Could ammonium nutrition increase plant C-sink strength under elevated CO_2 conditions?

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Abstract

Atmospheric carbon dioxide (CO_2) is increasing, and this affects plant photosynthesis and biomass production. Under elevated CO_2 conditions (eCO_2), plants need to cope with an unbalanced carbon-to-nitrogen ratio (C/N) due to a limited C sink strength and/or the reported constrains in leaf N. Here, we present a physiological and metabolic analysis of ammonium (NH_4^+)-tolerant pea plants (*Pisum sativum* L., cv. snap pea) grown hydroponically with moderate or high NH_4^+ concentrations (2.5 or 10 mM), and under two atmospheric CO_2 concentrations (400 and 800 ppm). We found that the photosynthetic efficiency of the NH_4^+ tolerant pea plants remain intact under eCO_2 thanks to the capacity of the plants to maintain the foliar N status (N content and total soluble proteins), and the higher C-skeleton requirements for NH_4^+ assimilation. The capacity of pea plants grown at 800 ppm to promote the C allocation into mobile pools of sugar (mainly sucrose and glucose) instead of starch contributed to balancing plant C/N. Our results also support previous observations: plants exposed to eCO_2 and NH_4^+ nutrition can increase of stomatal conductance. Considering the C and N source-sink balance of our plants, we call for exploring a novel trait, combining NH_4^+ tolerant plants with a proper NH_4^+ nutrition management, as a way for a better exploitation of eCO_2 in C3 crops.

1. Introduction

To successfully achieve the goal of sustainable agriculture, it is necessary to maintain equilibrium between the highest possible yield and product quality whilst ensuring minimal environmental impact. Nitrogen (N) fertilisation is the main driver for crop yield, but its intensified application leads to severe environmental pollution, including nitrate (NO_3^-) leaching, emissions of nitrous oxide (the most significant ozone-depleting compound and a powerful greenhouse gas), as well as other forms of N that are toxic for the environment [1]. It is therefore necessary to optimise the application of N fertilizers by better understanding the nutritional requirements of plants. Through nitrification processes, NO_3^- is the most common form in aerated soils; it could therefore be argued that modern breeding should select for efficient NO_3^- assimilation metabolism rather than ammonium (NH_4^+). Nitrification inhibitors (NI), which can stabilise large concentrations of NH_4^+ in the soil, pose new issues: in combination with NI, genotypes that efficiently exploit NH_4^+ nutrition will have an advantage and, thus, this could be considered an advantageous trait in terms of breeding. This capability needs to be evaluated in different environmental scenarios.

Actual atmospheric CO_2 concentrations (aCO_2) are increasing despite the urgency of globally reducing the emissions of CO_2 and other greenhouse gases [1]. The effect of increasing aCO_2 on plant responsiveness, particularly photosynthetic performance, has been studied extensively for decades (reviewed by [2]). More specifically, Rubisco (ribulose-1,5-bisphosphate carboxylase–oxygenase) carboxylation in C3 plants is suboptimal at aCO_2, and the predicted increase in CO_2 will enhance photosynthesis rates. However, the initial stimulation of photosynthesis is usually reversed in the long term: a phenomenon known as photosynthetic acclimation to elevated CO_2 concentrations (eCO_2) or photosynthetic down-regulation [3]. Among the multiple hypotheses put forward for explaining this biochemical limitation, two are more widely accepted by the scientific community. The first is the C-sink/source hypothesis [4], where the ability of Rubisco to fruitfully exploit an environment with higher substrate for carboxylation is constrained by the limited plant capacity of increasing C sink strength, therefore, plants reduce the photosynthetic capacity to adjust the whole plant C balance. The second one is based on the reduction in the plant N content under eCO_2 because of a C dilution effect [5], phenomenon recently attributed to a decrease in N acquisition across different environments [6], probably in line with NO_3^- assimilation constrains occurring under eCO_2 [7]. In this context, [8] provide a new photosynthetic model for computing the benefit of the C derived from photorespiration on net CO_2 assimilation rates, in plants receiving NO_3^- as their N source and under certain conditions, such as triose phosphate limitations. The contrains of NO3- assimilation under eCO2 has been described in many species and experiments, including bench experiments in Arabidopsis [9] and wheat plants in field conditions [10]. Nevertheless, other authors such us [11] indicate that the depleted NO_3^- assimilation capacity under eCO_2 is a source of debate.

Increasing NH_4^+ nutrition in crops with respect to NO_3^- has been studied for more than three decades, mainly due to its reduced environmental impact and the fact that less energy is required for plant N assimilation. However, NH_4^+ nutrition may be stressful because high doses of NH_4^+ can be toxic to plants: reduced plant growth with high external NH_4^+ concentrations is a classic effect of NH_4^+ toxicity [12]. More specifically, NH_4^+ nutrition is known to lead to a strong plant C/N imbalance promoted by excessive internal NH_4^+ accumulation with a concomitant high C-skeleton and energy demand (reviewed by Esteban [13]). In fact, by increasing the C-skeleton availability, i.e., higher light intensity, in pea and ammonium-fed wheat plants ([14][15]) or higher CO_2 levels in *Arabidopsis* and ammonium-fed wheat plants [16], the plant C/N balance is improved, and the NH_4^+ toxicity symptoms are alleviated or minimised.

In this context, the hypothesis that "strict NH_4^+ nutrition under eCO_2, could enhance C sink for satisfying the high C skeletons requirement for NH_4^+assimilation" arises. Furthermore, these conditions would help to maintaining a balanced plant C/N ratio with higher Rubisco carboxylation rates. For this purpose, we used NH_4^+ tolerant pea plants (*Pisum sativum* cv. snap pea; [17]), which were grown at a sufficient N (2.5 mM NH_4^+) and high NH_4^+ concentration (10 mM NH_4^+) and at two levels of CO_2. Here we suggest that NH_4^+ nutrition could be considered as a promising N source alternative to face eCO_2 conditions improving plant responsiveness by strengthening plant C sink. This article expects to give light about the C/N metabolites management by a NH_4^+-tolerant plant could be an strategy to cope with future CO_2 scenarios using NH_4^+ nutrition as alternative N source.

2. Material and Methods

Plant material, growth conditions and biomass determination

Seeds of an NH_4^+-tolerant pea variety (*Pisum sativum* L., cv. sugar snap, [17]) were surfaced sterilised, germinated at 26ºC in for 96 h in the dark, in a perlite:vermiculite (1:2) substrate and grown hydroponically. This genotype was chosen to avoid the masking effect of possible NH_4^+ toxicity symptoms that other genotypes could show, therefore making it possible to study only the effect of contrasting CO_2 concentrations under ample N fertilisation conditions. Modified nitrogen-free 'Rigaud Puppo' solution was used [18]: 1.15 mM K_2HPO_4; 2.68 mM KCl; 0.7 mM $CaSO_4$; 0.07 mM Na_2Fe-EDTA; 0.85 mM $MgSO_4$; 16.5 µM Na_2MoO_4; 3.7 µM $FeCl_3$; 3.4 µM $ZnSO_4$; 16 µM H_3BO_3; 0.5 µM $MnSO_4$; 0.1 µM $CuSO_4$; 0.2 µM $AlCl_3$; 0.1 µM $NiCl_2$; 0.06 µM KI. The solution was adjusted to pH 6.5 by adding H_3PO_4 and was then buffered with $CaCO_3$ (0.5 mM). NH_4^+ was supplied as $(NH_4)_2SO_4$ at two concentrations: 1.25 and 5 mM (i.e., 2.5 and 10 mM of NH_4^+ in nutrient solution). The one element that remains unbalanced between the treatments is sulphur which has been reported to have minor effects on the absorption of other mineral elements and, therefore, should not induce significant changes in the nutritional status of the plants [19]. The nutrient solution was replaced twice a week to maintain the N level, and the pH was kept at 6.5. The plants were cultured in two modified controlled-environment chambers (Heraeus-Votsch HPS-500, Norrköping, Sweden) at two different CO_2 concentrations: ambient CO_2 (aCO_2; 400 ppm) and elevated CO_2 (eCO_2; 800 ppm) ± 5%. The growth chamber conditions were 22/18ºC (day/night), 65 % relative humidity and with a photoperiod of 16 hours and 300 µmol m^{-2} s^{-1} photosynthetic photon flux density. The plants were grown under these conditions for 4 weeks. At the end of this period, the plants were collected for determinations. Three independent experiments were performed. The data presented is a combination of the three experiments.

At harvest, the plants were separated into roots and shoots. Samples were collected, frozen in liquid N_2 and stored at −80ºC. Dry weights (DW) were determined by drying the plant material in an oven at 80°C for 48 h until stabilisation.

Gas exchange determinations

Gas exchange in leaves was recorded in the last fully expanded leaf between 3 h and 8 h after the start of the photoperiod using a portable GFS-3000 gas exchange system (Walz, Effeltrich, Germany). The order of the measurement of the plants were complete randomized using a sample randon sample function in excel . The measurements were taken at an air flow rate of 300 ml min^{-1}, 25ºC, 1000 µmol $m^{-2}s^{-1}$ irradiance. Maximum photosynthesis (Amax) was recorded at 400 and 800 ppm CO_2, depending on the growing conditions. The A/Ci curves were constructed at 1000 µmol $m^{-2}s^{-1}$ irradiance and at the following CO_2 levels: 400, 250, 125, 250, 400, 600, 800, 1000, 1200 ppm CO_2. The A/Ci curves were

modelled according to [20]. Leaf steady-state respiration measurements were recorded 2 h after the sunset in the chamber. Inlet CO_2 was adjusted to the treatment; with an air flow rate of 300 ml min^{-1}, 0 µmol $m^{-2}s^{-1}$ irradiance.

Respiratory capacity of the roots

Root respiration measurements were taken using 0.05 g^{-1} fresh weight (FW) and 0.5 to 1 cm-long root cuttings using a Rank Oxygen electrode (LD2, Hansatech, UK) at 25ºC in a total volume of 4 mL of nutrient solution. The capacity of the cytochrome respiratory pathway was studied following the application of 20 mM SHAM to the electrode chamber. The residual pathway capacity was measured following the addition of 0.1mmol/L KCN. The alternative respiration capacity was calculated from the difference between total respiration minus cytochromic and residual respiration.

NH_4^+, N, C and mineral content in plant tissues

The soluble NH_4^+ content in the cells was obtained by centrifuging (20,000×g, 30 min) tissue (approx. 0.2 g FW) incubated in 1mL of milli-Q water at 80ºC in a bath for 5 min. The supernatants (leaves and roots) were stored at -20ºC until they were analysed using ion chromatography. The soluble cation content was determined using an isocratic method with a 20 mM methanesulphonic acid solution as the eluent in a Dionex-DX500 ion chromatograph (Dionex, CA, USA) with Ion Pac CG12A and Ion Pac CS12A columns. Detection was performed by conductivity as above. The NH_4^+ content was expressed as mg g^{-1} DW.

The content of N and C (%; w/w), as well as the other minerals, was calculated from the dry material. Leaves and roots were ground in a mixer mill (MM200, Retsch, Haan, Germany). 2–3 mg of DW were placed into tin capsules and analysed through Dumas combustion in an elemental analyser CNS 2500 (CE Instruments, Milan, Italy). The N_2 and CO_2 produced were detected by thermal conductivity. Acetanilide was used as a standard in the total N content parameter. The C/N ratio was calculated by dividing the percentage of C by the percentage of N. The mineral content was determined after acid digestion using inductively coupled plasma/optical emission spectrometry (iCAP 6500 Duo, Thermo Fisher Scientific, Cambridge, UK). The N-use efficiency (i.e., internal NUE) was calculated as the ratio between the plant biomass (g) and the amount of N absorbed per plant (g).

Compounds related to C and N metabolism

i) Soluble sugar, starch, and organic acid content

158 Soluble carbohydrates (fructose, glucose, and sucrose) were extracted from roots and leaves (0.2 g of
159 FW) in boiling ethanol (80%, volume/volume). The ethanol-insoluble residue was dried and the starch
160 extracted, and the glucose produced by the amyloglucosidase enzyme was analysed as for soluble
161 carbohydrates [21]. Soluble sugars were expressed as μmol g^{-1} of DW, and starch was expressed as
162 μmol of glucose g^{-1} DW. Fucose 0.5 mM was used as the internal standard in the extracts.

163 For organic acid determination, frozen (-80ºC) pea leaf or root samples (0.2 g) were homogenised to a
164 fine powder in liquid N using a mortar and pestle. A 1.5 mL aliquot of 5% (weight/volume)
165 trichloroacetic (TCA) acid in water was added. The extracts were kept frozen at -20ºC until use.
166 Succinate, malate, α-ketoglutarate, oxaloacetate, and citrate contents were determined via ion
167 chromatography in a DX-500 system (Dionex Corporation) by gradient separation using a
168 DionexIonPac AS11 (4 mm×250 mm) column and a Dionex ASRS Ultra II (4 mm) suppressor column
169 with the Dionex Ion-Pac ATC-3 (9 mm×24 mm) ion trap, and a pre-column Dionex Ion-Pac AG11 (4
170 mm × 50 mm). The samples were injected with an AS40 autosampler (Dionex) at a 1:20 dilution in
171 milli-Q water. A 2 mL min. flow of solvent (methanol 18% NaOH 0.2 mM) was applied, and organic
172 acid separation was performed using a NaOH gradient (from 0.2 mM to 35 mM) for 16 min. Detection
173 involved a conductivity method in the electrochemical detector ED 40 (Dionex). Organic acid content
174 was expressed as mg g^{-1} DW.

175 **ii) Amino acid content**

176 Frozen plant tissue (0.1 g) was ground with liquid N_2 and homogenised with 1 ml HCl 1M. The extract
177 was centrifuged at 16000×g and 4ºC for 10 min. The supernatant was then pH adjusted to 7 with NaOH
178 and stored at -20ºC. The amino acids were derivatised at room temperature for between 12 and16 h with
179 FITC dissolved in 20 mM acetone/borate, at pH 10. Single amino acids were determined by high-
180 performance capillarity electrophoresis using a Beckman Coulter PA-800 apparatus (Beckman Coulter
181 Inc., Brea, CA, USA). The potential applied was -20 kV. The background buffer was 80 mM borax, 45
182 mMα-cyclodextrin, at pH 9.2. Because of the analytical method used, the asparagine and proline content
183 pooled in the same pick. The units were expressed as μmol g^{-1} DW.

184

185 ## Proteins and enzymatic activity determination

186 **i) Total soluble protein and RuBisCo protein contents**

187 Frozen leaves (0.2 g) were homogenised in a mortar with liquid N_2 in a 0.1 M phosphate buffer, at pH
188 7. The samples were centrifuged at 20,000×g and 4ºC for 20 min, then 5 μg of total soluble protein from
189 the supernatants was loaded onto SDS-PAGE gel (12.5% acrylamide), and stained with Gel-Code Blue
190 Stain reagent (Pierce Biotechnology, Inc., Rockford, USA). To estimate the large subunit of RuBisCo

content, densitometry analysis was conducted using the program Quant 1 in Gel Doc 2000 (Bio-Rad, USA).

The total soluble protein (TSP) content was calculated using the Bradford method. The units were expressed as mg protein g^{-1} DW.

ii) Activity and protein gel blot analysis of glutamine synthetase enzyme

Glutamine synthetase (GS; EC 6.3.1.2) activity was determined using a glutamyl hydroxamate (GHM) synthesis-based biosynthetic assay (following [22]) and expressed as µmol GHM g^{-1} dry weight (DW) min^{-1}. The total soluble protein was calculated according to the Bradford method and expressed as mg protein g^{-1} DW.

Plant samples were ground with liquid N_2 and then homogenised with 1.5:1 (volume/weight) extraction buffer (50 mM Tris–HCl pH 8; 1 mM EDTA; 10 mM 2-mercaptoethanol; 5 mM dithiothreitol;10 mM $MgSO_4$; 1 mM cysteine; 0.6% polyvinylpolypyrrolidone). Phosphatase inhibitor cocktails 1 and 2 (Sigma–Aldrich, St. Louis, MO, USA) were added to a final concentration of 2.5 µL mL^{-1} each. The extracts were centrifuged at 20,000×g and 4 °C for 30 min.

SDS-PAGE was run with the following antibody: anti-GS IgG, which was raised in rabbit against a specific peptide from pea GS (Acc. # CAJ87510.1; [17]) and used at a 1:2000 dilution overnight at 4°C. A peroxidase conjugated goat anti-rabbit IgG, followed by luminescence detection with the ECLTM Plus kit (AmershamBiosciences, Buckinghamshire, UK), was used in foliar tissues, and analkaline phosphatase labelled goat-anti-rabbit IgG was visualised with NBT-BCIP (Sigma–Aldrich) in root samples.

Statistical analysis

All statistical analyses were performed using the Statistical Product and Service Solutions software package (SPSS, USA) version 15.0 for Windows. The data was analysed using a one- and two-way analysis of variance (ANOVA) for all parameters. In the one-way analysis of variance (one-way ANOVA), the Duncan post-hoc test was used as a method to separate treatment means; in the post-hoc tests displayed in the figures and tables the letters represent the significant differences between treatments. Moreover, a two-way analysis of variance (two-way ANOVA) test was used in order to study the effect of two factors, NH_4^+ concentration, and CO_2 level, on the parameters analysed and the interaction between these factors (CO_2 x NH_4^+); significant differences and interaction between the factors are indicated with asterisks in the figures and tables. All the statistical analyses were conducted at a significance level of 5% ($p \leq 0.05$).

3. Results

Plant growth and C-N content of NH_4^+ fed pea plants and eCO_2

Under eCO_2, the shoot and root biomass increased for both NH_4^+ concentrations relative to aCO_2 (Figure 1). Nevertheless, pea plant biomass accumulation was independent of the external NH_4^+ concentration supplied at each CO_2 level (Figure 1). In fact, there was no plant growth reduction at the higher NH_4^+ concentration (Figure 1). The plants showed no symptoms of stress: there was no appreciable browning of the roots or necrotic tips; no lesions on the stem or leaves; and no chlorosis or vascular browning in any treatment (Supplemental Figure S1). Plants grown under eCO_2 increased their shoot biomass by 86% at 2.5mM NH_4^+ and 32% at 10 mM NH_4^+; meanwhile the increase observed in root biomass was 28% and 50% at 2.5mM and 10 mM NH_4^+, respectively, in comparison with aCO_2. Although 10 mM NH_4^+ enhanced shoot biomass at day 14, the shoot biomass accumulation was equal at the endpoint, after 21 days (Supplemental Table 1).

The C content was the same between the treatments in both tissues. Remarkably, the N content was higher at 10 mM NH_4^+ at both CO_2 levels. Our treatments did not influence the leaf NH_4^+ content. Contrastingly, in the roots, more NH_4^+ was found at 10 mM NH_4^+ at both CO_2 levels (Table 2).

Increased C source under eCO_2 and ammonium nutrition: photosynthetic performance, carbohydrate availability and leaf and root respiration rates

The maximum photosynthetic rates (Amax) were higher under eCO_2 (Table 1). Remarkably, the maximum velocity of the Rubisco carboxylation rate (Vc_{max}) and maximum electron transport rate (J_{max}) did not differ significantly between the treatments. Curiously, an increase in stomatal conductance (g_s) was observed under eCO_2 and 10 mM NH_4^+, concomitant to the increase in dark respiration (R_D) of the leaf and Amax (Table 1). Note that this significant increase in R_D coincided with a higher sucrose content in leaves, which could be offering elevated respiratory substrate in this organ.

The contrasting CO_2 levels had a profound influence on the carbohydrate pools (i.e., soluble sugars and starch). Plants grown under eCO_2 showed a significantly decreased leaf starch pool with respect to aCO_2, which was accompanied by a concomitant release of soluble sugars, fructose, glucose and sucrose in the root, which was especially important at 10 mM NH_4^+ (Figure 2). This suggests a shoot-to-root translocation of C driven by the greater availability of C and the higher external NH_4^+ concentration. This provides a clue to the important C and N sink that the roots can represent under these growth conditions, to help maintain the C/N ratios independently from the CO_2 concentrations. Moreover, of the leaf soluble sugars, only sucrose presented a significant increase under eCO_2 and at 10 mM NH_4^+.

The CO_2 treatment did not impact the tricarboxylic acid content, while the N treatment did. In leaves, malate and citrate levels were reduced under high NH_4^+ conditions at both CO_2 concentrations, while the α-ketoglutarate content presented the contrary response. (Supplemental Figure S2). In roots, higher levels of succinate and α-ketoglutarate were found under high NH_4^+ conditions at both CO_2 concentrations. Meanwhile, the malate and citrate content in the root only increased concomitantly with the NH_4^+ concentration under aCO_2. As for root respiration, the cytochrome respiration was not modified by the treatments while the alternative pathway decreased at high NH_4^+ conditions under eCO_2 (Supplemental Figure S3); this could be induced as a way to dissipate excess energy. Residual respiration was not affected in pea plants.

Increased N assimilation derived from eCO_2 and high NH_4^+ levels is accompanied by decreased total amino acid accumulation and increased total soluble protein

The glutamine synthetase (GS) activity in leaves was about twice as high as in roots. In leaves, no differences were found between treatments (Table 3). In roots, the GS activity was significantly higher under eCO_2. When both NH_4^+ and CO_2 concentrations were high, the root GS polypeptide content was increased under eCO_2 (Table 3; Supplemental Figure S4). This increase in the activity and content of GS in the roots under eCO_2 was not accompanied by an increase in the total amino acid content (Table 3). Plants exposed to eCO_2 showed a reduced total amino acid content in both leaves and roots, independently of the external NH_4^+ concentration (Table 3).

In terms of the amino acid profile, the major amino acids (serine, alanine, cysteine and asparagine/proline) represented 78% in leaves and 87% in roots. In leaves, a decrease in the serine and asparagine/proline content was observed at both ammonium and glutamine levels at 10 mM NH_4^+ under eCO_2 (Supplemental Figure S5). The serine content, the precursor of which is 3-phosphoglycerate, is strongly diminished under eCO_2. This decrease in serine coincides with an increase in the levels of alanine, an amino acid that is synthesized from pyruvate; this is synthesised in the glycolytic pathway in a process subsequent to 3-phosphoglycerate. With regard to the major amino acids in the root, a decrease in the content of serine and glutamine was observed at both levels of ammonium and cysteine at 2.5 mM NH_4^+, this decrease coinciding with an increase in asparagine in the roots under eCO_2.

Finally, the total soluble protein (TSP) content in leaves increased under eCO_2. Interestingly, this was not due to the variation in the levels of the large subunit of Rubisco (RbLs), which in turn was lower under eCO_2 (Table 1). The N treatment did not influence either the leaf TSP or RbLs. Nevertheless, the root TSP content was greater under the high NH_4^+ concentration, while no differences were found between CO_2 levels (Table 3).

4. Discussion

Elevated CO_2 leads to increased photosynthetic activity and plant biomass regardless of external and internal NH_4^+ concentrations

A plant's capacity to tolerate NH_4^+ nutrition has been attributed to several factors, including their genetic background, i.e., the inter- and intraspecific variation, among other things. [17] described intraspecific variation for pea plant (*Pisum sativum* L.) sensitivity to NH_4^+ nutrition, concluding that *Snap pea* plants could be considered a reference cultivar for NH_4^+ tolerance, since the biomass production of these plants is not affected by NH_4^+ concentrations. In our study, Snap pea plant growth was also independent of the NH_4^+ concentration supplied, and the plant biomass variation observed was driven by the availability of C (i.e., eCO_2). Similarly to [17], we found a large accumulation of NH_4^+ in the roots, although the root GS was similar (without concomitant changes in the leaves). Interestingly, in contrast with [17], who claim that the Snap pea's tolerance is based on its greater root respiration, in our case the root respiration remained unvaried between the different NH_4^+ levels under aCO_2. For that reason, our plants could have been promoting the 'sequestration' of NH_4^+ in the root cell vacuoles, preventing its transport to the leaves, which are more sensitive to NH_4^+ accumulation [23,24], as occurs in NH_4^+-tolerant reference species such as rice [25]**.** We believe that the discrepancies between the results of [17] and our study are related to a very tight control of the pH during our experiment.

Although in plants with C3 photosynthetic metabolism exposure to eCO_2 increases photosynthesis rates, often such stimulation is partially reversed in an adaptation process known as 'photosynthetic acclimation' [4]. Photosynthetic acclimation is accompanied by alterations in the gas exchange characteristics that are indicative of a decreased carboxylation capacity [26]. In our study, the A_{max} of plants exposed to eCO_2 increased regardless of the NH_4^+ dose and presented greater GS activity. Furthermore, these plants maintained unaltered Vc_{max} and J_{max} rates. While A_{max} increased and TSP (together with leaf N) were not altered by growth at eCO_2, our study showed that the Rubisco content decreased significantly under eCO_2. Due to the fact that plants tend to maximise resource-use efficiency, the reduction in the Rubisco content would imply a reallocation of the N away from the CO_2 fixation machinery into more limiting processes, such as carbohydrate synthesis and non-photochemical processes[26]. Such processes may contribute to an increase in sink activity. Our study confirmed a reduction in N allocation to Rubisco (a major leaf N storage form), leading to more NUE for photosynthesis and biomass accumulation. This is a remarkable observation, as our previous work on pea plants in eCO_2 with NO_3^- as their N source registered a large leaf N and TSP reduction, precipitating Vc_{max} [27]. Furthermore, the decline in leaf N and TSP reduction is a phenomenon overwhemingly observed under eCO_2 in multiple plant species, including wheat, *Arabidopsis*, and many others (Leakey et al., 2009; Jauregui et al., 2016; Rubio Asensio & Bloom, 2017). In contrast, newer publications have reported the capacity of the plants to maintain leaf N status under eCO_2 when NH_4^+ is the N source

received by the plants (extensively reviewed in [16][9][28][29]). Our results therefore fuel the debate over the impact different sources of N have in eCO_2 [11], including a novel nuance: plants that are able to tolerate high levels of NH_4^+ under aCO_2 successfully exploit high levels of NH_4^+ under eCO_2, evading leaf N, TSP and RbLs depletion and thus, maintaining an advantageous photosynthetic response to eCO_2. In this way, we can suggest that exploiting NH_4^+-tolerant plants in combination with NH_4^+-based nutrition could be a strategy for dealing with the reported reduction in the nutritional content of crops [30] in higher CO_2 concentrations than at present: we detected no N or other mineral depletion, as P or S could not be detected during the vegetative stage, and those minerals could potentially be allocated into the harvested tissues. Although an NH_4^+-rich environment is complex to address in croplands due to nitrification –even when current formulation of nitrification inhibitors are used– these conditions can be achieved in greenhouses through fertigation.

Maintaining the leaf N status and favouring C allocation into mobile sugars over starch accumulation is an efficient strategy for overcoming photosynthetic acclimation under eCO_2

The responsiveness of the photosynthetic machinery to eCO_2 has been previously associated with increases in leaf carbohydrate that induce photosynthetic protein repression, leading to a down-regulation of photosynthetic capacity [31]. Within this context, an efficient whole-plant partition-allocation of C between mobile carbohydrates and end products, and the development of new sink tissue determines plant responsiveness under eCO_2 [32][33]. Indeed, the capacity of our plants to avoid photosynthetic acclimation may be linked to the efficient C-allocation strategy adopted, prioritising short-term and mobile C-storage pools (soluble sugars and tricarboxylic acids) over the long-term storage (starch). The mobile C pools released from starch under eCO2 were especially relevant at high $NH4^+$ concentrations, these being principally long-term transport sugars, such as sucrose (in leaves and roots), as well as immediately consumable/usable/available sugars, such as glucose and fructose (mainly in the roots). Furthermore, starch accumulation under eCO_2 is a recurrent observation with NO_3^- nutrition [30][5], including in our previous experiments with the same genotype of pea plants [27]. Contrastingly, with NH_4^+ nutrition, in our experiment we found a significant reduction of starch content under eCO_2 relative to aCO_2. This decreased starch content was undoubtedly related to the reallocation of C into the mobile pools of sugars and the higher growth rates observed under eCO_2.

Might the stomatal conductance be enhanced with NH_4^+ nutrition under eCO_2?

Stomatal closure is the first physiological response of plants exposed to increasing CO_2 concentrations (Flexas et al., 2007), and in multiple species and experiments the g_s parameter is seen to typically drop under eCO_2 [35][36]. In fact, this response is often associated with photosynthetic acclimation as CO_2 levels rise. However, our experiment shows that pea plants exposed to eCO_2 present greater g_s activity than those grown under aCO_2. Despite strict NH_4^+ nutrition being usually underrepresented in the

published literature, this atypical increase in g_s under eCO_2 has also been reported when plants receive NH_4^+ nutrition without noticeable symptoms of NH_4^+toxicity [37][28]. Additionally, [29] reported increased g_s under high NH_4^+ concentrations compared to low NH_4^+ levels, arguing that N depletion was the cause of this drop. Interestingly, other leaf conductance, such us mesophyll conductance, is also enhanced with NH_4^+ nutrition compared to NO_3^- [29]. Despite the relevance of this observation for whole plant C management, neither the molecular mechanisms nor the signalling cascade underlying the differential effect of the N forms on plant conductance has been elucidated. For instance, general plant leaf responses to eCO_2 include an increase in epidermal and guard cell size, an increase in stomatal area, a decrease in epidermal and stomatal density, and decreased stoma opening [38]. In this regard, [39] demonstrated that the interaction of eCO_2 and the N source can influence the stomatal and epidermal anatomy in wheat plants, not only due to the increased CO_2 concentration, but also to the N source. These authors showed that wheat plants growing at 600 ppm CO_2 and with NH_4^+ as the sole N source exhibited a smaller stomatal opening area and lower stomatal density than those grown with NO_3^-. Furthermore, they also observed that NH_4^+ toxicity notably affects the morphological traits of wheat leaves, including their size and shape [36]. Thus, the interesting question arises of whether NH_4^+ tolerant cultivars, which are not affected by toxicity symptoms of NH_4^+, such as this snap pea variety [17], could avoid stomatal anatomy alterations under eCO_2. Further investigation is needed to address this question in depth.

Root NH_4^+ assimilation is an important C sink at eCO_2 and high NH_4^+ concentrations

Our data supports the idea that there is a strengthening of the root C sink in eCO_2 under high NH_4^+ concentrations. At 2.5 mM NH_4^+, the relative increase in biomass is mainly due to shoot growth, while at 10 mM it is mainly due to root growth. Besides, NH_4^+ transamination from glutamine to asparagine can be a means of transporting the root-assimilated ammonia to shoot. The increased ability of leaves to deliver photoassimilates under eCO_2, helps root for NH_4^+ assimilation, as accordingly shows its increased GS activity, strengthening its C sink. This is especially evident in plants grown with 10 mM NH_4^+ under eCO_2, with higher leaf sucrose and glucose levels than with 2.5 mM NH_4^+, and higher C skeletons (sugars and carboxylic acids) in roots, leading root C allocation to support primary NH_4^+ assimilation. In the literature, increased C allocation in roots of plants grown under NH_4^+ nutrition has been usually observed [13]. This observation has been attributed to the high requirement of C skeletons to obtain an extra energy input as well as to incorporate NH_4^+ into organic compounds, avoiding its accumulation in plant cells. Up to now, this strategy has only been considered as a mechanism of NH_4^+ tolerance in some plant species. Here it can be considered as a strategy for overcome to eCO_2 maintaining biomass production and N status of crop plants.

5. Conclusion

We have described the physiological mechanisms underlying the response of an NH_4^+ tolerant pea plant grown hydroponically under solely NH_4^+ nutrition and elevated CO_2 conditions (eCO_2). The NH_4^+ tolerant pea plants overcome photosynthetic acclimation to eCO_2 and increased their biomass, maintaining leaf N status by reallocating surplus C for the primary assimilation instead of starch formation. In terms of root plasticity, NH_4^+ nutrition could be additionally considered as a strong N source, able to increase C sink strength in conditions of eCO_2. In this way, improved C source / sink balance, maintained photosynthetic capacity and kept plant N status. A proper NH_4^+ nutrition management could motivate plants to strenghten the C demand from their sinks organs and metabolic process, which in turn constitute a plant trait to avoid photosynthetic acclimation in C3 crops under eCO_2 conditions.

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7. CRediT author statement

Ivan Jauregui: Conceptualization, Investigation and Writing- Original draft preparation, Writing - Review & Editing. Mikel Rivero-Marcos: Visualization and Writing - Review & Editing. Iker Aranjuelo: Investigation and Writing- Reviewing and Editing. Pedro M. Aparicio-Tejo: Funding acquisition, Conceptualization, Supervision and Writing - Review & Editing. Berta Lasa: Data curation, Statistics, Visualization and Writing - Review & Editing. Idoia Ariz: Conceptualization, Data Curation and Writing - Review & Editing

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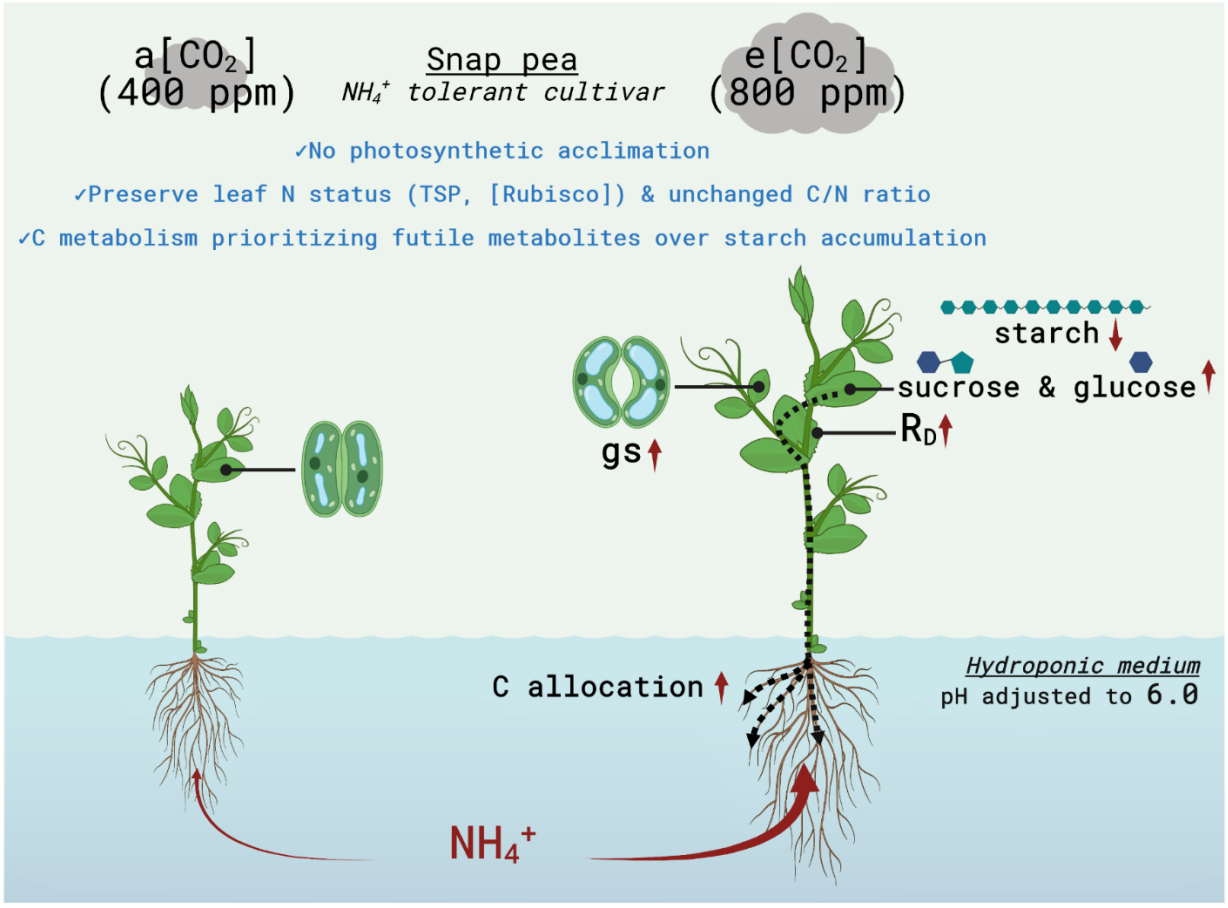

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550 Graphical Abstract (created with *biorender.com*)

Highlights

- We explore a novel strategy to cope with the widely reported reductions of leaf nitrogen content in plants exposed to atmospheric elevated CO_2 (eCO_2) using ammonium nutrition and ammonium-tolerant pea plants.
- Our Pea-ammonium tolerant avoids acclimation to eCO_2.
- Pea-ammonium tolerant plants favour C allocation into futile C pools over starch accumulation, and, preserves leaves nitrogen status.
- We bring light into an underrepresented physiological response of plants exposed to eCO_2: stomatal conductance can increase in eCO_2 if the source of nitrogen is ammonium.
- Besides, we found that we can consider the roots tissues as a source of C because of the energy cost of NH_4^+ assimilation.

Table 1. Effect of CO_2 level (Ambient, 400 *versus* Elevated, 800 µmol mol^{-1}) and NH_4^+ concentration (2.5 versus 10 mM) in *Pisum Sativum* plants (cv. *snap pea)* on Photosynthesis (A, µmol CO_2 $m^{-2}s^{-1}$), Rubisco maximum carboxylation rate (Vc_{max}, µmol CO_2 $m^{-2}s^{-1}$), maximum electron transport rate contributing to RuBP regeneration (J_{max}, µmol CO_2 $m^{-2}s^{-1}$), stomatal conductance (g_s, mol CO_2 $m^{-2}s^{-1}$), dark respiration (R_D, µmol CO_2 $m^{-2}s^{-1}$), Rubisco Large Subunit content (RbLs, optical density units). Each value represents the mean of biological replicates ± SD, n=3 and n=11 for Rubisco content. Statistical analysis was made by a two factors Analysis of the Variance (ANOVA). The asterisk (*) represent significant differences and *n.s.*, no significant differences ($P \leq 0.05$).

		A	Vc_{max}	J_{max}	g_s	R_D	RbLs
aCO_2	2,5 mM NH_4^+	18 ± 1 b	77 ± 9 a	150 ± 10 a	183 ± 6 b	-0.49 ± 0.02 a	68 ± 9 a
	10 mM NH_4^+	17 ± 1 b	78 ± 4 a	150 ± 6 a	186 ± 10 b	-0.60 ± 0.13 a	58 ± 4 a
eCO_2	2,5 mM NH_4^+	27 ± 1 a	72 ± 5 a	127 ± 6 a	203 ± 20 ab	-1.12 ± 0.07 b	48 ± 6 b
	10 mM NH_4^+	28 ± 2 a	70 ± 4 a	136 ± 7 a	240 ± 6 a	-1.56 ± 0.03 c	46 ± 3 b
	CO_2	*	n.s.	n.s.	*	*	*
	NH_4^+	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	CO_2 x NH_4^+	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 2. Effect of CO_2 level (Ambient, 400 *versus* Elevated, 800 µmol mol^{-1}) and NH_4^+ concentration (2.5 versus 10 mM) in *Pisum Sativum* plants (cv. *snap pea)* on ammonium content (mg g^{-1} DW) nitrogen (%), C/N ratio in shoot and root. Each value represents the mean of biological replicates ± SD, n=3 and n=6 for NH_4^+ content.

Statistical analysis was made by a two-way Analysis of the Variance (ANOVA). The asterisk (*) represent significant differences and *n.s.*, no significant differences ($P \leq 0.05$).

.		LEAF			ROOT		
		NH_4^+	N	C/N	NH_4^+	N	C/N
aCO_2	2,5 mM NH_4^+	0.66 ± 0.1 a	4.6 ± 0.4 a	9.4 ± 0.8 a	4.2 ± 0.5 b	4.8 ± 0.0 b	7.6 ± 0.3 a
	10 mM NH_4^+	0.80 ± 0.2 a	5.4 ± 0.3 a	7.9 ± 0.5 a	9.0 ± 1.1 a	6.2 ± 0.3 a	6.3 ± 0.4 b
eCO_2	2,5 mM NH_4^+	0.72 ± 0.1 a	4.4 ± 0.6 a	10.0 ± 1.5 a	4.1 ± 0.6 b	4.4 ± 0.2 b	8.7 ± 0.5 a
	10 mM NH_4^+	0.85 ± 0.1 a	5.5 ± 0.3 a	7.7 ± 0.4 a	7.0 ± 0.4 a	6.3 ± 0.2 a	6.2 ± 0.3 b
	CO_2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	NH_4^+	n.s.	*	n.s.	*	*	*
	CO_2 x NH_4^+	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3. Effect of CO_2 level (Ambient, 400 *versus* Elevated, 800 µmol mol^{-1}) and NH_4^+ concentration (2.5 versus 10 mM) in *Pisum Sativum* plants (cv. *snap pea)* on free amino acids content (µmol g^{-1} DW), total soluble protein (TSP) (mg g^{-1} DW) and GS activity (µmol GHM g^{-1} DW min^{-1}) in leaf and root. Each value represents the mean of biological replicates ± SD, n=6. Statistical analysis was made by a two factors Analysis of the Variance (ANOVA). The asterisk (*) represent significant differences and *n.s.*, no significant differences ($P \leq 0.05$).

		LEAF			ROOT		
		Total free amino acids content	TSP	GS activity	Total free amino acids content	TSP	GS activity
aCO_2	2,5 mM NH_4^+	1428 ± 87 a	178 ± 20 c	83 ± 5 a	1050 ± 104 a	76 ± 5 b	35 ± 3 b
	10 mM NH_4^+	1567 ± 187 a	195 ± 25 bc	85 ± 6 a	729 ± 38 b	105 ± 7 a	41 ± 4 ab
eCO_2	2,5 mM NH_4^+	998 ± 74 ab	255 ± 15 ab	90 ± 3 a	851 ± 24 b	81 ± 3 b	46 ± 3 a
	10 mM NH_4^+	1273 ± 80 b	272 ± 21 a	93 ± 3 a	740 ± 9 b	104 ± 7 a	51 ± 3 a
	CO_2	*	*	n.s.	n.s.	n.s.	*
	NH_4^+	n.s.	n.s.	n.s.	*	*	n.s.
	CO_2 x NH_4^+	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.