Down-regulation of N₂-fixation in alfalfa under elevated CO₂ alters rubisco content and decreases nodule metabolism *via* nitrogenase and tricarboxylic acid cycle.

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Abstract

Although responsiveness of N₂-fixing plants to elevated CO₂ conditions have been analyzed in previous studies, important uncertainties remain in relation to the effect enhanced CO₂ in nodule proteomic profile and its implication in leaf responsiveness. The aim of our study was to deepen our understanding of the relationship between leaf and nodule metabolism of N₂-fixing alfalfa plants after long term exposure to elevated CO₂. After 30-day exposure to elevated CO₂, plants showed photosynthetic down-regulation with reductions in the light-saturated rate of CO₂ assimilation (A_{sat}) and the maximum rate of rubisco carboxylation (V_{cmax}). Under elevated CO₂ conditions, the rubisco availability limited potential photosynthesis by around 12 %, which represented the majority of the observed fall in V_{cmax} . Photosynthetic down-regulation has been associated with decreased N availability even if those plants are capable to assimilate N₂. Diminishment in shoot N demand (as reflected by the lower rubisco and leaf N content) suggests that the lower aboveground N requirements affected negatively nodule performance. In this condition, specific nodule activity was reduced due to an effect on nodule metabolism that manifested as a lower amount of nitrogenase reductase. Moreover, the nodule proteomic approach also revealed that nodule functioning was altered simultaneously in various enzyme quantity apart from nitrogenase. At elevated CO₂, the tricarboxylic acid cycle was also altered with a reduced amount of isocitrate synthase protein. The nodule proteome analysis also revealed the relaxation of the antioxidant system as shown by a decline in the amount of catalase and isoflavone reductase protein.

Key words: Alfalfa, carbon dioxide, nodule, proteomic.

Abbreviations: 2-DE, two-dimensional electrophoresis; A_{sat} , light-saturated rate of CO₂ assimilation; C_i/C_a , leaf-to-ambient CO₂ concentration; DM, dry matter; PPFD, photosynthetic photon flux density;

1 R/S, root to shoot ratio; RbcS, rubisco small subunit mRNA; RH, relative humidity; RLS, rubisco large
2 subunit; RSS: rubisco small subunit; TCA, tricarboxylic acid cycle. TNC, total non-structural
3 carbohydrates.

1 **Introduction**

2
3 Since the beginning of the industrial revolution in the 18th century, the amount of CO₂ emitted by
4 humans has been the consequence of industrial development and a quadrupled population during the
5 last one hundred years (Krausmann et al., 2009). In 2009, atmospheric CO₂ concentration reached 387
6 $\mu\text{mol mol}^{-1}$ and, according to the predictions of the Intergovernmental Panel on Climate Change
7 (IPCC), at the end of the present century this concentration may be around 700 $\mu\text{mol mol}^{-1}$ (IPCC,
8 2007). The primary effect of increasing CO₂ in C₃ plants is a short term photosynthetic enhancement,
9 and consequently, plant productivity (Daepf et al., 2000). Nevertheless, frequently this response to CO₂
10 is not maintained over the long term and photosynthesis declines (Aranjuelo et al., 2009; Erice et al.,
11 2006a; Ainsworth and Rogers, 2007). Previous studies with alfalfa showed that this photosynthetic
12 down-regulation was attributed to a decreased photosynthetic efficiency without limitation of CO₂
13 supply to mesophyll cells (Aranjuelo et al., 2005; Erice et al., 2006b).

14
15 In the context of elevated CO₂ environments, legumes are particularly interesting due to their symbiotic
16 relationship with N₂-fixing bacteria, providing them N autonomy. Symbiotic N₂ fixation is narrowly
17 related to photosynthesis (Hartwig and Sadowsky, 2006), many studies have confirmed that most N₂-
18 fixing legumes increase their level of N₂ fixation per plant under elevated CO₂ conditions (Hartwig,
19 1998; Rogers et al., 2006). However, some studies suggest that reduced rubisco activity after long-term
20 exposure to CO₂ is associated with lower leaf N demand resulting in decline of nodule proteins and key
21 metabolic activities like malate dehydrogenase and phosphoenolpyruvate carboxylase. A common plant
22 response under elevated CO₂ is the enhancement of non-structural carbohydrates entailing the
23 inhibition of the expression of genes that encode for different proteins belonging to the photosynthetic
24 apparatus, resulting in decreased photosynthetic capacity and notable declines in the amount of rubisco
25 (Drake et al., 1997; Moore et al., 1999) and in photosynthetic capacity. This rubisco depletion has been

1 associated with significant decreases in the transcripts levels of genes encoding the small subunit of
2 rubisco (RbcS) (Nie et al., 1995; Van Oosten and Besford, 1994; 1995). Nevertheless, in spite of these
3 studies the link between carbohydrate repression of gene expression and control of photosynthetic
4 acclimation remains elusive (Cheng et al., 1998). This coupling between C and N cycle causes
5 biological symbiotic N₂ fixation to be regulated by photosynthesis (carbon supply) and N demand
6 (Aranjuelo et al., 2007).

7
8 Some authors have reported that the decrease in rubisco content and acclimation to elevated CO₂ is
9 accompanied by a lower leaf nitrogen content (Nakano et al., 1997), which may suggest that
10 photosynthetic down-regulation is due to the nitrogen status (Farage et al., 1998; Geiger et al., 1999).
11 Other studies have shown that elevated CO₂ concentration increase legume nodule growth (Phillips et
12 al., 1976; Murphy, 1986; Aranjuelo et al., 2009) despite specific nodule activity (SNA) could remain
13 unchanged (Cabrerizo et al., 2001).

14
15 The mechanisms that determine the response of photosynthesis to future climatic atmospheric
16 conditions of increased CO₂ concentration are of crucial interest in predicting the impact of global
17 change on the Earth's terrestrial ecosystems (Cheng et al., 1998). To achieve a more comprehensive
18 picture of the proteins related to nodule elevated CO₂ responses we performed protein separation by
19 two-dimensional electrophoresis (2-DE). This method was developed as an important approach in
20 evaluating plant responsiveness under limited growth conditions (Desclos et al., 2008, 2009). Djorjevic
21 (2004) reviewed the proteomic characterization of *Sinorhizobium meliloti* as free-living cells and
22 bacteroid but the knowledge about the plant proteome in the *Medicago* root nodule remains scarce
23 (Larrainzar et al., 2007).

1 A number of studies have shown the photosynthetic down-regulation and some of them have related
2 this phenomenon with carbohydrate accumulation and the plant's demand for nitrogen, but less
3 attention has been paid to the role of both rubisco subunits under elevated CO₂ in relation with nodule
4 metabolism. The aim of the present study was to determine the quantitative changes in rubisco large
5 (RLS) and small subunits (RSS) and their significance to the elevated CO₂ acclimation context in
6 alfalfa. The relationship between leaf and nodule metabolism has also been studied by 2-DE to find out
7 the proteins of interest involved in nodule response to long-term alfalfa CO₂ exposure. The present
8 work combines the physiological and biochemical measurements with nodule proteomic analyses in
9 order to contribute to the understanding of plants photosynthetic acclimation to elevated CO₂ in strictly
10 N₂-fixing alfalfa.

11

12

Material and methods

Plant material

Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were sterilized in a solution of HgCl₂ (0.1%, w/v) and germinated in Petri dishes. One week-old seedlings were transferred into 2 L pots (4 plants per pot) containing a mixture of inert perlite and vermiculite (2/1, v/v). During the first month, plants were inoculated three times with *Sinorhizobium meliloti* strain 102F78 (The Nitragin Co. Milwaukee, WI, USA) and irrigated alternatively with Hoagland N-free solution (Hoagland and Arnon, 1950) and distilled water (three times per week) to avoid salt accumulation in the substratum. Plants were grown in a greenhouse at 25/15 °C (day/night) with a 14h photoperiod under natural daylight, supplemented with fluorescent lamps (Sylvania Decor 183, Professional-58W, Germany) providing a photosynthetic photon flux density (PPFD) of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. When plants were 30 days old, they were transferred to a growth chamber (Conviron PGV 36, Winnipeg, Canada) and randomly assigned to the atmospheric CO₂ concentration (ambient -approximately 400 $\mu\text{mol mol}^{-1}$ - or elevated - 700 $\mu\text{mol mol}^{-1}$). The conditions in the growth chamber were 25/15 °C (day/night), 40% RH, 14h photoperiod and 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD.

Growth parameters

After one month exposure to ambient or elevated CO₂ concentration, 60 days-old plants were separated into leaves, stems, roots and nodules. Dry mass (DM) of each organ and root-to-shoot ratio (R/S) were obtained after drying in an oven at 80 °C for 48h.

Gas exchange parameters

Gas exchange parameters were measured in fully expanded young leaves after 30 days exposure to CO₂ treatments (60-days-old plants) using a LI-COR 6400 portable photosynthesis system (LICOR

1 biosciences, Lincoln, Nebraska, USA). The light-saturated rate of CO₂ assimilation (A_{sat}) was
2 estimated at a PPFD of 1200 μmol m⁻² s⁻¹ using equations developed by von Caemmerer and Farquhar
3 (1981). Measurements from net photosynthesis and intercellular CO₂ were used to assess the maximum
4 rate of rubisco carboxylation (V_{cmax}) employing the mathematic model developed by Ethier and
5 Livingstone (2004) and Sharkey et al. (2007). Leaf-to-ambient CO₂ concentration (C_i/C_a) was also
6 calculated for both ambient (400 μmol mol⁻¹) and elevated (700 μmol mol⁻¹) CO₂ concentrations.

8 *Analysis of non-structural carbohydrates*

9 Leaf total soluble sugars and starch concentration were quantified by grinding and filtering 200 mg of
10 leaf fresh weight from leaves harvested after one month treatment imposition in a cold mortar using an
11 extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at
12 28,710 × g for 15 min at 4 °C. The TSS quantification was performed in supernatant, whereas starch
13 was measured using the pellet as described by Jarvis and Walker (1993). TSS levels were measured
14 using the method of Yemm and Willis (1954). Total non-structural carbohydrates (TNC) were
15 calculated as the addition of starch and total soluble sugar concentration.

16
17 Since elevated CO₂ alters leaf morphology, biochemical parameters are often underestimated when
18 represented per mass unit (Sanz-Sáez et al., 2010). We have expressed TNC measurements on an area
19 basis.

21 *Analysis of N*

22 N concentration in leaves, stems and roots was measured as described by Meuriot et al., (2003) using a
23 C/N analyzer linked to an isotope ratio mass spectrometer (IRMS, Roboprer CN and mass
24 spectrometer, PDZ Europa Scientific Ltd., Crewe, UK).

Specific nodule activity (SNA) was calculated as described by Brioua and Wheeler (1994), being the ratio between plant total nitrogen content and nodule DM.

RNA isolation, synthesis of cDNA and Quantitative real-time RT-PCR

Total RNA was isolated from alfalfa leaves by phenol/chloroform extraction (Kay et al., 1987). RbcS gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, California, USA). cDNAs were obtained from 2.5 µg of total DNase-treated RNA in a 20 µL reaction containing 500 ng random hexamer primer, 0.5 mM each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1x first strand buffer (Invitrogen, Carlsbad, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primers sets used to amplify RbcS were: primer forward 5'-TTCGGAGCCACTGATTCTTCTC-3' and primer reverse 5'-ACTGCACTTGACGAACATTGTC-3'. Each 25 µL q-PCR reaction contained 1 µL of a dilution 1:10 of the cDNA, 200 nM dNTPs, 400 nM each primer, 3 mM MgCl₂, 2.5 µL of 1x SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl).

The PCR program consisted in a 4 min incubation at 95 °C to activate the hot-start recombinant *Taq* DNA polymerase, followed by 30 cycles of 45 s at 94 °C, 45 s at 69 °C, and 50 s at 72 °C, where the fluorescence signal was measured. The results obtained on the different treatments were standardized according to the alfalfa tubulin gene expression levels, which was analyzed with primer forward 5'-GAAGCAAGCGGTGGAAGATATG-3' and primer reverse 5'-CCAAATGGACCAGAACGCAAAC-3', and showed stable expression under the conditions tested in this study

Real-time PCR experiments were carried out at least with four independent RNA samples, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by

1 using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in
2 all PCR reactions.

4 ***Rubisco semi-quantification***

5 Extract from protein quantification was precipitated using sodium deoxycholate-trichloroacetic acid
6 protocol described by Peterson (1983). The resulting pellet was air dried and resuspended in Laemmli
7 lysis buffer (Laemmli, 1970) and boiled for 10 min to denature proteins. SDS-PAGE was performed
8 using 150 g L⁻¹ acrylamide separation gel and stained with silver nitrate (Blum et al., 1987). Gel images
9 were scanned and analysed using the ImageQuant TL software (GE Amersham Biosciences, UK). The
10 relative proportion of rubisco large (RLS) and small (RSS) subunits was calculated respect the ambient
11 CO₂ concentration large subunit.

13 ***Nodule proteomic characterization***

14 Frozen nodule samples (200 mg fresh weight) were ground with liquid nitrogen and resuspended in 2
15 mL of cold acetone containing 10% TCA (v/v). After centrifugation at 16,000 g for 3 min at 4 °C, the
16 supernatant was discarded and the pellet was rinsed with methanol, acetone, and phenol solutions as
17 previously described by Wang et al. (2003). The pellet was stored at -20 °C or immediately
18 resuspended in 200 µl of R2D2 rehydration buffer [5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)
19 dimethyl-ammonio]-1-propane-sulphonate, 2% N-decyl-N,N-dimethyl-3-ammonio-1-propane-
20 sulphonate, 20 mM dithiothreitol, 5 mM TRIS (2-carboxyethyl) phosphine, 0.5% IPG buffer (GE
21 Healthcare, Saclay, France), pH 4 to 7 (Mechin et al., 2003)]. The total soluble protein (*TSP*)
22 concentration was determined by the method of Bradford (1976) using BSA as standard. The two-
23 dimension electrophoresis (2-DE) was conducted according to what described by Desclos et al. (2008).

Image analysis of 2-DE gels

Images of the two-dimensional gels were acquired with the ProXPRESS 2D proteomic Imaging System and analysed using Phoretix 2-D Expression Software v2004 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Gels from four independent biological replicates were used. An average gel, representative of each group, was automatically selected by the software with a parameter for spots to be present on more than two-thirds of the gels. The software automatically selected the average gel with the most spots as the image for the reference gel, and unmatched spots from the remaining average gel were added to the reference gel which was subsequently used for spot matching to average gels. Warping and matching were automatically performed and only adjusted on those gels where darker images led to both incorrect warping and matching. *Mr* and *pI* were calculated using Samespots software calibrated with commercial molecular mass standards (precision protein standards prestained Bio-Rad) run in a separate marker lane on the 2-DE gel. ANOVA ($P < 0.05$) was performed using MiniTAB to compare the relative abundance of the total volume of all detected spots for each gel.

Protein identification by ESI-LC MS/MS

Excised spots were washed several times with water and dried for a few minutes. Peptide extracts were then dried and dissolved in starting buffer for chromatographic elution, which consisted of 3% CH₃CN and 0.1% HCOOH in water. Peptides were enriched and separated using lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic, Billerica, USA). For protein identification, tandem mass spectrometry peak lists were extracted and compared with the protein database using the MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine. Tandem mass spectrometry spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 for MS/MS fragments.

1 The LC MS/MS data were converted into DTA-format files which were further searched for proteins
2 with MASCOT Daemon. Only peptides matching an individual ion score >51 were considered.
3 Proteins with two or more unique peptides matching the protein sequence were automatically
4 considered as a positive identification. Among the positive matches based on one unique peptide, the
5 fragmentation spectrum from each peptide was manually interpreted using the conventional
6 fragmentation rules. In particular, we looked for a succession of at least five *y*- and/or *b*-ions, specific
7 immonium ions, specific fragment ions (proline and glycine), and signatures of any modifications
8 carried by the peptides. For protein identification, two strategies were used to mine the maximum
9 information. Measured peptides were searched in the NCBI nr-protein sequence database viridiplantae
10 (green plants) and bacteria. Once the proteins were identified, we proceeded to their presumed
11 biological function according to Bevan et al. (1998).

13 ***Statistical analysis***

14 Statistical analysis was performed using SPSS software 12.0 (SPSS, Chicago, Illinois, USA). Data
15 were subjected to one-way analysis of variance (ANOVA) to determine significant differences between
16 the CO₂ treatments. The results were considered significant at $P < 0.05$.

Results

After 30 days exposure to ambient or elevated atmospheric CO₂ concentration no differences in leaf, stem root, nodule dry mass (DM) as well as in R/S were observed (Table 1). Measured gas exchange parameters (A_{sat} and $V_{\text{c}_{\text{max}}}$) showed significant decreases under elevated CO₂ ($F = 25.8$; $P = 0.007$ and $F = 33.9$; $P = 0.004$ respectively) but no differences were found when comparing Ci/Ca for both 400 or 700 $\mu\text{mol mol}^{-1}$ (Figure 1).

Leaf TNC concentration was increased by elevated CO₂ ($F = 22.5$; $P = 0.003$) (Table 2). After CO₂ exposure leaf N concentration was decreased under elevated CO₂ ($F = 10.8$; $P = 0.017$) whereas stem or root N as well as total N concentration were unaffected by CO₂ (Table 2). SNA was significantly decreased by elevated CO₂ ($F = 13.3$; $P = 0.011$) (Table 2).

Elevated CO₂ led to significant decreased rubisco small subunit mRNA (RbcS) ($F = 221.4$; $P < 0.000$) (Figure 2A). Rubisco large subunit (RLS) content decreased significantly under elevated CO₂ concentration but rubisco small subunit (RSS) increased ($F = 15.33$; $P < 0.000$) (Figure 2B-C) which resulted in decreased RLS/RSS ratio ($F = 44.52$; $P < 0.000$) (Figure 2D).

The effect of rising CO₂ on the nodule protein pattern in alfalfa plants was studied using 2-DE (Figure 3). Twelve proteins have been identified with different expression under ambient and elevated CO₂ concentration. Eight of those proteins were down-regulated by CO₂ and 4 were up-regulated (Table 3). These proteins were classified into different groups according to their presumed biological function. The down-regulated proteins were classified into six groups: metabolism (1) Energy processes (1), transporters (2), disease/defence (1), unclassified (2) and secondary metabolism (1). Up-regulated proteins were classified into two: energy (2) and unclassified (2). The roles of these proteins are

discussed in the following section with regard to changes in physiological and biochemical changes observed in nodulated alfalfa under elevated CO₂.

Discussion

The primary effect of rising atmospheric CO₂ concentration (IPCC, 2007) in C₃ plants is the enhancement of photosynthesis but after long-term exposure the photosynthesis acclimation overcomes (Erice et al., 2006a; 2006b; Aranjuelo et al., 2009). Despite the theoretical initial increase of photosynthesis, in our study plant growth measured as dry mass (DM) accumulation did not show significant differences (Table 1). According to the A_{sat} and V_{cmax} results, plants grown under elevated CO₂ concentration showed a clear acclimation which led V_{cmax} to a fall of 17.2% (from 66 to 54, Figure 1). In alfalfa, leaf conductance is acclimated to elevated CO₂ simultaneously to photosynthesis as revealed from Ci/Ca results (Figure 1) confirming the previous reports (Aranjuelo et al., 2005; Erice et al., 2006b).

Studies about down-regulation have related the accumulation of total non-structural carbohydrates (TNC) with limitation of photosynthetic enzymes (Moore et al., 1999; Rogers and Ellsworth, 2002) including rubisco (Aranjuelo et al., 2005). The present work shows that TNC accumulates in leaves (Table 2) by elevated CO₂ (Erice et al., 2006b; Sanz-Sáez et al., 2010) and this may be the cause of the significant decrease in rubisco small subunit gene expression (RbcS) (Figure 2A). Down-regulation of RbcS by elevated CO₂ has been repeatedly reported (Majeau and Coleman, 1996; Cheng et al., 1998; Gesch et al., 1998) but the regulation of transcriptional and/or posttranscriptional processes (e.g. mRNA stability) will determine the level of rubisco protein at elevated CO₂ (Cheng et al., 1998). In the present study the reduction of RbcS expression was not accompanied by lower rubisco small subunit

1 quantity (RSS) which increased under elevated CO₂ conditions (Figure 2B and 2C). Similar results
2 with posttranscriptional regulation of protein content were obtained in tomato (Van Oosten and
3 Besford, 1995) or *Chlamydomonas reinhardtii* probably because the inhibition of translation of RbcS
4 mRNA (Winder et al., 1992).

5
6 It is considered that due to the relative quantity of rubisco large (RLS) and small subunits (RSS) and
7 the lowest specificity of RSS (Jordan and Ogren, 1981), differences in kinetic properties of rubisco and
8 thus photosynthesis, results from changes in RSS (Andersson and Blacklund, 2008). At ambient CO₂
9 conditions RLS doubled RSS quantity confirming that RSS is the subunit that may limit rubisco
10 carboxylation capacity (Figure 2B and C). Nevertheless, at elevated CO₂ conditions, RLS/RSS ratio
11 was inverted (Figure 2D) which entails that RLS quantity was lower than that for RSS (Figure 2B and
12 C). Thus, in such conditions RLS could limit photosynthesis. Under elevated CO₂, down-regulation
13 corresponding to rubisco quantity limitation can be calculated as the decrease from RSS at ambient
14 CO₂ to RLS at elevated CO₂. This decrease, 12.1%, corresponds to the 70.2% of V_{cmax} observed total
15 drop (17.2%). According to the obtained results one of the conclusions is that most of the
16 photosynthetic acclimation in alfalfa plants cultivated in growth chambers under elevated CO₂ is
17 related to changes in RLS, the limiting subunit.

18
19 Some studies have related the decrease in rubisco protein under elevated CO₂ with limited N
20 availability (Farage et al., 1998; Geiger et al., 1999; Rogers and Ainsworth, 2006). Symbiotic N₂
21 fixation could counterbalance the CO₂-induced N limitation in the rhizosphere (Haase et al., 2007).
22 Nevertheless, N₂-fixation in alfalfa has been revealed insufficient to support the N demand at elevated
23 CO₂ concentration in previous works (Aranjuelo et al., 2005) resulting in reduced leaf N (Sanz-Sáez et
24 al., 2010). In the present study alfalfa plants showed reduced leaf N but not stem or root which led to
25 no differences in total N (Table 2). The reduced leaf N was linked to lower specific nodule activity

(SNA) (Table 2). This fact may be related with a previously observed drop of nodule protein content as well as decreases in plant and bacteroid enzymatic activities like malate dehydrogenase or phosphoenolpyruvate decarboxylase (Aranjuelo et al., 2008). Nevertheless, variability on the response of SNA to elevated CO₂ has been extensively reported. Some authors showed an increase of SNA in alfalfa with root CO₂ enrichment (Fischinger et al., 2010) whereas shoot CO₂ feeding shows a short- not long term- effect on SNA (Vance and Heichel, 1991; Cabrerizo et al., 2001).

Nodule proteomic profile under elevated CO₂ confirmed the affectation of nodule metabolism reducing nitrogenase reductase (Figure 3; Table 3). Nitrogenase reductase, frequently referred to as Fe-protein (Howard and Rees, 1994), as part of the nitrogenase enzyme complex catalyzes the N₂ fixation reaction (Dixon and Kahn, 2004; Seefeldt et al., 2009). The lowered leaf N demand of photosynthetic acclimated plants, as observed under water limited conditions (Serraj et al., 1998; 1999), may decline N-transporting solutes and favours the accumulation of products associated with the N₂ fixation (ureides) in the nodules leading to an inhibition of nitrogenase activity in the bacteroids. This reduction of nitrogenase activity in the case of alfalfa grown under elevated CO₂ was the consequence of nitrogenase reductase depletion (Figure 3) (Table 3).

Nodules grown in elevated CO₂ also showed the reduction in bacteroid citrate synthase (Figure 3) (Table 3), the enzyme which catalyzes the oxaloacetate condensation with the acetyl-CoA, the way of citrate synthesis (Popova and Pinheiro de Carvalho, 1998). It catalyzes the initial stage of tricarboxylic acid cycle (TCA) and together with isocitrate dehydrogenase may be the crucial point of the TCA rate regulation (Wiegand and Remington, 1986; Chen and Gadal, 1990). Changes in TCA and its anaplerotic reactions may alter not only the source of redox equivalents for the electron transfer chain, but also as a source of intermediates for lipogenesis, organic and amino acid synthesis. Thus, TCA is considered as a central point of bacteroid intracellular metabolism decreasing energy supply to

1 nitrogenase and other energy demanding processes (Popova and Pinheiro de Carvalho, 1998). Increased
2 CO₂ treatment also reduced catalase content (Figure 3) (Table 3). Aranjuelo et al. (2007) showed that
3 down-regulated alfalfa plants decreased leaf antioxidant enzymes activity as catalase, superoxide
4 dismutase and glutathione reductase. This relaxation of antioxidant system was related to lower growth
5 rate resulted from photosynthesis acclimation (Erice et al., 2007). This observation agrees with the
6 repression of isoflavone reductase (Figure 3) (Table 3), a protein involved in the production of
7 isoflavone phytoalexins, which accumulates under biotic or abiotic stresses (Salekdeh et al., 2002; Kim
8 et al., 2003) and along with antioxidants play a crucial role in cell signaling or maintaining the redox
9 status of cells (Lee et al., 2009). This metabolic reduction in the nodules is also supported by the
10 repression of bacteroid ABC transporter (Figure 3) (Table 3) implicated in urea (Wang et al., 2008),
11 peptide transient (Stacey et al., 2002) and legume nodule development (Marx, 1996) which may be also
12 inhibited by the lower leaf N demand under elevated CO₂.

13
14 Besides, the nodules of alfalfa plant grown at elevated CO₂ concentration showed increased carbonic
15 anhydrase (Figure 3) (Table 3). This enzyme with the combined activity of phosphoenolpyruvate
16 carboxylase (Vance et al., 1994) and malate dehydrogenase (Schulze et al., 2002) transforms
17 phosphoenolpyruvate into oxaloacetate and malate (Atkins et al., 2001). The higher carbonic anhydrase
18 activity is needed to maintain a continuous supply of malate (Gálvez et al., 2000), and thus C and
19 energy available for bacteroid consumption (Aranjuelo et al., 2009). Parallel to this effect,
20 bicarbonate/CO₂ equilibrium on convective gas flow into legume nodules and concluded that carbonic
21 anhydrase increase would enhance O₂ transport into the central zone (Thumfort, 1996).

22
23 In summary, after long-term exposure alfalfa plants acclimated to elevated CO₂ showing decreases in
24 A_{sat} and V_{cmax}. This down-regulation was related to leaf TNC accumulation which may reduce RbcS
25 expression. Nevertheless, CO₂ did not decline RSS content probably due to posttranscriptional

processes including mRNA stability and/or the translation of RbcS mRNA. Under elevated CO₂ RLS decreased at photosynthesis limiting levels, reducing the potential photosynthesis around 12%, that is most (70%) of the observed V_{cmax} total drop in these plants. Photosynthetic down-regulation has been associated to decreased N availability. Limited bacteroid metabolism, as it was reflected by lower citrate synthase, would require lower O₂ demand. Increased carbonic anhydrase is related to changes in O₂ permeability which may damage N₂-fixation through the nitrogenase reductase, sensitive to O₂ concentration. Oxygen permeability alteration may lead to oxidative free radical production and together with antioxidant system inhibition (catalase and isoflavone reductase) may be involved in the SNA drop under elevated CO₂. Moreover, a reduced bacteroid N transient capacity due to ABC transporter depletion could entail nitrogenase activity decline by feedback mechanism and the showed leaf N diminution. On the light of these results new perspectives in the study of N₂-fixing plants acclimated to elevated CO₂ are open. Further investigations are needed concerning the role of bacteroid isocitrate synthase as key enzyme of TAC, the function of carbonic anhydrase in the nodule inner cortex and its implication regulating the O₂ concentration and the affectation of nitrogenase reductase as well as N transport from bacteroids to infected plant cells.

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