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# The effect of elevated atmospheric CO<sub>2</sub> concentration and nutrient supply on gas exchange, carbohydrates and foliar phenolic concentration in live oak (*Quercus virginiana* Mill.) seedlings

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**Abstract** – We determined the direct effects of atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) on leaf gas exchange, phenolic and carbohydrate allocation in live oak seedlings (*Quercus virginiana* Mill.) grown at present (370 µmol·mol<sup>-1</sup>) or elevated (520 µmol·mol<sup>-1</sup>) [CO<sub>2</sub>] for 6 months in open-top chambers. Two soil nitrogen (N) treatments (20 and 90 µmol·mol<sup>-1</sup> total N, low N and high N treatments, respectively) were imposed by watering the plants every 5 d with modified water soluble fertilizer. Enhanced rates of leaf-level photosynthesis were maintained in plants subjected to elevated [CO<sub>2</sub>] over the 6-month treatment period in both N treatments. A combination of increased rates of photosynthesis and decreased stomatal conductance was responsible for nearly doubling water use efficiency under elevated [CO<sub>2</sub>]. The sustained increase in photosynthetic rate was accompanied by decreased dark respiration in elevated [CO<sub>2</sub>]. Elevated [CO<sub>2</sub>] led to increased growth rates, while total non-structural carbohydrate (sugars and starch) concentrations were not significantly affected by elevated [CO<sub>2</sub>] treatment. The concentration of phenolic compounds increased significantly under elevated [CO<sub>2</sub>]. (© Inra/Elsevier, Paris.)

**elevated [CO<sub>2</sub>] / gas exchange / nitrogen / phenolics / *Quercus virginiana* / total non-structural carbohydrates**

**Résumé** – Effet d'une concentration atmosphérique élevée en CO<sub>2</sub> et d'un apport nutritionnel sur les échanges gazeux et les concentrations en hydrate de carbone et composés phénoliques foliaires chez de jeunes plants de *Quercus virginiana* Mill. Les effets directs de deux concentrations en CO<sub>2</sub> (370 µmol mol<sup>-1</sup> et 520 µmol mol<sup>-1</sup>) sur les échanges gazeux, les composés phénoliques et l'allocation d'hydrate de carbone ont été étudiés sur des semis de *Quercus virginiana* Mill. Pendant six mois dans des chambres à ciel ouvert. Deux traitements du sol N (20 et 90 µmol·mol<sup>-1</sup> des traitements totaux de N, traitements faibles en azote et traitement fort en azote respectivement) ont été imposés en arrosant les semis tous les cinq jours avec de l'engrais hydrosoluble modifié. Une augmentation de la photosynthèse a été mise en évidence chez les semis soumis à une concentration élevée en CO<sub>2</sub> dans les deux traitements de N. Une combinaison de taux plus élevé de la photosynthèse et de la conductibilité stomatique diminuée étaient responsable du quasi-doublement de l'efficacité d'utilisation de l'eau en CO<sub>2</sub> élevé. L'augmentation soutenue du taux de photosynthèse a été couplée à une diminution de la respiration en CO<sub>2</sub> élevé. Les semis ont utilisé le carbone supplémentaire principalement pour la croissance alors que les concentrations en hydrates de carbone non structuraux totaux (sucres et amidon) n'ont pas été affectées par le traitement élevé de CO<sub>2</sub>. (© Inra/Elsevier, Paris.)

**azote / échange de gaz / enrichissement en dioxyde de carbone / hydrates de carbone non-structuraux total / phénoliques / *Quercus virginiana***

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## 1. Introduction

In response to elevated atmospheric carbon dioxide ( $\text{CO}_2$ ) concentration ( $[\text{CO}_2]$ ), tree species often exhibit increases in carbon (C) assimilation rates [36, 39], instantaneous water use efficiency [25, 40] and growth [5, 53]. Elevated  $[\text{CO}_2]$  may also reduce dark respiration [56]. Total non-structural carbohydrates (TNC) have been generally shown to increase under elevated  $[\text{CO}_2]$ , but it also appears that this is a species-specific response [29, 50]. The magnitude of these responses may be affected by nutrient levels [15, 17].

In most temperate and boreal sites plants are often limited by suboptimal soil nitrogen (N) availability [26]. Under conditions of optimum  $[\text{CO}_2]$  combined with nutrient resource limitation, which restrict growth to a greater extent than photosynthesis, plants tend to show an increase in C/N ratios and an excess of non-structural carbohydrates [6]. This excess may then be available for incorporation into C-based secondary compounds such as phenolics [30]. The C-nutrient balance hypothesis predicts that the availability of excess C at a certain nutrient level leads to the increased production of C-based secondary metabolites and their precursors [46].

$\text{CO}_2$ -enriched atmospheres often induce reduction in the N concentration of plant tissues, which has been attributed to physiological changes in plant N use efficiency [5, 37, 38]. On the other hand, there is increasing evidence that the reduction in tissue N concentrations of high  $\text{CO}_2$ -grown plants is probably a size-dependent phenomenon resulting from accelerated plant growth [11]. It has also been documented that reductions in plant tissue N concentrations may substantially alter plant–herbivore interactions [32]. In fact, insect herbivores consume greater amounts of high  $\text{CO}_2$ -grown foliage apparently to compensate for their reduced N concentration [16]. This may play an important role in seedling survival and competitive ability.

The increase in plant productivity in response to rising  $[\text{CO}_2]$  is largely dictated by photosynthesis, respiration, carbohydrate production and their differential allocation between plant organs and the subsequent incorporation into biomass [22]. For this reason many studies have investigated the effects of elevated  $[\text{CO}_2]$  on plant primary metabolism [14], but relatively few studies have investigated the response of plant secondary metabolite concentrations to increasing  $[\text{CO}_2]$  and its interaction with N availability [31].

The aim of this study was to investigate how  $\text{CO}_2$  availability alters total phenolics, TNC (starch plus sugars) and to determine how elevated  $[\text{CO}_2]$  influences gas exchange of live oak seedlings (*Quercus virginiana* Mill.). Live oak is an important species in dwindling

southeastern United States natural ecosystems, and is able to withstand wind storms and hurricanes because of its deep and strong root system. Extrapolation from studies on seedlings to mature trees should be performed only with extreme caution. However, the seedling stage represents a time characterized by high genetic diversity, great competitive selection and high growth rates [9] and as such may represent one of the most crucial periods in the course of tree establishment and forest regeneration. Indeed, a small increase in relative growth at the early stage of development may result in a large difference in size of individuals in the successive years, thus determining forest community structure [3].

The null hypotheses tested in this study were: that elevated  $[\text{CO}_2]$  would have no effect on gas exchange, phenolics and TNC of live oak seedlings; and that interactions of  $\text{CO}_2$  with soil resource limitations (N) would have no effect on these variables.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Acorns of live oak were collected in late November from three adult (open-pollinated) trees growing in the campus gardens of the University of Florida (29°43' N and 82°12' W; Gainesville, FL, USA). Seeds of each tree were broadcast in individual trays filled with growing medium (mixture of peat, vermiculite, perlite and bark) and moistened regularly. The containers subsequently were placed in a growth chamber (day/night temperature, 25 °C; day/night relative humidity [RH], 80 %; photosynthetic photon flux density [PPFD], 800  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; photoperiod, 16 h). Germination took place at ambient  $[\text{CO}_2]$  level in the containers. Seedlings emerged in all trays after 10 days.

After 2 weeks of growth in the trays, 40 seedlings per family were transplanted into black PVC containers (Deepots®; 25 cm high  $\times$  5.5 cm in averaged internal diameter, 600  $\text{cm}^3$ ) and maintained in the growth chamber. The tubes were filled with a mixture (v/v) of 90 % sand and 10 % peat; a layer of stones was placed in the base of each tube. Seedlings in the growth chamber were watered daily. While plants were growing in the growth chamber, the first stage of growth was supported by adding commercial slow-release Osmocote (18/18/18, N/P/K); the nutrient additions were given in two pulses of 3 g each, applying the first after 1 week of growth in the tubes and the second after 6 weeks. Before moving the seedlings to the open-top chambers, the superficial layer of Osmocote was removed from the tubes and the latter flushed repeatedly for 1 week with deionized water

in order to remove accumulated salts and nutrients. During the 1st month of growth the seedlings were fumigated twice with a commercial fungicide.

Four months after germination (17 March), the seedlings were moved to six open-top chambers. Each chamber received one of two  $\text{CO}_2$  treatments: ambient  $[\text{CO}_2]$  or  $150 \mu\text{mol}\cdot\text{mol}^{-1}$  exceeding ambient  $[\text{CO}_2]$ . Details of the chamber characteristics and the  $\text{CO}_2$  treatment application may be found elsewhere [20, 27]. Overall mean  $[\text{CO}_2]$  was 370 or  $520 \mu\text{mol}\cdot\text{mol}^{-1}$  at present or elevated  $\text{CO}_2$  concentrations (daytime), respectively.

Ten days after transferring the plants to the open-top chambers, two different nutrient solution treatments were initiated and seedlings of each family were randomly assigned to a  $\text{CO}_2 \times$  nutrient solution treatment combination. Thus, the two  $\text{CO}_2$  treatments were replicated three times, with the two nutrient solution treatments replicated twice within each  $\text{CO}_2$  treatment. The seedling containers were assembled in racks and wrapped in aluminum foil to avoid root system heating, and set in trays constantly containing a layer of nutrient solution to avoid desiccation and minimize nutrient loss, thus limiting nutrient disequilibrium [24].

Plants were fertilized every 5 days to saturation with one of the two nutrient solutions obtained by modifying a water-soluble Peters fertilizer (Hydro-Sol<sup>®</sup>, Grace-Sierra Co., Milpitas, CA, USA): complete nutrient solution containing high N ( $90 \mu\text{mol}\cdot\text{mol}^{-1} \text{NH}_4\text{NO}_3$ ), or a nutrient solution with low N ( $20 \mu\text{mol}\cdot\text{mol}^{-1} \text{NH}_4\text{NO}_3$ ). Both nutrient solutions contained [in  $\mu\text{mol}\cdot\text{mol}^{-1}$ ]:  $\text{PO}_4$  (20.6), K (42.2), Ca (37.8), Mg (6),  $\text{SO}_4$  (23.5), Fe (0.6), Mn (0.1), Zn (0.03), Cu (0.03), B (0.1) and Mo (0.02), and were adjusted to pH 5.5; every 5 weeks supplementary Peters (STEM) micronutrient elements ( $0.05 \text{ g}\cdot\text{L}^{-1}$ ) were added. Deionized water was added to saturation every other day in order to prevent salt accumulation. Plant containers were moved frequently in the chambers to avoid positional effects.

## 2.2. Gas exchange

Measurements of stomatal conductance ( $g_s$ ) and C exchange rate ( $CER$ ) were made at the growth  $[\text{CO}_2]$  with a portable gas-analysis system (LI-6200, Li-cor Inc., Lincoln, NE, USA) on mid-canopy fully expanded leaves of the same stage of development of randomly selected plants; each time labeled leaves (two per plant) were measured twice. Measurements were performed on different occasions during the experiment, starting from d 5 of exposure (after plant acclimation to the new environment) to d 178, to investigate the time-course of gas

exchange. Measurements of daytime  $g_s$  and photosynthetic rate ( $A_n$ ) were performed under saturating light conditions (PPFD  $1\ 200\text{--}1\ 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), between 10:00 to 15:00 hours (temperature  $25\text{--}35^\circ\text{C}$ ). Measurements of dark respiration ( $R_d$ ) were performed on d 178 ( $CER$  was measured before sunrise, 04:00–06:00 hours). Intrinsic water use efficiency ( $WUE$ ) was calculated as  $A_n/g_s$ . On several occasions, in order to investigate daily course during sunny days,  $CER$  were monitored from predawn to dusk. Air temperature, RH and PPFD in the leaf cuvette were kept at growth conditions.

Groups of six different plants were selected for harvest (d 7) from each treatment for growth measurements, at the start of  $\text{CO}_2$  and nutrient treatments and continued every 5–7 weeks until September. Harvested plants were analyzed for total phenolic concentration (fresh leaves) and total non-structural carbohydrates after oven drying plant material at  $65^\circ\text{C}$  to constant weight.

## 2.3. Phenolics analysis

Equal-aged leaves (three per plant) were taken for total phenolic compounds analysis. Leaves were treated in liquid N at the field site, then transported to the laboratory and stored in the freezer at  $-20^\circ\text{C}$  until analysis. The leaf blades were punched on either side of the main vein. Five punches ( $0.2 \text{ cm}^2$  each) per leaf were analyzed for phenolics by modifying the insoluble polymer-bonding procedure of Walter and Purcell [55]. Other punches from the remaining leaf blades were used for dry weight (DW) determination, as described earlier. Leaf tissue was homogenized in 5.0 mL of hot 95 % ethanol, blending and boiling for 1–2 min. Homogenates were cooled to room temperature and centrifuged at  $12\ 000 \text{ g}$  for 30 min at  $28^\circ\text{C}$ . Supernatants were decanted and evaporated to dryness in N at  $28^\circ\text{C}$ . Eight milliliter aliquots of the sample in 0.1 M phosphate buffer ( $\text{KH}_2\text{PO}_4$ , pH 6.5) were mixed with 0.2 g of Dowex resin (Sigma Chemical Co., St. Louis, MO, USA) by agitating for 30 min ( $200 \text{ g}$ ,  $28^\circ\text{C}$ ). Dowex, a strong basic anion-exchange resin (200–400 dry mesh, medium porosity, chloride ionic form), was purified before use by washing with 0.1 N NaOH solution, distilled water and 0.1 N HCL and, finally, with distilled water. Absorbance at 323 nm ( $A_{323}$ ) was measured spectrophotometrically both before and after Dowex treatment, representing the absorbance by phenolic compounds. Phenolic concentration ( $\text{mg}\cdot\text{g}^{-1}$  DW) was determined from a standard curve prepared with a series of chlorogenic acid standards treated similarly to the tissue extracts and comparing changes in absorbance measured for the standards and those caused by the treatment.

## 2.4. Carbohydrates analysis

The amount of TNC, including starch and sugars, was carried out using the anthrone method. Previously dried plant materials were separated and ground in a Wiley mill fitted with 20 mesh screen. Approximately 100 mg of finely ground tissue were extracted three times in boiling 80 % ethanol, centrifuged and the supernatant pooled. The pellet was digested at 40 °C for 2 h with amyloglucosidase from *Rhizopus* (Sigma Chemical Co.) and filtered. Soluble sugars and the glucose released from starch were quantified spectrophotometrically following the reaction with anthrone.

## 2.5. Statistical analysis

Three-way analysis of variance (ANOVA) with harvest time, [CO<sub>2</sub>] and N availability as the main effects was conducted for all parameters except for those relative to the last harvest date which were tested by two-way ANOVA. Two- and/or three-way interactions were included in the model.

## 3. Results

### 3.1. Gas exchange

All gas exchange parameters showed variations ( $P < 0.0001$ ) with the course of the growing season and the relative stage of development of the leaves (*figure 1*).

Periodic measurements throughout the growing season indicated a consistent ( $P < 0.0001$ ) pattern of higher photosynthetic rate in leaves grown at higher [CO<sub>2</sub>] (when measured at the growth environment; *figure 1* and *table I*), with the greatest differences occurring by the end of the experiment. Plants grown in low N had lower ( $P < 0.0001$ ) photosynthetic rates when compared with

high N plants (*figure 1* and *table I*). There was no significant interaction between N and CO<sub>2</sub> treatment (*table I*). The effects of N and CO<sub>2</sub> treatment increased over time (*figure 1*) and the interaction between measurement date and N ( $P < 0.001$ ) or CO<sub>2</sub> ( $P < 0.05$ ) treatment was significant (*table I*).

Stomatal conductance, overall, was significantly reduced ( $P < 0.0001$ ) at higher [CO<sub>2</sub>] (*figure 1* and *table I*), although, by the end of experiment, the differences between CO<sub>2</sub> treatments tended to be lower when compared with the other measurement dates. Nutrient availability did not significantly affect stomatal conductance (*figure 1* and *table I*), even if high N plants showed higher values by the end of the experiment.

The increases in photosynthetic rate and decreases in stomatal conductance combined to increase (about doubled,  $P < 0.0001$ ) leaf-level water use efficiency with [CO<sub>2</sub>] at every date measured (*figure 1* and *table I*). Nutrient availability had a significant ( $P < 0.0001$ ) and positive effect on intrinsic water use efficiency (*figure 1* and *table I*) and resulted in a significant ( $P < 0.05$ ) interaction between N and CO<sub>2</sub> treatment (*table I*).

The increase in leaf-level water use efficiency with increasing [CO<sub>2</sub>] was confirmed by examining the slopes of the lines shown in the graph of photosynthetic rate against leaf conductance (*figure 2*). The regressions between CO<sub>2</sub> treatments were significantly different ( $P < 0.001$ ) and showed a lack of acclimation of photosynthetic rate under elevated CO<sub>2</sub> concentration. The effect of N treatment on the regression slope was less evident.

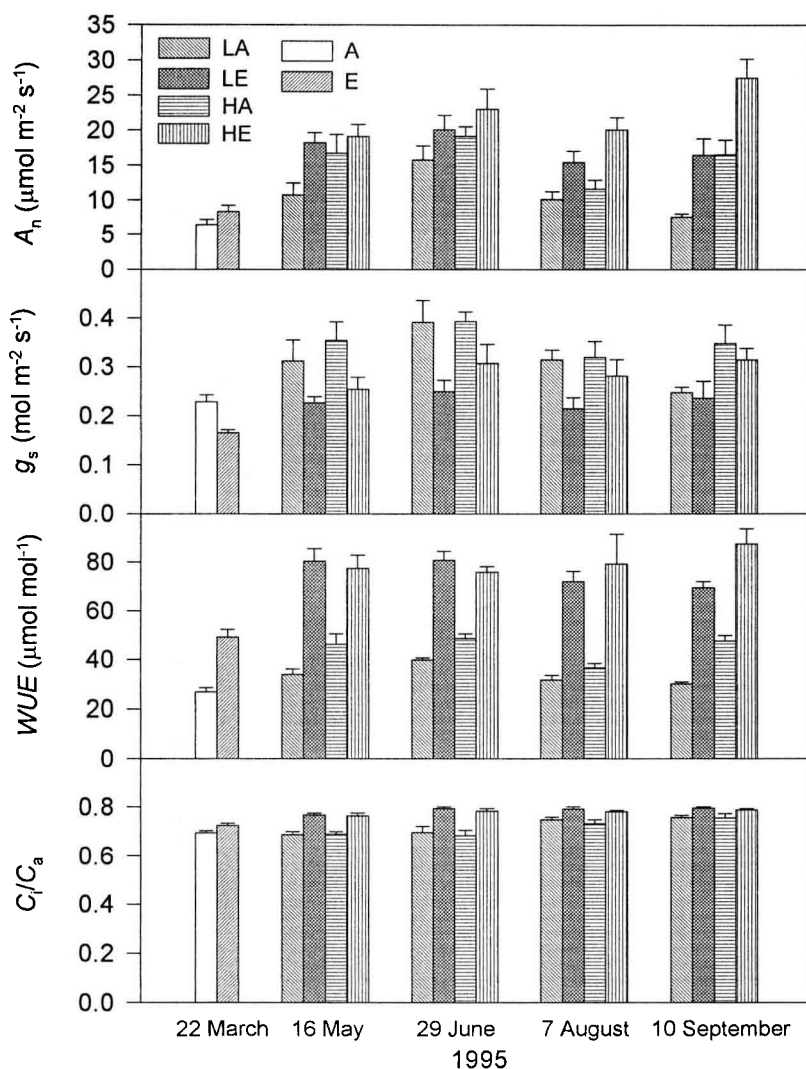
The  $C_i/C_a$  ratio intercellular [CO<sub>2</sub>] to ambient [CO<sub>2</sub>] ratio increased ( $P < 0.0001$ ) in plants grown at higher [CO<sub>2</sub>] (*figure 1* and *table I*). N availability had less effect on the  $C_i/C_a$  ratio (*figure 1* and *table I*).

Diurnal patterns of *CER* confirmed the positive effect of elevated [CO<sub>2</sub>] on photosynthetic rate, over most of

**Table I.** Analysis of variance (ANOVA) results showing the effects ( $P$ -value) of harvest date, [CO<sub>2</sub>] and N availability, and interaction amongst effects on gas exchange and foliar phenolic concentration of live oak seedlings.

Parameter	Date (4) <sup>1</sup>	[CO <sub>2</sub> ] (1)	N (1)	Date × [CO <sub>2</sub> ] (4)	Date × N (4)	[CO <sub>2</sub> ] × N (1)
$A_n$	0.0001	0.0001	0.0001	0.0131	0.0007	0.5370
$g_s$	0.0001	0.0001	0.1678	0.2604	0.0602	0.3187
<i>WUE</i>	0.0001	0.0001	0.0001	0.0099	0.0002	0.0311
$C_i/C_a$	0.0001	0.0001	0.0530	0.0001	0.8073	0.4514
Phenolics	0.0001	0.0001	0.9812	0.4195	0.9789	0.7977

Interaction Date × [CO<sub>2</sub>] × N was never significant. <sup>1</sup> Degrees of freedom.  $A_n$ : photosynthetic rate;  $g_s$ : stomatal conductance; *WUE*: water use efficiency;  $C_i/C_a$ : Intercellular [CO<sub>2</sub>] to ambient [CO<sub>2</sub>] ratio.



**Figure 1.** Gas exchange parameters as a function of plant age (days after start of treatment, 17 March),  $[\text{CO}_2]$ , nutrient treatment. Values are the mean ( $\pm$  standard error) at each date ( $n = 8-12$  plants), two leaves per plant measured twice. Treatments are referred to by symbols in the legend (A: ambient; E: elevated; LA: low N and ambient  $[\text{CO}_2]$ ; LE: low N and elevated  $[\text{CO}_2]$ ; HA: high N and ambient  $[\text{CO}_2]$ ; HE: high N and elevated  $[\text{CO}_2]$ ).  $C_i/C_a$ : intercellular  $[\text{CO}_2]$  to ambient  $[\text{CO}_2]$  ratio;  $WUE$ : water use efficiency;  $g_s$ : stomatal conductance;  $A_n$ : photosynthetic rate.

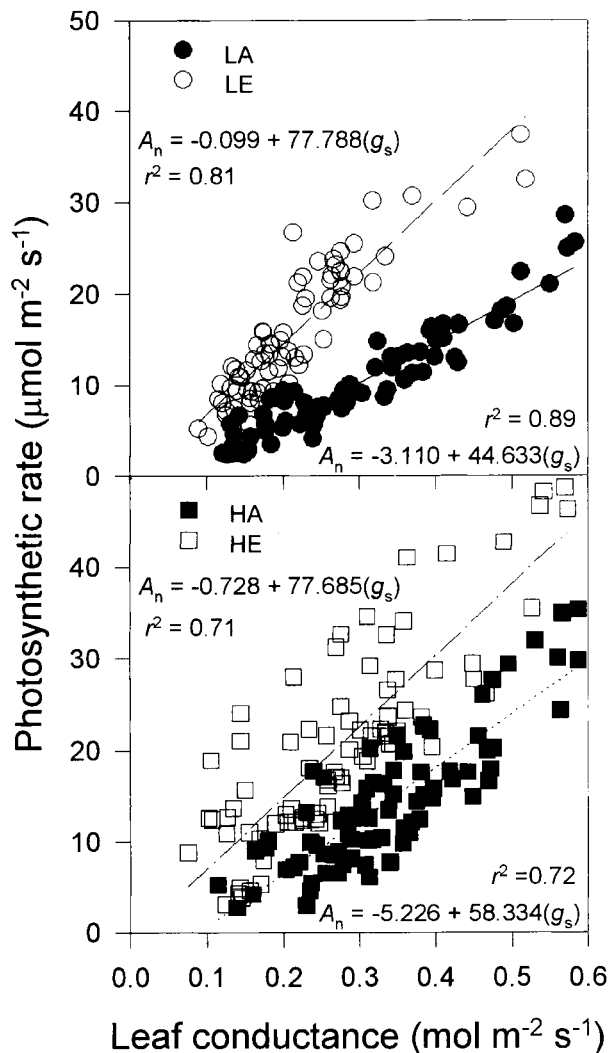
the day (data not shown). Plants grown at elevated  $[\text{CO}_2]$  had lower predawn dark respiration regardless of N availability.

When leaves were stratified as either old (spring leaves) or new (summer leaves) and analyzed as two groups, new leaves had higher ( $P < 0.0001$ ) photosynthetic rates (25–30 %), predawn dark respiration (30–70 %) and stomatal conductance (20–30 %), regardless of N or  $\text{CO}_2$  treatment (table II). Intrinsic water use efficiency was not influenced significantly by age. N availability significantly ( $P < 0.001$ ) affected all parameters but predawn dark respiration. The latter, in particular,

decreased 45 and 62 % in old and new leaves, respectively, in response to increasing  $[\text{CO}_2]$ .

### 3.2. Phenolics

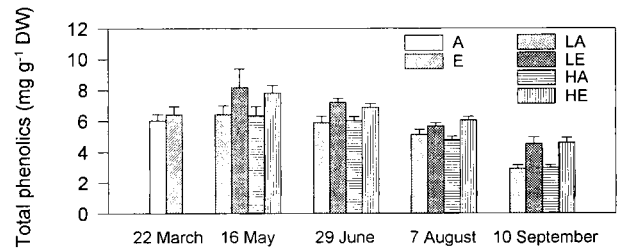
Overall, total phenolic compound concentration was increased significantly ( $P < 0.0001$ ) by elevated  $[\text{CO}_2]$  (figure 3 and table I), although the increment was much more evident by the end of the experiment (35 %) than during the previous harvests. Harvest date, in fact, had clear influences on the phenolic concentration ( $P < 0.0001$ ). N availability did not influence phenolic concentration significantly, and there were no significant interactions.



**Figure 2.** The dependence of net photosynthetic rate ( $A_n$ ) on leaf conductance under saturating light conditions in the open-top chambers. Lines represent results of linear regression (see equations in the figure). Treatments are referred to by symbols in the legend (LA: low N and ambient  $[CO_2]$ ; LE: low N and elevated  $[CO_2]$ ; HA: high N and ambient  $[CO_2]$ ; HE: high N and elevated  $[CO_2]$ ).  $g_s$ : stomatal conductance.

### 3.3. Carbohydrates

Generally, soluble sugars, starch and TNC concentrations were significantly affected by time of harvest (tables III and IV). However, carbohydrate concentration was not significantly affected by both N and  $CO_2$  treatment (tables III and IV). Although the interactions



**Figure 3.** Total foliar phenolic concentration in leaves as a function of plant age (days after start of treatment, 17 March),  $[CO_2]$ , nutrient treatment. Values are the mean ( $\pm$  standard error) at each date ( $n = 6-9$  plants), three leaves per plant. Treatments are referred to by symbols in the legend (LA: low N and ambient  $[CO_2]$ ; LE: low N and elevated  $[CO_2]$ ; HA: high N and ambient  $[CO_2]$ ; HE: high N and elevated  $[CO_2]$ ). DW: dry weight.

between harvest day and  $CO_2$  (and N) treatment were sometimes significant, it is not possible to identify a specific trend. The effect of  $CO_2$  and N treatments on carbohydrate concentration in the tap and fine roots sampled at the end of the experiment was also not significant (table V).

### 4. Discussion

Both atmospheric  $CO_2$  and nutrient supply greatly affected the photosynthetic rate of *Q. virginiana* seedlings. The increase in ambient  $[CO_2]$  elicited a similar increase in photosynthesis in both nutrient treatments [45]. The higher values of net assimilation rate at higher N supply are consistent with those reported in other studies [34, 43]. The effect of elevated  $[CO_2]$  on the photosynthetic rate persisted during the whole study period, despite reductions in N concentration [52]. The relatively low starch content of leaves in all treatments might suggest that there was no limitation to photosynthesis at elevated  $[CO_2]$  imposed by excessive carbohydrate loading. The absence of downward photosynthetic acclimation is similar to the findings of other studies on woody species [2]. No downward trend of photosynthesis was shown through length of exposure, portion of growing season and age of foliage [10, 19]. Declines in response to elevated  $[CO_2]$  have been reported to occur in older foliage [18, 21], late in the growing season [44] and after weeks of exposure to elevated  $[CO_2]$  [12, 48, 54]. Our findings contrast with responses in many experiments with potted plants [14, 47] in which the observed declining response to  $CO_2$  enrichment was attributed to sink limitations, including inadequate rooting volume in pots as well as

**Table II.** Gas exchange characteristics of single leaves selected from live oak seedlings grown in ambient or CO<sub>2</sub>-enriched air and with low or high N availability.

Treatment	$A_n$ ( $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$R_d$ ( $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$g_s$ ( $\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$WUE$ ( $\mu\text{mol}\cdot\text{mol}^{-1}$ )
OLD				
Low N ambient [CO <sub>2</sub> ]	8.96 (0.86)	1.11 (0.26)	0.28 (0.02)	31.89 (1.45)
Low N elevated [CO <sub>2</sub> ]	13.67 (1.26)	0.87 (0.36)	0.19 (0.01)	72.48 (2.63)
High N ambient [CO <sub>2</sub> ]	13.60 (1.23)	1.67 (0.36)	0.32 (0.02)	42.66 (2.33)
High N elevated [CO <sub>2</sub> ]	20.04 (2.02)	0.66 (0.22)	0.26 (0.02)	81.93 (5.59)
NEW				
Low N ambient [CO <sub>2</sub> ]	12.96 (1.22)	3.42 (0.56)	0.35 (0.02)	35.26 (1.31)
Low N elevated [CO <sub>2</sub> ]	19.87 (1.53)	1.39 (0.36)	0.26 (0.02)	75.12 (2.63)
High N ambient [CO <sub>2</sub> ]	17.66 (1.45)	3.28 (0.63)	0.39 (0.02)	45.14 (1.99)
High N elevated [CO <sub>2</sub> ]	27.35 (1.76)	1.14 (0.28)	0.35 (0.02)	83.23 (5.46)
Probability ( <i>P</i> -value)				
N	0.0001	0.9852	0.0001	0.0002
[CO <sub>2</sub> ]	0.0001	0.0001	0.0001	0.0001
Age	0.0001	0.0001	0.0001	0.3441

Data are means ( $\pm$  standard error) of 23–28 samples (OLD: spring leaves and NEW: summer leaves) measured at the end of August. The probability level (*P*) (ANOVA) is also reported (interaction between N, [CO<sub>2</sub>] and age was never significant except Age  $\times$  [CO<sub>2</sub>] for  $R_d$ ,  $P = 0.0123$ ).  $A_n$ : photosynthetic rate;  $R_d$ : dark respiration;  $g_s$ : stomatal conductance;  $WUE$ : water use efficiency.

**Table III.** Analysis of variance (ANOVA) results showing the effects (*P*-value) of harvest date, [CO<sub>2</sub>] and N availability, and interaction amongst effects on sugar, starch and total non-structural carbohydrate (TNC) concentrations in foliage, stem and roots of live oak seedlings.

Parameter	Organ	Date (4) <sup>1</sup>	[CO <sub>2</sub> ] (1)	N (1)	Date $\times$ [CO <sub>2</sub> ] (4)	Day $\times$ N (4)	[CO <sub>2</sub> ] $\times$ N (1)	Date $\times$ [CO <sub>2</sub> ] $\times$ N (4)
Sugars	Foliage	0.0003	0.3864	0.4950	0.1831	0.4138	0.2904	0.0417
	Stem	0.0002	0.7504	0.2298	0.0022	0.5712	0.2739	0.2193
	Roots	0.0451	0.9565	0.1118	0.4175	0.9890	0.2159	0.3348
Starch	Foliage	0.0001	0.4304	0.4518	0.5069	0.7394	0.2566	0.1012
	Stem	0.2243	0.6528	0.3699	0.9361	0.4936	0.6757	0.8173
	Roots	0.0001	0.2046	0.3933	0.0636	0.0728	0.2834	0.0071
TNC	Foliage	0.0004	0.3359	0.5503	0.2242	0.3848	0.2314	0.0278
	Stem	0.0002	0.9823	0.1464	0.0101	0.6018	0.2657	0.5416
	Roots	0.0001	0.2547	0.0456	0.0136	0.1720	0.0587	0.0337

<sup>1</sup> Degrees of freedom.

changing developmental sink strength. Samuelson and Seiler [49] found that seedlings of *Abies fraseri* growing in 1 000 cm<sup>3</sup> pots showed no depression in net photosynthesis after 12 months of exposure to elevated [CO<sub>2</sub>] while in 172 cm<sup>3</sup> pots photosynthetic acclimation was evident after 5 months. *Q. virginiana* seedlings grew in 600 cm<sup>3</sup> pots for about 6 months. However, the large tap-root, characteristic of seedlings of this species,

showed a positive response to elevated [CO<sub>2</sub>] and this might constitute an adequate sink for additional C. CO<sub>2</sub> stimulated growth of all plant compartments of *Q. virginiana* seedlings (the accumulation of total biomass increased 30–40 % by the end of the experiment) [52]. Greater C assimilation in response to CO<sub>2</sub> often stimulates new sinks for C [23]. The diurnal measurements of photosynthetic rate confirmed that, on a daily basis, an



**Table IV.** Sugar ( $\text{mg}\cdot\text{g}^{-1}$  dry weight [DW]), starch ( $\text{mg glu eq}\cdot\text{g}^{-1}$  DW, mg of glucose equivalent) and total non-structural carbohydrate (TNC) concentrations in foliage, stem and roots of live oak seedlings grown in ambient or  $\text{CO}_2$ -enriched air and with low or high N availability.

Parameter	Organ	22 March <sup>1</sup>		LA	29 June			LA	10 September		
		A	E		LE	HA	HE		LE	HA	HE
Sugars	foliage	53.7	58.2	72.6	74.0	73.7	90.6	71.6	69.8	75.1	69.2
		(3.7)	(1.4)	(4.4)	(3.9)	(8.3)	(5.9)	(4.2)	(3.2)	(2.2)	(2.2)
	stem	43.7	36.5	44.6	53.1	46.5	61.0	37.8	36.0	49.6	37.3
		(1.4)	(2.9)	(1.9)	(2.6)	(3.2)	(2.7)	(2.9)	(0.8)	(7.6)	(1.6)
	roots	37.8	42.6	45.7	48.0	57.3	46.0	47.0	46.2	50.9	52.0
		(1.4)	(3.2)	(3.5)	(3.4)	(5.1)	(3.2)	(2.1)	(3.2)	(3.9)	(2.9)
Starch	foliage	2.8	2.8	1.9	2.1	1.9	1.9	4.6	5.0	4.0	5.5
		(0.6)	(0.7)	(0.2)	(0.2)	(0.2)	(0.1)	(0.7)	(0.6)	(0.9)	(0.4)
	stem	2.8	4.1	3.0	6.2	9.1	8.1	3.9	4.0	3.9	3.9
		(0.0)	(0.4)	(0.5)	(3.9)	(3.9)	(3.2)	(0.5)	(0.3)	(0.7)	(0.3)
	roots	42.3	60.7	28.1	15.8	15.0	19.9	16.9	17.2	15.7	14.9
		(4.9)	(3.5)	(7.0)	(2.4)	(3.9)	(3.9)	(1.5)	(1.6)	(2.2)	(1.4)
TNC	foliage	56.5	61.0	74.5	76.1	75.6	92.5	76.1	74.7	79.1	74.7
		(4.3)	(0.7)	(3.9)	(4.1)	(8.3)	(5.9)	(3.9)	(2.8)	(2.7)	(2.4)
	stem	46.5	40.6	47.5	59.3	55.6	69.1	41.7	40.0	53.5	41.2
		(0.9)	(3.2)	(2.3)	(4.9)	(5.6)	(3.5)	(3.0)	(0.9)	(7.7)	(1.6)
	roots	83.1	103.3	73.8	63.8	72.4	65.9	61.8	65.1	62.8	67.4
		(2.1)	(4.2)	(5.7)	(3.8)	(5.9)	(4.3)	(0.9)	(1.0)	(6.2)	(2.4)

<sup>1</sup> On first harvest date N treatment was not applied yet. Data are means ( $\pm$  standard error) of six samples. A: ambient  $[\text{CO}_2]$ ; E: elevated  $[\text{CO}_2]$ ; LA: low N and ambient  $[\text{CO}_2]$ ; LE: low N and elevated  $[\text{CO}_2]$ ; HA: high N and ambient  $[\text{CO}_2]$ ; HE: high N and elevated  $[\text{CO}_2]$ .

**Table V.** Sugar ( $\text{mg}\cdot\text{g}^{-1}$  dry weight [DW]), starch ( $\text{mg}\cdot\text{glu}\cdot\text{eq}\cdot\text{g}^{-1}$  DW) and total non-structural carbohydrate (TNC) concentrations in tap- and fine-roots of live oak seedlings grown in ambient or  $\text{CO}_2$ -enriched air and with low or high N availability.

Treatment	Sugars	Starch	TNC
		Tap-root	
Low N ambient $[\text{CO}_2]$	42.3 (2.1)	26.4 (2.0)	68.7 (2.5)
Low N elevated $[\text{CO}_2]$	41.0 (3.6)	26.0 (1.8)	67.0 (3.2)
High N ambient $[\text{CO}_2]$	45.1 (2.7)	25.1 (2.9)	70.2 (4.7)
High N elevated $[\text{CO}_2]$	44.8 (2.7)	21.2 (2.1)	66.0 (3.1)
		Fine-roots	
Low N ambient $[\text{CO}_2]$	51.8 (2.6)	7.4 (0.9)	59.2 (1.9)
Low N elevated $[\text{CO}_2]$	51.4 (2.4)	8.3 (1.7)	59.8 (1.6)
High N ambient $[\text{CO}_2]$	56.6 (5.3)	6.3 (1.4)	62.9 (5.9)
High N elevated $[\text{CO}_2]$	59.1 (2.8)	8.5 (0.8)	67.6 (3.3)

Data are means ( $\pm$  standard error) of six samples collected at the end of the experiment (September). Nitrogen and  $[\text{CO}_2]$  effects and interaction  $\text{N} \times [\text{CO}_2]$  were never significant (ANOVA).

increase in C gain was maintained in elevated  $[\text{CO}_2]$  [51]. There was also no indication from the pattern of the photosynthetic rate over the course of the day that there

was an accumulation of carbohydrates in the afternoon in elevated  $[\text{CO}_2]$  causing temporary feedback inhibition.

Stomatal conductance of *Q. virginiana* generally decreased with  $\text{CO}_2$  enrichment in both N treatments (less evidently at the end of the experiment). Nutrient availability did not affect stomatal conductance except for the last harvest day. Stomatal response to  $\text{CO}_2$  is a common phenomenon and stomatal conductance in many plants decreases in response to increasing atmospheric  $[\text{CO}_2]$  [2, 9, 14], despite several documented exceptions [8, 19, 33]. At elevated  $[\text{CO}_2]$ , intercellular  $[\text{CO}_2]$  should rise if stomata close consistently, consequently leading to an increase in assimilation rate. Indeed, in *Q. virginiana* seedlings the ratio of intercellular to atmospheric  $[\text{CO}_2]$  increased up to 14 % at elevated atmospheric  $[\text{CO}_2]$ .

As a result of increased assimilation rate and decreased stomatal conductance, water use efficiency of leaves increased strongly at elevated  $[\text{CO}_2]$  in *Q. virginiana* seedlings. This increase is a common response to elevated  $[\text{CO}_2]$  [13, 14, 35]. A significant interaction between nutrient supply and  $[\text{CO}_2]$  led to a higher proportional increase in water use efficiency in seedlings grown in elevated  $[\text{CO}_2]$  with a low nutrient supply [45].

The effect of nutrient supply and CO<sub>2</sub> treatment on assimilation rate and stomatal conductance did not change when spring and summer leaves of *Q. virginiana* were compared, despite a large effect of leaf age (the latter not evident for water use efficiency). This finding may support the hypothesis of a lack of acclimation of gas exchange at elevated [CO<sub>2</sub>]. Dark respiration as measured on spring (maintenance respiration only) and summer leaves at the end of the experiment was significantly reduced by [CO<sub>2</sub>] but not affected by nutrient supply. Dark respiration was affected by age, and the interaction between CO<sub>2</sub> treatment and nutrient supply was significant, resulting in a larger reduction due to CO<sub>2</sub> treatment in summer leaves (recently expanded) in which the growth respiration component should be still important. Direct (short-term) and indirect (long-term) inhibition of respiration by CO<sub>2</sub> is a common, although not universal phenomenon [1, 7]. Lower leaf N, and presumably protein, was observed in *Q. virginiana* seedlings [52] and, therefore, it is possible that the amount of energy needed for leaf construction may be reduced in elevated [CO<sub>2</sub>] relative to ambient [CO<sub>2</sub>] [56]. However, reduced leaf N concentration in plants grown at elevated [CO<sub>2</sub>] does not necessarily indicate parallel differences in construction costs [57].

*Q. virginiana* seedlings were using photosynthates mainly for growth [52] and thus non-structural carbohydrates (sugars and starch) did not accumulate in any plant compartment. Soluble sugars and starch concentrations in stem and roots have already been found not to increase in other experiments [4, 28]. In contrast with our findings, starch and total non-structural carbohydrate accumulation in foliage (and other compartments) of plants grown at elevated [CO<sub>2</sub>] is a much more common phenomenon [2, 42, 43], although it has been reported to be a strong species-specific response [29, 50]. We sampled the plant material in the afternoon and Wullschlegel et al. [56] found no large differences between ambient [CO<sub>2</sub>] and ambient + 150 µmol·mol<sup>-1</sup> [CO<sub>2</sub>] (a similar CO<sub>2</sub> treatment to that used in our experiment) in starch and sucrose of leaves of yellow poplar and white oak seedlings collected in the evening.

The response of foliar phenolic concentration to CO<sub>2</sub> enrichment has been found to be variable [28, 31, 41]. In our experiment the CO<sub>2</sub> effect on increasing phenolic concentration took place without a parallel increase in total non-structural carbohydrates at elevated [CO<sub>2</sub>] that otherwise would have presumably diluted phenolics. An increase in the C/N ratios, which also occurred in our plant material [52], due to a decrease in N content in seedlings grown under elevated [CO<sub>2</sub>], is in accordance with increases in C-based compounds [32]. The increased foliar phenolic concentration in conjunction

with increased C/N ratios may alter the performance of herbivores of *Q. virginiana* in the regeneration phase, in view of projected increases in atmospheric [CO<sub>2</sub>]. Foliar phenolics decreased following leaf maturation [28]. Nutrient treatment did not affect phenolic concentration. This is in contrast with the C-nutrient balance hypothesis [6], which predicts that plants adjust physiologically to low nutrient availability by reducing growth rate and showing a high concentration of secondary metabolites. Nevertheless, several different responses to CO<sub>2</sub> enrichment reported in the literature and nutrient availability effects on C-based secondary compounds are in apparent contradiction with the C-nutrient balance hypothesis [28]. It is possible that when growth is suppressed under insufficient N supply conditions for new tissue formation, recycling of the enzymatic N required for secondary metabolism may occur, making increased phenolic accumulation possible [28]. The lack of response found in the present study can be attributed to the low N treatment not being sufficiently growth limiting [52].

Results from this study suggest that the establishment and growth of *Q. virginiana* on sites with poor nutrition will benefit substantially from elevated [CO<sub>2</sub>] as a result of more C gain. The sustained increase in photosynthetic rate, coupled with decreased dark respiration in elevated [CO<sub>2</sub>], provides the potential for increased C acquisition by the whole crown. Raised [CO<sub>2</sub>] may have a real impact on the defensive chemistry of *Q. virginiana* seedlings.

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