Nonstructural carbohydrate-balance response to long-term elevated CO$_2$ exposure in European beech and Norway spruce mixed cultures: biochemical and ultrastructural view

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Non-structural carbohydrate-balance response to long-term elevated CO$_2$ exposure in European beech and Norway spruce mixed cultures: biochemical and ultrastructural responses

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Abstract

Two dominant central European tree species (*Fagus sylvatica* and *Picea abies*), in a mixed culture in semi-open glass domes, were used to simulate the reaction of forests to long-term elevated CO$_2$ (EC) in a mountainous area (Beskydy Mountains, the Czech Republic). We investigated the effects of EC on soluble carbohydrate levels and composition. Starch content was evaluated using two methods: biochemical (glucose content after enzymatic hydrolysis) and stereological (starch grain proportion, size, and number in chloroplasts). In beech and spruce foliage, no significant changes in total soluble carbohydrate levels were observed. In spruce, starch content determined biochemically increased under EC, while no changes were detected in beech. The starch content determined stereologically increased only in beech. In spruce, EC exposure caused comparable starch increases in current-year and previous-year needles, although the former had a higher starch content and numerous larger starch grains regardless of CO$_2$ concentration. In both species, the biochemical determination of carbohydrates exhibited greater individual tree uniformity, in contrast to large intraspecies variability. No changes in leaf soluble carbohydrates under long-term elevated CO$_2$ demonstrate the ability of the studied tree species to efficiently allocate the photosynthates among the sinks. Thus, no photosynthetic downregulation via carbohydrate level signalling can be expected.

Keywords chloroplast ultrastructure; CO$_2$ enrichment; forest trees; soluble carbohydrates; starch
Introduction

Forests of the temperate and boreal zones serve as significant sinks for atmospheric CO$_2$. Elevated atmospheric CO$_2$ can enhance forest carbon sequestration by stimulating plant growth, though to an uncertain degree due to the accompanying environmental changes (e.g. Anderson-Teixeira et al. 2013). Several factors have to be considered in carbon storage studies, i.e. processes involved in carbon sequestration, such as photosynthesis, transport, or growth, exhibiting specific responses to particular environmental features (Korner 2015), and environment-induced changes in carbon allocation, use, and transport differing between the above- and below-ground tissues in trees (Bader et al. 2010). To predict future carbon sequestration potential in forest ecosystems, we need to characterize the physiological processes and structural changes that underlie the overall forest response. The reactions of plant communities in regions with diverse climates are different. However, most studies focused on temperate-zone trees, and thus information for the globally significant boreal and tropical forests is scarce (reviewed by Jones et al. 2014). Other complications arise from various reactions of different tree species (Bader et al. 2010) and their interactions in forest communities (e.g. Korner 2015).

Recently, many papers reporting different aspects of the effects of elevated CO$_2$ (EC) on plant growth, development, and stress responses have been published for different plant species. However, most of the biochemical data, especially that of the carbohydrate status, relate to model herbaceous plants or crops. Substantially fewer articles have characterized the responses of tree species, focusing mainly on
Acer, Betula, Fagus, Pinus, Populus, and Quercus (Lindroth 2010), and have rarely reported the results observed under long-term EC. Only a few papers have considered the trees dominant in boreal-zone forests. Stinziano and Way (2014) summarized data on responses of boreal tree species to EC, and concluded that more than four studies had been performed only for eight species and, importantly, had mostly been conducted using seedlings. The experiments using trees that focused on carbohydrate status (especially those distinguishing soluble sugars and starch) were often conducted using just a few different genotypes or even using clonally multiplied plants (Bader et al. 2010; Davey et al. 2006). The effect of EC on starch accumulation was usually investigated either by biochemical analysis or ultrastructural measurements, but comparison of data achieved by both approaches is scarce. Much information is still missing. However, only a small proportion of the results can serve as a basis for estimating the reactions of the true forest community to EC.

Our study, in contrast to the previously published ones, focused on detailed characterization of the effects of EC on foliage carbohydrate balance (distinguishing individual soluble carbohydrates and starch), reflecting the current leaf energy status. We used mixed stands of two dominant species in European forests representing different plant functional types, European beech and Norway spruce, grown in a mountainous region under ambient CO₂ (AC) and EC. For starch determination, we used two different rarely combined approaches, biochemical and ultrastructural approaches, to obtain a more complex view on the carbohydrate status of the tree foliage. We tested the following hypothesis: Long-
term tree exposure to EC, even in juvenile tree canopy, causes carbohydrate metabolism to completely adjust to higher carbon availability, which results from equilibrated photosynthate production and consumption.

Materials and methods

Experimental design and plant material

The experiments were conducted at the Bílý Kříž experimental ecological study site in the Beskydy Mountains (Czech Republic; 49°30´N, 18°32´E; 908 m a.s.l.). The climatic conditions of the experimental site are cool (annual mean temperature 4.9°C) and humid conditions, with mean precipitation of 1 100 mm and an average number of days with snow cover of 160 (Urban et al. 2001). Long-term CO₂ enrichment was applied to 5-year-old Norway spruce (Picea abies L. Karst) and European beech (Fagus sylvatica L.) trees since 2005, and all the biochemical and anatomical analyses presented in this study were conducted in 2009, after nearly four seasons of exposure to elevated CO₂ concentration (EC). Trees were exposed to ambient (385 µmol (CO₂) mol⁻¹; AC) or elevated (700 µmol (CO₂) mol⁻¹; EC) CO₂ concentrations using two spacious (10 m x 10 m x 7 m) semi-open glass domes with adjustable windows. A description of the facility and the detailed climatic characteristics are given in Urban et al. (2001). In each glass dome, 60 beech trees and 35 spruce trees were planted in an equilateral triangle layout (1.20 m side length).

Trees were grown with grass understory in a native soil identical to the surrounding forest: ferric podzols overlying a Mesozoic Godula sandstone (flysch-type) bedrock. The soil within the domes is characterized by high humus
and total nitrogen contents, with C:N ratio in the range of 24.3–26.3 and moderate availability of base cations (analysed in 2009). Trees of both species grew in a mixed canopy under both CO$_2$ conditions. The pseudo-replication design (Hurlbert 1984) based on a single dome for each treatment required the use of individual trees as replicates for the purpose of statistical analyses. Samples were collected in August 2009 from randomly selected individual trees. From each species and CO$_2$ condition, five trees were sampled both for anatomical and biochemical analyses (six sub-samples per tree). For biochemical analyses, additional individual trees from each species and CO$_2$ condition were sampled: two and five samples for soluble carbohydrate and starch analyses, respectively. In total, 7 and 10 trees per species and CO$_2$ treatment were sampled for soluble carbohydrates and starch, respectively. Sampling: tree height - approx. 100 cm (spruce) and 120 cm (beech), nearly no shading between individuals; sun-exposed S/SW-oriented shoots. For biochemical analysis, current-year (C) and previous-year (C+1) needles and beech leaves were immediately placed into liquid N$_2$ and stored at -70°C. For ultrastructural analysis, 1-mm$^2$ leaf blades or 1-mm needle segments from the middle parts were fixed in 5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.3).

Determination of non-structural carbohydrate content

Freeze-dried samples were divided into two: one half was weighed and homogenized in a ball mill (Retsch MM301, Germany, stainless steel balls (5 mm), 25.s$^{-1}$, 2 min) for starch analysis, and the non-milled half was weighed and used for soluble carbohydrate analysis.
Both halves were extracted in 80% methanol (v/v; 75°C, 10 min), and then, the solvents were evaporated and the residues were dissolved in ultrapure MilliQ water. The solutions were centrifuged and filtered. Carbohydrate measurements: high-performance liquid chromatography (HPLC), 80°C, MilliQ water; flow rate 0.5 mL.min\(^{-1}\); pre-column: Hema-Bio 1000 Q+SB and column: IEX Pb\(^{2+}\) (Watrex, CZ); refractometric detection (Shodex RI-71).

For starch determination, the residue after methanol removal was dissolved in MilliQ water. Thereafter, the water phase was removed, the residual pellet was washed three times, and the starch was hydrolysed by using a mixture of α-amylase (30 U.mL\(^{-1}\)) and amyloglucosidase (60 U.mL\(^{-1}\)), both from Sigma Aldrich. The solvent was evaporated and the pellet was redissolved in MilliQ water. Glucose content was determined by HPLC as soluble carbohydrates, except for the column (IEX Ca\(^{2+}\) form, Watrex, CZ). Detailed analysis procedures are reported by Lipavska al. (2000). The amounts of individual soluble carbohydrates and starch (as glucose amount after enzymatic cleavage) are expressed as µg of carbohydrate per unit leaf dry mass.

**Chloroplast ultrastructure and stereological measurements**

Fixed samples were washed in 0.1 M phosphate buffer (pH 7.3), post-fixed in 2% (w/v) buffered osmium tetroxide, dehydrated with ethanol series followed by propylene oxide, and embedded in resin (Spurr 1969). Ultrathin sections were cut on an ultramicrotome (UC7, Leica Microsystems, Austria) and post-stained with uranyl acetate and lead citrate. The grids were viewed with a JEOL JEM-1011 (JEOL, Tokyo, Japan) electron microscope and chloroplast images captured at
x 25 000 magnification using a digital camera (Veleta, Olympus, Münster, Germany).

For stereological measurements, samples from five trees per species and treatment, and seven individual chloroplasts from the first layer of palisade/mesophyll parenchyma cells per sample were evaluated. Measurements of proportion, area, number of starch grains, and total area of the chloroplast cross-sections were carried out using a point grid plug-in in Ellipse 2.07 software (ViDiTo, Košice, Slovakia).

Statistical analysis

Differences between non-structural carbohydrate contents and chloroplast ultrastructural parameters were evaluated using a nested ANOVA design (NCSS 9.0 software, LCC Kaysville, Utah, USA). The CO$_2$ treatment and species were designed to be the between-subjects fixed-effect factors; the individual studied tree was a random-effects nested factor (subjects) within the treatment; and in case of spruce needle age, the C and C+1 needles were the within-subject fixed-effect factors. This design was used because multiple measurements were made on the same tree. In addition, the significance of the difference between the trees exposed to AC or EC was determined for European beech and Norway spruce separately using the same design. Sample number (n) represents the total number of replicates per treatment used for statistical analysis (including sub-samples per tree). The Tukey-Kramer Multiple-Comparison Test was applied in both cases and data were tested at P < 0.05; the F value is presented.

Results
Summarizing the effects of CO$_2$ concentration, species, and their interaction resulted in following findings: If averaged across CO$_2$ treatments, the species significantly differed in starch content (for both biochemical and stereological evaluations) and chloroplast size, but showed relatively similar soluble carbohydrate levels (Table 1). If averaged across species, elevated CO$_2$ treatment induced significantly higher starch content (for both biochemical and stereological evaluations) and starch grain number, though soluble carbohydrates remained at a similar level. The interaction effect of the two factors, CO$_2$ concentration and species, was significant only in case of chloroplast cross-sectional area (Table 1).

In the following sections, the CO$_2$-induced responses are presented for individual species and characteristics separately.

**Biochemical determination of soluble carbohydrates and starch**

Carbohydrate contents exhibited only very slight changes resulting from exposure to EC (Fig. 1), with no significant increases in soluble carbohydrate levels (P < 0.05, F = 0.25) for beech and similarly for spruce (P < 0.05, F = 0.57) under EC.

In both species, the average amount of the total soluble carbohydrates was very similar (60 to 75 µg.mg$^{-1}$ dry weight). In beech, the carbohydrate spectrum consisted predominantly of sucrose (80%), approximately 10% of inositol, <10% of fructose, and a very small amount of glucose. In spruce, it consisted of about 60% sucrose, 10% glucose, pinitol, and fructose, and a very small amount of inositol. In both species, no EC-induced changes were detected in the proportions of individual sugars.
A slightly different situation was revealed for starch levels (Fig. 2), where starch deposition tended to increase under EC, particularly in spruce (spruce - $P = 0.056346$, $F = 4.16$; beech - $P = 0.141031$, $F = 2.37$). Interestingly, the starch increase under EC was only slightly lower in beech (31%) compared to spruce (34%).

In spruce, starch content was significantly higher in the C needles than in the C+1 needles ($P < 0.05$, $F = 10.58$), and EC caused comparable increases in starch contents of both the C and the C+1 needles, although statistically significant only in the C needles (Fig. 3).

**Chloroplast ultrastructure**

Chloroplasts with starch grains, plastoglobuli, and well-developed thylakoid systems were observed in all treatments (Fig. 4a-f). Thylakoid membranes were lightly stained, and large globules, probably containing tannin-like substances, were frequent. Plastoglobuli in beech chloroplasts were large and lightly stained, while in spruce they were small and much darker. In the C+1 needles, plastoglobuli were numerous (data not shown) and often formed clusters.

Chloroplast cross-sectional area (Fig. 5a, b) range was 6.2-8.0 $\mu$m$^2$ in beech and 9.4-11.4 $\mu$m$^2$ in spruce. EC caused a significant decrease in chloroplast area only in beech ($P < 0.05$, $F = 5.69$). In spruce, there was almost no difference in chloroplast area between the C and the C+1 needles.

Stereological evaluation revealed a lower proportion of starch in beech (8 to 17%) than in spruce chloroplasts (33 to 40% in the C needles and 13 to 16% in the C+1 needles). EC significantly enhanced the proportion of starch ($P < 0.05$, $F = 10.70$).
in beech (Fig. 5c). For spruce (Fig. 5d), a slight difference in the proportion of starch \( (P < 0.10, F = 8.86) \) was found between chloroplasts from the C and the C+1 needles.

The number of starch grains (counted on chloroplast cross-sections) ranged between 0.9 and 1.3 in beech (Fig. 5e) and 1.1 and 1.5 in spruce (Fig. 5f). Under EC, the number of starch grains tended to increase \( (P < 0.1, F = 4.88) \) only in beech.

Starch grains were smaller in beech \( (0.5-0.8 \, \mu\text{m}^2) \) than in spruce both in the C \( (3.0-3.9 \, \mu\text{m}^2) \) and the C+1 \( (1.1-1.4 \, \mu\text{m}^2) \) needles (Fig. 5g, h). In beech (Fig. 5g), starch grains under EC were significantly larger \( (P < 0.05, F = 7.71) \). In spruce, they were larger in the C needles compared with the C+1 needles (Fig. 5h); however, the difference was significant only under EC \( (P < 0.05, F = 7.72) \).

**Intraspecies variability**

Biochemical data were obtained from 7 to 10 trees of both species, with six samples per tree. Carbohydrate values of the samples of each tree exhibited high similarity \( (P < 0.001, F = 7.12-13.62) \). On the contrary, comparison of average values of individual trees exhibited large variability in soluble carbohydrate and starch contents. This is presented in Fig. 6 and in the supplementary material S1\(^2\) for beech and spruce (selected contrasting trees), respectively, showing the starch level homogeneity within a tree (beech, \( F = 13.62; \) spruce, \( F = 12.63 \)) in contrast to large differences between individual trees under both AC and EC.

**Discussion**

\(^2\) Supplementary data are available with the article through the journal Web site
Both studied species, European beech and Norway spruce, dominate in the forests across central Europe. However, each of them represent a different plant functional type: European beech is a winter-deciduous woody broad-leaved species, while Norway spruce is an evergreen woody needle-leaved species, thus differing considerably in their phenology, leaf structure, and leaf physiological traits such as photosynthetic capacity (Niinemets et al. 2015). In this study, aiming to estimate complex canopy reactions, we grew them with grass understory in a local natural forest soil for four years under AC and EC in glass domes with negligible chamber effect (Lhotakova et al. 2012). We are aware of the pseudo-replication problem, which might influence the results because of the location of individual trees within the dome as well as different tree genotypes. However, the aim of our study was to characterise the reaction of the whole tree community, similar to natural canopy where individuals are also of different genotypes and with various microclimatic conditions. Though the experimental site is located in a temperate zone, the most of the conditions (high precipitation, air humidity, and humus content) resemble boreal ecosystems. Moreover, in the Beskydy Mountains, several areas of boreal-like spruce forest stands are protected. Thus, our results represent a contribution to the scarce knowledge of boreal forest EC-induced responses, especially at carbohydrate status level. Carbohydrate balance integrates photosynthetic activity and allocation of assimilates to sinks and their consumption. In the presence of excess sugars, the hexokinase (HXK) sugar-sensing pathway (with trehalose-P and TOR kinase
signalling) coordinates plant response and induces photosynthesis acclimation
(e.g. Smeekens et al. 2010).
EC-induced increase in CO₂ assimilation rate was reported by Holisova et al.
(2012) in a parallel study. Notably, we found no significant changes in total
soluble carbohydrates in both tree species under EC (Fig. 1, Table 1), which was
confirmed in the following years (2010 and 2011, data not shown). Similar results
were published by Bader et al. (2010) for a mature beech in a species-rich canopy
exposed to EC for eight years. However, leaf sugars and starch are often
correlated with dark respiration (e.g. Griffin et al. 2001; Tissue et al. 2002), and
thus are perceived as strong predictors of its rate. As Holišová et al. (2012),
concurrently with our study, observed no increase in dark respiration in both
spruce and beech under EC, we propose that in EC-treated trees the surplus
carbohydrates are efficiently used directly to support growth and developmental
processes in sink organs.
Unchanged soluble carbohydrates indicate that no acclimation is in progress
(Yadav et al. 2013). However, while working on the same plant material as used
in this study, Kosvancova et al. (2009) observed a significant decrease in total
Rubisco content under EC in 2007-2008 interpreted as a photosynthesis
acclimation. Furthermore, in 2008, Urban et al. (2012) found a more pronounced
acclimation in previous-year needles than in current-year needles under EC.
Based on our results showing no difference in soluble carbohydrates, also in
previous-year needles under AC and EC (data not shown), we suggest that during
the following season, 2009, the trees balanced their assimilation/utilisation rates probably due to enhanced carbohydrate consumption in sinks.

Soluble carbohydrate balance is tightly connected to assimilatory (source) starch levels. We used two complementary approaches for starch quantification: biochemical determination and stereological measurement. Biochemical data showed a trend of starch increase in EC spruce and beech foliage (Fig. 2, Table 1). Stereological data revealed significantly higher starch accumulation in beech but not in spruce (Fig. 5). Increase in starch grain number or area under EC has frequently been reported (Oksanen et al. 2001; Palomäki et al. 1996; Sallas et al. 2003).

The different starch quantification methods provide complementary views. Usually, either biochemically or ultrastructurally acquired data are used. Our results point to the importance of consideration of both the method and sampling design. Ultrastructural data describe starch grain size and number and their proportions in the chloroplast, allowing estimation of grain surface and its impact on potential mobilisation rate. Biochemical data express more accurately the total starch amount that a tree has at disposal.

Both methods confirmed higher starch in the C needles than in the C+1 needles under both CO\textsubscript{2} conditions in spruce (Fig. 3 and 5), similarly to Griffin et al. (2000) but conversely to Zha et al. (2002) in Pinus sp. The discrepancy could result from sampling designs or species/seasonal variability in either starch itself or other deposited compounds influencing starch proportion. Interestingly, in spruce needles under AC and EC, Urban et al. (2012) observed a lower $A_{\text{max}}$ in
the C+1 needles compared to the C ones, which could have resulted in lower carbohydrate production, and subsequently lower starch accumulation in the C+1 needles. Smaller starch grains in chloroplasts of the C+1 spruce needles described by Kivimäenpää et al. (2003) were also (at least partly) explained by lower stomatal conductance and photosynthesis of the C+1 needles. Zagirova (2003) considered the decreased size of starch granules in chloroplasts of the C+1 fir needles as a structural aspect of source-sink relations. Nevertheless, most studies do not regard elevated starch as a driver for acclimation (e.g. Yadav et al. 2013), though some authors propose that starch could be somehow involved in triggering acclimation (e.g. Rey and Jarvis 1998). Most importantly, the majority of the studies neither distinguish between individual sugar levels nor even between soluble carbohydrates and starch (e.g. Bader et al. 2010; Rolo et al. 2015).

Starch grain size and shape might affect restriction of spatial CO$_2$ diffusion as hypothesised by Makino (1994), or influence the rate of starch cleavage occurring predominantly on the grain surface (Dhital et al. 2015). We observed EC-induced increases in all tested starch grain parameters, though significant only in beech (Fig. 5).

Generally, the reactions to EC depend strongly on species, genotype, age, mineral nutrient availability, different growth strategies, climate etc. (Cseke et al. 2009; Korner 2015). Young trees, in contrast to old forests, can increase photosynthesis by up to 50% under EC (e.g. Davey et al. 2006), but their reaction is species-specific (Bader et al. 2010; Korner et al. 2005). Although our “model forest” consisted of juvenile 9-year-old trees, its reactions to EC correspond to those of
mature trees (e.g. Korner et al. 2005). We propose that the negligibly higher
sugars under EC foreshadow the transition from an EC-perceptive stage to a
juvenile but well-adapted one, close to a mature forest status. The locality
characteristics (climate and minerals), decisive for sink activity/potential, were
not limiting.

Although the reactions to EC are presumably similar in evergreen conifer and
deciduous broad-leaf species, the conifers usually show a much stronger
assimilatory response to EC (Bauer et al. 2001). We found total starch levels to be
higher in spruce than in beech, irrespective of CO$_2$ level (Fig. 2, Table 1),
reflecting inherent metabolic traits of both species, together with higher soluble
carbohydrates in spruce (P < 0.001) but higher proportion of sucrose in beech
(Fig. 1). Surplus carbohydrates in evergreens are supposedly converted into starch
reserves, while in deciduous species, long-distance-transportable sucrose is
favoured.

The results discussed so far are given as average values for plant sets of different
genotypes (Fig. 1-5). In both species, comparison of individual trees revealed
great intraspecies differences (Fig. 6 and supplementary material S1), hidden
when average values are used. Some studies confirm that genetic traits
dramatically change the range of the responses (e.g. Cseke et al. 2009; Riikonen et
al. 2008). Cseke et al. (2009) reported similar EC-induced response in two aspen
genotypes in terms of photosynthesis, but dramatic differences in soluble
carbohydrates and starch, with the levels in one genotype under AC being similar
to the other one under EC. Analogously, in Fig. 6 and supplementary material S1
we show a large disparity in starch levels between individuals under the same CO₂ treatment. Moreover, this variance was also found for soluble carbohydrates (data not shown). Notably, we observed uniform values for replicates in individual trees and treatments.

To accurately estimate the EC-induced response of the whole canopy, attention should be paid to the fact that it not only integrates environmental conditions with species presence, abundance, and age, but also with genotype-dependent variability between individual trees involved. It is therefore highly advisable to realize that an analysis of the impacts of EC on selected parameters – no matter how detailed and sophisticated - could be misleading if carried out using a small group of plants.

Conclusion

In a mixed canopy of Norway spruce and European beech, 4-year EC exposure did not induce significant changes in soluble carbohydrates in both species, while in spruce, the starch content increased. These results indicate absence of ongoing photosynthetic acclimation, due to well-functioning allocation of carbon to storage and metabolic sinks. Great intraspecies variability in both species underline the necessity to evaluate large groups of genotype-divergent individuals to reliably estimate the responses of forest ecosystems.

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References


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<th>Starch content [µg·mg(^{-1})]</th>
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<th>Starch proportion [%]</th>
<th>Starch grain profile number</th>
<th>Starch grain profile size [µm(^2)]</th>
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<td>0.00000 (68.65)**</td>
<td>0.000004 (50.28)**</td>
<td>0.000006 (46.71)**</td>
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<td>0.403783 (0.74)</td>
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<td>0.193201 (1.76)</td>
<td>0.028817 (5.84)*</td>
<td>0.600262 (0.29)</td>
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**Table 1.** Effects of species, CO\(_2\) concentration, and their interaction on non-structural carbohydrates and chloroplast ultrastructure of Norway spruce and European beech. Two-way nested ANOVA. P values are reported with F values in brackets. Significant effects are marked with * if P < 0.05, and ** if P \(\leq 0.01\).
Figure captions

**Fig. 1** Content of soluble non-structural carbohydrates (mean ± SE) in leaves/needles as affected by elevated CO$_2$ concentration. a) European beech (n = 36), b) Norway spruce (n = 38). Significant differences are indicated by asterisks between the columns (* significant at P < 0.05). Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm

**Fig. 2** Starch content (mean ± SE) in leaves/needles as affected by elevated CO$_2$ concentration. a) European beech (n = 60), b) Norway spruce (n = 60). Significant differences are indicated by asterisks between the columns (* significant at P < 0.05). Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm

**Fig. 3** Starch content in Norway spruce of current- (C) and previous-year (C+1) needles (mean ± SE) as affected by elevated CO$_2$ concentration (n = 60; P < 0.05). Significant differences are indicated by asterisks between the columns (* significant at P < 0.05). Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm

**Fig. 4** Transmission electron micrographs of chloroplast cross-sections from the first layer of palisade/mesophyll parenchyma cells in leaves/needles grown under ambient (a,c,e) or elevated (b,d,f) CO$_2$ concentration. (a,b) European beech; (c-f) Norway spruce: (c,d) chloroplasts from current-year (C) needles; (e,f) chloroplasts from previous-year (C+1) needles. Note the larger starch inclusions in beech chloroplasts under elevated CO$_2$ concentration and in spruce chloroplasts in the C needles; GT, granal thylakoids; IGT, intergranal thylakoids; ST, starch inclusion; PG, plastoglobulus. Bar = 1 µm in all panels. Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm

**Fig. 5** Ultrastructural parameters of chloroplasts (means ± SE) in leaves of European beech (a,c,e,g) and needles of Norway spruce (b,d,f,h) as affected by elevated CO$_2$ concentration,
evaluated in the first layer of palisade/mesophyll parenchyma cells. (a,b) Chloroplast cross-sectional area (µm²); (c,d) proportion of starch in chloroplasts (%); (e,f) number of starch inclusions per chloroplast cross-section; (g,h) cross-sectional area of an average starch inclusion (µm²); (n = 35). Significant differences are indicated by asterisks between the columns (* significant at P < 0.05). Ambient CO₂ concentration, 385 ppm; elevated CO₂ concentration, 700 ppm.

**Fig. 6** Representation of variability in starch contents between individual trees of European beech within treatments. (a,c,e) Ambient CO₂ concentration; (b,d,f) elevated CO₂ concentration. Note the relatively homogenous values characterizing particular plants (1-6 represent individual samples per tree). Ambient CO₂ concentration, 385 ppm; elevated CO₂ concentration, 700 ppm.
Fig. 1 The content of soluble non-structural carbohydrates (mean±SE) in leaves/needles as affected by elevated CO$_2$ concentration. a) European beech (n=36), b) Norway spruce (n=38). Significant differences are indicated by asterisks between the columns (* significant at P < 0.05). Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm.
Fig. 2 The starch content (mean±SE) in leaves/needles as affected by elevated CO$_2$ concentration. a) European beech (n=60), b) Norway spruce (n=60). Significant differences are indicated by asterisks between the columns (* significant at P < 0.05). Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm
Fig. 3  The starch content in Norway spruce of current- (C) and previous-year (C+1) needles (mean±SE) as affected by elevated CO$_2$ concentration (n=60; P \(<\) 0.05). Significant differences are indicated by asterisks between the columns (* significant at P \(<\) 0.05). Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm
Fig. 4 Transmission electron micrographs of chloroplast cross-sections from the first layer of palisade/mesophyll parenchyma cells in leaves/needles grown under ambient (a,c,e) or elevated (b,d,f) CO$_2$ concentration. (a,b) European beech; (c-f) Norway spruce: (c,d) chloroplasts from current-year (C) needles; (e,f) chloroplasts from previous-year (C+1) needles. Note larger starch inclusions in beech chloroplasts from elevated CO$_2$ concentration and in spruce chloroplasts from C needles; GT, granal thylakoids; IGT, intergranal thylakoids; ST, starch inclusion; PG, plastoglobulus. Bar = 1 µm in all panels. Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm
Fig. 5 Ultrastructural parameters of chloroplasts (means±SE) in leaves of European beech (a,c,e,g) and needles of Norway spruce (b,d,f,h) as affected by elevated CO₂ concentration, evaluated in the first layer of palisade/mesophyll parenchyma cells. (a,b) Chloroplast cross-sectional area (µm²); (c,d) proportion of starch in chloroplasts (%); (e,f) number of starch inclusions per chloroplast cross-section; (g,h) cross-sectional area of an average starch inclusion (µm²); (n=35). Significant differences are indicated by asterisks between the columns (* significant at P < 0.05). Ambient CO₂ concentration, 385 ppm; elevated CO₂ concentration, 700 ppm.
Fig. 6 Representation of variability in starch contents between individual trees of European beech within treatments. (a,c,e) ambient CO$_2$ concentration; (b,d,f) elevated CO$_2$ concentration. Note the relatively homogenous values characterizing particular plants (1-6 means individual samples per tree). Ambient CO$_2$ concentration, 385 ppm; elevated CO$_2$ concentration, 700 ppm.
(a) glucose content [µg/mg dry mass]
(b) glucose content [µg/mg-1 dry mass]
(c) glucose content [µg/mg dry mass]
(d) glucose content [µg/mg-1 dry mass]
(e) glucose content [µg/mg dry mass]
(f) glucose content [µg/mg-1 dry mass]