Ann. For. Sci. 56 (1999) 549–561

Original article

Responses to elevated atmospheric CO_2 concentration and nitrogen supply of *Quercus ilex* L. seedlings from a coppice stand growing at a natural CO_2 spring

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(Received 15 September 1998; accepted 1 March 1999)

Abstract – *Quercus ilex* acorns were collected from a population of trees with a lifetime exposure to elevated atmospheric CO_2 concentration (CO_2), and after germination seedlings were exposed at two [CO_2] (370 or 520 μmol mol^{-1}) in combination with two soil N treatments (20 and 90 μmol mol^{-1} total N) in open-top chambers for 6 months. Increasing [CO_2] stimulated photosynthesis and leaf dark respiration regardless of N treatment. The increase in photosynthesis and leaf dark respiration was associated with a moderate reduction in stomatal conductance, resulting in enhanced instantaneous transpiration efficiency in leaves of seedlings in CO_2-enriched air. Elevated [CO_2] increased biomass production only in the high-N treatment. Fine root/foliage mass ratio decreased with high-N treatment and increased with CO_2 enrichment. There was evidence of a preferential shift of biomass to below-ground tissue at a low level of nutrient addition. Specific leaf area (SLA) and leaf area ratio (LAR) decreased significantly in leaves of seedlings grown in elevated [CO_2] irrespective of N treatment. Leaf N concentration decreased significantly in elevated [CO_2] irrespective of N treatment. As a result of patterns of N and carbon concentrations, C/N ratio generally increased with elevated [CO_2] treatment and decreased with high nutrient supply. Afternoon starch concentrations in leaves did not increase significantly with increasing [CO_2], as was the case for morning starch concentrations at low-N supply. Starch concentrations in leaves, stem and roots increased with elevated [CO_2] and decreased with nutrient addition. The concentration of sugars was not significantly affected by either CO_2 or N treatments. Total foliar phenolic concentrations decreased in seedlings grown in elevated [CO_2] irrespective of N treatment, while nutrient supply had less of an effect. We conclude that available soil N will be a major controlling resource for the establishment and growth of *Q. ilex* in rising [CO_2] conditions. © 1999 Éditions scientifiques et médicales Elsevier SAS.

carbon physiology / elevated [CO_2] / natural CO_2 springs / nitrogen / *Quercus ilex*

Résumé – Réponses de jeunes plants de *Quercus ilex* L. issus d'une population poussant dans une zone naturellement enrichie en CO_2, à une concentration élevée de CO_2 dans l'air et à un apport d'azote. Des jeunes plants de *Quercus ilex* L., issus d'une population d'arbres ayant poussé dans une concentration élevée de CO_2, ont été exposés à deux concentrations en CO_2 (370 μmol mol^{-1} ou 520 μmol mol^{-1}) en combinaison avec deux fertilisations du sol en azote (20 et 90 μmol mol^{-1} N total) dans des chambres à ciel ouvert pendant six mois. L'augmentation de concentration en CO_2 stimule la photosynthèse et la respiration nocturne des feuilles indépendamment du traitement en azote. Les augmentations de photosynthèse et de la respiration nocturne des feuilles ont été associées à une réduction modérée de conductance stomatique, ayant pour résultat d'augmenter l'efficience transpiratoire instantanée des feuilles des jeunes plants cultivés en CO_2 élevé. L'augmentation de concentration du CO_2 accroît la production de biomasse seulement dans le traitement élevé en azote. Le rapport des racines fines à la masse de feuillage a diminué avec le traite-

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ment en azote et a augmenté avec l'enrichissement en CO_2 .La surface spécifique de feuille (SLA) et les taux de la surface de feuille (LAR) ont diminé de manière significative pour les feuilles des jeunes plants développés sous une concentration élevée de CO_2, indépendamment du traitement en azote. La concentration en azote des feuilles a diminué de manière significative dans le traitement élevé en CO_2, indépendamment du traitement en azote. En raison des configurations des concentrations d'azote et de carbone, le taux C/N a augmenté avec le traitement élevé en CO_2 et diminué avec l'apport d'azote. Dans l'après-midi, les concentrations en amidon des feuilles n'ont pas augmenté de manière significative avec l'augmentation du CO_2, comme pour les concentrations en amidon dans le cas du traitement limité en azote du matin. Les concentrations en amidon dans les feuilles, la tige et les racines ont augmenté dans le cas du traitement avec une concentration élevée en CO_2 et diminué avec l'apport en azote. Les concentrations en sucre n'ont pas été affectées sensiblement par les traitements de CO_2 ou de N. Les concentrations phénoliques foliaires totales ont diminué pour les jeunes plants qui ont poussé dans le traitement en CO_2 élevé, indépendamment du traitement en N. Nous concluons que la disponibilité en azote dans le sol jouera un rôle majeur dans l'établissement et la croissance de *Q. ilex* dans un environnement caractérisé par un accroissement de la concentration en CO_2 dans l'air. © 1999 Éditions scientifiques et médicales Elsevier SAS.

azote / CO_2 élevé / physiologie du carbone / *Quercus ilex* / sources naturelles de de CO_2

1. Introduction

Atmospheric carbon dioxide concentration [CO_2] is currently increasing at a rate of about 1.5 μmol mol^{-1} annually [52], as a result of increasing fossil fuel consumption and deforestation. Moreover, models of future global change are in general agreement predicting levels reaching 600–800 μmol mol^{-1} by the end of next century from present levels ranging from 340 to 360 μmol mol^{-1} [12].

CO_2-enriched atmospheres have been shown to increase photosynthetic carbon gain, the growth of plants and concentrations of total non-structural carbohydrates, although there is evidence of species-specific responses (see reviews [1, 2, 7, 16, 42]). The impact of increased [CO_2] on plant growth is modified by the nutrient level: growth enhancement in elevated [CO_2] has often been shown to decline under nutrient stress. Indeed, enhanced growth may increase plant nutrient requirement, but many Mediterranean sites are considered to have low nitrogen (N) availability. On the other hand, it has been proposed that plants adjust physiologically to low nutrient availability by reducing growth rate and showing a high concentration of secondary metabolites [5]. The carbon–nutrient balance hypothesis predicts that the availability of excess carbon at a certain nutrient level leads to the increased production of carbon-based secondary metabolites and their precursors [39]. For instance, the often observed increase in C/N ratio under elevated [CO_2] has led some authors to suggest that [CO_2] increases might produce changes in the concentration of carbon-based secondary compounds [29], thus affecting plant–herbivore interactions. Changes in N availability may also alter per se the concentrations of carbon-based secondary chemicals [18].

A major effect of CO_2-enriched atmospheres is the reduction in the N concentration of plant tissues, which has been attributed to physiological changes in plant N use efficiency (e.g. [4, 31]). On the other hand, there is increasing evidence that reductions in tissue N concentrations of elevated CO_2-grown plants is probably a size-dependent phenomenon resulting from accelerated plant growth [10, 46]. It has also been documented that reductions in plant tissue N concentrations under elevated [CO_2] may substantially alter plant–herbivore interactions [30] as well as litter decomposition [13]. In fact, insect herbivores consume greater amounts of elevated CO_2-grown foliage apparently to compensate for their reduced N concentration; again, litter decomposition rates may be slower in elevated [CO_2] environments because of the altered balance between N concentrations and fiber contents.

Quercus ilex L. is the keystone species in the Mediterranean environment. *Q. ilex* forests, once dominant, have shrunk as a result of fires and exploitation for firewood and timber over thousands of years. The ability of *Q. ilex* to compete at the ecosystem level as [CO_2] continues to increase is of concern. While many studies have looked at seedling response to elevated [CO_2], nothing is known of progeny of trees growing for long term in a CO_2-enriched atmosphere. Extrapolation from studies on seedlings growing in elevated [CO_2] to mature trees should be made only with extreme caution. However, the seedling stage represents a time characterized by high genetic diversity, great competitive selection and high growth rates [7] 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].

As the increase in plant productivity in response to rising [CO_2] is largely dictated by photosynthesis, respiration, carbohydrate production and the subsequent

incorporation of the latter into biomass [24], the objectives of this study were i) to investigate how CO_2 availability alters whole-plant tissue N concentration, ii) to examine the effects of increased [CO_2] on carbon allocation to the production of biomass, total phenolic compounds and TNC (total non-structural carbohydrates, starch plus sugars), and finally iii) to determine how elevated [CO_2] influences gas exchange rate in progeny of *Q. ilex* trees growing in a CO_2-enriched environment under two different levels of N. The parent trees grow in poor soil nutrient conditions under long-term CO_2 enrichment and their carbon physiology has been the object of a previous study [48]. We hypothesized that the juvenile stage would behave like acclimated parent trees when grown in similarly poor soil nutrient conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Acorns of *Q. ilex* were collected in December from adult (open-pollinated) trees, growing in the proximity of the natural CO_2 spring of Bossoleto and which have spent their entire lifetime under elevated [CO_2]; the CO_2 vent is located in the vicinity of Rapolano Terme near Siena (Italy) (for details see [28]). Seeds were immediately sent to USA and sown in PVC pipe tubes (25 cm height × 5.5 cm averaged internal diameter, 600 cm^3). After germination, seedlings were thinned to one per pot. The tubes were filled with a mixture (v/v) of 90 % sand and 10 % peat, a layer of stones was placed at the base of each tube. 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 one pulse of 2 g, applied after 1 month of growth in the tubes. Soil nutrients in terrestrial systems suggest that N mineralization is sometimes limited to short periods early in growing season; furthermore, by giving an initial pulse of nutrients, we created a situation in which plant requirements for nutrients were increasing (due to growth) while supply was decreasing (due to uptake) [10], a phenomenon that may occur particularly in natural systems low in soil N. During the first month of growth (January 1995) the seedlings were fumigated twice with a commercial fungicide.

Two hundred and forty seedlings were grown for 6 months in six open-top chambers located at the School of Forest Resources and Conservation, University of Florida, Austin Cary Forest, approximately 10 km northeast of Gainesville. Each chamber received one of two CO_2 treatments: ambient [CO_2] or 150 μmol mol^{-1} exceeding ambient [CO_2]. The chambers were 4.3 m tall and 4.6 m in diameter, covered with clear polyvinylchloride film and fitted with rain-exclusion tops. Details of the chamber characteristics may be found in [23]. The CO_2, supplied in liquid form that vaporized along the copper supply tubes, was delivered through metering valves to the fanboxes of three chambers. The CO_2 treatment was applied during the 12 h (daytime) the fans were running with delivery being controlled by a solenoid valve connected to a timer. The CO_2 was delivered for about 15 min after the fans were turned off in the evenings in order to maintain higher concentrations in the chambers. The [CO_2] was measured continuously in both the ambient and elevated [CO_2] chambers using a manifold system in conjunction with a bank of solenoid valves that would step through the six chamber sample lines every 18 min. Overall mean daily [CO_2] for the above treatments was 370 or 520 μmol mol^{-1} at present or elevated [CO_2], respectively (for details see [26]). The [CO_2] during the night remained higher in the CO_2-enriched chambers, since the fans were turned off, avoiding air mixing.

At the beginning of March (1995), two different nutrient solution treatments were initiated. Within a chamber, equal numbers of pots (21) were randomly assigned to a high- or low-N treatment. Before starting the nutrient treatment, 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. 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 limiting nutrient disequilibrium [25].

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®, Grace-Sierra Co., Yosemite Drive Milpitas, CA, USA): complete nutrient solution containing high N (90 μmol mol^{-1} NH_4NO_3), or a nutrient solution with low N (20 μmol mol^{-1} NH_4NO_3). Both nutrient solutions contained PO_4 (20.6 μmol mol^{-1}), K (42.2 μmol mol^{-1}), Ca (37.8 μmol mol^{-1}), Mg (6 μmol mol^{-1}), SO_4 (23.5 μmol mol^{-1}), Fe (0.6 μmol mol^{-1}), Mn (0.1 μmol mol^{-1}), Zn (0.03 μmol mol^{-1}), Cu (0.03 μmol mol^{-1}), B (0.1 μmol mol^{-1}) and Mo (0.02 μmol mol^{-1}), and were adjusted to pH 5.5. Every 5 weeks supplementary Peters (S.T.E.M.) micronutrient elements (0.05 g dm^3) 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 in order to avoid positional effects.

2.2. Gas exchange measurement

Measurements of stomatal conductance (g_s) and leaf carbon exchange rate were made with a portable gas analysis system (LI-6200, Li-cor Inc., Lincoln, NE, USA) on upper-canopy fully expanded leaves of the same stage of development of randomly selected 18 plants for each $CO_2 \times N$ treatment combination (all measurements were made in duplicate and each leaf was measured twice). Measurements of daytime g_s and photosynthetic rate (A) were performed under saturating light conditions (PAR 1 000–1 500 μmol m^{-2} s^{-1}), between 10:00 to 15:00 hours on August 27–29 (air temperature 27–30 °C, relative humidity 70–75 %). Leaf dark respiration (R_d) was measured before sunrise (04:00–06:00 hours) on August 26–28 (air temperature, 23–25 °C). Instantaneous transpiration efficiency (ITE) was calculated as A/g_s. Air temperature, relative humidity and PPFD in the leaf cuvette were kept at growth conditions.

2.3. Biomass allocation

Heights and root-collar diameters were measured on all the plants (240) on September 4. On September 6 all plants were harvested and were separated into leaves, stem, and coarse (> 2 mm) and fine (< 2 mm) roots. Surface area of each leaf and total foliage area of each seedling were measured with an area meter (Delta-T Devices Ltd, Cambridge, UK). Plant material was dried at 65 °C to constant weight and dry mass (DW) measurements were made. Leaf area ratio, LAR (m^2 g^{-1}), was calculated as the ratio of total leaf area to total plant dry mass; specific leaf area, SLA (m^2 g^{-1}), as the ratio of total leaf area to leaf dry mass; partitioning of total plant dry mass, LWR, SWR and RWR (g g^{-1}), as the fraction of plant dry mass belonging to leaves, stem and roots, respectively. In addition, root/shoot dry mass ratio, RSR (g g^{-1}), was determined.

2.4. Carbohydrate, carbon and N analysis

The amount of total non-structural carbohydrates (TNC), including starch and sugars, was measured using the anthrone method on 12 seedlings for each $CO_2 \times N$ treatment combination. These seedlings were harvested either at dawn or in late afternoon, and immediately (after leaf area measurements) placed into a drier (see above). Previously dried plant materials (leaves, stem and roots) were ground in a Wiley mill fitted with 20 mesh screen. Approximately 100 mg of ground tissue was 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., USA) and filtered. Soluble sugars and the glucose released from starch were quantified spectrophotometrically following the reaction with anthrone. All samples were prepared in duplicate.

Total carbon and N concentrations (mg g^{-1} DW) were determined for all 240 seedlings (leaves, stem and roots) by catharometric measurements using an elemental analyser (CHNS 2500, Carlo Erba, Milano, Italy) on 5–9 mg of powder of dried samples.

2.5. Phenolic analysis

Equal-aged leaves (three leaves per plant) were taken from all 240 seedlings, the day before the harvest, for total phenolic compounds analysis. Leaves were put into liquid N at the field site, then transported to the laboratory and stored in the freezer at –20 °C until analysis. The leaf blades were punched on either side of the main vein. Five punches (0.2 cm^2 each) per leaf were analyzed for phenolics by modifying the insoluble polymer bonding procedure of Walter and Purcell [51]. Other punches from the remaining leaf blades were used for dry mass determination, as described above. Leaf tissue was homogenized in 5.0 cm^3 of hot 95 % ethanol, blending and boiling for 1–2 min. Homogenates were cooled to room temperature and centrifuged at 12 000 g for 30 min at 28 °C. Supernatants were decanted and evaporated to dryness in N at 28 (C. Aliquots (8 cm^3) of the sample in 0.1 M phosphate buffer (KH_2PO_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 g, 28 °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 the Dowex treatment, representing the absorbance by phenolic compounds. Phenolic concentration 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.

2.6. Statistical analysis

Individual measurements were averaged per plant, and plants measured with respect to each $CO_2 \times N$ treatment combination were averaged across the open-top chambers. Statistical analyses consisted of two-way

analysis of variance (ANOVA) for randomized design and Duncan's mean separation test for the measured parameters (5 % significant level); CO_2 and N were treated as fixed variables. A preliminary analysis showed that differences between chambers within the same CO_2 treatment were never significant. Proportions and percentages were transformed using the arcsine of the square root prior to analysis.

3. Results

3.1. Gas exchange rate

Increasing $[CO_2]$ had a significant effect on leaf carbon exchange rate (*table I*). Comparison of assimilation rates at the growth $[CO_2]$ showed that increasing $[CO_2]$ from 370 to 520 μmol mol^{-1} resulted in 33 % increase in A for plants grown with low-N supply and in 36 % increase for plants grown with high-N supply. Nutrient supply also significantly affected the response of A. Plants grown with high-N supply had 25 and 29 % higher A than plants grown with low-N supply at ambient and elevated $[CO_2]$, respectively. There was no strong interaction between CO_2 and N treatment (P = 0.084), i.e. increase in $[CO_2]$ elicited a similar increase in A in both N treatments.

Comparison of g_s at the growth $[CO_2]$ showed that increasing $[CO_2]$ from 370 to 520 μmol mol^{-1} led to a 14 % decrease in g_s for plants grown with low-N supply and to 10 % decrease with plants grown with high-N supply (*table I*). Nutrient supply treatment and the interaction between CO_2 and N treatment did not affect significatly g_s.

As a result of increases in A and decreases in g_s, ITE of leaves increased with $[CO_2]$ in both N supply treatments (*table I*). ITE was significantly different among the four $CO_2 \times N$ treatment combinations (ITE was higher with high-N supply), and there was a significant interaction between CO_2 and N treatment ($P < 0.05$) reflected by a marked increase in ITE in plants grown in elevated $[CO_2]$ with a high-N supply.

The ratio of internal $[CO_2]$ (C_i) to ambient (i.e. external) $[CO_2]$ (C_a) decreased ($P < 0.0001$) with both CO_2-enrichment and high-N supply, while the interaction between CO_2 and N treatment was not significant (*table I*).

The increase in A was associated with a significant increase in R_d (*table I*). Comparison of R_d at the growth $[CO_2]$ showed that increasing $[CO_2]$ from 370 to 520 μmol mol^{-1} led to 48 % increase for plants grown with low-N supply and to 36 % for plants grown with high-N supply. The increase in N supply and the interaction between CO_2 and N treatment had less of an effect on the increase in R_d.

3.2. Growth and biomass partitioning

Basal stem diameter, number of leaves per plant and foliage area were increased by elevated $[CO_2]$ treatment only when *Q. ilex* seedlings were grown in the high-N treatment (*table II, figure 1*). Shoot length and individual leaf area were not influenced by $[CO_2]$ treatment but increased with N supply. After 6 months of $CO_2 \times N$ treatment combination there were significant increases in the dry mass of roots and coarse roots of seedlings grown in elevated $[CO_2]$ compared to seedlings grown at

Table I. Gas exchange characteristics of leaves selected from *Quercus ilex* seedlings grown in ambient or CO_2-enriched air and with low or high N availability. Data are means (± SE) of 18 samples (seedlings); a total of 36 leaves were sampled per treatment and two leaves per plant were averaged. Means in the same column followed by the same letter are not statistically significant at $P \leq 0.05$. The probability level (P) is also reported. See text for abbreviations.

Treatment	A (μmol m^{-2} s^{-1})	R_d (μmol m^{-2} s^{-1})	Parameter g_s (mol m^{-2} s^{-1})	*ITE* (μmol mol^{-1})	C_i/C_a (μmol μmol^{-1})
low N ambient CO_2	09.20(0.60)a	0.52(0.10)a	0.37(0.02)a	25.91(1.89)a	0.90(0.01)a
low N elevated CO_2	13.68(0.73)b	0.99(0.11)bc	0.31(0.02)ab	44.19(1.12)b	0.84(0.01)b
high N ambient CO_2	12.30(0.45)b	0.66(0.14)ab	0.33(0.02)ab	38.46(1.68)c	0.86(0.01)b
high N elevated CO_2	19.18(0.86)c	1.04(0.13)c	0.30(0.02)b	65.05(1.87)d	0.80(0.01)c
Source of variation			Probability		
N	0.0001	0.4249	0.2633	0.0001	0.0001
CO_2	0.0001	0.0009	0.0429	0.0001	0.0001
$N \times CO_2$	0.0842	0.7020	0.6363	0.0156	0.7941

Table II. Results of ANOVA showing the *F*-ratio and statistical significance of the effects of CO_2, N and interaction $CO_2 \times N$ on growth parameters of *Quercus ilex* seedlings. See text for abbreviations.

Parameter	Source of variation		
	CO_2	N	$CO_2 \times N$
Foliage DW	17.193****	152.086****	14.156***
Stem DW	9.106**	156.663****	15.147***
Roots DW	38.501****	22.897****	2.116n.s.
Total DW	28.794****	115.660****	10.922**
Coarse roots DW	21.644****	0.150n.s.	0.037n.s.
Fine roots DW	25.898****	7.877**	7.225**
Fine root/foliage mass ratio	9.604**	21.062****	0.000n.s.
LWR	8.973**	31.812****	1.233n.s.
SLA	29.516****	0.070n.s.	1.583n.s.
LAR	37.358****	9.923**	2.447n.s.
RSR	15.653***	80.172****	5.438*
RWR	15.804***	85.789****	4.677*
SWR	11.629***	82.370****	5.317*
Total leaf area per plant	0.680n.s.	173.448****	14.138***
Leaf area per leaf	0.736n.s.	11.675***	0.039n.s.
Number of leaves per plant	1.369n.s.	55.057****	13.831***
Shoot lenght	0.009n.s.	145.447****	6.907**
Stem basal diameter	4.120*	56.561****	9.907**

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, n.s. not significant $P > 0.05$.

ambient $[CO_2]$, irrespective of N treatment. The interaction between CO_2 and N treatment was significant for total, stem, fine root and foliage biomass. As a result of this, effects of CO_2-enriched air on whole seedling growth, stem, fine root and foliage biomass were significant only in the high-N treatment (*figures 1* and *2*). Fine root/foliage mass ratio decreased with N treatment and increased with CO_2 enrichment (*table II, figure 2*).

As a result of increased allocation to below-ground tissue, RSR and RWR were increased significantly by CO_2 treatment, while SWR and LWR were decreased, only at a low level of N supply (*table II, figure 3*). More biomass was partitioned to above-ground tissue in the high-N treatment irrespective of CO_2 treatment; as a result RSR and RWR decreased, while conversely, SWR and LWR increased significantly at a high level of N supply. SLA and LAR decreased significantly in leaves of seedlings grown in elevated $[CO_2]$ irrespective of N treatment, while N supply affected LAR (only in elevated $[CO_2]$ but not SLA (*table II, figure 3*).

3.3. Carbon and N concentrations

Overall, carbon concentrations in leaves, stem and roots were not significantly affected by either CO_2 or nutrient treatment (*table III*). Leaf N concentration decreased significantly in elevated $[CO_2]$ irrespective of nutrient treatment, while N concentrations in stem and roots were decreased by elevated $[CO_2]$ in the high-nutrient treatment. Nutrient supply treatment affected N concentration significantly in leaves irrespective of CO_2 treatment, and in stem and roots only in the ambient $[CO_2]$ treatment. As a result of patterns of N and carbon concentrations, C/N ratio generally increased with elevated $[CO_2]$ and decreased with high nutrient supply (*table III*).

3.4. Total non-structural carbohydrate and total phenolic concentrations

Morning starch concentrations were higher ($P < 0.01$) in leaves of seedlings grown in CO_2-enriched air (*table IV*), but particularly at low level of N supply. Afternoon starch concentrations did not increase significantly with increasing $[CO_2]$. Both morning and afternoon sugars concentration did not increase significantly with rising $[CO_2]$. Both morning and afternoon starch concentrations decreased ($P < 0.001$) with increasing N addition while sugars concentration was not affected by N treatment (*table IV*).

Overall starch concentrations in leaves, stem and roots increased with rising $[CO_2]$ and decreased with N addition (*table V*). The concentration of sugars was not

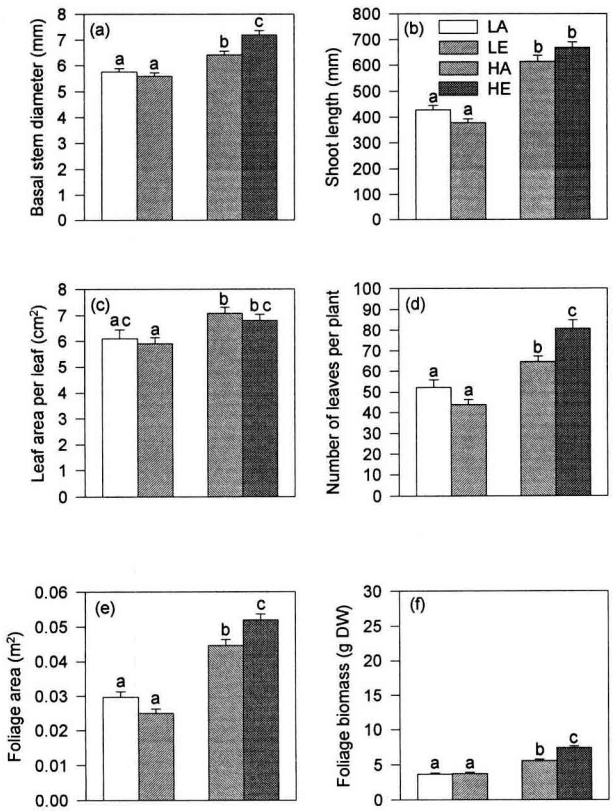

Figure 1. Basal stem diameter, seedling height, leaf area and number of leaves per plant, foliage area and biomass as a function of [CO_2] and nutrient level. Values are the mean (± SE, n = 40). Treatments are referred to by symbols in the legend (LA, low-N level and ambient [CO_2], LE, low-N level and elevated [CO_2], HA, high-N level and ambient [CO_2], HE, high-N level and elevated [CO_2]. Columns marked by the same letter are not statistically significant at $P \leq 0.05$.

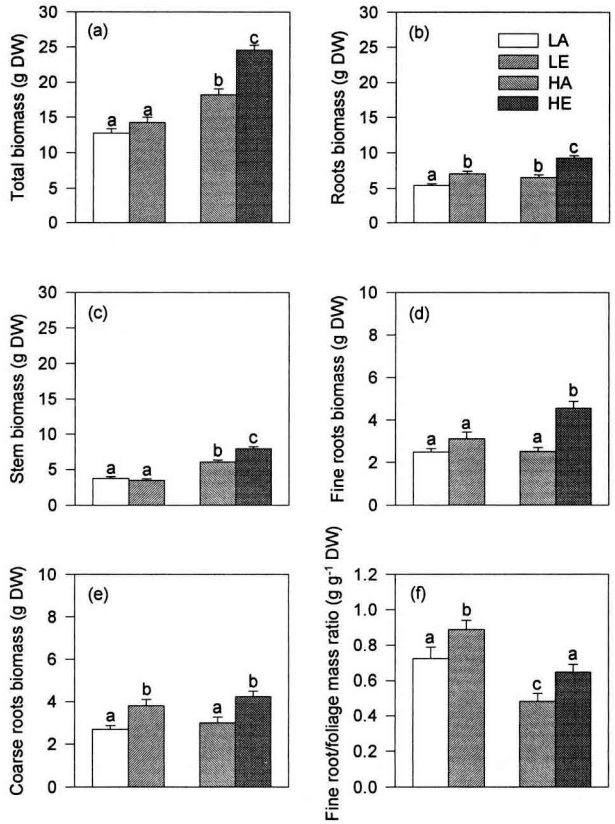

Figure 2. Whole plant, roots, stem, fine roots and coarse roots biomass, and fine root/foliage mass ratio as a function of [CO_2] and nutrient level. Values are the mean (± SE, n = 40). Treatments are referred to by symbols in the legend (LA, low-N level and ambient [CO_2], LE, low-N level and elevated [CO_2], HA, high-N level and ambient [CO_2], HE, high-N level and elevated [CO_2]. Columns marked by the same letter are not statistically significant at $P \leq 0.05$.

affected significantly by either CO_2 or N treatment. As a result, TNC concentrations were influenced by both CO_2 enrichment and N treatment because of changes in starch concentrations.

Total phenolic concentrations decreased in leaves of seedlings grown in elevated [CO_2] irrespective of N treatment, while N supply treatment and the interaction between CO_2 and N treatment had less of an effect (*figure 4*).

4. Discussion

Photosynthesis of *Q. ilex* seedlings was stimulated by elevated [CO_2] even in the low level of supplemental fertilization and despite declining foliar N concentration, as for other broad-leaved trees (e.g. [34]). The increase in leaf dark respiration expressed on a leaf area basis in CO_2-enriched air may be correlated with the enhanced carbohydrate content [44]. Although many studies show significant reductions in plant respiration in elevated [CO_2] (e.g. [47]; see [1] for a review), accordingly with these seedlings, parent *Q. ilex* trees at the natural CO_2 spring in Italy grow in a N poor soil and have also been found to show higher photosynthesis and dark respiration than trees at ambient [CO_2] [9, 48]. Stomatal response to CO_2 is a common phenomenon and stomatal conductance in many plants decreases in response to increasing [CO_2] (see reviews [1, 7, 42], and references cited therein). In our study, however, elevated [CO_2] treatments did not strongly alter leaf conductance.

Table III. N and carbon (C) percentages, and C/N ratios in leaves, stem and roots of *Quercus ilex* seedlings grown in ambient or CO_2-enriched air and with low or high N availability. Data are means (± SE) of 40 samples (seedlings). Means within the same column and plant compartment followed by the same letter are not statistically significant at $P \leq 0.05$. The probability level (P) is also reported.

Treatment	Parameter N %	C %	C/N ratio
Leaves			
Low N ambient CO_2	1.33(0.04)a	38.53(1.05)a	29.78(1.05)a
Low N elevated CO_2	1.20(0.04)b	40.36(0.83)a	35.65(1.45)b
High N ambient CO_2	1.70(0.04)c	38.38(0.99)a	23.09(0.83)c
High N elevated CO_2	1.43(0.05)a	38.21(0.90)a	27.30(0.96)a
Source of variation		Probability	
N	0.0001	0.3926	0.0001
CO_2	0.0001	0.2277	0.0001
N × CO_2	0.1175	0.2908	0.4596
Stem			
Low N ambient CO_2	0.55(0.03)a	37.08(0.94)a	71.55(3.56)ab
Low N elevated CO_2	0.62(0.04)ab	39.71(0.81)b	76.24(5.35)b
High N ambient CO_2	0.70(0.04)b	38.39(0.83)ab	61.00(3.32)a
High N elevated CO_2	0.59(0.03)a	38.31(0.86)ab	70.66(3.74)ab
Source of variation		Probability	
N	0.5639	0.1420	0.0802
CO_2	0.1229	0.9616	0.0495
N × CO_2	0.0156	0.1187	0.5493
Roots			
Low N ambient CO_2	0.53(0.02)a	35.66(0.98)a	73.65(4.56)a
Low N elevated CO_2	0.58(0.04)a	38.62(0.76)b	77.83(5.21)a
High N ambient CO_2	0.68(0.03)b	36.11(1.01)ab	58.05(3.79)b
High N elevated CO_2	0.58(0.03)a	36.44(1.01)ab	68.64(3.89)ab
Source of variation		Probability	
N	0.4623	0.0836	0.0960
CO_2	0.0165	0.3706	0.0056
N × CO_2	0.0174	0.1682	0.4759

Table IV. Sugars and starch concentrations, and their ratios, in leaves of *Quercus ilex* seedlings grown in ambient or CO_2-enriched air and with low or high N availability, collected at dawn and late afternoon. Data are means (± SE) of 6 samples (seedlings). Means within the same column followed by the same letter are not statistically significant at $P \leq 0.05$.

Treatment	Parameter Sugars (mg g^{-1} DW) *afternoon*	*morning*	Starch (mg g^{-1} DW) *afternoon*	*morning*	Starch/Sugars (ratio) *afternoon*	*morning*
Low N ambient CO_2	53.12(8.21)a	72.50(10.63)a	184.35(41.52)ab	108.77(24.16)a	4.83(1.81)a	1.48(0.24)a
Low N elevated CO_2	71.89(16.13)a	72.13(4.30)a	228.57(38.76)b	234.04(27.80)b	3.57(0.56)ab	3.40(0.60)b
High N ambient CO_2	69.52(6.37)a	52.68(5.94)b	122.62(23.68)a	67.91(14.58)a	1.77(0.31)b	1.25(0.23)a
High N elevated CO_2	71.54(3.62)a	59.07(2.23)ab	141.60(19.19)ab	96.35(23.40)a	1.97(0.23)ab	1.67(0.46)a

Similar results have been reported for other species when plants were grown at high irradiances (e.g. [6, 21, 31, 49]. Stomatal sensitivity to CO_2 in our seedlings, grown at full irradiances with an adequate supply of soil water, may have been reduced [16]. Indeed, the ratio of internal $[CO_2]$ (demand) to external $[CO_2]$ (supply) decreased with CO_2 enrichment while intercellular $[CO_2]$ remained relatively constant, despite at elevated $[CO_2]$ intercellular $[CO_2]$ should rise if stomata close consistently. This implies that as a result of strongly increased assimilation

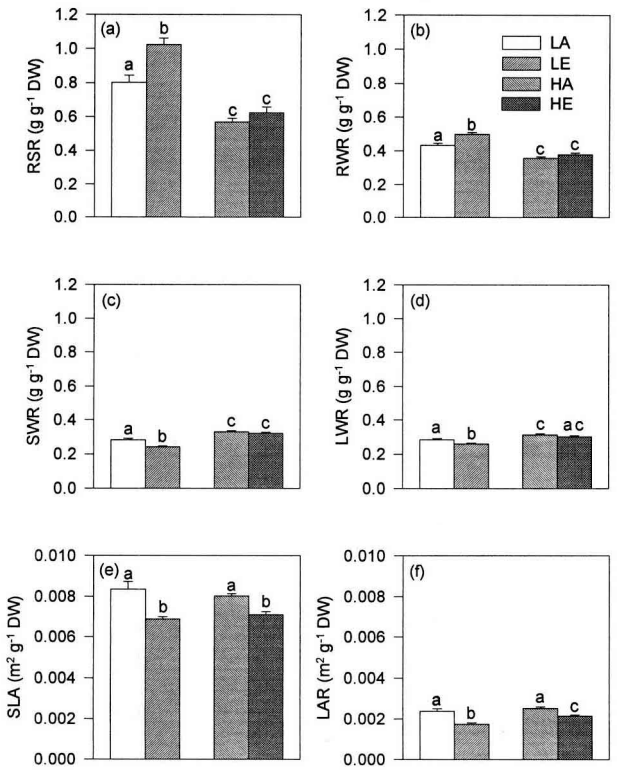

Figure 3. Root/shoot ratio (RSR), fraction of plant dry mass belonging to leaves (LWR), stem (SWR) and roots (RWR), specific leaf area (SLA), and leaf area ratio LAR as a function of $[CO_2]$ and nutrient level. Values are the mean (± SE, n = 40). Treatments are referred to by symbols in the legend (LA, low-N level and ambient $[CO_2]$, LE, low-N level and elevated $[CO_2]$, HA, high-N level and ambient $[CO_2]$, HE, high-N level and elevated $[CO_2]$. Columns marked by the same letter are not statistically significant at $P \leq 0.05$.

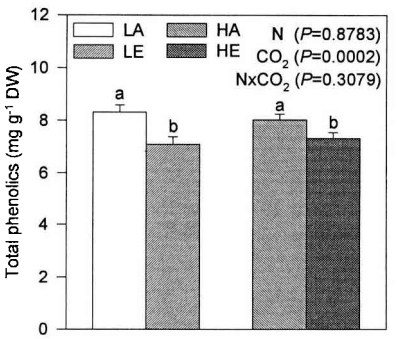

Figure 4. Total phenolic concentration as a function of $[CO_2]$ and nutrient level. Values are the mean (± SE, n = 40); three leaves per plant. Treatments are referred to by symbols in the legend (LA, low-N level and ambient $[CO_2]$, LE, low-N level and elevated $[CO_2]$, HA, high-N level and ambient $[CO_2]$, HE, high-N level and elevated $[CO_2]$. Results of ANOVA are also reported. Columns marked by the same letter are not statistically significant at $P \leq 0.05$.

rate and, secondarily decreased stomatal conductance, instantaneous transpiration efficiency of leaves markedly increased at elevated $[CO_2]$ [15].

The elevated $[CO_2]$ treatment increased seedling growth only when nutrient availability was high. Similar findings have been reported for *Pinus taeda* L. [20], *Betula populifolia* Marsh., *Fraxinus americana* L., *Acer rubrum* L. [3] and *Pinus palustris* Mill. [38]. However, positive growth responses to CO_2-enriched air even under conditions of low soil nutrient availability have been reported for *Castanea sativa* Mill. [17], *Pinus ponderosa* Dougl. ex Laws. [27], *Eucalyptus grandis* W. Hill ex Maiden [11] and *Quercus virginiana* Mill. [46]; in this latter case the experimental conditions were the same as in the present study. These contrasting results (even between studies with identical experimental protocols) indicate that the interactive effects of CO_2 and nutrient availability are species dependent. The lack of a growth response to elevated $[CO_2]$ in seedlings in the low-N treatment is of interest because suboptimal concentrations of N are common in the Mediterranean environment. Indeed, responses in the parent *Q. ilex* trees at the natural CO_2 springs in Italy do not appear to be clearly more evident than in trees at ambient $[CO_2]$ [22].

Coarse root (and total root) biomass responded positively to elevated $[CO_2]$ irrespective of nutrient availability, while fine root biomass increased significantly under low nutrient availability. Partitioning of resources was reflected by adjustments in shoot and root growth and in RSR. Low nutrient supply enhanced overall biomass partitioning to roots (higher RWR and RSR, lower SWR), while high-N availability resulted in a greater proportion of biomass being distributed to stem and leaves [19, 32, 38]. Preferentially induced distribution of photosynthates below-ground as carbon supply increases in response to CO_2-enriched air is a common phenomenon [7, 38, 43]. Such a pattern was detected in our experiment at a low level of nutrient supply only, which was reflected by increased RSR and RWR. This may allow seedlings in CO_2-enriched air to explore the soil in order to attain more resources such as water and nutrients to meet growth demands. Conversely, seedlings grown in ambient $[CO_2]$ had a greater proportion of biomass distributed to above-ground tissues at low level of nutrient supply only, which was reflected by decreased RSR and increased SWR and LWR. Increased stem biomass in seedlings grown under elevated $[CO_2]$ and high nutrient availability was associated with increased stem diameter

Table V. Sugars, starch and total non-structural carbohydrate (TNC) concentrations in leaves, stem and roots of *Quercus ilex* seedlings grown in ambient or CO_2-enriched air and with low or high N availability. Data are means (± SE) of 12 samples (seedlings). Means within the same column and plant compartment followed by the same letter are not statistically significant at $P \leq 0.05$. The probability level (P) is also reported.

Treatment	Parameter		
	Sugars (mg g^{-1} DW)	Starch (mg g^{-1} DW)	TNC (mg g^{-1} DW)
Leaves			
Low N ambient CO_2	62.81(7.04)a	146.56(25.58)a	209.37(25.27)a
Low N elevated CO_2	72.01(7.96)a	231.31(22.75)b	303.31(24.68)b
High N ambient CO_2	61.10(4.86)a	95.26(15.61)a	156.36(18.99)a
High N elevated CO_2	65.31(2.76)a	118.97(15.96)a	184.28(17.24)a
Source of variation		Probability	
N	0.4950	0.0002	0.0003
CO_2	0.2701	0.0110	0.0077
N × CO_2	0.6839	0.1425	0.1375
Stem			
Low N ambient CO_2	31.72(2.54)a	75.67(12.29)ab	107.39(12.21)ab
Low N elevated CO_2	30.18(1.90)a	101.49(10.55)b	131.67(9.78)b
High N ambient CO_2	25.52(2.27)a	51.73(8.28)a	77.28(8.59)a
High N elevated CO_2	26.54(3.05)a	83.15(19.40)ab	70.66(3.74)ab
Source of variation		Probability	
N	0.0529	0.1190	0.0732
CO_2	0.9186	0.0369	0.0520
N × CO_2	0.6121	0.8364	0.7780
Roots			
Low N ambient CO_2	57.21(8.99)a	301.59(38.40)a	358.80(32.97)a
Low N elevated CO_2	37.31(2.53)b	514.32(59.88)b	551.64(59.63)b
High N ambient CO_2	39.15(3.73)b	164.29(18.12)c	203.44(17.48)c
High N elevated CO_2	38.42(4.05)b	202.56(39.60)ac	240.98(41.31)c
Source of variation		Probability	
N	0.1251	0.0001	0.0001
CO_2	0.0637	0.0043	0.0071
N × CO_2	0.0841	0.0423	0.0635

and height, while in the low soil nutrient availability treatment, seedlings in elevated [CO_2] were even shorter than those in ambient [CO_2].

These findings support the idea that plants allocate photosynthate to tissues needed to acquire the most limiting resources [8]. Such shifts to below- and/or aboveground tissues, may have implications during the regeneration phase in terms of competition for light and water with other woody species of the Mediterranean vegetation. CO_2 treatment also increased the fine root/foliage mass ratio while N treatment had the opposite effect [46]. This change in allocation might represent a substitution between potential carbon assimilation and nutrient acquisition [34]. However, conflicting results are reported in the literature [37, 45]. LAR and LWR decreased in response to elevated [CO_2] suggesting that canopy-level adjustment in carbon assimilation did occur in these seedlings. It must be pointed out that our seedlings grew in pots and growth responses to elevated [CO_2] may sometimes be influenced by pot size, though the issue of pot size is far from being resolved. Soil nutrient disequilibrium (which we tried to minimize) may be more important than pot size in affecting growth response to elevated [CO_2]. Plants in natural environments do not have unlimited below-ground resources with which to maximize growth in elevated [CO_2] [1], and the presence of shallow bedrock at the site of origin of *Q. ilex* parent trees is, in this sense, a good example.

The observed increased leaf biomass and area in response to CO_2 enrichment (at a high level of soil nutrient availability), as a result of an increase in leaf number rather than leaf size, could affect whole-plant photosynthetic capacity [38]. Decreases in SLA have been

observed in plants grown in CO_2-enriched air (e.g. [14]) and have been attributed to an additional cell layer [40] or starch accumulation [36]. In our study, lower SLA at elevated [CO_2] was partly attributable to higher carbohydrate concentrations, a large part of which was starch.

The reduction in N concentration in leaves of seedlings grown at elevated [CO_2], irrespective of the N availability in the soil, agrees with other studies on tree species [7, 42]. Similarly, mature *Q. ilex* trees grown long term under elevated [CO_2], from which the acorns were collected, showed a decreased N concentration in leaves when compared to trees grown at ambient [CO_2] [48]. We found, however, that N concentration in stem and roots did not decrease in the CO_2-enriched air and low-N treatment combination. The decrease in tissue N concentration may be an indirect and size-dependent effect of elevated [CO_2] [10, 46], alternatively it has been proposed that starch accumulation dilutes N and lowers its concentration in the tissues (e.g. [53]). Carbon concentration was not strongly affected by either treatment (despite an increase in carbon concentration in the CO_2-enriched air and low-N treatment combination). The C/N ratio in leaves was enhanced by increasing [CO_2] and diminishing nutrient supply; this trend was confirmed in stem and roots but to a lesser extent. The increase in the C/N ratio could increase carbon storage and the concentration of carbon-based secondary compounds [30], and nutrient cycling in this as in other *Quercus* species [50]. In the current experiment foliar phenolic concentrations decreased in CO_2-enriched air. A variety of phenolic concentration responses to CO_2 enrichment have been observed (e.g. [35]). The parallel increase in TNC at elevated [CO_2] may have caused a dilution of phenolics. If increased [CO_2] reduces in the long-term both the N and phenolic concentrations of leaves the consequences for interactions between *Q. ilex* and insect herbivores may be great. Nevertheless, tannin concentrations in leaves of the parent trees were positively affected by elevated CO_2 [48].

Enhancement of mineral nutrient supply alone (which caused significant growth stimulation) reduced starch and TNC concentrations in leaves, stem and roots. CO_2 enrichment stimulated the accumulation of TNC, by strongly increasing starch formation, starch storage seems to be particularly important in *Q. ilex*. The lack of carbon flow to soluble sugars suggests a limitation in the partitioning of carbon to this intermediate [54]. Rising concentrations of TNC in the leaves may occur, among other reasons [43], because of reductions in sink activity (e.g. as a consequence of limiting resources other than CO_2). The accumulation of starch in leaves, particularly in the CO_2-enriched air and low-N treatment combination, however, was not accompanied by the reduction in photosynthetic rate, suggesting that the demand for carbohydrates in these seedlings is high.

This study, which represents the first one on progenies of trees grown long term in CO_2-enriched air, reports data similar to those conducted on mature trees at the natural CO_2 spring in Italy in soil with poor N availability [48], in that lack of a growth responses to elevated [CO_2] in seedlings in the low-N treatment occurred despite increased rate of net photosynthesis. All the parameters studied in the present experiment (except for carbon-based defense compounds) follow the findings on parent trees exposed to CO_2-enriched air when seedlings grown in elevated [CO_2] and low-N availability are taken into account. It is possible to hypothesize that the available soil N will be a major controlling resource for the establishment and growth of this species in rising [CO_2]. *Q. ilex* stands established in soils poor in N will probably not exhibit a larger increase in above-ground productivity in the predicted CO_2-enriched atmosphere, but below-ground processes and interactions between trees and tree-feeding insects might be altered.

Acknowledgement: The technical assistance of Dave Noletti is greatly appreciated.

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