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ORIGINAL PAPER

Photosynthetic down-regulation in N_2-fixing alfalfa under elevated CO_2 alters rubisco content and decreases nodule metabolism via nitrogenase and tricarboxylic acid cycle

Gorka Erice · Alvaro Sanz-Sáez · Ricardo Aroca · Juan Manuel Ruíz-Lozano · Jean-Christophe Avice · Juan José Irigoyen · Manuel Sanchez-Diaz · Iker Aranjuelo

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Abstract Although responsiveness of N_2-fixing plants to elevated CO_2 conditions have been analyzed in previous studies, important uncertainties remain in relation to the effect enhanced CO_2 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 N_2-fixing alfalfa plants after long-term exposure to elevated CO_2. After 30-day exposure to elevated CO_2, plants showed photosynthetic down-regulation with reductions in the light-saturated rate of CO_2 assimilation (A_{sat}) and the maximum rate of rubisco carboxylation (Vc_{max}). Under elevated CO_2 conditions, the rubisco availability limited potential photosynthesis by around 12 %, which represented the majority of the observed fall in Vc_{max}. Photosynthetic down-regulation has been associated with decreased N availability even if those plants are capable to assimilate N_2. 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 CO_2, 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.

Keywords Alfalfa · CO_2 · Nodule · Proteomic

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G. Erice · J. J. Irigoyen · M. Sanchez-Diaz
Departamento de Biología Vegetal, Sección Biología Vegetal, Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño, Facultades de Ciencias y Farmacia, Universidad de Navarra, c/Irunlarrea 1, Pamplona, Navarra, Spain
e-mail: gerisor@gmail.com

A. Sanz-Sáez
Departament de Biologia Vegetal, Facultat de Biologia, Universidad de Barcelona, Av. Diagonal, 645, 08028 Barcelona, Spain

R. Aroca · J. M. Ruíz-Lozano
Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Profesor Albareda 1, 18008 Granada, Spain

J.-C. Avice
INRA, UMR INRA-UCBN 950 Ecophysiologie Végétale, Agronomie & nutritions N.C.S., Institut de Biologie Fondamentale et Appliquée, IFR 146 ICORE, Esplanade de la Paix, 14000 Caen, France

I. Aranjuelo (✉)
Instituto de Agrobiotecnología, Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Campus de Arrosadía, 31192 Mutilva Baja, Spain
e-mail: iker.aranjuelo@gmail.com

Introduction

Since the beginning of the industrial revolution in the 18th century, the increase in atmospheric CO_2 has been mainly of anthropic origin. This has been the consequence of industrial development and a quadrupling of the human population during the last one hundred years (Krausmann et al. 2009). In 2009, atmospheric CO_2 concentration reached 387 μmol mol^{-1} and, according to the predictions of the Intergovernmental Panel on Climate Change, at the

end of the present century this concentration may reach 700 μmol mol^{-1} (IPCC 2007). The primary effect of increasing CO_2 in C_3 plants is a short-term photosynthetic enhancement, and consequently, an increase in plant productivity (Daepp et al. 2000). Nevertheless, this response to CO_2 is frequently not maintained over the long term and photosynthesis declines (Erice et al. 2006a; Ainsworth and Rogers 2007; Aranjuelo et al. 2009), a phenomenon named acclimation of photosynthesis. Previous studies with alfalfa showed that this photosynthetic down-regulation was attributed to decreased photosynthesis (Aranjuelo et al. 2005; Erice et al. 2006b). The "capacity" to adjust C fixation with C requirements has been described as a key process conditioning photosynthetic performance under elevated [CO_2] (Ainsworth et al. 2003; Ziska 2008; Sanz-Sáez et al. 2010). According to these studies, when growth under elevated [CO_2] conditions leads to an imbalance between C fixation and C requirements, plants decrease their photosynthetic rates to balance C source activity and sink capacity (Aranjuelo et al. 2009). From this point of view, the presence of a high carbohydrate demand, such as the nodules, should enable the N_2-fixing plants a better adjustment of leaf carbohydrate content.

Previous studies (Serraj et al. 1998; Rogers et al. 2006) have postulated that legumes, since they are capable of fixing atmospheric N_2, will have an advantage in plant growth over non-N_2-fixing plants. In the symbiotic relationship between the plant and the bacteria, the plant provides photoassimilates, which supply the energy and C skeletons (in the form of sucrose) that are transformed into dicarboxylic acids in the nodules (Lodwig et al. 2003). Organic acids provide reductant to both the N_{ase} complex and the respiratory chain that fuels N_{ase} with the ATP necessary for N_2 fixation (Streeter 1987). The bacteroid returns ammonium (NH_4^+) to the host that is assimilated in the form of amino acids, which are partitioned to the rest of the plant through the xylem according to their requirements (Udvardi and Day 1997). According to previous studies (Ainsworth et al. 2003; Rogers et al. 2006), legumes might be able to overcome photosynthetic acclimation because of their ability to reset the balance of C and N metabolism under elevated [CO_2]. Although positive productivity and N_2 fixation responses to elevated [CO_2] are common (Soussana and Hartwig 1996; Lee et al. 2003), such a response is not universal. According to observations made from other studies (Sanz-Sáez et al. 2010; Hungate et al. 2004; West et al. 2005), the possession of nodules did not avoid leaf carbohydrate build up with a consequent decrease in the photosynthetic capacity. It remains unclear why some studies show stimulation of N_2 fixation and plant growth under elevated [CO_2] and others not.

Previous studies conducted in N_2-fixing alfalfa plants exposed to other stressful growth conditions (Naya et al. 2007; Becana et al. 2010; Aranjuelo et al. 2011) remark that the main processes limiting nodule functioning are: (1) carbohydrate availability, (2) accumulation of nitrogenous compounds, (3) O_2 permeability, and accumulation of reactive oxygen species (ROS). 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, the larger photosynthetic rates of plants exposed to 1,000 μmol mol^{-1} CO_2 were translated into larger nodule carbohydrate levels. According to the second theory the decrease in nitrogenase activity has been associated with the accumulation of nitrogenous compounds (Hartwig 1998; Serraj et al. 1999). The accumulation of these compounds induces a negative feedback mechanism with a consequent inhibition of N_{ase} activity (Hartwig 1998; Serraj et al. 1998; King and Purcell 2005). 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 (Serraj et al. 1999; King and Purcell 2005; Aranjuelo et al. 2011). Results from experiments manipulating N sink strength demonstrate a clear effect on N_{ase} activity (Schulze 2004) and references therein. Finally, even if it has not been described in alfalfa nodules exposed to elevated CO_2, it should not be discarded that oxidative stress could also be involved in the worse nodule performance (Naya et al. 2007; Becana et al. 2010).

Although responsiveness of N_2-fixing plants to elevated CO_2 has been previously studied, those studies have been mainly focused in shoot performance, paying less attention to nodule. Moreover, the influence of increased CO_2 on nodule protein profile and functioning remains unclear. Within this context, the main goal of our study was to characterize elevated CO_2 effect in nodule functioning and its implication in shoot responsiveness to elevated CO_2 conditions. For this purpose, exclusively N_2-fixing alfalfa plants were exposed to elevated exposed to elevated (700 μmol mol^{-1}) and ambient (385 μmol mol^{-1}) CO_2. In order to test the effect of CO_2 on nodule and shoot performance, we characterized nodule and shoot carbohydrate (soluble sugars and starch), protein and N content. At the nodule level, 2-D proteomic profile analyze was carried out, whereas in leaves, Rubisco (representing up to 50 % of leaf soluble protein) content and expression were determined.

Materials and methods

Plant material

Alfalfa (*Medicago sativa* L. Cv. Aragón) seeds were sterilized in a solution of $HgCl_2$ (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 three times per week with either Hoagland N-free solution (Hoagland and Arnon 1950) or distilled water alternatively to avoid salt accumulation in the substratum. Plants were grown in a greenhouse at 25/15 °C (day/night) with a 14-h photoperiod under natural daylight, supplemented with fluorescent lamps (Sylvania Decor 183, Professional-58 W, Germany) providing a photosynthetic photon flux density (PPFD) of 400 µmol m^{-2} s^{-1}. When plants were 30 days old, they were transferred to two growth chambers (Conviron PGV 36, Winnipeg, Canada), four pots per growth chamber, and randomly assigned to the appropriate atmospheric CO_2 concentration (one growth chamber was maintained at ambient CO_2 concentration—around 385 µmol mol^{-1}—and the other chamber was maintained at elevated CO_2 concentration—700 µmol mol^{-1}). The conditions in the growth chamber were 25/15 °C (day/night), 40 % RH, 14-h photoperiod and 600 µmol m^{-2} s^{-1} PPFD.

Growth parameters

After 1 month exposure to ambient or elevated CO_2 concentrations, 60-day-old plants were separated into leaves, stems, roots and nodules. The dry mass (DM) of each organ and the root-to-shoot ratio (*R*/*S*) were obtained after drying in an oven at 80 °C for 48 h. Fresh weight aliquots of leaves and nodules were also stored at −80 °C for further biochemical and molecular analysis.

Gas exchange parameters

Gas exchange parameters were measured in fully expanded young apical leaves after 30-day exposure to CO_2 treatments (60-day-old plants) using a LI-COR 6400 portable photosynthesis system (LICOR Biosciences, Lincoln, Nebraska, USA). The A-Ci determinations were conducted from 60 to 1,400 µmol mol^{-1} CO_2 at a saturating PPFD of 1,200 µmol m^{-2} s^{-1}. The light-saturated rate of CO_2 assimilation (A_{sat}) was estimated using equations developed by von Caemmerer and Farquhar (1981). Measurements from net photosynthesis and intercellular CO_2 were used to assess the maximum rate of rubisco carboxylation (Vc_{max}) employing the mathematical model developed by Ethier and Livingston (2004) and Sharkey et al. (2007). Leaf-to-ambient CO_2 concentration (Ci/Ca) was also calculated for both ambient (400 µmol mol^{-1}) and elevated (700 µmol mol^{-1}) CO_2 concentrations.

Analysis of non-structural carbohydrates

Leaf total soluble sugars (TSS) and starch concentration were quantified from leaves harvested after 1 month of treatment by grinding and filtering 200 mg of leaf fresh weight in a cold mortar using an extraction buffer containing 50 mM K-phosphate (pH 7.5). The extract was filtered and centrifuged at 28,710×*g* for 15 min at 4 °C. The TSS quantification was performed with the supernatant, whereas starch was measured using the pellet as described by Jarvis and Walker (1993). TSS levels were measured using the method of Yemm and Willis (1954).

Analysis of N and determination of specific nodule activity

N concentration corresponding to leaf, stem, root and nodule samples was determined with 1.5 mg of ground dry subsamples (4 replicates for each sample). All these determinations were conducted at the Laboratoire d'Ecophysiologie Végétale, Agronomie et nutritions N, C, S, Université de Caen Basse-Normandie (France) using an elemental analyzer (EA 3000, EuroVector, Milan, Italy).

As it has been described above, experimental plants were grown in an inert substrate and were fertilized with a N-free Hoagland solution. This is why, it can assured all the N present in plants is derived from the nodule

N_2 fixation

Taking this into account, SNA was estimated as an indicator of plant N fixation integrator (Brioua and Wheeler 1994). SNA was determined as the ratio between plant total nitrogen content and nodule DM. The nodules harvested at the end of the month growing in elevated CO_2 were functional according to the red active external color and the obtained SNA values. No senescent nodules or separated from the root were observed in the experimental plants.

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 using an 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 of random hexamer primer, 0.5 mM of each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1× first strand buffer (Invitrogen, Carlsbad, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer 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 1:10 dilution of the cDNA, 200 nM dNTPs, 400 nM of each primer, 3 mM $MgCl_2$, 2.5 µL of 1× SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted of 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 for the different treatments were standardized according to β-tubulin gene expression levels, which were 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 with at least four independent RNA samples, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Negative controls without cDNA were used in all PCR reactions.

Rubisco semi-quantification

Extracts from protein quantification were precipitated using the sodium deoxycholate-trichloroacetic acid protocol described by Peterson (1983). The resulting pellet was air dried and resuspended in Laemmli lysis buffer (Laemmli 1970) and boiled for 10 min to denature proteins. For SDS-PAGE, 4 µg of soluble proteins was prepared and performed using a 150 g L^{-1} acrylamide separation gel and stained with silver nitrate (Blum et al. 1987). Gel images were scanned and analyzed using ImageQuant TL software (GE Amersham Biosciences, UK). The relative proportion of rubisco large (RLS) and small (RSS) subunits was calculated in reference to the abundance value of RLS observed in the ambient CO_2 concentration.

Nodule proteomic characterization

Frozen nodule samples (200 mg fresh weight) were ground with liquid nitrogen and resuspended in 2 mL of cold acetone containing 10 % TCA (v/v). After centrifugation at 16,000*g* for 3 min at 4 °C, the supernatant was discarded and the pellet was rinsed with methanol, acetone, and phenol solutions as previously described by Wang et al. (2003). The pellet was stored at –20 °C or immediately resuspended in 200 µL of R2D2 rehydration buffer [5 M urea, 2 M thiourea, 2 % 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate, 2 % *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulphonate, 20 mM dithiothreitol, 5 mM TRIS (2-carboxyethyl) phosphine, 0.5 % IPG buffer (GE Healthcare, Saclay, France), pH 4–7 (Mechin et al. 2003). The total soluble protein (TSP) concentration was determined by the method of Bradford (1976) using BSA as standard. Two-dimensional electrophoresis (2-DE) was conducted according to the method of Aranjuelo et al. (2011).

Image analysis of 2-DE gels

Images of the two-dimensional gels were acquired with the ProXPRESS 2D proteomic Imaging System (Perkin-Elmer, Courtaboeuf, France) and analyzed using Progenesis SameSpots software v3.0 (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 performed automatically and only adjusted on those gels where darker images led to both incorrect warping and matching. *M*r and p*I* 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. Proteins with higher relative abundance at ambient CO_2 concentration than elevated CO_2 concentration were considered down-regulated, whereas opposite were considered up-regulated.

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 % CH_3CN 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

Table 1 Effect of ambient (400 μmol mol^{-1}) or elevated CO_2 (700 μmol mol^{-1}) on leaf, stem, root, and nodule dry matter (DM) and the root/shoot ratio (*R/S*) in nodulated alfalfa plants after 30 days of treatment exposure

	Leaf DM (g $plant^{-1}$)	Stem DM (g $plant^{-1}$)	Root DM (g $plant^{-1}$)	Nodule DM (g $plant^{-1}$)	*R/S*
Ambient CO_2	0.32 ± 0.04	0.46 ± 0.05	0.28 ± 0.04	0.036 ± 0.005	0.50 ± 0.03
Elevated CO_2	0.34 ± 0.03	0.41 ± 0.05	0.37 ± 0.04	0.034 ± 0.003	0.45 ± 0.05

The DM of each organ was obtained after drying in an oven at 80 °C for 48 h. *R/S* ratio was calculated dividing the root DM and the shoot DM. Values represent the mean ± SE ($n = 4$)

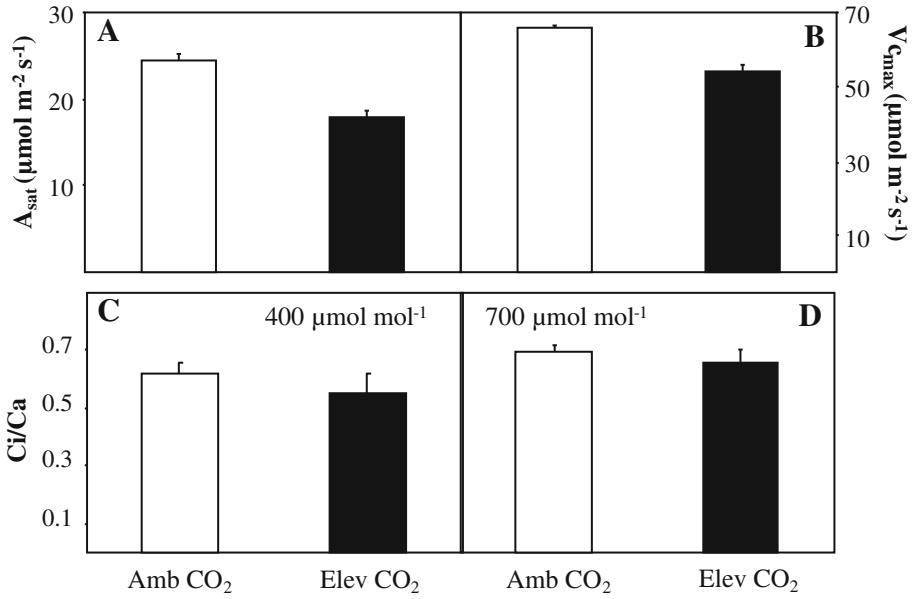

Fig. 1 Effect of elevated CO_2 on **a** saturating maximum photosynthetic rate (A_{sat}), **b** maximum carboxylation velocity of rubisco (Vc_{max}) and **c**, **d** leaf-to-ambient CO_2 concentration (Ci/Ca) at 400 or 700 μmol mol^{-1}, respectively, in fully expanded young apical leaves of nodulated alfalfa plants after 30 days of treatment exposure. Gas exchange measurements were performed at saturated light of 1,200 μmol m^{-2} s^{-1} and conducted from 60 to 1,400 μmol mol^{-1} of CO_2. Values represent the mean ± SE ($n = 4$)

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.

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

Results

After 30-day exposure to ambient or elevated atmospheric CO_2 concentration there were no differences observed in leaf, stem, root, or nodule dry mass, nor were there differences in *R/S* ratio (Table 1). The measured gas exchange parameters (A_{sat} and Vc_{max}) decreased 26.4 and 16.9 %, respectively, under elevated CO_2 (but no differences were found when comparing Ci/Ca for both 400 and 700 μmol mol^{-1} (Fig. 1).

Leaf TSS concentration was unaffected by elevated CO_2 but starch was increased by 12.8 % (Table 2). After elevated CO_2 exposure, leaf N concentration and total N concentration were decreased a 39.33 and 32.69 %, respectively, by elevated CO_2 whereas stem, root or nodule N was unaffected (Table 2). Specific nodule activity decreased a 37.2 %under elevated CO_2 (Table 2).

Elevated CO_2 decreased a 75 % the expression of rubisco small subunit mRNA (*RbcS*) (Fig. 2a). SDS-PAGE performed on leaf soluble proteins revealed that rubisco large subunit (RLS) content decreased 58.97 % under elevated CO_2 concentration but rubisco small subunit (RSS) content increased a 44.96 % (Fig. 2b, c). This

Table 2 Effect of ambient or elevated CO_2 (700 μmol mol^{-1}) on total soluble sugars (TSS), starch, leaf, stem and root N concentration, total N, and specific nodule activity (SNA) calculated as the ratio between plant total mg of N and nodule DM in nodulated alfalfa plants after 30 days of treatment exposure

	TSS (mg g^{-1} DM)	Starch (mg g^{-1} DM)	Leaf N (mg g^{-1} DM)	Stem N (mg g^{-1} DM)	Root N (mg g^{-1} DM)	Nodule N (mg g^{-1} DM)	Total N (mg $plant^{-1}$)	SNA (mg N nodule DM^{-1})
Ambient CO_2	43.49 ± 3.64	42.21 ± 1.37	16.16 ± 2.14	9.68 ± 1.09	6.46 ± 0.24	66.25 ± 0.41	13.64 ± 1.46	0.43 ± 0.03
Elevated CO_2	34.91 ± 1,92	48.41 ± 1.30	9.63 ± 0.85	7.75 ± 0.93	6.72 ± 0.84	61.68 ± 0.05	9.18 ± 0.67	0.27 ± 0.02

Values represent the mean ± SE ($n = 4$)

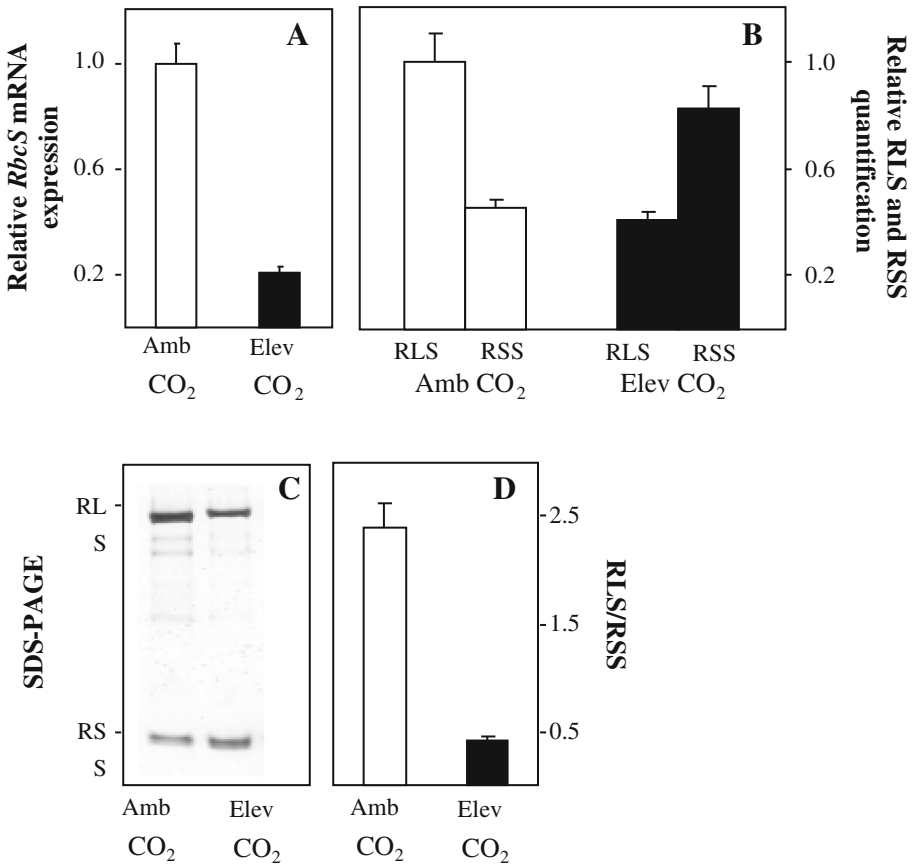

Fig. 2 Effect of elevated CO_2 on **a** the relative rubisco small subunit mRNA accumulation (*RbcS*), **b** the relative rubisco large (RLS) and small (RSS) subunit protein quantification (the value 1 corresponds to the abundance observed for RLS under ambient CO_2), **c** leaf SDS-PAGE profile, **d** and RLS/RSS protein ratio in nodulated alfalfa plants after 30 days of treatment exposure. Values represent the mean ± SE ($n = 4$)

resulted in a decrease in the RLS/RSS ratio of 83.2 % (Fig. 2d).

The effect of rising CO_2 on the nodule protein profile in alfalfa plants was studied using 2-DE (Fig. 3). Twelve proteins have been identified with different expression under ambient and elevated CO_2 concentration. Eight of those proteins were down-regulated by CO_2 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), secondary metabolism (1) and unclassified (2). Up-regulated proteins were essentially related to energy (2) or were unclassified (2). The roles of these proteins are discussed in the following section with regard to physiological and biochemical changes observed in nodulated alfalfa under elevated CO_2.

Discussion

Despite the theoretical initial increase in photosynthesis, in our study, plant growth measured as dry mass accumulation was similar in both [CO_2] (Table 1) (Aranjuelo et al.

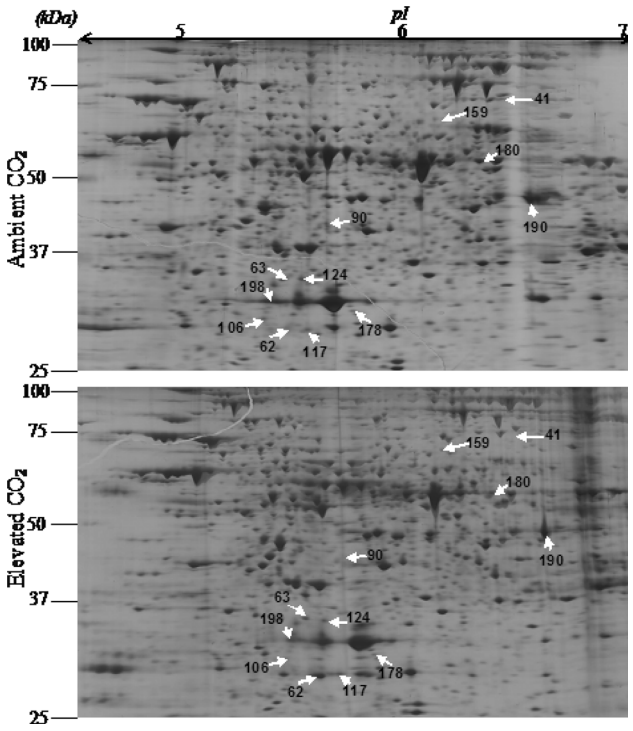

Fig. 3 Effect of elevated CO_2 in silver-stained two-dimensional gels of proteins extracted from *Sinorhizobium meliloti* strain 102F78 alfalfa nodules after 30 days of treatment exposure. In the first dimension, 125 µg of total proteins were loaded on an 18-cm IEF strip with a linear gradient of pH 4–7. The second dimension was conducted in 12 % polyacrylamide (w/v) gels (20 × 20 cm) (for details see "Materials and methods"). The gel image analyses were conducted with Progenesis SameSpots software v3.0 and the subsequent mass spectrometry analyses identified up to 12 proteins (*marked by arrows*) that were involved in the nodule response to elevated CO_2

2005, 2008, 2009). According to the A_{sat} and Vc_{max} results, plants grown under elevated CO_2 concentration showed a clear acclimation that led to a reduction of 17.2 % of Vc_{max} (from 66 to 54 µmol m^{-2} s^{-1}, Fig. 1). Studies about photosynthesis down-regulation have related the accumulation of total non-structural carbohydrates, including TSS and starch, with limitation of photosynthetic enzymes (Moore et al. 1999; Rogers and Ellsworth 2002) including rubisco (Aranjuelo et al. 2005, 2008). In the present work, even if leaf TSS concentration was unchanged starch was increased in response to elevated CO_2 (Table 2). Despite TSS usually shows variable response to increasing CO_2, starch is enhanced in most of the cases (Erice et al. 2006b; Sanz-Sáez et al. 2010). Starch has been described as a buffer to sucrose metabolism (Moore et al. 1999) and its accumulation is suggestive of higher sugar production. This greater carbohydrate availability may be the cause of the lower rubisco small subunit gene expression (*RbcS*) (Fig. 2a). Down-regulation of *RbcS* by elevated CO_2 has been reported repeatedly (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 CO_2 (Cheng et al. 1998). In the present study, the reduction of *RbcS* expression was not accompanied by a lower quantity of RSS, which actually increased under elevated CO_2 conditions (Fig. 2b, c). Similar results with posttranscriptional regulation of protein content were obtained in tomato (Van Oosten and Besford 1995) or *Chlamydomonas reinhardtii,* probably due to the inhibition of translation of *RbcS* mRNA (Winder et al. 1992).

Even in nodulated plants, some studies have related the decrease in rubisco protein under elevated CO_2 with limited N availability (Farage et al. 1998; Geiger et al. 1999). In the present study, although alfalfa plants stem, root or nodule N content was similar, reduced leaf N led to a depleted total N (Table 2). The reduced leaf N was linked to impaired nodule functioning under elevated CO_2 conditions, as reflected by the lower SNA (Table 2) (Aranjuelo et al. 2005). This fact may be related to a previously observed drop in 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 in the response of SNA to elevated CO_2 has been extensively reported. Some authors have reported an increase of SNA in alfalfa with root CO_2 enrichment (Fischinger et al. 2010), whereas in most reported experiments shoot CO_2 assimilation shows neither a short- nor a long-term effect on SNA (Vance and Heichel 1991; Cabrerizo et al. 2001).

The nodule proteomic profile under elevated CO_2 confirmed the alteration of nodule metabolism that reduced nitrogenase reductase (Fig. 3; Table 3). Nitrogenase reductase, frequently referred to as Fe-protein (Howard and Rees 1994), is part of the nitrogenase enzyme complex and catalyzes the N_2 fixation reaction (Dixon and Kahn 2004; Seefeldt et al. 2009). The lowered leaf N demand of photosynthetically acclimated plants, as observed under water-limited conditions in soybean (Serraj et al. 1998, 1999), may lead to a decline in N-transporting solutes. It also favors the accumulation of products associated with N_2 fixation in nodules leading to an inhibition of nitrogenase activity in bacteroides. The depletion in nitrogenase reductase content (Fig. 3; Table 3) suggests that the lower shoot N demand negatively affected the availability of the activity of this protein and consequently affected nitrogenase activity and N_2 fixation similarly.

In nodules grown under elevated CO_2, the bacteroid citrate synthase was also repressed (Fig. 3; Table 3). This enzyme catalyzes oxaloacetate condensation with acetyl-CoA, a step in citrate synthesis (Popova and de Carvalho 1998), and is involved in the initial stage of the tricarboxylic acid (TCA) cycle. Citrate synthase in association

Table 3 Annotation of identified down/up-regulated spots following 30 days of elevated CO_2 exposure in silver-stained 2-DE gels of alfalfa nodules

No.	B/V	Spot % volume variations	Exp. p*I*/M_r	Theor. p*I*/M_r	PM	SC (%)	Score ($P < 0.05$ corresponding to score >51)	Protein name/organism/NCBI accession no.	Putative function
Down-regulated									
198	B	15	5.11/30.01	5.35/31.99	6	33	360	Nitrogenase reductase/*Sinorhizobium meliloti* 1021/gil16262902	01. Metabolism
190	B	20	6.41/44.72	6.02/47.97	13	38	635	Type II citrate synthase/*Sinorhizobium meliloti* 1021/gil15965262	02. Energy
41	B	86	6.40/69.61	6.26/68.16	1	1	58	ABC transporter, ATP-binding protein/*Roseovarius nubinhibens* ISM/gil83952290	07. Transporters
124	B	47	5.39/31.04	4.33/10.70	1	19	51	YebA/*Klebsiella pneumoniae*/gil38639575	07. Transporters
180	B	24	6.29/58.77	6.52/62.93	4	10	194	Catalase/*Sinorhizobium meliloti*/gil1698550	11. Disease/Defence
90	B	61	5.20/40.23	6.19/47.46	1	1	55	Hypothetical protein Sare_4839/*Salinispora arenicola* CNS-205/gil159040327	13. Unclassified
106	V	52	5.21/28.65	8.61/36.35	1	2	51	Predicted protein/*Chlamydomonas reinhardtii*/gil159462486	13. Unclassified
63	V	71	5.23/33.49	5.39/35.39	4	16	99	Isoflavone reductase/*Medicago sativa*/gil19620	20. Secondary metabolism
Up-regulated									
62	V	71	5.36/29.01	5.78/29.85	3	15	88	Carbonic anhydrase/*Lotus japonicus*/gil28625017	02. Energy
117	V	48	5.58/29.02	6.02/30.13	5	20	164	Carbonic anhydrase/*Medicago sativa* subsp. × varia/gil1938227	02. Energy
159	B	38	6.31/72.68	10.75/25.17	2	4	52	Hypothetical protein NGO1977/*Neisseria gonorrhoeae* FA 1090/gil59802286	13. Unclassified
178	V	27	5.75/27.82	9.62/218.41	6	1	52	Predicted protein/*Physcomitrella patens* subsp. Patens/gil168015024	13. Unclassified

Spot no. represents the number of proteins assigned. Spot volume (%) is an estimation of relative protein abundance. The p*I* and molecular mass (M_r) values shown are the theoretical and experimental values. SC represents the protein sequence coverage (%) score, which is the Mascot score of the in-solution digestion protocol. PM indicates for each spot, the number of peptide matched according to the results of research on the NCBInr-protein sequence databases. Putative function, the predicted protein function is assigned according to the NCBInr-protein sequence database of Bacteria (B) or Viridiplantae (V)

with isocitrate dehydrogenase may be a crucial point in TCA cycle regulation (Wiegand and Remington 1986; Chen and Gadal 1990). Changes in the rate of the TCA cycle and its anaplerotic reactions may not only alter the source of redox equivalents for the electron transfer chain but also act as a source of intermediates for lipogenesis, organic acids and amino acids synthesis. Thus, TCA is considered as a central point of bacteroid intracellular metabolism, decreasing energy supply to nitrogenase and other energy demanding processes (Popova and de Carvalho 1998). Therefore, its depletion could have contributed to the poor nodule performance of nodules exposed to elevated CO_2. The proteomic characterization (Fig. 3; Table 3) also revealed a decrease in catalase content. Aranjuelo et al. (2007) showed that in alfalfa plants exposed to elevated CO_2, the activity of leaf antioxidant enzymes such as catalase, superoxide dismutase and glutathione reductase decreased. This relaxation of the antioxidant system was related to the lower growth rate that resulted from photosynthesis acclimation (Erice et al. 2007). This observation agrees with the repression of isoflavone reductase (Fig. 3; Table 3), a protein involved in the production of isoflavone phytoalexins that accumulates under biotic or abiotic stresses (Salekdeh et al. 2002; Kim et al. 2003) and along with antioxidants plays a crucial role in cell signaling or maintaining the redox status of cells (Lee et al. 2009). This metabolic reduction in the nodules is also supported by the repression of a bacteroid ABC transporter with a low score of 58 (Fig. 3; Table 3). This transporter is implicated in urea production (Wang et al. 2008), peptide transients (Stacey et al. 2002) and legume nodule development (Marx 1996) and all of which may also be inhibited by the lower leaf N demand under elevated CO_2.

Besides, the nodules of alfalfa plants grown at elevated CO_2 concentrations showed increased carbonic anhydrase content (Fig. 3; Table 3). This enzyme with the combined activity of phosphoenolpyruvate carboxylase (Vance et al. 1994) and malate dehydrogenase (Schulze et al. 2002) transforms phosphoenolpyruvate into oxaloacetate and malate (Atkins et al. 2001). The higher carbonic anhydrase activity is needed to maintain a continuous supply of malate (Gálvez et al. 2000), and thus C and energy available for bacteroid consumption (Aranjuelo et al. 2009). The up-regulation of this protein revealed that the poor nodule performance was not caused by limitations on C supply (in form of organic acids, the main C source for nodules) to the nodules. In parallel to this effect, bicarbonate/CO_2 equilibrium on convective gas flow into legume nodules would enhance O_2 transport into the central zone (Thumfort 1996).

In summary, after long-term exposure, N_2-fixing alfalfa plants acclimated to elevated CO_2 showed decreases in A_{sat} and Vc_{max}. This down-regulation was related to leaf carbohydrate accumulation which may reduce *RbcS* expression. Nevertheless, increased CO_2 did not decrease the RSS content, probably due to posttranscriptional processes including mRNA stability, the improvement of translation of *RbcS* mRNA and/or the inhibition of proteolysis (resulting to a modification of RSS turn-over). Under elevated CO_2, RLS decreased at photosynthesis limiting levels, reducing the potential photosynthesis by around 12 %, which is the majority of the observed total drop in Vc_{max} in these plants. Photosynthetic down-regulation has been associated with decreased N availability even if those plants are capable to assimilate N_2. Reduction in SNA suggests that the poor nodule performance was involved in the leaf N decrease. Nodule functioning was altered simultaneously in various enzyme quantities including nitrogenase. Limited bacteroid metabolism, as reflected by lower citrate synthase, would require lower O_2 demand. Increased carbonic anhydrase is related to changes in O_2 permeability that may damage N_2 fixation through nitrogenase reductase, which is sensitive to O_2 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 drop in SNA under elevated CO_2. Moreover, a reduced bacteroid N transient capacity due to ABC transporter depletion could result in a decline in nitrogenase activity via a feedback mechanism and lead to leaf N diminution. In the light of these results, new perspectives on the study of N_2-fixing plants acclimated to elevated CO_2 have been revealed. Further investigations are needed concerning the role of bacteroid isocitrate synthase as a key enzyme of the TCA cycle, the function of carbonic anhydrase in the nodule inner cortex, and its implication in regulating the O_2 concentration and the alteration of nitrogenase reductase as well as N transport from bacteroides to infected plant cells.

Author contribution Gorka Erice and Iker Aranjuelo designed, performed and conducted the experiment and wrote the manuscript. Alvaro Sanz-Sáez participated in the experiment execution and biochemic determinations. Ricardo Aroca and Juan Manuel Ruíz-Lozano conducted Rubisco content and expression analyses, Jean-Christophe Avice participated in the nodule 2D proteomic characterization and Juan José Irigoyen and Manuel Sanchez-Diaz participated in the experimental design and manuscript redaction.

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