Photosynthesis, N_2 fixation and taproot reserves during the cutting regrowth cycle of alfalfa under elevated CO_2 and growth.

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Abstract

Future climatic conditions, including rising atmospheric CO_2 and temperature, water availability limitations, etc. may increase photosynthesis and, consequently, plant production. A larger knowledge on legume performance under predicted growth conditions will be crucial for the safeguard crop management and the extended cultivation surface of those plants in the near future. N_2 fixation, is a key process conditioning plant responsiveness to varying growth conditions. It is likely that under future environments, due to the higher photosynthate availability, as a consequence of the higher growth rate under elevated CO_2. However, as described in the literature,

photosynthesis performance is frequently down-regulated (acclimated) under long-term exposure to CO_2, especially when interacting with stressful temperature, and water availability conditions. As growth responses to elevated CO_2 are dependent on sink-source status, it is generally accepted that down-regulation occurs in situations with insufficient C sink plant capacity. Alfalfa management involves the cutting of shoots, which alters the source-sink relationship and thus the photosynthetic behaviour. As the growth rate decreases at the end of the pre-cut vegetative growth period, nodulated alfalfa plants showed photosynthetic down-regulation, but during regrowth following defoliation, acclimation to elevated CO_2 disappears. The shoot harvest also leads to a drop in mineral N uptake and C translocation to the roots resulting in a reduction in N_2 fixation due to the dependence on photosynthate supply to support nodule function. Therefore, the production of new shoots during the first days following cutting requires the utilization of reduced C and N compounds previously stored in reserve organs. The stored reserves are mediated by phytohormones such as methyl jasmonate and abscisic acid and in situations where water stress reduces shoot production this potentially enables the enhancement of taproot protein levels in nodulated alfalfa, which may lead to these plants growing in better conditions in the following cut/regrowth cycle. The further knowledge of legume performance under predicted climate change conditions will be crucial the development of varieties with better adaptation in order to achieve greater and more efficient production values. Furthermore, for this purpose it is going to be necessary to improve existing methodologies and create new ones for phenotype characterization. Such knowledge will provide key information for future plant breeding programmes.

I. Introduction

Since the beginning of the industrial revolution in the 18th century, the amount of CO_2 emitted by man has been the consequence of economic development and a population that has quadrupled to 6.4 billion during the last one hundred years (Krausmann et al., 2009). In 1750, the atmospheric concentration of CO_2 was around 280 ppm, nowadays it reached 389 ppm and currently increases at 1.9 ppm per year on average (Intergovernmental Panel on Climate Change; IPCC, 2007). According to the predictions of the IPCC, at the end of the present century this concentration may be around 700 ppm.

The primary effect of increasing CO_2 is photosynthetic enhancement in C_3 plants, and consequently increased plant productivity (Daepp et al., 2000). So in the absence of other growth limiting factors such as mineral nutrition or water availability, photosynthesis may be enhanced under elevated CO_2 conditions (Campbell et al., 1988; Chaves and Pereira, 1992). Increasing CO_2 will favour carboxylation over photorespiration of ribulose-1,5-bisphosphate (RuBP) (Andrews and Lorimer, 1987). Moreover, the reduction in stomatal conductance by elevated CO_2 will lead to a lower leaf transpiration, and consequently, to a higher water use efficiency (WUE) (Drake et al., 1997).

Due to the greenhouse effect, elevated CO_2 also has indirect effects related to the increase in temperature. By the end of the present century, global warming may lead to a temperature increase of 4ºC (IPCC, 2007). As highlighted by previous studies (Drake

and Leadley, 1991; Long, 1991; Morison and Lawlor, 1999; Aranjuelo et al., 2005ab, 2008), plant performance under elevated CO_2 is affected by ambient temperature. Those studies showed that temperature enhancement, increased photosynthetic performance of plants exposed to 700 ppm due to the kinetic properties of rubisco (Long, 1991) and partly due to better utilisation of the end products of photosynthesis through increased sink metabolism at elevated temperatures. Enhanced C sink strength of plants exposed to 700 ppm and avoided leaf carbohydrate build up with the consequent avoidance of photosynthetic down-regulation and the increase in plant biomass. Another point of major concern in elevated CO_2 and temperature studies is the effect of temperature increase in plant population dynamics (Bélanger et al. 2002; 2005). In mid- and high latitudes, characterized by seasonal periods of dormancy (winter) and active growth (summer), plant phenology is mainly driven by temperature (Chmielewski et al., 2004). Thus, an indirect effect of increasing air temperatures means the prolongation of the growing season (Menzel and Fabian, 1999; Chmielewski and Rötzer, 2001).

Most climate scenarios are predicted to be affected by climate change, and in the case of the Mediterranean area (IPCC, 2007) resulting in increasing the aridity with subsequent water stress for the crops. Water deficit is the most important environmental factor limiting photosynthesis, plant growth and production in the Mediterranean climate (Chaves et al., 2002). Moreover, several experiments have revealed that the plant production response to elevated CO_2 can be affected significantly by soil water availability (Owensby et al., 1999; Körner, 2000; Volk et al., 2000).

The interaction between CO_2 concentration and the climate parameters (temperature, precipitation, relative humidity, etc.) makes studies combining different growth conditions fundamental to evaluation of the possible effects of future climatic conditions. The objective of this review is to summarize the current knowledge concerning the alfalfa response to elevated CO_2, temperature and drought conditions in order to identify the target processes that condition photosynthesis behaviour, N_2 biological fixation and consequently plant growth under predicted climate change conditions. This information might be applied to define the different factors conditioning plant adaptation to varying growth conditions in order to design new strategies and assign priorities in future breeding programs by providing important information for establishing selection criteria.

II. Nitrogen biological fixation is stimulated by increasing CO_2

The increase in crop productivity under elevated CO_2 as a consequence of the higher photosynthetic rates, is dependent on other essential elements, and low productivity resulting from nutrient limitations is not usually remedied by growth in higher atmospheric CO_2 (Pritchard and Amthor, 2005). In several cases, N availability is a critical factor, limiting plant growth or increasing the response to elevated CO_2. Although the influence of CO_2 on plant growth and photosynthetic activity has been studied extensively (Long et al. 2004; Nowak et al. 2004; Ainsworth and Long 2005; Erice et al. 2006b; Aranjuelo et al. 2005ab; 2008; 2009; Sanz-Sáez et al. 2010), less attention has been given to the role of nodule activity in plant performance under elevated CO_2. Nodule activity depends on photosynthates supplied by the plant, which are used by the nitrogenase enzyme as a source of energy and reducing power to fix N_2

(Larrainzar et al., 2009; Aranjuelo et al. 2011). This cycle coupling causes nitrogenase activity in plants to be regulated by photosynthesis (carbon supply), N availability (N source strength) and N demand (N sink strength) (Aranjuelo et al., 2007). Similarly, the products of N_2 fixation are exported throughout the plant via the xylem to other organs where N is required, for example, protein and or osmoregulant synthesis (Ladrera et al., 2007; Aranjuelo et al. 2011). This coupling results in the regulation of nitrogenase activity in plants by photosynthesis (carbon supply), nitrogen availability (N source strength), and N demand (N sink strength). This growth enhancement associated with the exposure to elevated CO_2 may lead to a higher N demand, so it is expected that the plant response to CO_2 may be limited by N availability (Hartwig, 1998; West et al., 2005). In this context, legumes are particularly interesting due to their symbiotic relationship with N_2-fixing bacteria, which provides them with N autonomy. That is why, under the N limitation situations that usually occur in nature, growth under elevated CO_2 is more stimulated in legumes than in non-legumes so long as the phosphate availability is high enough (Hebeisen et al., 1997; Ainsworth and Rogers, 2007). Many studies have confirmed that most N_2-fixing legumes increase their level of N_2 fixation per plant under elevated CO_2 conditions (Hartwig, 1998; Rogers et al., 2006). This phenomenon is possible because of the higher growth rate under elevated CO_2. Furthermore, if current photosynthesis determines nitrogenase activity, then an increase or decrease in the atmospheric CO_2 concentration would also cause an immediate increase or decrease in nitrogenase activity (Hartwig, 1998). Most authors agree on the fact that exposure of N_2 fixative plants to elevated CO_2 will stimulate symbiotic fixation through a greater supply of carbohydrates to the nodules.

III. Initial CO_2 stimulus on photosynthesis and N_2 fixation is followed by a down-regulation (acclimation)

As mentioned above under optimal growth conditions, elevated CO_2 concentration will favour the carboxylation over oxygenation carried out by rubisco, with consequent gains in biomass and yield. Nevertheless, frequently this response to CO_2 is not maintained over the long term and photosynthesis declines (Aranjuelo et al., 2005; 2009a; Erice et al., 2006a; Ainsworth and Rogers, 2007). This process, often referred as "down-regulation" (Saralabai et al., 1997), may be due to two main causes: 1) Stomatal limitations like those resulting from lower leaf conductance at elevated CO_2 concentration (Sánchez-Díaz et al., 2004); or 2) Metabolic limitation, usually attributable to a reduced carboxylation activity efficiency (Aranjuelo et al., 2005; Erice et al., 2006b) and/or a reduced amount of rubisco at elevated CO_2 (Urban et al., 2003; Aranjuelo et al., 2005).

One of the parameters that could influence photosynthetic down-regulation is the modification of C sink-source status (Urban et al., 2003). Growth responses to elevated CO_2 depend on the ability of plants to develop new sinks or expand the existing ones (Ceulemans, 1997; Aranjuelo et al., 2009a). In this sense, many studies suggest that down-regulation is the consequence of an insufficient C sink plant capacity (Ainsworth et al., 2004; Morgan et al., 2001; Erice et al., 2006a) as has been revealed in recent studies analysing C isotopic composition ($\delta^{13}C$) (Aranjuelo et al., 2008). At the whole-plant level this results when photosynthesis exceeds the capacity of sink organs to utilize photosynthate (Lewis *et al*., 2002; Aranjuelo *et al.*, 2009a). The enhancement of

non-structural carbohydrates and the inhibition of the expression of genes that encode for different proteins belonging to the photosynthetic apparatus are both suppressed through possible increases in hexose cycling within the leaf, resulting in decreased photosynthetic capacity and a notable decline in the amount of rubisco (Drake et al., 1997; Moore et al., 1999) and in photosynthetic capacity. From this point of view, the reduction in photosynthetic rates would be 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 (Lewis et al. 2002; Aranjuelo et al., 2009ab). Consequently, when plants exposed to elevated CO_2 have limitations in increasing C sink strength, plants decrease their photosynthetic activity to balance C source activity and sink capacity (Thomas and Strain, 1991).

According to some authors (Serraj et al., 1998; Lüscher et al., 2000; Rogers et al., 2006), since legumes are capable of fixing atmospheric N_2, they will have an advantage in plant growth over non-N_2-fixing plants. Moreover, these plants have an extra sink for additional C to be transferred to nodules, thus enhancing N_2 fixation (Udvardi and Day, 1997), which avoids leaf carbohydrate accumulation and therefore acclimation of photosynthesis (Erice et al., 2006b). Root nodules are strong sinks for carbohydrate (Walsh et al., 1987), in soybean the respiratory rate of a nodulated system may be an order of magnitude greater than for an equivalent non-nodulated root system (Vessey et al., 1988). This could be the reason why non-nodulated soybean plants undergo photosynthetic acclimation under elevated CO_2, whereas nodulated plants do not show the decrease in the CO_2 assimilation rate (Ainsworth et al., 2004).

However, some studies suggest that the increase in photosynthates is not reflected by an improvement of nodule activity. According to this, N_2 fixation is regulated by a N feedback mechanism related to increased N compounds in the vascular tissue that down-regulates or prevents nodulation and nodule growth, thus leading to a decreased N_2 fixation (Hartwig et al., 1994; Almeida et al., 2000). In a similar way to the imbalance between leaf C sources and sinks, a consequence of the decrease in leaf N and N-transporting solutes observed in exclusively N_2 fixing alfalfa plants exposed to high CO_2 is an accumulation of products that are associated with N_2 fixation in nodules, which in turn leads to inhibition of nitrogenase activity in the bacteroids (Aranjuelo et al., 2008; 2009a) (Figure 1).

Figure 1

This fact could explain why strictly N_2 fixing alfalfa plants may undergo photosynthetic acclimation (Aranjuelo et al., 2005; Erice et al., 2006a and 2006b). A recent study (Sanz-Sáez et al., 2010) deals with a hypothesis concerning putative nodule activity limitation in elevated CO_2 conditions and its implication in the decrease in photosynthetic efficiency. One characteristic of plants grown under elevated CO_2 conditions is the increase in the C/N ratio in plant tissues (Rogers et al., 1999; Hom, 2002; Torbert et al., 2004), which may indicate the inability to increase biological N_2 fixation or mineral N uptake. In this study it has been demonstrated that the addition of increased doses of mineral N (NH_4NO_3) initiates, finally, the disappearance of photosynthetic acclimation as the C/N ratio is recovered in plant tissues.

IV. Alfalfa management alters N_2 fixation and the response to CO_2

Alfalfa, as forage crop, is submitted to periodic cutting for animal breeding. During vegetative growth, shoots are the source organs providing photosynthates to young developing shoots, roots and nodules, which are the main C sink organs. However total or partial removal of the photosynthetic area requires the mobilization of C and N reserves from roots to shoots (Avice et al., 2003), which means an inversion between source and sink organs due to the disappearance of aerial source organs and the formation of new sinks with developing shoots.

A previous study conducted by Ainsworth et al. (2004) in soybean mutants confirmed that the cutting-regrowth cycle affects on photosynthetic acclimation are consistent with the source-sink balance hypothesis of photosynthetic down-regulation. Another study carried out in alfalfa plants exposed to elevated CO_2 (Erice et al., 2006b) where the growth effect on photosynthetic performance was studied showed that at the end of the vegetative pre-cut growth period, nodulated alfalfa plants showed photosynthetic down-regulation measured as net photosynthesis or as rubisco reduction and/or inactivation. In contrast, no changes in net photosynthesis were obtained during regrowth after cutting (Erice et al., 2006b) suggesting that photosynthetic down-regulation disappeared during regrowth. Plants have a high root/shoot ratio after cutting, and the presumed high respiration activity can function as a strong sink for photosynthates produced in the new growing shoots. In such a situation, sugar accumulation in the leaves, the negative feed-back control of photosynthesis (Drake et al., 1997; Moore et al., 1999), and therefore down-regulation, is avoided and enhanced yield under elevated CO_2 may take place.

Although aboveground organs cutting will might improve photosynthetic performance, shoot cutting also will have a important implications in nodule performance. Once shoot cutting has been undertaken, removing the photosynthetic tissues, mineral N uptake (Kim et al., 1991) and N_2 fixation in nodules are dramatically reduced (Davidson et al., 1990; Kim et al., 1993). Then, C supply to the roots is drastically reduced (Moustafa et al., 1969) while N_2 fixation is highly dependent on a continuous supply of photosynthates from the shoots and from the degradation of starch stored in roots to support nodule function (Ta et al., 1987; Ryle et al., 1986). In this situation, after shoots are cut, the temporary cessation of plant growth and the formation of new nodules as well as changes in their structure and function are triggered during the first days of regrowth (Wilson et al., 1942; Butler et al., 1959; Boucaud and Bigot, 1989; Ta et al., 1990).

Perennial legumes lose their nodules after the grazing or harvesting of shoots (Wilson, 1942; Butler et al., 1959; Whiteman, 1970) and new nodules are formed during the regrowth phase (Vance et al., 1979). The depletion of nodular tissue is concomitant with a fall of up to 88% in N_2 fixation, 24 h after cutting (Vance et al., 1979). Numerous reports have demonstrated a rapid decline of nodule nitrogenase activity following shoot removal in alfalfa (Phillips et al., 1983; Macdowall, 1983; Ta et al., 1990) and in other forage legumes (Ryle et al., 1985; Gordon and Kessler, 1990).

Harvesting shoots also induces a rapid increase in nodule protease activity, with marked reductions in nodule soluble protein concentration including leghemoglobin, and by disorganization and bacteroid loss in nodule cortical cells. However, 13 days after

cutting, protease activity decreases and leads to increases in the soluble protein content as well as the nitrogenase activity (Vance et al., 1979), which remains low until day 10 of regrowth (Kim et al., 1993).

After harvesting, infected cells containing bacteroids degenerate while the apical meristem, the vascular bundles and the infection threads of the nodules remain intact. In some forage legumes such as clover, *Desmodium* or *Phaseolus*, the loss of nodules that is induced by harvesting or grazing requires the reinfection of roots for new nodule to form with no recovery from senescence occurring. In contrast to this observation, alfalfa has the capacity to recover its nodules from the degenerated post-harvest status. This adaptive trait of alfalfa may explain why alfalfa has a rapid regrowth potential as compared to many other forage legumes (Vance et al., 1979).

V. The nitrogen reserves from taproots are key organic nutrients for the regrowth of alfalfa

As mentioned above, the defoliation of the aerial parts impacts drastically on photosynthesis capacity and on the N acquisition processes by the large decrease of the nitrogenase activity as well as the decline of absorption of mineral N forms from the soil (Kim et al., 1991, 1993; Ourry et al., 1994). Therefore, the production of new shoots during the first days following cutting requires utilization of reduced C and N compounds, the availability of organic reserves being a critical factor in this phase of the growth.

The fast shoot regrowth requires mobilization of organic C and N reserves previously accumulated in perennial tissues or specific storage organs (Volenec et al., 1996; Ourry et al., 2001). In leguminous forages like alfalfa or white clover it has been demonstrated that the spring shoot growth, as well as the shoot regrowth after mechanical cutting or pasturing, strongly depends on root N reserves (Hendershot and Volenec, 1993ab; Avice et al., 1996ab; Barber et al., 1996). It is well known that the endogenous N reserves, mainly amino acids and proteins, are largely used for the regrowth of the aerial parts, whereas the C reserves are mainly used for sustaining the respiratory metabolism in the belowground organs (roots and nodules) (Ta et al., 1990; Avice et al., 1996b).

Because defoliation triggers a reduction in N_2 fixation and mineral N assimilation, the mobilization of endogenous N reserves is crucial to meet the N requirements of the aerial buds during the first days following cutting. Among the different forms of soluble N compounds, amino acids and proteins represent the greatest fractions in the main organ of storage of the alfalfa, i.e. the taproot. In addition, specific proteins called vegetative storage proteins (VSPs) have been identified in taproots of alfalfa, and are characterized by their large accumulation during the autumn period (Staswick, 1994; Volenec et al., 1996; Ourry et al., 2001; Bewley, 2002). These VSPs are four polypeptides of 57, 32, 19 and 15 kDa (Hendershot and Volenec, 1993ab; Gana et al., 1998) that can represent up to 40% of the total soluble proteins in the taproot.

These proteins are rapidly degraded when the shoot growth in spring resumes, or after practising the cut of the aerial parts. For instance, during the 10 first days of regrowth after cutting, the protein concentration in taproot declines by 40% while the VSPs are

preferably degraded, presenting a rate of mobilization from 60% to 80%, and are largely used to provide N (and C) to the regrowing shoots (Hendershot and Volenec, 1993b; Avice et al., 1996a; Corre et al., 1996; Gana et al., 1998) (Figure 2).

Figure 2

In leguminous forages like alfalfa or white clover, several studies in controlled environments and field conditions have demonstrated that the availability of N reserves, and particularly the concentration in VSPs, is closely related to shoot growth potential in both legumes (Ourry et al., 1994; Volenec et al., 1996; Avice et al., 1997; Goulas 2001; Justes et al., 2002). Alfalfa plants, which posess the highest protein concentration and level of VSP accumulation, consequently exhibit the fastest growth of the buds, the greatest rate of expansion leaf area index and the highest shoot production in spring (Justes et al., 2002).

It is well established that the accumulation of VSPs is mediated by phytohormones. Methyl jasmonate induces accumulation of these protein reserves (1) by increasing the expression of genes of the VSPs in the short term (Staswick, 1994), and, (2) by altering the N distribution through modification of the relationship between source and sink organs in the long term (Meuriot et al., 2003). In addition, abscisic acid (ABA), which is also associated with some physiological responses induced by short photoperiods (Li et al., 2003), led to a reduction in mineral N absorption and enhancement of N allocation towards the roots. As a result, ABA promotes the accumulation of N reserves in taproots, through large increases in the root biomass and the concentration of VSPs (Avice et al., 2003). It is interesting to relate these observations with the effects of water

stress on N management in alfalfa. Indeed, in strictly N_2 fixing alfalfa plants subjected to drought (an abiotic stress leading to the synthesis of ABA), it has been observed that water stress reduced the shoot biomass production by 30%, but it increased the VSP concentration by 78% (Erice et al., 2007). Thus, it could be hypothesized that in alfalfa submitted to water deficit, the induction of VSP may be mediated by ABA. This increase in N reserves in the perennial organs (taproot), especially in the form of VSPs, can be an important adaptive trait towards tolerance of low water availability conditions, as they often occur in Mediterranean climates. Due to the close relationship between the availability of protein reserves and shoot growth potential, this capacity to accumulate and salvage N compounds in taproots under drought conditions would allow the plants to grow in better conditions in the following cycle of cut/regrowth or when the water status becomes more favourable (Erice et al., 2007).

The level of accumulation of N reserves in the taproot can also depend on the strains of *Sinorhizobium meliloti* with which the plant establishes an efficient symbiosis to fix N_2. In this respect, it has been demonstrated recently that the interaction between different strains from *S. meliloti* and alfalfa of Canadian origin can alter the plant tolerance to cold, as well as the plant response to high CO_2 (Bertrand et al., 2007). For that reason, one of the factors to consider in the tolerance of alfalfa to future climatic conditions based on the predictions of the IPCC (IPCC, 2007), could be the selection of more efficient *S. meliloti*/alfalfa genotype pairs. A study carried out with a genotype of Mediterranean alfalfa has shown that production not only depends on the alfalfa genotype and its efficiency but also on the strain of *S. meliloti*, which can condition the response of the symbiosis to abiotic factors like the temperature or the atmospheric

concentration of CO_2, two parameters that are very susceptible to predicted climate changes.

VI. Utility of C and N isotopes in Climate Change Studies

The need to develop methods to quantify plant responsiveness to elevated CO_2 in relatively small-scale and short-term experiments presents both challenges and opportunities for plant research (Pataki et al., 2003; Bowling et al., 2008). Measurements of the abundance of various C and N isotopes in samples have been used as unique tools that integrate physical and physiological processes over space and time (Avice et al., 1996b; Aranjuelo et al., 2008; 2009b). Isotope techniques (used in combination with gas exchange and other techniques) stand out prominently among the few tools available to partition photosynthesis and respiration, and to quantify allocation and partitioning (Deléens et al., 1994; Ciais et al., 1995; Yakir and Sternberg 2000; Bowling et al., 2008). Plants grown in environments with modified isotopic composition incorporate the tracer in C/N-containing compounds of the plant (Avice et al., 1996b; Nogués et al., 2004) providing essential information about the C and N sinks to which the recently fixed C/N is delivered. Isotopes can then be used to trace the fate of C and N newly incorporated into the plant under elevated CO_2 conditions and to calculate the residence time of C and N in various pools (Pataki et al., 2003; Aranjuelo et al., 2009b). As it has been explained before, a major adaptive mechanism to deal with in the near future is to maintain C sink strength under increasing CO_2 conditions. So the identification of plant processes conditioning C sink will be a key point in future plant breeding programmes. The application of C and N stable isotopes on this topic have

been previously described (Avice et al., 1996b; Aranjuelo et al., 2008; 2009b; Molero et al., 2011). In this sense, the application of $^{12}CO_2$ labelling in a study conducted in slow growing plants (Aranjuelo et al., 2009b) revealed that the C sink/source imbalance was conditioned by plant growth, the leaf C residence time and a by the capacity to deliver recently fixed C to respiration. Despite the relevance of C loss through respiration, little attention has been given to this topic in plants (Avice et al., 1996b; Aranjuelo et al., 2011). Previous studies conducted by Aranjuelo et al. (2009) under elevated CO_2 conditions revealed that those plants, invested an important amount of recently assimilated C in respiration processes. Furthermore, the same study highlighted that the "ability" to respire recently assimilated C may contribute towards preventing carbohydrate build-up and consequently to the avoidance of photosynthetic acclimation. According to this study, alfalfa plants exposed to growth conditions (varying CO_2, temperature, water availability, etc.) where C allocation and respiration ability are limited should be more susceptible to photosynthetic acclimation (Figure 3) than alfalfa plants grown in environments that enable the allocation of C away from leaves (shoots and roots) and/or respiration of the recently fixed C.

Figure 3

Similarly, a previous study (Aranjuelo et al., 2008) where exclusively N_2 fixing alfalfa plants were exposed to elevated CO_2 conditions (with a modified isotopic composition) revealed that, after two weeks of exposure to CO_2 labelling, all the C present in the total organic matter was derived from C fixed during the labelling period. However, the total renewal of C did not contribute towards avoidance of a build-up carbohydrate that caused photosynthetic down-regulation. Furthermore, this study revealed that larger C

sink associated to the presence of nodules (Ainsworth and Rogers, 2007) in plants grown at elevated CO_2 did not contribute towards overcoming photosynthetic acclimation.

Studies conducted with N isotopes ($\delta^{15}N$) have provided important information about their utility as integrative parameters of N metabolism (Pataki et al., 2003; Desclos et al., 2009). The use of methods of double ^{13}C and ^{15}N labelling in alfalfa plants during regrowth, enabled the characterization of C and N management (Avice et al., 1996b). The authors demonstrated that 8 days after cutting, 80% of the N and only 6% of the C of the new shoots were mobilized from the root and the crown, whereas around 40% of the C was used for the root respiratory production of energy (Avice et al., 1996b). Such information is of high relevancy in elevated CO_2 studies. As it was mentioned before, in addition to the C balance, N management is a target factor conditioning plant performance under elevated CO_2, especially in N_2 fixing plants. In this sense, a recent study (Molero et al., 2011) with conducted in exclusively N_2 fixing alfalfa plants labelled with ^{12}C and $^{15}N_2$ revealed key information concerning the C and N exchange between leaves and nodules. The isotopic enrichment of ^{15}N 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 is in the form of NH_3 (Molero et al., 2011). This study also allowed the identification of the distribution of ^{12}C and $^{15}N_2$ among amino acids and between the plant and the symbiont in different amino acid metabolic pathways. Furthermore, for the first time, the ^{12}C and $^{15}N_2$ labelling revealed that GABA and glycine C-transporting aminoacids from the leaves to the nodules.

VII. Conclusions and perspectives

Initial CO_2 stimulus on photosynthesis through higher supply of carbohydrates to the nodules would also cause an immediate increase in nitrogenase activity. Nevertheless, frequently this response to CO_2 is not maintained at the long-term and photosynthesis declines. Decreased net photosynthesis is related to leaf carbohydrate accumulation consequence of an insufficient sink plant capacity. The coupled C and N cycling makes nitrogenase activity to be regulated by photosynthesis, N availability and N demand. Despite N_2 fixing plants have an extra sink for additional C in alfalfa photosynthates increase under elevated CO_2 is not reflected by the improvement of nodule activity. An accumulation of products associated with the N_2 fixation in the nodules was observed leading to the inhibition of nitrogenase activity in the bacteroids. Alfalfa forage management of cutting-regrowth removing the shoots alters the source-sink status of the plants during regrowth. In this phase of plant development enhanced yield under elevated CO_2 may take place. Cutting provokes the cessation of nodule formation as well as their structure and function remaining low until day 10 of regrowth. Plant production and response to CO_2 is dependant on the mobilization of the organic C and N reserves (mainly VSP) previously accumulated in taproots. In alfalfa submitted to water deficit the strong induction of VSP, possibly mediated by ABA, can be an important adaptive trait to tolerate drought conditions allowing plants to grow in better conditions in the following cutting-regrowth cycle. In this sense the selection of more efficient *S. meliloti* strains may affect alfalfa tolerance to future climatic conditions determining the response to abiotic factors like the temperature or the atmospheric CO_2 concentration. The further clarification of C and N management between leaves and

nodules is a key topic in Climate Change studies. In this sense, stable isotopes might contribute to clarify the C and N management of plants and its implications in photosynthetic and nodule activity (in N_2 fixing plants) and consequently in plant performance. Although in recent years the use of stable isotopes in studies related to Climate Change effects in plants has increased (specially C stable isotopes), the appropriateness of this type of study reveals the need to strengthen the use of stable isotopes as a methodological tool.

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