



Unravelling the mechanisms that improve photosynthetic performance of N₂-fixing pea plants exposed to elevated [CO₂]



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ABSTRACT

Although the predicted enhanced photosynthetic rates of plants exposed to elevated [CO₂] are expected to increase carbohydrate and plant growth, recent findings have shown a complex regulation of these processes. The aim of this study was to determine the effect of elevated [CO₂] on pathways leading to the main forms of leaf C storage (starch) and export (sucrose) and the implications of this increased [CO₂] on photosynthetic performance of exclusively N₂ fixing plants. For this purpose, exclusively N₂-fixing pea plants were exposed to elevated [CO₂] (1000 μmol mol⁻¹ versus 360 μmol mol⁻¹ CO₂). The data obtained highlighted that plants exposed to elevated [CO₂] were capable of maintaining hexose levels (involved in Rubisco down regulation) at control levels with the consequent avoidance of photosynthetic acclimation. More specifically, in plants exposed to elevated [CO₂] there was an increase in the activity of pathways involved in the main forms of leaf C storage (starch) and export (sucrose). Furthermore, the study highlighted that although starch content increased by up to 40% under elevated [CO₂], there was also an increase in the proteins and compounds involved in starch degradation. Such a finding, together with an increase in the activity of proteins involved in sucrose synthesis revealed that these plants up-regulated the sucrose synthesis pathway in order to meet the large nodule photoassimilate requirements. As a consequence, the study highlighted the relevance of controlling the activity of pathways that determine leaf cellular carbohydrate availability and how this is linked with C-demanding organs such as nodules.

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1. Introduction

Plant photosynthetic performance is conditioned by changing environmental conditions. Most plants buffer these changes by adapting the activity of proteins involved in photosynthesis (Leakey et al., 2009). Another target point to balance leaf carbohydrate

availability is the mobilization of photoassimilates (mainly in the form of sucrose) towards C-demanding organs and/or by retaining part of the photosynthate as starch (Gibson et al., 2009). The effect of atmospheric [CO₂] in photosynthetic performance has been studied extensively during recent decades (Leakey et al., 2009). Because the current [CO₂] in the atmosphere is generally limiting for C₃ photosynthesis, it has been suggested that the predicted [CO₂] increase will enhance the photosynthetic rate of plants (Farquhar et al., 1980). However, the initial stimulation has been described as being partially reversed in an acclimation process often referred to as “down-regulation” (Leakey et al., 2009). According to the model proposed by Krapp et al. (1993) and Moore et al. (1999), as a result of a larger atmospheric [CO₂] (C_a) there would be an increase in the photosynthetic rates of plants and consequently in the availability of a major leaf photoassimilate form such as sucrose. More specifically, the enhanced HK catalytic activity initiates the transduction response that induces the repression of the promoter activities of Rubisco and other photosynthetic genes (Krapp et al., 1993; Moore et al., 1999).

The leaf soluble sugar level is conditioned by the leaf's metabolic activity, storage processes, and the sink strength of other plant organs (Moore et al., 1999). Therefore, the leaf sucrose content may reflect the balance of C sink/source demand. In this sense, starch

Abbreviations: ADPG, ADP-glucose; A_n, net photosynthetic rate; A₃₆₀, A_n determined at 360 μmol mol⁻¹ CO₂; A₁₀₀₀, A_n determined at 1000 μmol mol⁻¹ CO₂; C_a, atmospheric [CO₂]; Gluc, glucose; Gluc1P, glucose-1-phosphate; Gluc6P, glucose-6-phosphate; Fruc, fructose; Fruc6P, fructose-6-phosphate; Fruc-2,6bP, fructose-2,6-bisphosphate; HK, hexokinase; PGM, phosphoglucosmutase; PHI, phosphohexose isomerase; Pi_{chl}, chloroplastic phosphate; Pi_{cyt}, cytosolic phosphate; N_{red}, reduced N content; SP, starch phosphorylase; Suc, Sucrose; TOA, total organic acid; TP, triose phosphate; TSP, total soluble protein; TSS, total soluble sugar; UDPG, UDP-glucose; UDPG-PPi_{ase}, uridine diphosphoglucose pyrophosphorylase; α-Amy, α-amylase; β-Amy, β-amylase; 3PGA, 3-phosphoglyceric acid.

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has also been proposed as a buffer for sucrose metabolism that might minimize leaf sucrose cycling (Moore et al., 1999). Although the synthesis of sucrose and starch takes place in the cytosol and chloroplast, the C fluxes through these pathways are highly regulated in order to avoid the inhibition of photosynthetic activity (Stitt et al., 2010). Starch synthesis in leaves has been traditionally considered to be exclusive to chloroplasts and the synthesis of sucrose exclusive to the cytosol (Neuhaus et al., 2005). According to these studies, starch is considered as the end product of a unidirectional pathway where ADP-glucose pyrophosphorylase (APG) exclusively catalyses the synthesis of ADP-glucose (ADPG). However, the pathway leading to starch synthesis has been recently questioned (Stitt et al., 2010). Studies conducted by Baroja-Fernández et al. (2004) observed that ADPG can also be synthesized in the cytosol by sucrose synthase and afterwards imported into the chloroplast for starch synthesis.

Previous studies have highlighted that responsiveness of plants to elevated $[\text{CO}_2]$ is restricted under N limiting conditions. The negative acclimation of photosynthesis may be the result of lowered leaf N content due to limitations in soil N availability conditions (Theobald et al., 1998). Indeed, it has been suggested that a persistent increase in plant biomass production under elevated $[\text{CO}_2]$ can only be maintained by an increase in N uptake (Soussana and Hartwig, 1996). Furthermore, there is evidence that the carbohydrate-mediated repression of photosynthetic genes is more severe in nitrogen deficient plants (Aranjuelo et al., 2013). According to some authors (Serraj et al., 1998; Rogers et al., 2006), because legumes are capable of fixing atmospheric N_2 they will have access to atmospheric N_2 and they will have an advantage in plant growth over non- N_2 -fixing plants, especially in N limited soils. Moreover, legumes 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 (Eric et al., 2011).

Although Aranjuelo et al. (2013) showed that exclusively N_2 fixing pea plants were capable of maintaining improved photosynthetic rates, in contrast to the photosynthetic acclimation observed in NO_3^- -fed plants under high $[\text{CO}_2]$, the role played by nodules in this remains unclear. The aim of this study is to characterize nodule C sink demand and its implications in regulation of the synthesis of two major leaf carbohydrates (sucrose and starch) and to ascertain the mechanisms involved in this enhanced performance under elevated $[\text{CO}_2]$. For this purpose, nodule activity together with enzymes and intermediate compounds involved in the synthesis of leaf sucrose and starch were monitored in exclusively N_2 fixing pea plants exposed to elevated ($1000 \mu\text{mol mol}^{-1}$) and ambient ($360 \mu\text{mol mol}^{-1}$) $[\text{CO}_2]$.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was conducted with exclusively N_2 -fixing pea (*Pisum sativum* L. cv Frilene) plants, inoculated with *Rhizobium leguminosarum* biovar viciae strain NLV8, which is hup-, grown in 2.5 L plastic pots (one plant per pot) filled with 3:2 (v/v) vermiculite-perlite. Sixteen plants per treatment were grown in controlled-environment chambers (Heraeus-Votsch HPS-500, Norrköping, Sweden) at 25/18 °C (day/night) with a photoperiod of 16 h of $480 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) and a relative humidity of 70/80% (day/night). The cabinets provided two independent T-RH combined probes (41372VC/VF RM Young Co., Traverse City, MI, USA) connected to an external microprocessor (LI-1000, LiCor, Lincoln, Nebraska, USA). Plants were irrigated with a drip system, with N-free nutrient solution (Rigaud

and Puppo, 1975). Half of the randomly selected plants were placed into an elevated $[\text{CO}_2]$ cabinet where they were exposed to $1000 \mu\text{mol mol}^{-1}$, whereas the other half were placed in a cabinet with ambient $[\text{CO}_2]$ ($\approx 360 \mu\text{mol mol}^{-1}$). CO_2 bottles were provided by Praxair (Pamplona, Spain). Air entered the cabinets from a compressor installed at the top of the building, and was filtered by four air filters (coarse- $5 \mu\text{m}$ and $1 \mu\text{m}$ \emptyset particle and $0.01 \mu\text{m}$ \emptyset particle physical filters and a charcoal chemical filter) to prevent anomalous components. Cabinets were equipped with an infrared CO_2 analyser (polytron-IRGA, Dragäer, Lübeck, Germany) connected to a microprocessor located inside the cabinet. $[\text{CO}_2]$ was analyzed and controlled every second. All the determinations were conducted after 4 weeks of exposure to elevated $[\text{CO}_2]$ conditions.

2.2. Sampling and plant growth determinations

Plant sampling was always carried out 5 h after the onset of the photoperiod. For plant growth determinations, samples were dried at 70 °C for 48 h in order to obtain the dry biomass (DM).

2.3. Gas exchange determinations

Fully expanded apical leaves were enclosed in a Li-Cor 6200 gas exchange portable photosynthesis system (Li-Cor, Lincoln, Nebraska, USA). Net photosynthesis (A_n) was determined according to Long and Hallgreen (1985) in all the plants at 360 (A_{360}) and 1000 (A_{1000}) $\mu\text{mol m}^{-2} \text{s}^{-1}$ CO_2 . Stomatal conductance (g_s) was determined as described by Harley et al. (1992).

2.4. Metabolic compound determinations

2.4.1. Leaf determinations

Rubisco activity and content was determined in leaves that were harvested at mid-morning and immediately plunged into liquid nitrogen. The samples were stored at -80°C , awaiting analysis. The leaf tissue was powdered in liquid nitrogen and homogenized in a cold mortar with an extraction buffer containing 100 mM Bicine-NaOH (pH 7.8), 10 mM MgCl_2 , 1 mM $\text{Na}_2\text{-EDTA}$, 5 mM DTT and 2% PVPP (Aranjuelo et al., 2005). An aliquot of the extract was used to determine the chlorophyll content (Arnon, 1949). Another aliquot was clarified by centrifugation at $20,000 \times g$, and used to determine enzyme activity by measuring the oxidation of NADH at 340 nm (Sharkey et al., 1991). For Rubisco initial and total activity assays, an NADH-coupled spectrophotometric procedure was followed (Pérez et al., 2005). The activation state was calculated by considering the initial activity as a percentage of the total activity.

Rubisco protein content was determined according to Aranjuelo et al. (2005). Aliquots of the dissociated extracts, containing 9 μg of protein, were applied to each well. The gels were stained in 0.1% (w/v) Coomassie blue dissolved in 5:5:2 (v/v/v) water-methanol-acetic acid overnight, and subsequently destained in 12.5% (v/v) isopropanol and 10% (v/v) acetic acid. Finally, the gels were scanned with a BioRad GS-700 densitometer. The protein content was determined with Molecular Analyst software (BioRad). Rubisco activity determinations (with the exception of Rubisco and glycolate oxidase) were conducted in a buffer containing 50 mM MOPS (pH 7.2), 20 mM KCl, 5 mM MgCl_2 , 1 mM $\text{MK}_2\text{-EDTA}$ and 5 mM DTT according to Scott and Kruger (1995). After centrifugation at $25,000 \times g$ for 20 min the supernatant was filtered through a 5 mL Bio GEL P6DG (BioRad) column and centrifuged at $180 \times g$ for one minute. The supernatant was equilibrated with a solution containing 50 mM MOPS (pH 7.2), 20 mM KCl and 5 mM MgCl_2 . The enzyme determinations were carried out

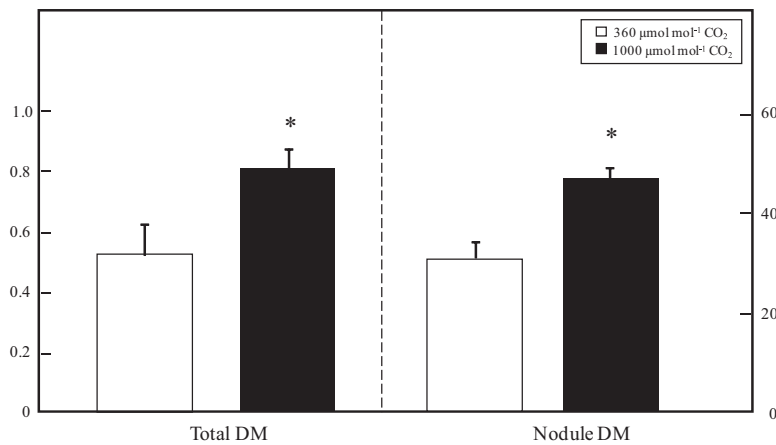


Fig. 1. Effect of elevated CO_2 (1000 versus $360 \mu\text{mol mol}^{-1}$) exposure on Total dry biomass (DM; g plant^{-1}) and nodule DM (mg plant^{-1}) in exclusively N_2 -fixing pea plants (*Pisum sativum* L.). Each value represents the mean of 16 replicates \pm SE. The asterisks (*) indicate significant differences from the corresponding control values at $P \leq 0.05$.

through the spectrophotometric (Shimadzu UV-1601, Shimadzu Corporation, Japan) determination of NADPH production.

Leaf and nodule extracts were homogenized in a solution containing 80% (v/v) ethanol that was heated until boiling. The hydroalcoholic phase was evaporated through a Turbovap (Zymark, Carmel, IN, USA) and resuspended with 4 mL of distilled water. The sample was centrifuged at $2300 \times g$ for 10 min and the supernatant and the pellet were stored separately at -80°C .

Sucrose (Suc), glucose (Gluc) and fructose (Fruc) contents were determined in the above mentioned supernatant fraction of leaf samples. For this purpose we used the protocol developed by Gordon et al. (1987). Starch content was determined in the corresponding pellets according to Cabrerizo et al. (2001).

Leaf contents of glucose-6-phosphate (Gluc6P), glucose-1-phosphate (Gluc1P), fructose-6-phosphate (Fruc6P), fructose-2,6-bisphosphate (Fruc-2,6bP), triose phosphate (TP), 3 phosphoglyceric acid (3PGA), and UDP-glucose (UDPG) were determined after leaf extraction according to Lorenc-Plucińska (1998) using a Waters 2690 high performance liquid chromatograph (Waters Millipore Corp., Milford, MA, USA) coupled to a electrochemical flux cell (VT-O3, ANTEC Leydin, Leiden, The Netherlands). The columns (CarboPac PA-10 precolumn and the PA-10 analytical column provided by the Dionex Corporation CA, USA) were set at 30°C . The flux was fixed at 1 mL min^{-1} with a linear gradient.

Uridine diphosphoglucose pyrophosphorylase (UDPG-PPiase, EC 2.7.7.9) activity was determined according to Gordon et al. (1990) and adenosine diphosphoglucose pyrophosphorylase (ADPG-PPiase, EC 2.7.7.27) was determined according to Rao et al. (1990). Phosphoglucomutase (PGM, EC 5.4.2.2) and phosphohexoisomerase (PHI, EC 5.3.1.9) were determined according to De Veau et al. (1992). Hexokinase (HK, EC 2.7.1.1) was determined as described by Levi and Preiss (1978). For fructose bisphosphatase (FBPase, EC 3.1.3.11) the cytosolic form was determined as described by Rufty et al. (1984). For phosphofructokinase (PFK, EC 2.7.1.11) analyses the extract was added to 1 mL of buffer containing 50 mM HEPES-NaOH (pH 6.8), 5 mM MgCl_2 , 0.15 mM NADH, 0.5 mM ATP, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1.2 mM F6P, 1 u aldolase, 1 u TPI and 2 u of glycerol phosphate dehydrogenase. Aldolase (Ald, EC 4.2.2.13) was determined by adding the extract to a reaction medium containing 50 mM HEPES-NaOH (pH 6.8), 5 mM MgCl_2 , 0.15 mM NADH, 1 mM F1,6bP and 1 mM of glycerol phosphate dehydrogenase. Starch phosphorylase (SP, EC 2.4.1.1) was analyzed according to the description of Rao et al. (1990). Determination of α -amylase (α -Amy EC 3.2.1.1) and β -amylase (β -Amy EC 3.2.1.2) were as described by De Veau et al. (1992).

2.4.2. Nodule determinations

Nitrogen fixation was determined as total nitrogenase activity (TNA). Plant root systems were sealed and H_2 evolution was measured in an open flow-through system under $\text{N}_2:\text{O}_2$ (79:21) in accordance with Witty and Minchin (1998) using an electrochemical H_2 sensor (Qubit System Inc., Kingston, Canada). The H_2 sensor was calibrated with high purity gases (Praxair, Madrid) employing a gas mixer (Air Liquid, Madrid, Spain) flowing at the same rate as the sampling system (500 mL min^{-1}).

Determination of total soluble proteins (TSP) and total soluble sugars (TSS) from aliquots of nodule extract were performed in the reaction medium and assay conditions described for the leaves. The total organic acid (TOA) extraction protocol was as described by Cabrerizo et al. (2001). The extracts were filtered with Millex filters (Millipore, Billerica, MA, USA) and injected in a DX-500 (Dionex, Salt Lake City, UT, USA) on an ion chromatograph equipped with an IonPac AS11 (Dionex,) column connected to an ATC-1 (Dionex) protecting column and an AG11 precolumn (Dionex).

2.5. Reduced N content

Reduced N (N_{red}) (including by total organic N and ammonium) was determined in shoot, root and nodule samples with the Kjeldahl method (Aranjuelo et al., 2005). Plant level N_{red} was calculated as the sum of shoot, root and nodule N_{red} .

2.6. Statistical analyses

Data was processed by one way analysis of variance (ANOVA) using the statistical software package SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Means \pm standard errors (SE) were represented. The results were accepted as significant at $P \leq 0.05$.

3. Results

Our study showed that exposure to elevated $[\text{CO}_2]$ increased total DM and nodule DM (Fig. 1). Gas exchange determinations conducted at the corresponding growth conditions (Table 1) revealed that plants exposed to $1000 \mu\text{mol mol}^{-1}$ CO_2 had the highest photosynthetic rates (A_n). However, when A_n was determined in all the plants at 360 (A_{360}) and 1000 (A_{1000}) $\mu\text{mol mol}^{-1}$ $[\text{CO}_2]$, no significant differences associated with growth $[\text{CO}_2]$ were observed (Table 1). g_s was not affected by the $[\text{CO}_2]$ (Table 1). At the protein level, elevated $[\text{CO}_2]$ did not modify TSP or Rubisco protein content. Table 1 also shows that the Rubisco activation state of plants

Table 1

Effect of elevated CO₂ (1000 versus 360 μmol mol⁻¹) exposure on leaf photosynthesis determined at 360 (A₃₆₀, μmol CO₂ m⁻² s⁻¹), 1000 (A₁₀₀₀) μmol mol⁻¹ [CO₂], and at the corresponding growth conditions (A_n) together with stomatal conductance (g_s, mol CO₂ m⁻² s⁻¹), leaf total soluble protein (TSP, mg g⁻¹), Rubisco content (g g⁻¹ protein), activation state (%) and plant level reduced N (N_{red}, g plant⁻¹) content determined at the corresponding growth conditions in exclusively N₂-fixing pea plants (*Pisum sativum* L.). Each value represents the mean of 8 replicates ± SE. The asterisks (*) indicate significant differences from the corresponding control values at P ≤ 0.05.

[CO ₂]	A ₃₆₀	A ₁₀₀₀	A _n	g _s	TSP	Rubisco	Activ. state	N _{red}
360 μmol mol ⁻¹	8.73 ± 0.52	12.61 ± 0.20	8.73 ± 0.52	0.96 ± 0.06	95.08 ± 2.49	0.48 ± 0.03	60 ± 3	3.70 ± 0.09
1000 μmol mol ⁻¹	8.01 ± 0.15	11.42 ± 0.53	11.42 ± 0.53*	0.92 ± 0.15	88.60 ± 1.62	0.49 ± 0.05	69 ± 3*	5.11 ± 0.47*

Table 2

Effect of elevated CO₂ (1000 versus 360 μmol mol⁻¹) exposure on leaf sucrose (Suc), starch, glucose (Gluc), fructose (Fruc) content (expressed in mg g⁻¹ DM) chloroplast and cytosol phosphate (Pi_{chl} and Pi_{cyt} respectively, mM), together with their ratio (Pi_{chl}/Pi_{cyt}, %) in exclusively N₂-fixing pea plants (*Pisum sativum* L.). Each value represents the mean of 8 replicates ± SE. The asterisks (*) indicate significant differences from the corresponding control values at P ≤ 0.05.

[CO ₂]	Suc	Starch	Gluc	Fruc	Pi _{chl}	Pi _{cyt}	Pi _{chl} /Pi _{cyt}
360 μmol mol ⁻¹	61.55 ± 5.45	74.40 ± 3.84	3.45 ± 0.49	1.58 ± 0.35	1.66 ± 0.19	21.66 ± 2.10	7.69 ± 0.91*
1000 μmol mol ⁻¹	90.35 ± 3.33*	104.04 ± 6.84*	6.75 ± 2.02	1.9 ± 0.47	2.52 ± 0.29*	42.28 ± 6.17*	5.08 ± 0.46

Table 3

Effect of elevated CO₂ (1000 versus 360 μmol mol⁻¹) exposure on leaf glucose-6 phosphate (Gluc6P), fructose-6-phosphate (Fruc6P), glucose-1 phosphate (Gluc1P), fructose-2,6-bis phosphate (Fruc-2,6bP), UDP-glucose (UDPG), phosphoglyceric acid (3PGA) and triose phosphate (TP) contents (expressed in μM) in exclusively N₂-fixing pea plants (*Pisum sativum* L.). Each value represents the mean of 8 replicates ± SE. The asterisks (*) indicate significant differences from the corresponding control values at P ≤ 0.05.

[CO ₂]	Gluc6P	Fruc6P	Gluc1P	Fruc-2,6bP	UDPG	3PGA	TP
360 μmol mol ⁻¹	462.70 ± 60.78	4.35 ± 0.43	19.37 ± 3.25	524.56 ± 66.83*	1129 ± 40	21160 ± 2393	4366 ± 580
1000 μmol mol ⁻¹	865.09 ± 43.16*	4.19 ± 0.43	20.00 ± 5.14	398.54 ± 52.43	1354 ± 76*	165574 ± 2768	3904 ± 645

exposed to 1000 μmol mol⁻¹ CO₂ was larger than in plants grown at 360 μmol mol⁻¹ [CO₂]. Exposure to elevated [CO₂] increased plant level N_{red} (Table 1).

At the leaf level, although [CO₂] did not modify Glc, Fru and TP content significantly, the sucrose and starch content increased 56 and 40%, respectively (Table 2). Although Pi_{chl} and Pi_{cyt} increased in plants under elevated [CO₂], their ratio (Pi_{chl}/Pi_{cyt}) decreased by 34% in such plants (Table 2). The analyses of phosphate sugars (Table 3) highlighted that even though [CO₂] did not significantly modify Gluc1P, Fruc6P and 3PGA content, Gluc6P and UDPG increased (87 and 20%, respectively) and Fruc-2,6bP decreased (24%).

The quantification of the activity of enzymes involved in sucrose metabolism (Fig. 2) showed that the activities of aldolase, PFK and FBPase_{cyt} were not altered by [CO₂]. On the other hand, HK and UDPG-PPi_{ase} activities increased in plants exposed to 1000 μmol mol⁻¹ CO₂ (Fig. 2). In relation to proteins involved in starch metabolism, our data (Fig. 2) showed that ADPG-PPi_{ase} was the only enzyme whose activity decreased (by 18%) in plants exposed to 1000 μmol mol⁻¹ [CO₂]. The activity of proteins such as PHI, PGM, SP, α-Amy and β-Amy increased in plants exposed to 1000 μmol mol⁻¹ CO₂ (Fig. 3).

Determinations conducted at the nodule level showed that elevated CO₂ increased TNA, TSP, TSS and TOA by 100, 32, 52 and 25% respectively.

4. Discussion

Although positive productivity and N₂ fixation responses to elevated [CO₂] have been previously described (Soussana and Hartwig, 1996; Sanz-Sáez et al., 2012; Aranjuelo et al., 2013), the way that nodule carbohydrate requirements are implicated in leaf carbohydrate availability and photosynthetic performance needs to be elucidated. Gas exchange determinations conducted at the corresponding growth condition revealed that the larger DM production of these plants was the consequence of their larger photosynthetic rates. Photosynthetic performance under elevated [CO₂] has been previously described as being diminished by down-regulation of the photosynthetic machinery, a process frequently described as photosynthetic acclimation (Ainsworth et al., 2004). In order to test

possible photosynthetic acclimation phenomena, A_n was determined at 360 and 1000 μmol mol⁻¹ CO₂. The absence of significant differences for A_n, determined at the same [CO₂] showed that the photosynthetic activity was not diminished under elevated [CO₂] conditions (Pérez et al., 2005). N availability is a critical factor, limiting photosynthetic performance and plant growth of plants exposed to elevated CO₂ conditions. In this sense, low leaf [N] has been described to lead to either a proportional (Geiger et al., 1999) or a selective (Reviere-Rolland et al., 1996) reduction in Rubisco. Such inhibition has been widely described in non N₂ fixing plants such as wheat (Pérez et al., 2005), barley (Sicher and Bunce, 1997) and rice (Gesch et al., 2003). As mentioned above, the biological N₂ fixation provides an unlimited N source to legumes that would confer a larger responsiveness to elevated [CO₂] in these plants than in non N₂ fixing plants (Hui et al., 2002; Aranjuelo et al., 2013). Our study showed that pea plants exposed to 1000 μmol mol⁻¹ [CO₂] were capable of overcoming photosynthetic acclimation thanks to their capacity to match their N requirements. Moreover, the absence of significant differences in reduced N content (comprising total organic N and ammonium), total soluble protein and Rubisco protein content would contribute to an explanation of photosynthetic acclimation being absent in plants exposed to elevated [CO₂].

The capacity of plants to adjust photosynthetic activity alongside leaf C demand is a major point conditioning photosynthetic performance under elevated [CO₂] (Ainsworth et al., 2004). It has been noted that N₂-species show a larger stimulation of growth and photosynthetic rates in response to elevated CO₂ than non-fixing species (Ainsworth and Rogers, 2007). In addition to the unlimited access to atmospheric N₂, the large nodule C requirements would contribute towards adjustment of the leaf C sink/source (Arrese-Igor et al., 1999; Cabrerizo et al., 2001). However, our study showed that although Gluc and Fruc levels were not affected by [CO₂], exposure to 1000 μmol mol⁻¹ [CO₂] increased the availability of the two major carbohydrates, Suc (56% increase) and starch (40% increase). Similar enhancements have been described in a recent study (Aranjuelo et al., 2013) where N₂ fixing and NO₃⁻ fed pea plants were exposed to elevated [CO₂]. Although carbohydrate content increased in elevated [CO₂], it is also notable that according to Aranjuelo et al. (2013), Suc and starch increases were much more marked (366 and 76%, respectively) in NO₃⁻ fed pea plants. These

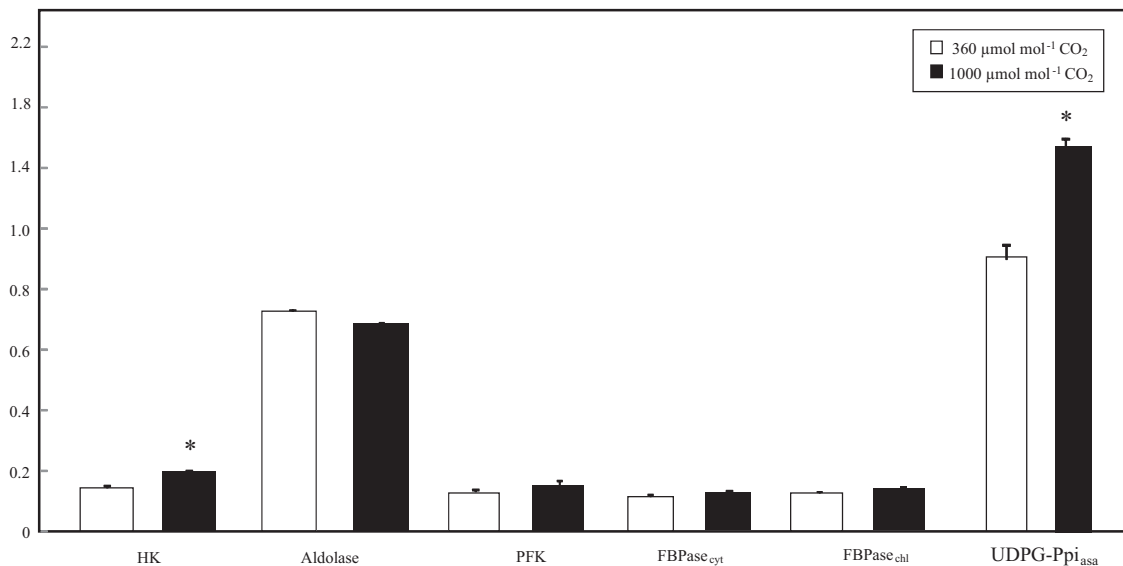


Fig. 2. Effect of elevated CO_2 (1000 versus $360 \mu\text{mol mol}^{-1}$) exposure on proteins involved in leaf sucrose metabolism such as hexokinase (HK), aldolase, phosphofructokinase (PFK), cytosolic and chloroplastic fructose bisphosphatase (FBPase_{cyt} and FBPase_{chl} respectively) together with uridine diphosphoglucose pyrophosphorylase (UDPG-Ppi_{ase}) activity (expressed in $\text{mmol NAD min}^{-1} \text{mg protein}^{-1}$) in exclusively N_2 -fixing pea plants (*Pisum sativum* L.). Each value represents the mean of 8 replicates \pm SE. The asterisks (*) indicate significant differences from the corresponding control values at $P \leq 0.05$.

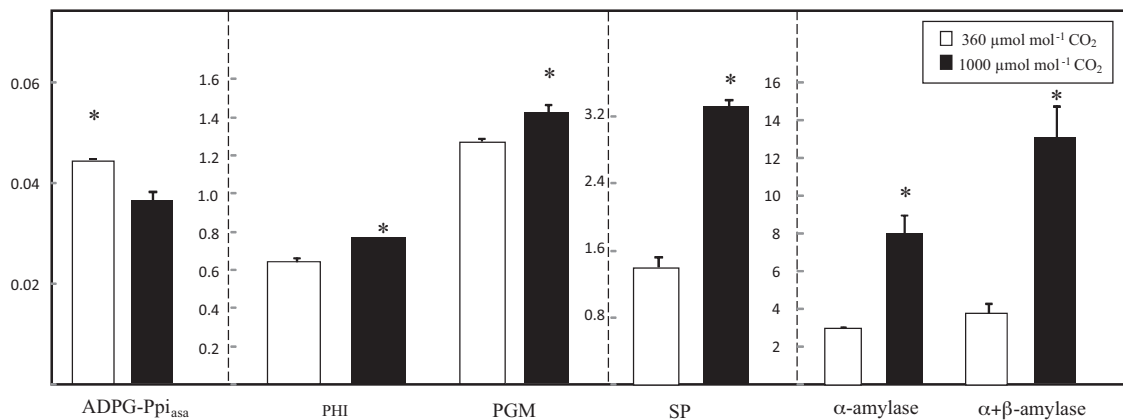


Fig. 3. Effect of elevated CO_2 (1000 versus $360 \mu\text{mol mol}^{-1}$) exposure on proteins involved in leaf starch metabolism such as adenosine diphosphoglucose pyrophosphorylase (ADPG-Ppi_{ase}), phosphohexoisomerase (PHI, $\text{mmol NADPH min}^{-1} \text{mg protein}^{-1}$) and phosphoglucosyltransferase (PGM, $\text{mmol NADPH min}^{-1} \text{mg protein}^{-1}$) together with starch phosphorylase (SP), α -Amylase (α -amylase) and α - β -amylase (α + β -amylase) activities (expressed in $\mu\text{g glucose min}^{-1} \text{mg protein}^{-1}$) in exclusively N_2 -fixing pea plants (*Pisum sativum* L.). Each value represents the mean of 8 replicates \pm SE. The asterisks (*) indicate significant differences from the corresponding control values at $P \leq 0.05$.

results suggest that despite carbohydrate levels increasing in plants exposed to elevated $[\text{CO}_2]$, such an increase was not strong enough to cause the inhibition of Rubisco.

The detection of a 56% increase in sucrose content could be associated with the fact that Suc is the main form of translocated carbon in most plants and the main substrate for sink development (Stitt et al., 2010). The large carbohydrate requirements of nodules (as reflected by the increase in the nodules' total soluble sugar and organic acid content) would explain the increase in the availability of this photoassimilate. A major regulator of Suc synthesis is the Suc phosphate synthase (SPS). SPS activity has been described as being increased by Gluc6P and inhibited by Fruc-2,6 bP (Stitt et al., 2010). The increase in the Suc precursors, Gluc6P and UDPGlc, together with the enhanced activities of UDPG-Ppi_{ase} and PGM detected in plants exposed to $1000 \mu\text{mol mol}^{-1} \text{CO}_2$ confirmed that such plants invested a large amount of C in the synthesis of this major C transport form. The fact that the Fruc6P content was 250 times lower than the UDPG content confirmed the role of Fruc6P as a key compound in the regulation of Suc synthesis. Fruc6P levels depend on the activity of FBPase and PFK. Because Fruc-2,6

bP has been described (Jacquot et al., 1995) to promote PFK at the expense of FBPase and consequently against Suc synthesis, the lower Fruc-2,6 bP content detected in plants exposed to elevated $[\text{CO}_2]$ confirmed that the synthesis of Suc was enhanced in these plants (Fig. 5). As observed above, the larger leaf Suc content could have contributed (together with starch) to photoassimilate partitioning towards nodules. The synthesis of Suc is also conditioned by triose phosphate (TP) and Pi content (Stitt, 1990). Interestingly, the 34% decrease in the $\text{Pi}_{\text{chl}}/\text{Pi}_{\text{cyt}}$ suggests that (compared with plants under ambient $[\text{CO}_2]$) Suc synthesis was promoted in plants under elevated $[\text{CO}_2]$ at the expense of starch synthesis.

As observed by Stitt et al. (2010), the 40% increase in leaf starch content of elevated $[\text{CO}_2]$ leaves could be considered to be an overflow product that is synthesized when the rate of photosynthesis exceeds the rate of synthesis of other products. Our metabolomic and proteomic approach highlighted that starch synthesis was tightly regulated in leaves of plants exposed to $1000 \mu\text{mol mol}^{-1} \text{CO}_2$. More specifically, our data revealed that the activity of proteins involved in the degradation of starch was larger than the activity of enzymes involved in its synthesis. Starch remobilization can

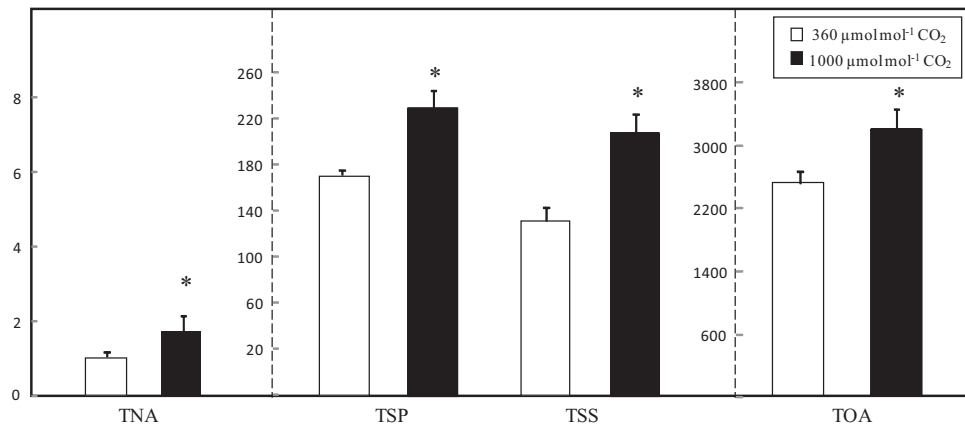


Fig. 4. Effect of elevated CO_2 (1000 versus $360 \mu\text{mol mol}^{-1}$) exposure on nodule total nitrogenase activity (TNA) (expressed as $\mu\text{mol H}_2\text{g}^{-1} \text{DM min}^{-1}$), together with total soluble protein (TSP) (expressed as $\text{mg}^{-1} \text{g DM}^{-1}$), total soluble sugar (TSS) (expressed as $\mu\text{mol glucose g}^{-1} \text{DM}^{-1}$) and total organic acid (TOA) content (expressed as $\mu\text{g g}^{-1} \text{DM}$) in exclusively N_2 -fixing pea plants (*Pisum sativum* L.). Each value represents the mean of 8 replicates \pm SE. The asterisks (*) indicate significant differences with the corresponding control values at $P \leq 0.05$.

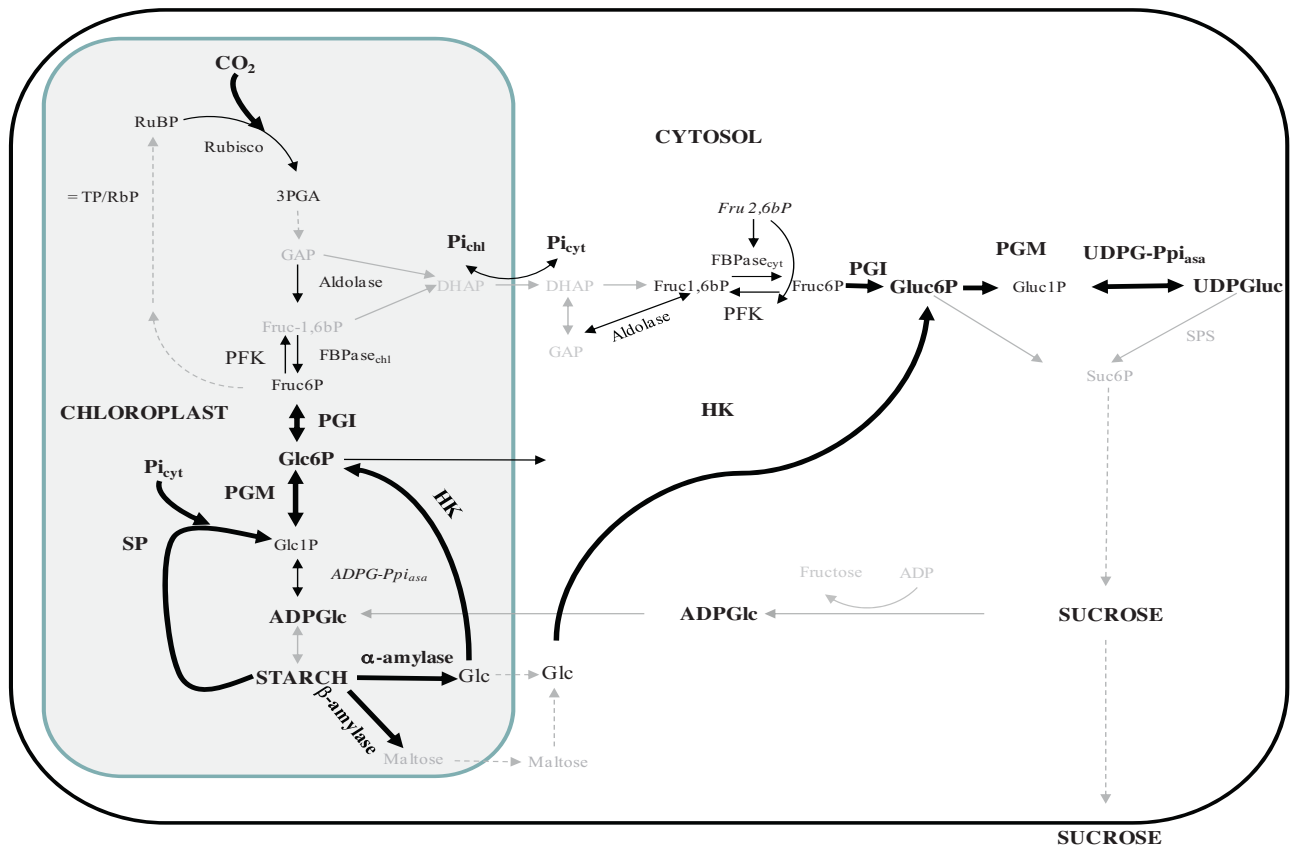


Fig. 5. Most visible changes in carbon primary metabolism in leaves of exclusively N_2 -fixing pea plants (*Pisum sativum* L.) grown at elevated and ambient $[\text{CO}_2]$ (1000 versus $360 \mu\text{mol mol}^{-1}$). Thick bold type proteins and compounds arrows represent pathways enhanced under elevated $[\text{CO}_2]$, whereas italicized ones correspond to down-regulated ones. Grey coloured compounds and proteins were not determined.

occur through two pathways: (i) phosphorolytic (producing hexose phosphate) and (ii) hydrolytic (producing sugars such as glucose and maltose), with the hydrolytic pathway being the more dominant one (Sharkey et al., 2004). Our data revealed that the increase in starch phosphorolytic degradation was confirmed by enhancement of the SP, PGM and PHI activities. The hydrolytic degradation involves an initial attack by β -amylase leading to maltose synthesis that can be translocated to the cytosol where it is converted to glucose through the cytosolic disproportionating enzyme, DPE2 (Niittylä et al., 2004). Starch degradation can also be carried out by α -amylase, which leads to glucose synthesis. The cytosolic

glucose is converted to Gluc6P by the HK that it is directed towards Suc synthesis (Stitt et al., 2010). The fact that under elevated $[\text{CO}_2]$ α + β amylase and HK activity increased by 244 and 35%, respectively, together with the 86% increase in Gluc6P revealed that a proportion of the C remobilized from starch was directed towards Suc synthesis (Fig. 5). The occurrence of a futile cycle as a consequence of the simultaneous synthesis and breakdown has been previously described (Scott and Kruger, 1995; Baroja-Fernández et al., 2001). Furthermore, proteins involved in starch breakdown have been observed to be activated during the day (Zeeman et al., 2004). According to Stitt and Heldt (1981), starch synthesis and

degradation acts as a buffer to Suc metabolism. Furthermore, the futile cycle has been described as enabling the rapid metabolic channelling of carbohydrates towards other pathways (TCA cycle etc.) in response to specific physiological and biochemical needs. In this context, our data suggest that products of phosphorolysis were invested in Suc synthesis (see discussion below) (Fig. 5).

As observed by Moore et al. (1999), leaf cellular sugar levels are not only a function of their metabolism within the cell, but are also affected by storage processes, metabolic activities of surrounding cells, and activities of distant cells in sink tissues. This is a matter of great concern because N₂ fixation relies on a plant-bacteroid interchange of resources (Arrese-Igor et al., 1999; Gálvez et al., 2005). Our study showed (Fig. 4) that exposure to an elevated [CO₂] increased total nitrogenase activity (Ortega et al., 1992; Sanz-Sáez et al., 2012). The fact that such an increase was not reflected in a larger N_{red} content could be explained by the enhanced N compound demand (as reflected by the enhanced DM) by the aboveground organs under elevated [CO₂] conditions (Rogers et al., 2006). In agreement with previous studies (Arrese-Igor et al., 1999; Cabrerizo et al., 2001), the increase in nodule TSS and TOA of plants exposed to elevated [CO₂] confirmed that the greater photosynthetic rate in legumes grown under high CO₂ conditions (Bertrand et al., 2007) enabled the larger supply of organic C to nodules (Fig. 4). C partitioning towards nodules would contribute to overcoming leaf carbohydrate build-up, with the consequent avoidance of photosynthetic acclimation. The increase in photoassimilates also contributed to the larger nodule DM of plants exposed to enhanced [CO₂]. The fact that TSP content also increased under elevated [CO₂] conditions (in an organ with high metabolic activity such as nodules) suggests that in order to cover aboveground plant N demand (as reflected by the Rubisco content data) the activity of the TCA cycle (source of energy and carbon skeletons for Aa synthesis) was also increased.

5. Conclusions

In summary, our study highlighted the relevance of Suc and starch metabolism in the leaf C sink/source balance and its implications in photosynthesis and growth in exclusively N₂-fixing pea plants exposed to elevated [CO₂]. The study showed that plants exposed to 1000 μmol mol⁻¹ CO₂ were capable of balancing carbohydrate levels and consequently overcoming photosynthetic acclimation due to their capacity to store (in the form of starch) and allocate (in the form of Suc) the excess photoassimilates to nodules. The characterization of starch metabolism revealed that in spite of the larger starch content under elevated [CO₂], part of it was remobilized towards Suc synthesis. Starch remobilization together with the increase in the activity of proteins involved in Suc synthesis would be explained by the large photoassimilate requirements of nodules exposed to elevated [CO₂]. Such an increase in the nodule carbohydrate content enabled the enhancement of nitrogenase activity matching the plant N-demand.

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