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Effects of long-term exposure to elevated CO_2 and N fertilization on the development of photosynthetic capacity and biomass accumulation in *Quercus suber* L.

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ABSTRACT

The effects of long-term (4 year) CO_2 enrichment (70 Pa versus 35 Pa) and nitrogen nutrition (8 mM versus 1 mM NO_3^-) on biomass accumulation and the development of photosynthetic capacity in leaves of cork oak (*Quercus suber* L., a Mediterranean evergreen tree) were studied. The evolution of photosynthetic parameters with leaf development was estimated by fitting the biochemical model of Farquhar *et al.* (*Planta* 149, 78–90, 1980) with modifications by Sharkey (*Botanical Review* 78, 71–75, 1985) to A–C_i response curves. CO_2 enrichment had a small reduction effect on the development of the maximum CO_2 fixation capacity by Rubisco (V_{Cmax}), and no effect over maximum electron transport capacity (J_{max}), day-time respiration (R_d) and Triose-P utilization (TPU). However, there was a statistically significant effect of N fertilization and the interaction CO_2 × N over the evolution of V_{Cmax}, J_{max} and TPU. Relative stomatal limitation (estimated from A–C_i curves) was higher (+20%) for plants grown under ambient CO_2 than for plants grown under elevated CO_2. There was a significant effect of CO_2 and N fertilization over total biomass accumulation as well as leaf area. Plants grown at elevated CO_2 had 27% more biomass than plants grown at ambient CO_2 when given high N. However, for plants grown under low N there was no significant effect of CO_2 enrichment on biomass accumulation. Plants grown under low N also had significantly higher root : shoot ratios whereas there were no differences between CO_2 treatments. The larger biomass accumulation of *Q. suber* under elevated CO_2 is attributable to a higher availability of CO_2 coupled to a larger leaf area, with no significant decrease in photosynthetic capacity under CO_2 enrichment and elevated N fertilization. For low N fertilization, the effects of CO_2 enrichment over leaf area and biomass accumulation are lost, suggesting that in native ecosystems with low N availability, the effects of CO_2 enrichment may be insignificant.

Key-words: cork-oak (*Quercus suber* L); growth; long-term CO_2 enrichment; modelling. N fertilization; photosynthesis.

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Abbreviations: A, net CO_2 assimilation; C_a, external CO_2 partial pressure; C_i, intercellular CO_2 partial pressure; g_s, stomatal conductance; J_{max}, maximum electron transport rate; Rubisco, RUBP carboxylase/oxygenase; TPU, triose-P utilization; V_{Cmax}, maximum Rubisco activity; R_d, dark respiration.

INTRODUCTION

Cork oak (*Quercus suber* L) is an evergreen tree that is the mainstay of multiple-use agroforestry systems with great socio-economic and environmental value in the western Mediterranean region, especially in the Iberian Peninsula and North Africa. Because these systems are quite restricted in global terms, there has been a lack of investigation on how global changes (namely an increased concentration of CO_2 in the atmosphere) will influence tree physiology and growth.

In the past, most studies with enriched CO_2 environments were conducted over short periods of exposure, generally not exceeding more than one growing season, when the potential for CO_2 effects over growth and acclimation are maximal (for reviews see, e.g. Poorter 1993; Drake & Gonzàlez-Meler 1997). C_3 plants show a significant stimulation of net CO_2 assimilation under short-term CO_2 enrichment with accumulation of carbohydrates (Neuhaus *et al.* 1990; Ludewig *et al.* 1998; Griffin *et al.* 2000). These may be involved in the down-regulation of genes coding for photosynthetic enzymes (e.g. rbcS), soluble proteins and therefore of photosynthetic capacity (Spalding 1994; Moore *et al.* 1998, 1999). However, mRNA transcripts of enzymes, involved in the utilization and export of triose-P, which are modulated by nitrogen and phosphate in a gene-specific manner, have been reported to be induced by sugars, with similar trends for enzyme content and activity (Nielsen *et al.* 1998; Stitt & Krapp 1999).

However, the effects of global climate change at the ecosystem level will be dependent on longer-term responses of tree species. Only recently, attention has been drawn to long-term and life-term exposure effects of elevated CO_2 on some Mediterranean type evergreens (see, e.g. Medlyn *et al.* 1999; Stylinski *et al.* 2000; Tognetti, Raschi & Jones 2000). Using a meta-analysis technique, Medlyn *et al.*

(1999) evaluated the results of several long-term field experiments in enriched CO_2 atmospheres across Europe. They found that light-saturated photosynthesis was stimulated in the elevated CO_2 atmosphere (usually twice ambient concentration), but a 10–20% acclimation of photosynthesis occurred when elevated and ambient CO_2 grown plants were measured at the same CO_2 concentration. This resulted largely from a regulation of the potential electron transport rate (J_{max}) and the maximum Rubisco activity (V_{cmax}). The reduction in these parameters was linked to the effects of elevated CO_2 on leaf nitrogen concentration. However, in a life-long CO_2 experiment on a natural CO_2 spring in Italy, Stylinski *et al*. (2000) saw no statistically significant effect of elevated CO_2 over V_{Cmax}, J_{max} and measured maximum photosynthesis rates of *Quercus pubescens* (40–50-year-old trees). Thus, they concluded that there was no evidence of photosynthetic acclimation (on the contrary V_{Cmax} and J_{max} were higher in elevated CO_2 grown plants as compared to control plants) early in the growing season when, presumably, sink demand is higher (Stylinski *et al*. 2000).

Experiments with CO_2 enrichment, suggest that responses to elevated CO_2 (acclimation) may be set by an optimization strategy in allocating nitrogen from the photosynthetic machinery to leaf-area expansion and growth (Maroco, Edwards & Ku 1999; Poorter & Nagel 2000). Thus, N may play an important role and modulate the potential for photosynthetic acclimation, sink–source imbalance, and growth responses to elevated CO_2 (McGuire, Melilo & Joyce 1995; Paul & Driscoll 1997; Rogers *et al*. 1998; Niinemets *et al*. 1999). Even though there are studies on the responses of Mediterranean evergreen oaks to long-term exposure to elevated CO_2 (Tognetti *et al*. 1998; Stylinski *et al*. 2000; Tognetti *et al*. 2000), few have utilized controlled soil conditions, namely nutrient addition. Furthermore, the effects of elevated CO_2 on photosynthesis and growth may change during the course of the year, and/or from year to year depending on genetic, physiological, biochemical and morphological traits and the environmental interactions with these plant traits (Miller *et al*. 1997; Turnbull *et al*. 1998; Medlyn *et al*. 1999; Griffin *et al*. 2000; Norby *et al*. 2001). It is therefore of interest to study these effects in different plant-developmental stages.

The developmental control of photosynthesis in elevated versus ambient CO_2 is rarely reported for trees. However, it is potentially of great importance to understand the physiological and ecological responses of trees to global changes in climate. We test the hypothesis that photosynthetic acclimation to elevated CO_2 would develop during leaf ontogeny and is dependent upon N availability. Four year-old plants were suitable for this type of study because these plants have one growth flush in April–May as mature trees, in contrast to 1–2-year-old cork oak plants, which may produce new leaves for most of the growing season. In slow growing-species of the Mediterranean ecosystem, the interaction of elevated CO_2 and nitrogen availability are especially important in determining the short- versus long-term responses, because remobilization of N from storage organs versus nutrient absorption may be limiting for new leaf expansion and metabolism, especially under conditions of elevated carbohydrates availability. In this paper, we studied the effects of long-term exposure (4 years) to elevated CO_2 partial pressure (70 Pa) versus ambient CO_2 (35 Pa) and its interaction with N availability over the development of photosynthetic capacity in expanding leaves, as well as its effects over long-term biomass accumulation and partition.

MATERIALS AND METHODS

Plant material and growth conditions

Plants were grown from selected seeds in 10 L pots filled with 1 : 1 mixture of sand and compost. To avoid constrains to root growth, plants were transferred to 30 L containers after the second growth season by transferring the 10 L soil cylinder into the larger soil volume. Plants were exposed for 4 years to ambient (35 Pa) versus elevated (70 Pa) CO_2 inside two side-by-side small greenhouses with controlled temperature, light and relative humidity. The CO_2 partial pressures inside the greenhouses were controlled by on-line infra-red gas analyser sampling coupled to an automatic CO_2 injection system (Aralab, Lisbon, Portugal) which was able to maintain the CO_2 partial pressures at the target set point ±3 Pa. Temperature, light and relative humidity variation inside the greenhouses mimicked the 30 year daily average for Lisbon (38°42′ N, 9°11′ W) with maximum daily temperature of 35 ± 3 °C, minimum of 5 ± 2 °C, average relative humidity of 50 ± 5%, and maximum photosynthetic photon flux density of 1800 ± 200 μmol m^{-2} s^{-1} (80% of maximum PPFD outside the greenhouse). Plants were fertilized once a week with a modified Hoagland solution to deliver either 1 mM NO_3^- (Low N) or 8 mM NO_3^- (High N) and watered with distilled water every other day. To avoid position effects, plants were rotated once a week inside the greenhouse and between the two greenhouses once per month. All measurements were made during spring time, when the growth flush of new leaves occurs.

Gas exchange and biochemical modelling of C_3 photosynthesis

Gas exchange measurements were performed with an open flow gas exchange system (Li-6400; Licor Inc, Lincoln, NB, USA). A–C_i response curves were obtained by increasing CO_2 concentrations of 15, 25, 35, 50, 70, 90, 120 Pa sequentially at a leaf temperature of 21 °C and PPFD of 1200 μmol m^{-2} s^{-1} (which was saturating for photosynthesis) in two leaves per plant. After flushing, leaves were measured at developmental stages corresponding to 1/3, 2/3 and 3/3 of full expansion. One-year-old-leaves (fully developed in the previous year) were used as controls. Percentages of maximum leaf expansion were determined by following leaf area evolution of the measured leaves at weekly intervals. Data collected from leaves at the same developmental stage (as evaluated from leaf expansion) were used in the analysis, to control for the effects of elevated CO_2 and N over leaf

growth. The biochemical model of Farquhar, von Caemmerer & Berry (1980) with modifications by Sharkey (1985) was fitted to A–C_i response curves using the NON-LIN procedure from SAS 6·12 (SAS Institute, Cary, NC, USA) as described in Maroco *et al*. (2001) in order to estimate the maximum Rubisco CO_2 fixation capacity (V_{Cmax}), maximum electron transport rate (J_{max}) and triose-P utilization rates (TPU). Relative stomatal limitation was calculated from A–C_i response curves as described in Farquhar *et al*. (1980).

Sugars, soluble protein, chlorophylls, N and P determinations

For sugar, protein and chlorophyll determinations, leaf-discs from leaves opposite to those used for gas exchange measurements, were collected at 1200 h solar time, immediately weighted, frozen in liquid N and stored at –80 °C until further assay. Total soluble and insoluble sugars concentrations were determined as described by Stitt *et al*. 1989). Soluble protein was extracted as described in Maroco *et al*. (2001) and measured using Biorad's protein assay kit according to the manufacturer's instructions (Biorad, Hercules, CA, USA). Total chlorophylls, chlorophyll *a* and chlorophyll *b* were measured as described previously (Maroco *et al*. 1999). For N and P determinations whole mature leaves were dried in an oven at 80 °C, ground to a fine powder and assayed for N and P by gas chromatography and plasma emission spectroscopy as described by Niinemets *et al*. (1999).

Biomass and leaf area determinations

Plant biomass was determined by weighing after separation into components (roots, stems, leaves and cork) and drying for 48 h in an oven at 80 °C. Roots were carefully extracted, washed and dried to constant weight. Leaf area measurements were carried out with a portable leaf area measurement system (Li-3000; Licor Inc.).

Statistical analysis

Data is presented as the averages of four to eight replicates per treatment (CO_2 and N) and leaf-developmental stage for each set of measurements except for N and P contents which were measured only in fully developed and well-exposed leaves. Tests of significance for the effects of CO_2 and N versus leaf developmental stage and its interactions were performed with the multivariate analysis of variance (MANOVA) approach to the repeated-measures analysis of variance (StatSoft 1999). A MANOVA was used to test the significance of the CO_2 and N treatments and its interaction over biomass, leaf area, specific leaf weight (SLW), root : shoot ratio, N, and P contents. Suitable data transformations were performed to assure homogeneity of variances and normal distribution of data. Statistically significant effects were assumed when $P < 0{\cdot}05$.

RESULTS

Biomass and leaf area

Total biomass (as well as its components: leaves, stems and roots) accumulation under elevated CO_2, as compared to ambient CO_2, was stimulated by approximately 30% when plants were grown with high N (Table 1). However, there were no effects of CO_2 enrichment on biomass accumulation for plants grown at low N. In addition, plants grown at elevated CO_2 and high N produced a significant amount of cork, which is an important asset for cork oak (Table 1). Total leaf area was significantly higher under elevated CO_2 but the differences between elevated versus ambient CO_2 were smaller for the low N, as compared to high N-grown plants (Table 1). Elevated CO_2 did not significantly affect the root : shoot ratios but this ratio was approximately twice as high in low N, as compared to high N grown plants. There were no significant differences in SLW for either the CO_2 or the N treatments.

Evolution of photosynthetic capacity in developing leaves

There was a significant effect of leaf age and N fertilization over the development of Rubisco maximum activity (V_{Cmax}) (Fig. 1a versus 1b). However, the approximately 20% reduction observed in V_{Cmax} in elevated versus ambient CO_2 grown plants in fully mature leaves, had a low statistical sig-

Table 1. Dry biomass accumulation, leaf area, specific leaf weight (SLW) and root : shoot ratio in *Q. suber* plants exposed for 4 years to elevated (70 Pa) and ambient CO_2 (35 Pa) under different N fertilization regimes: high N (8 mM) and low N (1 mM). Data is shown as average ± SE of 4–6 replicates per treatment

CO_2 (Pa)	N (mM)	Leaves (g)	Stems (g)	Cork (g)	Roots (g)	Leaf area (m^2)	SLW (g/m^2)	Root:shoot (g/g)
70	8	96·4 ± 4·4	248·1 ± 27·2	10·2 ± 3·0	562·8 ± 47·4	0·71 ± 0·06	140·982 ± 8·446	1·70 ± 0·15
70	1	45·0 ± 4·2	33·1 ± 6·6	2·7 ± 1·0	341·9 ± 48·4	0·32 ± 0·02	140·028 ± 5·624	2·31 ± 0·23
35	8	79·2 ± 11·3	73·2 ± 11·0	4·0 ± 1·1	443·9 ± 42·8	0·53 ± 0·07	134·797 ± 7·748	1·72 ± 0·25
35	1	37·0 ± 2·5	43·6 ± 5·8	4·2 ± 0·8	301·9 ± 26·8	0·28 ± 0·02	135·216 ± 7·055	2·43 ± 0·17
Statistical significance (P)								
CO_2		0·037	0·001	0·027	0·123	0·007	0·26	0·761
N		<0·001	<0·001	0·009	0·001	0·002	0·65	0·007
CO_2 × N		0·428	<0·001	0·135	0·434	0·029	0·56	0·829

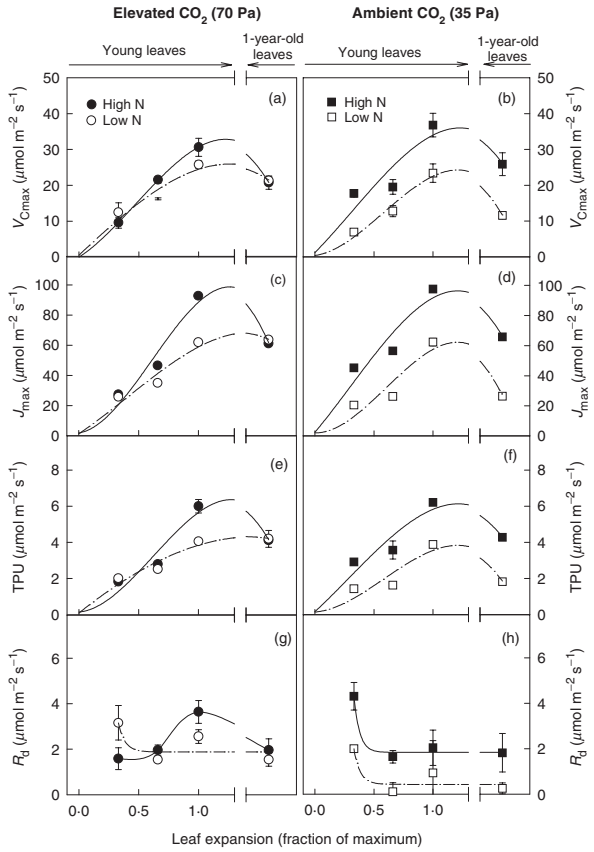

Figure 1. Evolution of V_{Cmax}, J_{max}, TPU and R_d on developing leaves at 1/3, 2/3 and 3/3 of maximum leaf area (young leaves) and in leaves of the previous year (1-year-old leaves) from *Q. suber* plants exposed for 4 years to elevated (70 Pa) or ambient (35 Pa) CO_2 under high (8 mM) and low (1 mM) N fertilization. Data points are the model estimates ± SE.

nificance ($P = 0{\cdot}08$) after accounting for the overall effects of age and N. There was no statistically significant reduction in RuBP regeneration capacity (as estimated from J_{max}) in elevated versus ambient CO_2 grown plants, whereas there was a significant reduction in J_{max} for the low N as compared with the high N-grown plants (Fig. 1c versus 1d). The capacities for utilization of Triose-P (TPU) (Fig. 1e versus 1f) as well as estimated dark respiration (R_d) were significantly affected by the N fertilization but not by CO_2 enrichment (Fig. 1g versus 1h). Relative to leaf development, effects of N fertilization were only significant after two-thirds of leaf expansion for plants grown under elevated CO_2, whereas for plants grown under ambient CO_2, the N fertilization treatments resulted in significant differences at the earlier stages of leaf development for both V_{Cmax}, J_{max} and TPU (Fig. 1a–f). Another striking effect of N fertilization was observed in the 1-year-old leaves. Whereas for plants grown under elevated CO_2, there were no significant differences in V_{Cmax}, J_{max} and TPU, plants grown under ambient CO_2 showed significant differences between the high and low N treatments for these photosynthetic parameters (Figs 1a–f; $P < 0{\cdot}05$). Development rates of the photosynthetic capacity (as estimated from the slopes of the parameters versus leaf expansion from Fig. 1) were not significantly affected by the CO_2 treatments after accounting for the N effects. However, these rates were significantly lower for the low N-grown plants.

Relative stomatal limitation (RSL) of photosynthesis, estimated from A–C_i curves, increased with leaf age (Fig. 2) and was 20% higher for fully expanded leaves of plants grown under ambient CO_2 relative to RSL from leaves grown under elevated CO_2 (Fig. 2). In addition, plants grown under ambient CO_2 and with low N had significantly lower RSL than plants fertilized with high N. However, the differences between the N regimes were not apparent for the plants grown under elevated CO_2.

Soluble and insoluble sugars

Concentrations (per unit of leaf area) of both soluble (suc, fru, glu) and insoluble sugars (starch) were significantly affected by leaf age, CO_2 and N treatments (Fig. 3a & b; $P < 0{\cdot}05$). The largest differences in soluble sugar contents were observed at the early leaf developmental stages between CO_2 treatments, whereas for fully developed leaves and 1-year-old leaves, there were no statistically significant differences (Fig. 3a & b). Plants grown with higher N fertilization had generally higher contents of soluble sugars although this was not always the case for all the leaf ages sampled. Starch concentration was significantly higher in plants grown under elevated CO_2 and elevated N. However, for plants grown under ambient CO_2, there was no statistically significant differences in starch content between N treatments (Fig. 3c & d).

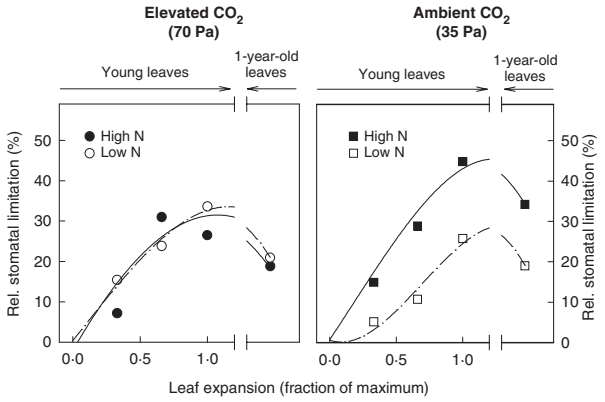

Figure 2. Evolution of relative stomatal limitation on developing leaves at 1/3, 2/3 and 3/3 of maximum leaf area (young leaves) and in leaves of the previous year (1-year-old leaves) from *Q. suber* plants exposed for 4 years to elevated (70 Pa) or ambient (35 Pa) CO_2 under high (8 mM) and low (1 mM) N fertilization. Data points were estimated from A–C_i curves as described in material and methods.

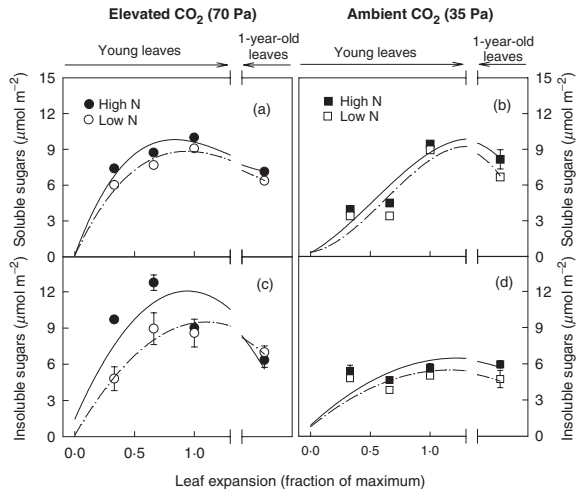

Figure 3. Soluble (sucrose, fructose and glucose) and insoluble (starch) sugar concentrations in developing leaves at 1/3, 2/3 and 3/3 of maximum leaf area (young leaves) and in leaves of the previous year (1-year-old leaves) from *Q. suber* plants exposed for 4 years to elevated (70 Pa) or ambient (35 Pa) CO_2 under high (8 mM) and low (1 mM) N fertilization. Data points are the average ± SE of four different leaves.

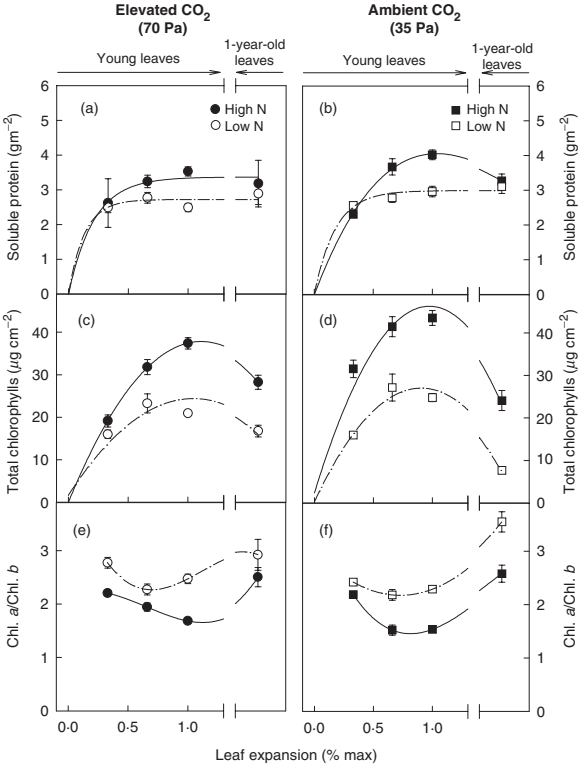

Figure 4. Evolution of soluble protein, total chlorophyll and ratio of chlorophyll a to chlorophyll *b* in developing leaves at 1/3, 2/3 and 3/3 of maximum leaf area (young leaves) and in leaves of the previous year (1-year-old leaves) from *Q. suber* plants exposed for 4 years to elevated (70 Pa) or ambient (35 Pa) CO_2 under high (8 mM) and low (1 mM) N fertilization. Data points are the average ± SE of four different leaves.

Protein, chlorophylls, nitrogen and phosphorus contents

Soluble protein content was significantly reduced in low N-grown plants as compared to elevated N-grown plants (Fig. 4a & b; $P = 0{\cdot}006$). There was an 18% reduction of soluble proteins in full-expanded young leaves from plants grown under elevated CO_2 as compared with ambient CO_2 when grown with high N, although this reduction was not statistically significant ($P = 0{\cdot}23$). For plants grown under low N as well as for 1-year-old-leaves from both treatments there were no differences in soluble proteins (Fig. 4a & b). The same trend was observed in total chlorophylls (Fig. 4c & d). Plants grown under low N had significantly lower chlorophyll contents (both chl *a* and chl *b*) but the reduction in chlorophyll content observed under elevated CO_2 (–16·5%, $P = 0{\cdot}20$) was not statistically significant. Ratios of chl *a* to chl *b* were significantly lower in plants grown with high N (Fig. 4e & f) but again there were no significant differences between the two CO_2 treatments. Concentration of N (% dry-matter) in fully expanded mature leaves was significantly lower in the low N and elevated CO_2-grown plants than in high N and ambient CO_2-grown plants (Fig. 5). Phosphorus concentration (% dry-matter) was also affected by N fertilization but not CO_2. Plants grown at high N showed the lowest P contents (Fig. 5).

DISCUSSION

Long-term biomass accumulation

The potential for biomass stimulation under long-term elevated CO_2 is dependent on nutrient availability, namely on N (Poorter 1998). We observed no differences in total biomass and leaf area of elevated CO_2 versus ambient CO_2-grown plants under low N fertilization, whereas for high N

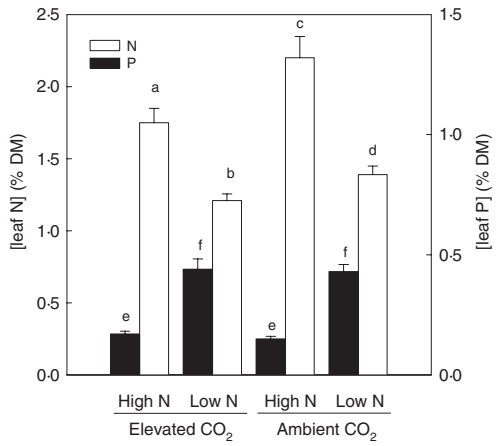

Figure 5. Nitrogen (N) and phosphorus (P) contents of mature leaves from *Q. suber* plants exposed for 4 years to elevated (70 Pa) or ambient (35 Pa) CO_2 under high (8 mM) and low (1 mM) N fertilization. Data points are the average ± SE of four different leaves. Different letter suffixes indicate statistically significant differences at $P = 0{\cdot}05$.

there was approximately 30% stimulation in biomass (Table 1). This was lower than found in average in a set of temperate zone or boreal forest trees grown in elevated CO_2 across Europe (Medlyn *et al*. 1999). However, as found in our study, there was a trend for lower responses to elevated CO_2 under low nutrient availability than otherwise. The response of slow growing species, as *Q. suber*, to elevated CO_2 was shown to be strongly limited by N supply (Poorter 1998). Responses to elevated CO_2 under low nutrient availability result in a large accumulation of carbohydrates that cannot be used for structural growth and these carbohydrates may be involved in signalling N deficiency through source–sink imbalance and down-regulation of photosynthesis (Paul & Driscoll 1997; Poorter 1998). For fast-growing species (mainly herbaceous), N deficiency may be driven by a dilution effect of N due to accelerated growth at elevated CO_2. In this scenario, acclimation may be an indirect result of N diversion for growth, rather than being a direct effect of N supply on photosynthesis (Farage, McKee & Long 1998; Rogers *et al*. 1998) and with *Pinus ponderosa* (a slow-growing species) Johnson, Ball & Walker (1997) suggest that simple growth dilution explain most nutrient responses.

There is evidence suggesting that signals derived from N deficiency act to regulate nitrogenous compounds uptake, organic acid synthesis and starch accumulation, modulate the sugar-mediated repression of photosynthetic genes and even to modulate allometric relations (reviewed by Stitt & Krapp 1999). However, it is not clear how these signals may interact in the long term. Studies with long-term CO_2 enrichment have not experimentally tested, or did not demonstrate, the effects of nutrient availability in acclimation processes (Tissue, Thomas & Strain 1993; Griffin *et al*. 2000). In this study, we observed that the response of a slow-growing species to elevated CO_2 was strongly limited by N. This is contradictory to a previous review study of 10 year research data by Idso & Idso 1994) where the authors claimed that growth stimulation by elevated CO_2 might not be limited by less than optimal nutrient availability [see also McGuire *et al*. (1995)]. In addition, allometric relations, namely the root : shoot ratio, was not affected by CO_2 but rather by N availability, with plants grown at lower N availability displaying higher root to shoots ratios (+ 35–40%). This is contradictory to a report by Norby (1994) in woody species were stimulation of root : shoot was about 6% due to elevated CO_2, with no effect due to the nutrient status. In another review by McGuire *et al*. (1995) where N levels were not manipulated, the increases in root : shoot under low N availability were considered statistically insignificant, but deemed to be a statistical artefact due to the low power of the statistical test. In contrast, in studies that actively manipulate N availability (like ours), there was a significant stimulation of root : shoot ratios under low N with contradictory effects of CO_2 (Ceulemans & Mousseau 1994; McGuire *et al*. 1995; Saxe, Ellsworth & Heath 1998). Elevated CO_2 may stimulate the growth of fine roots whereas N may stimulate the growth of coarse roots (Tingey *et al*. 1996) but limiting pot size may limit root growth, and change allometric relations (Arp 1991; Sage 1994). If pot size is limiting for root growth, then the root : shoot of elevated CO_2-grown plants may be altered in comparison with ambient CO_2-grown plants. In this study, with a slow-growing tree species, root : shoot ratios (as well as root biomass) were significantly affected by N availability, rather then CO_2, with no evidence for pot-size limitations (see Table 1).

Nutrient, protein and sugar contents of the leaves

The stimulation of biomass observed under elevated CO_2 and high N occurs in spite of a decrease in N and protein concentration in the leaves of elevated CO_2 relative to ambient CO_2-grown plants (Figs 4 and 5). Reductions in the contents of N and P under elevated CO_2 may be the result of a larger leaf area in elevated CO_2- grown plants. These results are consistent with other reports where N and protein content in leaves and woody tissues have been reported to decrease in response to long-term exposure to elevated CO_2 (Tissue *et al*. 1993; McGuire *et al*. 1995; Tissue, Griffin & Ball 1999). The decrease in N and P contents of the leaves has effects on C metabolism at both protein metabolism as well as the energetics of the photosynthetic system (namely for ATP synthesis). Plants grown under low N had higher P content compared to plants grown under high N, with no significant effect of the CO_2 treatment (Fig. 5). This may be the result of a lower utilization of P in the synthesis of energetic compounds, which are limited by enzyme activity and content. The efficiency of N and P utilization under elevated CO_2 seems to be interrelated and foliar P : N ratios for optimum photosynthesis may increase for elevated CO_2 conditions (Niinemets *et al*. 1999). Consistently, we found a statistically significant increase of P : N ratios for plants grown under elevated CO_2 as compared to ambient CO_2-grown plants when the plants were grown with high N ($0{\cdot}10 \pm 0{\cdot}01$ versus $0{\cdot}07 \pm 0{\cdot}004$, respectively, $P = 0{\cdot}01$). However, under low N, there were no statistically significant differences in the P : N ratio between elevated versus ambient CO_2-grown plants ($0{\cdot}36 \pm 0{\cdot}04$ versus $0{\cdot}31 \pm 0{\cdot}02$, respectively). Signals derived from N deficiency may be involved in molecular and biochemical responses of sugar metabolism in short-term exposure to elevated CO_2 (Stitt & Krapp 1999). Several years of exposure to elevated CO_2 and N deficiency may result in a diversion of N from the metabolic machinery to other sinks. Consistently, we observed the larger sugar contents (soluble and insoluble) in the high N treatment. However, differences in sugar contents between CO_2 regimes were present only at the early stages of leaf development, when the demand for N for leaf-growth and metabolism is larger.

Effects over the photosynthetic capacity development and relative stomatal limitation of photosynthesis

Results from modelling of A–C_i curves suggest that maximal Rubisco activity is down regulated by about 20% in mature

leaves, whereas there were no differences in capacities for RUBP regeneration (estimated from J_{max}) and TPU under elevated CO_2 as compared to ambient CO_2-grown plants (Fig. 1). Decreases in Rubisco activity with long-term acclimation mimics the shorter term effects of elevated CO_2 (Tissue *et al*. 1993; Turnbull *et al*. 1998; Medlyn *et al*. 1999; Tissue *et al*. 1999; Griffin *et al*. 2000). However, these results are contradictory to the slight increase in Rubisco capacity (although not statistically significant) observed in a life-term CO_2 enrichment experiment with *Q. pubescens* (Stylinski *et al*. 2000). No effect of elevated CO_2 was observed over maximum electron transport capacity as well as triose-P utilization. This contrasts with the decrease in activity of phosphoribulose kinase (which activity can be approximated by J_{max}) observed in short-term experiments with fast-growing species (Maroco *et al*. 1999; Medlyn *et al*. 1999; Griffin *et al*. 2000). Inversely, fructose 1, 6 bis-P phosphatase and ADP-glucose pyrophosphorylase, which are key enzymes in the utilization of triose-P, have been reported to show no significant reduction or even increase in activity under elevated CO_2 (Ludewig *et al*. 1998; Maroco *et al*. 1999). These results were consistent with our estimates of TPU.

There were no statistically significant differences in the developmental rates of photosynthetic capacities of leaves growing under elevated versus ambient CO_2. However, the development of photosynthetic capacity was delayed when plants were grown under low N. This is contrary to one report with tobacco where exposure to elevated CO_2 resulted in an earlier shift of maximum photosynthetic capacity and a subsequent faster senescence (Miller *et al*. 1997). However, in a recent study with six C_3 grassland species, Craine & Reich (2001) found an increase in leaf area and longevity under elevated CO_2 but not under elevated N. In our study, under elevated CO_2, 1-year-old leaves showed no significant differences in photosynthetic capacity at high or low N. However, for ambient CO_2-grown leaves, there was a significant decrease in photosynthetic capacity under low N which may be associated with remobilization and export of N from old to new expanding leaves. Growth under elevated CO_2 actually delayed senescence of old-leaves (as evaluated visually) and this effect (increased leaf area duration) may improve assimilate availability for growth and biomass accumulation.

Effects of elevated CO_2 on dark respiration are still controversial. Elevated CO_2 may inhibit or stimulate dark respiration (for a review see, e.g. Poorter *et al*. 1992; Tjoelker *et al*. 2001). In this study, we did not see any consistent difference in dark respiration, as estimated by modelling, between CO_2 treatments after accounting for the N effects. The value of R_d was consistently lower during the development of the leaf for the low N plants as compared to the high N plants when grown at ambient CO_2, but these differences were not statistically significant for plants grown under elevated CO_2 .

Relative stomatal limitation of photosynthesis, as estimated from A–C_i curves, was 20% higher in mature leaves grown at ambient CO_2 than in plants grown at elevated CO_2 (Fig. 2). Again, the effects of N nutrition were not apparent in the elevated CO_2-grown plants, whereas for ambient CO_2-grown plants, there was a significantly higher RSL for plants grown at high N. A decrease in RSL under elevated CO_2 may be due to a higher availability of substrate for photosynthesis, which coupled with a reduction in photorespiration may overcome the reduction in stomatal conductance observed at elevated CO_2. If there is no reduction in stomatal density, a reduction in stomatal conductance will be set by intercellular CO_2 concentration, which is correlated with the functioning of the photosynthetic system under elevated CO_2 (Jarvis, Mansfield & Davies 1999). On the other hand, if photosynthesis is restricted due to a reduction in photosynthetic enzymes (e.g. Rubisco) under low N fertilization, the limitation imposed by stomata over the CO_2 assimilation process will be relatively lower as observed in our study at ambient CO_2 and low N. In the long term, a lack of sensitivity of stomata to CO_2 under elevated CO_2 may characterize woody species (Saxe *et al*. 1998).

CONCLUDING REMARKS

The long-term stimulation of biomass in *Q. suber* grown at 70 Pa CO_2 for 4 years (approximately 30% in comparison to the ambient CO_2-grown plants) resulted from the higher CO_2 availability and from the shift in the partition of N from the photosynthetic machinery (mainly from Rubisco) to growth and leaf area expansion. N, rather than CO_2, limited the development of the photosynthetic capacity in newly forming leaves, promoted the investment of photoassimilates towards the roots, and changed the optimal P : N ratio for photosynthesis. Data presented in this study suggest that the potential for biomass stimulation by elevated CO_2 in *Q. suber* is very much dependent on N availability. Therefore, in natural Mediterranean ecosystems – where N availability is generally low – biomass stimulation by elevated CO_2 may be insignificant due to N limitation for photosynthesis and growth, rather than to acclimation to elevated CO_2 partial pressures.

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