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The influence of ozone and nutrition on $\delta^{13}C$ in *Betula pendula*

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Abstract In the cellulose of stems and leaves, $\delta^{13}C$ was investigated in a birch clone (*Betula pendula*), which was exposed throughout the growing season to either <3 (control) or 90/40 nl O_3 l^{-1} (day/night). Each regime was split into plants under high or low nutrient supply. $\delta^{13}C$ was increased (becoming less negative), in stems rather than leaves, by both high nutrition (+2‰) and O_3 stress (+1‰). Whereas high nutrition raised the water-use efficiency (WUE) while lowering the CO_2 concentration in the inner leaf air space (c_i), WUE decreased and c_i increased under O_3 stress. Therefore, only the nutritional effect on the carbon isotope fractionation was reproduced by the model of Farquhar et al. (1982) which estimates WUE by means of $\delta^{13}C$ based on c_i. c_i was not biased by 'patchiness' in respect to stomatal opening. The latter was verified by microscopical analysis and the complete water infiltration of the birch leaves through the stomata, independent of the diurnal course of the leaf conductance for water vapour. Under low nutrient supply, the activity of phosphoenol pyruvate carboxylase (PEPC) was roughly doubled by ozone to about 1.3% of the total carboxylation capacity (by PEPC + rubisco), and was increased to 1.7% under high nutrition. The fractionation model, extended to account for varying activities of the carboxylating enzymes, indicated that stimulated PEPC was the cause of elevated $\delta^{13}C$, although c_i was increased under O_3 stress. The stimulation of PEPC and, as a consequence, elevated $\delta^{13}C$ are discussed as part of a whole-plant acclimation to O_3 stress.

Key words $\delta^{13}C$ · Ozone · Nutrition · Betula pendula · Carboxylation

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Introduction

During photosynthesis, plants discriminate against $^{13}CO_2$ in favour of $^{12}CO_2$ and, as a consequence, their biomass becomes more negative in $\delta^{13}C$ (with respect to the PDB standard; Craig 1957) than atmospheric CO_2. Farquhar et al. (1982) proposed a model which explains the variation of $\delta^{13}C$ in C_3 plants by changes in the CO_2 concentration of the intercellular leaf air space, c_i. Via c_i, WUE (water-use efficiency) is estimated from $\delta^{13}C$ and related to the extent to which stomata limit photosynthesis relative to biochemical carboxylation. The model has proven useful for the investigation of plants under drought stress (Winter 1981; Ehleringer et al. 1992).

Air pollutants may reduce both stomatal conductance and carboxylation efficiency (Reich and Amundson 1985; Dann and Pell 1989; Matyssek et al. 1991), therefore $\delta^{13}C$ analysis may determine the relevance that such changes at the leaf level have for plant production. Responses of a wide range of woody and herbaceous plants to air pollutants have been investigated, and in nearly all cases, $\delta^{13}C$ was increased (Freyer 1979; Greitner and Winner 1988; Martin et al. 1988; Boutton and Flagler 1991; Saurer et al. 1991; Matyssek et al. 1992; Elsik et al. 1993). According to Farquhar et al. (1982), such an increase should indicate lowered c_i. However, in some cases, physiological measurements revealed an increased c_i, especially in plants exposed to O_3 (Boutton and Flagler 1991; Saurer et al. 1991; Matyssek et al. 1992; Elsik et al. 1993). Thus, under O_3 stress a different mechanism may underlie alterations of $\delta^{13}C$ than that found with drought. Matyssek et al. (1992) suggested that the increase in phosphoenol pyruvate (PEP) carboxylase (PEPC) activity, observed in O_3-exposed C_3 plants such

as pine and poplar (Lüethy-Krause et al. 1990; Landolt et al. 1994), could cause elevated $\delta^{13}C$. The O_3 impact may be modified by plant nutrition which, in turn, links $\delta^{13}C$ to the water relations if nutrient availability determines stomatal regulation (Schulze 1986).

The aim of this study is to assess the functional basis of elevated $\delta^{13}C$ in *Betula pendula*, when WUE is lowered and c_i is raised under O_3 stress. How would nutrition affect $\delta^{13}C$ in such a scenario? Besides gas exchange, the envisaged mechanistic explanation of the $\delta^{13}C$ level must address the activity of the carboxylating enzymes and the anatomy of the leaves. Can PEPC be uncovered as one determinant of changes in $\delta^{13}C$ under O_3 stress?

Theory

According to Farquhar et al. (1982), the carbon isotope composition of C_3 plants, $\delta^{13}C_p$, basically relates to the CO_2 concentration in the intercellular air space of the leaf mesophyll, c_i:

$$\delta^{13}C_p=\delta^{13}C_a-a-(b-a)c_i/c_a \quad \text{Eq. 1}$$

where $\delta^{13}C_a$, approximately –8‰, is the isotope composition of atmospheric CO_2; a (4.4‰) is the diffusive ^{13}C fractionation of CO_2 passing through stomata; b (27‰) is the net ^{13}C fractionation by enzymatic carbon fixation; c_a is the atmospheric CO_2 concentration, about 340 µl l^{-1}. $\delta^{13}C_p$ typically ranges between –20‰ and –35‰ (Deines 1980). The negative correlation between $\delta^{13}C_p$ and c_i was experimentally verified by Evans et al. (1986) and related to the ratio of CO_2 assimilation rate versus transpiration rate (i.e. WUE), reflecting the degree of stomatal limitation on photosynthesis. Such a relationship may not apply if the biochemical fractionation b is uncertain: $\delta^{13}C_p$ may increase although c_i is elevated. That b is equal to 27‰ results from fractionation by the carbon-fixing enzymes ribulose-bisphosphate carboxylase/oxygenase (rubisco; b_2 = 29‰; in the chloroplasts) and PEPC (b_1 = –5.7‰; in the cytosol; Farquhar et al. 1989b):

$$b=\beta b_1+(1-\beta)b_2 \quad \text{Eq. 2}$$

where β = proportion of total carboxylase activity due to PEPC. A value for b of 27‰ is associated with a value for β of 0.05 (i.e. 5%), as shown for wheat (Holbrook et al. 1984). β has been regarded as constant when interpreting $\delta^{13}C_p$ by means of Eq. 1. To cope with changes in β, Eq. 1 can be rewritten as:

$$\delta^{13}C_p=\delta^{13}C_a-a-[\beta b_1+(1-\beta)b_2-a]\, c_i/c_a \quad \text{Eq. 3}$$

$\delta^{13}C_p$ now relates to the product of c_i and β, and Eq. 3 is evaluated with a two-dimensional 'Taylor expansion' (see Appendix). Typical values are chosen as starting conditions (index 0): $(\delta^{13}C_p)_0$ = –27.4‰ (Farquhar et al. 1989a), β_0 = 0.05 and c_{i0} = 231 µl l^{-1} (by solving Eq. 3 for c_i). By means of the Taylor expansion the degree is calculated to which $\delta^{13}C_p$ changes (denoted as $\Delta\delta^{13}C_p$) in response to changing c_i (Δc_i) and β ($\Delta\beta$) relative to the starting conditions. Note that the symbol 'Δ' is used here to denote simple differences in each parameter (and not isotopic fractionation between CO_2 and plant biomass in the sense of Farquhar et al. 1989a).

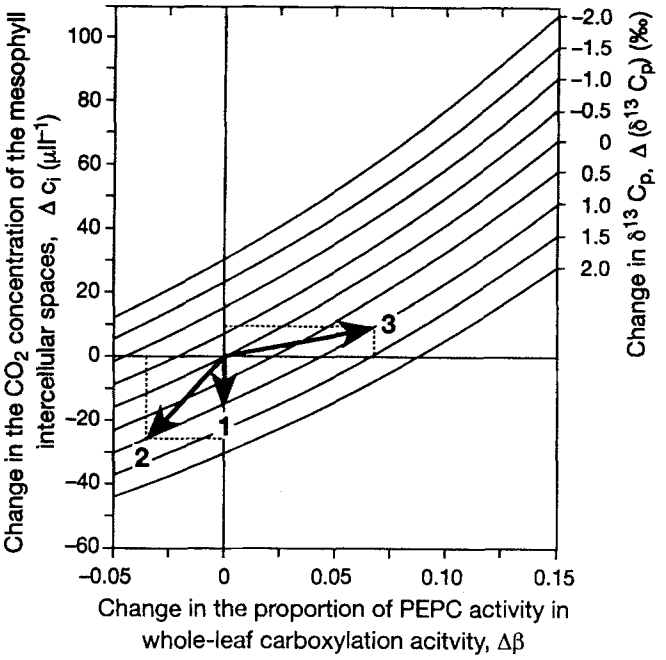


Fig. 1 Relationship between changes in the CO_2 concentration of the intercellular spaces of the leaf mesophyll, Δc_i, in the proportion of phosphoenol pyruvate carboxylase (PEPC) activity in whole-leaf carboxylation activity, Δβ, and in the $^{12}C/^{13}C$ isotope fractionation reflected in plant biomass, $\Delta(\delta^{13}C_p)$; 'Δ' denotes simple differences in each parameter (see Theory). Numbers on the right hand side of the figure give $\Delta(\delta^{13}C_p)$ for each of the curves depicted (i.e. $\Delta c_i/\Delta\beta$ relationships; curves derived from Eq. 4, which represents the $^{12}C/^{13}C$ fractionation model after evaluation by the Taylor expansion; see Appendix). Each curve graphically denotes all those possible combinations of Δβ and Δc_i which yield the *same* isotopic fractionation $\Delta(\delta^{13}C_p)$. This means that for a given $\Delta(\delta^{13}C_p)$ value pairs of $\Delta\beta/\Delta c_i$ must fall on the corresponding $\Delta(\delta^{13}C_p)$ curve to comply with the fractionation model. The three examples (*arrows* 1–3 and *dashed lines*) demonstrate such relations between the three parameters for a given $\Delta(\delta^{13}C_p)$ = + 1‰

Figure 1 illustrates the above relationship. For a given $\Delta(\delta^{13}C_p)$ value, pairs of $\Delta\beta/\Delta c_i$ must fall on the corresponding $\Delta(\delta^{13}C_p)$ curve to comply with the fractionation model. Three examples are given for $\Delta(\delta^{13}C_p)$ = + 1‰ (Fig. 1, *arrows*). These are: (1) c_i decreases while β stays constant; this example is consistent with Eq. 1 (unchanged PEPC activity). (2) Both c_i and β decrease. (3) Both β and c_i increase. Changes in β as low as 0.01 do affect $\delta^{13}C_p$, while case (3) will be examined in particular for its relevance under O_3 impact by allowing the fractionation model to account for variable β. The above calculations only marginally depend on the starting values $(\delta^{13}C_p)_0$, β_0 and c_{i0}. Figure 1 therefore covers a wide range of combinations of the parameters $\delta^{13}C_p$, β and c_i for the analysis of differences between treatments ($\delta^{13}C_p$ = $\delta^{13}C$ in the following). The analysis

inherently relates $\delta^{13}C$, which integrates metabolism throughout its seasonal course, to the short-term in vivo determination of leaf gas exchange and in vitro assessment of enzyme activity. Given this database, it will be tested to what extent β, when predicted by the model as a function of measured $\delta^{13}C$ and c_i, is consistent with β as directly derived from the measured enzyme activities.

Material and methods

Plants and treatments

From 13 April through to 28 September 1992, cuttings of one birch clone (*Betula pendula*, Roth) were grown in 10-l pots filled with sand and a basal layer of inert synthetic clay beads (1 plant/pot). Plants were transferred into the Birmensdorf field fumigation chambers on 6 May. Clone and fumigation chambers were the same as described in Matyssek et al. (1991, 1992; see also Landolt et al. 1989). On clear sunny days, a shading roof limited the photon flux density to a maximum of about 600 µmol m^{-2} s^{-1} to prevent over-heating in the open-top chambers; the roof was not employed under overcast or cloudy conditions, nor at dawn and dusk. Plants were separated into two O_3 treatments (80 plants/treatment, 8 plants/chamber): continuous exposure to charcoal-filtered air with either <3 nl O_3 l^{-1} (control; regarded as 'O_3-free') or 90/40 nl O_3 l^{-1} (day/night, i.e. 7 a.m.–9 p.m./9 p.m.–7 a.m., until 28 September). O_3 was generated from pure oxygen (Fischer, model 502) and, in both treatments, continuously monitored with a 'Monitor Labs 8810' analyser. Each O_3 regime was split into high and low nutrient supply by watering plants with either a 0.05% or 0.005% fertilizer solution (Hauert, Nährsalz Typ A/Anzucht), which contained macro- and micro-nutrients (Table 1). The concentration ratio between N and the other macro-nutrients was similar to that suggested by Ingestad and Lund (1986) for birch. The plants did not show any symptoms of nutrient deficiency or toxicity. In late September, the foliage of the main stem was analysed for nutrients (before autumnal discolouration) by classes of macroscopical O_3 injury (according to Günthardt-Goerg et al. 1993): cations, S and P by ICP (ARL 3580), and N with a Carlo Erba NA1500 analyser.

$\delta^{13}C$ analysis

On 28 September, the main stem and stem foliage of each tree were harvested and dried to constant weight (at 65°C), separating leaves according to macroscopical O_3 injury. The analysis concentrated on the injury classes 0 (no O_3 injury) and 2–3 (O_3-induced discolouration and small necroses; cf. Günthardt-Goerg et al. 1993). The plant material was ground to a fine powder. Cellulose was isolated from the samples as described in Matyssek et al. (1992), combusted to CO_2, and $\delta^{13}C$ was determined by mass spectrometry (Finnigan MAT 250, Germany). The precision of the $\delta^{13}C$ analysis (including sample preparation) was 0.1‰.

Table 1 Nutrient concentrations of the high nutrient supply fertilizer (0.05% solution, "HF"). Note that proportions of low nutrient supply (0.005% solution, "LF") were similar to the high nutrient supply, but with all concentrations diluted by a factor of 10

Macro-nutrients (mM)						Micro-nutrients (µM)					
N	P	S	K	Ca[a]	Mg	Fe	Mn	Cu	Zn	Mo	B
6.5	0.9	0.2	2.0	0.2	0.2	11.0	5.6	3.4	1.9	0.6	9.2

[a] Added to fertilizer with Ca = 2.5 µM background concentration

Leaf gas exchange

Attached complete leaves were measured with a thermo-electrically climate-controlled cuvette system (Walz, Germany) as described by Matyssek et al. (1991). The CO_2 concentration of the O_3-free system air was adjusted to 340 ± 5 µl l^{-1} by a mass flow-controlled CO_2-dispensing system. Gas exchange rates were based on the one-sided leaf area (Delta-T area meter MK2, UK). Two kinds of measurements were conducted on different tree individuals as part of a larger study on leaf gas exchange (S. Maurer and R. Matyssek, unpublished data): (1) diurnal courses of leaf gas exchange were recorded from the plants exposed in the fumigation chambers. Ambient temperature and air humidity were continually reproduced inside the gas exchange cuvette, whereby the photon flux density was reduced by the cuvette lid by about 8%. (2) 'Steady-state' in gas exchange was determined at 11 a.m. as the response to a 90-min exposure to $I > 1000$ µmol photons m^{-2} s^{-1}, $T_l = 19$°C and $\Delta w = 10$ mmol mol^{-1} (i.e. the leaf/air difference in the mole fraction of water vapour). The evening before this determination, plants randomly chosen from treatments were brought to the measuring site close to the chambers and shielded from direct sunlight and rain during the experiment.

'Patchiness' in stomatal opening (cf. Terashima et al. 1988) was investigated according to Beyschlag and Pfanz (1990). Several times during the day, stomatal conductance (g_{H2O}) was assessed with a CO_2/H_2O-diffusion porometer (Walz, Germany). After each measurement, leaves were detached, and immediately thereafter their water uptake by infiltration through the stomata was gravimetrically determined (applied pressure adjusted to 0.3 MPa with

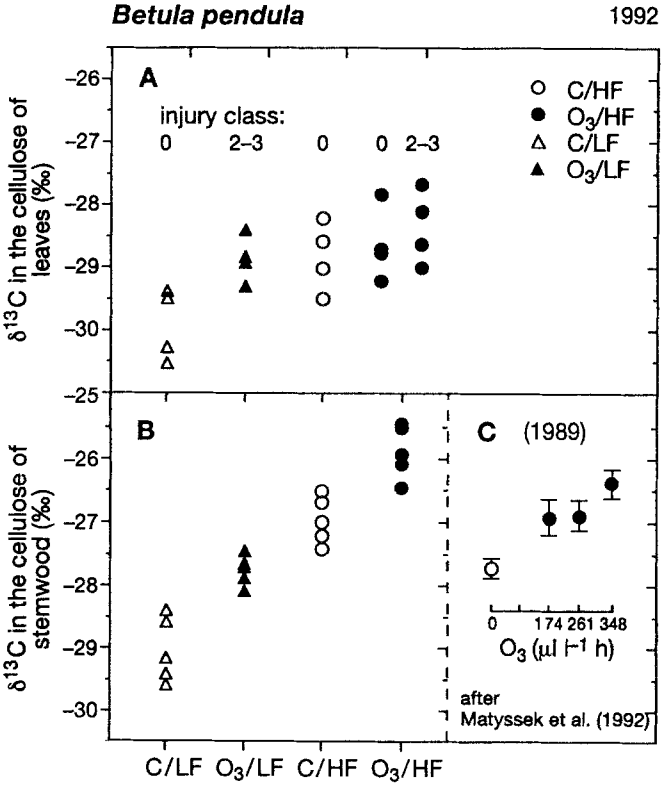


Fig. 2 $\delta^{13}C$ in the cellulose of leaves (**A**) and stemwood (**B**) by experimental treatment (*C* O_3-free control; *O_3* exposure to 90/40 nl O_3 l^{-1} during 7a.m.-9p.m./9p.m.-7a.m., O_3 dose throughout experiment = 241 µl l^{-1} h; *LF* low-fertilized; *HF* high-fertilized). Each data point represents one individual tree; injury classes of leaves according to Günthardt-Goerg et al. (1993). No leaves of class 0 were available in LF plants on September 28, the date of harvest. **C** $\delta^{13}C$ in the stemwood cellulose of HF plants as related to the O_3 dose from an experiment conducted during the growing season of 1989 (reported in Matyssek et al. 1992)

a manometer). The extent of infiltration was also examined with a binocular microscope. Stomatal opening and leaf anatomy were investigated by light and low-temperature scanning electron microscopy (Scheidegger et al. 1991; Günthardt-Goerg et al. 1993).

Enzyme activity

Whole leaves were homogenized in 100 mM phosphate buffer (pH 7.5; 5% polyvinyl-pyrrolidon, 0.5% Triton X-100) to determine the activity of rubisco and PEPC according to Schmieden-Kompalla et al. (1989). A Tris buffer (pH 8.0; 50 mM Tris, 20 mM $MgCl_2$, 10 mM KCl, 20 mM $NaHCO_3$, 5 mM DTE, 0.25 mM EDTA), enriched with $H^{14}CO_3^-$ to yield about 200×10^3 dpm/500 µl (from a $NaH^{14}CO_3$ stock solution of 1 mCi; Amersham, USA), was used for the enzymatic tests. In the case of PEPC, the reaction was started by adding 50 µl PEP to the test assay (400 µl buffer + 50 µl enzyme extract). Rubisco was pre-incubated in the buffer for 10 min before 50 µl RuBP-Na was added (PEP and RuBP-Na 0.6 mM each in assay). The reactions were terminated with 100 µl HAc after 1 (rubisco) or 10 min (PEPC). The residue of the solutions obtained after vacuum evaporization was re-dissolved in water before radioactivity was measured in a liquid scintillation counter (Philips 4540).

Results

Ozone increased the $\delta^{13}C$ value of leaves at low rather than high nutrient supply in contrast to the corresponding control plants in O_3-free air (Fig. 2A). In addition, high fertilization increased $\delta^{13}C$ along with the concen-

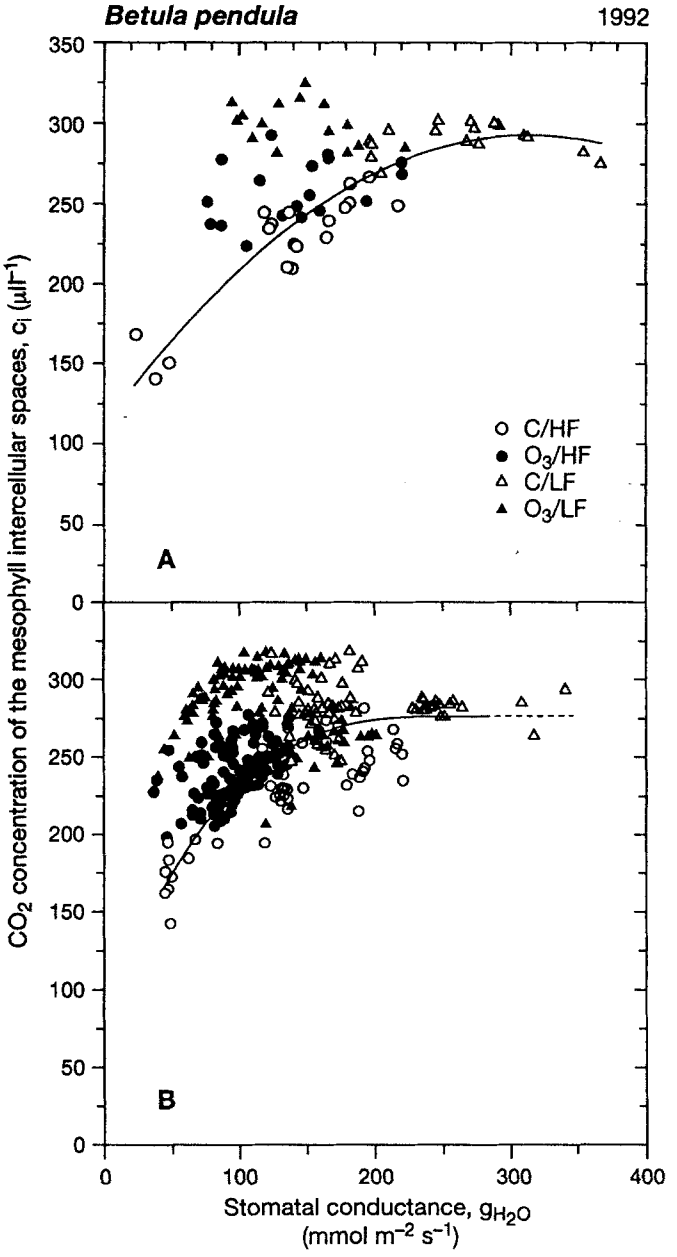


Fig. 3A,B Relationship between CO_2 concentration of the intercellular spaces in the leaf mesophyll, c_i, and the stomatal conductance for water vapor, g_{H2O}. Data from **A** 'steady-state' responses of gas exchange after 90-min exposure to constant light, temperature and humidity conditions (see Methods), and from **B** diurnal courses of gas exchange of the plants exposed in the Birmensdorf field fumigation chambers. In **A**, g_{H2O} of low-fertilized control plants statistically differs from that under ozonation and high nutrient supply at $p < 0.01$ each. For graphical reasons, only data are depicted in **B** which were measured between 10 a,m.–4 p.m. at photon flux densities >150 µmol m^{-2} s^{-1} (at low light, c_i increased with decreasing g_{H2O}). *Lines* fitted through the data represent polynomial curves derived from control plants in O_3-free air (see Table 3 for the significance levels in c_i between treatments); treatment abbreviations as shown in Table 2

Table 2 Nutrient concentrations of leaves in late September. Total foliage on tree stems analysed by classes of macroscopical O_3 injury according to Günthardt-Goerg et al. (1993) (0 no injury, 1 light-green dots spread over leaf, 2 light-green or bronze-green discolouration, 3 bronze-green discolouration with small necrotic areas; means ± standard deviation, *SD*, of 5–7 trees/treatment; class 1, i.e. early injury, not shown

Treatments				
O_3 concentration day/night (nl l^{-1})	0/0	0/0	90/40	90/40
Fertilization				
Cf. Table 1	High	Low	High	Low
Concentration (%)	0.05	0.005	0.05	0.005
Abbreviation	C/HF	C/LF	O_3/HF	O_3/LF
Class of visible O_3 injury in leaves	0	0	2–3	2–3
Nutrient concentrations (mg g^{-1})				
K	27.5±2.0	12.0±1.3	30.5±2.6	16.8±3.1
Ca	5.7±0.5	13.8±1.5	5.2±0.3	10.1±0.7
Mg	3.2±0.3	4.3±0.3	2.5±0.1	3.6±0.5
Fe (µg g^{-1})	253±59	57±16	317±33	53±8
N	33.9±3.0	18.4±4.0	22.8±3.0	17.0±2.0
P	8.7±0.4	2.9±0.3	8.6±0.6	3.4±0.6
S	1.7±0.1	0.9±0.2	1.1±0.1	1.0±0.1

Table 3 Effects of ozone and nutrition on $\delta^{13}C$ and c_i of *Betula pendula*. Differences between treatment mean values of $\delta^{13}C$ in the cellulose of stemwood and leaves and of c_i from 'steady-state' responses (see Methods) and diurnal courses of leaf gas exchange (calculated from $\delta^{13}C$ and c_i as shown each in Figs. 2 and 3; treatment abbreviations as in Table 2). Differences are significally differed from zero (*t*-test) when indicated by asterisk. The standard deviations of the differences (SD_Δ) were calculated from SD_1 and SD_2 of the respective mean values as: $\pm SD_\Delta = (SD_1^2 + SD_2^2)^{0.5}$

	Effect of ozone		Effect of nutrition	
	O_3/HF-C/HF	O_3/LF-C/LF	C/HF-C/LF	O_3/HF-O_3/LF
$\Delta(\delta^{13}C)\pm SD_\Delta$ (‰)				
Wood	1.1±0.6**	1.3±0.6**	2.1±0.6***	1.9±0.5***
Leaves	0.4±0.9	1.1±0.7*	1.2±0.8*	0.5±0.7
$\Delta c_i \pm SD_\Delta$ ($\mu l l^{-1}$)				
Steady state	34±41**	10±16*	−65±38***	−42±23***
Diurnal course	9±35*	5±28	−51±34***	−47±30***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

trations of most nutrients in the leaves (Table 2). Only the concentrations of Ca and Mg were lowered at high nutrient supply, in conjunction with the non-metals N and S, under O_3 exposure, whereas K and Fe concentrations tended to be raised by ozone. The effect of both O_3 exposure and high nutrition in elevating $\delta^{13}C$ became most evident in the cellulose of stemwood (Fig. 2B) which, unlike leaves of different age (there is indeterminate shoot growth in birch), integrates the production and carbon isotope fractionation of the total foliage throughout the growing season. The $\delta^{13}C$ of the plants grown with high nutrition confirmed the findings obtained from a similar experiment in 1989 (Matyssek et al. 1992; Fig. 2C), where the increase of $\delta^{13}C$ in the stem cellulose depended on the O_3 dose accumulating during the growing season.

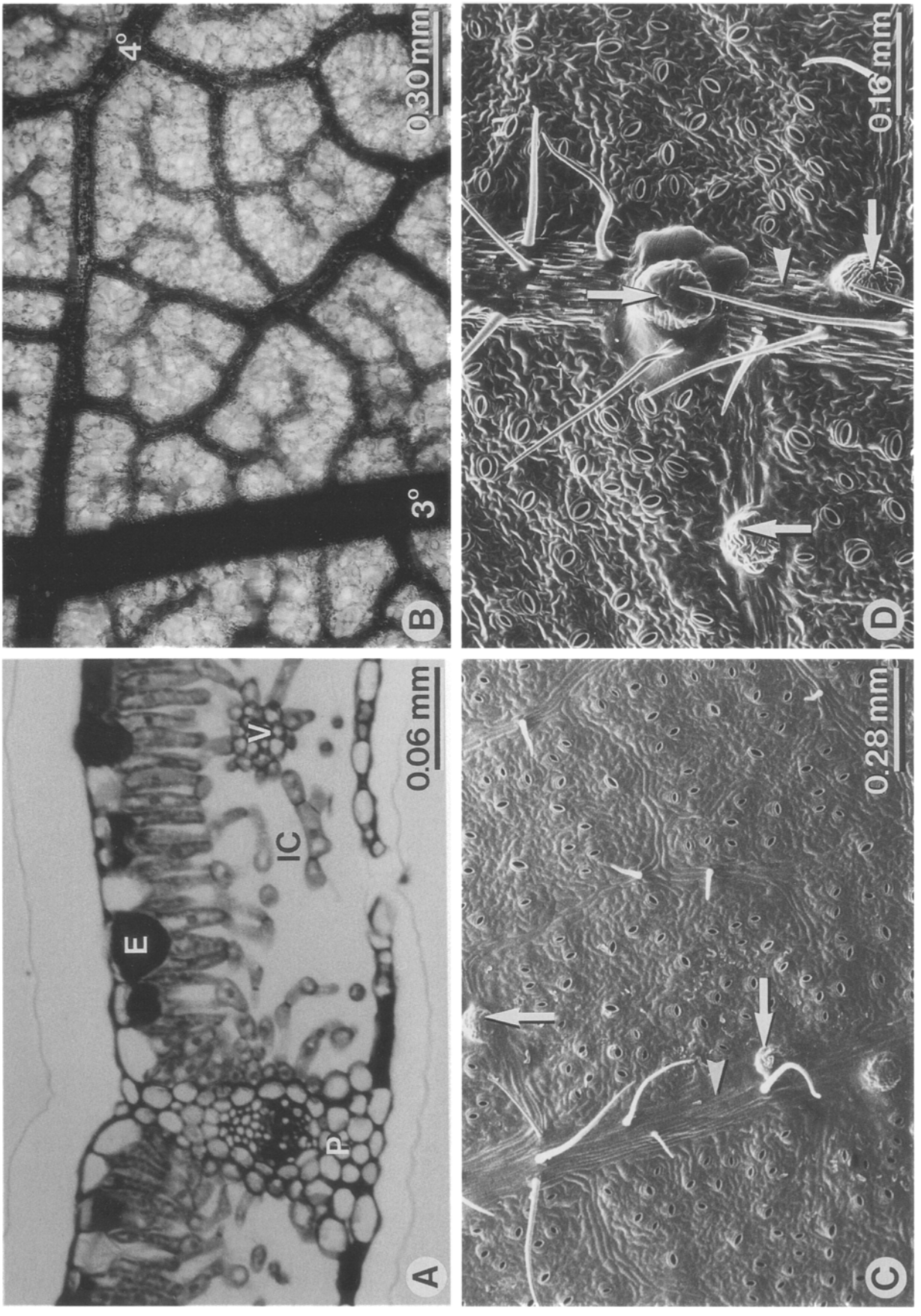
The 'steady-state' responses of leaf gas exchange (Fig. 3A) showed g_{H2O} to be highest in low-fertilized plants under O_3-free air, whereas high nutrition enhanced WUE as reflected by the significantly lowered c_i (Table 3; Fig. 3A). O_3 exposure reduced g_{H2O} especially under low nutrition, while c_i remained unchanged or tended to increase in both nutrient regimes (Fig. 3A; Table 3). Thus, c_i indicated a decrease in stomatal limitation on CO_2 uptake and in WUE under O_3 stress, because the CO_2 assimilation rate declined in proportion more than the g_{H2O} (cf. Matyssek et al. 1991, 1992). Averaging c_i from the 'steady-state' gas exchange of all leaves measured in a treatment, increases in c_i of 10–34 $\mu l\ l^{-1}$ related to a rise in $\delta^{13}C$ by about 1‰ in the stem cellulose of the O_3-exposed plants (Table 3). In contrast, high nutrition raised $\delta^{13}C$ by about 2‰, but c_i was distinctly lowered (by about 42–65 $\mu l\ l^{-1}$). The relationship of Fig. 3A was also indicated under the variable conditions of diurnal gas exchange courses (Fig. 3B), where mainly the nutritional effect on c_i was confirmed (Table 3).

The shade-adapted birch leaves (shading roof) had large inner air spaces (Fig. 4A,B), which increased further due to O_3-induced cell collapse (cf. Matyssek et al. 1991; Günthardt-Goerg et al. 1993), and the distribution of stomatal width was homogenous within and across the intercostal fields between leaf veins (Fig. 4C,D). Consequently, water infiltration through the stomata was complete, while the water uptake did not correlate with g_{H2O} during the diurnal course (not shown). Thus, 'patchiness' in stomatal opening did not occur, and as a result, the c_i data given in Table 3 were applicable to the Farquhar model (Eq. 1). The model predicted the increase of $\delta^{13}C$ in the stem cellulose caused by high nutrient supply on the basis of *lowered* c_i for both O_3 regimes, as reflected in Fig. 5A and B by the overlap between the ranges of Δc_i and $\Delta\delta^{13}C$ (see legend for details and Table 3). Thus, raised $\delta^{13}C$ was the consequence of stomatal limitation on CO_2 uptake. However, the latter mechanism failed to explain the increase of $\delta^{13}C$ in the presence of *elevated* c_i under O_3 stress.

Under low rather than high nutrition, the activity of rubisco tended to increase in the young leaves at the top of the stem, but tended to be lowered at the stem base of O_3-exposed plants in comparison to the control (Fig. 6A,B). PEPC activity was increased by ozone, especially in the leaves of the low-fertilized plants (Fig. 6C,D). Here, ozone doubled the proportion, β, of PEPC activity in total carboxylase activity (PEPC + rubisco) from 0.006 to 0.013 (Table 4). Under high nutrition, the O_3-caused $\Delta\beta$ was about + 0.003. By means of the fractionation model (Eqs. 3, 6; see Appendix) $\Delta\beta$ yielded + 0.018 for low, and + 0.009 for high-fertilized plants under O_3 stress. The dashed lines in Fig. 7A and B give the minimum changes necessary in β, which the model requests, to comply with the measurements (see legend for details). Thus, with β as the variable depending on the actual $\delta^{13}C$ and c_i, the model calculates trends in $\Delta\beta$ similar to the measured O_3-caused changes in β. The higher calculated than measured values of $\Delta\beta$ indicate that the PEPC activity in vivo (responsible for the $\delta^{13}C$ level found in the plant biomass) may be even higher than the activity determined in vitro. The PEPC activity should be considered, therefore, as one factor determining the $\delta^{13}C$ response of plants to O_3 stress.

Discussion

According to the model by Farquhar et al. (1982), $\delta^{13}C$ analysis relies on c_i as a measure of the CO_2 concentra-


A
E
IC
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P
0.06 mm
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4°
3°
0.30 mm
C
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D
0.16 mm

tion in the inner leaf air space. However, c_i, as calculated from leaf gas exchange, may be biased by 'stomatal patchiness' (Terashima et al. 1988). The latter may or may not be a general phenomenon (Cheeseman 1991; Lauer and Boyer 1992), but seems to be linked to heterobaric leaf anatomy. The shade-adapted birch leaves had large inner air spaces reflecting a transition to homobaric anatomy (Neger 1918; Björkman 1981). For this reason and the fact that plants were not limited by water supply, 'patchiness' did not occur. Thus, the obtained c_i allowed the use of the Farquhar model.

$\delta^{13}C$ was increased by a high supply of macro- and micro-nutrients. In parallel, leaves displayed low c_i and high WUE as already known for trees under conditions of non-limiting Mg nutrition (Küppers et al. 1985; Beyschlag et al. 1987). Given lowered c_i as caused by drought, the increase in $\delta^{13}C$ by high nutrition was predicted by the Farquhar model (Eq. 1; cf. Winter 1981; Farquhar and Richards 1984). Thus, $\delta^{13}C$ may allow the estimation of WUE in plants differing in nutrient supply, although reports about nutritional effects on $\delta^{13}C$ are inconsistent (Hubick 1990; Condon et al. 1992; Schmidt et al. 1993). The low WUE of low-fertilized birches resulted from high g_{H2O} and high transpiration, which may also have contributed to the elevated Ca and Mg concentration in the leaves (cf. Table 2). The capacity of both high nutrition and drought to increase WUE and $\delta^{13}C$ may conflict with the idea that water shortage narrows stomatal pores via reduced nutrient transport to the leaves (Schulze 1986). Nutrition apparently determined the level of $\delta^{13}C$, at which ozone became effective (cf. Fig. 2).

Ozone may inhibit photosynthesis by narrowing stomata and impeding CO_2 diffusion into the leaf, and/or by lowering the carboxylation capacity of the mesophyll (Winner et al. 1988). If the stomata solely limit photosynthesis, a decreasing g_{H2O} reduces c_i and increases WUE. In such a case, ozone would affect leaf gas exchange in a manner similar to drought, raising $\delta^{13}C$ via a lowered c_i. Such a mechanism has indeed been reported for O_3-exposed plants (Greitner and Winner 1988), but cannot be generalized for this kind of stress (contrasting with Farquhar et al. 1989b). Ozone does impair the carboxylation capacity of the mesophyll by reducing the amount and activity of rubisco (Dann and Pell 1989) or through extensive tissue destruction (Matyssek et al. 1991). If the CO_2 uptake rate declines proportionately more than g_{H2O} then WUE decreases, whereas c_i rises in parallel with $\delta^{13}C$ (Saurer et al. 1991; Matyssek et al. 1992). Given this scenario, the Farquhar model, with a constant β as originally proposed, does not apply to O_3 stress. Other factors, e.g. dark respiration, which indeed was increased under O_3 impact (S. Maurer and R. Matyssek, unpublished data), seem to have negligible or inconsistent effects on $\delta^{13}C$ (Troughton et al. 1974; Farquhar et al. 1982).

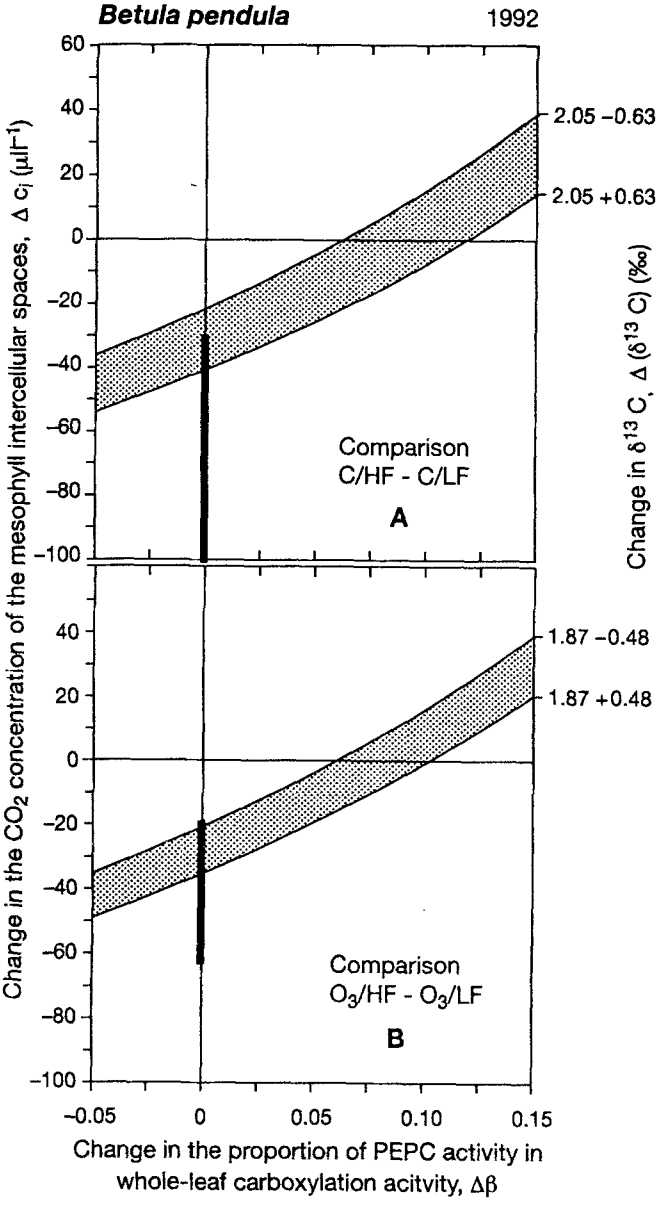

Fig. 4A–D Vein structure and stomatal pores in shade-adapted birch leaves (abaxial side, high-fertilized, no visible injury). **A** Cross-section showing epidermal cells with mucilaginous layer (*E*), spongy parenchyma with large intercellular space (*IC*), bundle sheath of parenchyma cells (*P*) around quarterny leaf vein, and veinlet (*V*). **B** Reticulum of tertiary (*3°*), quarterny (*4°*) and higher-order veins confining the intercostal fields of the leaf lamina. **C, D** 'Low-temperature' scanning electron microscopy: homogenous distribution of stomatal width within and across intercostal fields, no 'patches' of stomata contrasting in aperture. Terminology of leaf anatomy according to Hickey (1973), *short arrow* 3° vein, *long arrow* peltate scales

Fig. 5A,B Relationship between Δc_i, $\Delta\beta$, and $\Delta(\delta^{13}C)$ as introduced in Fig. 1. The measured $\Delta(\delta^{13}C)$ is given as the *hatched area*, which represents mean ± standard deviation (SD_Δ) according to the data of Table 3. Mean ± SD_Δ of the measured Δc_i is given as the *bold vertical bar* along the ordinate (Δc_i from 'steady-state' gas exchange; Table 3). **A** The effect of nutrition on $\Delta(\delta^{13}C)$ of control, and **B** of O_3-exposed plants. In both cases, the Δc_i-bar overlaps with the $\Delta(\delta^{13}C)$-area, which means that, according to Fig. 1, the measurements are consistent with the prediction by the Farquhar model when based on constant β (i.e. $\Delta\beta = 0$, cf. Eq. 1); treatment abbreviations as shown in Table 2

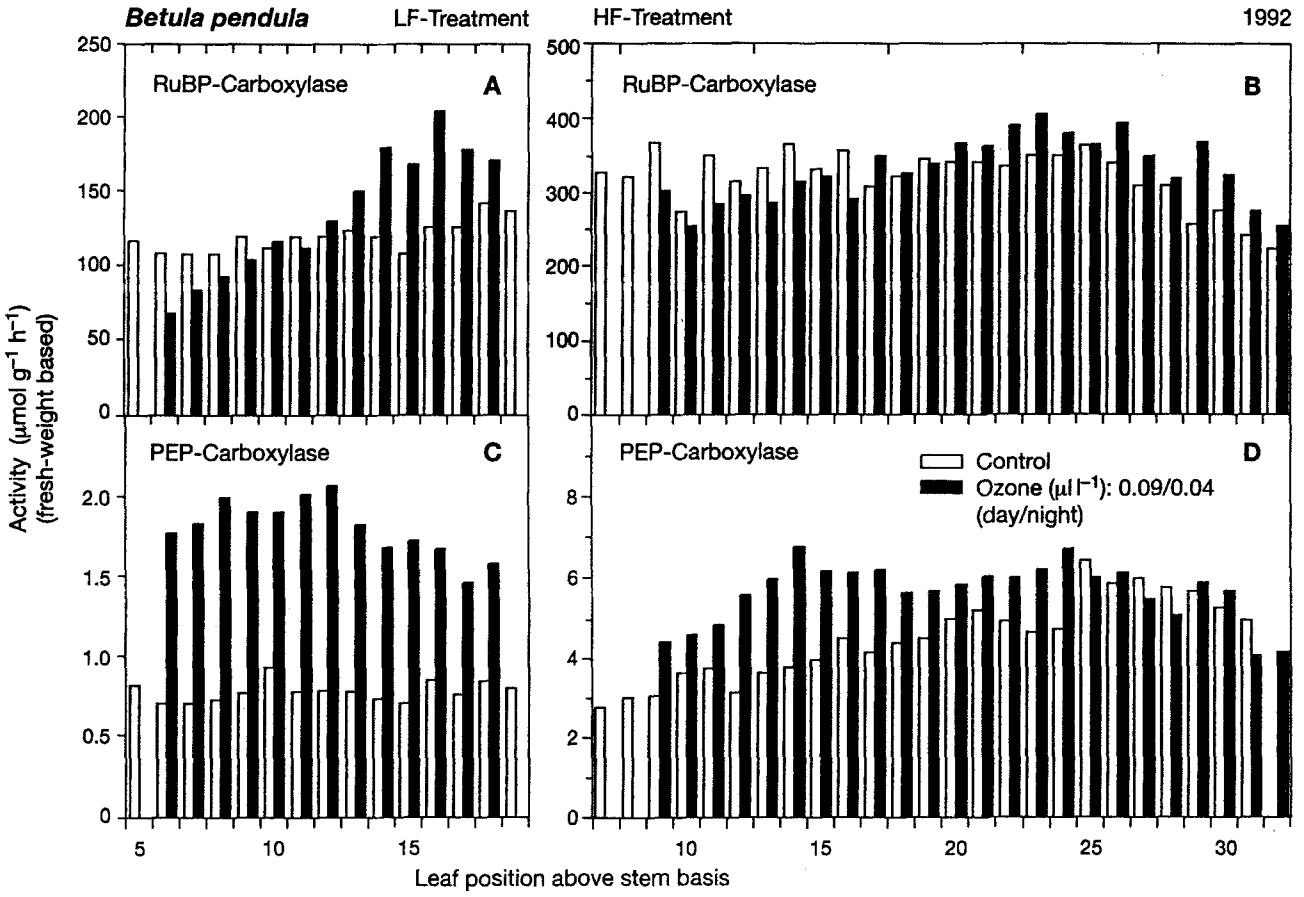

Fig. 6A–D Activity of the carboxylating enzymes as related to the leaf position above the stem basis. **A**, **B** RuBP carboxylase (rubisco); **C**, **D** phosphoenol pyruvate carboxylase (PEPC) of low- (**A**, **C**) and high- (**B**, **D**) fertilized plants (mean values of five plants/treatment each). Differences between O_3-exposed and control plants are statistically significant at $P < 0.05$ (Kruskal-Wallis test) at the leaf positions 6, 15, 16, 18 in (**A**); 29 in (**B**); 6, 7, 8, 9, 11, 12, 14, 15, 16, 17 in (**C**); 9, 12, 14 in (**D**)

Table 4 Effects of ozone and nutrition on β of *Betula pendula*. Proportion of phosphoenol pyruvate carboxylase activity in whole-leaf carboxylation activity, β (mean ± SD across all leaf positions in a treatment as based on the data shown in Fig. 6; treatment abbreviations as in Table 2). In the low-fertilized plants, the effect of ozonation on β was significant at $P < 0.001$

	Treatment			
	C/HF	O_3/HF	C/LF	O_3/LF
β±SD	0.014±0.005	0.017±0.005	0.006±0.002	0.013±0.007

In this study, O_3-induced increases in $\delta^{13}C$ were shown to correlate with increases in PEPC activity. This enzyme was stimulated by ozone, as is also seen in other tree species (Lüethy-Krause et al. 1990; Landolt et al. 1994). The less pronounced PEPC stimulation at high rather than low nutrient supply is consistent with the light increase of $\delta^{13}C$ in the leaves of high-fertilized plants (cf. Figs. 2A, 6D). Although PEPC activity is always much lower than that of rubisco, PEPC discriminates much less against ^{13}C and therefore has the capacity of raising $\delta^{13}C$. Wiskich and Dry (1985) regard the CO_2 fixation by PEPC in C_3 plants to be involved in anaplerotic reactions which feed the pool of oxalacetate in the mitochondria. Stimulation of PEPC is assumed to reflect an increased demand for substrate and energy in the plant, and such a demand does exist under O_3 stress. Repair and detoxification processes are stimulated in O_3-exposed plants (Wolfenden and Mansfield 1991; Polle 1995), may increase the O_3 tolerance of the foliage (Günthardt-Goerg et al. 1993) or maintain leaf formation (Matyssek et al. 1993; R. Matyssek et al. 1995), and can be associated with enhanced respiratory costs (e.g. Reich 1983). The enhanced demand for assimilates in the leaves changes whole-plant carbon allocation at the expense of root growth (Matyssek et al. 1992, 1993) and may be interpreted as an acclimation response to ozone, limiting reductions in biomass production under O_3 stress (Mooney and Winner 1991). Thus, increased PEPC activity and, as a consequence, elevated $\delta^{13}C$ may be viewed as part of such acclimation. If the Farquhar model accounts for changes in the PEPC/rubisco activity ratio (Eqs. 3, 6; see Appendix), stimulated PEPC may explain increased $\delta^{13}C$ under O_3 stress, even though c_i is elevated. Estimations of WUE based on $\delta^{13}C$ may take into consideration changes in the activity ratio of these carboxylating enzymes.

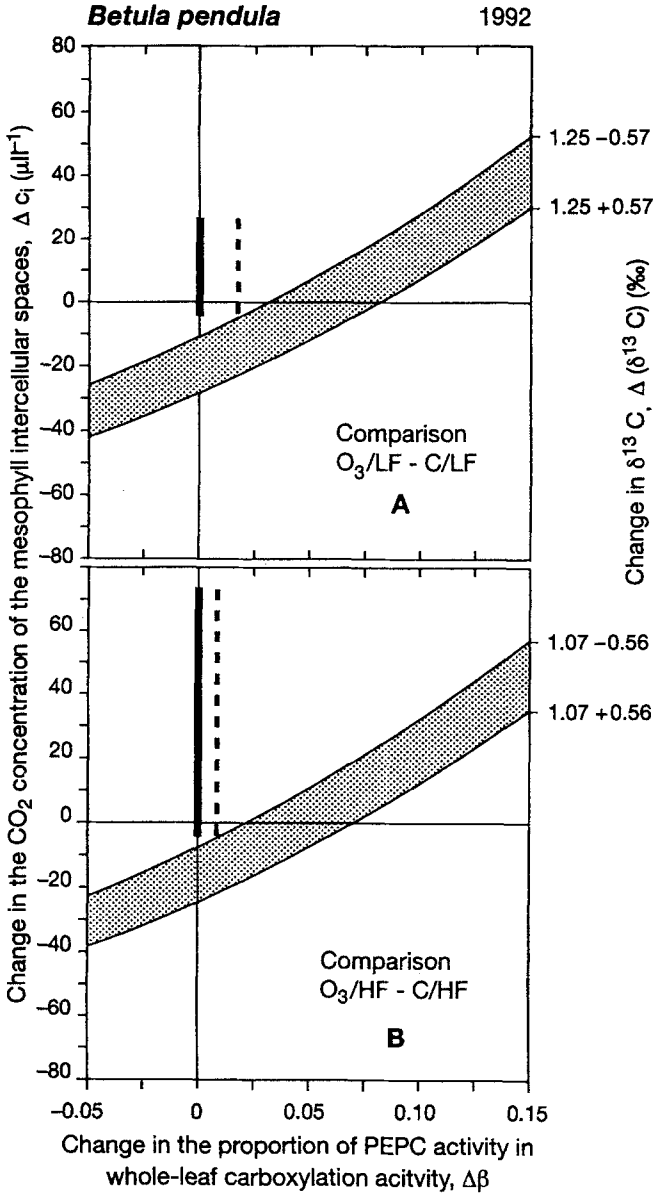

Fig. 7 As Fig. 5, but (**A**) and (**B**) show the effect of ozone on $\Delta(\delta^{13}C)$. As the *solid* Δc_i *bars* do not overlap with the $\Delta(\delta^{13}C)$-area, the measurements are, according to Fig. 1, not consistent with the prediction by the Farquhar model when based on constant β (i.e. $\Delta\beta = 0$, Eq. 1). Instead, the *broken* Δc_i bars give the minimum changes necessary in β, which the model yields and requests (Eqs. 3, 6; see Appendix) to comply with the measurements. The model yields $\Delta\beta = +0.018$ for low, and +0.009 for high-fertilized plants under O_3 stress on the basis of measured $\Delta(\delta^{13}C)$ and Δc_i; $\Delta\beta$ derived from in vitro measurements was 0.007 and 0.003, respectively (see Table 4); treatment abbreviations as shown in Table 2

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Appendix

We discuss the isotope fractionation, Eq. 3, and regard $\delta^{13}C_p$ as a function of the two variables β and c_i:

$$\delta^{13}C_p(\beta,c_i)=\delta^{13}C_a-a-[\beta\, b_1+(1-\beta)\, b_2-a]c_i/c_a \quad \text{(Eq.3)}$$

The Taylor expansion of this function near the point (β_0,c_{i0}), the 'starting conditions' (index zero), is given by:

$$\delta^{13}C_p(\beta_0+\Delta\beta,c_{i0}+\Delta c_i)=\delta^{13}C_p(\beta_0,c_{i0})+\partial/\partial\beta[\delta^{13}C_p(\beta_0,c_{i0})]\Delta\beta+\partial/\partial c_i[\delta^{13}C_p(\beta_0,c_{i0})]\Delta c_i+\partial^2/\partial\beta\partial c_i[\delta^{13}C_p(\beta_0,c_{i0})]\Delta\beta\Delta c_i$$

(+ derivations of order ≥2 which are zero for the function in question), (Eq. 4)

where e.g. $\partial/\partial\beta[\delta^{13}C_p(\beta_0,c_{i0})]$ is the partial derivation to β of $\delta^{13}C_p$ at the point (β_0,c_{i0}) and $\Delta\beta$ (respectively Δc_i) is a small change in β (respectively c_i).

Denoting changes in $\delta^{13}C_p$,i.e. the difference $\delta^{13}C_p\cdot(\beta_0+\Delta\beta,c_{i0}+\Delta c_i)-\delta^{13}C_p(\beta_0,c_{i0})$, as $\Delta\delta^{13}C_p$ and abbreviating $\partial/\partial\beta\cdot[\delta^{13}C_p(\beta_0,c_{i0})] = K_1$, $\partial/\partial c_i[\delta^{13}C_p(\beta_0,c_{i0})] = K_2$, $\partial^2/\partial\beta\partial c_i\cdot[\delta^{13}C_p(\beta_0,c_{i0})] = K_3$, Eq. 4 is rewritten as:

$$\Delta\delta^{13}C_p=K_1\Delta\beta+K_2\Delta c_i+K_3\Delta\beta\Delta c_i, \quad \text{(Eq. 5)}$$

Equation 5 indicates the extent to which $\delta^{13}C_p$ changes in response to changing c_i and β relative to the starting conditions. Typical values are chosen as starting conditions: $(\delta^{13}C_p)_0 = -27.4‰$ (Farquhar et al. 1989a), $\beta_0 = 0.05$ (Holbrook et al. 1984) and $c_{i0} = 231\ \mu l\cdot l^{-1}$(by solving Eq. 3 for c_i); $a = 4.4‰$, $b_1 = -5.7‰$, $b_2 = 29‰$, $c_a = 350\ \mu l\,l^{-1}$(see section Theory). K_1, K_2 and K_3 are then evaluated as described above and the result is:

$K_1=-(b_1-b_2)c_{i0}/c_a=22.9‰$, $K_2=-[\beta_0 b_1+(1-\beta_0)b_2-a]/c_a=-0.065‰$, $K_3=-(b_1-b_2)/c_a=0.099‰(\beta\,1\ l^{-1})$

Finally, Eq. 5 is solved for Δc_i:

$$\Delta c_i=(\Delta\delta^{13}C_p-K_1\Delta\beta)/(K_2+K_3\Delta\beta) \quad \text{(Eq. 6)}$$

This formula allows the calculation of the curves in Fig. 1 (e.g. set $\Delta\delta^{13}C_p=+1‰$ and vary $\Delta\beta$ from –0.05 to +0.05).

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