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Carbon allocation in shoots of alpine treeline conifers in a CO_2 enriched environment

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Abstract With a new approach we assessed the relative contribution of stored and current carbon compounds to new shoot growth in alpine treeline conifers. Within a free air CO_2 enrichment experiment at the alpine treeline in Switzerland, ^{13}C-depleted fossil CO_2 was used to trace new carbon in the two tree species *Larix decidua* L. and *Pinus uncinata* Ramond over two subsequent years. The deciduous *L. decidua* was found to supply new shoot growth (structural woody part) by 46% from storage. Surprisingly, the evergreen *P. uncinata*, assumed to use current-year photosynthates, also utilized a considerable fraction of storage (42%) for new wood growth. In contrast, the needles of *P. uncinata* were built up almost completely from current-year photosynthates. The isotopic composition of different wood carbon fractions revealed a similar relative allocation of current and stored assimilates to various carbon fractions. Elevated CO_2 influenced the composition of woody tissue in a species-specific way, e.g. the water soluble fraction decreased in pine in 2001 but increased in larch in 2002 compared to ambient CO_2. Heavy defoliation applied as an additional treatment factor in the second year of the experiment decreased the lipophilic fraction in current-year wood in both species compared to undefoliated trees. We conclude that storage may play an important role for new shoot growth in these treeline conifers and that altered carbon availability (elevated CO_2, defoliation) results in significant changes in the relative amount of mobile carbon fractions in woody tissue. In particular, stored carbon seems to be of greater importance in the evergreen *P. uncinata* than has been previously thought.

Keywords ^{13}C · Elevated CO_2 · *Larix decidua* · *Pinus uncinata* · Reserves

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Introduction

Since the 1960s a number of studies have been conducted to address seasonal patterns of carbohydrate formation, its allocation to different organs and storage, as well as the use of storage during spring growth using the ^{14}C labelling technique (e.g. Hansen 1967; Gordon and Larson 1968; Kandiah 1979; Hansen and Beck 1990, 1994; Lacointe et al. 1993). In *Pinus resinosa*, previous season's needles were found to play a major role for the carbon supply to new needle growth

(Gordon and Larson 1968). For another evergreen conifer, *Pinus sylvestris*, Hansen and Beck (1990) found that storage did not matter for new tissue formation including branch wood, as the photosynthetic activity of 1-year-old needles sufficiently supplied new growth before the new shoot generation became autonomous (Hansen and Beck 1994). In contrast, in the deciduous *Malus* ssp. (Hansen 1997; Kandiah 1979) and *Juglans regia* (Lacointe et al. 1993), some incorporation of stored carbon into new tissue growth was detected, but the largest proportion of storage was metabolically respired. In all of these ^{14}C studies, trees were relatively young (1–8 years), pot-grown and pruned with the exception of *P. sylvestris* (Hansen and Beck 1990, 1994). Trees were pulse labelled with radioactive ^{14}C and after chase periods of various length, the distribution of ^{14}C was assessed by detecting the amount of radioactivity in the organs of interest. Cerasoli et al. (2004) used stable ^{13}C labelling to assess the fate of winter storage in saplings of the evergreen *Quercus suber* L. Winter assimilates were stored in first year leaves and translocated to new leaves, and additionally to fine and coarse roots during spring flush. In another study using ^{13}C labelling, an increased mobilization of stored carbon was observed in transplanted seedlings of *Pinus nigra* Arn. in response to lifting of bare-root tree seedlings grown in nursery beds as opposed to container-grown seedlings (Maillard et al. 2004). However, in parallel to the ^{14}C-studies, no mature trees were used. Labelling with ^{13}C occurred in growth chambers and in non-continuous cycles over about a month.

The stable isotope technique used in this study provides a different approach to test the role of storage for new tissue formation. Within a free air CO_2 enrichment (FACE) experiment at the alpine treeline (Hättenschwiler et al. 2002) including the two tree species European larch (*Larix decidua* L.) and Mountain pine (*Pinus uncinata* Ramond), the use of fossil CO_2 for enrichment enabled us to trace the carbon within trees. Due to the different carbon isotopic composition ($^{13}C/^{12}C$ ratio) of fossil CO_2, carbon isotopic analysis allowed us to estimate the proportion of new and old carbon used for new shoot growth during two consecutive growing seasons (2001, 2002).

The fact that trees are continuously exposed to elevated CO_2, allows the stable carbon isotope ratio to integrate over the whole growth period of the respective tissue. This is an important advantage over the studies done so far that can only show the distribution of ^{14}C or ^{13}C applied within a much shorter time frame. The labelling used here can reveal the mixing ratio of storage formed sometime before and the new carbon acquired at any time after the onset of CO_2 enrichment. In fact, it was shown that reserves formed in summer and autumn are used sequentially and for different purposes (Lacointe et al. 1993).

Here, we aimed to investigate the allocation of stored and recent carbon compounds for current-year growth of mature (27-year-old larch and 29-year-old pine), field grown treeline conifers. We hypothesized a negligible utilization of stored carbohydrates for evergreen pine as previous season's needles are expected to sufficiently supply new shoot growth with current photosynthates. In contrast, we expected a considerable utilization of reserves for deciduous larch. To test whether different carbon fractions were distinctly supplied by reserves versus current photosynthates, wood bulk material, lignin + cellulose, cellulose and the water soluble fraction were analysed separately for $\delta^{13}C$. In addition, different age classes of pine needles were investigated for a comparison with twig wood.

The separation of different carbon fractions also allowed us to test potential CO_2 effects on the proportion of different fractions in wood formed under elevated CO_2 compared to that formed under current ambient CO_2.

As an additional treatment factor, half of all trees were defoliated by 80%. The combination of CO_2 enrichment and defoliation, similar to strong herbivore pressure, enabled us to study possible interactive effects of these factors on the relative contribution of different carbon fractions.

Materials and methods

Study site and plant material

The CO_2 enrichment experiment is situated at the alpine treeline near Davos (Switzerland, 9°52′E/46°46′N) at an elevation of 2,180 masl. In 1975, a large scale reforestation was started with individual tree seedlings of the three species *Larix decidua* L., *Pinus uncinata* Ramond and *Pinus cembra* L. The trees have a similar age (*L. decidua* 27 and *P. uncinata* 29 years old at the start of our experiment) and originate from high elevation provenances. Tree height ranges from 0.8 to 1.5 m. The experimental site is NE-exposed with a slope of 25–30%. The soil was classified as a Ranker and the average temperatures in January and July are –5.8 and 9.4°C, respectively (Schönenberger and Frey 1988). The vegetation period starts in mid June with bud break of the trees and ends at the end of September with larch needle senescence. The understory vegetation is dominated by dwarf shrubs, mainly

Vaccinium myrtillus, *V. uliginosum* and *Empetrum hermaphroditum*. Common herbaceous species are *Melampyrum pratense*, *Gentiana punctata* and *Homogyne alpina*.

Experimental design

A total of 40 trees were chosen in early June 2001, 20 individuals each of larch (*L. decidua*) and pine (*P. uncinata*). Around each tree a 1.1 m^2-plot was established including the understory vegetation. Half of the plots (i.e. 10 per tree species) were randomly assigned to an elevated CO_2 atmosphere during the growing season (26 June to 20 September 2001: mean ± SD of 566 ± 42 ppm, 14 June to 22 September 2002: 569 ± 25 ppm) with the remaining half as control plots at ambient CO_2 concentration (370 ppm). Plots were delimited by three wooden posts and a horizontal, hexagonal stainless steel frame. From each frame, 24 laser-punched drip irrigation tubes (inner diameter of 4.3 mm, laser holes of 0.5 mm every 15 cm; Drip Store Inc., Escondido, CA, USA) were hung vertically around the ring (15 cm apart from each other) and stabilized with a 3 mm stainless stell rod inside. Through these holes, oriented to the centre of the plot, CO_2 jets were injected. Each plot was fed by four supply tubes of pure CO_2 from each of the cardinal directions. CO_2 was continuously measured in five reference plots by four sampling lines mounted in mid-canopy height and as well in each of four cardinal directions from the centre of the plot. CO_2 was and kept constant by means of a monitoring and regulating system (Hättenschwiler et al. 2002).

In 2002 a second treatment to influence the trees' carbon balance was applied. Each of five trees from both CO_2 treatments and both species was defoliated by approximately 80%. In larch, we cut all short shoots with scissors just above the meristem leaving every fifth shoot along each branch and the terminal shoots untouched. In pine, the inner 80% needles from each of the four latest annual increments were cut (Handa et al. 2005).

Sampling and sample preparation

Before tree bud burst, wood samples from twig sections produced in 2000 and 2001 were taken on 12/13 June 2002. We sampled subdominant twigs from mid-canopy positions (40–80 cm above ground), avoiding the leading shoots towards the plot margin and corresponding to the positions of the CO_2 sampling lines in the reference plots. To obtain sufficiently large samples, 4–6 twigs from each larch tree and 2 twigs from each pine tree were cut. Wood samples from twig sections produced in 2002 were collected in the same way at the end of the growing season, on 23/24 September 2002. In addition, needles from the last three pine needle generations were collected. Needles from 2002 were taken directly from the cut twig section. Five pairs of needles produced in 2000 and 2001 were collected from the same twig.

From all wood samples, the bark was removed immediately after sampling and only the remaining xylem was used for further analysis. All samples were predried in a microwave (30 s at 650 W, then 1 min at 450 W) to stop enzymatic activity before they were dried to constant weight (48 h at 80°C) and ground.

Gas exchange measurements were taken on 22–24 August 2001 (Hättenschwiler et al. 2002) from 1 intact current-year lateral shoot per tree under light saturating conditions using a LI-COR 6400 (LI-COR, Lincoln, NE, USA) with a conifer cuvette (LI-6400-05).

Of the xylem samples from 2001 and 2002, we used 50–70 mg to separate different carbon fractions, starting with the water soluble one. Samples were mixed with 5 ml deionized water in a round centrifuge tube (PPCO 12 cm, Nalge Nunc International, Rochester, NY, USA), heated up to 60°C on a heating block, and mixed from time to time using a Vortex mixer (Vortex Genie 2, Bender & Hobein AG, Zürich, Switzerland). After 15 min of extraction, the samples were centrifuged for 10 min at 12,000*g* (Kontron Centrifuge, Berthold Hermle KG, Gosheim, Württ, Germany) and the supernatants were collected using a pasteur pipette. The pellets were treated again in the same way as described above twice before the supernatants of each sample were combined and filtered (Nalgene Syringe Filters 0.45 μm, Nalge Company). The liquid was filled in nickel cups and evaporated in a drying oven at 70°C to determine the dry weight of the water soluble fraction.

From the remaining pellet, pure cellulose was extracted using the procedure of Brenninkmeijer (1983). This method involves the removal of the lipophilic fraction by washing with a (2:1) toluene to ethanol-solution, delignification with a solution of $NaClO_2$ (0.5%) and acetic acid (0.15%), and the purification of the remaining material with NaOH (4%). According to Brenninkmeijer this extraction method is relatively rigorous, and approximately 20% of the cellulose is lost in favour of its purity. As we were forced to run the procedure with considerably less starting material (50 instead of 2,000 mg as recommended by Brenninkmeijer), the relative loss might be even higher, but data are still useful for a comparison of the relative contributions under ambient and elevated CO_2 conditions.

For a comparison of $\delta^{13}C$ between years and species, the purity of cellulose rather than the recovery is important.

In this study the water soluble fraction, the lipophilic fraction, cellulose, and lignin were distinguished as different 'carbon fractions'. The size of the lipophilic fraction was determined as the difference in sample dry weight after the removal of the water soluble fraction and before delignification, the second step of the Brenninkmeijer extraction method. Likewise, the lignin fraction was determined as the difference in dry weight before and after delignification.

Monitoring of air $\delta^{13}C$

We analysed the carbon isotopic composition of fossil CO_2 used for enrichment using a Gasbench (Gasbench II, Thermo Finnigan, Bremen, Germany) and an isotope ratio mass spectrometer (Delta Plus XL, Thermo Finnigan, Bremen, Germany). A mean $\delta^{13}C$, ± SE of –30.52 ± 0.39‰ was measured. The average CO_2 concentration in elevated CO_2 plots (566 ppm in 2001 and 599 ppm in 2002) represents a CO_2 addition of 196 and 199 ppm, respectively. The isotopic composition of the source air in elevated CO_2 plots was calculated using the following mixing ratio:

$$\delta^{13}C_{\text{elevated plot}} = \frac{\delta^{13}C_{\text{air}} \times c_{\text{air}} + \delta^{13}C_{\text{fumigation}} \times c_{\text{fumigation}}}{c_{\text{elevated plot}}} \quad (1)$$

that is in our case for the year 2001:

$$\begin{aligned}\delta^{13}C_{\text{elevated plot}} &= \frac{(-7.9‰ \times 370\,\text{ppm}) + (-30.5‰ \times 196\,\text{ppm})}{566\,\text{ppm}} \\ &= -15.7‰, \end{aligned} \quad (2)$$

where 370 ppm is the concentration and –7.9‰ the $\delta^{13}C$ of current air CO_2. Analogically, 196 ppm is the CO_2 concentration and –30.5‰ the $\delta^{13}C$ of fumigation CO_2 in elevated CO_2 plots where it adds to current air CO_2. The resulting difference in $\delta^{13}C$ between ambient and elevated CO_2 plots is –7.8‰ (–15.7 + 7.9‰). For 2002, the second year of CO_2 enrichment, the resulting $\delta^{13}C$ of elevated CO_2 is –15.8 and the difference in $\delta^{13}C$ between ambient and elevated CO_2 plots is –7.9‰. Since the carbon isotope ratio of annual plants reflects the isotopic composition of source air, the isotope signal of such test plants grown in CO_2-enriched air can be used as an indicator of the long-term mixing ratio of the new source air (Pepin and Körner 2002). In addition to our direct monitoring of the CO_2 concentration and the calculation of $\delta^{13}C$ of new source air in elevated CO_2 plots we used Garden cress (*Lepidium sativum* L.) to compare the response in $\delta^{13}C$ of larch and pine with that of an annual plant. Cress seeds were sown in small plastic pots on three dates over the summer 2002. The pots were mounted on the inside of the posts delimiting each plot on mid-canopy height. A pot with cress was placed within six elevated CO_2 and three ambient CO_2 plots, as the variation of $\delta^{13}C$ values within ambient plots is expected to be lower. The plants were harvested after a month. The $\delta^{13}C$ of C_3-plants is not only affected by source air, but also by fractionation processes within the plant, namely the kinetic fractionation (a) occurring in the gaseous phase during CO_2 uptake of the leaves and the biochemical fractionation (b) by the enzyme Rubisco, responsible for the fixation of carbon. Both processes discriminate against the heavier ^{13}C isotope and depend on the ratio between the CO_2 concentration in the substomatal cavities (p_i) and outside the leaf (p_a). According to Farquhar et al. (1982) the discrimination (Δ) can be expressed as:

$$\Delta = a + (b - a)\frac{p_i}{p_a}. \quad (3)$$

The $\frac{p_i}{p_a}$ is mainly affected by stomatal conductance and photosynthetic capacity. As elevated atmospheric CO_2 can affect both (Gunderson and Wullschleger 1994; Norby et al. 1999), the relative impact of either of these processes is difficult to calculate. Thus, ideally we would have used a C_4-plant for the monitoring of enriched plot air isotopic composition, as C_4-plants are relatively insensitive to changes in the $\frac{p_i}{p_a}$-ratio (Farquhar 1983). The harsh treeline environment, however, did not allow the use of any C_4-plant. We calculated an average difference in $\delta^{13}C$ of –6.58 ± 0.63‰ (mean ± SE) between elevated and ambient CO_2 grown cress, which is 1.3‰ less than the calculated difference of –7.9‰ (in 2002). This discrepancy could be due to lower CO_2 concentrations at the border of the plots (where cress pots were mounted) compared to the locations where air samples for the control of CO_2 concentration were taken or due to changes in $\frac{p_i}{p_a}$ leading to a shift in discrimination. The contribution of reserves stored in the cress seeds that were formed under ambient conditions may be neglectable.

Carbon isotopic analysis

The carbon isotope ratio was determined in xylem bulk material, the water soluble fraction, cellulose and the

fraction consisting of cellulose and lignin resulting after the extraction of lipophilic components. In plant bulk material, the proportion of different carbon fractions can vary among plant tissues. The fact that lignin for instance, has a more negative $\delta^{13}C$ value than cellulose (by 2–4‰) can thus hamper the comparison of bulk material across different tissues or across plant species. For this reason, the use of pure cellulose (representing the largest fraction of plant material) has been proposed (Wilson and Grinsted 1977), which allows a composition-independent comparison of $\delta^{13}C$ of different tissues. The carbon isotopic composition was analysed with an elemental analyser (EA-1110, Carlo Erba Instrumentazione, Milan, Italy) to produce CO_2, H_2O and N_2 under excess oxygen at 1,020°C, a GC-Column to separate the gases, and an isotope ratio mass spectrometer (Delta S, Thermo Finnigan, Bremen, Germany). The inlet into the mass spectrometer was governed by a variable opensplit interface (Conflo II, Thermo Finnigan, Bremen, Germany). Standards were measured with each series to ensure the precision of the measurements (±0.1‰). The carbon isotopic composition is given as $\delta^{13}C$ in ‰ relative to the Pee Dee Belemnite Standard PDB.

Calculation of new carbon (f_{new})

Based on the differences in $\delta^{13}C$ between ambient and elevated CO_2, the proportion of new carbon (f_{new}) in each carbon fraction was calculated (Eq. 4, Table 3). It represents the proportion of carbon acquired after the onset of CO_2 enrichment which in 2001 equals the proportion of current-year carbon ($1–f_{new}$ = old carbon). In tissues formed in 2002, f_{new} represents a mixture of carbon assimilated in 2001 and 2002. Thus, given a constant contribution of storage to new shoot growth, f_{new} should increase from 2001 to 2002. For each of the different carbon fractions, the mean difference in $\delta^{13}C$ between pairs of ambient and elevated CO_2 grown trees was divided by the difference in source air which roughly represents the maximum potential difference in plant material.

$$f_{new} = \frac{\delta^{13}C_{elevated\ trees} - \delta^{13}C_{ambient\ trees}}{\delta^{13}C_{elevated\ CO_2} - \delta^{13}C_{ambient\ CO_2}} \times 100, \qquad (4)$$

where $\delta^{13}C_{elevated\ CO_2} = -15.7‰$ in 2001 and –15.8‰ in 2002 (from Eq. 2) and $\delta^{13}C_{ambient\ CO_2} = -7.9‰$. The values for f_{new} are based on the assumption that $\frac{pi}{pa}$ remains constant at ambient and elevated CO_2. To check for possible changes in $\frac{p_i}{p_a}$, we used the data from gas exchange measurements of both tree species taken between 22 and 24 August 2001.

Data analysis

Shoot xylem bulk and cellulose $\delta^{13}C$ of consecutive years was analysed using a repeated measures ANOVA. Single trees were treated as subjects, ''year'' was the repeated factor with two levels (2001 and 2002) within each subject. ''Species'' and ''CO_2'' were fixed between subject factors. The $\delta^{13}C$ values of xylem bulk from the year 2000 were used as a co-variable to account for pretreatment differences among trees. ''Defoliation'' was excluded from the model as it showed no effect on the $\delta^{13}C$ values. All interactions were left in the model except those between the co-variable and the fixed between subject factors. In order to test the hypotheses concerning the importance of stored carbon compounds for new shoot growth in both larch and pine, a separate ANOVA was carried out for each species using the same model as described above.

In larch, the year × CO_2 interaction was significant. Thus, the differences in $\delta^{13}C$ (in f_{new}) between CO_2 treatments in the years 2001 and 2002 were compared using paired t tests. The differences between CO_2 treatments were determined for arbitrary pairs of trees one grown under ambient, one under elevated CO_2 conditions which makes them somewhat artificial (applies to the next paragraph as well). We carried out an additional test comparing the change in $\delta^{13}C$ between the years 2001 and 2002 within trees grown under elevated CO_2 conditions only. Hereby, in order to account for climatic variability between years, we corrected the $\delta^{13}C$ data of elevated CO_2 grown trees by the change in $\delta^{13}C$ between years observed in ambient CO_2 grown trees (with respect to the baseline 2000).

For all carbon fractions, the $\delta^{13}C$ values were used to calculate the change in $\delta^{13}C$ under elevated CO_2 for pairs of trees and then the fraction of new carbon. The change in f_{new} from 2001 to 2002 was analysed with paired t tests.

To compare the needle $\delta^{13}C$ with wood $\delta^{13}C$ from twigs in pine we carried out a repeated measures ANOVA with ''plant organ'' (twig, needles) as repeated factor within an individual tree. ''CO_2'' was the only between subject factor in the model.

To analyse the relative contribution of the fractions, each fraction was treated separately. In a three-way ANOVA the effects of tree species, CO_2, and defoliation were analysed for the year 2002. Then, the effect of CO_2 was tested in a one-way ANOVA for each species in 2001 and the effects of CO_2 and defoliation were tested with a two-way ANOVA for each species in 2002. For pine under elevated CO_2, a part of the data from 2001 fractions were lost due to a failed extraction. To test for the effect of the year in larch

Table 1 $\delta^{13}C$ in xylem bulk and cellulose from *L. decidua* and *P. uncinata* grown at either ambient (Amb.) or elevated (Elev.) atmospheric CO_2 concentrations (means ± SE, *N* in parentheses if ≠ 10). In addition, the difference (Diff.) between the treatments is given (± SE of the difference)

	L. decidua			*P. uncinata*		
	Amb. CO_2 $\delta^{13}C$‰	Elev. CO_2	Diff. ‰	Amb. CO_2 $\delta^{13}C$‰	Elev. CO_2	Diff. ‰
Xylem bulk						
2000	–28.30	–29.12	–0.82	–27.39	–27.72	–0.33
	±0.28	±0.31(9)	±0.42	±0.26	±0.11	±0.29
2001	–27.53	–32.14	–4.61	–26.96	–30.87	–3.91
	±0.10	±0.52	±0.53	±0.26	±0.35	±0.44
2002	–28.83	–34.66	–5.83	–27.25	–31.57	–4.32
	±0.21	±0.51	±0.55	±0.29(8)	±0.71	±0.77
Cellulose						
2001	–25.85	–30.07	–4.23	–25.14	–30.58	–5.44
	±0.11	±0.48(9)	±0.50	±0.35(9)	±0.48(8)	±0.59
2002	–27.65	–33.71	–6.06	–26.11	–31.41	–5.30
	±0.27	±0.55	±0.61	±0.30(8)	±0.69	±0.75
Lignin + cellulose						
2001	–27.52	–31.99	–4.47	–26.33	–31.17	–4.84
	±0.10(9)	±0.52(9)	±0.53	±0.23	±0.54(8)	±0.60
2002	–28.92	–34.83	–5.91	–26.52	–31.63	–5.11
	±0.35	±0.49	±0.58	±0.37(8)	±0.74	±0.83
Water soluble						
2001	–27.31	–31.06	–3.76	–27.64	–29.41	–1.77
	±0.13	±0.38(9)	±0.40	±0.19(9)	±0.47(7)	0.38
2002	–28.23	–33.62	–5.39	–27.04	–32.19	–5.15
	±0.24	±0.30	±0.51	±0.31(8)	±0.67(9)	0.74

Twig material from years 2000 (pretreatment) and 2001 was collected on 12/13 June 2002, material from 2002 was collected on 23/24 September 2002

over both years we used the repeated measures model from above. The software used for the statistical analysis was SPSS for Windows release 11.0.1 (SPSS Inc., Chicago, USA).

Results and discussion

$\delta^{13}C$ and new carbon in twigs from larch and pine

Irrespective of the CO_2 concentration the trees grew in, larch had generally lower $\delta^{13}C$ values than pine (Table 1). However, the difference in $\delta^{13}C$ between the two species varied among years for both, xylem bulk and cellulose (year × species interaction $P = 0.005$ and $P = 0.010$, respectively). These results indicate species specific isotope discrimination not related to CO_2 treatments, but possibly influenced by physiological responses to interannual variation in environmental conditions which were not addressed specifically in this study.

As expected from the use of ^{13}C depleted CO_2 to enrich the atmosphere of elevated CO_2 plots, both tree species exhibited significantly lower $\delta^{13}C$ in xylem bulk and cellulose ($P < 0.001$) when grown in elevated CO_2 compared to trees at ambient CO_2 (Table 2). In larch, but not in pine, this CO_2 effect increased from 2001 to 2002 (year × CO_2 interaction, Table 2, Fig. 1). When instead of the differences in $\delta^{13}C$ between ambient and elevated CO_2 grown trees (determining f_{new}), the change in $\delta^{13}C$ from 2001 to 2002 of elevated CO_2 grown larch trees was analysed (corrected data, see Data analysis section), the result remained the same ($P = 0.094$ for xylem bulk and $P = 0.027$ for cellulose).

A substantial use of carbon reserves in both tree species is indicated by relatively small absolute differences in $\delta^{13}C$ between ambient and elevated CO_2 grown trees (Table 1) compared to the average difference observed for cress (–6.58‰) and calculated for air (–7.8‰). In 2001, the structural material (i.e. lignin + cellulose) was composed of 54% new carbon (f_{new}) in larch, and 58% in pine, accordingly 46 and 42% of carbon derived from storage, respectively. We found similar proportions of f_{new} in cellulose, lignin + cellulose (representing structural carbohydrates), and in xylem bulk in both species (Table 3). In larch, f_{new} of the water soluble fraction was also similar to that of structural fractions. All carbon fractions in larch twig wood contained significantly more f_{new} in the second year than in the first year, except for the bulk

Table 2 Analysis of variance for $\delta^{13}C$ in xylem bulk and cellulose from twigs

	Source of variance	Xylem bulk		Cellulose	
		F	*P*	*F*	*P*
L. decidua					
Within subjects	Year	29.766	0.000	45.776	0.000
	Year × CO_2	5.261	0.036	7.225	0.017
	Year × bulk2000	0.913	0.353	1.046	0.323
Between subjects	CO_2	150.227	0.000	108.698	0.000
	Bulk2000	7.084	0.017	4.837	0.044
P. uncinata					
Within subjects	Year	0.869	0.366	1.791	0.206
	Year × CO_2	0.422	0.526	0.061	0.809
	Year × bulk2000	0.001	0.982	0.066	0.802
Between subjects	CO_2	47.633	0.000	73.162	0.000
	Bulk2000	3.645	0.076	3.123	0.103

Each species was analysed separately, and $\delta^{13}C$ of pretreatment xylem bulk from the year 2000 was used as a co-variable

xylem material, where the change in f_{new} was only marginally significant. In pine, f_{new} was comparatively small for the water soluble fraction in 2001, because of a less negative $\delta^{13}C$ under elevated CO_2 than expected. Pine might have been photosynthetically active for some time before the CO_2 enrichment started in 2001. The early season photosynthetic products, which were most probably part of the water soluble fraction would then bear an isotope signature more similar to ambient CO_2 conditions. This may also explain the rather low f_{new} in pine bulk material compared to the structural fractions. Apart from an increase in f_{new} in the water soluble fraction, there was no significant change in f_{new} from 2001 to 2002 in pine twig wood. Cellulose was the carbon fraction showing the most pronounced difference between larch and pine. Larch had lower f_{new} than pine in 2001, but considerably higher f_{new} than pine in 2002. The lignin + cellulose fraction, which accounts for 81–93% of xylem bulk material in larch and 65–72% in pine showed f_{new} values intermediate to those in xylem bulk and cellulose, with the exception of pine in 2002. The similar amounts of f_{new} in the extracted carbon fractions are evidence for constant carbon investments from current and older photosynthates to these distinct carbon pools.

Table 3 Proportions of new carbon f_{new} in different carbon fractions from twigs in the years 2001 and 2002 for *L. decidua* and *P. uncinata*

	f_{new} (%)[a]		N[b]	*T*	*P*
	2001	2002			
L. decidua					
Xylem bulk	59	74	10	1.965	0.081
Lignin + cellulose	54	77	8	2.362	0.050
Cellulose	54	78	9	3.070	0.015
Water soluble	47	68	9	2.945	0.019
P. uncinata					
Xylem bulk	49	57	8	0.747	0.479
Lignin + cellulose	58	67	6	0.461	0.664
Cellulose	64	62	5	–0.069	0.948
Water soluble	23	56	5	2.908	0.044

[a] f_{new} means new carbon from photosynthesis after the onset of CO_2 enrichment (with a different $\delta^{13}C$)

[b] N for paired ambient-elevated differences of both years. Note, N is different from that in Table 1 because only pairs of trees with data in both years were used

With a greater use of stored carbon compounds, we expected a larger difference in f_{new} between 2001 and 2002. Over time, f_{new} would of course approach 100% due to replacement of stored carbon compounds from 2000 and earlier by new carbon compounds from 2001 and later. In contrast, if exclusively current-year photosynthates are used for new shoot growth, f_{new} should be 100% in both years. Lacointe et al. (1993) have shown that reserves are used according to the ''Last In First Out'' (LIFO) system, where the most recently formed reserves are used first. In that case, for trees grown at elevated CO_2, use of storage in 2001 would involve material formed under ambient CO_2, i.e. unlabelled material, in 2000 or earlier. However, use of storage in 2002 would primarily involve reserves formed under elevated CO_2 in 2001 with a carbon isotopic composition very similar to that of current material. Thus, if only reserves from the previous year were used, f_{new} would approach 100% rather fast. Effectively, in larch, f_{new} in wood increased in the second year of CO_2 treatment compared to the first year, whereas it remained constant in pine. This finding fits the concept of different storage use in evergreen and deciduous tree species. For the evergreen *P. sylvestris* it was shown that stored reserves were not important for new shoot growth (Hansen and Beck 1990). Rather, the current photosynthates produced by previous years needles supplied new growth in evergreen conifers in spring (Kozlowski and Winget 1964; Gordon and Larson 1968; Hansen and Beck 1994). For a range of deciduous trees, in contrast, growth of new shoots and leaves—at least in the early stage—is supported by stored reserves (Gäumann 1935; Hansen 1967, 1971; Kandiah 1979; Lacointe et al. 1993). Gäumann (1935) reported that in mature beech trees (approx. 110 years old) two fifths of the carbohydrates used for leaf formation and elongation of canopy shoots were supplied from storage.

However, in absolute terms pine used almost as much storage as larch for the formation of structural material in 2001 (42% in pine vs. 46% in larch), and notably in the second year, f_{new} in wood of the evergreen pine was smaller than that in the deciduous

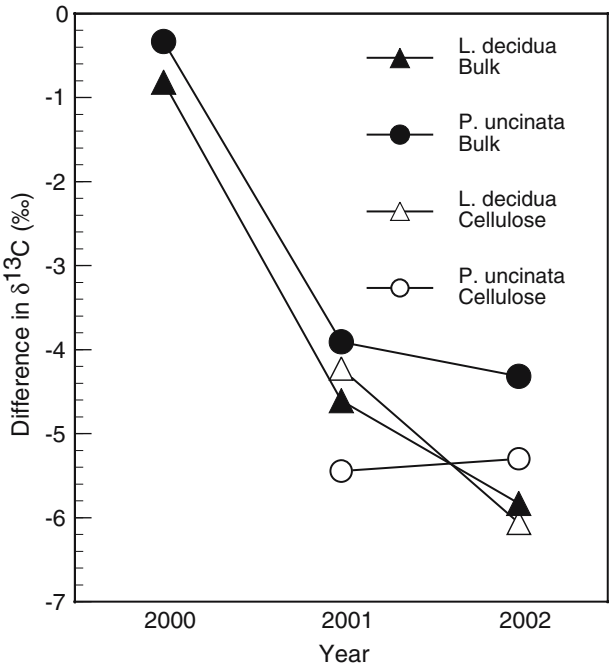

Fig. 1 Differences in twig $\delta^{13}C$ between trees grown at ambient and elevated atmospheric CO_2 concentrations in 2001 and 2002 in twig samples from the years 2000 (pre-treatment) and 2001 (sampled on 12/13 June 2002), and 2002 (sampled on 23/24 September 2002). *Triangles* represent measurements from larch and *circles* from pine, *closed symbols* represent $\delta^{13}C$ of xylem bulk material and *open symbols* $\delta^{13}C$ of extracted cellulose (no extraction for 2000)

larch. This suggests a greater contribution of stored carbon in pine compared to larch in 2002, and it seems that pine mobilized even older carbon reserves (from 2000 or earlier). If reserves are used according to LIFO, the use of storage older than the previous year reserves would imply a severe carbon limitation in pine. Our trees at the alpine treeline might have responded in a way different from those of Hansen and Beck (1990) who made their experiments in the Botanical Garden in Bayreuth where conditions were more favourable. Furthermore, Hansen and Beck (1990) only used autumn labelling to assess reserve use in spring, and the fate of autumn reserves may not be representative for the fate of the total reserves formed during the whole season. For example, autumn reserves in deciduous walnut trees were mainly used for respiratory purposes whereas reserves formed the previous summer were incorporated into structural material (Lacointe et al. 1993). In evergreen Cork oak, winter storage was shown to be entirely allocated to new growth rather than respired (Cerasoli et al. 2004).

A different physiological response in pine by lower $\frac{p_i}{p_a}$ and improved water use efficiency under elevated CO_2 could be a possible alternative explanation for a relatively low f_{new} in pine. An decrease in $\frac{p_i}{p_a}$ would decrease stomatal ^{13}C discrimination which could explain higher $\delta^{13}C$ in pine to some extent. However, this was not supported by independent calculations of $\frac{p_i}{p_a}$ from needle gas exchange measurements (Handa et al. 2005).

It is interesting to note that even in wood formed in the year 2000, before CO_2 enrichment, we found a slight difference in $\delta^{13}C$ between trees later exposed to ambient and elevated CO_2. This difference in $\delta^{13}C$ has the same direction as the one in response to elevated CO_2 observed in 2001 and 2002 (mean ± SE of –0.82 ± 0.42‰ in larch and –0.33 ± 0.29‰ in pine). This may indicate some horizontal transport of new carbon to older wood parts through ray parenchyma cells. For larch these are the only place where reserves are stored whereas in Pine reserves can also be stored in needles. This could explain the larger ambient-elevated difference in $\delta^{13}C$ in larch compared to pine in 2000.

In summary, the initial hypothesis that larch supplies a significant part of new shoot growth by old carbon reserves is supported. However, the hypothesis that new shoot growth is exclusively supplied by current photosynthates in pine is rejected based on the data presented here.

$\delta^{13}C$ and new carbon in pine needles

At ambient CO_2, pine needles showed essentially the same $\delta^{13}C$ values as wood xylem bulk (Fig. 2, left panel) across all needle age classes. In contrast, $\delta^{13}C$ was significantly lower in needles than in wood when grown at elevated CO_2 in both years of CO_2 treatment (Table 4). In 2002, the difference in bulk $\delta^{13}C$ was –6.18 ± 0.81‰ (mean ± SE) in needles grown at elevated CO_2 compared to ambient CO_2, whereas it was only –4.32 ± 0.77‰ (mean ± SE) in bulk xylem wood. The pattern observed for cellulose differed somewhat from that in bulk material (Fig. 2, right panel), in part because of the offset in $\delta^{13}C$ between needles of trees later grown at ambient and elevated CO_2 (+0.99 ± 0.32‰, mean ± SE) already present in 2000, before the CO_2 enrichment started. In 2002, the difference in cellulose $\delta^{13}C$ in needles grown at elevated CO_2 compared to ambient CO_2 was –6.30 ± 0.84‰ (mean ± SE), whereas it was –5.30 ± 0.75‰ (mean ± SE) in wood cellulose. In 2001 and 2002, needle and wood cellulose were less different than needle and wood xylem bulk. However, taking into account the pre-treatment offset, the difference in needle cellulose would be approximately –7.3‰. Needle and wood cellulose differed significantly in $\delta^{13}C$ at either CO_2 concentration, but were similarly affected

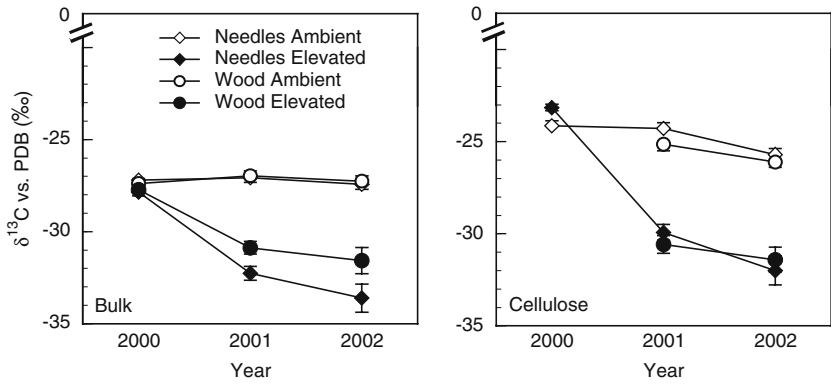

Fig. 2 $\delta^{13}C$ in needles (*diamonds*) and wood (xylem bulk, *circles*) of *P. uncinata* from 2000 to 2002 grown at ambient CO_2 (*open symbols*) or elevated CO_2 (*closed symbols*) in 2001 and 2002. Needle samples were collected on 23/24 September 2002, for wood see Fig. 1. Means and standard errors of bulk material (*left*) and cellulose (*right*) are shown

by elevated CO_2 in 2001 (Table 4). In 2002, however, there was no difference between needle and wood cellulose $\delta^{13}C$ but needle cellulose was more responsive to elevated CO_2 (organ × CO_2 interaction, Table 4). Compared to the f_{new} values in twig material (Table 3), the proportion of new carbon in needle material was generally higher and increased in 2002. For needle bulk material f_{new} was 66% in 2001 and 78% in 2002, for cellulose it was 71 and 85%, respectively.

The contribution of f_{new} in pine needles was substantially larger than in wood, suggesting that pine needle growth is mostly supported from current year photosynthates either of the newly growing needles themselves or of older needle age classes, as it was shown earlier (Hansen and Beck 1990, 1994).

The relative contribution of different carbon fractions in twigs

All fractions distinguished here differed widely between species ($P < 0.001$). In fact, the effect of species was larger than the CO_2 effect. In general, the relative amount of the water soluble and the lipophilic fraction were considerably larger in pine than in larch, at the expense of the structural fractions, cellulose and lignin (Fig. 3).

Enrichment of CO_2 did not change the relative amount of cellulose in either species (Table 5). This is not surprising, because an enhanced production of cellulose would rather lead to larger shoot increments than to a higher cellulose density. In their studies on tissue chemistry of *Pinus palustris* seedlings under elevated CO_2, Entry et al. (1998) and Runion et al. (1999) also did not observe any change in stem cellulose content. Likewise, elevated CO_2 had a minor influence on the relative amount of lignin, with only a slight decrease in larch in 2002. Similarly, lignin did not change in response to elevated CO_2 in *Picea abies* (Hättenschwiler et al. 1996) or in *Pinus sylvestris* (Kilpelainen et al. 2003). Even the mobile fractions, which were expected to be particularly responsive to elevated CO_2, showed only sporadic and inconsistent CO_2 effects in the two species and years. At elevated CO_2, there was a tendency for a higher lipophilic fraction in larch ($P = 0.087$) in 2001, but one for a reduced lipophilic fraction in pine in 2002 ($P = 0.092$). The inconsistent effects in larch and pine in their water soluble fraction are reflected in a significant species × CO_2 interaction in both years ($P = 0.012$ in 2001 and $P = 0.009$ in 2002). In 2001, pine twigs showed a negative CO_2 effect in the water soluble fraction, larch twigs showed an increase in the water soluble fraction in 2002. It is a common observation, that non structural carbohydrates (NSC) increase under elevated CO_2 conditions (Körner 2000, for a review) and also did so in the present experiment (Handa et al. 2005). However, it should be noted that here we measured the relative contribution of different fractions (which together summed up to 100%) and not their absolute concentrations independent of other compounds. Moreover, the separation of our water soluble fraction was not very specific, and a subsequent NSC analysis showed for example that only about 40% thereof were actually NSC (whereof 90% were NSC-

Table 4 Analysis of variance for $\delta^{13}C$ in *P. uncinata* needles compared to wood

Year	Source of variance	Xylem bulk		Cellulose	
		F	*P*	*F*	*P*
2001	CO_2	118.568	0.000	79.043	0.000
	Organ	21.133	0.000	8.380	0.013
	Organ × CO_2	15.354	0.001	0.106	0.750
2002	CO_2	37.177	0.000	31.712	0.000
	Organ	30.476	0.000	0.282	0.604
	Organ × CO_2	17.432	0.001	14.338	0.002

$6 \le N \le 10$ per CO_2 level and organ

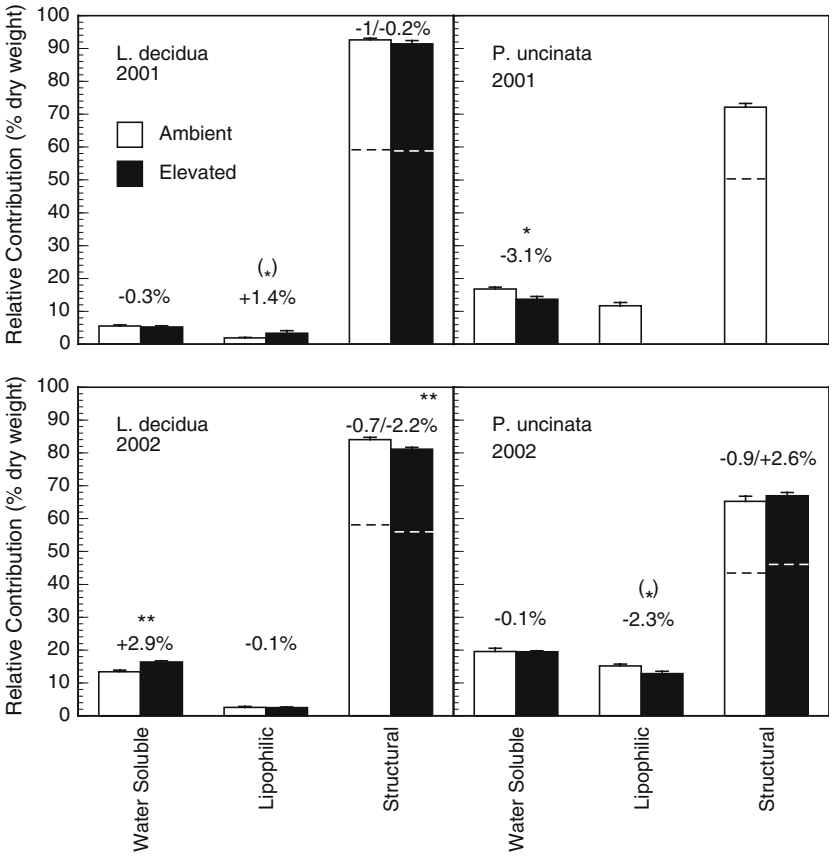

Fig. 3 Relative contribution of carbon fractions in twigs from *L. decidua* and *P. uncinata* grown at either ambient (*open bars*) or elevated (*shaded bars*) atmospheric CO_2 concentrations. *Upper panel*: 2001, *lower panel*: 2002. The structural fraction includes lignin (*lower part of bar*, *right percentage change*) and cellulose (*upper part of bar*, *left percentage change*). Note that the values for lignin and cellulose are over-/underestimated, respectively. *Asterisks* represent the significance levels of CO_2 effects (** for $P < 0.001$, * for $P < 0.01$, (*) for $P < 0.1$)

Table 5 Analysis of variance for the relative contributions of carbon fractions in twigs

Year	Fraction	Source of variance	*L. decidua*[a]		*P. uncinata*[b]	
			F	*P*	*F*	*P*
2001	Water soluble	CO_2	0.295	0.594	10.146	0.007
	Lipophilic	CO_2	3.290	0.087		
	Cellulose	CO_2	2.093	0.166		
	Lignin	CO_2	0.043	0.838		
2002	Water soluble	CO_2	22.902	0.000	0.091	0.767
		Defoliation	0.417	0.528	0.553	0.471
		CO_2 × defol.	1.306	0.207	0.638	0.439
	Lipophilic	CO_2	0.024	0.879	3.301	0.092
		Defoliation	1.283	0.274	3.115	0.101
		CO_2 × defol.	0.130	0.723	0.036	0.853
	Cellulose	CO_2	0.918	0.352	1.031	0.328
		Defoliation	0.546	0.471	1.190	0.295
		CO_2 × defol.	0.506	0.487	0.404	0.536
	Lignin	CO_2	18.366	0.001	2.550	0.134
		Defoliation	1.238	0.282	0.293	0.598
		CO_2 × defol.	0.401	0.536	0.037	0.851

Data analysed for each species in each year separately

[a] Larch 2001: $n = 10$ ambient, $n = 9$ elevated, 2002: $n = 10$ for all treatments

[b] Pine 2001: $n = 9$ ambient, $n = 7$ elevated, 2002: $n = 8$ ambient, $n = 9$ elevated, $n = 6$ defoliated, $n = 11$ control

sugars, namely fructose, glucose and sucrose). The remaining 60% might be other sugar species, water soluble proteins, and small particles such as cellular fragments (<0.45 μm) from the pellet that were able to pass the filter.

Defoliation performed in 2002, did not significantly affect any carbon fraction in either larch or pine (Table 5). However, when the effect of defoliation, was analysed across both tree species, there was a significant reduction of the lipophilic fraction irrespective of CO_2 treatment (P = 0.033, Fig. 4).

The cellulose extraction method used here is well-suited for stable isotope analysis, the main goal of this study, but less so for an accurate quantitative determination of cellulose. This is illustrated by the generally low proportions of cellulose. To obtain a highly pure cellulose for isotopic analysis we accepted some loss during purification. Using this method, we obtained only 29 and 22% cellulose for larch and pine, respectively, instead of the commonly reported 40–45% for softwoods (Robson and Hague 1993; Brendel et al. 2000). In contrast to cellulose, the lignin fraction was overestimated because it was calculated as the difference between the yield before delignification and the cellulose fraction.

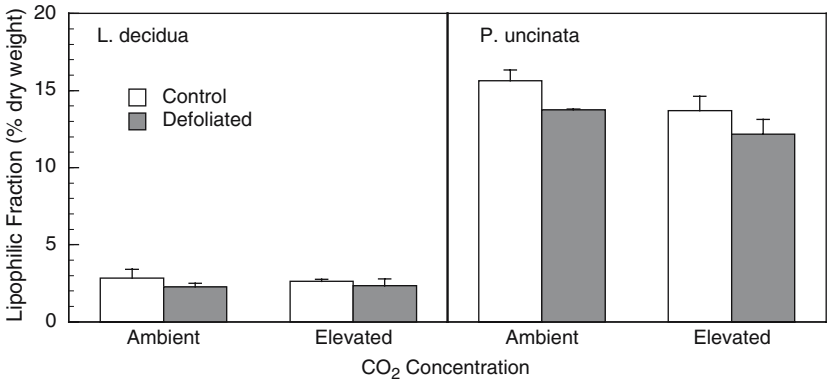

Fig. 4 The effect of defoliation in 2002 on the relative contribution of the lipophilic fraction in twigs. *Bars* represent means and standard errors for *L. decidua* (*left*) and *P. uncinata* (*right*) grown at either ambient or elevated CO_2 conditions

For a comparison of the contribution of different fractions between years, our sampling dates in spring 2002 for the 2001 material and autumn 2002 for the 2002 material, were not ideal. For the structural fractions, that do not change once they are formed, this may not be much of a problem, but the mobile fractions are likely to differ considerably between seasons and thus spring and autumn harvest.

Conclusions

This study provides evidence that stored carbon compounds contribute considerably to new wood formation in both *L. decidua* and *P. uncinata* growing at treeline. In 2001, approximately 40–50% of the carbohydrates used for new twig growth in larch and pine were supplied from storage. While both tree species apparently allocate reserves to new twig growth, pine needles were formed almost exclusively from current photosynthates. These results shed new light on earlier studies with ^{14}C pulse labelling, which concluded that stored carbon is not important for new growth in evergreen conifers. The constant relative contribution of stored carbon to different fractions of woody tissue in both species examined, provide evidence for a stable allocation of storage and current-year photosynthates across vastly different carbon pools including lignin and mobile carbon compounds.

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