Life-long growth of *Quercus ilex* L. at natural CO$_2$ springs acclimates sulphur, nitrogen and carbohydrate metabolism of the progeny to elevated pCO$_2$

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**ABSTRACT**

The aim of the present study was to analyse whether offspring of mature *Quercus ilex* trees grown under life-long elevated pCO$_2$ show alterations in the physiological response to elevated pCO$_2$ in comparison with those originating from mature trees grown at current ambient pCO$_2$. To investigate changes in C-, (for changes in photosynthesis, biomass and lignin see Polle, McKee & Blaschke 2001), N-, and S-metabolism soluble sugar, soluble non-proteinogenic nitrogen compounds (TSNN), nitrate reductase (NR), thiols, adenosine 5’-phosphosulphate (APS) reductase, and anions were analysed. For this purpose *Q. ilex* seedlings were grown from acorns of mother tree stands at a natural spring site (elevated pCO$_2$) and a control site (ambient pCO$_2$) of the Laiatico spring, Central Italy. Short-term elevated pCO$_2$ exposure of the offspring of control oaks lead to higher sugar contents in stem tissues, to a reduced TSNN content in leaves, and basipetal stem tissues, to diminished thiol contents in all tissues analysed, and to reduced APS reductase activity in both, leaves and roots. Most of the components of C-, N- and S-metabolism including APS reductase activity which were reduced due to short-term elevated pCO$_2$ exposure were recovered by life-long growth under elevated pCO$_2$ in the offspring of spring oaks. Still TSNN contents in phloem exudates increased, nitrate contents in lateral roots and glutathione in leaves and phloem exudates remained reduced in these plants. The present results demonstrated that metabolic adaptations of *Q. ilex* mother trees to elevated pCO$_2$ can be passed to the next generation. Short- and long-term effects on source-to-sink relation and physiological and genetic acclimation to elevated pCO$_2$ are discussed.

**Key-words**: amino compounds; cysteine; ecotypic differentiation; elevated pCO$_2$; glutathione; oak; phloem transport; sugar.

**Abbreviations**: CHES, 2-(cyclohexylamino)-ethansulphonacid; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; γ-EC, γ-glutamylcysteine; mBBr, monobromobimane; NR, nitrate reductase; PVPP, polyvinylpyrrolidone; TSNN, total soluble non-proteinogenic nitrogen.

**INTRODUCTION**

Rising atmospheric pCO$_2$ affects physiological processes and, consequently, growth and development of plants. The results of investigations into the effects on the carbohydrate and nitrogen metabolism as well as on water, and nutrients uptake have been published (Kirschbaum et al. 1994; Saxe, Ellsworth & Heath 1998; Stitt & Krapp 1999; Ward & Strain 1999; Bassirirad 2000). Physiological traits and growth characteristics under elevated pCO$_2$ were mostly obtained from plant populations grown for multiple generations at the current ambient pCO$_2$. The exposure to elevated pCO$_2$ was performed as a completely new environment in a one-step change. Therefore, the results achieved do not necessarily reflect the effects of the rapid, but continuous increase in atmospheric pCO$_2$ mixing ratio since the industrial revolution which is also expected to continue for the next century (cf. Andalo, Godelle & Moussau 1999). Strong differences between plant species, the experimental design, the time of exposure, the use of adult plants or seedlings, the water and nutrient supply were observed (Saxe et al. 1998).

For estimating the consequences of elevated pCO$_2$, genetically fixed and long-term physiological acclimation processes have to be distinguished. Both regulate plant metabolism to achieve homeostasis in a changing environment by feedback and/or transcriptional control. A new equilibration may be reached after weeks, years, or even centuries (Shugert et al. 1986; Davis 1989). However, only genetically manifested changes will be passed to the next generation. Naturally pCO$_2$ enrichment sites are a tool to address this question and to estimate the impact of increasing global pCO$_2$ on natural ecosystems in predictive models. The time scale of exposure to elevated pCO$_2$ has been sufficiently long to allow both physiological and genetic adaptation. The aim of the presented experiment was to analyse whether offspring of mature trees grown under life-long elevated pCO$_2$ show alterations in the physiological
response to elevated $pCO_2$ in comparison with those originating from mature trees grown at current ambient $pCO_2$.

Polle, McKee & Blaschke (2001) determined, in a reciprocal experimental design, the biomass, photosynthesis, foliar carbohydrate concentrations and structural biomass, including lignin, during leaf maturity in Quercus ilex seedlings, which were grown from acorns of mother tree stands at a natural spring site (elevated $pCO_2$; spring oaks) and a control site (ambient $pCO_2$; control oaks) in Laiatico, Central Italy. Under ambient $pCO_2$ the biomass and foliar carbon did not differ between spring or control oak seedlings. Under elevated $pCO_2$ the whole tree biomass, total leaf area and stem diameter were significantly increased in both sets of seedlings. However, above-ground biomass accumulation showed a higher stimulation in spring compared with control oak seedlings. In contrast, a higher stimulation of below-ground biomass was observed in control oak seedlings. Independent of the seed source a higher photosynthetic rate, but no significant differences in non-structural carbohydrates were observed under elevated $pCO_2$. However, lignification of leaves was delayed during short-term exposure to elevated $pCO_2$, but not after long-term exposure. These results indicate that life-long exposure of the parents mediated acclimation to elevated $pCO_2$ (Polle et al. 2001).

To determine the acclimation of Q. ilex seedlings to elevated $pCO_2$ in more detail we investigated, in the same experiment, soluble compounds and key enzymes of the main metabolic pathways, namely the carbohydrate, nitrogen and sulphur metabolism in different tissues and in phloem exudates. The following hypotheses were investigated: (1) how the origin of the acorns either from ambient $pCO_2$ (control oaks) or from elevated $pCO_2$ (spring oaks) affects physiological parameters. This was investigated after growth of both progenies under ambient $pCO_2$. (2) How elevated $pCO_2$ mediates short-term effects on oak seedlings. This was studied with control oak seedlings by comparing seedlings under ambient and elevated $pCO_2$. (3) How physiological parameters are adapted to life-long growth under elevated $pCO_2$. These effects were analysed by comparing control oaks and spring oaks grown under elevated $pCO_2$.

MATERIALS AND METHODS

Seed sampling sites and plant material

Acorns of Quercus ilex L. were collected from the natural CO$_2$ spring (spring site) ‘Laiatico’ in Tuscany, Italy as described by Polle et al. (2001). The spring is located approximately 10 km north-west of the city of Volterra, Italy (43°24′N, 10°50′E) within the village of Laiatico (Hättenschwiler et al. 1997; Schulte et al. 1999). The geography of the site was characterized by Stylinski et al. (2000). The $pCO_2$ in the spring varies with windspeed and convective turbulence. A maximum $pCO_2$ of 2000 p.p.m. were measured (Schulte et al. 1999). This $CO_2$ spring was selected because contamination with $H_2S$ and $SO_2$ reached maximum values of 60 and 4 p.p.b., respectively, which are low in comparison with other $CO_2$ springs. The average $pH_2S$ and $pSO_2$ of 20 and 1-5 p.p.b., respectively, did not reach concentrations thought to mediate visible symptoms of injury (De Kok et al. 1983; De Kok, Stuiver & Stulen 1998). In fact, no symptoms of $H_2S$ and $SO_2$ damage were observed at the field site. However, it has to be assumed that atmospheric sulphur is taken up by the leaves, and used as an additional sulphur source (De Kok et al. 1997; Herschbach et al. 2000). If this influx is significant, elevated contents of sulphate (from $SO_2$) and thiols (from $H_2S$) are to be expected. As the leaves, bark and wood from Q. ilex collected from the Laiatico spring did not differ significantly from tissues of plants collected at the control site in sulphate and thiol contents (Schulte 1998), it was assumed that atmospheric sulphur did not influence sulphur metabolism considerably.

Other acorns were collected at a distance of about 200 m from the spring site under ambient $pCO_2$ (control site). The hill exposition, the morphology, the soil structure, composition and hydrology as well as the vegetation of the control site were similar to the spring site. For a detailed description of the sites see Raisies (1998) and Tognetti, Cherubini & Innes (2000). Acorns from the spring and the control site were of the same size class.

Acorns of Q. ilex from both the control and spring site were germinated and cultivated in growth tubes, 8 cm in diameter and 40 cm in length (Seegmüller & Rennenberg 1994). To avoid uncontrolled mycorrhization the substrate was inoculated with Laccaria laccata (Polle et al. 2001). Seedlings were grown for a total of 8 months in a controlled environment (growth chambers HPS 1500; Heraeus Vötsch, Hanau, Germany) at day/night (16/8 h) with a photosynthetic active radiation (PAR) of 225–250 $\mu$E m$^{-2}$ s$^{-1}$ at 80 cm height, 20/15 °C temperature and 60/70% relative air humidity. Every two days 20 mL water was added to each seedling. The oak seedlings grown from acorns from both the spring site and the control site were exposed either under elevated (700 p.p.m.) or ambient $pCO_2$ (350 p.p.m.). To avoid chamber effects two environmental growth chambers were used for each treatment.

Sampling of different tree tissues

After 8 months biometric parameters of the seedlings were determined by Polle et al. (2001). Mature leaves were collected from the youngest fully developed leaf thrust. The trunk was dissected into stem and roots. The stem was divided into apical and basipetal parts from which bark and wood were separated. Lateral roots were dissected from the main root which then was divided into bark and wood. All samples were weighed, frozen in liquid nitrogen and stored at −80 °C until analysis.

Collection of phloem exudate

Phloem exudates were collected from apical and basipetal stem bark slices. Bark slices of approximately 200 mg fresh
weight were washed in 20 mM ethylenediaminetetraacetic acid (EDTA) and allowed to equilibrate in different incubation solutions. The incubation solution for thios at 4 °C contained 20 mM EDTA, 1 mM cyanide at pH 5-9 and polyvinylpyrrolidone (PVPP) at a PVPP/bark fresh weight rati of 1 based on Herschbach, Jouanin & Rennenberg (1998). Exudation of soluble amino compounds were performed as described by Schneider et al. (1996). Patterns of sucrose exudation were analysed as a control and were independent of the equilibration solution applied. After 5 h of incubation sucrose exudation was nearly completed and amounted to 90–95% compared to 12 or 24 h exudation, respectively. Since acid invertase activity was below 4% of the corresponding bark slices, cellular and apoplastic contamination were disregarded.

Analysis of thios

In a modified method from Herschbach et al. (1998) thios in phloem exudates were determined as the sum of the respective reduced and oxidized forms. Phloem exudates were centrifuged at 16 000 g and 4 °C for 10 min. Aliquots of 200 μL of the supernatant were adjusted to pH 8.3 ± 0.2 by adding 75 μL of 200 mM CHES (2-(cyclohexylamino)-ethansulfonicacid), pH 9.1. Reduction of thios was initiated by addition of 15 μL 15 mM dithiothreitol (DTT) and terminated after 60 min by addition of 20 μL 30 mM monobromobimane (mBBr) for derivatization. After 15 min derivatization was stopped by acidification with 250 μL 5% (v/v) acetic acid to stabilize mBBr-thiol derivatives. Aliquots of this solution were used for high-performance liquid chromatography (HPLC) analysis.

For thiol analysis tree tissues were homogenized in a mortar under liquid N2 and extracted from approximately 50 mg frozen powder. Samples were transferred into precooled (4 °C) vials containing 1.5 mL 0.1 N HCl plus 100 mg insoluble PVPP and were centrifuged at 16 000 g and 4 °C for 15 min. Aliquots of 200 μL of the supernatant were adjusted to pH 8.3 ± 0.2 with 150 μL 500 mM CHES pH 9.4. Oxidized thios were reduced for 60 min by adding 15 μL 5 mM DTT. Derivatization was performed with 20 μL 30 mM mBBr for 15 min. Subsequently, thiol derivatives were stabilized with 250 μL acetic acid (5% v/v). Thiol derivatives were separated and quantified after HPLC analysis by fluorescence detection as described by Schupp & Rennenberg (1988). Peaks were identified and quantified using a standard solution containing 0.2 mM cysteine (Cys), 0.1 mM γ-glutamylcysteine (γEC) and 1 mM glutathione (GSH) in 0.01 M HCl. Recovery of Cys, γEC, and GSH amounted 62–92%, 87–104%, and 82–96%, respectively.

Analysis of soluble amino compounds

For determination of soluble amino compounds phloem exudates were adjusted to pH 2.2 with 1 N HCl. Aliquots of 70 μL were taken for free soluble amino compounds analysis. Tissue samples were powdered under liquid nitrogen and free amino compound were extracted based on Winter, Lohaus & Heldt (1992) and were analysed as described by Schneider et al. (1996). Aliquots of 70 μL of the extract were taken for automatic amino acid analysis (LKB 4151; Alpha Plus; Pharmacia, Freiburg, Germany).

Amino compounds were separated on a PEEK (Ultrapac 8, lithium form L-1352, pore size 8–9 μm, 250 × 4.6 mm; Pharmacia) column according to Schneider et al. (1996) with a system consisting of five lithium-citrate buffers. After post-column derivatization with ninhydrin absorption of the amino-ninhydrin derivatives was measured at 440 and 570 nm. Peaks were identified and quantified using an amino acid standard solution containing 39 amino compounds (each 0.5 mM) plus ammonium (Sigma, Munich, Germany). Recovery of amino compounds was determined by adding aliquots of the amino acid standard solution to the extraction solution of tissues and amounted to 80–100%.

Sugar analysis

Twenty milligram PVPP was added to 1 mL of a 1:40 dilution of phloem exudates at 4 °C for 60 min. Recovery was determined with 20 μL of a 0.5 M standard solution containing myo-inositol, glucose, fructose and sucrose and ranged from 50 to 100%. Tissues were extracted from aliquots of frozen powder in 1 mL bi-distilled water, containing 100 mg PVPP; for analysis, extracts were diluted 1:40. Recovery was determined by adding 4 μL of the standard solution to the extraction medium and ranged from 77 to 104%. Aliquots of the diluted phloem exudates and tissue extracts were used for sugar analysis by an automated sugar analyser (DX 500; Dionex, Idstein, Germany). Myo-inositol, sucrose, glucose and fructose were separated on an anion exchange column (CarboPac PA 1, 4 × 250 mm; Dionex) with an isocratic NaOH system. Separation was carried out with 36 mM NaOH free of carbohydrate within 36–50 min at a flow rate of 1 mL min⁻¹. Sugars were detected by pulsed amperometry. Peaks were identified and quantified using a standard solution containing 0.1 mM myo-inositol, sucrose, glucose and fructose. Subsequent to each separation, the column was regenerated with 200 mM NaOH free of carbohydrate and equilibrated for 24 min with 36 mM NaOH at a flow rate of 1 mL min⁻¹.

Anion analysis

Anions were extracted from tissue samples powdered in liquid N2 in a mortar. Aliquots of 150 mg were suspended in 2 mL of twice-distilled water containing 20 mg insoluble PVPP to bind phenolic compounds. After shaking for 1 h at 4 °C, samples were boiled for 15 min, centrifuged for 5 min and again for 10 min, at 16 000 g × 4 °C (Centrifuge 5402; Eppendorf, Engelsdorf, Germany). The clear supernatant was used for anion analysis by anion exchange chromatography. Anions were separated on a IonPac column (AS9-SR, 250 × 4 mm; Dionex) eluted with a mixture of 1.8 mM Na2CO3 and 1.7 mM NaHCO3 at a flow rate of
1·1 mL min\(^{-1}\). Detection of anions was achieved by a conductivity detector module (CDM; Dionex).

**Determination of APS reductase and nitrate reductase activities**

Both enzymes, nitrate reductase (NR, E.C. 1·6.6·1/1·6.6·2) and the adenosine 5’phosphosulphate (APS) reductase (APR, E.C. 1·8.99.) were extracted as described by von Ballmoos, Nussbaum & Brunold (1993) and von Ballmoos et al. (1998), respectively, for leaves (\(n=12\)) and roots (\(n=10\)). One part of leaf or root material was homogenized in 10 parts extraction buffer for 20 and 5 s with a polytron. Crude extracts were filtered through two layers of miracloth and used for determination of both NR and APR activities as well as protein contents.

The NR activity was determined after von Ballmoos et al. (1993, 1998). The nitrite produced was detected spectrophotometrically at 546 nm with the diazotization reaction in a 1:1 mixture of the enzyme assay and 1% (w/v) sulphanilamide in 1·5 M HCl/0·2% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride. The APS reductase activity was determined as the formation of \(^{35}\)S-sulphite from AP\(^{35}\)S in the presence of the artificial carrier dithioerythritol (DTE) based on Brunold & Suter (1983). The enzyme assay contained 50 \(\mu\)L 1 M TRIS/HCl pH 9, 200 \(\mu\)L 2 M MgSO\(_4\), 50 \(\mu\)L H\(_2\)O, 10 \(\mu\)L 0·2 M DTE and 80 \(\mu\)L 1:2 diluted leaf or root extract. The reaction was initiated by the addition of 10 \(\mu\)L 3·75 mM AP\(^{35}\)S (22·2 kBq \(\mu\)mol\(^{-1}\)) and 100 \(\mu\)L 1 M Na\(_2\)SO\(_3\). After 30 min incubation at 37 °C the reaction tube was transferred into a 20 mL scintillation vial containing 1 mL 1 M triethanolamine pH 9–11 and 200 \(\mu\)L 1 M H\(_2\)SO\(_4\), were added to liberate \(^{35}\)S-sulphite as \(^{35}\)SO\(_2\). After 12–24 h at room temperature the reaction tube was removed and the trapping solution was mixed with 2 mL scintillation fluid. The \(^{35}\)S radioactivity was detected by liquid scintillation counting (Betamatic; Kontron Instruments, Zurich, Switzerland). Protein contents were determined according to Bradford (1976) using BSA as a standard.

**Data analysis**

Data shown are means ± SD of samples from six oak seedlings of each treatment and progeny trees. Student’s \(t\)-test and the multifactorial Duncan test (SPSS for Windows, 7·0; SPSS Inc., Chicago, IL, USA) were applied to determine significant differences between treatments and progenies.

**RESULTS**

**Total soluble sugar in tree tissues and phloem exudates**

Elevated \(p\)CO\(_2\) and the origin of the acorns did not influence the composition of the samples of individual sugar compounds (data not shown). Therefore, total soluble sugar contents are presented.

(1) Under ambient \(p\)CO\(_2\) the origin of acorns influenced soluble sugar only in apical stem tissues (Fig. 1A & B). In comparison with spring oaks, control oaks had 1·8 and 2·9 times higher total soluble sugar contents in the apical and basipetal bark, respectively. In other tissues and in phloem exudates total sugar contents were unaffected by the origin of the acorns and the \(p\)CO\(_2\) treatment.

![Figure 1. Total soluble sugar contents in tissues and phloem exudates of *Quercus ilex* seedlings. *Quercus ilex* seedlings were grown from acorns of mother tree stands with natural elevated \(p\)CO\(_2\) (B, D; spring oaks) or from acorns of mother tree stands with natural ambient \(p\)CO\(_2\) (A, C; control oaks). Both progenies were either grown under ambient (A, B) or under elevated \(p\)CO\(_2\) (C, D). Sugars in tissues were extracted in water and quantified in tissues as well as in phloem exudates by anion exchange chromatography. Different letters indicate statistically significant differences between treatments.](image-url)
(2) Short-term CO₂ effects were observed in basipetal stem and in root tissues (Fig. 1A & C). Sugar contents approximately doubled under elevated pCO₂, but were similar in phloem exudates.

(3) Life-long elevated pCO₂ will lead to a recovery of total soluble sugar contents except in the basipetal bark (Fig. 1C & D). Although total soluble sugar concentrations in phloem exudates seem to increase in the basipetal stem bark, this effect was not statistically significant.

(4) When spring oaks were reset to ambient pCO₂, soluble sugar contents decreased by 34% in the apical and by 83% in basipetal bark, respectively. This values were below the level of control oaks under ambient pCO₂ (Fig. 1B & D). Sugar contents in phloem exudates, however, remained unaffected.

Composition and contents of total soluble non-proteinogenic nitrogen compounds and NR in tree tissues and phloem exudates

(1) Under ambient pCO₂ total soluble non-proteinogenic nitrogen compounds (TSNN) in the stem bark and in the root wood of spring oaks was approximately twice that of control oaks (Fig. 2A & B). The provenance of the acorns did not affect protein contents and NR activities in roots and leaves, respectively (Fig. 7, Treatment A & B).

(2) Short-term exposure to elevated pCO₂ decreased TSNN in the leaves close to zero when the protein content slightly increase and the NR activity also slightly, but not significantly, increased. Although TSNN in phloem exudates was unaffected (Fig. 2A & C) in basipetal stem wood TSNN decreased five-fold and in the root bark and wood 10- and 3-3-fold, respectively. In contrast TSNN increased approximately two-fold in the basipetal stem bark and the apical stem wood. Short-term elevated pCO₂ did not change protein content in roots, and the NR activity was slightly, but not significantly, reduced (Fig. 7, Treatment A & C).

(3) Life-long growth under elevated pCO₂ recovered TSNN in leaves and in tissues of the main root to the level of control oaks under ambient pCO₂ (Fig. 2A & D). But, the protein content increased in leaves during life-long compared to short-term growth under elevated pCO₂, although the NR activity was not different (Fig. 7, Treatment C,D). Phloem contents of TSNN seemed to increase after life-long elevated pCO₂ over that level of control oaks under elevated pCO₂, but this effect was statistically significant for basipetal phloem only (Fig. 2C & D). Recovery of TSNN contents was not observed in the apical stem wood, which remained enhanced after life-long growth under elevated pCO₂. In the roots the unchanged TSNN and protein content was accompanied by an decreased NR activity in comparison to control oaks under ambient pCO₂ (Fig. 7, Treatment A & D).

(4) After resetting spring oaks to ambient pCO₂ in basipetal and apical stem bark, as well as in the root wood, TSNN contents increased (Fig. 2B & D). Simultaneously, the protein content and the NR activity was decreased in leaves whereas in roots the NR activity increased to the level of control oaks (Fig. 7, Treatment B & D). Export of TSNN content, i.e. the content in basipetal phloem exudates remained slightly enhanced although this effect was not statistically significant.

Figure 2. Total soluble non-proteinogenic nitrogen (TSNN) contents in tissues and phloem exudates of Quercus ilex seedlings. Quercus ilex seedlings were grown from acorns of mother tree stands with natural elevated pCO₂ (B, D; spring oaks) or from acorns of mother tree stands with natural ambient pCO₂ (A, C; control oaks). Both progenies were either grown under ambient (A, B) or under elevated pCO₂ (C, D). Amino compounds separated by anion exchange chromatography were identified and quantified as ninhydrin derivatives at 570 nm. Different letters indicate statistically significant differences between treatments.
Amino compounds in TSNN

Three amino compounds, namely Asn, Arg, and Asp, dominated the TSNN pool of all oak seedlings independent of pCO₂ and the provenance of the acorns. In the leaves, stem tissues, and root tissues these compounds accounted for more than 80% of TSNN (Fig. 3). In lateral roots also ammonium and in phloem exudates ammonium plus Gln contributed significantly to the respective TSNN pool.

1. Under ambient pCO₂, significant differences between the provenance of the acorns were observed for the three main amino compounds (Fig. 3A & B). In leaves of spring oaks Asp was 52% lower compared with control oaks. In basipetal stem tissues Asn contents were 256% and Asp contents 200% higher in the bark of spring oaks, but Asn was increased to 74% and Asp increased to 167% in the wood. In basipetal phloem exudates of spring oaks Asn, Asp and ammonium were significantly higher, and in addition Arg and Gln tended to be increased.

2. Short-term exposure to elevated pCO₂ also affected the composition of the three most abundant amino compounds (Fig. 3A & C). In leaves, all three amino compounds decreased dramatically. In basipetal tissues Arg increased in the stem bark (by 400%), but Asn (to 21%) and Asp (to 8%) decreased in the root bark. Wood Asn contents increased in the apical stem (to 270%), but decreased in basipetal stem parts (to 22%). In the basipetal stem wood Arg also decreased (to 16%). In the wood of the roots the contents of all three amino compounds decreased. In the apical and basipetal phloem exudates Asn, Arg, and Asp decreased, whereas Gln increased to 300 and 125%, respectively.

3. In spring oaks acclimation to elevated pCO₂ was observed in several tissues. In comparison with the control oaks under elevated pCO₂, contents of Asn, Arg, and Asp in leaves, in the root bark and in basipetal phloem exudates were high when spring oaks were cultivated under elevated pCO₂ (Fig. 3C & D). In contrast, in basipetal stem bark the contents of all three amino compounds were lower in spring oaks compared with the control oaks under elevated pCO₂, but were similar in the apical stem bark. In the wood, significantly higher Arg contents were found in basipetal stem parts of spring oaks. Asp, Gln and NH₄⁺ contents were higher in phloem exudates from apical and basipetal stem sections of spring oaks. In basipetal phloem exudates Asn and Arg were also enhanced.

4. When spring oaks were cultivated under ambient pCO₂ the high contents of the amino compounds observed under elevated pCO₂ were maintained or even further enhanced. The latter was observed especially in basipetal tissues. Asn increased in the bark (to 513%), and both Asn (to 240%), and Arg (to 190%) increased in the wood (Fig. 3B & D). Higher Arg contents were found in the root wood (to 201%). In contrast, Asn (to 31%) and Arg (to 47%) decreased in the wood of the apical stem under ambient compared to elevated pCO₂. Under ambient pCO₂ Asn and Arg were enhanced in apical, but were reduced in basipetal phloem exudates. Asp and Gln were decreased in both apical and basipetal phloem exudates of spring oaks under ambient pCO₂.

All the differences described are based on µmol N per g tissue fresh weight. When these three main amino compounds were expressed as percentage of TSNN, then differences between the CO₂ regime and/or the origin of the oak seedlings were not observed. The percentage distribution of main TSNN compounds was similar in all tissues and in phloem exudates (data not shown).

Thiol composition and contents in tree tissues and phloem exudates

1. The origin of the acorns did not affect the GSH and the Cys content in the oak tissues under ambient pCO₂. Only in the basipetal stem bark was the Cys content higher in tissues of spring than control oaks (Figs 4, 5A & B). Furthermore, the APR activity did not vary between the two provenance of the acorns, either in the roots or in the leaves (Fig. 7). In apical and in basipetal phloem exudates, however, the GSH content was significantly lower in spring than in control oaks.

2. In all tissues, the short-term exposure to elevated pCO₂ reduced GSH and Cys contents between 37 and 57% and between 35 and 59%, respectively (Figs 4, 5A & C). Surprisingly, the APR activity in leaves was reduced to 26% (Fig. 7). Cys contents in phloem exudates were not affected by short-term elevated pCO₂. However, in common with the leaves, the GSH contents were decreased dramatically to 8 and 15% in apical and basipetal phloem exudates, respectively, whereas both GSH and Cys contents in the lateral roots and Cys contents in the stem bark were unaffected. Nevertheless, the APR activity in lateral roots was also reduced by short-term exposure to elevated pCO₂ to 14% (Fig. 7).

3. Life-long exposure to elevated pCO₂, however, led to a recovery of GSH and Cys contents in all stem tissues to the level of the control oaks under ambient pCO₂. Only Cys and GSH in the leaves remained diminished (Figs 4, 5C & D) although the APS reductase activity recovered (Fig. 7). The GSH content in phloem exudates increased but did not reach the level of controls under elevated pCO₂ whereas the Cys contents were not changed by life-long exposure to elevated pCO₂ (Figs 4, 5C & D). GSH in lateral roots was even further increased above the level of control oaks under ambient pCO₂. Under these conditions, the APS reductase activity in the roots recovered (Fig. 7).

4. Cultivating spring oaks under ambient pCO₂ caused GSH and Cys to increase to 189 and 220%, respectively, in the leaves, to reach the level of control oaks under ambient pCO₂. However, APS reductase activity in leaves and roots did not change when spring oaks were cultivated under ambient pCO₂ (Fig. 7). The GSH content in phloem exudates, however, doubled under these conditions, but did not reach the level of control oaks under ambient pCO₂ (Fig. 4B & D). In the basipetal stem bark, Cys increased to 175% and in lateral roots GSH decreased to 29%. In all
Figure 3. Main amino compounds in tissues and in phloem exudates of *Q. ilex* seedlings. Quercus ilex seedlings were grown from acorns of mother tree stands with natural elevated pCO₂ (B, D; spring oaks) or from acorns of mother tree stands with natural ambient pCO₂ (A, C; control oaks). Both progenies were either grown under ambient (A, B) or under elevated pCO₂ (C, D). Amino compounds were identified as described in Fig. 2. Different letters indicate statistically significant differences between treatments.
other tissues Cys and GSH contents were not affected (Figs 4, 5B &D).

**Anions in tree tissues**

1. Sulphate, nitrate and phosphate contents did not vary between control and spring oaks. Both provenance had comparable anions contents in all tissues (Fig. 6A & B).

2. Short-term exposure to elevated pCO₂ affected anion contents in root tissues but not in above-ground tissues (Fig. 6A & C). Sulphate in root wood declined to 29% and nitrate in the root bark and in lateral roots decreased to 31 and 38%, respectively.

3. Life-long exposure to elevated pCO₂ increased the sulphate content in the root wood and the nitrate content in the root bark and the contents recovered to the level of that of the control oaks under ambient pCO₂ (Fig. 6C & D). The reduced nitrate content in the lateral roots by short-
Life-long growth under elevated $pCO_2$ acclimates S, N and C metabolism

Figure 6. Sulphate, nitrate and phosphate contents in tissues of *Q. ilex* seedlings. *Quercus ilex* seedlings were grown from acorns of mother tree stands with natural elevated $pCO_2$ (B, D; spring oaks) or from acorns of mother tree stands with natural ambient $pCO_2$ (A, C; control oaks). Both progenies were either grown under ambient (A, B) or under elevated $pCO_2$ (C, D). Anions were separated by anion exchange chromatography and detected by conductivity measurements. Different letters indicate statistically significant differences between treatments.
term elevated pCO2 was maintained after life-long elevated pCO2. Phosphate in root tissues increased to the level of control oaks under ambient pCO2.

(4) When spring oaks were grown under ambient pCO2, the sulphate contents of the leaves increased (to 165%, Fig. 6B & D) and, in addition, the nitrate in basipetal stem wood and lateral roots increased to 151 and 213%, respectively. The phosphate contents in the root bark decreased to 51%.

**DISCUSSION**

**Short-term effects of elevated CO2 are reversed at life-long elevated pCO2**

The present study demonstrates that short-term exposure of *Q. ilex* to elevated pCO2 affects source-to-sink relations of C-, N-, and S-metabolism. Leaves are the source-organs for carbohydrates and therefore cover the energy demand of the whole plant. Increased photosynthesis during short-term elevated pCO2 exposure seems to be sufficiently high to maintain soluble carbohydrates in the leaves at the level of control oaks (Fig. 1, Polle et al. 2001). This was also found in response to life-long elevated pCO2 in the present study and in the field (Blaschke et al. 2001). Polle et al. (2001) assumed that sugar export has to increase to allow enhanced growth. In the present study sugar contents of phloem exudates were not enhanced after short-term or life-long elevated pCO2 (Fig. 1). This difference to spring oaks at the field at the Laiatico side (Blaschke et al. 2001) may be due to the use of seedlings in contrast to adult trees in the field. Thus, the increase in soluble sugar of basipetal stem and root tissues after short-term elevated pCO2 which is reversed under life-long elevated pCO2 exposure can only be explained by an enhanced sugar export. It may be assumed that the transport velocity in the phloem, and, hence, the mass flow of sugar may have been increased in the seedlings under these conditions.

Leaves are also the main source for reduced sulphur (Brunold 1993). In the light-dependent sulphate assimilation APS reductase catalyses the first reduction step converting sulphate to sulphite. Therefore, the decreased APS reductase activity may have diminished the flux through the sulphate reduction pathway and, consequently, through reduced sulphur pools in the leaves under short-term elevated pCO2 (Figs 4, 5 & 7). Apparently, the source strength of the leaves for reduced sulphur was diminished and GSH and Cys contents in stem tissues of *Q. ilex* was reduced. Under life-long elevated pCO2, the APS reductase activity in the leaves, but not the GSH content, recovered to the level of the control. However, the reduced sulphur contents in stem and root tissues were restored under these conditions. Apparently, growing tissues along the stem and in the roots were preferentially refilled with reduced sulphur for growth and development.

Diminished soluble thiol (Figs 4 & 5) and TSNN (Fig. 2) contents of the leaves in combination with a faster growth and a higher biomass production under short-term elevated pCO2 (Polle et al. 2001) may be explained by nutrient dilution or nutrient deficiency. Short- and medium-term changes in the need and in the supply of nitrogen are rapidly noticeable from changes of the cycling pool of TSNN (Rennenberg & Gessler 1999). Therefore, TSNN contents determined in the present study were an appropriate measure for the nitrogen state of the tree. Since the cycling pool of TSNN connects the sites of nitrogen uptake with the sites of nitrogen demand (Gessler et al. 1998c), the unchanged TSNN content in phloem exudates in control oaks after short-term pCO2 exposure supports the assumption that nutrient dilution and not nutrient limitation was the reason for diminished TSNN in leaves and basipetal stem tissues (Fig. 2). After life-long elevated pCO2 the NR activity in the leaves increased (Fig. 7). Consequently, TSNN contents recovered to the level of control oaks under ambient pCO2 in the leaves and to even higher levels in phloem exudates.

Apparent, the leaves feed a surplus of TSNN into the cycling pool (Schneider et al. 1996; Gessler et al. 1998a, b, c) to support enhanced shoot growth under life-long elevated pCO2 (Polle et al. 2001). From field studies with beech it is highly probable that increasing TSNN contents in the phloem indicate a more than sufficient nitrogen supply (Schneider et al. 1996; Gessler et al. 1998a). Therefore, enhanced TSNN in apical wood under life-long elevated pCO2 suggests that this tissue constitute a preferential sink for storage of excess nitrogen.

The increased biomass accumulation after short-term elevated pCO2 exposure was accompanied by a shift in the root-to-shoot ratio in favour of root growth (Polle et al. 2001). Preferential root growth is generally observed when nutrient or water availability becomes growth limiting (Curtis & Wang 1998). Seegmüller and coworkers showed that increased root growth of *Q. robur* after short-term exposure to elevated pCO2 is sufficient to mediate enhanced sulphate uptake (Seegmüller & Rennenberg 1994; Seegmüller et al. 1996). Enhanced root growth also requires a sufficient supply with soluble amino compounds for protein synthesis. Despite a dilution of reduced nitrogen and sulphur in stem tissues, lateral root contents were largely unaffected after short-term elevated pCO2 in the present study (Figs 2, 4 & 5). As the phloem contents of TSNN were not affected this may be the result of enhanced mass transport of TSNN into the roots mediated by enhanced transport velocity as also assumed for carbohydrate transport. In woody plants nitrate assimilation in the roots can contribute significantly to the budget of reduced nitrogen of the whole tree (cf. Stitt & Krapp 1999). Therefore, reduced NR activity and nitrate content in lateral roots may indicate a shift in nitrate assimilation from the root to the shoot (Fig. 7).

**Genetic and/or physiological acclimations to elevated CO2?**

Biometric analysis clearly indicated that growth of spring and control oak seedlings did not differ under ambient pCO2, but was enhanced under elevated pCO2 (Polle et al. 2001). Similar results were observed in a comparable exper-
Life-long growth under elevated pCO₂ acclimates S, N and C metabolism

Growth under elevated pCO₂ acclimates S, N and C metabolism. However, when spring seeds of Agrostis canina were reset to ambient pCO₂, the greater biomass production and the higher initial relative growth rate retained. This was partially explained by a higher seed weight (Fordham & Barnes 1999). In the present study, soluble compounds and key enzymes of the C-, N- and S-metabolism largely were unaffected by the provenance of the oak seedlings under ambient pCO₂. Apparently, the reserves within the cotyledons must be similar for the initial growth of both progenies. Nevertheless, life history interacted with physiological parameters under elevated pCO₂. Apparently, adaptation mechanism responsible for the observed acclimation to elevated pCO₂ can be transferred to the following generation.

Genetic adaptation is based on molecular changes. Physiological acclimation to achieve a new ‘quasi steady-state’ at the whole plant level is realized by regulatory processes. Plants that are native to natural CO₂ springs have adapted to elevated pCO₂ over a long time exceeding the life span of trees. Because of the high variability of populations (Houpis et al. 1999; Lindroth, Roth & Nordheim 2001), none of the studies with herbaceous plants (Fordham et al. 1997; Andalo et al. 1999; Fordham & Barnes 1999) can distinguish between physiological or genetic acclimation to elevated pCO₂. Site-specific ecotypes, degree of fitness, and genetic isolation are only a few prerequisites for genetic adaptation. The number of generations under elevated pCO₂ of the mother tree stands at the Laiatico spring and the question whether the area around the spring is large enough to allow the development of a population is unknown (Andalo et al. 1999). Although trees are more immobile than herbaceous plants and the formation of small climatic populations is feasible the proof of genetic acclimation to elevated pCO₂ needs molecular and further biochemical studies.

C-, N-, and S-metabolism of plants are directly interrelated (Noctor et al. 1997; Paul & Foyer 2001). Therefore, regulation of the respective metabolic pathways aimed to reach a new ‘quasi steady state’ in a changed environment depends on each other. The unaffected phosphate contents in all tissues of Quercus ilex seedlings under elevated pCO₂ indicates that such changes are not necessarily required for all metabolic pathways (Fig. 6). The specific reactions of C-, N-, and S-metabolism observed as a consequence of life-long exposure to elevated pCO₂ will depend on age, season and tissue. They have to accommodate, but also to coordinate C-, N-, and S-metabolism under these particular

**Figure 7.** Protein contents, and NR and APS reductase activities in leaves and roots of Q. ilex seedlings. Quercus ilex seedlings were grown from acorns of mother tree stands with natural elevated pCO₂ (B, D; spring oaks) or from acorns of mother tree stands with natural ambient pCO₂ (A, C; control oaks). Both progenies were either grown under ambient (A, B) or under elevated pCO₂ (C, D). *In vitro* NR activity was measured as nitrite production. APS reductase activity was measured as production of [³⁵S]sulphite released from [³⁵S]APS in the presence of DTT. Different letters indicate statistically significant differences between treatments.
environmental conditions. To support this assumption it will also be helpful to perform the presented experiment under natural conditions at the Laiaatico site, so that broader environmental factors having influence will be considered. However, the question remains from the present study, in which way the observed adaptations of C-, N-, and S-metabolism to elevated pCO₂ can be achieved if not by genetic acclimation.

ACKNOWLEDGMENTS

We are grateful to Dr Antonio Raschi for providing the acorns of Q. ilex. The authors gratefully acknowledge Michael Stalder for excellent technical assistant. This study was financially supported by the Deutsche Forschungsgemeinschaft (contracts Re 515/6 and He 3003/1) and by the European Commission (MAPLE-Project EV5VCT940432).

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