species (ROS) have proven to be the key factors modulating N_2 fixation at the physiological/biochemical levels. However, with the exception of water availability and [CO_2], it should also be considered that nodule performance has not been characterised in detail under other limiting growth conditions. This highlights the necessity to conduct further studies considering these factors. Finally, we also observe that a better understanding of these metabolic effects of changing environment in nodule functioning would require an integrated and synergistic investigation based on widely used and novel protocols such as transcriptomics, proteomics, metabolomics and stable isotopes.

Key words: C/N metabolism, climate change, nodule, N_2 fixation, omic methodologies

Abbreviations: AAT, aspartate aminotransferase; Asn, asparagine; BNF, biological N_2 fixation, CA, carbonic anhydrase; DM, dry mass; DR, dehydroascorbate reductase; GOGAT, glutamine oxoglutarate amid otransferase; Gln, glutamine; Glu, glutamate; GOT, glutamate oxaloacetate transaminase; GS, glutamine synthetase; ICDH, isocitrate dehydrogenase; IPCC, Intergovernmental Panel on Climate Change; LCGI, Legume Crops Genome Initiative; MDH, malate dehydrogenase; N_{ase}, nitrogenase; NifH, nitrogenase reductase; OAA, oxaloacetate; PBM, peribacteroidal membrane; PEP, phosphoenolpyruvate; PEPc, phosphoenol pyruvate carboxylase; ROS, reactive oxygen species; SNA, specific nodule activity; SOD, superoxide dismutase; SS, sucrose synthase; TCA, tricarboxilic acids; TSP, total soluble proteins; TSS, total soluble sugars.

Introduction

According to the predictions of the Intergovernmental Panel on Climate Change (IPCC, 2007), most climate scenarios are expected to be affected by climate change. It is predicted that by the end of this century, atmospheric CO_2 levels will increase from the present 400 to 700 μmol mol^{-1}. The increase in the concentration of this greenhouse gas will cause ambient temperatures to rise by 1.8 - 4.0 ºC. Rising temperature will increase evapotranspiration rates and exacerbate low water availability and salinity problems commonly observed in environments such as the Mediterranean, where current annual potential evapotranspiration is often nearly twice the amount of rainfall (Sabaté et al., 2002). This is a matter of major concern, because water deficit, together with soil N content and salinity, is the most important environmental factor limiting plant growth and production in the Mediterranean climate (Chaves et al., 2002; Annicchiarico et al., 2011). Most studies analyzing the Climate Change effect on plant growth have considered CO_2 concentration, temperature, water availability and salinity separately. This is especially relevant because, in natural conditions, plants are frequently exposed to interacting environmental stressful growth conditions. If the goal of studies analyzing plant growth under changing growth conditions is to understand how will they perform in near future, it is important to reproduce, as accurately as possible, such growth conditions. Otherwise, extrapolations derived from growth conditions that will not be real in the near future for agricultural systems will not be trustworthily. In order to better understand how plant growth will be affected, it will be essential to strictly scrutinise environmental particularities that preclude or permit them. Unfortunately, most of the literature is based on studies characterizing single stress factors. This is a matter of great concern, because it is well known that the effect of combined stresses on plant growth causes alterations that cannot be predicted if they are analysed alone

(Valladares and Pearcy, 1997). For example, Chaves and Pereira (2004) observed that although photochemical processes are very resistant to low water availability, a down regulation of the photosynthetic apparatus occurs when plants are exposed simultaneously to drought and elevated temperature conditions.

During the 1960s and 1970s, enhancement of crop productivity was enabled by improvements in N fertilisation. In the last 40 years, the amount of synthetic nitrogen (N) applied to crops has risen dramatically from 12 to 104 Tg / year (Mulvaney et al., 2009), resulting in significant increases in yield but with considerable impacts on the environment throughout the world. The impacts of N in the environment are becoming increasingly apparent due to excessive fertilising regimes. To alleviate these problems, European policies have set, for instance, acceptable pollution limits from farming (Shortle and Abler, 2001). Among such policies it is recommended that grain crops be rotated with legumes. Legumes represent the second major crop of agricultural importance worldwide and cover about 14% of total land under cultivation (FAOSTAT 2010). These plants represent an important source of protein and calories for humans and animals (Rogers et al., 2009). In addition, and compared with other crops, legumes represent a particular plant group thanks to their capacity of to fertilise soils through the fixation of atmospheric N_2 (Hirsh, 2004). The symbiotic relationship between *Rhizobiaceae* family bacteria and legumes provides access to atmospheric N_2. Biological N_2 fixation (BNF) provides to the legumes and the surrounding plants an additional N source that is of great value in impoverished soils. As observed by Peoples et al., 1995, this symbiotic relationship is the main source of N_2 fixation in terrestrial ecosystems (provides 50% of BNF) and reduces the need to fertilise soils with chemical compounds, which leads to additional economic and environmental benefits.

The symbiotic relationship between the plant and bacteria takes place in the root nodules. Within the nodule, the bacteria are isolated from the host cell by the peribacteroidal membrane (PBM) that regulates the exchange between both symbionts (Day et al., 2001). The regulation of nitrogenase activity and consequently plant N availability is conditioned by C supply to the bacteroids (Galvez et al., 2005; Larrainzar et al., 2009). Therefore, there is a tight dependency on C and N metabolism between bacteroids and the plant. The host provides photoassimilates, which supply the energy and C skeletons required by the bacteroid to fix N_2 by nitrogenase (N_{ase}). Sucrose is partitioned to the nodules through the phloem where it is cleaved by sucrose synthase, enters glycolysis and is transformed into dicarboxylic acids, mainly in the form of malate and succinate (Lodwig and Poole, 2003). Within the bacteroid malate is oxidised by the TCA cycle to provide reductant to both the N_{ase} complex and the respiratory chain that fuels N_{ase} with the ATP necessary for N_2 fixation (Kouchi and Yoneyama, 1986; Streeter, 1987). The bacteroid returns ammonium (NH_4^+) to the host that is assimilated in the form of glutamine (Gln), and this is further metabolised into other N transport forms, depending on the legume species, such as asparagine (Asn) or to purine derivatives known as ureides that are partitioned to the rest of the plant through the xylem according to their requirements (Udvardi and Day, 1997; Larrainzar et al., 2009; Molero et al., 2011). It has also been suggested that some amino acids are supplied by the plant to the bacteroid in order to produce Ala or Asp from the transamination of oxaloacetate or pyruvate (Lodwig et al., 2003; Prell and Poole, 2006). According to Lodwig et al. (2003), the plant provides glutamate (Glu) to the bacteroid where it is used as a transamination donor to produce amino acids. Provision of amino acids would regulate nodule NH_4^+ content, shutting it down when amino acid levels are too high.

A large number of the studies analysing nodule performance have been conducted specifically in nodules, without giving much consideration to the rest of the plant. This is a matter of great concern, because as mentioned above it has long been known that N_2 fixation relies on the interchange of plant-bacteroid resources (Hardy and Havelka, 1976; Sprent et al., 1988). Labelling experiments have shown that photosynthates are rapidly (within 1 h) transferred to the nodules (Voisin et al., 2003abc). This inter-organ coupling implies that factors altering leaf performance will affect nodule functioning and vice versa (Aranjuelo et al., 2007; 2011; Rubio et al., 2002). These findings highlight the great importance of considering plant-bacteria interactions as a whole.

Another point that should be considered is that a large number of studies analysing nodule performance have been conducted under optimal growth conditions. However, as mentioned before, legumes are frequently exposed to varying temperature, relative humidity, soil water, and nutrient availability conditions that limit plant and nodule functioning (Aranjuelo et al., 2007; 2008; Sanz-Sáez et al., 2010). Indeed, the majority of the literature that describes nodule functioning under stressful growth conditions has focused on drought effects, whereas other environmental variables such as temperature, salinity, and CO_2 have received less attention. However, because the objective of such studies is to further understand the key factors conditioning nodule performance in natural conditions, it is important to simulate growth conditions as realistically as possible. This is why it is important to extend the research of this organ across a wider spectrum of growth conditions. Furthermore, in addition to the separate analyses of stressful growth conditions, it is important to analyse the interaction between different stresses all together. As described in previous studies (Aranjuelo et al. 2007; 2008;

2009; Sanz-Sáez et al., 2010), the effect of combined stresses on plant growth causes alterations that cannot be predicted if they are analysed alone, such as those resulting from synergistic and antagonistic phenomena.

In summary, the main goal of this article is to review the current state of knowledge of nodule performance under stressful growth conditions (analysed separately and its interaction) as a base for understanding plant responses in a changing environmental context and to highlight needs of further research strategies. We will also emphasise the need to consider the other organs of the plant (rather than the nodule) and the interactions among them. We will also highlight the application of new methodologies that can further increase our knowledge of processes regulating nodule functioning and the symbiotic exchange with the plant. This information might be applied to further understand plant-nodule communication and regulation, which will guide future plant breeding programmes aiming to develop legume varieties better adapted to the different Climate Change Scenarios.

2. Processes conditioning N_2 fixation in legumes

N_2 fixation in legumes is strongly related to the physiological state of the host plant. The diversity of results described in the literature (Aranjuelo et al., 2007; 2008; Gálvez et al., 2001; 2005a; Purcell et al., 2004; Schulze et al., 2004; Serraj et al., 2003bc) highlights the complexity of nodule performance under varying environmental conditions. The main processes limiting nodule functioning are: (i) carbohydrate availability, (ii) accumulation of nitrogenous compounds, (iii) O_2 permeability, and (iv) accumulation of reactive oxygen species (ROS).

2.1. Carbohydrate availability. As mentioned above, regulation of BNF is related to C supply by the host, mostly in the form of malate, for bacteroid respiration. Since nodules are a strong sink, they require a large amount of carbon: it has been estimated that during the dayup to 45% of photoassimilates may be exported towards the nodules (Gordon et al., 1987). The C supply might be reduced due to a decline in photosynthesis at the leaf level that is observed under soil moisture deficiency, high temperature, and salt stress. On the other hand, C supply could be increased under elevated CO_2 concentrations (see below). Previous studies (Arrese-Igor et al., 1999; Gálvez et al., 2005; Gordon et al. 1999) describe that under stressful growth conditions where photosynthetic activity is inhibited there is a decrease in carbohydrate supply to the bacteroids with a consequent diminishment in N_{ase} activity. This is due to the downregulation of one of the enzymes responsible for the cleavage of sucrose in nodules: sucrose synthase (SuSy) (Arrese-Igor et al., 1999). Conversely, when photosynthetic rates increase, there is an increase in N_2 fixation due to the larger amount of photosynthetically derived organic carbon supplied to nodules (Arrese-Igor et al., 1999; Aranjuelo et al., 2008; Rogers et al., 2009).

2.2. Accumulation of nitrogenous compounds. The decrease in nitrogenase activity has been associated with the accumulation of nitrogenous compounds (Hartwig et al., 1994; Serraj et al,. 1999). The accumulation of these compounds can originate from decreases in carbohydrate fluxes to the nodules or the impairment of xylem transport and the consequent decreases in the transport of nitrogenous compounds to the plant (Serraj et al., 1999; Aranjuelo et al. 2008). The accumulation of these compounds induces a negative feedback mechanism with a consequent inhibition of N_{ase} activity (Serraj et al., 1998; 2001; King and Purcell, 2005; Hartwig et al., 1994). Also, the accumulation of N

compounds could originate from the reduced aboveground N demand and could cause the accumulation of N_2 fixation products in the nodules with a consequent inhibition of N_{ase} activity (Aranjuelo et al., 2011; King and Purcell, 2005; Larrainzar et al., 2007; Schulze 2004a; Serraj 2003b; Serraj and Sinclair 1996). Results from experiments manipulating N sink-strength demonstrate a clear effect on N_{ase} activity (Schulze 2004 and references therein).

2.3. O_2 permeability. Although O_2 is required in respiration processes by the nodule, O_2 regulation is critical for BNF since most N_{ase} are sensitive to its presence (Becana et al. 2010). Nodule permeability to O_2 via the regulation of the O_2 diffusion barrier has been suggested as a key factor conditioning N_{ase} performance (Hunt and Layzell, 1993). Previous studies (Serraj and Sinclair, 1996; Purcell and Sinclair, 1994) showed that water stress causes a diminishment in the permeability to O_2 diffusion, which leads to a reduction in nodule respiration and therefore a lower production of energy via ATP synthase. The reduction in O_2 availability to the bacteroid may also be associated with a decrease in the concentration of leghemoglobin, which could be degraded by reactive oxygen species (ROS) (Marino et al., 2007).

2.4. Oxidative stress. Another mechanism responsible for nitrogen fixation inhibition is oxidative stress (Gogorcena et al., 1995; Porcel et al., 2003; Naya et al. 2007). Some environmental conditions, such as drought or salinity, are responsible for nodule senescence and also cause an O_2 content imbalance, which is necessary to ensure a successful nodule performance (Zahran 1999). According to Witty et al. (1986), the decrease in O_2 permeability led to an O_2 restriction to the bacteroid. The imbalance in O_2 control is associated with the formation of ROS, which could produce cellular

damage (Naya et al., 2007). ROS production and removal is a complex process that requires a tight biochemical control involving enzymatic and non-enzymatic detoxification mechanisms that have been developed by plants (Marino et al. 2006; Aranjuelo et al., 2013a). As recently reported, drought increases the expression of genes involved in the detoxification of O_2 radicals such as cytosolic CuZn-superoxide dismutase (SOD), and gluthatione reductase, etc. However, other studies report a decrease in antioxidant activity under drought conditions (Gogorcena et al., 1995; Porcel et al., 2003). However, in a large number of these studies the response of nodule antioxidants have not been analysed at the molecular level and in most of the studies N_{ase} activity was not monitored, making it difficult to establish a relationship between the decrease in antioxidant protection and the loss of nodule function.

Although all these factors have been described as essential in nodule performance, drought is the only stressful environmental factor where they have been extensively studied.

3. Water availability

Soil moisture deficiency has a pronounced effect on N_2 fixation because nodule initiation, growth, and activity are all more sensitive to water stress than general root and shoot metabolism. The particular way in which water stress is developed might be of special importance not only for understanding the response to drought, but also in evaluating the plant's capacity to acclimation (Kaiser, 1987). As highlighted in a recent study conducted with *Lotus japonicus* exposed to different water stress conditions Sanchez et al. (2012), the metabolic response of these plants was tightly linked to the stress-dose. The differences in the severity level of the applied drought and the species analysed could partially explain the different results described in the literature (Ramos

et al., 1999; Hungria and Vargas 2000; Gálvez et al., 2005; Naya et al., 2007; Sanchez et al., 2012).

3.1 Mild water stress

Studies conducted with *Glycine max* (Durand et al., 1987), *Phaseolus vulgaris* (Ramos et al., 1999), *Medicago truncatula* (Larrainzar et al., 2009), *Medicago sativa* (Naya et al., 2007a) and *Pisum sativum* (Gálvez et al., 2005a), where the plants were exposed to withholding water stress, showed that nodule functioning varied as drought intensity increased. In most cases, after 3 days of withholding water, N_{ase} activity started to decrease (when compared with the control plants) and this decrease became more marked as the days went by. As it is shown in Fig. 1, where we have summarized the main findings provided by the literature on nodule and plant performance under water stress conditions, at the leaf level, water stress causes photosynthetic inhibition caused by the stomatal closure (reflected by the depleted g_s) and the impairment of Rubisco Aranjuelo et al. (2011). The decrease in SS activity results in an accumulation of sucrose and a reduced concentration of organic acids, mainly in the form of malate, which causes a shortage of substrates for bacteroid respiration (González et al., 2001; Gálvez et al., 2005). As a consequence, a transient accumulation of oxygen in the infected region would take place, leading to an increase in the resistance of the oxygen diffusion barrier in order to avoid nitrogenase damage (see above). Both the depletion of respiratory substrates and the consequent closure of the oxygen diffusion barrier would cause the observed decline in BNF. However in *Medicago* species, observations from other studies (Ramos et al., 1999; Larrainzar et al., 2009; Naya et al. 2007) suggest that organic acids, together with soluble sugar content, increased in early droughted nodules. Under early drought conditions there is an increase in N compounds that could have

induced N feedback inhibition that affected N_{ase} activity negatively (Ladrera et al., 2007; Larrainzar et al., 2009). As it is shown in Fig. 1, the lower leaf N demand has been described to cause the nodule amino acid accumulation nodules. Also, the decrease in N_{ase} could be caused by the decline in nodule proteins such as N_{ase} Fe protein (NifH), oxidoreductase (FixC) and transmembrane proteins (LpdA) (Larrainzar et al., 2009). Results obtained by (Naya et al., 2007) showed that oxidative stress was also involved in the diminished BNF. The upregulation during drought of a number of genes involved in antioxidant protection, together with the accumulation of peroxidised lipids and oxidatively modified proteins in droughted nodules, has revealed that such nodules are exposed to oxidative stress.

3.2 Severe water stress

Data obtained from *M. truncatula* and *M. sativa* (Larrainzar et al., 2009; Aranjuelo et al., 2011; Naya et al., 2007) indicated that under severe water stress C availability was not involved in the regulation of BNF in these plants under drought conditions. These studies revealed that even though drought inhibited photosynthetic activity at the leaf level, there was not any C shortage (in the form of soluble sugar and organic acid compounds) in their nodules (Fig. 1). Furthermore, these studies suggested that, similarly to what is described in leaves, there is an increase in nodule soluble sugars (sucrose, raffinose), sugar alcohols (galactinol, myo-inositol, pinitol) and organic acids (fumaric acid, malate) with osmoregulantory activity. The accumulation of organic solutes could constitute an adaptive response to water stress, given that this mechanism is involved in the restoration of turgor, the reduction of oxidative damage induced by free radicals, and also the stabilisation of membrane structure and enzymes (Chen and Murata, 2002). The accumulation of specific organic solutes (osmotic) is a characteristic

response of plants subjected to prolonged severe water stress. In this sense, plants have been shown to redirect a significant amount of carbohydrates to stabilise nodule and leaf water status (Patonnier et al., 1999; Chia et al., 2000; Streeter 2003; Valliyodan and Nguyen 2006; Sweetlove et al., 2010; Zhang et al., 2011; Sanchez et al. 2012). The study carried out in *Medicago sativa* (Naya et al., 2007; Aranjuelo et al., 2013a) indicated that nodules subjected to soil moisture deficiency had an accumulation of soluble sugars and organic acids, proteins that are part of the TCA cycle were involved in the lower respiration rates of the nodules. It is likely that such accumulation is derived from the mobilisation of starch derived carbohydrates in nodules, namely sucrose. Such results suggest that under severe drought conditions the TCA cycle did not operate to its optimal aerobic capacity due to the lower nodule permeability to O_2 (Lodwig and Poole, 2003). However, the absence of significant changes in photosynthetic efficiency and the respiratory cost of N_2 fixation reveal that droughted plants adjusted to such lower inputs to sustain nodule catabolism according to the lower plant N demand (Aranjuelo et al., 2013a).

As it has been described above, the relevancy of oxidative stress in nodule functioning is a matter of major concern. As reported by Naya et al. (2007), drought induces increased expression of genes involved in the detoxification of O_2 radicals. Under severe drought conditions, diminished respiratory rates, high cytosolic concentration of leghemoglobin, the abundance of catalytic Fe and the presence of redox proteins (with the ability to transfer electrons to O_2) would justify the importance of regulating ROS content (Becana et al., 2010). However, other studies (Gogorcena et al., 1995; Porcel et al., 2003) show that severe water stress induces a decrease in antioxidant activity. In addition to the enzymatic mechanisms, the metabolomic characterisation conducted in

droughted nodules also showed that the content of compounds with ROS scavenging capacity like ascorbic acid and proline also increased under severe drought conditions (Becana et al., 2010; Van Den Ende and Valluru, 2009). It should be noted that before oxidative damage can be caused by ROS, these molecules already play a crucial role in oxidative signalling during drought stress, at both the transcriptional and post-translational levels (Marino et al., 2006).

In a recent study conducted in *Medicago sativa* (Aranjuelo et al., 2011), it was observed that deleterious drought effects on leaf N status (mainly regarding Rubisco) could have negatively affected nodule functioning. This study showed that together with a decrease in Rubisco content, in droughted leaves there was also a down-regulation of proteins involved in Rubisco assembly (putative Rubisco binding-protein). The depletion at the leaf level of Rubisco and amino acid content (with the exception of proline) suggests that under these unfavourable conditions there was a mobilisation of N from the main leaf N reservoir (i.e. Rubisco) toward below ground organs such as the primary root and nodules. The fact that the enzymes, proteosome b1 subunit (proteolytic activity) and glutamine synthetase (involved in the GS-GOGAT cycle where assimilated NH_3 is converted to glutamic acid, Glu, and glutamine, Gln), were up-regulated under drought conditions suggests that there was reallocation of N derived from Rubisco to other organs (Gordon et al., 1999; Aranjuelo et al., 2011). Such data suggest that the reduced aboveground N demand caused amino acid build up in the nodules. Several compounds such as glutamine, asparagine, aspartate and ureides have been suggested to be involved in a N feedback mechanism (Serraj et al., 2001; King and Purcell 2005; Larrainzar et al., 2009; Sulieman and Schulze, 2010). Ureide accumulation is part of a general response to stress, in particular because ureides play a key role in cell protection under

oxidative stress conditions (Brychkova et al., 2008), such as the nodule senescence induced by drought (Puppo et al., 2005; Yamaguchi et al., 2010). The accumulation of amino acids has also been associated with stabilisation of protein structure (Schobert and Tschesche, 1978) and osmoregulation (Irigoyen et al., 1992; Larrainzar et al., 2009; Joshi et al., 2010). Together with amino acid osmoregulants, the increases in sugars, sugar alcohols and organic acids with osmoregulant activity have been described as linked with the stabilisation of nodule and leaf water status (Patonnier et al., 1999; Chia et al., 2000; Streeter 2003; Valliyodan and Nguyen, 2006).

3.3 Sustained low water availability

Although withholding water is the most common method for short-term experiments, sustained or cyclic water stress is also essential to simulate more realistic responses to drought (Pennypacker et al., 1990). In this sense, studies were low water availability plants were watered with lower water content since the beginning of the experiment should also be considered. Previous studies conducted by our group (Aranjuelo et al. 2007; 2009) where exclusively N_2 fixing alfalfa plants were grown under full *versus* low (≈ at 50 % of field capacity) water levels showed that although low irrigation strongly decreased total dry matter, these plants adapted their growth rate to the available water content without suffering any water stress, as revealed by their relative water content (Aranjuelo et al., 2007; 2009). Interestingly, although no significant differences were observed in leaf gas exchange determinations, the leaf N, total soluble proteins (TSP) and Rubisco contents were negatively affected by low water availability. The obtained results highlighted the fact that the lower N shoot demand negatively affected nodule TSP content and the activity of enzymes involved in N_2 assimilation, such as malate dehydrogenase (MDH) and aspartate aminotransferase (AAT). The

lower MDH suggests that malate availability could have been depleted in those nodules, with the consequent effect in respiration. The lower investment of photoassimilates in nodule DM production of droughted plants also contributed to the lower N_2 fixation at the plant level.

In summary, current knowledge of droughted nodule performance reveals that even under moderate water stress conditions, carbohydrate shortage has a key role in depleted N_2 fixation, under severe water stress conditions, oxidative stress and N compound accumulation in nodules are likely to be the main factors explaining the poor nodule performance (Fig. 1). Furthermore, in moderate and severe stress conditions, but mainly under the latter, the plants accumulate specific compounds involved in osmoregulatory and antioxidant processes. Although little information is available, under sustained limited water availability conditions the available data suggest that lower shoot N demand is also involved in the reduced nodule performance. Several reports have suggested that N-fixing plants of *M. sativa* (Antolín et al., 1992), *P. vulgaris* (Lodeiro et al., 2000), *P. sativum* (Frechilla et al., 2000), and soybean (Kirova et al., 2008) can be more tolerant to drought than nitrate-reducing plants, but despite the obvious agronomic interest of this observation, the physiological reasons underlying such a response remain largely unknown.

4. Salinity

Salt stress has been included among the major stressful environments conditioning the performance of legumes in arid and semi-arid regions mainly due to the effect of salt on nodule functioning (Yamaguchi and Blumwald, 2005). This is a matter of major

concern since almost 40% of world's land surface might be subjected to potential salinity problems (Zahran, 1999).

In general, rhizobia are more salt tolerant than their respective plant host, with some bacterial strains being able to grow in media with 300-700 mM NaCl (Mpepereki et al., 1997; Zahran, 1999). However, the salinity response of legumes varies greatly and depends on soil properties, the developmental growth stage and legume species (Cordovilla et al., 1994; 1995abc). The process of nodule formation is particularly sensitive to salt stress because under such conditions root hair curling is inhibited and bacterial colonisation and infection highly reduced (Zahran and Sprent, 1986). High salinity has been described to affect plant growth and symbiotic relationships in legumes (Tejera et al., 2004; López et al., 2008; 2009; 2010). According to these studies, shoot development is more sensitive than the roots. In a recent study conducted by Ben Salah et al. (2009) where two *Medicago ciliaris* lines (with different tolerance to salinity) were exposed to salt stress, it was shown that although plant growth was inhibited by 21% in the tolerant line and 73% in the sensitive line, N_2 fixation was depressed in these lines by 60% and 86% respectively. Although salinity has been described to deleterious for plant growth, as it is remarked by the question mark of Fig. 2, to our knowledge, leaf and root performance has been scarcely studied. Similar to previous observations for droughted nodules, nodule functioning under salinity has been shown to be conditioned by carbohydrate flux and oxidative stress (Serraj 2002; Tejera et al., 2004; López and Lluch 2008; López et al., 2008; Ben Salah et al., 2009; 2010). In salt sensitive plants (*Medicago ciliaris*), it was shown that soluble sugar content (including sucrose) decreased in nodules (Ben Salah et al., 2009). Sucrose synthase and alkaline/neutral invertase determinations indicated that the lower sucrose content of

these plants was caused by decreased enzyme performance. Furthermore, as it is shown in Fig. 2, the lower malate availability (main form of C supply to the bacteroid) suggested that C supply was involved in salinity-derived deleterious effects on N_2 fixation. Such a decline could lead to a shortage of substrates for bacteroidal respiration and consequently to reduced N_{ase} activity. The reduction in N_2-fixing activity by salt stress is usually attributed to a reduction in respiration of the nodules (Walsh, 1995). However, other studies have also revealed that inhibited N_2 fixation is not always related to a lower C availability. Ben Salah et al. (2010) observed that in salt tolerant plants salinity increased the availability of the soluble sugar content in their nodules. Furthermore, opposite to the observations in the salt sensitive line, (Ben Salah et al., 2010) showed that malate content also increased in the tolerant *Medicago ciliaris*. These authors remarked that the better performance of salinity tolerant plants was related to their ability to conserve photosynthetic activity and to maintain higher sucrolytic activity. According to this study, the activity of enzymes involved in sucrose breakdown increased in the tolerant line with a consequent increase in nodule sucrose content, which is opposite to what was observed in the salinity sensitive *Medicago ciliaris* lines. Although the increase in TSS has been frequently related to osmoregulatory processes (Chen and Murata 2002; Zhu 2002) however according to López et al. (2008) the accumulation of osmoregulants is a consequence of damage produced by salt, rather than a protection strategy.

In addition to the salt effect in nodule functioning, ion accumulation (mainly Na^+ and Cl^-) also induces cytotoxicity. Unless ions are stored in vacuoles, they have been described to induce damage of cellular components, disturbance of enzymatic activities and overproduction of ROS (Munss and Tester, 2008). Oxidative stress has also been

implicated in reduced performance of nodules grown in elevated salinity conditions (Tejera et al., 2004; Borucki and Sujkowska, 2008; Garg and Manchanda, 2008; Ben Salah et al. 2010) although to a much lesser extent than observed in droughted nodules. As it is shown in Fig. 2, the reduction in N_2-fixing activity by salt stress is also attributed to a reduction in cytosolic protein production by nodules, specifically leghemoglobin, (Delgado et al., 1994) or a degradation of leghemoglobin (López et al., 2008; Ben Salah et al. 2010). Such degradation could be explained by the up-regulation of proteases (with affinity for leghemoglobin) in the infected cells that would then produce catalytic Fe to react with H_2O_2 to produce ROS. It has been reported that there are significant differences in the antioxidant enzymes that protect nodular tissue in nodules exposed to salinity (Puppo and Halliwell, 1988; Tejera et al., 2004; Ben Salah et al., 2010). These studies have revealed that the down-regulation of the activity of enzymes such as superoxide dismutase (SOD), dehydroascorbate reductase (DR) and peroxidase could have negatively affected the integrity of the peribacteroidal membrane and consequently leghemoglobin content.

Although there are some discrepancies, the consensus in the literature is that carbohydrate availability and oxidative stress are the target points that modulate nodule functioning under salinity stress conditions (Fig. 2). Similar to droughted nodules, the reported studies also indicate an increase in osmoregulatory compounds.

5. High temperature

Despite its relevance, studies examining the effects of temperature on plant nodule performance are scarce and mainly focused on aboveground organ performance, giving little attention to nodule functioning (Zahran et al., 1999; Djedid et al., 2011).

Compared with Figs. 1 (drought), 2 (salinity) and 4 (CO_2), absence of detailed knowledge on nodule and root functioning under changing temperature conditions are reflected in Fig. 3. As it is shown in Fig. 3, our current knowledge on this topic shows that at the leaf, level, high temperature has been described to affect negatively photosynthetic performance. Stomatal closure and inhibited Rubisco activity would explain the depleted photosynthetic activity. At the belowground level, the optimum temperature range for root-nodule symbiosis for temperate legumes is between 15 and 25 °C, while for tropical legumes upper limits range between 27 and 40 ºC (Hungria and Franco, 1993a; Aranjuelo et al., 2007). Temperature might affect N_2 fixation directly or indirectly. Direct inhibition by temperature is a consequence of decreased nodule development, functionality and accelerated nodule senescence (Piha and Munns, 1987; Zhang et al., 1997; Aranjuelo et al., 2007). Indirect inhibition is related to temperature effects on root hair formation depression, reduction of nodulation sites and modified adherence of bacteria to root hairs (Frings, 1976; Hungria and Vargas, 2000).

The root infection process has been described as the component most affected by high temperature, with sensitivity located at the nodulation sites (Hungria and Franco, 1993a; Hungria and Vargas, 2000). As observed by previous studies (Pankhurst and Gibson 1973), elevated temperature has been described to inhibit the number of sites for nodulation, adherence of bacteria to root hairs, root-air penetration and infection-thread formation. The acceleration of nodule senescence has been implicated under elevated temperatures (Hungria and Franco, 1993). In a previous study conducted by (Aranjuelo et al., 2007) with exclusively N_2 fixing alfalfa plants exposed to elevated temperature conditions, it was shown that elevated temperature affected plant N content negatively. The absence of significant differences in nodule dry mass revealed that such a decrease

in N content was explained by the lower specific nodule activity (SNA) of the plants. Furthermore, the analyses of nodule plant and bacteroid fractions highlighted that the bacteroid fraction was more sensitive to temperature increase than the plant fraction (Aranjuelo et al., 2007). Although the lower photosynthetic rates of treatments exposed to elevated temperature decreased the leaf soluble sugar content, at the nodule level no significant differences were observed in this parameter (Fig. 3). The fact that in elevated temperature nodules MDH activity decreased to 50 % at the bacteroid level suggests that less malate entered mitochondria with a consequent effect on the tricarboxylic acid cycle and energy obtention for bacteroid consumption. When analysing this enzyme it must also be considered that the MDH also forms a complex with the AAT enzyme whose activity also increases in elevated temperature plants. In contrast to these findings, Hungria et al. (1989) observed that elevated temperature negatively affected enzymes involved in amino acid biosynthesis such as glutamine synthetase and glutamate synthetase and lowered synthesis of ureides.

Nevertheless, the limited studies available do show that poor nodule functioning in temperature stressed plants is not due to carbohydrate limitation (Fig. 3). Testing the potential limitations of nodule respiration and oxidative stress therefore require further investigation.

6. Elevated CO_2

Several authors (Serraj et al., 1998; Luscher et al., 2000; Rogers et al., 2006) have postulated that legumes, because they are capable of fixing atmospheric N_2, will have an advantage in plant growth over non- N_2-fixing plants. It has been noted that N_2- species show a larger stimulation of growth and photosynthetic rates in response to elevated

CO_2 than non-fixing species (Ainsworth and Rogers, 2007; Aranjuelo et al., 2013b). As it is represented in Fig. 4, the greater photosynthetic rate in legumes grown under high CO_2 conditions (Bertrand et al., 2007) would imply that there is a larger supply of organic C to nodules (Arrese-Igor et al., 1999; Cabrerizo et al., 2001). However, the initial stimulation in photosynthetic rates frequently disappears in a process described as "photosynthetic down-regulation" (Long et al., 2004; Ainsworth and Long, 2005; Aranjuelo et al., 2005; Erice et al., 2007). Imbalance between the photoassimilate source and the demand by the plant induces inhibition of the expression of genes that encode for different proteins belonging to the photosynthetic apparatus such as Rubisco, as well as a reduction in photosynthetic capacity (Long et al., 2004; Ainsworth and Long, 2005). Studies conducted in *Medicago sativa* exposed to elevated CO_2 have revealed that there is a specific decrease in Rubisco content (Aranjuelo et al., 2008; 2009).

The reduction in photosynthetic rates has been described as being conditioned by a plant's ability to develop new sinks (e.g. new vegetative or reproductive structures, enhanced respiratory rates) or to expand the storage capacity or growth rate of existing sinks (Aranjuelo et al., 2008). Taproots represent another potential C sink in legumes such as alfalfa (Erice et al., 2007). This specific storage organ contains the most important C and N (in perennial legumes) pools in the form of non-structural carbohydrates, soluble proteins and amino acids (Volenec et al., 1996; Avice et al., 2003; Meuriot et al., 2004b; Pembleton et al., 2010). Among the soluble proteins, vegetative storage proteins (VSP) represent up to 40% of the total soluble proteins (Avice et al., 1996b). A previous study analysing root performance under elevated CO_2 conditions in exclusively N_2 fixing alfalfa plants (Erice et al., 2007) highlighted a

specific increase in the VSP content. However, since this study did not characterise nodule and leaf function in those plants, the implications of VSP content in relation to nodule performance are unknown.

As mentioned above, photoassimilate partitioning toward nodules is a key point conditioning nodule functioning (Voisin et al., 2003ab). Since legumes form a symbiotic association with N_2-fixing bacteria, have an extra sink for any additional C that can be exchanged with the bacterial symbiont to enhance N_2 fixation (Udvardi and Day, 1997; Bertrand et al., 2007; Aranjuelo et al., 2013b). Studies conducted in exclusively N_2 fixing alfalfa (Aranjuelo et al., 2008; Sanz-Sáez et al. 2010) and pea plants (Cabrerizo et al., 2001) exposed to elevated CO_2 confirmed that nodule C sink strength (reflected as a larger dry mass and carbohydrate content) increased. However, such increases did not contribute to overcoming leaf carbohydrate build-up, with a consequent reducing effect on the photosynthetic capacity of these plants (Cabrerizo et al., 2001; Aranjuelo et al., 2008; Sanz-Sáez et al., 2010; Gillespie et al. 2012).

Concerning nodule functioning under elevated CO_2 conditions, a previous study conducted in *Pisum sativum* showed that although more N_2 was fixed at the plant level, the specific N_2 fixation of the nodules was not improved (Cabrerizo et al., 2001). Furthermore, as it is shown in Fig. 4, the larger photosynthetic rates of plants exposed to 1000 μmol mol^{-1} CO_2 were translated into larger nodule carbohydrate levels. Such an increase was explained by the elevated activity of enzymes involved in C metabolism like sucrose synthase, UDPG pyrophosphorylase and PEPc. However, this study also showed that specific N_2 fixation, together with the activity of enzymes involved in N metabolism such as glutamate synthase and aspartate aminotransferase, was not affected

by elevated CO_2 exposure. On the other hand, a study conducted in *Medicago sativ*a exposed to 700 μmol mol^{-1} CO_2 revealed that although plant level N_2 fixation increased under elevated CO_2, the larger photoassimilate availability did not contribute to increases in specific nodule N_2 fixation (Aranjuelo et al., 2008). Moreover, carbohydrate availability decreased in nodules of plants exposed to elevated CO_2. According to the same study, the fact that MDH, ATT, PEPc and isocitrate dehydrogenase (ICDH) activities decreased suggests that the deteriorated respiratory mechanism also was involved in the decline in nodule performance. Furthermore, as observed by other studies (Schulze et al., 1998; Nomura et al., 2006; Fischinger and Schulze, 2010), the lower activity of these enzymes might be linked to the decrease in dicarboxylic acids with key C-skeleton functions, which includes the malate required for N assimilation (see Fig. 4). These studies suggested that organic acid limitations were mainly attributed to compounds involved in N assimilation. This disparity in the results reflected in the up/down-regulation of the same compounds of Fig. 4, could be explained by the fact that legume responsiveness to the predicted CO_2 enhancement has been described as dependent on environmental conditions, plant species and bacteria strain (water availability, temperature, etc.) (Serraj et al., 1998; West et al., 2005; Aranjuelo et al., 2008; 2009; Fischinger et al., 2010; Bertrand et al. 2011). The lower shoot demand of plants exposed to elevated [CO_2] could be also involved in reduced nodule functioning. According to Serraj et al. (1999), exposure of *Glycine max* to elevated CO_2 that when the shoot N demand decreases, the concentration of N-transporting solutes declines with a consequent accumulation of products associated with the N_2 fixation in the nodules that negatively affected N_{ase} activity (Fig. 4). Although in *Pisum sativum* exposed to elevated CO_2 a decrease in protein and free amino acid content was also observed at the leaf level, which was opposite the

observations of (Serraj et al. 1999), no significant differences were observed in amino acid content in nodules (Cabrerizo et al., 2001).

In addition to the photoassimilates partitioned from aboveground organs towards nodules, recent studies (Fischinger and Schulze, 2010; Fischinger et al., 2010) suggest a role of direct nodule CO_2 fixation in nodule and plant functioning under elevated CO_2. As it is shown in Fig. 4, legume nodules fix substantial amounts of CO_2 largely through the combined activity of carbonic anhydrase (CA) and PEPc, resulting in carboxylation of phosphoenolpyruvate (PEP) (Fischinger et al. 2010). In order to analyse the role of nodule CO_2 fixation, these authors grew plants in a hydroponic system where the CO_2 concentration could be differentiated between above and below ground organs. After 3 weeks of exposure to high CO_2 conditions the authors observed that N_2 fixation increased in the plants. Furthermore, the nodule and xylem amino acid content was also observed to increase under these conditions. The increase was attributed to bigger nodules and more efficient N_2 fixation. The enhancement of N_2 fixation was translated into a larger biomass production in plants exposed to elevated CO_2. Interestingly, the same authors also highlighted that the additional C skeletons provided by PEP (Fischinger and Schulze 2010) improved the N assimilation and transport to shoots. As shown in Fig. 4, according to the model proposed by (Fischinger and Schulze, 2010), instead of entering the TCA cycle, PEP is carboxylated and transformed into oxaloacetate (OAA), which is required for aspartate and asparagine synthesis. The stimulation of such a pathway under elevated CO_2 conditions would imply a more efficient use of C, N and energy.

Finally, we would also like to observe that in spite of previous characterisation of oxidative status at the leaf level (Erice et al. 2007; Aranjuelo et al., 2008; Gillespie et al. 2012), to our knowledge no oxidative stress characterisation has been conducted in nodules of legumes exposed to elevated CO_2 conditions. As also observed at the leaf level, we should not ignore that antioxidant status was improved in the nodules of the plants in these studies. Such findings highlight the importance of improving our knowledge on this topic.

Despite some discrepancies, in general terms the literature indicates that larger leaf and or plant level photosynthetic rates do not always reflect a larger nodule photoassimilate availability. Bibliography highlights the relevance of [CO_2] to the organic acid content and respiration of nodules. Aboveground protein depletion suggests that the accumulation of N compounds could have also affected nodule functioning.

7. Nodule performance under interacting abiotic conditions

Most experiments analysing nodule functioning under changing environmental conditions have been conducted in optimal growth conditions. However, analyses of the CO_2 effect and its interaction with other environmental conditions are of great relevance because the responsiveness of plants to enhanced CO_2 has been shown to differ with temperature, and soil nutrient availability, etc. (Aranjuelo et al., 2006; Erice et al., 2006). Moreover, in the field different stresses often occur simultaneously, such as high temperatures and drought periods, especially in semi-arid or drought-stricken areas. Investigations performed on field crops as well as on model plants subjected to combined heat and drought stress have shown that the combination of these two stresses has a stronger detrimental effect on plant growth and productivity compared to each

single stress (Valladares and Pearcy, 1997; Aranjuelo et al., 2006; Erice et al., 2006; Annicchiarico et al., 2011). Since the main goal of those studies is to further understand plant performance under predicted climate scenarios, it is important to conduct studies as realistically as possible. This is why studies where plants are subjected to interacting growth conditions are of great relevance.

7.1 Elevated temperature and water availability

A previous study conducted by Aranjuelo et al. (2007) analysed, the effect of elevated temperature (25 *versus* 28.5 ºC) and sustained low water availability (watered at 50 % of control plants) in nodule performance. At the leaf level, the temperature increase inhibited photosynthetic performance as a result of the lower Rubisco activity. However, water availability had no effect on these parameters. TSS were also lower in the leaves. At the nodule level, the plant fraction (compared to bacteroid fraction) was more sensitive to temperature and water availability interactions. More specifically, our data showed that although MDH and ATT activities increased in elevated temperature under optimal water availability conditions, under low water availability a temperature increase negatively affected PEPc and ATT activities. The absence of differences in PEPC and ATT -specific activities showed that lower enzyme activities associated with elevated temperature and drought were a consequence of depleted protein content. These limitations might explain the inhibitory effect of elevated temperature on nitrogen fixation. The absence of a temperature effect on TSS in nodule tissue in this experiment suggests that decreased N content was not caused by a reduction in carbohydrate supply.

7.2 Elevated CO_2 and temperature effect

In a previous study conducted by (Aranjuelo et al., 2008), exclusively N_2 fixing alfalfa plants were exposed to elevated CO_2 (≈ 400 *versus* ≈ 700 μmol mol^{-1}) and temperature (≈ 19 *versus* ≈ 24 ºC). This study showed that elevated CO_2 only increased plant biomass in elevated temperature conditions as a consequence of their larger photosynthetic rates. The study showed that although plants fixed more N_2 at the plant level, the nodule N_2 fixation efficiency decreased, especially in elevated temperature treatments. The lower efficiency in elevated CO_2 and temperature conditions was explained by the depletion of nodule TSP content. Although the temperature increase affected Rubisco content positively, the data suggested that such an increase did not overcome the elevated CO_2-associated depletion in Rubisco content. Consequently. it is very likely that regardless of ambient temperature, the lower shoot N demand was also involved in the reduced nodule functioning. On the other hand, diminishment of nodule starch content under elevated CO_2 conditions suggests that C skeleton availability could be also linked to the lower N_2 fixing efficiency. Interestingly, this study indicated that the larger amount of photoassimilates was invested in the production of more nodule biomass and not in carbohydrate partitioning towards nodule metabolism.

7.3 Elevated CO_2 and water availability

The interaction of both factors in N_2 fixation has been considered in previous studies (Cabrerizo et al. 2001; Serraj 2003a; Aranjuelo et al. 2008, Rogers et al. 2009). Serraj (2003a) observed in *Glycine max* exposed to elevated CO_2, that although no significant differences were detected in dry matter (DM) under fully watered conditions, droughted plants exposed to 700 μmol mol^{-1} CO_2 produced more biomass than the corresponding ambient CO_2 treatments. Furthermore, N_2 fixation proved to be more drought tolerant than CO_2 fixation; it was only when the applied drought was severe that N_2 fixation

decreased. The fact that even under ambient CO_2 conditions the soluble sugar content increased in drought conditions negated carbohydrate limitation as the main factor conditioning N_2 fixation. Drought associated decreases in N_2 fixation have been linked to increases in ureides, amides and other amino acids (Serraj et al., 2001; Serraj, 2003a; Rogers et al., 2009). Decreased ureide levels detected in *Glycine max* nodules exposed to elevated CO_2 and drought conditions by (Serraj, 2003a) showed that the maintenance of shoot N demand contributed towards overcoming N feed-back inhibition of N_{ase}. The higher carbohydrate levels detected in plants in this study suggests that more C was available for synthesis or transport of N compounds such as ureides, and amino acids etc. Although (Serraj, 2003a) did not provide any water status data, the lower stomatal opening of plants exposed to elevated CO_2 should also be taken into account because it suggests that in many experiments elevated CO_2 increased the time to reach a particular water stress (De Luis et al., 1999; Rogers et al., 2009). Therefore, when analysing the factors explaining the CO_2 x H_2O interaction and nodule functioning, such points should be considered. A previous study conducted by (Aranjuelo et al., 2009) where exclusively N_2 fixing *Medicago sativa* plants were grown under elevated CO_2 and sustained low water availability (watered at 50 % of pot capacity), it was evident that the CO_2 associated increase in DM was only observed in fully watered plants. Interestingly, the data also suggested that the reduction in shoot N demand (reflected by the TSP and especially Rubisco depletion) affected nodule activity negatively (MDH and GOT) particularly in water-limited conditions. The higher content of TSS, especially under optimal water availability conditions, means that the concept of C limitations on nodules can be discarded.

8. New methodologies for a greater understanding of nodules performance

Knowledge of the molecular and physiological basis of plant-microbe interactions and their responses to abiotic stress is of vital importance because it should lead to better and more efficient nitrogen-fixing cultivars. In recent years the use of novel methodologies (such as molecular genetics, metabolic analysis and isotope tracing) have enabled significant progress in understanding the C and N exchange between plants and nodules (Larrainzar et al., 2007; Marino et al., 2007; Aranjuelo et al. 2013a). However, as mentioned above, much remains to be learned about the biochemical and physiological basis of the functioning of nodules and their interaction with plants. Therefore, experiments combining different methodologies of studying plants and microbes in an integrated way are preferred so that a broader view can be seen. Among other methodologies, recent advances in nodule proteomics, metabolomics and fluxomics have provided novel information concerning nodule functioning within a Climate Change context.

8.1 Transcriptomics

Molecular bases involved in adaptations to different abiotic constraints can be explored using genomic tools (such as transcriptomics) in order to have a genome-wide scale of stress responses (Gruber et al., 2009; Sanchez et al., 2010; Kang et al., 2011; Zahaf et al. 2012).

Recently, transcriptome analysis of legumes have identify different genes that respond to drought (Buitinik et al., 2006; Chen et al., 2008; Foito et al., 2009; Kang et al., 2011), salt stress (Zahaf et al., 2012; Postnikova et al., 2013), high temperatures (Soares-Cavalcanti et al., 2012) and elevated CO_2 (Ainsworth et al., 2006). In general, hundreds or thousands of gene expression appear to be altered under changing environmental conditions. Several groups of stress-regulated genes have been found to be altered under

droughted. As an example genes involved in ABA biosynthesis and osmotic adjustment, antioxidants, genes encoding vegetative storage proteins and raffinose biosynthesis (Foito et al., 2009; Kang et al., 2011). The importance of the expression of genes related with root growth under salt conditions has also been remarked as an important adaptive factor (Zahaf et al., 2012). In general, complementary transcriptomics and proteomics or metabolomics studies tend to be more integrative to understand global responses to different stresses.

In this sense, the current Gene Expression Atlas project (MtGEA) dealing with the characterization of gene expression profiles for the majority of *M. truncatula* genes covering different organs (such as roots, nodules, stems, petioles, leaves, flowers, etc.) will be characterized when subjected to various kinds of abiotic and biotic stresses. Once the annotation of the *M. truncatula* genome will be complete (Boscari et al., 2013), this could represent an useful resource for legume functional genomics, which will aid gene function determination, biological discovery, and molecular breeding efforts. (Benedito et al., 2008: He et al., 2009).

8.2 Proteomics

Proteome is a reference to the total set of proteins encoded by the genome of an organism; "proteomics" can be understood as the global study of the proteins comprising the proteome, including the changes in structure and abundance in response to developmental and environmental conditions. Currently, most of the studies analysing nodule protein performance in N_2 fixing plants have been focused on specific proteins such as N_{ase}, PEPc, ATT, MDH, ICDH, GS and GOGAT. However, recent proteomic characterisations conducted in *Medicago truncatula* (Larrainzar et al., 2007; 2009) and *Medicago sativa* (Aranjuelo et al., 2013a) exposed to drought conditions reveal that in addition to those proteins, the nodule protein profile was much more

affected. In case of *Medicago truncatula* a decline in the levels of bacteroid proteins involved in BNF and C metabolism was observed, along with an up-regulation in protein biosynthesis, probably as an adaptation to the water deficit imposed. In contrast, the variations in enzymes related to N assimilation were found to not correlate with the reduction in BNF, suggesting that these enzymes do not have a role in the regulation of N_2 fixation. In the case of *Medicago sativa*, the proteomic approach revealed the stimulation of the anaplerotic pathway, which could have contributed towards sustaining the provision of C skeletons for amino acid synthesis (e.g. glutamate and proline). These studies highlight the fact that the direct evaluation of protein expression through proteomic analyses and the application of integrated system approaches are highly advantageous for the identification of key proteins involved in plant responsiveness to varying environmental conditions. However, despite their relevance, proteomic characterisations conducted in N_2 fixing nodules are still scarce.

8.3 Metabolomics

The metabolism of plants is highly flexible and can be conditioned by different factors (genetic, environmental, spatial, etc.) that will determine the identity and abundance of different metabolites (Fiehn et al., 2008). Under drought conditions, many metabolites, such as hexoses, are believed simply to accumulate (Muller et al. 2011, and references therein). Further, minor sugars (e.g. trehalose and mannitol), amino acids (e.g. proline), and organic acids (e.g. malate, fumarate, and isocitrate) also appear to accumulate under water restriction. Although recent publications include a more detailed metabolomic characterisation (Larrainzar et al., 2009; Aranjuelo et al., 2011; Kang et al, 2011), metabolite patterns and their coordinated changes between plant compartments are unclear. The influence of drought on nodule metabolic pathways and associated changes

in metabolite exchange between nodules and other plant organs (which may also cause some metabolic pools to vary) are still uncertain. Although, metabolomics is expected to provide new insights into plant's performance, metabolic profiling gives a snapshot of one plant/organ/tissue state at a given moment.

8.4 Stable isotopes

Different technologies have been used to monitor metabolic fluxes (Suthers et al., 2007; Sekiyama and Kikichi, 2007) and the use of labelling strategies combined with stable isotope analysis seems to be a good tool to enhance our understanding of metabolic dynamics. The stable isotopic composition of carbon ($\delta^{13}C$) has proved to be a very useful tool that provides relevant information on the exchange of C/N between the different organs of legumes (Avice et al., 1996a; Voisin et al., 2003b; Meuriot et al., 2004a; Aranjuelo et al., 2008; Fischinger and Schulze, 2010), cereals (Schnyder 1992; Gebbing et al., 1998; Aranjuelo et al., 2011). Plants grown in environments with modified isotopic composition incor-porate the tracer in C/N-containing compounds of the plant (Avice et al., 1996; Molero et al., 2011) providing essential information about the C and N sinks to which the recently fixed C/N is delivered. Isotopes enable two powerful approaches: (1) dynamic analysis of time-course data for the distribution of an isotopic label, and (2) steady-state analysis of metabolic labelling patterns under conditions of isotopic steady state. Pulse labelling of the organ or the cell with a tracer, such as ^{15}N and ^{13}C, enables analysis of the further partitioning of the label into different compounds of different plant organs (Aranjuelo et al. 2009b; 2011; 2013a). During recent years, new protocols have been developed to analyse the isotopic composition of specific compounds such as proteins, amino acids and sugars (especially glucose, fructose and sucrose) (Voisin et al., 2003; Molero et al., 2011). The

development of those protocols has provided key information concerning the C and N exchange between leaves and nodules. A recent study (Molero et al., 2011) conducted in exclusively N_2 fixing alfalfa plants labelled with ^{12}C and $^{15}N_2$ revealed that the isotopic enrichment of $^{15}N_2$ in amino acids was greater for leaves than for nodules, suggesting that part of the fixed N_2 was recruited to protein synthesis in the nodule or was in the form of NH_3 (Molero et al., 2011). Furthermore, this study also allowed the identification of the distribution of C and N among amino acids, and between the plant and the symbiont, in different amino acid metabolic pathways. Furthermore, for the first time, ^{12}C and $^{15}N_2$ labelling revealed that GABA and glycine were major C-transporting amino acids from the leaves to the nodules.

9. Future and perspectives

Biological nitrogen fixation (BNF) constitutes one of the potential N-source solutions for farmers using little or no fertiliser, and it plays a key role in sustainable legume production. In order to have a real impact on farmers' fields, it is important to fully understand the mechanisms by which legumes resist or tolerate drought, high temperature, salinity etc. The fact that as it is summarized in this manuscript, carbohydrate availability and oxidative stress are common processes conditioning nodule performance under drought and salinity conditions, highlights the relevancy of those factors in the correct nodule functioning. Similarly, shoot N demand has also been identified as a target point limiting nodules performance in plants exposed to drought and elevated [CO_2]. Although the current knowledge identifies carbohydrate availability, oxidative stress and shoot N demand like key processes conditioning nodule performance, absence of complete characterization in wide spectra of environmental conditions reveals the need to increase our knowledge in this topic. A

better knowledge on those processes could be of great importance for future breeding programmes.

The advances in our understanding of abiotic resistance together with the application of genomics - i.e. molecular markers developed during genomics projects (i.e. Legume Crops Genome Initiative (LCGI), Gepts et al., 2005) - will assist breeders in developing new, resistant cultivars. Breeding programs that aim to release commercially successful varieties typically grow different genotypes in a target set of environments in order to undertake phenotypic selection for forage yield, better performance under favorable or unfavorable conditions. The combination between current phenotypic selection and the recent incorporation of molecular marker assisted breeding could accelerate alfalfa improvement (Li and Brummer, 2012). Therefore it is important to consider the genetic variability in plant and bacteria resistance/tolerance to abiotic stresses individually and the genetic variability in the interaction between the plant and the bacteria. Recently, techniques have been developed to utilize marker assisted selection in alfalfa breeding programs (Mengoni et al., 2000; Flajoulot et al., 2005). As it has been shown in this review, For this propose, the understanding of molecular mechanisms involved in the response of plants to the combination of two of the most deleterious stresses could lead to the development of new strategies and tools for enhancing stress tolerance via genetic manipulation, as already demonstrated by the improved plant stress tolerance achieved by ectopic expression or over-expression of several stress induced genes. There is the need to further analyse plant stress responses at the molecular level due to the complexity of events associated with the sensing of stress and the activation of specific pathways. This complexity is even greater when a combination of different stresses such as water x N availability x temperature x CO_2 are simultaneously applied. The

application of "omic" methodologies (in combination with the more classical agronomic and physiological studies) might contribute to the elucidation of mechanisms responsible for plant responsiveness to changing climate The combination of physiology, metabolomics, proteomics and gene expression analyses will provide us key information concerning the plant mechanisms that condition the best or worst performance under a wide range of Climate Change conditions.

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Figure legends

Figure 1. Model representing the most visible changes in carbon and nitrogen primary metabolism of leaves, roots, and nodules of exclusively N_2 fixing plants exposed to drought. This figure is a tentative summary representing the main findings described in the literature. Thick and broken arrows represent (respectively) increased and decreased pathways. Arrows up (↑) and down (↓) represent compounds whose content is up or down regulated. Aa, amino acids; A_n, net photosynthesis; Asn, asparagine; C_i, intercellular CO_2 concentration; ETC, electron transport chain; g_s, stomatal conductance; Lb, leghemoglobin; N_{ase}, nitrogenase; Pro, proline; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; Suc, sucrose; SuSy, sucrose synthase; TCA, tricarboxylic acid pathway; VSP, vegetative storage protein.

Figure 2. Model representing the most visible changes in carbon and nitrogen primary metabolism of leaves, roots, and nodules of exclusively N_2 fixing plants exposed to salinity. This figure is a tentative summary representing the main findings described by the bibliography. Broken arrows represent decreased pathways. Arrows up (↑) and down (↓) represent compounds whose content is up or down regulated. Question marks (?) refer to parameters whose performance is scarcely known. Asn, asparagine; ETC, electron transport channel; Lb, leghemoglobin; N_{ase}, nitrogenase; ROS, reactive oxygen species; Suc, sucrose; SuSy, sucrose synthase; TCA, tricarboxylic acid pathway.

Figure 3. Model representing the most visible changes in carbon and nitrogen primary metabolism of leaves, roots, and nodules of exclusively N_2 fixing plants exposed to elevate temperature. This figure is a tentative summary representing the main findings described by the bibliography. Broken arrows represent decreased pathways. Arrows up (↑) and down (↓) represent compounds whose content is up or down regulated. Question marks (?) refer to parameters whose performance is scarcely known. A_n, net photosynthesis; Asn, asparagine; ATT; aspartate aminotransferase; C_i, intercellular CO_2 concentration; ETC, electron transport channel; g_s, stomatal conductance; Lb, leghemoglobin; MDH, malate dehydrogenase; N_{ase}, nitrogenase; RWC, relative water content; Suc, sucrose; TCA, tricarboxylic acid pathway; TSP, total soluble proteins.

Figure 4. Model representing the most visible changes in carbon and nitrogen primary metabolism of leaves, roots, and nodules of exclusively N_2 fixing plants exposed to elevated CO_2. This figure is a tentative summary representing the main findings

described by the bibliography. Thick arrows represent enhanced pathways. Arrows up (↑) and down (↓) represent compounds whose content is up or down regulated. Question marks (?) refer to parameters whose performance is scarcely known. Aa, amino acid; A_n, net photosynthesis; Asn, asparagine; Asp, aspartate; C_i, intercellular CO_2 concentration; ETC, electron transport channel; g_s, stomatal conductance; Lb, leghemoglobine; N_{ase}, nitrogenase; OAA, oxalacetate; PEP, phosphoenolpyruvate; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; Suc, sucrose; SuSy, sucrose synthase; TCA, tricarboxylic acid pathway; TSS, total soluble sugars; VAZ, violoxhantine, enteraxantine and zeaxanine cycle; VSP, vegetative storage protein.

Figure 1.

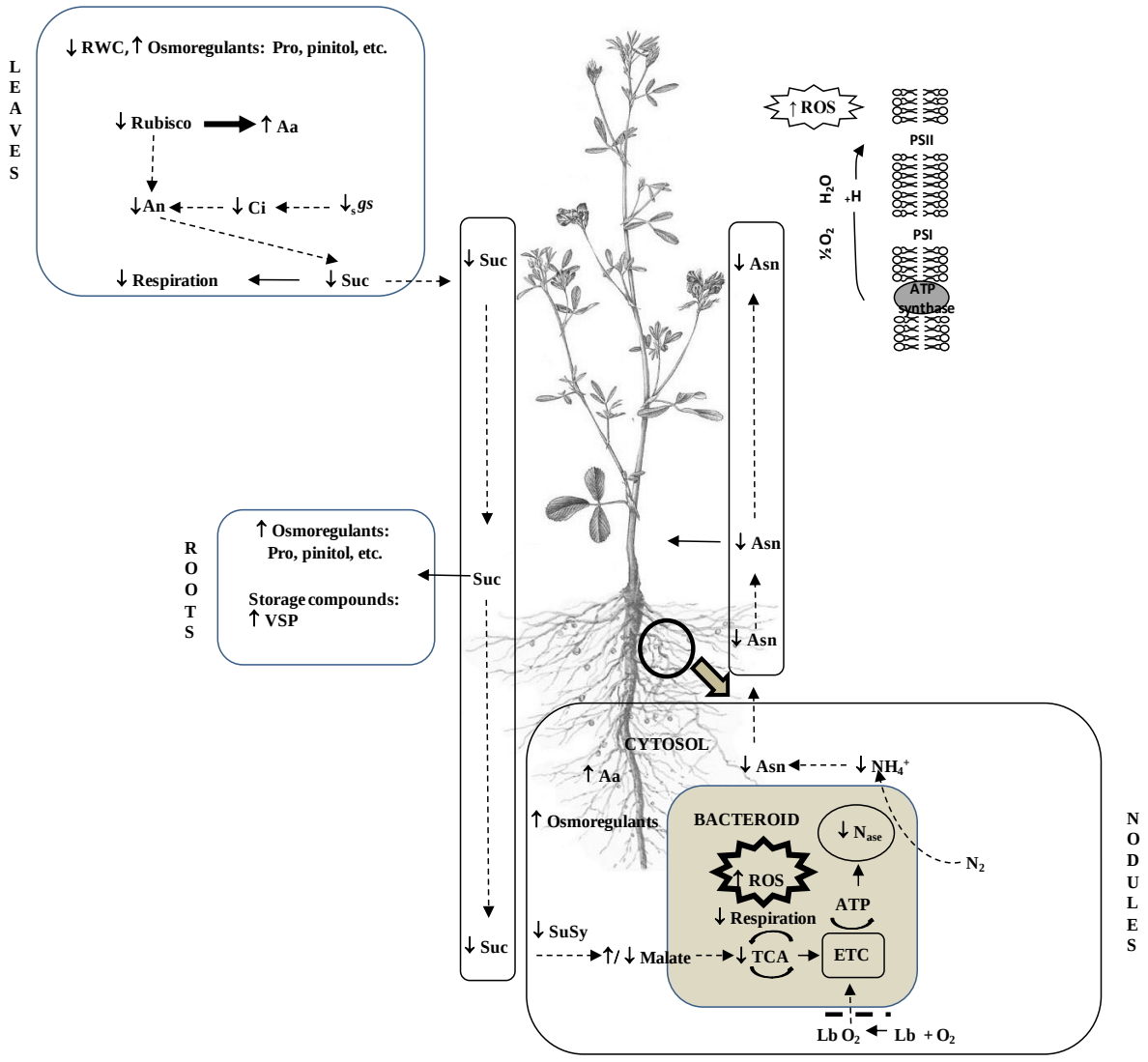
LEAVES
↓ RWC, ↑ Osmoregulants: Pro, pinitol, etc.
↓ Rubisco
↑ Aa
↓An
↓ Ci
↓sgs
↓ Respiration
↓ Suc
↓ Suc
↑ ROS
PSII
PSI
ATP synthase
H2O
+H
½ O2
↓ Asn
↓ Asn
↓ Asn
ROOTS
↑ Osmoregulants: Pro, pinitol, etc.
Storage compounds: ↑ VSP
Suc
↓ Suc
CYTOSOL
↑ Aa
↓ Asn
↓ NH4+
↑ Osmoregulants
BACTEROID
↓ Nase
N2
↑ ROS
ATP
↓ Respiration
↓ SuSy
↑/↓ Malate
↓ TCA
ETC
Lb O2
Lb + O2
NODULES

Figure 2.

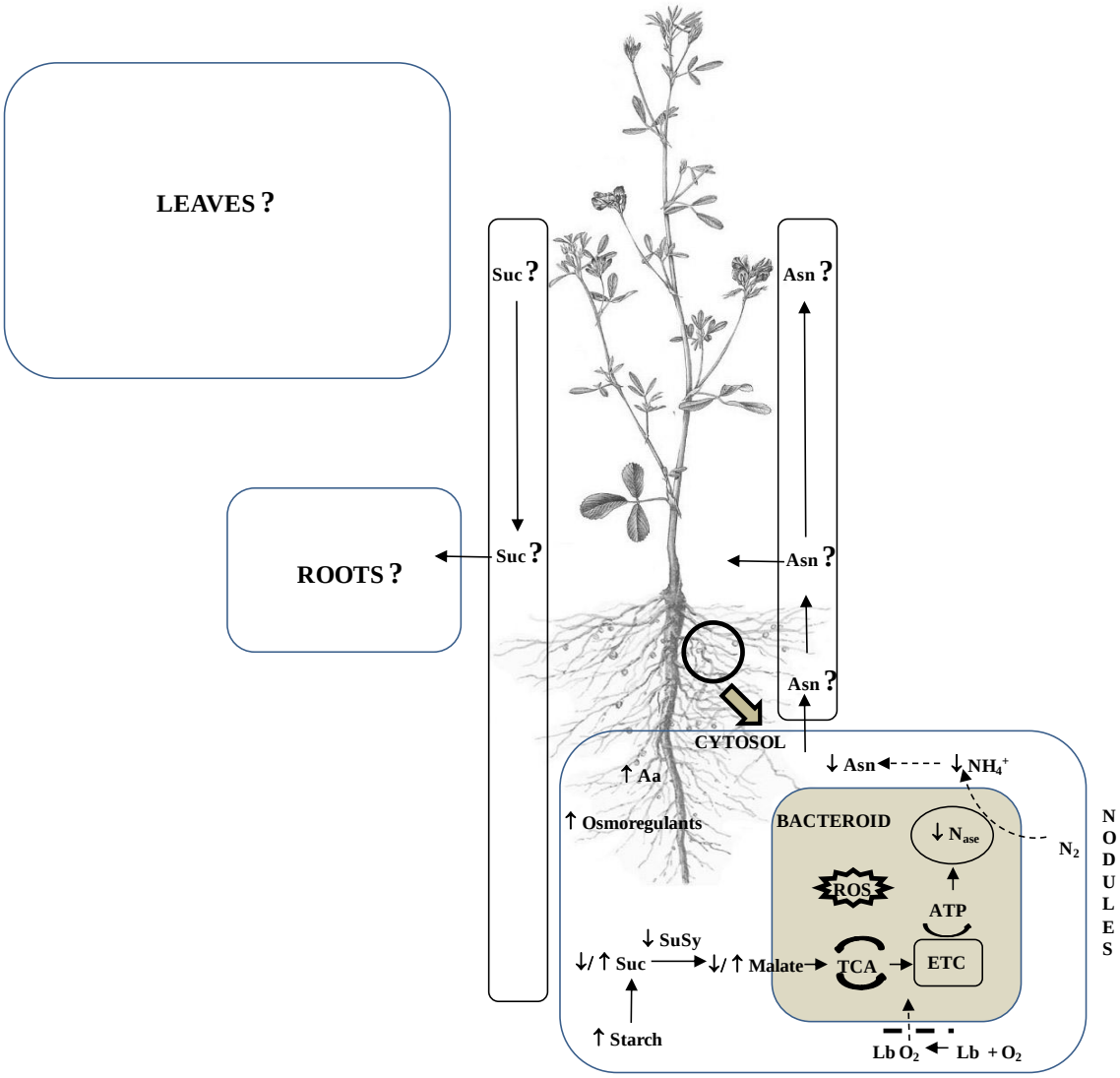
LEAVES ?
ROOTS ?
Suc ?
Suc ?
Asn ?
Asn ?
Asn ?
CYTOSOL
↓ Asn
↓ NH_4^+
↑ Aa
↑ Osmoregulants
BACTEROID
↓ N_{ase}
N_2
ROS
ATP
↓ SuSy
↓/↑ Suc
↓/↑ Malate
TCA
ETC
↑ Starch
Lb O_2
Lb + O_2
NODULES

Figure 3.

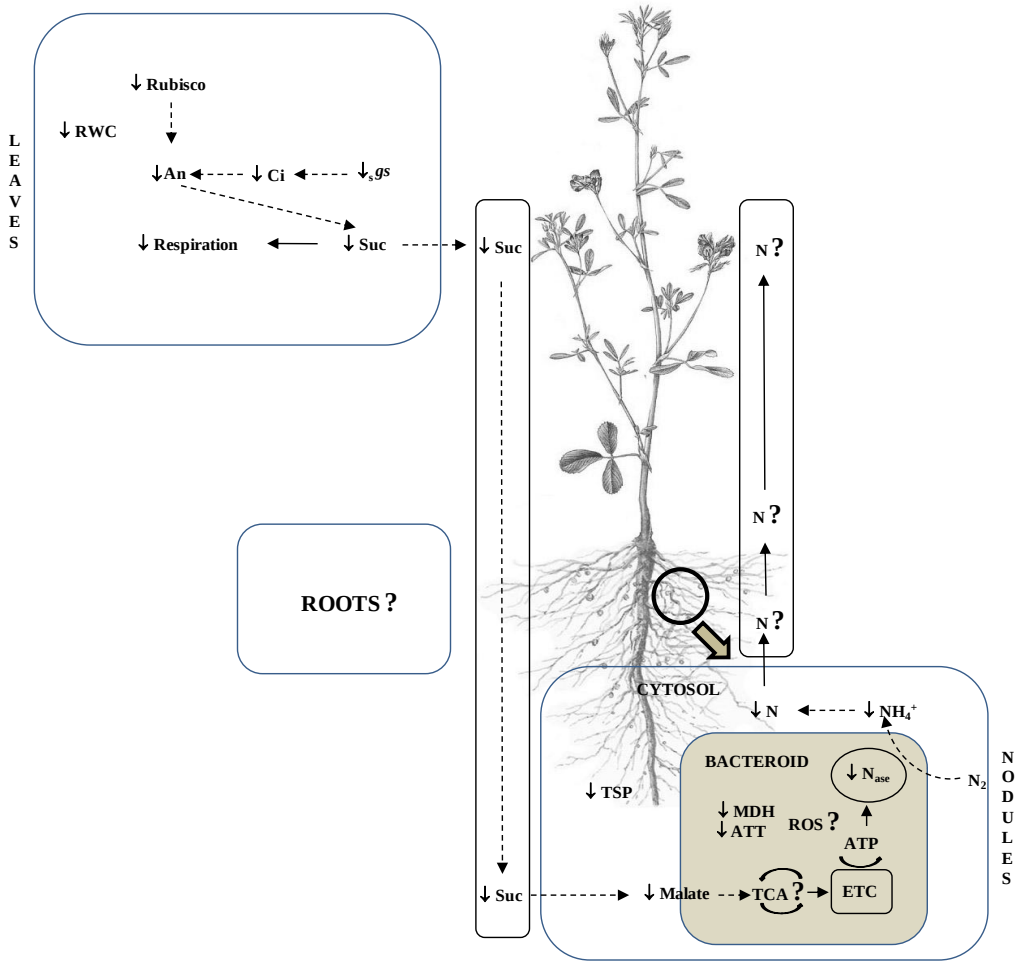
LEAVES
↓ Rubisco
↓ RWC
↓An
↓ Ci
↓$_s$gs
↓ Respiration
↓ Suc
↓ Suc
N ?
N ?
N ?
ROOTS ?
CYTOSOL
↓ N
↓ NH_4^+
BACTEROID
↓ N_{ase}
N_2
↓ TSP
↓ MDH
↓ ATT
ROS ?
ATP
↓ Suc
↓ Malate
TCA ?
ETC
NODULES

Figure 4

Figure 4.