Research highlights

The main goal of this study was to characterize the relevancy of N source in photosynthetic performance, and consequently in plant growth under elevated CO_2 conditions.

For this purpose, exclusively N_2 fixing *versus* NO_3^- -fed pea plants were grown under ambient (360 μmol mol) and elevated (1000 μmol mol) [CO_2].

Our study showed that although N_2 fixation matched plant N requirements (with the consequent increase in photosynthetic rates), in NO_3^--fed plants exposure to elevated [CO_2] negatively affected N assimilation with the consequent photosynthetic down-regulation.

CORRESPONDING AUTHOR INFO

Name: Iker Aranjuelo

Instituto de Agrobiotecnología, Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Campus de Arrosadía, E-31192-Mutilva Baja, Spain.

Fax: +0034 948 232191

E-mail address: iker.aranjuelo@unavarra.es

Pea plant responsiveness under elevated [CO_2] is conditioned by the N source (N_2 fixation *versus* NO_3 fertilization)

Iker Aranjuelo[1*], Pablo M. Cabrerizo[1], César Arrese-Igor[2] and Pedro M. Aparicio-Tejo[1,2]

[1] Instituto de Agrobiotecnología (IdAB), Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Campus de Arrosadía, E-31192-Mutilva Baja, Spain.

[2] Dpto. Ciencias del Medio Natural, Universidad Pública de Navarra Campus de Arrosadía, E-31006-Pamplona, Spain.

Running title: N_2 fixation *versus* N fertilization under elevated [CO_2]

Corresponding author:

Name: Iker Aranjuelo

Present address: Instituto de Agrobiotecnología (IdAB), Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Campus de Arrosadía, E-31192-Mutilva Baja, Spain.

Phone: +34 948 168000

Fax: +34 948 232191

E-mail address: iker.aranjuelo@unavarra.es

ABSTRACT

The main goal of this study was to test the effect of [CO_2] on C and N management in different plant organs (shoots, roots and nodules) and its implication in the responsiveness of exclusively N_2-fixing and NO_3^--fed plants. For this purpose, exclusively N_2-fixing and NO_3^--fed (10 mM) pea (*Pisum sativum* L.) plants were exposed to elevated [CO_2] (1000 μmol mol^{-1} *versus* 360 μmol mol^{-1} CO_2). Gas exchange analyses, together with carbohydrate, nitrogen, total soluble proteins and amino acids were determined in leaves, roots and nodules. The data obtained revealed that although exposure to elevated [CO_2] increased total dry mass (DM) in both N treatments, photosynthetic activity was down-regulated in NO_3^--fed plants, whereas N_2-fixing plants were capable of maintaining enhanced photosynthetic rates under elevated [CO_2]. In the case of N_2-fixing plants, the enhanced C sink strength of nodules enabled the avoidance of harmful leaf carbohydrate build up. On the other hand, in NO_3^--fed plants, elevated [CO_2] caused a large increase in sucrose and starch. The increase in root DM did not contribute to stimulation of C sinks in these plants. Although N_2 fixation matched plant N requirements with the consequent increase in photosynthetic rates, in NO_3^--fed plants, exposure to elevated [CO_2] negatively affected N assimilation with the consequent photosynthetic down-regulation.

Keywords: Carbon, CO_2, legumes, nitrogen, sink

1. **Introduction**

Current CO_2 concentration present in the atmosphere is limiting for C_3 photosynthesis, primarily because of the bifunctional nature of Rubisco and its low affinity for CO_2 (Bowes, 1993). Assuming that there are no other limitations, an increase in atmospheric [CO_2] (C_a) will increase photosynthesis through its direct effect on Rubisco (Bowes, 1993; Farquhar et al., 1980). However, although the initial stimulation of net photosynthesis associated with elevated [CO_2] is sometimes retained during long-term exposure to this gas (Rogers et al., 2004; Ainsworth et al., 2004), it is often partially reversed in an acclimation process often referred to as 'downregulation' (Ainsworth et al., 2002; Ainsworth and Rogers, 2007; Aranjuelo et al., 2011; Leakey et al., 2009b; Long et al., 2004).

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., 2004; Aranjuelo et al., 2011; 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., 2011; Aranjuelo et al., 2009). From this point of view, the reduction in photosynthetic rates would be conditioned by a plant's ability to develop new sinks (e.g. new vegetative or reproductive structures, enhanced respiratory rates), or to expand the storage capacity or growth rate of existing sinks. 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. According to previous studies

(Ainsworth et al., 2004; Davey et al., 2004; Rogers et al., 2009b), 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]. According to Leakey et al. (2009a) the large C sink strength of nodules will avoid leaf carbohydrate build up. Arrese-Igor et al. (1999) observed that the greater photosynthetic rates of plants grown under elevated [CO_2] conditions imply that a larger amount of photosynthetically derived organic carbon is supplied to nodules, where it is used by the nitrogenase enzyme in the bacteroid fraction within the nodules. This could be the reason why non-nodulated soybean plants undergo photosynthetic acclimation under elevated [CO_2], whereas nodulated plants do not show a decrease in the CO_2 assimilation rate (Ainsworth et al., 2004). In non N_2-fixing plants, the C sink/source is balanced by reducing Rubisco content, which leads to lower photosynthetic activity, making Rubisco derived N available for other N sinks

Although positive productivity and N_2 fixation responses to elevated [CO_2] are common (Soussana and Hartwig, 1996; Lee et al., 2003; Feng et al., 2004), 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., 2005b; Aranjuelo et al., 2008), 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. One possible explanation might be the fact that CO_2 x N_2 fixation differs depending on the species analyzed (West et al., 2005a; Rogers et al., 2009a). The second explanation could be the variability of environmental conditions such as temperature, water availability, N availability etc. (Serraj et al., 1998; Serraj et al., 1999; Erice et al., 2007).

Experiments conducted under controlled (Stitt and Krapp, 1999) and field conditions (free air CO_2 enrichment, FACE; (Ainsworth and Long, 2005) have highlighted that C sink development is restricted under N limiting conditions. N limitation seems to be more marked in non N_2-fixing plants than in N_2-fixing plants. As observed by Taub et al. (2008), in non-legumes N concentration decreases between 10-14 % at elevated CO_2 (540-958 μmol mol^{-1}) whereas in N_2-fixing plants such as soybean only decreased 1.5 %. The negative acclimation of photosynthesis may be the result of lowered leaf N content due to low soil N availability conditions (Theobald et al., 1998). In this sense, it has been suggested that a persistent increase in plant biomass production under elevated [CO_2] can only be maintained by an increase in N uptake (Soussana and Hartwig, 1996; Feng et al., 2004). As a response to N availability limitations, plants exposed to elevated [CO_2] have been described to invest a greater proportion of root biomass to explore a greater soil volume (Arndal et al. 2013). However, it should be also remarked that root nutrient uptake under elevated [CO_2] have shown inconsistent patterns, due to differences in experimental protocols and species specific responses (Bassirirad 2000).

There is evidence that the carbohydrate-mediated repression of photosynthetic genes is more severe in nitrogen deficient than in nitrogen replete plants (Long et al., 2004; Stitt and Krapp, 1999). According to Theobald et al. (1998), N assimilation cannot match CO_2 fixation, with a consequent carbohydrate build up and depletion of N content. According to Bloom et al. (2002; 2010), C compound accumulation is the consequence of depletion in NO_3^- assimilation and N content. Since N_2 fixing plants are largely independent of soil N availability, they may become more competitive than non fixing plants in elevated CO_2 conditions (Feng et al., 2004; Lüscher et al., 1998). Although N_2 fixation costs are larger than for NO_3^- assimilation (5.2-18.8 *versus* 2.5 g C g^{-1} N;

Minchin and Witty 2005), as revealed by Kaschuk et al. (2010) the C costs of biological N_2 fixation might be compensated by increased photosynthesis and consequently enhanced plant growth.

The aim of this study was to check whether the source of N is a major factor involved in C and N management in legumes and what implications it has for the responsiveness of photosynthetic performance under elevated [CO_2] conditions. For this purpose, exclusively N_2-fixing *versus* NO_3^--fed pea plants were exposed to elevated [CO_2] conditions. In order to test the effect of [CO_2] and N source on photosynthetic performance, we characterized shoot, root and nodule carbohydrate (sucrose, glucose, fructose and starch), amino acid, protein content and N content. As mentioned above, previous studies that have explored the responsiveness of N_2 fixing plants to environments with high [CO_2] levels have mainly focused on leaf characterization. Although belowground organs have received comparatively little attention, due to the tight link between plant level C and N exchange further study will the key to understanding the plant level responsiveness to predicted [CO_2] scenarios.

2. Material and Methods

2.1 Plant material and growth conditions

The experiment was conducted with pea plants (*Pisum sativum* L. cv Frilene) grown in 2.5 L plastic pots (one plant per pot) filled with 3:2 (v/v) vermiculite-perlite. Exclusively N_2 fixing pea plants were inoculated with *Rhizobium leguminosarum* biovar *viciae* NLV8. Plants were placed in two growth cabinets (Heraeus-Votsch HPS-500, Norrkoping, Sweden) at 25/18ºC (day/night) with a photoperiod of 16 hours at 480 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and a relative humidity of

70/80 % (day/night). The cabinets were fitted with two independent T-RH combined probes (41372VC/VF RM Young Co., Traverse City, MI, USA) connected to an external micro-processor (LI-1000; LiCor, Lincoln, Nebraska, USA). Plants were irrigated by a drip system, with N-free nutrient solution (Rigaud and Puppo, 1975) for N_2-fixing plants and 10 mM KNO_3 for nitrate fed plants. In order to confirm that such KNO_3 content was not limiting for plant growth, during the last week of the experiment a set of additional plants was watered with 20 mM KNO_3 nutrient solution. The absence of differences in growth, and no reduction in N and NO_3^- content confirmed that 10 mM was not limiting. Half of the randomly selected plants were placed into an elevated [CO_2] cabinet where they were exposed to 1000 μmol mol^{-1}, whereas the other half were placed in a cabinet where the [CO_2] was set at 360 μmol mol^{-1}. The [CO_2] variation was within 20 μmol mol^{-1} CO_2. Air entered the cabinets from a compressor installed at the top of the building, and was filtered by four air filters (coarse-5 μm and 1 μm Ø particle and 0.01 μm Ø particle physical filters and charcoal chemical filter) to prevent anomalous components. Cabinets were equipped with an infrared CO_2 analyzer (polytron-IRGA, Dragäer, Lübeck, germany) connected to a micro-processor located inside the cabinet. [CO_2] was analyzed and controlled every second. All the determinations were conducted after 4 weeks of exposure to elevated [CO_2] conditions. Both cabinets provided uniform air mixing and two independent T-RH combined probes in each of the cabinets were connected to an external microprocessor. To prevent temporary [CO_2] changes within cabinets, plants were handled inside the cabinet by means of two windows installed at the glass door that allowed measurements without opening the cabinet's doors.

2.2 Sampling and plant growth determinations

Eighteen plants per treatment combination were harvested after four weeks of planting. For each plant, shoots, roots and nodules were weighed separately. Plant sampling was always carried out 5 hours after onset of the photoperiod. For plant growth determinations, samples were dried at 70 ºC for 48 h in order to measure the dry matter (DM). Leaf surface area was determined with a LICOR 3100 leaf area meter (LICOR, Inc., Lincoln, NE).

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). Determinations were conducted at 25 ºC, 480 μmol m^{-2} s^{-1}PPFD, 70 % RH and at the corresponding growth [CO_2].

2.4 Sugar, starch and total soluble proteins (TSP) content

Shoot, root 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 2.300 g for 10 minutes and the supernatant and the pellet were stored separately at -80 ºC. Sucrose, glucose (Glc) and fructose (Fru) were determined in the supernatant fraction. Soluble sugar and starch content was determined in the pellet according to the method of Cabrerizo et al.(2001). Protein concentration was determined in the supernatant fraction using the Bradford Assay (Bio-Rad) (Aranjuelo et al., 2005).

2.5 Amino acid content

Leaf fresh material was exhaustively extracted in boiling 80% (v/v) ethanol. Ethanol-soluble extracts were dried in a Turbovap LV evaporator (Zymark Corp, Hopkinton, MA, USA) and soluble compounds were re-dissolved with 4 ml of distilled water, and mixed and centrifuged at 20000 *g* for 10 min. Free amino acids were assayed using the acid ninhydrin method of Yemm and Willis (1954).

2.6 Nitrate (NO_3^-) and reduced N (N_{red}) content

Nitrate content was determined in the soluble fraction described above for soluble sugar and organic acid content. The samples were injected into a capillary electrophoresis system (Beckman P/ACE system 5500, Beckman Instruments, Inc. CA, USA). The determinations were conducted with a -25 KV voltage at 25 ºC using a photodiode detector with a 254 nm wavelength. The buffer used was composed of 2.25 mM pyromellitic acid, 6.5 mM NaOH, 0.75 mM hexamethonium hydroxide and 1.6 triethanolamine with the pH adjusted to 7.7. Reduced N_2 (N_{red}) was determined with the Kjeldahl method (Aranjuelo et al., 2005). Plant N content (PNC) refers to plant N content, calculated as the sum of N_{red} and NO_3^-.

2.7 Statistical analyses

Data were processed by two-way analysis of variance (ANOVA) to test the effects of [CO_2] and N nutrition using the statistical software package, SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The results were accepted as significant at $P < 0.05$. When differences between treatments ([CO_2] and/or N nutrition interactions) were significant according to the ANOVA analysis, least significant differences (LSD) were evaluated using Fisher's LSD test ($P < 0.05$).

3. Results

Growth parameters revealed that regardless of [CO_2], NO_3^--fed plants produced more total DM than the corresponding N_2-fixing plants (Table 1). Under elevated [CO_2], total DM increased by 71 and 111 % in N_2 fixing and NO_3^--fed plants, respectively (Table 1). Even if, elevated [CO_2] and NO_3 fertilization increased shoot biomass, roots was only significantly affected by elevated [CO_2] in NO_3^-fed plants (Fig. 1). Although exposure to 1000 μmol mol^{-1} [CO_2] increased nodule DM (Fig. 1), the number of nodules was not significantly affected by [CO_2] enhancement (Table 1).

As shown in Fig. 2A, the responsiveness of photosynthetic rates (A_n) to elevated [CO_2] varied depending on the N source. In N_2 fixing plants A_n increased, whereas in NO_3^-fed plants no significant effect was observed. N source had no significant effect in A_n . Exposure to elevated [CO_2] increased A_n/TSP by 48 and 177 % in N_2-fixing and NO_3^--fed plants, respectively (Fig. 2.B). Although stomatal conductance (g_s) was not affected by [CO_2] in N_2-fixing pea plants, in NO_3^--fed plants its values increased under 1000 μmol mol-1 [CO_2] (Fig. 2C). As shown in Figure 2D, transpiration (T_r) was unaffected in NO_3^--fed plants, but in N_2-fixing plants its value diminished in plants grown under 1000 μmol mol^{-1} [CO_2].

Exposure to elevated [CO_2] increased sucrose content in all organs (Fig. 3A) but such an effect was more marked in NO_3^-fed than in N_2-fixing plants. The largest increase was detected in leaves where its content increased by 56 and 366 % in N_2-fixing and NO_3^--fed plants, respectively. Although glucose was not altered by [CO_2] in the different organs, irrespective of the N treatment, it is remarkable that no glucose was

detected in NO_3^--fed roots (Fig. 3B). Fructose content (Fig. 3C) diminished in the shoots of NO_3^-fed plants and the nodules of N_2-fixing plants exposed to elevated [CO_2], with no significant differences observed in other organs. No fructose was detected in NO_3^--fed roots. As shown in Figure 2D, [CO_2] enhancement increased the fructose content by 40 % in N_2-fixing plants and by 75 % in NO_3^--fed shoots, with no significant differences recorded in roots of the other N treatment. Elevated [CO_2] effect in starch content was conditioned by N source form. Nodules were the only organ where elevated [CO_2] increased (82 %).

The quantification of the plant N content (PNC) revealed that elevated [CO_2] was conditioned by N source. The PNC data also highlighted that while no differences were detected in elevated [CO_2] in N_2-fixing plants, in NO_3^--fed plants the PNC decreased by 15 %. Similar results were observed in the shoots for reduced N content (N_{red}). Although at the root level there was no [CO_2] effect detected in the N_{red} content, it is remarkable that N_{red} values at both CO_2 levels were larger in NO_3^--fed plants than N_2-fixing plants. N_{red} was stimulated in nodules of N_2-fixing plants exposed to elevated [CO_2]. Table 2 also shows that shoot and root NO_3^- was not affected by [CO_2] in NO_3^--fed plants.

Finally, as shown in Fig. 4A, the total soluble protein (TSP) content diminished by 14 % in N_2-fixing plants and 87 % in NO_3^--fed plants. [CO_2] effect in leaf TSP was modulated by N source. Although under ambient [CO_2] conditions, TSP was larger in NO_3^--fed plants, under elevated [CO_2] TSP content was larger in N_2-fixing plants. The [CO_2] effect on amino acid content was similar in both N treatments, and varied depending on the organ analyzed (Fig. 4B). Whereas in shoots the amino acid content

decreased under 1000 μmol mol^{-1} [CO_2], in roots no significant differences were observed.

4. Discussion

Our study showed that [CO_2] interacted N source form, being NO_3^-fed plants the ones that produced more DM than the corresponding N_2-fixing plants. Under both ambient and elevated [CO_2] such increase was more marked in roots (108 and 104 % respectively) than in shoots (50 and 29 % respectively). Furthermore, the fact that in NO_3^-fed plants exposed to elevated [CO_2] the increase in root DM was larger than that in shoots, suggests that those plants invested more photoassimilates to root biomass in order to explore a greater soil volume. Such investment would be explained by the N necessity of those plants. These results highlight the relevance of analyzing belowground organ growth.

The absence of significant differences in photosynthetic (A_n) activity of NO_3^--fed *versus* N_2-fixing plants, revealed that the larger DM of NO_3^--fed plants (compared with the corresponding N_2-fixing plants) was caused by their higher plant level photosynthetic rates. The fact that NO_3^--fed plants had more leaf area implied that, as described previously (Aranjuelo et al., 2005), they were capable of fixing more CO_2 at the plant level with a consequent increase in plant growth. Interestingly, gas exchange data also revealed that the [CO_2] affected photosynthetic activity differently in N_2-fixing and NO_3^-fed plants. Although A_n increased in N_2-fixing plants grown at 1000 μmol mol^{-1}, in the case of NO_3^-fed plants, exposure to elevated [CO_2] had no significant effect on A_n. Previous results obtained in studies analyzing plant growth and photosynthetic

activity (Rogers et al., 2004; Ainsworth and Long, 2005; Aranjuelo et al., 2005; Ainsworth and Bush, 2011a) highlight the fact that photosynthetic activity under elevated [CO_2] does not always increase and depends on exposure time, plant species and limiting environmental conditions. The reduction in Rubisco carboxylation capacity could be caused by stomatal and/or non-stomatal limitations of photosynthesis. Gas-exchange measurements showed that leaf conductance (g_s) was not affected (N_2-fixing plants) or increased (NO_3^--fed plants), which implies that plants grown under elevated [CO_2] conditions had a higher intercellular CO_2. Photosynthetic non-stomatal limitation may be caused by a diminishment in the Rubisco protein content (Urban 2003). Although total soluble protein (TSP) content decreased under both forms of N supply, such diminishment was much more marked in the NO_3^--fed plants (47 %) than in the N_2-fixing plants (12 %). Although Rubisco was not quantified in this study, because Rubisco might represent up to 30 % of TSP (Aranjuelo et al., 2005) it is very likely that under elevated [CO_2] there was also a decrease in Rubisco content. The fact that plants exposed to 1000 μmol mol^{-1} CO_2 had larger (48 and 177 % per N_2-fixing and NO_3^--fed plants, respectively) A_n/TSP revealed that these plants improved their Rubisco carboxylation efficiency under elevated [CO_2] (Ainsworth and Rogers, 2007).

A frequent acclimation response is the accumulation of leaf carbohydrates in elevated [CO_2] (Ainsworth et al., 2004; Long et al., 2004; Sanz-Sáez et al., 2010; Moore et al., 1999). The fact that in the NO_3^--fed plants the increase in the concentration of shoot sucrose (366 %) was much larger than in the corresponding N_2-fixing plants (56 %) revealed that this large accumulation in leaf photoassimilates was involved in the photosynthetic down-regulation of NO_3^--fed plants. Sucrose, together with starch, represent the major forms of leaf cellular carbohydrate storage. Leaf sucrose content

may reflect the balance of C sink/source demand (Long et al., 2004). In this sense, starch has also been proposed as a buffer for sucrose metabolism that might minimize leaf sucrose cycling (Moore et al., 1999; Stitt et al., 2010). According to these authors, when there is a sink limitation under elevated [CO_2] the plants increase their starch sink strength. Our data confirmed that starch accumulation was more marked in the shoots of NO_3^--fed (75 %) than in the corresponding N_2-fixing plants (40 %) exposed to elevated [CO_2]. Leaf carbohydrate levels are conditioned by metabolic activity, storage processes and the sink strength of the other plant organs (Aranjuelo et al., 2011; Moore et al., 1999). Sucrose is the main form of translocated C in most plants and the main substrate for sink limitation. The analyses of sucrose content in belowground organs increased in N_2-fixing (44 and 24 % in roots and nodules) and NO_3^- -fed (457 %) plants exposed to elevated [CO_2]. The increase in starch content of N_2-fixing (51 and 82 % in roots and nodules) and NO_3-fed roots (145 %) highlighted that, especially in NO_3^- -plants, roots represented a major C sink under elevated [CO_2].

Our data revealed that although N_2-fixing plants were capable of maintaining plant level N content (PNC) under elevated [CO_2], in the case of NO_3^--fed plants such content decreased by 15 %. Furthermore, the reduced N (N_{red}) data of different organs revealed that the diminishment in N content of NO_3^--fed plants exposed to 1000 μmol mol^{-1} [CO_2] was explained by the lower leaf N content. The fact that NO_3^- content was not affected by [CO_2] revealed that the lower shoot N content was caused by the depletion of N assimilation in NO_3^--fed plants exposed to elevated [CO_2]. The decrease in shoot amino acid content of these plants also confirmed such down-regulation in N assimilation. NO_3^- assimilation takes place essentially in aboveground organs (Gonzalez et al., 1996), where the nitrate reductase activity is regulated by carbohydrate content

and light (Redinbaugh et al., 1996). According to Reich et al. (2006), Norby et al. (2001) and Hungate et al. (2003), N assimilation cannot match CO_2 fixation, with the consequent carbohydrate build up and depletion in N content, and (Bloom et al., 2002) described that CO_2 fixation interferes with NO_3^- assimilation. According to these studies, reduction of NO_2^- to NH_4^+ and the amino acid compete with starch for the availability of ferredoxin required for both processes. In agreement with previous findings (Ezquer et al., 2010a), our data reveal that exposure to elevated [CO_2] in NO_3^--fed plants was favoured over amino acid assimilation (Li et al., 2011; Ezquer et al., 2010b; Yamakawa and Hakata, 2010).

Concerning N_2-fixing plants, although under elevated [CO_2] conditions PNC matched the values obtained in NO_3^--fed plants, it is remarkable that under ambient [CO_2] that the N content was lower in N_2-fixing than in NO_3^--fed plants. The absence of significant N fertilization differences in PNC under 1000 μmol mol^{-1} [CO_2] reveals that under ambient [CO_2] conditions the nodules might have had C limitations that conditioned the level of N_2 fixation in these plants. Such limitations were overcome under elevated [CO_2]. In relation to the [CO_2] effect in N_2-fixing plants, the absence of significant differences in the PNC and N_{red} values of N_2-fixing plants under elevated [CO_2] showed that, in contrast to the observation in NO_3^--fed plants, nodules were capable of matching the N requirements of such plants (Rogers et al., 2006; Cabrerizo et al., 2001). Despite the larger DM (increased 71 %), the lack of any detectable differences in PNC revealed that N_2 fixation was increased under elevated CO_2 conditions. The larger nodule DM, together with the increase in nodule sucrose (24 %) and starch (82 %) confirmed that the increased photosynthetic rate and level of photoassimilates enabled the improvement

in N_2 fixation by those plants. These results highlight the relevance of nodules as a major C sink.

5. Conclusion

In summary, our study highlighted the relevance of the relationship of leaf performance under elevated [CO_2] to other organs such as roots and nodules. Although in both N_2-fixing and NO_3^--fed plants exposure to 1000 μmol mol^{-1} CO_2 increased plant growth, gas exchange data revealed that N_2-fixing plants were capable of maintaining enhanced photosynthetic rates, whereas in the case of NO_3^--fed plants, photosynthetic activity was down-regulated. Our data highlighted that in NO_3^--fed plants exposed to elevated [CO_2], starch formation prevailed over NO_3^- assimilation with consequent limitations in leaf N content. Such results reveal that, under elevated [CO_2], NO_3^--fed plants will require large N fertilization levels so to overcome photosynthetic down-regulation. The overcoming of photosynthetic acclimation of N_2-fixing plants was explained by their avoidance of excessive leaf carbohydrate build up. The large C requirements of the nodules enabled avoidance of large photoassimilate accumulation. Furthermore, even though the limited C availability under ambient [CO_2] limited the N content in N_2-fixing plants, the nodules' C sink requirements under elevated [CO_2] were fulfilled by the enhanced photosynthetic and photoassimilate availability of these plants. In the other hand, despite their larger DM, PNC data showed that NO_3^--fed plants exposed to elevated [CO_2] had N limitations that conditioned photosynthetic performance. In contrast to the nodules, the larger root DM of NO_3^--fed plants did not enable the maintenance of control N and photoassimilate levels.

Acknowledgements

This work has been funded by the Spanish National Research and Development Programme (AGL2011-30386-CO2-01/02). Iker Aranjuelo was the recipient of a Ramón y Cajal research grant (Ministerio de Economía y Competitividad).

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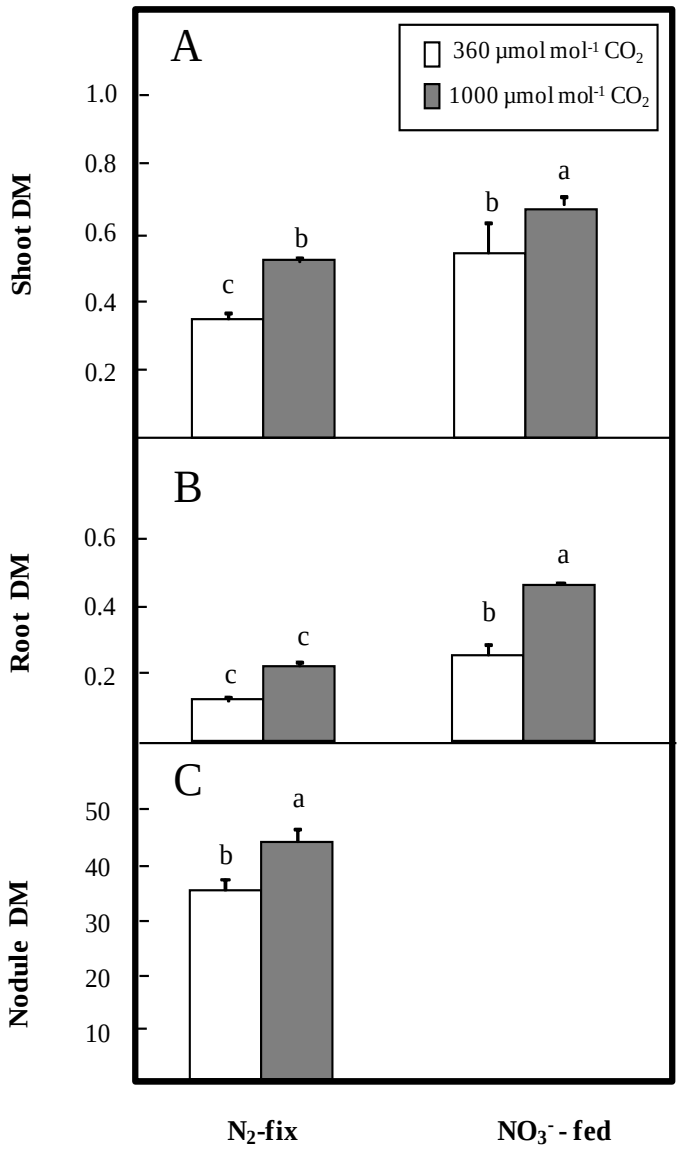
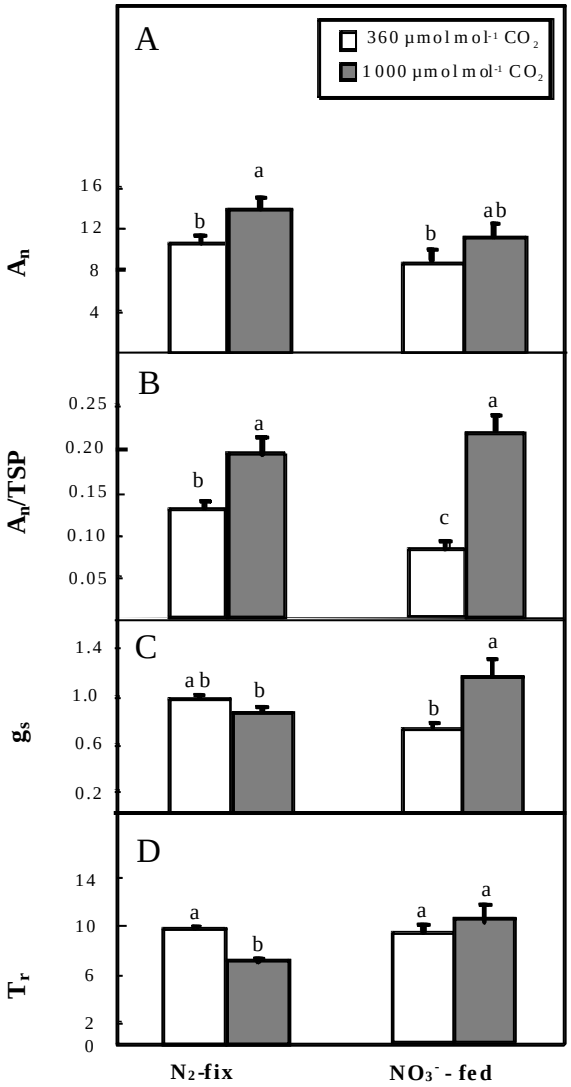
Figure 1.



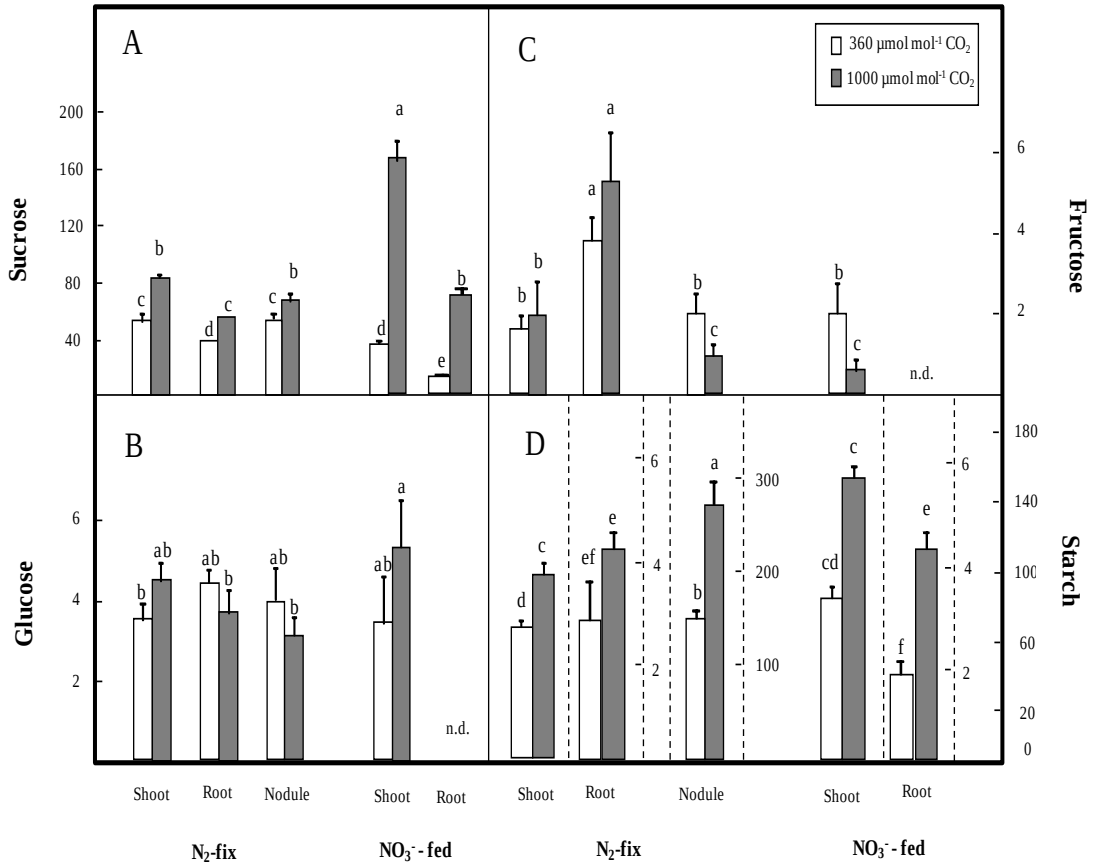
Parameter	Two-way ANOVA
Shoot DM	CO_2, N
Root DM	CO_2 x N
Nodule DM	CO_2

Figure 2.



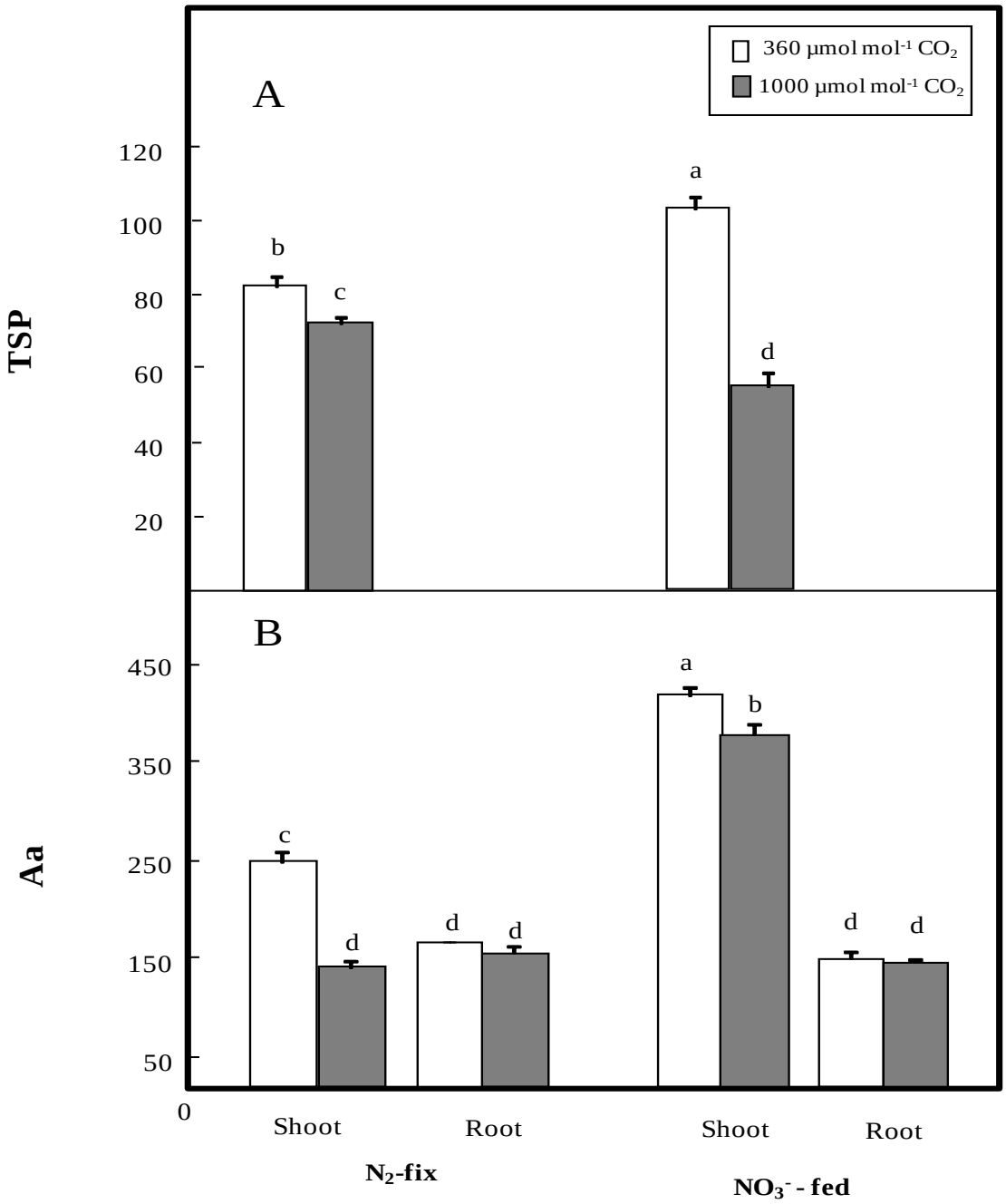
Parameter	Two-way ANOVA
A_n	CO_2
A_n / TSP	CO_2
g_s	CO_2 x N
T_r	n.s.

Figure 3.



Parameter	Two-way ANOVA
Sucrose	
Shoot	CO_2
Root	CO_2
Nodule	CO_2
Glucose	
Shoot	n.s.
Root	N
Nodule	n.s.
Fructose	
Shoot	n.s.
Root	N
Nodule	CO_2
Starch	
Shoot	CO_2 x N
Root	CO_2 x N
Nodule	CO_2

Figure 4.



Parameter	Two-way ANOVA
TSP	
Shoot	CO_2 x N
Aa	
Shoot	CO_2 x N
Root	n.s

FIGURE LEGENDS

Figure 1. Effect of exposure to elevated CO_2 (1000 *versus* 360 μmol mol^{-1}) on shoot dry matter (DM, g $plant^{-1}$), root DM (g $plant^{-1}$) and, nodule DM (DM, mg $plant^{-1}$) determined in exclusively N_2-fixing (N_2-fix) *versus* nitrate-fed (NO_3^--fed) pea plants (*Pisum sativum* L.). Each value represents the mean of 18 replicates ± SE. Each value represents the mean of 4 replicates ± SE. A two-way ANOVA was used to test [CO_2] and/or N source (N) effect and their interaction (CO_2 x N). The different letters represent significant differences between average values at P = 0.05. n.s. refers to non significant differences.

Figure 2. Effect of exposure to elevated CO_2 (1000 *versus* 360 μmol mol^{-1}) on net photosynthesis (A; A_n, μmol CO_2 m^{-2} s^{-1}), A_n, and the total soluble protein ratio (B; A_n/TSP), stomatal conductance (C; gs, mol H_2O, m^{-2} s^{-1}) and transpiration (D; Tr, mol H_2O, m^{-2} s^{-1}) determined in exclusively N_2-fixing (N_2-fix) *versus* nitrate-fed (NO_3^--fed) pea plants (*Pisum sativum* L.). Each value represents the mean of 4 replicates ± SE. A two-way ANOVA was used to test [CO_2] and/or N source (N) effect and their interaction (CO_2 x N). The different letters represent significant differences between average values at P = 0.05. n.s. refers to non significant differences.

Figure 3. Effect of exposure to elevated CO_2 (1000 *versus* 360 μmol mol^{-1}) on leaf, root and nodule sucrose (A), glucose (B), fructose (C) and starch (D) content (mg g^{-1}) determined in exclusively N_2-fixing (N_2-fix) *versus* nitrate-fed (NO_3^--fed) pea plants (*Pisum sativum* L.). Each value represents the mean of 4 replicates ± SE. A two-way ANOVA was used to test [CO_2] and/or N source (N) effect and their interaction (CO_2 x N). The different letters represent significant differences between average values at P = 0.05. n.s. refers to non significant differences. n.d. refers to non-detectable data.

Figure 4. Effect of exposure to elevated CO_2 (1000 *versus* 360 μmol mol^{-1}) on leaf and root total soluble protein (A; TSP, mg g^{-1}) and amino acid (B; Aa, μmol g^{-1}) content determined in exclusively N_2-fixing (N_2-fix) *versus* nitrate-fed (NO_3^--fed) pea plants (*Pisum sativum* L.). Each value represents the mean of 4 replicates ± SE. A two-way ANOVA was used to test [CO_2] and/or N source (N) effect and their interaction (CO_2 x N). The different letters represent significant differences between average values at P = 0.05. n.s. refers to non significant differences.

Table 1. Effect of exposure to elevated CO_2 (1000 *versus* 360 μmol mol^{-1}) on total dry matter (DM, g $plant^{-1}$), the root/shoot ratio and nodule number ($plant^{-1}$) determined in exclusively N_2-fixing (N_2-fix) *versus* nitrate-fed (NO_3^- -fed) pea plants (*Pisum sativum* L.). Each value represents the mean of 18 replicates ± SE. A two-way ANOVA was used to test [CO_2] and/or N source (N) effect and their interaction (CO_2 x N). The different letters represent significant differences between average values at P = 0.05. n.a. refers to not applicable analyses.

	[CO_2]	**Total DM**	**Root/Shoot**	**Nodule number**
N_2-fix	360 μmol mol^{-1}	0.51 ± 0.03 c	140.50 ± 25.25 b	118.54 ± 7.31 a
	1000 μmol mol^{-1}	0.89 ± 0.05 b	173.54 ± 18.00 b	136.58 ± 6.21 a
NO_3^--fed	360 μmol mol^{-1}	0.90 ± 0.08 b	240.20 ± 22.11 a	-
	1000 μmol mol^{-1}	1.90 ± 0.15a	191.35 ± 26.24 b	-
Two-way ANOVA		CO_2 x N	CO_2 , N	n.a.

Table 2. Effect of exposure to elevated CO_2 (1000 *versus* 360 μmol mol^{-1}) on reduced N content (N_{red}, %), nitrate content (mM) and plant N content determined in exclusively N_2-fixing (N_2-fix) *versus* nitrate-fed (NO_3^- -fed) pea plants (*Pisum sativum* L.). Each value represents the mean of 5 replicates ± SE. A two-way ANOVA was used to test [CO_2] and/or N source (N) effect and their interaction (CO_2 x N). The different letters represent significant differences between average values at P = 0.05. n.a. refers to not applicable analyses.

	[CO_2]	**N_{red}**			**NO_3^-**		**PNC**
		Shoot	**Root**	**Nodule**	**Shoot**	**Root**	
N_2-fix	360 μmol mol^{-1}	4.06 ± 0.18 b	2.49 ± 0.16 b	0.51 ± 0.03 c	-	-	43.31 ± 1.93 bc
	1000 μmol mol^{-1}	4.97 ± 0.18 b	2.64 ± 0.10 b	0.89 ± 0.05 b	-	-	44.59 ± 2.15 bc
NO_3^--fed	360 μmol mol^{-1}	5.45 ± 0.18 a	3.85 ± 0.12 a	-	13.50 ± 1.82 a	31.40 ± 1.38 a	49.39 ± 1.78 a
	1000 μmol mol^{-1}	4.16 ± 0.18 c	4.20 ± 0.06 a	-	9.80 ± 1.95 a	26.76 ± 1.21 a	42.40 ± 1.25 b
Two-way ANOVA		CO_2 x N	N	CO_2	n.a.	n.a.	CO_2 x N