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

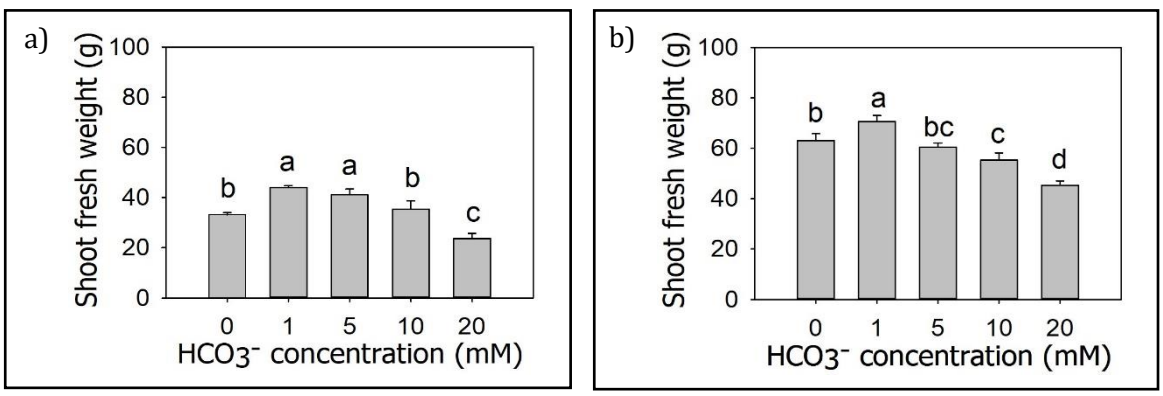

Figure 1. Lettuce (a) and pepper (b) shoot fresh weight after two weeks of growth under different $HCO_3^-$ concentrations. Bars=mean ±SEM (n=8 for lettuce, n=9 for pepper). Different letters indicate significant ($p < 0.05$) differences between treatments. (Experiment 1).

$CO_2$ 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).

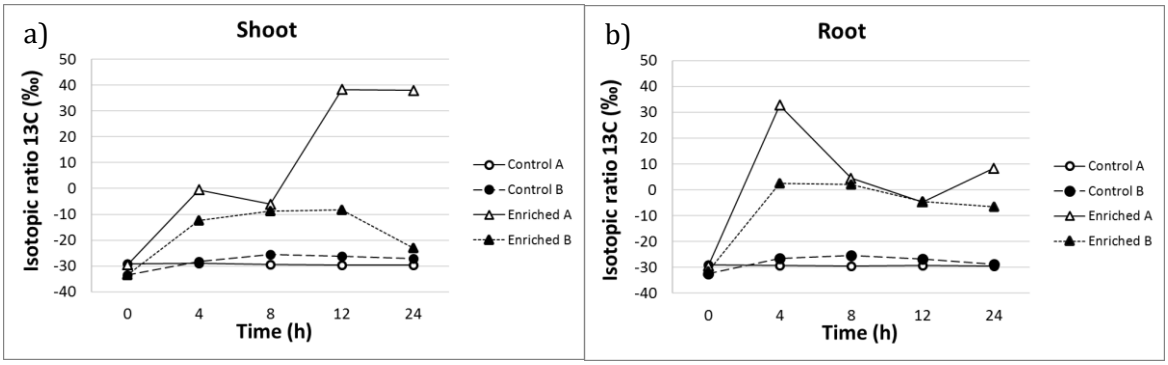


Figure 2. δ13C (‰) for shoots (a) and roots (b) containing 0 or 1 mM $NaH^{13}CO_3$ *versus* time for DIC uptake by lettuce. Points are from individual plants grown in two replicate experiments (Experiments 2A and B).

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).

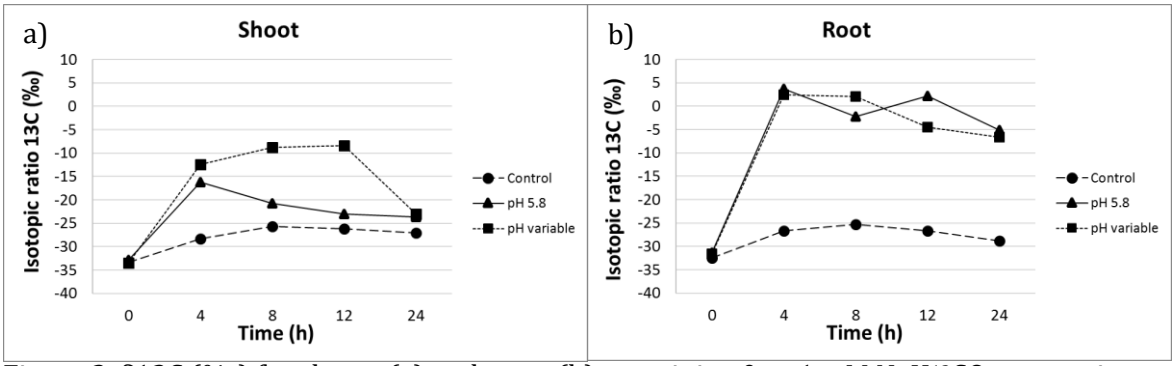


Figure 3. δ13C (‰) for shoots (a) and roots (b) containing 0 or 1 mM $NaH^{13}CO_3$ *versus* time for DIC uptake by lettuce. Each point is from an individual plant (Experiment 2B).

**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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