Nitrogen source as key factor conditioning responsiveness of *Arabidopsis* plants to elevated CO_2 conditions

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CERTIFICAN:

Que el trabajo descrito en la presente memoria titulado "**Nitrogen source as a key factor conditioning responsiveness of *Arabidopsis* plants to elevated CO_2 conditions**", que presenta D. **Iván Jáuregui Mosquera** para optar al grado de Doctor con mención de "Doctor Internacional", ha sido desarrollado bajo su dirección en el laboratorio de Fisiología Vegetal, del Departamento de Ciencias del Medio Natural de la Universidad Pública de Navarra.

Revisado el trabajo, autorizamos la presentación de la citada Tesis Doctoral, dado que reúne las condiciones necesarias para su defensa.

En Pamplona, a

Fdo. Pedro Mª Aparicio Tejo

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Results obtained in this study have been presented in the following publications:

Iván Jáuregui, **Pedro Mª Aparicio-Tejo**, **Concepción Avila**, **Rafael A. Cañas**, **Iker Aranjuelo**. "Root-shoot mineral transport as key target processes involved in *Arabidopsis* responsiveness to elevated [CO_2] conditions" Plant, Cell and Environmental (Under review)

Iván Jáuregui, **Pedro Mª Aparicio-Tejo**, **Concepción Avila**, **Marina Rueda-López**, **Iker Aranjuelo**. "Ensuring energy availability, a target process conditioning plant responsiveness to elevated CO_2" Physiologia Plantarum (Under review)

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A mis padres Carlos y Esther.

A mi hermana Laura.

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ABBREVIATIONS

ABSTRACT:

The amount of carbon dioxide (CO_2) in Earth's atmosphere has exceeded 400 parts per million (ppm) during three continuous months of observations in 2014, which is an absolute record for the last 800,000 years. As a consequence, plants, as photosynthetic organisms, will inevitably be influenced by the changed growing conditions that result from the increased [CO_2]. The effect of increased [CO_2] on plants has been widely studied and goes far beyond favouring carboxylation of Rubisco and generating higher growth rates. The present thesis aims to highlight the relevance of different nitrogen sources in the response of *Arabidopsis thaliana,* a C_3 model plant, to elevated [CO_2]. For this purpose, this has been an integrated study of leaf and root organs combining techniques that range from plant physiology to molecular biology. Under nitrate nutrition and elevated [CO_2] conditions, *Arabidopsis thaliana* plants increase their biomass and maximum photosynthetic rates; nevertheless, the total soluble protein, Rubisco content and leaf N content reveal a general decrease in leaf N availability (chapter I). Although the expression of nitrate transporters was substantially upregulated in roots, plants did not efficiently transport nitrate and other minerals from roots to leaves, which compromised leaf N and mineral status. Therefore, our results suggest that the diminution of transpiration rates causes a reduction in the xylem flux, which inexorably generates an imbalance in the transport of nitrate and mineral elements between organs under elevated [CO_2]. Moreover, root nitrate assimilation (based on the amino acid content) is favoured in order to overcome N limitations due to the reduction in leaf nitrate assimilation. In chapter II plant performance under elevated [CO_2] and ammonium nitrate conditions was characterized and it was found that biomass doubled due to substantially increased photosynthetic rates. Gas exchange characterization revealed that these plants overcame photosynthetic acclimation. Plants maintained Rubisco concentrations at control levels alongside enhanced energy efficiency. The increments found in leaf carbohydrates and organic acid content linked to enhanced respiration rates supported the fact that the plants under elevated [CO_2] maintained their energy status. The transcriptomic analysis enabled the identification of photoassimilate allocation and remobilization as fundamental processes used by

the plants to avoid photosynthetic acclimatization. Moreover, based on the relationship between plant carbon status and hormone functioning, the transcriptomic analyses provided an explanation of why phenology accelerates under elevated [CO_2]. Finally, in chapter III the relevance of ammonium nutrition under elevated [CO_2] was analysed; for this purpose a double nitrate reductase mutant (NR mutant, *nia1-1/chl3-5* defective) was used. These results highlight that plants under elevated [CO_2] which preferentially assimilate ammonium as their only N source maintain leaf growth and photosynthetic rates similar to plants receiving ammonium nitrate. However, under ambient [CO_2] concentrations, ammonium toxicity symptoms emerge and development is extremely constrained. In elevated [CO_2] conditions, an NR mutant maintained the energy supply for the Calvin cycle pathway and managed efficient photoassimilate transport between plant tissues. Furthermore, the data suggest active ammonium assimilation in leaves due to the exceptional conditions (C skeletons, ATP, adequate pH homeostasis and no photoinhibition) of these plants under elevated [CO_2]. Hence, the results obtained in the present doctoral thesis aim towards the incorporation of the source of nitrogen as key in the response at the elevated [CO_2] of *Arabidopsis thaliana* plants. Consequently, the present doctoral thesis aims to determine whether the incorporation of the correct source of nitrogen is the key component in how *Arabidopsis thaliana* plants respond to elevated [CO_2].

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INTRODUCTION

1.– Elevated [CO_2] conditions, the new scenario for plant development.

Over the long term, carbon (C) exchanges among the atmosphere, biosphere, pedosphere and hydrosphere have been balanced. Nevertheless, as reported by the Intergovernmental Panel on Climate Change (IPCC, 2013) this biogeochemical balance has been broken due to anthropogenic forcing factors. From the beginning of the industrial revolution the atmospheric carbon dioxide (CO_2) concentration has been increasing from 280 parts per million (ppm) to its historical maximum of 400 ppm this year (US Department of Commerce NOAA, 2014). The IPCC foresees that the [CO_2] will rise to around 700 ppm by the end of this century if this progression continues. Nonetheless, humans are also part of the solution with implementation of policies based on scientific recommendations.

It has been reported that the increments in atmospheric [CO_2] since pre-industrial times have had a direct impact on crop yields (Mayeux et., 1997), influence the timing of growth (Reyes-Fox *et al.*, 2014) and will probably affect food quality (Myers *et al.*, 2014). Elevated [CO_2] stimulate the carboxylation rates and depresses the oxygenation rates of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Sage, 1994). Therefore, plants broadly increase their growth rates during short-term exposure (Long et al., 2004; Amthor, 1995). Nevertheless this response is not as clear in long-term exposures (de Graaff et al., 2006; Long et al., 2006; Ainsworth & Long, 2005). The current atmospheric [CO_2] is limiting for Rubisco activity in C_3 plants (Farquhar *et al.*, 1980) and the available information suggests that the predicted short-term exposure to elevated [CO_2] will enhance photosynthetic rates (Leakey *et al.*, 2009) and growth in C_3 plants (Li *et al.*, 2008; Long *et al.*, 2004). Nevertheless, long-term exposure has been associated with the phenomenon broadly known as photosynthetic acclimation (Aranjuelo et al., 2013a; Ainsworth and Rogers, 2007; Stitt and Krapp, 1999), described as modification of the

photosynthetic efficiency of plants exposed to elevated CO_2 (compared with plants grown in 400 ppm CO_2). The downregulation of potential photosynthetic rates constrains the expected stimulation of growth in plants exposed to future atmospheric CO_2 concentrations.

2.– Unravelling the divergence between the classical visions of carbon and nitrogen under elevated [CO_2].

Plants adjust their carbon fixation and photosynthate utilization capacity. The coordination between C fixation and utilization has been described as a key process that regulates photosynthesis under elevated [CO_2]. One of the most cited hypotheses to explain acclimation to elevated CO_2 is known as the "source/sink hypothesis", and it theorizes that photosynthetic rates are limited by an insufficient plant sink strength (Arp, 1991). The accumulation of non-structural carbohydrates is sensed by hexokinase, and this represses expression of genes coding the photosynthetic apparatus and finally, induces acclimation (Long *et al.*, 2004; Moore *et al.*, 1999). At the whole plant level, photosynthetic rates are tightly coordinated with the ability to maintain and develop new sink tissues. The most suitable strategy for C reallocation in sink tissues defines the type of plant development under elevated CO_2 (Aranjuelo *et al.*, 2011). Under the source-sink hypothesis, respiration cooperates by consuming the excesses non-structural carbohydrates, avoiding this accumulation (Leakey *et al.*, 2009). Markelz et al. (2013) showed that in elevated [CO_2], *Arabidopsis thaliana* plants with higher photosynthetic rates also showed greater respiration rates and this work also established the mechanism at the transcriptomic level. Thus, the enhanced respiration rates will support the high energy-demanding Calvin cycle pathway to maintain the highly dynamic photosynthetic machinery under elevated [CO_2].

Nitrogen (N) availability and management has also been described as determining photosynthetic performance under raised [CO_2] (Langley & Megonigal, 2010; Stitt & Krapp, 1999). It has been reported that photosynthetic acclimation is tightly dependent on N dosage: the effect is evident when plants are N-limited, but not observed in well-fertilized conditions (Reich & Hobbie, 2012; Geiger et al., 1999). Under elevated [CO_2] it has been documented that N content is

reduced (in varying degrees), in all plant tissues (Cotrufo et al. 1998), in all culture conditions (Poorter et al. 1997) and has been corroborated as a conserved response across a wide range of plant species (Loladze, 2014). The association between a reduction in nitrogen content and the performance of plants under elevated [CO_2] has been an object of intense debate. On one hand, these results support the thesis that nitrogen use efficiency is improved in plants exposed to elevated CO_2. On the other hand, analysing this evidence through the prism of plant physiology, their performance under elevated CO_2 indicates plant growth limitations due decrements in N content. Such reductions directly diminish protein content (Taub et al. 2008), Rubisco content (Long *et al.*, 2004) or chlorophyll (Ainsworth & Long, 2005); ultimately, depletion of the organic N content (Bloom et al., 2010) generates physiological perturbations that constrain the expected "fertilization effect of CO_2".

3.– Nitrate assimilation is constrained under elevated [CO_2] conditions in C_3 plants. The opportunity for ammonium nutrition.

In the last decade an outstanding observation has been made, that the form of N applied plays a crucial role in the responsiveness of plants to elevated CO_2 (Bloom *et al.*, 2014). Rachmilevitch et al., (2004) described that the reduction in photorespiratory rates under elevated CO_2 inhibits leaf nitrate (NO_3^-) assimilation in wheat and *Arabidopsis thaliana* plants and Bloom et al. (2010) stated three possible hypotheses to explain this reduction based on: 1) a lower photorespiration rate under elevated CO_2 that limits NO_3^- reduction to nitrite due to the lack of NADH from inefficient export of malate to the chloroplasts (Searles & Bloom, 2003); 2) an increase in CO_2 fixation that produces stromal acidification that consequently reduces NO_2^- transport into the chloroplast (Rachmilevitch *et al.*, 2004); 3) increases in the competition between enzymatic activities for ATP and Fd reductant compounds that constrain NO_3^- assimilation (Foyer *et al.*, 2012). The reduction in photorespiration rates in elevated CO_2 causes a decrease in NO_3^- assimilation, diminishing plant organic N compounds, shortening the growth rate and compromising food quality (Loladze, 2014). Consequently, it could be expected that plants would vary the strategy of organic N incorporation under elevated CO_2, promoting the

assimilation of NO_3^- by the roots (Chapter I) and prioritizing ammonium uptake (Matt *et al.*, 2001) and leaf ammonium assimilation (Chapter III) to ensure optimal nutrition status.

When both N inorganic forms are available for plant uptake, ammonium (the reduced form) has been proposed as the preferred nitrogen source because of its moderate costs in carbon catabolism (Bloom *et al.*, 1992), energy and water (Andrews *et al.*, 2013) for assimilation. Nevertheless, in many plants species, when ammonium is supplied as the sole N source "ammonium toxicity" may emerge (Ariz *et al.*, 2011; Britto & Kronzucker 2002). There are multiple hypotheses proposed to explain the symptoms of ammonium syndrome: cations and root pH disturbance (Britto & Kronzucker, 2002), intercellular pH disruption (Britto et al., 2001), uncoupling of phosphorylation (Gerendás et al., 1997) and absence of sufficient C skeletons for ammonium assimilation (Britto & Kronzucker, 2005). It is unquestioned that mixing very small concentrations of nitrate with ammonium alleviates ammonium toxicity (Hachiya *et al.*, 2012). To investigate this further, modern molecular biology enables the subtle steps in metabolic pathways to be uncovered through the silencing of key genes. In this regard, with the goal of not changing the stoichiometry in the nutrient solution and growth plants under ammonium-based nutrition, the nitrate reductase-defective *nia1-1/chl3-5* genotype of *Arabidopsis thaliana* (Wilkinson & Crawford, 1993) was used. In the current context of increases in the concentration of atmospheric [CO_2], understanding the complexity of the response of plants to ammonium nutrition and mixed ammonium nutrition can help translate it from research into plant physiology (Chapter II; Chapter III) towards its application in crop production.

4.– Root–to–shoot integration and transport management. Uncovering plant performance under elevated [CO_2]

Exposure to elevated [CO_2] has been described as increasing root growth (Arp, 1991) and modifying the shoot to root ratio (Farrar & Williams, 1991). Besides, roots have a central role in nutrient uptake/assimilation and likewise represent a relevant C sink. Thus, although elevated

[CO_2] alters root architecture, produces physiological perturbations and drives the acquisition of mineral elements, the role of roots is not adequately integrated into plant responsiveness under such conditions. The regulation of genes in plants is highly interconnected and potentially coordinated for different types of metabolite signalling throughout the whole plant (Lejay *et al.*, 2008; Wang *et al.*, 2001). Hence, an integrated and dependent response between leaves and roots is expected. A whole-genome transcriptomic approach allows identification of integrated response patterns in the performance of plants under elevated CO_2. The complexity of the scheme needs to be studied in detail.

When carbohydrate-demanding tissue or metabolic reactions are quenched by sugar transportation (Lemoine *et al.*, 2013) or by energy compounds (Noguchi *et al.*, 2001), the plant needs to reduce carbon fixation to match demand, otherwise an imbalance will be created due to accumulation of non-structural carbohydrates. The distribution of sugars throughout the whole plant the can be affected at the level of individual cells, at the short distances between cells and at the long distances via phloem (Ku et al., 1999). In all cases, the transport could be limited by tissue-specific gene expression and by energy-dependent transport requirements (Williamset al., 2000). The allocation of photoassimilates along sink strength has been studied in elevated CO_2 conditions using genotypes with different harvest indexes (Aranjuelo *et al.*, 2013) or modifications of regrowth capacity in plants (Fischer *et al.*, 1997; Erice *et al.*, 2011). Nevertheless, the genomic mechanism and physiological causes of the limiting transport in elevated CO_2 conditions are not clear and need to be elucidated. The end point of this transport disturbance is that carbohydrate accumulation has the potential to regulate the signalling cascade of plant hormones such as abscisic acid (ABA), which directly affect plant performance (Eveland & Jackson, 2012). Understanding whole-plant carbon partitioning and its implications will provide valuable knowledge to overcome long-term CO_2 acclimation.

Plants modulate stomatal opening in order to optimize the rate of [CO_2] diffusion into the leaf for photosynthesis and to minimize water loss. Stomatal closure in elevated [CO_2] has been well

characterized in previous studies (Ainsworth & Rogers 2007). Such closure has an important effect on transpiration and therefore, water transport through the plant. Elevated [CO_2] has been observed to reduce transpiration (Taub & Wang 2008) and downward xylem mineral transport (Teng et al. 2006). In *Arabidopsis thaliana* plants, nitrate has been described as being absorbed from roots, loaded into the xylem and transported to the chloroplast where it is reduced (Andrews, 1986). Root-to-shoot long distance nitrate transport is primarily driven via the xylem, and therefore is highly dependent on the transpiration rate. Consequently, the transpiration rate is a key process that conditions plant performance under elevated [CO_2] (Long et al. 2004). Likewise, xylem loading delay in plants exposed to elevated CO_2 has been described (McGrath & Lobell, 2013) as reducing mineral element contents and disturbing plant mineral stoichiometry (Loladze, 2002) that therefore could trigger physiological perturbation. Unequivocally, mineral element contents are reduced in plants exposed to elevated [CO_2] (Loladze, 2014; Duval et al., 2011; Poorter et al. 1997; Manderscheid et al. 1995). Mineral elements are used as structural components in a wide range of molecules and cells, regulate electrochemical balance in the cell (Baxter, 2009) and thousands of enzymes would be unable to work without their particular co-factor (Hänsch & Mendel, 2009). Hence, the physiological reorganization of enzymatic reactions in elevated CO_2 conditions could promote a competition for mineral elements as cofactors. A recent meta-analysis of Free-Air CO_2 Enrichment experiments (FACE) described decreases in protein content and iron (Fe) and zinc (Zn), as cofactor mineral elements, in different plant species as a key responses to elevated [CO_2] (Myers *et al.*, 2014). Thus, the interdependence between the processes of transpiration and mineral handling in plant performance under elevated CO_2, is an important area to research. Nevertheless, the genomic basis of these processes has not yet been studied in elevated CO_2 conditions. Such evidence could constrain plants performance under elevated [CO_2].

Along with the mechanisms involved in root acquisition and whole plant distribution of mineral elements can alter essential functions in plant metabolism. The multidirectional movement of nitrate from the different compartments in the cell across the plant is mediated by a large number of genes (Wang et al. 2012) that are potentially modulated at the transcriptional and post

transcriptional level (Orsel et al.2002). Several nitrate transporters, for example NRT 1.1 and NRT1.2, have been described as being involved in regulating stomatal opening (Kanno et al. 2012). Moreover, nitrate has been described to act as an osmotic anion that depolarizes guard cells (Guo et al. 2002) or mediates cellular ABA uptake (Andrews et al., 2013). Furthermore, another relevant field of study is how plants manage efficient whole-plant nitrate transport under conditions where transport via xylem flow is restricted, such as exposure to elevated [CO_2]. In this scenario, the involvement of NRT1.5 in long-distance nitrate transport (Krapp *et al.*, 2014) could be fundamental due to its key responsibility in nitrate xylem loading. There is a scientific consensus that nitrate acts as a signal molecule (Canales et al. 2014; Alvarez et al. 2012), regulating multiple processes such as nitrogen metabolism, energy metabolism, glycolysis and amino acids (Wang et al. 2007) in cooperation or antagonism with other cascades (Krapp et al. 2014). Therefore, a study of nitrate at the whole-plant level is essential given the interconnectedness between nitrate-dependent processes throughout different plant organs and the physiological changes under elevated [CO_2].

OBJECTIVES

Plants sense, respond and adapt to rising atmospheric [CO_2]; understanding how they behave in this scenario is a present challenge for plant physiology, and the results will be relevant for agronomy.

The overall objective of the present document is to extend the knowledge on the physiological response and the metabolism modifications that modulate *Arabidopsis thaliana* plants grown under different nitrogen sources and elevated [CO_2]. This general aim is developed in three parts, corresponding to the different chapters of the thesis:

- Chapter I: the goal of this study was to characterize the mechanisms and implications of the root-to-shoot performance of *Arabidopsis thaliana* plants fed nitrate under elevated [CO_2].
- Chapter II: the goal of this study was to examine the impact of elevated [CO_2] exposure on shoot and root C/N metabolism and its implications for photosynthesis and development in *Arabidopsis thaliana* plants grown with ammonium nitrate as N source.
- Chapter III: the goal of this study was revealing the mechanisms involved in development of *Arabidopsis thaliana* performance under elevated [CO_2] and principally ammonium-assimilation using a double nitrate reductase-defective mutant (*nia1-1/chl3-5*) and comparing this to wild type ammonium nitrate-based nutrition.

MATERIAL AND METHODS

Plant material and experimental design

Experiment 1.

The experimental work was conducted using *Arabidopsis thaliana,* Columbia 0 ecotype. Seeds were germinated on the surface of 0.65% agar in a seed holder system by Araponics (Araponics SA, Belgium) to perform the experiment on plants grown under hydroponic conditions. Seeds in seed holders were initially placed in a germination chamber in dark conditions for 48h at 24ºC, with saturated humidity and distilled water as solution. Plants were then moved to a growth chamber for 13 days at 150 µmol m^{-2} s^{-1} PAR, 16/8 h photoperiod, 22-18ºC thermoperiod, saturated humidity conditions and ambient [CO_2] concentration. Distilled water was replaced every 2-3 days. When plants were 15 days old, they were selected for uniformity and then transferred to 8 liter hydroponic containers, each container holding 8 plants. Puppo & Rigaud, (1975) solution was used and changed as follows: K_2HPO_4 was added instead of KH_2PO_4; pH was adjusted to 5.8 with H_3PO_4 and was buffered with $CaCO_3$ (0.5 mM). The nitrogen (N) source was KNO_3 at 0.75 mM concentration. The solution was replaced every 3-4 days. Plants were cultured in two different controlled-environment chambers (Heraeus-Votsch HPS-500, Norrkoping, Sweden) with the modification described by Aranjuelo *et al.*, (2014a) at two different [CO_2] levels: 400 ppm (ambient CO_2) and 800 ppm (elevated CO_2). The growth chamber conditions were 22/18ºC (day/night), 16 h photoperiod, 80% RH and 200 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD). All determination were conducted after 5 weeks exposure to CO_2 treatments, previous to first flower were buds visible, when the more advanced phenology treatment (elevated [CO_2]) were in 3.9 growth stage ontology from the scale described by Boyes (2001).

Experiment 2

In this experiment, the growth conditions were identical than in the previous experiment 1 except that the nitrogen was applied as NH_4NO_3 at 0.75 mM concentration.

Experiment 3.

The experiment was was conducted using *Arabidopsis thaliana* plants, Columbia 0 ecotype and a mutant double nitrate reductase defective (*nia1-1/chl3-5*) named as G´4-3 by Wilkinson & Crawford, (1993). Germination and plant growth environmental conditions were identical than in experiment 1 except:

Nitrogen was applied as NH_4NO_3 at 1 mM concentration

Photoperiod was short day, 8/16 h

All determination were conducted after 4 weeks exposure to CO_2 treatments, when the more advanced phenology treatment (elevated [CO_2]) were in 3.7 growth stage ontology from the scale described by Boyes (2001).

Plant growth determinations and gas exchange measurements

Plant sampling was carried out 4 h from the beginning of the light period. Shoots and roots were collected (12 plants per treatment) and were placed immediately into liquid nitrogen and stored at –80ºC, awaiting analysis. For plant growth determinations, samples were dried at 70°C for 48 h in order to obtain the dry biomass (DM).

Gas exchange measurements were carried out with the LiCor 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA) in fully expanded leaf with the following parameters: 25ºC leaf temperature, 500 μm s^{-2} flow, 10-20% humidity.

Determinations were conducted between 4 h and 7 h after the onset of the photoperiod. Electron transport rate and transpiration were obtained with Li-COR. Maximum photosynthetic rates were made at 1000 μmol m^{-2} s^{-1} PPFD and net photosynthetic rate at 200 μmol m^{-2} s^{-1} PPFD. The light-saturated rate of CO_2 assimilation was measured at saturated light conditions (1000 μmol m^{-2} s^{-1} PPFD) in order to estimate the maximum carboxylation velocity of Rubisco (Vc_{max}) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) using the model of (Harley et al.1992); the selected points for this model curves were 8, from 99 to 1200 μmol mol^{-1} CO_2. Dark respiration (Rd) measurement was performed 30 min before the dark period started; measurements were made in automatic mode and therefore, the leafs were placed in the chamber for at last 10 min. The rate of electron transport rate (ETR), transpiration (Tr), intercellular [CO_2], (Ci) and the relative quantum yield of PSII at the steady state, (ΦPSII) were obtained with LiCOR. The electron flux for photosynthetic carbon reduction (ETRc) and the electron flux for photorespiratory carbon oxidation (ETRc) were measured as described by Eppron et al. (1995).

Metabolite determinations

Soluble sugar, starch and organic acid content

Frozen plant tissue (0.1 g) was homogenized in 1 ml of 80% ethanol and was sonicated for 25min at 30ºC using an ultrasonic bath (Selecta, Spain). The extract was centrifuged at 16000g and the supernatant collected; the same procedure repeated 2 additional times. The supernatant was evaporated through a Turbovap® (Zymark, Carmel, IN, USA) and the solid phase was dried at 70ºC over 24 hours to measure starch content. After evaporation, the sample was resuspended with 1.5 ml of distilled water in an ultrasonic bath.

Sucrose, glucose and fructose content were determined in the supernatant fraction with a capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) consisting of a fused silica capillary of 50µ ø internal length and 31.4/38.4 cm. The buffer used for the detection, was a solution of 10 mM benzoic acid and 0.5 mM MTAB, pH 12 (adjusted with NaOH). The method of analysis was performed at a voltage of -15 kV and 20°C. Detection was indirectly to a wavelength of 225 nm. Internal standard used fucose (Fuc) which was added to the extract to be tested (0.5 mM final concentration of Fuc).

Starch content was determined in the dry pellet and homogenized in a mortar with milli-Q water. The total material was homogenized with botex and ultrasonic bath; then was incubated 1 h at 100 ° C. Each of the extracts was cooled and added 30% vol/vol of amyloglucosidase solution (41 mg of previously desalted enzyme amyloglucosidase in 50 ml of NaAc buffer, 8.55 mM, pH 4.5) to hydrolyse all the starch present in the sample and then quantifying as glucose by capillary electrophoresis. Amyloglucosidase was desalted on acrylamide gel columns Bio-Rad Bio-Gel P-6-DG desalting buffer (250 mM MOPS, 25 mM $MgCl_2$, 100 mM KCl, pH 7). The extracts were homogenized after the addition of amyloglucosidase solution and incubated at 50°C for about 12 h. Later the extracts were centrifuged at 4800 g, the supernatant recovered and stored at -20°C until the time of determination by capillary electrophoresis.

The organic acids malate, citrate, oxalate and succinate were determined in the supernatant fraction with isocratic ion chromatography. The extracts were filtered with Milex filters (Millipore, Billerica, MA, USA) and injected in a DIONEX-DX500 (Dionex Corporation, CA, USA) system equipped with an ED40 electrochemical detector. IonPac AS11 column connected to an ATC-1 protecting column and an AG11 pre-column (all chromatography equipment from Dionex, Salt Lake City, UT, USA) was used.

Amino acid content

Frozen plant tissue (0.1 g) was ground with liquid N_2 and homogenized with 1 ml HCl 1M. The extract was centrifuged at 16000g and 4ºC for 10 min. Then the supernatant was pH adjusted to 7 with NaOH and stored at -20ºC. Amino acids were derivatized at room temperature between 12-16 h with FITC dissolved in 20 mM acetone/borate, pH 10. Single amino acids were determined by high performance capillarity electrophoresis using a Beckman Coulter PA-800 apparatus (Beckman Coulter Inc., Brea, CA) with laser-induced fluorescence detection (argon ion: 488 nm) according to Ariz *et al.* (2012). Samples were derivatized with fluorescein isothiocyanate and separation was performed in a 50 μm i.d. × 43/53.2 cm fused-silica capillary at a voltage of 30 kV and a temperature of 20ºC. The migration buffer was 80 mM borax (pH 9.2) containing 45 mM alpha-cyclodextrin. Samples were injected using a pressurized method (5 s). Internal standard norvaline was used. The protocol did not enable the separate analyses of glycine and serine, so they were quantified together.

Mineral determinations

Nitrate and ammonium content

Nitrate (NO_3^-) and ammonium (NH_4^+) content was detected in the soluble fraction of the metabolite analysis described above with the isocratic ion chromatography technique using a DIONEX-DX500 (Dionex Corporation, CA, USA). For NO_3^- content IonPac AS11 column connected to an ATC-1 protecting column and an AG11 pre-column was used (all chromatography equipment from Dionex, Salt Lake City, UT, USA). For NH_4^+ content, IonPack CG12A and CS12A columns was used.

Carbon, Nitrogen and C/N ratio

Carbon (C) and N content (%) was determined in the dry material in a CNS 2500 elemental analyser (CE Instruments, Milan, Italy). The C/N ratio was calculated from N and C percentage data (g 100 g^{-1}DW).

Mineral content

The macronutrients potassium, calcium, phosphorus, sulphur, and magnesium as well as the microelements iron, zinc, molybdenum, manganese, copper and nickel were determined after acid digestion using ICP/OES (inductively coupled plasma/optical emission spectrometry: iCAP 6500 Duo, Thermo Fisher Scientific, Cambridge, United Kingdom)

Protein determinations

Total soluble protein content and Rubisco

Frozen plant tissue (0.1 g) was ground with N_2 and homogenized with extraction buffer (Tris-HCl 50 mM pH 8, EDTA 1 mM, 2-mercaptoethanol 10 mM, DTT 5 mM, $MgSO_4$ 10 mM, cysteine 1 mM, PVPP 0,5%, PMSF 1mM). It was then centrifuged at 16000g and 4ºC for 10 min and the supernatant was collected. Total soluble protein was quantified using the Bradford micro-essay (Bradford, 1976). For SDS-PAGE, 5 µl of total soluble protein was mixed and denatured with the following loading buffer (Tris-HCl 62 mM, pH 6.8, glycerol 50%, 2-mercaptoethanol 5%, SDS 2.3% and blue bromophenol 0.1%) and was boiled at 100ºC for 5 min. Samples were added to acrylamide gels (12.5% m/v) and run at 125 V for 1 hour with running buffer (Tris 25mM, glycine 192 mM, SDS 0.1 mM). Gels were stained with Code Blue

Stain reagent (Pierce Biotechnology, Inc., Rockford, EEUU). The Rubisco large subunit was quantified with the "Quant 1" software in GelDoc 2000 (Bio-Rad). Gel data were standard normalized and reflected as percentage taking the content obtained in the 400 ppm [CO_2] treatment as a reference.

Gene expression

RNA isolation and microarray analysis

Frozen plant material (0.4 g) was ground with N_2 and was used for RNA isolation. Three biological replicates were made for each tissue. Total RNA was isolated according to Liao *et al.* (2004). RNA concentration and purity was determined spectrophotometrically (NanoDrop ND-1000A UV-Vis spectrophotometer) with the quality of samples determined according to Canales *et al.* (2011): only samples with a 1.9–2.1 A260/A280 nm ratio and higher than a 1.9 A260/A230 nm ratio were used. Supplementary checking of RNA quality was done by agarose gel electrophoresis.

An Agilent 44 K *Arabidopsis* microarray and one-colour Cy3 labelling were used. The microarray slides were hybridized, stained and washed as recommended by the manufacturer's standard protocol (Agilent Technology, California, EEUU). Hybridized slides were scanned using a GENEPix 4100A Microarray scanner (Molecular Devices, CA, USA) with a 5 μm resolution.

qPCR validation

Validation of gene expression in the microarray results was performed by Real-time PCR on a CFX3 Real-Time System C1000 thermal cycler (Bio-Rad) with a qPCR SsoFast^{TM} EvaGreen^{R} Supermix (Bio-Rad) under the following conditions: 95ºC for 3 min (1 cycle); 95ºC for 1 s and 60ºC for 5 s (40 cycles). Gene-specific primer sequences for amplification are described in Supplementary Table 1. After the final cycle, a melting curve analysis was performed over a temperature range of 65-95ºC in 0.5ºC increments to verify the reaction specificity. Five ng of reverse-transcribed cDNA was used as the template for each reaction. The raw fluorescent data for each reaction were fitted with the MAK2 model, which requires no assumptions regarding the amplification efficiency of a qPCR assay (Boggy and Woolf, 2010). The initial target concentrations (D0 parameter) for each gene were deduced from the MAK2 model using the qPCR package of "R statistic environment software" (Ritz and Spiess, 2008) and normalized to the reference gene (Actin, At1g07940).

Statistical analysis

Statistical analysis for all parameters analysed with the exception of microarrays analysis was performed by one-factor ANOVA (SPSS v.12.0; SPSS Inc., Chicago, IL, USA). The results were accepted as significant with $P\ value \leq 0.05$.

For the microarray analysis, three replicates per treatment and tissue were performed. Background correction was performed with the quantile between arrays method (Bolstad *et al.*, 2003). A linear model was fitted with limma (Smyth, 2004) and corrected by adjusting P values using the Benjamini and Hochberg method (1995). Leaf and root tissue were compared separately. For this study, the significant difference in gene expression was $Q\ value \leq 0.05$ and $\log_2$ fold change ≥ 0.5.

RESULTS AND DISCUSION:

Experiment 1: Root–shoot mineral transport as key target processes involved in *Arabidopsis* responsiveness to elevated [CO_2] conditions

Results:

The study showed that elevated [CO_2] increased total biomass in *Arabidopsis thaliana* plants grown with nitrate as the unique nitrogen source. The increment found in shoots was 40% and in root biomass, 25% (Figure 1; Supplemental data thesis Figure 1). Gas exchange determinations revealed that the maximum photosynthesis rates were enhanced by 45% and transpiration diminished 30% (Table 1) in plants exposed to 800 ppm [CO_2]. In addition, leaf dark respiration (Rd) was enhanced 40%.At the protein level, Rubisco content showed a notable decrease close to 35% (Table 1), leaf total soluble protein (TSP) was decreased by 15% and root TSP by 20% in plants grown at elevated [CO_2].

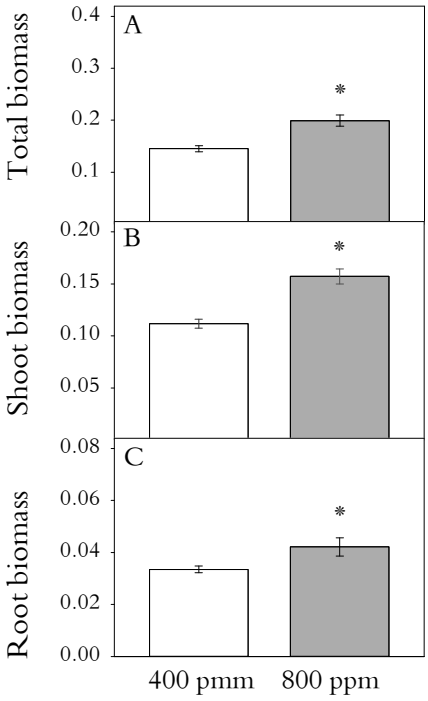

Figure 1. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on total biomass (A), shoot biomass (B) and root biomass (C) in g of dry weight (DW). Values represent the mean of 12 replicates ± SD.

Table 1. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on net photosynthesis (A max μmol CO_2 $m^{-2}s^{-1}$), transpiration (Tr, mmol $m^{-2}s^{-1}$), dark respiration (Rd, μmol CO_2 $m^{-2}s^{-1}$), leaf and roots total soluble protein (TSP mg prot g DW^{-1}) and Rubisco content (%; 100% DO represent 400 ppm [CO_2]). Values represent the mean of 6 replicates ± SE.

	Amax	Tr	R_d	leaf TSP	root TSP	Rubisco content
400 ppm	5.64 ± 0.28	**3.43** ± 0.3 *	1.20 ± 0.08	**120** ± 7 *	**58** ± 3 *	**100%** *
800 ppm	**8.02** ± 0.39 *	2.39 ± 0.21	**1.91** ± 0.07 *	102 ± 6	47 ± 3	66%

Leaf N content decreased 20% (Figure 2) under elevated [CO_2] and leaf carbon content increased 5%; therefore, the C/N ratio was modified. Root N and C content had no significant differences and a C/N ratio imbalance was not observed. At the same time, nitrate content was increased remarkably in roots but not in shoots. As expected, the leaf ammonium content was higher in plants due to higher photorespiration activity in plants exposed to 400 ppm [CO_2]. Related to elemental content, elevated [CO_2] reduced the macronutrients, potassium, calcium, phosphorus and sulfur; at the same time calcium and magnesium were increased in leaves. In roots, increases in calcium and a decrease in magnesium were also found in ambient [CO_2] concentrations. Also, a clear dynamic reduction in mineral elements as protein cofactors was observed (Figure 4) for iron, zinc, molybdenum, manganese, copper and nickel in the leaves of plants grown in elevated [CO_2]. However, in roots an accumulation of iron and copper was observed in elevated [CO_2] conditions, although the manganese and nickel content was higher under ambient [CO_2].

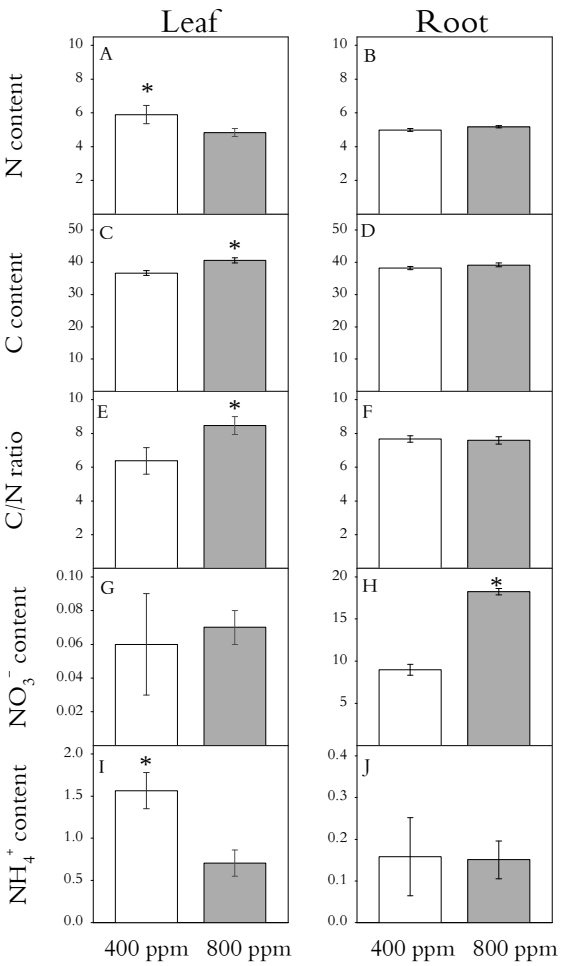

Figure 2. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on leaf (A) and root N content (B) in g/100g; leaf (C) and root C content (D) in g/100g; leaf (E) and root C/N ratio (F) in %; leaf (G) and root nitrate, NO_3^- content (H) in mg g^{-1} DW; leaf (I) and root ammonium, NH_{4+} content (J) in mg g^{-1} DW. N and C content values represent the mean of 3 replicates ± SE; NO_3^- and NH_4^+ represent the mean of 6 replicates ± SE.

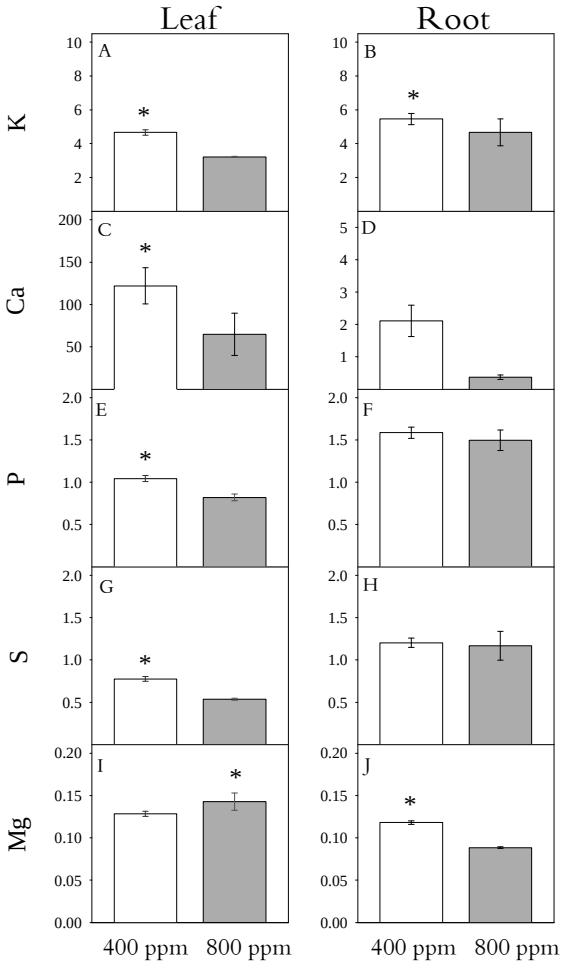

Figure 3. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on macronutrients: leaf (A) and root (B) potassium content, K; : leaf (C) and root (D) calcium content, Ca; leaf (E) and root (F) phosphorus content, P; leaf (G) and root (H) sulfur content, S; leaf (I) and root (J) potassium content, Mg. Each value represents the mean of 3 replicates ± SE.

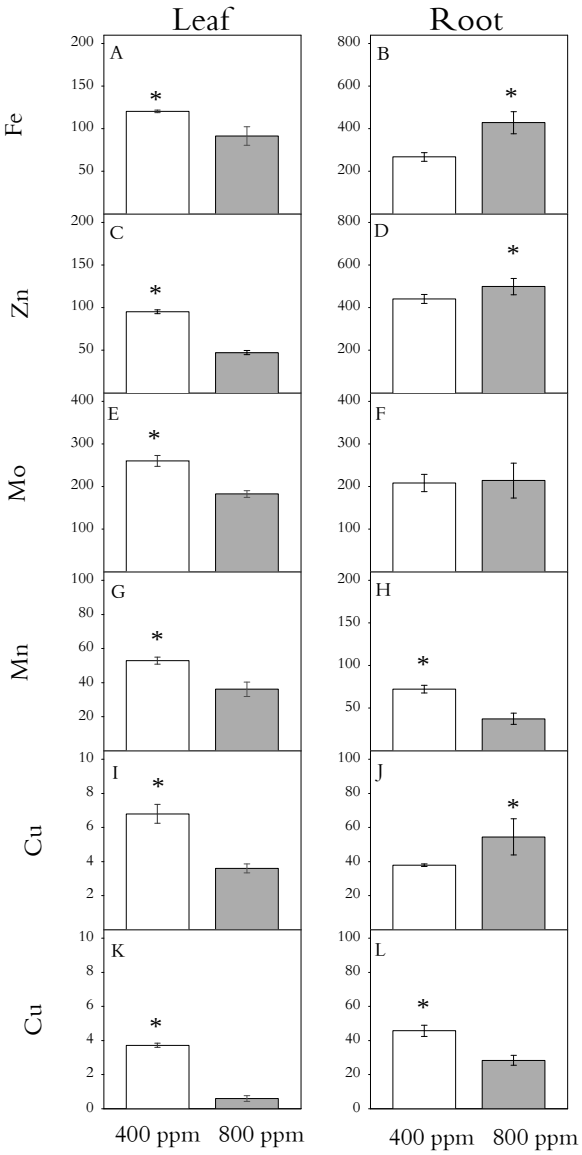

Figure 4. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on mineral elements as protein cofactors including: leaf (A) and root (B) iron content, Fe; leaf (C) and root (D) zinc content, Zn; leaf (E) and root (F) molybdenum content, Mo; leaf (G) and root (H) manganese content, Mn; leaf (I) and root (J) copper content, Cu; leaf (K) and root (L) nickel content, Ni. Each value represents the mean of 3 replicates ± SE.

Leaf carbohydrate content was modified in plants exposed to 800 ppm (Figure 5): glucose and fructose increased and there was a remarkable 450% rise in starch content. However, there was no significant change in sucrose levels. At the same time in plants exposed to elevated [CO_2], root glucose was increased, while sucrose and fructose content were unaltered. Root starch content slightly diminished.

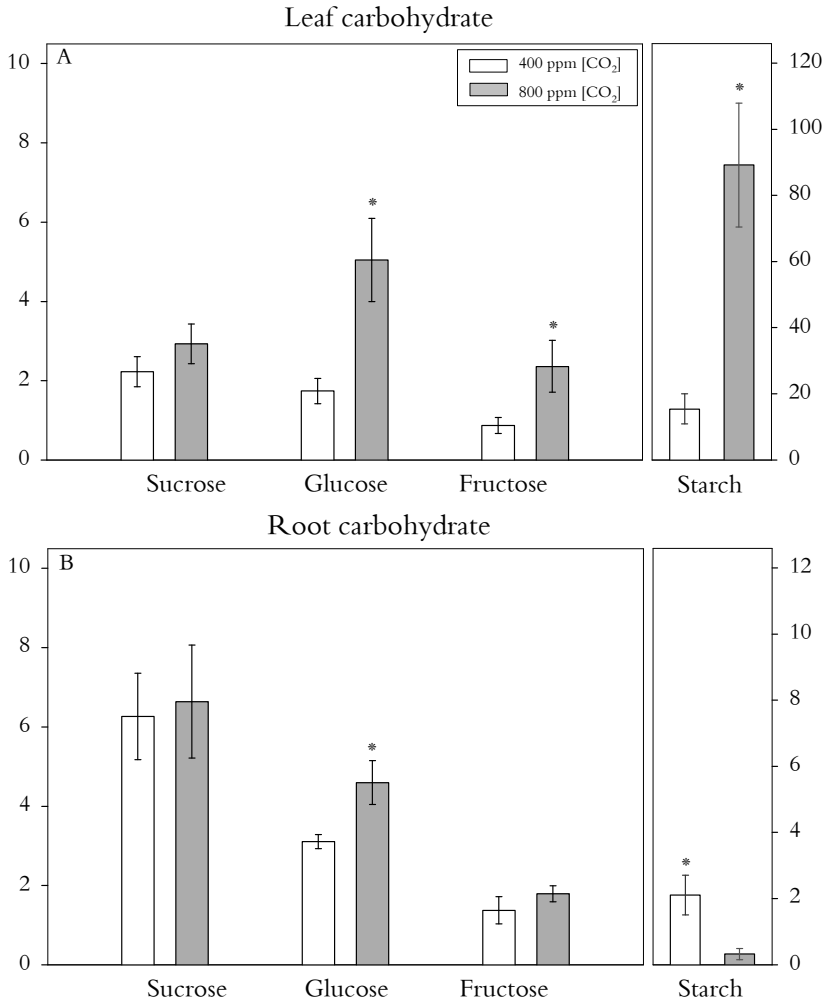

Figure 5. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on shoot (A) and root (B) carbohydrate content: sucrose, glucose, fructose (mg g^{-1} of dry weight) and starch (mg glucose g^{-1} of dry weight). Sugar values represent the mean of 4 replicates ± SE; starch values represent the mean of 6 replicates ± SE.

Concerning the leaf organic acid content (Figure 6), plants exposed to elevated [CO_2] diminished succinate, malate, oxalate and citrate content in leaf tissues. Meanwhile in root tissue, the organic acid content was unvarying, while a slight significant decrease detected for oxalate content.

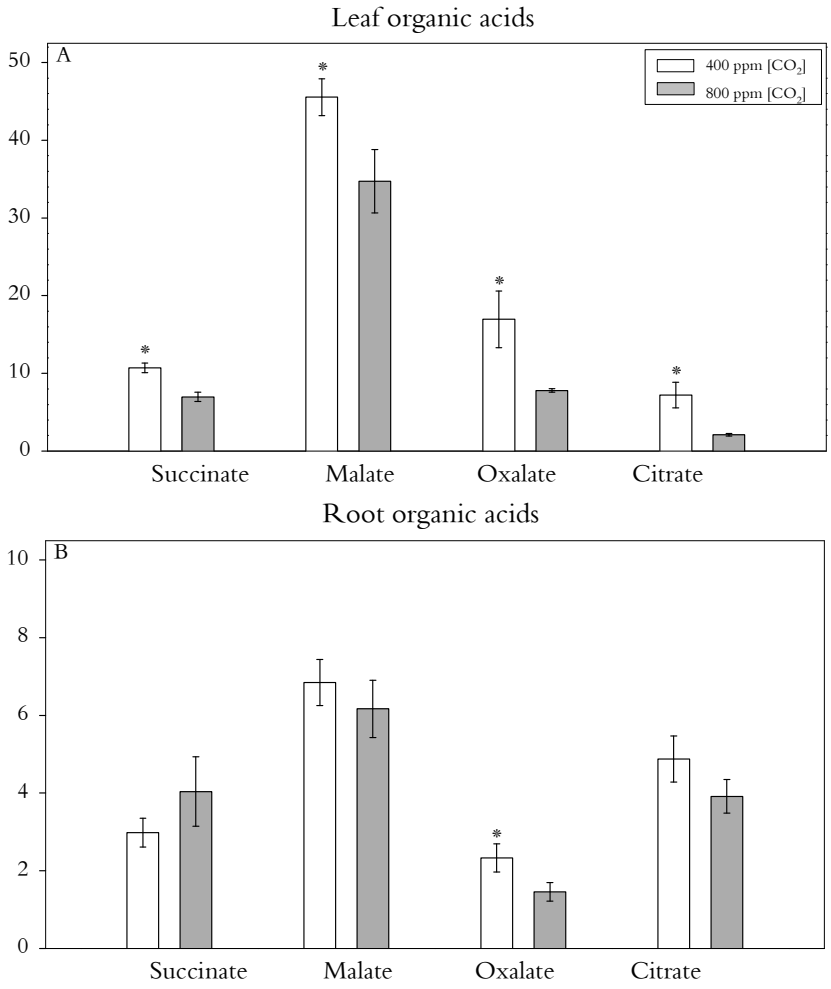

Figure 6. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on shoot (A) and root (B) organic acid content including succinate, malate, oxalate and citrate; mg g^{-1} of dry weight. Values represent the mean of 4 replicates ±SD ; starch values represents the mean of 6 replicates ± SD

The leaf amino acid content increased under elevated CO_2 conditions (Figure 7). In particular, glutamate and aspartate were increased substantially. Increases in asparagine and decreases in tyrosine, valine and threonine were also found. Simultaneously, the root total amino acid content decreased in elevated [CO_2]. Although the root glutamine content increased remarkably in elevated [CO_2], other amino acids such as alanine, glycine+serine, threonine, GABA, tyrosine, tryptophan, phenylalanine, methionine, leucine and isoleucine decreased in elevated [CO_2].

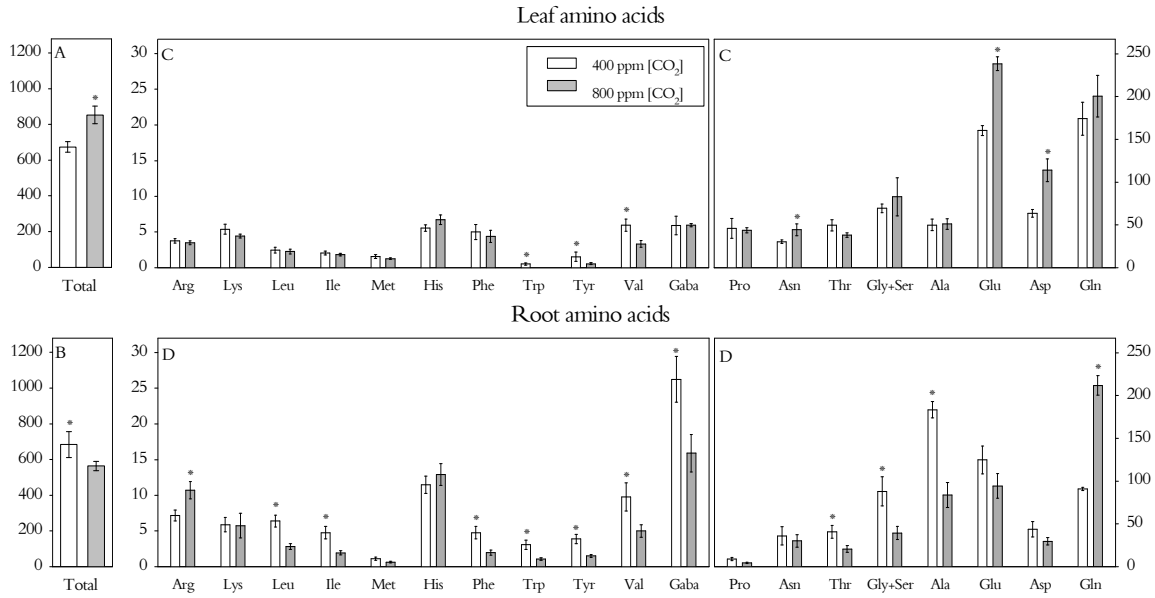

Figure 7. Effect of elevated [CO_2] (800 *versus* 400 µmol mol^{-1}) in *Arabidopsis thaliana* plants (col. 0) in shoot and root tissue on total amino acid content (A-B) and amino acids profile (C-D); µmol g^{-1} DW. Each value represents the mean of 4 replicates ± SE

The gene expression analysed with microarrays highlighted the fact that elevated [CO_2] induced more genes in roots than in leaves: 48 genes were differentially expressed in leaves and 174 in roots (Table 2). A large proportion (90%) of significantly induced genes were upregulated in roots of the plants exposed to 800 ppm [CO_2]. Because the root is associated with transport, the nitrate transporters, NRT 1.5 (at1g32450) and NRT 2.2 (at1g08100), are highly up-regulated. Moreover, the bZIP transcription factor (at5g65210) was overexpressed in roots of plants under

elevated [CO_2]. Genes related to zinc, phosphate and iron transport were up-regulated in roots at elevated [CO_2] conditions (Supplemental Table 1). The zinc transporter (at5g5920) is highly overexpressed in roots. Phosphate transporter PHO1 (at2g03260), glycerol-3-phosphate transporters 2 (at4g25220) and 3 (at1g30560) and phosphate transporter 1 (at2g30070) were overexpressed in roots of plants grown under elevated [CO_2]. Moreover, the metal handing functional group has a relevant role in the roots of plants exposed to elevated [CO_2]. Iron acquisition, transport, and homeostasis were up-regulated in elevated [CO_2]: vacuolar iron transporter homologs 1 (at1g21140) and 2.1 (at3g25190) and nicotinamide synthase 1 (at5g04950) were upregulated in roots. Simultaneously, ferric reduction oxidase (at1g01580) was overexpressed in roots and ferritin-1 (at5g01600) was down-regulated in leaves at elevated [CO_2]. Furthermore, the anion channel, SLAH1 (at1g62280), was overexpressed in root tissue (Supplemental Table 1).

It is noteworthy that the primary metabolism was not affected by elevated [CO_2] (Table 2). However, overexpression of genes related to root respiration such as PEP-kinase (at1g08650), succinate dehydrogenase (at2g18450), ubiquinol-cytochrome C reductase (at2g01090), glycerate 3-phosphate transporters 2 (at1g30560) and 3 (at4g25220), and transketolase (at3g60750) was found under elevated [CO_2]. At the same time in leaf tissue, pyruvate kinase 4 (at3g49160) and phosphoglucomutase (at1g70820) were down-regulated in elevated [CO_2]. Simultaneously, β carbonic anhydrase 3 (at1g23730) was noticeably induced in leaves of plants grown under elevated [CO_2] conditions (Supplemental Table 1). Finally, at root tissue highlight CBL-interacting serine/threonine-protein kinase family (CIPK) (at2g34180, at1g29230, at2g38490).

Table 2. Ontology-based overview of the global responses of transcript levels of *Arabidopsis thaliana* plants (col. 0) exposed to [CO_2] (800 versus 400 ppm) in shoot and root.

	Leaf			**Root**		
	Total	**up**	**down**	**Total**	**up**	**down**
Major CHO metabolism	**0**	0	0	**1**	1	0
Glycolysis	**2**	0	2	**1**	1	0
Gluconeogenesis/ glyoxylate cycle	**1**	1	0	**0**	0	0
Oxidative pentose phosphate cycle	**0**	0	0	**1**	1	0
Tricarboxylic acid cycle	**1**	1	0	**1**	1	0
Electron transport/ ATP	**0**	0	0	**2**	2	0
Cell Wall	**0**	0	0	**6**	5	1
Lipid metabolism	**1**	0	1	**2**	1	1
Amino acids metabolism	**1**	1	0	**0**	0	0
Metal handling	**1**	0	1	**5**	4	1
Secondary metabolism	**1**	1	1	**1**	0	0
Hormones metabolism	**1**	0	1	**2**	1	1
Co-factor/Vitamine	**1**	0	1	**1**	1	0
Stress	**1**	1	0	**16**	15	1
Redox	**2**	1	1	**1**	1	0
Miscellaneous	**3**	0	3	**8**	6	2
RNA	**5**	3	2	**11**	10	1
DNA	**1**	0	1	**1**	1	0
Protein	**4**	2	2	**20**	19	1
Signalling	**0**	0	0	**25**	24	1
Cell	**0**	0	0	**3**	3	0
microRNA	**0**	0	0	**2**	2	0
Development	**1**	0	1	**8**	7	1
Transport	**5**	1	4	**10**	10	0
Unknown	**15**	5	10	**45**	42	2
Total	**47**	17	31	**173**	158	13

Discussion:

1. Plant growth and photosynthetic performance

Plant responses to elevated [CO_2] have been widely documented in the literature (Terashima et al. 2014; Aranjuelo et al. 2011; Long et al. 2004; Stitt & Krapp 1999). Nevertheless, some uncertainty remains about the mechanisms leading to these responses and the implication of roots. Indeed, conclusions drawn by other authors in this area mostly focus on information from leaf tissue, and this may give an incomplete picture of the physiological processes involved given the interconnectedness of shoot and root tissues (Ainsworth & Bush, 2011; Lejay et al. 2008; Wang et al. 2003). The link between photosynthetic activity and the plant C requirements has been described as a key process conditioning the response of plants under elevated [CO_2] (Ainsworth & Rogers, 2007; Moore et al. 1999). As shown in Table 1, elevated [CO_2] increased the maximum photosynthetic rates in *Arabidopsis thaliana* plants and therefore their biomass production. Despite of the lower Rubisco content, the plants gown under 800 ppm [CO_2] were capable to increase photosynthetic rates. Rubisco down-regulation has been linked to leaf carbohydrate accumulation. Our study detected a notable increase in leaf non structural carbohydrates glucose, fructose and starch (Figure 5) in plants grown under elevated [CO_2] conditions that could have affected Rubisco protein content (Table 1A). It is worth noting that starch accumulation has been described as being negatively correlated with biomass in 94 *Arabidopsis* accessions (Sulpice *et al.*, 2010). Such an effect shows that excess starch biosynthesis in plants exposed to 800 ppm causes an inefficient management of fixed C, and ultimately, could generates physiological perturbations. The noticeable 450% increase in leaf starch content in plants under elevated [CO_2] revels that growth performance was lower than could have been expected. The growth of *Arabidopsis* at 800 ppm [CO_2] led to modifications of the shoot C/N ratio (Figure 2E) due to the increase in C (growth and starch content), but it was also accentuated by a decrease in the N content due to a reduction in TSP and Rubisco (Table 1). In our growth conditions, in the plants exposed to elevated [CO_2] we have found a decrease of 20% in leaf N content while no differences in root tissue. [CO_2]. Those plant N reduction has been subject to

intense debate (Loladze 2014; Taub & Wang 2008; Cotrufo et al. 1998). Here we propose a discussion between both metabolic pathway involved in those limitation, the transport and the assimilation.

2.– Transpiration and mineral transport

Transpiration (*Tr*), which is the dominant process that controls water transport in plants (De Boer & Volkoc, 2003), has been described as a key parameter conditioning N transport in plants (Matimati et al 2014). In our grown conditions, plants exposed to elevated [CO_2] reduce transpiration rates about 30% (Table 1). Recently, McGrath & Lobell (2013) corroborates that the decrements of transpiration rates reduces nutrient translocation along whole-plant and affect plant performance under elevated [CO_2]. In addition to Tr, nitrate partitioning through the plant has been described to be also regulated by a large number of nitrate transporters (Wang et al. 2012).

Fundamentally, our data remark that elevated [CO_2] induced large-scale transcriptomic changes in roots, highlighting the integrative and co-dependent response of the plant. Patterson et al. (2011) observed a similar tissue pattern in *Arabidopsis thaliana* plants grown with NO_3^- at ambient [CO_2]. Our transcriptomic approach underscored that nitrogen transport was strongly affected by [CO_2]: NRT 2.2 and NRT 1.5 were highly overexpressed at elevated [CO_2] conditions. AtNRT 2.2 is a head member of HATS (high affinity nitrate transporters) and makes a relevant contribution to the influx of root nitrate uptake (Li et al. 2007). NRT1.5, expressed in pericycle cells (Lin et al. 2008), is the principal xylem nitrate-loading transporter and had a vital importance in long-distance nitrate transport along roots to shoots tissues (Chen et al. 2012). Additionally, the CIPK family genes are overexpressed in roots, and this group has been proposed as playing a role in the post-translational regulation of nitrate transporters by phosphorylation and is involved in multiple signalling pathways (Krapp et al., 2014; Ho et al. 2009;). However, despite of those gene induced response, in the roots of plants exposed to elevated [CO_2] we found a awfully 200% increase in nitrate content (Figure 2H). Those result

suggests the existence of a physical barrier that limit nitrate transport; the gene overexpression could be a direct effect of this nitrate accumulation. Therefore, the inadequate root-to-shoot nitrate transport, either manifesting as inadequate xylem loading or a reduction in N transport caused a leaf N deficiency, and consequently, acute plant performance under elevated [CO_2].

Besides the reduction of N, data obtained by Loladze (2014; 2002) carried out using whole kind of plant species in a meta-analysis shown that, unequivocally, plants exposed to elevated [CO_2] reduce leaf mineral elements concentration (with C exception). Our data showed that elevated [CO_2] caused a reduction in leaf macroelements (with the exception of a slide increase in magnesium) (Figure 3) as well as micronutrients used as protein co-factor minerals iron, zinc, molybdenum, manganese, cupper and nikel (Figure 4). Besides, in root of plants performance under elevated [CO_2] we found increments in iron, zinc, and cupper. The two most cited hypothesis to explain that response are: 1.- higher carbon assimilation resulting a dilution via carbohydrates of mineral elements (Gifford et al. 2000; Kuehny et al. 1991); 2.- leaf mineral elements of plant exposed to elevated [CO_2] is constrained by the decrease in the transpiration rate that reduce xylem flux (Taub and Wang 2008; Teng et al. 2006; Poorter et al. 1997). Accompanying N and nitrate transport reduction, we have found a delay in transport direction from roots to leaf in potassium, phosphorus, sulphur, iron, zinc and copper. It has been reported that the reduction in mineral elements as calcium or zinc was attributed to changes in transpiration rate (Baxter, 2009). The interpretation of those evidence does not appear to be other than a decrease in xylem flow throughout the plant. Additionally to those ionomic response, our transcriptomic approach underlined the importance of genes related to mineral acquisition, transport, handling and homeostasis, especially in root of plants exposed to elevated [CO_2]. It has been reported that elevated [CO_2] did not alter plant phosphorus acquisition (Jin et al. 2013). Nevertheless, our results indicate a decrease in the leaf phosphorus content and overexpression in root of genes as PHO1, phosphate transporter 1 and glycerol-3-phosphate transporter 2 and 3. The essential role of phosphorus in photosynthesis, respiration, energy production and storage suggested that substantial changes between different metabolic pathways influenced the response of plants under elevated [CO_2]. At the same time, a 50% increase in iron

content was found in roots alongside strong gene induction of iron transporter and nicotianamine synthase genes. It has been reported that these genes are induced by nitrate content (Wang et al. 2003) and, indeed, nitrate was highly accumulated in roots in our experiment. Besides, the Ferritin-1 chloroplastic gene, which is involved in iron storage in vacuoles (Briat et al.2010), was downregulated in leaf tissue exposed to elevated [CO_2] while this leaf metal content decrease. Iron is required for many key enzymes involved in the nitrogen cycle such as NR, NiR, and Fd. Furthermore, the information suggests plants exposed to 800 ppm have higher iron requirements and that the iron is prioritized towards active proteins. Those data noted an imbalance in iron transport between the root and leaf. The 100% decline in zinc content observed in the leaves of plants exposed to elevated [CO_2] was in line with the extreme overexpression of β-carbonic anydrase (discussed later). Hence, the accumulation of those mineral element under elevated [CO_2] was supported with the overexpression of zinc transporter (Supplemental Table 1). Canales et al. (2014) recently published a meta-analysis of the nitrate response of the root transcriptome. The network analysis described the bZIP transcription factor (overexpressed in our experiments in the roots of plants exposed to elevated [CO_2]) as a key component of nitrate regulation. The systemic gene response during the management of mineral elements, which paralleled the mineral content, emphasized their decisive role in mineral transport, that determine *Arabidopsis* plants performance under elevated [CO_2].

3.– Exposure to elevated [CO_2] constrains leaf nitrate assimilation and promote root nitrate assimilation in *Arabidopsis thaliana* plants.

Photosynthetic performance in elevated [CO_2] has been linked to the inhibition of leaf NO_3^- assimilation (Bloom et al. 2014; Rachmilevitch et al. 2004). Three different hypotheses have been proposed to explain this phenomenon. First, Bloom et al (2010) showed that the lower photorespiration rates caused by elevated [CO_2] might constrain the malate shuttle needed for NO_3^- reduction. We found a diminution of leaf malate content in elevated [CO_2] conditions (Figure 6A). The downregulation of genes encoding pyruvate kinase and phosphoglucomutase suggests that organic acid synthesis was depressed at 800 ppm [CO_2]. Phosphoglucomutase is an

important protein that regulates carbon flow through starch metabolism and the energetic processes of the TCA acid cycle and the pentose phosphate pathway (Periappuram et al. 2000). At the same time, pyruvate kinase is a key enzyme that stimulates the flux of carbon into the TCA (Stitt and Krapp 1999). These results, together with the increases in leaf starch content found, suggest that plants actively send C to carbohydrate biosynthesis and that the depletion of organic acids could constrain the high energy requirements of plants exposed to elevated [CO_2] (Aranjuelo et al. 2013). The second hypothesis that has been proposed for the inhibition of nitrate assimilation in elevated [CO_2] is competition between high energy-demanding enzymatic reactions for the bound reductant power compounds. Moreover, it has been proposed that the availability of ATP becomes diminished in plants grown under non photorespiratory conditions (Foyer et al. 2012), Undoubtedly, energy competition could be a bottleneck in plants at elevated [CO_2]. The third hypothesis regarding the inhibition of leaf nitrate assimilation in elevated [CO_2] is based on a decline in NO_2^- influx into chloroplasts. In leaves, nitrite enters the chloroplast via neutral acid HNO_2 or via a transporter (Eichelmann et al. 2011). The chloroplast stroma under elevated [CO_2] will become more acidic due to increased [CO_2] assimilation movement (Raven, 1997), and this would constrain nitrite transport. Our results indicate no differences in the expression of genes encoding leaf nitrate reductase, nitrite reductase or nitrite transport. However, in accordance with other authors, we detected a strong induction of genes encoding carbonic anhydrase in leaves (Prins et al. 2011; Fabre et al. 2007). Bloom et al. (2002) demonstrated that HCO_3^- reduces nitrite transport into the chloroplast, and consequently, carbonic anhydrase might alleviate stromal acidification in order to improve nitrite transportation. Moreover, carbonic anhydrase has been proposed as an essential enzyme that controls gas exchange between plants and the atmosphere (Flexas et al. 2008). Therefore, β-carbonic anydrase (highly overexpressed in leaf organ) might play an important role in elevated [CO_2] due to the influence of transpiration, xylem fluxes and soluble nutrient translocation.

Amino acid synthesis requires the coordination of triose phosphate oxidation and nitrogen reduction or amino acid donors. Hence, any perturbation in such energetically dependent processes could alter their distribution (Foyer et al. 2011). Interestingly, our results revealed that

elevated [CO_2] increased leaf amino acids due to enhancement of the total pool of glutamate and aspartate (Figure 7C), which is in agreement with other authors (Nunes-Nesi et al. 2010). There is evidence that shows how the amino acids, glutamate and aspartate, disturb the root-to-shoot nitrogen transport due to an inhibitory effect on nitrate influx (Andrews et al. 2013; Vidmar et al. 2000) and glutamate has been reported as inhibiting nitrate reductase in tobacco plants (Fritzet al. 2006). Despite of root amino acid content decreased under elevated [CO_2], a remarkable 200% increase in glutamate content (Figure 6B) was found. This result are in line with Kruse et al. (2002), who proposed that elevated [CO_2] increased root nitrate assimilation, and taking into account potential inhibition of leaf nitrate under elevated [CO_2], an augmentation of root nitrogen assimilation would be expected to accommodate the nutritional needs of such plants. The NADPH required for root nitrate assimilation is provided by glycolysis and the pentose phosphate cycle (Figure 6B). Our data showed there was a preference for glucose over C-storage in the form of starch (Figure 5B). Furthermore, transcriptomic analysis underlined an upregulation of genes encoding proteins related to the respiration cycle such as sucrose-phosphatase, transketolase, succinate dehydrogenase and glycerate 3-phosphate transporters. Glutamine accumulation has been proposed to increase nitrate reductase activity in barley by regulating the phosphorylation status of the enzyme, probably by acidification of the cytosol due to its transport (Fan et al. 2006). In line with this idea, phosphoenolpyruvate carboxylase kinase, highly overexpressed in roots of plants exposed to elevated [CO_2], provides carbon skeletons for glutamine synthesis (Taylor et al. 2010).

Conclusion:

In this study we highlighted the importance of integrated studies that explore root-to-shoot interactions to understand plant performance under future [CO_2] conditions. Although plants exposed to elevated [CO_2] show increased growth, mineral content analyses revealed that depleted nutrient content affected negatively TSP and Rubisco content. Moreover, the reduction in protein content together with the overwhelming accumulation of starch confirmed the limitations of plant development. Although the NRT 1.5 root nitrate transporter (principal actor in nitrate loading in the phloem) and NRT 2.2 are noticeably up-regulated, the large-scale nitrate accumulation in the roots exacerbated leaf nitrogen deficiency and limited the beneficial effect of future atmospheric [CO_2] concentrations in C_3 plants. Exposure to elevated [CO_2] reduced transpiration, which in turn caused a delay and a reduction in the transport of mineral elements between tissues. Thus, the slowdown of export of co-factor mineral elements from roots to leaves triggers plant performance under elevated [CO_2]. Furthermore, we propose a reorganization of nitrate assimilation between tissues: root nitrogen assimilation is enhanced to offset a shutdown of nitrogen metabolism in the leaves of plants exposed to elevated [CO_2].

Experiment 2: Ensuring energy availability, a target process conditioning plant responsiveness to elevated CO_2

Results:

The study showed that elevated [CO_2] overwhelmingly stimulated *Arabidopsis thaliana* biomass. As shown in Figure 1, increments of 110% were found in shoots and 115% in roots (Supplemental data thesis Figure 2). Likewise, light-saturated [CO_2] assimilation (A_{max}) was enhanced about 50%, the Rubisco maximum carboxylation rate (Vc_{max}) by 35%, the maximum electron transport rate contributing to RuBP regeneration (J_{max}) by 50%, and the leaf dark respiration (Rd) was enhanced 55% in plants under elevated [CO_2] conditions. In contrast, transpiration rates (Tr) and electron transport rate (ETR) did not show significant differences under elevated [CO_2] (Table 1).

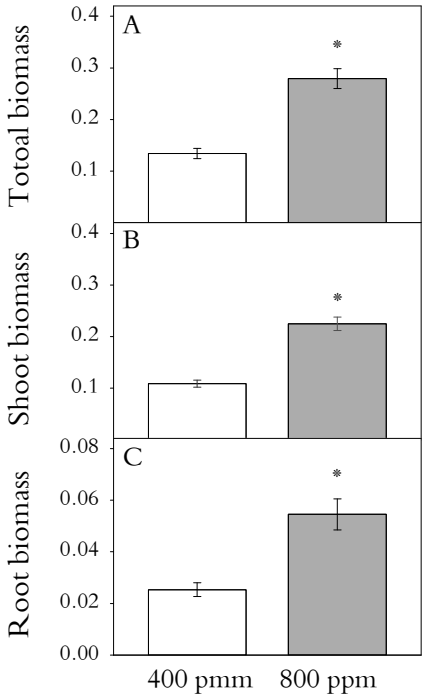

Figure 1. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on total biomass (A), shoot biomass (B) and root biomass (C) in g. Values represent the mean of 12 replicates ± SE.

Table 1. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on maximum photosynthesis (Amax), Rubisco maximum carboxylation rate (Vcmax) and maximum electron transport rate contributing to RuBP regeneration (Jmax), and dark respiration (Rd) in µmol CO_2 $m^{-2}s^{-1}$; transpiration (Tr) in mmol $m^{-2}s^{-1}$); electron transport rate (ETR), in $\mu mol^{-2}s^{-1}$. Each value represents the mean of 6 replicates ± SE.

	Amax	Vcmax	Jmax	Rd	Tr	ETR
400 ppm	5.49 ± 0.28	**22.5 ± 1.0**	46.9 ± 1.8	**1.08 ± 0.07**	**3.07 ± 0.30***	**50.7 ± 1.8**
800 ppm	**8.78 ± 0.43** *	30.1 ± 1.3 *	**70.6 ± 1.7** *	1.88 ± 0.12 *	2.53 ± 0.20	52.3 ± 3.7

At the protein level, leaf TSP showed a slight decrease (8%) and there were no significant differences in root TSP of plants growing under elevated [CO_2]. Meanwhile, the Rubisco content did not differ significantly (Table 2)

Table 2. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on total soluble protein (TSP mg prot g DW^{-1}) and Rubisco content (%). Each value represents the mean of 6 replicates ± SE.

	Leaf TSP	Rubisco content	Root TSP
400 ppm	**136.9** ± 4.1*	**100%**	66.3 ± 8.8
800 ppm	125.3 ± 6.7	100%	58.6 ± 2.9

Quantification of the plant nitrogen (N) content revealed the leaf N content was not altered in *Arabidopsis thaliana* plants exposed to elevated [CO_2], no changes were found in carbon (C) content and therefore, no imbalances were found in the C/N ratio. However, a slight increase in root N content and no differences in C content led to a slight decrease in the C/N ratio under elevated [CO_2] (Figure 2). The inorganic N compound content at elevated [CO_2] was associated with a reduction in nitrate content in leaves but not in roots. At the same time, the ammonium content was higher in roots of plants exposed to elevated [CO_2], but not in leaves (Figure 2).

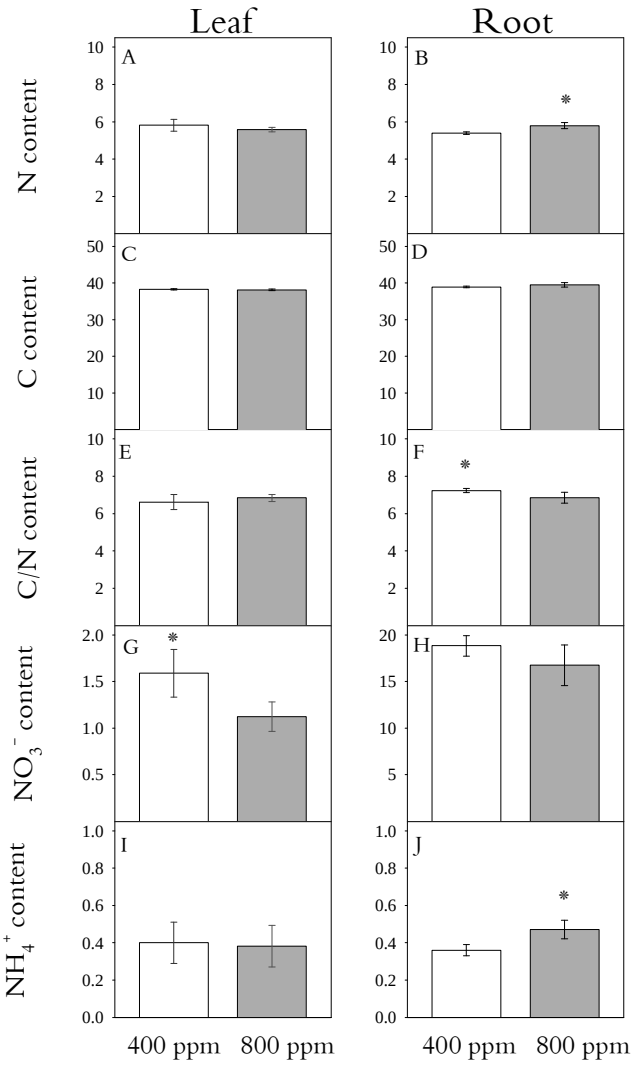

Figure 2. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on leaf (A) and root (B) nitrogen content in g/100g; leaf (C) and root (D) carbon/nitrogen ratio in %; leaf (E) and root total amino acid content in mmol g^{-1} DW; leaf (G) and root (H) nitrate content in mg g^{-1} DW; leaf (I) and root (J) ammonium content in mg g^{-1} DW. N content value represents the mean of 3 replicates ± SE. Total amino acid nitrate and ammonium content represent means of 4 replicates ± SE

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The effect of [CO_2] on the total amino acid content depends on the plant tissue observed: leaf amino acids increased, whereas in roots there was a decrease under elevated [CO_2] (Figure 3). The amino acid composition profile highlights more individualized dynamics. In leaf tissue, increments in lysine, leucine, isoleucine, phenylalanine, alanine, asparagine, threonine, alanine, aspartate, glutamine and the pool composed by glycine + serine were found at elevated [CO_2] while only a slight decrease in histidine was found. On the other hand, in root organs, decreases in lysine, leucine, isoleucine, phenylalanine, tryptophan, tyrosine, valine, GABA, proline,threonine, glutamate, aspartate, glutamine and the glycine+serine pool were found (Figure 3).

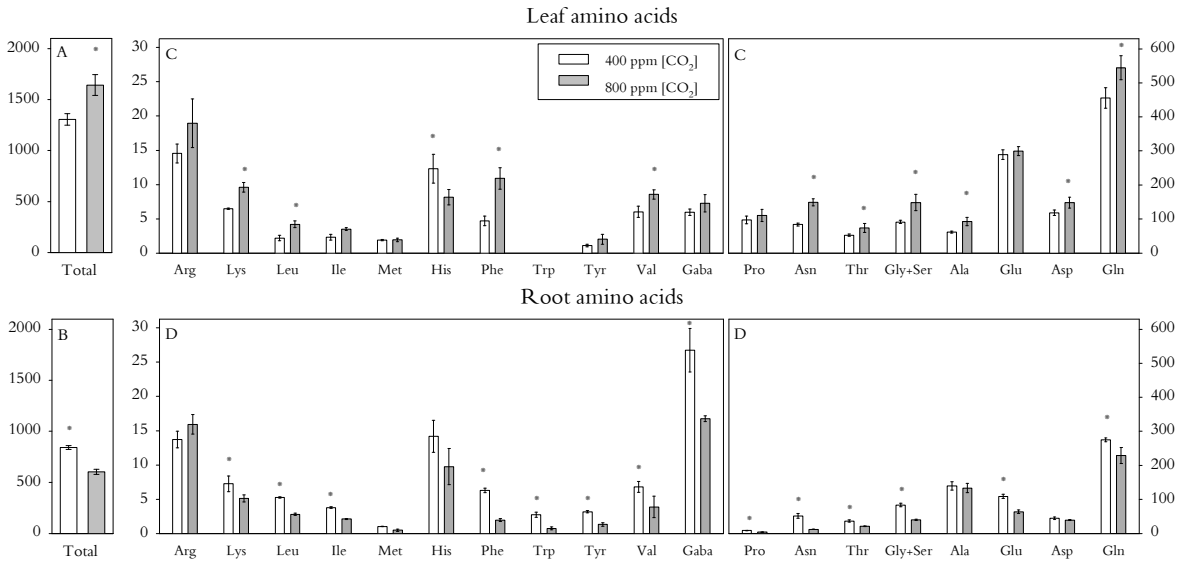

Figure 3. Effect of elevated [CO_2] (800 *versus* 400 µmol mol^{-1}) in *Arabidopsis thaliana* plants (col. 0) in shoot and root tissue on total amino acid content (A-B) and amino acids profile (C-D) ;µmol g^{-1} DW. Each value represents the mean of 4 replicates ± SE

Elevated [CO_2] also modified the organic acid distribution between tissues: a large 260% increase in malate, 245% in succinate and 90% in oxalate was found in the leaf, while no significant difference in leaf citrate was found at elevated [CO_2]. At the same time, a decrease of 60% in malate, 45% in oxalate and 55% in citrate content was found under elevated [CO_2] (Figure 4).

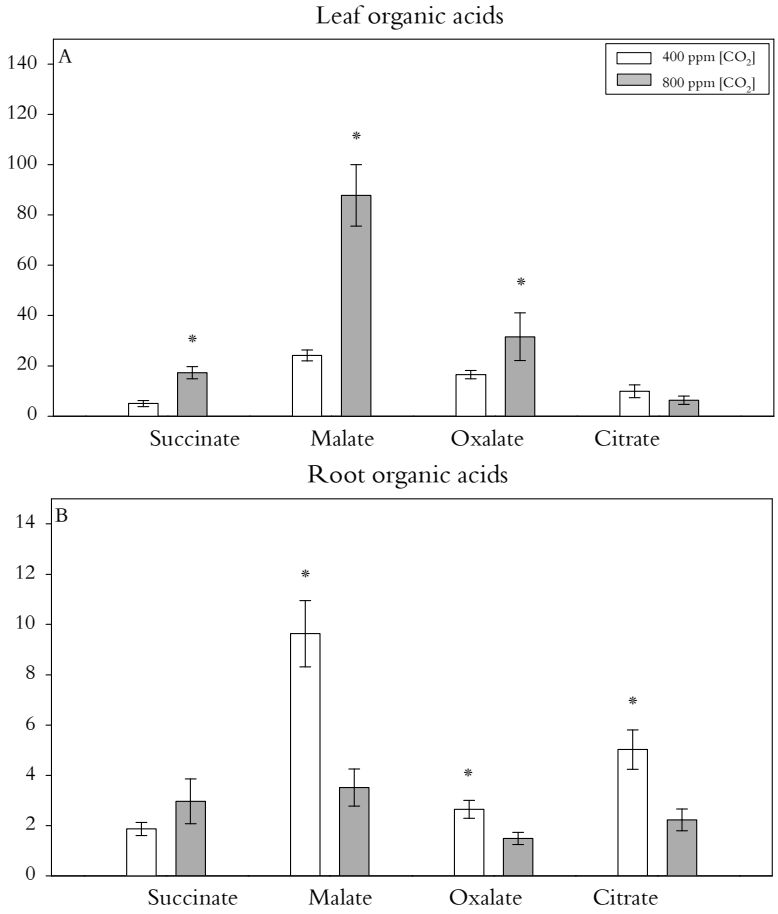

Figure 4. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on shoot (A) and root (B) organic acid content: succinate, malate, oxalate and citrate; mg g^{-1} DW. Values represent the mean of 4 replicates ±SE

The carbohydrate determinations highlight that the exposure to elevated [CO_2] modifies leaf sugars and starch content, whereas no significant differences were found in root tissue. Regarding leaf tissue, sucrose increased 140%, fructose 275%, starch 175% and glucose decreased 50% in elevated [CO_2] conditions (Figure 5).

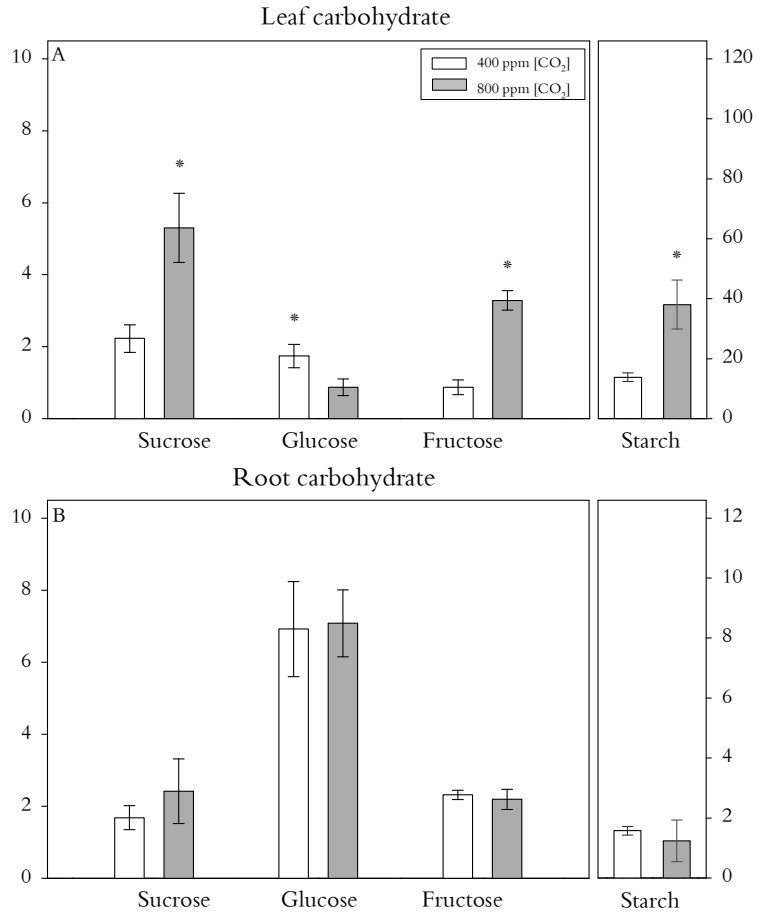

Figure 5. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* plants (col. 0) on shoot (A) and root (B) carbohydrates content: sucrose, glucose, fructose (mg g^{-1} DW) content and starch (mg glucose g^{-1} DW) content. Sugar values represent the means of 6 replicates ± SE; the starch value represents the mean of 6 replicates ± SE.

The transcriptomic analysis using microarrays revealed that exposure to [CO_2] largely influenced the plant above-ground metabolism: 255 genes were affected in leaves while only 48 were affected in roots. The results emphasize that in leaf tissue, 90% of genes were overexpressed under elevated [CO_2] conditions. Strikingly, in root tissue the response is contrary to this: 95% of the genes were downregulated under elevated [CO_2] conditions.

When genes were organized by functional annotation (Table 2), in leaf organs at elevated [CO_2] genes of the following categories were induced predominantly: protein modification, RNA modification, signalling, stress and development. When we descend from the quantification of the functional group to the unitary expression of each gene across we found biologically highlighting the following genes: sucrose synthase, SUS 3, (at4g02280); glucose-6-phosphate/phosphate translocator 2, GPT2 (At1g61800); galactinol synthase 2, GolS2 (at1g56600); alpha-amylase 1, AMY1 (at4g25000); putative phospholipid-transporting ATPase 5 (at1g72700) and ATPase family protein members (at3g28510, at3g28540, at4g02480); protein phosphatase 2C 77 (at5g57050), ABI2 (at5g57050); G-box-binding factor 3 (at2g46270); translocator protein homolog (at2g47770) and senescence-associated gene, SAG29 (at5g13170). At the same time, despite the lower expression overall, in roots of plants grown with ambient [CO_2] conditions the group linked to stress was increased. In addition, we highlight the following genes due to its biological relevance in our experimental conditions: asparagine amidohydrolase 1, (at3g16150); dihydrolipoamide branched chain acyltransferase (at3g06850); 2-oxoisovalerate dehydrogenase, putative (at1g21400).

Table 3. Ontology-based overview of the global responses of transcript levels of *Arabidopsis thaliana* plants (col. 0) exposed to [CO_2] (800 versus 400 ppm) in shoot and root organs

	Leaf			**Root**		
	Total	**down**	**up**	**Total**	**down**	**up**
Photosynthesis	**2**	2	0	**1**	0	1
Major CHO metabolism	**3**	0	3	**0**	0	0
Minor CHO metabolism	**1**	0	1	**0**	0	0
Glycolysis	**3**	2	1	**0**	0	0
Gluconeogenese/ glyoxylate cycle	**1**	0	1	**1**	1	0
Tricarboxylic acid cycle	**0**	0	0	**1**	1	0
Cell wall	**3**	0	3	**1**	1	0
Lipid metabolism	**7**	0	7	**1**	1	0
Amino acids metabolism	**1**	0	1	**3**	3	0
Metal handling	**1**	1	0	**0**	0	0
Secondary metabolism	**3**	1	2	**3**	3	0
Hormones metabolism	**8**	1	7	**3**	3	0
Tetrapyrrole synthesis	**1**	0	1	**0**	0	0
Stress	**15**	1	14	**8**	8	0
Redox	**2**	0	2	**0**	0	0
C-1 metabolism	**1**	0	1	**0**	0	0
Miscellaneous	**15**	3	12	**4**	3	1
RNA	**26**	2	24	**2**	2	0
DNA	**3**	0	3	**1**	1	0
Protein	**38**	2	36	**3**	3	0
Signalling	**22**	0	22	**1**	1	0
Cell	**10**	1	9	**0**	0	0
micro RNA	**1**	0	1	**0**	0	0
Development	**12**	1	11	**2**	2	0
Transport	**9**	1	8	**2**	2	0
Unknown	**64**	5	59	**11**	11	0
Total	**254**	23	231	**48**	46	2

Discussion:

The ability to maintain higher photosynthetic rates in plants exposed to elevated [CO_2] has been widely discussed in the literature (Leakey et al. 2009a; Ainsworth and Long 2005; Stitt and Krapp 1999). The close thorough relationship between C fixation and N performance is considered as a key factor in that response. Recent studies support the concept that elevated [CO_2] inhibits leaf nitrate assimilation in C_3 plants, diminishes organic nitrogen compounds and consequently slows the development of plants (Bloom et al. 2014; Bloom et al. 2010). Within this context, elevated [CO_2] has been described as favouring ammonium uptake or root nitrate assimilation (Matt et al. 2001). In this study we have evidenced that *Arabidopsis thaliana* plants exposed to elevated [CO_2] and grown with NH_4NO_3 as the nitrogen source are able to overcome photosynthetic acclimation. Our data showed that plants exposed to 800 ppm [CO_2] doubled their total biomass, either in the shoot or root organs. This notable increase in growth has been supported by the stimulation of carbon fixation in these plants: maximum photosynthesis rates (A_{max}, increased 50%), maximum rate of Rubisco carboxylase activity (Vc_{max}, raised 35%) and the maximum electron transport rate contributing to RuBP regeneration (J_{max} showed an increment of 50%), as shown in Table 1. Nevertheless, when plants exposed to elevated [CO_2] were grown with nitrate as the sole source of nitrogen, they were incapable of overcoming photosynthetic acclimation (Bloom et al. 2010). However, other studies (Bloom et al. 2014) conducted in plants fertilized with ammonium and exposed to elevated [CO_2] showed that plants could sidestep photosynthetic acclimation. Therefore, these results indicate the crucial influence of the N source on the photosynthetic responsiveness of plants grown in elevated [CO_2].

It is generally consistent that elevated [CO_2] diminishes transpiration (Ainsworth and Rogers, 2007). This transpiration reduction under elevated [CO_2], which is due to stomatal closure, is involved in a reduction in the xylem flux (Taub and Wang, 2008) and consequently, diminished leaf mineral content (Loladze, 2014). Recently, (McGrath & Lobell, 2013) have described that

the reduction in transpiration in elevated [CO_2] produces inefficient xylem loading and root-to-shoot transport; moreover, in Chapter I has been described how those reduction in transpiration rates constrains the expected stimulation of raised [CO_2]. Moreover, transpiration flow is essential for long distance nitrate allocation throughout the whole plant (Wang et al. 2012) and could be a handicap for the development of the plant in elevated [CO_2] conditions. Nevertheless, although we found a 15% reduction in transpiration, it did not match with reduction in N content (Table 1) as found in Chapter I. This phenomenon could be explained by the broad ecological correlation among leaf photosynthesis capacity and the transpiration rate (Flexas et al. 2013). Galmés et al. (2014) described the tight relationship between Rubisco content and mesophyll conductance by which the plant adjusts the stomatal opening to maximize the concentration of carbon necessary for Rubisco. The reduction of transpiration rates found in *Arabidopsis thaliana* plants grown with NH_4NO_3 under elevated [CO_2] conditions is simply an adjustment to maximize the capacity for C fixation in these plants.

Plant nitrogen status is a key parameter that influences plant metabolic changes, photosynthesis and consequently growth in plants exposed to elevated [CO_2] (Geiger et al. 1999). Moreover, the kind of nitrogen source that is available to plants has a direct influence on their energy status. Bloom et al. (1992) explained that plants use 15% of their available C to assimilate N when ammonium is the source; whereas C usage can reach 23% when only nitrate is available. As shown in Figure 2A, leaf N content was not modified in high [CO_2] conditions, and therefore there is no C/N imbalance. Related to protein content, a slight decrease of 8% was found in leaves as other authors has found (Markelz et al. 2013) while in roots organ that decrease was not significant. Moreover, the Rubisco content was equal between treatments. This extends the evidence that the larger response of the photosynthetic apparatus observed in ammonium nitrate fed plants at 800 ppm could be explained by their capacity to maintain Rubisco and TSP content at control values. The enhancement of leaf amino acid content (Figure 3A) confirmed that plants exposed to 800 ppm [CO_2] had no N availability problems, as other authors have found (Geiger et al. 1999). Meanwhile, in root organs, amino acid metabolism is diminished in elevated [CO_2] conditions. Decreases were found in the majority of amino acid profiles under elevated [CO_2]

(Figure 3D), in concordance with the downregulation of genes related to amino acid conversion (asparagine amidohydrolase 1; dihydrolipoamide branched chain acyltransferase; and 2-oxoisovalerate dehydrogenase, putative), together with the decrement in organic acid compounds (Figure 4B) supports the idea that *Arabidopsis thaliana* plants demand more carbon skeletons for amino acids synthesis in elevated [CO_2]. As a consequence, all this data suggest that plants exposed to elevated [CO_2] are energetically more efficient in nitrogen assimilation under ammonium nitrate conditions.

Respiration is a key physiological process in sustaining the growth and biomass production of plants and ecosystems (Tcherkez et al. 2012). Whereas chloroplasts in plant cell use photosynthesis to produce the carbohydrate substrate on which they depend, glycolysis and respiration provide ATP, reducing equivalents, and metabolic intermediates used in biosynthesis elsewhere in the cell (Araújo et al. 2014). Our study showed that an increase of 50% was detected for dark respiration (Table 1) as other studies focused on the enhanced leaf respiration at elevated [CO_2] in *Arabidopsis thaliana* plants has found (Watanabe et al,. 2014; Markelz et al. 2013). The role of enhanced respiration could be to maintain the energy requirements of costly enzymatic reactions such as photosynthesis and nitrate assimilation (Foyer et al. 2012) or to make photoassimilate transport from source to sink tissues more effective (Leakey et al. 2009a). In addition, the high rates of respiration consume excess photoassimilates, preventing their accumulation, which produces the phenomenon of photosynthetic acclimation to elevated [CO_2] under the prism of the source-sink hypothesis (Stitt & Krapp, 1999). Furthermore, leaf organic acids were remarkably increased at elevated [CO_2] conditions, particularly the malate content (Figure 4A), which is fundamental for leaf nitrate assimilation (Andrews et al. 2013). The highest respiration performance, linked to the build-up of carbohydrates and organic acids in plants exposed to elevated [CO_2], denotes a higher energy status that is capable of maintaining the greatest growth rates.

Plants exposed to elevated [CO_2] must handle the assimilation of carbon and the subsequent photoassimilate utilization efficiently. It has been suggested that inadequate management of source and sink interactions is a key factor in photosynthetic acclimation of plants exposed to elevated [CO_2] (Ainsworth et al. 2004; Aranjuelo et al. 2013). In our experiment, leaf fructose, sucrose and starch (Figure 5A) increased substantially in plants exposed to elevated [CO_2], but at the root level (Figure 5B), their content was not affected by [CO_2]. Starch has been described as an integrated biomass biomarker (Sulpice et al. 2009) that reflects C allocation efficiency throughout the whole-plant. Although starch the content increased 250% in elevated [CO_2], like a previous study conducted with *Arabidopsis thaliana* plants under elevated [CO_2] (Li et al. 2008), it did not reflect an imbalance between C storage and growth as others have noted (Aranjuelo et al. 2013). At the same time, sucrose is the major form of C translocation between organs (Ku et al. 1999; Ward et al. 1998), and the rate of sucrose transport was involved in photosynthetic downregulation at elevated [CO_2] among different plant species (Ainsworth and Bush 2011).

In recent years several studies have characterized the transcriptomic response of *Arabidopsis thaliana* plants performance under elevated [CO_2] (Markelz et al. 2013; Queval et al. 2012; Li et al. 2008; Li et al. 2006). However, these studies were focused on shoot characterization, and did not pay attention to the [CO_2] effect on root tissue. Our study revealed that the stimulation of plant metabolism produced significant overexpression of leaf transcription under elevated [CO_2], whereas a repression of genes was observed in roots (Table 3). Interestingly, although the plants were capable of maintaining higher carbon fixation rates, our study highlighted that the expression of genes involved in the Calvin-Benson's cycle was not affected by elevated [CO_2]. The fundamental factor in the maintenance of rates of photosynthesis seems to be the management and transport of photoassimilates (Arp, 1991). In our analysis, SUS3 and GPT2, were noticeably overexpressed in leaves of plants exposed to elevated [CO_2]. Sucrose synthase is a key plant enzyme involved in sucrose catabolism, the first step towards C mobilization into multiple metabolic pathways and cellulose biosynthesis (Baroja-Fernández et al. 2012; Bieniawska et al. 2007). It has been reported that transgenic tobacco plants over-expressing

maize SUS3 exhibited increases in photosynthesis rates and that this contributed to development or maintenance of sink tissue (Baxter et al. 2003). Thus, this information suggests that SUS3 plays a lead role in the management of C during the enzymatic and sink organ demands found under elevated [CO_2] conditions, and that this ultimately sustains the performance of the affected plants. GPT2 has been described as being involved in carbon skeleton delivery for starch biosynthesis and sustaining the oxidative pentose phosphate pathway (Kunz et al. 2010), which increased rapidly after sucrose feeding treatment (Usadel et al. 2008) and were also found in *Arabidopsis thaliana* plants exposed to elevated [CO_2] (Li et al. 2008). Athanasiou et al. (2010) proposed GPT2 as an indispensable enzyme for enhanced photosynthetic rates and overcoming dynamic acclimation based on the extraordinary induction of this gene when plants were transferred from low to high light conditions. Similarly, the role of this gene has also been highlighted in *Glicine max* plants exposed to elevated [CO_2] (Leakey et al. 2009b). Besides SUS3 and GTP2 as outstanding members in the maintenance of high rates of photosynthesis, other players related to carbohydrate metabolism have been described as being relevant to plant performance under elevated [CO_2]. Similar to the findings of other authors (Li et al. 2008), AtGolS2 was highly induced in elevated [CO_2] and indicated an active metabolism related to raffinose biosynthesis in *Arabidopsis thaliana* plants. The raffinose family of oligosaccharides has been described as having an osmoprotectant role against ROS in chloroplasts (Nishizawa et al. 2008). In this context, the increase in raffinose biosynthesis could be related to the enhanced activity of the photosynthetic machinery activity in plants exposed to 800 ppm [CO_2] and its associated increase in the susceptibility to oxidative stress. The overexpression of AtGolS2 would be actively involved in maintaining high performance functions in the chloroplast. Indeed, elevated [CO_2] conditions underline the expression of leaf AMY 1, an enzyme responsible for starch degradation in dead cells (Doyle et al. 2007). Linked to the function of carbon skeleton recycling, an upregulation of AtPLT5 was also found. It has been reported that it plays a role in providing carbohydrates to sink tissues (Reinders et al. 2005) and due to its location in the plasma membrane it is important in the acquisition of carbohydrates from the apoplast (Klepek et al. 2005). AtPLT5 is an energy dependent H^+ symporter and its requirements could be filled by the overexpression of genes related to ATP transport and ATPase family.

The abundance of transcripts encoding genes of ABA-dependent responses have been found in leaves of plants exposed to elevated [CO_2] (Kaplan et al. 2012; Queval et al. 2012). It has been reported that sugar induced an integrated response with ABA (Eveland and Jackson 2012; Li et al. 2006; Smeekens 2000). Moreover, phloem carbohydrate export is known to control reproductive development and underlie flower induction (Corbesier, 2002) and senescence (Wingler & Roitsch, 2008). Our results are in line with this network cascade and we found several actors related to this response. The type 2C protein phosphatase 77, which were overexpressed in leaves of plants under elevated [CO_2], is the dominant regulator of the ABA response (Zhang et al. 2009). Moreover, Pinheiro et al. (2011) reported that correlated with the sugar pathway genes, SUS3 and AMY1 (at4g25000), genes overexpressed in leaf of plants exposed to elevated [CO_2] in our analysis. Moreover, other transcription factors such as G-box binding factor 3 also correlated with these sugar genes (Pinheiro & Chaves, 2011) and translocator protein homolog is involved in amplifying ABA signalling (Guillaumot et al. 2009). Together with the overexpression of SAG29 (at5g13170) found supports the fact that elevated [CO_2] induced senescence (Springer et al. 2007). Recently, a meta-analysis was published that explains how elevated [CO_2] shortens the plant's vegetative period (Reyes-Fox *et al.* 2014). Our results provide an integrated explanation: plants fulfill all carbon requirements rapidly in elevated [CO_2] conditions leading to a cascade of ABA signalling that directly affects the timing of their phenology.

Conclusions:

In summary, our study highlights the relevance of a mixed ammonium nitrate nutrition for plants exposed to major [CO_2] concentrations: it enables plants to overcome the broadly expected photosynthetic acclimation to [CO_2]. The ability of it to sustain higher photosynthetic rates over extended periods of time is supported by the capacity of plants to adjust their protein content and maintain nitrogen status without deficiencies. The major contents in carbohydrates found together with the optimum conditions of the photosynthetic apparatus and the whole-plant successful energy status, make *Arabidopsis thaliana* plants cultivated with ammonium nitrate capable of exploiting future CO_2 conditions. Furthermore, our study underlines respiration and the transport of end products, along with the major actors glucose-6-phosphate/phosphate translocator 2 (GPT2) and sucrose synthase 3 (SUS3) genes, as the key metabolic processes for avoiding the collapse between the source and sink (classical causes of photosynthetic downregulation). In this way we have discerned one possible relationship leading to advancement of senescence in plants under elevated [CO_2]: the large-scale C status of affected plants is the switch that promotes a cooperative response with ABA signalling and leads to an acceleration in the timing of phenology.

Experiment 3:

Results:

Arabidopsis thaliana plants grown with ammonium as unique source were unable to develop in our growth conditions (Supplemental data thesis, Figure 5-6-7). despite of pH of 5.8 and low N dosage of ammonium (NH_4^+) used. In this line, it's widely accepted that addition of nitrate alleviates the ammonium toxicity in *Arabidopsis thaliana* plants (Hachiya *et al.*, 2012). An effective strategy to evaluate the importance of nutrition mainly based on ammonium, without affect N stoichiometry in the solution, is used a double nitrate reductase defective mutant (*nia1-1/chl3-5*) were kindly provided by Dr. N.M. Crawford (Wilkinson & Crawford, 1993), which has reduced 200% leaf nitrate reductase activity and was not detected in root tissue (Hoffman *et al.*, 2004). qPCR

Our study revealed that, regardless of analyzed genotype, exposure to elevated [CO_2] overwhelm increased shoot, root and total biomass (Figure 1). The increments in biomass found for wild type plants in shoots were 350% and 550% on n root organ biomass. Related to G3´5 mutant plants (which we will name NR mutant from now) elevated [CO_2] notably increased biomass, a large 1450% for shoots and 1820% for roots organs (Figure 1). Comparing the differences between both ecotypes it was possible to observe a significant decrease in the growth in NR mutant when plants were exposed to ambient concentrations of [CO_2]: 75% reduction in shoots and 80% in roots. Nevertheless, those differences were moderate in plants performance under elevated [CO_2]: no differences were found for above-ground and 50% in below-ground tissue.

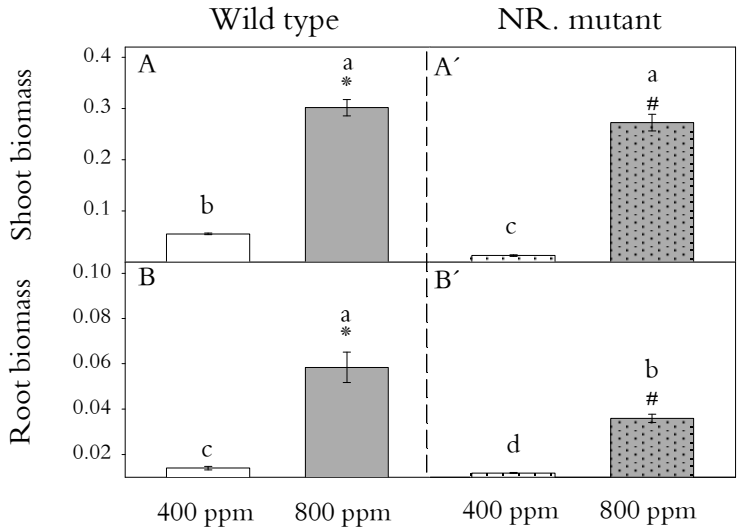

Figure 1. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* wild type plants (col. 0) and NR mutant (NR double mutant G´4-3, nia1- 1/nia2-5) genotype on shoot (A-A´) and root (B-B´) biomass in g. Values represent the mean of 12 replicates ± SE.

Leaf C content (Figure 2) slide decreased on wild type plants exposed to elevated [CO_2]. NR mutants plants exposed to elevated [CO_2] increased C content; NR mutant grown under ambient conditions shown the lower C content. Nevertheless, N content increase under NR mutant performance under 400 ppm [CO_2]. Related to C/N ratio, same pattern as C content were found and NR mutant plants grown under actual [CO_2] presented the lower ratio.

Exposure to elevated [CO_2] had clear beneficial effect on the carbon fixation activity, regardless of the genotype used (Figure 3). The photosynthetic rates at growth conditions (An) were enhanced by 150% for wild type plants and 420% for NR mutant The maximum rate of Rubisco carboxylase activity (Vc_{max}) showed an increase of 30% in wild type and 130% for NR mutant. Likewise, the maximum electron transport rate contributing to RuBP regeneration (J_{max}) showed similar increments, 30% for wild type and 175% for NR mutant plants. The photosynthetic rates of the NR mutant at 400 ppm was lower than wild type plants in those conditions, as well as Vcmax and Jmax. Surprisingly this phenomenon was not repeated when plants where grown at 800 ppm, and no differences between genotype was observed in

parameters A, Vcmax or Jmax. In like manner as leaf intercellular [CO_2] (Ci) were approximately twice, nearly to 800 ppm, under elevated [CO_2]. Leaf dark respiration rates were larger in plants exposed to elevated [CO_2]. Comparing genotypes, were observed a reduction of 80% (in ambient [CO_2] concentrations) and 20% (at elevated [CO_2]) in dark respiration of NR mutant plants comparing with wild type. Electron transport rate (ETR) was raised in plants exposed to elevated [CO_2]: in wild type plants increased 60% and in NR mutant up to 200% (Figure 4). Those differences was not found comparing genotypes performance under elevated [CO_2]; nevertheless, ETR noticeably diminished in NR mutant comparing to wild type plants under ambient [CO_2] conditions. Same pattern was found for electron flux for photosynthetic carbon reduction (ETRc) and electron flux for photorespiratory carbon oxidation (ETRo): elevated [CO_2] exposition increase both parameters regardless of genotype, and were appreciably reduced in NR mutant plants under ambient [CO_2]. When we observed ETRc/ETRo ratio we denoted that under elevated [CO_2] conditions electron trough carboxylation is promoted. At the same time, we observed a slide decrease of those transport on NR mutant comparing with wild type plants under ambient [CO_2], result not detected under elevated concentrations. In wild type plants elevated [CO_2] produce a 30% increase on the relative quantum yield of PSII at the steady state, ΦPSII. However, between both genotypes exposed to elevated [CO_2] the ΦPSII did not change. NR mutant plants exposed to elevated [CO_2] increased 150% ΦPSII comparing with plants exposed to atmospheric concentrations.

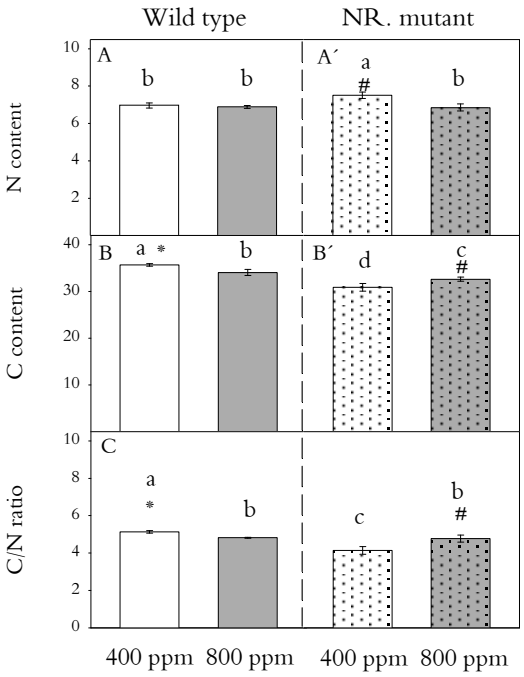

Figure 2. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* wild type plants (col. 0) and NR mutant (NR double mutant G´4-3, nia1- 1/nia2-5) genotype on shoot carbon, C, (A-A´), nitrogen, N, (B-B´) content in % and carbon/nitrogen ratio, C/N (C-C´). Values represent the mean of 3 replicates ± SE.

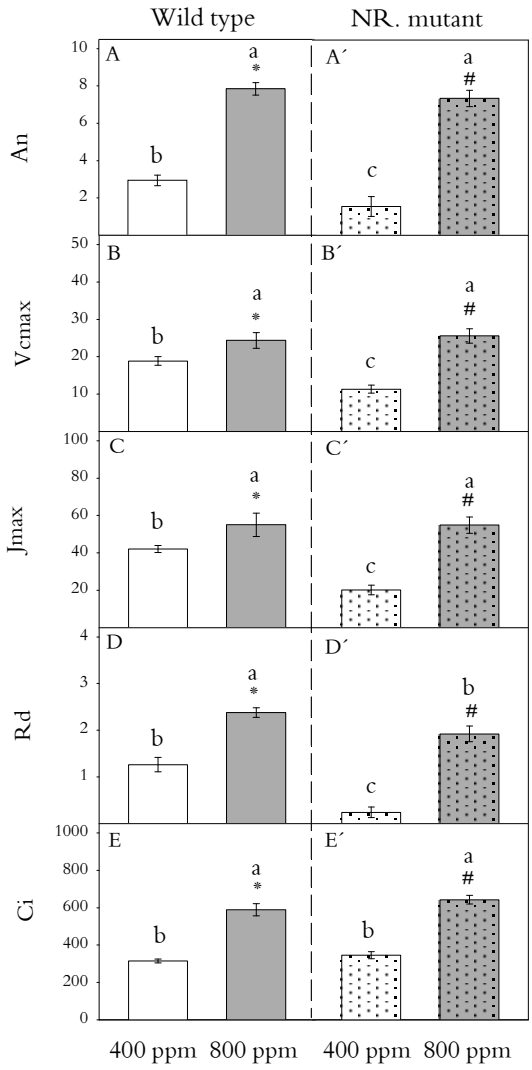

Figure 3. Effect of elevated [CO2] (800 versus 400 ppm) in *Arabidopsis thaliana* wild type plants (col. 0) and NR mutant (NR double mutant G´4-3, nia1-1/nia2-5) genotype on net photosynthesis, An, (A-A´), maximum rate of Rubisco carboxylase activity, Vcmax (B-B´), maximum electron transport rate contributing to RuBP regeneration, Jmax, (C-C´), leaf dark respiration, Rd (D-D´) and intercellular [CO_2], Ci, in μmol CO_2 $m^{-2}s^{-1}$. Values represent the mean of 6 replicates ± SE.

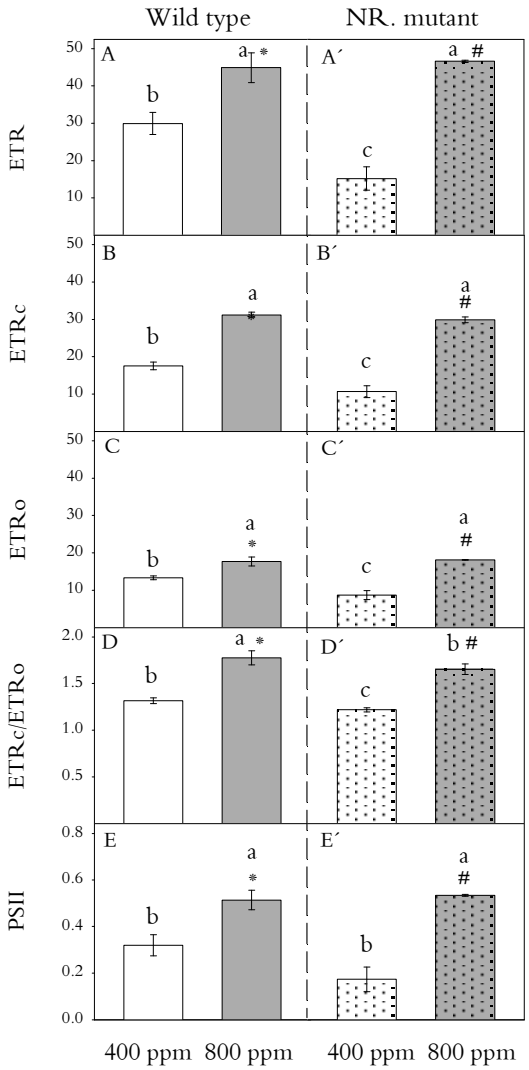

Figure 4. Effect of elevated [CO2] (800 versus 400 ppm) in *Arabidopsis thaliana* wild type plants (col. 0) and NR mutant (NR double mutant G´4-3, nia1-1/nia2-5) genotype on electron transport rate, ETR, (A-A´), electron flux for photosynthetic carbon reduction, ETRc, (B-B´), for photorespiration, ETRo, (C-C´) in μmol^{-2} s^{-1}; ratio ETRc/ETRo, (D-D´); and the relative quantum yield of PSII at the steady state, ΦPSII, (E, E´).Values represent the mean of 6 replicates ± SE.

Leaf carbohydrate (Figure 4) content was modified in both genotypes exposed to 800 ppm [CO_2]: while fructose, glucose and starch content increased no significant change was found in sucrose levels. At the same time, in root tissue we found a slide decrease of starch in those conditions. Meanwhile, NR mutant plants showed increments on sucrose and glucose under elevated [CO_2]. Starch content is outstanding in both CO_2 treatments and not significant differences were found. In root tissue we found decreases on sucrose glucose and fructose on plant performance under elevated [CO_2].

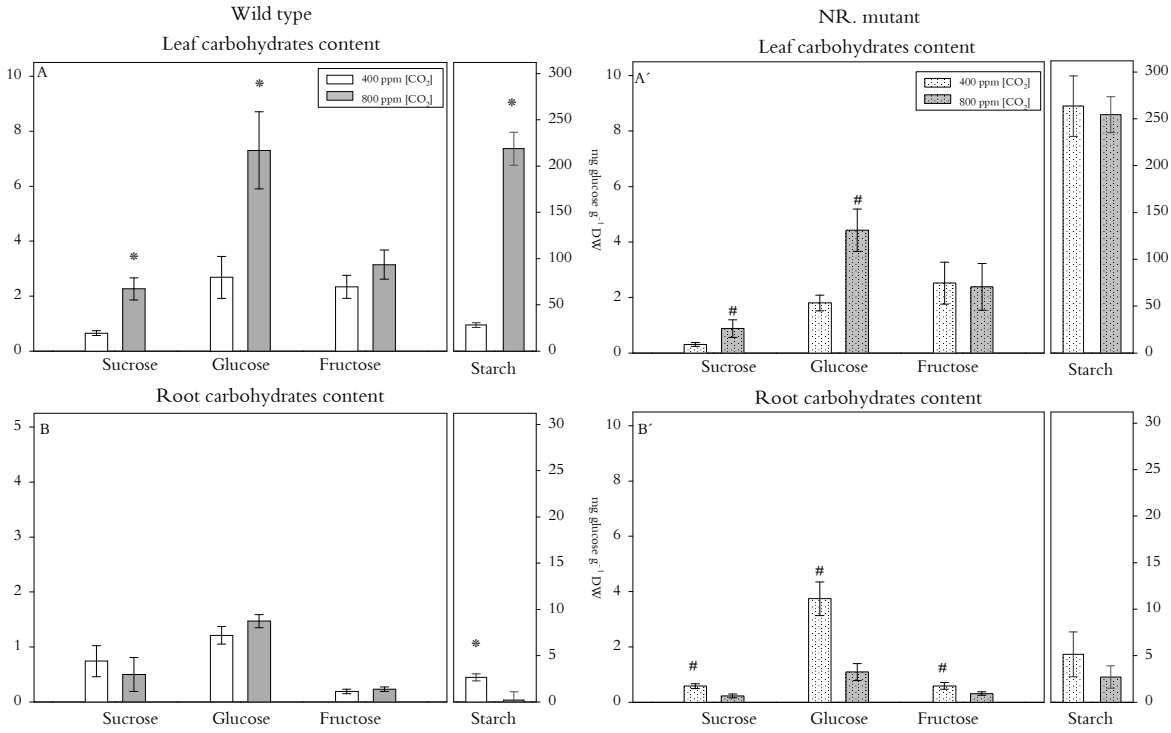

Figure 5. Effect of elevated [CO2] (800 versus 400 ppm) in *Arabidopsis thaliana* wild type plants (col. 0) and NR mutant (NR double mutant G´4-3, nia1- 1/nia2-5) genotype on leaf (A-A´) and root (B-B´) on carbohydrate content: sucrose, glucose, fructose; mg g^{-1} DW and starch, mg glucose g-1 DW. Values represent the mean of 4 replicates ± SE.

The total amino acid content increased in both tissue on wild type plants under elevated [CO_2] conditions (Figure 5). At the leaf organ, the major amino acids such us glutamine, alanine, threonine and asparagine were increased substantially; meanwhile, glycine, serine and aspartate content were reduced. Acompaying, minor amino acids content such us lysine, histidine, phenylalanine and GABA increased while triptohan and valine decreased. Simultaneously, on root tissue increases glutamine, alanine, asparagine, glycine, threonine and proline were found. Related to minor amino acids elevated [CO_2] conditions increase arginine content while decrease lysine, phenylalanine, tryptophan and tyrosine. Total amino acids content increased in leaf tissue in NR-mutant plants increased under elevated [CO_2], whereas in root tissue is reduced (Figure 6). In leaf organ we had found a substantial increments on glutamine, asparagine and lesser extent on threonine and alanine on plant performance under elevated [CO_2]; glycine and serine content were reduced in those conditions. In root tissue under elevated [CO_2] we found a reduction in the large part of major amino acids: glutamine, asparagine, glutamate, alanine, aspartate, glycine, serine and proline. Related to minor amino acids increases in arginine, ileucine, histidine and valine were found while GABA content decrease.

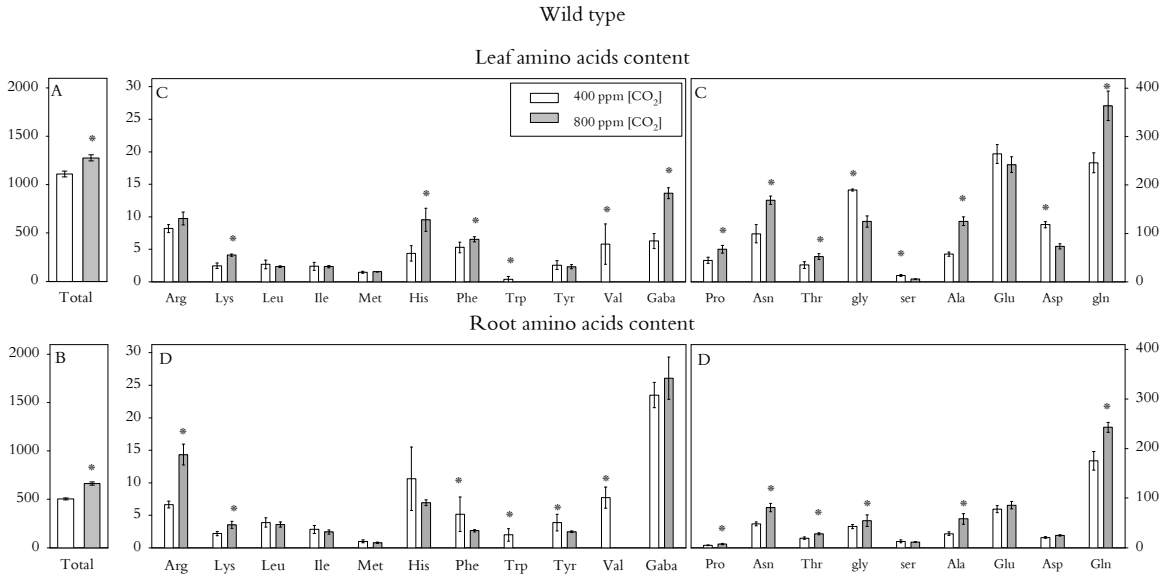

Figure 6. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants (col. 0) in shoot and root tissue on total amino acid content (A-B) and amino acids profile (C-D) ;µmol g^{-1} DW. Each value represents the mean of 4 replicates ± SE

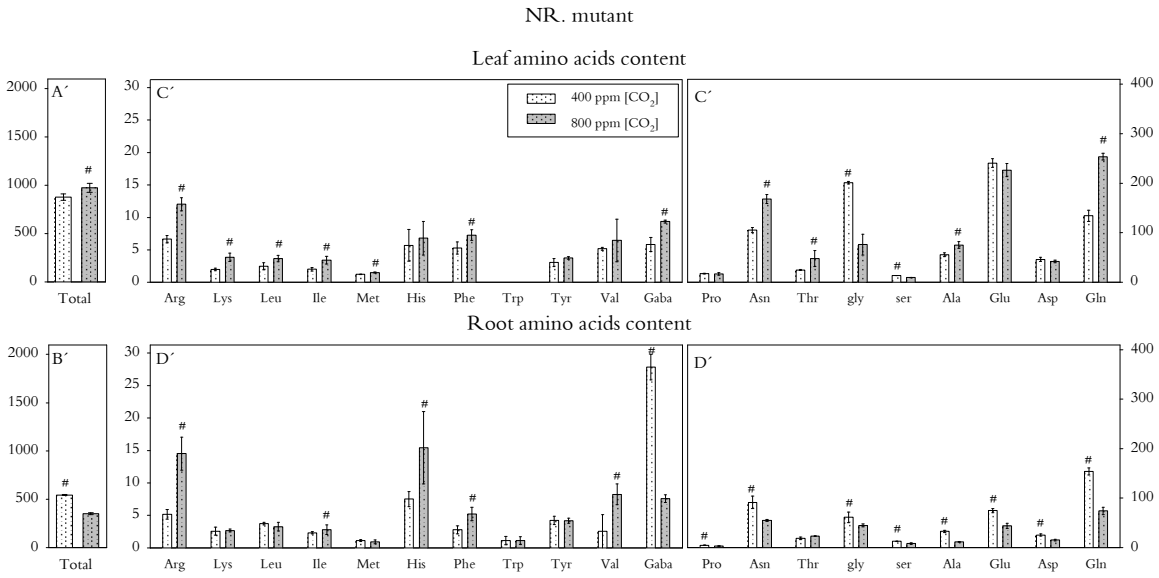

Figure 7. Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* plants NR mutant (NR double mutant G´4-3, nia1- 1/nia2-5) genotype in shoot and root tissue on total amino acid content (A´-B´) and amino acids profile (C´-D´) ;µmol g^{-1} DW. Each value represents the mean of 4 replicates ± SE

Table 1. Ontology-based overview of the global responses of transcript levels of *Arabidopsis thaliana* wild type plants exposed to [CO_2] (800 versus 400 ppm) in shoot and root organs.

	Leaf tissue			**Root tissue**		
	Total	down	up	**Total**	down	up
Photosynthesis	**1**	0	1	**3**	3	0
Major CHO metabolism	**1**	0	1	**1**	0	1
Minor CHO metabolism	**1**	1	0	**8**	2	6
Glycolysis	**0**	0	0	**2**	0	2
Fermentation	**0**	0	0	**0**	0	0
gluconeogenese/ glyoxylate cycle	**0**	0	0	**0**	0	0
Oxidative pentose phosphate cycle	**0**	0	0	**0**	0	0
Tricarboxylic acid cycle	**1**	1	0	**0**	0	0
Electron transport/ ATP	**1**	0	1	**3**	3	0
Cell wall	**4**	0	4	**23**	19	4
lipid metabolism	**5**	1	4	**20**	12	8
N-metabolism	**0**	0	0	**1**	1	0
Amino acid metabolism	**4**	0	4	**6**	3	3
S-assimilation	**0**	0	0	**3**	3	0
Metal handling	**2**	0	2	**6**	4	2
Secondary metabolism	**8**	1	7	**16**	7	9
Hormone metabolism	**10**	5	5	**22**	16	6
Co-factor and vitamine metabolism	**1**	0	1	**4**	0	4
Tetrapyrrole synthesis	**1**	0	1	**2**	0	2
Stress	**17**	2	15	**43**	20	23
Redox	**0**	0	0	**7**	1	6
Polyamine metabolism	**0**	0	0	**1**	1	0
Nucleotide metabolism	**0**	0	0	**5**	4	1
Biodegradation of Xenobiotics	**0**	0	0	**3**	1	2
C1-metabolism	**0**	0	0	**2**	1	1
Miscellaneous	**19**	3	16	**61**	28	33
RNA	**19**	5	14	**93**	49	44
DNA	**1**	0	1	**7**	3	4
Protein	**20**	8	12	**108**	41	67
Signalling	**23**	5	18	**37**	16	21
Cell	**5**	0	5	**23**	12	11
microRNA	**0**	0	0	**1**	0	1
Development	**4**	2	2	**32**	19	13
Transport	**6**	1	5	**44**	19	25
Unknown	**58**	19	39	**269**	180	89
Total	**212**	54	158	**856**	468	388

Table 2. Ontology-based overview of the global responses of transcript levels of *Arabidopsis thaliana* NR mutant (NR double mutant G´4-3, *nia1- 1/nia2-5*) exposed to [CO_2] (800 versus 400 ppm) in shoot and root organs.

	Leaf tissue			**Root tissue**		
	Total	down	up	**Total**	down	up
Photosynthesis	**29**	27	2	**30**	4	26
Major CHO metabolism	**15**	3	12	**3**	1	2
Minor CHO metabolism	**19**	9	10	**17**	6	11
Glycolysis	**6**	2	4	**7**	7	0
Fermentation	**2**	0	2	**1**	0	1
gluconeogenese/ glyoxylate cycle	**1**	0	1	**0**	0	0
Oxidative pentose phosphate cycle	**6**	3	3	**8**	7	1
Tricarboxylic acid cycle	**7**	2	5	**4**	2	2
Electron transport/ ATP	**9**	5	4	**10**	8	2
Cell wall	**33**	16	17	**36**	27	9
lipid metabolism	**46**	20	26	**32**	15	17
N-metabolism	**2**	0	2	**4**	3	1
Amino acid metabolism	**29**	19	10	**24**	10	14
S-assimilation	**2**	1	1	**2**	1	1
Metal handling	**11**	4	7	**13**	6	7
Secondary metabolism	**51**	18	33	**37**	29	8
Hormone metabolism	**33**	10	23	**36**	26	10
Co-factor and vitamine metabolism	**7**	4	3	**5**	2	3
Tetrapyrrole synthesis	**15**	14	1	**4**	1	3
Stress	**104**	18	86	**65**	27	38
Redox	**26**	16	10	**15**	6	9
Polyamine metabolism	**2**	1	1	**0**	0	0
Nucleotide metabolism	**22**	11	11	**8**	4	4
Biodegradation of Xenobiotics	**3**	0	3	**3**	1	2
C1-metabolism	**3**	0	3	**2**	2	0
Miscellaneous	**144**	48	96	**95**	46	49
RNA	**164**	92	72	**148**	68	80
DNA	**25**	19	6	**12**	7	5
Protein	**284**	151	133	**156**	64	92
Signalling	**106**	26	80	**56**	33	23
Cell	**51**	28	23	**38**	20	18
microRNA	**8**	2	6	**11**	9	2
Development	**64**	29	35	**47**	19	28
Transport	**92**	19	73	**51**	31	20
Unknown	**526**	299	227	**399**	232	167
Total	**1947**	916	1031	**1379**	724	655

Discussion:

Despite the wealth of research done on the response of plants to elevated [CO_2] (Terashima et al. 2014; Aranjuelo et al. 2011; Stitt & Krapp 1999) important uncertainly remain on the plant level due to the diverse mechanisms involved in the adaptation of plants to those conditions. Rachmilevitch et al. (2004) has proposed that leaf nitrate assimilation is reduced in conditions where photorespiration is reduced, as occur in elevated [CO_2]. This phenomenon could be the cause of the decline in leaf nitrogen content on C_3 plants performance under this conditions (Poorter *et al.*, 1997). Bloom et al., (2014; 2002) proposed ammonium nutrition as a tool to compensate the reduction of nitrate assimilation; nevertheless, *Arabidopsis thaliana* plants grown with ammonium as unique source exert the typical effects of "ammonium toxicity" (Britto & Kronzucker, 2002). When in elevated [CO_2] conditions *Arabidopsis thaliana* plants receive mixed ammonium nitrate as N source, plants were able to avoid photosynthetic acclimation and maintain nitrogen and protein content as plants grown at ambient [CO_2] conditions (see Chapter II) . Afterwards we propose a discussion between the mechanism involved in both *Arabidopsis thaliana* plants (wild type and NR mutant) performance under elevated [CO_2].

1.– *Arabidopsis thaliana* wild type plants performance under elevated [CO_2] and ammonium nitrate nutrition

Our results show that exposure to elevated [CO_2] remarkably enhanced biomass (Figure 1A,B; supplemental data thesis Figure 3). As shown in Figure 2B, plants exposed to elevated [CO_2] maintain leaf N content as plants growth in ambient concentrations. That notable increases in growth has been supported by the stimulation of photosynthetic rates. Moreover, the increments found in maximum rate of Rubisco carboxylase activity (Vcmax) and maximum electron transport rate contributing to RuBP regeneration (Jmax), corroborated that *Arabidopsis thaliana* plants exposed to elevated [CO_2] and grown with NH_4NO_3 as the nitrogen source were capable

to overcome photosynthetic acclimation (Figures 3A, 3B), as has been described in Chapter II and likewise other authors has found (Markelz et al., 2013).

It has been described that transcripts are modified rapidly and massively in response to changes in N (Alvarez et al., 2012; Wang et al., 2007) and C (Queval et al., 2012; Usadel et al., 2008). However, studies conducted in long term exposure to elevated [CO_2] in leaf tissues did not greatly alter gene expression on rice (Fukayama et al., 2011), sugarcane (De Souza et al., 2008), maize (Prins et al., 2011; Kim et al., 2006), poplar (Tallis et al., 2010), soybean (Leakey et al., 2009) and in *Arabidopsis thaliana* plants (see Chapter I and II; Markelz et al., 2013; Leakey et al., 2009). Contrary to expectations, the outstanding status of the photosynthetic apparatus were not associated with increases of genes expression encoding related to carbon assimilation. Nevertheless, in leaf tissue we found upregulated genes related to light harvest as Photosystem II subunit P-2 (at2g30790), electron transport rate as Cytochrome b-c1 complex subunit 7-2 (at5g25450), utilization of light energy as zeaxanthin epoxidase (at5g67030). Thus results, together with the higher electron transport rate parameters (Figure 4) and the enhancement of dark respiration (Figure 3D), support the fact that plants exposed to elevated [CO_2] grown with mixed NH_4NO_3 as N source were more efficient in managing plant energy status, as our group has already proposed (see Chapter II). Linked to energy status, the increments found in sugar and starch content together with the expression of genes as alpha-amylase 1 (at4g25000), a kind of amylase secreted from chloroplast and responsible for starch degradation in dead cells (Doyle et al. 2007), suggest a more profitable movement between C pools in leaf of plants exposed to elevated [CO_2]. Another biological response greatly overexpressed at transcriptional level in leaf of plants exposed to elevated [CO_2] was linked to the synthesis of cell wall and lipid metabolism, in concordance with higher growth rates found. A root level, highlight the sugar content (Figure 5B) and genes related to sugar handling pathways (at5g51970, at3g47800, at1g60140), glucose transport (at5g16150) and glycolysis (at5g47810, at3g22960), framework that points toward more suitable whole-plant C status on plant performance under elevated [CO_2]. Related to N metabolism, we had found higher total amino acids (Figure 6) as well as amino acids responsible of N mobilization content in both tissues under elevated [CO_2]. Additionally, underline mo-

molybdopterin cofactor sulfurase (at5g55130), co-factor for nitrate reductase in root and shoot tissue, that strengthen the proposed reorganization of nitrate assimilation between tissues under elevated [CO_2] detailed in Chapter I.

2.– *Arabidopsis thaliana* NR mutant exposed to elevated CO_2 and ammonium nitrate nutrition:

NR mutant plants performance under ambient [CO_2] exhibit evident overall suppression of growth in shoot and roots (Supplemental data thesis Figure 4), rates that were negligible comparing to wild type plants grown at ambient [CO_2], and *ende* to NR mutants grown at 800 ppm (Figure 1). Accompanying, the certainly lower photosynthetic rates, lower electron transport rate, diminished ETRc/ETRo ratio and reduced relative quantum yield of PSII at the steady state attested that those plants suffer photosynthetic disorders. When we analyzed metabolic compounds content, underline the accumulation of starch (Figure 5A´) on plants grown in ambient [CO_2] (that reaches values of plans exposed to elevated [CO_2]). This build-up, that negatively correlated with biomass as reported by Sulpice *et al.*, (2010), could be cause of plant developmental disturbances. Together, all that results emphasized that NR mutant grown at ambient [CO_2] presents symptoms associated to ammonium toxicity (Britto & Kronzucker, 2002) that seriously disturb plant development. Nevertheless, it's noteworthy that NR mutant plants exposed to elevated [CO_2] awfully increase shoot biomass, comparative to wild plants grown in the same conditions (Figure 1A). This outstanding growth rates were sustained by the successful condition of the photosynthetic apparatus as A_{growth}, V_{cmax} and J_{max} parameters indicated (similar as wild type plants under same conditions). Our data evidence that NR mutant plants were able to utilized optimally the increments in [CO_2] and avoid photosynthetic acclimation in the same manner as observed in wild type plants exposed to elevated [CO_2].

The ability for NR mutant plants under elevated [CO_2] to maintain the greatest rates of photosynthesis need to be supported by the capacity of the plants to ensure the energy demanding for C fixation. As shown in figure 5A´, this plants shown higher sugar content

comparing to plants performance under ambient [CO_2]. Linked, microarrays analysis presented upregulation of genes encoding for energetic metabolic pathways. Firstly, on pentose phosphate pathway highlighting transaldolase (at5g13420), 6-phosphogluconolactonase (at1g13700) and glucose-6-phosphate dehydrogenase (at1g24280), gene expression that powerful correlate with enzyme activity and growth (Keurentjes *et al.*, 2008). Secondly, on glycolysis pathway headline transcripts encoding phosphofructokinase (at4g26270, at2g22480), that performs the committed step in the glycolytic pathway, and phosphoglucomutase (at1g70820), that control carbon flow through starch metabolism or TCA acid cycle and the pentose phosphate pathway (Periappuram et al. 2000). Finally, on tricarboxylic acid cycle starring a strong induction of fumarase hydralase 2 (at5g50950), key enzyme proposed as generate flexible carbon storage and pH homeostasis (Pracharoenwattana *et al.*, 2010), aconitate hydratase (at2g05710), gene highly upregulated under elevated [CO_2] that stand for greater respiration rates (Watanabe *et al.*, 2014) as well as distinguished TCA members as 2-oxoglutarate dehydrogenase (at5g65750), malate dehydrogenase (at2g19900) and citrate synthase (at2g42790). Moreover, NR mutant exposed to elevated [CO_2] showed greater dark respiration rates (Figure 3D´) that, linked to the gene induction described, suggest that those plants were more conveniently maintain an outstanding energy status. Accompanying, the adequate manage/transport of photoassimilates along sink tissues are crucial and concomitant processes for maintaining high photosynthetic rates in plant´s exposed to elevated CO_2 (Lemoine et al., 2013; Ainsworth & Bush, 2011). Related to sugar metabolism highlight the overexpression genes responsible of starch remobilization as amylases (at4g15210, at4g25000, at3g23920), glucan-water dikinase (at4g24450) and alpha-glucan phosphorilase (at3g46970, at3g29320), scheme that suggest an active turnover of those starch content not observed under ambient conditions; hence, the starch breakdown closely correlation with biomass increments (Gibon et al., 2009). Besides, the sugar transport group was overexpressed at elevated [CO_2], supported by beta-fructofuranosidase (at1g12240, at1g62660) and sugars transporters (at3g18830, at4g02050, at1g08920) put forward that the satisfactory distribution of sugars between source/sink tissues prevents accumulation, the classical hypothesis that explain photosynthetic acclimation under elevated [CO_2] (Ainsworth et al., 2004; Arp, 1991) and sustain efficiently the outstanding C fixation rates of those plants.

In other to maintain a satisfactory N status, NR mutant plants primarily use ammonium assimilation pathway; an important question is how can guarantee the requirements of C skeletons and energy for this enzymatic reaction. Hence, it has been reported for plants growth under ambient [CO_2] conditions that the assimilation of novo ammonium mainly occur in roots (Marschner, 2012). In root of NR mutant plants exposed to elevated [CO_2] we observed an induction of glutamate dehydrogenase 2 (at5g07440), that could cooperate with GS-GOGAT pathway providing 2-oxoglutarate and reducing equivalents in N excess conditions (Labboun *et al.*, 2009). Nevertheless, we also found a repression of glutamate synthase 1 (at5g53460) and 2 (at2g41220) together with increments in all N related amino acids, as well as ammonium transporters AMT1;2 (at1g64780) and AMT1;3 (at3g24300). Likewise, expression of genes as dicarboxylate transporter 1 (at5g12860), involved with DIT2-1 in primary ammonia assimilation (Renné *et al.*, 2003), and outstanding glycolysis members as phosphoenolpyruvate carboxylase 3 (at3g14940), 1 (at1g08650), phosphoglycerate/bisphosphoglycerate mutase (at1g78050), pyruvate kinase (at2g36580), triosephosphate isomerase (at3g55440) and phosphoglycerate kinase family (at1g79550, at1g22170) targeting to increased ammonium assimilation in roots at ambient [CO_2]. Otherwise, it has been reported that there are a transport of NH_4^+ from the roots via xylem (Schjoerring et al., 2002), reaching 11% of total ammonium in oilseed rape specie (Finnemann & Schjoerring, 1999). We observed a indicative induction of genes encoding cytosolic glutamine synthetase (at5g16570, at5g37600), involved in the novo ammonium assimilation (Thomsen et al., 2014), on leaf tissue of NR mutant plants exposed to elevated [CO_2]. Together, we had found higher glutamine amino acid content, as well as other amino acids responsive in N transport as asparagine and arginine (Figure 7D´). Moreover, plants exposed to elevated [CO_2] seems to be more successful and satisfied the energy and C demanding for GS-GOGAT in leaf tissue as has been previously discussed. All those results suggest an active ammonium novo assimilation in leaf tissue on plants exposed to elevated [CO_2]. The relationship between promotion of ammonium assimilation organ and plant sensibility has been reported in bibliography (Lasa et al., 2002): sensitive ammonium plants mainly assimilated ammonium in shoots. Nevertheless, GS1 seems to be a key enzyme in detoxification ammonium (El Omari *et*

al., 2010), and ammonium tolerant model specie as rice exert absence of GS1;1 in root tissue (Tabuchi et al., 2007), promoting assimilation in leaf tissues. In the aboveground tissue of plants exposed to elevated [CO_2], with outstanding C and energy status, seems to be not unreasonable promote the assimilation of ammonium in shoot, as long as plants would be able to maintain intracellular pH, because the incorporation of one NH_4^+ molecule into amino acid generates 1.33 H^+ (Raven, 1986). Furthermore, pH homeostasis has been proposed as a critical factor for ammonium toxicity, because plants should waste ATP for pumping protons across membrane; it is known as fructile transmembrane NH_4^+ hypothesis (Britto et al., 2001). In root tissue this excess might excreted (Runge, 1983), but in the leaf the administration of this acidification turns out to be slightly more complicated. Under elevated [CO_2], the extent photosynthesis and respiration rates found could favor leaf ATP:NH_4^+ stoichiometry along cell or "proton economy" (Britto & Kronzucker, 2005). On leaf of plants exposed to elevated [CO_2] highlight the overexpression of an extensive family of vacuolar transporter, involved in the active removal of intracellular H^+, as cation exchanger 3 (at3g51860), 7 (at5g17860) and 16 (at1g64170), as well as outstanding genes members encoding antiporter mineral element transport (Martinoia et al., 2007), useful mechanism for excreted proton from cytosol.

Photoinhibitory processes in photosynthetic organism is another factor that has been reported in the ammonium syndrome (Dai et al., 2014; Drath et al., 2008; Guo et al., 2007; Britto & Kronzucker, 2002), consequence observed on florescence analysis (Figure 4E´) on NR mutant plants exposed at ambient [CO_2]. The photoinhibition can be seen as a dynamic balance between causes that inactive PSII and its repair. It has been proposed that interruption of Calvin cycle accelerate photodamage on PSII (Takahashi and Murata, 2005) and the production of ROS and photodamage is linked with the repression of D1 protein (Mulo et al., 2008), key enzyme involved on reassembly of the photodamaged PSII centers. In our transcriptomic approach we had found relevant overexpressed genes encoding light-harvesting antennas, and encoding D1 protein (at1g05385) as well as enzymes involved in in ROS detoxification enzymes (Podgórska *et al.*, 2013) as peroxidases, catalases and glutation peroxidases in ambient [CO_2]. Moreover, stechiometry of ATP need to be balanced with the rates of production/consumption, if no,

photoinibition damage emerge (Walker et al., 2014). Understand the mechanism of those photoinhibition overcome for NR mutants plants exposed to ambient [CO_2] seems to be a complicated task in our experimental design. Nevertheless, we attested that those photoinhibitory response was not observe on plant exposed to elevated [CO_2].

Conclusions:

In summary, the results indicate that *Arabidopsis thaliana* plants grown in ammonium-based nutrition under elevated [CO_2] show a potential development that is comparable to ammonium nitrate-based plants: aerial biomass and rates of photosynthesis are similar between wild-type and NR mutants plants. We established that NR mutant (NR nia1-1/chl3-5 defective) plants exposed to elevated [CO_2] and ammonium nutrition were overwhelmingly improved in their development due to their capacity to satisfy energy-demands for the Calvin cycle and perform efficient transport of photoassimilates between source and sink tissues. Furthermore, we identified at the gene expression level a response that suggests effective leaf ammonium assimilation by NR mutants exposed to elevated [CO_2]. The high C and energy status, which suggested that there were no restrictions in C skeletons or ATP, linked to the reduction in photorespiration electron-consumption, as well as an appropriate leaf pH homeostasis together indicated the existence of satisfactory conditions for such ammonium assimilation. Moreover, opposite to the observations under ambient [CO_2] in NR mutants, photoinhibitory processes did not appear under elevated [CO_2]. Nevertheless, the mechanisms used to avoid ammonium toxicity under exposure to elevated [CO_2] remain unclear and additional experiments are required to address this question. The results indicate that implementation of ammonium nutrition would overcome photosynthetic acclimation under future [CO_2] conditions.

GENERAL DISCUSSION:

C_3 plants growth at elevated [CO_2] classically has been described to increase photosynthetic rates (Ainsworth & Rogers, 2007) and growth (Long *et al.*, 2004), reduce stomatal closure (Leakey et al., 2009), decrease nitrogen (Cotrufo *et al.*, 1998) and protein content (Taub *et al.*, 2008). All these physiological and biochemical modifications produced a overall change in the metabolism of the plants, and frequently leads to the phenomenon known as photosynthetic acclimation or photosynthetic downregulation. Several hypotheses have been literate to explain those response: carbon dilution hypothesis (Kuehny et al. 1991); inadequate availability for plants to allocate carbon between source/sink (Aranjuelo et al., 2013; Moore et al., 1999; Krapp et al., 1993); mineral element constraints plants metabolism, via carbohydrate dilution (Poorter et al., 1997) or due to reduction transpiration rates (McGrath & Lobell, 2013); the general reduction of nitrogen content in leaf hold down protein content that limited plant performance, via carbon dilution (Taub & Wang, 2008) or by decrease N uptake due to reduction xylem flux (Hetherington & Woodward, 2003). Accompanying, the assimilation of nitrate in leaf of C_3 plants is reduced in conditions where photorespiration is inhibited (Bloom et al., 2010; Rachmilevitch et al., 2004). Those experimental evidence opened new frontiers of study: the N source apply has relevance for plant´s response to elevated [CO_2] conditions. With the information compiled in this thesis is achievable to said that the final response of plants performance under elevated [CO_2] owes to the action of several biological modifications simultaneously that cannot be explained with a sole hypothesis.

Our results shown that, independently of preferential nitrogen source utilized, elevated [CO_2] increases shoot and root biomass on *Arabidopsis thaliana* plant. This increment were supported with the capacity of plants to maintain higher rates of photosynthesis comparing to plants exposed to ambient [CO_2] concentrations. As shown in chapters II and III, when plants where fertilized with ammonium in the solution, the photosynthetic apparatus was in noteworthy

conditions, and plants were able to take more advantage of the large ambient [CO_2] (as Vcmax and Jmax parameters attested). Linked to photosynthetic capacity, we had confirmed that under nitrate conditions plants notably reduced total protein and Rubisco content (although we were aware that the concentration of nitrogen is different); meanwhile, under ammonium nitrate conditions this content were not substantially changed (comparing to plants growth under ambient [CO_2]). Together, we had denoted that gene expression encoding for Calvin cycle pathway has not necessary been coordinated with higher photosynthetic found (Chapters I,II and III). Those observation suggest the existence of a steward transcriptional mechanism that conserve primary metabolism expression via compensatory mechanisms when plants are exposed to elevated [CO_2] in long term and no stressful conditions. The physiological response of photosynthetic apparatus discerned go beyond to modifications on genes encoding carbon fixation enzymes.

Plant performance under elevated [CO_2] altered N metabolism and the N source supplied proved to be a target factor in those response. At leaf organ, our data confirmed that plants receiving nitrate had lower leaf N content under elevated [CO_2] (Chapter I). Whereas, this diminution were not found in plants were ammonium assimilation was favoured (Chapter III). At root level, plants grown with nitrate as unique source (Chapter I) and exposed to elevated [CO_2] strengthen root nitrate assimilation in order to avoid leaf N reduction. Furthermore, under nitrate conditions we had found a large nitrate accumulation and gene expression encoding nitrate transporters NRT2.1 and NRT1.5 in root tissue; we had proposed that the decreases in transpiration reduces xylem flux, nitrogen transport (as well as other minerals as cofactors), causing adverse conditions for plant development. Nevertheless, under primary ammonium-nutrition plants (NR mutant plants) and elevated [CO_2], we discussed a reorganisation between tissues for ammonium anabolism and leaf build up within the context of energy economy and absence of associated toxicity problem (Chapter III) . These results evidence that it is necessary to include the N source factor into meta-analysis how disclosing that leaf N content decreased in plants exposed to elevated [CO_2]; likewise, could explain why N content not groped in ammonium assimilation preferred species as rice (Loladze, 2014)

Elevated [CO_2] exposition has been frequently described to causes a general increase in total soluble sugars. In the present study one interesting response based on transcriptomic approach was identified: transport and allocation of end products along sink tissue is linked with remarkable photosynthetic apparatus performance; anon, *Arabidopsis thaliana* plants receiving ammonium in the solution more conveniently done those processes. As described in chapter II, the overexpression of representative genes for sugar transport and management avoid the collapse of end products between the source and sink. Moreover, it was also described that the large-scale C status of those plants concur together with ABA signalling and promote advancement in plant phenology towards flowering. Accompanying, elevated [CO_2] exposition increased starch content in *Arabidopsis thaliana* plants, regardless of N source applied. Even though, the functionality of these increases seems to be different depending on the source of N tested. In nitrate fertilized plants, exposition to elevated [CO_2] provoked an exceptional increment in starch content and has been described that could constrain photosynthetic activity and plant development (Sulpice *et al.*, 2010). Nevertheless, when ammonium was present in the nutritional solution these increases were not identified as restrictive for development. Although the concentrations of starch in plants grown with ammonium nitrate in chapter III are similar to those obtained when grown with nitrate (Chapter I), those plants were grown under short day photoperiod, and therefore due to circadian clock, those accumulation were not comparable. Nonetheless, the gene expression highlight an active starch breakdown on wild type and for NR mutants plants exposed to elevated [CO_2], comparing to the starch accumulation of those plants under ambient [CO_2].

Leaf respiration rates were consistently enhanced under elevated [CO_2] (Watanabe et al., 2014; Markelz et al., 2013; Leakey et al., 2009). Nevertheless, we had denoted differences on energy status between plants performance with nitrate or ammonium/ammonium nutrition mixture nutrition on organic acid content and transcriptomic data. In this document we highlighted that the ability of plants exposed to [CO_2] to maintain the outstanding photosynthetic rates must be closely linked to the ability of plants to ensure energy status. Once again, plants receiving ammonium in the nutrient solution more conveniently preserved an efficient energy status.

Under nitrate conditions (see chapter I) and elevated [CO_2] the limitations on malate content probably constrain leaf nitrate assimilation; the downregulation of genes as phosphoglucomutase provide hint indicating that plants prioritize carbohydrates biosynthesis, which causes depletion of organic acids. Meanwhile in ammonium nitrate nutrition (see chapter II) we had found higher organic acid, particularly the malate content. Likewise, data obtained in NR mutant plants suggest denotes a higher energy status based on the upregulation of genes encoding pentose phosphate, glycolysis and tricarboxylic acid pathway (chapter III) that contribute to maintain the outstanding photosynthetic rates found

GENERAL CONCLUSIONS:

To summarize, the general conclusions drawn from this thesis are:

1. Nitrate based nutrition provokes an overwhelming reduction in leaf N content, total soluble proteins and Rubisco content in *Arabidopsis thaliana* plants exposed to elevated [CO_2]. Nevertheless, this situation was not found in plants receiving ammonium in the solution.

2. Under nitrate nutrition and elevated [CO_2] conditions, a reduction in the root-to-shoot mineral and nitrate transport was detected. Such diminishment correlated with the reduction in transpiration rates and constrained *Arabidopsis thaliana* development. In addition, the reduction in leaf nitrate assimilation is an attempt to overcome the promotion of root nitrate assimilation.

3. *Arabidopsis thaliana* plants exposed to mixed ammonium nitrate nutrition and elevated [CO_2] were able to maintain highly efficient photosynthesis. Aspects of metabolism across the whole plant including energy, respiration and end product transport all cooperated together in this response.

4. The performance of primarily ammonium-assimilating plants (*Arabidopsis thaliana* double nitrate reductase defective mutant *nia1-1/chl3-5*) under elevated [CO_2] showed leaf development, photosynthetic rates and transcriptomic responses comparable to the growth of wild type plants under ammonium nitrate. Likewise, opposite to the observations under ambient [CO_2], NR mutant plants sidestepped "ammonium toxicity".

5. Undoubtedly, the nitrogen source supplied to *Arabidopsis thaliana* under raised [CO_2] determined plant performance.

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Supplemental data thesis:

Figure 1: Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* (col. 0) grown with nitrate as N source (Chapter I).

Figure 2: Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* (col. 0) grown with ammonium nitrate as N source (Chapter II).

Figure 3: Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* wild type plants exposed to [CO_2] (800 versus 400 ppm) grown with ammonium nitrate as N source (Chapter III).

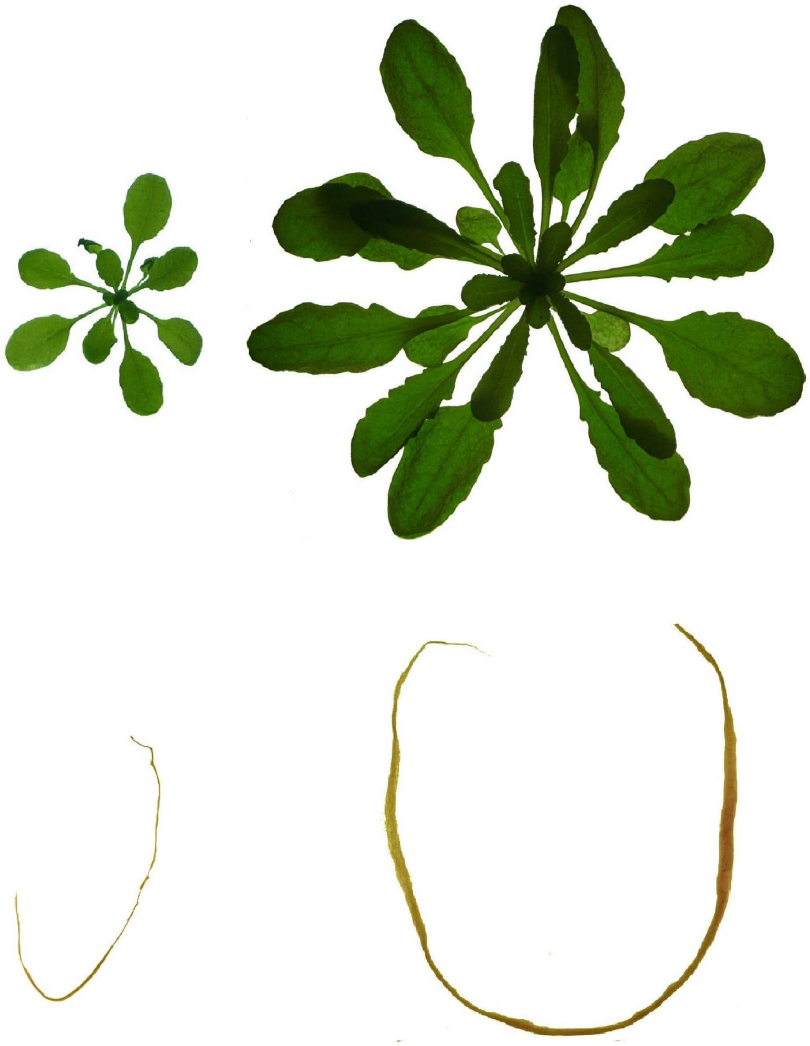


Figure 4: Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* NR mutant (NR double mutant G´4-3, *nia1- 1/nia2-5*) genotype plants exposed to [CO_2] (800 versus 400 ppm) grown with ammonium nitrate as N source (Chapter III).

Figure 5: Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* (Col 0) grown with ammonium as unique N source (0.75 mM).

Figure 6: Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* (Col 0) grown with nitrate, ammonium nitrate and ammonium as N source at 1 mM (0.75 mM).

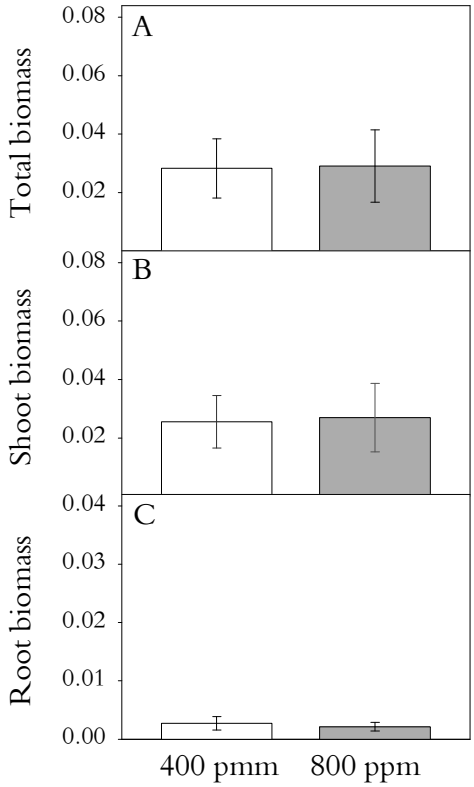
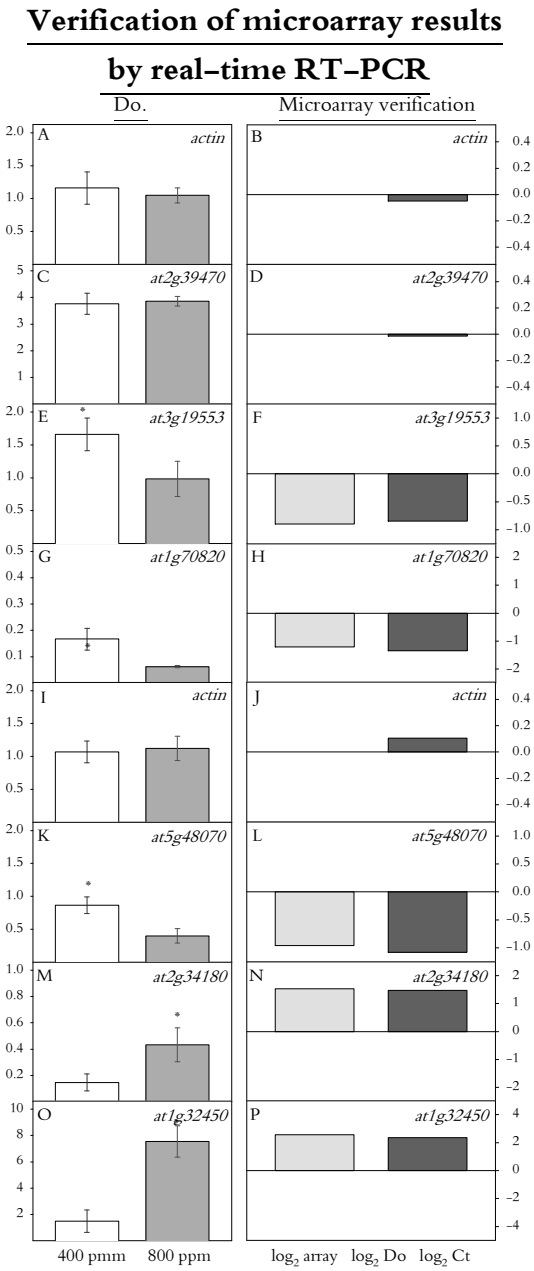
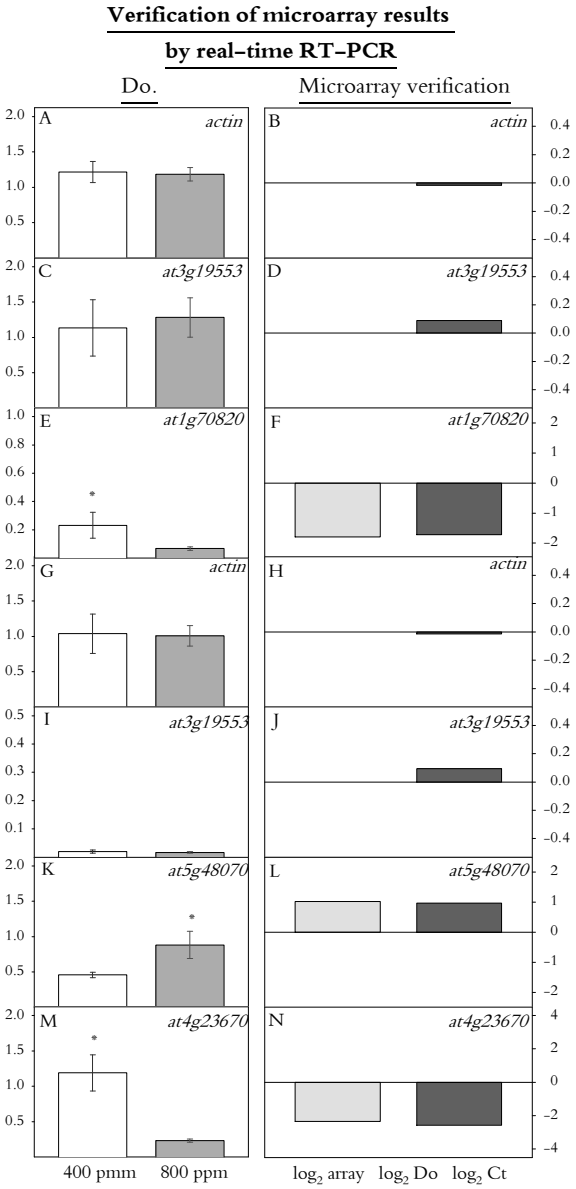


Figure 7: Effect of elevated [CO_2] (800 versus 400 ppm) in *Arabidopsis thaliana* (Col O) grown with ammonium as unique N source (0.75 mM). Each value represents the mean of 6 replicates ± SE



Supplemental Figure1 . Verification of Microarray Results by real-time RT-PCR of Arabidopsis thaliana plants (col. 0) exposed to [CO_2] (800 versus 400 ppm). The figures shown a comparison of the results obtained in the microarray log_2 and Do parameter.



Supplemental Figure2. Verification of Microarray Results by real-time RT-PCR of *Arabidopsis thaliana* plants (col. 0) exposed to [CO_2] (800 versus 400 ppm) on leaf (A-F) and root (G-N) tissues. The figures shown a comparison of the results obtained in the microarray log_2 and Do parameter.

Chapter III

Supplemental Table 2 . Primer sequences used in real-time PCR NO3

Gene	TAIR	Sense primer	Antisense primer
Actin	At1g07940	ATCTCTCAGCACATTCCAACAG	TATTGCCACCATCATCTCAAGC
PsbP-like protein 2	At2g39470	TCTCTGTGGTTACAAAACTGTCTG	AACTCAGAATCTAACTCACAATGCC
Polyamine uptake transporter 5	At3g19553	GCACAAGTCTTTAAAAGCGCA	CAGGTACAAACCCTCCTGCT
Phosphoglucomutase	At1g70820	CCAGGTTTCAGTCTTTCTCGTT	CTGACCGGGCCGACAATC
Xyloglucan endotransglycosylase 9	At5g48070	TCAAGGTGCTCCTCCAGAGT	CGCAAATACTCAGCTAGTGCC
CBL-interacting protein kinase 13	At2g34180	GCCGATCATCGACTGCTTCA	CCTTGCTCCTTGTCCACCTT
NRT 1.5	At1g32450	GATGACTATGACACCGAGAG	AAAAGACTGATCCGATTTAGCC

Supplemental Table 1. Primer sequences used in real-time PCR NH4NO3

Gene	TAIR	Sense primer	Antisense primer
Actin	At1g07940	ATCTCTCAGCACATTCCAACAG	TATTGCCACCATCATCTCAAGC
Polyamine uptake transporter 5	At3g19553	GCACAAGTCTTTAAAAGCGCA	CAGGTACAAACCCTCCTGCT
Phosphoglucomutase	At1g70820	CCAGGTTTCAGTCTTTCTCGTT	CTGACCGGGCCGACAATC
Polyketide cyclase/dehydrase and lipid transport superfamily protein	at4g23670	CATTGAAGGCCACGTCAACA	AGAAACTCCACAAGACCCCC
Xyloglucan endotransglycosylase 20	at5g48070	TCAAGGTGCTCCTCCAGAGT	CGCAAATACTCAGCTAGTGCC

Chapter III