The influence of root-zone bicarbonate and carbon dioxide enrichment on lettuce, pepper and tomato growth

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Declaration

Except where reference is made to other sources, I declare that the contents in this thesis are my own work and have not been previously submitted, in part or in full, for the award of a higher degree elsewhere.

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Abstract

Enhancing atmospheric CO_2 levels in commercial glasshouses is a widely used technique to increase productivity, but has high-energy costs and detrimental environmental impacts due to frequent ventilation of the glasshouse (to prevent plant diseases) releasing CO_2 into the atmosphere. Previous studies suggest that enrichment of the root zone (RZ) with CO_2 (RZ CO_2) may be a more economic and sustainable alternative to aerial CO_2 enrichment. This thesis aimed to compare the effects of RZ dissolved inorganic carbon (DIC) enrichment by adding either bicarbonate (HCO_3^-) or gaseous CO_2 to hydroponic and aeroponic systems, and to determine the physiological and molecular mechanisms by which plants respond to RZ DIC.

Supplying hydroponically grown plants with high bicarbonate concentrations (20 mM) inhibited growth of lettuce, pepper and tomato. However, lower concentrations increased biomass accumulation in lettuce (10% increase at both 1 mM and 5 mM HCO_3^-) and pepper (10% increase at 1 mM HCO_3^-), but had no effect in tomato. Exposing plants to 1 mM $NaH^{13}CO_3^-$ significantly increased shoot δ13C values over time, therefore confirming the uptake of DIC by the roots. Root δ13C values also significantly increased over time, however higher values at the beginning of $NaH^{13}CO_3^-$ exposure suggested root-to-shoot transport of DIC. Nutrient solution pH did not affect root carbon uptake, but shoot δ13C values were lower in those plants exposed to lower pH levels (5.8) compared to those exposed to fluctuating pH (between 6.3 and 6.7), suggesting differences in root-to-shoot transport of DIC. Thus, root carbon uptake was independent of the form in which CO_2 was provided (gaseous CO_2 at pH 5.8; HCO_3^- at higher pHs). Adding 1 mM HCO_3^- to hydroponically grown plants did not change foliar nutrient content, but K, P, N, Zn, Cu and Mn concentrations decreased at 20 mM HCO_3^-, suggesting nutrient deficiencies could limit growth.

Applying 2000 ppm RZ CO_2 to hydroponically grown lettuce, tomato and pepper did not affect biomass accumulation. Applying 1500 ppm CO_2 to the RZ of aeroponically grown lettuce increased shoot biomass between 19-25% (in 4 independent experiments) compared to those grown with 400 ppm RZ CO_2. However, leaf gas exchange measurements were inconsistent and therefore increased biomass could not be attributed to higher photosynthetic rates. In another 3 independent experiments, applying 1500 ppm CO_2 to the RZ of aeroponically grown lettuce did not stimulate biomass accumulation,

probably because the plants were exposed to higher night temperatures. Similarly, pepper and tomato did not show any biomass response to elevated RZ CO_2, suggesting that the responses to RZ CO_2 concentration are environment- and species-dependent. Nutrient analysis indicated that aeration with high RZ CO_2 decreased lettuce foliar Mg and S concentrations, whereas root N concentrations were higher than control plants.

Multi-hormone analysis of foliar and root tissues revealed that lettuce plants showed few differences in hormone status following RZ CO_2 enrichment. High RZ CO_2 increased foliar jasmonic acid concentration of lettuce, but the physiological significance of this change is not clear. Pepper plants showed significantly higher foliar 1-aminocyclopropane-1-carboxylic acid and lower trans-zeatin and salicylic acid concentrations, as well as lower root N6-(Δ2-isopentenyl) adenine and higher salicylic acid and gibberellic acid concentrations. These hormonal responses were associated with lower leaf area expansion of pepper plants exposed to elevated RZ CO_2.

Finally, transcriptome analysis of lettuce plants indicated that fatty acid biosynthesis, amino acid biosynthesis and carbon metabolism appeared to be the major pathways enriched in roots exposed to elevated RZ CO_2. In addition, proteins related to the cell walls and membranes seemed enhanced under elevated RZ CO_2. Although increased CO_2 concentration around the roots caused major transcriptomic restructuring, the aerial parts of the plants showed limited transcriptomic changes.

Taken together, this thesis is the first study of the responses of several horticultural species to elevated RZ CO_2 within different growing systems in order to decipher the impact that elevated RZ CO_2 has on crop productivity. Although bicarbonate enrichment of hydroponics and RZ CO_2 enrichment of aeroponics stimulated biomass accumulation of lettuce in many experiments, further work is required to fully understand the physiological response mechanisms to RZ CO_2. Whether the root transcriptomic changes in response to elevated RZ CO_2 represent an adaptive response to their environment requires a better temporal understanding of changes in specific genes. Ultimately, whether these changes are functionally significant to shoot growth seems to be strongly environmentally regulated.

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List of Abbreviations

Abb.	Definition	Abb.	Definition
^{12}C	Carbon-12	**K**	Potassium
^{13}C	Carbon-13	**KEGG**	Kyoto Encyclopedia of Genes and Genomes
^{14}C	Carbon-14 or radiocarbon	**Leu**	Leucine
A	Assimilation rate	**Lys**	Lysine
ABA	Abscisic acid	**Met**	Methionine
ACC	1-aminocyclopropane-1-carboxylic acid	**Mg**	Magnesium
Ala	Alanine	**Mn**	Manganese
Arg	Arginine	**MS**	Mass spectrometry
Asn	Asparagine	**N**	Nitrogen
Asp	Aspartic acid	**$NaH^{13}CO_3$**	Sodium bicarbonate-13C
B	Boron	**NaOH**	Sodium hydroxide
BA	6-benzylaminopurine	**NH_4^+**	Ammonia
CA	Carbon anhydrase	**NMR**	Nuclear magnetic resonance
Ca	Calcium	**NO_3^-**	Nitrate
Ci	Intercellular CO_2 concentration	**NT**	Night-temperature
Cit	Citrulline	**Orn**	Ornithine
CK	Cytokinins	**P**	Phosphorus
CO_2	Carbon dioxide	**PEP_C**	Phosphoenolpyruvate carboxylase
CO_3^{-2}	Carbonate	**pH**	Potential of Hydrogen
Cu	Copper	**Phe**	Phenylalanine
Cys	Cysteine	**PPFD**	Photosynthetic Photon Flux Density
DEGs	Differentially expressed genes	**Pro**	Proline
DFTS	Deep flow technique system	**RNA**	Ribonucleic acid
DIC	Dissolved inorganic carbon	**RZ CO_2**	Root-zone carbon dioxide
DO	Dissolved oxygen	**RZ DIC**	Root-zone dissolved inorganic carbon
E	Transpiration rate	**RZ HCO_3^-**	Root-zone bicarbonate
EC	Electrical conductivity	**S**	Sulfur
eCO_2	Elevated carbon dioxide	**SA**	Salicylic acid
Fe	Iron	**Ser**	Serine
GA_3	Gibberellic acid	**Thr**	Threonine
GABA	ɣ-aminobutyric acid	**Trp**	Tryptophan
Gln	Glutamine	**Tyr**	Tyrosine
Glu	Glutamic acid	**tZ**	Trans-zeatin
Gly	Glycine	**Val**	Valine
GO	Gene Ontology	**Zn**	Zinc
g_s	Stomatal conductance	**ZR**	Zeatine riboside
H_2CO_3	Carbonic acid	**$\delta^{13}C$**	Delta C thirteen
HCL	Hydrochloric acid		
HCO_3^-	Bicarbonate		
His	Histidine		
IAA	Indole-3-acetic acid		
Ile	Isoleucine		
iP	Isopentenyladenoside		
JA	Jasmonic acid		

Chapter 1 General Introduction

1.1 Context

The world greenhouse cultivation area (permanent structures covered with glass or plastic) is estimated to be around 497,815 ha of which some 173,561 ha are spread throughout Europe (Hickman, 2018). Commercial protected cultivation originated in northern Europe due to the need to protect crops from adverse weather and overcome the problem of cultivating cold-sensitive species, allowing year-round production (Wittwer, et al., 1995). All greenhouse cultivation systems comprise fundamental climate control components. However, their design and complexity are variable depending on the local climate and the socio-economic environment. Air temperature, humidity, wind, solar radiation and aerial CO_2 concentration are the most important parameters that can be controlled in a greenhouse (Hanan, 1998) and technologies must be adapted to the local requirements to allow the control of each one.

Photosynthesis uses light energy to convert CO_2 and water into sugars, which are required for plant growth and respiration. Plant growth and net primary production depend on the balance between the photosynthesis and respiration rates (Prentice et al., 2001 ; Oijen et al., 2010). More than one third of the CO_2 in the atmosphere is exchanged annually with the terrestrial biosphere by passage through stomata into leaves and dissolution in leaf water (Farquhar et al., 1993; Ciais et al., 1997), and about half of this amount is fixed in photosynthesis. In northern Europe, greenhouse operators often inject extra CO_2 into the aerial environment to enhance crop photosynthesis and dry-matter accumulation under low radiation conditions (Nederhoff, 2004). In general, supplementation between 700-1000 ppm CO_2 is used, however, depending on the crop, light intensity, temperature, growth stage of the crop and vent position the levels could be increased up to 1500 ppm CO_2 (Mohyuddin, 1990 ; Dunn et al., 2017).

Sources of CO_2 for enrichment include boiler, combined heat and power (CHP) and burner exhaust gases and liquefied pure gas (Adams et al., 2009). Flue gases from natural gas boilers are widely used in the UK as a source of CO_2 for enrichment. This practice has high energy costs of £200K per annum for a 5 Ha glasshouse (Pratt, 2011). Moreover, when the humidity is very high (>80%) inside the glasshouse, the vents open to minimise plant disease development and the CO_2 released into the atmosphere (Buffington et al., 1987; Körner et al., 2003) . If the vents are completely open, it is challenging to maintain the CO_2

concentrations inside the greenhouse and almost all the CO_2 supplied into the air environment will escape through the vent (Figure 1.1). With the recent increased interest in global warming and climate change, many governments have set a maximum CO_2 emission levels for various industries, including the greenhouse sector (Defra, 2012). Despite the efforts of growers to minimize spending and maximize production through technical improvements, it is necessary to consider other systems such as localized root-zone CO_2 (RZ CO_2) enrichment, to improve the production without harming the environment.

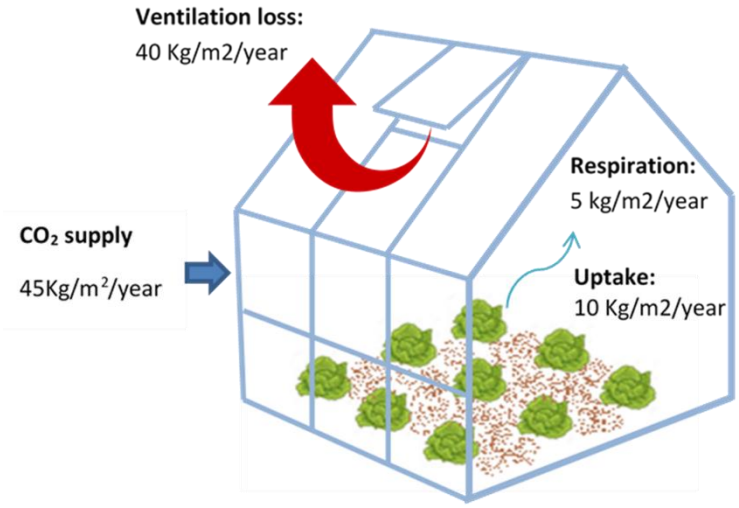


Figure 1.1. CO_2 uptake by plants, CO_2 loss by respiration and CO_2 loss by venting in a glasshouse with a supply rate of 45 kg/m2/year under high ventilation rates**.** *Modified from Wageningen University & Research, Business Unit G. Horticulture. 2016.*

1.2 The movement of CO_2 in plant roots and rhizosphere

Generally, soil CO_2 concentrations greatly exceed that of the atmosphere (Van Veen et al., 1991), which currently is around 410 ppm (NASA, 2019). The soil volume surrounding plant roots is called “rhizosphere” and is inhabited by a unique population of microorganisms influenced by the chemicals released from plant roots (Hartmann et al., 2008). The physical, chemical and biological properties of the soil adjacent to the root are very different compared with the bulk soil (Gregory, 2006; Hinsinger et al., 2009). The soil surrounding the roots regulates processes such as, water uptake, nutrient acquisition, gaseous exchange and microbial proliferation (Helliwell et al., 2017). Rhizosphere

processes play a key role in C sequestration and nutrient cycling in terrestrial ecosystems and has been identified as one of the key fine-scale components in the overall C cycle (Helal & Sauerbeck, 1989; Van Veen et al., 1991; Coleman et al., 1992). Root respiration and microbial respiration, including decomposition of soil organic material, are major contributors to the total soil CO_2 efflux (Kuzyakov, 2006). Concentrations of CO_2 in the soil vary with depth (Johnson et al., 1994, Duenas et al., 1995), with soil water content (Bouma et al., 1997), soil type (Duenas et al., 1995) and time of the year (Johnson et al., 1994) and range from 2000 to 5000 ppm but may become as great as 20% when soils are poorly aerated (De Jong & Schappter, 1972; Norstadt & Porter, 1984).

In most higher plants, atmospheric CO_2 enters the leaves via stomatal pores which also serve as variable valves to control the loss of H_2O vapour when the uptake of CO_2 happens. The CO_2 can either be fixed in those parts of the plant or translocated to the roots. In the roots, it can be respired, laid down in the process of growth or exuded with subsequent utilization by microorganisms (Warembourg & Paul, 1973). Although aquatic plants have mechanisms by which CO_2, derived from sediments and respiration from roots, moves from below-ground to the shoot (Brix, 1990; Colmer, 2003b), terrestrial plants are thought to capture insignificant amounts of CO_2 through their roots. However, there are some exceptions, such as the terrestrial plant *Stylites andicola*, which lacks stomata and captures almost all of the CO_2 via its roots (Keeley et al*.,* 1984) .

The evidence that plants can take up CO_2 via their roots is quite old (Ruben & Kamen, 1940; Stolwijk & Thimann, 1957) and since then many experiments have tested this hypothesis. Even though around 40% of the Earth terrestrial land area is covered by grasslands (Adams et al., 1990; White et al., 2000), most work has focused on the carbon flux in forest ecosystems, due to their important role in the global carbon cycle and hence most of the knowledge regarding the carbon movement in the root-soil interface derives from these studies. Carbon balances are dominated by two carbon fluxes: photosynthesis and ecosystem respiration. Ecosystem respiration is divided into autotrophic respiration (leaves, stems and roots) and heterotrophic respiration (fungi, bacteria and animals) (Hanson et al., 2000). Plants are the most important autotrophs that contribute to the CO_2 flux from soil through root respiration. The CO_2 respired by roots diffuses from inside the roots outward and is released into the soil pore space. Some of this CO_2 can be dissolved in the soil producing dissolved inorganic carbon (DIC) which is controlled by the partial

pressure of CO_2 (pCO_2), pH and temperature (Clark & Fritz, 1997). DIC is formed through the following reaction:

$$\text{Soil } CO_{2\,(g)} = CO_{2\,(aq)} + H_2O = H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \quad [1]$$

Total system DIC is the sum of dissolved CO_2 gas (CO_2), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) (Karberg, 2005).

$$[DIC] = [CO_2\,(aq)] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}] \quad [2]$$

By convention:

$$[H_2CO_3] = [H_2CO_3] + [CO_2\,(aq)] \quad [3]$$

The soil inorganic carbon comprised the CO_2 in the gas phase, the liquid phase solution containing HCO_3^- and CO_3^- and the carbonate in the solid phase. Carbon dioxide is a weak acid that slowly dissolves basic minerals such as $CaCO_3^+$. When the CO_2 dissolves in water, at the same time, hydrolysis of HCO_{3-} is converted into pedogenic carbonates with other salts, usually with Ca^+ and Mg^{2+}(Bai et al., 2017).

Solution pH determines the reaction direction of carbonates, and thus the proportion of the carbonate species present in the solution (Figure 1.2). The prevalent form of carbonates at pH ≤6.36 is H_2CO_3, at pH between 6.36 and 10.33 is HCO_3^-, and CO_3^{2-} is predominant at pH >10.33 (Lindsay, 1979). The solubility of CO_2 increases from pH 5, because at this given pH a proportion of DIC exists as HCO_3^-, and CO_3^{2-} (Golterman and Clymo, 1969).

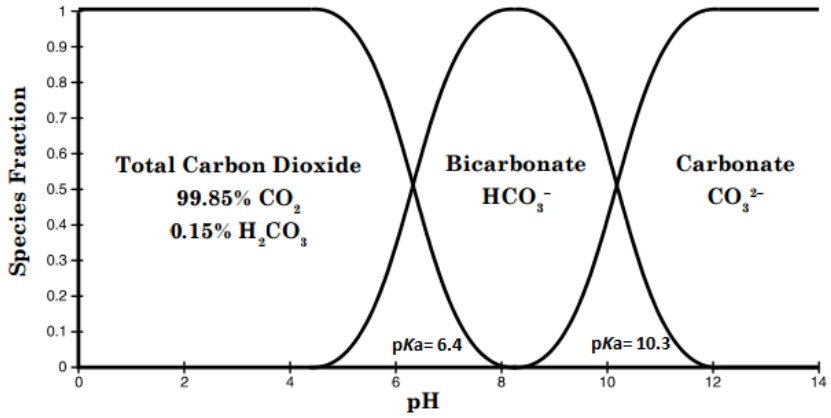


Figure 1.2. Distribution of total carbon dioxide, bicarbonate and carbonate vs. pH. Source: Wojtowicz, 2001.

Interestingly, recent studies in trees have demonstrated that some of the CO_2 respired by the roots can be dissolved in the xylem sap and translocated to the shoots (Bloemen et al., 2013).

1.1.2 The use of isotopes to measure carbon flows and metabolic pathways in plants.

Carbon isotopes have been very useful to study CO_2 efflux from soils (Kuzyakov, 2006), carbon flow processes in photosynthesis (Nelson, 1963), the translocation of different photosynthates to various parts of the plant (Kriedemann, 1969) and also for identifying major pathways in plant primary metabolism (Fernie et al., 2005). The most well-known labelling study is probably the elucidation of the Calvin-Benson cycle using $^{14}CO_2$ in the green algae *Chlorella* (Calvin, 1962).

There are three carbon naturally occurring isotopes with molecular weights of 12, 13 and 14. ^{12}C and ^{13}C are stable isotopes and their relative natural abundances in the carbon of the atmospheric carbon dioxide are 98.9% and 1.1% respectively. ^{14}C is an unstable radioactive isotope present at <0.01%. Labelling with radioactive ^{14}C has been widely used to study the allocation of photoassimilated carbon in different crop (Ho & Shaw, 1977; Farrar & Farrar, 1985) and tree (Pumpanen et al., 2009) species. It is relatively cheap, has a long life and is easily handled (Warembourg et al., 1973; Kölling et al., 2013). However, is not usually analysed by mass spectrometry (MS) and measurements of carbon partitioning into specific metabolites is laborious.

Since the MS was developed, labelling experiments with ^{13}C have increased in frequency. The advantages of using ^{13}C labelling are its compatibility with the MS and nuclear magnetic resonance (NMR) techniques and the lack of radioactivity. Moreover, ^{13}C can allow metabolite identification (Krishnan et al., 2005) , provide positional information for the isotope label (Huege et al., 2007) and allows carbon allocation towards sink tissues to be determined (Cliquet et al.,1990).

The ^{13}C content is usually determined with a mass spectrometer which measures the ratio (*R*) between ^{13}C and ^{12}C and is compared to some standards. *R* values are commonly converted to values of $\delta^{13}C$ and the units are called “per mil” or ‰.

$$R = {}^{13}CO_2/{}^{12}CO_2 \qquad [1]$$

$$\delta^{13}C = [\,(R \text{ sample} / R \text{ standard}) - 1] \times 1000 \qquad [2]$$

The standard generally used in determination of carbon isotopic ratios is carbon in carbon dioxide obtained from limestone, called Pee Dee Belemnite (PDB), from the pee dee formation in South Carolina and with a R of 0.0112372 (Craig, 1957).

The small atomic mass dependent differences in the physical and chemical behaviour of isotopes (such as $^{12}CO_2$ and $^{13}CO_2$) causes an unequal distribution of isotopes between different substances of phases in biogeochemical processes in a phenomenon known as *isotopic fractionation* (Hoefs, 2004). Since ^{13}C is heavier than ^{12}C, it forms stronger chemical bonds and a kinetic fractionation occurs, because faster enzymatic reaction rates occur with substrates containing the lighter isotopic forms than reactions involving the heavier isotopic forms. Isotopic fractionation in plants is commonly quantified as a carbon isotope discrimination factor (Δ) and is defined as the depletion of ^{13}C due to the preference for the lighter isotope ^{12}C. Δ differs from $\delta^{13}C$ in that it indicates the change of the isotopic composition induced solely by the plant, being independent of the isotopic composition of the standard and it also eliminates the variation as a result of the starting value of the atmospheric CO_2 used in photosynthesis.

$$\Delta = \frac{Ra - Rp}{Rp} = \frac{^{\delta}a - ^{\delta}p}{1 + ^{\delta}p} \qquad [3]$$

*R*a indicates the $^{13}C/^{12}C$ ratio of CO_2 in air and *Rp* is that of the plant carbon (Farquhar & Richards, 1984).

Most natural materials such as plant biomass have a negative $\delta^{13}C$ value compared to the standard, and these vary between different plant species and plants growing in different environments (Nier and Gulbransen, 1939; Wickman, 1952) (Figure 1.3).

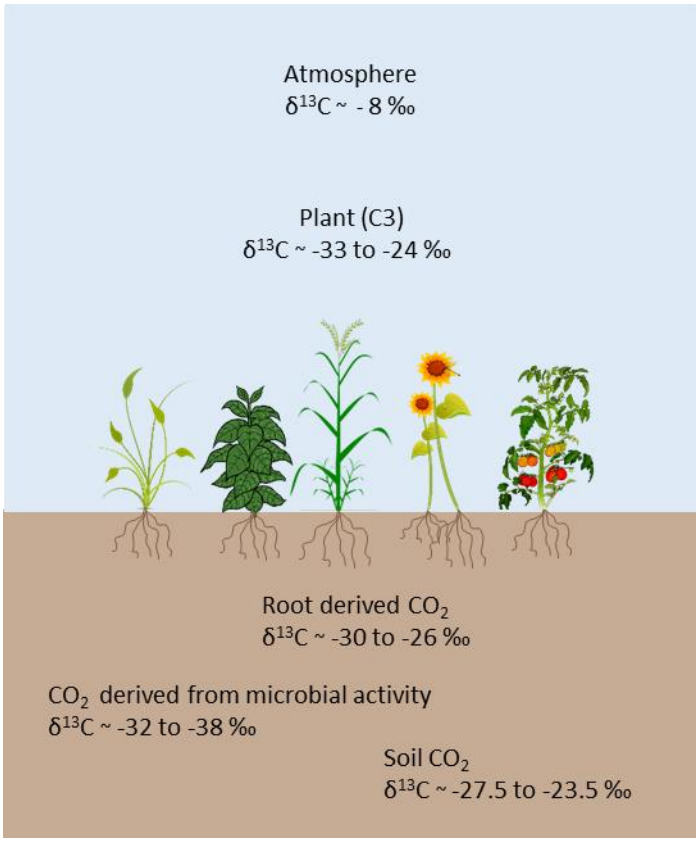


Figure 1.3. $\delta^{13}C$ values in C3-plant dominated ecosystems. Modified from Gillon et al., 2012.

It has long been known that carbon isotope fractionation characteristics differ according to the photosynthesis type (C3 *versus* C4) of the plant (Bender, 1968), since kinetic fractionation occurs in the initial process of carbon dioxide fixation during photosynthesis: C3 plants incorporate CO_2 through the enzyme ribulose bisphosphate carboxylase (Rubisco) to form a three-carbon compound and they show a higher ^{13}C depletion ($\delta^{13}C$ values between -33 to -24 ‰) compared to C4 plants that initially incorporate CO_2 through the enzyme phosphoenolpyruvate carboxylase (PEP_C). Within the leaves of C4 plants, a two-step CO_2 incorporation process occurs, such that CO_2 is first taken up by PEPc and then the carboxylation product is transported from the outer mesophyll cells to the inner bundle sheath layer where decarboxylation and refixation occurs by Rubisco (Figure 4.1). Values in C4 plants are in the range of -16 to -10 ‰ (Farquhar et al., 1989).

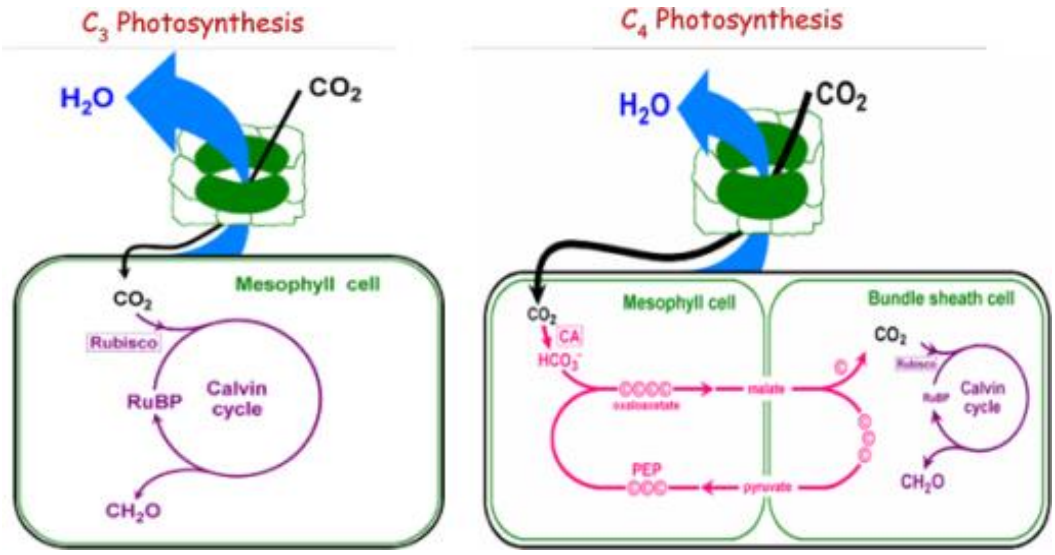


Figure 1.4: Diagram of C3 and C4 plant photosynthesis pathways (Wang, et al., 2012).

1.1.3 Using Isotopes to demonstrate the incorporation of DIC via root phosphoenolpyruvate carboxylase activity

The transport of DIC from the rhizosphere to inside the cell roots is still not well known. However, when the pH in the solution is high, the predominant form HCO_3^- may be transported actively, co-transported with H^+ or exchanged with OH^- or NO_3^- (Ben-Zioni et al., 1971; Barneix et al., 1984). DIC could also be assimilated as CO_2 or H_2CO_3 after being converted externally to HCO_3 and CO_2 or H_2CO_3 (Raven, 1984; Lucas, 1985). In the case of CO_2 gas, some studies have shown that it may diffuse through aquaporins located in the roots (Sade et al., 2010 ; Maurel, 2008).

Ruben's (1940) experiments were probably the first to demonstrate $C^{11}O_2$ uptake by excised barley roots. After this, many studies have shown the uptake of DIC through the roots using both labelled ^{14}C or ^{13}C in a variety of crops and trees (Table 1.1).

Table 1.1. Studies on the uptake and transport of DIC using ^{13}C or ^{14}C isotopic labelling in soil and hydroponically grown plants.

Method	Type of system, species	Main Finding	References
^{13}C soil DIC labelling	Potted seedlings, *Pinus taeda*	Soil DIC uptake contributed 0.8 % to plant carbon gain	Ford et al., 2007
	Field-grown, conifers	Soil DIC uptake did not affect stem CO_2 efflux	Ubierna et al., 2009
^{14}C soilless DIC labelling	Hydroponics, *Pisum sativum* and *Hordeum vulgare*	The CO_2 uptake by roots was small	Stolwijk & Thimann, 1957
	Hydroponics, *Salix*.sp	^{14}C label was found in the leaves and shoots	Vapaavuori & Pelkonen, 1985
	Hydroponics, *Salix*.sp	^{14}C label was found in the leaves and shoots	Vuorinen et al., 1989
	Hydroponics, *Salix*.sp and *Hordeum vulgare*	CO_2 and HCO_3^- enrichment led to higher PEPc activity	Vuorinen & Kaiser, 1997
	Hydroponics, *Nicotiana*.sp	^{14}C label was fixed in vascular photosynthetic cells	Hibberd & Quick, 2002
	Hydroponics, *Solanum lycopersicum*	^{14}C label was found in amino and organic acids in leaf, stem and root	Cramer & Lips, 1995
	Hydroponics, *Solanum lycopersicum*	CO_2 enrichment led to a higher ^{14}C label in the roots, shoot and xylem sap. The carbon was mostly in organic compounds	Cramer & Richard, 1999
	Hydroponics, *Solanum lycopersicum*	^{14}C partitioning to amino acid synthesis was increased	Viktor & Cramer, 2005

$C^{14}O_2$ and $HC^{14}O_3$ labelling revealed that most of the fixation was into C4 acids, suggesting the presence of PEP_C in the roots of pea and barley (Stolwijk & Thimann, 1957). The activity of PEP_C was measured *in vivo* as ^{14}C incorporation into organic acids in maize roots (Cramer et al., 1993) and as ^{14}C incorporation into acid stable products in tomato roots (Cramer & Lips, 1995) , as *in vivo* dark CO_2 fixation rate in roots of willow and barley (Vuorinen & Kaiser, 1997) and *in vitro* in tomato plants (Cramer et al.,1999; Viktor & Cramer, 2005). *In vivo*, the incorporation of ^{14}C and dark CO_2 fixation were higher (50-90%) in plants enriched either with CO_2 or HCO_3^-. *In vitro*, enrichment with RZ CO_2 did not influence leaf or root PEPc activity in NO_3^- fed tomato plants. Moreover, it made only a small contribution (<5%) to the total carbon budget of the plant (Viktor & Cramer 2003, 2005). Almost all tissues of C3 plants contain PEP_C but the activities are low compared to C4 and CAM plants. Several studies suggested that the generation of NADPH by the sequential action of PEP_C have important functions such as the synthesis of fatty acids in the roots of seedlings (Latzko et al., 1983). PEP_C catalyses the formation of oxaloacetate from phosphoenolpyruvate and HCO_3^- with Mg^{2+} as an obligate cofactor (Figure 1.5). It is a ubiquitous enzyme in plants and plays an important C fixation role in C4 and CAM plants in photosynthetic carbon assimilation (O'Leary, 1982; Chollet et al., 1996). In C3 plants and in non-photosynthetic tissues, PEP_C also has important functions; including replenishment of tricarboxylic acid cycle intermediates (Stitt, 1999; Champigny & Foyer, 1992) and regulating cytoplasmic pH (Sakano, 1998). The rate of DIC incorporation by PEP_C in roots is influenced by the form of N supplied, the concentration of DIC in nutrient solutions and salinity stress (Schweizer & Erismann, 1985; Arnozis et al., 1988; Vuorinen et al., 1989, 1992; Cramer et al., 1993). Despite the key role of PEP_C in root metabolism, the regulation of root PEP_C is still not fully understood (Jeanneau, 2002).

Carbonic anhydrase (CA) catalyses the reversible hydration of CO_2 (Rengel, 1995). In C3 leaves, CA activity has been found in both the stroma of chloroplasts (87% of total cellular activity) and the cytosol (13%), but also at high rates in root nodules where it is responsible for equilibrating CO_2 and HCO_{3-} (Atkins et al., 2001). Under elevated RZ CO_2, tomato roots did not show any significant differences in CA activity compared to plants aerated with 0 ppm RZ CO_2, however leaf CA activity was 30% lower (Viktor and Cramer, 2005). These authors also suggested that this downregulation was due to increased CO_2 availability in the leaves.

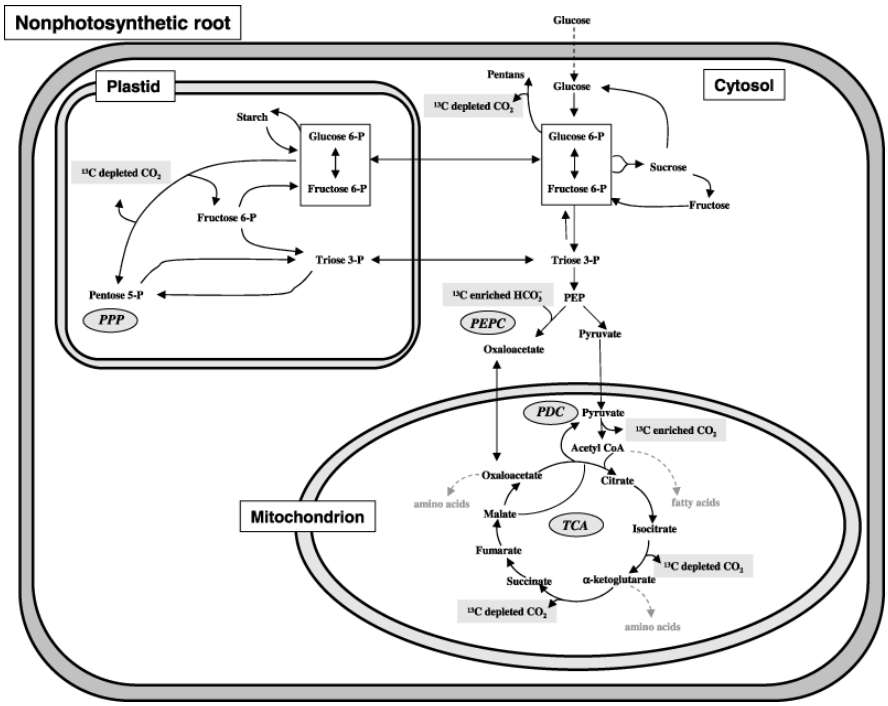


Figure 1.5. Pathway of anaplerotic carbon incorporation into the TCA cycle in plant roots. Showing the potential carboxylation and decarboxylation reactions contributing to the carbon isotope composition of the net CO_2 flux from a nonphotosynthetic root. PPP, pentose phosphate pathway; PEP_C, phosphoenolpyruvate carboxylase reaction; PDC, pyruvate dehydrogenation complex; TCA, tricarboxylic acid cycle (Wingate, 2007).

1.3 Soilless culture system to study RZ CO_2 enrichment

Different methods including non-isotopic and isotopic techniques have separated several CO_2 sources that contribute to the total carbon flux from soil. However, many of the published results are biased by the experimental system in which individual factors or processes were examined (Jones & Darrah, 1993; Meharg, 1994). While the contribution of soil organic matter from root and shoot residues after plant death is well known, the organic carbon derived from living roots has not been sufficiently investigated. One of the main issues was the different results obtained from the use of soilless culture (Schonwitz & Ziegler, 1988; Meharg & Killham, 1991) or sterile soils (Warembourg, 1975; Merbach et al., 1990, 1991) compared to those where soil was utilized, because the

interactions of the roots with the mineral soil matrix and the soil microorganisms lead to the different C allocation and sequestration by roots. Also, the different tracer techniques lead to different results (Kuzyakov & Domanski, 2000). Thus, there is still no accurate method to quantify a source separately from all others (Kuzyakov, 2006). For this reason, measuring the CO_2 concentration surrounding the roots when external CO_2 is added to the soil is challenging, because of interactions with other parameters such as heterotrophic respiration. Consequently, most of previous studies using crops have exposed the roots to different CO_2 concentrations in soilless culture systems (SCS).

Soilless culture can be defined as "any method of growing plants without the use of soil as a rooting medium, in which the inorganic nutrient absorbed by the roots are supplied via the irrigation medium" (Savvas et al., 2013). Among the different systems available, hydroponics is a method in which plant roots are suspended in either static, continuously aerated nutrient solution or in a continuous flow or mist of nutrient solution (Jones, 2004). Nutrient solutions comprise potassium (K), nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), sulphur (S) and micronutrients. The main advantages that hydroponics confers compared to soil are more efficient nutrition regulation, better pest and disease control, efficient use of water and fertilizers, easy and low-cost sterilization of the medium, availability in regions without arable lands and higher density planting which can increase yields per hectare (Resh, 2013).

There are several kinds of hydroponics in the market, however this thesis focuses on the classic static deep flow technique system (DFTS) (Jensen & Collins, 1985) (Figure 1.6). In DFTS, plants are grown with the roots suspended in a nutrient solution of approximately 5-15 cm depth (van Os et al., 2008) in which electrical conductivity (EC) and pH can be accurately controlled. On a commercial scale, plants are generally fixed in large styrofoam blocks floating on the nutrient solution. Therefore, DFT hydroponics are very suitable for RZ DIC enrichment as they are a simple and easy way to implement the treatments.

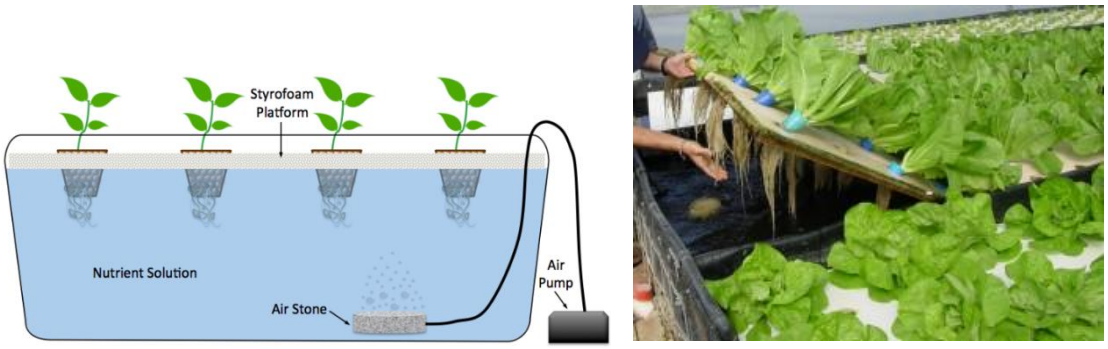


Figure 1.6. Deep flow hydroponic technique. (Image taken from https://aquaponics.com and https://offgridgorilla.com).

Another promising technique for the future is thought to be aeroponics (Figure 1.7). It is widely used in laboratory studies, but less used in large-scale commercial production (Resh, 2004). It is a similar technique to the hydroponics, except that plant roots are suspended in air and sprayed with nutrient solution. Compared to hydroponics, one of its key advantages is the aeration, as the roots are essentially growing in air, avoiding the lack of oxygen that may occur in hydroponics systems in the absence of adequate aeration. Also, it is an easy method to apply and monitor a known concentration of CO_2 gas to the root-zone of plants. Both hydroponics and aeroponics have been used to apply either carbonate (HCO_3^-) ions or gaseous CO_2 (Table 1.2).

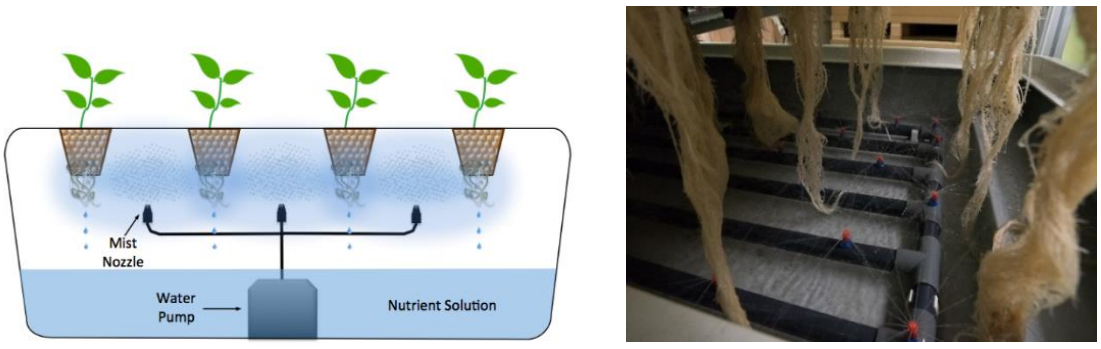


Figure 1.7. Aeroponic system. (Image taken from https://www.aessensegrows.com/en/why-aeroponics)

Table 1.2. Studies where bicarbonate or carbon dioxide were applied in hydroponics and aeroponic systems.

System	DIC specie supply	Concentration	References
Hydroponics	Bicarbonate	1, 5, 10, 20 mM	Bialczyk et al., 1992, 1994, 2004, 2005
	Bicarbonate	1, 5, 10, 20 mM	Yang et al., 1994
	Bicarbonate	1, 5, 10, 20 mM	Alhendawi et al., 1997
	Bicarbonate	0, 5, 10, 20 mM	Al Mansouri et al., 2014
	Carbon Dioxide	5000,6000, 8000 ppm	Cramer et al., 1995,1996, 1999, 2001, 2003, 2005
	Carbon Dioxide	15000 ppm	Boru et al., 2003
Aeroponics	Carbon Dioxide	370,2500,5000, 10000ppm	Zhao et al., 2010
	Carbon Dioxide	360,2000,5000,10000 ppm	He et al., 2004, 2007, 2010, 2016.
	Carbon Dioxide	2500, 5000 ppm	Li et al., 2009

1.4 Root-zone DIC enrichment effects on growth and yield

According to previous studies, the effects of elevated RZ CO_2 on plant growth depend on plant species, pH, air temperature, irradiance, mineral nutrition, abiotic stress, the RZ CO_2 treatment time, CO_2 concentration applied and the RZ CO_2 concentration. For this reason, the results obtained regarding growth and yield have been very contradictory and therefore controversial. The last meta-analysis including 358 experiments demonstrated that elevated RZ CO_2 significantly increased yield by 2.9% (Enoch & Olsen, 1993). Since 1993, new technologies and increased interest in the topic has lead to new experiments and a better understanding of the effects of both high concentration of RZ CO_2 and HCO_3. However, these studies have sometimes shown opposing results (Figure 1.8).

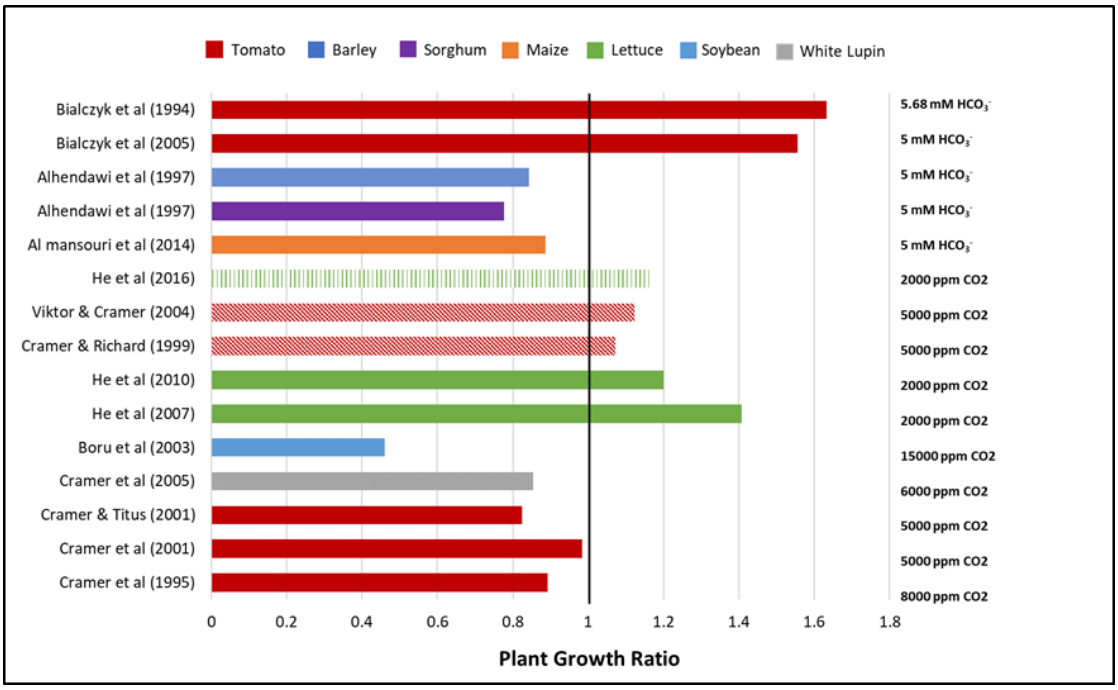


Figure 1.8. Growth of plants with elevated HCO_3^- and CO_2. Data are plotted as a ratio of enriched (5-6 mM HCO_3^-) and (2000-15000 ppm CO_2) to control (0 mM HCO_3^-) and (360 ppm CO_2).

Despite the limited average yield enhancement by elevated RZ CO_2 previously shown (Enoch & Olesen, 1993), since then some authors have reported an average yield plant growth ratio of 1.3 and 1.5 when applying elevated RZ CO_2 and HCO_3- under specific environmental conditions. Adding 5.68 mM HCO_3^- (0.0025% CO_2) to the nutrient solution at pH 6.8 increased dry weight and leaf blade area of tomato plants grown hydroponically compared to those plants grown without HCO_3^- (Bialczyk et al., 1994). With the same

experimental conditions and species, when nitrate (NO_3^-) and ammonium (NH_4^+) was supplied as a nitrogen source at an optimum ratio (4:1), adding 5 mM HCO_3^- to the medium, increased whole plant relative growth rate (RGR) and leaf area but decreased shoot to root ratio (Bialczyk et al., 2005). Thus, root growth was sensitive to the HCO_3^-, indicating root carboxylate accumulation in the presence of high HCO_3^- and transport of the remaining carbon pool to the shoots affecting their biomass and leaf area.

Different light intensities, air temperatures, salinity, N source and their combination have produced variable results of RZ CO_2 enrichment on hydroponic tomato biomass and RGR. At high irradiances (1500 µmol $m^{-2}s^{-1}$), 37/19 °C and 2 mM $NaNO_3^-$, high RZ CO_2 (5000 ppm) increased the biomass of both salinized (100 mM NaCl) and control plants compared to those aerated with 0 ppm RZ CO_2. The lack of biomass accumulation in control plants aerated with 0 ppm RZ CO_2 was attributed to the inhibition produced by CO_2-free aeration. On the other hand, under moderate irradiances (<1000 µmol $m^{-2}s^{-1}$) and 2 mM $NaNO_3^-$, elevated RZ CO_2 (5000 ppm) increased growth rates only of plants grown at high day temperatures (35°C) or salinized plants (100-150 mM NaCl) at more moderate day temperatures (28 °C) at 60% day/night relative humidity. In these cases, the RZ CO_2 effect increment on growth rate was 18% and 7% respectively (Cramer & Richard, 1999; Cramer & Lips 1995). These effects were comparatively small compared to the salinity-induced growth inhibition.

In the absence of a specific applied stress (low irradiances of 450 µmol $m^{-2}s^{-1}$, maximum day temperatures of 25 °C and additionally 2mM NO_3^-), high (5000 ppm) RZ CO_2 for 15 days increased mean relative growth rate (RGR) by 12% compared to those plants exposed to lower (380 ppm) RZ CO_2 (Viktor & Cramer, 2005). According to these researchers, the variability in biomass accumulation could be because of an anaplerotic provision of C for amino acid synthesis in the roots of plants forced to uptake NO_3^- under salinity conditions or because of a light dependent mechanism. They also concluded that high RZ CO_2 induces small changes in RGR of the plants in the absence of specific stresses such as salinity (Cramer & Richard, 1999) or aluminium (Cramer & Titus, 2001).

Therefore, RZ CO_2 enrichment of hydroponically-grown tomato plants does not provide any considerable benefit to plant biomass accumulation. However, these studies suggested significant biomass enhancement in certain situations where abiotic stresses are implemented. Thus, it could be interesting to investigate further the interaction

between abiotic stresses mentioned above and others such us drought, as the concentration of soil DIC in arid and semi-arid regions is often high (Bai et al., 2017).

In aeroponically grown lettuce, the effects of elevated RZ CO_2 were more consistent, although slightly variable under different environmental conditions. Applying high RZ CO_2 (2000, 10000 and 50000 ppm) to aeroponically grown lettuce in a glasshouse where all plants were exposed to fluctuating air temperature (20-36 °C) and light intensity (300-2100 µmol $m^{-2}s^{-1}$) during the day at pH 6.3, increased shoot dry weights by ~ 30%, ~ 60% and ~70% respectively after two weeks of treatment, compared to control plants exposed to ambient rhizosphere concentrations of 360 ppm CO_2 (He et al., 2007). Similar growth enhancement occurred when the same species were grown in controlled environment (CE) rooms, with elevated RZ CO_2 (2000, 10000 and 50000 ppm) increasing shoot dry weights (~20% to ~50%) under constant air temperatures of 28/22°C and irradiance of 650 µmol $m^{-2}s^{-1}$ at pH 6.5 compared to plants aerated with ambient CO_2 (360 ppm). Elevated RZ CO_2 had a greater effect at higher air temperatures of 36/30°C than more moderate temperatures (28/22°C), although the final biomass weight was higher at 28/22°C.

Root-zone temperature also affected the response to elevated RZ CO_2. High RZ CO_2 (2000, 10000 and 50000 ppm) promoted shoot and root growth to a greater extent at ambient RZ temperatures (A-RZT) of 26-38°C than a cooler temperature of 20°C-RZT. However, the final shoot fresh weight in each elevated RZ CO_2 concentration was greater at 20°C-RZT than at A-RZT and significantly higher (~20-40%) compared to ambient RZ CO_2 (360ppm) (He et al., 2013, 2016). In these studies, growth enhancement was mainly attributed to higher photosynthetic CO_2 assimilation and to a greater NO_3^- uptake (details in next section 1.5 and 1.6). Thus, variation in root-zone temperature alters plant response to elevated RZ CO_2, likely by affecting nitrate uptake. In soils, N availability and uptake is positively correlated with temperature (Dong et al., 2001).

Negative effects of RZ CO_2 enrichment have also been reported. Hydroponically grown maize, sorghum and barley in a nutrient solution supplied with 5, 10, 20 mM $NaHCO_3^-$ at pH 8 (predominant form HCO_3^-), markedly decreased plant dry weight by ~20 to ~40% compared to those plants grown without bicarbonate. In all these species, HCO_{3-} decreased shoot and root dry weight and decreased the uptake and translocation of iron (Fe) with clear visual chlorosis symptoms in plants grown at 10 and 20 mM (Alhendawi et

al., 1997). In similar experiments 5, 10, 20 mM bicarbonate (at pH 8) decreased plant dry weight of hydroponically grown maize by ~10 to ~40 % (Al Mansouri & Alhendawi, 2014), which was associated with an inhibition of root respiration Solution pH was maintained constant at pH 8 and 7 for HCO_{3-} treated and control plants respectively, therefore the assumption was that the high pH was not the direct cause of the plant biomass decreased but the high bicarbonate concentrations at this given pH level. Bicarbonate was added as Na salt, however the Na concentration added (no more than 20 mM Na) was unlikely to inhibit growth as its uptake was relatively low compared to major macronutrients. Moreover, Bialczyk et al., (1994, 2004) used both $KHCO_{3-}$ and $NaHCO_{3-}$ in their work and the positive effects in biomass accumulation were seen with both types of salt.

Applying high concentrations (10 mM) of bicarbonate or CO_2 (5000 ppm) to tomato plants grown in bags filled with a mixture of pine sawdust and wood in polytunnels had no effect on dry weight (Cramer et al., 2001). However, 6000 ppm RZ CO_2 decreased the dry weight of hydroponically grown white lupin by 15% compared to ambient RZ CO_2 of 360 ppm (Cramer et al., 2005). The range of experimental designs, plant species and environmental conditions could have led to the variable results shown in plant biomass accumulation. Although positive effects of RZ CO_2 enrichment may be interpreted as an effect of increased foliar CO_2 following CO_2 release from a leak in the root-zone, all the studies above have stated that IRGA measurements at the base of the shoot did not detect additional aerial CO_2 concentrations compared to ambient (although this data was not shown).

1.5 Effects of root-zone CO_2 enrichment on leaf gas exchange parameters

Some studies showing increased dry weight under high RZ CO_2 have correlated increased biomass accumulation with changes in gas exchange parameters (Assimilation rate (*A*), stomatal conductance (g_s), transpiration rate (*E*) and intercellular CO_2 concentration (*Ci*)). Tomato plants grown under low irradiances (500 µmol $m^{-2}s^{-1}$) at continuous 40% and 80% RH did not show significant differences in photosynthetic rates (Cramer & Richard, 1999). However, at 80% RH lower *E* and g_s were observed with elevated RZ CO_2 (5000 ppm) than plants grown at 360 ppm RZ CO_2. They concluded that the carbon supply through the xylem partially met the demand for carbon for photosynthesis, closing the stomata without detrimental effects on growth. Under high irradiances (~1000 µmol $m^{-2}s^{-1}$), *A* and

g_s were significantly lower in plants grown under 5000ppm RZ CO_2 compare to plants grown under 360 ppm RZ CO_2. In the same environmental conditions but with salinity the RZ CO_2 treatments did not alter these parameters, although values were significantly lower than non-salinised plants. Stomatal limitation of salinized plants was significantly lower in the CO_2 enriched treatment compared to the control (Cramer & Richard, 1999). These authors concluded that partial stomatal closure did not affect photosynthate availability due to root-assimilated carbon increasing biomass accumulation in plants grown under salinity, high temperature and high irradiance conditions. Plants grown in high DIC had 13-fold higher concentrations of xylem-transported carbon compounds moving to the shoot, representing 1% and 10% of the CO_2-assimilating photosynthetic activities of these plants grown at 360 ppm and 5000 ppm RZ CO_2.

At high RZ CO_2 (2000, 10 000, 50 000 ppm) and high irradiance (> 600 µmol $m^{-2}s^{-}1$), g_s was lower, *A* was higher and midday leaf water content (RWC) increased compared to aeroponically-grown lettuce plants grown at ambient RZ CO_2 (360 ppm) at both 28/22°C and 36/30°C air temperatures, indicating that partial stomatal closure did not constrain photosynthesis . The authors concluded that higher RZ CO_2 minimised leaf water deficits, enhancing NO_3^- supply thus increasing total reduced N and Rubisco amounts, thus stimulating photosynthetic rate and dry matter accumulation (He et al., 2010).

1.6 Effects of root-zone DIC enrichment on nutrient uptake, carboxylates and amino acids.

Most plants exposed to high air CO_2 concentrations exhibit enhanced growth rates thus creating greater nutrient demand, especially for N (Rogers et al., 2006; Sicher & Bunce, 2008). However, RZ DIC enrichment causes variable effects on root and shoot nutrient concentrations. At least part of these changes can be attributed to RZ DIC enrichment effects on solution pH and therefore nutrient uptake (Figure 1.9).

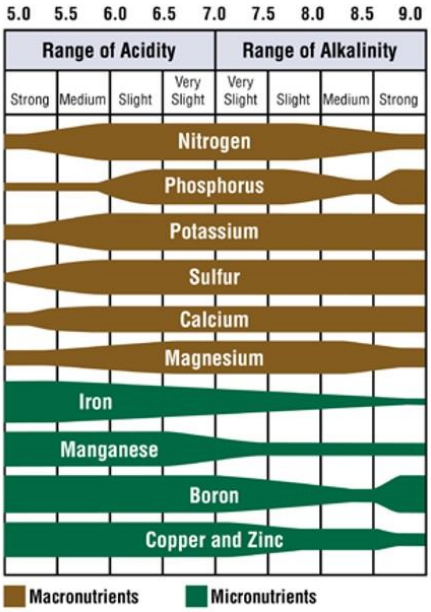


Figure 1.9. Nutrient availability changes with the pH of mineral soils. Nutrients are most available when the band is wide and less when is narrow. *Source:* Brady and Weil, 2007.

In general, increasing the concentration of HCO_{3-} by 5, 10, 20 mM decreased the uptake of potassium (K), nitrate (NO_3^-), magnesium (Mg), sulfur (S) , phosphorus (P) and iron (Fe), with exception of root calcium (Ca) uptake, in sorghum and maize plants when the pH of the nutrient solution was ~ 8 . The decreased in uptake of nutrients was correlated also with a lower shoot and root biomass (Alhendawi et al., 1997; Al mansouri et al., 2014). On the other hand, 5.68 mM HCO_{3-} increased tomato biomass accumulation, leaf blades and roots nitrogen (N) content, K content in all tissues and Ca content in roots, shoot and leaf blades. Although P content was not different from the control (Bialzyck et al., 1994). While the variability of some elemental concentrations could be due to the different species, nutrient solutions and experimental design used, it seems that bicarbonate application increases Ca in all cases. The formation of $CaHCO_3^-$ is very common in calcareous soils because Ca precipitates with HCO_3^-, therefore it could be expected that in a hydroponic system with high pH were the [HCO_3^-] is high, this also occurs.

Other studies where RZ CO_2 gas was applied were focused mainly in nitrogen metabolism with no major data related to other nutrient elements. Tomato plants grown for 60 days at elevated RZ CO_2 (2500, 5000 and 10000 ppm) decreased root N, P, K, Ca and Mg concentrations after 60 days, compared to those exposed to RZ ambient CO_2 of 370 ppm (Zhao et al., 2010). CO_2 assimilation and NO_{3-} assimilation are matched, since NO_{3-}

assimilation can progress only when CO_2 assimilation provides the carbon skeletons for the synthesis of the various amino acids. Therefore, free amino acids are precursors involved in transferring N assimilated in the root cell to the shoot.

The assimilation of DIC by roots affects carbon and nitrogen metabolism of the whole plant. During growth, the nitrogen demand for the formation of cellular matter is met by inorganic nitrogen mainly from assimilation in the roots of the NO_3^- or ammonia (NH_4^+) present in water or soil. In aerobic soils where nitrification can occur, NO_3^- is usually the predominant form absorbed by the roots. A proton gradient across the plasma membrane drives the uptake of nitrate against a concentration gradient and is either reduced, stored in the vacuoles or translocated to the shoot for reduction and vacuolar storage. Cytosolic nitrate reductase (NR) produces nitrite (NO_2^-) which enters the plastid (chloroplast in the leaves) and is reduce to ammonium by nitrite reductase (NIR) (Figure 1.10).

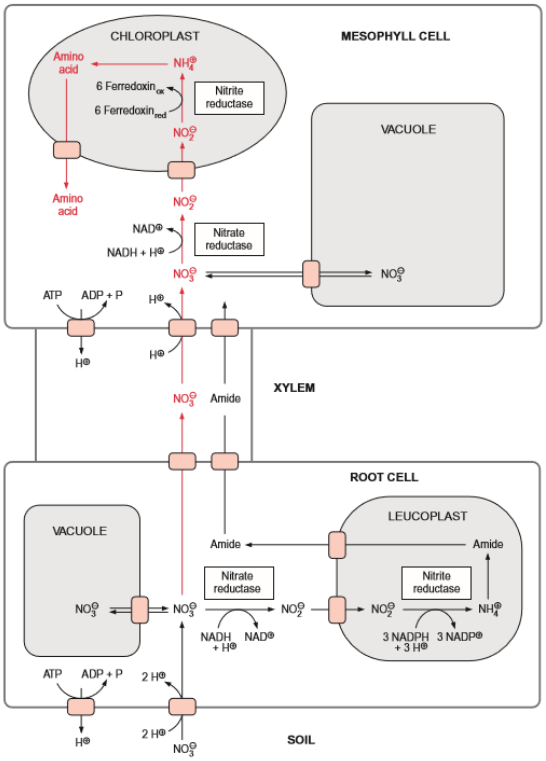


Figure 1.10. Nitrogen assimilation in the roots and leaves of a plant (Heldt, 2011).

NH_4^+ is fixed by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway where nitrogen metabolism must interact with carbon metabolism, since GS activity requires energy in the form of ATP and GOGAT uses C skeletons and reductant in the form of 2-oxoglutarate (2OG) which is synthesised either by an isocitrate dehydrogenase or an aspartate aminotransferase (AspAT). AspAT origin produces 2OG at the expense of

glutamate (Glu) leading to the production of aspartate (Asp) from oxaloacetate (OAA) synthesized via PEPc (Lancien et al., 2000) (Figure 1.11). This pathway is crucial, since the glutamine (Gln) and Glu produced are donors for the biosynthesis of major N-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Lea & Ireland, 1999). Therefore, the sources of NH_4^+ for the synthesis of amino acids, are both, NO_3^- after being reduced to NH_4^+ and also the one directly taken as NH_4^+.

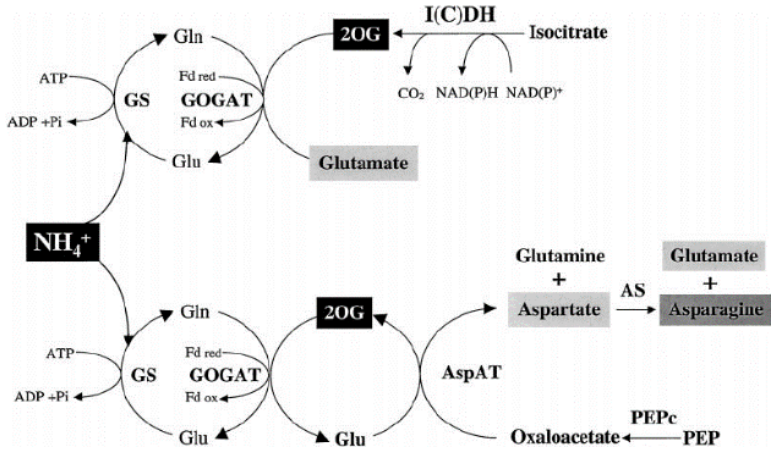


Figure 1.11. Primary amino acid synthesis by the GS/GOGAT cycle in plants. Lancien et al., 2000.

Elevated RZ CO_2 (5000 ppm) stimulated the activity of GS in roots and leaves of hydroponically grown tomato plants which also was correlated with an increased in the NR activity and higher total and reduced NO_3^- in roots. However, root GOGAT activity was unaltered and its activity was higher than GS only when salinity stress (100 mM NaCl) was applied meaning that both enzyme activities were not strongly coupled (Cramer et al., 1999). They suggested that GS activity was higher because NO_3^- uptake and assimilation increased under elevated DIC. In salinity condition and with RZ CO_2 enrichment, NO_3^- uptake of salinized (100 mM NaCl) plants was higher than in non-salinize tomato plants. Carbon labelling ($NaH^{14}CO_3$) of those plants showed an increase in the proportion of ^{14}C allocated to amino acids, which indicated that DIC provides carbon skeletons for the formation of amino acids (Cramer & Lips, 1995). It was hypothesized that because in salinity conditions proline accumulation occurs, proline synthesis from glutamate might depend on C provision from PEPc which had higher *in vitro* activity than control plants (Cramer et al., 1999).

On the other hand, the total concentration of amides and amino acids in the xylem sap of tomato plants grown at 5 mM HCO_3^- and NH_4^+ was 2.6 times greater compared to those fed with NO_3^-. Glutamine was the main component in the xylem sap of all plants exposed to different treatments. RZ bicarbonate (5 mM HCO_3^-) enrichment increased the concentration of amides and amino acids in the xylem sap about 17% when supplied with NO_3^- and by 56% when supplied with NH_4^+ (Bialzyck, 2004).

Elevated (6500 ppm) RZ CO_2 increased short-term (6-8 hours) NO_3^- uptake in NR-deficient mutants of barley (Cramer et al., 1996), and in wild-type tomato plants aerated with 5000 ppm CO_2 over 6-12 hours (Van der Merwe & Cramer, 2000). DIC was proposed to directly stimulate NO_3^- uptake rather than indirectly affect NR activity and subsequent amino acid synthesis. Increased rhizosphere DIC may stimulate NO_3^- uptake through a direct exchange of cytoplasmic HCO_3^- for NO_3^-. The cytoplasmic pH is assumed to have a pH 7, thus the predominant inorganic carbon species would be HCO_3^-. If there is external CO_2 enrichment, the CO_2 would convert to HCO_3^- , producing a positive gradient for the movement of HCO_3^- from the cytoplasm to the external solution, which could modify NO_3^- uptake (Van der Merwe & Cramer, 2000). However, RZ CO_2 concentration had no influence on long-term NO_3^- uptake (15 days) , although a greater initial uptake could have contributed to a higher relative growth rate of NO_3^- fed plants grown with 5000 ppm RZ CO_2 than those grown at 380ppm RZ CO_2 . In this case, increased growth rate in NO_3^- fed plants with elevated RZ CO_2 was associated with transfer of root-derived organic acids to the shoot and conversion to carbohydrates, while carbon allocation into amino acids was lower compared to NH_4^+ fed plants (Viktor & Cramer, 2005).

Elevated RZ CO_2 increased leaf NO_3^- and total reduced N concentration in aeroponically growth lettuces, which was correlated with increased *A* (He et al., 2010).They deduced that the productivity could also have been partially due to DIC incorporation which allowed greater incorporation of N into amino acids in the roots as a consequence of a greater supply of anaplerotic carbon for protein synthesis.

^{14}C labelling studies have recovered only a small proportion (<5%) of carbon in the inorganic form in root and shoot tissues (Cramer et al., 1999), indicating that carbon derived from DIC is delivered to the shoot in the form of an organic compound. C and N absorbed by the roots can also be used in carboxylate synthesis (Bialczyk & Lechowski, 1995; Bialczyk et al., 2004), with malate, citrate , fumarate and succinate identified in

hydroponically-grown tomato xylem sap. Adding 5mM HCO_3^- to the medium increased the concentration of carboxylates, mainly malate (Bialczyk et al., 2004). Malate is a key product in plant metabolism and participates in processes such as respiration and energy generation, photosynthesis (both C3 and C4), fatty acid oxidation, lignin biosynthesis, stomatal function, nitrogen fixation and amino acid biosynthesis, ion balance, uptake of phosphorus and iron and aluminium tolerance (Gietl, 1992; Kochian, 1995; Martinoia and Rentsch, 1994). Oxaloacetate may be reduced to malate (Cramer & Lips, 1995) or be aminated to aspartate and asparagine (Cramer et al., 1993). Under deficit irrigation, malate concentration increases in xylem sap causing alkalinisation and indirectly producing stomatal closure (Korovetska et al., 2014), which could also be linked with the stomatal closure shown in previous studies where elevated RZ CO_2 was applied.

Xylem sap pH, nitrogen uptake, carbohydrates, amino acids, and carboxylates could all play a signalling role under elevated RZ CO_2. Many of these compounds could also interact with other compounds such as phytohormones. Some studies showed an increased uptake of NO_3^- while others a decreased in the uptake by elevated DIC. Moreover, there is a different effect of DIC in the uptake of NO_3^- and NH_4^+. While further research is required to elucidate the effect of high RZ CO_2, it is also necessary to understand better the interaction between DIC and N/C metabolism.

1.7 Effects of RZ CO_2 enrichment on phytohormones

Enoch and Olsen (1993) suggested that CO_2 could act as a plant hormone or at the very least influence plant hormone systems, based on the interaction that ethylene (ET) has with CO_2 and HCO_{3-} , as CO_2 can block or promote physiological effects of ET. Ethylene is a plant hormone involved in different processes such as stimulation of germination (Corbineau, 2014), positive regulator of root hair development (Song et al., 2016), negative regulator of root nodulation (Guinel, 2015), promotion, inhibition or induction of organs senescence and abscission, differential cell growth, stress responses and resistance to necrotrophic pathogens (Davies, 2004). In closed environments, elevated ET levels can cause shortened height, epinasty, leaf rolling, premature leaf senescence, and sterility (Abeles et al., 1992;Morison & Gifford, 1984). ET in the soil can inhibit root growth of various plants (Visser et al., 1997, Pierik et al., 1999). However, in some cases high CO_2 concentrations (2-10% CO_2) inhibits the biological activity of ET (Sisler & Wood, 1988).

The interaction between CO_2, ethylene and other hormones such auxins and cytokinins are complex and there is limited research focusing on the relationship between elevated ambient CO_2 (eCO_2) and plant hormones. eCO_2 (700 pm) increased biomass production (29%) of *Arabidopsis thaliana*, while IAA (indole-3-acetic acid, by 13.7%), GA_3 (gibberellic acid, by 55.4%), ZR (zeatin riboside, by 15.6%), DHZR (dihydrozeatin ribosidem, by 55.9%) and iP (isopentenyladenosine, by 74.6%) increased and ABA (abscisic acid, by 15.2%) content decreased (Teng et al., 2006).

Contrary to this eCO_2 (550 ppm) increase the abundance of transcripts of ABA-responsive genes of *Arabidopsis thaliana* (Li et al., 2006). eCO_2 also downregulates jasmonic acid (JA), ET and enhanced salicylic acid (SA) signalling (DeLucia et al, 2012) In a hydroponic tomato, root IAA content and ET evolution was increased by eCO_2 by 26.5% and 100% respectively (Wang et al., 2009). The same effects were found in previous studies were in response to the elevated CO_2 conditions, IAA concentration significantly increased in tomato roots, promoting the growth of roots and stimulating ET production by 1-aminocyclopropane-1-carboxylic acid synthase activation (Abeles et al., 1992; Kende, 1993). These studies applied suboptimal ambient CO_2 concentrations, and the responses found could be of relevance when applying high RZ CO_2.

There is apparently just a single study which measured hormones in plants grown with RZ CO_2 enrichment. Elevated RZ CO_2 (2500, 5000 ppm) in aeroponically grown muskmelon decreased significantly the root growth while xylem sap IAA, tZ (trans-zeatin) and GA_3 were significantly decreased and ABA concentration significantly increased (Li et al., 2009). Further studies seem necessary to understand how CO_2 interacts with phytohormones, as both play a key role in crop productivity.

1.8 Conclusion and aims of the thesis

Overall, in hydroponic tomato plants, high RZ CO_2 and optimum concentrations of HCO_3^- increased biomass accumulation. This effect was highlighted when plants were exposed to high irradiances, salinity stress or high shoot temperatures. However, positive effects also were obtained at lower irradiances when combined with other environmental parameters such as high temperature and salinity (Cramer et al., 1996, 1999). Moreover, in aeroponically grown lettuce plants supplied with elevated RZ CO_2 increased shoot and

root growth when plants were exposed to high temperatures and moderate irradiances (He at al., 2007, 2010). However, RZ CO_2 enrichment also decreased growth in some studies (Figure 1.8). In each of these studies, there was no consistent single effect on plant growth. Multiple effects were correlated with DIC uptake, including altered leaf gas exchange, effects on nutrient uptake, direct utilization of C and N for the synthesis of carboxylates and amino acids and their transport and metabolism in the plant. Therefore, there is a complex relationship between elevated RZ DIC and other factors, such as temperature, irradiance and nutrient availability which are related to plant physiological processes and their response to high RZ DIC. These relationships are still not fully understood.

This thesis aims to understand the effects of elevated RZ CO_2, and HCO_{3-} on growth and physiological responses of tomato, pepper and lettuce plants. The effects of high RZ DIC on biomass accumulation have been very variable in the past and more studies are needed to understand the interaction between biomass accumulation in different systems and environmental conditions (Chapter 2). Much attention has been paid on nitrogen assimilation of RZ DIC enriched plants, but it is necessary to have a broader view on the effects on other nutrients and amino acids (Chapter 3). There seems to be important interactions between CO_2 and some phytohormones, but there is a lack of information on the possible mechanisms (Chapter 4). Since no clear conclusion about the growth regulatory effects of enriched RZ DIC on plants exists, non-targeted gene expression studies provide an opportunity to establish putative plant signalling mechanisms (Chapter 5) that could be tested in future work.

Chapter 2. Effects of elevated root-zone dissolved inorganic carbon on biomass and leaf gas exchange in lettuce, pepper and tomato.

2.1 Introduction

Although it is well known that CO_2 is absorbed through the stomata in the leaf, many studies have observed that roots are able to take up dissolved inorganic carbon contained in soils as well as gaseous CO_2 respired by the roots (Chapter 1). Some of these studies have correlated below-ground carbon availability with increased growth of horticultural species such as potato, tomato, lettuce and maize (Arteca et al., 1979; Cramer et al., 1999; He et al., 2010). Plant roots and shoots may respond differently to different RZ HCO_{3^-} and CO_2 concentrations via developmental, growth and other physiological changes, as reflected in the considerable variability in plant growth responses (Figure 1.8). However, few studies have compared the effects of different RZ carbon sources on plant growth within a single growth facility.

Although the effects of increased atmospheric CO_2 concentrations have been extensively studied, little is known about the belowground effects of DIC. The plant RZ may be ideal for the localised enrichment of the growing environment with HCO_3^- or CO_2. Previous studies have highlighted the potential of this approach for increasing crop growth and productivity. Elevated RZ CO_2 (5000 ppm) increased the biomass of hydroponically grown tomato by up to 2-fold (Cramer et al., 1999) whilst increasing the concentration of CO_2 in the RZ to supra-optimal levels benefits plant growth and productivity. Enriching the RZ to 45000 ppm CO_2 of hydroponically grown potato substantially increased tuberization, and increased stolon length, number of tubers per stolon, and overall dry weight (Arteca et al., 1979). CO_2 enrichment (2000-50000 ppm) of the RZ of aeroponically grown lettuce increased shoot and root dry weight by ~1.6-fold and ~1.8-fold respectively (He et al., 2010). However, to our knowledge, the effects of enriching the RZ to the optimal CO_2 concentrations (700-1500ppm) currently used for the large-scale CO_2 enrichment of the aerial environment (or to levels considered sub-optimal with respect to the aerial environment) has not been considered.

Plant photosynthetic responses to elevated RZ CO_2 have demonstrated contradictory results. Elevated RZ CO_2 showed no changes or lower photosynthesis and lower stomatal conductance (Cramer et al., 1999), whereas aeroponically grown lettuce increased photosynthesis and decreased stomatal conductance at high RZ CO_2 (He et al., 2010). While photosynthesis and biomass accumulation were not directly correlated, maximal *A* increased with the total reduced N concentration of the shoot under different elevated RZ CO_2 concentrations (He et al., 2010). The lower *A* and g_s in tomato plants was interpreted as a "down-regulation" of the photosynthetic system (Cramer et al., 1999). Because an additional carbon supply to the plant might alter leaf gas exchange and therefore biomass accumulation, gas exchange parameters should be measured in trying to explain how RZ CO_2 increases biomass accumulation.

In this chapter, pepper, lettuce and tomato were used to assess the effects of RZ DIC on growth. Tomato and pepper are mostly grown outdoors in southern European countries (Spain and Italy) where the environmental conditions are optimal for their development. In northern European countries which experience severe weather, they are mainly grown in greenhouses in hydroponic systems. Lettuce, on the other hand, grows well in cold weather and is mainly grown in open fields in northern Europe. However, because hydroponics confers significant pest and disease control advantages, more growers are shifting to soilless culture cultivation. Most experiments that have studied the uptake of DIC by plant roots have used soilless culture systems. These approaches avoid the difficulty of measuring the overall DIC applied in the RZ of soil-grown plants, where other factors such CO_2 release from microbial respiration could compromise the experimental design. While most authors have retained the same experimental designs in their studies (Chapter 1), none have investigated different approaches for RZ DIC enrichment in different environments, with a variety of horticultural species. Therefore, the aim of this chapter is:

1. To explore the effects of elevated RZ DIC on plant physiological responses in a range of UK environmental conditions in the greenhouse, and in a controlled environment with regulation of temperature, light and relative humidity.
2. To determine if (and in which chemical form) the plants are taking up carbon through the roots.
3. To investigate whether high RZ DIC affects leaf gas exchange.

2.2 Materials and methods

2.2.1 Plant Material

Crisphead lettuce (*Lactuca sativa* cv. Consul; cv. Antartika; cv. Donovan) and butterhead lettuce (*Lactuca sativa* cv. Sunstar) seeds were purchased from Moles Seeds (Essex, UK) and Hazera Seeds (Lincolnshire, UK), respectively. Pepper (*Capsicum annuum* (L.) cv. Bellboy F1) and tomato (*Solanum lycopersicum* (L.) Mill. cv. Alisa Craig) seeds were purchased from Moles Seeds (Essex, UK).

For the hydroponic system, seeds were grown in 84-cell plug trays (Length 52 cm x Width 32 cm x Depth 5 cm, plug size 3.8 cm square) containing vermiculite and germinated in the glasshouse or controlled environment room (CE room) depending on the experiment location (section 2.2.5). Plants were transferred to hydroponic culture at 2-4 leaf stage, after rinsing the roots in distilled water (dH_2O) water.

For the aeroponic system, seeds were individually sown in 150-cell plug trays in 2 cm x 2 cm x 4 cm rockwool cubes (Growell, Ltd, UK) and germinated in the glasshouse or CE room depending on the experiment location. Plants were transferred to the system at the 4 leaf-stage in lettuce and at 2-4 leaf-stage in tomato and pepper.

2.2.2 Hydroponic system design for RZ HCO_{3^-} and CO_2 application

To determine the effect of HCO_{3^-} enrichment in the rhizosphere, deep flow hydroponics system (DFTS) were built for each crop (Figure 2.2). Individual 16 L boxes and lids (Wilkinson's Stores, Lancaster, UK) of 17.2 cm height, 42.7 cm width and 32.8 cm depth, were painted black to avoid light penetration and therefore the growth of algae. Prior to the nutrient solution preparation, the major element concentrations in the water of the glasshouse and CE room were analysed (Table 2.1), to determine whether these were suitable for general hydroponic use (Smith, 1999; Whipker et al., 2003). Each box contained 14 L of half-strength Hoagland solution. The composition of the nutrient solution was 0.5 mM NH_4NO_3, 1.75 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2.01 mM KNO_3, 1.01 mM KH_2PO_4, 0.5 mM $MgSO_4{\cdot}7H_2O$, 1.57 µM $MnSO_4{\cdot}5H_2O$, 11.3 µM H_3BO_3, 0.3 µM $CuSO_4{\cdot}5H_2O$, 0.032 µM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1.04 µM $ZnSO_4{\cdot}7H_2O$ and 0.25 mM NaFe EDTA. (Hoagland & Arnon, 1950). Details of the nutrient solution preparation can be found in Appendix 2,

Table 1. Bicarbonate was added in the form of $NaHCO_{3-}$ at 1, 5, 10 and 20mM to the nutrient solution.

Table 2.1. Elemental analysis of tap water used for hydroponic culture in the Lancaster glasshouse and CE room.

Element	Level	Desired level[1]
Sodium (Na)	13.04 ppm	<50 ppm
Calcium (Ca)	10.55 ppm	<71 ppm
Potassium (K)	0.65 ppm	<10 ppm
Magnesium (Mg)	1.48 ppm	< 5ppm
Phosphorus (P)	1.87 ppm	< 5ppm
Sulphur (S)	2.20 ppm	< 30ppm

[1] Source: Whipker, et al., 2003.

The lids (43 x 33 cm) were modified with four 2.5 cm holes in each quadrant to hold four plants per box (4 x 0.14 m^2). In the middle of the lid, an additional hole was cut to accommodate a closed cell foam piece through which an external diameter 6 mm pipe was inserted.

The end of the pipe outside the box was connected to an aquarium air pump (All Pond Solution Ltd, Middlesex, UK) which continuously supplied ambient air (Flow rate: 3.2 L min^1) to add O_2 to the nutrient solution as well as stirring it. The medium was changed every 3-4 days and the pH was maintained at 6.4 (at which CO_2 and HCO_{3-} concentrations are equivalent) by adjusting the pH via dropwise addition of 1N HCl or NaOH once every day. The initial EC of the nutrient solution was 1000 ± 2 µS/cm. There was minimal variation over the course of the experiments with average values of 1042 ± 9.8 µS/cm for 0 mM; 1118 ± 8.4 µS/cm for 1 mM; 1481 ± 16.3 µS/cm for 5 mM; 1952 ± 21.7 µS/cm for 10 mM; and 2870 ± 33.9 µS/cm for 20 mM. A DO700 hand-held multi-parameter instrument was used to monitor daily changes of pH (Figure 2.1, A) (Extech Instrument, MA, USA). Single daily measurements of total alkalinity were taken after acid addition using the Alkalinity test kit, HI-3811 (Hanna Instruments, Ltd, USA). Bicarbonate is depleted after 3-4 days, meaning re-application is necessary when nutrients are replaced (Figure 2.1, B).

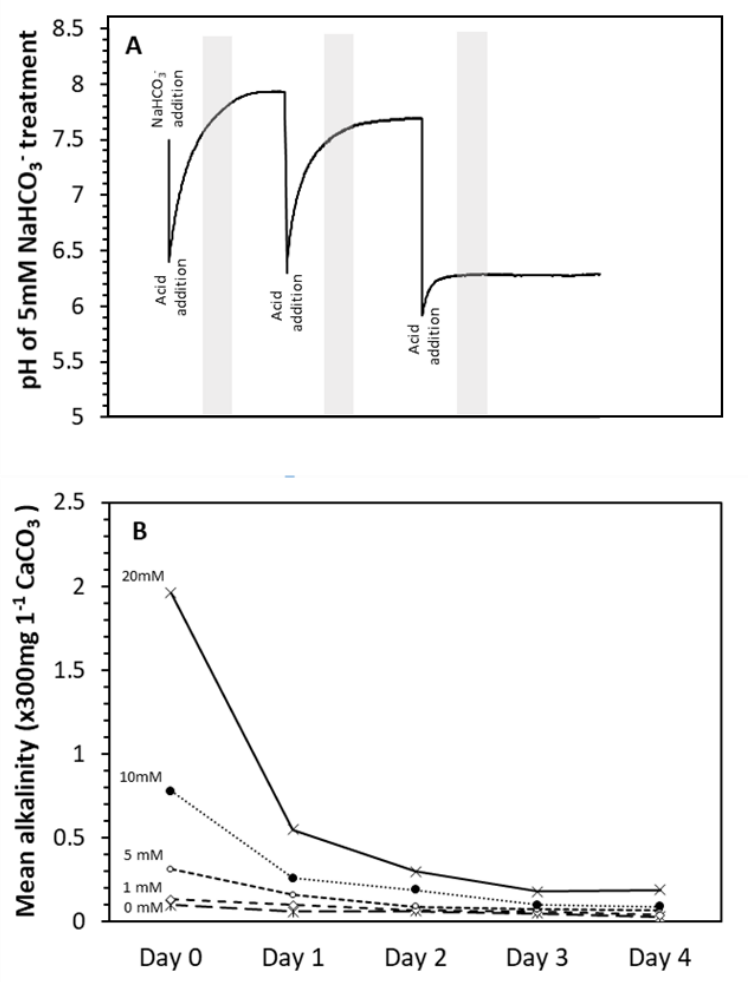


Figure 2.1. Effects of 5 mM $NaHCO_3^-$ on nutrient solution pH (A), and total alkalinity (B). Grey section indicates night time period. Alkalinity measurements were done at 9 am every morning.

The hypocotyls of the plants were inserted through a closed foam cell in each hole and they were grown for 10-15 days.

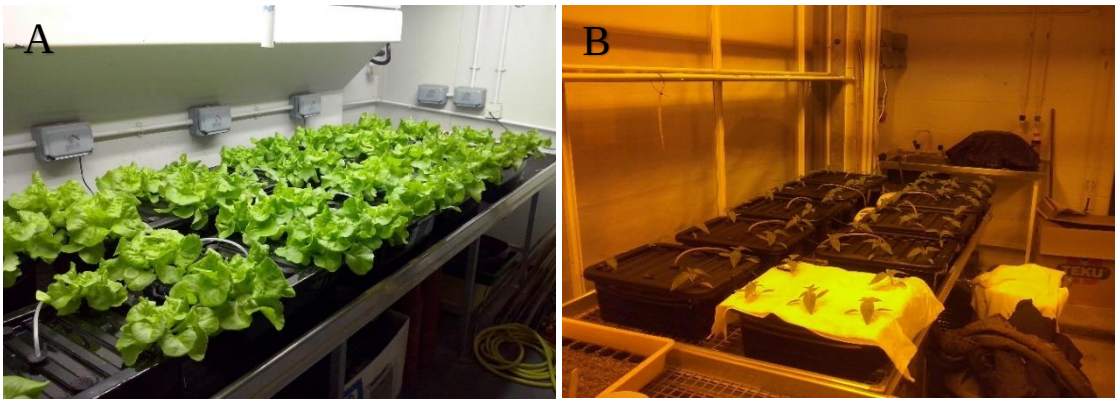


Figure 2.2. Lettuce (A) and pepper (B) hydroponic systems in the CE room and glasshouse respectively.

To determine the effects on biomass of elevated RZ CO_2 (2000 ppm), the same hydroponic system above was used with some modifications to apply CO_2 into the nutrient solution. The pipe connected to the aquarium air pump was inserted through a hole in the lid and the end of the pipe was connected to a tank air curtain (All Pond Solution Ltd, Middlesex, UK) , to create a curtain of air bubbles across the tank and therefore distribute the bubbles along the box. The CO_2 was supplied as described in Section 2.2.4.

2.2.3 Hydroponic system design to determine $NaHCO_3^-$ uptake

Two time-course experiments were developed (Table 2.2). The hypocotyls of the plants were inserted through a closed cell foam collar and the nutrient solution in each pot was constantly aerated through a 6 mm pipe connected to an air pump.

In the non-recirculating system, ten lettuce plants (*Lactuca sativa* cv. Sunstar) were each placed in a 300 mL jar with nutrient solution (Figure 2.3). After 3 days, 1 mM ^{13}C labelled sodium bicarbonate was added to four jars at 08.30. The experimental protocol is summarised in Table 2.1. Two plants were harvested at 08.00 (non-enriched controls) and another two plants (control and enriched) were harvested 4, 8, 12 and 24 hours after the $NaH^{13}CO_3$ was added. At harvest, plants were divided into leaves and roots, which were rinsed 3 times in dH_2O to remove any nutrient solution.

Table 2.2. Time - course of bicarbonate labelling experiment.

Time	**08.00 hrs**	**08.30 hrs**	**12.00 hrs**
Action	Harvest two non-enriched plants	Add 1 mM ^{13}C labelled sodium bicarbonate to nutrient solution	Harvest two enriched plants and two non-enriched plants
Time	**16.00 hrs**	**20.00 hrs**	**8.00 hrs+1Day**
Action	Harvest two enriched plants and two non-enriched plants	Harvest two enriched plants and two non-enriched plants	Harvest two enriched plants and two non-enriched plants

Figure 2.3. Lettuce in hydroponics system for $NaH^{13}CO_3$-labelling experiments.

Three recirculating hydroponic systems were used, each containing 5 lettuce plants, with each one placed in a 300 mL jar (Figure 2.4):

1) Control system with half-strength Hoagland solution with the pH 5.8 manually adjusted at the beginning of the treatment.
2) Labelled ($NaH^{13}CO_3$ addition) system with naturally fluctuating pH (Figure 2.5).
3) Labelled ($NaH^{13}CO_3$ addition) system with pH constantly controlled at pH 5.8 using a pH automatic controller (pH Kontrol 01, Prosystem Aqua).

$NaH^{13}CO_3$ addition occurred at 08.30, three days after plants were introduced to the systems. Prior to adding the label at 08.00, three plants, one from each system, were harvested and divided into leaves and roots, which were rinsed 3 times in dH_2O. The same procedure was performed 4, 8, 12 and 24 hours after the $NaH^{13}CO_3$ was added.

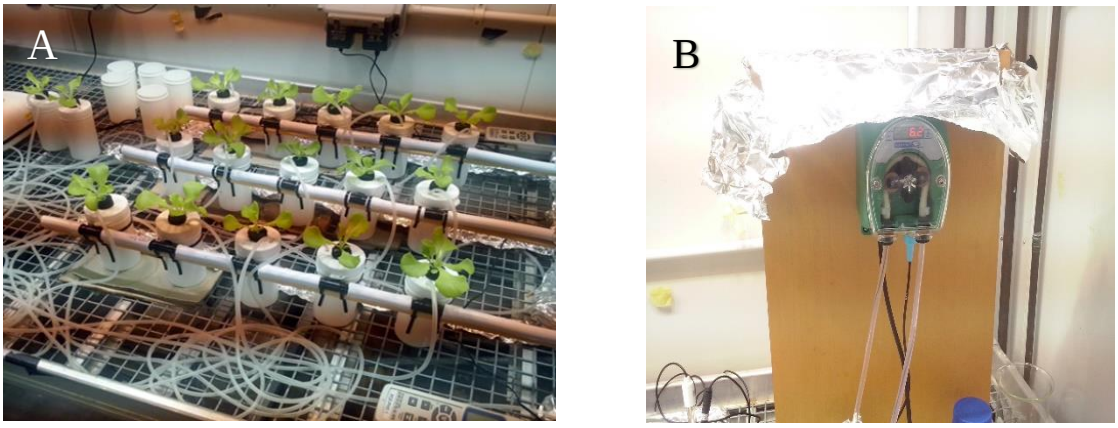


Figure 2.4. Lettuce in a recirculating hydroponic system (A) and the pH automatic controller (B).

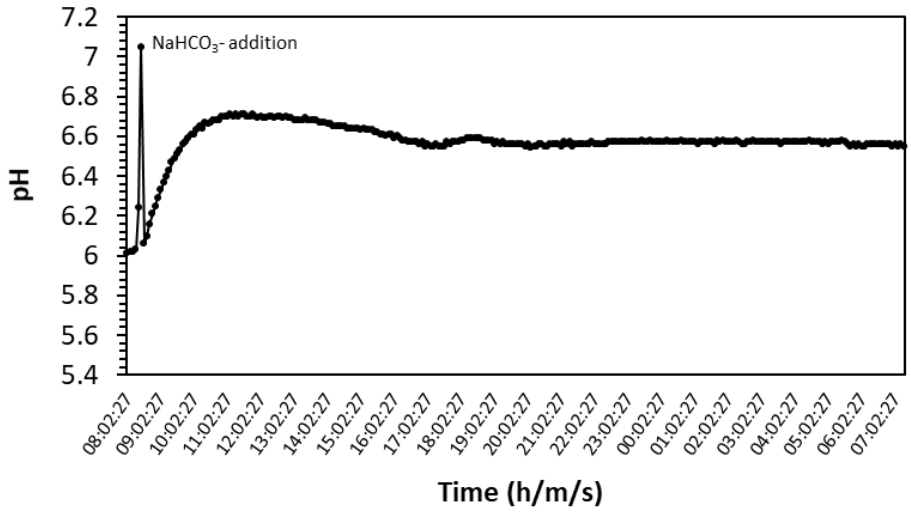


Figure 2.5. pH values *versus* time in the labelled ($NaH^{13}CO_3$ addition) system. Maximum pH after adding 1 mM $NaH^{13}CO_3$ was 7.05. The prevalent form of carbonates at pH ≤6.36 is CO_2 and H_2CO_3, at pH between 6.36 and 10.33 is HCO_3^-, and CO_3^{2-} is predominant at pH >10.33 (Lindsay, 1979).

2.2.4 Aeroponic system design

Two Platinum Aero Pro 8 (Platinum aero pro-8, Platinum Hydroponics, Rf. 038-006-2191) systems were modified for RZ CO_2 application (Figure 2.6). Each system contained 8 mapitot pots of 30 x 25 x 25 cm and 12 L capacity. The pots were placed in their own 60 L tank of 60 x 110 cm. A water pump (All Pond Solution Ltd, Middlesex, UK) placed in the tank, delivered Hoagland nutrient solution to each pot through a 6 mm tube drip inserted in the left-upper corner of each lid. A nebulizer (Leroy Merlin, Spain Ref.16936710) (flow rate: 12-14 L h^{-1}) was connected to this tube to mist the roots continuously.

Figure 2.6. Platinum Aero Pro 8 system modified to apply CO_2 gas into the root zone.

The nutrient solution drained back to the tank, making it a re-circulating system. In the middle of each lid, a 5 cm hole contained a mesh pot to hold the plant. The pH was monitored daily to have a near-constant pH between 6 – 6.4 by manually adjusting each day with dropwise 1N HCl or NaOH addition (Figure 2.7).

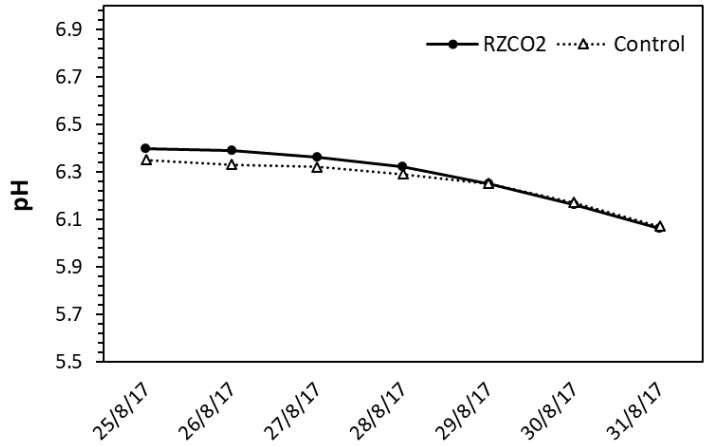


Figure 2.7. Nutrient solution pH variation in control and RZ CO_2 enriched aeroponic tanks.

One bin was supplemented with CO_2 (1500 ppm) while the other was supplied only with compressed air (400 ppm). The CO_2 gas flowed from an industrial grade size VK cylinder (BOC group Ltd, Guildford, UK) to a flow meter through an external diameter 6 mm pipe (Air Hose Clear Polyurethane, RS Components, UK) .The CO_2 delivered from the outlet connection of the flow meter was pre-mixed with ambient air by a Y piece push-in fitting (Legris 3140 Pneumatic Y Tube-to-Tube Adapter, RS Components, UK) and a serpentine tube. The compressed ambient air was supplied through a 6 mm tube connected to the faucet of the glasshouse (Figure 2.8, A).

The pre-mixed air ended in a sealed mixing box (Figure 2.8, C) where the air was passively mixed and extracted by an air pump (All Pound Solution Ltd, Middlesex, UK). The CO_2 concentration inside the box was monitored continuously using a CO_2 gas analyser (PP Systems, WMA-4) (Figure 2.8, B). The enriched air was distributed to each pot by a series of 6 mm pipes.

Figure 2.8. VK CO_2 cylinder and the glasshouse faucet where the compressed air was supplied from (A). CO_2 gas analyser (B) and mixing box which delivered the mixed air to each pot in the system, connected to the analyser (C).

To prevent leakages in the bin, each lid was sealed with self-adhesive rubber foam (Rubber staff Ltd, Bath, UK) around the rim. To check for leakages, the air above the lid, at the shoot base, and around the system was routinely sampled with a LI-COR 6400 with no significant difference compared to the ambient air (Figure 2.9). The hypocotyls of the plants were inserted through a collar made with a layer of parafilm, aluminium foil and an impermeable CO_2 sealant (Qubitac, Qubit Systems Inc., Canada) in the mesh pot. In some cases, when the hypocotyl was sufficiently long, a close cell foam was used instead.

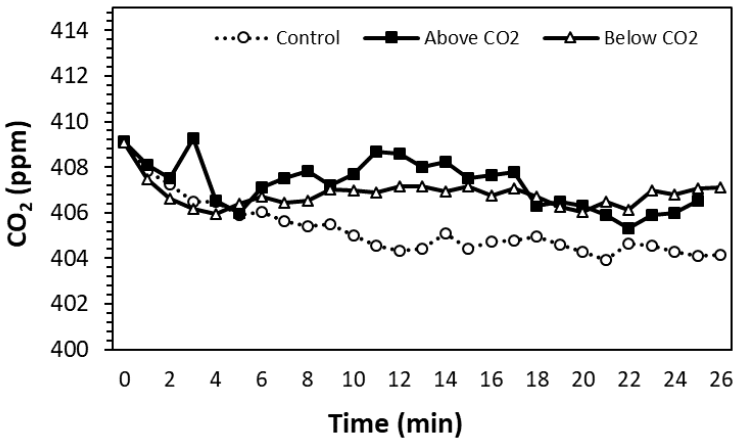


Figure 2.9. LI-COR measurements checking for leaks (greater than ambient CO_2 in the greenhouse = Control) above and below the shoot base when applying 2000 ppm CO_2. Each point represents individual measurement per minute done by the LI-COR.

Nutrient solution dissolved oxygen concentrations (DOCs) and temperature of one mapitot pot from each of the treatments was measured during 24 h in October 2018 using a dissolved oxygen meter (PCE-PHD1, PCE instruments Ltd, UK). Because of limited instrumentation, the first 24 h of measurements occurred in the pot submitted to elevated

RZ CO_2 and the second 24 h of measurements occurred in the pot under ambient RZ CO_2. On both days, DOC increased during the night period compared to day DOC values. However, under RZ CO_2 when the lights switched on and the temperature started increasing during the first hours of the morning, the DOC decreased from 6.5 mg/L to 4 mg/L and the values recovered to around 5 mg/L during late night. Nutrient solution temperature on the first day was slightly higher during the day, reaching a maximum of 32°C compared to the 30°C on the second day. In general, under elevated RZ CO_2 the oxygen was slightly lower. However, these DOCs measured in the nutrient solution were within acceptable limits according to previous studies on lettuce grown in deep flow hydroponics. Dissolved oxygen concentrations of at least 4 mg/L were recommended for optimum lettuce growth and development, although neither root damage nor delay of shoot growth was observed among treatments receiving different DO concentrations (2mg/L – 17mg/L) (Goto et al., 1997). In the worst-case scenario, with high greenhouse temperatures, DOCs were maintained above 3 mg/L (Figure 2.10, A&B). On average, the temperature inside the box was three and five degrees higher during the day and night respectively, compared to ambient air temperature (Figure 2.10, C).

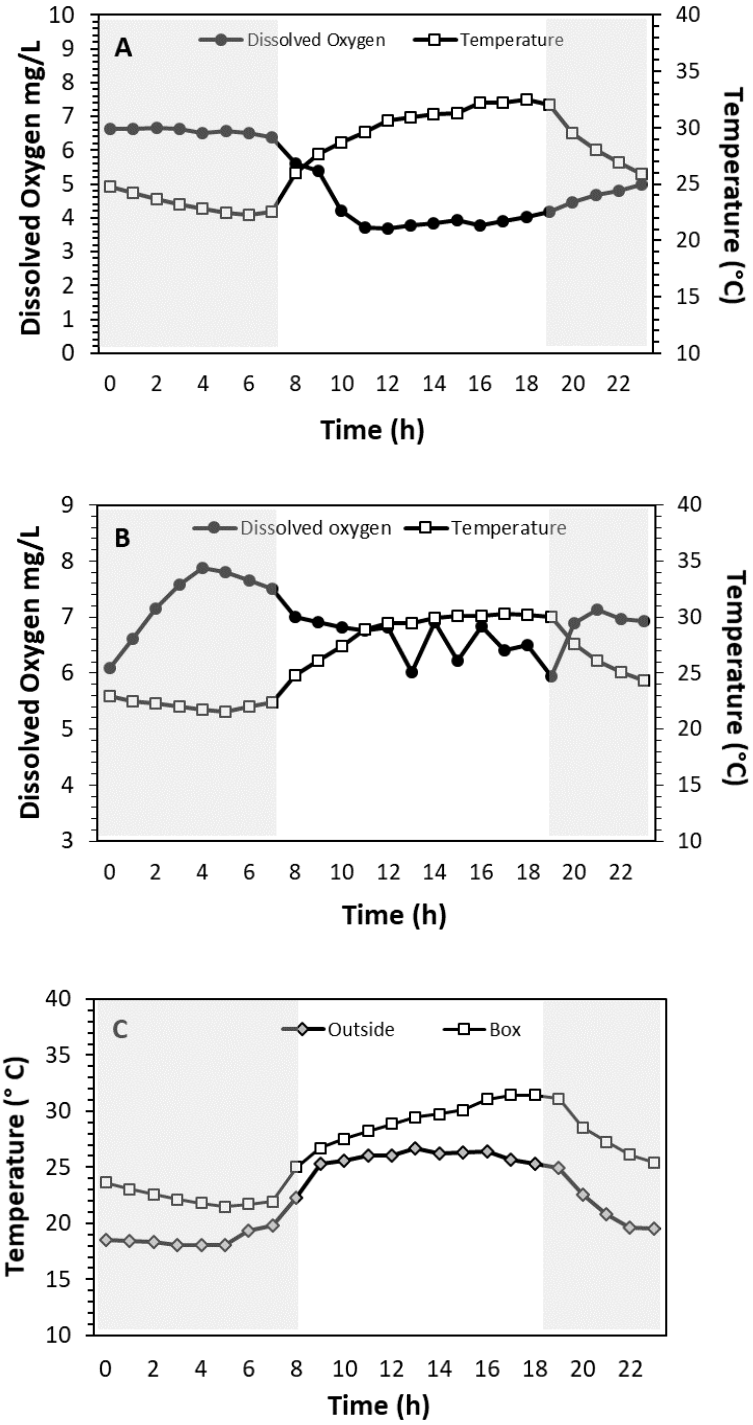


Figure 2.10. Dissolved oxygen concentration (DOC) and temperature of the box with elevated RZ CO_2 for a period of 24h (A) and the control box with ambient RZ CO_2 (B) along with ambient glasshouse temperature (C) during 7 (A) and 8 (B, C) of October 2018. Grey sections in the graphs indicate night-time. Each point is the mean value per hour with day and night indicated by lack of shading and grey shading respectively.

To better understand the nutrient solution dissolved oxygen concentration dynamics (Figure 2.10), the mean day/night temperature was plotted against the dissolved oxygen concentration in the box. Irrespective of RZ CO_2 enrichment, nutrient solution temperature was negatively correlated with oxygen levels. At the same temperatures (25-29°C), oxygen levels were lower under elevated RZ CO_2 than the control box, which means that an elevated RZ CO_2 and high temperatures deplete oxygen levels in the RZ, probably by stimulating respiration rate.

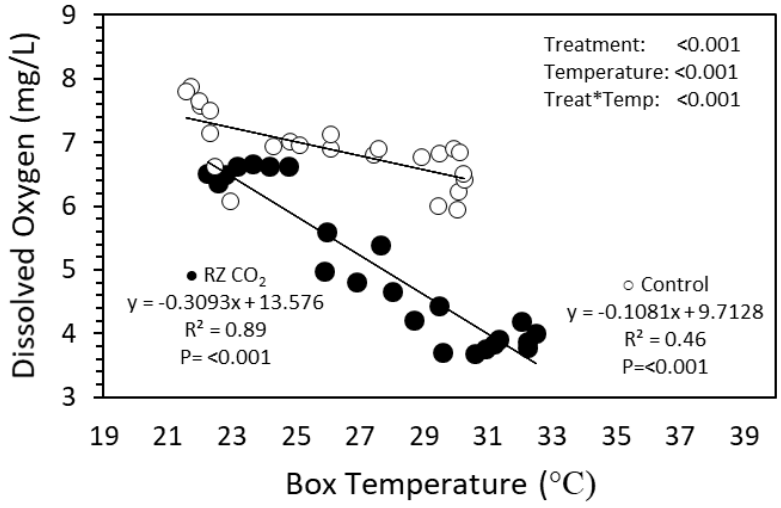


Figure 2.11. Dissolved oxygen (mg/L) plotted against mean box temperature. The regression equation, R^2 and significance of the regression (P value) are given. p-values determined by ANCOVA for each main effect (*x*-variable and treatment) and their interaction are reported. Primary data were derived from Figure 2.10.

2.2.5 Location of the studies

Multiple experiments in different systems assessed plant growth (Table 2.3). Six hydroponic experiments applying either bicarbonate or CO_2 were performed, while ten aeroponic experiments applying CO_2 gas were carried out, with different measurements made according to instrument availability and to supply plant material for biochemical and genetic analysis.

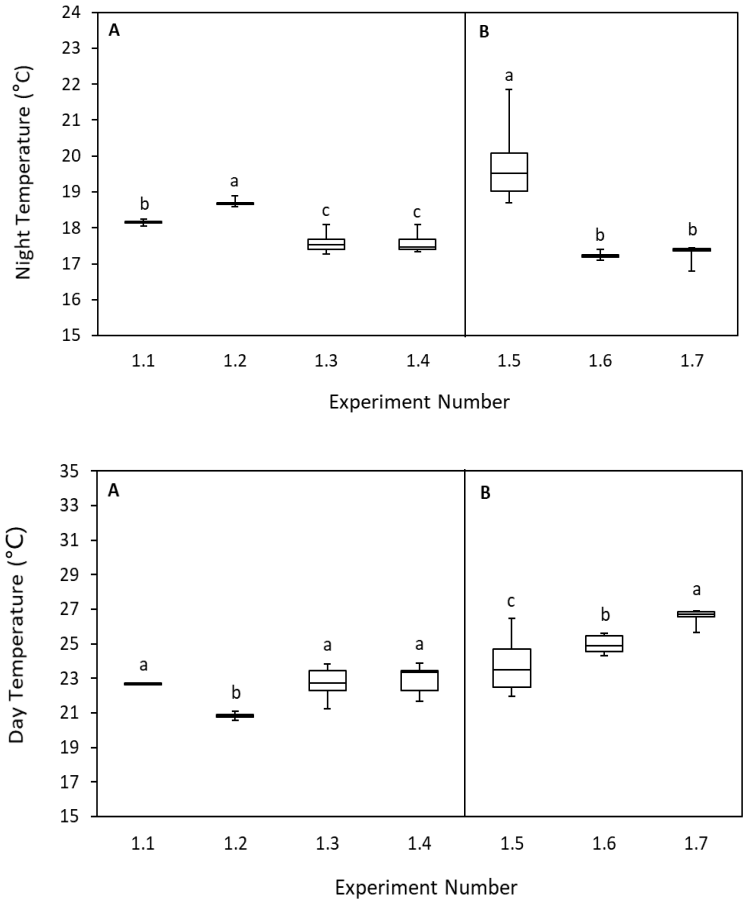
Initially and because the system was previously used in our facilities, HCO_{3^-} enrichment effects in hydroponics was tested. As optimal lettuce growing conditions substantially differ from tomato and pepper, lettuce was grown in the CE room, mainly during summer period as the glasshouse temperature tends to increase during this season. After testing the different crops under RZ HCO_{3^-} enrichment, the CO_2 delivery system was developed. The system was used first to test the RZ CO_2 enrichment in the three crops in hydroponics. Finally, when the aeroponic system was built, different locations were tested according to facility availability and always taking into account the different growth requirements for lettuce compared to tomato and pepper. Since RZ CO_2 and HCO_{3^-} enrichment more often stimulated growth of lettuce and pepper, more experiments were performed with these crops compared to tomato.

Table 2.3. System type, experiment number, crop type, treatment applied, location, date from sowing to harvest and measurements done in each experiment.

System	Experiment Number	Crop	Treatment	Location	Date	Measurements
Hydroponic	1.1	Lettuce (Butterhead)	Bicarbonate	CE room	01/11/15 - 15/12/15	Biomass
	1.2	Lettuce (Butterhead)	Bicarbonate	CE room	02/05/16 - 10/06/16	Biomass, Nutrients
	1.3	Pepper	Bicarbonate	Glasshouse	01/11/15 - 12/12/15	Biomass
	1.4	Tomato	Bicarbonate	Glasshouse	01/11/15 - 03/12/15	Biomass
	1.5	Lettuce	CO_2	Glasshouse	01/05/16 - 21/06/16	Biomass, Gas exchange
	1.6	Tomato	CO_2	CE room	25/08/16 - 24/09/16	Biomass, Gas exchange
	1.7	Pepper	CO_2	CE room	25/10/16 - 11/11/16	Biomass, Gas exchange
Aeroponic	2.1	Lettuce (Butterhead)	CO_2	Glasshouse	16/11/16 - 15/12/16	Biomass
	2.2	Lettuce (Crisphead)	CO_2	Glasshouse	27/01/17 - 27/02/17	Biomass
	2.3	Lettuce (Crisphead)	CO_2	CE room	26/06/17 - 27/07/17	Biomass, Phytohormones
	2.4	Lettuce (Crisphead)	CO_2	CE room	10/08/17 - 03/09/17	Biomass, Gas exchange, Nutrients
	2.5	Lettuce (Crisphead)	CO_2	Glasshouse	11/09/18 - 16/10/18	Biomass, Gas exchange, Amino acids
	2.6	Lettuce (Crisphead)	CO_2	Glasshouse	14/10/18 - 13/11/18	Biomass
	2.7	Lettuce (Crisphead)	CO_2	Glasshouse	13/11/18 - 13/12/18	Biomass, RNA-seq
	2.8	Pepper	CO_2	Glasshouse	11/10/16 - 10/11/16	Biomass
	2.9	Pepper	CO_2	Glasshouse	20/10/17 - 17/11/17	Biomass
	2.10	Pepper	CO_2	Glasshouse	26/10/17 - 27/11/17	Biomass, Gas exchange, Nutrients, Phytohormones
	2.11	Pepper	CO_2	Glasshouse	18/06/18 - 12/07/18	Biomass, Leaf expansion
	2.12	Tomato	CO_2	Glasshouse	11/11/16 - 30/11/16	Biomass

The glasshouse was naturally lit with automated supplementary lighting supplied by ten high-pressure sodium lamps (600 W Greenpower, Osram, St Helens, UK) when the Photosynthetic Photon Flux Density (PPFD) was < 400 μmol m^{-2} s^{-1} for a 12 h photoperiod (08.00 hrs to 20.00 hrs). The illumination in the CE room was provided by twelve 400 W metal halide lamps (HQI-T 400N, Osram, St Helens, UK) for a 12 h photoperiod (8.00 hrs to 20.00 hrs). The temperature, humidity and CO_2 concentration in the CE room and glasshouse were recorded by Electron II C sensor (HortiMax B.V. Pijnacker, Netherlands).

Hydroponically grown lettuce, tomato and pepper were subject to different environmental conditions. In the CE room, mean day/night temperature was set as ~23/18°C for lettuce and ~26/18°C for tomato and pepper. As expected, mean day/night temperature was more variable in the glasshouse than the CE room and temperatures varied between ~24-29°C during the day and ~18-22°C during the night. However, the relative humidity was similarly variable in both CE room and glasshouse ranging from ~25-60% during the day and ~35-75% during the night (Figure 2.12).



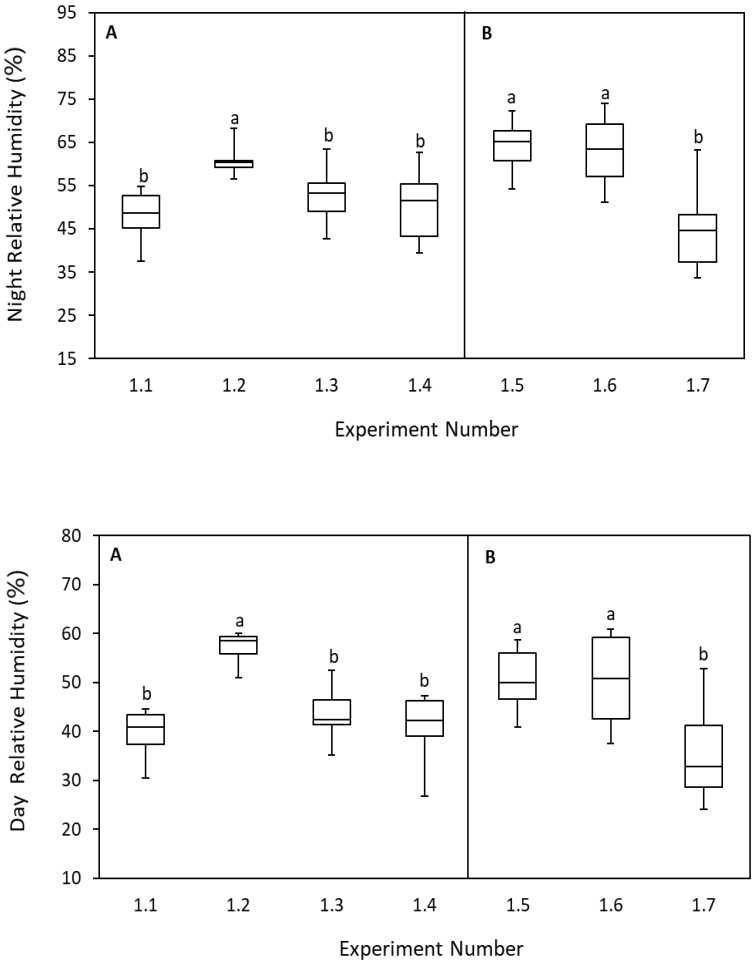
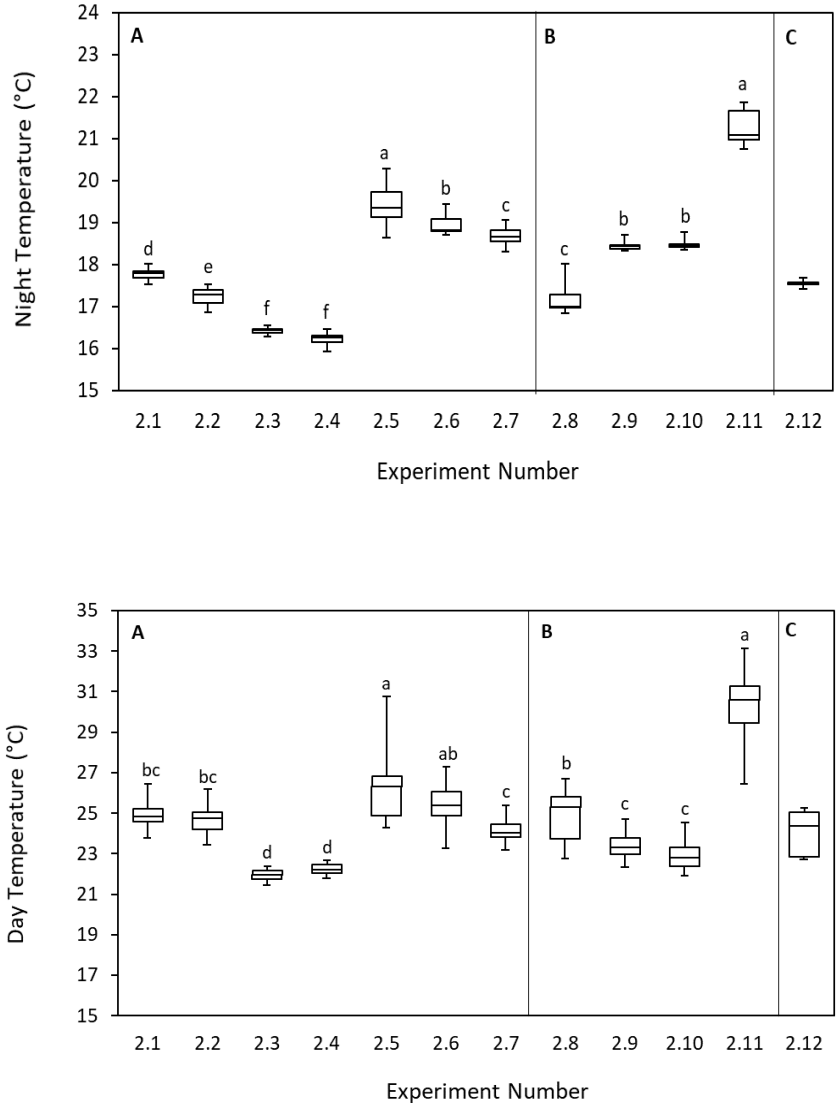


Figure 2.12. Mean day/night temperature and relative humidity during each experimental period for RZ HCO_{3^-} (A) and CO_2 (B) enrichment in hydroponics. The horizontal line within the box represents the median. The boundaries of each box represent the lower 25th and upper 75th percentiles. The spacing of components within the box indicates skewness in the data. The whiskers represent the sample minimum and maximum values. Values indicated with different letters indicate statistically significant differences according to one – way ANOVA test ($p<0.05$), followed by LSD post-hoc analysis. Different letters indicate statistically significant differences.

Aeroponically grown lettuce, pepper and tomato were also exposed to different environmental conditions as 10 out of 12 experiments in this system were carried out in the glasshouse (Table 2.4). In the glasshouse, mean day RH% was between 25-35% while in the CE room was between 60-70%. Mean night RH% in the glasshouse was between 35-60% and in the CE room between 70-85%. Day and night temperatures showed the greatest differences between experimental locations. In the glasshouse, mean day temperatures varied from 23 to 32°C and night temperatures from 17 to 22°C. In the CE room, temperatures were constant around 22°C during the day and 16.5°C during the night (Figure 2.13).



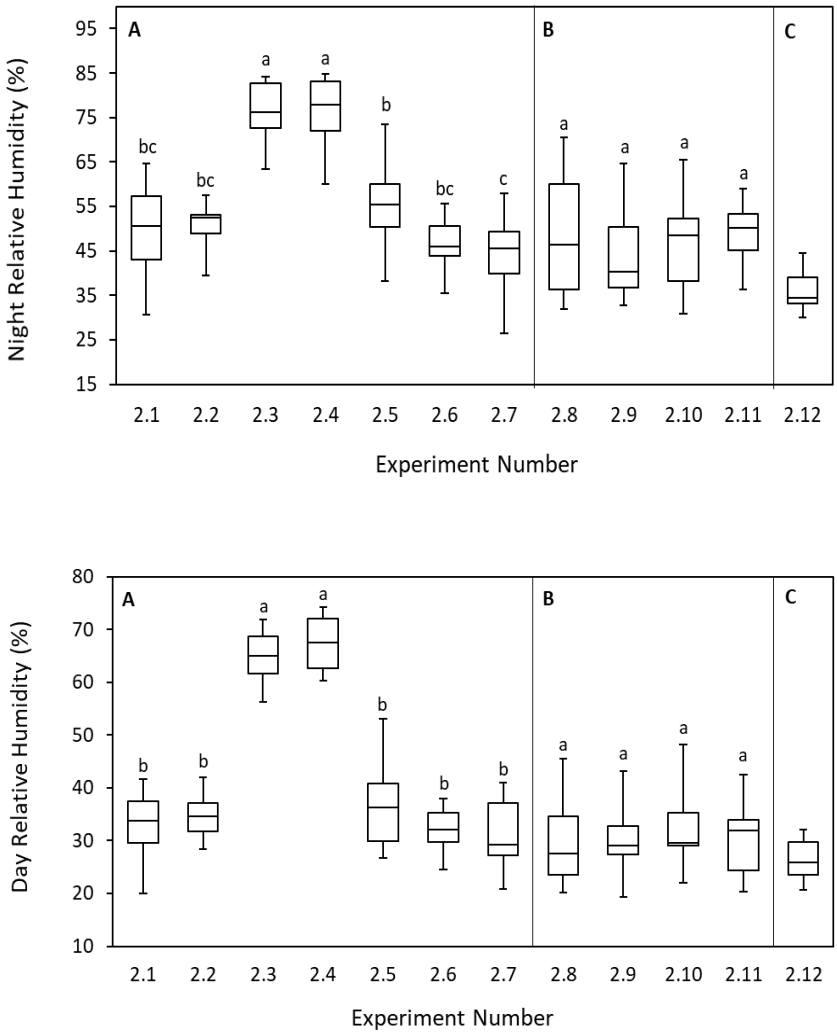


Figure 2.13. Mean day/night temperature and relative humidity during each experimental period for lettuce (A), pepper (B) and tomato (C) grown aeroponically. The horizontal line within the box represents the median. Values indicated with different letters indicate statistically significant differences according to one – way ANOVA test ($p<0.05$), followed by LSD post-hoc analysis. Different letters indicate statistically significant differences

2.2.6 Plant measurements

After plant removal from the bins, they were separated into shoot and root tissues. Roots were dried with a tissue before fresh weight determination. Shoot and root fresh weight were measured using a two decimal point scientific balance. Leaf area was determined using a leaf area meter (Li-cor Model 3100 Area Meter, Cambridge, UK). All tissues were dried at 70°C for 4 d and then reweighed.

2.2.7 Gas exchange measurements

Gas exchange measurements were performed using a portable Li-6400XT photosynthesis system. Conditions within the leaf chamber were set according to the ambient environmental conditions. All gas-exchange measurements were performed during two consecutive days immediately before harvesting. In addition, since the irregular and delicate surface of crisphead type lettuce leaves were difficult to access, fully-developed leaves (numbers 6 and 7 from the base of the plant) were measured.

2.2.8 $NaHCO_3^-$ uptake determination

All the plant material was freeze-dried and ground to a fine powder using a pestle and mortar, which along with the steel spatula, were washed with ethanol before each use to avoid cross-contamination. For all biomass fractions, two mg subsamples were wrapped in foil capsules and combusted at 950^{o}C in an Elementar Vario MICRO elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany). In this process, the carbon in samples is converted entirely to CO_2 and the isotopes analysed on an isotope ratio mass spectrometer (Isoprime 100 IRMS, Isoprime Ltd., Stockport, UK). Standards were wheat flour standard, and two in house standards calibrated against international standards. Three standards were run along with every 12 batch samples, with 13 standards run in total. The mean values of the standards was δ13C = -28.12 ± 0.01, therefore the error while measuring was minimum. Since δ13C values in C3 plants fall between -33 and -24‰ (Farquhar et al., 1989), values greater than -24‰ could be considered as significantly enriched as a result of bicarbonate uptake.

2.2.9 Data analysis

To compare shoot, root fresh and dry weight and leaf area in the hydroponic experiments, a nested ANOVA was carried out to test the differences between the treatments and the variability of the plants within the boxes and the variability of the boxes within the treatments, followed by LSD post-hoc analysis.

For the aeroponic experiments, treatment differences were determined using an Independent Samples Student's t-test at the $P < 0.05$ level. Furthermore, a univariate (two-way) ANOVA was performed for leaf gas exchange parameters to test both the individual effects of day and treatment effects and the interactions between day and treatment.

2.3 Results

2.3.1 Bicarbonate effects in lettuce, pepper and tomato grown hydroponically.

In the experimental design, there were no differences between the boxes within the treatments according to the nested ANOVA performed (Table 2.4), which means that the differences were mainly due to the treatments and not because of the position of the box in the glasshouse or CE room.

Table 2.4. Results of nested ANOVA (P Values reported) comparing shoot fresh weight, leaf area, shoot dry weight and root dry weight between treatments and boxes (nested within treatments) separately for each experiment 1.1, 1.3 and 1.4. Significant results are in boldface ($P<0.05$).

		Shoot fresh weight	**Leaf area**	**Shoot dry weight**	**Root dry weight**
Lettuce Experiment 1.1	Treatment	**0.001**	**0.001**	**0.001**	0.214
	Box(Treatment)	0.688	0.437	0.165	**0.020**
Tomato Experiment 1.3	Treatment	0.082	0.103	0.092	0.084
	Box(Treatment)	0.458	0.190	0.714	0.141
Pepper Experiment 1.4	Treatment	**0.005**	**0.004**	**0.022**	0.079
	Box(Treatment)	0.350	0.496	0.161	0.572

Lettuce shoot fresh weight, dry weight and leaf area increased by ~10% when the roots were exposed at 1 mM and 5 mM HCO_3^-. Root dry weight was significantly lower in control plants and those exposed to 1 mM HCO_3^- compared to other treatments. However, total plant dry weight (data not shown) was ~ 10 % higher under 1 and 5 mM whereas at 20 mM was 20% lower.

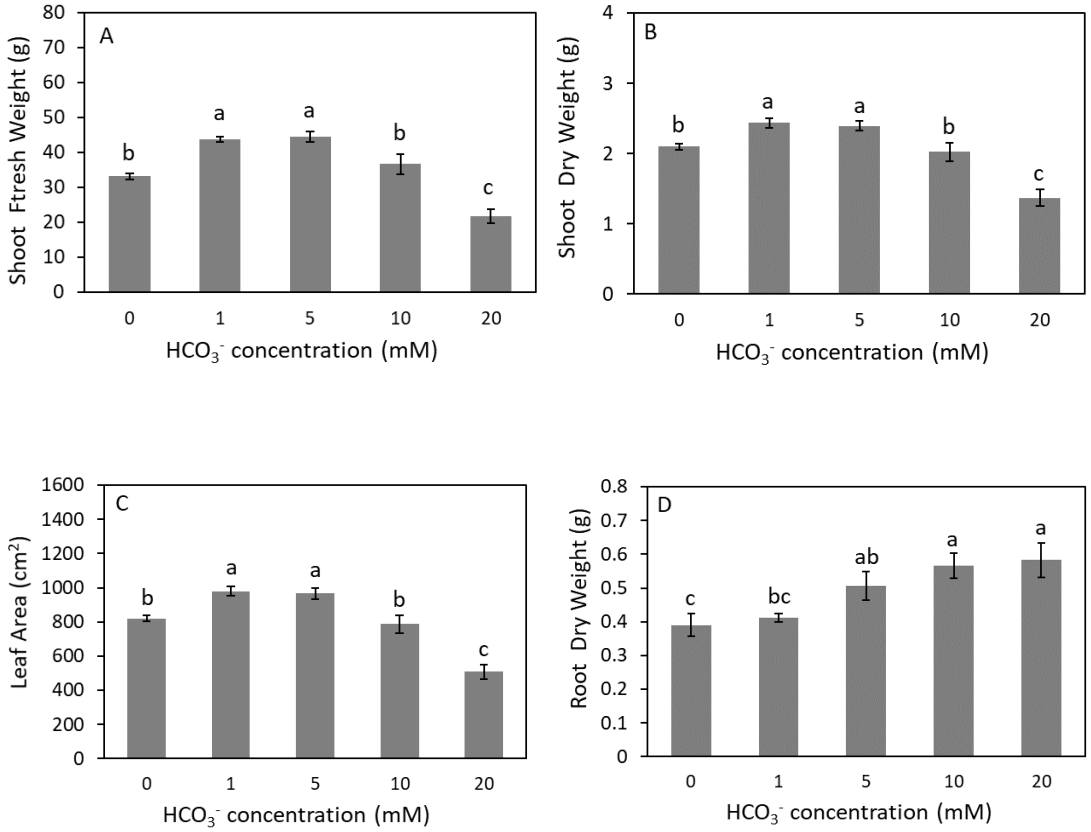


Figure 2.14. Shoot fresh (A) and dry weight (B), leaf area (C) and root dry weight (D) of lettuce plants under different $NaHCO_3^-$ concentrations. Values indicated with different letters indicate statistically significant differences according to one – way ANOVA test ($p<0.05$), followed by LSD post-hoc analysis. Bars represent means ± SE (n=8).

Shoot fresh weight, shoot dry weight, and leaf area increased significantly in pepper plants exposed to 1 mM bicarbonate compared to control plants. Furthermore, high HCO_3^- concentrations (>10 mM) decreased all parameters compared to control plants. However, root dry weight of control plants was significantly lower than those exposed to all bicarbonate concentrations.

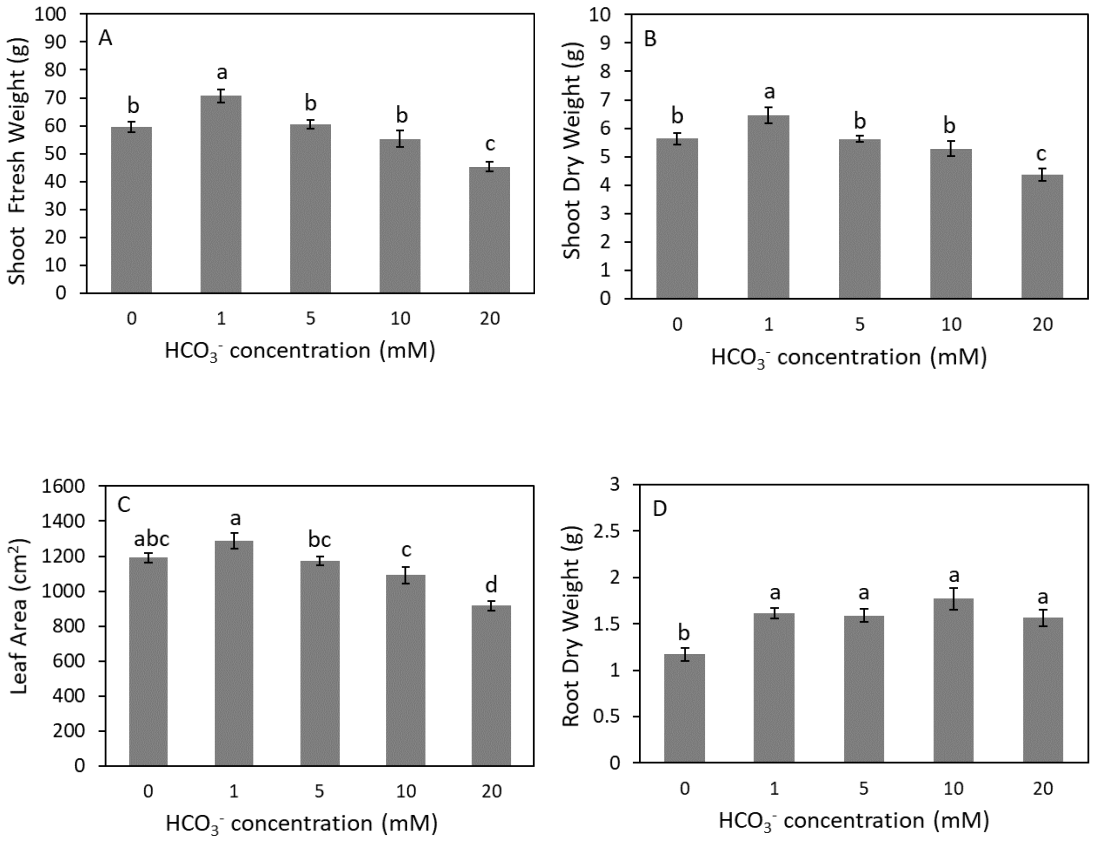


Figure 2.15. Shoot fresh (A) and dry weight (B), leaf area (C) and root dry weight (D) of pepper plants under different HCO_3^- concentrations. Values indicated with different letters indicate statistically significant differences according to one – way ANOVA test ($p<0.05$), followed by LSD post-hoc analysis. Bars represent means ± SE (n=9).

Tomato shoot fresh weight, dry weight and leaf area did not show any significant differences from control plants when applying bicarbonate between 1-10 mM. However, 20 mM HCO_3^- decreased those parameters. Root dry weight increased when increasing [HCO_3^-] from 1-10 mM, but was significantly smaller compared to those treatments when exposed to 20 mM HCO_3^- .

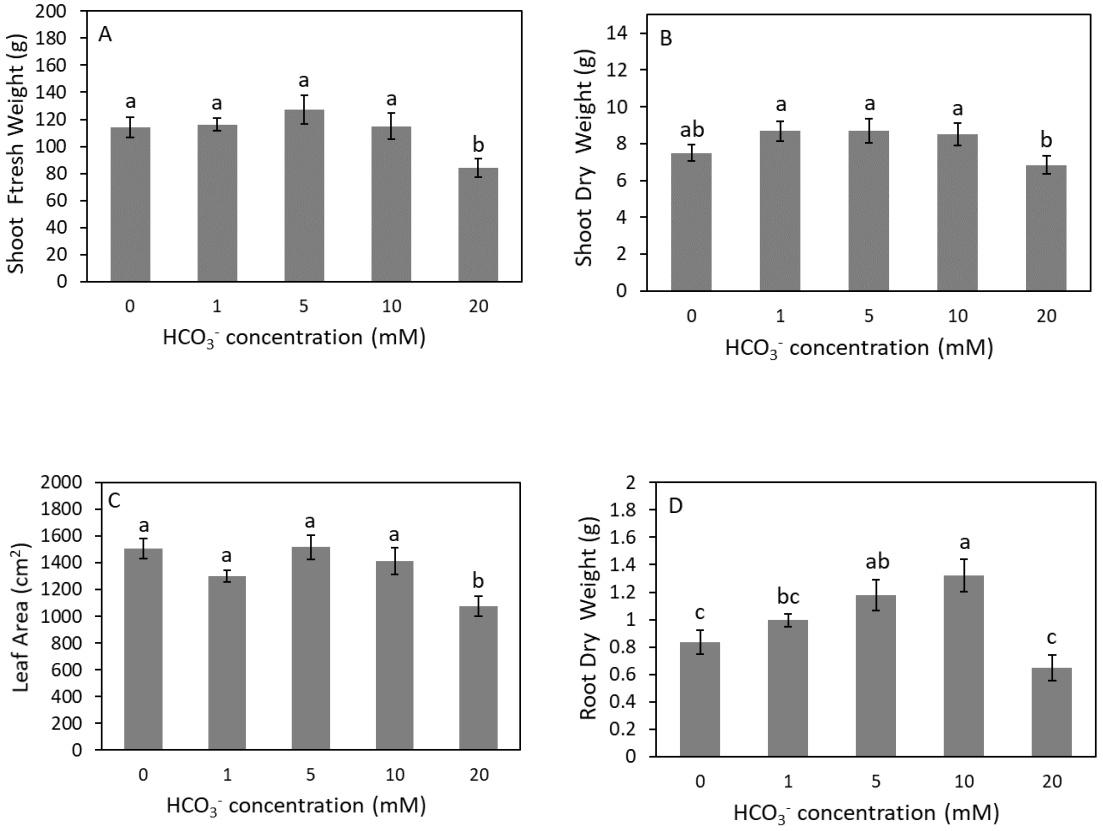


Figure 2.16. Shoot fresh and dry weight, root dry weight and leaf area of tomato plants under different HCO_{3-} concentrations. Values indicated with different letters indicate statistically significant differences according to one – way ANOVA test ($p<0.05$), followed by LSD post-hoc analysis. Bars represent means ± SE (n=12).

2.3.2 *$NaHCO_3^-$ uptake determination*

The $\delta^{13}C$ values of roots increased greatly between 0 and 4 hours immediately after adding labelled bicarbonate, indicating higher DIC uptake at the beginning of the experiment. In contrast, shoot $\delta^{13}C$ values increased significantly between 4 and 12 hours in bicarbonate-enriched plants in both experiments. Continued increases in shoot $\delta^{13}C$ values while root $\delta^{13}C$ values stabilised or decreased (between 4 and 24 hours after addition of bicarbonate) suggests DIC transport from the root to the shoot (Figure 2.17).

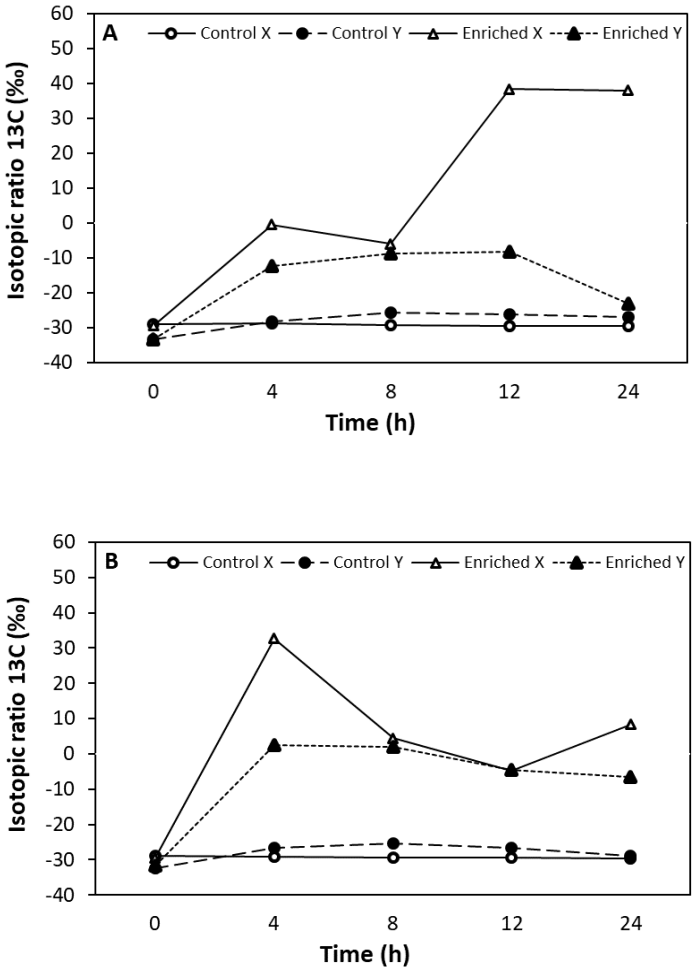


Figure 2.17. δ13C (‰) for leaves (A) and roots (B) containing 0 or 1 mM $NaH^{13}CO_3$ *versus* time for DIC uptake by lettuce. Points are from individual plants grown in two replicate experiments (X and Y).

Root $\delta^{13}C$ values in plants exposed to different solution pHs were similar, indicating DIC incorporation is independent of the form of carbon taken up, since the ^{13}C will be in the form of CO_2 at pH 5.8 , while at naturally fluctuating pH (between 6.3 and 6.7) the ^{13}C will be predominantly in the form of HCO_3^-. Between 2 and 4 hours after bicarbonate addition, greater ^{13}C translocation from the roots to the shoot occurred when nutrient solution pH was allowed to naturally fluctuate, as indicated by the higher shoot $\delta^{13}C$ values (Figure 2.18).

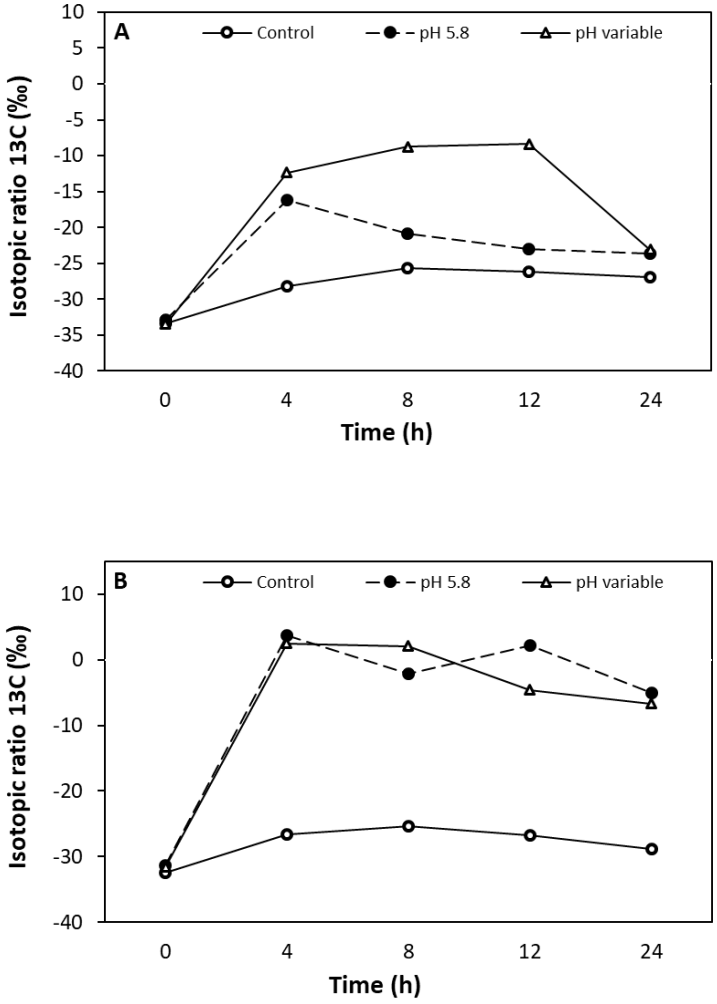


Figure 2.18. δ13C (‰) for leaves and roots containing 0 or 1 mM $NaH^{13}CO_3$ *versus* time for DIC uptake by lettuce. Points are from individual plants.

2.3.3 Carbon dioxide effects in lettuce, pepper and tomato grown hydroponically.

Shoot fresh and dry weight, leaf area or root dry weight of lettuce and pepper grown hydroponically under elevated RZ CO_2 concentration of 1500 ppm did not significantly differ from control plants grown under ambient RZ CO_2 of 400 ppm (Table 2.5). Tomato shoot fresh and dry weight and leaf area, were ~20% higher with CO_2 treatment compared to control ones, although these differences were not statistically significant.

Table 2.5. Shoot fresh weight, total leaf area, shoot dry weight and root dry weight for RZ CO_2 and control lettuce, tomato and pepper plants. Data are means ± SE of 8 replicates. Analysis was performed using an Independent Sample T-Test, P-value < 0.05.

		Shoot Fresh Weight (g) ± SE	Total leaf Area (cm) ± SE	Shoot Dry Weight (g) ± SE	Root Dry Weight (g) ± SE
Lettuce Experiment 1.5	**RZ CO_2**	24.18 ± 1.93	706 ± 5	*nd*	*nd*
	Control	24.58 ± 2.07	697 ± 5	*nd*	*nd*
Tomato Experiment 1.6	**RZ CO_2**	3.74 ± 0.24	215 ± 1	0.69 ± 0.04	1.50 ± 0.08
	Control	3.04 ± 0.33	165 ± 2	0.54 ± 0.06	1.34 ± 0.08
Pepper Experiment 1.7	**RZ CO_2**	1.60 ± 0.12	71 ± 7	0.27 ± 0.01	0.29 ± 0.13
	Control	1.53 ± 0.09	64 ± 2	0.27 ± 0.02	0.27 ± 0.15

Since the day of measurement significantly affected some of the gas exchange parameters, measurements were analysed per day. Pepper and tomato grown in the CE room at elevated RZ CO_2 and constant temperatures/relative humidity did not exhibit significant differences in CO_2 assimilation rate (*A*), stomatal conductance (g_s), internal CO_2 (*Ci*) or transpiration (*E*), compared to control plants. On the other hand, lettuce grown in the glasshouse at high temperatures and higher RZ CO_2 had significantly higher (10-20%) *A*, g_s, *E* and *Ci*, compared to control plants, although this was not reflected in greater biomass (Table 2.6).

Table 2.6. P-values of two-way ANOVA examining the effects of two different days; the control and CO_2 treatments and the interaction between both on CO_2 assimilation (A), Stomatal conductance (g_s), Internal CO_2 (Ci) and Transpiration (E) of pepper, tomato and lettuce. Significant results are in boldface.

Pepper		**Day**	**Treat**	**Day*Treat**
	A (µmol m^{-2} s^{-1})	0.747	0.434	0.827
	g_s (mol m^{-2} s^{-1})	0.358	0.381	0.835
	Ci (µmol mol^{-1})	0.056	0.874	0.419
	E (mmol m^{-2} s^{-1})	**<0.001**	0.889	0.174
Tomato				
	A (µmol m^{-2} s^{-1})	0.408	0.507	0.558
	g_s (mol m^{-2} s^{-1})	**0.001**	0.347	0.468
	Ci (µmol mol^{-1})	**<0.001**	0.073	0.689
	E (mmol m^{-2} s^{-1})	**<0.001**	0.148	0.353
Lettuce				
	A (µmol m^{-2} s^{-1})	0.138	**0.034**	0.248
	g_s (mol m^{-2} s^{-1})	**0.016**	**0.031**	0.237
	Ci (µmol mol^{-1})	**0.013**	**0.012**	0.199
	E (µmol m^{-2} s^{-1})	0.937	**0.005**	0.184

2.3.4 Carbon dioxide effects in lettuce, pepper and tomato grown aeroponically.

2.3.4.1 Lettuce

Of seven similar experiments, four of them showed that elevated RZ CO_2 significantly increased dry shoot biomass by about 19-25% compared to those grown with 400 ppm RZ CO_2, regardless of the variety and location of the experiment (Table 2.7). However, the last three experiments did not show any change in dry shoot biomass between treatments. On the other hand, root dry weight did not significantly differ between treatments in any of the experiments. In Experiments 2.5, 2.6 and 2.7, root dry weight was not determined because samples were taken for further analysis (Table 2.3).

Table 2.7. Shoot fresh weight, shoot dry weight and root dry weight mean values of lettuce plants grown aeroponically. Growth enhancement % is based on the difference increased of shoot dry weight. Data are means ± SE of 8 replicates. Asterisks indicate significant differences between treatments (* P <0.05 ; ** P<0.001).

	Treatment	Shoot fresh weight (g) ± SE	Shoot dry weight (g) ± SE	Root dry weight (g) ± SE	Increase (%)
Experiment 2.1	**Control**	25.08 ± 2.04	1.24 ± 0.09	0.32 ± 0.03	23%
	RZ CO_2	28.38 ± 2.01	1.53 ± 0.09*	0.32 ± 0.02	
Experiment 2.2	**Control**	46.37 ± 1.45	2.48 ± 0.07	0.51 ± 0.13	19%
	RZ CO_2	51.94 ± 2.89	2.97 ± 0.19*	0.42 ± 0.13	
Experiment 2.3	**Control**	62.47 ± 5.19	3.01 ± 0.25	0.75 ± 0.15	25%
	RZ CO_2	76.34 ± 4.60	3.77 ± 0.19*	0.72 ± 0.21	
Experiment 2.4	**Control**	71.30 ± 4.28	3.40 ± 0.21	0.65 ± 0.17	22%
	RZ CO_2	86.86 ± 2.56**	4.17 ± 0.12**	0.56 ± 0.11	
Experiment 2.5	**Control**	112.86 ± 3.3	5.53 ± 0.2	*nd*	0%
	RZ CO_2	113.15 ± 5.8	5.60 ± 0.2	*nd*	
Experiment 2.6	**Control**	21.50 ± 1.3	1.54 ± 0.1	*nd*	0%
	RZ CO_2	21.70 ± 2	1.54 ± 0.1	*nd*	
Experiment 2.7	**Control**	66.85 ± 3.6	3.02 ± 0.2	*nd*	0%
	RZ CO_2	60.99 ± 2.1	2.91 ± 0.1	*nd*	

Experiments 2.3 and 2.4 were chosen to assess the impact of RZ CO_2 on gas exchange parameters, as the greatest impact on shoot dry weight occurred in those experiments. A two-way ANOVA assessed the effects that different days, treatments and their interaction had on leaf gas exchange parameters (Table 2.8). Since there was a significant day effect in several parameters, the data was analysed separately for two different days.

Table 2.8. P-values of two-way ANOVA examining the effects of two different days; the control and CO_2 treatments and the interaction between both on CO_2 assimilation (A), Stomatal conductance (g_s), Internal CO_2 (Ci) and Transpiration (E) of Experiments 2.3 and 2.4. Significant results are in bold text.

Experiment 2.3		**Day**	**Treat**	**Day*Treat**
	A (µmol m^{-2} s^{-1})	0.642	**0.009**	0.329
	g_s (mol m^{-2} s^{-1})	0.388	**0.025**	0.381
	Ci (µmol mol^{-1})	0.606	0.232	0.931
	E (mmol m^{-2} s^{-1})	**<0.001**	**0.006**	0.667
Experiment 2.4				
	A (µmol m^{-2} s^{-1})	**0.006**	0.692	0.616
	g_s (mol m^{-2} s^{-1})	**0.007**	0.790	0.408
	Ci (µmol mol^{-1})	**<0.001**	0.920	0.553
	E (mmol m^{-2} s^{12})	**0.001**	0.978	0.562

Experiment 2.3 generally had higher *A*, g_s, *Ci* and *E* than Experiment 2.4 on both days. Although RZ CO_2 increased *A* (10-15%), g_s (10-20%) and *E* (10-17%) in Experiment 2.3, no treatment differences were detected in Experiment 2.4. However, in Experiment 2.4, *A* increased (10%), g_s increased (20%) , *Ci* increased (10%) and *E* increased (25%) on the second day of measurement compared to the first day (Figure 2.19). Thus, there was no consistent effects of RZ CO_2 enrichment on leaf gas exchange.

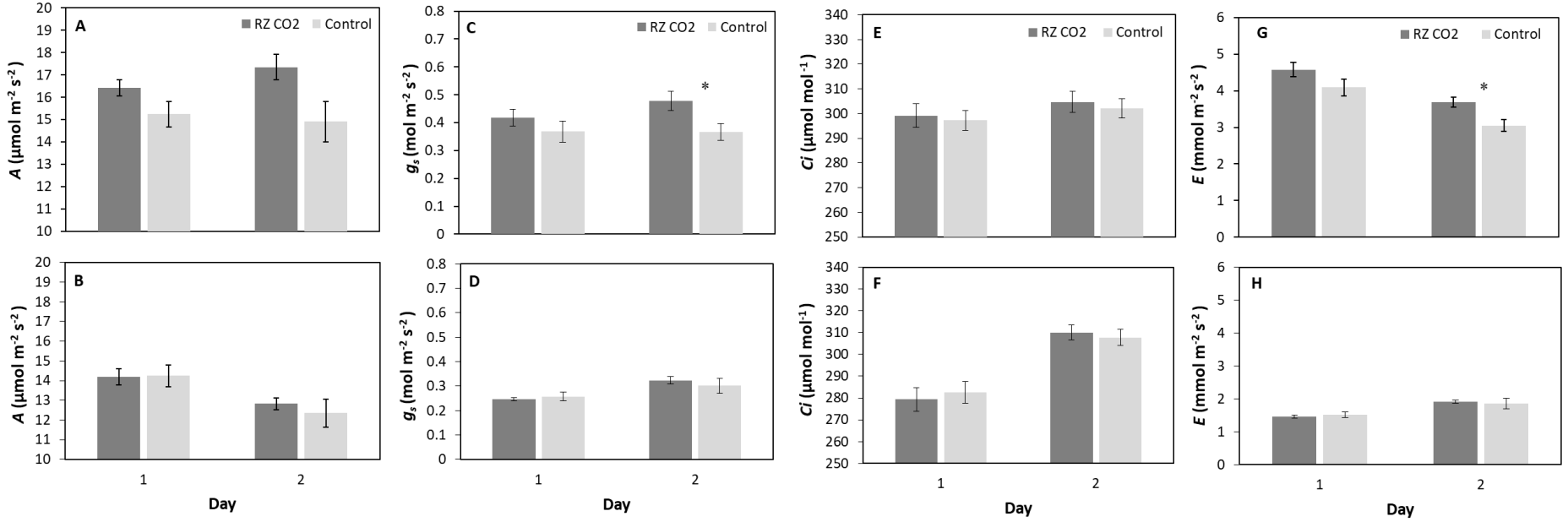


Figure 2.19. RZ CO_2 enrichment effects in Experiments 2.3 (top panels – A, C, E, G) and 2.4 (lower panels – B, D, F, H) on CO_2 assimilation rate (A and B), stomatal conductance (C and D), internal CO_2 (E and F) and transpiration rate (G and H) measured in two consecutive days immediately before harvest. Data are means ± SE of 5 replicates. Asterisks indicate significant differences between treatments on individual days.

2.3.4.2 Pepper

Applying 1500 ppm CO_2 to the root-zone of aeroponically grown pepper did not significantly change shoot dry weight, total leaf area and root dry weight in the first three experiments (2.8 , 2.9 and 2.10), compared to control plants grown at ~ 400 ppm RZ CO_2. Experiment 2.11 showed lower shoot fresh/ dry weight, total leaf area and root dry weight compared to control plants (Table 2.9).

Table 2.9. Shoot fresh weight, total leaf area, shoot dry weight and root dry weight for RZ CO_2 and control pepper plants. Data are means ± SE of 8 replicates. Analysis was performed using an Independent Sample T-Test, P-value < 0.05. "*nd*" = not determined.

	Treatment	Shoot fresh weight (g) ± SE	Shoot dry weight (g) ± SE	Total leaf Area (cm) ± SE	Root dry weight (g) ± SE
Experiment 2.8	**Control**	1.54 ± 0.09	0.27 ± 0.01	65 ± 2	0.27 ± 0.01
	RZ CO_2	1.60 ± 0.12	0.27 ± 0.02	71 ± 7	0.29 ± 0.01
Experiment 2.9	**Control**	*nd*	3.12 ± 0.24	544 ± 30	0.96 ± 0.18
	RZ CO_2	*nd*	3.30 ± 0.28	541 ± 33	1.21 ± 0.23
Experiment 2.10	**Control**	9.52 ± 0.38	1.10 ± 0.05	215 ± 7	0.64 ± 0.17
	RZ CO_2	9.44 ± 0.39	1.10 ± 0.03	215 ± 9	0.54 ± 0.07
Experiment 2.11	**Control**	10.50 ± 0.75	1.26 ± 0.1	276 ± 21	*nd*
	RZ CO_2	8.47 ± 0.76	1.01 ± 0.1	219 ± 16	*nd*

2.3.4.3 Tomato

RZ CO_2 enrichment did not stimulate biomass of aeroponically grown tomato. In contrast, shoot fresh/dry weight and leaf area tended to decrease ~10% in RZ CO_2 enriched plants (Table 2.10).

Table 2.10. Shoot fresh weight, total leaf area, shoot dry weight and root dry weight for RZ CO_2 and control tomato plants. Data are means ± SE of 8 replicates. Analysis was performed using an Independent Sample T-Test, P-value < 0.05.

	Treatment	Shoot fresh weight (g) ± SE	Shoot dry weight (g) ± SE	Total leaf area (cm^2) ± SE	Root dry weight (g) ± SE
Experiment 2.12	**Control**	14.63 ± 1.54	1.93 ± 0.19	529 ± 51	0.88 ± 0.06
	RZ CO_2	13.29 ± 0.59	1.72 ± 0.08	458 ± 17	0.89 ± 0.05

Since RZ CO_2 enrichment stimulated biomass accumulation of aeroponically grown lettuce in some, but not all experiments (Table 2.7), dry weight increase was correlated with environmental conditions in aiming to understand this variability. High night temperatures were negatively correlated with RZ CO_2-mediated growth promotion (Figure 2.20), but there were no other significant relationships between growth and day temperature or day/night relative humidity or day/night VPD (data not shown).

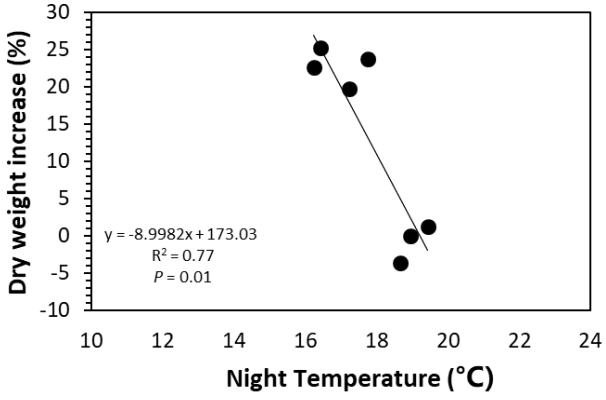


Figure 2.20. Lettuce shoot dry weight increase (%) plotted against mean (A) Night Temperature. The regression equation, adjusted R^2 and significance of the regression (P value) are given. Primary data were derived from Table 2.7 (dry weight increase) and Figure 2.12 (environmental conditions).

2.4 Discussion

Gaseous CO_2 enrichment of nutrient solution (1500 ppm CO_2) did not increase biomass of hydroponically grown lettuce, tomato or pepper plants. Previously, positive effects of gaseous CO_2 enrichment were detected in stressed (salinity stress, high temperatures, high irradiance) plants or at higher RZ CO_2 concentrations (5000ppm, 10000ppm, 500000ppm). In addition, the right NO_3^-/NH_4^+ ratio concentration in the nutrient solution combined with elevated bicarbonate or CO_2 may influence the effect of DIC on growth (Cramer et al., 1993, Bialczyk et al., 2004). However, the experiments described here aimed to study direct effects of DIC, independent of other interactive effects such as salinity, high temperatures, high irradiance or altered nitrogen content in the nutrient solution. Furthermore, the CO_2 concentrations used in this research were lower (~1500 ppm). For this reason, the lack of significant results obtained in some experiments here (Tables 2.5, 2.9 and 2.10) may result from optimal growing conditions (thus CO_2 could not alleviate stress) or perhaps the low CO_2 levels applied were not high enough to promote growth.

Aeroponics are a good system to study the effect of CO_2 since there are no physical barriers when applying the gas to the RZ. Comparable previous studies at higher ambient temperatures (>28/22°C) and PPFD (> 600 µmol $m^{-2}s^{-1}$) (He et al. 2007, 2010, 2016) showed that two weeks of applying elevated RZ CO_2 (2000 ppm) to aeroponically grown lettuce increased shoot growth (~20%) compared to plants aerated with ambient CO_2 (360 ppm). Here, growing plants under elevated RZ CO_2 (1500 ppm) at lower irradiances (<600 µmol $m^{-2}s^{-1}$) and lower temperatures (<25/17°C) for ten days enhanced shoot growth by a similar percentage of ~20% (Experiments 2.1-2.4, Table 2.5). Despite the positive impact in biomass accumulation in those cases, in some experiments growth stimulation was not observed (Experiments 2.5-2.7, Table 2.5). This lack of response might be associated with higher night-time temperatures (NT) (>17°C) in the glasshouse as biomass accumulation under high RZ CO_2 was greater at lower NTs (Figure 2.20). However, increasing NT from 17 to 34°C with a constant day temperature of 25°C and a constant application of elevated air CO_2 (1200 ppm), NT respiration was enhanced by 2% with no increased in carbon gain or dry mass of lettuce plants (Frantz et al., 2004). Many studies confirmed that higher NTs increase respiration rates (*R*) in diverse species including soybean (Bunce, 2005), lettuce,

tomato (Frantz et al., 2004), and rice (Cheng et al., 2009; Kanno et al., 2009; Kanno & Makino, 2010; Mohammed & Tarpley, 2010). Whether the yield decline reported in several studies of aerial CO_2 enrichment (Peng et al., 2004; Nagarajan et al., 2010; Welch et al., 2010) can be attributed to NT effects on *R* is unclear, since many environmental variables (relative humidity, rainfall, air movement) also vary along with NT and temperature effects on *R* do not always affect crop biomass (Peraudeau et al., 2015).

Lettuce and other crops fix carbon during the day through photosynthesis and respire some of the accumulated carbon predominantly during the night. Whole plant *R* comprises 30-70% of carbon losses fixed from photosynthesis (Loveys et al., 2002), with roots contributing 30-50% of this (Porter et al., 1990). Root respiration decreased exponentially as soil CO_2 concentration increased from 130 ppm to 7015 ppm, however assuming a respiratory quotient of 1, oxygen concentrations at 7000 ppm would decrease from 21% to just 20.3% (Qi et al., 1994). In a controlled environment, hydroponically grown tomato under elevated RZ CO_2 (> 2000ppm) increased oxygen consumption and decreased CO_2 release over a 7 h period (van der Westhuizen and Cramer 1998), but long-term effects of RZ CO_2 enrichment on biomass accumulation were not assessed. In Experiment 2.5 where there were no biomass differences between treatments, RZ oxygen concentrations decreased from early morning when the temperature started to rise. However, when the temperature declined at the end of the day, it seemed that the recovery of oxygen levels on the plant under elevated RZ CO_2 was difficult to achieve as compared to the control plant (Figure 2.10, A&B).

Although elevated RZ CO_2 significantly increased root dry weight (He et al., 2010), no effect was detected here, likely because the small pot size (30 cm depth) limited root growth compared to earlier studies with 1 m depth (He et al., 2016). The effects of high RZ CO_2 concentrations occurred after few days of treatment: decreased stomatal conductance (g_s), increased assimilation rate (*A*), less water loss, higher midday leaf relative water content (RWC), higher sink capacity (larger root systems enhanced NO_3^- uptake and increased the capacity for utilizing photoassimilate) and higher levels of reduced NO_3^- (He et al. 2010). In experiments that examined the RZ CO_2 effect on lettuce growth, leaf gas exchange measurements were inconsistent and not correlated with increased growth. The environmental conditions in the UK were very different from the experiments carried out by He et al (2010) in New Zealand where the temperatures in the glasshouse reached to

36 °C with a very high light intensity (300-2100 µmol $m^{-2}s^{-1}$) and the CE room conditions were set to the standard growing conditions for New Zealand. This led to the conclusion that although elevated RZ CO_2 can affect leaf gas exchange, this does not directly increase growth and other parameters such as nutrient uptake or metabolic signals might be more directly correlated with the increase in growth. Furthermore, whole plant carbon assimilation (Jauregui et al., 2018) could more closely reflect changes in biomass growth under elevated RZ CO_2.

On the other hand, RZ CO_2 enriched aeroponically grown pepper and tomato did not show any differences compared to control plants (Table 2.7 and 2.8). When aeroponically grown tomato was enriched with 2500, 5000, 10000 ppm RZ CO_2 for 60 days, root length, plant height and stem diameter were significantly lower than control plants exposed to 370 ppm CO_2 (Zhao et al., 2010). They observed decreased root macronutrient (Ca, N, P, K, Mg) concentrations and also decreased PM-H^+ ATPase and V-H^+ ATPase activities, which are involved in exporting protons to apoplast and pumping H^+ into the vacuole and both are important for intracellular pH regulation. Their decreased activity could lead to cytoplasmic acidosis which can limit root growth (Drew, 1997). However, it is difficult to compare the results obtained here to previous results, as the CO_2 concentrations and duration of treatment differed.

Root uptake of DIC has been repeatedly demonstrated (Vuorinen et al., 1992, Hibberd et al., 2002; Cramer et al., 1995, 1999; Bialczyk et al., 1992), although its effects *in planta* are not well known. These studies did not implement different pH levels in the nutrient solution to understand better the form of DIC that roots were taking up. Inorganic carbon absorbed through the roots is converted to organic and amino acids which are exported to the shoots, where they are decarboxylated to augment photosynthesis (Bialczyk, et al., 1992, 1995, Cramer, et al., 1995, 1999, Viktor & Cramer, 2005). However, since this small contribution to the total carbon budget of the plant cannot explain the stimulation of growth (Viktor & Cramer, 2003), it is necessary to explore other routes that can promote the plant growth.

To determine the effect of xylem-transported CO_2 derived from the root system on aboveground carbon assimilation and CO_2 efflux, sunflower plants were grown hydroponically in the open air or in a closed box (to observe whether plants with no label

were enriched) and the dynamics of $NaH^{13}CO_{3-}$ (25 mM) uptake (for 1 hour) investigated at low (4.8-5.5) and high (8.3-8.5) nutrient solution pH. At low pH, most (87-90%) of the label taken by the roots diffused from the plant shoot and some (10-13%) was re-fixed by photosynthesis. At high pH, bicarbonate did not readily escape from the aerial part of the plant because plants enclosed in the box had no ^{13}C label. Moreover, the small proportion of ^{13}C label that moved from roots to shoots indicated that RZ bicarbonate uptake was restricted at high pH (Shimono et al., 2019). Similarly, infusing $^{13}CO_2$-labeled aqueous solution into the base of 7-yr-old field-grown eastern cottonwood (*Populus deltoides*) trees determined that 17% of the CO_2 infused was assimilated in woody and leaf tissues (Bloemen et al., 2013). The low pH study (Shimono et al., 2019) agrees with the slight increase in $\delta^{13}C$ in the recirculated system performed in the CE room, where complete air exchanges could be lower than the glasshouse (Figure 2.16). Despite this $\delta^{13}C$ enrichment, treated plants showed significantly greater labelling than control plants. On the other hand, at higher nutrient solution pH (6.7) there was greater translocation of DIC from root to shoot (Figure 2.18), contrary to the previous results (Shimono et al., 2019). In conclusion, lettuce plants absorbed both CO_2 and bicarbonate, with a greater translocation of bicarbonate to the shoot. Whether these leaves release CO_2 to the atmosphere requires isotopic measurements of atmosphere gases.

High (> 10 mM) bicarbonate concentrations decreased biomass accumulation (Figures 2.14-2.16) as reported previously (Chapter 1), and when nutrient solution pH is high (> 8) and unregulated, bicarbonate uptake may be restricted as previously shown (Shimono et al. 2019). In contrast, bicarbonate enrichment of hydroponic solutions (1 mM and 5 mM HCO_3^-) increased shoot growth of lettuce and pepper plants. Previously, bicarbonate enrichment of hydroponically grown rice (Yang et al., 1994) and tomato (Bialczyk et al., 1994, 2004) stimulated growth, with increased xylem sap concentrations of amides and amino acids with the right proportions of bicarbonate (5 mM) and N (NO_3^- 4: NH_4^+ 1) concentrations in the nutrient solution. DIC assimilation in root cells may affect synthesis of amides and amino acids supplying carbon skeletons to NH_4^+ incorporation and regulating the activity of some enzymes of ammonium metabolism (Bialczyk et al., 2004). Whether additional RZ DIC affected amino acids and nutrient concentrations is addressed in Chapter 3.

2.5 Conclusion

Applying low concentrations (1-5 mM) of bicarbonate stimulated biomass accumulation in lettuce and pepper, but not tomato, indicating species dependent effects of root DIC supply. Isotope labelling experiments demonstrated that root DIC uptake was pH independent, meaning that roots can absorb both CO_2 and bicarbonate. However, translocation to the shoot was greater at higher pH levels of 6.4. Applying RZ DIC in the form of CO_2 gas (1500-2000 ppm) to hydroponically grown lettuce, pepper and tomato did not promote shoot fresh or dry weight. However, applying CO_2 gas (1500 ppm) to aeroponically grown lettuce increased shoot dry weight, especially when environmental conditions were controlled and constant during the treatment. Under more changeable glasshouse conditions, inconsistent results were obtained, with no growth promotion in plants exposed to high night temperatures. Experiments with larger hydroponics and aeroponics systems, and continuous regulation of pH/EC/DO, and factorial combinations of different environmental conditions (photoperiod, temperature, light intensity and relative humidity) would provide additional data sets to complement the results obtained in this chapter. Since leaf gas exchange measurements generally did not show any significant differences between treatments, alternative mechanisms of growth promotion are evaluated in subsequent chapters.

Chapter 3. Elevated root-zone dissolved inorganic carbon changes nutrient and amino acids concentrations in lettuce and pepper.

3.1 Introduction

Plant growth requires nutrient uptake from the soil. There are 17 essential elements for higher plants divided in two classes. The macronutrients are the ones required by the plant in larger amounts and those required in smaller amounts are called micronutrients. Macronutrients include Nitrogen (N), Phosporus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), and Sulfur (S). Micronutrients include boron (B), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo), chlorine (Cl), and nickel (Ni). The amount of macronutrients absorbed by vegetables is in the order of K>N>Ca>P>Mg (Maruo, 2013) and the ratio differs from one vegetable to another. Enriching the RZ with DIC can alter nutrient uptake and utilization efficiencies in the plant.

Atmospheric CO_2 is the main form of inorganic carbon assimilated by terrestrial photosynthetic organisms. As water percolates through the soil it becomes enriched with CO_2 from plant and microbial respiration. Dissolution of CO_2 in water produces carbonic acid, which dissociates into bicarbonate and carbonate. High concentrations of HCO_3^- in the soil can decrease plant productivity, particularly in soils with low bioavailability of plant nutrients, high calcium content and high alkalinity (Zhao & Wu, 2017; Poschenrieder et al., 2018). In calcareous soils with high pH (>7), the availability of Fe and other essential micronutrients such as Zn, Mn, and Cu are usually low due to their precipitation as oxides or carbonates (Poschenrieder et al., 2018). On the other hand, Ca, Mg and K are less available and aluminium (Al), Fe and Mn can cause plant toxicity in acidic soils (Fageria & Nascente, 2014).

In soilless culture systems, a well-prepared nutrient solution will provide to the plant every element in an optimum ratio. A nutrient solution should (Jones, 2014):

- Contain all essential elements in ion form.
- Ensure the concentration of each ion is in a good range for plant growth.
- Have a pH around 5.5-6.5.
- Minimise any fluctuations in ion concentration and pH.
- Be well oxygenated.
- Be free of pathogens in the solution.

When adding HCO_3^- into the nutrient solution, the pH increases, and it should be corrected with the appropriate buffer to avoid any precipitation and make all the elements available to the plant. On the other hand, CO_2 is highly soluble in water and follows normal solubility laws according to temperature and pressure of the solution. As CO_2 dissolves in water, the solution contains unhydrated CO_2 at about the same concentration (~10 µM) by volume as in the atmosphere. At pHs lower than 8, CO_2 dissolves by a slow reaction (a halftime of approximately 15 s) resulting in the weak acid H_2CO_3 that dissociates rapidly into bicarbonate and carbonate (Wetzel, 2001).

At pH 8, applying between 10-20 mM HCO_3^- to the nutrient solution of hydroponically grown maize plants decreased various shoot nutrient concentrations: K (10-20%), Ca (40-50%), NO_3^- (20-30%) and S (10%), while Mg increased by 10-20%. In addition, applying 5 mM HCO_3^- decreased K (10%), NO_3^- (20%), S (10%) and P (25%) while Mg increased significantly by 20% and Ca increased by 10% although not significantly. On the other hand, in the root tissue K (30%), NO_3- (~40%), S (22%) and P (20%) decreased when applying 5-20 mM HCO_3^-, while Ca and Mg increased 2-3-fold (300%) and 23% respectively, and P increased by 30% with adding just 5 mM HCO_3^- (Alhendawi et al., 2014). Moreover, same authors found that adding ≥ 5 mM HCO_3 decreased shoot and root Fe concentration by 20-70% in maize, sorghum and barley (Alhendawi et al., 1997). On the contrary, at pH 6.5, adding 5.68 mM HCO_3^- to the nutrient solution increased root and shoot NO_3^- (20-30%), K (~25-40%) and Ca (25-30%) tissue content in tomato (Bialzyck et al., 1994). Zn deficiency symptoms have also been associated with high HCO_3^- concentrations in "Zn-inefficient" rice cultivars while "Zn-efficient" rice cultivars sustained root growth in the presence of high HCO_3^- (Yang et al.,1994).

Most of the RZ CO_2 literature has focused on nitrogen uptake and metabolism since it is linked to carbon metabolism. Nitrogen is taken up by plants in the inorganic form as nitrate, ammonium ion, ammonia gas or (in legumes) as a nitrogen gas before microbial nitrogen fixation occurs.The nitrogen content of amino acids is all derived from ammonia, which occupies the principal role in plant nitrogen metabolism (Lea & Ireland, 1999). In most plants, amino acids are the main chemical forms in which nitrogen is transported (Tegeder, 2014). Their synthesis also depends on carbon skeletons produced by photosynthesis and sugar catabolism (Heldt, 1996). Amino acid biosynthesis, degradation and transport are strongly regulated to meet demand in response to the availability of nitrogen and carbon (Pratrelli & Pilot, 2014). The essential amino acids (leucine, isoleucine, methionine, phenylalanine, arginine, histidine, tryptophan, valine, threonine, and lysine) are synthesized only by plants, while non-essential amino acids (alanine, β-alanine, asparagine, cysteine, glutamine, aspartic acid, glycine, proline, serine and tyrosine) are synthesized by both plants and humans. Amino acids, besides being the main constituents of proteins, are involved in many physiological processes, such as growth and development (Ortiz-Lopez et al, 2000), pH control (Snedden et al., 1992), generation of metabolic energy (Gailili et al., 2014) , resistance to abiotic and biotic stress (Zeier, 2013) and plant signalling (Hausler et al., 2014). Therefore, it seems important to understand the impacts of RZ CO_2 enrichment on plant nitrogen metabolism.

Although studies have been carried out under elevated air CO_2, little is known about the plant amino acid concentration under elevated RZ CO_2. Elevated aerial CO_2 (700 ppm) increased the concentrations of glutamine (2-3 fold), glutamate (50%) and minor amino acids (2-3 fold) in the leaves of young tobacco plants. Root amino acid concentrations in those plants were changed mainly due to a disproportionate increase of glutamine (44-92%) and alanine (2-3 fold). This increase in amino acids was correlated also with increased NO_{3-} uptake (Geiger et al., 2002). Hydroponically grown tomato plants showed changes in xylem sap amino acid concentrations when grown under different NO_3-/NH_4+ and bicarbonate concentrations. In all treatments, the xylem sap of NH_4+ supplied seedlings had 2.6-fold higher total amino acid concentrations than plants supplied with NO_3-. Glutamine, glutamate, asparagine, and aspartate constituted 69% of this fraction in plants supplied with NO_3- and about 77% in plants grown with NH_4+. Applying 5 mM bicarbonate increased total amino acids concentration by about 17% in the case of NO_3- and 56% in the case of NH_4+ (Bialczyk et al., 2004). In addition, more ^{14}C label (supplied as $NaH^{14}CO_3$

for up to 1 h) was incorporated into organic acids, carbohydrates and amino acids under elevated RZ CO_2 (5000 ppm) than ambient RZ CO_2 (360 ppm) in hydroponically grown tomato seedlings. Incorporation was greater in the organic acid fraction than the total amino acid and carbohydrate fractions. However, under salinity stress (100 mM NaCl), more label was allocated into amino acids (Cramer and Lips, 1995). In a second labelling study, ^{14}C localized in the amino acid fraction was not strongly influenced by the concentration of the RZ CO_2 (0 ppm or 5000 ppm CO_2) but label incorporation into amino acids was greater in NH_4^+ than NO_3^- - fed plants where the diversion into organic acids and carbohydrates and relative growth rate was greater (Viktor and Cramer, 2005). From those experiments, it was concluded that:

A) Both NO_3^- ions (after they are reduced to NH_4^+) and direct uptake of NH_4^+ions provide sources for amino acid synthesis.
B) Elevated DIC increases root PEPc activity, to a greater extent under NH_4^+ nutrition even though incorporation of NO_3^- is also increased under high RZ DIC. In all cases growth increased is greater under NO_3^- nutrition compared to NH_4^+ nutrition.

C) Oxaloacetate derived from PEPc may (1) be converted to malate by malate dehydrogenase (EC 1.1.1.37); (2) be transaminated by aspartate transaminase (EC 2.6.1.1) to form aspartate; or (3) enter the tricarboxylic acid cycle to form 2-oxoglutarate, which may in turn enter the glutamine synthetase glutamate synthase (GSGOGAT; EC 6.3.1.2; 1.4.7.1) pathway for synthesis of glutamate and glutamine.

Although tissue nutrient concentrations have been measured in the past when applying high RZ HCO_3^- concentrations, little is known regarding the effects of elevated RZ CO_2 on nutrient concentrations. Therefore, the aim of this chapter is to investigate how bicarbonate added to a hydroponic nutrient solution and RZ CO_2 in an aeroponic system affect the nutrient concentration in lettuce and pepper plants. In addition, amino acid profile was analysed in aeroponically grown lettuce plants since there seems to be no information regarding individual amino acid concentrations in aeroponically grown crops under elevated RZ CO_2.

3.2. Material and Methods

3.2.1 Plant material and treatments

Crisphead lettuce (*Lactuca sativa* cv. Consul) and pepper (*Capsicum annuum* (L.) cv. Bellboy F1) seeds were purchased from Moles Seeds (Essex, UK) and butterhead lettuce (*Lactuca sativa* cv. Sunstar) seeds from Hazera Seeds (Lincolnshire, UK).

For the hydroponic system (Experiment 1.2 /Chapter 2), seeds were grown in 84-cell plug trays (Length 52 cm x Width 32 cm x Depth 5 cm, plug size 3.8 cm square) containing vermiculite and germinated in a CE room. The CE room temperature ranged between 18 - 21 °C and relative humidity ranged between 40 - 60%. Lights were 102-Watt LED light strips (B100 series, Valoyaâ, Finland) providing an average PPFD across the growing area of 189 µmol m^{-2} s^{-1} with a 16 hour photoperiod. Plants were transferred to hydroponic culture at 2-4 leaf stage, after rinsing the roots in water. Each box contained 14 L of half-strength Hoagland solution. Bicarbonate was applied in the form of $NaHCO_3^-$ at 0, 1 and 20 mM. The medium was changed every 3 - 4 days and $NaHCO_3^-$ re-added. Daily, the pH was adjusted dropwise with HCl or NaOH to ~6.4 for the duration of the experiment which lasted 10 days (Figure 2.1).

For the aeroponic system, seeds were individually sown in 150-cell plug trays in 2 cm x 2 cm x 4 cm rockwool cubes (Growell, Ltd, UK). Pepper was germinated in the glasshouse (Experiment 2.9 / Chapter 2) and lettuce in the CE room (Experiment 2.4 / Chapter 2). Plants were transferred to the system at the 4 leaf-stage in lettuce and at 2-4 leaf-stage in pepper. RZ CO_2 (1500 ppm) was applied to one of the bins and to the other bin, ambient RZ CO_2 was added using the same delivery system described in Chapter 2. The pH was controlled everyday between 5.8-6.3 with the necessary dropwise application of HCl or NaOH.

3.2.2 Plant measurements

Shoot fresh weight was determined after 10 days of treatment, along with leaf area using a leaf area meter (Li-cor Model 3100 Area Meter, Cambridge, UK). Roots were collected, rinsed with dH_20 and dried with absorbent paper. Both shoot and root material was then dried at 70°C for 4 days to record dry weight and stored in airtight containers to provide samples for nutrient analysis.

For amino acid analysis, lettuce grown aeroponically under elevated RZ CO_2 was used (Experiment 2.5 / Chapter 2), since aeroponically grown lettuce had a greater response to RZ CO_2 enrichment than in other crops and systems. A leaf disc of 2.5 cm diameter from leaf number 6 was collected from each plant and immediately frozen in liquid nitrogen. Roots were cut, rinsed with dH_20, dried with tissue and immediately frozen in liquid nitrogen. Both shoots and roots were kept at -80°C until delivery to the laboratories undertaking the measurements (Palacký University, Czech Republic).

3.2.3 Nutrient analysis

For the bicarbonate experiment, the median four plants were taken from each of the 0, 1 and 20 mM $NaHCO_3^-$ treatments and sent to NRM Technologies Ltd.â (Bracknell, UK) for C013 Plant Foliar Suite Analysis, incorporating analysis for: total N, P, K, Mg, Ca, S, Cu, Mn, Zn, Fe and B.

For the lettuce aeroponic experiment, macronutrients (Ca, K, Mg, Na, P and S) were analysed via acid microwave digestion followed by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Nitric acid (HNO_3) was used to decompose all organic matter to CO_2. Ball-milled (MM400, Retsch, Haan, Germany) oven dried leaf and root tissue (0.25 g) was weighed in acid-washed and rinsed reaction vessels. Five mL of 100 % HNO_3 (Aristar grade) was added and left for 15 min in a fume hood until the initial reaction was finished. Vessels were sealed and weighed and then placed in the rotor in a MARS 6 microwave (CEM, Buckingham, UK). Vessels were heated to 200°C over 15 min and then held at 200°C for another 15 min. After cooling down, vessels were weighed again to note weight loss. Samples and blank solutions were then diluted in two steps to first 20 % HNO_3 and second to the final concentration of 2 % HNO_3 by using MilliQ water. To analyse nutrients, ICP-OES (iCAP 6300, Thermo Scientific, Massachusetts, USA) with axial view configuration was used. To validate the digestion, tomato and spinach leaves samples with known nutrient concentrations were run and the recovery detected through the ICP-OES was used to calculate final sample concentration. The element reference standard solutions were prepared daily from 1000 mg L^{-1} stock solutions. The same protocol was used for leaves and roots of pepper plants grown under elevated and ambient RZ CO_2.

In each experiment, shoot and root nutrient content was calculated multiplying tissue nutrient concentration by its dry mass. When nutrient concentrations of both tissues were not available, only shoot nutrient content was determined.

3.2.4 Total Nitrogen and NO_3^- analysis

Total leaf and root nitrogen in % was analysed using an Elemental Analyser (VARIO- El elemental analyser). Oven-dried leaf and root tissue samples were wrapped in aluminium capsules and dropped into a furnace held at 905°C onto CuO with a pulse of O_2 and a constant flow of Helium carrier gas. N was converted to gas (N_2) and a pure copper reduction unit after the furnace reduced any conversion of NOx to N_2. N_2 was measured in a TCD (total dissolved carbon) detector positioned at the end of the elemental analyser and peak areas were compared to standards and amounts of N calculated.

NO_3^- concentration in the leaf tissue was measured using the method of Cataldo et al*.,* (1975). Oven dry and ground samples (0.1 g) were suspended in 10 mL dH_20. The suspensions were incubated at 80°C for 2 h. After mixing, the samples were centrifuged at 1300g for 5 min and the supernatants were decanted and saved for analysis.

Aliquots (0.1 mL) of the extracts were pipetted into 10 mL tubes and mixed thoroughly with 0.4 mL 5% (w/v) salicylic acid-sulphuric acid. After 20 min at room temperature, 9.5 mL of 8% (w/v) NaOH were added to raise the pH above 12. Samples were cooled to room temperature for 20 min and absorbance at 410 nm was determined in an Ultrospec 2100 Pro UV/visible spectrophotometer (GE Healthcare UK Ltd, Little Chalfont, UK). Standards containing 10 to 120 mg/L in a 0.1 mL aliquot were analysed with each sample.

3.2.5 Amino acids analysis

For the free amino acids analysis, samples were collected and pooled together in liquid nitrogen. Around 50 mg FW of the pooled material was extracted. The extraction procedure was performed using an AccQTag Ultra derivatization kit (©Waters). All extracted samples were analysed using an ACQUITY UPLC® System and a XevoTM 122 TQ-S triple quadrupole mass spectrometer (©Waters) according to the annotation note of Waters Corporation (Milford, MA, USA) (Gray and Plumb, 2016). Calibration curves were constructed for each component analysed using internal standards: γ-aminobutyric acid (GABA), *L*-alanine (Ala), *L*-arginine (Arg), *L*-asparagine (Asn), *L*-aspartic acid (Asp), *L*-citrulline (Cit), *L*-glutamine (Gln), *L*-glutamic acid (Glu), *L*-glycine (Gly), *L*-histidine (His), *L*-ileucine (Ile), *L*-leucine (Leu), *L*-methionine (Met), *L*-phenylalanine (Phe), *L*-proline (Pro), *L*-serine (Ser), *L*-tryptophan (Trp) and *L*-tyrosine (Tyr), and the deuterium-labelled compounds *L*-glutamic acid-2,3,3,4,4-d_5, γ-aminobutyric acid-2,2,3,3,4,4-d_6 and *DL*-

leucine-2,3,3,4,5,5,5,5',5',5'-$d_{10,}$ all purchased from ©Sigma-Aldrich Inc. (Germany). Samples were analysed by Dra. Nuria De Diego (Palacký University, Czech Republic.)

3.2.6 Statistical analysis

One-way analysis of variance (ANOVA) was performed to analyse differences in nutrient concentrations between different bicarbonate concentrations, followed by LSD post-hoc analysis.

To determine treatment differences in aeroponically grown lettuce and pepper an Independent Samples Student's t-test at the $P < 0.05$ level was performed.

Pearson's Correlation was used to examine correlations between leaf and root nutrient concentrations and growth parameters. Prior to the analysis, normality of the residuals was tested using Shapiro-Wilk test (Shapiro and Wilk, 1965).

3.3 Results

Shoot dry weight and leaf area were 10 % and 20% higher at 1 mM HCO_3- and ~ 50 % lower at 20 mM compared to control plants (Table 3.1). Bicarbonate enrichment of the RZ (1 and 20 mM) significantly decreased shoot N (22%-33%), P (13%), K (14%-33%), Zn (20%-52%) and Cu (42%) concentrations, with significantly lower K and Zn concentrations at 20 mM than 1 mM. Furthermore, 20 mM HCO_3^- significantly decreased foliar Mn concentration by 28%. In contrast, bicarbonate enrichment of the RZ (20 mM) significantly increased Mg, Fe and B concentrations by 42%, 20% and 42% respectively (Figure 3.1). Thus, the concentration of bicarbonate affected the direction and magnitude of changes in different nutrient concentrations.

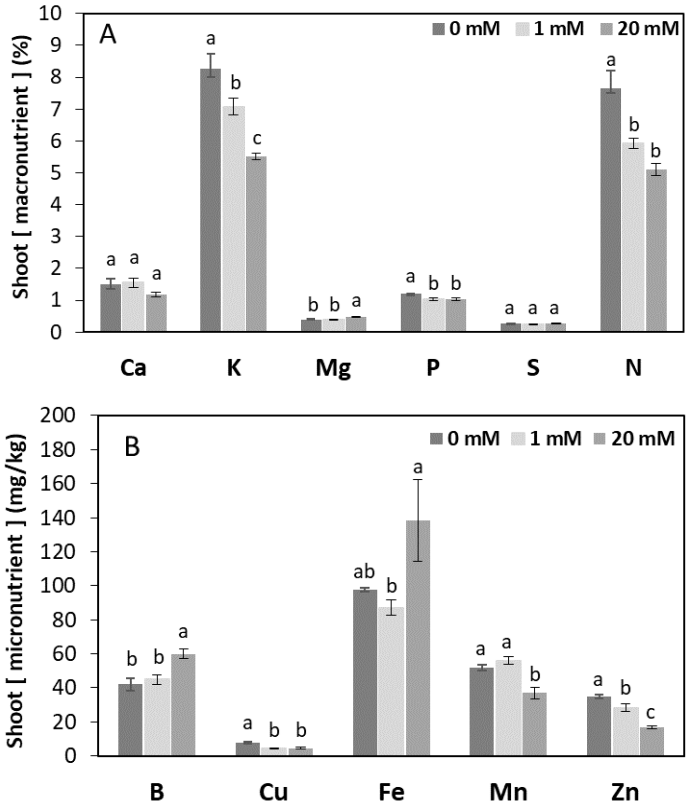


Figure 3.1. Lettuce shoot macronutrients concentration **(A)**: calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P) and nitrogen (N). Shoot micronutrients concentration **(B)**: boron (B), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn). Values indicated with different letters indicate statistically significant differences according to one – way ANOVA test ($p<0.05$), followed by LSD post-hoc analysis. Bars represent means ± SE (n=4).

Table 3.1. Shoot fresh and dry weights, leaf area and growth rate of lettuce plants grown under 0, 1 and 20 mM HCO_3^- (Experiment 1.2, table 2.5/ Chapter 2) . Values are means ± S.E. of 4 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$).

HCO_3^-	0 mM	1 mM	20 mM
Shoot fresh weight (g)	28.2 ± 0.7 [a]	32.3 ± 1.6 [a]	13.3 ± 0.9 [b]
Shoot dry weight (g)	1.70 ± 0.04 [a]	1.94 ± 0.09 [a]	0.80 ± 0.05 [b]
Leaf area (cm^2)	543 ± 32 [b]	677 ± 34 [a]	279 ± 16 [c]
Growth Rate (mg g^{-1} /d)	2.52 ± 0.03 [b]	3 ± 0.1 [a]	1.3 ± 0.1 [c]

Shoot macronutrient content was not affected by 1 mM HCO_3^- but was lower under 20 mM HCO_3^-. Micronutrient content was more variable, with Cu and Fe significantly lower under 1 mM HCO_3^- compared to control plants.

Table 3.2. Total nutrient content of plants exposed to 0, 1 and 20 mM HCO_3^-. Values are means ± S.E. of 4 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$).

mg	Ca	K	Mg	P	S	N
Control	25.5 ± 3.1[a]	140.1 ± 10.1[a]	6.7 ± 0.5[a]	20.2 ± 0.8[a]	4.6 ± 0.2[a]	129.3 ± 7.5[a]
1 mM	26.6 ± 4.7[a]	120.9 ± 16.1[a]	6.6 ± 0.8[a]	17.5 ± 2.0[a]	4.2 ± 0.5[a]	101.3 ±13.3[a]
20 mM	9.5 ± 1.1[b]	44.3 ± 3.7[b]	3.82 ± 0.4[b]	8.3 ± 0.7[b]	2.12 ± 0.2[a]	40.9 ± 3.6[b]

µg	B	Cu	Fe	Mn	Zn
Control	87.7± 4.3[a]	1.3± 0.1[a]	59.4 ± 3.5[a]	165.5 ± 4.6[a]	71.7 ± 7.5[b]
1 mM	95.8 ±12.9[a]	0.7± 0.1[b]	47.2 ± 3.6[b]	149.3 ±22[a]	77.3 ± 12.2[ab]
20 mM	30.1 ± 4.5[b]	0.4 ± 0.0[c]	13.6 ± 1.7[c]	112.5 ± 22.9[a]	48.4 ± 5.1[bc]

Shoot dry weight of lettuce grown aeroponically was 22% higher under RZ CO_2 (Experiment 2.4, Chapter 2). Foliar Ca, K, Mg and S concentrations were about 20%, 15%, 24% and 15% lower under RZ CO_2, while P was about the same concentration. In the roots, Ca was about 10% higher under RZ CO_2 compared to control plants. No major changes were found in the other macronutrients (Figure 3.2, A and B).

Regarding micronutrients, foliar Zn concentration was 15% higher under RZ CO_2 whereas Mn and Fe were lower by 10 % (Figure 3.2, C and D). The differences in the concentrations of other micronutrients were relatively small and no clear treatment differences were detected.

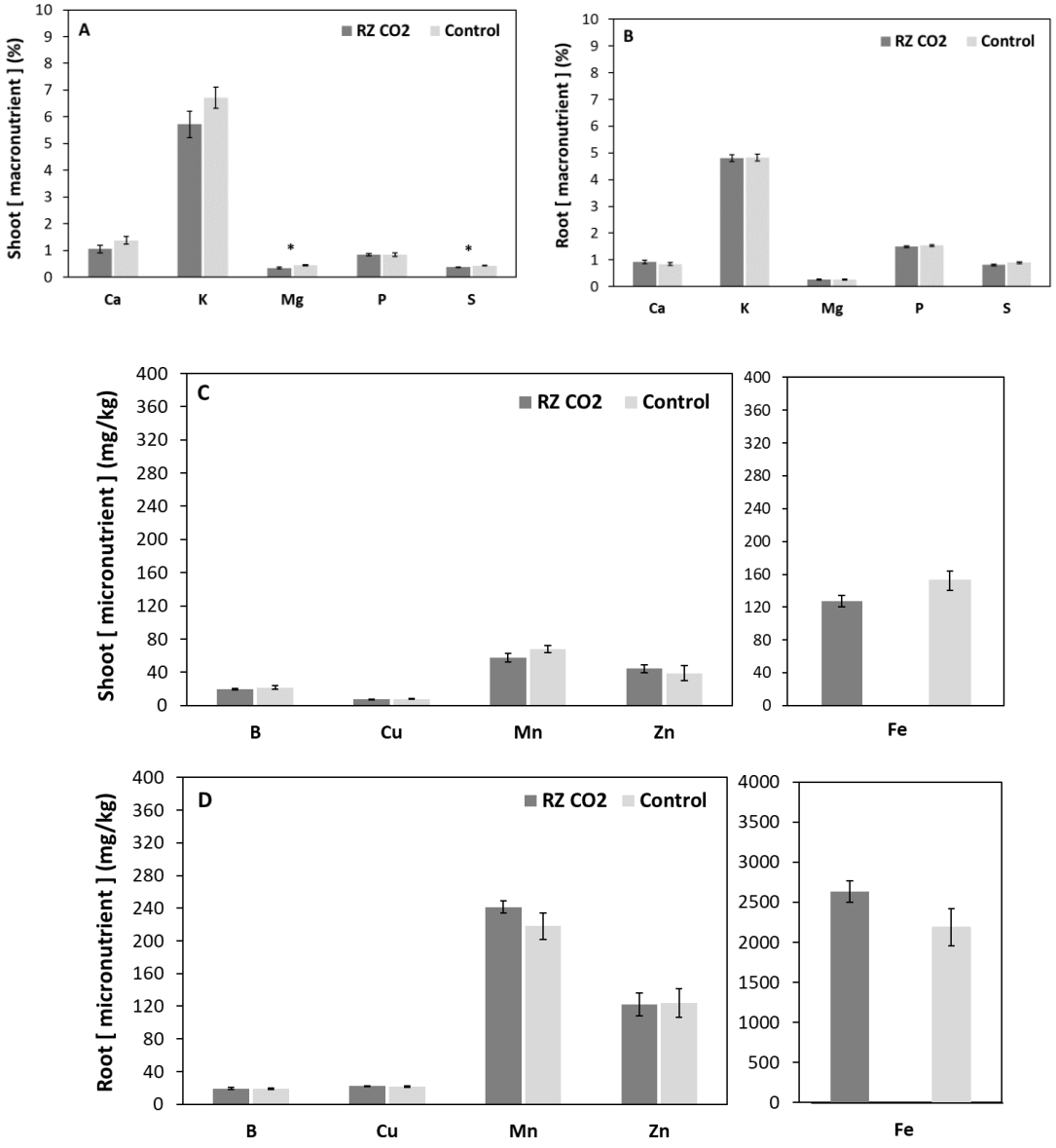


Figure 3.2. Shoot (A) and root (B) macronutrients concentrations: calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P) and Sulphur (S). Shoot (C) and root (D) micronutrients concentrations: boron (B), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn). Bars are means ± SE of 8 replicates. Asterisks indicate significant differences between treatments (Independent Sample T-test, p-value < 0.05).

Elevated RZ CO_2 tended to decrease total shoot N concentration by 5%, but significantly increased root N concentration by 5%. Elevated RZ CO_2 significantly increased root NO_{3-} concentrations and there was a greater proportion of NO_{3-}. Elevated RZ CO_2 tended to decrease C/N ratio in both roots and leaves (Figure 3.3).

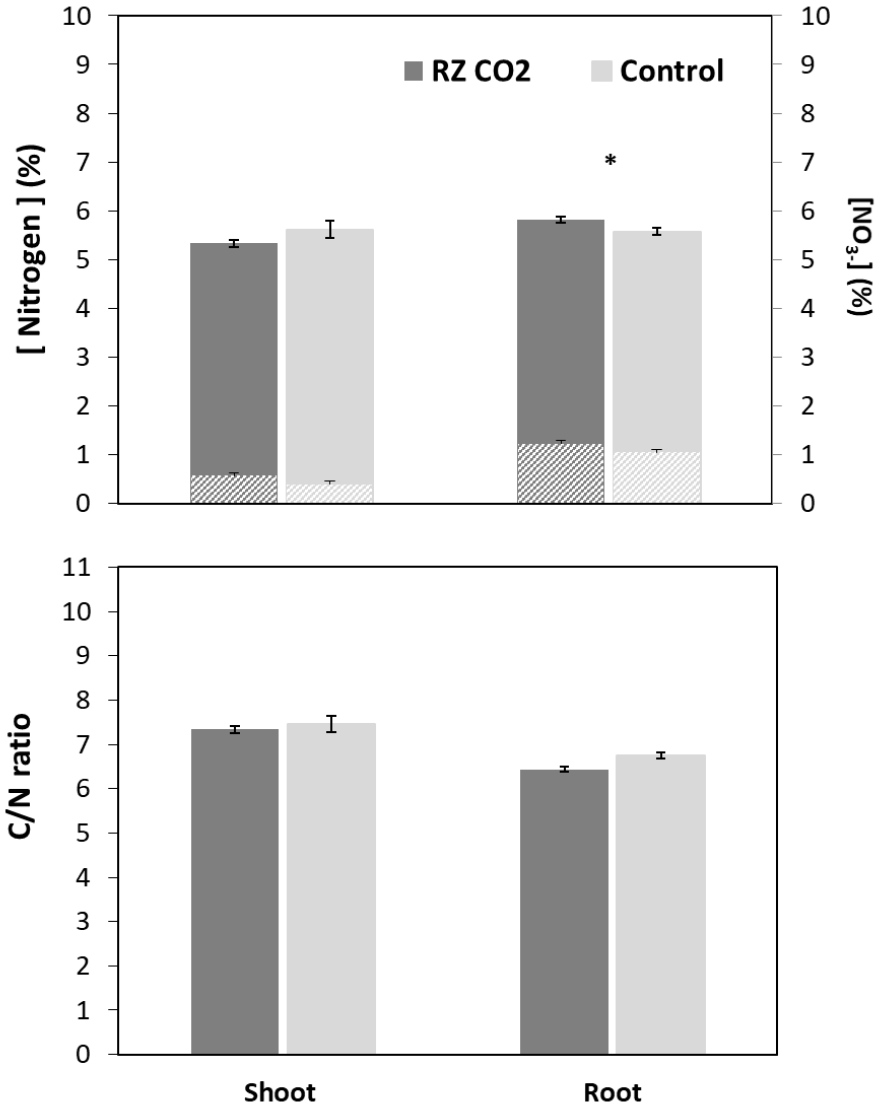


Figure 3.3. Shoot and root nitrogen and NO_{3-} concentration (%) and C/N ratio in lettuce plants exposed to high and ambient RZ CO_2. Bars are means ± SE of 8 replicates. Asterisks indicate significant differences between treatments (Independent Sample T-test, p-value < 0.05).

Under elevated RZ CO_2, more nutrients had tissue concentrations that were correlated to each other, compared to control plants (Table 3.3 and 3.4). For the macronutrients, in control plants, leaf Ca concentrations were positively correlated with leaf K and Mg concentrations. Under elevated RZ CO_2, leaf Ca was positively correlated with K, Mg, Mn, S and N. Moreover, leaf K and Mg concentrations were correlated, as were Mg and Mn concentrations, and S with N and Zn concentrations. These analyses suggest greater co-ordination of nutrient uptake and transport under elevated RZ CO_2.

Both treatments induced similar correlations in root nutrient concentrations. However, while Mg and Mn were predominantly correlated with Ca, Cu and Fe in control plants, under elevated RZ CO_2, S was positively correlated with B, Cu, K and P.

In control plants, nutrient concentrations were not correlated with either shoot dry weight or root dry weight. Under elevated RZ CO_2 in which plant biomass was greatest, shoot dry weight was significantly negatively correlated with root Ca and Mg concentrations, but was positively (although not significantly) correlated with shoot NO_{3^-} concentrations. Interestingly, the negative correlation between total shoot N and NO_{3^-} was higher under elevated RZ CO_2 compared to control plants.

Table 3.3. Pearson's correlation coefficients among macro- and micro- nutrient concentrations in roots and shoots, and root (RDW) and shoot dry weight (SDW) of lettuce plants exposed to elevated RZ CO_2.

		Root												Leaf													
Root	B	B																									
	Ca	0.66	Ca																								
	Cu	**0.85****	0.57	Cu																							
	Fe	**0.78***	0.46	**0.94****	Fe																						
	K	**0.88****	0.55	0.66	0.49	K																					
	Mg	0.65	**0.97****	0.48	0.37	0.6	Mg																				
	Mn	-0.66	-0.39	**0.8***	**0.92****	-0.29	-0.27	Mn																			
	P	0.7	0.29	**0.77***	0.61	0.7	0.19	-0.54	P																		
	S	**0.76***	0.69	**0.76***	0.56	**0.71***	0.62	-0.53	**0.82***	S																	
	Zn	-0.17	-0.69	-0.43	-0.32	-0.04	-0.54	0.34	-0.3	-0.57	Zn																
	N	0.02	-0.08	0	0.09	0.23	-0.05	0.07	0.03	-0.26	0.14	N															
	NO_{3-}	-0.18	0.31	-0.31	-0.59	0.07	0.35	0.65	-0.1	0.19	-0.32	-0.31	NO_{3-}														
Leaf	B	0.56	0.16	0.59	0.62	0.64	0.21	-0.39	0.42	0.28	0.11	0.61	-0.51	B													
	Ca	0.35	0.07	-0.02	-0.15	0.63	0.15	0.13	0.44	0.37	0.25	0.25	0.21	0.22	Ca												
	Cu	**0.8***	0.25	**0.9****	**0.8***	**0.71***	0.2	-0.61	**0.83***	0.65	-0.08	0.03	-0.34	0.65	0.14	Cu												
	Fe	0.7	0.38	0.58	0.45	**0.88****	0.45	-0.23	0.6	0.61	0	0.36	-0.07	**0.84****	0.55	0.66	Fe											
	K	-0.12	-0.34	-0.41	-0.44	0.16	-0.3	0.37	0.11	-0.21	0.42	0.49	0.1	-0.04	**0.76***	-0.21	0.07	K									
	Mg	0.39	0.08	0.04	-0.11	0.65	0.14	0.09	0.52	0.44	0.21	0.19	0.23	0.21	**0.99****	0.21	0.57	**0.72***	Mg								
	Mn	0.35	-0.08	0.31	0.17	0.52	-0.08	-0.2	**0.74***	0.59	0.04	0.03	-0.07	0.38	0.71	0.49	0.63	0.34	**0.76***	Mn							
	P	0.4	0.04	0.15	0	0.5	0.07	0.19	0.31	0.1	0.37	-0.01	0.27	-0.01	0.4	0.38	0.16	0.38	0.45	-0.02	P						
	S	0.56	0.2	0.33	0.1	**0.85****	0.29	0.06	0.65	0.6	0.13	0.19	0.24	0.51	**0.82***	0.52	**0.86****	0.39	**0.85***	**0.78***	0.42	S					
	Zn	0.04	-0.2	-0.26	-0.46	0.4	-0.02	0.6	0.05	0.07	0.53	-0.08	0.45	0.1	0.61	0.06	0.45	0.36	0.62	0.43	0.45	**0.72***	Zn				
	N	0.15	-0.02	-0.21	-0.19	0.37	0.1	0.08	0.09	0.1	0.34	0.37	-0.05	0.29	**0.84****	-0.14	0.46	0.65	**0.79***	0.55	-0.12	0.56	0.44	N			
	NO_{3-}	-0.82	-0.87	-0.7	-0.56	**0.97****	-0.87	0.36	-0.72	**0.86***	0.49	-0.47	-0.03	**0.93***	-0.79	-0.66	**0.93***	0.01	-0.82	-0.58	-0.11	-0.82	-0.19	-0.8	NO_{3-}		
	SDW	-0.6	**0.76***	-0.53	-0.49	-0.63	**0.73***	0.37	-0.38	-0.47	0.49	-0.57	0	-0.52	-0.29	-0.29	-0.54	-0.11	-0.27	-0.07	-0.04	-0.33	0.25	-0.26	0.82	SDW	
	RDW	-0.05	-0.09	-0.21	-0.17	-0.19	-0.14	-0.2	0.11	0.18	0.01	-0.48	0.01	-0.53	0.36	-0.25	-0.33	0.29	0.37	0.34	-0.35	-0.05	-0.05	0.38	0.62	0.27	RDW

1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0 -0.1 -0.2 -0.3 -0.4 -0.5 -0.6 -0.7 -0.8 -0.9 -1

**Correlation significant at 0.01 level (2-tailed) *Correlation significant at 0.05 level (2-tailed)

Table 3.4. Pearson's correlation coefficients among macro- and micro- nutrient concentrations in roots and shoots, and root (RDW) and shoot dry weight (SDW) of lettuce plants exposed to ambient RZ CO_2.

		Root												Leaf												
Root	B	B																								
	Ca	-0.58	Ca																							
	Cu	-0.38	0.9**	Cu																						
	Fe	-0.37	0.73	0.87*	Fe																					
	K	0.88**	-0.49	-0.39	-0.48	K																				
	Mg	-0.35	0.96**	0.94**	0.78*	-0.33	Mg																			
	Mn	-0.34	0.8*	0.91**	0.8*	-0.43	0.87*	Mn																		
	P	0.33	0.22	0.36	-0.01	0.41	0.36	0.48	P																	
	S	-0.19	0.53	0.44	0.01	-0.1	0.53	0.57	0.78*	S																
	Zn	0.41	0	-0.03	-0.06	0.37	0.06	-0.32	-0.21	-0.27	Zn															
	N	0.73	-0.29	-0.12	0.06	0.76*	-0.11	-0.24	0.04	-0.51	0.43	N														
	NO_{3-}	-0.53	0.2	0.21	-0.1	-0.33	0.04	0.15	0.26	0.46	-0.39	-0.64	NO_{3-}													
Leaf	B	0.1	-0.02	0.16	0.14	0.38	0	0.09	0.36	-0.06	-0.34	0.45	0.27	B												
	Ca	-0.45	0.55	0.19	-0.14	-0.2	0.4	0.06	0.08	0.56	0.22	-0.47	0.3	-0.33	Ca											
	Cu	0.24	-0.01	0.16	0.1	0.54	0.02	-0.06	0.28	-0.17	0.06	0.62	0.17	0.92**	-0.2	Cu										
	Fe	0.61	0.03	0.26	0.19	0.74	0.21	0.16	0.55	-0.01	0.2	0.81*	-0.21	0.72	-0.31	0.82*	Fe									
	K	-0.39	0.25	-0.18	-0.21	-0.21	0.1	-0.25	-0.4	0.07	0.21	-0.27	-0.19	-0.49	0.77*	-0.39	-0.49	K								
	Mg	-0.14	0.46	0.15	-0.22	0.04	0.41	0.11	0.34	0.7	0.25	-0.34	0.09	-0.4	0.91**	-0.28	-0.16	0.68	Mg							
	Mn	0.38	0.16	0.36	0.21	0.37	0.35	0.57	0.87*	0.52	-0.39	0.26	-0.04	0.48	-0.27	0.31	0.65	-0.5	0.01	Mn						
	P	0.19	-0.48	-0.37	-0.1	0.33	-0.51	-0.57	-0.48	0.84*	0.16	0.6	-0.12	0.54	-0.48	0.64	0.33	-0.2	-0.66	-0.32	P					
	S	0.56	0.24	0.31	0.14	0.72	0.41	0.19	0.58	0.21	0.41	0.71	-0.35	0.4	0.09	0.58	0.87*	-0.1	0.29	0.58	0.03	S				
	Zn	-0.17	0.55	0.76*	0.63	0	0.59	0.64	0.5	0.27	-0.21	0.18	0.4	0.75	-0.1	0.7	0.63	-0.5	-0.18	0.53	0.11	0.43	Zn			
	N	0.2	-0.52	-0.45	-0.23	-0.22	-0.46	-0.19	-0.29	-0.22	-0.1	-0.25	-0.28	-0.64	-0.44	-0.75	-0.54	-0.2	-0.3	-0.18	-0.22	-0.59	-0.68	N		
	NO_{3-}	0.07	-0.19	-0.19	-0.11	0.15	-0.27	-0.56	-0.57	-0.62	0.72	0.3	0	-0.02	0.05	0.31	0.04	0.09	-0.18	-0.72	0.65	-0.02	-0.09	-0.19	NO_{3-}	
	SDW	-0.05	-0.24	0.06	0.19	0.02	-0.26	-0.03	-0.06	-0.4	-0.31	0.22	0.4	0.74	-0.62	0.65	0.33	-0.7	0.81*	0.07	0.69	-0.15	0.56	-0.18	0.27	SDW
	RDW	0.12	0	0.02	0.05	0.29	0.08	0.23	0.44	0.19	-0.62	0.29	-0.17	0.6	-0.21	0.35	0.44	-0.1	-0.07	0.71	0.02	0.37	0.31	-0.29	-0.7	0.09	RDW

1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0 -0.1 -0.2 -0.3 -0.4 -0.5 -0.6 -0.7 -0.8 -0.9 -1

**Correlation significant at 0.01 level (2-tailed) *Correlation significant at 0.05 level (2-tailed)

With RZ CO_2 enrichment, lettuce had higher shoot Zn (50%), Cu (22%) and P (35%) contents, and significantly higher N (25%) content. Root nutrient content did not significantly differ between treatments (Table 3.5).

Table 3.5. Lettuce shoot and root nutrient content under elevated RZ CO_2 and control plants. Different letters and bold text indicate significant differences between treatments ($p<0.05$).

	Shoot		**Root**	
	RZ CO_2	**Control**	**RZ CO_2**	**Control**
mg				
Ca	43.4 ± 5.9[a]	44.8 ± 5.1[a]	4.1 ± 0.6[a]	5.1 ± 1.3[a]
K	237.9 ± 21.3[a]	216.4 ± 18.4[a]	21.7 ± 2.8[a]	24.5 ± 7.8[a]
Mg	14.1 ± 1.0[a]	15.3 ± 0.6[a]	1.2 ± 0.2[a]	1.6 ± 0.4[a]
P	**34.6 ± 2.3[a]**	**25.6 ± 1.9[b]**	6.7 ± 0.9[a]	7.8 ± 2.5[a]
S	15.3 ± 0.6[a]	13.6 ± 0.7[a]	3.5 ± 0.5[a]	4.6 ± 1.4[a]
N	**225 ± 6.7[a]**	**179.9 ± 6.8[b]**	26.9 ± 3.7[a]	28.9 ± 9.5[a]
µg				
B	84.8 ± 4.2[a]	72.7 ± 12.4[a]	8.7 ± 1.7[a]	10 ± 2.4[a]
Cu	**29.9 ± 0.95[a]**	**24.5 ± 1.5[b]**	10.2 ± 1.5[a]	11.5 ± 3.2[a]
Fe	528.8 ± 25.2[a]	466.8 ± 44.3[a]	1234.1± 207.9[a]	1286.5 ± 371.3[a]
Mn	238.9 ± 22.8[a]	211.6 ± 18.5[a]	110.3± 14.6[a]	127.6 ± 34.3[a]
Zn	**436.8 ± 26.4[a]**	**288.5 ± 43.9[b]**	87.7 ± 16.7[a]	92.6 ± 20.1[a]

Pepper plants did not show any significant treatment differences in foliar macronutrient and micronutrient concentrations. In the roots, Zn and Fe were 12% and 15% higher in plants grown under RZ CO_2 (Figure 3.4).

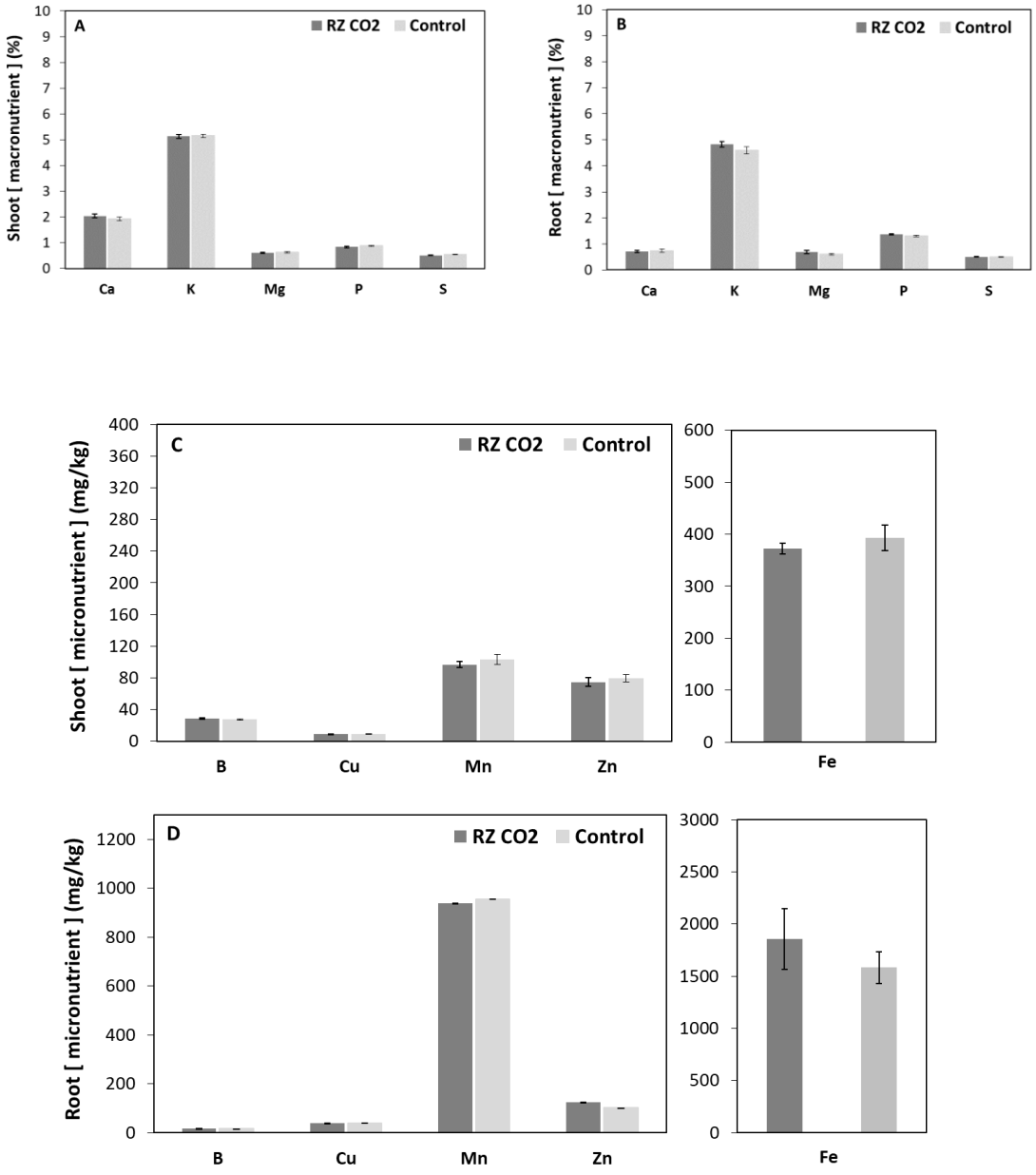


Figure 3.4. Shoot (*A*) and root (*B*) macronutrients concentrations: calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P) and Sulphur (S). Shoot (*C*) and root (*D*) micronutrients concentration**s**: boron (B), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn). Bars are means ± SE of 8 replicates. Asterisks indicate significant differences between treatments (Independent Sample T-test, p-value < 0.05).

Although the differences were not significant in pepper, elevated RZ CO_2 had similar effects on leaf and root nitrogen concentration as in lettuce. Shoot N concentration decreased by 4%, but increased root N concentration by 5%. C/N ratio was lower in the roots but higher in the leaves under RZ CO_2 (Figure 3.5).

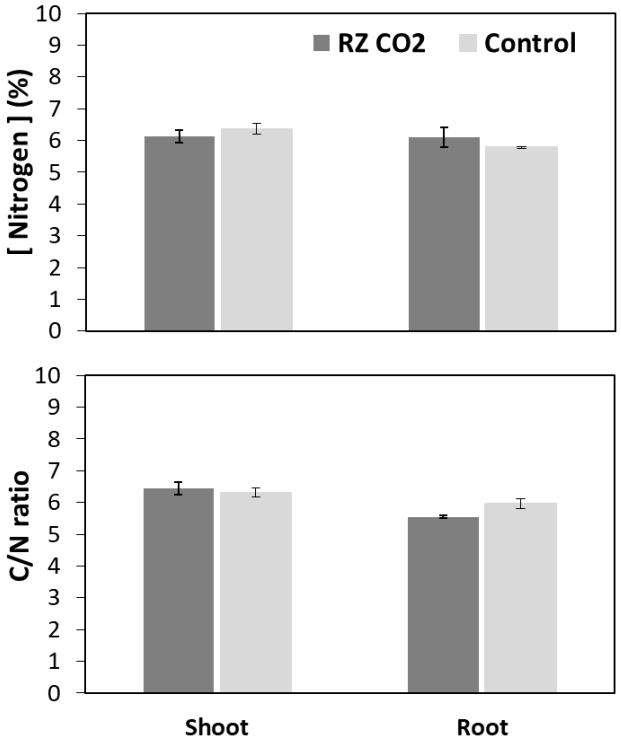


Figure 3.5. Shoot and root nitrogen concentration (%) and C/N ratio in pepper plants exposed to high and ambient RZ CO_2. Bars are means ± SE of 8 replicates. Asterisks indicate significant differences between treatments (Independent Sample T-test, p-value < 0.05).

Foliar glutamine (Gln), asparagine (Asn), histidine (His), glycine (Gly), lysine (Lys), tyrosine (Tyr), valine (Val), proline (Pro) and isoleucine (Ile) concentrations were higher under elevated RZ CO_2 compared to control plants, while arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), cysteine (Cys), alanine (Ala), serine (Ser), threonine (Thr), methionine (Met), leucine (Leu) and tryptophan (Trp) were lower. Among minor amino acids, citrulline (Cit) and ornithine (Orn) increased and GABA decreased under RZ CO_2 (Table 3.6).

Table 3.6. Amino acids concentrations (pmol/mg FW) in the leaves and roots of lettuce grown aeroponically under elevated RZ CO_2. Total amino acids are given in nmol/mg FW. Data are means ± SE of 8 replicates. Analysis was performed using an Independent Sample T-Test, P-value < 0.05, with significant treatment differences highlighted in bold text.

Shoot				Root			
Amino acid	**RZ CO_2**	**Control**	**% change**	**Amino acid**	**RZ CO_2**	**Control**	**% change**
Ser	**221240±30.1***	**321800±24.8**	**-31.25**	**Pro**	1521570± 0.6	1551940±0.6	**1.9**
Pro	121090±7.2	115190±9.8	**5.12**	**Ser**	35780±0.0	33430±0.0	**7.0**
Ala	42770±8.5	53850±7.5	**-20.58**	**Glu**	35680±0.1	64350±0.1	**-44.5**
Gln	**12950±1.2***	**9800±0.8**	**32.14**	**Ala**	10700±0.3	11920±0.3	**-10.2**
Glu	10470±1.6	14210±1.5	**-26.32**	**Orn**	2850±0.0	2030±0.0	**40.4**
Orn	**2460±0.6***	**730±0.1**	**236.99**	**Gln**	2450±0.04	2460±0.04	**-0.4**
Tyr	1227±0.2	1066±0.1	**15.10**	**Tyr**	2310±0.5	2220±0.5	**4**
Met	**1144±0.1***	**1838±0.2**	**-37.76**	**Thre**	2260±0.0	2500±0.0	**-9.6**
Asn	721±0.05	629±0.04	**14.63**	**Arg**	1650±0.1	2870±0.1	**-42.5**
Phe	574±0.02	556±0.02	**3.24**	**Val**	880±0.02	1240±0.03	**-29**
Val	**556±0.05***	**427±0.05**	**30.21**	**Asn**	470±0.2	450±0.2	**4.4**
Thre	**471±0.0***	**679±0.0**	**-30.63**	**Met**	390±0.0	230±0.0	**69.5**
Leu	**235±0.01***	**262±0.01**	**-10.31**	**Ile**	283±0.0	291±0.0	**-2.7**
Asp	**216±0.01***	**272±0.01**	**-20.59**	**Asp**	270±0.0	280±0.0	**-3.5**
Ile	**151±0.01***	**131±0.01**	**15.27**	**Trp**	210±0.0	220±0.0	**-4.5**
Arg	128±0.01	152±0.01	**-15.79**	**Leu**	200±0.0	180±0.0	**11.1**
Trp	**47±0.0***	**61±0.0**	**-22.95**	**Phe**	161±0.5	157±0.5	**2.5**
Gly	**33±0.0***	**24±0.0**	**37.50**	**GABA**	110±0.03	70±0.03	**57.1**
Lys	28±0.0	18±0.0	**55.56**	**His**	50±0.0	60±0.0	**-16.6**
His	**20±0.0***	**16±0.0**	**25.00**	**Lys**	47±0.06	52±0.06	**-9.6**
Cit	**15±0.0***	**10±0.0**	**50.00**	**Gly**	12±0.0	10±0.0	**20**
GABA	**10±0.0***	**20±0.0**	**-50.00**	**Cit**	10±0.0	5±0.0	**100**
OH-Pro	10±0.0	10±0.0	**0.00**	**Cys**	0.4±0.0	0.39±0.0	**2.5**
Cys	**0.3±0.0***	**0.6±0.0**	**-50.00**	**OH-Pro**	0.1±0.06	0.3±0.06	**-66.6**
Total	416.6	521.7			1618.3	1676.9	

3.4 Discussion

In general, high concentrations of bicarbonate (≥ 5mM) in the rhizosphere reportedly decreased foliar K, Mg, S, P and Fe concentrations, while high leaf tissue Ca concentrations occurred when applying bicarbonate and in plants grown in calcareous soils at high pH (Alhendawi et al., 1997, Al mansouri et al., 2014; Bialczyk, 1994). In this study, applying 20 mM HCO_3^- concentration did not change Ca concentration, but K, P, N, Zn, Cu and Mn concentrations decreased remarkably while B, Mg and Fe were increased significantly (Figure 3.1). According to the adequate range of shoot nutrient concentrations in lettuce (Table 3.7), at 20 mM HCO_3^- only Zn was below the limits while B was above the limits. Although P concentrations exceeded the recommended range, this was independent of bicarbonate concentrations applied. Thus bicarbonate enrichment of the nutrient solution should have caused minimal nutritional stress.

Table 3.7. Early-heading leaf macro- and micro-nutrient optimum concentration ranges in butterhead lettuce. Grey sections indicate nutrient concentrations range under 20 mM HCO_3^-

Status	**Macronutrients (%)**					
	N	**P**	**K**	**Ca**	**Mg**	**S**
Deficient	< 4	0.4	5	1	0.3	
In range	4 - 6	0.4 - 0.6	5 - 7	1 - 2	0.3 – 0.6	0.2 - 0.3
High	> 6	0.6	7	2	0.6	
	Micronutrients (mg/kg)					
	B	**Mn**	**Cu**	**Zn**	**Fe**	
Deficient	15	20	5	40	< 50	
In range	15-30	20-40	5 – 10	40 – 60	50 – 150	
High	30	40	10	60	> 150	
Toxic	> 100	> 250				

Sources: Hartz and Johnstone (2007); Hochmuth et al., (2018).

Zinc influences many biological processes, including carbohydrate metabolism and cell proliferation. It also serves as an integral component of some enzyme structures, such as carbonic-anhydrase, alcohol dehydrogenase, and glutamate dehydrogenase (Rehman et al., 2012). Zn deficiency rapidly inhibited plant growth and development, and photosynthesis of many plants, including rice (Ajay & Rathore, 1995) and spinach (Randall & Bouma, 1973). However, photosynthetic and growth responses to Zn deficiency differ from one species to another, meaning that some plants adapt better to such conditions (Zhao & Wu, 2017). Zn deficient lettuce plants have a rosette appearance and growth is restricted. Later, necrotic spots with a dark margin appear along leaf edges (Roorda van

Eysinga & Smilde, 1981). Although plants in this study showed no signs of necrosis, decreased Zn availability may have restricted shoot growth of plants grown at 20 mM HCO_3^-.

Boron is an essential micronutrient for higher plants and is required at different concentrations for optimal growth in different species. Boron plays an important role in some plant functions including metabolic pathways, sugar translocation, pollen germination, hormone action, root development, normal growth and functioning of the apical meristem, water translocation from roots to the upper part of the plant body and membrane structure and body (Liu et al., 2000; Lou et al., 2001). Although B concentrations at 20 mM HCO3- did not reach toxic levels (60mg/kg vs >100mg/kg), foliar B accumulation can reduce crop yield (Ozturk et al., 2010).

Mg levels were higher in some studies at high (>5 mM) HCO_3^- concentrations in white lupin (Bertoni et al., 1992), peach rootstocks (De la Guardia & Alcantara, 2002) and tomato (Siddiqi et al., 2002), while in others the Mg concentration remained unchanged or decreased such as in tomato, tobacco, maize and olive trees. Although Mg deficiency in soils can inhibit plant growth (Verbruggen and Hermans, 2013), high soil Mg concentrations do not damage the crop growth but might hinder K uptake (Senbayram et al., 2015), which may explain the decrease of K under 20 mM HCO_3^- concentration. The physiological and molecular importance of Mg has been underestimated in the last decades as Mg deficiencies in agriculture are not easily recognized and therefore little research has been done regarding Mg nutrient metabolism in the plant (Cakmak & Yazici, 2010). Despite Mg concentrations being in range under high HCO_3^- concentrations, deciphering the physiological meaning of the increase will need further studies comparing interaction between Mg and HCO_3^- response-curves.

High levels of HCO_3^- (> 5 mM) usually decrease Fe concentrations (Zhou et al., 1984; Alhendawi et al., 1997) causing leaf chlorosis. However, 20 mM HCO_3^- increased foliar Fe concentrations (Figure 3.1, B). Although the plants were not showing any visual chlorosis symptoms, chlorotic leaves actually have higher Fe concentrations of Fe than green leaves (Morales et al*.,* 1998; Roosta et al., 2014). This phenomenon, called the “chlorosis paradox”, is caused by Fe precipitation as insoluble compounds in Fe- deficient leaves (Mengel, 1994; Romheld, 2000). Longer periods of elevated RZ HCO_3^- treatment will

probably cause leaf chlorosis. In addition, the inhibition of leaf expansion may diminish the dilution of Fe within the leaves, resulting in higher Fe concentrations.

At 1 mM HCO_3^-, N, P, K, Cu and Zn showed smaller decreases (8-20%) in foliar concentrations than the 20 mM treatment. These decreases in nutrient concentration were likely a consequence of similar nutrient uptake but greater growth, with shoot weight increasing by 10 %. Bicarbonate-induced growth promotion at 1 mM may have diluted tissue N concentration, in contrast with previous results in tomato where 5 mM HCO_3^- was suggested to promote NH_4^+ incorporation into amides and amino acids using carbon skeletons supplied from HCO_3^- (Bialczyk et al., 2004). Tomato and lettuce plants have different nutrient requirements for their growth and development, therefore it is probable that nitrogen metabolism differs between these species when exposed to bicarbonate.

Despite pepper plants not showing any significant variability among macro- and micro-nutrients under elevated RZ CO_2, aeroponically grown lettuce showed significantly lower shoot Mg and S concentrations under high RZ CO_2 although root macronutrient concentrations did not differ between RZ CO_2 treatments. Also, K, Ca, Fe and Mn were lower and Zn was higher compared to control plants, but not significantly (Figure 3.2). Under elevated air CO_2, nutrient concentrations can decline as a consequence of increased photosynthesis and carbohydrate production adding biomass and thus diluting other nutrient elements (Loladzle, 2002). Nevertheless, no consistent and significant changes in photosynthesis rates were found during the experiments (Chapter 2), although whole plant CO_2 assimilation and fluorescence measurements could reveal unforeseen effects of changes in nutrient concentration under elevated RZ CO_2.

It is also well known that circadian clocks regulate rhythmic growth and physiology (Hsu & Hammer, 2014). Transpiration is the main driver of nutrient movement through the xylem. This rhythmic process depends on the regulation of stomatal conductance and the activity of aquaporins, both of which are regulated by the circadian clocks (Dodd et al., 2004, 2005). In our experiments, sampling was done consistently during the morning (9-11 am) under moderate climatic conditions. Any perceived bias in the results is hypothetical in the absence of additional data, so it is suggested that sampling at different times of day would be helpful to evaluate the robustness of the obtained data sets.

An early effect of Mg deficiency in plants is the disturbed partitioning of assimilates between roots and shoots because the supply of sink organs with photosynthetic products is impaired, and sugars accumulate in source leaves (Senbayram et al., 2015). The most commonly known function of Mg in plants is probably its role as the central atom of the chlorophyll molecule in the light-absorbing complex of chloroplasts and its contribution to photosynthetic fixation of carbon dioxide. However, the Mg bond to chlorophyll makes up only a small part of the total Mg fraction. Depending on plant Mg status, between ~20-35% of the element is localised in the chloroplast, with the remainder present in more mobile forms (Cakmak & Yazici, 2010). Because of its high phloem mobility, Mg can easily be translocated to actively growing parts of the plant where it is needed for chlorophyll formation, enzyme activation for protein biosynthesis, and phloem export of photosynthates to ensure vegetative and generative growth (Senbayram et al., 2015). Although visual symptoms occur in older leaves, no visual changes were observed in our plants as the concentration was in range according to Table 3.2.

Despite only 0.1% of plant dry matter existing as sulfur, it is an essential macronutrient for protein structure and is fundamental in a large array of compounds with critical catalytic and electrochemical functions. Higher plants acquire S predominantly in the form of anionic sulfate from the soil. In plastids, sulfate is reduced to sulfite which then combines with O-acetyl- Ser to form Cysteine (Lesutek et al., 2000). Then, sulfur is converted either into methionine or directly incorporated into proteins and glutathione (Saito, 1999). Despite Mg and S being in optimal ranges in both treatments at the time of harvest (Mg: 3.3-4.4 g/Kg; S: 3.5-4.2 g/kg), a significant interaction between Mg and S might be occurring at elevated RZ CO_2. Shoot Mg and S concentrations were highly correlated (Table 3.3), perhaps because these elements were added to the nutrient solution as $MgSO_4^-$.

Nutrient absorption by plants is usually referred to as ion uptake or ion absorption because plants absorb the ionic form. It was suggested that cation-cation and anion-anion interactions occur mostly at the membrane level and are competitive. On the other hand, cation-anion interactions occur at both the membrane and in cellular processes after absorption. However, these cellular processes are poorly understood (Hiatt & Leggett, 1972 ; Fageria, 2001). Under elevated RZ CO_2, nutrient concentrations in shoot tissues and between root/shoot tissues were more frequently correlated than in control plants

(Tables 3.3 and 3.4). Overall, every element was positively correlated to each other, however NO_{3-} was negatively correlated with K and S in the root tissue and B and Fe in shoot tissue. Total N was also negatively correlated with NO_{3-}, probably because the conversion of NO_{3-} into other compound such as amino acids, organic acids or carbohydrates.

Despite the differences in nutrient concentration in plant tissues discussed above, there were fewer differences in nutrient content between treatments. Shoot P, N, Cu and Zn contents were significantly higher under elevated RZ CO_2 compared to control plants while no significant differences were detected in root nutrient contents, probably because there was no difference in root dry biomass (Table 3.5).

Increased net photosynthesis and decreased shoot nitrogen and water use at elevated atmospheric CO_2 fundamentally alter these source-sink relationships. Therefore elevated RZ CO_2 was also expected to vary these relationships. Shoot to root communication of leaf N status is necessary to optimize carbohydrate allocation in roots among growth, N uptake and inorganic N assimilation. Coordination of N transport from root to shoot and of carbohydrate transport from shoot to root is fundamental for maintaining a C/N ratio through the plant that is optimal for plant growth and development (Martin et al., 2002; Zheng, 2009). Nitrogen concentration in leaves were lower and root concentrations were higher under elevated RZ CO_2. C/N ratio was lower in roots and was similar in shoot compared to control plants (Figure 3.3). At low soil NO_3^- concentrations, root C/N ratios are high, and roots have enough carbohydrates to assimilate most of the NO_3^- they absorb and thus they deliver little NO_3^- to the shoot. At high soil NO_3^- concentrations, root C/N ratios are low and a greater proportion of absorbed NO_3- remains unassimilated in the root and therefore is transported to the shoot (Andrews, 1986a; Andrews, et al., 1992) The N data agree with previous studies under elevated RZ CO_2, with initial stimulation of NO_3- uptake but no overall effects after prolonged (15days) RZ CO_2 application (He et al., 2010 ; Viktor & Cramer, 2005). In addition, numerous studies have reported positive interactions between N and P which increases P absorption and higher yields (Fageria, 2001). To date C,N and P ratios under elevated aerial CO_2 are not well understood (Wang et al., 2019). Studies demonstrated that phosphate (Pi) concentrations are sensed by the roots to function as a signal to report their own availability. Plants employ multifaceted signaling systems comprising local and systemic machineries to respond to changes of

external Pi (Chiou & Lin, 2011). Also, N sensing in the roots has been linked to extensive changes in cytokinin signalling (Ruffel et al., 2011). Therefore, elevated RZ CO_2 might play a role in changing the signalling mechanisms to regulate nutrient concentrations.

Zn uptake tends to display a linear pattern with its concentration in the nutrient solution or in the soils (Thoresby & Thornton, 1979; Kabata-Pendias & Pendias, 2001), with roots loading the shoot tissues via the xylem (Broadley et al., 2007). Zn translocation to the root xylem occurs via symplast and apoplast (Brennan, 2005; Broadley et al., 2007), but high Zn levels have also been detected in the phloem, denoting that this metal is translocated through both xylem and phloem tissues (Pearson et al., 1995; Haslett et al., 2001). Under elevated atmospheric CO_2, shoot zinc content decreased probably due to dilution effects caused by greater growth (Fangmeier et al., 1997). Some studies have reported that under elevated atmospheric CO_2, phytoextraction of heavy metals (including Cu, Pb, Cd and Zn) is enhanced (Li et al., 2013). Perhaps RZ CO_2 had the same effects in lettuce plants increasing the uptake of Cu and Zn, without reaching toxic levels.

In aeroponically grown lettuce, growth did not differ between treatments (Experiment 2.5, Chapter 2), and the total amount of amino acids in the leaf tissue was (20%) lower in plants grown under elevated RZ CO_2 than in control plants. Serine was the most abundant amino acid, and together with proline, alanine, glutamine and glutamic acid, they constituted about 98 % of the total amino acids. Serine is a polar amino acid that plays a fundamental role in plant metabolism, development and cell signalling. It participates in the biosynthesis of biomolecules such as amino acids (glycine, methionine and cysteine), nitrogenous bases, phospholipids and sphingolipids. In addition, serine is the main source of one-carbon units for the methylation reactions of nucleic acids and proteins. The biosynthesis of serine proceeds from different pathways, one associated to the glycolate pathway within photorespiration, and two non-photorespiratory pathways, the phosphorylate and the glycerate pathways (Figure 3.7). The last two are thought to be minor pathways, but they represent the only serine formation in non-photosynthetic tissues, in darkness or in plants with low or no photorespiration (Ros et al., 2014). Serine hydroxymethyltransferase (SHMT) interconverts glycine to serine with the equilibrium slightly in favour of glycine production. In leaves, the reaction is driven in the direction of serine synthesis because of the high glycine concentrations produced from photorespiration. Roots synthesize glycine instead of consuming it (Ho & Saito, 2001).

Daytime depletion of serine in the leaves occurred in plants overexpressing serine:glyxoxilate aminotransferase (SGAT), which participates in the conversion of glyoxylate and serine to glycine in the glycolate pathway. This depletion was also associated with activating the phosphorylate pathway during the day, but particularly at night during which foliar serine concentrations recover to normal levels (Modde et al., 2017). Leaves of lettuce plants exposed to elevated RZ CO_2 had significantly lower serine and higher glycine concentrations compared to control plants (Table 3.1) suggesting a possible downregulation of photorespiratory pathway in plants under high RZ CO_2 and alternatively the activation of another route.

Ornithine was 2-fold higher in leaves exposed to elevated RZ CO_2 compared to control plants. Although ornithine is not involved in protein synthesis and is present in very small quantities, it plays a critical role in N metabolism and in plant growth, drought and stress responses. It is synthesized from glutamate and is a key intermediate in the biosynthesis of arginine, proline, polyamines and alkaloids. Overexpressing the S1NAGS1 gene in tomato plants increased cellular ornithine concentrations two to nine-fold, accompanied by a slight increase in citrulline and a decrease in arginine (Kalamaki et al., 2009a), which was associated with enhanced drought and salt tolerance. The same trend of amino acid concentrations was found in the leaves (Table 3.6), which might be linked also with a stress response caused by either higher night temperatures or exposure of the roots to decreased oxygen concentrations from early in the morning to mid-night (Figure 2.10 , Chapter 2). It was postulated that plants accumulate a non-toxic metabolite such as ornithine, that can readily be converted to osmo-protective molecules upon stress induction (Kalamaki et al.,2009b). Examining Figure 3.6 suggests that there is a connection between foliar serine depletion during the day (because the photorespiration pathway is repressed and the phosphorylated pathway activated) that leads to production of ornithine and proline through glutamate, due to the lower concentrations of glutamate found under elevated RZ CO_2.

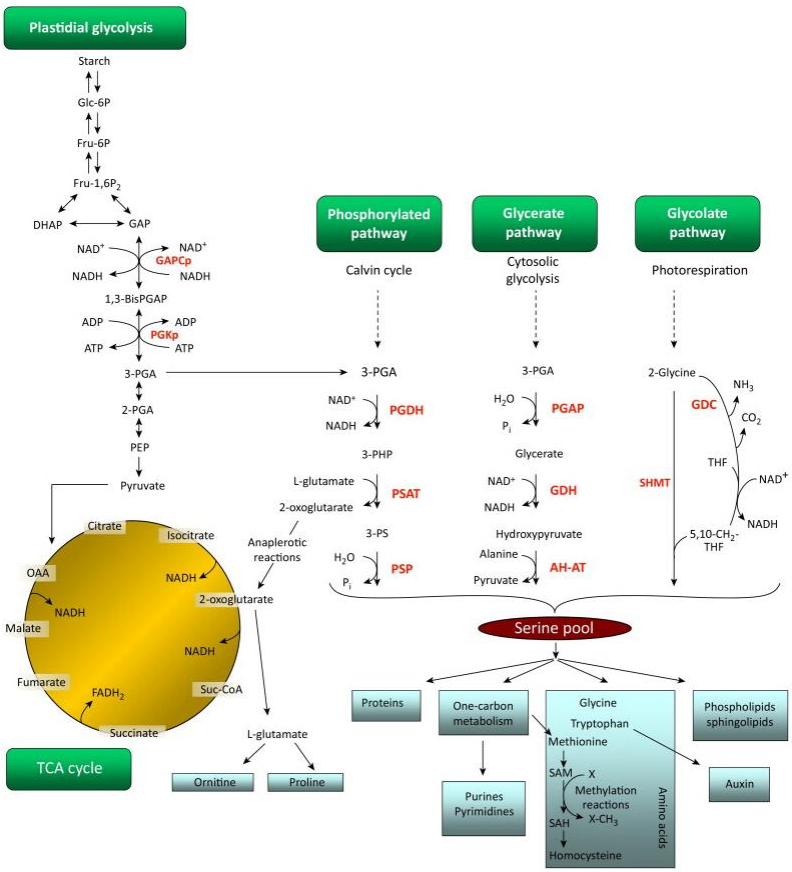


Figure 3.6. Schematic representation of serine (Ser) biosynthesis in plants and connecting phosphorylated pathway, photorespiratory pathway (glycolate pathway) and plastidial glycolysis. Broken lines indicate several enzymatic reactions (Ros et al., 2014).

3.5 Conclusions

HCO_{3^-} addition to hydroponic media promoted growth at 1-5 mM, but must be used moderately as growth suppression is observed at higher concentrations. Higher bicarbonate concentrations decreased N, P, Cu, K, Mn and Zn concentrations, perhaps because high solution pH (Chapter 2) decreased nutrient availability. On the contrary, growth promotion at 1 mM HCO_{3^-} could not be attributed to higher nutrient concentrations.

While nutrient concentration did not vary between treatments in pepper, elevated RZ CO_2 decreased foliar Mg and S concentrations in aeroponically grown lettuce even though shoot Mg and S content did not differ between treatments. Elevated RZ CO_2 increased root N concentrations but shoot N concentrations did not change. Higher shoot N, P and

Zn content indicates greater uptake of those elements. Correlations between nutrient concentrations are complex and seem to be enhanced under elevated RZ CO_2. Therefore, greater biomass accumulation under elevated RZ CO_2 might be partially due to better co-ordination of nutrient uptake. Finally, the decreased serine concentration under elevated RZ CO_2 accompanied with changes in glycine, ornithine, glutamate and proline concentrations, suggest that serine biosynthesis pathways may influence plant productivity.

Chapter 4. Phytohormone profiles of lettuce and pepper plants exposed to elevated root-zone carbon dioxide.

4.1 Introduction

Stimulated by the global importance of a continuous increase in atmospheric CO_2 levels, a large number of studies have focused on high CO_2 effects on plant growth and performance. Results have been variable even within the same species and plant growth is not always enhanced (Hasegawa et al., 2013; Luo et al., 2006). Some studies investigating the effects of atmospheric CO_2 enrichment have determined the concentrations of phytohormones *in planta*, as they are important in regulating plant growth and development (Chapter 1). In some studies, elevated CO_2 (eCO_2) enhances total leaf area by stimulating greater leaf expansion (Taylor et al.,1994 ; Ferris et al., 2001). In general, elevated atmospheric CO_2 increased leaf IAA, GA_3, ZR, DHZR, iPA and ethylene concentrations (Teng et al., 2006; Wang et al., 2009; Seneweera et al., 2003), while ABA and JA concentrations decreased (Li et al., 2006; Guo et al., 2012). However, opposite results have been observed when applying RZ CO_2 to aeroponically grown muskmelon, where xylem sap IAA, tZ and GA_3 concentrations decreased and ABA increased compared to control plants, but tissue concentrations were not measured (Li et al., 2009). Since there is limited information on RZ CO_2 effects on phytohormone concentrations, information on foliar and root response to eCO_2 are briefly reviewed.

Auxin regulates embryo development, stem cell maintenance, root and shoot architecture, and tropic growth responses (Davies, 2010a) and reactions to environmental changes (Benkova, et al., 2003). In response to eCO_2, leaf size and anatomy changes, often increasing the total leaf area per plant, single leaf area and leaf thickness (Pritchard, et al., 1999). Regarding leaf development alone, IAA effects include control of the initiation of leaf primordia (Reinhardt, et al., 2000), control of vascular differentiation (Sieburth, 1999), as well as control of leaf expansion during both the cell division leaf growth phase (Ljung, et al., 2001), and the cell enlargement leaf growth phase (Braun, et al., 2008). Increased carbohydrate production under eCO_2 appears to increase biosynthesis of IAA in the shoot which consequently is transported to the roots and stimulates root growth and root hair development in *Arabidopsis thaliana* (Hachiya, et al., 2014; Niu, et al., 2011) and tomato

seedlings (Wang et al., 2009). In contrast, with greater shoot and root growth, IAA decreased in roots of sweet pepper exposed to high CO_2 (Piñero et al., 2013). In general, under eCO_2, shoots and roots grow unequally, such that root to shoot ratios are usually increased (Sigurdsson et al., 2001, Ainsworth & Long, 2005). IAA positively regulates lateral root growth and an accumulation of sugars in leaves stimulates IAA biosynthesis and promotes its transport from shoot to root (Sairanen et al.,2012; Lilley et al.,2012). Because plants accumulate higher carbohydrate concentrations under eCO_2, IAA was proposed to act as a long-distance signal from shoot to roots.

Cytokinins (CKs) have a chemical structure similar to adenine, and promote cell division and regulate embryogenesis, vascular tissue development, root architecture, and light responses (Kieber & Schaller, 2014). The most common form of naturally occurring CK in plants is zeatin (Sakakibara, 2006). CK concentrations are highest in meristematic regions and continuously growing parts of roots, young leaves, developing fruits, and seeds (Shani et al., 2006, Taiz & Zeiger, 2010). Elevated CO_2 caused a greater increase in tZ type- CK delivery to cotton leaves at low (2 mM) than at high (12 mM) nitrogen concentrations, compared to plants exposed at ambient CO_2 (Yong et al., 2000). Similarly, under high CO_2 the delivery of CK from the roots to the shoots was higher than in control plants (Schaz et al*.,* 2014). However, IAA can inhibit CK biosynthesis and signalling (Moubayidin, 2009) and higher IAA levels under eCO_2 may be responsible for decreased CK concentrations (Hachiya et al., 2014). Since synergistic interactions between IAA and CKs can regulate nodule organogenesis (Hwang et. al., 2012), light-mediated leaf initiation, and organ positioning (Yoshida, 2011), it is not clear what effect eCO_2 will have on their responses. As leaf development progresses, leaf proliferation is gradually replaced by leaf expansion and differentiation as the main processes driving leaf growth to reach its final size and shape (Vanhaeren et al., 2016). Leaves develop from the shoot apical meristem (SAM) and CKs play a role in SAM maintenance (Gordon et al., 2009). In addition, a fine coordination of local IAA and CKs responses regulates and stabilizes leaf initiation and early studies also showed that CKs stimulated plant cell division (Skoog & Miller, 1957). However, while it is well known that IAA acts as a positive regulator of organ initiation, CKs have complex effects which seem to depend on species and developmental context (Bar & Ori, 2014). The action of CKs can also be tissue-specific. In isolated pumpkin cotyledons, BA activated cell division in palisade mesophyll and upper epidermis without affecting their growth;

while stimulating the growth of spongy mesophyll and lower epidermal cells without inducing cell division (Fofanova & Khokhlova, 1983; Kulaeva et al., 1984). In addition, studies with excided leaf discs of pepper plants showed that adding 5μM of BA (N_6-benzyladenine) promoted expansion of the disc after 24h. This expansion was not mediated through changes in net uptake of CKs or utilization of carbohydrates (Nielsen & Ulvskov, 1992).

Gibberellins are involved in several important biochemical and morphogenetic responses which include elongation of stems, leaves, and reproductive organs (Colebrook et al., 2014). Elevated CO_2 stimulated leaf growth by triggering both cell expansion and cell division (Taylor et al., 2003; Luomala et al., 2005). Elevated CO_2 reverted a growth reduction of *Arabidopsis* treated with the GA biosynthesis inhibitor paclobutrazol (Ribeiro et al., 2012). It was suggested that the effect of eCO_2 on plant growth might be partially coupled with the effects of GA. In support, eCO_2 increased gibberellin concentrations in several species such as orchids (Li et al., 2002), *Arabidopsis* (Teng et al., 2006) and *Populus* (Liu et al., 2014). GAs can stimulate leaf expansion by increasing cell length and cell number (Yang et al. 1996), with enhanced wall extensibility largely promoting cell expansion (Cosgrove & Sovonick-Dunford 1989). A proteome analysis suggested that following exogenous GA_3 application, rice leaves could sense GA_3 and transmit a signal to activate cell growth and division (Wang et al., 2013). Whether eCO_2 can activate this signal, is still unknown.

Jasmonic acid and salicylic acid play key roles in regulating plant defence responses to abiotic and biotic stresses (Riemann et al.,2015 ; Khan et al., 2015). Elevated CO_2 enhanced SA-dependent defence and decreased JA-dependent defence (Zavala et al., 2013 ; Sun et al., 2016). On the contrary, eCO_2 increased JA-biosynthesis pathway metabolites in guard cells of *B. napus* and *Arabidopsis*, with JA-Ile and JA signalling believed to play an essential role in the stomatal closure induced by eCO_2 (Geng et al., 2016). Although ABA has been implicated in stomatal responses to elevated CO_2, as some ABA-deficient or ABA-insensitive mutants are compromised in CO_2-induced stomatal closure (Webb & Hetherington, 1997), in other cases stomatal closure seemed ABA-independent (Merilo et al., 2015; Xue et al., 2011; Geng et al., 2016).

Ethylene and CO_2 interactions have been long studied mainly because ethylene can "force" fruit ripening, which is antagonised by high CO_2 concentrations. Ethylene is a gaseous hormone that is synthesized in almost all plant tissues in the presence of oxygen (Lin et al., 2010). It is regarded as a multifunctional phytohormone that promotes or inhibits growth and senescence processes depending on its concentration, timing of application, and the plant species (Pierik et al., 2006; Reid, 1995). Key enzymes in ethylene biosynthesis are 1- aminocyclopropane – 1 – carboxylic acid (ACC) synthase and ACC oxidase which catalyze the reactions from S-adenosylmethionine to ACC, and from ACC to ethylene respectively. CO_2 is an essential cofactor for ACC oxidase (Dong et al.,1992). High CO_2 might decrease ethylene production rates in fruits by at least partially blocking the conversion of ACC to ethylene (Rothan & Nicolas, 1994). Also the role of ethylene in leaf growth and development has been confirmed using ethylene inhibitors, and genetically using ethylene insensitive mutants lacking the key enzymes of ethylene biosynthesis (Bleecker et al., 1998; Oh et al., 1997). It was proposed that increased ethylene production might be central in promoting growth when leaf glucose concentrations are high, such as rice growing under eCO_2 (Seneweera et al., 2003).

Increased leaf area in response to eCO_2 (Taylor et al., 1994) may result from greater rates of cell division, increased cell expansion, or a combination of these processes. As mentioned above, phytohormones including CK, IAA and GA_3 are involved in cell division, cell elongation and protein synthesis, while RZ CO_2 enrichment might impact the concentration of plant hormones and therefore plant growth. Thus, determining whether RZ CO_2 affects phytohormone concentrations *in planta*, and their putative physiological importance in regulating leaf expansion, will further our understanding of the mechanisms by which RZ CO_2 affects growth.

4.2 Material and Methods

4.2.1 Plant Material and treatments

Crisphead lettuce (*Lactuca sativa* cv. Consul) and pepper (*Capsicum annuum* cv. Bellboy F1) seeds were purchased from Moles Seeds (Essex, UK).

Both crops were grown aeroponically system as indicated, for pepper (Experiment 2.9) and lettuce (Experiment 2.3) at different times as described in Chapter 2. Seeds were individually sown in 150-cell plug trays in 2 cm x 2 cm x 4 cm rockwool cubes (Growell, Ltd, UK) and germinated in the glasshouse. Plants were transferred to the system at the 4 leaf-stage in lettuce and at 2-4 leaf-stage in pepper. Elevated (1500 ppm) RZ CO_2 was applied to one of the bins and to the other bin, ambient RZ CO_2 was added using the same delivery system described in Chapter 2. The pH was controlled everyday between 5.8-6.3 with the necessary dropwise application of HCL or NaOH.

4.2.2 *Multi-hormone analysis*

Aeroponically grown lettuce and pepper samples were taken between 9-10am in the morning, immediately frozen in liquid nitrogen and stored at –20°C before being freeze-dried for 48 h. The samples were then ground and weighed (50 mg) to allow extraction with 0.5 mL extraction buffer (methanol:water 80:20 v/v) for 0.5 h at 4°C. Solids were separated by centrifugation (20 000 *g*, 15 minutes) and re-extracted for 30 minutes at 4ºC in an additional 0.5 mL of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †C_{18} cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of the plant pigments and evaporated at 40ºC under vacuum either to near dryness or until organic solvent was removed. The residue was dissolved in a 1 mL methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA). Ten µL of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for

each analyzed component (1, 10, 50, and 100 µg L^{-1}) and corrected for 10 µg L^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%. Samples were analysed by Dr. Alfonso Albacete in CSIC (Murcia, Spain) (Albacete et al., 2008). Five out of the 11 hormones (ACC, tZ, ABA, JA and SA) were detected in both leaf and root tissue of lettuce and pepper plants, whereas ZR, iP, GA_3 and IAA were detected just in pepper shoots and roots.

4.2.3 Leaf area expansion measurements in plants grown aeroponically

To determine leaf expansion rates in aeroponically grown pepper, the length (L) and width (W) of one leaf per plant was measured with a ruler, every day for 12 days two times per day, at 9 am and at 5 pm.

The estimated leaf area was calculated multiplying W x L. Leaf expansion rate (LER) was calculated as follow:

$LER = (lnLA_2) - (lnLA_1) / t_2 - t_1$

Where LA_1 and LA_2 are the estimated leaf areas and t_1-t_2 is time (d) between two consecutive days (Liu et al., 2003).

At harvest, actual leaf area of that leaf was measured with the leaf area meter, in addition to the total leaf area of each plants (Experiment 2.11, Table 2.9).

4.2.4 Leaf area expansion in response to ACC, GA_3 and BA application

Leaf discs (8 mm diameter) were harvested using a cork borer from the base and tip of the leaves between 9 – 10 am and incubated for 1 h on a solution containing 10 mM Mes-KOH (pH 6.5), 10 mM KCl and 10 mM sucrose. Different concentrations of each hormone were applied based on the work done by Nielsen and Ulvskov, (1992). After 1 h incubation, the discs were transferred to a similar solution with the following concentrations of each hormone to perform dose-curve responses:

1) 0.1, 10 and 100 µM GA_3
2) 5, 50 and 500 µM BA
3) 0.1, 10 and 100 µM ACC

Control treatments did not include GA_3, BA or ACC. Two more assays were carried out with selected concentrations based on the dose curve responses and to test whether the response was consistent between independent assays. Petri dishes were incubated in the glasshouse under ambient conditions. Two perpendicular diameters were measured on each disc using a ruler and projector with nine-fold magnification.

In another experiment, instead of collecting leaf discs, 100 μM of each hormone were sprayed for 10 days on soil-grown pepper leaves to understand better how these hormones affect leaf expansion in situ. At the end of the experiment, total leaf area was determine using a leaf area meter (Li-cor Model 3100 Area Meter, Cambridge, UK) and shoot fresh weight was recorded. All tissue was dried at -70°C for at least 72h and then re-weighted.

4.2.5 Statistics

To determine differences on leaf and root phytohormone concentrations between treatments, an Independent Samples Student's t-test at the $P < 0.05$ level was performed.

Pearson's Correlation was used to examine correlations between leaf and root phytohormones and gas exchange parameters. Prior to the analysis, normality of the residuals was tested using Shapiro-Wilk test (Shapiro and Wilk, 1965).

One-way analysis of variance (ANOVA) was performed to analyse differences between hormone concentrations applied in each leaf-disc assay followed by LSD post-hoc analysis.

4.3 Results

Compared to control lettuce plants grown aeroponically at ambient RZ CO_2, elevated RZ CO_2 had little effect on leaf phytohormone concentrations. ACC was 20 % lower and SA 15 % higher under RZ CO_2 although these changes were not statistically significant. On the other hand, JA concentrations significantly increased by 30%. In contrast, tZ and ABA did not show any differences between treatments. Root phytohormone concentrations did not differ significantly between treatments, even though ABA was 30% lower under RZ CO_2.

In pepper, RZ CO_2 enrichment decreased leaf *t*Z concentrations by 50%, but increased leaf ACC concentrations by ~60 %. Shoot and root SA concentrations showed opposing changes to RZ CO_2 enrichment, with leaf SA concentrations decreasing by 35% while root SA concentrations increased by 50 % (Figure 4.1). Root iP and GA_3 concentrations also significantly differed between treatments. While iP was 30% lower under high RZ CO_2, GA_3 was 10% higher compared to control plants (Figure 4.2).

Although phytohormone concentrations showed limited responses to RZ CO_2 enrichment, pepper was more responsive than lettuce.

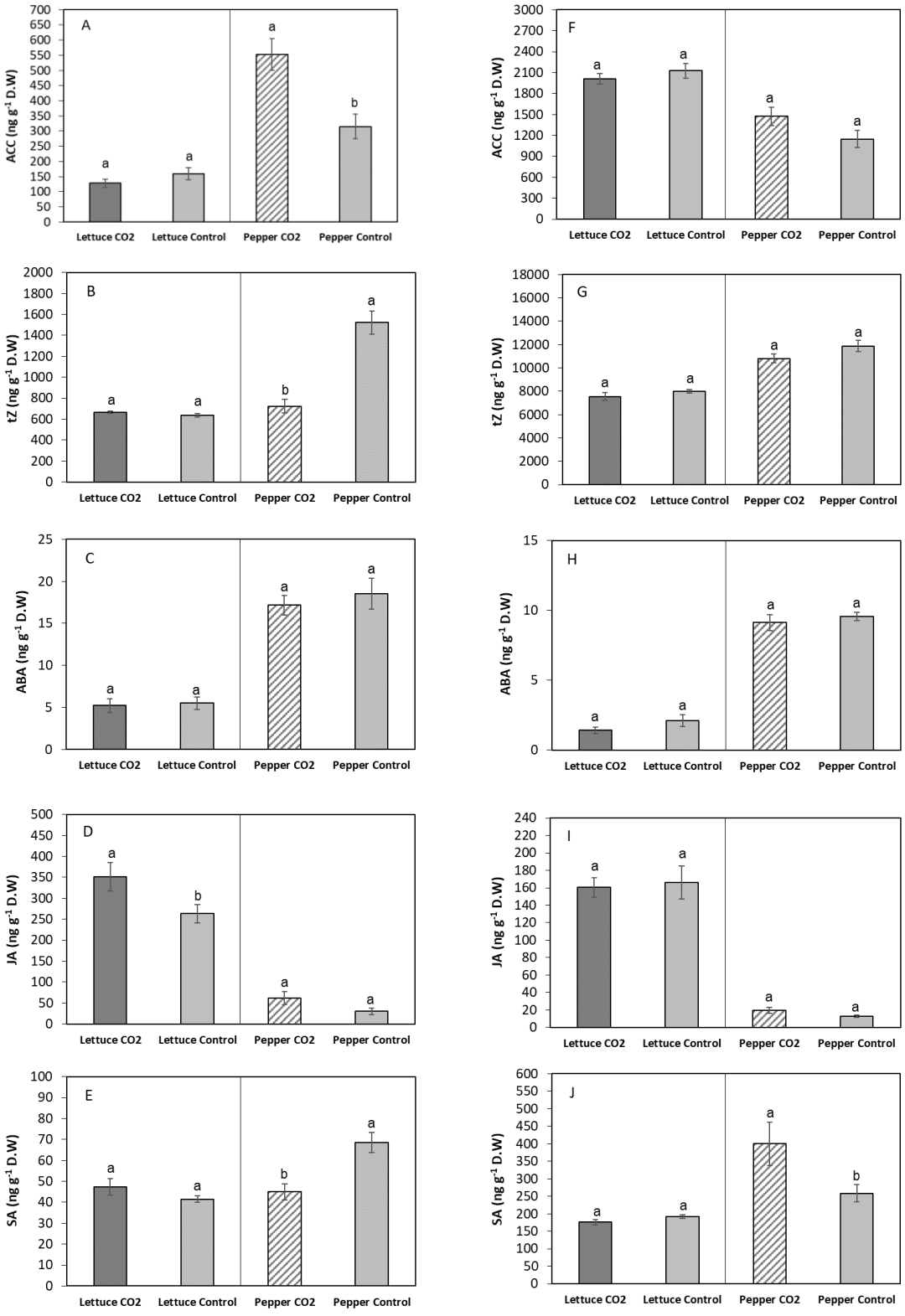


Figure 4.1. Lettuce and pepper leaf (A, B, C, D, E) and root (F, G, H, I, J) phytohormone concentrations under high RZ CO_2 and ambient CO_2. Bars are means ± SEM of 8 replicates, with different letters indicating significant ($P < 0.05$) differences within a species.

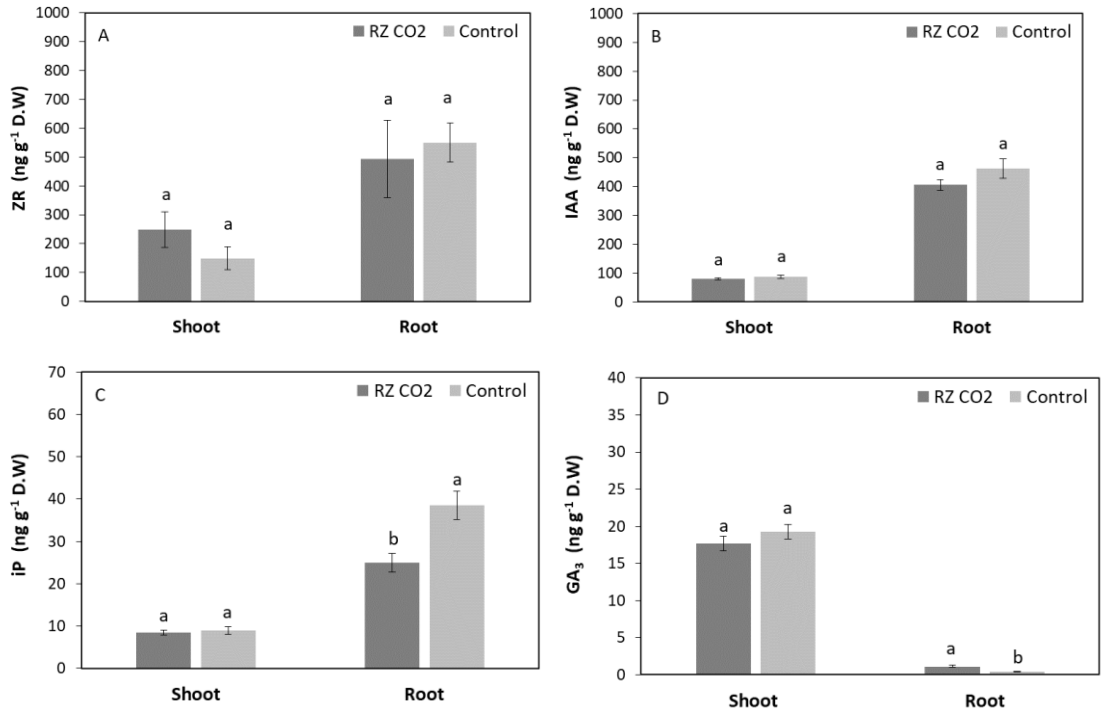


Figure 4.2. Pepper leaf and root ZR (A), IAA (B), iP (C) and GA_3 (D) phytohormone concentrations under high RZ CO_2 and ambient CO_2. Bars are means ± SEM of 8 replicates, with different letters indicating significant ($P < 0.05$) differences between treatments.

In pepper, leaf phytohormone concentrations showed more positive correlations when grown under high RZ CO_2 than under control conditions (Table 4.1 and 4.2). Although RZ CO_2 enrichment had no significant effect on leaf gas exchange (data not shown) or foliar ABA concentration, ABA was positively correlated with *A* and foliar JA concentration. While foliar ACC concentration was positively correlated with *A*, g_s and *E,* it was not correlated with any other hormone. Under elevated RZ CO_2, leaf tZ was positively correlated with leaf SA concentrations while leaf iP was positively correlated with root IAA, ABA and SA concentrations (Table 4.1). However, foliar tZ concentrations were not correlated with leaf gas exchange.

Table 4.1. Pearson's correlations between leaf and root phytohormones and gas exchange parameters of pepper plants grown under elevated RZ CO_2.

		Leaf									Root									Gas exchange				
Leaf	ACC	ACC																						
	tZ	0.25	tZ																					
	ZR	0.47	0.16	ZR																				
	iP	0.27	0.05	0.68	iP																			
	GA_3	-0.16	0.31	0.13	0.01	GA_3																		
	IAA	0.29	0.34	0.6	0.36	0.69	IAA																	
	ABA	0.48	-0.05	-0.21	-0.65	-0.21	0.05	ABA																
	JA	0.53	-0.21	-0.19	-0.47	-0.72	-0.41	0.8*	JA															
	SA	0.34	0.75*	-0.11	-0.17	0.08	0.35	0.44	0.19	SA														
Root	ACC	-0.21	0.59	-0.48	-0.27	0.53	0.14	-0.08	-0.37	0.52	ACC													
	tZ	0.22	0.7	0.17	0.25	0.64	0.61	-0.15	-0.53	0.58	0.49	tZ												
	ZR	0.07	-0.04	0.16	0.73	0.2	0.62	-0.71	-0.63	0.34	0.15	0.41	ZR											
	iP	-0.09	0.77	0.52	0.68	0.39	0.34	-0.73	-0.69	0.32	0.29	0.83*	0.01	iP										
	GA_3	0.05	0.24	-0.7	0.37	0.2	-0.43	-0.67	-0.66	0.06	0.66	0.69	0.24	0.39	GA_3									
	IAA	0	0.26	0.43	0.85**	0.03	0.06	0.86*	-0.58	-0.18	0.04	0.24	0.46	0.86*	0.81	IAA								
	ABA	0.2	-0.28	0.56	0.94**	-0.07	0.22	-0.63	-0.42	-0.39	-0.45	0.08	0.73	0.34	0.39	0.71*	ABA							
	JA	0.2	0.81*	0.07	0.37	-0.33	-0.18	-0.04	0.20	-0.6	-0.53	-0.46	0.36	-0.79	0.42	0.09	0.64	JA					
	SA	0.34	-0.28	0.67	0.87**	-0.02	0.31	-0.46	-0.26	-0.42	-0.4	-0.09	0.75	0	0.24	0.64	0.9**	0.66	SA				
Gas exchange	*A*	0.84*	0.61	0.55	0.01	0.12	0.69	0.84*	0.75	0.64	-0.06	0.52	-0.1	0.19	-0.32	-0.33	-0.16	-0.32	-0.03	*A*				
	Gs	0.82*	0.3	0.69	0.61	-0.34	0.48	0.36	0.60	0.42	-0.43	0.41	0.38	0.5	-0.12	0.22	0.47	0.16	0.4	0.73	*Gs*			
	Ci	0.44	-0.16	0.52	0.88**	-0.55	0.04	-0.3	0.13	-0.07	-0.61	0.15	0.49	0.69	0.2	0.64	0.84*	0.56	0.61	0.13	0.76*	*Ci*		
	E	0.88**	0.38	0.68	0.53	-0.22	0.57	0.47	0.61	0.49	-0.34	0.49	0.38	0.45	-0.12	0.12	0.37	0.09	0.35	0.82*	0.99**	0.66	*E*	

*Correlation is significant at the 0.05 level (2-tailed) **Correlation is significant at the 0.01 level (2-tailed)

Table 4.2. Pearson's correlations between leaf and root phytohormones and gas exchange parameters of pepper plants grown under ambient RZ CO_2.

		Leaf									Root									Gas exchange				
Leaf	ACC	ACC																						
	tZ	-0.47	tZ																					
	ZR	-0.29	0.32	ZR																				
	iP	0.62	-0.20	-0.10	iP																			
	GA_3	-0.53	0.41	0.63	0.71*	GA_3																		
	IAA	-0.29	0.28	0.79*	0.25	0.12	IAA																	
	ABA	-0.37	-0.24	-0.17	0.72*	0.22	-0.36	ABA																
	JA	0.51	0.01	-0.09	-0.19	0.17	-0.43	0.32	JA															
	SA	-0.28	-0.58	-0.26	-0.09	-0.21	0.01	0.35	-0.63	SA														
Root	ACC	-0.39	0.69	0.59	-0.66	0.72*	0.20	0.32	0.65	-0.55	ACC													
	tZ	-0.23	0.51	0.70	0.00	0.55	0.39	-0.25	0.27	-0.69	0.59	tZ												
	ZR	-0.06	0.41	-0.50	0.21	-0.25	-0.27	-0.54	-0.57	0.00	-0.46	-0.24	ZR											
	iP	0.12	0.25	-0.04	0.27	0.16	-0.14	0.74*	-0.27	-0.38	-0.20	0.31	0.74*	iP										
	GA_3	-0.55	0.85	-0.32	0.01	-0.26	-0.01	0.08	-0.29	-0.74	0.09	0.33	0.40	0.06	GA_3									
	IAA	-0.54	0.61	0.23	-0.03	0.38	0.20	-0.46	-0.49	-0.27	0.11	0.54	0.60	0.74*	0.67	IAA								
	ABA	0.24	0.24	0.02	-0.07	0.28	-0.12	-0.45	0.08	-0.33	0.14	0.07	0.37	0.53	-0.32	0.28	ABA							
	JA	-0.05	0.42	0.63	0.06	0.14	0.59	0.01	0.47	-0.51	0.63	0.62	-0.62	-0.39	0.25	-0.09	-0.07	JA					
	SA	-0.11	-0.05	0.71*	0.29	0.24	0.69	-0.22	-0.10	-0.09	0.16	0.64	-0.46	-0.06	-0.07	0.19	0.00	0.64	SA					
Gas exchange	*A*	-0.35	0.67	0.41	-0.73	0.74	-0.04	0.25	0.51	-0.55	0.97**	0.44	-0.17	0.08	-0.09	0.19	0.25	0.31	-0.25	*A*				
	Gs	-0.62	-0.02	-0.47	-0.02	-0.36	-0.02	0.00	-0.71	0.69	-0.55	-0.74	0.56	-0.08	0.98*	0.06	0.19	-0.58	-0.28	-0.46	*Gs*			
	Ci	0.07	-0.57	-0.70	0.30	-0.58	-0.32	0.04	-0.54	0.69	0.89**	-0.71	0.41	-0.05	0.34	-0.13	0.01	-0.68	-0.14	0.82*	0.77*	*Ci*		
	E	0.39	-0.56	-0.74	0.46	-0.63	-0.46	-0.09	-0.22	0.37	0.85*	-0.50	0.40	0.12	0.32	-0.11	0.11	-0.53	-0.07	0.81*	0.52	0.92**	*E*	

*Correlation is significant at the 0.05 level (2-tailed) **Correlation is significant at the 0.01 level (2-tailed)

Generally, although RZ CO_2 did not cause differences in growth compared to control plants, in some cases leaf area was lower under RZ CO_2 (Table 2.10, Chapter 2). Since there were more changes in phytohormone concentrations in response to RZ CO_2 in pepper than lettuce, leaf expansion was measured over 12 days (Experiment 2.11). Leaf expansion rate decreased from Days 1 to 7 in both treatments, which could not be be attributed to environmental conditions, since temperature and relative humidity were relatively constant along the 12 days (Fig. 4.3, A). Nutrient depletion may have caused this decrease as growth ceased on Day 7, the day before the nutrient solution was replaced. Over the entire experiment high RZ CO_2 decreased area of the measured leaf by 25% compared to control plants (Figure 4.3, C).

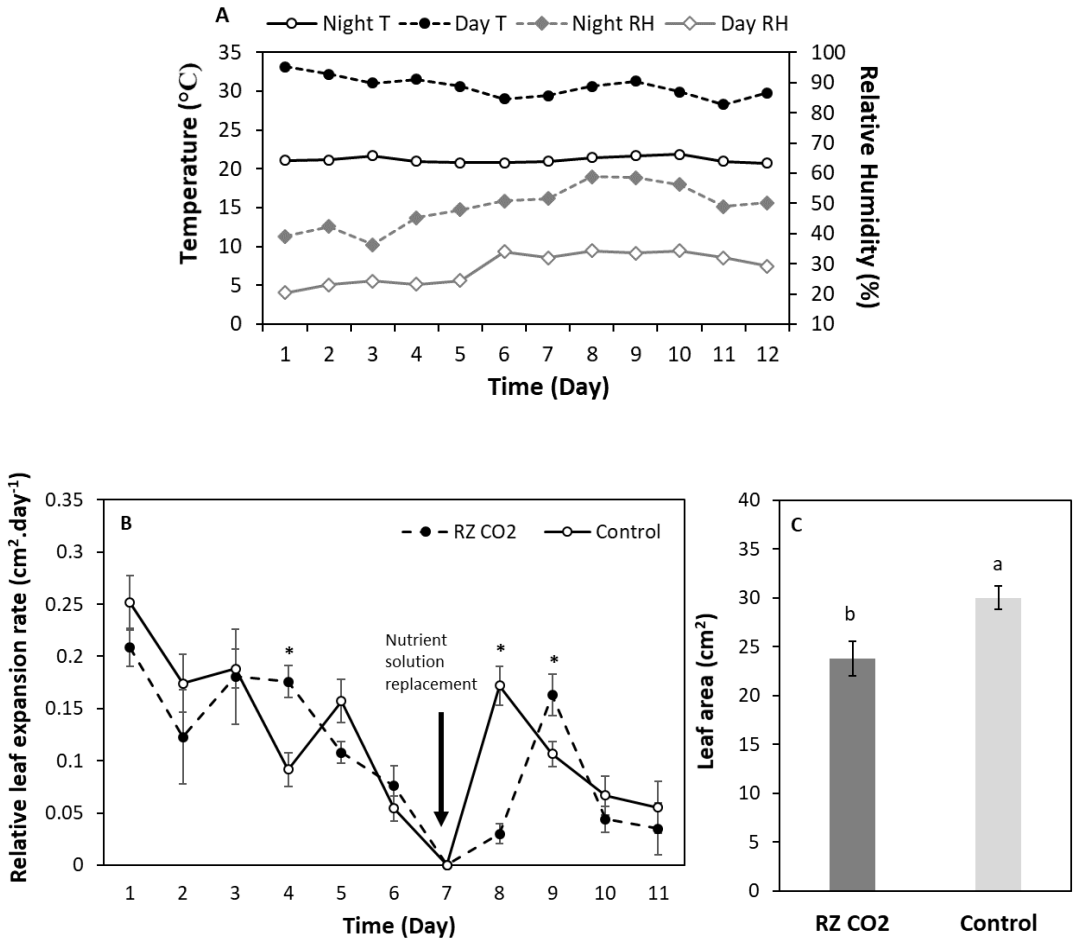


Figure 4.3. Night/ Day temperature (T) and relative humidity (RH) variation throughout the measurements (A). Daily pepper relative leaf expansion rate (B) and final leaf area (C) in plants exposed to high RZ CO_2 and ambient RZ CO_2. Bars are means ± SEM of 8 replicates. Asterisks and different letters indicate significant ($P < 0.05$) differences between treatments.

To better understand the impact of different phytohormones on pepper leaf expansion, leaf disc assays were performed. ACC, GA_3 and BA were selected, because of their role in regulating cell division and elongation and because RZ CO_2 affected these phytohormones (Figure 4.1, 4.2).

Initially a GA_3 dose-curve was performed using discs from the tip and the base of the leaf to see whether leaf-disc expansion differed with leaf position. Apical leaf-discs expanded little in comparison with discs taken from the base, meaning that expansion occurs mainly in the basal part of the leaf. In apical discs, increasing GA_3 concentrations from 0.1 to 1 µM increased leaf expansion more than at 10 µM (Figure 4.4). In basal discs, GA_3 concentration had no significant effect on leaf expansion.

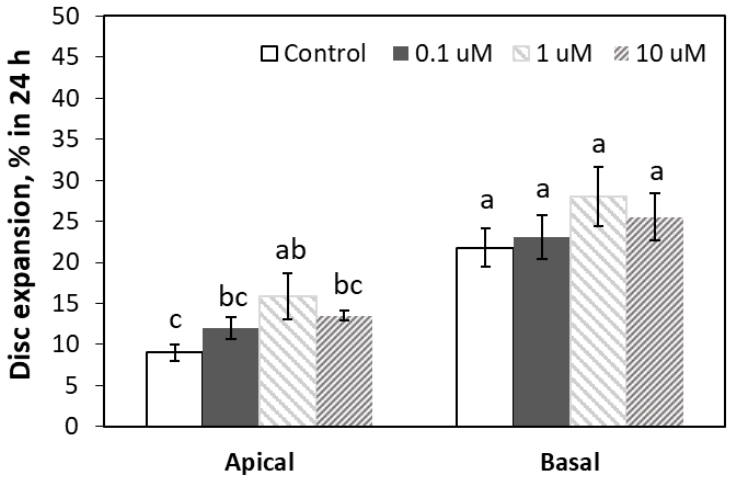


Figure 4.4. Pepper leaf area expansion rates of leaf disc taken from the tip and the base of the leaf after 24 h exposed to 0.1, 1 and 10 GA_3. Bars are means ± SEM of 10 replicates. Different letters indicate significant ($P < 0.05$) differences between treatments.

A second assay performed GA_3, BA and ACC dose- response curves with discs taken from the base of the leaf. Higher GA_3 concentrations (than applied previously) increased leaf expansion by 16% at 100 µM GA_3, with no significant effect at lower concentration. An intermediate cytokinin concentration (50 µM BA) increased leaf disc expansion by 25% compared to control leaf-discs (Figure 4.5), whereas a higher concentration (500 µM BA) had no significant effect. Leaf expansion was not affected across a broad range (0.1-100 µM) of ACC concentrations.

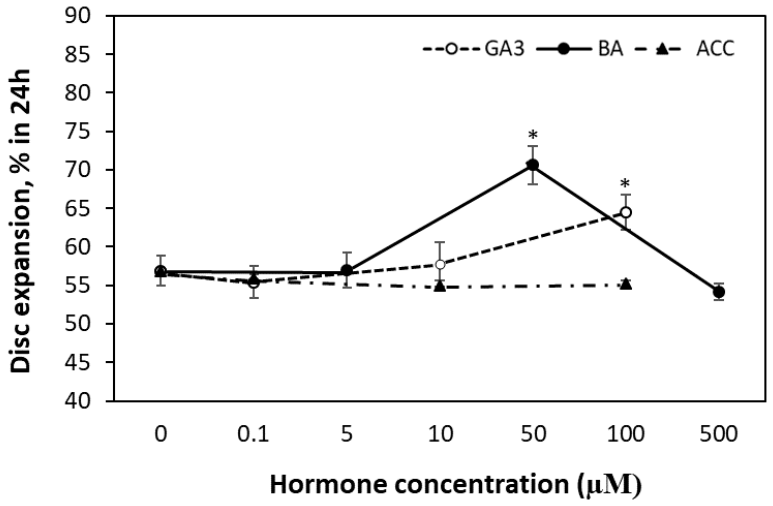


Figure 4.5. Pepper leaf area expansion rates of leaf disc after 24h exposed to GA3: 0.1,10 and 100µM; BA: 5, 50 and 500 µM or ACC: 0.1, 10 and 50 µM. Bars are means ± SEM of 10 replicates. Asterisk indicate significant ($P < 0.05$) differences within hormone treatments.

A third assay checked the consistency of response to GA_3, BA and ACC. In this case, 100 µM GA_3 and 100 µM ACC did not affect leaf expansion, while again 50 µM BA increased leaf-disc expansion significantly by 30% compared to control (Figure 4.6). Therefore, BA has a positive constant leaf expansion effect on pepper leaf-discs but not ACC and GA_3.

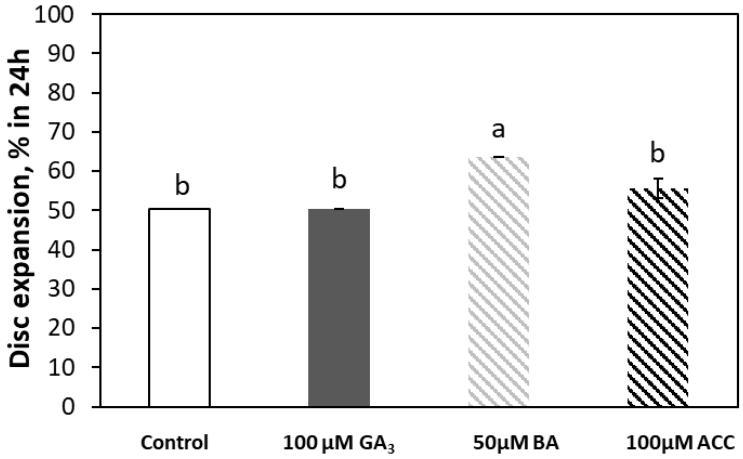


Figure 4.6. Pepper leaf area expansion rates of leaf disc after 24 h. Leaf discs were floated on treatment solutions containing from 100µM GA_3, 50 µM BA and 100 µM ACC. Bars are means ± SEM of 10 replicates. Different letters indicate significant ($P < 0.05$) differences between treatments.

To determine whether the effects of these hormones *in vivo* was similar to in excised leaf discus, the same hormones (ACC, BA and GA_3) were individually applied at a single concentration (100 μM). Foliar application of BA or GA_3 to soil grown peppers had no significant impact on shoot fresh weight, shoot dry weight or total leaf area. ACC application decreased shoot dry weight by 10% and total leaf area by 26% (Table 4.3).

Table 4.3. Shoot fresh weight, shoot dry weight and total leaf area of pepper plants sprayed with 100 μM of ACC , GA_3 and BA. Values are means ± S.E. of 4 replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$).

Treatment	Shoot fresh weight (g) ± SE	Shoot dry weight (g) ± SE	Total leaf area (cm_2) ± SE
ACC	79.93 ± 2.63^{a}	8.95 ± 0.15^{a}	858 ± 49^{c}
GA_3	90.05 ± 6.93^{a}	9.83 ± 0.65^{a}	1354 ± 126^{a}
BA	85.95 ± 2.55^{a}	10.13 ± 0.41^{a}	1129 ± 39^{abc}
Control	84.75 ± 4.48^{a}	9.94 ± 0.40^{a}	1161 ± 85^{ab}

4.4 Discussion

Although roots were growing in a high CO_2 environment, surprisingly there were more changes in leaf phytohormone concentrations than changes in root phytohormone concentrations (cf. Figure 4.1, 4.2). RZ CO_2 enrichment did not change lettuce root phytohormone concentrations, but increased root SA concentrations of pepper while decreasing shoot SA concentrations. These opposing tissue-specific responses may reflect enhanced basipetal transport of SA via the phloem from shoots to roots, but this hypothesis can only be assessed by girdling (phloem removal) the stem (e.g. Castro et al. 2019). Research into the signalling pathways initiated by SA have mainly focused on its role in plant defence and immunity (Vlot et al., 2009). However, in addition to biotic stresses, SA mediates responses to abiotic stresses such as drought, low temperature and salinity (Miura and Tada 2014). Generally, low concentrations (≤ 10 µM) of applied SA promote plant growth under unfavorable conditions, whereas high SA concentrations (≥ 100 µM) inhibit growth; the threshold between low and high concentrations depends on plant species and the method of treatment (Kovácic et al., 2009; Hayat et al., 2005; Hosseini et al., 2011). Elevated RZ CO_2 had no effect on shoot biomass or shoot and root nutrient concentrations (Chapter 3), yet root dry weight was (40%) lower under elevated RZ CO_2. Although comparatively less is known about the role of SA in plant root development, opposite results have been obtained when applying exogenous SA to the roots. Whereas high exogenous SA concentrations (> 150 µM) inhibits root growth of *Arabidopsis* primary and lateral root development (Armengot et al., 2014), lower concentrations (< 150 µM) increased root biomass in corn (Agtuca et al., 2013) and soybean (Gutierrez-Coronado et al., 1998). In addition, root SA was positively correlated with leaf iP but also with root ABA concentrations.

Phytohormonal profiling revealed a solitary difference between aeroponically-grown lettuce plants grown under ambient and elevated RZ CO_2: increased leaf JA concentrations under elevated RZ CO_2 (Figure 4.1, D). Jasmonates are derived from linolenic acid in a lipoxygenase-dependent process via the octadecanoid pathway (Wasternack and Hause et al., 2013). Increased in JA-biosynthesis pathway metabolites in guard cells were found under high CO_2 conditions, indicating that jasmonoyl- isoleucine (JA-Ile) biosynthesis pathway plays an essential role in stomatal closure induce by short term (1 h) high CO_2 (800ppm) application (Geng et al., 2016) while long-term CO_2 exposure decreased JA

production Although RZ CO_2 can induce stomatal closure (He et al., 2010), stomatal conductance did not differ between treatments here (Figure 2.19, Chapter 2). Since JA is usually regarded as a growth inhibitor (de Ollas et al., 2018), stimulation of lettuce growth under RZ CO_2 enrichment cannot be attributed to this hormonal difference. Nevertheless, since JA is involved in plant defence responses, the importance of these JA decreases should be investigated with factorial experiments imposing RZ CO_2 enrichment and pest/disease assays.

Furthermore, applying BA (a synthetic cytokinin) to leaves of intact pepper plants did not stimulate leaf area, while floating leaf-disc with 50 µM BA did increased significantly the area of the disc after 24h. Therefore, a decrease in foliar tZ could have also be involve in a decrease or lack response of pepper plant under high RZ CO_2. Decreased foliar tZ concentrations occur when nitrogen is depleted in the plant RZ (Rahayu et al. 2005), and may be induced in response to decreased shoot nitrogen concentrations. However, the magnitude of N depletion in pepper leaves (< 5% - Figure 3.6, chapter 3) is unlikely to cause significant changes in foliar CK concentrations, suggesting that alternative explanations must be sought to explain the decreased tZ concentration. Foliar tZ was positively correlated with SA (Table 4.1). As stated before, CKs and SA have been suggested to play a synergistic role in plant-defence immunity, although again, if no visual symptoms were found and control plants were in the same space, the elevated RZ CO_2 might be activating other response route.

In recent years, cytokinins have been determined to play an important role in defence against biotrophic pathogens. It was observed that applying exogenous low concentration levels (<10 uM) of cytokinins can help the biotrophic pathogen thrive by creating favourable physiological conditions, whereas high cytokinins concentrations (>100 µM) activate plants immunity through SA- dependent processes (Argueso et al., 2012). As no visual disease symptoms were detected in pepper shoot or roots, it might be that same response is activated from another type of biotic or abiotic stress due to high CO_2 levels in the roots. In addition, contrary to our results, ABA and SA have been well documented to be antagonistic in relation to plant defence responses to pathogens (Mohr and Cahill, 2007; Jiang et al., 2010). Therefore, it is unlikely that the response of these phytohormone changes were triggered by the presence of a pathogen.

Although in general pepper plants grown aeroponically did not show any significant differences in biomass or leaf area between treatments, one experiment showed that RZ CO_2 enrichment decreased biomass and leaf area (Table 2.10, Chapter 2). Phytohormonal profiling showed significantly higher leaf ACC concentrations and significantly lower leaf tZ and SA concentrations under elevated RZ CO_2 compared to the control treatment. These hormonal changes suggest that RZ CO_2 enrichment induces a long-distance stress-response in leaves. ACC is one of the most important intermediaries in ethylene biosynthesis, and its concentration increases in response to osmotic/ionic stress and other stresses including pathogen attacks and wounding (Morgan and Drew, 1997; Albacete et al., 2008). Often, but not always, ACC is transported from the roots to the shoot, when the roots are exposed to stress (McManus, 2012). In addition, tomato plants under flooding or lack of oxygen in the rhizosphere increased ACC transport from the roots to the shoots where it is converted into ethylene (Shiu et al., 1998). Long distance transport of ACC has also been suggested under other root stresses such as drought (Davies et al., 2000), nutrient stress (Lynch and Brown,1997) and salinity (Ghanem et al.,2008). Pepper plants grown under elevated RZ CO_2 may have been stressed due to higher temperatures and therefore higher respiration and lower oxygen availability in the rhizosphere (Chapter 2).

Ethylene as a plant hormone influences many aspects of plant growth and photosynthesis (Abeles et al., 1992 ; Pierik et al., 2006). Leaf disc assays were performed with the aim to understand the effects of each hormone in pepper grown without any imposed treatment, so we could better understand the lack on growth on those plants exposed to elevated RZ CO_2. While low ethylene concentrations (0.02 ppm) can increase growth, high concentrations (1 ppm) inhibit growth (Konings and Jackson, 1979). Moreover, ethylene inhibits leaf elongation in slow growing grass species (Fiorani et al., 2002) at high concentrations (>1 $\mu L\ L^{-1}$) compared to fast growing species. Applying saturating ACC concentrations to the leaves of pepper plants decreased leaf area compared to control plants (Table 4.3), which could also inhibit leaf growth in pepper grown under elevated RZ CO_2 (Figure 4.3). In addition, the lack of response of ACC in the leaf-disc assay was probably due to the extra ethylene production as wounded during disc excision.

ACC was positively correlated with photosynthesis rates, stomatal conductance and transpiration in pepper plants exposed to elevated RZ CO_2 (Table 4.1). Ethylene's involvement in regulating stomatal conductance and photosynthesis is still not well

understood, with ethylene mediating auxin-induced stomatal opening in *Vicia faba* (Merrit et al., 2001) but inhibits ABA induced stomatal closure in *Arabidopsis* (Tanaka et al., 2005). Furthermore, ABA has been correlated with synergistically closing the stomata with JA, and although leaf JA and ABA were positively correlated (Table 4.1) in our study stomatal conductance did not differ from control plants were this correlation was not happening (Table 4.2).

Finally, leaf-discs floated on low (< 100 μM) GA_3 concentrations did not promote leaf expansion compared to control plants, in agreement with Nielsen and Ulvskov (1992). However, same authors reported increased leaf expansion with a similar assay applying the same GA_3 concentration level (0.1 μM). They stated that low light irradiation (60 μmol. m-2 s-1) was limiting the GA_3 response and therefore higher irradiation was used (75 μmol m-2 s-1). As the assays were performed in the glasshouse variable light intensity through the day and between days could have been affecting the GA_3 response or perhaps, the wounding during disc excision could have promoted ethylene release which antagonised any GA_3-mediated growth increment (Achard et al., 2003).

4.5 Conclusions

Growth promotion (Experiments 2.1-2.4) or inhibition (Experiments 2.5-2.7) under elevated RZ CO_2 does not seem related to phytohormone changes in lettuce. On the other hand, pepper plants showed changes in foliar phytohormone (ACC, tZ and SA) concentrations, which were correlated with decreased leaf growth in some experiments. The high frequency of correlations between defence related phytohormones (SA and JA) found under elevated RZ CO_2 compared to control plants suggest that plants perceived either a biotic (for which there were no symptoms) or abiotic stress. The accumulation of ACC and the decreased in tZ as growth inhibitors may mask a positive effect of elevated RZ CO_2 on pepper growth.

Chapter 5. The effects of elevated root-zone carbon dioxide concentration on lettuce gene expression.

5.1 Introduction

Elevated RZ CO_2-induced biomass accumulation in aeroponic grown lettuce was variable however the consensus response was growth promotion (Table 2.7, Chapter 2). Increased growth was not associated with enhanced leaf gas exchange (Figure 2.19), nutrient and amino acid concentrations (Chapter 3) or favourable plant hormone status (Chapter 4). Whole genome transcriptome analysis has been widely used to elucidate the molecular mechanisms of plant stress responses (Allendorf et al., 2010; Hsieh et al., 2009). Consequently, analysis of genes differentially expressed under elevated and ambient RZ CO_2 may provide insights into gene functions and help understand the variable response among experiments.

Root and shoot gene expression analysis in response to eCO_2 in different crops and trees has been widely studied. In soybean, eCO_2 (500 ppm) showed changes in transcripts involved in cell growth and proliferation which was linked with stimulated respiratory breakdown of carbohydrates (Ainsworth et al., 2006). KEGG (Kyoto encyclopaedia of genes and genomes) pathway analysis of poplar grown under 550 – 720 ppm CO_2 revealed that enrichment of pathways related to eCO_2 were related to metabolism, including amino acids (glycine, serine and threonine metabolism), carbohydrates (glycolysis/gluconeogenesis), nucleotides (pyrimidine metabolism), cofactors and vitamins (lipoic acid metabolism) and energy (carbon fixation in photosynthetic organisms) (Liu et al., 2014). Applying ~700 ppm of aerial CO_2 to rice plants showed many up- and down-regulated genes related to signal transduction and transcription. Although changes were small (-0.75 to 0.75 log2 fold-change), genes that were up-regulated included those encoding for enzymes such as carbonic anhydrase, rubisco (RuBP), phosphoglycerate kinase and glyceraldehyde-3-phosphate. In contrast, genes that were down-regulated included those encoding enzymes for RuBP regeneration (fructose bisphosphate phosphatase, fructose bisphosphate aldolase, sedoheptulose bisphosphate phosphatase and phosphoribulokinase) and starch synthesis (ADP-glucose pyrophosphorylase and starch synthase) (Fukuyama et al., 2009).

These examples show that eCO_2 causes different response at the gene expression level within different plant species. Although the rhizosphere in the soil contains a high concentration of DIC that interacts with plant roots, gene expression analysis under elevated RZ CO_2 does not appear to have been studied. Therefore, a whole transcriptome sequencing study using RNA sequencing (RNA-seq) was performed in order to gain insights into the molecular mechanism responsible for the observed effects of high RZ CO_2 in lettuce plants. Studying differentially expressed genes (DEGs) related to high RZ CO_2 in aeroponics could further understand the effects of high RZ CO_2 and may contribute to the investigation of specific pathways.

5.2 Materials and Methods

5.2.1 Plant material and conditions

Crisphead lettuce (*Lactuca sativa* cv. Consul) seeds were purchased from Moles Seeds (Essex, UK). Seeds were individually sown in 150-cell plug trays in 2 cm x 2 cm x 4 cm rockwool cubes (Growell, Ltd, UK) and germinated in the glasshouse. Plants were transferred at 4-leaf stage to the aeroponic system described in Chapter 2. The glasshouse average day/night temperature ranged between 18 - 26 °C, and day/night relative humidity ranged between 20 - 60% (Figure 2.13, Chapter 2). The glasshouse was naturally lit with automated supplementary lighting supplied by ten high-pressure sodium lamps (600 W Greenpower, Osram, St Helens, UK) when the Photosynthetic Photon Flux Density (PPFD) was < 400 μmol m^{-2} s^{-1} for a 12 h photoperiod (08.00 hrs to 20.00 hrs).

5.2.2 Collection of plant material

Leaf tissue samples were collected between 10.30 and 11.30 hrs approx. 2 h after the start of the photoperiod and 10 days after the beginning of the treatment. For consistency with previous hormone and nutrient experiments, a leaf disc of 2.5 cm diameter was taken from the fully expanded leaf 6 of 16 (8 control and 8 treated) plants and immediately flash frozen in liquid nitrogen. Roots were excised from each plant, quickly dried with tissue paper and immediately flash frozen in liquid nitrogen. Both leaf and roots were stored at -80 °C.

5.2.3 Leaf RNA extraction

RNeasy Plant Mini Kit (Qiagen, Manchester, UK) was used to extract the RNA from leaf tissue following the Qiagen protocol. DNase treatment was applied to reduce DNA

contamination. After the extraction, 4 samples per treatment were pooled, therefore having 2 control samples and 2 RZ CO_2 samples. The RNA concentration was measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, DE, USA) and the ratio between A260 and A280 used to establish adequate RNA purity, in addition to monitoring for RNA degradation and contamination on 1% agarose gels.

5.2.4 Root RNA extraction

Phenol- Chloroform method and lithium chloride precipitation was used to isolate the RNA from the roots (Verwoerd et al., 1989). Roots were ground in nitrogen liquid to a fine powder. After grinding, 500 µl of hot extraction buffer (80°C) was added (phenol – 0.1 M LiCl, 100mM Tris-HCl pH=8, 10 mM EDTA, 1% (w/v) SDS (1:1)). The mixture was homogenized by vortexing for 30 seconds and 250 µl chloroform-isoamylalcohol was added and mixed. Tubes were centrifuged for 5 minutes at 10 000 g. The supernatant was then mixed with one volume of 4 M LiCL and the samples were store at 4 degrees overnight to allow RNAs to precipitate. After 10 min centrifugation at 10 000 g the pellets were dissolved in 250 µL H_2O, 0.1 volume of 3 M NaOAc pH=5.2 and 2 volumes of ethanol. After centrifugation the pellets were washed with ethanol and dried. DNase treatment was applied to reduce DNA contamination. The RNA concentration was measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, DE, USA) and the ratio between A260 and A280 was used to establish adequate RNA purity, in addition to monitoring for RNA degradation and contamination on 1% (w/v) agarose gels.

5.2.5 Library Preparation and Transcriptome Sequencing

RNA samples were sent on dry ice to Beijing Novogene Bioinformatics Technology Co. Ltd. (Beijing, China) for RNA sequencing analysis. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA) and RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired end reads were generated.

5.2.6. Bioinformatics analysis

5.2.6.1 Quality control

The original raw data from Illumina was transformed to Sequenced Reads by base calling. Raw data was recorded in a FASTQ file, which contains sequence information (reads) and corresponding sequencing quality information. Raw reads in the fastq format were first processed through in-house Perl scripts. Clean reads were obtained by removing reads containing adapter sequence, reads containing poly-N and low-quality reads from the raw reads. Q20, Q30, GC-content and sequence duplication level of the clean reads were calculated. At the same time, Sequencing Quality Scores (Q) were calculated based on the equation: $Q = -10\log10(e)$

where "e" is the estimated probability of the base call being wrong (Ewing et al., 1998; Richterich, 1998). Specifically, Q20 and Q30 were utilized as quality scores having an incorrect base call probability of 1 in 100 times and 1 in 1000 times, respectively. Guanine-cytosine (GC) content was calculated and was used for normalization of read counts, as GC abundance is heterogeneous across the genome making it difficult to separate the GC effect from true expression signal, and thus it may influence RNAseq quantification (Pickrell et al., 2010; Risso et al., 2011; Benjamini and Speed, 2012). All the downstream analyses were based on the clean reads which were determined by their error rate, Q20, Q30, and GC contents.

5.2.6.2 Mapping reads to the reference genome

Lettuce is a diploid species with 2n = 2x = 18 chromosomes and a genome size estimated to be ~ 2.5 Gb (Michaelson et al., 1991) and confirmed recently with the whole genome sequencing and assembly of *L. sativa* cv. Salinas at ~2.7 Gb (Reyes-Chin-Wo et al., 2017), which was used as the reference sequence in this RNA sequencing research.

Index of the reference genome was built using Bowtie v2.2.3 (Broad Institute, Cambridge, MA, USA) according to Langmead et al. (2009) and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 (Broad Institute, Cambridge, MA, USA), according to Trapnell et al. (2012). The reference genome and gene model annotation files were downloaded from the Lettuce Genome Resource website (lgr.genomecenter.ucdavis.edu). TopHat was selected as the mapping tool because it can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools.

5.2.6.3 Quantifying gene expression levels

Gene expression levels were estimated by calculating the number of fragments per kilobase of transcript per million fragments mapped (FPKM) and normalized using HTseq-count and DESeq2. HTSeq v0.6.1 was used to count the read numbers mapped to each gene. Then FPKM of each gene was calculated based on the length of the gene and read counts mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels (Trapnell, et al., 2010).

5.2.6.4 Differential expression analysis

To identify differentially expressed genes between the two treatments (control and elevated RZ CO_2), differential expression analysis was performed using the DESeq R package (1.18.0). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). Raw expression values were converted into log2 ratio. Genes with an adjusted *P*-value <0.05 were defined as differentially expressed.

5.2.6.5 Gene Ontology analysis

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected. The differentially expressed genes were classified into several biological process categories from GO annotation for each significant expression profile. The criterion of $P<0.05$ was used to screen for significant GO terms.

5.2.6.6 Pathways analysis

All of the DEGs contained in all significant expression profiles underwent pathway analysis. KEGG (www.genome.jp/kegg) annotations were assigned according to the KEGG database using KOBAS software (Peking University, Beijing, China) to test the enrichment of DEGs in particular KEGG pathways. Enrichment degree of KEGG was measured via the rich factor, q-value and gene counts enriched to this pathway. Rich factor is the ratio of DEGs in the related pathway divided by the number of all the annotated genes in this pathway. The

higher the rich factor is, the greater the degree of enrichment. q-value is the adjusted p-value after multiple hypothesis testing. The closer the q-value is to zero, the more significant the enrichment.

5.3 Results

RNA-seq of the 8 cDNA libraries resulted in a total of ~538 million raw reads, of which ~520 million clean reads were selected for further analysis. The clean Q30 base rate, a key parameter that represents the quality of sequenced base, was 93.2% and the average CG content percentage was 44.7%. When mapping the RNA-seq reads to the reference genome, 89.64-91.55% of the reads could be mapped and 86.66-87.92% could be mapped uniquely to the location (Table 5.1).

Table 5.1. Overview of Mapping Status. Clean reads were obtained by removing low quality reads from raw reads. Q20 and Q30 represent the percentages of bases whose correct base recognition rates are greater than 99% and 99.9% in total bases, respectively. GC represents the percentages of G and C in total bases. Total mapped represents total number of reads that was mapped to the reference genome. Multiple mapped is the number of reads that was mapped to multiple sites in the reference genome and uniquely mapped is number of reads that was uniquely mapped to the reference genome.

Sample name	Raw reads	Clean reads	Clean bases	Q20 (%)	Q30 (%)	GC (%)	Total mapped	Multiple mapped	Uniquely mapped
Leaves control rep 1	61107356	59732318	9G	97.78	93.58	45.94	54685452 (91.55%)	2835177 (4.75%)	51850275 (86.8%)
Leaves control rep 2	70035632	67852034	10.2G	97.71	93.4	45.89	61672059 (90.89%)	2874698 (4.24%)	58797361 (86.66%)
Leaves RZ CO_2 rep 1	63980830	62767210	9.4G	97.64	93.25	45.87	57400261 (91.45%)	2741284 (4.37%)	54658977 (87.08%)
Leaves RZ CO_2 rep 2	63761294	62061062	9.3G	97.8	93.62	45.77	56758175 (91.46%)	2815236 (4.54%)	53942939 (86.92%)
Root RZ CO_2 rep 1	67582384	64973144	9.7G	97.51	92.99	43.6	58577103 (90.16%)	1454158 (2.24%)	57122945 (87.92%)
Root RZ CO_2 rep 2	68859748	65955904	9.9G	97.39	92.73	43.66	59125106 (89.64%)	1460018 (2.21%)	57665088 (87.43%)
Root RZ control rep 1	80122146	76899054	11.5G	97.62	93.2	43.54	69228882 (90.03%)	1709778 (2.22%)	67519104 (87.8%)
Root RZ control rep 2	62849010	60096644	9G	97.66	93.25	43.56	54484218 (90.66%)	1303257 (2.17%)	53180961 (88.49%)

5.3.1 Differential gene expression in leaves and roots in response to RZ CO_2

To elucidate the underlying molecular mechanisms of response of lettuce to elevated RZ CO_2, the gene expression profiles of shoot and roots of plants exposed to elevated RZ CO_2 and ambient RZ CO_2 were compared and the significant DEGs identified. In leaves a total of 18261 transcripts were expressed in both RZ CO_2 and control treatments. In roots, a total of 21611 transcripts were detected in both groups (Figure 5.1).

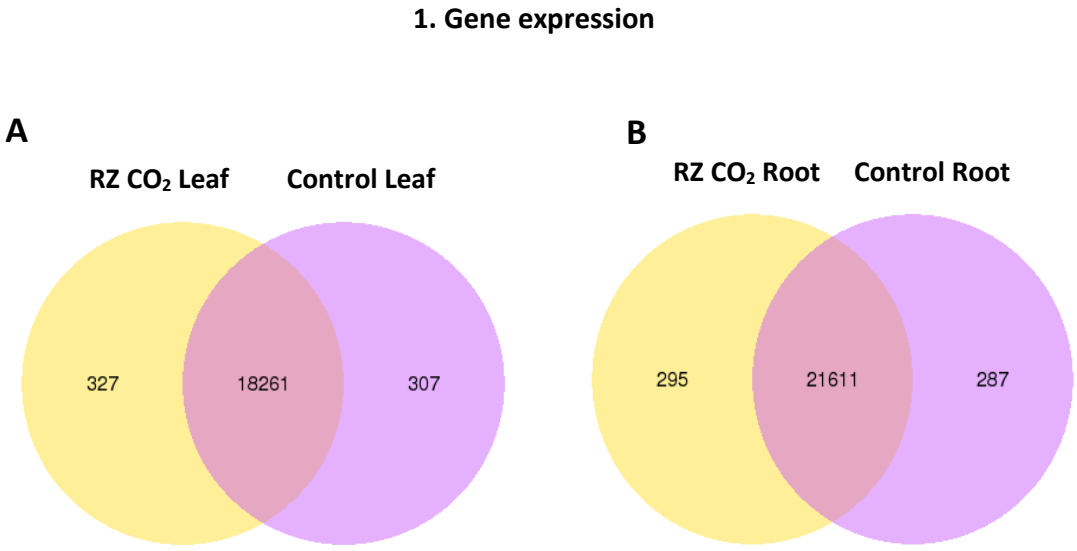


Figure 5.1. Venn diagrams to illustrate the number of express transcripts between the two groups. Number of transcripts for elevated RZ CO_2 (Yellow) and control (Purple) in leaves (A) and roots (B). The numbers in each circle represents the total number of genes in each comparison group, and the overlapping area of the circles represents the number of common genes between the two comparison groups.

Transcripts with an adjusted *P*-value <0.05 found by DESeq were assigned as differentially expressed genes (DEGs). Comparing the RZ CO_2-treated and control samples identified a total of 10 DEGs in the leaves, of which two were up-regulated and eight down-regulated. In contrast, with the low quantity of DEGs expressed in the leaves, in the roots a total of 294 DEGs were observed, of which 174 were up-regulated and 120 down-regulated (Figure 5.2).

2. Differentially Expressed Genes

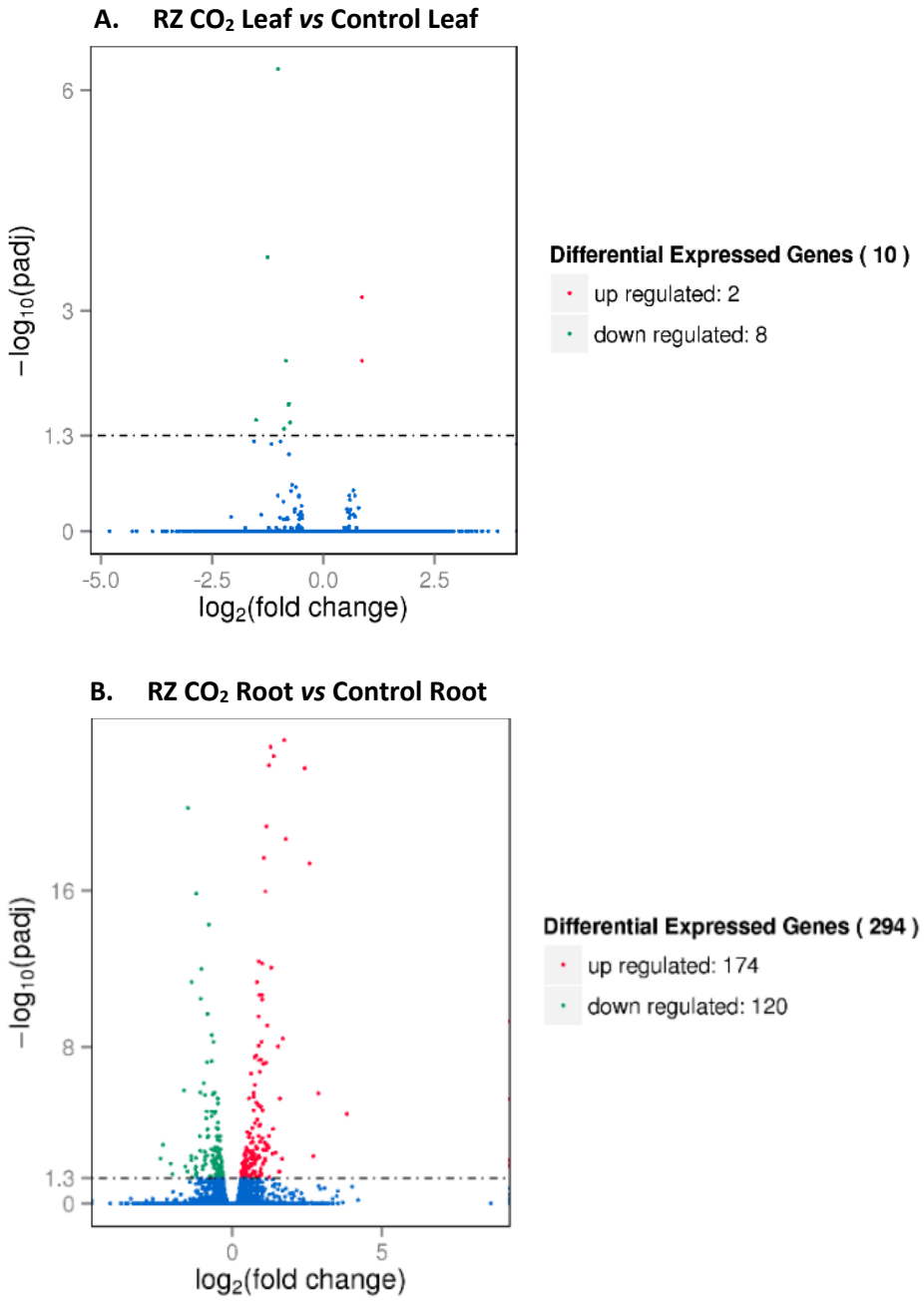


Figure 5.2. Volcano plots for all the DEGs in each comparison. DEGs in leaves (obtained by pairwise comparison of leaves (A) and roots (B) between treatments (RZ CO_2 and control). The x-axis shows the fold change in gene expression between different samples, and the y-axis shows the statistical significance of the differences. Significantly up and down-regulated genes are highlighted in red and green, respectively. No significant difference in gene expression is shown in blue.

5.3.2 GO enrichment analysis

To understand the function of the DEGs identified in Section 5.3.1, all the genes were mapped onto terms in the GO database (Figure 5.3). This helps assess if the group of DEGs has some functional signal. Nineteen significant GO terms were detected in roots, but no significant GO terms were detected in leaves. In roots, a large proportion of DEGs fell into the categories of biological process (GO: 0008150), metabolic process (GO: 0008152), single-organism process (GO:0044699), single-organism metabolic process (GO:0044710), oxidation-reduction process (GO:0055114), small molecule metabolic process (GO:0044281), carboxylic - acid metabolic process (GO: 0019752), oxoacid metabolic process (GO: 0043436), organic acid metabolic process (GO:0006082) and cellular amino acid metabolic process (GO:0006520). In the molecular function class, a large number of DGEs were annotated to catalytic activity (GO:00038240), oxidoreductase activity (GO:0016491), oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (GO:0016705), oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors, resulting in the reduction of molecular oxygen to two molecules of water (GO:0016717), heme binding (GO:0020037), tetrapyrrole binding (GO:0046906), cofactor binding (GO:0048037), iron ion binding (GO:0005506) and coenzyme binding (GO:0050662) .

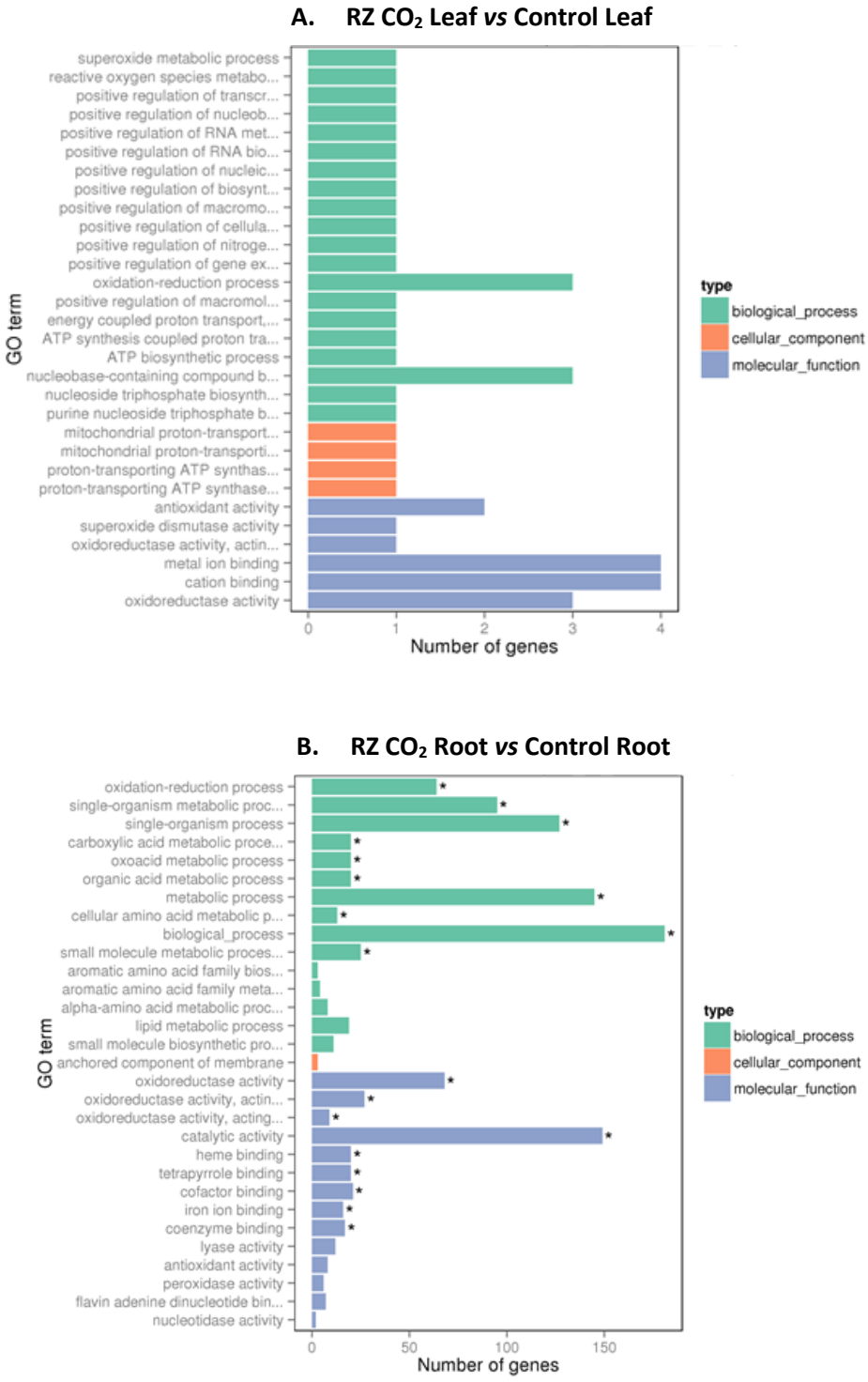


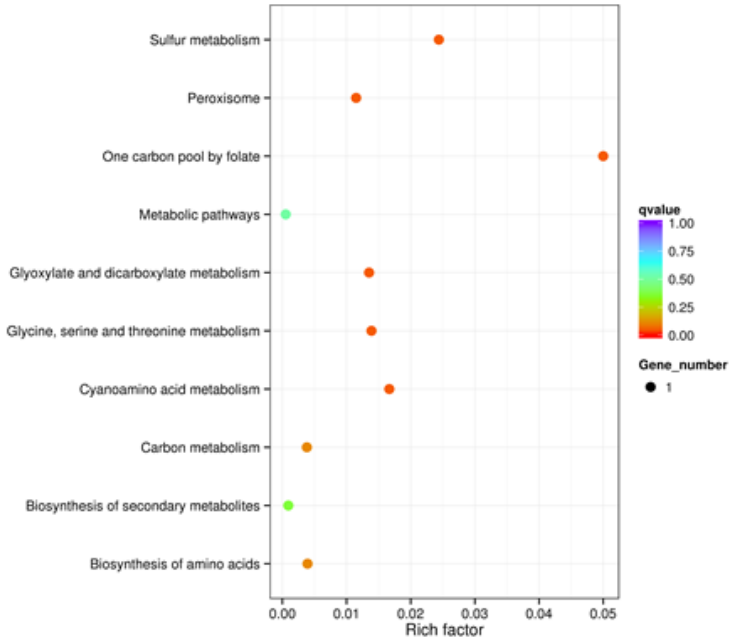
Figure 5.3. Gene ontology (GO) enrichment analysis of DEGs in lettuce leaf (A) and root (B) tissue comparing RZ CO_2 vs Control. The x-axis shows the enriched GO terms and the y-axis is the number of DEGs. Different colours are used to distinguish biological process (green), cellular component (orange) and molecular function (blue). Asterisk (*) indicates significantly enriched GO terms according to corrected p-value <0.05.

5.3.3 KEGG Pathway Enrichment Analysis of DEGs

All the DEGs were subjected to a KEGG pathway enrichment analysis (Figure 5.4). Down-regulated genes under elevated RZ CO_2 in leaves were involved in 10 distinct pathways. Of them, the top five were one carbon pool by folate (ath00670), sulfur metabolism (ath00920), cynoamino acid metabolism (ath00460), glycine, serine and threonine metabolism (ath00260) and glyoxylate and dicarboxylate metabolism (ath00630). Up-regulated genes were not found in leaves under elevated RZ CO_2.

The up-regulated genes in roots were involved in 54 distinct pathways. Of them, the top 10 were biosynthesis of unsaturated fatty acids (ath01040), metabolic pathways (ath011000), biosynthesis of secondary metabolites (ath01110), fatty acid (FA) metabolism (ath01212), phenylpropanoid biosynthesis (ath00940), phenylalanine, tyrosine and tryptophan biosynthesis (ath00400), carbon metabolism (ath01200) , carbon fixation in photosynthetic organisms (ath00710) and biosynthesis of amino acids (ath01230). The down-regulated genes were involved in 27 pathways. Of them, the top 10 were cutin, suberin and wax biosynthesis (ath00073), sulfur metabolism (ath00920), glycerolipid metabolism (ath00561), glycine , serine and threonine metabolism (ath00260), thiamine metabolism (ath00730), glycerophospholipid metabolism (ath00564), biosynthesis of secondary metabolites (ath01110), one carbon pool by folate (ath00670), regulation of autophagy (ath04140) and carotenoid biosynthesis (ath00906).

A. RZ CO_2 Leaf *vs* Control Leaf



B. RZ CO_2 Root *vs* Control Root

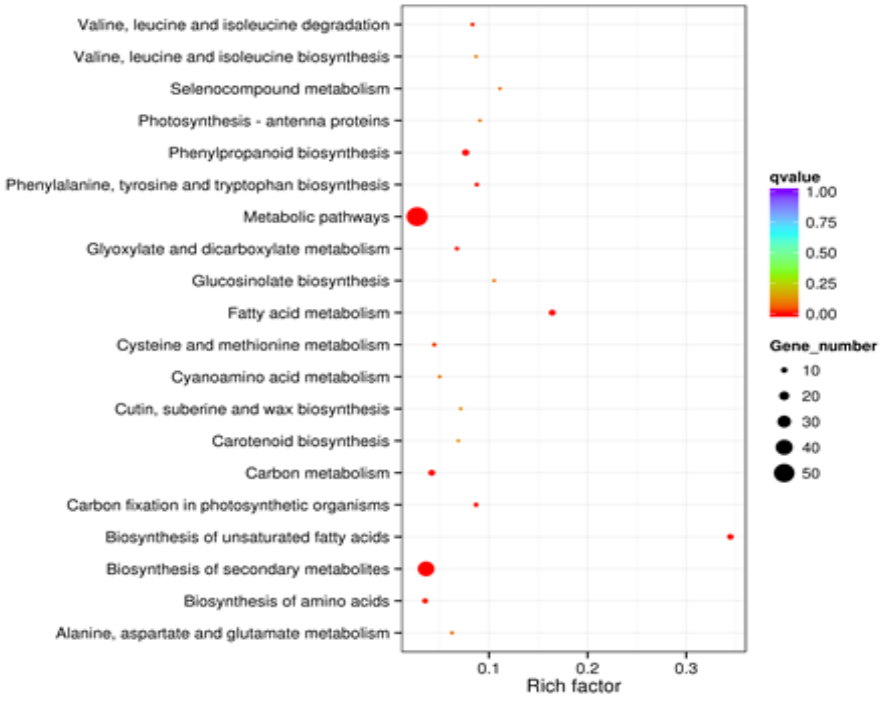


Figure 5.4. Top 20 KEGG pathway enrichment of up- and down-regulated DEGs in leaves (A) and roots (B). The number of genes in each pathway is equal to the dot size. The dot colour represents the q-value. The smaller the q-value, the redder the dot. Rich factor = number of DEGs enriched in certain pathway term/number of unigenes annotated in this pathway term.

5.3.3 RZ CO_2 related genes

Among the 10 DEGs between treatments in leaves, one gene encoding for superoxide dismutase [Fe], one gene encoding for stem-specific protein TSJT1 and one gene encoding for chaperone protein dnaJ 11, where significantly (>1 fold change, p<0.05) down-regulated in plants under elevated RZ CO_2. Another seven genes were also significantly down-regulated and two genes encoding for B-box zinc finger protein 32 and uncharacterized (LOC111919665) were up-regulated (Table 5.2).

Table 5.2. Significantly up- and down-regulated genes in leaves under elevated RZ CO_2. Difference of gene expressions in this list are all significant (P < 0.05). In red are shown normalized expression log2 fold-change greater than +1/-1. Abolute expression levels are given in read counts.

Description	Gene ID	log2 fold change	P value	Absolute expression level	
				Control	RZ CO_2
Serine hydroxymethyltransferase 6 - like	LOC111877987	-0.84	0.005	835	465
5'-adenylylsulfate reductase 3	LOC111891058	-0.79	0.019	3249	1884
Chaperone protein dnaj 8	LOC111892295	-0.77	0.018	1033	606
Ethylene-responsive transcription factor 5 - like	LOC111905844	-0.74	0.033	918	549
Superoxide dismutase [Fe]	LOC111905867	**-1.02**	0.000	2469	1219
Cytochrome P450 71A8 – like	LOC111907014	-0.88	0.040	403	219
B-box zinc finger protein 32	LOC111913899	0.87	0.004	412	754
Stem-specific protein TSJT1-like	LOC111916149	**-1.52**	0.030	195	68
Chaperone protein dnaJ 11	LOC111917860	**-1.25**	0.000	352	148

In roots, among the most enriched pathway terms (Figure 5.4B), 11 DEGs were up-regulated in FA metabolism, 12 in phenylpropanoid biosynthesis, 11 in carbon metabolism, 10 in biosynthesis of unsaturated FA and 9 in biosynthesis of amino acids. In the FA metabolism, seven genes encoding Δ12 fatty acid desaturase (FAD) -like proteins and two Δ12 acyl-lipid desaturase-like proteins were up-regulated under elevated RZ CO_2. In the phenylpropanoid biosynthesis pathway, a wide range of genes encoding for different proteins were up-regulated, among them, one gene, encoding beta-glucosidase-like protein, and two genes encoding the peroxidase 60 and 7-like proteins, were the genes with highest fold-change: 1.3, 1.4, and 1.2-fold, respectively. In the amino acid biosynthesis pathway, one gene encoding tyrosine aminotransferase-like had the highest fold change (1.3-fold). In addition, three genes involved in phenylalanine biosynthesis

encoding arogenate dehydratase and arogenate dehydrogenase 2 and two genes involved in leucine and isoleucine biosynthesis were overexpressed (Table. 5.3). Finally, in the carbon metabolism and carbon fixation metabolism pathways, four genes encoding for ribulose bisphosphate carboxylase small chain (rbcS), chloroplastic-like protein, among which the last 3 genes were not present in control roots, were significantly up-regulated under RZ CO_2 treatment (2.9-fold). One gene, encoding glyceraldehyde-3-phosphate dehydrogenase A and two genes, encoding chlorophyll a-b binding protein of LHCII type 1-like protein were also up-regulated under high RZ CO_2 . However, the read counts of those *loci* were lower compared to other genes encoding for proteins involved in other functions in the same carbon metabolism pathway such as Glumatame dehydrogenase B.

Table 5.3. Significantly up-regulated genes in roots under elevated RZ CO_2. Difference of gene expressions in this list are all significant ($P < 0.05$). In red are shown expression levels log2 fold-change greater than +1/-1. Absolute expression levels are given in read counts.

Description	Gene ID	log2 fold change	P value	Absolute expression level	
				Control	RZ CO_2
1. Fatty acid metabolism					
3-oxoacyl-[acyl-carrier-protein] synthase II	LOC111883554	0.48	0.02	622	867
1.1 Biosynthesis of unsaturated fatty acids					
Delta(12) fatty acid desaturase DES8.11-like	LOC111896575	**1.05**	0.00	2023	3634
Delta(12)-fatty-acid desaturase FAD2-like	LOC111909558	0.84	0.00	840	1506
Delta(12) fatty acid desaturase DES8.11-like	LOC111896570	0.88	0.00	1621	2991
Delta(12) fatty acid desaturase DES8.11-like	LOC111889078	0.84	0.00	330	681
Delta(12) fatty acid desaturase DES8.11	LOC111889077	0.67	0.03	3628	5766
Delta(12)-fatty-acid desaturase FAD2-like	LOC111900952	0.62	0.01	6760	10387
Delta(12)-acyl-lipid-desaturase-like	LOC111900953	0.60	0.00	7329	11081
Delta(12) fatty acid desaturase DES8.11-like	LOC111897874	0.72	0.00	4307	7081
Delta(12)-acyl-lipid-desaturase-like	LOC111915490	0.89	0.00	277	512
1.2 Valine, leucine and isoleucine degradation					
3-ketoacyl-CoA thiolase 2	LOC111901559	0.38	0.00	2429	3164
2. Phenylpropanoid biosynthesis					
Raucaffricine-O-beta-D-glucosidase-like	LOC111876277	0.84	0.00	247	442
Beta-glucosidase 13-like	LOC111876276	**1.29**	0.00	1139	2786
Cationic peroxidase 1-like	LOC111887994	0.83	0.00	1046	1864
Peroxidase 60-like	LOC111905466	**1.37**	0.00	72.8	189
Cytochrome P450 84A1-like	LOC111895133	0.57	0.00	1934	2863
Phenylalanine ammonia-lyase-like	LOC111894107	0.47	0.02	7549	10458
Mannitol dehydrogenase-like	LOC111883427	0.70	0.00	327	531
Peroxidase N1-like	LOC111896419	0.51	0.00	1490	2119
Peroxidase 7-like	LOC111915473	**1.22**	0.00	173	404
Caffeic acid 3-O-methyltransferase	LOC111903396	0.90	0.00	899	1677
Peroxidase N1-like	LOC111899521	0.69	0.00	257	414
Mannitol dehydrogenase-like	LOC111915590	0.69	0.00	5453	8820
3. Biosynthesis of amino acids					
Enolase	LOC111906568	0.64	0.00	415	648
3.1 Valine, leucine and isoleucine					
2-isopropylmalate synthase A-like	LOC111896991	0.61	0.01	309	470
Threonine dehydratase biosynthetic	LOC111906545	0.91	0.00	374	703
3.2 Phenylalanine, tyrosine and tryptophan					
Arogenate dehydratase/prephenate dehydratase 6	LOC111901426	0.39	0.02	1456	1914
Arogenate dehydratase/prephenate dehydratase 6	LOC111896614	0.43	0.01	1372	1847
Arogenate dehydrogenase 2	LOC111905077	0.74	0.01	178	297
Anthranilate synthase alpha subunit 1	LOC111919728	0.55	0.05	685	1001
3.3 Cysteine and methionine metabolism					
Tyrosine aminotransferase-like	LOC111906702	**1.31**	0.00	358	888
4. Carbon metabolism					
Threonine dehydratase biosynthetic	LOC111906545	0.91	0.00	374	703
Enolase	LOC111906568	0.64	0.00	415	648
Glutamate dehydrogenase B	LOC111893255	0.35	0.01	5844	7441
4.1 Valine, leucine and isoleucine degradation					
3-hydroxyisobutyryl-CoA hydrolase 1-like	LOC111913847	0.97	0.00	946	1852
4.2 Carbon fixation in photosynthetic organisms					
Glyceraldehyde-3-phosphate dehydrogenase A	LOC111886224	**2.71**	0.00	6	36
4.2.2 Glyoxylate and dicarboxylate metabolism					
Ribulose bisphosphate carboxylase small chain	LOC111908249		0.00	0	47
Ribulose bisphosphate carboxylase small chain	LOC111908250		0.00	0	29
Ribulose bisphosphate carboxylase small chain	LOC111908252		0.01	0	16
Ribulose bisphosphate carboxylase small chain	LOC111917913	**2.88**	0.00	8	63
4.2.3 Photosynthesis antenna proteins					
Chlorophyll a-b binding protein of LHCII type 1-like	LOC111914737		0.01	0	15
Chlorophyll a-b binding protein of LHCII type 1-like	LOC111908061	**3.83**	0.00	2	41

DEGs that were not involved in any of the previous named enriched pathways were also up-regulated. Among them, genes encoding ammonium transporter 1 member-like proteins, expansin-A7 like proteins, UPD-glycosyltransferase-like proteins cytochrome P450-like proteins and a few transcription factors were significantly up-regulated (Table 5.4).

Table 5.4. Significantly up-regulated genes in roots under elevated RZ CO_2. Difference of gene expressions in this list are all significant ($P < 0.05$). In red are shown expression levels log2 fold-change greater than +1/-1. Absolute expression levels are given in read counts.

Description	Gene ID	log2 fold change	P value	Absolute expresion level	
				Control	RZ CO_2
Ammonium transmembrane transporter activity					
Ammonium transporter 1 member 2-like	LOC111886484	**1.00**	0.00	543	1087
Ammonium transporter 1 member 1-like	LOC111875863	**1.00**	0.00	438	876
Cell wall organization					
expansin-A7-like	LOC111902505	**1.35**	0.00	41	104
expansin-A7-like	LOC111918733	**1.46**	0.00	64	175
expansin-A7-like	LOC111920885	**1.24**	0.05	63	150
Transferase activity					
UDP-glycosyltransferase 89B1-like	LOC111915618	**1.69**	0.00	64	206
UDP-glycosyltransferase 89B2-like	LOC111915635	**1.55**	0.00	127	371
UDP-glycosyltransferase 73C3-like	LOC111894110	**1.13**	0.00	167	367
UDP-glycosyltransferase 73E1-like	LOC111894085	0.81	0.01	162	283
UDP-glycosyltransferase 74G1-like	LOC111900864	0.41	0.03	968	1290
Transcription factors					
probable WRKY transcription factor 70	LOC111885363	0.49	0.01	591	829
probable WRKY transcription factor 70	LOC111898200	0.77	0.01	170	289
WRKY transcription factor 18-like	LOC111917191	**1.39**	0.00	408	1067
transcription factor MYB30-like	LOC111902163	**1.00**	0.00	121	244
Plant development and defence					
cytochrome P450 CYP72A219-like	LOC111916050	**1.67**	0.01	35	111
cytochrome P450 71A4-like	LOC111916769	**1.79**	0.00	377	1309
cytochrome P450 71B2-like	LOC111891421	0.47	0.03	570	790
cytochrome P450 71B9-like	LOC111891426	0.53	0.00	748	1079
cytochrome P450 704C1-like	LOC111909557	0.82	0.00	6378	11238
cytochrome P450 CYP72A219-like	LOC111916052	0.39	0.02	1340	1751

Regarding down- regulated genes in roots, among the most enriched pathways, two genes involved in cutin and suberin biosynthesis, one gene involved in gluconeogenesis, and two genes involved in glyoxylate and glycine biosynthesis were significantly down-regulated under high RZ CO_2. DEGs non-involved in any of the most enriched pathways were also significantly down-regulated. Five genes encoding PELPK1-like proteins, five genes related to nitrate, zinc and sulphate transporters-like proteins and seven genes involved in sulphur metabolism and sulphur deficiency response were down-regulated under high RZ CO_2 (Table 5.5).

Table 5.5. Significantly down-regulated genes in roots under elevated RZ CO_2. Difference of gene expressions in this list are all significant ($P < 0.05$). In red are shown expression levels log2 fold-change greater than +1/-1. Absolute expression levels are given in read counts.

Description	Gene ID	log2 fold change	P value	Absolute expression level	
				Control	RZ CO_2
1. Cutin, Suberin and wax biosynthesis					
Omega-hydroxypalmitate O-feruloyl transferase-like	LOC111895386	0.59	0.01	1522	1014
Cytochrome P450 86B1-like	LOC111884821	0.70	0.04	616	378
2. Sulfur metabolism					
5'-adenylylsulfate reductase 3	LOC111907858	0.33	0.01	7002	5558
5'-adenylylsulfate reductase 3	LOC111891058	0.58	0.00	7299	4866
3. Carbon metabolism					
3.1 Pyruvate metabolism, Glycolysis/Glucogenesis, Carbon fixation					
Phosphoenolpyruvate carboxykinase (ATP)-like	LOC111877438	0.36	0.01	3497	2734
4. Biosynthesis of amino acids					
4.1 Glycine, serine and threonine metabolism, Starch and sucrose metabolism					
Alanine--glyoxylate aminotransferase 2 homolog 2	LOC111896211	0.43	0.01	1231	911
4.2 Glycine, serine and threonine metabolism, carbon metabolism, glyxolate, dicarboxylate, cyaminoacid metabolism, one carbon fool by folate					
Serine hydroxymethyltransferase 6-like	LOC111877987	0.87	0.01	2137	1167
5. Cellular component proteins					
Protein PELPK1-like	LOC111890400	0.89	0.00	775	417
Protein PELPK1-like	LOC111899044	**1.21**	0.01	409	177
Protein PELPK1-like	LOC111899067	**1.34**	0.05	240	95
Protein PELPK1-like	LOC111899074	**1.20**	0.00	156	68
Protein PELPK2-like	LOC111893161	**1.05**	0.00	361	174
6. Transcription factors					
Ethylene-responsive transcription factor RAP2-11-like	LOC111894027	**1.60**	0.00	416	138
Ethylene-responsive transcription factor RAP2-11-like	LOC111903343	0.90	0.00	497	265
7. Transporter activity					
High-affinity nitrate transporter 3.1-like	LOC111907038	0.48	0.00	12058	8619
High-affinity nitrate transporter 3.1-like	LOC111907039	0.49	0.00	11009	7861
Zinc transporter 1	LOC111880346	0.46	0.00	4120	2994
Zinc transporter 4	LOC111906062	0.39	0.05	1592	1216
Sulfate transporter 1.3-like	LOC111908212	**1.05**	0.00	715	346
8. Plant development and defence					
Cytochrome P450 716B1-like	LOC111890912			103	40
Cytochrome P450 76A1	LOC111907130	**1.18**	0.03	1995	880
Cytochrome P450 78A5-like	LOC111889895	0.42	0.00	6877	5128
9. Sulfur deficiency response					
Protein SULFUR DEFICIENCY-INDUCED 2	LOC111915910	0.54	0.01	1037	716
Protein SULFUR DEFICIENCY-INDUCED 1-like	LOC111885583	**1.47**	0.00	738	266
Protein SULFUR DEFICIENCY-INDUCED 1-like	LOC111901564	0.83	0.00	855	481
Protein RESPONSE TO LOW SULFUR 3-like	LOC111903384	**1.35**	0.00	430	169
Protein RESPONSE TO LOW SULFUR 3-like	LOC111903383	0.82	0.00	1455	824

5.4 Discussion

This chapter aimed to gain an overall view of elevated RZ CO_2 effects on gene expression, which hitherto has never been attempted, to augment our mechanistic understanding of previous studies. Gene expression analyses revealed that 2 and 8 transcripts were up- and down-regulated in the leaves respectively, whereas 174 and 120 transcripts were up- and down-regulated in the roots respectively. Elevated RZ CO_2 had a greater impact on gene expression in the roots and in the long term these effects are expected to influence plant development. However, it is necessary to link these transcriptomic changes to physiological and morphological variables.

In roots, several DEGs encoding cell wall and membrane proteins such as expansin-like, PELPK-like proteins and zinc, nitrate, ammonium and sulphur transporters were differentially expressed between treatments (Table 5.4). Genes encoding expansin 7-like proteins were up-regulated under high RZ CO_2. Expansin genes are found throughout the entire plant kingdom and are considered cell-wall loosening proteins (molecular modification of the wall network that results in relaxation of wall stress) capable of mediating cell wall extension in acidic conditions without hydrolytic breakage of major structural components of the cell wall (Cho and Crosgove, 2002). The pH of the cell wall of growing cells is typically between 4.5 and 6, spanning the range where expansins are activated (McQueen-Mason et al., 1992). Acid-induced growth and expansin action are implicated in plant growth responses to hormones and external stimuli such as light, drought, salt stress and in morphogenetic processes such as root-hair formation (Cosgrove, 2005). Two families of expansins are recognized nowadays, α- and β-expansins. *Arabidopsis* contains 26 α and 5 β expansins (Cosgrove, 2000). Among them, two α-expansins, expansin-7 and 18 are expressed in initiating and tip-growing root hairs. In addition, exogenous ethylene positively affects root hair initiation, but endogenous ethylene is seemingly not required for normal root hair formation (Cho and Cosgrove, 2002). When roots were separated from the medium, promotion of ethylene biosynthesis could induce root hair initiation. It was concluded that expansin-7 genes can be activated through two different pathways, developmental or environmental/hormonal, that merged at or before transcriptional regulators that modulate the initiation of root hairs and the expression of the specific gene set. Root ACC concentrations (the precursor of ethylene) did not change in lettuce roots following elevated RZ CO_2 treatment (Chapter 4), which may indicate that the expression of these expansin genes was mediated by other

stimuli. Although root dry weight did not significantly differ from control plants (Chapter 2), high air CO_2 stimulated greater root branching in soil-grown plants (Del Castillo et al., 1989). Since root biomass did not differ between treatments, root architecture was not further studied. Although expression of specific expansin genes can be induced by several factors, the signal pathway and the molecular mechanisms underlying wall loosening is still not clear.

Contrary to upregulation of expansin genes, elevated RZ CO_2 downregulated five genes encoding cell wall PEPK1-2 like proteins, which have a primary function of providing structural support to the cell wall (Table 5.5). The gene, At5g09530 which encodes PELPK1 is reportedly induced by abiotic and biotic stresses (Kilian et al., 2007; Sottosanto et al., 2004; Ascencio-Ibanez et al., 2008), by ABA (Goda et al, 2008) and the allelochemicals gallic acid (Golisz et al., 2008) and benzoxazolin 2(3H)-one (Baerson et al., 2005). During normal development, in the absence of external stresses, At5g09530 transcripts have been found in mature vasculature of roots, and in the seed coat or endosperm of developing seeds (Schmid et al. 2005; Le et al.2010; Brady et al. 2007). Moreover, peptides of At5g09530 have been detected within root exudates (Basu et al. 2006). Further studies should seek to identify expression patterns of At5g09530 in response to elevated RZ CO_2.

Five genes encoding UDP-glycosyltransferases (UGTs) were also upregulated in response to elevated RZ CO_2. Plant UDP-glycosyltransferases typically transfer glucose from UDP-glucose to small acceptor molecules (Vogt and Jones, 2000). UGTs are thought to be cytoplasmic enzymes which glycosylate a broad array of aglycones, including plant hormones, all major classes of plant secondary metabolites and xenobiotics such as herbicides (Ross et al., 2001; Jones and Vogt, 2001). Transfer of a sugar onto the acceptor molecule can lead to changes in the activity of the acceptor as well as changes in its subcellular localization. For example, glycosylation of plant hormones such as auxins, abscisic acid, and gibberellins inactivates their biological activity (Kleczkowski and Shell, 1995). This also could explain why plant hormone concentrations showed limited changes under RZ CO_2 in lettuce plants (Chapter 4). Once conjugated, the glycosylated acceptor can exit the cytosol and enter a membrane compartment through recognition by transporters, such as those identified in the tonoplast membrane of the vacuole, which is considered to be the major compartment for storage of the glucoconjugates. Although

the role of UGTs in response to biotic stresses has been well characterized, their precise contribution remains unclear (Rehman et al, 2018).

Since the nutrient solution supplied was rich in NO_3^- compared to NH_4^+, up-regulation of genes encoding transporter proteins for NO_3^- was expected. Irrespective of plant (nitrogen) nutritional status, 15N-studies demonstrate that NH_4^+ is preferred up to 20-fold over nitrate by Arabidopsis plants. Interestingly and linked to the expansin-7 proteins, NH_4^+ transporters are preferentially expressed in root hairs (Lauter et al., 1996). Surprisingly, genes encoding ammonium transporter member 1-1, 2 (AMT1.1 and 1.2) like proteins were up-regulated under high RZ CO_2 while genes encoding high affinity nitrate transporters 3.1 (NRT3.1) like proteins were down-regulated. The AMT1.1 protein participates in root NH_4^+ acquisition, its long-distance transport to the shoots, and in re-uptake of apoplastic NH_4^+ derived from shoot photorespiration (Mayer and Ludewig, 2006).

On the other hand, high external NO_3^- concentration downregulates NRT3.1 and NRT2 expression. However, this down- regulation was mediated by another transporter named NRT1.1 and not through a mechanism involving N metabolites. Apart from being a mechanism against NH_4^+ toxicity, the mediated regulation of NRT 1.1 is hypothesized to satisfy a specific NO_3^- demand of the plant to the different roles that NO_3^- has on the plant, besides from being an N source (Krouk et al., 2006). In an acidic environment (<pH 5), the gradient is opposite at root plasma membranes, with passive flow of NO_3^- outwards and an inward gradient for NH_4^+. However, the pH of the medium was kept above 5.5 thus this could not be a plausible cause. High RZ CO_2 increased foliar NO_3^- concentrations and total root N concentration in lettuce compared to control plants (Chapter 3). Previous studies indicated greater short-term uptake of NO_{3-} under elevated RZ CO_2 but no long-term (15 days) differences in NO_{3-} uptake (He et al., 2010 ; Viktor and Cramer, 2005). Since the lettuce plants here were exposed to 10 days high RZ CO_2, NO_3^- uptake could have been diminished by the time of harvest.

Previous literature regarding RZ CO_2 enrichment did not mention altered sulphur metabolism, however, this analysis revealed five genes encoding sulphur deficiency response-like proteins and downregulation of two genes encoding 5'-adenylylsulfate reductase (APS) 3-like proteins under elevated RZ CO_2. APS reductase catalyzes a key reaction in the plant sulphate assimilation pathway leading to the synthesis of cysteine

and the antioxidant glutathione (Melinda et al., 2005). Moreover, the nutrient analysis performed in Chapter 3 demonstrated lower (15%) sulphur concentrations in lettuce shoots compared to control plants, but no significant treatment differences in roots. Since sulphur is an essential element for plants, it is plausible these changes in gene expression may indicate an incipient S deficiency prior to any significant change in S concentrations *in vivo.*

Elevated RZ CO_2 downregulated gene expression encoding zinc transporters, consistent with the 10% higher foliar zinc concentrations under elevated RZ CO_2 compared to control plants (Chapter 3). Indeed, zinc-transporters 1 and 4 are upregulated in response to a lack of zinc, with the root- and shoot-expressed zinc transporter-4 suggested to transport zinc intracellularly or between plant tissues (Grotz et al., 1998). The bioavailability of Zn in soil solution increases at low pH (Pedler et al., 2004) so it may be possible that after a fast zinc absorption, because changes in the pH of the cells, zinc transporters are doing a regulatory function to not absorb excess zinc.

There are 244 cytochrome P450 genes (and 28 pseudogenes) in the Arabidopsis genome, so it is not surprising to find DEGs of these proteins. They are monooxygenases with extremely diverse reactions, but usually based on activation and heterolytic cleavage of molecular oxygen with insertion of one of its atoms into the substrate and reduction of the other to form water (Bak et al., 2011). Plant P450s participate in a variety of biochemical pathways to produce primary and secondary metabolites such as phenylpropanoids, alkaloids, terpenoids, lipids, cyanogenic glycosides, and glucosinolates, as well as plant hormones (Mizutani, 2012). Under elevated RZ CO_2, 6 genes encoding for cytochrome P450-like proteins from different subfamilies were upregulated while another 3 genes from the same family were downregulated (Table 5.4 and 5.5).Further information on the molecular function of these enzymes is required to understand the functional significance of these changes in gene expression.

One of the most enriched DEG pathways in roots was the FA biosynthesis pathway (Figure 5.4, B). FAs are the main components of plant membrane lipids and seed storage. Plant FA concentrations vary in response to different environmental situations. In plant cells, the first step is considered to be that catalysed by acetyl-CoA carboxylase, which converts acetyl-CoA to malonyl-CoA (Harwood, 1988). Acetyl-CoA is not imported by plastids, therefore must be generated *de novo* in the stroma of plastids (Weaire et al., 1975;

Roughan et al., 1979). Synthesis of FAs requires a carbon source, NADPH and ATP which in photosynthetic tissues can be supplied from photosynthesis. However, in non-photosynthetic tissues such as roots, the synthesis of Acetyl-CoA depends on the import of metabolites from the cytosol. Once biosynthesized, these FAs are incorporated into the glycerolipid synthetic pathways in plastids or endoplasmic reticulum (ER) for assembly into galactoglycerolipids, sulfolipids and phospholipids. FADs introduce double bonds into the hydrocarbon chains of FAs to produce unsaturated fatty acids which play a key role in plant development and acclimatization to environmental stresses. FAD desaturations are oxygen dependent reactions, i.e. the concentration of oxygen in the cytosol affect the FA unsaturation pattern of lipids. They belong to a large gene family that contains conserved histidine regions and are encoded by nuclear genes but affect the desaturation in different subcellular localizations. Microsomal Δ12 desaturases (FAD2) are hydrophobic transmembrane proteins located in ER and primarily inserting a *cis* double bond between the C12 and C13 position of monounsaturated oleic acid (18:1) producing polyunsaturated linoleic-acid (18:2) (Los and Murata, 1998; Shanklin and Cahoon, 1998). FAD2 is the key enzyme accounting for FA biosynthesis in non-photosynthetic tissues such as roots (Miquel and Browse, 1992; Zhang et al., 2012). In the present study, RZ CO_2 upregulated seven genes encoding FAD2-like proteins (Table 5.3). Specifically, Δ12 fatty acids desaturases DES 8.11-like, were predominantly upregulated. Although there is limited information about the function of this desaturase, it seems to convert the double bond in the Δ12 position of linoleic acid esterified to phosphatidylcholine into groups with new functionalities (Fritsche et al., 1999).

Elevated RZ CO_2 also upregulated many genes in the phenylpropanoid biosynthesis pathway, which are a large class of secondary metabolites synthesized from primary metabolites, phenylalanine or tyrosine, through a series of enzymatic reactions. They can be divided into five groups, including flavonoids, monolignols, phenolic acids, stilbenes, and coumarins (Noel et al., 2005). Among the different genes expressed in this pathway, peroxidases seem to be predominantly expressed. They are involved in many physiological and biological processes, including the cross-linking of molecules in the cell wall, auxin oxidation, oxidation of cinnamyl alcohols prior to their polymerisation during lignin and suberin formation, and responses to biotic and abiotic stresses (Boudet, 1998; Siegel, 1993; Kawano, 2003). In plants, there are two major steps to produce lignin: monolignol biosynthesis and monolignol polymerization via free radical coupling. In *Arabidopsis*,

monolignols are synthesized from phenylalanine via the phenylpropanoid pathway and after biosynthesis monolignols are polymerized to produce lignin. It has been suggested that peroxidases and laccases are key enzymes catalysing monolignol polymerization and that there is a close relationship between these enzymes and lignin accumulation in secondary cell walls (Shigeto et al.,2015 ; Berthet et al., 2011). Enhanced air CO_2 concentrations (1000 ppm) increased the expression levels of lignin related genes in celery (*Apium graveolens*) leaves (Liu et al., 2018). Perhaps RZ CO_2 has the same effects in plant roots, or increased lignin biosynthesis could enhance tolerance to the abiotic stress caused by the elevated CO_2 in the rhizosphere.

Finally, elevated RZ CO_2 was expected to alter genes involved in carbon metabolism, yet genes encoding for PEP_C or CA were not expressed differentially between treatments as expected from the previous literature. Surprisingly, four genes encoding ribulose bisphosphate carboxylase small chain (rbcS), chloroplastic-like, one gene encoding glyceraldehyde-3-phosphate dehydrogenase A-chloroplastic and two genes encoding Chlorophyll a-b binding protein of LHCII type 1-like were up-regulated under high RZ CO_2. Also, one gene, encoding a phosphoenolpyruvate carboxykinase (ATP)-like protein, was down-regulated. Although the lower accounted reads on those genes suggest that those changes are not biologically relevant, it is rare to detect expression of genes related to photosynthesis in the roots. However, in hairy roots growing under light, added CO_2 (1-5%) and sugars, adaptation to photoautotrophy occurred with increased chlorophyll formation and Rubisco activity (Flores et al.,1993). The light intensity applied to induce chlorophyll and Rubisco in these roots was between 3-10 W/m^2. In the aeroponic system used in these studies (Chapter 2), the incident light intensity inside the box were the roots were growing, under the lights in the glasshouse, was 1% of the incoming light to the box and ranged between 1 – 6 W/m^2. Thus, it could be possible that under favourable conditions, roots shifted towards photoautotrophy. Another plausible explanation is the appearance of microalgae in the roots, however, taking into account the relative number of root cells versus number of photosynthetic organisms. Moreover, the roots appeared healthy and achlorophyllous. In addition, the raw clean read in roots were just 0.01% of that in the leaves. Even though this is a very small change compared to gene expression in the leaves, it is not clear if the roots could become completely photoautotrophic in long-term experiments.

Our results open the path to future avenues of research, including both *in vitro* and *in planta* studies. We provide candidate genes for studying multiple RZ CO_2 enrichment responses in lettuce that should be further verified with a real-time quantitative PCR. Targeted gene knockout experiments can generate inheritable mutant alleles that will be transmitted to the offspring. The generation of such knockout lines could be a powerful tool for the functional analysis of many genes of interest identified in this work. Our analysis only used studies with lettuce plants that were sensitive to the RZ CO_2 enrichment. Future work could also include analyzing the regulation of DEGs in studies with other plant varieties or other systems that can be affected by elevated RZ CO_2. For example, using a model genetic plant such as Arabidopsis which has been extensively studied, analysis can be repeated to identify candidate regulators of Arabidopsis response to elevated RZ CO_2.

5.5 Conclusions

This genome-wide transcriptional analysis has produced novel data regarding the effects of RZ CO_2 enrichment on gene expression. There were hundreds of DEGs, mainly in the roots. Nevertheless, changes in gene expression do not necessarily mean that the proteins they code are functional, since proteins frequently undergo post-translational modifications that affect their activity. However, this RNA-seq study can give important information about which pathways and pool of genes are most highly affected. Fatty acid biosynthesis, amino acid biosynthesis and carbon metabolism seem to be the major pathways enriched under elevated RZ CO_2. Also, proteins related to cell walls and membranes seems to be changed under elevated RZ CO_2.

Chapter 6. General discussion

Although multiple studies have previously applied RZ CO_2 enrichment in different growing systems (Figure 1.8), results have been variable with growth promotion and growth inhibition both noted. Part of the reason for this variability may be that each study has chosen a single growing system. Consequently, this study chose to work on hydroponic and aeroponic systems that applied different forms of RZ CO_2 enrichment (bicarbonate, gaseous CO_2) based on previous studies (Figure 1.8). These systems were also considered the most suitable to apply either bicarbonate or CO_2 gas since microbial numbers are lower than in soil and therefore the CO_2 concentrations applied will be little affected by microbial respiration (Sirsat & Neal, 2013). Since aerial CO_2 enrichment is widely applied to high value protected horticultural crops (eg. tomato, pepper and lettuce) that are mainly grown all year round in the UK in glasshouses (HDC, 2014), these species were selected for this study. Furthermore, there are concerns that aerial CO_2 enrichment of these crops liberates considerable quantities of CO_2 to the atmosphere when the glasshouses are ventilated, which is necessary to lower humidity to restrict foliar disease development. In addition, the few studies that have examined RZ DIC enrichment of these crops show variable results (Figure 1.8). Thus, it was relevant to the UK horticultural industry to test different environmental conditions (Table 2.3) to try to understand the variability of the results obtained in previous investigations, and potentially evaluate whether RZ CO_2 enrichment was more efficient than the current industry practice of aerial CO_2 enrichment.

Many studies of the physiological effects of bicarbonate have been carried out due to its presence in calcareous soils, limiting crop production in areas with high soil bicarbonate content (Poschenrieder at al., 2018). Plant growth and development largely depend on the combination and concentration of mineral nutrients available in the soil. Deficient or toxic levels of any one of them may decrease plant productivity. Hydroponics is a method of growing plants that provides all nutrients in their inorganic (readily available) form in a liquid solution with or without solid media. Hydroponic systems have been extensively used for exploring nutrient requirements and the toxicity of some elements in *Arabidopsis* and other plant species (Berezin et al., 2012; Kopittke et al., 2009). Here, bicarbonate enrichment at (~ 6.4 pH) of hydroponically-grown lettuce plants increased biomass accumulation at 1 and 5 mM HCO_3^-, while 20 mM reduced biomass accumulation (Figure

2.14). Pepper plants increased biomass accumulation at 1 mM HCO_3^- (Figure 2.15) while tomato did not show any response to bicarbonate enrichment (Figure 2.16). The decrease in growth under high HCO_3^- (>5 mM) agrees with several studies where decreased growth was reported (Alhendawi et al., 1997 ; Al Mansouri et al., 2014; Roosta et al., 2015), with decreased nutrient availability at high pHs (> 8) limiting biomass accumulation (Alhendawi et al., 1997,2004; Yang et al., 1994; Parra Terraza et al., 2012). On the contrary, bicarbonate addition at low concentrations (≤ 5mM) to the nutrient solution and at lower pH levels (<7) enhanced nutrient uptake and thus biomass accumulation in tomato plants (Bialzcyk et al., 1994). Despite increased crop nutrient concentrations in those studies, growing lettuce plants at 1 mM bicarbonate generally had no effect on tissue nutrient concentration and shoot nutrient content, while higher bicarbonate concentrations (>10 mM) decreased nutrient uptake (Figure 3.1). Thus high bicarbonate concentrations limit nutrient uptake and therefore productivity, while increased biomass at 1 mM bicarbonate cannot be attributed to a higher tissue nutrient concentration and content.

Since 1 mM HCO_3^- promoted lettuce growth, it was important to understand whether bicarbonate was absorbed from the roots and in which form it was taken up (Section 2.3.2). As described in Chapter 1, pH determines the reaction direction of carbonates in the solution, with more CO_2 in solution when the pH is lower than 6.4 and higher HCO_{3-} concentrations when the pH exceeds 6.4. When individual plants were placed in flasks with 1 mM $NaH^{13}CO_3$ added into the nutrient solution in the glasshouse, shoot $\delta^{13}C$ values increased from 0 to 4 h after labelling but the greatest increase occurred 8 to 12 h after labelling. This increased rate coincided with the transition between day and night hours. Root $\delta^{13}C$ values increased between 0 to 4 h after labelling and thereafter the values were slightly decreased, suggesting there is bicarbonate transport between the roots and the shoot, meaning that lettuce plants are able to transport C to the shoot. Previous studies have shown the uptake of DIC through the roots using both labelled ^{14}C or ^{13}C in a variety of crops and trees (Viktor and Cramer 2003, Vuorinen, et al. 1992), with incorporation of DIC into organic products in the roots occurring through the activity of phosphoenolpyruvate carboxylase (PEPc). Labelled organic acids are transported by the xylem to the shoots to provide a ready source of CO_2 via decarboxylation in the shoot, and the released CO_2 is re-fixed (via Rubisco) through photosynthesis. However, the small contribution of the root-derived carbon cannot always explain the observed increase in growth (Viktor and Cramer 2003).

To determine whether nutrient solution pH affected bicarbonate uptake, a second experiment placed lettuce plants in three bespoke recirculating hydroponic systems in a CE room. Two systems supplied nutrient solution amended with 1 mM $NaH^{13}CO_3$ and one remained as a control system. The pH of one bicarbonate-enriched system was continuously controlled at pH 5.8 (where CO_2 will be the main form) and the other was allowed to fluctuate naturally over the 24 h after the first pH correction (6.4) when the bicarbonate was added (mimicking the conditions under which earlier experiments were conducted – Figures 2.14-2.16). Since root $\delta^{13}C$ values increased irrespective of nutrient solution pH, it seems that roots absorbed both CO_2 and bicarbonate. However, translocation to the shoot (as indicated by shoot $\delta^{13}C$ values) was greater at fluctuating pH, with bicarbonate being the main form translocated (Figure 2.18). Further analyses of label in xylem sap collected from plants exposed to the different nutrient solution pHs seems warranted, but this would be difficult to achieve in a crop such as lettuce.

Gaseous RZ CO_2 enrichment was investigated in both hydroponics and aeroponics, as previously reported in the literature. RZ CO_2 enrichment (1500-2000 ppm) of lettuce, pepper or tomato grown hydroponically did not promote biomass accumulation (Table 2.5). A much higher RZ CO_2 (5000 ppm) concentration was required to promote biomass accumulation in tomato plants grown hydroponically, but only when an additional stress (eg. 150 mM NaCl salinity) was added (Cramer et al. 1999). However, since these studies aimed to decipher the effects of RZ CO_2 on plant growth without any additional stress, and given the lack of response of RZ CO_2, enrichment of hydroponic solutions with gaseous CO_2 was not further investigated.

In contrast, elevated RZ CO_2 enrichment of aeroponically grown lettuce showed the greatest response among all the systems and all the treatments. Of seven similar experiments performed in both glasshouses and CE rooms, four of them showed that shoot biomass increased by 19-25% under elevated RZ CO_2 compared to control plants (Table 2.7). Similarly, crisphead lettuce plants grown under 2000 ppm RZ CO_2 increased shoot biomass by around 20% (He et al., 2007, 2010). These authors demonstrated that elevated RZ CO_2 increased photosynthesis rates while stomatal conductance decreased. However, leaf gas exchange measurements were inconsistent between days and not significantly different between treatments here (Figure 2.19). Measuring individual leaf gas exchange of crisphead lettuce leaves is challenging because of their irregular surface.

Furthermore, single measurements of two leaves per plant does not represent whole plant gas exchange. Experiments measuring whole-plant gas exchange (Jauregui et al. 2018) could give a better idea of the effects of elevated RZ CO_2.

Furthermore, in three of the experiments carried out in the glasshouse, shoot biomass accumulation showed no response or even a decrease in response to elevated RZ CO_2 (Table 2.7). In trying to decipher the impact that ambient environmental conditions had on each experiment, it was noted that CE room conditions were constant, while glasshouse conditions varied greatly (Figure 2.12, 2.13). Figure 2.20 suggested that high NT might abolish growth promotive effects of high RZ CO_2 in those experiments. However, Frantz et al., (2004) indicated that night-time temperatures (between 20-31 degrees) *per se* did not affect lettuce biomass accumulation. Under elevated RZ CO_2, the oxygen levels in the aeroponic system declined during the day when the irradiance and temperatures are higher (Figure 2.10). Recovery of oxygen levels towards midnight probably allowed a slow recovery of root respiration rates. On the other hand, pepper and tomato grown aeroponically did not show significant differences between treatments (Tables 2.9, 2.10), suggesting that RZ CO_2 effects are species dependent as suggested in previous studies.

Relatively few studies have examined the impact of high RZ CO_2 on plant nutrient status. Applying long-term (60 days) elevated RZ CO_2 (2500 ppm) to aeroponically grown tomatoes decreased root N, P, K, Ca and Mg concentrations (Zhao et al., 2010). Although applying high RZ CO_2 to aeroponically grown pepper did not change leaf nutrient status, leaf Mg and S concentrations of lettuce decreased. The remaining macro- and micro-nutrient concentrations were unaltered in the leaves (Figure 3.2). Although root tissue N concentrations increased under high RZ CO_2, no other changes were observed in root nutrient concentrations (Figure 3.3). Increased root PEP_C activity and decreased leaf Ca- and Mg-ATPase activity was also observed along with lower root Mg concentrations under elevated RZ CO_2 (Zhao et al., 2010, 2015). Lower photosynthetic rates were attributed to root export of malic acid (due to increased PEP_C root activity) inhibiting leaf PEP_C activity and decreased Ca- and Mg-ATPase activity that greatly reduced the photochemical efficiency of PSII. In contrast, relatively short exposure (10 days) of lettuce to RZ CO_2 did not change leaf gas exchange parameters or Mg content (Figure 3.2, Table 3.5). While sulfur deficiency has also been linked with reduced photosynthesis rates (Gilbert et al.,1997), no change in S content occurred. Further measurements of photochemistry

(chlorophyll fluorescence) may provide complementary information to the conventional gas exchange analyses undertaken here.

As nitrogen and carbon metabolism are matched, it is not surprising that many previous studies have focused on nitrogen metabolism under elevated RZ CO_2. Elevated RZ CO_2 was suggested to stimulate short-term NO_3^- uptake (6-8h) while having no long-term (15 days) effect (Cramer et al., 1996). Increased growth rates in NO_3^- fed plants with elevated RZ CO_2 was associated with transfer of root-derived organic acids to the shoot and conversion to carbohydrates (Viktor and Cramer, 2005). Elevated RZ CO_2 increased leaf NO_3^- and total reduced N concentration in aeroponically growth lettuce, which was correlated with increased *A* (He et al., 2010). Elevated RZ CO_2 had no significant effects on leaf total N or NO_{3-} concentration of lettuce plants, although NO_{3-} concentration was higher (~30%) and total N was lower (10%) under elevated RZ CO_2 (Figure 3.3). A significant portion of N transported to shoots is recycled to roots via phloem transport of amino acids (Forde and Clarkson, 1999). Such transport of amino acids from shoots to roots in the phloem could cause feedback inhibition of root growth and NO_3^- assimilation (Marschner, 1986; Imsande and Touraine, 1994; Marschner et al., 1996). Leaf N concentrations decline under prolonged growth at elevated CO_2 (Oren et al., 2001). Photosynthetic acclimation can account for some of this decrease (Long et al., 2004), but fertilization with NH_4NO_3 eliminates it (Crous et al., 2010; Liu et al., 2011), showing that increased N supply can compensate for the effects of elevated CO_2 through enhanced root N uptake and plant N assimilation. This suggests that elevated CO_2 interrupts shoot to root N signalling. A shift from leaf NO_3^- assimilation to root NO_3^- assimilation requires translocation of more carbohydrate to the roots to provide sufficient energy and carbon skeletons for these processes (Zheng, 2009).

RZ CO_2 (6500 ppm) increased NO_{3-} uptake compared with ambient RZ CO_2 (Cramer et al., 1996). Under elevated RZ CO_2 NO_{3-} fed plants allocated greater ^{14}C Incorporation into organic acids and carbohydrates while NH_{4+} fed plants allocated greater label into amino acids (Viktor and Cramer, 2005). These authors suggested that incorporation of root DIC serves an anaplerotic function supplying carbon skeletons for amino and organic acids synthesis. The anaplerotic pathway makes use of inorganic C to build the C4 compounds leading to amino acid and protein synthesis (Jeanneau et al., 2002). Bialczyk et al., (2004) reported changes in amino acids profiles in plants fed with NO_{3-} and under high

bicarbonate concentrations but no studies have highlighted the influence of RZ CO_2 on amino acid profiles. In this study, aeroponically grown lettuce under elevated RZ CO_2 had lower (20%) total amino acids than control plants (Table 3.6), in an experiment where growth was not promoted. Serine constituted 50 % of total amino acids and with proline, alanine, glutamine and glutamic acid, constituted about 98 % of the total amino acids. Foliar serine concentrations were lower and glycine were higher under elevated RZ CO_2. These results, along with lower glutamate and higher ornithine and proline concentrations, might suggest decreased photorespiration and a shift to an alternative pathway of serine biosynthesis (Figure 3.7).

Previous studies suggested that ABA signalling could be involved in stomatal closure caused by elevated RZ CO_2 (Cramer et al.,1999). Thus applying RZ CO_2 (2500 ppm) to aeroponically grown muskmelon decreased xylem sap IAA, tZ and GA_3 concentrations while increasing ABA concentrations compared to control plants (Li et al., 2009), even though tissue concentrations were not measured. Despite these multiple changes in phytohormone signalling, lettuce plants exhibited a single increase in foliar JA concentration (Figure 4.1). On the other hand, elevated RZ CO_2 significantly increased foliar ACC concentrations of pepper plants, while tZ and SA concentrations significantly decreased. Furthermore, root SA and GA_3 concentrations increased whereas iP concentrations significantly decreased. Assessing the physiological significance of these hormonal changes is difficult in the absence of hormone biosynthesis mutants in the relevant species. Nevertheless, an excised leaf-disc assay indicated that CK applications enhanced leaf expansion, while foliar spraying of ACC decreased leaf expansion of intact pepper plants. These responses indicate that phytohormonal changes under elevated RZ CO_2 may hinder leaf expansion (and thus radiation interception and biomass accumulation) of pepper plants. However, phytohormone changes cannot explain the increased biomass of aeroponically grown lettuce plants.

Roots have evolved the ability to sense diverse environmental factors and use this information to drive changes in growth activities. Root growth and architecture are particularly sensitive to abiotic stresses including drought and salinity (Duan et al., 2015), although no studies appear to have been performed under elevated RZ CO_2. Transcriptomic analysis of aeroponically grown lettuce indicated that in roots several DEGs encoding cell wall and membrane proteins such as, expansin-like, PELPK- like proteins and

zinc, nitrate, ammonium and sulphur transporters were differentially expressed between treatments (Table 5.3, 5.4, 5.5). Besides, the most enriched pathway under elevated RZ CO_2 was fatty acid biosynthesis (Figure 5.4), which could be an acclimation response to a changing environment (Dong et al., 2016). In this study, upregulation of root lignin biosynthesis under elevated RZ CO_2 has parallels with enhanced lignin biosynthesis under water deficit (Yoshimura et al., 2007), even if the functional significance is not clear. Decreased root lignification in flooded plants exposed to low soil oxygen levels (Komatsu., et al.,2010) suggests that the low oxygen levels seen in aeroponic culture with elevated RZ CO_2 (Figure 2.10) were insufficient to stimulate expression of lignin biosynthesis genes. Although up-regulation of genes encoding for PEP_C or CA under elevated RZ CO_2 was expected, no DEGs were found. On the contrary and surprisingly, genes encoding for rbSC were up-regulated in the roots probably because environmental conditions inside the box (water, nutrients, elevated CO_2 and an irradiance of 1% of the total incident light) prompted changes consistent with a shift to photoautotrophic conditions.

Since the experiments included in this thesis did not explicitly assess the effects of short-term RZ CO_2 enrichment on crop quality, any future cost-benefit analysis on the horticultural viability of the practice should consider the impacts on crop yields only. However, assessing long-term RZ CO2 enrichment would be also important to decipher whether some of the changes seen in this work would negatively impact crop quality over a cropping cycle. In addition, environmental conditions should be carefully considered when RZ CO_2 is applied, as the great variability seen in different experiments probably affected growth. However, the experimental design aimed to evaluate the effect of RZ CO_2 enrichment on growth of the crop under greenhouse conditions typical of UK horticulture. More extensive growth facilities would have allowed multi-factor experiments to systematically test the interactions of RZ CO_2 enrichment with other environmental variables. In appropriate environmental conditions, data compiled in this thesis demonstrated significant benefits of RZ CO_2 enrichment in lettuce. Whether these yield benefits are commercially attractive depends on upscaling the studies within a commercial-scale facility.

Concluding remarks

- Bicarbonate enrichment of hydroponics enhanced growth of lettuce and pepper by ~10% at low (< 5 mM) HCO_{3^-} concentrations.
- Applying 1500 ppm RZ CO_2 to aeroponically grown lettuce plants stimulated growth by 19-25%, but in some cases higher NT temperatures might hinder biomass accumulation.
- The uptake of DIC through the roots of lettuce plants was demonstrated using $NaH^{13}CO_3$.
- 1 mM HCO_{3^-} did not significantly increase macronutrient and micronutrient concentrations, suggesting that growth promotion was not caused by altered plant nutrition.
- Applying 1500 ppm RZ CO_2 to aeroponically grown lettuce decreased Mg and S concentrations but shoot nutrient content was not altered. Shoot N and P content increased under elevated RZ CO_2.
- Although RZ CO_2 enrichment causes variations in some phytohormone concentrations (ACC, tZ, JA and SA), they do not seem related to the increased growth of lettuce plants. However, the decrease or lack of growth response in pepper plants might be due to higher leaf ACC concentrations.
- The higher number DEGs found in roots compared to leaves indicate an initial RZ CO_2 effects in roots. The greater expression of genes encoding fatty acids biosynthesis and cell wall proteins suggest that root morphology changes to adapt to new environmental conditions. Whether root function is also affected should be the focus of future studies.

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Appendices

Appendix 1

Root-zone CO_2 enrichment increases biomass accumulation in lettuce and pepper grown hydroponically and aeroponically

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Abstract

Enhancing CO_2 levels in commercial glasshouses is a widely used technique to increase productivity, but has high-energy costs and detrimental environmental impacts due to frequent ventilation of the glasshouse (to prevent plant diseases) releasing CO_2 into the atmosphere. Previous studies suggest that root-zone (RZ) CO_2 enrichment may be a more economic and sustainable alternative to aerial CO_2 enrichment. These experiments aimed to compare the effects of RZ CO_2 enrichment by adding either bicarbonate or gaseous CO_2 into hydroponic and aeroponic systems respectively, and to determine the physiological mechanisms by which plants respond to RZ CO_2. Root-zone CO_2 enrichment (1500 ppm) of aeroponically-grown lettuce increased shoot dry weight by around 20% compared to those grown with 400 ppm RZ CO_2. Supplying hydroponically grown plants with different HCO_3^- concentrations, that increased the levels of dissolved inorganic carbon (DIC), increased biomass accumulation of lettuce (10% increase at 1 mM and 5 mM HCO_3^-) and pepper (10% increase at 1 mM HCO_3^-). Plants exposed to 1 mM $NaH^{13}CO_3$ showed a significant increase of foliar δ13C values over time, therefore confirming the uptake of DIC by the roots. The δ13C values of roots increased significantly over time, however higher values at the beginning of $H^{13}CO_3^-$ exposure suggested root-to-shoot transport of DIC. Nutrient solution pH did not affect root carbon uptake, but shoot δ13C values were lower in those plants exposed to lower pH levels (5.8) compared to those exposed to fluctuating pH (between 6.3 and 6.7), suggesting differences in root-to-shoot transport of DIC. Thus, root carbon uptake was independent of the form in which CO_2 was provided (gaseous CO_2 at pH 5.8; HCO_3^- at higher pHs). How this additional carbon promotes plant growth is still unclear. Potential mechanisms of action such as increased rates of photosynthesis, altered amino acid concentrations and changes in phytohormone concentrations will be investigated in future studies.

Keywords: Bicarbonate, root-zone CO_2, hydroponics, aeroponics, plant growth, lettuce, pepper.

INTRODUCTION

Photosynthesis uses light energy to convert CO_2 and water into sugars, which are required for growth and respiration. Biomass accumulation is the difference between the photosynthesis rate and respiration rate. Greenhouse operators often inject extra CO_2 (700-1500 ppm) (Portree 1996) into the aerial environment to increase photosynthesis and dry-matter accumulation. However, when the humidity or the temperature is very high, the greenhouse is vented, and CO_2 is released into the atmosphere, which is economically wasteful and releases a greenhouse gas to the atmosphere.

In most higher plants, leaf stomata are the principal means of gas exchange, including the capture of CO_2. Although some aquatic plants assimilate large amounts of CO_2 from the sediments via roots, terrestrial plants are thought to capture insignificant amounts of CO_2 through their roots. However, the terrestrial plant *Stylites andicola*, which lacks stomata, captures almost all of the CO_2 via its roots (Keeley et al.1984), suggesting that some or perhaps all plants can obtain CO_2 from their roots.

Alteration of root-zone CO_2 concentrations has both positive and negative impacts on plant growth. The effects of altered root-zone (RZ) CO_2 depend on the enrichment system, plant species, pH, air temperature, irradiance, mineral nutrition, abiotic stresses such as high irradiance or salinity, the duration of RZ CO_2 enrichment, CO_2 concentration applied and the RZ CO_2 concentration (Enoch & Olesen 1993). Across 358 experiments, mean biomass increased by 2.9% when elevated RZ CO_2 was applied. Despite this low percentage, some authors have reported 1.8-fold more dry matter and leaf area in tomato plants, when 5.68 mM bicarbonate (HCO_3^-) (0.0025% CO_2) was added to a standard nutrient solution at pH 6.5 (Bialczyk *et al.* 1994). Also, adding 5 mM HCO_3^- to the nutrient solution containing modified nitrogen concentrations at an optimum ratio (NO^{3-} 4: NH^{4+} 1) and at pH 6.8 increased biomass of tomato by about 1.8-fold (Bialczyk *et al.* 2005). Cramer and Richards (1999) found that the biomass of both control and salinized (100 mM NaCl) tomato plants increased when the hydroponic solution was aerated with 5000 ppm CO_2 under high irradiance (1500 µmol m^{-2} s^{-1}) and high air temperatures (37/19°C) at pH 5.8. However, the effect of DIC was 40% greater in non-salinized than in salinized plants. When plants were grown at irradiances less than 1000 µmol m^{-2} s^{-1}, elevated rhizosphere DIC increased growth rates only of control plants grown at high temperatures (35°C) or salinized plants at more moderate temperature (28°C). Two weeks treatment with elevated RZ CO_2 (50000 ppm) in aeroponically grown crisphead type lettuce increased the growth (~1.6 fold) under 36/30°C and irradiance of 650 µmol $m^{-2}s^{-1}$ at pH 6.5 compared to plants aerated with ambient (360 ppm) CO_2 (He *et al*. 2010). Moreover, increasing RZ CO_2 in aeroponically grown lettuce alleviated midday depression of photosynthesis and therefore increased leaf area, shoot and root production (He *et al*. 2007). However, there is little consensus on the mechanisms by which root zone CO_2 concentration affects growth.

The positive effects of increased DIC concentration in the rhizosphere on plant growth can be due to increased DIC incorporation in root cells, enhanced NO_3^- uptake, decreased CO_2 release during root respiration or from changes in shoot gas exchange (Cramer & Richards 1999; Qi *et al.* 1994). However, negative effects also have been reported. Enrichment with 5, 10 and 20 mM HCO_3^- markedly decreased shoot and root dry weight of hydroponically grown barley, sorghum and maize maintained at pH 8 (Alhendawi *et al*. 1997). Aerating semi-hydroponically grown white lupin with 6000ppm RZ CO_2 decreased growth by ~27% compared to control plants grown at 360 ppm CO_2 (Cramer *et al*. 2005). These negative effects were related to decreased root elongation and nutrient uptake and diminished ion transport to aerial organs. However, some of these studies used pH levels as high as 7 or 8 (Alhendawi *et al.* 1997, Wanek *et al.* 2000) where the nutrient availability was likely suboptimal.

Previous studies have shown the uptake of DIC through the roots using both labelled ^{14}C or ^{13}C in a variety of crops and trees (Viktor & Cramer 2003, Vuorinen, *et al.* 1992), with incorporation of DIC into organic products in the roots occurring through the activity of phosphoenolpyruvate carboxylase (PEPc). Labelled organic acids are transported by the xylem to the shoots to provide a ready source of CO_2 via decarboxylation in the shoot, and the released CO_2 is re-fixed (via Rubisco) through photosynthesis. However, the small contribution of the root-derived carbon cannot always explain the observed increase in growth (Viktor & Cramer 2003).

Therefore, the aim of this study was to investigate the effect of DIC enrichment of the RZ on lettuce and pepper plants grown in different systems (deep flow hydroponics and aeroponics) and different environments with both HCO_3^- and optimal gas CO_2 concentration (similar to that applied in the aerial environment of commercial greenhouses). Since nutrient solution pH affects the relative proportions of dissolved CO_2 and HCO_3^- concentrations, the carbon uptake by lettuce roots exposed to different nutrient solution pHs was measured.

MATERIAL AND METHODS

Direct bicarbonate enrichment of hydroponics (Experiment 1)

To determine the effect of bicarbonate enrichment of the rhizosphere, deep flow hydroponics system (DFTS) were built for each crop between November and January 2015. Seeds of pepper (*Capsicum annuum* (L.) "Bellboy F1") and lettuce (*Lactuca sativa* L. var. capitata "Sunstar"), were grown in vermiculite and transferred to the hydroponic systems 23

days post germination, after rinsing the roots in water. Pepper were grown in the glasshouse [25°C/16°C day/night, photosynthetically active radiation (PAR) ~500 µmol m^{-2} s^{-1}] and lettuce in a controlled environment room (CE)[20°C/16°C day/night, PAR ~300 µmol m^{-2} s^{-1}] at Lancaster Environment Centre (Lancaster University, UK).

The DFTS consisted of individual 16 L boxes of 0.17 m height, 0.43 m width and 0.33 m depth. The boxes were completely opaque and contained 14 L of half-strength Hoagland solution (Hoagland & Arnon 1950). The composition of the nutrient solution was 0.5 mM NH_4NO_3, 1.75 mM Ca $(NO_3)_2 \cdot 4H_2O$, 2.01 mM KNO_3, 1.01 mM KH_2PO_4, 0.5 mM $MgSO_4 \cdot 7H_2O$, 1.57 µM $MnSO_4 \cdot 5H_2O$, 11.3 µM H_3BO_3, 0.3 µM $CuSO_4 \cdot 5H_2O$, 0.032 µM $(NH_4)6Mo7O_24 \cdot 4H_2O$, 1.04 µM $ZnSO_4 \cdot 7H_2O$ and 0.25 mM NaFe EDTA. Bicarbonate was applied in the form of $NaHCO_3$ at 0, 1, 5, 10 and 20 mM.

The lids (0.43 x 0.33 m) were modified with four 2.5 cm holes in each quadrant to hold four plants per box (4 x 0.14 m^{-2}). Two boxes were used for each treatment and were completely randomized. In the middle of the lid, an additional hole was cut to accommodate a closed cell foam piece through which an external diameter 6 mm pipe was inserted. The end of the pipe outside the box was connected to an aquarium air pump (All Pond Solution Ltd, Middlesex, UK) which continuously supplied ambient air (Flow rate: 3.2 L min^{-1}) to add O_2 to the nutrient solution as well as stirring it. The medium was changed every 3-4 days and the pH was maintained at 6.4 (at which CO_2 and bicarbonate concentrations are equivalent) by adjusting the pH via dropwise addition of 1N HCl or NaOH once every day.

Carbon uptake of hydroponically grown lettuce plants (Experiment 2)

To investigate whether the plants were taking up the carbon through the roots, two experiments measured changes in ^{13}C content in leaf and root tissue over time. Butterhead lettuce type seedlings (*Lactuca sativa* L. var. capitata "Sunstar") grown in vermiculite were transferred to two different water culture hydroponic systems (non-recirculating (A) and recirculating (B) system) at the 4-leaf stage. The hypocotyls of the plants were inserted through a closed cell foam collar and the nutrient solution in each pot was constantly aerated through a 6 mm pipe connected to an air pump.

In the non-recirculating system (Experiment 2A), ten lettuce plants (*Lactuca sativa* L. var. capitata "Sunstar") were each placed in a 300 mL jar with nutrient solution. After 3 days, 1 mM $NaH^{13}CO_3$ was added to four jars at 08.30. Two plants were harvested at 08.00 (non-enriched controls) and another two plants (control and enriched) were harvested 4, 8, 12 and 24 hours after the $NaH^{13}CO_3$ was added. At harvest, plants were divided into leaves and roots, which were rinsed 3 times in dH_2O to remove any nutrient solution.

Three recirculating hydroponic systems were used (Experiment 2B), each containing 5 lettuce plants, with each one placed in a 300 mL jar:

- Control system with half-strength Hoagland solution with the pH 5.8 manually adjusted at the beginning of the treatment.

4) Labelled ($NaH^{13}CO_3$ addition) system with naturally fluctuating pH.

5) Labelled ($NaH^{13}CO_3$ addition) system with pH constantly controlled at pH 5.8 using a pH automatic controller (pH Kontrol 01, Prosystem Aqua).

$NaH^{13}CO_3$ addition occurred at 08.30, three days after plants were introduced to the systems. Prior to addition of the label at 08.00, three plants, one from each system, were harvested and divided into leaves and roots, which were rinsed 3 times in dH_2O. The same procedure was performed 4, 8, 12 and 24 hours after the $NaH^{13}CO_3$ was added.

Direct gaseous CO_2 enrichment in aeroponic system (Experiment 3)

Four experiments were carried out between January and August 2017, two in a naturally lit (with supplementary lighting when PAR was < 400 µmol m^{-2} s^{-1}) glasshouse and two in an artificially lit controlled environment room (PAR ~300 µmol m^{-2} s^{-1} at bench height).

Crisphead lettuce (*Lactuca sativa* L. var. capitata "nidus jaggeri") and butterhead lettuce (*Lactuca sativa* L. var. capitata "Sunstar") types grown in Grodan rockwool were transferred to two aeroponic systems (Platinum aero pro-8) at the 4-leaf stage. The hypocotyls of the plants were inserted through a collar made with impermeable CO_2 sealant (Qubitac) in the lids of 12 L pots with one plant per pot and 8 plants per system. Nebulisers (flow rate: 12-14 L h^{-1}) misted roots with recirculated half-strength Hoagland's solution coming from a 60 L reservoir.

The pH was monitored every day to have a near-constant pH between 6 – 6.3 by manually adjusting each day with dropwise HCl or NaOH addition.

After transplanting, two different [CO_2], 400 and 1500 ppm, were applied into each bin. The system consisted of an enriched channel supplemented with CO_2 and a non-enriched channel supplied only with compressed air. The air from the enriched channel was completely mixed in a mixing box before entering the aeroponic system. The [CO_2] in the mixing box was monitored continuously using a CO_2 gas analyser (PP Systems, WMA-4). To prevent leakages, the lid was sealed with self-adhesive rubber foam around the rim. The air above the lid and at the shoot base was routinely sampled with a LI-COR 6400, with no significant difference compared to the ambient air.

Plant measurements

After plant removal from the bins, plants were separated into shoot and root tissues. All tissues were dried at 70°C for 4 d and then reweighed. The ^{13}C content is usually determined with a mass spectrometer, which measures the ratio (*R*) between ^{13}C and ^{12}C. All plant material was freeze-dried and ground to a fine powder using a pestle and mortar, which along with the steel spatula, were washed with ethanol before each use to avoid cross-contamination. For all biomass fractions, subsamples (2 mg) were wrapped in foil capsules and combusted at 950°C in an Elementar Vario MICRO elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany). In this process, the carbon in samples is converted entirely to CO_2 and the isotopes analysed on an isotope ratio mass spectrometer (Isoprime 100 IRMS, Isoprime Ltd., Stockport, UK). Standards were Elemental microanalysis wheat flour standard, and two in-house standards calibrated against international standards.

Statistical analysis

To compare the average dry biomass weight between treatments, the statistical software SPSS 21.0 (IBM, USA) was used to perform a Student's t-test at the $P < 0.05$ level.

RESULTS

Root-zone CO_2 enrichment effects on biomass accumulation

Vegetative growth and biomass accumulation of lettuce increased by 10% at 1 mM and 5 mM HCO_3^- whereas in pepper, this increase (of similar magnitude) was only visible at 1 mM HCO_3^- (Figure 1).

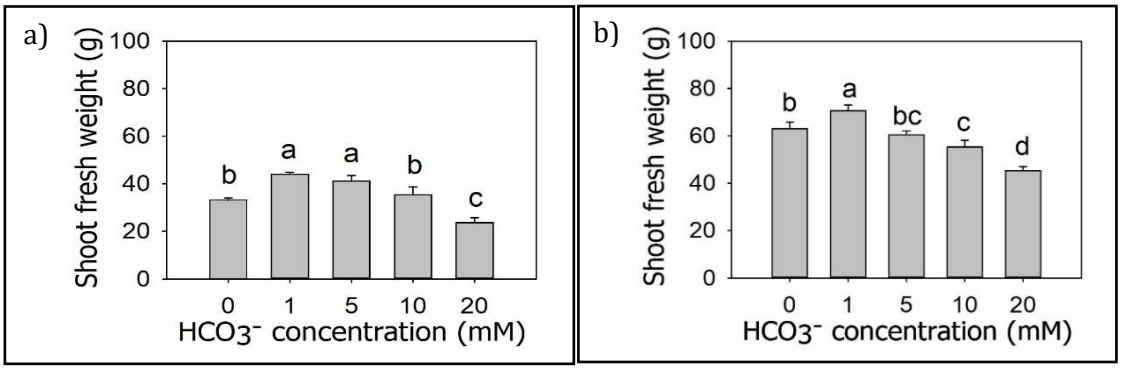

Figure 1. Lettuce (a) and pepper (b) shoot fresh weight after two weeks of growth under different HCO_3^- concentrations. Bars=mean ±SEM (n=8 for lettuce, n=9 for pepper). Different letters indicate significant ($p < 0.05$) differences between treatments. (Experiment 1).

CO_2 enrichment of the RZ significantly increased dry shoot biomass in lettuce by about 20% compared to those grown with 400 ppm root-zone CO_2 cultivated aeroponically, regardless of the variety and location of the experiment (Table 1).

Table 1. Shoot biomass increase (%) of RZ CO_2-enriched, aeroponically grown lettuce in the glasshouse and control environment (CE) room (NS, not significant). (Experiment 3).

Lettuce variety	Location	Increase
Butterhead (Sunstar)	Glasshouse	22% (NS)
Crisphead (Antartica)	Glasshouse	19% ($p<0.05$)
Crisphead (Consul)	CE room	25% ($p<0.05$)
Crisphead (Consul)	CE room	27% ($p<0.01$)

Tissue δ13C

The $\delta^{13}C$ values of roots increased greatly between 0 and 4 hours after addition of bicarbonate, indicating higher DIC uptake at the beginning of the experiment immediately after applying the treatment. In contrast, shoot $\delta^{13}C$ values increased significantly over 12 and 24 hours in bicarbonate-enriched plants. Continued increases in shoot $\delta^{13}C$ values while root $\delta^{13}C$ values stabilised or decreased (between 4 and 24 hours after addition of bicarbonate) suggests DIC transport from the root to the shoot (Figure 2).

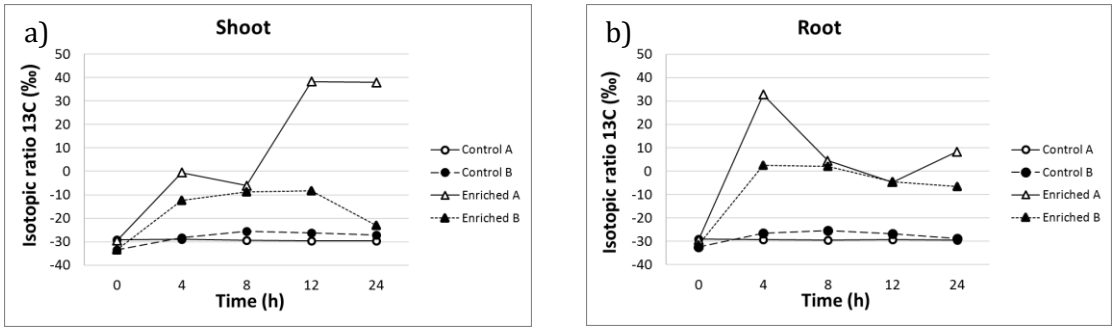

Figure 2. δ13C (‰) for shoots (a) and roots (b) containing 0 or 1 mM $NaH^{13}CO_3$ *versus* time for DIC uptake by lettuce. Points are from individual plants grown in two replicate experiments (Experiments 2A and B).

Root $\delta^{13}C$ values in plants exposed to different solution pHs were similar, indicating DIC incorporation is independent of the form of carbon taken up since the ^{13}C will be in the form of CO_2 at pH 5.8, while at naturally fluctuating pH (between 6.3 and 6.7) the ^{13}C will be in the form of HCO_3^-. Between 4 and 12 hours after HCO_3^- addition, greater ^{13}C translocation from the roots to the shoot occurred when nutrient solution pH was allowed to naturally fluctuate, as indicated by the higher shoot $\delta^{13}C$ values (Figure 3).

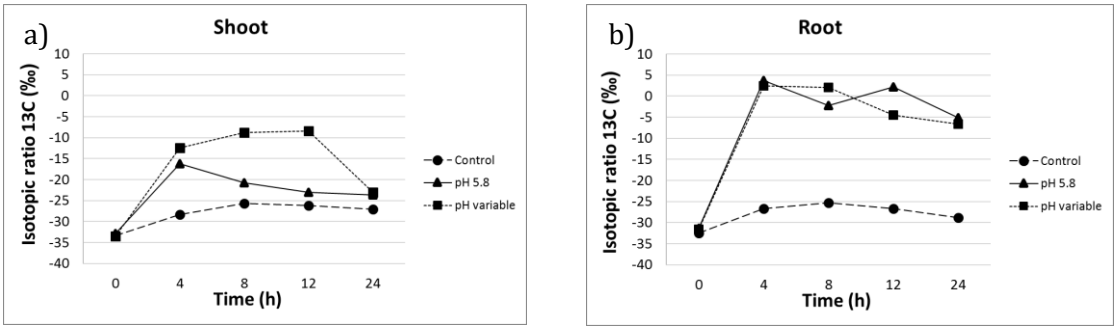

Figure 3. δ13C (‰) for shoots (a) and roots (b) containing 0 or 1 mM $NaH^{13}CO_3$ *versus* time for DIC uptake by lettuce. Each point is from an individual plant (Experiment 2B).

DISCUSSION

Many studies have focused on the impact of increasing atmospheric CO_2 on plant metabolism and physiology, however relatively few studies have considered the impact of rhizosphere CO_2 concentrations. It is almost certain that plant roots are exposed to high CO_2 concentrations in the soil. Moreover, past studies are contradictory since some indicated benefits of enriching the roots with CO_2 (Cramer *et al*. 1999; Van der Merwe *&* Cramer 2000; Viktor & Cramer 2003, 2005; He *et al*. 2007, 2010, 2016), while others showed no significant effect (Cramer *et al*. 2001; Bouma *et al*. 1997) and some even demonstrated negative effects of RZ CO_2 enrichment (Boru *et al*., 2003; Zhao *et al*. 2010; Li *et al*. 2009).

Bicarbonate enrichment of hydroponic solutions (1 mM and 5 mM concentration of HCO_3^-) increased shoot growth of lettuce and pepper plants (Figure 1a & b). Previously, bicarbonate enrichment of hydroponically grown rice (Yang *et al.* 1994) and tomato (Bialczyk *et al.* 1994, 2005) stimulated growth at similar bicarbonate concentrations. With the right proportions of bicarbonate (5 mM) and N (NO^{3-} 4: NH^{4+} 1) concentrations in the nutrient solution, xylem sap concentrations of amides and amino acids increase, thereby supplying carbon skeletons to NH^{4+} incorporation and regulating the activity of some enzymes of ammonium metabolism. Therefore, further work is needed to decipher if nitrogen uptake is the only process promoting the growth of bicarbonate enriched plants.

Comparable previous studies at higher ambient temperatures and PAR (He *et al*. 2007, 2010, 2016) showed that twelve days of applying elevated RZ CO_2 (2000 ppm) to aeroponically grown lettuce increased shoot growth (~18%) compared to plants aerated with ambient CO_2 (360 ppm). In our study, growing plants under elevated RZ CO_2 at (1500 ppm) at irradiance for ten days enhanced shoot growth (Table 1). Although elevated RZ CO_2 significantly increased root dry weight (He *et al.* 2010), no effect was detected in our study (data not shown). Effects of high RZ CO_2 concentrations occurred after few days of treatment: decreased stomatal conductance (g_s) , less water loss, higher midday leaf relative water content (RWC), higher sink capacity (larger root systems enhanced NO_3^- uptake and increased the capacity for utilizing photoassimilate) and higher levels of reduced NO_3^- (He *et al.* 2010). Further studies measuring leaf gas exchange are needed to compare with the conclusions of these previous studies.

The uptake of DIC through the roots has been repeatedly demonstrated (Vuorinen *et al.* 1992; Hibberd *et al.* 2002, Cramer *et al.* 1995, 1999; Bialczyk *et al.* 1992), although its effects on plant responses are not well known. Inorganic carbon absorbed through the roots is converted to organic and amino acids which are exported to the shoots, where they are decarboxylated to augment photosynthesis (Bialczyk *et al.* 1992, 1995; Cramer *et al.* 1995, 1999; Viktor & Cramer 2005). However, since this small contribution (<5%) to the total carbon budget of the plant cannot explain the stimulation of growth (Viktor & Cramer 2003), it is necessary to consider other mechanisms that can promote the plant growth.

Since the isotopic ratio of ^{13}C in the shoot differed when the nutrient solution was at pH 5.8 or as high as 6.7 (Figure 3), and previous studies linked the pH with cell wall extension, it is necessary to determine whether xylem pH variation affects plant growth. Although changes in rhizosphere pH in response to bicarbonate addition may not always change xylem sap pH (due to the buffering capacity of the xylem sap – Gollan *et al.* 1992), it is difficult to reconcile putative xylem sap alkalisation (which should inhibit leaf expansion – Bacon *et al.* 1998) with bicarbonate-induced growth promotion. Thus, it will be necessary to measure a range of growth promoting phytohormones, e.g. (auxins, cytokinins, abscisic acid, gibberellins and ethylene - Davies 2004) in plants exposed to different root-zone CO_2 concentrations, to investigate additional mechanisms of growth regulation.

CONCLUSIONS

Although the experiments with $NaH^{13}CO_3$ demonstrated DIC uptake through the roots, and both CO_2 (aeroponic) and bicarbonate (hydroponic) promoted lettuce growth, the underlying mechanisms are still unclear. Therefore, further studies which measure the concentrations of phytohormones and amino acids (as putative growth regulators) are needed.

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Appendix 2

Appendix 2 Table 1. Constituent chemicals of Hoagland's stock solution ($g.L^{-1}$). Stock solution was made in 1 L preparations for each Solution A, B & C. 500 mL of A and B and 50 mL of C were then added to 100 L of water to make a working solution of Hoagland's.

Source	Stock solution ($g.L^{-1}$)	Element	Final Concentration for half-strength solution (ppm)
A		N	97.9
NH_4NO_3	8	Ca	80.6
$Ca(NO_3)_2 \cdot 4H_2O$	82.6	K	125.3
KNO_3	35.7	P	22.7
B		S	22.7
KNO_3	5	Mg	17.2
KH_2PO_4	27.4	Mn	0.05
$MgSO_4 \cdot 7H_2O$	24.6	B	0.12
$MnSO_4\ 5H_2O$	0.053	Cu	0.02
H_3BO_3	0.14	Mo	0.005
$CuSO_4 \cdot 5H_2O$	0.015	Zn	0.17
$(NH_4)6Mo7O_24 \cdot 4H_2O$	0.008	Fe	0.25
$ZnSO_4 \cdot 7H_2O$	0.06		
C			
NaFe EDTA	36.71		